Antibiotics-induced transfer of resistance genes and emergence of new resistant bacteria in red swamp crayfish guts and culture sediments

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Research Article

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Abstract

Background

Antibiotic resistance genes (ARGs) can be transferred from environmental microbes to human pathogens, which could lead to treatment failure of bacterial infections. The aquaculture, which is polluted by current and past over-used antibiotics, is considered as a notorious reservoir of ARGs. However, the fact on the mobilization of ARGs in aquaculture systems, and their contributions to the emergence and dissemination of antibiotic resistant bacteria (ARB) remain elusive.

Results

We used crayfish as an aquatic animal model to analyze longitudinal changes of bacterial communities, ARGs and mobile gene elements (MGEs) in the gut and sediment samples in response to enrofloxacin administration. Our metagenomic analysis revealed significant perturbations of the microbiota and ARGs compositions in the gut compared to those in sediments, which is characterized by a obviously delayed recovery after antibiotic administration. The significantly positive correlation between the total abundance of ARGs and MGEs was observed and MGEs were frequently located close to ARGs in genomes of the bacteria from Enterobacteriaceae. These mobile ARGs had high intracellular and intercellular transferred potential by transposon-mediated recombination in gut and sediment samples in the pressure of antibiotics. In addition, multiple antibiotic resistant pathogens of Enterobacteriaceae and other new ARBs had emerged and proliferating under the pressure of antibiotic.

Conclusion

Our findings showed that MGEs facilitated the diffuse of ARGs through horizontal gene transfer mainly in the bacteria from Enterobacteriaceae family in gut and sediment environments. Especially, the transposase-mediated recombination was the major driver of the transfer for ARGs in the pressure of antibiotics. Simultaneously, the changes in community composition as the emerged and proliferating of the multiple resistant pathogenic bacteria played a crucial role in the augment of various ARGs.

Background

Infections with pathogenic bacteria which are resistant to antibiotics, especially to multiple antibiotics, are significant causes of morbidity and mortality in the world. It is reported that antibiotic resistance will account for 10 million cases of death per year by 2050, with a global cost of 100 trillion US dollars [1]. Antibiotic resistance has been recognized as a global health challenge. The resistance can spread among bacteria through horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs), which are usually located on mobile genetic elements (MGEs). The ARGs together with MGEs form mobile resistant units which can quickly be transferred from environmental bacteria to human pathogens(2,3) via HGT [2].
Majority (84%) of human-associated and mobile ARGs are already present in ESKAPE pathogens (i.e. *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) [3, 4]. Thus, to manage the global health challenge, it is important to understand the interactions between the environmental, animal- and human-associated bacteria (the One Health Concept) [5].

Undoubtedly, direct environmental antibiotic pollution through extensive use of antibiotics in breeding industry [6] and plant production [7] or via excretions (urine and feces) from humans and domestic animals [8] plays a crucial role in the development and spread of resistance. Previous studies have reported that there is an obvious increase in relative abundance of resistant bacteria and ARGs in environments with high levels of residual antibiotics as a result of use and excretion [9]. Recent data has highlighted soil and water environments as reservoirs and recipients of ARGs of clinical interest [10]. Previous meta-analyses showed that resistant bacteria contain distinct ARG contents at varying abundances in the gut, soil, and water, and had different ecological niches [11]. Therefore, quantifying the pathways and identifying the drivers of evolutionary expansion and spread of resistance gene factors across microbes in various habitats are key components in the management of the resistance.

Aquaculture is a high-growth industry with broad links to water, sediment, and other ecosystems. Intensification of production and increasing incidence of aquatic animal pathogens are both driving antimicrobial use [12]. By 2030, global antimicrobial use for human, terrestrial and aquatic food producing animal sectors is projected to reach 236,757 tons annually while aquatic food producing animal would use 13,600 tons which represents 5.7% of the global consumption, with a 33% increase between 2017 and 2030. The most used classes of antimicrobials include quinolones (27%), tetracyclines (20%), amphenicols (18%), and sulfonamides (14%) [13]. Application of antimicrobials in aquaculture provides a potentially wider environmental pathway for spread of drug and antimicrobial resistance with important ecosystem and health implications [14]. Highly diverse and abundant ARGs and MGEs were detected in shrimp and sh guts, as well as in aquaculture environments (water and sediments) [15, 16]. Thus, aquaculture was recognized as a significant reservoir for mobile resistance genes.

Evaluation of ARG variations in temporal and dissemination pattern in various ecological systems with artificial use of antibiotics is critical to the management of resistance. The red swamp crayfish (*Procambarus clarkii*) dig burrows in the cultivation process and its feces is usually used as high-quality fertilizer, which increases soil permeability and material circulation. Besides, rice-crayfish system has recently become the most economically important farmed aquatic species in China [17]. In this study, we used crayfish animal model to analyze the longitudinal changes of bacterial community, dynamics of ARGs and MGEs, and the spread of ARGs in the animal gut and sediment samples following the administration of enrofloxacin, a synthetic third generation quinolone broad-spectrum antibacterial drug which is frequently used in aquaculture. Our findings showed the pathways that mediate the expansion and spread of various resistance genes across microbes between the gut and sediment samples, and enrichment in the transfer potential of the ARGs as well as the emergence of antibiotic resistance bacteria, which affected the changes in microbial community structure.
Results

Shifts in bacterial communities to antibiotic exposure

To explore the alterations in the resistome in time dimension following the antibiotic administration, we first describe the microbial community changes in the guts of crayfishes and sediments. To assess these dynamic microbiota variances, gut content and sediment samples were collected from 8 time-points (3 repetitions) during the whole experiment for 16S rRNA sequencing (Fig. 1a) and the sequencing results were listed in Additional file1: Table S1.

The data revealed that the application of antibiotics resulted in a sharp decline of the microbial community as indicated by the alpha diversity in the guts, (Chao1 and dominance index, P=0.0083) and there was no restoration to baseline levels even after 28 days following antibiotic cessation (Fig. 1b). Although the microbial diversity was reduced, the reduction was not significant in the sediment samples (Fig. 1b). In addition, the sequencing data demonstrated a temporal change and segregation in microbial structure between different phases (Fig. 1c).

The antibiotic affected both the microbial composition in the guts and sediments, with persistent incomplete recovery at 42 days in the guts (Fig. 1d). Furthermore, Enterococaceae family demonstrated the highest sensitivity to the antibiotic treatment in the guts and the relative abundance was reduced from 29% to 0.2%. In contrast, administration of antibiotics for 14 days led to increase in Enterobacteriaceae from 20% to 34% in the gut (Fig. 1d). The data showed that Citrobacter genus, an environmental contaminant[18] was the most dominant genus and had similar trends as Enterobacteriaceae in the guts (Fig. 1e). In addition, a partial sequence that was identified as an uncultured bacterium, which was initially reported in the intestinal bacteria in wild and domesticated adult black tiger shrimp [19], was hardly affected in the guts (Fig. 1d). The findings showed that resistance may exist in some bacteria that cannot be cultured in vitro. There was led variation in the family level of bacteria in the sediments, and the microbiome was primarily dominated by Rhodocyclaceae (18%), Lactobacillaceae (18%) and Geobacteraceae (12%) throughout the entire experimental period. Enterobacteriaceae was the only family common to the guts and sediments and it was more stable in the sediments (Fig. 1d). In summary, the application of antibiotic altered the composition of the bacterial community and diversity in both the guts and sediments. Besides, the microbiome determinants in the guts were more affected than those in the sediments and demonstrated a marked delay in recovery.

Longitudinal changes of the ARGs in the gut and sediment microbiome in response to antibiotic administration

To understand dynamic changes of the ARGs between samples in different periods, we employed shotgun metagenomic sequencing on the gut contents and sediment samples to test the presence of ARGs. A total of 480 Gb of Illumina sequencing data was produced from the samples, and then we
acquired 14.74 million 150 bp paired-end reads per sample (on average) after host-subtraction and trimming (Additional file2: Table S2).

The data showed that there was a substantial increase in the total abundance of the ARGs in the crayfish guts and sediments microbiome, which reached a maximum value at 14 day. There was 6.97 times and 6.56 times enrichment in the guts and sediments, respectively, following the antibiotic administration (Fig. 2a). On the other hand, the relative abundance of ARGs in 99 different subtypes in the gut bacteria was higher than that in the sediments (85 subtypes) and the recovery time was 14 days later (Additional file1: Fig. S2). Most of the ARGs observed in the gut microbiome were predicted to confer multidrug, \(\beta\)-lactam and quinolone phenotypes which increased by 6.95, 6.31 and 7.49 times, respectively. In the sediments, sulfonamide, chloramphenicol, tetracycline, quinolone, and aminoglycoside resistance genes were the primary ARGs which were enhanced by over 8 times. In addition, the sediments contained more varieties of the ARGs which included 3 extra classes of rifamycin, bleomycin and trimethoprim (Fig. 2a).

Our data showed that the relative abundance of genes predicted to confer resistance to quinolone were dramatically increased after application of enrofloxacin which was mainly produced by \(qnrB\) and \(qnrS\) genes (Fig. 2b). Among these quinolone resistance genes, \(qnrB\) accounted for more than 95% in all samples of guts (Fig. 2b). The \(qnrB\) gene was associated with the highest risk in the ARG family as reported by the World Health Organization and as identified in various Enterobacterial species. The dominant quinolone resistance gene was \(qnrS\) which accounted for 95.9% in the sediments (Fig. 2b). The application of the enrofloxacin was associated with little changes in the proportions of quinolone resistance genes in total ARGs (Fig. 2b). Therefore, these data illustrated that enrofloxacin did not only potentially enrich quinolone resistance genes but also promoted emergence of other resistance genes to different antibiotics.

To characterize changes in the pattern of the ARG components, we specifically characterized core ARGs composition in the guts and sediments along the temporal continuum (Fig. 2c). The core ARGs were defined as ARGs with an average RPKM\(\geq10\) in guts and a RPKM\(\geq1\) in sediments. The core ARGs in the gut was substantially larger than that in sediments and included a wide range of genes of multidrug, \(\beta\)-lactam. On the other hand, sulphonamides and tetracyclines were mainly in sediments. Interestingly, the core ARGs such as \(sul1, sul2, tetA, acrB, aadA\) and \(floR\) were both present in the gut and sediment bacteria (Fig. 2c). The bloom of the ARGs were largely driven by the core ARGs in all samples (Fig. 2d). In general, there was massive increase in diversity and relative abundance of total ARGs in the gut and sediment microbiome while the ARGs in the gut microbiome had a more marked delay in the recovery following the antibiotic administration.

**Spatial co-occurrence of MGEs and ARGs after antibiotic administration**

Administration of antibiotics contributed to the enrichment of MGEs and the change of the relative abundance of MGEs showed similar tendency with the ARGs both in the guts and sediments (Fig. 3a). There was a clear increase in three types of MGEs (plasmid, intergase and IS), and the most abundant MGE group was IS at 14 days in the guts and intergase in the sediments, with differential patterns.
following antibiotic administration. At day 35, 21 days after the antibiotic exposure, the types of MGEs in guts and sediments were comparable to day 0 (Fig. 3b). In addition, there was positive correlation between the total abundance of ARGs and MGEs and which was stronger in sediments than in the guts. Besides, the abundance of ARGs was significantly correlated with the ISs in guts, but was correlated with plasmids, integrons and ISs in sediments (Fig. 3c). We also calculated the correlation between MGEs and the core ARGs, and showed that most of the core ARGs had significantly positive correlation with relevant MGEs in guts and sediments (r>0.9, Additional file1: Fig. S3). Therefore, tracking of such mobile genetic elements may provide a deeper insight into the extent of the spread of antibiotic resistance in the aquatic environment.

To determine which mobile ARGs were together with MGEs in a specific region and to what extent they were associated with the MGEs, we searched the 5-kb flanking regions of each mobile ARG for adjacent MGEs as integrase / recombinase and /or transposase. There was a tendency for an increased frequency of co-occurrence of MGEs and ARGs, with day 0 having 0.53% relative abundance of the total ARGs to 1.48% at day 14 in the guts. While there was no obvious change and the relative abundance of mobile resistant units maintained a high degree around 7.6% in all sediment samples, which may be due to the complicated environmental factors in sediments (Additional file1: Table S3). The main mobile resistant genes in the guts and sediments included clinically relevant genes which conferred resistance to antibiotic classes such as tetracyclines, sulphonamides, β-lactams, and aminoglycosides (Additional file1: Fig. S4a), which were widely disseminated genes. In addition, the most abundant ARGs forming mobile resistant units were sul2, floR and aadA (18.7%, 17.4% and 19% of mobile resistant units, respectively) in the guts while the domain mobile ARGs were aadA, tetA and sul2 (30.3%, 12%, 8.6%) in sediments. All these ARGs frequently coexisted with transposase and phage intergrase genes in the guts and sediments (Additional file1: Fig. S4a).

Similarly, there was a marked increase in the abundance of plasmids that carried the ARGs in the guts and sediments because of the antibiotic exposure (Fig. 3b). Besides, the percentage of ARGs in the plasmids increased from 2.78 to 9.41% in total ARGs in the guts and around 11.7-14.4% in the sediments (Additional file1: Table S3). The aadA, tetA, tetE and sul2 genes in the plasmids were both present in the gut and sediment samples and there were MGEs in the neighborhood which demonstrated that these genes had the highest dissemination potential (Additional file1: Fig. S4). The association showed that the widespread MGEs were present in the neighboring resistance genes and were closely associated with emergence and spread of ARGs among bacteria, especially the transposase.

**Mobile genetic context predicts dissemination potential of ARGs**

Bacteria in the gut and sediment samples shared several ARGs such as sul1, sul2, tetA and tetR, flanking with the same MGEs (sul1 with phage integrase, sul2, tetA, tetR with transposase) (Additional file1: Fig. S4a), indicating a possible existence of a flow of genetic elements between the guts and sediments making the ARGs widely diffuse in the presence of the antibiotic. Two different contigs with the same sequence of 4,053 bps composed of ARGs group (tetA, tetR, MFS) and a IS91 transposase gene were
found, and were classified as a plasmid sequence (identity=99.796%, evalue ≈ 0, bitscore = 5,272) with a chromosome sequence (identity=100%, evalue ≈ 0, bitscore=5303) of the species *Pseudomonas aeruginosa* in the sediment samples (Fig. 4a). We speculated that *tetR* and *tetA* genes were most likely to undergo intracellular transfer by transposon _IS91-mediated recombination between the plasmid and the chromosome in *Pseudomonas aeruginosa* in the sediment samples following the antibiotic pressure.

Furthermore, there was a contig with a sequence of 4127 bp harboring the *tetR* and *tetA* gene group and a nearby transposon of Tn3 family, had been classified as plasmid sequence of *Salmonella enterica* in the guts (identity=100%, evalue ≈ 0, bitscore=5303), and a different contig with the same sequence was observed in the sediments (identity=100%, evalue ≈ 0, bitscore=5303) (Fig. 4b). The transposon Tn3 has been shown to commonly carry antimicrobial passenger genes, recruit mobile integrons, and promote the exchange of genes [21]. This evidence proved that the *tetR* and *tetA* gene group had high intercellular transfer potential of *Salmonella enterica* through plasmids between the guts and sediments, which was a high prevalence species of antimicrobial resistance [22]. In addition, *sul2* gene, defined as a highly disseminated gene in bacteria based on 42 genera that contained it, was reported to exist in small non-conjugative plasmids or large transmissible plasmids [23]. In our study, the *sul2* gene was present in a gene fragment composed of two transposons of *Tnp IS 1595* and a *Tnp_1* in *Flavobacterium sp* plasmids _I3-2_ in guts (identity=99.95%, evalue ≈ 0, bitscore=3529). Similarly, the transposon of Tnp_IS 1595 with *sul2* gene existed in the chromosomes of *Myroides odoratimimus* (identity=99.49%, evalue ≈ 0, bitscore=1039) which also belong to the *Flavobacteriaceae* in the sediments, showing that the *sul2* gene may be transferred among *Flavobacteriaceae* between the guts and sediments since the *Tnp_IS 1595* transposon could insert itself into a new genome by the activity of its transposase (Fig. 4b). *Flavobacteriaceae* is a potential ancestral source of the tigecycline resistance gene *tet (X)* [24]. In addition, the relative abundance of all the contigs with mobile resistant units had an increase in the gut and sediment bacteria following the antibiotic administration (Additional file 4: Fig. S5). All of the contigs mentioned above are listed (Additional file 3: Table S4). These results showed that the MGEs could be used to predict potential future transfer of neighboring ARGs and induce dissemination of ARGs between the guts and sediments through HGT under antibiotic pressure.

**Correlation between the ARGs and bacterial taxa**

Correlation analysis revealed possible hosts of target genes in complex environmental scenarios if the target genes and the coexisting microbial taxon had significantly positive correlations. A comparison of the correlation analysis between the core ARGs and the top 20 relative abundance of bacteria in the family level showed that there was a stronger and dense correlation in the guts of crayfishes than in the sediments (Fig. 6). *Enterobacteriaceae* family was highly correlated with 17 ARGs (r>0.8, p<0.05), followed by *porphyromonadaceae* (which was highly correlated with 16 ARGs) and *Vibrionaceae* (15 ARGs) in the guts (Fig. 6a). The family with the highest correlation with 11 ARGs was *Sphingomonadaceae* (r=0.8, p<0.05) in the sediments (Fig. 6b). *Enterobacteriaceae* was the only family that had relatively high correlation with ARGs both in the guts and sediments (Fig. 6). Meanwhile, the total ARGs in the guts had a strong positive correlation with *Citrobacter freundii* (R²=0.7248) and
*Citrobacter braakii* (R²=0.7102) (Additional file 1: Fig. S6a). Furthermore, the *Citrobacter* maintained a relatively high proportion through the whole experiment. Our data also demonstrated that the ARGs were positively correlated with *Escherichia coli* (R²=0.7905) and *Salmonella enterica* (R²=0.7974) (Additional file 1: Fig. S6b). The species had been recognized as significant pathogens in patients with underlying diseases and belong to *Enterobacteriaceae* family.

To analyze potential host for the mobile resistant units, metagenomic sequences of bacteria in the guts and sediments were used to predict co-occurrence of ARGs with bacteria patterns. The network inference modeling demonstrated that the exchanges of the mobile resistant units were mainly detected in 31 genera of *Proteobacteria, Firmicutes* and *Bacteroidetes*, and the exchanges were more active in *Proteobacteria*. Importantly, our data showed that the majority of these highly disseminated genes such as *sul1, sul2* and *tetA* and improved flanking with MGEs, were strongly associated with more than 5 genera of *Proteobacteria* (Fig. 6c). The main host ranges at genus levels such as *Klebsiella, Salmonella, Escherichia* and *Aeromonas* belong to *Enterobacteriaceae* family, *Proteobacteria* phylum (Fig. 6c). *Klebsiella*, an invasive and resistant zoonotic pathogenic genus, had the most of mobile ARGs such as *aadA, aph(3')-I, floR, qnrS, sul1* and *tetA* [25], followed by *Escherichia* and *Salmonella*. These mobile ARGs appeared to be stably transferrable between genus of *Enterobacteriaceae* in their respective hosts. Previous data reported that *Enterobacteriaceae* were adapted to sharing genetic material and much important resistance due to mobile resistance genes [26]. Furthermore, recent demonstration of interphylum gene transfer further supported this phenomenon [27]. However, our study showed that the genes exchange network was only between different classes (Fig. 6c).

**Metagenomic reconstruction and distribution of ARGs in MAGs**

Reconstruction of bacterial genomes from metagenomic sequence provided a snapshot of taxonomic distribution of the ARGs among members of the gut and sediment microbial communities. In our study, a total of 96 genomic bins were obtained from all the samples and enabled a genome-based investigation of ARGs distribution. The average size of the bins was 2.07 Mb while the average length of N50 was 32,152 bp. As shown in Fig. 6, there was taxonomic identification of bins with completeness of > 70% and contamination of < 5%. Out of the identified bins, 37.5% were assigned to *Firmicutes*, 15.6% to *Proteobacteria* while 20.3% bins were identified as *Bacteroidetes*. Similarly, 65.7% of the metagenomic bins were identified at the genus or species level, while the remaining bins did not match to genome-sequenced reference strains (Additional file 4: Table S5). All the bins showed differences in the abundance in different days. 61 bins showed a higher abundance at the 18th day while 18 new bins were comparable to day 0 of antibiotic treatment.

Most core ARGs with high relative abundance conferring multidrug, β-lactamase, MLS and tetracycline were detected in the metagenomics bins in limited members of the bacterial phylogeny. These bins with ARGs were all assigned to *Proteobacteria* (4 bins) and *Firmicutes* (2 bins), with a dramatic enrichment following the antibiotic treatment while restoring to baseline at day 42 (Fig. 6a). In addition, the bins carrying ARGs such as *Citrobacter braakii, Morganella morganii_A and Hafnia paralvei* belonging to
Enterobacteriaceae, and Aeromonas veronii belonging to Aeromonadaceae were all zoonotic pathogens of Proteobacteria phylum and were associated with a higher rate of in-hospital mortality (Fig. 6a). Similarly, there was emergence of multiple antibiotics resistant bacteria (MRB) of Citrobacter braakii (7 ARGs), Morganella morganii_A (2 ARGs) and Anaerolineae bacterium CG2 (2 ARGs). The Citrobacter braakii accounted for 16% in all bins of the gut, and carried multidrug resistance genes (acrB, mdtC, tolC, emrB and emrA), MLS resistance genes (macB) and bata-lactam resistance genes (bla\textsubscript{CMY-100}, bla\textsubscript{CMY-74}), which mostly belonged to core ARGs (Fig. 6b). Our study showed that genomic bins of sediment7_2r.29 belonging to Anaerolineae bacterium CG.30 family and gut28_1.57 belonging to Clostridium genus were new ARBs, which suggested that these resistance genes may also be present in the hosts that we had not identified. In addition, some core ARGs such as ampC, acrB, bla\textsubscript{CMY-100}, bla\textsubscript{CMY-74} and emrB on bins showed an obvious high abundance at day 28, albeit temporarily (Fig. 6b), Therefore, the application of enrofloxacine did not only promote resistance to different antibiotics but also potentially enriched multiple antibiotics resistance pathogenic bacteria, thus contributing to the changes in community composition. The proliferating of these multiple resistant pathogenic bacteria played a crucial role in augmenting various ARGs under antibiotic pressure.

**Discussion**

**The change of microbial diversity correlated with increased resistance of bacteria**

We found that excess ARGs carried by Enterobacteriaceae partially explains the expansion of this group in crayfish gut microbiome following antibiotic exposures. Moreover, ARGs were highly enriched in Enterobacteriaceae in human gut microbiota. Enrichment of mobile ARGs in Enterobacteriaceae such as Citrobacter, klebsiella, Escherichia and Salmonella in the gut can be partially interpreted by the fact that many zoonotic clinical pathogens are Enterobacteriaceae, which are presumably exposed to more antibiotic selection pressures due to clinical treatments. These findings suggest that Enterobacteriaceae constitute a major ARG pool, potentially even a mobile ARG pool in naturally connected different environments, regardless of the origin of their ARGs.

Multiple antibiotic resistant bacteria (MAB) and other new antibiotic resistant bacterium (ARB) have emerged with a high relative abundance as a result of interactions between evolutionary units involved in antibiotic resistance[21]. Proliferations of these multiple resistant pathogenic bacteria promotes the spread of various ARGs under antibiotic pressures. Comparative genomic characterization of antimicrobial profiles and genomic characteristics of some MABs have been recently investigated[28]. Equally important, the loss of microbial diversity correlated with increased resistance of bacteria to antibiotics applied in certain environments[29]. Therefore, the need for implementing strategies to restore bacterial diversity after antibiotic exposures in environments may result in low resistance rates in future[29].

**The potential mobility of antibiotic resistance genes**
MGEs such as integrase / recombinase and/or transposase play important roles in acquiring and disseminating ARGs in any environment in which bacteria are in close contact, for example, in the guts of animals and humans or waste-water treatment plants [30]. Studies have assessed MGE cassettes from microbial communities to find novel mobile resistance genes since new ARGs often occur first by transfers to MGEs [31]. By assessing the neighboring genes of ARGs, we found that ARGs tend to be clustered together with MGEs, including conjugative plasmids, integrons and transposons, which form mobile resistant units as a result of increased antibiotic pressures. Of all the transferable ARGs in guts and sediments, they frequently coexisted with transposases, consistent with the finding that transposases are the most prevalent mobile genes in nature and are the major drivers for the spread of resistance [32].

The tetracycline and sulfonamide resistance genes such as tetA, tetR, sul1, and sul2, portions of core ARGs both in gut and sediments bacterial, were tend to be flanking transposase [31]. In our study, the tetA-tetR gene, flanked by Tn_IS91 and Tn3, was found on plasmids and chromosomes at the same time. IS91 is a prototype element of a family of bacterial insertion sequences that transposes by a rolling-circle mechanism. It is involved in dissemination and evolution of virulence and pathogenicity gene types. The tet(X4) gene, flanked by the IS91 transposase gene family on the chromosome in Shewanella species, has recently been reported [33]. Moreover, the archetype of the Tn3 family is among the earliest unit transposons to be identified in Gram-negative bacteria. The Tn3 transposon was exclusively detected during antibiotic inputs flanking tetracycline, beta-lactamase, quinolones, and florenicol resistance genes in low abundance. The tetA-tetR gene transfer can occur intracellularly via transposase-mediated recombination. Tetracycline and sulfonamide resistance genes were the most prevalent ARGs in aquatic animal [34] and aquaculture environments [35] as the large-scale applications in treating common bacterial infections. A high concentration of these resistance genes in environments may be due to their mobile character in part.

**Aquaculture are reservoirs of mobile resistance genes**

The high-level relative abundance of ARGs remained in the guts until 14 days after cessation of antibiotic exposures, indicating that ARGs could exist for a long time and could not be easily removed in aquaculture. In our study, several ARGs were also detected in the guts and sediments at day 0. And a previous study reported that tetracycline-resistance genes were found in sediments from aquaculture farms in which antibiotics were rarely applied [36] indicating that guts and sediments in aquaculture are reservoirs of mobile resistance genes and facilitate their mobilization in the environment.

Transmissible plasmids promote horizontal resistance gene transfers in the presence of antibiotics. Since many different ARGs circulate on plasmids, it was proposed that inhibiting plasmid transfers is a potential strategy for reducing antibiotic resistance rates [37]. In this study, when the bacterial genome database was searched for mobile resistant units, mobile ARGs were found to be in abundance on plasmids. For instance, the mobile tetA-tetR gene group in the Tn3 transposon was found on plasmids in the gut and sediment samples of *Salmonella enterica*. In addition, quinolone resistance qnrS gene was
found to be associated with plasmids, consistent with the plasmid-mediated spread mechanisms of *qnrS*. Thus, the reservoir for ARGs in crayfish guts has the potential for increasing during antibiotic exposures and later be mobilized to sediments under plasmid facilitation.

Since the aquatic environment can serve both as a natural reservoir and as a conduit for the spread of antibiotic resistance genes, antimicrobial overuse in aquaculture is associated with health implications given the potential for the transfer of ARGs from environmental bacteria to human pathogens [38]. It is vital to set up measures for public awareness and guidelines to prevent the spread of ARGs and to improve antibiotic resistance surveillance

**Conclusion**

Antibiotic-mediated alterations in bacterial structure as well as the decrease in microbial diversity correlates with increased antibiotic resistance in bacteria. The microbiome and ARG determinants in the gut were strongly affected, relative to those in sediments and induced a more markedly delayed recovery after antibiotic administration. Alterations of bacterial community composition due to emergence and proliferation of new antibiotic resistance bacteria (ARM) and multiple antibiotic resistance bacteria (MRB) enhanced the dispersion of various ARGs. In addition, MGEs facilitate the diffusion of ARGs through horizontal gene transfer, mainly in the gut and sediment environments of the *Enterobacteriaceae* family. The MGEs-mediated mobile resistant units showed an extraordinary ability of spreading in the *Enterobacteriaceae* family, and some MRBs of *Enterobacteriaceae* have emerged. Therefore, the *Enterobacteriaceae* probably constitutes a mobile ARG pool in nature, which is connected to different environments.

**Methods**

**Experimental setting**

The crayfish ponds investigated in this study were located in MianZhu, China (31°35′N, 104°20′E). Three ponds were approximately uniform in size (100 m$^2$) and depth (1 m). We had analyzed and adjusted the water parameters before the experiments to make sure that the rearing conditions were similar across the ponds. The congeneric larval crayfishes were inoculated with a stocking density of about 2,000 individuals into each pond on April 10th, 2019. The three ponds were identically managed, including water inputs, daily water exchange rate (~5%), feed type, and schedule. All animals were fed with a commercial non-medicated feed (Sichuan Runge Biotechnology Co., Ltd.) for 45 days, twice per day at 9 a.m. and 5 pm. After the adaptation phase, the crayfishes were fed for a period of 14 days with medicated feed (10-15 mg enrofloxacin hydrochloride per kg and day). After the treatment phase, all the crayfishes were fed again with non-medicated feed for 28 days (Fig. 1a). As expected, since all the crayfishes were adults, the effect of antibiotic treatment on the weight of the crayfishes was not significant ($p=0.7710$) (Additional file 1: Fig. S1a). Residue of enrofloxacin was found in muscle of the crayfishes fed with the medicated feed. It was 1703, 726, 150 and 41 ppb at day 14, 18, 21 and 28, respectively. After day 28, the antibiotic
concentration was under detection limit (Fig. S1b). The gut contents of three individuals from each pond and sediments samples of day 0 (pre-antibiotic phase); day 7 and 14 (antibiotics phase); day 18, 21, 28, 35 and 42 (post-antibiotic phase) were collected (Fig. 1a). The gut samples were collected at 4 h after the crayfish feeding in the morning. crayfish dissection and sampling were performed as described previously [39]. The sediment samples (200 g for each sample) were collected using a grab sampler from five sites from each pond, and then mixed well. All samples were stored at -80°C until genomic DNA was extracted and subjected to for 16S rRNA and metagenomics sequencing. Genomic DNA was extracted using the Fast DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions.

16S rRNA gene amplicon sequencing and analysis

The barcoded primer pair (forward: CCTAYGGGRBGCASCAG, reverse: GGACTACNNGGGTATCTAAT) was used to amplify the V3-V4 region of the 16S rDNA. The obtained amplicons were sequenced on the Illumina Hiseq 2500 platform, and paired-end reads with lengths of 250 bp were generated. Paired-end reads were merged using FLASH VI.2.7 and then imported to the tool QIIME 2 [40] to perform data filtration and chimera removal. Then the data were denoised with plugin deblur and the taxonomic classification of OTUs was performed using q2-feature-classier, which was trained for the used primers using the 99% OUT data set of the SILVA Release 138. Chao 1 and phylogenetic diversity indices were estimated from OTU tables after normalization to 7,584 reads per sample, which was the lowest number of reads per sample. All computational analyses were performed using the QIIME pipeline. The dissimilarity of the taxonomical clusters were analyzed using the Bray-Curtis dissimilarity measure and represented in a non-metric multidimensional scaling (NMDS) plot.

Metagenome assembly, annotation, binning

Metagenome sequencing was performed through Illumina Hiseq 2500 platform to generate pair-end reads with a length of 150 bp. The raw reads were analyzed for quality using FastQC [41]. Adapter removal and quality filtering were conducted using Trimmomatic [42]. Sequences with an average quality score below 30 or shorter than 50bp were discarded to got clean reads. And then the clean reads were mapped to the genome sequence of Procambarus virginalis to filter out the animal host genome sequences. MetaPhlAn2 [43] was used to classify the metagenomic reads to taxonomies and yield relative abundance of taxa identified in the samples.

The tool metaSPADES v 3.10 [44] was used for metagenome assembly. Each assembly was evaluated by Quast v5.0.2 [45], and only contigs larger than 200bp used in further analyses. Protein-coding genes were predicted using prodigal v2.6.3 [46] using the “meta” mode. Functional annotation was performed using emapper-2.1.5 [8] based on the EggNOG v5.0 database.

To calculated the depth of contigs, the clean reads from each sample were aligned against the contigs by BWA-MEM. The generated sam file were sorted by samtools and the duplicates were marked and removed by Picard. Then the bam file were used for metagenomic binning which performed using MetaBat2 [47]. A total of 1,318 MAGs were generated, and then genome dereplication was performed using dRep [48] with
the following parameters: -comp 75 -con 10. There were 96 MAGs were remained and followed taxonomic classification using gtdbtk-1.3.0 [49] classify workflow to be assigned to taxonomies and aligned based on a set of marker genes from GTDB-database. Phylogenetic analysis of the 96 MAGs were inferred by construction of a maximum-likelihood tree based on the alignments using the iqtree 2.0.3 [50] and visualized using the iTOL [51] webtool.

**Antibiotics resistance genes and mobile genetic elements analysis**

The ARGs were identified by Blastp against the SARG database [52] with E value ≤1e-10 \(^{-7}\) with a minimum identity of 95% over 40% query coverage. MGEs were predicted using HMMER v3.3.2 [15] and Blastp [53] through the Isfinder 2020-9 and integron v1.2 databases respectively. Hits with a maximum 1x 10-5 evalue a. Plasmidverify [54] was used to analyze the possibility of contig originating from plasmids. The MGEs were grouped into three groups as phage integron, IS, and plasmid. Position and co-occurrence of the ARGs and MGEs were analyzed using local shell scripts. Co-occurrence was considered positive if an ARG was found with a MGE in ten ORFs from upstream of downstream a mobile genetic element gene.

To determine the classification and identification of contig carrying resistance genes, the NCBI-nt database through Blastn (v2.12.0+) was used to define the species of the contigs. We used Blastn parameter with "-evalue 1e^{-5}\) and manually selected a coverage >40%. A hit with the highest score was used as the possible species classification. Network planning of the ARGs and their hosts were visualized by Cytoscape 3.8.2[55] using an edge-weighted curved layout.

**Data quantification and standardization**

To calculate the relative abundance of each genome, samtools [19] was used to sort the sam files and coverm was used to aligne and calculate the reads counts of each genome. The reads counts were normalized using the reads per kilobase mapped (RPKM) method to account for potential read count biases caused by differences in contig length or total library size. The relative abundance of ARGs and MGEs were calculated similar as MAGs.

**Statistical analyses and visualization**

Correlation between ARG density and other sample parameters were evaluated using Person’s and Spearman’s correlation tests. Both r values greater than 0.8 and p values less than 0.05 were used to show extremely significant correlation, while r values greater than 0.6 and p values less than 0.05 were defined as significant correlation. Correlations between the relative abundance of the ARGs and taxa in the metagenomic data were visualized by heatmap R package. The significance of differences in the compositions of ARGs, OTUs, in each of the gut and sediment sample categories was tested using analysis of similarities (ANOSIM) in the vegan R package.

**Abbreviations**
ARG: Antibiotic resistance gene

MGE: Mobile gene elements

MAB: Multiple antibiotic resistant bacteria

ARB: antibiotic resistant bacterium

Declarations

Ethical approval and consent to participate

All experiments involving animals were performed in accordance with Animal Management and Ethics Committee, Huazhong Agriculture University and following Ethical Review Body approval.

Ethical approval ID Number: HZAUSHR-2019-001.

Consent for publication

Not applicable.

Availability of data and materials

The raw RNA sequencing and Metagenome sequencing read datasets generated in this study are available in NCBI Read Archive (SRA) repository with SRA Bioproject number PRJNA818367 (https://dataview.ncbi.nlm.nih.gov/object/ PRJNA818367). Analyzed data are provided in the supplementary material.

Conflict of interest

The authors declare no competing interests.

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Authors’ contributions

Xuexia Jin participated in the experiment, wrote the main manuscript text and prepared figures; Sizhen Liu participated in biological analysis; Tong Liu and Zhenting Zhang participated in the preparation of experimental materials. Yunxiang liang, Jinshui Zheng and Nan Peng participated in experimental technical guidance and manuscript revision. All authors reviewed the manuscript.

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Figures
The perturbations of the microbiota compositions in the gut of crayfish and sediments as the application of antibiotic. a Experimental design. Three ponds of crayfish were supplemented with antibiotics for two weeks. After the antibiotic feeding period, the crayfish were fed for 4 weeks. Gut and sediment samples of days 0 (Pre-antibiotic phase), 7, 14 (Antibiotic phase), and 18, 21, 28, 35, 42 (Post-antibiotic phase) were subjected to 16sRNA gene and metagenomic sequencing. For all groups, feeding was performed twice a
day at 9 a.m. and at 5 p.m. β-α-diversity indices (including richness and dominance) for crayfish gut and sediment microbiota. c Non-metric Multi-Dimensional Scaling ordination (NMDS) of the gut and sediment microbiota showing the segregation in microbial structure between different phases using Bray-Curtis. Each dot represents one sample. d Bar plot for relative abundance of dominant bacteria at the family level of crayfish gut and sediment samples over time. e Changes in relative abundances of the Proteobacteria phylum and Enterobacteriaceae family in gut and sediment samples to antibiotic exposures.

Figure 2

Enrichment of ARGs coding for different antibiotic classes before, during and after enrofloxacin exposure in the gut of crayfish and sediment microorganisms. Changes in relative abundance of total ARGs (a) and quinolone resistance genes (b) in the gut and sediment microorganisms. List (c) and relative abundance (d) of core ARGs in the gut and sediment microorganisms.

Day 0 (Pre-antibiotic exposure period), 7, 14 (Antibiotic exposure period), 18, 21, 28, 35 and 42 (Post-antibiotic exposure period).
Figure 3

Effects of antibiotics on relative abundance of total MGEs before, during and after exposures and the correlations between total ARGs and MGEs from the gut of crayfish and sediment microorganisms. 

a Relative abundance of total ARGs and MGEs in the gut and sediment microbes. 

b Relative abundance of Plasmid, Intergon and Insertion groups. 

c Correlation between relative abundances of total ARGs and the MGE groups.
Day 0 (Pre-antibiotic exposure period), 7, 14 (Antibiotic exposure period), 18, 21, 28, 35 and 42 (Post-antibiotic exposure period).

Figure 4

**Predicted transfer of co-MGEs-ARGs between gut and sediment bacteria.**

a Intracellular mobility model of the transposon of *tetA-tetR* gene between plasmid and chromosome in sediment bacteria. A cell of an antibiotic resistance *Pseudomonas aeruginosa* species is presented (envelope and chromosome shown in blue; contains one plasmid).

b Intercellular mobility model of transposons of *tetA-tetR* and *sul2* genes between gut and sediment bacteria. Two cells of gut and sediment species are presented (including chromosome and plasmid).

Gene segments, including ARGs (violet), neighboring contexts information of ARGs (blue) and ARG-associated mobile genetic element (orange) are shown and the spacing distance between them noted. Black arrows indicate transfer processes, with those mediated by a transposase protein labeled Tnp.
Correlations between ARGs and microbiome in the gut of crayfish and sediment. Correlation of relative abundance analysis between core ARGs and microbiome (top 20) in the bacterial community of crayfish gut (a) and sediment (b). $r \geq 0.05$, * $0.6 \leq r < 0.8$; ** $0.8 \leq r < 1$. c Network analysis revealed co-occurrence patterns among ARGs co-occurring with MGEs and their potential hosts in crayfish guts and sediments. The thickness of each connection line (edge) between two nodes represents significant and strong pairwise correlations (identity>99, coverage >40, top three target). The nodes were colored by a modularity class. Size of the ARGs node was proportional to relative abundance while size of species node was proportional to the number of connections (degree).
Figure 6

**Phylogenetic distributions of ARGs based on bacterial taxa carrying ARGs.** a Bacterial taxa were identified based on reconstructed genomes assigned to 360 bins with $\geq 70\%$ completeness and $< 5\%$ contamination. Phylogenetic tree and taxonomic assignments of reconstructed bins are shown as the innermost layer. Taxonomic assignment was based on the average amino acid identity of encoded proteins to the most closely related reference genome sequence. Branches and labels with different colors represent different phyla, as indicated by the color code to the lower left. The second layer shows the samples from guts and sediments. The third layer depicts the relative abundance of bins, inside to outside 0, 7, 14, 18, 21, 28, 35 and 42 d. Relative abundance of bins in each sample was calculated from the average contig coverage obtained by re-mapping reads from samples and normalizing to the total reads in the sample as shown in the third layer. The heatmap in the most outside layer depicted the abundances of ARGs across bacterial families based on taxonomic classification of bins from the samples taken from gut and sediment. b The relative abundance profiles of ARGs in chromosomes of the selected bacterial taxa and the relative abundance of total genes (marked as 1–7 in Fig. 6a).

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.
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