Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jes

JES JOURNAL OF ENVIRONMENTAL SCIENCES

www.jesc.ac.cn

Metagenomic insights into microorganisms and antibiotic resistance genes of waste antibiotic fermentation residues along production, storage and treatment processes

Ziming Han[1,4], *Xiao Luan*[1], *Haodi Feng*[1], *Yanqin Deng*[3], *Min Yang*[2,4], *Yu Zhang*[1,2,4,*]

[1] *State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China*
[2] *National Engineering Research Center of Industrial Wastewater Detoxication and Resource Recovery, Beijing 100085, China*
[3] *Wuhan Agricultural Inspection Center, Wuhan 430016, China*
[4] *University of Chinese Academy of Sciences, Beijing 100049, China*

ARTICLE INFO

Article history:
Received 16 August 2022
Revised 24 October 2022
Accepted 24 October 2022
Available online 10 November 2022

Keywords:
Pharmaceutical manufacturing
Antibiotic mycelia residue
Antibiotic resistance bacteria
Thermal treatment
Solid waste

ABSTRACT

Antibiotic fermentation residue (AFR) is nutrient-rich solid waste generated from fermentative antibiotic production process. It is demonstrated that AFR contains high-concentration of remaining antibiotics, and thus may promote antibiotic resistance development in receiving environment or feeding farmed animals. However, the dominate microorganisms and antibiotic resistance genes (ARGs) in AFRs have not been adequately explored, hampering understanding on the potential antibiotic resistance risk development caused by AFRs. Herein, seven kinds of representative AFRs along their production, storage, and treatment processes were collected, and multiple methods including amplicon sequencing, metagenomic sequencing, and bioinformatic approaches were adopted to explore the biological characteristics of AFRs. As expected, antibiotic fermentation producer was found as the predominant species in raw AFRs, which were collected at the outlet of fermentation tanks. However, except for producer species, more environment-derived species persisted in stored AFRs, which were temporarily stored at a semi-open space. *Lactobacillus* genus, classified as Firmicutes phylum and Bacilli class, became predominant bacterial taxa in stored AFRs, which might attribute to its tolerance to high concentration of antibiotics. Results from metagenomic sequencing together with assembly and binning approaches showed that these newly-colonizing species (e.g., *Lactobacillus* genus) tended to carry ARGs conferring resistance to the remaining antibiotic. However, after thermal treatment, remaining antibiotic could be efficiently removed from AFRs, and microorganisms together with DNA could be strongly destroyed. In sum, the main risk from the AFRs was the remaining antibiotic,

* Corresponding author.
E-mail: zhangyu@rcees.ac.cn (Y. Zhang).

https://doi.org/10.1016/j.jes.2022.10.035

while environment-derived bacteria which tolerate extreme environment, survived in ARFs with high content antibiotics, and may carry ARGs. Thus, hydrothermal or other harmless treatment technologies are recommended to remove antibiotic content and inactivate bacteria before recycling of AFRs in pharmaceutical industry.

Introduction

The bulk antibiotics are mainly produced by bacteria or fungi by industrial fermentative process, resulting in a large number of discharges of antibiotic fermentation production wastewater and waste antibiotic fermentation residues (AFRs, also called as antibiotic mycelial residues). Pharmaceutical manufacturing effluents especially the production wastewater have been reported as one of the key sources of antibiotic contamination in the environment (Berendonk et al., 2015; He et al., 2021; Larsson, 2014). At the same time, AFRs consisting microbial biomass (i.e., mycelial cells of antibiotic producer species) and fermentation substrate, also contain high concentration of remaining antibiotics (Bewick, 1979). As the by-product routinely generated during the antibiotic production, 8-10 tons of wet AFR would be produced for every ton of antibiotic produced (Li et al., 2012). AFRs contained high content of organic matter and showed potential to use as organic fertilizer and soil amendment. However, high-concentration of residual antibiotic in AFR might promote antibiotic resistance development in receiving environment without the harmless treatment.

Bloom of antibiotic resistance genes (ARGs) in soils receiving raw AFRs was blamed for antibiotic input to soils, because antibiotic resistant bacteria in soils could get competitive advantage under antibiotic selective pressure caused by AFR soil application (Cai et al., 2019). It has been reported that tetracycline resistance genes and macrolide resistance genes were enriched in soils amended by oxytetracycline fermentation residue (Awad et al., 2022) and erythromycin fermentation residue (Zhang et al., 2020), respectively. With the aim of removing antibiotic from AFRs, hydrothermal treatment (Cai et al., 2020) has the advantage of relatively low cost and ease of indusial-scale study (Tang et al., 2020), and has been adopted to the harmless of erythromycin fermentation residue in industrial-scale (Han et al., 2022).

Except for remaining antibiotic, the antibiotic producer cells also composed AFRs. Natural antibiotic-producing organisms (bacteria or fungi) were isolated and cultivated for antibiotic industrial production. The producer organism is a kind of non-pathogenetic single species persisted in fermentation tank of pharmaceutical factory (Pernodet et al., 1993), and the living cells might be partially destroyed during the extraction step of antibiotic production. From this view, the environmental risk of antibiotic producer species in AFRs was negligible. Recently, a few studies attempted to detect ARGs in AFRs collected from storage sites and obtained positive results, resulting in the rethinking on genes and genetic contexts favored in AFRs along production and storage processes. For instance, *ermF, ermB, ermX, mefA* et al. were detected in erythromycin fermentation residue (Cai et al., 2020; Shen et al., 2019), diverse *tet* genes were detected in oxytetracycline fermentation residue (Liu et al., 2012), *ermB, ermF, ermT, ermX, msrD* were detected in spiramycin fermentation residue (Liu et al., 2014). However, the information on the origin (i.e., bacterial hosts) and dissemination risk of these ARGs in AFR is still not available. This hampered the risk assessment of AFRs.

Thus, to comprehensively understand the biological characterization and antibiotic resistance risk of AFRs, following questions should be clearly addressed: (1) What kinds of microorganisms or antibiotic resistance genes are present and dominated in AFRs? (2) What's the variation of microorganism and antibiotic resistance gene in AFRs during production, storage and treatment processes? (3) How to block the dissemination of ARGs during harmless treatment and resource utilization AFRs? To fill these gaps, biological characteristics including possible microorganism and antibiotic resistance gene-carrying bacteria in seven kinds of representative AFRs along their production, storage, and treatment processes were investigated. Amplicon sequencing and metagenomic-based assembly and binning methods could obtain taxonomy information of AFRs, reconstruct microbial genomes and exhibit the carried ARGs in each genome (Rice et al., 2020). These culture-independent methods and bioinformatic analyses have made it possible to explore the microbial composition and assess the antibiotic resistance risks of AFRs.

In this study, seven kinds of representative AFRs were collected along production, storage and thermal treatment periods from pharmaceutical companies which produced antibiotics by biological fermentation. The 16S rRNA gene amplicon sequencing and ITS2 gene amplicon sequencing were adopted for profiling bacterial and fungal composition of AFRs, respectively. Dominant bacterial species together with carried ARGs in AFRs were obtained using metagenomic sequencing and systematic bioinformatic analyses. This study tried to clarify the debate on antibiotic resistant bacteria in AFRs, and highlighted the thermal inactivation of microorganisms in AFRs benefited the mitigation on environmental antibiotic resistance dissemination.

1. Material and methods

1.1. *Collection of seven representative antibiotic fermentation residues (AFRs)*

Seven representative AFRs were collected at pharmaceutical companies (Table 1, Appendix A Table S1), and sample types consisted of raw, stored and treated AFRs (Fig. 1). The raw AFRs were collected at the outlet of fermentation tanks, and represented the original characteristics as much as possible. If the AFRs were temporarily stored at a semi-open space in the

Table 1 – Collected antibiotic fermentation residue (AFR) samples in this study.

AFRs	Antibiotic types	Sample types	Sequencing types
Paromomycin	Aminoglycoside	Raw residue	16S, meta
Oxytetracycline	Tetracycline	Raw residue	16S, meta
Spiramycin	Macrolide	Raw residue	16S, meta
Vancomycin	Vancomycin	Raw residue	- [d]
Penicillin	β-lactam	Raw, stored, treated residues [a]	16S, ITS2, meta
Cephalosporin	β-lactam	Treated residue [b]	-
Erythromycin	Macrolide	Stored, treated residues [c]	16S, meta

[a] Penicillin fermentation residue was treated by thermal drying under 100 - 110°C with a disc dryer for about 5 sec (the entire heating and cooling process lasted for at least 20 min).
[b] Cephalosporin fermentation residue was hydrothermally treated under 110 - 150°C for over 15 min.
[c] Erythromycin fermentation residue was hydrothermally treated under 130 - 170°C for over 15 min.
[d] No sequencing data.16S: 16S rRNA gene amplicon sequencing; ITS2: ITS2 gene amplicon sequencing; meta: metagenomic sequencing.

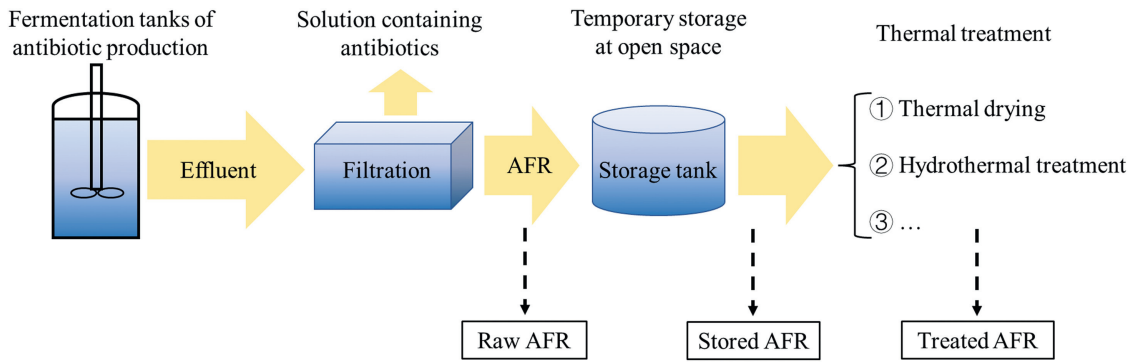

Fig. 1 – The production, storage and treatment processes of antibiotic fermentation residues (AFRs). Raw AFR was collected just after fermentation and filtration processes. Stored AFR was collected from the storage tank at semi-open space. The AFR treated by thermal approaches was viewed as treated AFR.

pharmaceutical facility, they were viewed as stored AFRs. If the AFRs were treated by a certain high-temperature technician for further utilization, they were viewed as treated AFRs.

Five antibiotics including paromomycin (classified as aminoglycoside), oxytetracycline (classified as tetracycline), spiramycin (classified as macrolide), vancomycin (classified as vancomycin) and erythromycin (classified as macrolide) are produced by bacterial fermentation. The raw paromomycin, oxytetracycline, spiramycin, and vancomycin fermentation residues, were collected at the pharmaceutical companies in Wuxi city, Shijiazhuang city, Wuxi city and Shijiazhuang city in China, respectively. Erythromycin fermentation residue was collected at a pharmaceutical company in Xinjiang Uygur Autonomous Region, China, and the sample types included stored and treated residues. The stored erythromycin fermentation residue was then hydrothermally treated under 130 - 170°C for over 15 min.

Two antibiotics including penicillin and cephalosporin are produced by fungal fermentation. Penicillin fermentation residue was collected at pharmaceutical companies in Xinjiang Uygur Autonomous Region, and it was temporarily stored in the facility for further treatment with a disc dryer at 100 - 110°C for about 5 sec, and the entire heating and cooling process lasted for at least 20 min. Thus, samples of penicillin fermentation residue consisted of three types including raw, stored and treated residues. Cephalosporin fermentation residue was collected at the same pharmaceutical company in Xinjiang Uygur Autonomous Region, and the sample type only included treated residue. It was hydrothermally treated under 110 - 150°C for over 15 min. More details on AFRs can be found in Appendix A.

1.2. *DNA extraction and amplicon sequencing of AFRs*

DNA extraction was performed by FastDNA SPIN Kit (MP Biomedicals, USA) according to the manufacturer's instructions. DNA concentration was measured by NanoDropOne (ThermoFisher, USA) and Qubit (ThermoFisher, USA). The V4-V5 region of the bacterial 16S rRNA gene was amplified using PCR with primers 515F (GTGCCAGCMGCCGCGGTAA) and 907R (CCGTCAATTCMTTTRAGTTT) (Fouhy et al., 2016). The fungal ITS2 gene was amplified using PCR with forward primer (GCATCGATGAAGAACGCAGC) and reversed primer (TCCTCCGCTTATTGATATGC) (Huang et al., 2020; White et al., 1990). Bacterial 16S rRNA gene amplicon sequencing was performed for all AFRs, while fungal ITS2 gene amplicon sequencing was performed for penicillin fermentation residue because its producer species was fungus. Amplicon sequencing was conducted on an Illumina NovaSeq PE250 platform at Guangdong Magigene Biotechnology Co. Ltd., China. The sequencing data were deposited in the NCBI BioProject database with accession number PRJNA868603 and PRJNA868588 for bacterial commu-

Table 2 – Amplicon sequencing of raw AFRs.

AFRs	Classification of producer species	Target gene of amplicon sequencing	Dominant genus and relative abundance
Paromomycin	Bacterium	16S rRNA gene	*Streptomyces* (77.64% ± 0.90%)
Oxytetracycline	Bacterium	16S rRNA gene	*Streptomyces* (95.86% ± 0.29%)
Spiramycin	Bacterium	16S rRNA gene	*Streptomyces* (88.34% ± 1.29%)
Vancomycin	Bacterium	16S rRNA gene	PCR failed
Penicillin	Fungus	ITS2 gene	*Penicillium* (98.83% ± 0.14%)

nity and fungal community, respectively. More details on amplicon sequencing can be found in Appendix A.

1.3. *Metagenomic sequencing of AFRs*

Metagenomic sequencing was performed on an Illumina X-ten platform with a paired-end 2×150 base read lengths at Guangdong Magigene Biotechnology Co. Ltd., China. Over 12 Gb of raw reads were obtained for each sample and were deposited in the NCBI BioProject database with accession number PRJNA868900. Trimmomatic software was used for quality control of raw sequencing data with parameters as follows: remove adapters (ILLUMINACLIP:TruSeq3-PE.fa:2:30:10), remove leading low quality or N bases (below quality 3) (LEADING:3), remove trailing low quality or N bases (below quality 3) (TRAILING:3), scan the read with a 5-base wide sliding window, cutting when the average quality per base drops below 20 (SLIDINGWINDOW:5:20), and drop reads below the 50 bases long (MINLEN:50) (Bolger et al., 2014; Yan et al., 2013), and FastUniq software was used to remove duplication (Xu et al., 2012). After quality control, the clean data was used for following analyses.

1.4. *Bioinformatic analyses*

The filtered reads of samples were pooled using "cat" command and then were assembled together by MEGAHIT with default k-mer (Li et al., 2015). Binning of assembled metagenomic sequencing was performed by metaWRAP pipeline (Uritskiy et al., 2018) with MaxBin2 for bin files (Wu et al., 2015) and Quant_bins module for abundance of each bin in each sample. Bins are reconstructed microbial genomes obtained from metagenomic sequencing reads, to obtain the dominant bacterial taxa together with their functional genes. Results and reads mapping rates of metagenomic assembly was shown in Appendix A Table S2. Considering the high mapping rates (>96% in raw AFRs and >70% in stored AFRs on average) (Appendix A Table S2), genetic contexts obtained from assembling and binning were representative. High-quality metagenome-assembled bacterial genome bins (genome quality = completeness – 5×contamination > 50%) (Parks et al., 2017) were chosen for taxonomy assignments via the GTDB-Tk software (Chaumeil et al., 2020) and for antibiotic resistance gene annotation by Resistance Gene Identifier (RGI) software of CARD (Alcock et al., 2019). The cut-off of "perfect" and "strict" algorithms of RGI were used, which have been widely used in previous studies (Gupta et al., 2020; Yu and Zhao, 2019). In RGI's algorithms, "perfect" means an 100% match to a CARD reference sequence, while "strict" includes ARGs with mutations or previously unknown variants (Jia et al., 2017). ARG annotation of this study was based on metagenome-assembled bacterial genome bins. The functional genes in bins were annotated via RAST (Aziz et al., 2008; Overbeek et al., 2014) and Prokka software (Seemann, 2014).

2. Results and discussion

2.1. *Microorganisms and ARGs of raw AFRs*

To explore the microbial composition and ARGs of raw AFRs, paromomycin, oxytetracycline, spiramycin, vancomycin and penicillin fermentation residues were collected at the outlet of fermentation tanks in pharmaceutical companies (Fig. 1, Table 1). Within them, the fermentation producer of penicillin is fungus, while others are bacteria (Table 2).

Streptomyces is known as one of the most common antibiotic-producing bacteria for the production of many antibiotics (Demain, 1974). As expected, *Streptomyces* was the most dominant bacterial genus in the collected paromomycin (relative abundance of 77.64% ± 0.90%), oxytetracycline (95.86% ± 0.29%) and spiramycin (88.34% ± 1.29%) fermentation residues according to the results of 16S rRNA gene amplicon sequencing. While the results of ITS2 gene amplicon sequencing showed that *Penicillium* (98.83% ± 0.14%) was the dominant fungal genus in penicillin fermentation residue (Table 2, Appendix A Table S3). In addition, for vancomycin fermentation residue, the PCR of 16S rRNA gene was failed. Possible reason was that the mycelial cells were destroyed due to the high pH during separation process of vancomycin from its fermentation residue (Zhao et al., 2016). It should be noted that although the raw residues were collected just at the outlet of fermentation tanks to avoid pollution as much as possible, the relative abundance of producer species was still less than 100%. The possible pollution might come from sampling stage and PCR.

To investigate the most dominant taxonomy and carried ARGs in raw AFRs, the dominant bin of each type of AFRs, which is defined as metagenome-assembled bacterial genome with highest abundance, was obtained by metagenomic sequencing together with assembly and binning approaches (Fig. 2, Appendix A Tables S4 and S5). The dominant bin obtained from paromomycin fermentation residue was classified as *Streptomyces chrestomyceticus*, which carried *AAC(3)-VIIa* and *ANT(4′)-Ib* conferring resistance to aminoglycoside. The dominant bin obtained from oxytetracycline fermentation residue was classified as *Streptomyces rimosus*, which car-

Fig. 2 – Metagenomic sequencing of raw AFRs. The dominant metagenomic-assembled bacterial genome of each type of AFRs, and the taxonomy and carried antibiotic resistance genes of these genomes were obtained.

ried *otr*(B) and *otr*(A) conferring resistance to tetracycline. The dominant bin obtained from spiramycin fermentation residue was classified as *Streptomyces ambofaciens*, which carried *ermZ*, *gim*A, *Erm*O and *srm*B conferring resistance to macrolide. Both binning results and 16S rRNA gene amplicon sequencing results showed *Streptomyces* was the most abundant bacterial taxon in paromomycin, oxytetracycline and spiramycin fermentation residues. Binning results provided more detailed taxonomic information (to species level) of these producer species than amplicon sequencing, and also revealed specific ARGs together with their surrounding genes. Only a few bins could be obtained from AFRs (Appendix A Table S2), a possible explanation was that the bacterial community composition of AFR was very simple.

Streptomyces-carried ARGs obtained in this study by metagenomic assembly and binning were also supported by previous studies (Demain, 1974). Bacterial producer of paromomycin appeared to possess aminoglycoside-modifying enzyme acetyltransferase (AAC) activity to protect itself against paromomycin (Cundliffe, 1989). Ribosomal modification-based oxytetracycline-resistance genes, *otr*(A) and *otr*(B), were determined in oxytetracycline-producing organism *Streptomyces rimosus* (Doyle et al., 1991; McMurry and Levy, 1998). The *ermZ*, encoding a methyltransferase modifying 23S rRNA conferring resistance to spiramycin (Karray et al., 2007); *gim*A, encoding a macrolide glycosyltransferase (Gourmelen et al., 1998); *erm*O (also called as *srm*A), encoding a macrolide methyltransferase (Pernodet et al., 1999); and *srm*B, encoding an ATP-dependent transport protein and conferring resistance to spiramycin (Pernodet et al., 1993; Schoner et al., 1992), were found in the spiramycin producer *Streptomyces ambofaciens*, and were responsible for its self-resistance to spi-

Table 3 – Amplicon sequencing of stored AFRs.

AFRs	Classification of producer species	Target gene of amplicon sequencing	Dominant genus and relative abundance
Penicillin	Fungus	ITS2 gene	*Penicillium* (36.10% ± 17.58%), *Kazachstania* (2.42% ± 1.05%), *Yarrowia* (2.95% ± 3.43%)
Penicillin	Fungus	16S rRNA gene	*Lactobacillus* (92.46% ± 3.99%), *Ralstonia* (1.55% ± 1.04%), *Aeromonas* (0.41% ± 0.34%)
Erythromycin	Bacterium	16S rRNA gene	*Lactobacillus* (73.27% ± 7.47%), *Proteus* (7.17% ± 6.25%), *Solobacterium* (3.53% ± 0.99%)

Penicillium genus was classified as Fungi (kingdom), Ascomycota (phylum), Eurotiomycetes (class), Eurotiales (order), and Aspergillaceae (family); *Lactobacillus* genus was classified as Bacteria (kingdom), Firmicutes (phylum), Bacilli (class), Lactobacillales (order), and Lactobacillaceae (family). More detailed classification information could be found in Appendix A Table S6.

ramycin. In addition, *Penicillium chrysogenum* was one of the common fungal producers of penicillin (Demain, 1974), which was in line with our amplicon sequencing results of penicillin fermentation residue.

In raw AFRs, a portion of antibiotic-producing cells might be still alive, and whether these cells poses environmental antibiotic resistance risk needs to be clarified. The antibiotic-producing bacteria need self-protective mechanisms to avoid suicide (Hopwood, 2007), and some ARGs existing in pathogens might link to the ARGs from antibiotic producers according to bioinformatic deduction (Jiang et al., 2017). However, these antibiotic producers themselves were just environmental microorganism and could be commonly found in soil, water and air samples; the link of ARGs between clinic pathogens and antibiotic producers was a long-term evolutional perspective. The intrinsic antibiotic resistance of producer species and the acquired/mobile antibiotic resistance of pathogenetic bacteria were highly different (Cox and Wright, 2013). Mobile genetic elements played key roles in the horizontal transfer of ARGs (von Wintersdorff et al., 2016). In the investigated raw AFRs, no mobile genetic element was adjacent to ARGs in genomes of antibiotic producers (Fig. 2), indicating the rapid horizontal gene transfer from antibiotic producers to environmental taxa (especially pathogenetic bacteria) tended to be very low probability. Thus, it was improper to overestimate the antibiotic resistance risks of antibiotic producers.

2.2. *Microorganisms and ARGs of stored AFRs*

In pharmaceutical factories, wet AFRs were often temporarily stored at a semi-open space, which allowed the contact between AFRs and surrounding environment. In this study, stored penicillin and erythromycin fermentation residues were collected (Table 1). According to results of ITS2 gene amplicon sequencing, relative abundance of *Penicillium*, the penicillin producer, was 36.10% ± 17.58% in stored penicillin fermentation residue (Table 3), which was lower than that in raw penicillin fermentation residue (98.83% ± 0.14%) (Table 2). The 16S rRNA gene amplicon sequencing of raw penicillin fermentation residue was failed, indicating the bacterial species in it was too low to detectable. In contrast, 16S rRNA gene amplicon sequencing of stored penicillin fermentation residue showed bacteria persisted in it, and *Lactobacillus*, assigning to Firmicutes at phylum level, Bacilli at class level, was predominant (Table 3, Appendix A Table S6). As for stored erythromycin fermentation residue, by 16S rRNA gene amplicon sequencing, the predominant bacteria were also classified as *Lactobacillus*, instead of the producer *Streptomyces* (Table 3, Appendix A Table S6).

To obtain more complete genomic context from stored residues, metagenomic sequencing together with assembly and binning approaches was utilized. From metagenomic data of stored penicillin fermentation residue, a high-quality bin (i.e., metagenome-assembled genomes) were identified and annotated to *Firmicutes* at phylum level, Bacilli at class level, and Lactobacillaceae at family level (Fig. 3). From metagenomic data of stored erythromycin fermentation residue, two high-quality bins, assigning to Firmicutes at phylum level, Bacilli at class level, and Lactobacillales at order level, were identified (Fig. 3). The bacterial classification results of 16S rRNA gene amplicon sequencing and metagenomic binning were similar (Table 3, Fig. 3). ARGs were annotated in these bins (Fig. 3, Appendix A Tables S7, S8). For bin.20 of stored penicillin fermentation residue, *VCC-1* conferring resistance to β-lactams, was obtained. Two bins of stored erythromycin fermentation residue carried genes conferring resistance to macrolide. In bin.2 (classified as *Paucilactobacillus vaccinostercus*) and bin.24 (classified as *Lactococcus lactis*), *efmA* (conferring resistance to macrolide) and *lmrC, lmrD* (conferring resistance to macrolide) were obtained, respectively. Microbial communities of AFRs were especial because of the simple microbial composition and high biomass content. Thus, in this study, ARG abundance in AFRs was calculated by relative abundance of ARG-carrying metagenome-assembled bacterial genome with the unit of "genome copies per million reads". In raw AFRs, relative abundance of antibiotic-producing species was over 4,000 copies per million reads (Appendix A Table S4), while in stored AFRs, relative abundance of environment-derived species was no more than 100 copies per million reads on average (Appendix A Table S7). In comparison, relative abundance of per ARG-carrying metagenome-assembled bacterial genome in erythromycin-polluted soils (Han et al., 2022) and low-anthropogenic-polluted Hadal Trench (Yang et al., 2021) was no more than 10 copies per million reads on average. This indicated the inactivation of antibiotic resistant bacteria in AFRS was essential.

As stored residues were open at surrounding environment, some environment-derived species would colonize and persist in stored residue. These environment-derived species fa-

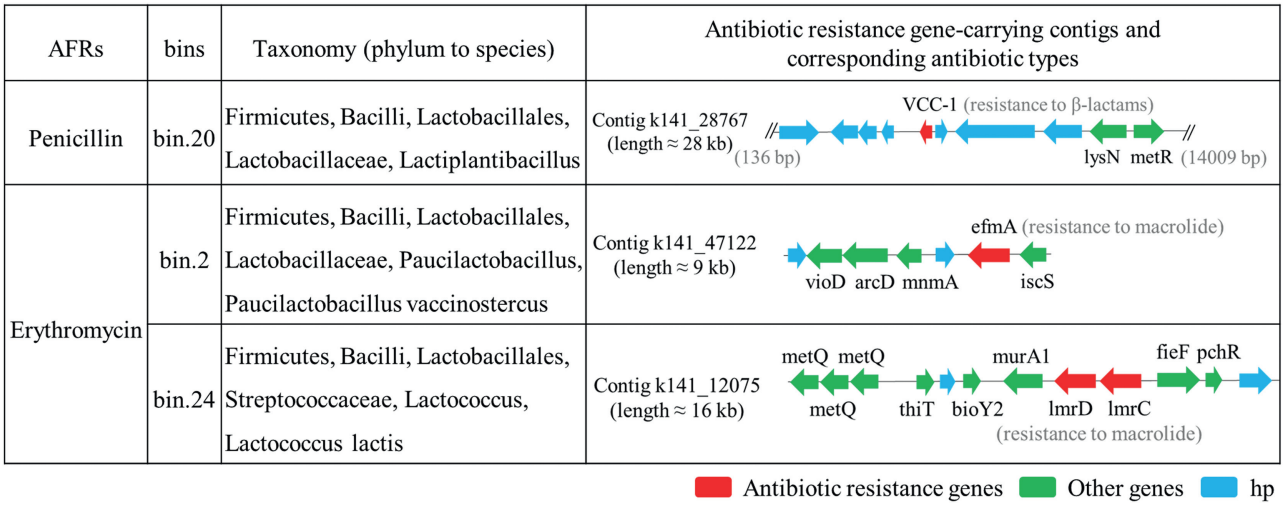

Fig. 3 – Metagenomic sequencing of stored AFRs. The ARG-carrying metagenomic-assembled bacterial genomes (bins) of each AFR sample.

vored such wet and nutrient-rich fermentation substrate and could survive under high concentration of antibiotic. Both binning results and 16S rRNA gene amplicon sequencing results showed environment-derived species of stored penicillin and erythromycin fermentation residues were annotated to Firmicutes at phylum level, Bacilli at class level. It has been reported that class Bacilli was also the dominant bacteria except for producer *Streptomyces* in oxytetracycline fermentation residue, via clone libraries of 16S rRNA gene (Liu et al., 2012). Species of Bacilli class and Lactobacillaceae family, or evolutionary similar species, had fermentation function (Green et al., 2021). Bins from stored penicillin and erythromycin fermentation residues carried β-lactams and macrolide resistance genes, respectively. Both of the antibiotic resistance ability and fermentation function might be responsible for their colonization in AFRs. Similar to environmental media polluted by antibiotics (Han et al., 2020; Zhang et al., 2015), antibiotics in stored residues selected the antibiotic resistant bacteria coming from surrounding environment, promoting antibiotic resistance development. Thus, the stored residues became the potential spot for accumulation, proliferation and dissemination of environment-derived antibiotic resistant bacteria. Effective treatment need to be conducted to inactivate these newly-colonizing, antibiotic resistance species in stored residues.

2.3. *Characteristics of thermal treated AFRs*

Thermal treatment was one of the common technics of AFRs aiming to remove remaining antibiotic for further safety resource utilization (Cai et al., 2020; Tang et al., 2020). In this study, treated penicillin, cephalosporin and erythromycin fermentation residues were collected, and they were treated by thermal drying at 100 - 110°C for about 5 sec, hydrothermal treatment at 110 - 150°C for over 15 min, and hydrothermal treatment at 130 - 170°C for over 15 min, respectively (Table 1). After thermal treatment, the microorganisms and DNA in residues were strongly destroyed, and the PCR attempt for 16S rRNA gene amplicon sequencing was failed (Appendix A Fig. S1). At the same time, the remaining antibiotic was effectively removed. Erythromycin concentrations was (1659 ± 202) mg/(kg TS) in erythromycin fermentation residue before thermal treatment, and reduced to (320 ± 26) mg/(kg TS) in treated erythromycin fermentation residue; penicillin G concentration was (1750 ± 113) mg/(kg TS) in penicillin fermentation residue before thermal treatment, and reduced to non-detectable level (detection limit = 1 μg/(kg TS)) in treated penicillin fermentation residue. More details on antibiotic concentrations in AFRs could be found in Appendix A Text S1. Thus, two aspects of impacts were achieved by thermal treatment of AFRs: (1) removing remaining antibiotics, and (2) inactivating microorganisms together with DNA.

In treated AFRs, the antibiotic and moisture were effectively removed by thermal treatment, and the DNA content from both antibiotic producer and environment-derived species reduced to non-detectable level. Utilization (e.g., soil application as organic fertilizer) of treated AFRs could reduce the addition of bacteria or genetic material present in raw or stored AFRs as much as possible, which assisted in blocking the antibiotic resistance dissemination during soil application of AFRs.

2.4. *Rethinking on antibiotic resistance risk of AFRs*

High antibiotic content was the major concern of AFRs' resource utilization because of public health threat caused by antibiotic pollution (Medlicott et al., 2020). In this study, we attempted to disentangle the AFRs' risks caused by antibiotic content, antibiotic producer species, and environment-derived bacteria persisting in AFRs, to answer the debate that "whether AFR soil application transfer ARGs". It was proved that the remaining antibiotic was the primary threat from AFRs, and the possible proliferation of antibiotic resistant bacteria in stored AFRs and environmental media receiving AFRs attributed to the select pressure of antibiotic.

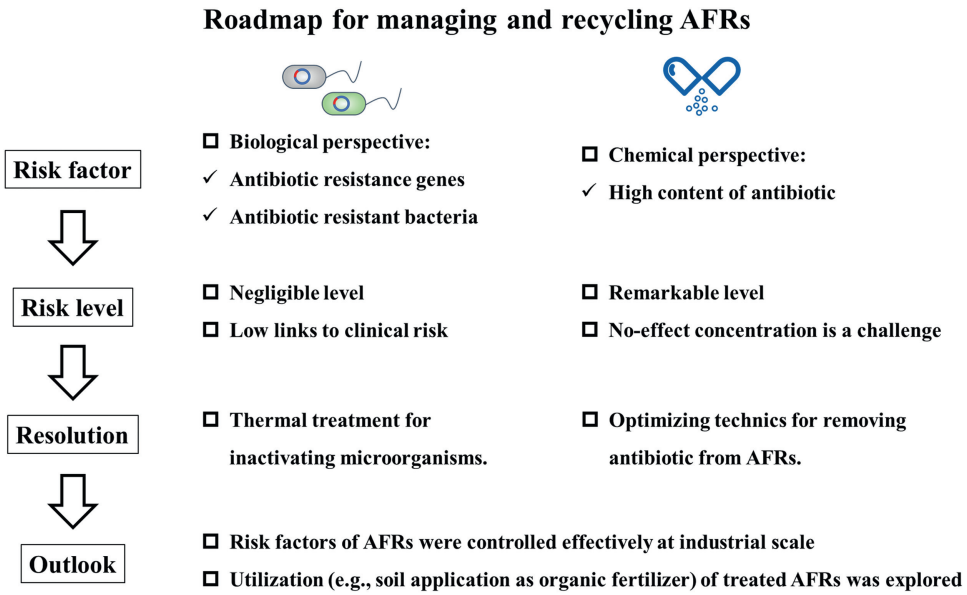

Fig. 4 – Roadmap for managing and recycling AFRs.

From this view, the fundamental risk factor of AFRs was high antibiotic content, and issues on ARGs and antibiotic resistant bacteria were its secondary consequence. ARGs enriched in stored AFRs because of their high content of antibiotics. Similarly, ARGs were enriched in soils receiving AFRs because antibiotics were introduced to soil via AFRs (Awad et al., 2022; Cai et al., 2019; Wang et al., 2018). Thus, effective treatment such as thermal treatment was suggested before usage or disposal of AFRs, because it could decrease antibiotic content and destroy microorganisms in AFRs. It should be noted that, in this study, a systematic metagenomic-based strategy was adopted for characterizing raw, stored and treated AFRs, and more validation by culture-based approach was needed in the future.

Understanding toward risks of antibiotics in environment media required two concepts. On the one hand, selective pressure of antibiotics promoted the risk for antibiotic resistance transmission, especially the horizontal gene transfer between non-pathogenetic bacteria to pathogen (Martinez et al., 2015). The mobile, clinical-relevant ARGs in environmental bacterial community meant higher health risks (Oh et al., 2018; Zhang, 2021). On the other hand, long-term persistent antibiotic pressure could become the driver of evolution of undiscovered forms of resistance, which eventually may pose additional challenges in the clinics (Bengtsson-Palme and Larsson, 2015). Environment media acted as a huge "reservoir" of antibiotic resistance determinants, and the environmental origin of many clinical-related ARGs have been confirmed (Larsson and Flach, 2021). Thus, risk of antibiotic resistance evolution could not be ignored under the topic of soil antibiotic contamination caused by the use of AFR without the harmless treatment.

2.5. *Roadmap for managing and recycling AFRs*

China has become one of biggest producer of bulk antibiotics (Ying et al., 2017), and thus bore heavy burden for safety disposal of AFRs. AFRs have been listed in China's National Catalogue of Hazardous Wastes since 2008, and thus the utilization as organic fertilizer or feed additive outside the pharmaceutical facility has been forbidden. Only some very high-cost methods, including landfilling and incineration, could meet the criterion for the disposal of Hazardous Wastes in China (Zhong et al., 2014). Plenty of economical and efficient approaches have been attempted for safety disposal of AFRs (Cai et al., 2020; Han et al., 2022; Shen et al., 2019; Zhang et al., 2020), however, the remaining antibiotic in AFRs could be decreased to what extent meant "safe" is still unclear.

Here, we attempted to propose some demands for future management of AFR recycling (Fig. 4). Firstly, risks of AFRs should be assessed by both chemical (remaining antibiotic content) and biological (antibiotic resistant bacteria) perspective, and ideal harmless treatment approach could inactivate the microorganisms in AFRs. Secondly, the reasonable safety threshold of remaining antibiotic content in treated AFRs is still a challenge. Predicted no effect concentration (PNEC) in aquatic media (Bengtsson-Palme and Larsson, 2016) is useful information, and long-term impacts of sub-inhibitory antibiotics on soil microbiome need systematic in-situ assessment (Andersson and Hughes, 2014; Han et al., 2022). Thirdly, development and optimization of effective technics in removing antibiotic from AFRs are still a key issue, and potential impact of antibiotic byproducts and transformation products also needed to be evaluated (Fan and He, 2011; Kamarei et al., 2014; Li et al., 2008).

3. Conclusion

This study performed a systematic exploration on microorganisms and ARGs in raw, stored and treated antibiotic fermentation residues (AFRs). Antibiotic producers were predominant in raw AFRs, while environment-derived antibiotic resistant bacteria could colonize and persist in stored residue.

The main risk factor of AFRs was the high antibiotic content, and thermal treatment was suggested to reduce remaining antibiotic and inactivate genetic contents in AFRs. These results clarified the debate on occurrence of ARGs in AFRs, and appealed the fundamental research on environmental impact of sub-inhibitory antibiotic and technology research on antibiotic removal for environmental friendliness and sustainability in the utilization of AFRs.

Declaration of Competing Interest

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

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 32141002). We thank Mr. Yunpeng Shen, Dr. Jing Zhang, and Ms. Xiumei Zhao from the State Environmental Protection Engineering Center for Harmless Treatment and Resource Utilization of Antibiotic Residues, Beijing CDC, and North China Pharmaceutical Co. Ltd., respectively, for their help in sampling and analysis of AFRs.

Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2022.10.035.

REFERENCES

Alcock, B.P., Raphenya, A.R., Lau, T.T.Y., Tsang, K.K., Bouchard, M., Edalatmand, A., et al., 2019. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res. 48 (D1), D517–D525.

Andersson, D.I., Hughes, D., 2014. Microbiological effects of sublethal levels of antibiotics. Nat. Rev. Microbiol. 12 (7), 465–478.

Awad, M., Tian, Z., Han, Z., Luan, X., Zhang, Y., Yang, M., et al., 2022. Application of the hydrothermally treated oxytetracycline fermentation residue in agriculture: concentrations of antibiotic and resistance genes in soil and plant. J. Soil. Sediment. 22 (4), 1095–1104.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., et al., 2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics 9, 75.

Bengtsson-Palme, J., Larsson, D.G.J., 2015. Antibiotic resistance genes in the environment: prioritizing risks. Nat. Rev. Microbiol. 13 (6) 396-396.

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ. Int. 86, 140–149.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., et al., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13 (5), 310–317.

Bewick, M.W.M., 1979. Use of antibiotic fermentation wastes as fertilizers for tomatoes. J. Agric. Sci. 92, 669–674.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30 (15), 2114–2120.

Cai, C., Hua, Y., Li, H., Li, L., Dai, L., Liu, H., et al., 2020. Hydrothermal treatment of erythromycin fermentation residue: Harmless performance and bioresource properties. Resour. Conserv. Recy. 161, 104952.

Cai, C., Liu, H., Dai, X., Whalen, J.K., 2019. Multiple selection of resistance genes in arable soil amended with cephalosporin fermentation residue. Soil Biol. Biochem. 136, 107538.

Chaumeil, P.A., Mussig, A.J., Hugenholtz, P., Parks, D.H., 2020. GTDB-Tk: A toolkit to classify genomes with the genome taxonomy database. Bioinformatics 36 (6), 1925–1927.

Cox, G., Wright, G.D., 2013. Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. Int. J. Med. Microbiol. 303 (6-7), 287–292.

Cundliffe, E., 1989. How antibiotic-producing organisms avoid suicide. Annu. Rev. Microbiol. 43, 207–233.

Demain, A.L., 1974. How do antibiotic-producing microorganisms avoid suicide. Ann. N. Y. Acad. Sci. 235, 601–612.

Doyle, D., McDowall, K.J., Butler, M.J., Hunter, I.S., 1991. Characterization of an oxytetracycline-resistance gene, otrA, of *Streptomyces rimosus*. Mol. Microbiol. 5 (12), 2923–2933.

Fan, C., He, J., 2011. Proliferation of antibiotic resistance genes in microbial consortia of sequencing batch reactors (SBRs) upon exposure to trace erythromycin or erythromycin-H_2O. Water Res. 45 (10), 3098–3106.

Fouhy, F., Clooney, A.G., Stanton, C., Claesson, M.J., Cotter, P.D., 2016. 16S rRNA gene sequencing of mock microbial populations-impact of DNA extraction method, primer choice and sequencing platform. BMC Microbiol. 16 (1), 123.

Gourmelen, A., Blondelet-Rouault, M.H., Pernodet, J.L., 1998. Characterization of a glycosyl transferase inactivating macrolides, encoded by gimA from *Streptomyces ambofaciens*. Antimicrob. Agents Chemother. 42 (10), 2612–2619.

Green, I.R., Oberg, C.J., Broadbent, J.R., Thunell, R.K., McMahon, D.J., 2021. Galactose-positive adjunct cultures prevent gas formation by *Paucilactobacillus wasatchensis* WDC04 in a model gas production test. J. Dairy Sci. 104 (10), 10540–10549.

Gupta, C.L., Tiwari, R.K., Cytryn, E., 2020. Platforms for elucidating antibiotic resistance in single genomes and complex metagenomes. Environ. Int. 138, 105667.

Han, Z., Feng, H., Luan, X., Shen, Y., Ren, L., Deng, L., et al., 2022. Three-year consecutive field application of erythromycin fermentation residue following hydrothermal treatment: cumulative effect on soil antibiotic resistance genes. Engineering 15 (8), 78–88.

Han, Z., Zhang, Y., An, W., Lu, J., Hu, J., Yang, M., 2020. Antibiotic resistomes in drinking water sources across a large geographical scale: Multiple drivers and co-occurrence with opportunistic bacterial pathogens. Water Res. 183, 116088.

He, Y., Tian, Z., Luan, X., Han, Z., Zhang, Y., Yang, M., 2021. Recovery of biological wastewater treatment system inhibited by oxytetracycline: rebound of functional bacterial population and the impact of adsorbed oxytetracycline on antibiotic resistance. Chem. Eng. J. 418, 129364.

Hopwood, D.A., 2007. How do antibiotic-producing bacteria ensure their self-resistance before antibiotic biosynthesis incapacitates them? Mol. Microbiol. 63 (4), 937–940.

Huang, G., Liao, J., Han, Z., Li, J., Zhu, L., Lyu, G., et al., 2020. Interaction between fungal communities, soil properties, and the survival of invading *E. coli* O157:H7 in soils. Int. J. Environ. Res. Public Health 17 (10), 3516.

Jia, B., Raphenya, A.R., Alcock, B., Waglechner, N., Guo, P., Tsang, K.K., et al., 2017. CARD 2017: expansion and

model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res 45, D566–D573.
Jiang, X., Ellabaan, M.M.H., Charusanti, P., Munck, C., Blin, K., Tong, Y., et al., 2017. Dissemination of antibiotic resistance genes from antibiotic producers to pathogens. Nat. Commun. 8, 15784.
Kamarei, F., Attar, H., Nikjah, S., Goodarzi, M., 2014. Determination of erythromycins in fermentation broth using liquid phase extraction with back extraction combined with high performance liquid chromatography. Arabian J. Chem. 7 (3), 292–296.
Karray, F., Darbon, E., Oestreicher, N., Dominguez, H., Tuphile, K., Gagnat, J., et al., 2007. Organization of the biosynthetic gene cluster for the macrolide antibiotic spiramycin in Streptomyces ambofaciens. Microbiology 153 (Pt 12), 4111–4122.
Larsson, D.G.J., 2014. Pollution from drug manufacturing: review and perspectives. Philos. Trans. R. Soc. B 369 (1656), 20130571.
Larsson, D.G.J., Flach, C.-F., 2021. Antibiotic resistance in the environment. Nat. Rev. Microbiol. 20 (5), 257–269.
Li, D., Liu, C.-M., Luo, R., Sadakane, K., Lam, T.-W., 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics 31 (10), 1674–1676.
Li, D., Yang, M., Hu, J., Zhang, Y., Chang, H., Jin, F., 2008. Determination of penicillin G and its degradation products in a penicillin production wastewater treatment plant and the receiving river. Water Res. 42 (1–2), 307–317.
Li, Z., Tian, B., Zuo, J., Yu, X., Shen, H., Wang, Y., et al., 2012. Progress in treatment and disposal technology of antibiotic bacterial residues. Chin. J. Environ. Eng. 30 (2), 72–75.
Liu, M., Ding, R., Zhang, Y., Gao, Y., Tian, Z., Zhang, T., et al., 2014. Abundance and distribution of Macrolide-Lincosamide-Streptogramin resistance genes in an anaerobic-aerobic system treating spiramycin production wastewater. Water Res. 63, 33–41.
Liu, M., Zhang, Y., Yang, M., Tian, Z., Ren, L., Zhang, S., 2012. Abundance and distribution of tetracycline resistance genes and mobile elements in an oxytetracycline production wastewater treatment system. Environ. Sci. Technol. 46 (14), 7551–7557.
Martinez, J.L., Coque, T.M., Baquero, F., 2015. What is a resistance gene? Ranking risk in resistomes. Nat. Rev. Microbiol. 13 (2), 116–123.
McMurry, L.M., Levy, S.B., 1998. Revised sequence of OtrB (tet347) tetracycline efflux protein from *Streptomyces rimosus*. Antimicrob. Agents Chemother. 42 (11), 3050.
Medlicott, K., Gordon, B., Montgomery, M., Tayler, E., Sutherland, D., Schmoll, O., et al., 2020. Technical brief on water, sanitation, hygiene and wastewater management to prevent infections and reduce the spread of antimicrobial resistance. WHO/FAO/OIE Recommendation Report.
Oh, M., Pruden, A., Chen, C., Heath, L.S., Xia, K., Zhang, L., 2018. MetaCompare: a computational pipeline for prioritizing environmental resistome risk. FEMS Microbiol. Ecol. 94 (7), fiy079.
Overbeek, R., Olson, R., Pusch, G.D., Olsen, G.J., Davis, J.J., Disz, T., et al., 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res. 42, D206–D214.
Parks, D.H., Rinke, C., Chuvochina, M., Chaumeil, P.-A., Woodcroft, B.J., Evans, P.N., et al., 2017. Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life. Nat. Microbiol. 2 (11), 1533–1542.
Pernodet, J.-L., Alegre, M.-T., Blondelet-Rouault, M.-H., Guérineau, M., 1993. Resistance to spiramycin in *Streptomyces ambofaciens*, the producer organism, involves at least two different mechanisms. J. Gen. Microbiol. 139 (5), 1003–1011.
Pernodet, J.-L., Gourmelen, A., Blondelet-Rouault, M.-H., Cundliffe, E., 1999. Dispensable ribosomal resistance to spiramycin conferred by srmA in the spiramycin producer *Streptomyces ambofaciens*. Microbiology 145 (9), 2355–2364.
Rice, E.W., Wang, P., Smith, A.L., Stadler, L.B., 2020. Determining hosts of antibiotic resistance genes: A review of methodological advances. Environ. Sci. Technol. Lett. 7 (5), 282–291.
Schoner, B., Geistlich, M., Rosteck, P., Rao, R.N., Seno, E., Reynolds, P., et al., 1992. Sequence similarity between macrolide-resistance determinants and ATP-binding transport proteins. Gene 115 (1), 93–96.
Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30 (14), 2068–2069.
Shen, Y., Zhuan, R., Chu, L., Xiang, X., Sun, H., Wang, J., 2019. Inactivation of antibiotic resistance genes in antibiotic fermentation residues by ionizing radiation: Exploring the development of recycling economy in antibiotic pharmaceutical factory. Waste Manage. 84, 141–146.
Tang, M., Gu, Y., Wei, D., Tian, Z., Tian, Y., Yang, M., Zhang, Y., 2020. Enhanced hydrolysis of fermentative antibiotics in production wastewater: Hydrolysis potential prediction and engineering application. Chem. Eng. J. 391, 123626.
Uritskiy, G.V., DiRuggiero, J., Taylor, J., 2018. MetaWRAP—a flexible pipeline for genome-resolved metagenomic data analysis. Microbiome 6 (1), 158.
von Wintersdorff, C.J., Penders, J., van Niekerk, J.M., Mills, N.D., Majumder, S., van Alphen, L.B., et al., 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Front. Microbiol. 7, 173.
Wang, B., Li, G., Cai, C., Zhang, J., Liu, H., 2018. Assessing the safety of thermally processed penicillin mycelial dreg following the soil application: Organic matter's maturation and antibiotic resistance genes. Sci. Tot. Environ. 636, 1463–1469.
White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols. Academic Press, San Diego, pp. 315–322.
Wu, Y.-W., Simmons, B.A., Singer, S.W., 2015. MaxBin 2.0: an automated binning algorithm to recover genomes from multiple metagenomic datasets. Bioinformatics 32 (4), 605–607.
Xu, H., Luo, X., Qian, J., Pang, X., Song, J., Qian, G., et al., 2012. FastUniq: a fast de novo duplicates removal tool for paired short reads. PLoS One 7 (12), e52249.
Yan, L., Yang, M., Guo, H., Yang, L., Wu, J., Li, R., 2013. Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. Nat. Struct. Mol. Biol. 20 (9), 1131–1139.
Yang, H., Liu, R., Liu, H., Wang, C., Yin, X., Zhang, M., et al., 2021. Evidence for long-term anthropogenic pollution: The hadal trench as a depository and indicator for dissemination of antibiotic resistance genes. Environ. Sci. Technol. 55 (22), 15136–15148.
Ying, G.-G., He, L.-Y., Ying, A.J., Zhang, Q.-Q., Liu, Y.-S., Zhao, J.-L., 2017. China must reduce its antibiotic use. Environ. Sci. Technol. 51 (3), 1072–1073.
Yu, M., Zhao, Y., 2019. Comparative resistomic analyses of *Lysobacter* species with high intrinsic multidrug resistance. J. Global Antimicrob. Resist. 19, 320–327.
Zhang, A.N., 2021. An omics-based framework for assessing the health risk of antimicrobial resistance genes. Nat. Commun. 12, 4765.
Zhang, Q.-Q., Ying, G.-G., Pan, C.-G., Liu, Y.-S., Zhao, J.-L., 2015. Comprehensive evaluation of antibiotics emission and fate in the river basins of China: Source analysis, multimedia modeling, and linkage to bacterial resistance. Environ. Sci. Technol. 49 (11), 6772–6782.

Zhang, Y., Liu, H., Dai, X., Cai, C., Wang, J., Wang, M., et al., 2020. Impact of application of heat-activated persulfate oxidation treated erythromycin fermentation residue as a soil amendment: Soil chemical properties and antibiotic resistance. Sci. Tot. Environ. 736, 139668.

Zhao, S., Peng, W., Zhang, J., Xiong, F., 2016. Filtration of vancomycin by ceramic membrane. China Brewing 35 (2), 119–122.

Zhong, W., Li, Z., Yang, J., Liu, C., Tian, B., Wang, Y., et al., 2014. Effect of thermal–alkaline pretreatment on the anaerobic digestion of streptomycin bacterial residues for methane production. Bioresour. Technol. 151, 436–440.