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Increasing Antimicrobial Resistance and Potential Human Bacterial Pathogens in an Invasive Land Snail Driven by Urbanization

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ABSTRACT: Our understanding of the role urbanization has in augmenting invasive species that carry human bacterial pathogens and antimicrobial resistance (AMR) remains poorly understood. Here, we investigated the gut bacterial communities, antibiotic resistance genes (ARGs) and potential antibiotic-resistant pathogens in giant African snails (*Achatina fulica*) collected across an urbanization gradient in Xiamen, China ($n = 108$). There was a lack of correlation between the microbial profiles of giant African snails and the soils of their habitats, and the resistome and human-associated bacteria were significantly higher than those of native snails as well as soils. We observed high diversity (601 ARG subtypes) and abundance (1.5 copies per 16S rRNA gene) of giant African snail gut resistome. Moreover, giant African snails in more urban areas had greater diversity and abundance of high-risk ARGs and potential human bacterial pathogens (e.g., ESKAPE pathogens). We highlight that urbanization significantly impacted the gut microbiomes and resistomes of these invasive snails, indicating that they harbor greater biological contaminants such as ARGs and potential human bacterial pathogens than native snails and soils. This study advances our understanding of the effect of urbanization on human bacterial pathogens and AMR in a problematic invasive snail and should help combat risks associated with invasive species under the One Health framework.

KEYWORDS: *antibiotic resistance genes, biological invasion, ESKAPE pathogens, human bacterial pathogens, metagenome-assembled genomes, resistome*
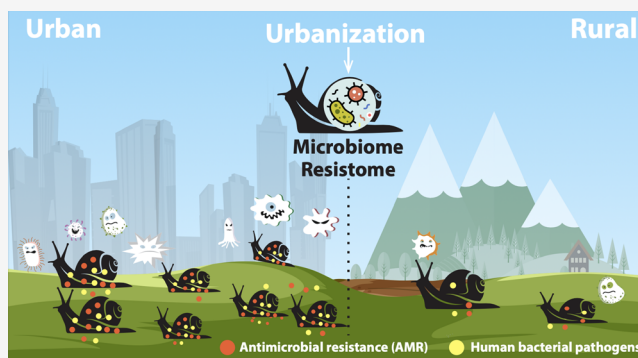
1. INTRODUCTION

Biological invasions contribute to the emergence of zoonotic diseases, introduce novel human pathogens, threaten native species diversity, and directly degrade important ecosystem components.[1] The acceleration of urbanization also has enormous impacts on biodiversity and reduces native ecosystem resistance to biological invasions.[2,3] Consequently, urbanization promotes an increased richness and abundance of zoonotic host species and their associated pathogens.[4,5] Urbanization also creates animal–human–environment interactions that can profoundly impact the epidemiology of emerging zoonotic diseases.[5,6] The ongoing COVID-19 pandemic and the Monkeypox outbreak are sobering reminders of the devastating consequences of the spillover of pathogens from wildlife to humans, especially in highly urbanized areas.[7,8] Of the emerging zoonoses, at least 70% have wildlife origins and most often emerge at human-wildlife interfaces.[5,7] A recent study provides evidence that biological invasions facilitate the emergence of zoonotic diseases.[1] Consequently, invasive animal-related zoonoses have become an emergent issue for urban public health.[9,10] However, little is known about how urbanization impacts biological contaminants (i.e., human bacterial pathogens and antimicrobial resistance (AMR)) via animal invasions.

The global use of antibiotics in the medical and animal husbandry sectors, and the shortage or inadequacy of wastewater management practices have led to high prevalence of antibiotic resistance genes (ARGs) in the urban environment.[11,12] This proliferation has triggered the creation and spread of multi-antibiotic resistant "superbugs" and increasing animal–human–environmental health risk.[13] The multifaceted interplay among urbanization, invasive animals, and biological contaminants can intensify the spread of ARGs and human pathogens in urban ecosystems.[9,10,14] Invasive animals often thrive in urban habitats and can act as reservoirs and vectors of biological contaminants, amplifying the spread of ARGs and public health threats.[2,14] In addition, the occurrence of invasive
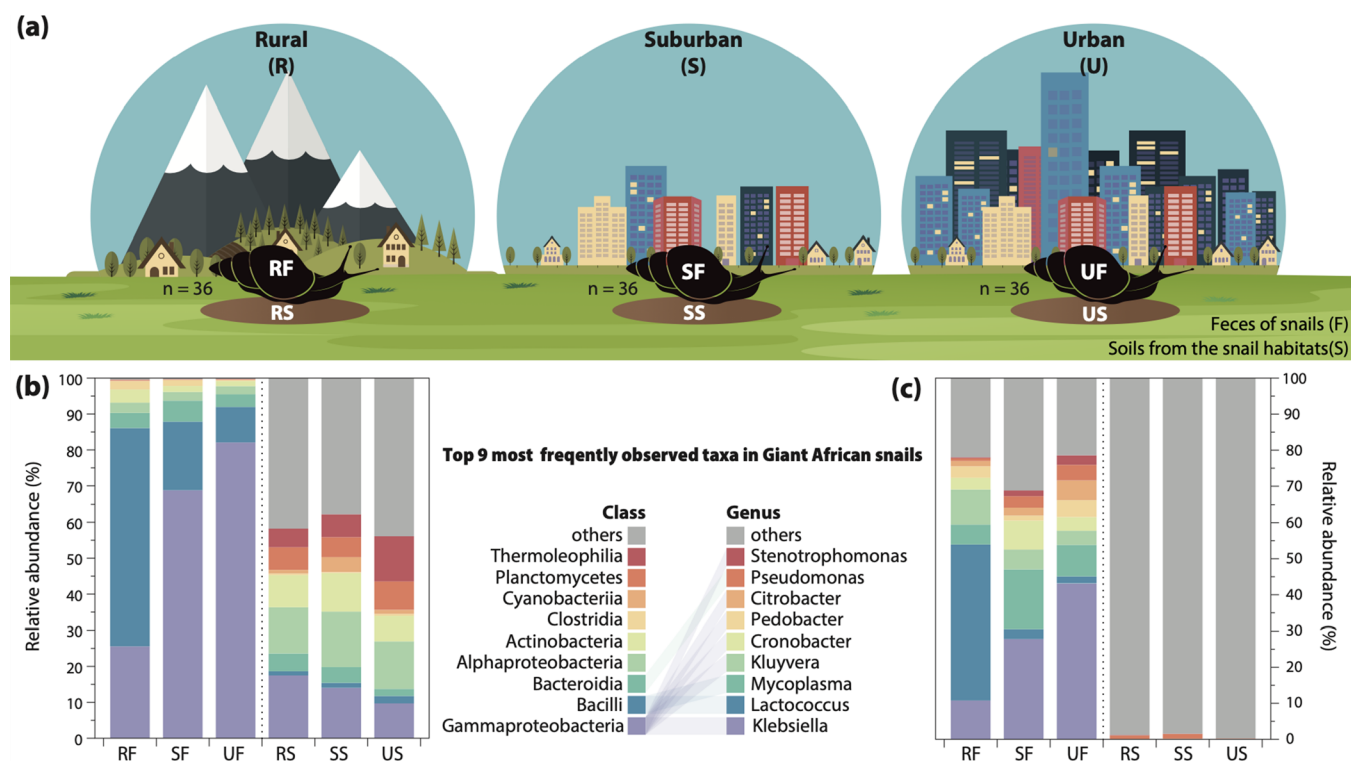
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Figure 1. Summary of the sampling approach and bacterial composition from giant African snail feces and their associated soils. (a) Graphical summary of the sampling approach. Bacterial composition of giant African snails and soils at class (b) and genus (c) levels. Only the nine most frequently observed taxa in giant African snails are shown in the legend, with the remaining lineages grouped into "others."

animals in urban environments may heighten the potential for genetic exchange between bacterial strains, which can generate more virulent, antibiotic-resistant bacteria transmissible to humans and other animals.[15] Considering these factors, it is important to recognize the environmental risks associated with invasive animals as they may serve as hotspots for horizontal gene transfer, facilitating the spread of AMR and pathogens. Despite the public health threats posed by ARGs from pathogens, few studies have examined the role of invasive animals in exacerbating human exposure to ARGs and other biological contaminants in urban settings.

The giant African snail (*Achatina fulica*, syn. *Lissachatina fulica*) is listed among the 100 world's worst invasive alien species.[16] It has been introduced to many regions around the world and has significant impacts on native ecosystems.[17] Giant African snail is known to carry various human pathogens and drug-resistant bacteria, which may pose a risk to human health.[18] For example, these snails have a major role in the global spread of rat lungworm (*Angiostrongylus cantonensis*), which causes zoonotic disease eosinophilic meningitis in humans. Healthy giant African snails are reported to harbor potentially pathogenic bacteria, plus they can acquire multiple antibiotic resistance genes (ARGs) from their surrounding environment and horizontal gene transfer of ARGs to human bacterial pathogens in their gut.[19,20] Considerable ecological and economic damage associated with giant African snails has caused wide public concern.[10,21] The global economy has incurred a cost of US $3.9 billion from invasive alien gastropods since 1966, with the giant African snail incurring costs of $17.3 million in Asia ($n = 7$), $13.0 million in North America ($n = 23$), and $0.6 million in South America ($n = 5$).[22] Urbanization has significant impacts on the living conditions of giant African snail (and other animals), including

changes in diet, exposure to antibiotics and pollutants, alterations in gut microbiota, and increased environmental stress.[23,24] Moreover, it is highly adaptable and has been shown to rapidly adapt to anthropogenically altered habitats,[25] resulting in its high abundance in many habitats, such as urban ecosystem.[26,27] As a result, giant African snails are known to thrive in cities and residential areas due to the favorable microclimate and the abundance of food sources.

Humans can be exposed to the biological contaminants carried by giant African snails through various routes, especially in urban areas.[10,18] Children in particular are at a higher risk of coming into direct contact with the snails while playing in parks or residential areas, where they may touch the snail or its excretions (e.g., feces, mucus), which carry human pathogens.[21,25] In addition, giant African snails can damage crops and gardens, potentially leading to contamination of food sources and exposure to pathogens or ARGs via this pathway. Ingestion of contaminated fruits, vegetables, and/or water sources can lead to exposure to the snail-borne pathogens.[28] In some subtropical and tropical countries, giant African snails are also human food sources.[10,29] Consuming snail meat can be another route of exposure. Therefore, human pathogens associated with giant African snails can be exposed to humans through direct contact with the snails, their excretions, and contaminated urban gardens, crops, and/or water sources.

Here, we hypothesize that the giant African snails harbor biological contaminants, such as ARGs and potential human bacterial pathogens, and that urbanization could be a factor contributing to the increased biological contamination of these invasive snails. To test these hypotheses, we collected giant African snails and their habitat soils as well as native snails from urban, suburban, and rural areas, respectively. We

analyzed the microbiomes and antibiotic resistomes in soils and snail feces by using 16S rRNA high-throughput sequencing, metagenomic sequencing, and high-throughput quantitative PCR (HT-qPCR). We constructed metagenome-assembled genomes (MAGs) from the gut microbiomes, including pathogenic, ARG-carried, VFs-carried, and novel MAGs. Our aims were to (1) determine whether the giant African snail gut is harboring potential human bacterial pathogens and AMR; (2) reveal the impacts of urbanization on the gut microbiomes and antibiotic resistome of the giant African snail; and (3) identify the high-risk pathogens carried with multiple ARGs. This work seeks to broaden our understanding of the environmental biological contamination posed by invasive snails.

2. METHODS

2.1. Collecting and Processing Samples. A total of 108 adult giant African snails and 720 native snails (*Bardybaena similaris*, Asian Tramp snails) were collected from nine sites along an urbanization gradient (3 rural, 3 suburban, 3 urban greenspaces) in Xiamen, China (24°28′47.4024″N and 118°5′21.9264″E) (Figures 1a and S1). At each greenspace, we collected 12 active snails of similar body sizes (5−7 cm) using a visual search method. These snails were collected in a separate sterilized wild-mouth jar that was sealed with breathable pafilm after collection. Since native snails are much smaller in size compared to giant African snails, we collected 80 native snails from each sampling site and placed them in sterile disposable plastic Petri dishes to obtain sufficient feces (Figure S1). Given the mobility of the snail, we overlayed a 15 m × 15 m plot over snail collection points, and five soil sampling points were selected from each snail habitat using a W-shaped path. Three replicate topsoil samples were collected from the 0−7 cm surface layer of each soil sampling point and stored on dry ice. All snails and soil samples were brought to the laboratory within 6 h of sampling. Snail fecal samples were collected from each snail for molecular analyses. To do this, we undertook a 60 h starvation treatment to allow complete excretion from snails. All feces per snail was pooled and stored at −20 °C until molecular processing. Detailed information about the sampling sites and procedures for measuring the physicochemical properties and metal content of the soils can be found in the Supporting Information.

2.2. DNA Extraction and 16S Gene Amplicon Sequencing. *2.2.1. DNA Extraction.* The FastDNA Spin Kit for soil (MP biomedicals, USA) was used to extract DNA from approximately 0.25 g of feces and 0.5 g of soil, respectively, according to the manufacturer's instruction. DNA quality and concentration quantifications of extracted DNA were done with a Qubit 3.0 fluorimeter (Invitrogen) prior to storing the extracted DNA at −20 °C until further analysis.

2.2.2. Amplicon Sequencing. The DNA of fecal and soil samples were used for analyses of bacterial diversity and composition. To characterize the richness and community composition of bacteria, the V4−V5 hypervariable region (515F/907R primer set) of the 16S genes was sequenced via Illumina MiseqPE300 and PacBio Sequel II platform (Majorbio, Shanghai, China), respectively. Species abundance at >0.01% reads was considered present in a sample. The 16S rRNA sequences were processed using Usearch (v.11.0)[30] and Quantitative Insight into Microbial Ecology (QIIME v.1.9.1)[31] and clustered into operational taxonomic units (OTUs) at a

sequence similarity level of 97%. Sequences were clustered into operational taxonomic units (OTUs) at a sequence 3% dissimilarity level using UPARSE pipeline.[32] OTUs with less than two sequences were removed, and their representative sequences were assigned to taxonomic lineages using the RDP classifier within the SILVA database (release 138).

2.2.3. Human Bacterial Pathogen. The potential bacterial pathogens were predicted by Bugbase software.[33] Human-associated bacteria were predicted by FAPROTAX.[34] To further analyze the potential human bacterial pathogens at the species level, the high-throughput qPCR (HT-qPCR) approach combined with SmartChip qPCR software (V 2.7.0.1) was used to further identify and quantify the marker genes of 33 typical human pathogens according to our previous studies.[35,36] Details of key quality control data for DNA extraction and qPCR following the MIQE and EMMI[37] guidelines could be found in the Supporting Information.

2.3. Metagenomic Assembly and Binning. *2.3.1. Shotgun Metagenomic Sequencing.* Shotgun metagenomic sequencing was performed with an Illumina NovaSeq (Illumina Inc., San Diego, CA, USA) using NovaSeq Reagent Kits. About 6 Gb (sequencing depth about 12Gb) trimmed 150-base-pair paired-end reads were generated per sample after quality check (FastQC, version 0.11.5),[38] quality filtering (Trimmomatic, v.0.33),[39] and host sequences decontamination (Bowtie2, v.2.2).[40] Binning was carried out using MetaWRAP v.1.2.1 with three binning methods (CONCOCT v.0.4.0, MaxBin v.2.2.2, and MetaBAT v.2.12.1).[41] The bins were assessed by CheckM v.1.0.5 as having both completeness ≥80% and contamination ≤5% were retained for pairwise dereplication comparison.[42] Taxonomy affiliation of each MAG was determined by GTDB-Tk v.0.3.2 classify_wf.[43] Open reading frames (ORFs) were predicted from MAGs using Prodigal v.2.6.3. Phylogenetic analysis of MAGs was conducted with FastTree v 2.1.10[44] based on a set of 120 bacterial domain-specific marker genes from GTDB, and the phylogenetic tree was visualized in iTOL.[45]

2.3.2. Quantification of Antibiotic Resistance Genes. For ARG quantification, trimmed paired-end reads were processed with the ARGs-OAP (v 2.3) pipeline using default settings to obtain the annotation of ARG profiles.[46] The cut-off values for ARGs were set as an alignment length > 75 nucleotides, an e-value <10^{-5}, and an identity >80%. ARG abundances were normalized against the ARG reference sequence length and the 16S rRNA gene from the metagenomic datasets and represented as "copies per 16S rRNA gene."[46]

2.3.3. ARG-Carrying MAGs. ARG-carrying MAGs were annotated with the Comprehensive Antibiotic Research Database (CARD 3.2.3) by their recommended tool (RGI, v 5.2.1).[47] In total, 85 ORFs annotated in 31 high-quality MAGs were identified as ARGs with resistance functions. The 78 high-quality MAGs were categorized as "multi-resistant (30)", single-resistant (2), and nonresistant, according to whether >1, =1, or = 0 ARG classes were annotated in the genome, respectively.

2.3.4. Virulence Factors. The MAGs were searched against the constructed virulence factor database by Blastn, and those genomes with an ORF with global nucleic acid identity greater than 80% to any virulence factor sequence were finalized as VF-carrying MAGs.

2.4. Data Analysis. Data analyses were performed in R studio (v.4.0.5). Principal coordinates analysis (PCoA) using the Bray−Curtis distance was carried out with vegan and
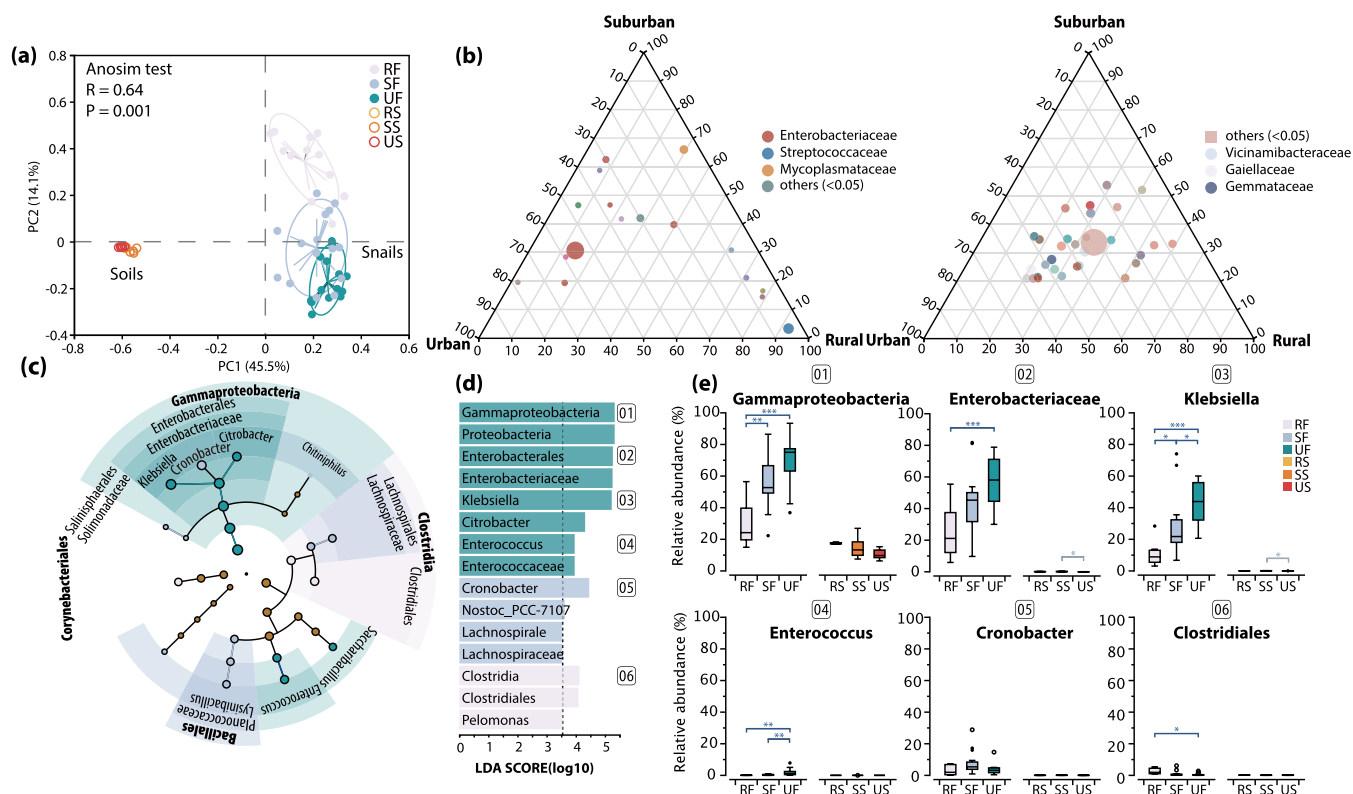
Figure 2. Effect of urbanization on bacterial composition in the gut of giant African snails and their associated soils. (a) Unconstrained PCoA ordination based on Bray−Curtis distances showing that the microbiomes of soil and snail are very disparate from each other (Anosim test, p = 0.001). (b) Ternary plots of OTUs in snail fecal (left) and soil (right) samples and their relative abundances. Each point represents a single OTU. The size of each circle indicates the relative abundance (weighted average) of the genus, and its color indicates the taxonomy at the family level. In a ternary plot, the placement of each circle corresponds to the proportional contribution of the indicated compartments to the overall relative abundance. (c) Cladogram based on LEfSe (Linear discriminant analysis Effect Size) analysis was used to investigate the significant differences in taxa. (d) LEfSe analysis for characteristic microbial species (from the class level to genus level, LDA score > 3.5). (e) Analysis of differences in the microbial taxa among the urban, suburban, and rural snail feces shown by LEfSe (LDA coupled with effect size measurements). Relative abundance of the top six microbial taxa in terms of the LDA score, (1) *Gammaproteobacteria*, (2) *Enterobacterales*, (3) *Klebsiella*, (5) *Enterococcus*, and (6) *Clostridiales*. Statistical significance was determined using the Mann−Whitney U test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

ggplot2 packages in R. ANOSIM based on sample distances was performed to test for differences in the microbiome and resistome composition. The Kruskal−Wallis H test with post-hoc test was used for multiple comparisons; for two-group comparisons, a two-tailed Wilcoxon (paired) or Mann−Whitney U-test (unpaired) test was used. Linear discriminant analysis effect size (LEfSe) analyses were used to identify different bacterial features among the urban (12), suburban (12), and rural snails (12) (LDA score > 3).

3. RESULTS

3.1. Gut Microbiomes of Giant African Snails Are Affected by Urbanization.

Our results showed a significant difference in the total bacterial biomass between the invasive and native snails (Figure S2a). Specifically, the total bacterial biomass in giant African snails is several hundred times greater than that in native snails, as indicated by the range of values between 2.7×10^{10} and 2.2×10^{11} for invasive snails and 3.0×10^8 to 5.4×10^8 (16S rRNA copies per gram feces) for native snails. In addition, the total bacterial biomass in giant African snails increased with urbanization, while native snails appear to be less abundant in rural and suburban environments compared to urban areas. For the snail gut metagenomic data, 97.1% of the reads on average were annotated as bacteria, 1.5% as viruses, 1.3% as eukaryotes, and 0.1% as archaea

(Figure S2b). For the bacteria, the relative abundance of *Gammaproteobacteria* and *Bacilli* class accounted for approximately 80% of the gut microbiome, and the *Gammaproteobacteria* were significantly more abundant in the urban and suburban snails compared with rural snails (Figure 1b). At the family level, *Enterobacteriaceae* was the most abundant bacterial family in the urban snails, accounting for approximately 59% of the total species, consisting mainly of the genus *Klebsiella* (43%) (Figure 1c). *Klebsiella* was significantly enriched in urban and suburban snails (Kruskal−Wallis post hoc test, $p <$ 0.001), while the rural snails were dominated by *Lactococcus* (Kruskal−Wallis post hoc test, $p <$ 0.001) (Figure S3). The top nine genera of snail gut bacteria (∼ 80%) represent only 1% of the soil (Figure 1c) despite the snail gut sharing many bacteria with the soil (Figure S4a). In addition, no significant differences were observed in the microbial α-diversity of soil (Figure S4b) and fecal samples (Figure S4c) across the urbanization gradient (Kruskal−Wallis post hoc test, $p >$ 0.05).

The differences in microbiome across the urbanization gradient (urban, suburban, and rural) of snails and soils were assessed via principal coordinates analysis (PCoA) based on the Bary−Curtis distances of the bacterial communities with snails and soils were divergent from each other and soils showing greater similarity than snails across sites (Figure 2a). According to the PCoA (Figure 2a), the gut microbiome in
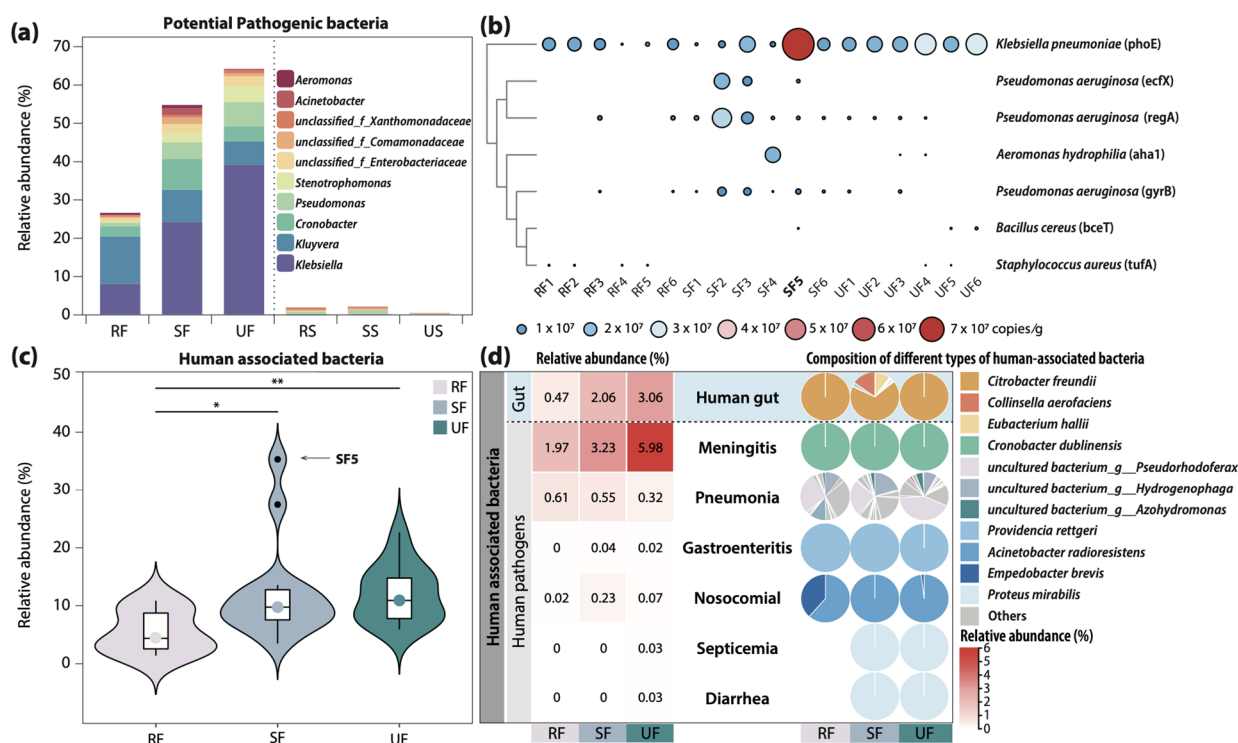
Figure 3. Effect of urbanization on potential pathogen profiles. (a) Relative abundance of potential pathogenic bacteria in fecal samples from giant African snails in rural (RF), suburban (SF), urban (UF) areas, as well as the corresponding soil samples from their respective rural (RS), suburban (SS), and urban (US) habitats, based on Bugbase software. (b) Abundance of human bacterial pathogen marker genes detected in the snails using HT-qPCR assay. (c) Violin plot of predicted human-associated bacteria in giant African snails based on FARPOTAX database. Statistical significance was determined using the Mann−Whitney U test. *$p \leq 0.05$, **$p \leq 0.01$. (d) Composition and abundance of human-associated bacteria (human gut and human pathogens) carried by giant African snails.

rural snails deviated from that of suburban and urban snails (Anosim test, urban vs rural $R = 0.91$, $p = 0.001$; suburban vs rural $R = 0.65$, $p = 0.001$), while suburban snails only partly overlapped with urban snails (Anosim test, $R = 0.23$, $p = 0.003$), indicating similarity between suburban and urban snail gut microbiome. Urban and suburban snail gut microbiome partially overlapped along the second axis but did not overlap with rural samples (Figure S5a). The gut microbiome of snails had a distinct urbanization signature, as evidenced by the fact that *Enterobacteriaceae* were mainly distributed in urban and suburban areas, while *Streptococcaceae* were mainly distributed in rural areas (Figure 2b). In contrast, the microbiome composition of the soil from the snail habitat was more similar and relatively evenly distributed among urban-suburban-rural areas (Figure 2b). Concurrently, there was no significant difference in physicochemical properties between urban, suburban, and rural soils in general (Figure S6).

LEfSe analysis demonstrated that urban and suburban snails were characterized by a significantly higher abundance of *Gammaproteobacteria* than rural snails at the class level. In the class *Gammaproteobacteria*, *Klebsiella* and *Citrobacter* (*Enterbacterales−Enterobacteriaceae*) were significantly higher in urban snails at the genus level (Figure 2c−e). Gut microbial variation in urban−suburban−rural snails evidenced the effects of urbanization on the composition of gut microbiome and confirmed the associations between *Gammaproteobacteria* and urban environments (Figure 2d). We found a strong correlation between some species of *Gammaproteobacteria* in the gut microbes of snails and the urbanization gradient (Figure 2e). This finding indicates that *Gammaproteobacteria*

in the gut of giant African snails appears to respond to urbanization.

3.2. Human-Associated Bacteria in Giant African Snails. Urbanization is associated with a shift in the composition of the gut microbiome of giant African snails toward commonly observed human-associated bacteria (Figure 3). Overall, the Bugbase analysis showed that genes associated with pathogenicity were enriched in the giant African snails harboring many potential pathogenic bacteria (Figure 3a). Seven pathogenic marker genes from five human pathogens were found in invasive snails, which targeted *Klebsiella pneumoniae* (*pho*E), *Pseudomonas aeruginosa* (*ecf*X, *reg*A, *gyr*B), *Aeromonas hydrophilia* (*aha*1), *Bacillus cereus* (*bce*T), and *Staphylococcus aureus* (*tuf*A) (Figure 3b). The marker gene for *K. pneumoniae* was present in all snails with the highest average relative abundance ranging from 5.9×10^5 to 6.9×10^7 copies/g (Figure 3b).

Human-associated bacteria carried by giant African snails consist of human gut and human pathogenic bacteria, according to the FAPROTAX database (Figures 3c,d and S7). The relative abundance of human-associated bacteria made up 9.5% of the total relative abundance of the bacterial community in urban snails, which was significantly higher than that in rural snails ($p < 0.01$). Human gut was mostly attributed to the OTUs affiliated with *Citrobacter freundii* (Figure 3d). Human pathogens were associated with numerous species, such as *Cronobacter dublinensis* (Meningtis) and *Acinetobacter radioresistens* (Nosocomial) (Figure 3d). It is important to stress that the abundance of human-associated bacteria harbored by giant African snails was significantly higher than that of native snails and soil (Kruskal−Wallis post
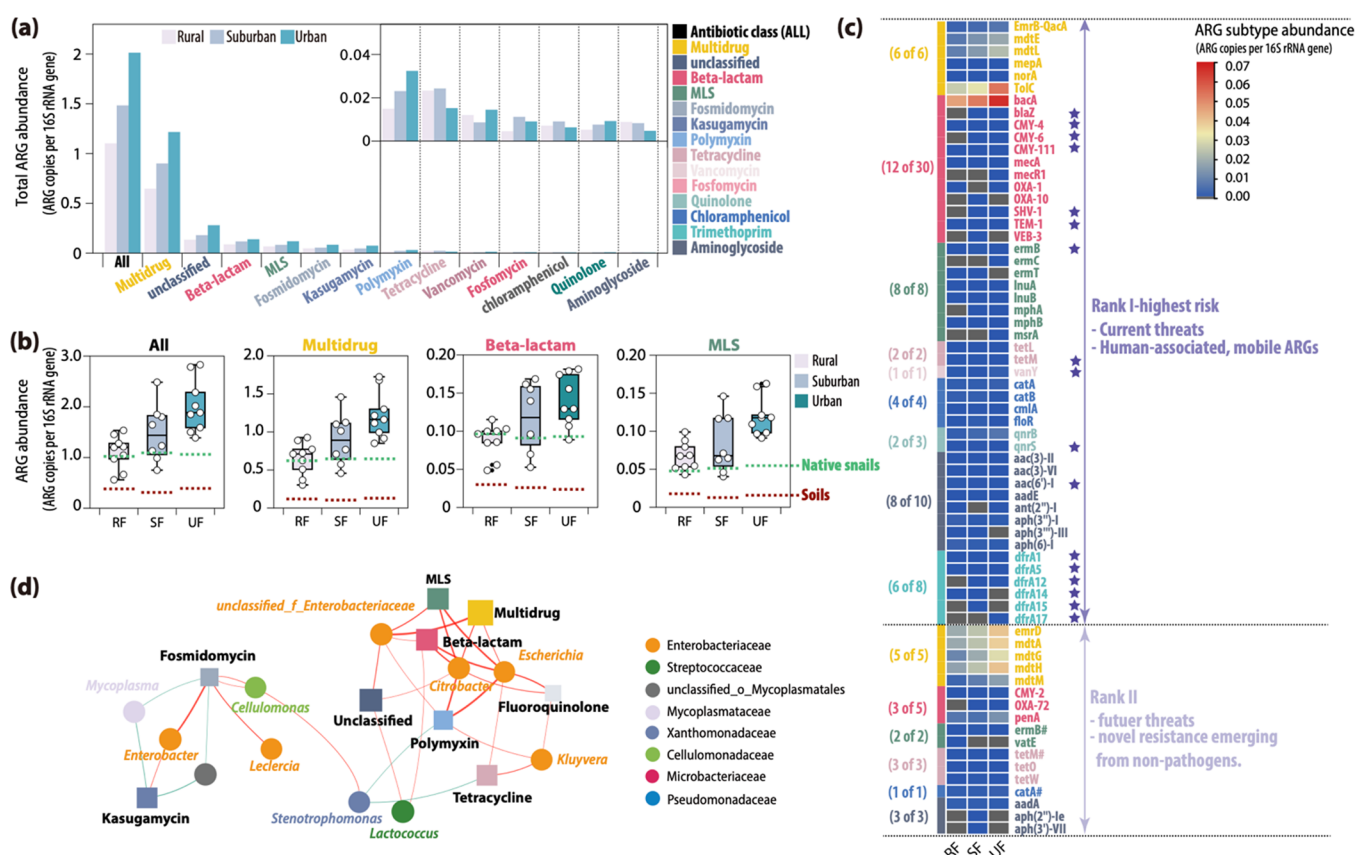
Figure 4. Effect of urbanization on ARGs in giant African snails. (a) Total abundance of different ARG types in rural (RF), suburban (SF), and urban (UF) giant African snails. (b) Total abundance of all, multidrug, beta-lactam and MLS resistance genes in giant African snails versus native snails and soil at the same site. (c) Heatmap showing the abundances of ARG subtypes from metagenome assemblies in giant African snails at the same locations. The abundance of ARGs was normalized into copies of ARGs per 16S rRNA copies (copies per 16S rRNA gene). X of Y in parentheses indicates that X resistance gene subtypes were detected from a total of Y in the high-risk ARG subgroup. (d) Network analysis revealing the co-occurrence patterns between ARG types and microbial taxa. The circle nodes represent the microbial taxa (labeled at genus level, colored at the family level), and the squares represent ARG types. The edges represent a significantly strong correlation (Spearman's rank correlation: |r| > 0.6, p < 0.01), and the thickness of the edges is proportional to the value of Spearman's correlation coefficient. Red and green edges denote Spearman's rank correlation coefficient r > 0.6 and r < −0.6, respectively.

hoc test, $p < 0.001$), at the same locations in urban and suburban areas (Figure S7). The giant African snails at the SF5 sample site area have shown an increased presence of human-related biocontamination, as indicated by a high number of *pho*E marker genes (Figure 3b) and the highest abundance of human-associated bacteria (Figure 3c).

3.3. Resistome Composition and Abundance in Giant African Snails. ARGs identified in giant African snails conferred resistance to 21 families of antibiotics, with abundances ranging from 3.0×10^{-6} to 1.2 (copies per 16S rRNA gene). Thirteen dominant types of ARGs (>99.5% of the ARGs resistome) constituted the whole resistome in the guts of giant African snails (Figure 4a). The urban giant African snails harbored a more abundant resistome (2.0 ARG per 16S rRNA gene) than suburban (1.5 copies per 16S rRNA gene) and rural snails (1.1 copies per 16S rRNA gene). ARGs encoding resistance to multidrug, unclassified type, beta-lactam, macrolide–lincosamide–streptogramin (MLS), fosmidpmycin, and kasugamycin were identified as major components of the resistome in giant African snails with all showing an increasing trend in relative abundance with urbanization (Figure 4). Our analysis reveals that resistomes exhibit less distinct separation than microbiomes (Figure S5a,b). However, we observed a positive correlation between

the urbanization degree and the increasing trends of both resistomes and microbiomes along the PC1 axis (Figure S5c,d). Moreover, the abundance of major components of the resistome in urban giant African snails was significantly higher than that in native snails as well as in local soils (Figure 4b). For example, the most abundant ARG type in urban giant African snails was multidrug, which was two-fold more abundant than that in native snails and 9-fold than that in local soils (Figure 4b). Similarly, all beta-lactam and MLS ARG types exhibited the same trend, with higher abundances observed in urban giant African snails.

A total of 601 subtypes of ARGs were detected, 336 of which were consistently detected in all the urban, suburban, and rural snail samples (Table S1). Not all ARGs pose a serious threat to public health, but some ARG subtypes have significant potential to multiple human health risk characteristics. Recent omics-based research identified the ARG families with significant potential to endanger public health in Rank I (73 subtypes, the highest risks, current threats, already present in pathogens) and Rank II (19 subtypes, future threats, not yet present in pathogens) ARG subtypes that pose the highest risk of contributing to new or multidrug resistance in pathogens, based on their gene mobility (carried by MGEs), human-
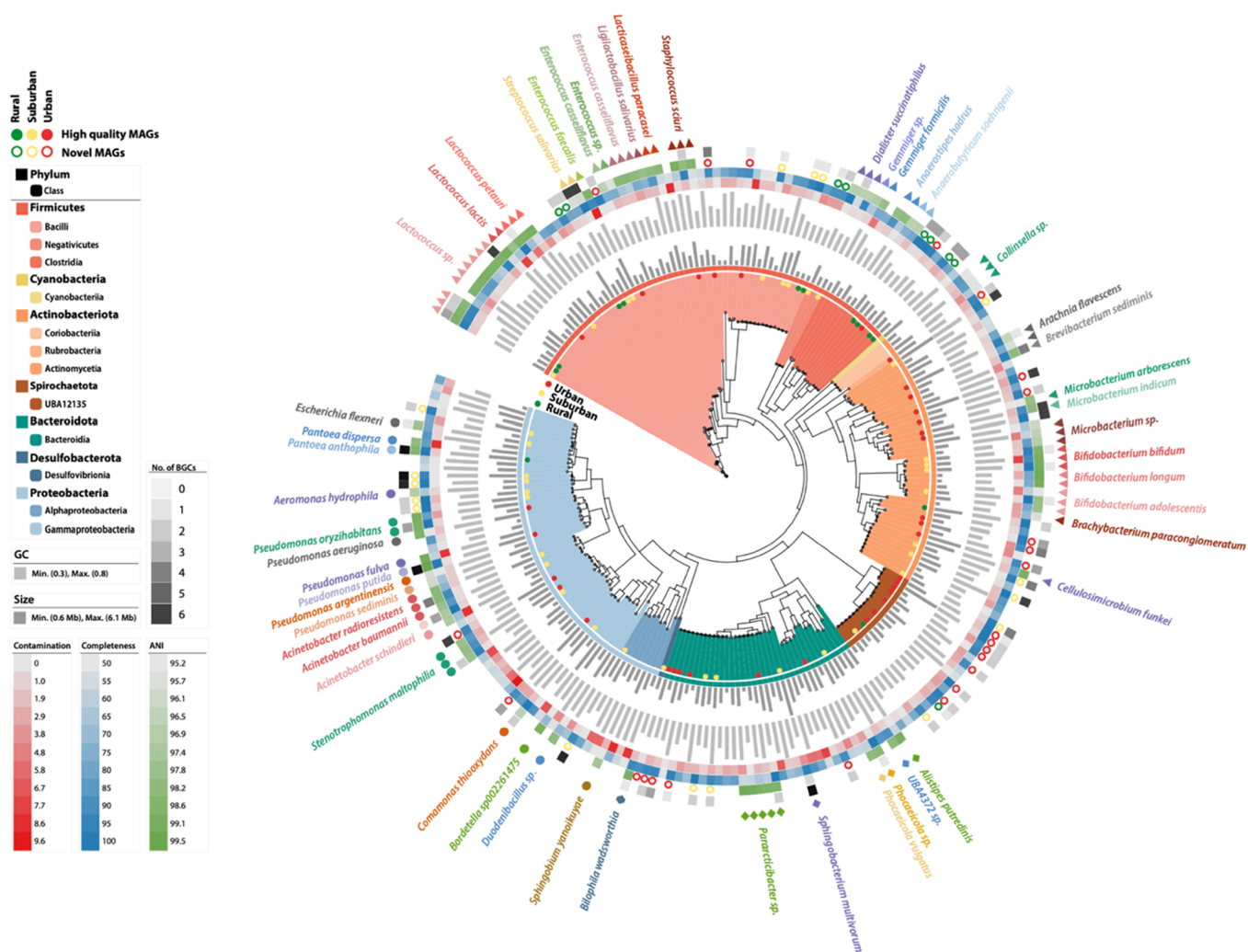
Figure 5. Phylogenetic tree of the 229 MAGs obtained from the gut of giant African snails. The colors and symbols used in the tree indicate various characteristics of the MAGs. Red, blue, and green heatmaps correspond to the level of contamination, completeness, and average nucleotide identity (ANI) of each MAG. Light gray and dark gray bars denote the GC and size of each MAG, respectively. The outermost symbols represent high-quality MAGs from the *Firmicutes, Actinobacteriota, Bacteroidota, Desulfobacterota, and Proteobacteria* phyla, with solid triangles, upside-down triangles, hexagons, rhombus, and ellipse representing each group, respectively. The hollow circles represent novel MAGs not previously identified. Furthermore, the snails are color-coded by habitat, with red, yellow, and green indicating urban (*n* = 12), suburban (*n* = 12), and rural (*n* = 12) snails, respectively.

associated, and pathogenic host (presence/absence in ESKAPE pathogens).[48]

According to the high-risk Rank I-II ARG list,[48] we found that giant African snails contained nearly half of the highest risk ARGs (49 of all 73 Rank I subtype), including 17 subtypes that were identified by the World Health Organization (WHO) as causing clinical problems and/or have been widespread on mobile genetic elements (total 37) (Figure 4c). We also identified 17 Rank II ARGs (19 in total), including three genes (*cat*A, *tet*M, and *erm*B) with Rank I homologous (Figure 4c). Giant African snails contained almost all high-risk multidrug, MLS, tetracycline, chloramphenicol, and vancomycin Rank I-II ARGs subtypes (Figure 4c). The abundance of multidrug families was the highest in both Rank I (such as *Tol*C, *mdt*E/L, and *Eme*B-*Qac*A) and Rank II (*emr*D and *mdt*A/G/H/M) high-risk ARG subtypes. Generally, rural snails had low abundance and urban snails had high abundance of these high-risk ARGs subtypes (Figure 4c), which was consistent with the trend of the overall abundance of multidrugs (Figure 4a). In addition, to initially investigate

the possible host information of ARG types/VFs, we used a network analysis approach to explore the co-occurrence patterns between ARG families/VFs (level 2) and microbial taxa (Figures 4d and S8). We found strong correlations with *Enterobacteriaceae* species observed with both multiple ARGs (Figure 4d) and VFs (Figure S8), suggesting that *Enterobacteriaceae* may be a potential host for resistant and pathogenic bacteria.

3.4. Pathogenic and Novel MAGs from the Giant African Snail. *3.4.1. High-Quality and Novel MAGs.* Two hundred and twenty-nine MAGs (with contamination of ≤10% and completeness of ≥50%) could be constructed. Among them, 78 MAGs with contamination ≤5% and completeness of ≥90% were considered as high-quality MAGs according to the Genomic Standards Consortium.[49] Consistent with the taxonomic profiling of the amplicon sequencing (Figure 1b,c), the reconstructed MAGs predominantly belonged to the phyla Proteobacteria (59/229) and Firmicutes (55/229) (Figure 5). By using an average nucleotide identity (ANI) threshold of >95% for species delineation, 92 of the 229 MAGs

could be assigned to a known species, and the rest could only be classified to a known genus (85/229) or family (34/229) (Table S2), suggesting the presence of potentially novel species carried by giant African snails. Many of the high-quality MAGs that were retrieved from giant African snails could not be assigned to the species level but still carried ARGs and VFs. These novel MAGs belonged to the class *Actinomycetia* (11/50), *Bacilli* (9/50), and *UBA12135* (9/50).

3.4.2. AMR and Human Bacterial Pathogens. Thirty high-quality MAGs carried a total of 60 ARGs (ARGs-carried MAGs), dominated by the multidrug resistance genes *ade*F, *Kpn*E/F/H, and *qac*G/J (Table S3). Meanwhile, we observed a positive correlation between ARGs and VFs (R^2 = 0.73 after removing two outliers (>1.5σ)) among high-quality MAGs that had ARGs (Figure S9). We also identified multiple potential human bacterial pathogens that carry both ARGs and VFs (Figure S10). For example, MAG 029 *Escherichia/Shigella flexneri* (MAG029) from suburban snails, an opportunistic human bacterial pathogen,[50] carried the highest number of ARGs (24), mostly of the multidrug resistance genes (Table S3). Opportunistic human bacterial pathogens, *Pantoea anthophila* (MAG228) from rural, *Citrobacter* spp. (MAG016) from suburban, and *Pseudomonas putida* (MAG173) and *Staphylococcus sciuri* (MAG151) from urban snails, all carried multiple ARGs and VFs. The pathogens from giant African snails were determined as a potential threat to humans by fully deciphering their underlying ARGs and VFs.

4. DISCUSSION

4.1. Effect of Urbanization on Giant African Snails.
Urbanization increases the potential for biological invasions, which in turn facilitates the spread of zoonotic diseases in cities.[1,2] Our study underscores the impact of urbanization on the composition of the microbiome and resistome of giant African snails, demonstrating increased similarity in urban areas. This sensitivity to urbanization sets them apart from native snails and the soils. Urban environments offer new habitats and food sources, fostering snail growth and reproduction.[51] Snail behavior and biology, including feeding and reproduction, may result in heightened exposure to biological contaminants compared to soils. In addition, the gut environment of snails, with its warm and moist conditions, could provide a suitable environment for the growth and survival of pathogens. While our study highlighted the significant impact of urbanization on the gut microbiome of giant African snails, native snails in the same environment did not exhibit such changes.

Our study found a significant enrichment of total bacteria, human-associated bacteria, and ARGs in giant African snails compared to native snails, particularly in urban areas. One possible explanation for this might be that bioaccumulation occurs and subsequently shifts the gut microbiomes of these snails.[52,53] Urbanization introduces contaminants, including heavy metals, pesticides, pharmaceuticals, and biological contaminants (e.g., ARGs) into the environment, which may accumulate in snail tissues and alter their gut microbiome and resistome.[54−56] Giant African snails, being highly adaptable compared to native species, are better suited for urban environments, potentially increasing their exposure to contaminants and enhancing their invasive potential and competitiveness.[18,57] Previous studies have indicated that giant African snails serve as ideal hosts for numerous pathogens, primarily due to their extensive urban distribution

and exposure to sources such as landfill sites, waste disposal areas, and feces from homeless individuals and rodents.[10,58] The high reproductive capacity of these invasive species further contributes to the continuous increase in biologically contaminated snails, resulting in a pervasive presence of biological contaminants among urban giant African snails,[58,59] in line with the findings of our study. Conversely, native snails, which are generally smaller and slower growing, probably have a reduced capacity for bioaccumulation, leading to a lower impact on their gut microbiome.[60,61] Additionally, variations in reproductive strategies and biological traits between giant African snails and native snails could account for the observed disparities between these two groups.[62]

4.2. Harboring Human Bacterial Pathogens.
Our study revealed a marked increase in the abundance of human-associated bacteria in giant African snails residing in urban areas. We identified *Enterobacteriaceae* taxa, such as *Klebsiella pneumoniae*, *Cronobacter dublinensis*, and *Escherichia−Shigella*, in high abundances within these snails, which are frequently implicated as infectious agents in clinical settings.[63] *Enterobacteriaceae*, a common human pathogen, is widespread in these snails and ranks among the predominant pathogens affecting humans globally.[18,64] Giant African snails have an omnivorous feeding habit, favoring human and animal excrement, rotting garbage, decaying plants, and even dead snails, contributing to their carriage of a wide range of human bacterial pathogens.[17,18] The presence of parks, gardens, and other managed urban greenspaces that offer food and water sources serve as key habitats for these snails, leading to their exposure to a diverse range of food sources that allow them to acquire human-associated bacteria through various pathways.[65] Previous studies have supported this observation, indicating that the gut microbiome composition of wildlife living in urban environments is similar to that of humans.[66]

The relative abundance of *Gammaproteobacteria* in the gut of giant African snails is positively associated with the level of urbanization, suggesting that it may serve as an indicator of environmental contamination. As urbanization intensifies, *Gammaproteobacteria* in the environment thrive and subsequently accumulate within giant African snails, reflecting the increased contamination levels. A previous study reported that *Gammaproteobacteria* in the guts of soil invertebrates is a potential indicator of environmental contamination.[67] Notably, key families within this class, such as *Enterobacteriaceae* and *Xanthomonas*, exhibit significant increases in abundance in the gut of giant African snails as urbanization intensifies. Moreover, our study revealed a strong correlation between the abundance of these families and ARGs and VFs, suggesting that the increased abundance of *Gammaproteobacteria* may lead to an increase in ARGs and VFs. A recent study has shown that the gut microbiome of snails sampled in the 1980s had lower abundance of *Enterobacteriaceae* than snails sampled in the 2000s, with only 0.3% in 1980 compared to 82% in samples from 2000.[68] This finding supports the idea that the prevalence of *Enterobacteriaceae* in snail gut may be responsive to escalating urbanization over time, reinforcing the role of *Gammaproteobacteria* as an indicator of environmental contamination. Consequently, the heightened abundance of *Gammaproteobacteria* in giant African snails may be a response to the increasing levels of contaminants introduced into the environment as urbanization advances.

4.3. Harboring Antibiotic Resistomes.
Consistent with our hypothesis, we show that the gut of the giant African snails

had high resistome diversity (601 ARG subtypes) and abundance (1.53 copies per 16S rRNA gene) which is a real-world problem as this resistome confers resistance to almost all dominant types of antibiotics. Furthermore, a comparison of our results to a similar study that examined livestock manure revealed that the giant African giant snail had a higher diversity of ARG subtypes (601) than broilers (545), swine (427), laying (419), dairy cow (288), and cattle (232). Moreover, the relative abundance of total ARG in giant African snails was comparable to that of broiler manure (1.59 copies per 16S rRNA gene), which exhibited the highest relative abundance among the animal samples tested.[69] Therefore, giant African snails could be an underappreciated reservoir for ARGs that has the potential to transfer AMR from soil and other environmental substrates to humans. Moreover, our results indicate that the abundance of several high-risk ARGs was associated with urbanization, including *erm*B, *tet*M, *qnr*B, CMY-4/6/111, and *dfr*A1/12/14. Urban areas may be a potential source of transfer of antibiotic resistance to snails.[3,70] These results provide crucial insights into the complex interplay between the resistome of giant African snails, urbanization, and the spread of antibiotic resistance.

Furthermore, our study revealed that urbanization significantly shaped the snail gut resistome and enriched several ARGs including multidrug, beta-lactam, and MLS. These results highlight the potential for urbanization to serve as an agent of selection for the transfer of AMR into the gut of these snails and suggest that the accumulation of biological contaminants in more urban areas may increase the risk of transfer to humans.[55,56] The dominant ARGs carried by giant African snails were significantly different from those reported in soil, human gut, and other soil fauna. For instance, tetracycline resistance genes are ubiquitous and abundant in the human gut worldwide,[71,72] aminoglycoside and beta-lactam resistant genes have been shown to be the most abundant ARG types in urban park soil with reclaimed water irrigation,[20] and multidrug resistance genes, vancomycin, and beta-lactam resistant genes are the most diverse and dominant ARGs in collembola.[24,73] However, ARGs conferring resistance to multidrug, beta-lactam, and MLS resistance genes were found to be dominant in the gut microbiome of the giant African snails we studied. Our study revealed that multidrug resistance accounted for 60% of the total ARG abundance in giant African snails, exceeding other environmental samples such as urban streams,[74] livestock manure,[69] and untreated urban sewage.[75] This high ratio of multidrug resistance in giant African snails sets it apart from related studies and calls for further attention, especially given the increasing prevalence of multidrug-resistant pathogenic bacteria worldwide, which poses significant challenges to disease control.[75]

The greater abundance of *Enterobacteriaceae* with urbanization could contribute to the increased ARGs in giant African snails. Indeed, we identified many clinically relevant bacteria that present a global human health threat, such as the multidrug-resistant ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp) pathogens in our snail samples.[50,76] Additionally, based on the host tracking results from metagenomic assembly analysis, these ARGs are in 38% of the high-quality MAGs, including the ESKAPE pathogen *Pseudomonas aeruginosa*. Most of the ARG-carrying MAGs in the gut of giant African snails are multidrug-resistant bacteria. The emergence of large numbers of drug-resistant

bacteria in clinical settings as well as in the environment has been recognized as a global health problem.[50,77] In addition, the ARG-carrying MAGs identified as human bacterial pathogens further confirmed that these pathogens also harbored more virulence factors. Multidrug-resistant human bacterial pathogens thrive in giant African snails and can be transmitted to and from healthy humans, other animals, and the environment—especially in urban settings. It is therefore noteworthy that both high diversity and abundance of high-risk ARGs and potential human bacterial pathogens are present simultaneously in our snail samples. The gut microbiome of our giant African snails serves as an important reservoir, which could potentially transfer via horizontal gene transfer to human bacterial pathogens and contribute further to the emergence of clinical antibiotic-resistant bacteria.[19,20] Taken together, urbanization plays a significant role in increasing the biological contamination in invasive snails, associated with a higher prevalence of ARGs and potential human bacterial pathogens, thereby posing risks to public health.

5. ENVIRONMENTAL IMPLICATIONS

Our study found a correlation between the presence of ARGs and potential human pathogens in giant African snails and urbanization. Our findings demonstrate that these giant African snails in urban areas are a reservoir of ARGs and potential human bacterial pathogens and consequently pose potential risks to human health. However, limitations in distinguishing between relic DNA and viable bacteria within the gut microbiome of giant African snails were noted, emphasizing the need for more accurate methods in future studies to accurately assess the risk posed by giant African snails to human health. Urbanization may lead to increased risks of disease transmission and negative impacts, requiring greater attention to the problem of biological invasions, particularly in urban areas with zoonotic hosts, such as this snail species. Our study provides novel insights into the impact of urbanization on the gut microbiomes and resistomes of giant African snails, offering early warning signals of potential ecological and health risks posed by other invasive species.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c01233.

> Additional information on sampling sites information; physicochemical properties and metal content of soils and quality control; microbial taxonomy; bacterial communities; α-diversity; soil properties; human-associated bacteria abundance; network analysis; ARG-VF correlation; and 601 ARGs, 229 MAGs, and 30 high-quality MAGs from giant African snails (PDF)

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Notes

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