

**Tire wear particle microbiome-derived extracellular vesicles
function as potent vectors amplifying antimicrobial
resistance risk in soil ecosystems**

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Abstract

Tire wear particles (TWPs), a ubiquitous and characteristic pollutant of the Anthropocene, are emerging as a critical environmental hotspot for pathogenic bacteria and antibiotic resistance genes (ARGs). Despite concentrating these threats, the nanoscale mechanisms driving ARG dissemination from TWPs remain elusive. Here, we identify bacterial extracellular vesicles (EVs) as a critical, yet overlooked vector for resistance transfer within the TWP platisphere. We found that the TWP microhabitat stimulated a significantly higher proportion of colonizing potential pathogens to release EVs (46.3%) compared to bulk soil and water combined (7.3%). Moreover, these TWP-derived EVs were selectively enriched with critical ARGs, notably those conferring multidrug and tetracycline resistance. Functionally, we confirmed that these ARG-loaded EVs could transfer resistance traits, significantly enhancing the survival of *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* DH5 α under antibiotic stress. Together, our findings demonstrate that TWPs induce colonized pathogens to release ARG-carrying EVs, which subsequently enhance the phenotypic antibiotic resistance of recipient pathogenic bacteria. This study reveals that TWP-derived EVs serve as a bridge between environmental and clinical resistomes, representing a new urbanization-driven pathway in the antimicrobial resistance crisis.

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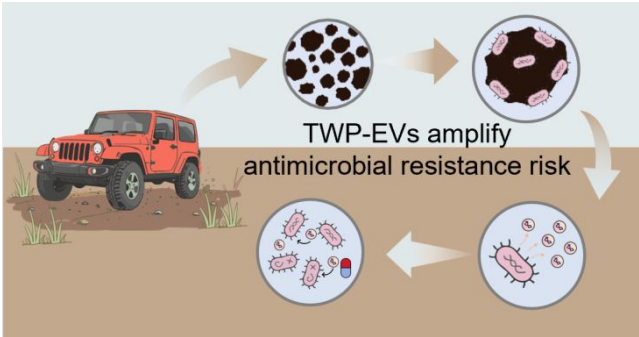
48 **TOC**

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51 **Synopsis:** Tire wear particle-derived extracellular vesicles enhance pathogen resistance,
52 revealing a new urbanization-driven pathway for antimicrobial resistance dissemination.

53 **Keywords:** Tire wear particles; Urbanization; Antimicrobial resistance; Pathogens;
54 Extracellular vesicles



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Introduction

Tire wear particles (TWPs), a major byproduct of urbanization, are accumulating globally with estimated emissions of 2.89 million tons per year¹. Resulting from tire-road friction, these particles are dispersed into aquatic ecosystems through atmospheric deposition, rain erosion, and surface runoff. Subsequently, TWPs accumulate in farming systems, particularly in paddy fields, via contaminated irrigation water and the land application of road-side sediments. The heavy environmental accumulation of TWPs poses significant ecotoxicological risks². TWPs are primarily composed of a synthetic and natural rubber matrix; furthermore, their leachates, primarily composed of toxic additives such as antiozonant N-phenyl-N'-(1,3-dimethylbutyl)-p-phenylenediamine (6-PPD), 6-PPD-quinone, and zinc, are known to cause oxidative stress, reproductive harm, and mortality in aquatic and soil organisms³. However, the ecological risks of TWPs are not limited to their direct chemical toxicity. More critically, TWPs also serve as novel microhabitats⁴ that readily adsorb co-existing pollutants and antibiotics⁵, creating unique multi-stress niches for pathogens and actively promoting the enrichment of pathogenic bacteria and antibiotic resistance genes (ARGs)^{6, 7}. While the enrichment function of TWPs is increasingly recognized, the full scope of the biological risks posed by TWP-derived pathogens-particularly the mechanisms by which ARGs are mobilized and transferred from this microhabitat-remains poorly understood⁸.

This knowledge gap is potentially bridged by bacterial Extracellular Vesicles (EVs). EVs are nanoscale, lipid-bilayer structures that act as key mediators of cell-to-cell

communication⁹. Importantly, EVs are known to function as "long-distance weapons" that can initiate or exacerbate infectious diseases¹⁰. For instance, EVs from *Pseudomonas aeruginosa* can cross the blood-brain barrier (BBB), a site the bacteria may not have yet colonized, to induce neuro-inflammation and disrupt the barrier's integrity. Moreover, EVs from various environmental settings have been found to package numerous ARGs¹¹⁻¹⁴. Consequently, the pathogen-rich TWP microhabitat likely represents an overlooked reservoir where these EV-mediated threats are concentrated. This raises a critical question: Do resident pathogens selectively package priority resistance genes into EVs, thereby establishing a novel and potent mechanism for ARG dissemination¹⁵.

To address this critical question, we (1) established a six-month microcosm experiment with TWP-treated paddy soil-water and employed metagenomics to characterize pathogens and ARGs in both TWP-derived microbiota and EVs; and (2) developed an EV-recipient co-culture model to functionally validate that TWP-derived EVs enhance pathogen survival under antibiotic stress. By functionally demonstrating that TWP-derived EVs enhance pathogen resistance, this study reveals a novel and previously overlooked pathway linking urbanization to the antimicrobial resistance crisis, underscoring the urgent need for a One Health-based assessment framework (**Fig. 1a**).

Materials and methods

Sampling and experiment design

A six-month soil microcosm incubation (January 10-July 10, 2023) was conducted in Xiamen, Fujian Province, China. For each microcosm, 3 kg of paddy soil was placed

into a plastic container and the moisture was adjusted to 75% of field capacity with sterile water. The soils were pre-incubated for two weeks to activate microbial activity. Afterward, a 5 cm water layer was maintained above the soil surface throughout the experiment by replenishing sterile water twice weekly.

To simulate the introduction of TWPs into paddy fields, approximately 8 g of TWPs were enclosed in nylon mesh bags (50 μm pore size; 6 cm $\times$ 4 cm) and positioned at the soil-water interface. This yields an environmentally relevant overall concentration of 2.67 g/kg^{16, 17}, though we acknowledge localized concentrations around the bags are inherently higher.

The TWPs used in this study were produced from a discarded car tire, with a median particle diameter of approximately 200 μm , consistent with the environmental observed size range (nanoscale to $\sim$ 220 μm)¹⁸. Prior to the experiment, the TWPs were surface-sterilized by soaking in 75% ethanol for 30 min, followed by thorough rinsing with sterile deionized water to eliminate indigenous microbial interference. At the end of the 180-day incubation, nylon bags were carefully retrieved using sterilized tweezers, opened, and the TWPs were transferred to sterile 50 mL centrifuge tubes. Surrounding soil and overlying water were also collected from physically distant areas to minimize localized TWP effects. All samples were divided into two subsamples: one for EVs extraction and the other for direct microbial DNA analysis.

Co-incubation of EVs and recipient bacteria

Co-incubation assays were performed following a previously described method¹⁹ with minor modifications. For more details, please refer to **Text S1**.

EVs isolation, purification and characterization

EVs were isolated from soil, microcosm water, and TWP surfaces using a combination of differential centrifugation and iodixanol density gradient ultracentrifugation. Briefly, pre-processing was tailored to each matrix: soil EVs were recovered by suspending the samples in PBS followed by centrifugation to remove soil particles; water was processed through sequential filtration and tangential-flow filtration (TFF); and TWP-derived EVs were collected through a standardized vortexing and shaking protocol.

For soil, 800 g was suspended in sterile PBS (1:5), shaken (180 rpm, 10 °C, 8 h), centrifuged (8,000 ×g, 20 min, 4 °C), and sequentially filtered (0.45/0.22 µm PVDF).

For microcosm water, 2 L of water was filtered through 8, 3, 0.8, 0.45, and 0.22 µm membranes. Filtrates were concentrated by tangential-flow filtration (100 kDa MWCO).

For TWP-derived EVs, surface material was rinsed, mixed with 200 mL PBS, vortexed (2 h), then shaken (180 rpm, 10 °C, 4 h); the supernatant was centrifuged (8,000 ×g, 20 min) and filtered (0.22 µm). All precleared filtrates were centrifuged (12,000 ×g, 45 min, 4 °C) and ultracentrifuged (120,000 ×g, 90 min, 4 °C) to pellet EVs.

Crude EVs were purified by iodixanol density gradients (15–45%, prepared from 60% stock in 3.6% NaCl, 10 mM HEPES, pH 7.5) and ultracentrifuged (120,000 ×g, 16 h, 4 °C). Fractions of 1.10–1.19 g mL⁻¹ were pooled, diluted 5× in PBS, and re-pelleted (120,000 ×g, 90 min, 4 °C). Given the challenges of achieving absolute EV purity from complex environmental matrices, we acknowledge these extracts are essentially "EV-enriched." For simplicity, we use the term "EVs" throughout this manuscript.

Isolated EVs were treated with DNase I to remove externally adsorbed nucleic acids,

ensuring that both the downstream metagenomic sequencing results and the phenotypic effects observed in subsequent functional assays were strictly attributable to the internal vesicle-associated cargo. Furthermore, morphology was characterized using transmission electron microscopy (TEM) following a previously described protocol with minor modifications.

Metagenomic sequencing

The DNA extraction procedures for bacteria and EVs are detailed in **Text S2**. DNA libraries were prepared and sequenced on an Illumina HiSeq 4000 platform (Majorbio Bio-pharm, Shanghai, China). More details about sequencing, taxonomy, and gene annotation can be found in the supplemental information (**Text S3**).

Antibiotic susceptibility test

Antibiotic susceptibility was evaluated following the Clinical and Laboratory Standards Institute (CLSI) guidelines. Tetracycline (Solarbio, Beijing, China) was used as the representative antibiotic for all phenotypic susceptibility assays. The suspensions were spread evenly on LB agar plates using sterile swabs. Antibiotic disks were placed on the agar surface with at least 24 mm spacing between centers. Plates were incubated at 37°C for 16-18 hours, after which inhibition zones were measured. Susceptibility was determined by comparison with CLSI reference values. All assays were performed in biological triplicates.

Statistical analysis

All analyses were performed in IBM SPSS Statistics 25 (IBM Corp., Armonk, NY) and R 4.0.2. Data are reported as mean $\pm$ SD. Group differences were evaluated by one-way

ANOVA with Tukey's post hoc test. Correlations were computed in R, and plots were produced with ggplot2. Statistical significance was set at $P < 0.05$.

Results and discussion

Pathogenic origin and virulence cargo of TWP-derived EVs

We established a TWP-treated paddy-soil microcosm to assess pathogenic traits at both the microbial and EV levels. EVs were isolated from three compartments: TWP particles, bulk soil, and water (**Fig. S1**). By comparing against pathogenic bacterial databases²⁰, we profiled potential pathogens across all samples. The diversity of both potential pathogens (208-325 taxa) and risk pathogens (49-59 taxa) among EV-associated taxa was significantly lower than in the corresponding environmental samples (289-323 and 59-63, respectively; $P < 0.05$, Welch's T, **Fig. S2**). Yet, their abundances were substantially higher ($P < 0.05$, Welch's T). On average, potential pathogens represented 20.3% of EV-associated taxa but only 1.3% of bulk microbial communities, while risk pathogens accounted for 1.8% of EV-associated taxa versus 0.4% in the bulk microbes (**Extended data 1**). The substantial enrichment demonstrates that the microbial community actively producing EVs harbors a higher pathogenic load in our experimental system. We further investigated whether this hazardous potential was universal, or if TWPs selectively foster EV-source communities as a novel microhabitat with even greater pathogenic potential.

Our investigation confirmed this selective role, revealing pronounced habitat-specific patterns. The richness of EV-source taxa from TWPs was significantly lower than from soil and water (198-236 vs 296-325, $P < 0.05$, ANOVA). However, this less diverse

community was substantially more pathogenic: In the TWP microhabitat, a remarkable 46.3% of all potential pathogens were identified as EV-source taxa, in stark contrast to only 7.3% in bulk soil and water combined (**Fig. 1b**, $P<0.05$, ANOVA). This selective enrichment extended to high-risk pathogens: their relative abundance in TWP EVs reached 3.3%, a threefold increase compared to soil-water EVs ($P<0.05$, ANOVA), despite a lower richness of detected taxa (49-53 vs 55-59, $P<0.05$). This strongly suggests that the TWP microhabitat is not a passive reservoir, but a selective niche that disproportionately promotes the release of pathogenic EVs. We attribute this modulation to the unique environmental stressors (e.g., chemical leachates and surface properties) of the TWP microhabitat, which likely trigger colonizing pathogens to substantially upregulate EV production. This enhanced release of pathogen-derived EVs may have important public health implications. Given that pathogens exploit EVs to modulate immune responses²¹⁻²⁴, our findings identify TWPs as a critical abiotic driver in the soil system. Mirroring the pathogen profile, the diversity of virulence factor genes (VFGs) was significantly lower in TWP-derived EVs than in soil-water EVs ($P<0.05$, ANOVA). Yet, the relative abundance of VFGs was significantly higher in TWP-derived EVs ($P<0.05$, ANOVA, **Fig. S3**). Crucially, the magnitude of this enrichment in EVs far exceeded that of the source communities. Specifically, the VFG abundance in the bulk microbial communities of the TWP habitats showed a moderate enrichment of only 1.9-fold compared to the soil-water environment. However, this modest concentration was dramatically amplified within the EV fraction, where the VFG abundance in TWP-derived EVs was 6.1-fold higher than the soil and water-

derived EVs. This stark discrepancy suggests that VFG abundance in TWPs-EVs is not driven solely by the genetic profile of the source community, but likely involves a selective packaging process or the preferential vesiculation of VFG-carrying bacteria. Previous assessments of TWP-associated ecological risks have mainly focused on chemical pathways, such as the leaching of additives like 6-PPD^{25, 26}. Our findings reveal a previously overlooked biological dimension, in which pathogenic bacteria colonizing tire surfaces release VFG-enriched EVs, thereby escalating the ecological risk profile of TWPs from chemical contaminants to microbially mediated health threats.

Prevalence and composition of ARGs in TWP-associated EVs

To assess the associated resistance risk, we next profiled ARG composition and abundance in TWP-derived EVs. Based on SARGs database annotation, 45-82 ARGs were detected in EVs. EV-borne ARGs were dominated by multidrug, β -lactam, triclosan, aminoglycoside, and tetracycline classes (**Fig. 1c**). Specifically, multidrug resistance subtypes such as *adeJ*, *adeK*, and *adeI* exhibited the highest relative abundances (**Fig. 1d**). EVs from different habitats carried distinct ARG loads. Spatially, TWP-derived EVs contained significantly higher ARG abundances than those from bulk soil and water (one-way ANOVA, $P < 0.05$; **Fig. 1c**) with multidrug and tetracycline categories showing the most pronounced increases (Welch's ANOVA, $P < 0.05$; **Fig. 1c**). This pattern suggests that EVs derived from TWPs represent potential hotspots and amplifiers for the selection and enrichment of ARGs. Mechanistically, dense biofilms forming on TWP surfaces likely enhance cell-cell interactions and horizontal gene transfer, thereby increasing ARG load²⁷⁻²⁹. In parallel, co-selection

pressures from organic contaminants and metals accumulating on TWP surfaces may induce oxidative stress³⁰⁻³². Such stress has been reported to promote EV biogenesis and selective ARG packaging^{25, 33-36}, thereby further boosting ARG abundance in TWP-derived EVs. Unlike bulk soil, which is largely stationary, TWPs are highly mobile particles that are readily transported by surface runoff and atmospheric pathways³⁷. Because EVs can mediate interspecies gene transfer, enabling their cargo to cross taxonomic barriers, TWP-EVs may act as vehicles that disseminate their ARG cargo over broader spatial scales³⁸. As TWPs migrate along the urban road-surface runoff-agricultural irrigation continuum, they link these environments into a coupled human-environmental transmission chain. However, because our extracts are essentially "EV-enriched," these effects might be overestimated. Developing higher-purity isolation methods for environmental EVs is essential for precise future evaluations. Given the selective enrichment of clinically relevant multidrug and tetracycline resistance genes, these findings underscore a critical healthcare risk: TWP-EVs likely constitute a stealthy and pervasive vector for antimicrobial resistance, efficiently bridging environmental reservoirs with human exposure pathways.

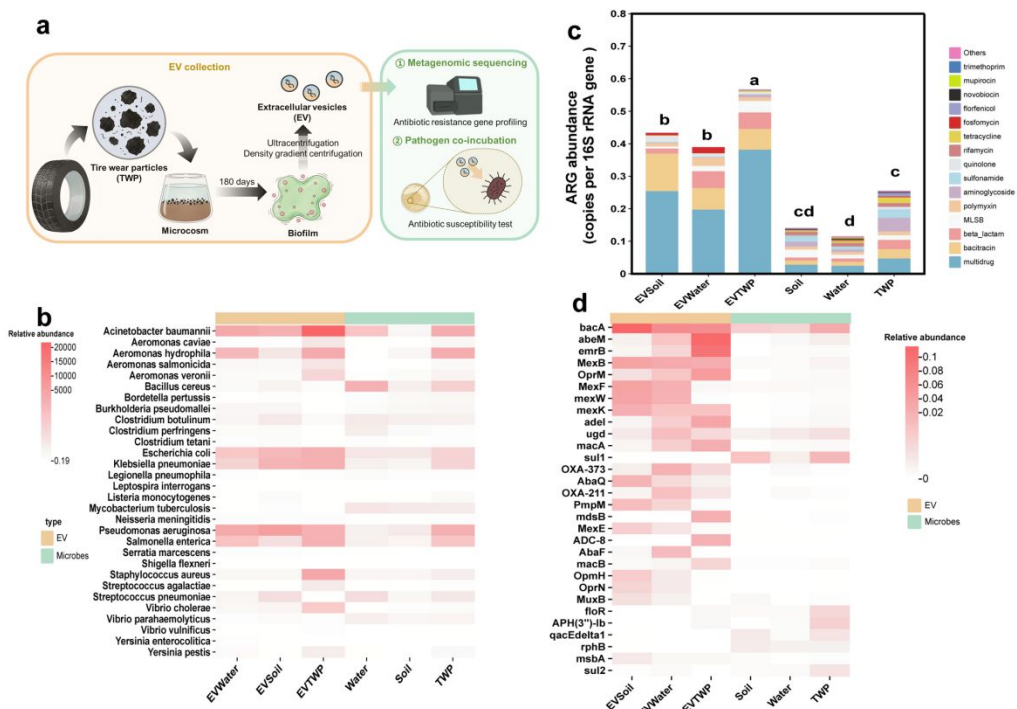


Fig. 1. Enriched pathogenic profiles and ARG loads in TWP-derived EVs.

(a) Schematic illustration of the experimental procedure. TWPs were introduced into a paddy microcosm system and incubated for 180 days. EVs were subsequently extracted from the tire-associated environment. The collected EVs were analyzed via two approaches: (i) metagenomic sequencing to determine the ARG profiles, and (ii) co-incubation of TWP-derived EVs with pathogenic bacteria followed by antibiotic susceptibility testing to assess resistance acquisition. (b) Heatmap depicting the relative abundance of the top 50 potential pathogen species ranked by total abundance across all samples. (c) Total relative abundance of different ARG types in EVs and microbes from various habitats, expressed as copies per 16s rRNA gene. Bars indicate mean values, and error bars represent standard deviation. Different lowercase letters above the bars indicate significant differences in total ARG relative abundance among groups (one-way ANOVA with Tukey's post hoc test, $P < 0.05$). Groups sharing at least one

common letter are not significantly different ($P > 0.05$). (d) Distribution and expression of top 30 abundant ARG subtypes.

TWP-derived EVs promote pathogen growth under antibiotic stress

Building upon genomic evidence identifying TWP-derived EVs as higher-risk reservoirs for ARGs and pathogens compared to other environmental media, we next performed functional assays to verify whether this genomic potential translates into phenotypic resistance. Specifically, we examined whether TWP-derived EVs can promote antibiotic resistance in potential pathogens. We co-incubated TWP-EVs with *P. aeruginosa* PAO1 and *E. coli* DH5 α . We then measured tetracycline susceptibility via disk diffusion, quantified by inhibition-zone diameters. Tetracycline was selected because tetracycline-class ARGs are enriched in TWP-derived EVs versus soil-water EVs. Crucially, exposure to TWP-EVs induced a significant reduction in inhibition-zone diameters for both *P. aeruginosa* PAO1 ($P < 0.05$, t test; **Fig. 2a, c**) and *E. coli* DH5 α ($P < 0.05$, Welch's t-test; **Fig. 2b, d**). Furthermore, TWP-EVs significantly upregulated genes associated with clinical antibiotic resistance mechanisms (e.g., encoding efflux pumps and membrane permeability proteins)³⁹ (**Figure S4**). These results suggest that TWP-derived EVs facilitate the survival of pathogenic microorganisms under antibiotic pressure. This may be attributed to three EV-mediated mechanisms: (1) potential HGT of ARGs; (2) induction of stress response pathways in recipient bacteria via EV-contained compounds; and (3) direct delivery of functional resistance-conferring enzymes. Notably, unlike conjugation or transduction-which depend on cell-cell contact or phage host specificity-EVs can confer resistance through

both HGT-dependent and HGT-independent routes, positioning them as versatile vectors that may complement classical HGT mechanisms in environmental hotspots such as the TWP plastisphere. Such functional acquisition of resistance is clinically concerning, as it directly increases the likelihood of antibiotic treatment failure and exacerbates the healthcare burden^{40 41}. Together, these findings indicate that TWP-derived EVs function as an emerging urbanization-linked vector that bridges environmental and clinical resistomes, amplifying threats under the One Health framework. This threat is further compounded by their remarkable mobility; with studies showing environmental EVs can migrate up to 15 meters in soil matrices, TWP-EVs pose a severe risk as long-distance vectors for ARG dissemination⁴². Future studies incorporating a wider range of antibiotics and pathogens will be essential to fully evaluate these clinical risks. Additionally, exploring TWP-EVs at various time points remains essential to evaluate the short-term risks of TWPs upon their initial release into the environment.

In summary, this study provides new evidence for a specific EV-mediated pathway by which TWPs accelerate the dissemination of ARGs. Our findings from the microcosm and functional co-culture models demonstrate that the TWP microhabitat acts as a potent driver, significantly inducing the release of ARG-enriched EVs from colonized pathogens. This EV-mediated transfer subsequently results in a functional enhancement of phenotypic antibiotic resistance in recipient bacteria. As TWPs continuously wash from urban roads via surface runoff into agricultural irrigation systems, this physical transport chain introduces these localized biological risks into broader ecosystems. By

identifying TWP-derived EVs as a critical bridge between the environmental and clinical resistomes, our work reveals a novel, urbanization-driven pathway that exacerbates the antimicrobial resistance crisis. Moving forward, incorporating this biological pathway into comprehensive risk assessments will be essential for informing robust public health strategies within the One Health framework.

While this study characterizes the risks associated with a mature and stable platisphere, further investigation into the effects during the initial and long-term phases of TWP exposure remains essential for a comprehensive evaluation.

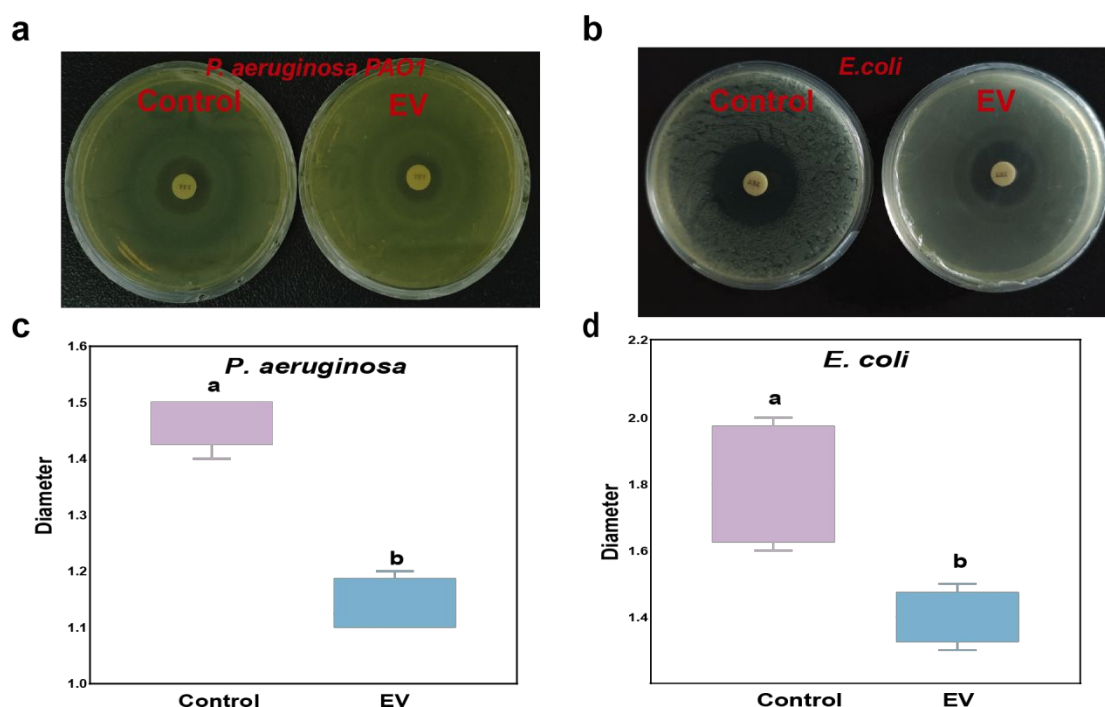


Fig. 2. TWP-derived EVs enhance the antibiotic resistance of potential pathogenic bacteria. (a, b) Reduction in tetracycline inhibition zones of *P. aeruginosa* PAO1 (a) and *E. coli* DH5α (b) following the addition of TWP-derived EVs compared to the Control (CK). (c, d) Quantitative comparison of inhibition zone diameters for *P. aeruginosa* PAO1 (c) and *E. coli* DH5α (d) treated with or without TWP-EVs. Different

lowercase letters above the bars indicate significant differences ($P < 0.05$) as determined by t test. All experiments were performed in triplicate, and data are presented as mean $\pm$ standard deviation (SD).

Author Contributions

Y.F.Q, and D.Z developed and designed the research. Y.F.Q, J.Y.P and F.Y.L, performed the experiments. Y.L, J.Y.P, and Q.S.H contributed to sample collecting. Y.F.Q and D.Z wrote the manuscript. Y.F.Q., L.Q.S., and H.Z.L. performed the visualization. D.Z, Z.G.Y., and Q.S.H edited and revised the manuscript. All authors reviewed and approved the results and the revisions made to the manuscript.

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Data availability

The datasets generated during the current study are available in the [NCBI Sequence Read Archive (SRA)] repository, [accession number: PRJNA1198724].

Supporting information

Supporting Information: Additional experimental details, materials, and methods, including co-incubation, DNA isolation, and metagenomic sequencing; EV

characterization; pathogen diversity and VFG abundance in EVs versus environments; and recipient bacteria transcriptional responses.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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