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Short-Term Benzalkonium Chloride (C_{12}) Exposure Induced the Occurrence of Wide-Spectrum Antibiotic Resistance in Agricultural Soils

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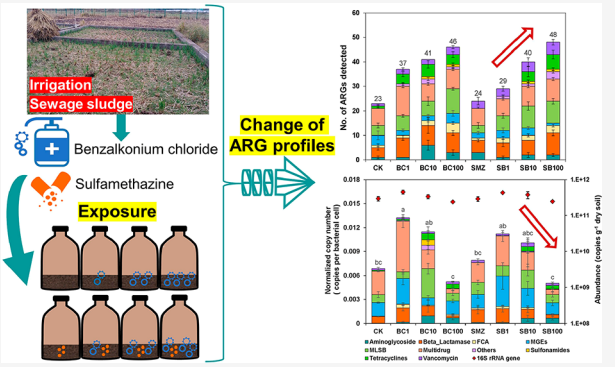
ABSTRACT: Antibiotic resistance genes (ARGs) are global pollutants that pose a potential risk to human health. Benzalkonium chloride (C_{12}) (BC) disinfectants are thought to exert selection pressure on antibiotic resistance. However, evidence of BC-induced changes in antibiotic resistance in the soil environment is lacking. Here, we established short-term soil microcosms to investigate ARG profile dynamics in agricultural soils amended with sulfamethazine (SMZ, 10 mg kg^{-1}) and gradient concentrations of BC (0–100 mg kg^{-1}), using high-throughput quantitative PCR and Illumina sequencing. With the increase in BC concentration, the number of ARGs detected in the soil increased, but the normalized ARG abundance decreased. The added SMZ had a limited impact on ARG profiles. Compared to broad-spectrum fungicidal BC, the specificity of SMZ significantly affected the microbial community. Network analysis found that low–medium BC exposure concentrations resulted in the formation of small but strong ARG co-occurrence clusters in the soil, while high BC exposure concentration led to a higher incidence of ARGs. Variation partitioning analysis suggested that BC stress was the major driver shaping the ARG profile. Overall, this study highlighted the emergence and spread of BC-induced ARGs, potentially leading to the antimicrobial resistance problem in agricultural soils.



KEYWORDS: *antibiotic resistance genes, benzalkonium chloride, sulfamethazine, coselection, agricultural soils*

1. INTRODUCTION

The high concentrations and high doses of disinfectants and antibiotics used during the COVID-19 pandemic seriously threaten human health.[1−3] Although it is generally accepted that antibiotic selection is the main pressure resulting in the enrichment of antibiotic resistance genes (ARGs),[4,5] recent studies have shown that nanoparticles,[6] herbicides,[7] microplastics,[8] and non-nutritive sweeteners[9] might also function additively or synergistically to promote antibiotic resistance. Notably, as antibiotics are restricted or even banned in animal production, more disinfectants are used to maintain adequate sanitation to prevent bacterial diseases.[10] Disinfectant usage is higher (even an order of magnitude higher) than antibiotics, especially during the COVID-19 pandemic.[3,11] Therefore, widely used disinfectants, such as quaternary ammonium compounds (QACs), have exacerbated the problem of antimicrobial resistance due to co- and cross-resistances.[12,13]

Furthermore, QACs and antibiotics have been reported to have several identical resistance mechanisms. There is a possibility that quaternary ammonium disinfectants may drive the coselection of ARGs.[13] Cross-resistance refers to QACs and antibiotics using one resistance mechanism of action, such as multidrug efflux pumps.[14,15] For example, the use of QAC disinfectants increases the expression of energy-driven NorA, MdeA, and ArcAB-TolC multidrug efflux systems, which can expel QACs and antibiotics from bacterial cells, consequently resulting in enhanced resistance to antibiotic agents.[16,17] Additionally, coresistance occurs when the genes encoding resistance to QACs and antibiotics are situated on the same mobile genetic element (MGE), such as a plasmid, transposon, or integron.[13] Class I integrons can carry a gene cassette that encodes resistance to different antibiotics in variable areas. QAC resistance genes (*qacE*△*1*) and sulfonamide resistance genes (*sul1*) often co-occur in the conserved region of class I integrons.[18] Thus, ARGs may develop horizontal gene transfer of QAC resistance genes, expanding the resistant flora,[19] especially under the background of compound pollution of

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sulfonamides and QACs. To date, studies based on freshwater microcosms,[20] membrane biofilm reactors,[21] and bacterial isolates[22] have shown that QAC exposure promotes microbial resistance to antibiotics. Nevertheless, few studies have focused on the changes in antibiotic resistance induced by QACs in the soil environment.

Medical, livestock, and poultry sewage sludge are contaminated with disinfectants and antibiotics, and as a consequence, wastewater irrigation and sludge application result in the release of these compounds into the soil.[23−25] An 88-year-long study found that the concentration of quaternary alkylammonium disinfectants in soils rose exponentially and accumulated from 2.3 $\mu g\ kg^{-1}$ to 131.2 $\mu g\ kg^{-1}$ after long-term wastewater irrigation.[26] Moreover, QACs can accumulate on the surface and probably also in the interlayers of clay minerals in soils, increasing their persistence and causing the prolonged exposure of soil microbes to subinhibitory concentrations of QACs.[27,28] Consequently, from the perspective of human health and environmental stability, it is important to explore the influence of antibiotic and QAC induction on the selection and spread of antibiotic resistance in the soil environment.

This study was designed to explore the coselection effects of short-term exposure to sulfamethazine (SMZ) and different concentrations of benzalkonium chloride (C_{12}) (BC, a widely used class of QACs)[29] on the agricultural soil resistome through a soil microcosm experiment. As a typical sulfonamide antibiotic, sulfamethazine (SMZ) was detected with higher frequency and concentrations than other sulfonamide drugs in soil fertilized with manure.[30,31] High-throughput quantitative PCR (HT-qPCR) and bacterial Illumina sequencing were used to characterize the response of the ARG profile and microbiome to BC and SMZ stress. In this study, we tested the following two hypotheses: (1) BC exposure can enhance resistance levels with increasing BC concentrations, and (2) SMZ and BC synergistically promote the coselection of soil ARGs. This work helps to elucidate the enrichment and decrease of ARG profiles and the driving factors in agricultural soil environments under BC stress, as well as explore the synergistic effect of SMZ and BC under the background of combined pollution.

2. MATERIALS AND METHODS

2.1. Soil Sampling and Chemicals. According to the standard multipoint mixed sampling method, a composite soil sample with five subsamples (about 2 kg for each) was collected at a depth of 0 to 20 cm from a rice-wheat rotation test field, which has no history of organic fertilizer application in the past five years, at Ningbo (N 29°47′24″, E 121°22′3″), Zhejiang Province, China, in November 2020. The composite soil sample was thoroughly mixed, sieved (2 mm), and then used for the soil microcosm experiment. The soil was classified as a silty loam (46.4% sand, 53.1% silt, and 0.5% clay), with a pH of 5.75, total carbon 16.5 g C kg^{-1}, and total nitrogen 1.9 g N kg^{-1}. The pH value was measured at a soil and water suspension ratio of 1:5 (*w*/*v*). Total soil carbon and nitrogen content was determined with a CNS Element Analyzer (Vario MAX C/N, Elemental, Germany). Benzalkonium chloride (C_{12}) (BC, CAS: 139-07-1) and sulfamethazine (SMZ, CAS: 57-68-1) were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) and Dr. Ehrenstorfer GmbH (Augsburg, Germany), respectively.

2.2. Experiment Design. The soil microcosm experiment was performed in 200-mL brown jars. Eight treatments were established, with three replicates containing 40 g of soil per jar: three treatments with 1, 10, and 100 mg BC kg^{-1} soil (BC1, BC10, and BC100, respectively), one treatment with 10 mg SMZ kg^{-1} soil (SMZ), three treatments with combined SMZ and BC that were designed with quality ratios of 10:1 (SMZ 10:BC 1 mg kg^{-1}, SB1), 1:1 (SMZ 10:BC 10 mg kg^{-1}, SB10), and 0.1:1 (SMZ 10: BC 100 mg kg^{-1}, SB100), and a control treatment (CK) with an equal volume of deionized water.[32] The added concentrations of SMZ (10 mg kg^{-1}) and BC (1 and 10 mg kg^{-1}) were within the same order of magnitude as those found in agricultural soils with the long-term implementation of wastewater irrigation or the application of manure.[26,30,33] Moreover, reported QAC concentrations in sewage sludges were higher than in soils,[23] so we chose a high concentration of BC (100 mg kg^{-1}) to observe an ecotoxic effect.

The soil moisture content was kept at 40% of the maximum water-holding capacity, and soils were preincubated for 7 d at 25 °C to activate soil endogenous microorganisms. After 7 d, SMZ and BC were applied to the preincubated soils in the corresponding jars. The samples were mixed thoroughly to ensure that a uniform distribution of SMZ and BC was applied. All treated samples were adjusted to 60% of the maximum water-holding capacity, incubated at 25 °C, and destructively sampled at 28 d. Distilled water was regularly added during this period to maintain constant soil moisture. The aerobic conditions were maintained via refreshing the air in the jars every 2 d.

2.3. DNA Extraction and Bacterial 16S rRNA Illumina Sequencing. Total genomic DNA was extracted from approximately 0.5 g of frozen soil using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). After measuring the integrity by 1.5% agarose gel electrophoresis, DNA concentration was further determined by spectrophotometric analysis using the NanoDrop2000 (Thermo Scientific, USA). The DNA was stored at −20 °C before use. The soil bacterial 16S rRNA gene (V4 to V5 region) was amplified with the universal primers 515F (5′-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′)[34,35] on an Illumina MiSeq platform (Majorbio, Shanghai, China). The sequencing data were analyzed by USEARCH,[36] including demultiplexing, quality-filtering, and taxonomy classification. The UNOISE algorithm was used to perform denoising of amplicon reads and generated the zero-radius operational taxonomic units (ZOTUs).[37] Each ZOTU representative sequence was taxonomically assigned according to the rRNA sequences in the Silva database (SSU132),[38] using the Ribosomal Database Project Classifier algorithm with a confidence threshold of 0.8.[39] Raw sequences have been deposited at the National Center for Biotechnology Information Sequence Read Archive under Bioproject accession number PRJNA785286.

2.4. HT-qPCR and Data Processing. HT-qPCR was performed via a SmartChip Real-time PCR system (Wafergen Inc., USA). A total of 296 primer sets (Table S1) were adopted to detect major classes of ARGs [including aminoglycoside, beta-lactamase, MLSB (macrolide-lincosamide-streptogramin B), multidrug, FCA (fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol), sulfonamides, tetracyclines, and vancomycin], 10 MGEs, and 16S rRNA genes, which were validated and used in a previous study.[40] The specific methods and amplification procedures involved in the above experiments are described in the study of Chen et al.[41]

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

The HT-qPCR results were preprocessed on SmartChip qPCR Software to remove the wells with multiple melting peaks or amplification efficiencies beyond the range of 1.8−2.2. A threshold cycle (Ct) of 31 was used as the detection limit. The abundance and fold change (FC) values of ARGs were determined referring to the study of Chen et al.[41] The average number of 16S rRNA genes per bacterium is currently estimated at 4.1 based on the Ribosomal RNA Operon Copy Number Database (rrnDB version 4.3.3).[42] The normalized copy number of ARGs per bacterial cell was calculated after bacterial cell numbers were estimated by dividing 16S rRNA gene copy numbers by this value.[43]

2.5. Statistical Analysis. The data collation and statistical analyses were conducted using Microsoft Excel 2016 and SPSS (v.25, IBM). The Procrustes test, Mantel test, principal coordinate analysis (PCoA), heatmap, and bubble chart were performed or created using R software (version 3.5.3) with the "vegan", "pheatmap", and "ggplot2" packages. To explore the co-occurrence patterns among ARGs, MGEs, and microbial taxa, a correlation network was established in the R environment using the "Hmisc" and "igraph" packages. If Spearman's correlation coefficient (R^2) was >0.8 and the P value was <0.01, a correlation between any two items was regarded as statistically robust.[44,45] The P values were adjusted with multiple testing corrections using the Benjamini-Hochberg method[46] to reduce the chances of obtaining false-positive results. Gephi 0.9.2 software was used to visualize the network graphs using the Fruchterman-Reingold layout algorithm.[47] Canoco 5.0 software was used for redundancy analysis (RDA) to reveal the dominant impact factors for the ARG profiles.

3. RESULTS

3.1. Diversity and Abundance of the Antibiotic Resistome. An HT-qPCR array was employed to evaluate the changes in the diversity and abundance of ARGs in agricultural soils treated with SMZ (10 mg kg^{-1}) and different BC concentrations (0, 1, 10, and 100 mg kg^{-1}) in the laboratory. A total of 85 unique ARGs and eight MGEs were detected across all the samples. The detection number ranged from 23 to 48, and the highest detection number was found in the SB100 treatment (Figure 1a). The detection number in the SMZ treatment was similar to that of the control soil (CK). The treatment with added BC significantly increased the occurrence of ARGs in the soil, and the detection number increased with increasing BC concentrations.

The normalized abundance of ARGs was 0.0069 copies per bacterial cell in the CK treatment (Figure 1b). Compared with the CK treatment, the low (BC1 and SB1) and medium (BC10 and SB10) BC concentration treatments increased the normalized abundance of ARGs, but only the BC1 treatment, with 0.013 copies per cell, reached a significant level (P < 0.05). A similar trend was observed between the normalized abundance (ARG copies per bacterial cell) and absolute abundance (ARG copies per gram of dry soil) of ARGs in all the treatments (Figure S1). The absolute abundance of ARGs ranged from 2.99×10^8 to 1.44×10^9 copies per gram of dry soil and was positively correlated with the absolute abundance of 16S rRNA genes (P < 0.001). We found a negative concentration effect in treatments with different concentrations of BC in which, as the BC exposure concentration increased, the increase in the total abundance of resistance genes gradually decreased (Figure 1b). In addition, SMZ increased

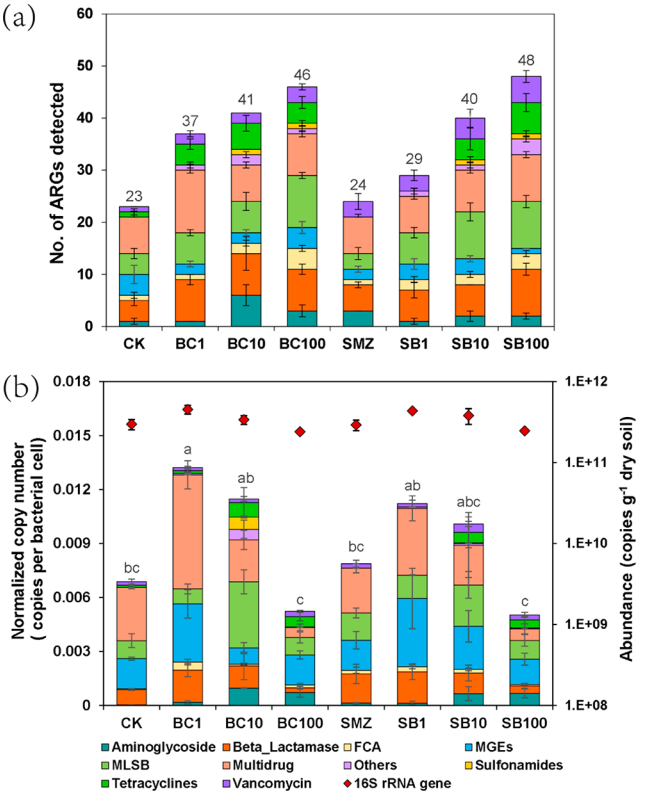

Figure 1. (a) The detected number of resistance genes. (b) The normalized abundance (normalized to cell number) of resistance genes and absolute abundance of 16S rRNA genes. Different letters above the bars indicate a significant difference at P < 0.05 (ANOVA). FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol; MLSB: macrolide-lincosamide-streptogramin B. CK, unamended soil; SMZ, soil amended with sulfamethazine (10 mg kg^{-1}); BC1, BC10, and BC100, soil amended with 1, 10, and 100 mg kg^{-1} benzalkonium chloride (C_{12}), respectively; SB1, SB10, and SB100, soil with combined SMZ (10 mg kg^{-1}) and benzalkonium chloride (C_{12}) (1, 10, and 100 mg kg^{-1}, respectively).

the total abundance of resistance genes slightly, but there was no significant difference from the CK treatment. Comparing treatments of 10 mg kg^{-1} equal concentration (SMZ, BC10, and SB10), BC has a more significant effect on resistome than SMZ. The combined effect of SMZ and BC did not synergistically increase the detection number and abundance of ARG, but it transformed the ARG profile to some extent.

In this study, the most dominant types of ARGs found in agricultural soils were Multidrug, MLSB, and beta-lactamase resistance genes. Compared with the CK treatment, exposure to BC at low concentrations (BC1 and SB1) resulted in a significant increase in the abundance of MGEs and FCA resistance genes (Figure S2), suggesting that low concentrations of BC may increase the potential of horizontal transfer of ARGs. Moreover, the BC10 and SB10 treatments increased the abundance of aminoglycoside and MLSB resistance genes markedly. The application of BC at high concentrations (BC100 and SB100) also significantly increased the abundance of aminoglycoside resistance genes but led to a remarkable decline in the abundance of multidrug resistance genes. Therefore, a higher abundance of ARGs could be observed at a lower BC concentration.

3.2. Enrichment and Decrease of the Antibiotic Resistome. A heatmap comparing the fold change (FC)

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

values of resistance genes of different treatments and the CK treatment is displayed in Figure 2. Compared with the CK

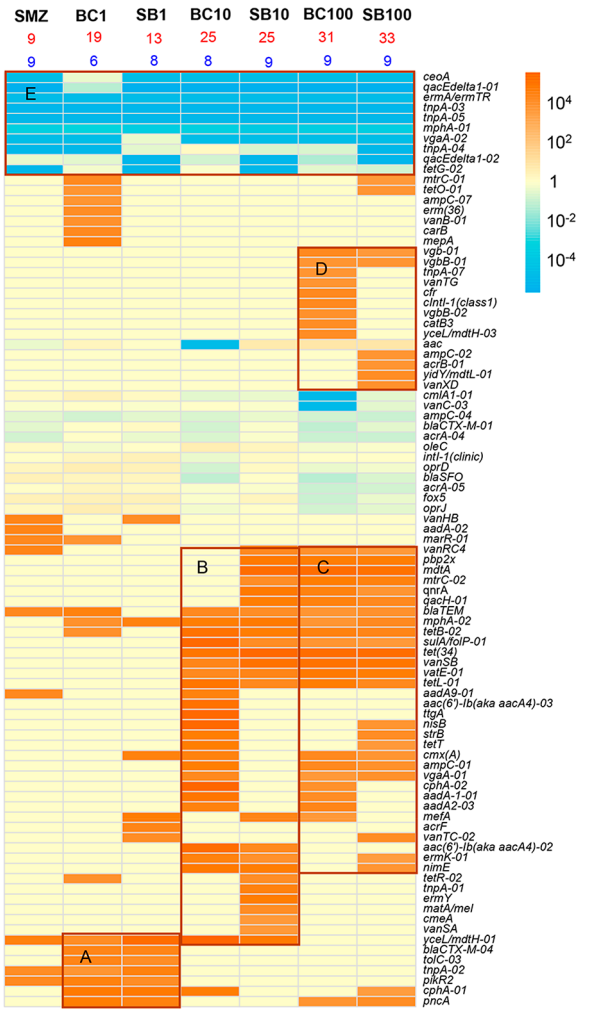

Figure 2. Log-transformed fold change (FC) value of resistance genes in soils with benzalkonium chloride (C_{12}) (BC) and sulfamethazine (SMZ) exposure in microcosm experiments. With the CK treatment samples as reference samples, the FC value of each resistance gene was calculated via a ΔΔCt comparison method. The number on the top of each column refers to the number of significantly enriched (red) or decreased (blue) resistance genes in different treatments. SMZ, soil amended with SMZ (10 mg kg^{-1}); BC1, BC10, and BC100, soil amended with 1, 10, and 100 mg kg^{-1} BC, respectively; SB1, SB10, and SB100, soil with combined SMZ (10 mg kg^{-1}) and BC (1, 10, and 100 mg kg^{-1}, respectively).

treatment, a total of 68 unique resistance genes were significantly enriched in at least one sample among all the detected 93 unique resistance genes. Between 9 and 33 unique ARGs were enriched among different treatments, with which the SB100 treatment enriched 33 unique ARGs. We observed that most ARGs were enriched, while some ARGs were not enriched and some even decreased (marked with E in the heatmap). The enriched ARG subtype sets in the low concentration (BC1 and SB1) and high concentration (BC100 and SB100) treatment groups were different. The former enriched the A cluster marked in the heatmap, while the latter enriched the C and D clusters. The medium concentration (BC10 and SB10) treatment groups enriched the B cluster marked in the heatmap. The maximum enrichment of a beta-lactamase resistance gene, *cphA-02*, reached 375373-fold in the BC10 treatment. Furthermore, other unique ARGs, such as *ttgA*, *yceL/mdtH-01*, *nisB*, *sulA/folP-01*, and *tet(34)*, were enriched >200000-fold. The MLSB resistance gene *mphA-02* was enriched in all treatment samples, except for SMZ. Surprisingly, among the seven sulfonamide resistance genes tested, only the gene *sulA/folP-01* was enriched following SMZ exposure.

3.3. Co-Occurrence Patterns in the Antibiotic Resistome. Four networks were established based on strong (R^2 > 0.8) and significant (P < 0.01) correlations to evaluate the ARG subtypes and MGE co-occurrence patterns for the different treatments (Figure S3). Topological properties widely used in network analysis were calculated to characterize the intricate inter-relationships between the nodes (Table S2). The high BC concentration treatment (BC100+SB100) network contained more nodes (64) and stronger connections (edges: 236) than did the control network (nodes: 33, edges: 50), meaning that SMZ (10 mg kg^{-1}) and BC (100 mg kg^{-1}) enhanced the connection densities between resistance genes and prompted the co-occurrence network pattern to become more complex. Notably, the average clustering coefficient and modularity values were higher for the BC1+SB1 and BC10+SB10 networks than for the control network, suggesting that treatments of low (1 mg kg^{-1}) and medium (10 mg kg^{-1}) BC concentrations produced a stronger "small world" topology. Each network was separated into six modules (except for the BC10+SB10 network, with nine modules), in which nodes had greater numbers of correlations with each other. The most highly connected node within the module was considered the network "hub".[48] For instance, the MLSB resistance gene *mphA-02*, associated with 32 unique ARGs, was the hub of Module 1 in the control network. However, in soil exposed to a high BC concentration (100 mg kg^{-1}), the BC100+SB100 network yielded three hubs associated with 63 unique ARGs in Module 1, including that the "*pikR2*", "*tetR*", and "*vanHB*" genes belonged to the MLSB, tetracycline, and vancomycin resistance genes, respectively.

3.4. Effects of Treatments on Soil Bacterial Community Composition. The effects of different treatments on the overall distribution pattern of the bacterial communities in the soil were characterized via PCoA analysis based on the Bray–Curtis distance (Figure 3a). The treatments with added SMZ (SMZ, SB1, SB10, and SB100) were separated from treatments with no SMZ. The treatments with the addition of BC alone (BC10 and BC100) and CK treatment clustered together. The SMZ caused a significant shift (Adonis test, P = 0.001) in the overall bacterial community. In particular, comparing treatments of 10 mg kg^{-1} equal concentration (SMZ, BC10, and SB10), the BC10 treatment was clustered together with the CK treatment, while the SMZ and SB10 treatments clustered. It means that SMZ has a greater impact on the microbial community structure than BC at equal concentrations. A heatmap was constructed to show the bacterial community composition for the different treatments (Figure 3b), which further confirmed the effect of SMZ on the bacterial community. The bacterial community change pattern (family level) was similar among the treatments with added SMZ (SMZ, SB1, SB10, and SB100), with significant increases (P < 0.001) in the abundances of Acidobacteriaceae (Subgroup 1), Micromonosporaceae, and Burkholderiaceae but significant decreases (P < 0.001) in the abundances of Xanthomonadaceae, Isosphaeraceae, Nitrosomonadaceae, and Solibacteraceae

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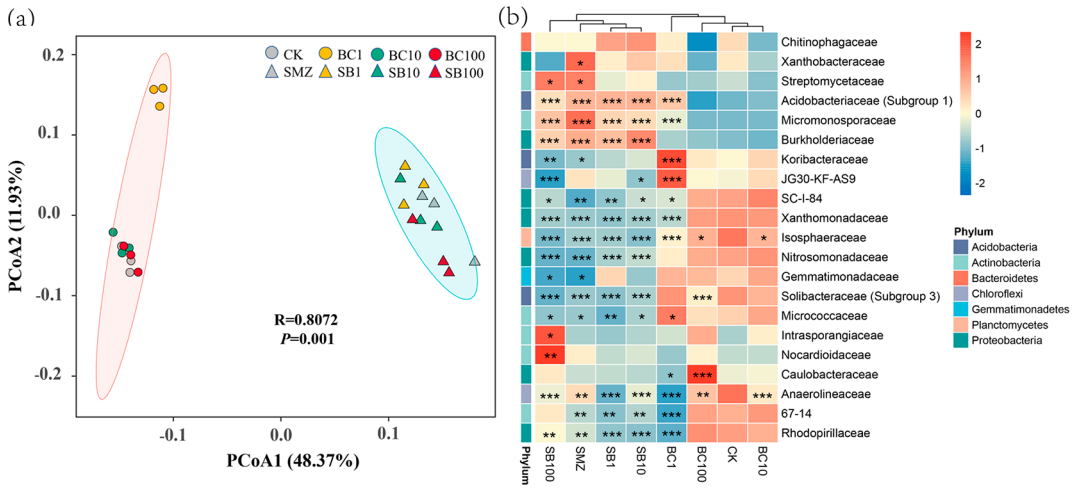

Figure 3. (a) Principal coordinate analysis (PCoA) based on the Bray−Curtis distance showed the overall distribution pattern of bacterial communities in soil. CK, unamended soil; SMZ, soil amended with sulfamethazine (10 mg kg^{-1}); BC1, BC10, and BC100, soil amended with 1, 10, and 100 mg kg^{-1} benzalkonium chloride (C_{12}), respectively; SB1, SB10, and SB100, soil with combined SMZ (10 mg kg^{-1}) and benzalkonium chloride (C_{12}) (1, 10, and 100 mg kg^{-1}, respectively). (b) Heatmap displaying the composition of bacterial communities at the family level (relative abundance > 0.5% in any sample). The asterisk indicated significant differences between CK and other treatments (LSD test; *P < 0.05; **P < 0.01; ***P < 0.001).

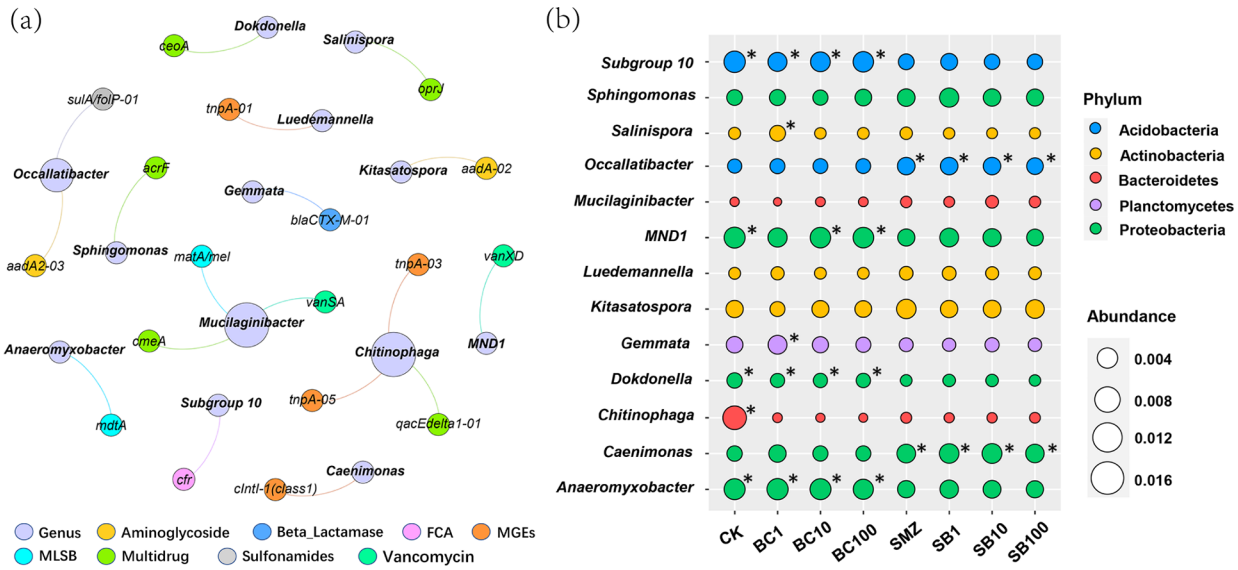

Figure 4. (a) Network analysis displaying co-occurrence patterns between antibiotic resistome and microbial taxa at the genus level. A connection represents a strong (Pearson's R^2 > 0.8) and significant (P < 0.01) correlation. All significant correlations in the data set were positive. The nodes are colored according to ARG classification and genus, and node size is weighted according to the number of edges connected with others. FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol; MLSB: macrolide-lincosamide-streptogramin B. (b) The relative abundance of the possible host of ARGs in different treatments. Bubbles are scaled by area to illustrate relative abundance. Asterisks (*) indicate a statistically significant difference (P < 0.05). CK, unamended soil; SMZ, soil amended with sulfamethazine (10 mg kg^{-1}); BC1, BC10, and BC100, soil amended with 1, 10, and 100 mg kg^{-1} benzalkonium chloride (C_{12}), respectively; SB1, SB10, and SB100, soil with combined SMZ (10 mg kg^{-1}) and benzalkonium chloride (C_{12}) (1, 10, and 100 mg kg^{-1}, respectively).

(Subgroup 3). In addition, BC (1 mg kg^{-1}) alone (BC1) significantly increased the abundances of Micromonosporaceae, Koribacteraceae, JG30-KF-AS9, and Acidobacteriaceae (Subgroup 1) but decreased the abundances of Xanthomonadaceae, Isosphaeraceae, Anaerolineaceae, 67-14, and Rhodopirillaceae.

3.5. Co-Occurrence Patterns among Antibiotic Resistome and Microbial Taxa. Network analysis methods have been applied to explore co-occurrence patterns between the antibiotic resistome and microbial taxa to identify potential ARG hosts in complicated environments.[49] As shown in Figure 4a, 13 bacterial genera had significant co-occurrence relationships with 18 ARGs and were identified as potential hosts of these ARGs. Thereinto, three genera were identified as the potential hosts of multiple types of ARGs. For instance, the genus *Mucilaginibacter* was identified as the possible host of an MLSB resistance gene, *matA/mel*, a multidrug resistance gene, *cmeA*, and a vancomycin resistance gene, *vanSA*. The genus *Chitinophaga*, classified as Bacteroidetes, was speculated to be the possible host of one multidrug resistance gene (*qacEdelta1-01*) and two transposase genes (*tnpA-03* and *tnpA-05*). In addition, the genera *Anaeromyxobacter*, *Caenimonas*, *Dokdo-*

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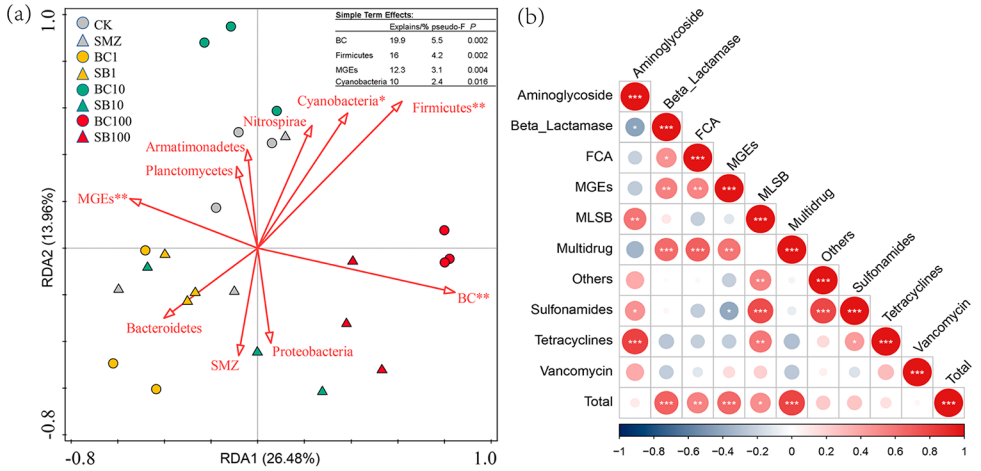

Figure 5. (a) Redundancy analysis (RDA) revealing the correlation of major microbial phyla (relative abundance > 0.1% in any sample), benzalkonium chloride (C_{12}) (BC), sulfamethazine (SMZ), MGEs, and ARGs. All variables in the model were evaluated for collinearity using the variance inflation factor. CK, unamended soil; SMZ, soil amended with SMZ (10 mg kg^{-1}); BC1, BC10, and BC100, soil amended with 1, 10, and 100 mg kg^{-1} BC, respectively; SB1, SB10, and SB100, soil with combined SMZ (10 mg kg^{-1}) and BC (1, 10, and 100 mg kg^{-1}, respectively). (b) Pearson correlation coefficients between the normalized abundance (normalized to cell number) of MGEs and ARGs. The color of the circle represents the positive or negative correlation coefficient. The size of the circle represents the degree of correlation. Significance levels are represented by *($P < 0.05$), **($P < 0.01$), and ***($P < 0.001$).

nella, *Gemmata*, *Kitasatospora*, *Luedemannella*, *MND1*, *Salinispora*, *Sphingomonas*, and *Subgroup 10* were only significantly correlated with one ARG. These potential host microorganisms were affiliated with five phyla, including Acidobacteria, Actinobacteria, Bacteroidetes, Planctomycetes, and Proteobacteria (Figure 4b). The relative abundances of the 13 genera varied among different treatments. Compared to CK and single different BC concentration treatments, treatments with SMZ added (SM, SB1, SB10, and SB100) significantly reduced the relative abundances of the genera *Anaeromyxobacter*, *Dokdonella*, and *Subgroup 10* but increased the relative abundance of the genera *Occallatibacter* and *Caenimonas*.

3.6. Contribution of Different Factors to Variations in the ARG Profile. The contributions of the bacterial community (based on the abundances of major phyla), BC, SMZ, and MGEs to the changes in the ARG profiles were explored by performing RDA analysis (Figure 5a). BC (P = 0.002), Firmicutes (P = 0.002), MGEs (P = 0.004), and Cyanobacteria (P = 0.016), all of which significantly correlated with the ARG profiles, were considered as important explanatory variables. RDA1 and RDA2 explained 40.44% of the total variance in the ARG profiles. Variation partitioning analysis was carried out to decode the contributions of various factors to ARG profile changes. The different BC concentrations accounted for 24.28% of the total variation in the ARG profile and were a major driver shaping the ARG profile in soil. Bacterial community composition explained 8.10% of the ARG profile variations. Meanwhile, the Procrustes test based on the Bray−Curtis distance showed that the ARG profiles had a weak correlation with the bacterial community (M^2 = 0.757, P = 0.003, permutations 9999), which was further confirmed by the Mantel test (Pearson's r = 0.188, P = 0.026). MGEs alone contributed only 4.74% of the total variance. Nevertheless, Pearson correlation analysis showed that there was a highly significant correlation between the normalized abundances of total ARGs and MGEs (r = 0.655, $P < 0.001$). We also discovered that MGEs had positive correlations with the beta-lactamase ($P < 0.01$), FCA ($P < 0.01$), and multidrug ($P < 0.01$) resistance genes and a negative correlation with sulfonamide ($P < 0.05$) resistance genes. More information about the correlation is provided in Figure 5b.

4. DISCUSSION

4.1. Fate of the Antibiotic Resistome in Soil under Short-Term BC Exposure. The findings of this study, in which the BC exposure caused coselection for increased bacterial resistance to multiple antibiotics in the bacterial community, followed previous results.[20,22,50,51] Intriguingly, a unique concentration effect was found in the changes of ARG profiles in agricultural soils exposed to different concentrations of BC for 28 d, which was slightly different from our hypothesis (1). For example, the number of ARGs detected in the soil environment increased with the increase of BC exposure concentration (Figure 1a), but the normalized ARG abundance decreased. Compared with the CK treatment, the total normalized abundance of ARGs increased significantly when exposed to low concentrations of BC alone, reaching 0.013 copies per bacterial cell. However, as the BC exposure concentration increased, the increase in the total normalized abundance of ARGs gradually decreased. As a disinfectant, the appropriate concentration of QAC is considered to be one of the key factors in the selection of resistant bacteria.[52] Coselection for antimicrobial resistance only occurs when the bioavailable QAC concentrations are below the critical biocidal concentration.[53] Low BC concentrations have low biological toxicity and might be utilized as a source of carbon and energy by some microorganisms.[54] Thus, we observed a significant increase in the bacterial abundance of the BC1 and SB1 treatments (Figure 1b). Because ARGs are carried by soil bacteria, the total ARG abundance increased under low BC concentrations (BC1 and SB1). In contrast to low concentrations, high BC concentrations are highly toxic to organisms. However, the bacterial abundance decreased slightly but did not reach a significant level compared with the CK treatment in the BC100 and SB100 treatments, which were speculated to be related to the adsorption and storage of QACs in the soil. QACs are cationic surfactants, and their high adsorption affinity for soil particles will cause their bioavailability to

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

decrease, thereby reducing mineralization and their acute toxicity.[55] However, the adsorption and isolation of soil increase the persistence of BC, and the gradual release of BC induces the occurrence of more antibiotic resistance. As we observed, the highest number of ARGs was detected at high BC concentrations (BC100 and SB100). Therefore, bacteria that survived at high BC concentrations may consist of two parts: a few resistant bacteria with a number of ARGs and some inert bacteria with little or no ARGs that were not affected by high concentrations of BC.

Beyond the shifts in the abundance and diversity of total ARGs, exposure to different concentrations of BC also elicited changes in a wide spectrum of specific ARG types. From the heatmap of the FC value of ARGs (Figure 2), we found that diverse ARGs showed different response patterns to BC exposure. Cluster A was enriched at low-concentration BC exposure but not at medium and high BC concentrations, indicating that these ARGs might be carried by bacteria that are sensitive to medium and high BC concentrations.[20] The medium BC concentration enriched cluster B. The high BC concentration significantly enriched clusters C and D, which might be ascribed to the low abundance of soil indigenous ARGs selected or the acquisition of new ARGs in BC-resistant bacteria coselected by BC exposure.[13] The numbers of significantly enriched ARGs increased with the BC concentration. The apparent decrease in cluster E might be due to the killing or inhibition of some soil bacteria by SMZ and BC.[56,57] Therefore, BC could have a striking influence on the soil ARG spectrum, enriching or decreasing specific types of ARGs with different BC concentrations.

4.2. BC-Induced Coselection of the Antibiotic Resistome. The remarkable responses of soil ARG abundance and diversity to BC exposure prompted us to explore the drivers of soil ARG spectrum changes. RDA and variation partitioning analysis showed that BC stress was the main factor in soil ARG spectrum changes, rather than the microbial community, as widely believed.[48,58] Such observations might be related to disinfectant cross-resistance and coresistance.[13] In this study, we observed that under exposure to SMZ and different concentrations of BC, the relative abundance of encoded antibiotic deactivate genes increased to some extent in the soil (Figure S4). In addition, the abundance of ARGs encoding cellular protection increased at medium and high BC concentrations (Figure S4). This might be caused by the destruction of cell membranes by medium and high concentrations of BC, causing oxidative stress in bacteria.[59] Oxidative stress appeared to be a key factor in enhancing ARG expression to trigger ARG proliferation.[21]

The significant co-occurrence patterns of ARGs and MGEs exposed to different BC concentrations in the network analysis provided further insights into the potential BC stress-induced coselection of ARGs. High BC concentrations in the network might apply adequate selective pressures on the co-occurrence of ARGs and MGEs, thus resulting in higher co-occurrence incidences. Moreover, the ARGs with the same resistance mechanism or a resistance mechanism encoded by the same genetic elements generally co-occurred in the same module.[60] For example, five ARGs (*aadA-1-01*, *aadA2-03*, *cphA-02*, *cfr*, and *vgbB-02*) with antibiotic deactivation mechanisms co-occurred strongly in Module 5 of network BC100+SB100 (Figure S4). It was noteworthy that the genes encoding the integrase and transposase were observed to have a strong correlation with many ARGs in the network module, which indicated the occurrence of horizontal gene transfer and the coselection of ARGs.[61] Previous research has shown that QACs have a high coselective potential compared with other nonantibiotic substances due to the close genetic proximity of additional resistance mechanisms that can be coselected via coresistance.[62] For instance, environmental bacteria were screened for multiple antibiotic resistance mechanisms under the use of 50 mg/L benzalkonium chloride, including the co-occurrence of benzalkonium chloride tolerance and ARGs on the same mobile DNA molecule, mutations in the *pmrB* gene, and the induction of *mexCD-oprJ* multidrug efflux pump overexpression.[22] Therefore, BC dominated the coselection for ARGs in agricultural soils through a variety of resistance mechanisms and pathways.

4.3. MGEs, SMZ, and the Bacterial Community Shaped the ARG Profile. The lower ratio of variation explained by MGEs (only 4.74%) revealed that horizontal gene transfer of ARGs was less vital than other factors under BC exposure in this study. The total abundance and diversity of ARGs stored in the agricultural soil used for the microcosm experiment in this study were much lower than in other soil environments where manure or irrigation wastewater was applied for a long time, which may lead to a relatively low horizontal gene transfer potential of ARGs. Nevertheless, the positive correlation between MGEs and total ARGs or different types of ARGs (such as beta-lactamase resistance genes) indicated that the potential of horizontal gene transfer in the spread and enrichment of ARGs should not be neglected.

Unlike most studies in which the bacterial community was the major driving force for ARGs,[58,63,64] the bacterial community in this study contributed only an 8.10% change in the ARG profile. Although the Procrustes and Mantel tests showed a weak correlation between the overall bacterial community and the distribution characteristics of ARGs, the network analysis showed that specific bacterial taxa were associated with specific ARGs. These bacterial populations were considered to be the potential hosts of ARGs.[45,49] Network analysis suggested that five bacterial phyla, including Acidobacteria, Actinobacteria, Bacteroidetes, Planctomycetes, and Proteobacteria, were potential hosts of ARGs, and these taxa were also reported in previous studies.[65,66] Moreover, the external environment could affect the vertical gene transfer of ARGs by changing the abundance of the bacterial hosts.[33,67] According to the results of the current study, SMZ stress significantly affected the abundance of some potential ARG hosts. In particular, the treatments with SMZ all significantly increased the relative abundance of *Occallatibacter*, the potential host for the sulfonamide resistance gene *sulA/folP-01*. In addition, the Firmicutes have been reported to carry and spread ARGs.[68] These findings supported our conclusion that Firmicutes was an important explanatory variable for ARG spectrum changes in agricultural soils exposed to BC. Compared to BC (the active ingredient of broad-spectrum fungicide), a single antibiotic targets a specific microbiome and causes changes in the bacterial community structure more easily.[69] Different from our hypothesis (2), SMZ and BC had a limited synergy in promoting the coselection of soil ARGs (Figure 1), but SMZ shaped the ARG profile by influencing microbial communities. However, it may be because SMZ mainly selects for sulfonamide resistance genes; but among the 295 ARG profiles tested, sulfonamide resistance genes only account for seven, so the effect of SMZ on the ARG profiles did not appear to be so great. BC had less influence on the

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

microbial community structure, which meant that BC affected the enrichment and spread of ARGs in a more complicated way. In the future, more in-depth research could be conducted on this mechanism.

This study provides empirical evidence that short-term BC exposure can promote the development of bacterial antibiotic resistance in agricultural soils. SMZ significantly changed the bacterial community structure but had a limited effect on the overall ARG abundance. Furthermore, BC and SMZ had no obvious interaction in shaping the ARG profile in soils. BC coselected ARGs through multiple resistance mechanisms and pathways, while SMZ mainly affected vertical gene transfer of ARGs by changing the bacterial host abundance. Therefore, under long-term BC exposure and the accumulation of BC in soil, the coselection effect induced by BC inevitably accelerated the occurrence and spread of ARGs. We believe our findings will contribute to future assessments of public health risks linked with QAC-induced antibiotic resistance. In addition, this study stressed it is still necessary to focus on the lower BC concentration range in future research.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c04730.

Table S1, information on 296 genes detected in gene chip; **Table S2**, topological properties of co-occurring ARG subtypes and MGEs networks; **Figure S1**, absolute abundance of ARGs and MGEs in different treatments; **Figure S2**, heatmap of overall ARGs compositions (copies per bacterial cell) in soil; **Figure S3**, ARG subtypes and MGE co-occurrence networks under low, medium, and high concentrations of benzalkonium chloride (C_{12}) (BC) in soil; and **Figure S4**, relative frequencies of ARG groups classified based on resistance mechanisms (PDF)

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Notes

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX