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Understanding the spread of antibiotic resistance in vegetables cultivated with sewage sludge: implications for food safety and human health

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Abstract

The conventional practice of using sewage treatment plant (STP) derived sludge as a fertilizer poses significant negative impacts on agroecosystems. Since sludge has diverse contaminants, including heavy metals (HMs), antibiotics (ABs) and antibiotic resistance genes (ARGs), its application in the agricultural fields contaminates the food and hence causes human health risks via the food chain. The transfer of ABs and ARGs from sludge to soil and then to plants can influence the development of antibiotic resistance (AR) in plant endophytes, and leads to variations in their characteristics. In a pot experiment, vegetable carrot (*Daucus carota*) and spinach (*Spinacia oleracea*) were amended with sludge samples from three sewage treatment plants (STPs) with varying treatment capacities and both above and below-ground parts of the plants were analysed for the presence of specific ABs (amoxicillin, azithromycin, chloramphenicol, ciprofloxacin, tetracycline), ARGs (*blaCTX-M, blaGES, blaNDM, ermF, qnrS, Sul1*), and mobile genetic elements (MGEs) (*intl1*, *IS26*). Among the characterized culturable endophytic bacteria (EB), 22 exhibited resistance to various antibiotics (highest against ampicillin, ciprofloxacin, chloramphenicol) and heavy metals (highest against lead, nickel, and chromium). Most importantly, seven multiple antibiotic-resistant endophytic bacteria (MAREB) exhibited resistance to all tested heavy metals (HMs). Additionally, all MAREB tested positive for biofilm production, and a notable proportion (72.72%) of these endophytes displayed mobility, with strong auto-aggregation ranging from 16.67 to 92.61%. The biofilm formation dynamics among these MAREB exhibited a Gaussian distribution pattern, increasing with higher antibiotic concentrations. Notably, five MAREB demonstrated survival at clarithromycin concentrations up to 150 μg ml^{-1}. The study revealed the presence of ABs (μg kg^{-1}) and ARGs (copies kg^{-1}) in all parts of both vegetables, ranging from 2.87 to 314.88 and 1×10^{5} to 3.2×10^{10}, respectively. MAREB displayed various advantageous features to support plant growth under different stress conditions. Moreover, 51.09% of the identified EBs were reported as both plant and human-associated pathogens, and 9.09% were solely human pathogens. Transfer factor (TF), translocation factor (TLF), and bioconcentration factor (BCF) values were correlated with higher ABs and ARGs abundance in the root and shoot compartments of both vegetables. The risk assessment for ABs and ARGs highlighted children are particularly vulnerable to prolonged adverse health risks from consuming these vegetables. Therefore, this research is imperative for understanding the co-selection mechanisms, the need for improvement of the existing treatment systems in contaminants removal, and the evaluation of the presence of ABs and ARGs in sludge before its application in agricultural fields.

Keywords Sewage sludge, Antimicrobial resistance, Endophytic bacteria, Co-selection, Risk assessment

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Introduction

Ever since the discovery of penicillin in 1928, antibiotics have been used extensively in aquaculture, veterinary medicine, and human medicine worldwide to prevent or treat microbial infections (Gworek et al. 2021). Most notably, on a global scale, human consumption amounts to 15.7 defined daily doses (DDDs) per 1,000 individuals (Sriram et al. 2021). Global antibiotic consumption by humans increased by 65% from 2000 to 2015, and if present patterns persist, it could surge by 200% by the year 2030. (Scaria et al. 2021). The fact that exposure of pathogens to antibiotics encourages the selective proliferation of resistant bacteria is widely recognized. Thus, excessive or imprudent use of antimicrobials is one of the prime factors contributing to the global development of antimicrobial resistance (AMR). Globally, an estimated 1.27 million deaths due to bacterial AMR were reported in 2019 (Murray et al. 2022). Without effective interventions to address AMR, projections suggest that this figure could rise to around 10 million deaths annually by 2050 (Salam et al. 2023; Murray et al. 2022). In North America, annual infections linked to antibiotic resistance surpass 2 million cases, resulting in 23,000 deaths (US Department of Health and Human Services, CDC 2017). Similarly, Europe sees over 700,000 instances of antibiotic-resistant infections annually (Cassini et al. 2019), leading to 33,000 deaths and an economic burden of €1.5 billion (European Centre for Disease Prevention and Control 2009). With a 36% surge in antibiotic use from 2000 to 2010, global mortality due to infectious diseases now stands at 20% (Laxminarayan et al. 2016; Martens et al. 2017). In the United States, over 2.8 million antibiotic-resistant infections occur annually, causing more than 35,000 deaths (Annual Epidemiological Report 2020). In India, child mortality due to antibiotic-resistant bacterial infections occurs every nine minutes, with over 50,000 infants at risk of sepsis from ineffective treatments (Subramanium and Girish 2020).

Antibiotics are not fully metabolized within the body, and a substantial portion (ranging from 17 to 90%) administered to humans and animals is excreted without changing (Carvalho et al. 2016). Consequently, these partially metabolized compounds are excreted and make their way into sewage systems and sewage treatment plants (STPs). Antibiotics have different half-lives, ranging from 0.43 to 3466 days, but most last less than 100 days (Cycoń et al. 2019). Nonetheless, numerous studies have indicated that the treatment methods employed in sewage treatment plants (STPs) are inadequate for the complete removal of heavy metals (HMs), antibiotic residues, antibiotic-resistant bacteria (ARBs), and antibiotic resistance genes (ARGs) (Tan et al. 2023). Consequently, these substances tend to accumulate in the STP-derived sludge samples, posing potential hotspots for the selection of ARBs and ARGs and their subsequent release into the environment (Gupta et al. 2022). Sewage sludge, a semi-solid residue generated after the wastewater treatment process, has a significant capacity to adsorb antibiotics, promoting their accumulation. Consequently, managing sewage sludge in an environmentally beneficial manner proves extremely challenging.

On a global scale, approximately 50 million tonnes of sewage sludge are produced annually. Projections suggest a 24% rise by 2030 and a 51% increase by 2050 (Ehalt Macedo et al. 2022). In India, the estimated generation of sewage sludge is 7.34 kg capita^{-1} year^{-1} or 144 kg per million litres of sewage daily (Belani et al. 2023). The production of sewage sludge is expected to reach 150–200 million tonnes worldwide by 2025, up from an estimated current production of 100–125 million tonnes (Vaithyanathan and Cabana 2021). Sewage sludge is typically disposed off through landfill or incineration. However, the Council Directive 86/278/EEC (CEC 1986) promotes agricultural reuse to prevent adverse impacts on soil, vegetation, animals, and humans. The escalating production of sludge, coupled with stringent disposal regulations, presents challenges for waste management authorities in ensuring proper sludge management (Sharma et al. 2022a, b), placing substantial strain on the environment. It is now recognized as a significant contributor to the growing environmental issue of antibiotic resistance, fuelled by antibiotic-resistant bacteria (ARBs), antibiotic resistance genes (ARGs), mobile genetic elements (MGEs), along with antibiotics (ABs) and their metabolites (Zhang et al. 2022). Despite some researchers highlighting potential risks of soil contamination by pathogens, heavy metals, and emerging contaminants found in the sludge (Wu et al. 2023).

Numerous prior studies have consistently highlighted a significant prevalence of multidrug-resistant bacteria (MDR), ARGs, and MGEs in STPs and their effluents like reclaimed wastewater and sludge (Zheng et al. 2023). Additionally, several researches have also revealed elevated levels of ABs, ARGs, ARBs, and HMs in agroecosystems fertilized with sewage sludge compared to unamended environments (Urbaniak et al. 2024). Soil is recognized as a substantial reservoir of antibiotics and ARGs originating from both natural and human-induced activities, as the application of sludge directly introduces ARBs and ARGs into the soil (Rathinavelu et al. 2024). The World Health Organization (WHO) has highlighted ARGs as a significant public health issue (Hembach et al. 2022). ARGs dissemination isn't solely driven by antibiotics; various other factors are also involved. MGEs, often found near ARGs, facilitate their transfer to other microbes via horizontal gene transfer (HGT) (Li et al.

2024). For example, the intl1 and IS26 can capture and mobilise ARGs from the environment and integrates them into gene cassettes (Wang et al. 2024a, b). Environmental intl1, IS26, and ARG-carrying bacteria are concerned about their potential to transfer ARGs. Furthermore, bacteria that possess heavy metal resistance genes (HMRGs) frequently also harbour ARGs, as environmental HMs serve as factors that favor the selection of ARGs (Yan et al. 2024). Even after discontinuation of its use, ARGs can persist in the soil for extended periods, potentially transferring to the plant microbiome and entering the food chain (Xiao et al. 2023). The accumulation and spread of ARGs in the natural environment pose a risk of transferring these genes to pathogenic bacteria in clinical settings, potentially compromising the effectiveness of antibiotic treatments (Cherian et al. 2023). Fruit vegetables and cereal crops have demonstrated a lower capacity for the uptake of contaminants of emerging concern (CECs) compared to leafy and root vegetables (Christou et al. 2019). Consequently, the consumption of vegetables serves as a potential route of exposure to bacteria-carrying ARGs and pathogens (Klaui et al. 2024).

While previous studies have explored the presence of ABs and ARGs in STPs and effluent water (Li et al. 2023; Zhao et al. 2024), some research has touched upon the effects of irrigation water and animal manure on plant ABs and ARGs uptake (Gudda et al. 2023; Abdellah et al. 2023), while others have investigated the impact of wastewater and animal manure on the levels of ARGs and ARBs in farmland (Wang et al. 2024a, b; Granados et al. 2024). Another recent study by Bhattacharjee et al. (2024) also highlighted the role of earthworms in ARGs transmission in soil–plant systems. However, these investigations have often neglected to fully consider the critical role that sludge may play in fostering AMR by influencing the characteristics of the endophytes. The rhizosphere, known as the most nutrient-rich region around a plant, can facilitate the proliferation of bacterial population, potentially leading to the transmission of antibiotic resistance determinants from sludge to plants through endophytes in various ways (Gao et al. 2020). Several studies have documented antibiotic resistance in endophytic populations in crops fertilized with manure (Zhang et al. 2023). However, it remains unknown whether the antibiotic resistance of endophytic populations can be directly influenced by environmental antibiotic pollution, especially in the edible parts of vegetables. Additionally, the impact of amending biosolids on the phenotypic antibiotic susceptibility profiles of these resistant endophytic bacteria is unclear, and there is a close relationship between endophytes and human pathogens (Karmakar et al. 2019). The consumption of raw food products or vegetables, such as salads, may pose risks to human health (Lepecka et al. 2022). While there is ample evidence regarding the contamination of soils amended with sludge by ABs and ARGs, there is a lack of information concerning the crucial connection between the uptake of ABs and ARGs by vegetables and their mobility. This scarcity of data makes it challenging to draw definitive conclusions about the potential human health impacts of sludge application. Therefore, this study aims to unravel the intricate relationships between sludge characteristics, ABs/ARGs exposure, and the transmission of AMR from soil to endophytes and ultimately to humans. Therefore, the study gave insights into the potential convergent evolution induced by sludge exposure, as well as the elucidation of the pathogenic characteristics of resistant endophytes. The examination of virulence properties, such as auto-aggregation, motility, and biofilm formation dynamics under varying antibiotic concentrations and exposure times, provided a holistic perspective of the environmental implications of antibiotic resistance, particularly concerning sludge-amended soil–plant systems.

Understanding these aspects is crucial for comprehending the spread of antibiotic resistance in agroecosystems. To fully grasp the dynamics of antibiotic resistance transmission from soil to plants to humans, it is essential to understand the various factors driving the transmission of AB and ARGs after land application. Beyond the scientific implications, this study may provide valuable insights for policymakers, agricultural stakeholders, and public health authorities and highlight the economic significance by emphasizing the tangible benefits of addressing AMR in agricultural settings. In addition, the findings may have the potential to safeguard agricultural productivity, reduce healthcare costs associated with antibiotic-resistant infections, and ensure the sustainability of food production systems. Hence, interdisciplinary collaboration and targeted intervention aimed at mitigating the economic burden of AMR while safeguarding public health and food security on a global scale are crucial. Therefore, this study aims to fill the gap in the existing knowledge with the objectives (a) to examine the prevalence of ARGs in the sludge-amended soils and in the below and above-ground plant parts, (b) identify the characteristics of endophytic bacterial isolates from soil-grown vegetables, (c) assess the potential risks to human health linked with ARGs and ABs in the vegetables, and (d) examine the factors that influence the dissemination of antibiotic resistance within soil–plant ecosystems.

Materials and methods

Experimental design and sample collection

Three distinct sewage treatment plants, STP1 (Bhagwanpur) (25.27274 N, 83.00519 E), STP2 (Dinapur)

(25.34762 N, 83.04844 E), and STP3 (Dinapur) (25.34762 N, 83.04844) of Varanasi city, India, were chosen for the study. The selected plants exhibit varying treatment capacities, with STP1 capable of treating 8 million litres per day (MLD), STP2 with a capacity of 80 MLD, and STP3 having the highest treatment capacity at 140 MLD. During September 2022, dried sewage sludge samples were collected in triplicate from the drying tanks of each plant, transported to the laboratory, and stored at 4 °C. For analyses, samples were dried at 60 °C in an air oven, crushed, and sieved through a 4 mm sieve before characterization and examination for evaluating different parameters. Pot experiments were conducted from October 2022 to January 2023 using soil obtained from Banaras Hindu University's agricultural research farm, Varanasi (25°18′ N, 83°03′ E), an area untouched by sewage sludge or wastewater irrigation for the past five years. The sandy loam soil was collected from the surface (0–30 cm) and screened through a 4-mm mesh before use. Four treatment groups, each with three replicate pots, were designed for two selected vegetables, either including or excluding sewage sludge (25%) addition in soil. In this research, Carrot (*Daucus carota*) and spinach (*Spinacia oleracea)* were chosen due to their wide consumption as raw or lightly cooked root and leafy vegetables. Each clay pot (30 cm diameter, 25 cm height) was initially sown with eight spinach and carrot surface sterilized seeds, with only the three healthiest plants kept after germination. Approximately 4.0 kg of soil was employed in each pot, followed by overnight incubation and watering. Watering (80–100 ml) based on water-holding capacity occurred every third day, with no pesticide or fungicide use and manual weed removal. Soil and vegetable samples were collected 81 days post-sowing. Harvested vegetables were transported in individual bags, stored in a chiller, and processed for molecular and bacteriological analyses.

Soil and sludge physicochemical properties and DNA extraction

Fresh sludge samples underwent physicochemical analysis as follows: pH and electrical conductivity (EC) were determined in 1:2.5 (soil:water) suspensions using methods outlined by Jackson (1973). EC was measured using a digital conductivity meter (Systronics, India), while pH was assessed using a pH meter (CyberScan pH 510, Eutech Instruments Pte Ltd). Bulk density (BD) and water holding capacity (WHC) were measured according to the method proposed by Blake (1965) and Piper (1945), respectively. For nutrient analysis, organic carbon (OC) was determined using the Walkley and Black method (1934), total nitrogen (N) was quantified using micro-Kjeldhal techniques (Jackson 1958), available phosphorus (AP) was assessed via the ascorbic acid method (Olsen et al. 1965), and available potassium content was measured using the 1 N ammonium acetate method (Nelson and Heidel 1952). To analyze total micronutrients, heavy metal/metalloids, and sludge samples were digested using aqua regia (HCl: HNO3; 3:1) as described by Nieuwenhuize et al. (1991). Subsequently, heavy metals (Cd, Cr, Co, Ni, and Pb), micronutrients (Cu, Mn, Fe, and Zn), and metalloid (As) levels were determined using an Atomic Absorption Spectrophotometer (AAS) (Agilent FS-240) following the method outlined by Lindsay and Norvell (1978). Soil and sludge DNA was isolated using a FastDNA Spin kit for soil (MP Biomedicals, CA, USA) as per the manufacturer's protocols, while phyllosphere and root DNA were extracted using a DNeasy Plant Mini kit (Qiagen, Hilden, Germany). The DNA concentration and quality were verified utilizing a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific) following extraction and 1.2% agarose gel electrophoresis was employed to corroborate the results. The isolated DNA was kept at − 20 °C until further examination.

Isolation and enumeration of total cultivable bacteria (TCB) and endophytic bacteria (TCEB)

Total soil bacterial count was determined via serial dilution (10^{-5}–10^{-8}) method in 0.9% saline and spread plating on Luria Bertani (LB) agar plates supplemented with cycloheximide (50 g ml^{-1}) (Patra et al. 2024). After 48 h of incubation at 37 °C, colony-forming units (CFU) were enumerated in triplicate. Isolation of TCEB from vegetable samples has been done by using the method described by Chebotar et al. (2022) with slight modification; vegetables were completely cleaned under running tap water, air-dried, and then immersed in 20% hydrogen peroxide for 30 min before being rinsed three times in sterile milli-Q water for 3 min. They were then submerged in 70% ethanol for 1 min before being washed, as described above. Sterilized samples were confirmed by applying wash water to LB agar and checking for colony development. Subsequently, 3 g of disinfected vegetables were finely diced and pulverized with quartz sand in a sterile mortar. The resulting tissue was mixed with 10 ml of sterile saline water and subjected to serial dilution up to 10^{-4}. Each 0.1 ml of the diluted solution was plated on LB agar and incubated for 3 days at 28 °C, and CFUs of total cultivable endophytic bacteria (TCEB) were enumerated. Single colonies were selected, examined for purity under a microscope, and stored at − 80 °C in sterile 50% glycerol for subsequent analysis.

Antibiotic resistance pattern analysis in EB

Fifty-four EB isolates were selected based on distinct colony appearance. Antibiotic susceptibility (AST) for 17 antibiotics was measured using the Kirby Bauer Disc Diffusion technique. Overnight-grown culture broth (0.1 ml) from the log phase was swabbed on Muller Hinton Agar plates using a sterile cotton swab (Hi-media, PW1184). Discs of erythromycin (E) 15 µg, ampicillin (A) 10 µg, penicillin-G (P) 10U, chloramphenicol (C) 30 µg, cefoxitin (FOX) 30 µg, kanamycin (K) 30 µg, ciprofloxacin (CF) 5 µg, cotrimoxazole (CO) 30 µg, gentamycin (G) 10 µg, ofloxacin (OFX) 5 µg, rifampicin (RIF) 5 µg, streptomycin (S) 10 µg, tetracycline (TE) 30 µg, imipenem (IPM) 10 µg, azithromycin (AZM) 15 µg, vancomycin (VA) 5 µg, norfloxacin (NX) 10 µg, procured from HI Media, and zones of inhibition were measured after 24 h of incubation at 37 °C. Recent Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines were followed to categorize isolates as resistant (R), intermediate (I), or susceptible (S). Multiple antibiotic-resistant endophytic bacteria (MAREB) were those resistant to at least three antibiotic classes, and the multiple antibiotic resistance index (MARI) was determined for each isolate. MARI readings over 20% indicate a high-risk contamination source.

Heavy metal tolerance, auto-aggregation, co-aggregation, motility, dynamics of biofilm formation assay of MAREB isolates

Heavy metal tolerance assay was performed according to a slightly modified, previously published protocol (Gupta et al. 2012), shortly MAREB were tested for tolerance to ten heavy metals using LB medium (4 ml) containing increasing concentrations of metal salts (Pb $(NO_3)_2$, $CuSO_4{\cdot}5H_2O$, $MnSO_4{\cdot}H_2O$, $K_2Cr_2O_7$, $CdCl_2$, $CoCl_2{\cdot}6H_2O$, ZnSO4·7H2O, $HgCl_2$, $NiCl_2{\cdot}6H_2O$, and $AgCl_2$) at concentrations ranging from 0.25 to 1.5 mg ml^{-1}. Tolerance was evaluated by observing growth within a 12 to 36 h window, with absorbance readings at 600 nm surpassing 0.6 after 36 h of incubation at 37 °C, indicating a positive result. The minimum inhibitory concentration (MIC) was determined to be the lowest concentration of heavy metals that hindered bacterial growth. Each experiment was conducted three times, and average data were reported.

Auto-aggregation, co-aggregation, motility, and dynamics of biofilm formation assays of MAREB were performed by the standard method described in our previous research (Patra et al. 2024). The antibiotics selected for the biofilm formation assay were Amoxicillin, Azithromycin, Ciprofloxacin, Chloramphenicol, and Tetracycline, as these are commonly used in the studied area. Additionally, Vancomycin and Clarithromycin were included as last-resort antibiotics and anti-biofilm agents.

Molecular detection of ARGs, metal resistance genes (MRGs) and mobile genetic elements (MGEs) in MAREB isolates

Genomic DNA from endophytic bacterial isolates was extracted using the MasterPure Complete DNA & RNA Purification Kit (Lucigen, USA). Plasmid DNA extraction utilized the QIAprep Spin Miniprep Kit (Qiagen, Germany), following the manufacturer's instructions. Qualitative PCR was conducted to identify genes associated with antibiotic resistance *rpoB516* (Rifampicin), *rpoB526* (Rifampicin), *rpoB531* (Rifampicin), *blaTEM* (ampicillin), *blaGES* (ampicillin), *blaCTX* (ampicillin), *tetQ* (tetracycline), *tetM* (tetracycline), *tetW* (tetracycline), *Sul1* (cotrimoxazole, erythromycin), *ermF* (erythromycin), *blaNDM* (imipenem), *qnrS* (ciprofloxacin), along with *16 s rRNA* and mobile genetic elements (MGEs) like *IS26* and class 1 integron (*intl1*) gene and heavy metal resistance *merA* (mercury), *silE (silver)*, *cZcD* (cobalt, zinc, cadmium), *nikA* (nickel), *copB* (copper), *arsA* (arsenic) (Nurpermatasari et al. 2018; Wu et al. 2007; Stedtfeld et al. 2018; Aminov et al. 2001; Hu et al. 2008; Enne et al. 2001; Garder et al. 2014; Colmer-Lluch et al. 2014; Smith et al. 2006; Wu et al. 2014b, a; Barraud et al. 2010; Liebert et al. 1997; Percival et al. 2008; Roosa et al. 2014; Margaryan et al. 2013; Argudin et al. 2013; Yang et al. 2018). PCR was performed as follows: initial denaturation at 94 °C (5 min), 35 cycles of denaturation (1 min), and annealing (1 min) at the temperatures listed in Supp. Table S2, extension (30 s) at 72 °C, and a final extension step (7 min) at 72 °C. Gel electrophoresis (1.2%) was exercised to examine amplified PCR results with the appropriate DNA ladder.

Characterization of endophytic bacteria for beneficial traits

The endophytic behavior of the test bacterium was investigated with adaptations from the method described by Bressan and Borges (2004), employing spinach as the host plant. Seeds, sanitized on their surfaces, were soaked for 12 h in a 10 ml solution of EB (OD 0.5 at 600 nm) with 0.25% carboxymethyl cellulose (CMC). Control seeds were treated similarly but soaked in sterile water with CMC. Following this, seeds were plated according to the protocol outlined by the International Seed Testing Association (ISTA 2006) and incubated under controlled conditions: 14 h light, 10 h dark, 65% relative humidity, at 25 ± 2 °C, and monitored daily for 7 days. Upon completion, seedlings were sampled, surface sterilized, and crushed in 0.1 M PBS. The resulting solution was serially diluted to 10^{-4} and plated on LB agar supplemented

with antibiotics resistant to the specific EB strain. Incubation at 37 °C facilitated the observation of bacterial colonies, with results compared against control samples. The identity of the bacteria was confirmed through morphological and biochemical assays. Assays, including IAA production, root colonization, phosphate solubilization, siderophore production, cellulose production, HCN production, protease production, ACC deaminase, in vitro antagonism, and biofilm production, were conducted in triplicate following established methods described in Karmakar et al. (2019).

Molecular identification of MAREB isolate phylogenetic analysis

Genomic DNA was extracted and utilised to amplify the V1-V9 region of the 16S rRNA gene via standard PCR with universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGATT-3′). Amplification was confirmed on a 1.2% agarose gel. Purification of PCR products was done using the MinElute PCR purification kit, followed by sequencing with an Applied Biosystems 3500 Genetic Analyzer. Raw sequences were edited and assembled using Auto-Assembler. Consensus sequences (~1500 bp) were compared to the National Centre for Biotechnology Information (NCBI) Gen Bank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST), and the identified sequences were submitted to Gene Bank. Similar bacterial strains were selected from NCBI, and all sequences were aligned using MEGA 11.0 software (Tamura et al. 2021) for multiple sequence alignment. The phylogenetic tree was constructed using the neighbor-joining statistical method with bootstrap values derived from 500 replications in MEGA 11.0 software. The final tree visualization was done using the Interactive Tree of Life (iTOL) version 6.0 from the European Molecular Biology Laboratory (EMBL) web tool (https://itol.embl.de/).

Extraction and quantification of the Antibiotics

Antibiotics (purity > 98%) (amoxicillin, azithromycin, chloramphenicol, ciprofloxacin, tetracycline) and formic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Solvents (HPLC grade) (methanol, acetonitrile and water) were purchased from Merk (Darmstadt, Germany). Each antibiotic (10 mg ± 0.1 mg) was carefully weighed and then transferred to a 25 ml volumetric flask containing approximately 10 ml of solvent, with the concentration calculated accounting for the compound's purity. Specifically, azithromycin was dissolved in a solution of methanol, water, and formic acid (in a ratio of 50:49.99:0.01), while the other antibiotics were dissolved in a solution of acetonitrile, water, and formic acid (in a ratio of 50:49.99:0.01). Firstly, stock standard antibiotics (1000 µg ml^{-1}) solution was prepared and then subsequently diluted in different concentrations (750, 500, 250, 100, 50, 25, 10, 1 µg ml^{-1}) and stored at -18 °C in the dark. A UV–VIS spectrophotometer was used to scan the prepared stock solution in order to determine the wavelength at which the chosen antibiotic had the highest absorption.

Extraction of target antibiotics from soil, sewage sludge, and plant parts was carried out using a standard protocol according to Moudgil et al. (2019) and Barreiro et al. (2022) with some minor modifications. The extraction solvent varied across different matrices; nevertheless, regardless of the matrix type (soils, sludges, roots, and leaves), 2 g of sample was weighed into a 50 ml centrifuge tube containing 20 ml of extraction solvent. The antibiotics were extracted with a mixture of acetonitrile/methanol: water: formic acid (50:49.95:0.05), whereas from sewage sludge, the antibiotic residues were extracted with acetonitrile. Following the addition of the extraction solvent, the Falcon tubes were centrifuged at 4500 rpm for 15 min. Subsequently, a fraction of the supernatant (1 ml) was filtered through a 0.22 µm nylon syringe filter (Merck Millipore Ltd.) and then transferred to an amber vial for analysis via HPLC. The samples were examined using a Waters (717 plus Autosampler) HPLC (Waters Corporation, Milford, USA) outfitted with a reverse phase C18 column (SunFire® 4.6 mm × 250 mm × 5 µm) and a photodiode array (PDA) detector (Waters 2998). Phase A (0.01% acidified water with formic acid) and Phase B (0.01% acidified acetonitrile/methanol with formic acid) were combined to create a gradient mode mixture that allowed for the separation of the analytes. The flow rate was maintained at 1 ml min^{-1}, the samples at 8 °C and the column temperature at 43 °C. Physiochemical properties of the selected Abs and the generated standard curve utilised for quantification of antibiotics were represented in Supp. Table S1 and Fig S1.

Absolute quantification of selected ARGs and MGEs by quantitative PCR (qPCR)

The preparation of standard curves involved using plasmids carrying target genes in the pGEM-T vector (Thermo Fisher Scientific, USA) that contained gBLOCK fragment (IDT technologies, Belgium) for each target gene. The plasmid DNA carrying target ARGs was serially diluted ten times to construct seven-point qPCR standard curves with copy counts ranging from 102 to 108. A final volume master mix of 20 µl was prepared, consisting of 10 µl Maxima SYBR Green/ROX qPCR master mix (2x) (Thermo Fisher Scientific, USA), 1 µl of each primer (10 µM), 0.2 µl of BSA (20 mg/ml), 6.8 µl of

nuclease-free water, and 1 µl of DNA template (2.5 ng/µl). The PCR protocol was set as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Finally, a melt curve analysis was conducted with a temperature ramp from 60 to 95 °C. All qPCR experiments were conducted in 96-well plates under standard conditions and following the manufacturer's protocols (Applied Biosystems). Each reaction was repeated three times with an additional non-template control. Based on the standard curves, the application (Applied Biosystems 7500 v 2.3) determined the qPCR efficiencies. The PCR efficiencies ranged from 80.115% to 114.32%, with R^2 values above 0.992 on all calibration curves (Fig. S2). Six ARGs with two MGEs and 16 s rRNA were chosen for absolute quantification. All of the primers used in this experiment were synthesized by Sigma Aldrich, India, which are described in Supp. Table S2. The 16 s rRNA gene was also quantified so that the gene abundance could be adjusted to the complete bacterial community. Therefore, the absolute gene abundance for sludge amended pot soil and different compartments of vegetables (root, shoot) obtained was determined for respective sampling days and the time of harvesting, respectively.

Estimation of bioconcentration, transfer, translocation factors and dietary intake

The plant's ABs and ARGs accumulation from soil was measured using the bioconcentration factor (BCF), calculated as the ratio of analyte concentration in crop tissue to soil, based on dry weight.

$$\text{Bioconcentration factor (BCF)} = \frac{C_{\text{vegetables}}}{C_{\text{soil}}} \quad (1)$$

where, $C_{\text{vegetable}}$ is the concentration of ABs/ARGs present in edible parts of the plant (here, the leaf is the main edible part for spinach, and the root is for carrot).

The transfer factor (TF) indicates the movement of antibiotics from sludge-amended soil to the plant's roots, while the translocation factor (TLF) signifies the transportation of antibiotics from soil to shoots.

$$\text{Transfer factor (TF)} = \frac{C_{\text{root}}}{C_{\text{soil}}} \quad (2)$$

where C_{root} is the concentration of ABs/ARGs in the plant root and C_{soil} is the concentration in the soil.

$$\text{Translocation factor (TLF)} = \frac{C_{\text{shoot}}}{C_{\text{soil}}} \quad (3)$$

where C_{shoot} is the concentration of ABs/ARGs present in the shoots.

Human exposure to antibiotics and ARGs from edible vegetables

The assessment involved estimating human exposure to ABs and ARGs from the consumption of edible vegetables. The calculations were based on the absolute concentrations of ABs and ARGs without accounting for degradation or proliferation. The estimated daily intake (EDI) of antibiotics and ARGs was determined using the specified formulas. Equations 4 and 5 were employed to calculate the EDI of ABs (ng $kg^{-1}d^{-1}$) and ARGs/MGEs (copies kg^{-1} d^{-1}) from vegetable consumption for each treatment, respectively.

$$\text{EDI}_{\text{antibiotics}}(\text{ng/kg}) = \frac{\text{DIV} \times \text{C} \times (1 - \text{w})}{\text{BW}} \quad (4)$$

$$\text{EDI}_{\text{ARGs}}(\text{copies/kg}) = \frac{\text{DIV} \times \text{C} \times (1 - \text{w})}{\text{BW}} \quad (5)$$

Annual human exposure (AHE) of ABs and ARGs was estimated based on the daily consumption of these vegetables by using the following formula

$$\text{AHE}_{\text{antibiotics}}(\text{ng/kg}) = \text{C} \times \text{DIV} \times \text{BW} \times \text{T} \quad (6)$$

$$\text{AHE}_{\text{ARGs}}(\text{copies/kg}) = \text{C} \times \text{DIV} \times \text{BW} \times \text{T} \quad (7)$$

Hazard quotient (HQ) is calculated from EDI to threshold levels that are acceptable daily intake (ADI) as shown in Eq. 8, sourced from reputable authorities, such as the Joint FAO/WHO Expert Committee on Food Additives (JECFA), European Agency for the Evaluation of Medicinal Products (EMEA), etc. based on toxicological or microbiological effects for long-term exposure to ABs form air, drinking water, or food. To address combined antibiotic exposure risks, the Hazard Index (HI) was calculated, summing HQ for each antibiotic by using Eq. 9. If the value of HQ or HI exceeds one, it suggests a potential health risk. Consumption of vegetables contaminated with antibiotics represents just one route of human exposure. Thus, an HQ_{AB} greater than 0.1 signifies a potential hazard.

$$\text{Hazard quotient (HQ)} = \frac{\text{EDI}}{\text{ADI}} \quad (8)$$

$$\text{Hazard index (HI)} = \sum_{i=1}^{n} \text{HQ}_i = \text{HQ}_1 + \text{HQ}_2 + \cdots + \ldots \text{HQn} \quad (9)$$

where BW is the assumed body weight, which is 70 kg for adults and 16.2 kg for children (Hawrami et al. 2020), C is the ABs, ARGs or MGEs concentration present in the edible portion of vegetables. DIV is the daily vegetable

intake, which is 0.342 and 0.232 kg d^{-1} for spinach and for carrot, it is nearly 0. 276 and 0.228 kg d^{-1} for adults and children, respectively. W is the water content (%) of edible tissues (91.4% spinach, 88.3% carrot), and T is the exposure time (365 days).

Statistical analysis

The selected target genes (ARGs and MGEs) and 16S rRNA gene (denoting total bacterial population) were denoted as "log transformed gene copy number" in every gram of weight normalised to the DNA extraction yield in the soil and vegetable samples. IBM SPSS Statistics 25 software (Chicago, USA) was applied to analyse the variance of the experimental data. Using Tukey's Honest Significant Difference (HSD) tests, the average for each treatment was examined for a significant difference ($p \leq 0.05$). GraphPad Prism 8 and Origin 2024 software were utilized to create all of the statistical graphs. Using Microsoft Excel 2016 was exercised to calculate the average, standard deviations (S.D.) and standard errors (S.E.) of all the data were determined.

Results

Physicochemical characteristics of sludge and soil and the effects of sludge amendment on soil and plant growth parameters

The physicochemical properties of sludge, soil, and amended soil, showing variations among them, are presented in Supp Table S3. Specifically, D2S exhibited higher bulk density (BD), water holding capacity (WHC), organic carbon (OC), available phosphorus (AP), and total nitrogen (N) ratio while having lower electrical conductivity (EC) and moisture content (M) compared to the other two. Heavy metal concentrations in the sludge followed the order D2S > D1S > BS, except for Mn, which is higher in BS. Concentrations (mg kg^{-1}) of As, Cd, Cr, Co, Cu, Fe, Ni, Pb, Zn, and Mn range from (4.56–11.45), (23.39–213.36), (47.52–83.05), (58.65–86.24), (243.12–436.45), (474.33–778.09), (61.85–90.54), (42.09–89.0), (147.86–1173.0), and (192.92–257.15). The average metal concentration in the control soil followed the order Fe (225.6) > Mn (121.62) > Zn (89.42) > Cu (31.54) > Ni (10.07) > Co (7.19) > Pb (6.54) > Cr (2.12) > As (0.82) > Cd (0.48). Prior to the plantation experiment, all types of sludge-amended soils generally exhibited higher values for most physicochemical properties compared to control agricultural soils, except for soil pH, which decreased. In comparison to control pot plants, treated pot plants showed a significant increase in all plant growth parameters for both spinach and carrots. Particularly for D2S sludge-amended soil, there was an approximately 1.8-fold increase in spinach shoot height (cm), a 1.9-fold increase in root length (cm), and a 1.35-fold increase in leaf area (cm^2 $leaf^{-1}$) (Supp Table S4). Similar changes were observed for carrots, with a 1.3-fold increase in both leaf area and shoot height and a 2.15-fold increase in root length. The ratio of spinach shoots to roots showed no discernible change throughout the sludge treatment.

Isolation of TCB and TCEB

The presence of TCB and ampicillin-resistant populations (CFU g^{-1}) was assessed in sewage sludge, sludge-amended soil, and control soil. D2S amended soil exhibited a higher TCB count (30.29×10^7), followed by D1S (25.24×10^7) and BS (23.59×10^7). The TCEB population in BS, D1S, and D2S amended plants ranged from 4.54×10^5 to 5.23×10^6. In comparison to controls, cultivable ampicillin-resistant endophytic bacteria levels for BS, D1S, and D2S treated samples were (2.74×10^4), (3.28×10^4), and (3.6×10^4) for spinach plants, and for carrots, decreased to (1.7×10^4), (2.74×10^4), and (3.24×10^4), respectively. Considering colony characteristics, a total of 52 (40 EB from the treated plant and 12 EB from the control plant) cultivable ampicillin-resistant bacterial colonies were isolated from both plant samples for antibiotic susceptibility testing.

Antibiotics and heavy metal resistance pattern of endophytic bacteria

A diverse antibiotic resistance (AR) pattern was observed among these 52 EB, with the zone of inhibition varying from 0 to 33 mm in radius. The Multiple Antibiotic Resistance Index (MARI) ranged from 0 to 0.88 across all 52 isolates. Notably, high susceptibility was identified for vancomycin and ofloxacin, while the lowest susceptibility was observed against penicillin G and ciprofloxacin among the 17 tested antibiotics. The percentage resistance to respective antibiotics were A-100%, AZM-71.15%, FOX-28.85%, C-80.77%, CF-86.54%, CO-42.31%, G-42.30%, K-61.54%, P-90.38%, OFX-15.38%, RIF-53.85%, S-30.76%, TE-76.92%, E-67.30%, IPM-44.23%, VA-25% and NX-36.54%. Further analysis focused on MAREB isolates, characterized by diverse colony traits, resistance to over six antibiotic classes, and elevated MARI values ranging from 0.52 to 0.88. MARI values for the 22 selected MAREB isolates are provided in Fig. 1**.** Among these MAREB isolates, a significant proportion exhibited resistance to multiple heavy metals, with high resistance observed against Pb (100%), Cr (86.36%), Ni (86.36%), Cu (81.82%), Mn (81.81%), Ag (77.27%), Zn (77.27%), Co (72.72%), Cd (68.18%), and Hg (40.91%). Notably, seven bacteria (E1R, E3R, E6R, E7R, E9R, E11L, E16L, E18L, and E21L) demonstrated resistance to all tested heavy metals, while isolates E12L and E14L displayed resistance to 3 and 2 heavy metals, respectively (Fig. 1).

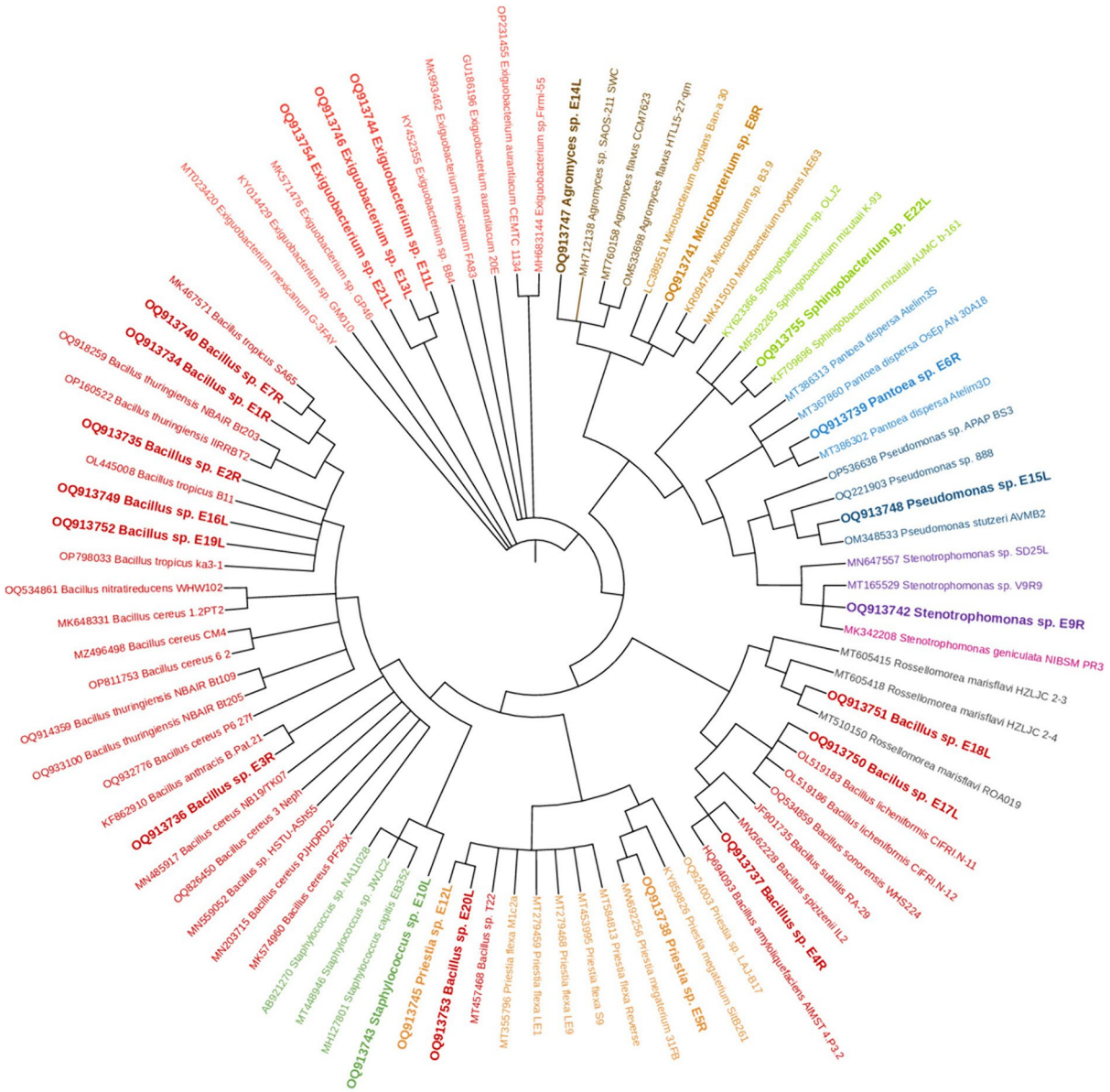

Fig. 1 Neighbor-joining tree based on distance analysis representing the relationship between the *16S rRNA* sequences of 22 multidrug resistance endophytic bacterial isolates from three different sludge amended soil grown vegetables (spinach and carrot) 66 reference sequences (*16S rRNA gene*) of the related species from NCBI GenBank. Strain with same genus has been represented in same colour. Bootstrap values generated from 500 replicates are shown at the nodes

Characterization of the MAREB isolates, phylogenetic analysis and BIOLOG assay

Utilizing 16S rRNA sequencing, the isolates were identified as follows: *Bacillus* sp. strain E1R, *Bacillus* sp. strain E2R, *Bacillus* sp. strain E3R, *Bacillus* sp. strain E4R, *Priestia* sp. strain E5R, *Pantoea* sp. strain E6R, *Bacillus* sp. strain E7R, *Microbacterium* sp. strain E8R, *Stenotrophomonas* sp. strain E9R, *Staphylococcus* sp. strain E10L, *Exiguobacterium* sp. strain E11L, *Priestia* sp. strain E12L, *Exiguobacterium* sp. strain E13L, *Agromyces* sp. strain E14l, *Pseudomonas* sp. strain E15L, *Bacillus* sp. strain E16L, *Bacillus* sp. strain E17L, *Bacillus* sp. strain E18L, *Bacillus* sp. strain E19L, *Bacillus* sp. strain E20L, *Exiguobacterium* sp. strain E21L, and *Sphingobacterium* sp. strain E22L. In total, there were 10 distinct genera distributed among five different orders and spanning four bacterial phyla. The dominant phyla were Firmicutes and Proteobacteria, accounting 72.72% of the identified bacteria, with 16 isolates. Within this, three isolates (13.64%) belonged to Proteobacteria, two (9.09%) to

Actinobacteria, and one (4.54%) to Bacteroidetes. Additionally, the Biolog assay revealed three distinct clusters, showcasing overlapping metabolic pathways among six selected isolates (supplementary Table S6 and Fig. S3). The accession numbers for the deposited 16S rRNA gene sequences in GenBank ranged from OQ913734 to OQ913755, and a phylogenetic tree was constructed and presented in Fig. 2. A literature survey categorized these bacteria into three groups: (i) bacteria associated with both plant and human (51.09%), (ii) plant-associated bacteria (31.82%), and (iii) human pathogenic bacteria (9.09%).

Characterization of MAREB for plant beneficial traits

The outcomes of beneficial traits documented by MAREB are depicted in Fig. S4. Out of the 22 bacterial isolates, all exhibited root colonization in spinach, with 68.18% testing positive for IAA production, 63.64% for siderophore production, and 63.64% showing ACC-deaminase activity. Additionally, 54.54% displayed a positive reaction in the HCN test, 63.64% exhibited cellulose activity, and 72.27% tested positive for protease assay. Notably, all isolates (100%) were found to produce biofilm, and the majority demonstrated phosphate solubilization capability. In dual culture assays, it was observed that 63.63% of the examined bacteria suppressed the growth of *C. truncatum*, while 68.18% exhibited inhibitory effects on both *A. flavus* and *F. oxysporum*, underscoring their potential to generate antifungal substances.

Auto-aggregation, co-aggregation and motility properties of MAREB

MDR isolates displayed increasing auto-aggregation percentages over a 24-h incubation period (Table S5). Notably, *Bacillus* sp. strain E3R (92.61%) and *Exiguobacterium* sp. strain E21L (90.69%) exhibited the highest auto-aggregation percentages, followed by several other strains such as *Bacillus* sp. strain E4R (83.61%), *Bacillus* sp. strain E16L (85.27%), and *Priestia* sp. strain E5R (68.51%). Conversely, strains like *Bacillus* sp. strain E20L (39.49%), *Bacillus* sp. strain E2R (30.82%), and *Bacillus*

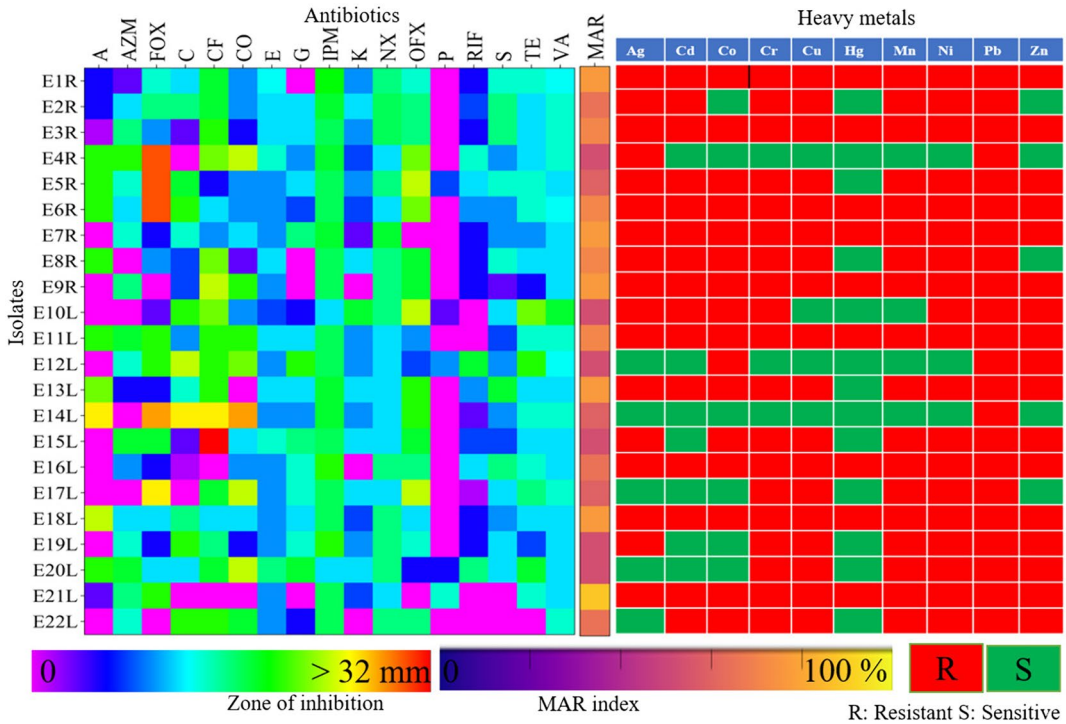

Fig. 2 Heat map representing antibiotic and heavy metal resistance in EB recorded by MAREB. The sensitivity of bacteria to antibiotics is represented as a zone of inhibition (mm) in radius. MAR index was calculated using the breakpoint value (Sandhu et al. 2016). The breakpoint was given by CLSI and EUCAST was used to determine bacterial antibiotic resistance. Bacteria Categorization of heavy metal resistance/susceptibility was done following different scales as explained in material and methods. [Antibiotics denoted as ampicillin (A), cotrimoxazole (CO), ciprofloxacin (CF), erythromycin (E), chloramphenicol (C), streptomycin (S), rifampicin (RIF), gentamycin (G), ofloxacin (OFX), tetracycline (TE), cefoxitin (FOX), kanamycin (K), azithromycin (AZM), penicillin-G (P), imipenem (IPM), norfloxacin (NX) and vancomycin (VA) and heavy metals denoted as silver (Ag), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), mercury (Hg), manganese (Mn), nickel (Ni), lead (Pb), zinc (Zn)]

sp. strain E17L (16.67%) showed lower auto-aggregative properties. Co-aggregation investigations revealed pronounced aggregation on combining specific strains, such as *Microbacterium* sp. strain BI8 with *Bacillus* sp. strain E19L and *Agromyces* sp. strain E14L with *Exiguobacterium* sp. strain E21L. Visual auto-aggregation assays confirmed the presence of visible flocs for high auto-aggregative MAREB strains, while minimal flocs were observed for other strains after incubation. Similarly, co-aggregation sets of low aggregative strains displayed visible flocs. Approximately 68.18% of MAREB isolates exhibited motility, displaying a swarming motility pattern on 0.3% agar media plates (Table S5). Non-motile strains included *Bacillus* sp. E4R, *Bacillus* sp. E7R, *Microbacterium* sp. strain E8R, *Staphylococcus* sp. E10L, *Agromyces* sp. E14L, *Bacillus* sp. E18L, and *Sphingobacterium* sp. E22L.

Biofilm formation dynamics of MAREB

ATCC 35984, characterized by its Ica-positive slime-producing trait, and ATCC 12228, lacking both Ica and slime production, were utilized as positive and negative controls, respectively. The cut-off value (ODc) for each plate was determined by calculating three standard deviations above the mean optical density (OD) of the negative control: ODc = average OD of the negative control + (3 × SD of the negative control). Results indicated that following 24 h of incubation, 36.36% of isolates exhibited minimal biofilm formation ($0.542 < ODs < 1.462$), 18.18% displayed moderate biofilm formation ($1.462 < ODs < 2.594$), while the remaining 45.45% demonstrated robust biofilm formation ($2.594 < ODs$). Biofilm-forming ability is detailed in Table S5, with the tissue culture plate technique confirming positive biofilm formation for all isolates. Biofilm dynamics in sludge isolates revealed a Gaussian distribution pattern, with increased antibiotic concentrations (amoxicillin, azithromycin, ciprofloxacin, chloramphenicol, tetracycline, vancomycin, and clarithromycin) generally enhancing biofilm production (Figs. S7, S8, S9, S10, S11, S12, S13). Notably, when antibiotics were added after biofilm development, significant resistance was observed. In contrast, introducing antibiotics prior to bacterial inoculation led to reduced biofilm formation at higher concentrations for most isolates. However, E3R, E6R, E11L, E15L, and E21L showed consistent biofilm dynamics across various antibiotic sets. Interestingly, five isolates (E2R, E10L, E14L, E18L, and E21L) demonstrated survival at clarithromycin concentrations up to 150 g ml^{-1} in Set A, showcasing notable resistance.

Qualitative assessment of ARGs, MRGs and MGEs in MAREB

All 22 selected MAREB isolates underwent testing to assess the presence of antibiotic and heavy metal-resistant genes, along with mobile genetic elements. Among the 14 antibiotic resistance genes examined, the respective percentages of MAREB isolates showing positive results were as follows: *blaNDM*-81.82%, *qnrS*-72.73%, *Sul1*-63.64%, *blaGES*-13.64%, *ermF*-9.09%, *Intl1*-59.09%, *IS26*-81.82%, *tetW*-36.36%, *blaTEM*-95.09%, *rpoB516*-59.09%, and *tetQ*-81.82% (Fig. 3a) However, no PCR amplification was detected for *tetM*, *rpoB526*, *rpoB531*,and *blaCTX* genes. Among all 22 MAREB isolates, E11L and E21L were identified as harbouring the highest number of antibiotic resistance genes (10) in their genomic DNA, while E12L and E22L carried three and two antibiotic resistance genes, respectively. Additionally, regarding heavy metal-resistant genes, positive results were obtained for *nikA* (77.27%), *copB* (77.27%), and *czcD* (59.09%), with none of the tested bacteria containing *merA1*, *silE*, and *arsA* genes. Out of the 22 isolates,

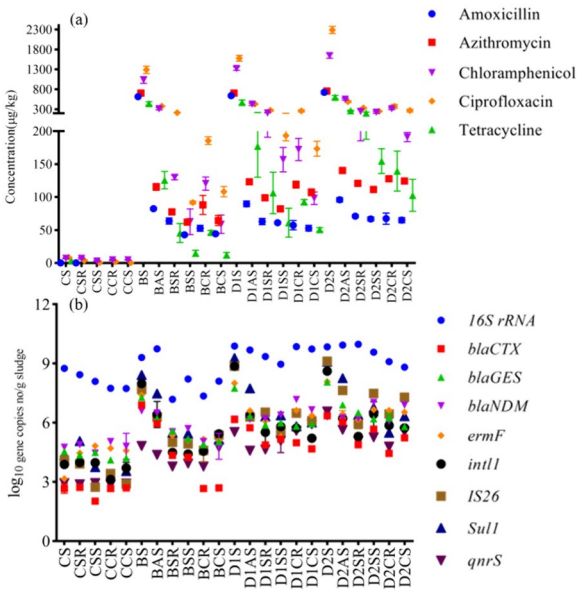

Fig. 3 **a** Concentration of antibiotics throughout the soil plant system **b** Abundance of absolute ARGs and MGEs throughout the soil plant system (CS: control soil; CSR: control soil grown spinach root; CSS: control soil grown spinach shoot; CCR: control soil grown carrot root; CCS: control soil grown carrot shoot; BS: STP1 derived sludge; BAS: BS amended soil; BSR: BS amended soil grown spinach root; BSS: BS amended soil grown spinach shoot; BCR: BS amended soil grown carrot root; BCS: BS amended soil grown carrot shoot; D1S: STP2 derived sludge; D1AS: D1S amended soil; D1SR: D1S amended soil grown spinach root; D1SS: D1S amended soil grown spinach shoot; D1CR: D1S amended soil grown carrot root; D1CS: D1S amended soil grown carrot shoot; D2S: STP3 derived sludge; D2AS: D2S amended soil; D2SR: D2S amended soil grown spinach root; D2SS: D2S amended soil grown spinach shoot; D2CR: D2S amended soil grown carrot root; D2CS: D2S amended soil grown carrot shoot

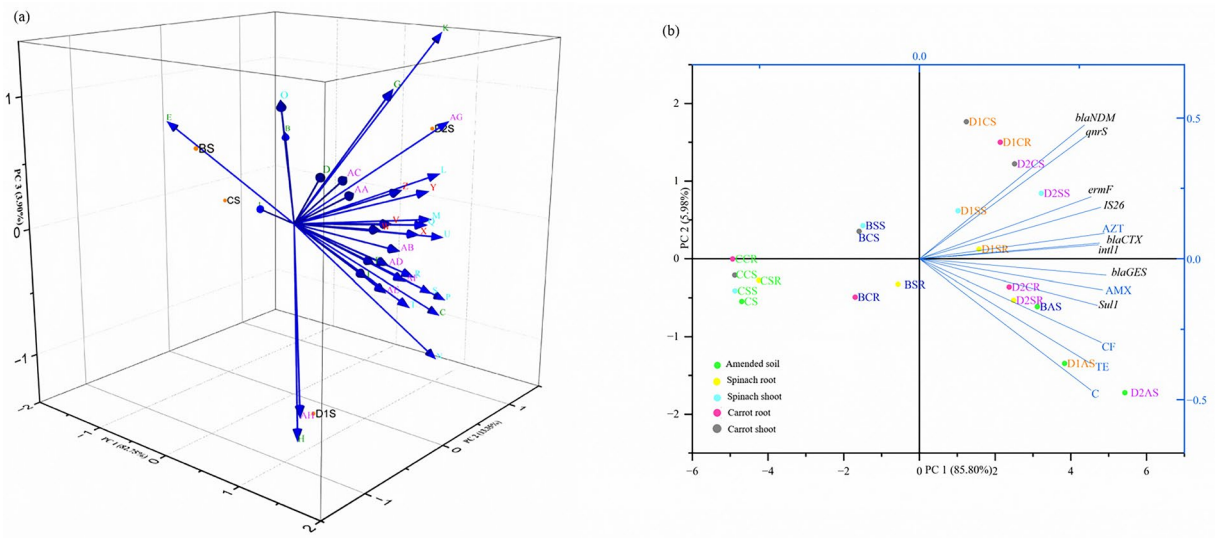

Fig. 4 **a** 3D plot of the first three components resulting from the principal component analysis (PCA) of the different parameters of STP derived sludge environment containing physicochemical properties of the sludge, concentration of selected antibiotics and the absolute abundance of ARGs and MGEs (B: bulk density; C: water holding capacity; D: moisture; E: pH; F: electrical conductivity; G: organic carbon; H: total N (%); I: available P; J: available K; K: TBC; L: Fe; M: Cu; N: Zn; O: Mn; P: Cd; Q: Cr; R: Ni; S: Pb; T: Co; U: As; V: Amoxicillin; W: Azithromycin; X: Chloramphenicol; Y: Ciprofloxacin; Z: Tetracycline; AA: *blaCTX*; AB: *blaGES*; AC: *blaNDM*; AD: *ermF*; AE: *intI1*; AF: *IS26*; AG: *qnrS*; AH: *Sul1*). **b** Principal component analysis (PCA) showing the correlations of ABs concentration, ARGs, MGEs gene abundance, in sludge, amended soil and different plant compartments (CS: control soil; CSR: control soil grown spinach root; CSS: control soil grown spinach shoot; CCR: control soil grown carrot root; CCS: control soil grown carrot shoot; BS: STP1 derived sludge; BAS: BS amended soil; BSR: BS amended soil grown spinach root; BSS: BS amended soil grown spinach shoot; BCR: BS amended soil grown carrot root; BCS: BS amended soil grown carrot shoot; D1S: STP2 derived sludge; D1AS: D1S amended soil; D1SR: D1S amended soil grown spinach root; D1SS: D1S amended soil grown spinach shoot; D1CR: D1S amended soil grown carrot root; D1CS: D1S amended soil grown carrot shoot; D2S: STP3 derived sludge; D2AS: D2S amended soil; D2SR: D2S amended soil grown spinach root; D2SS: D2S amended soil grown spinach shoot; D2CR: D2S amended soil grown carrot root; D2CS: D2S amended soil grown carrot shoot)

13 were found to have plasmids, but no antibiotic resistance genes or heavy metal-resistant genes were amplified in the plasmid DNA.

Abundance of ABs, ARGs and MGEs in sludge, soil and plant and establishment of correlation

In the control agricultural soil samples analysed, most ABs (Amoxicillin, Azithromycin, Tetracycline) were undetectable, with concentrations below the limits of detection (LOD) and quantification (LOQ) (Fig. 4a). However, chloramphenicol and ciprofloxacin residues were present in very low concentrations (μg kg^{-1}), specifically 7.10 and 8.55, respectively. In sludge, the mean antibiotic concentrations (μg kg^{-1}) varied in the order D2S > D1S > BS, with ranges as follows: tetracycline (445.47 to 602.87), amoxicillin (619.50 to 725.87), azithromycin (707.89 to 756.93), chloramphenicol (1033.98 to 1645.79), and ciprofloxacin (1291.04 to 2287.40). Antibiotic residues were also analysed in amended pot soil after crop harvesting (Fig. 4a). Correlation analysis revealed a significant relationship between physicochemical properties, HMs content, ABs concentration, and abundance of ARGs ($r = 0.635$, $p \leq 0.05$) in the sludge (Fig. 5a). Concentrations of ABs in amended soil and various compartments of vegetables (root and shoot) were assessed (Fig. 4a). Overall concentrations (μg kg^{-1}) of antibiotics in amended soil varied: amoxicillin (82.39 to 95.98), azithromycin (115.57 to 140.47), chloramphenicol (315.69 to 554.41), ciprofloxacin (382.41 to 493.65), and tetracycline (125.74 to 266.96). D2S amended soil showed significantly higher antibiotic concentrations compared

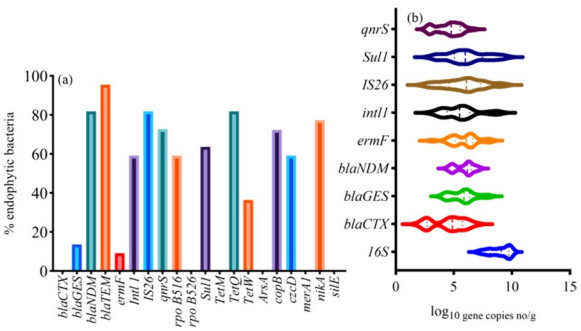

Fig. 5 **a** Percentage of MAREB strains carrying selected ARGs, MGEs and HMRGs, and **b** the violin plot represents the overall absolute abundance ARGs and MGEs throughout the all studied compartments

to other groups ($p \leq 0.05$), while no significant difference was observed between BS and D1S groups ($p \geq 0.05$). All five targeted ABs were detected in variable concentrations in both above and underground edible parts. Ciprofloxacin exhibited the highest concentration, followed by chloramphenicol (2.87 to 314.88), tetracycline (N.D to 209.19), azithromycin (N.D to 127.72) and amoxicillin (N.D to 71.05). Distribution of antibiotics in edible parts varied for spinach (leaves > roots > shoots) and carrot (roots > leaves > shoots). Significant differences were observed in the uptake of ciprofloxacin at the four treatment levels for both vegetables. Additionally, a very low concentration of antibiotic residues (ciprofloxacin and chloramphenicol) was detected in the unexposed (control) plant samples.

qPCR amplification efficiencies for all target genes ranged from 80.115 to 114.32%, demonstrating good linearity (Supplementary Fig. S2). The absolute copies (copies/g) of antibiotic resistance genes (ARGs) varied from 105.23 to 1.3×10^9. In sludge samples, ARGs ranged from 2.2×10^7 to 1.3×10^9. The dissemination of ARGs from sludge to soil to plants was 1 to 3 times lower than in soil and sludge samples. In amended pots, mean ARG copies varied from 2.5×10^4 to 2.0×10^8, while for spinach and carrot plants, it ranged from 6.2×10^3 to 3.2×10^7 and 6.1×10^3 to 2.2×10^7, respectively. IS26 copies varied by 100-folds from sludge to amended soil to plants, being the most abundant ARG (5.5×10^2 to 1.3×10^9), while *qnrS* had a lower concentration (2.8×10^2 to 3.9×10^6). Under sludge treatment, total copy numbers of target ARGs in bulk soil increased significantly for all treatment groups ($p \leq 0.05$), and the dynamics of the ARGs dissemination have been presented in Figs. 3b, 4b. The abundance of ARGs and MGEs in the treatment group was two to four-fold higher than in the control group, following the antibiotic trend CS < BS < D1S < D2S (Fig. S5). Importantly, *intl1* and *IS26* copies were significantly higher in all treated plant samples. For spinach, significantly higher ARGs abundance was observed in shoot tissues compared to roots; however, no significant variation was observed for carrot roots and shoots. The PCA biplot elucidated the impact of sludge amendment and antibiotics (ABs) concentration on the abundance of antibiotic resistance genes (ARGs) across all tested plant compartments. A robust correlation was evident between ABs and ARGs concentrations in the D1S and D2S amended systems, while a distinct association was observed in the BS amended and CS systems (Fig. 5b).

Translocation of ABs in sludge amended soil–plant system and human health risk assessment

Mobility of ABs

Various methods have been employed to assess the mobility of antibiotics (ABs) in the soil–plant system. The subsequent sections provide detailed discussions on each of these indices.

Transfer factor (TF)

The Transfer Factor (TF) indicates the potential presence of a specific antibiotic residue in a plant compartment. Supplementary Fig S6a illustrates the TF values for all tested ABs in three sludge-amended soil–plant systems compared to a control soil–plant system. In the spinach plant system, TF values for antibiotics range as follows: amoxicillin (1.29 to 1.43), azithromycin (0.67 to 0.86), chloramphenicol (0.22 to 0.53), ciprofloxacin (0.54 to 0.68), and tetracycline (0.36 to 0.78). In the carrot plant system, TF values vary for amoxicillin (0.63 to 0.70), azithromycin (0.76 to 0.91), chloramphenicol (0.34 to 0.57), ciprofloxacin (0.27 to 0.76), and tetracycline (0.36 to 0.52). Overall, higher antibiotic accumulation is observed in all spinach plants grown in amended soil due to the higher percentage of residue in sludge-amended soil compared to the control plant. The TF values follow the order CS > BS > D1S > D2S, and amoxicillin, azithromycin, and ciprofloxacin are the main antibiotics contributing to TF values in all systems. Comparable findings have been observed for Antibiotic Resistance Genes (ARGs) and Mobile Genetic Elements (MGEs). Enhanced accumulation of ARGs is apparent in vegetables grown in amended soil, with *blaCTX*, *qnrS*, and *intl1*, *IS26* identified as the principal ARGs and MGEs influencing Translocation Factor (TF) values across all systems (data not shown).

Translocation factor (TLF)

The TLF values for selected antibiotics in sludge-amended soil–plant systems and the control soil are presented in Fig. S6b. Antibiotic TLF values in the soil–plant system range as follows: amoxicillin (0.67 to 0.97), azithromycin (0.80 to 0.92), chloramphenicol (0.41 to 0.77), ciprofloxacin (0.22 to 0.75), and tetracycline (0.33 to 0.74). In the carrot plant system, TLF values vary for amoxicillin (0.84 to 0.96), azithromycin (0.73 to 0.97), chloramphenicol (0.23 to 0.61), ciprofloxacin

(0.28 to 0.72), and tetracycline (0.26 to 0.73). Considering these values, the selected antibiotics can be ranked in ascending order as TET < CIF < C < AMX < AZT for all amended plant systems in spinach. In the carrot system, both the BS and D1S show a similar TLF pattern as TET < C < CIF < AZT < AMX, whereas for the D2S plant system, it is as C < CIF < TET < AMX < AZT. Sludge-amended soil exhibits significantly higher TLF values than control soil, following the order: CS < BS < D1S < D2S. AMX and AZT consistently display the highest TLF values across the system, indicating a high level of translocation of these antibiotics to the shoots. Sludge-amended soil exhibits notably higher Translocation Factor (TLF) values for Antibiotic Resistance Genes (ARGs) and Mobile Genetic Elements (MGEs). Among these, *blaCTX*, *qnrS*, *intl1*, and *IS26* demonstrate the highest translocation rates across all observed cases.

Bio-concentration factor (BCF)

The Bio-Concentration Factor (BCF) values for selected heavy metals in both control and sludge-amended soil–plant systems for spinach were amoxicillin (0.52 to 0.70), azithromycin (0.54 to 0.79), chloramphenicol (0.13 to 0.91), ciprofloxacin (0.12 to 0.51), and tetracycline (0.12 to 0.58). In the carrot plant, amoxicillin (0.54 to 0.68), azithromycin (0.56 to 0.89), chloramphenicol (0.15 to 0.34), ciprofloxacin (0.07 to 0.55), and tetracycline (0.10 to 0.38) (Fig. S6c). Based on these BCF values, ABs can be ranked in ascending order for all treatments as CIP < TET < AMX < AZT < C for spinach and C < TET < CIP < AMX < AZT for carrot, respectively. The treatment groups consistently demonstrate higher Bioconcentration Factor (BCF) values compared to the control, gradually increasing in the following order: CS < BS < D1S < D2S. This same trend is observed in the case of Antibiotic Resistance Genes (ARGs), with *blaCTX*, *qnrS*, *intl1*, and *IS26* significantly contributing to the Bioconcentration Factor values.

Health risk analysis

Hazard quotient (HQ) and hazard index (HI) of ABs

HQ values for adults and children were determined against each selected antibiotic (AB) using their ADI values, providing insights into potential health risks. The corresponding HQ values are presented in Supplementary Table S5, indicating the HQ values for detected antibiotics in both amended and non-amended soil-grown vegetables (Spinach, carrot). The risk associated with the five antibiotics varied across different targets, with overall HQ values ranging from 0.0005 to 3.8163 throughout the system. The HQ values for detectable antibiotics in control plants were significantly lower than the threshold of ≥ 0.1, which is considered a potential hazard to human health in this assessment. For treated plants, HQ values for most antibiotics (AZT, AMX, TET, and C) were consistently below 0.1 for both adults and children, regardless of the amendment. However, HQ values for CIF exceeded 0.1 in both plant types and for both adult and children's populations. Cumulative risk assessment, represented by Hazard Index (HI) values in Table 1, indicated a high HI value (> 0.1) for both treated plants ranging from (0.0025 to 4.6535) with variations in the order: BS < D1S < D2S. Moreover, higher HQ and HI values were observed for the children population compared to adults.

EDI and AHE of AB, ARGs and MGEs

One pathway of human exposure to ABs and ARGs is through the consumption of vegetables. Threshold levels were set based on ADI values sourced from literature or

Table 1 Hazard Quotient (HQ) and Hazard Index (HI) values of different selected Antibiotics (ABs) (AMX: amoxicillin; AZT: azithromycin; C: chloramphenicol; CF: ciprofloxacin; TE: tetracycline) for adult and children for Sludge amended soil- plant system, where A denotes adult population and C denotes children population

Human exposure		AMX		AZT		C		CF		TE		Hazard index (HI)	
Treatment	Plant	HQ (A)	HQ (C)	HQ (A)	HQ (C)	HQ (A)	HQ (C)	HQ (A)	HQ (C)	HQ (A)	HQ (C)	HI (A)	HI (C)
CS	Spinach (shoot)	0	0	0	0	0.0005	0.0014	0.0024	0.0070	0	0	0.0029	0.0084
	Carrot (root)	0	0	0	0	0.0009	0.0030	0.0016	0.0059	0	0	0.0025	0.0090
BS	Spinach (shoot)	0.0902	0.0869	0.0153	0.0449	0.0105	0.0309	0.3030	0.7543	0.0021	0.0061	0.4211	0.9232
	Carrot (root)	0.1023	0.3651	0.0175	0.0625	0.0108	0.0388	0.3327	1.1874	0.0019	0.0067	0.4652	1.6605
D1S	Spinach (shoot)	0.1282	0.3757	0.0203	0.0594	0.0263	0.0772	0.5410	1.5859	0.0085	0.0250	0.7244	2.1234
	Carrot (root)	0.1497	0.4351	0.0291	0.1040	0.0181	0.0647	0.5331	2.9791	0.0078	0.0278	0.7379	3.6107
D2S	Spinach (shoot)	0.1404	0.4116	0.0186	0.0805	0.0385	0.1129	0.7072	2.0728	0.0217	0.0635	0.9264	2.7413
	Carrot (root)	0.1497	0.5343	0.0338	0.1206	0.035	0.1259	1.0691	3.8163	0.0158	0.0564	1.3034	4.6535

Table 2 Estimated Daily Intake (EDI) and Annual Human Exposure (AHE) values of different selected Antibiotics (ABs) (AMX: amoxicillin; AZT: azithromycin; C: chloramphenicol; CF: ciprofloxacin; TE: tetracycline) for adult and children for Sludge amended soil- plant system, where A denotes adult population and C denotes children population

Human exposure		AMX				AZT				C			
Treatment	Plant	Daily (ng)		Annual (µg)		Daily (ng)		Annual (µg)		Daily (ng)		Annual (µg)	
		A	C	A	C	A	C	A	C	A	C	A	C
CS	Spinach (shoot)	0	0	0	0	0	0	0	0	0.001 ± 0.0003	0.003 ± 1.1E−05	25.1 ± 1.2	3.94 ± 0.4
	Carrot (root)	0	0	0	0	0	0	0	0	0.002 ± 0.0001	0.007 ± 0.0004	33.2 ± 1.89	6.34 ± 0.36
BS	Spinach (shoot)	0.02 ± 0.0001	0.02 ± 0.0005	375.2 ± 10.23	58.9 ± 1.42	0.03 ± 0.0008	0.08 ± 0.002	541.6 ± 2.10	85.0 ± 2.87	0.03 ± 0.006	0.08 ± 0.002	548.0 ± 22.56	86.03 ± 4.23
	Carrot (root)	0.02 ± 0.002	0.07 ± 0.003	312.7 ± 14.03	59.8 ± 2.68	0.03 ± 0.003	0.11 ± 0.004	455.3 ± 23.84	87.0 ± 2.58	0.03 ± 0.005	0.09 ± 0.002	415.0 ± 10.56	79.34 ± 3.54
D1S	Spinach (shoot)	0.02 ± 0.0004	0.07 ± 0.001	533.1 ± 15.93	83.7 ± 2.50	0.03 ± 0.0004	0.1 ± 0.001	716.5 ± 13.73	112.5 ± 2.15	0.06 ± 0.006	0.19 ± 0.018	1370.1 ± 49.2	215.1 ± 10.12
	Carrot (root)	0.03 ± 0.001	0.09 ± 0.006	372.7 ± 15.22	71.2 ± 3.77	0.05 ± 0.002	0.18 ± 0.008	757.3 ± 36.33	144.8 ± 5.60	0.04 ± 0.002	0.16 ± 0.010	692.6 ± 67.47	132.4 ± 6.90
D2S	Spinach (shoot)	0.03 ± 0.001	0.08 ± 0.003	584.0 ± 25.96	91.7 ± 4.07	0.03 ± 0.0005	0.14 ± 0.002	970.5 ± 16.82	152.4 ± 2.64	0.1 ± 0.010	0.28 ± 0.030	2003.6 ± 114.98	314.5 ± 15.75
	Carrot (root)	0.03 ± 0.001	0.11 ± 0.004	457.6 ± 14.68	87.5 ± 5.48	0.06 ± 0.0006	0.2 ± 0.001	878.3 ± 10.14	167.9 ± 7.93	0.09 ± 0.003	0.31 ± 0.04	1348.2 ± 49.02	257.8 ± 8.37

CF				TE			
Daily (ng)		Annual (µg)		Daily (ng)		Annual (µg)	
A	C	A	C	A	C	A	C
0.0003 ± 5.3E−07	0.001 ± 1.5E−06	7.43 ± 0.02	1.16 ± 0.005	0	0	0	0
0.0002 ± 0.0001	0.0008 ± 1E−05	3.80 ± 0.04	0.72 ± 0.008	0	0	0	0
0.04 ± 0.003	0.11 ± 0.003	802.81 ± 8.89	126.03 ± 3.97	0.006 ± 0.0001	0.02 ± 0.005	129.5 ± 6.26	20.3 ± 1.23
0.05 ± 0.008	0.18 ± 0.012	762.8 ± 25.34	145.8 ± 3.54	0.006 ± 0.0003	0.02 ± 0.003	85.7 ± 4.56	16.4 ± 0.89
0.08 ± 0.001	0.24 ± 0.005	1687.8 ± 39.69	265.0 ± 6.81	0.025 ± 0.007	0.07 ± 0.002	533.5 ± 19.24	83.75 ± 3.18
0.08 ± 0.004	0.45 ± 0.039	1222.6 ± 80.32	233.7 ± 15.35	0.023 ± 0.0008	0.083 ± 0.002	357.35 ± 18.21	68.32 ± 4.05
0.11 ± 0.004	0.31 ± 0.014	2206.0 ± 100.68	346.3 ± 15.80	0.065 ± 0.007	0.19 ± 0.023	1351.4 ± 80.89	212.15 ± 12.72
0.12 ± 0.017	0.45 ± 0.03	1913.7 ± 52.30	365.8 ± 14.95	0.05 ± 0.011	0.17 ± 0.029	724.21 ± 58.42	138.45 ± 12.46

Data represented as Mean ± S.E

Table 3 Estimated Daily Intake (EDI) and Annual Human Exposure (AHE) values of different selected Antibiotic resistance genes (*ermF*, *qnrS*, *Sul1*, *blaCTX*, *blaGES*, *blaNDM*, *intI1*, *IS26*) for adult and children for Sludge amended soil- plant system, where A denotes adult population and C denotes children population

Treatment		CS				BS			
Human exposure	Plant	Daily (copies kg^{-1})		Annual (copies kg^{-1})		Daily (copies kg^{-1})		Annual (copies $kg^{-1)}$	
		A	C	A	C	A	C	A	C
ermF	Spinach (shoot)	2.8e+4±3.7e+3	8.3e+4±1e+4	5.9e+11±7.7e+10	9.3e+10±1.2e+9	6.3e+4±70.65	2.7e+5±305.45	1.3e+12±4.4e+10	2e+11±7.3e+9
	Carrot (root)	2.4e+4±4e+3	8.6e+4±16,282.30	3.7e+11±6.9e+10	7.1e+10±1.3e19	7e+4±197.40	2.5e+5±572.35	1e+12±1.9e+11	2e+11±3.7e+10
qnrS	Spinach (shoot)	3.7e+2±8.95	1.1e+3±26.24	7.8e+9±1.8E+8	1.2e+9±2.9E+7	3.6e+3±70.68	1.5e+4±305.41	7.6e+10±1.4e+9	1.2e+10±2.3e+8
	Carrot (root)	3.6e+2±77.66	1.3e+3±277.20	5.5e+9±1.1E+8	1e+9±2.2e+8	2.8e+3±55.17	1e+4±196.94	4.3e+10±8.4e+8	8.3e+9±1.6e+8
Sul1	Spinach (shoot)	2.4e+3±197.44	7.1e+3±578.74	5.0e+10±4.1e+9	7.9e+9±6.4e+8	1.5e+5±60.8	6.5e+5±280.9	3.1e+12±1e+11	4.9e+11±1.5e10
	Carrot (root)	1e+3±33.23	3.8e+3±118.62	1.6e+10±5.0e+8	3.1e+9±9.7e+7	2.7e+4±647.66	9.8e+4±2311.8	4.2e+11±9.9e+9	8e+10±1.8e+9
blaGES	Spinach (shoot)	1.3e+4±488.05	3.8e+4±1430.59	2.7e+11±1e+10	4.3e+10±1.5e+9	6.6e+4±927.14	2.8e+5±4006.2	1.3e+12±1.9e+10	2.1e+11±3e+9
	Carrot (root)	6.2e+3±58.69	2.2e+4±209.51	9.5e+10±8.9e+8	1.8e+10±1.7e+8	3.5e+4±1571.8	1.2e+5±5610.7	5.3e+11±2.4e+10	1e+11±4.5e+9
blaCTX	Spinach (shoot)	4.4e+1±52.36	1.2e+2±185.45	9.1e+8±2.4e+7	1.4e+8±8.5e+6	7.6e+3±131.32	3.3e+4±567.4	1.6e+11±2.7e+9	2.5e+10±4.2e+8
	Carrot (root)	2.2e+2±52.45	8e+2±203.12	3.4e+9±3e+7	6.5e+8±5.7e+6	4.4e+3±13,300.2	1.5e+4±47,474.8	6.7e+10±2e+9	1.2e+10±3.8e+8
blaNDM	Spinach (shoot)	1.2e+4±317.4	3.5e+4±930.36	2.5e+11±6.6e+9	4e+10±1e+9	2.1e+5±1e+4	9.3e+5±4.3e+4	4.4e+12±2e+11	7e+11±3.2e+10
	Carrot (root)	5.1e+4±440.86	1.8e+5±1573.66	7.8e+11±6.7e+9	1.5e+11±1.2e+9	1.6e+5±5877.6	5.7e+5±2e+4	2.4e+12±8.9e+10	4.7e+11±1.7e+10
Intl1	Spinach (shoot)	4.4e+4±77.86	1.2e+4±228.24	9.1e+10±1.6e+9	1.4e+10±2.5e+8	1e+4±394.49	4.6e+4±1704.62	2.2e+11±8.2e+9	3.5e+10±1.2e+9
	Carrot (root)	6.7e+2±29.36	2.3e+3±104.82	1e+10±4.4e+8	1.9e+9±8.5e7	1.6e+4±321.2	5.8e+4±1147.06	2.5e+11±4.9e+9	4.8e+10±9.3e+8
IS26	Spinach (shoot)	2.6e+2±11.63	7.7e+2±34.25	5.5e+9±2.4e+8	8.6e+8±2.3e+7	3.6e+4±1.8e+3	1.5e+5±8.2e+3	7.5e+11±2.2e+10	1.1 e+11±3.5e+9
	Carrot (root)	1.4e+3±35.81	5.1e+3±127.84	2.1e+10±5.4e+8	4.2e+9±1e+8	1.8e+4±572.46	6.6e+4±2043.42	2.8e+11±8.7e+9	5.4e+10±1.6e+9

D1S				D2S			
Daily (copies kg^{-1})		Annual (copies kg^{-1})		Daily (copies kg^{-1})		Annual (copies kg^{-1})	
A	C	A	C	A	C	A	C
8.4e+4±1943.94	2.4e+5±5698.0	1.7e+12±4e+10	2.7e+11±6.3e+9	1.9e+5±1.1e+5	5.7e+5±3.2e+5	4.1e+12±2.3e+11	6.4e+11±3.6e+10
1.9e+6±488.5	6.8e+6±1432.2	2.9e+13±5.3e+11	5.8e+12±1e+11	2.4e+4±303.02	8.6e+4±1081.66	3.7e+11±4.6e+9	1.1e+8±1.4e+6
5.3e+4±1634.1	1.5e+5±4789.6	1.1e+12±3.3e+10	1.7e+11±5.3e+9	7.8e+4±1311.49	2.3e+5±3844.23	1.6e+12±2.7e+10	2.5e+11±4.2e+9
1.8e+5±645.66	6.6e+5±2309.8	2.8e+12±1.6e+11	5.4e+11±3.1e+10	2.9e+4±3730.6	1e+5±13,316.56	4.5e+11±5.7e+10	1.4e+8±1.7e+7
9.2e+5±925.23	2.7e+6±4004.5	1.9e+13±1.2e+12	3e+12±1.8e+11	2.3e+6±7.2e+4	6.8e+6±2.1e+5	4.8e+13±1.4e+12	7.6e+12±2.3e+11
3e+5±1545.31	1e+6±5546.23	4.6e+12±1e+11	8.9e+11±1.9e+10	1.4e+5±3178.7	5.2e+5±11,346.5	2.2e+12±4.8e+10	7.1e+8±1.5e+7
4.1e+5±8.2e+3	1.2e+6±2.4e+4	8.7e+12±1.7e+11	1.3e+12±2.6e+10	6.5e+5±4997.6	1.9e+6±14,649.1	1.3e+13±1e+11	2.1e+12±1.6e+10
3.1e+5±5.5e+3	1.1e+6±1.9e+4	4.8e+12±8.5e+10	9.2e+11±1.6e+10	1e+6±5.4e+3	3.9e+6±1.9e+4	1.6e+13±8.3e+10	5.2e+9±2.6e+7
6e+4±849.08	1.7e+5±2488.84	1.2e+12±1.7e+10	1.9e+11±2.7e+9	2e+5±2.4e+3	5.8e+5±7.1e+3	4.1e+12±5e+10	6.5e+11±7.9e+9
4.4e+4±99.91	1.5e+5±356.63	6.7e+11±1.5e+9	1.2e+11±2.9e+8	1.2e+4±412.3	4.5e+4±1471.9	1.9e+11±6.3e+9	6.1e+7±1.9e+6
1e+6±547.8	2.9e+6±1656.4	2.1e+13±1.1e+12	3.2e+12±1.2e+11	4.6e+6±1.4e+4	1.3e+7±4.1e+4	9.6e+13±2.9e+11	1.5e+13±4.5e+10
6.8e+6±3.9e+4	2.4e+7±1.4e+5	1e+14±6e+11	2e+13±1.1e+11	5.5e+5±1.8e+3	1.9e+6±6.7e+3	8.5e+12±2.9e+10	2.6e+9±9.1e+6
2.9e+5±1792.7	8.6e+5±5255.03	6.1e+12±3.7e+10	9.5e+11±5.8e+9	1.1e+6±8.1e+4	3.3e+6±2.3e+5	2.3e+13±1.6e+12	3.7e+12±2.6e+11
2.4e+5±9846.9	8.7e+5±3.5e+4	3.7e+12±1.5e+11	7.1e+11±2.8e+10	3.3e+5±2.7e+3	1.1e+6±9.8e+3	5.1e+12±4.2e+10	1.6e+9±1.3e+7

Table 3 (continued)

D1S				D2S			
Daily (copies kg^{-1})		Annual (copies kg^{-1})		Daily (copies kg^{-1})		Annual (copies kg^{-1})	
A	C	A	C	A	C	A	C
1.7e+5 ± 2.6e+3	5e+5 ± 7.8e+3	3.6e+12 ± 5.5e+10	5.6e+11 ± 8.7e+9	1.3e+7 ± 5.2e+5	3.9e+7 ± 1.5e+6	2.8e+14 ± 1e+13	4.4e+13 ± 1.7e+12
1.3e+6 ± 5.3e+4	4.9e+6 ± 1.9e+5	2.1e+13 ± 8.1e+11	4e+12 ± 1e+11	1.2e+7 ± 3.8e+6	4.6e+7 ± 1.3e+7	1.9e+14 ± 5.9e+13	6.2e+10 ± 1.8e+10

Data represented as Mean ± S.E

authoritative bodies such as the Evaluation of Medicinal Products (EMEA) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The ADIs for the five selected antibiotics were established, considering their microbiological effects. Tables 2 and 3 present EDI values of ABs and ARGs detected in vegetables in the current study, with variations observed in the order BS < D1S < D2S. The EDI values (ng kg^{-1} day^{-1}) of AMX, AZT, C, CF, and TET across all treatments in both vegetables and populations (adults and children) ranged from (0.02 to 0.11), (0.03 to 0.2), (0.001 to 0.31), (0.0002 to 0.45), and (0.006 to 0.1), respectively. Subsequently, the Average Hazardous Exposure (AHE) value (ng kg^{-1} $year^{-1}$) varied from (58.9 to 584.0), (85 to 970.5), (3.94 to 2003.6), (0.72 to 2206), and (16.4 to 1351.4). Among the vegetables assessed, the EDI through spinach consumption was the highest for both adults and children. Notably, in this study, the EDI values for all ABs were below the ADI values, except for CF in children.

Similarly, the EDI values (copies kg^{-1} bw^{-1} day^{-1}) for sludge-treated pot-grown vegetables were significantly higher than the control (Table 3). The ranking of ARGs in terms of EDI values was *qnrS* < *blaCTX* < *blaGES* < *ermF* < *intl1* < *Sul1* < *blaNDM* < *IS26*. The corresponding EDI values varied as *ermF* (2.4×10^{-4} to 6.8×10^{-6}), *qnrS* (3.6×10^{-2} to 2.3×10^{-5}), *Sul1* (1×10^{-3} to 6.8×10^{-6}), *blaGES* (6.2×10^{-3} to 3.9×10^{-6}), *blaCTX* (4.4×10^{-1} to 5.8×10^{-5}), *blaNDM* (1.2×10^{-4} to 1.3×10^{-7}), *intl1* (6.7×10^{-2} to 3.3×10^{-6}), and *IS26* (2.6×10^{-2} to 4.6×10^{-7}). Subsequently, the AHE values (copies kg-1 bw-1 year-1) ranged as *ermF* (7.1×10^{-10} to 2.9×10^{-13}), *qnrS* (1.1×10^{-9} to 2.8×10^{-12}), *Sul1* (3.1×10^{-9} to 4.8×10^{-13}), *blaGES* (1.8×10^{-10} to 1.6×10^{-13}), *blaCTX* (1.4×10^{-8} to 4.1×10^{-12}), *blaNDM* (4×10^{-10} to 9.6×10^{-13}), *intl1* (1.9×10^{-9} to 2.3×10^{-13}), and *IS26* (8.6×10^{-8} to 2.8×10^{-14}).

Discussion

Impact of sludge application on soil and plant growth parameters

The physicochemical characteristics (bulk density, water holding capacity, electrical conductivity, moisture, heavy metals, organic matter content, nutrients, pH, persistent organic pollutants (POPs), etc. of STP-derived sludge are important because they influence the enrichment of ABs and ARGs in the sludge. These characteristics impact the mobility, transfer, and translocation of ABs and ARGs within the sludge, soil, and plant systems. Understanding these properties helps in assessing the potential risks associated with the presence of ABs and ARGs in the environment, offering insights into their behaviour and potential impact on human health through food chain exposure. The observed differences in physicochemical characteristics among STPs with high and low treatment capacities suggested that the nature of mixed liquor suspended solids (MLSS) is influenced by the aeration systems, as STP3 utilizing submerged aeration while the other two using surface aeration. Cu, Zn, Cd, and Pb were found in high concentrations and varied greatly, while the amounts of Ni and Mn varied less. The reason for the higher abundance of Mn in BS sludge possibly may be that the lower treatment capacity leads to reduced dilution effects, allowing Mn to concentrate more in the sludge. A higher abundance of heavy metals in the D1S and D2S may suggest a potential impact of aeration system variation on overall treatment efficiency and sewage characteristics as they primarily treat urban wastewater from the main city, influencing metal accumulation and nutrient availability in the treated sludge. Similar results were found in studies by Nyashanu et al. (2023). Agricultural soil had a neutral pH, while sludge samples were slightly acidic. It might be due to organic matter decomposition and microbial digestion in sewage treatment plants. Nitrification may also contribute to acidity by converting ammonia to nitrate. Minimal variation was observed in organic carbon and other parameters across the three samples, except for phosphorus (P). Elevated levels of organic matter, available phosphorus, and nitrogen detected in the sludge suggested the potential for higher bacterial counts (Turek et al. 2019). The data obtained from sludge samples for physicochemical properties, AB and ARGs were utilised to generate a 3D PCA plot (Fig. 5a), in which it forms 3 different clusters; among these D1S and D2S showed a strong positive correlation with most of the physicochemical parameters, HM contents, AB contents and ARGs somewhere indicating the co-selection of the antimicrobial resistance in sludge environment (Lu et al. 2022). Therefore, wastewater treatment plants should implement stringent operational standards to ensure the quality of sewage sludge used in agriculture, addressing growing concerns about the safety of the food chain. The utilization of sewage sludge in agricultural soil is known to enhance soil nutrients and promote crop growth. In our study, all amendments led to increased growth parameters (height, root length, and biomass) for both vegetables (Supplementary Table S4), indicating that the addition of nutrient-rich sludge likely boosted plant growth and microbial activity, as supported by a previous study by Albalasmeh et al. (2020). The acidic pH and higher electrical conductivity (EC) may be attributed to sludge acidity, while the reduction in heavy metal content could be a result of organic matter complexing and plant nutrient uptake (Al Mamun et al. 2022).

Antibiotics and heavy metals resistance pattern of endophytic bacteria

The TCEB identified the endophytic bacterial population in vegetables, and screening for multi-antibiotic resistance highlighted the presence of MAREB. Initial screening with Ampicillin, a widely used antibiotic with developed resistance, revealed a diverse range of bacteria as vegetable endophytes, exhibiting varied resistance patterns to 17 common antibiotics for human and animal infections. Alarmingly, all bacteria exhibited a multiple antibiotic resistance index (MARI) of 0.2 (20%). The varied resistance patterns to antibiotics (ABs) and heavy metals (HMs) observed in these bacteria are associated with factors such as their source, concentration, duration of exposure to pollutants, and adaptive mechanisms developed to counteract the detrimental effects of antibiotics and heavy metals. Karmakar et al. (2019) and Pan et al. (2024) showed convergent evolution within heavy metal and antibiotic-resistant endophytic bacterial isolates, indicating co-selection of antimicrobial resistance (AMR) by heavy metals like Cu and Cd in vegetables. Vertical and horizontal gene transfer between introduced sludge multidrug-resistant bacteria and native bacteria, are crucial factors as reported in our previous study Patra et al. (2024). Notably, bacteria resistant to all selected heavy metals also exhibited resistance to quinolones, tetracycline, rifamycin, macrolides, aminoglycosides, and β-lactams, suggesting that sewage from surrounding cities contains residues of both antibiotics and heavy metals. Even after treatment, incomplete removal of these residues in sludge imposes selection pressure on resistant bacterial communities to survive through mechanisms such as membrane permeability, alteration of antibiotics and heavy metals, increased efflux, changes in target sites, and sequestration of antibiotics and heavy metals (Salmonowicz et al. 2023; Sharma et al. 2022a, b) as reported by previous researchers. It can be concluded that MDR in sludge transfers antibiotic and heavy metal resistance genes to native soil bacteria, which, upon acquiring resistance, may subsequently transfer these traits to endophytic isolates in sludge-amended soil-grown vegetables.

Identification and evaluation of MAREB for beneficial traits in plants

The cultivable MAREB isolated from vegetable samples exhibited taxonomic diversity (Fig. 2), distinguishing this study from others that typically focus on limited genera. Previous research highlighted the prevalence of genera *Lysinebacillus*, *Pseudomonas,* and *Serratia* in contaminated lettuce and *Bacillus, Enterobacter*, *Providentia*, *Bravibacillus*, *Pseudomonas*, *Achromobacter*, *Microbacterium*, *Acinetobacter*, and *Pantoea* in polluted soil-grown vegetables. Notably, 50% of MAREB identified in our study are both plant-associated and human pathogenic bacteria, and showed abundant plant beneficial traits, posing a transmission risk linked to the sludge's richness in ABs, ARGs and MGEs. This phenomenon may be attributed to the chemically complex, nutrient-rich rhizosphere environment that selectively supports bacterial growth. Endophytic bacteria, requiring positive interactions with host plants for prolonged harboring, often developed resistance to ABs and HMs (Aleynova and Kiselev. 2023).

Therefore, this study indicates the intricate interaction and transmission ambiguity between fresh produce and human pathogenic microorganisms (HMPs). Recent studies further demonstrate the persistence of human pathogenic bacteria in *Arabidopsis* sp. and lettuce leaves and the colonization of several pathogens in *R. sativus*, with *L. monocytogenes* (Jacob et al. 2021; Szymańska et al. 2024).

Assessment of aggregation, motility and dynamics of biofilm formation by MAREB

Notably, 72.72% of MAREB showed significant mobility and strong auto-aggregation, both of which are essential for the production of biofilms. In line with other research showing the significance of motility in biofilm production, non-motile MAREB were unable to build robust biofilms (Boas et al. 2024). In order for bacteria to respond to environmental cues and develop strong biofilm structures for increased survival and resistance, motility is essential for biofilm formation. This capacity to move also promotes genetic exchange (horizontal gene transfer) and increases stress tolerance. Furthermore, biofilm formation in MAREB was aided by all of the chosen antibiotics at lower concentrations. This suggests that sub-lethal antibiotic concentrations cause biofilm formation through a variety of pathways, including those that aid in bacterial adaptation and survival and auto-inducer molecule production and antibiotic signalling. Bhattacharya et al. (2019) reported similar results for multiple drug-resistant bacteria that were isolated from the Indian Sundarban estuary. Therefore, MAREB with aggressive biofilm formation on vegetables poses a significant health risk to consumers, as ingestion of such contaminated produce can potentially introduce antibiotic resistance and persistent infections into the human microbiome, complicating treatment options and increasing the spread of resistant pathogens (Galie et al. 2018).

Molecular detection of ARGs, HMRGs, and MGEs genes

The qualitative analysis of ABs and HMs resistance genes in MAREB revealed amplification in chromosomal DNA,

suggesting intrinsic chromosomal encoding of these traits rather than plasmid-mediated transfer. Possible reasons include genomic DNA mutations contributing to acquired resistance or loss of plasmid segments (Carroll and Wong 2018). Quantitative assessment of selected ARGs highlighted significant variations in gene abundance among sludge, soil, and vegetable samples, possibly due to high pharmaceutical residue concentrations in sewage treatment plant (STP) sludge, providing selection pressure for resistance development and facilitating mobile genetic element-mediated gene transfer. Notably, intl1 and IS26 showed positive correlations with various ARG families (Fig. 5b), indicating their role in gene migration between species in sludge and environmental reservoirs, leading to increased ARGs and MGE abundance in amended soil and vegetables.

Influence of sludge application on the presence of ABs and ARGs in soil and vegetables

A higher presence of studied antibiotics in sludge compared to soils observed in the present study, potentially due to degradation and dilution, especially for tetracyclines and amoxicillin, which have lower adsorption by soil components. Selected vegetables exhibited notably higher antibiotic concentrations, consistent with previous reports of antibiotic accumulation in various crops such as cherry tomato, lettuce, cucumber, oats, rice, and corn (Ahmed et al. 2015; Pan and Chu 2017). Agricultural practices involving wastewater, sewage sludge and animal manure elevated antibiotic levels in crops, with reports of antibiotics like ciprofloxacin and ofloxacin detected in carrots and soil (Wu et al. 2014b, a). Concerns persist regarding the translocation of antibiotics into edible crops due to their differing molecular structures, resistance, adsorption, and half-lives in soils, as indicated by recent research on pharmaceutical translocation within plant tissues (Sun et al. 2021). This study uncovered a notable presence of antibiotic resistance genes (ARGs) in sludge, amended soil, and plants, suggesting ARGs dissemination even without antibiotic selection pressure. A strong positive correlation between MGEs and ARGs highlights the impact of sludge farming on the soil resistome. The elevated mean of ARGs (copies g^{-1}) (2.6×10^{9} and 2.4×10^{8}) observed in spinach leaves and carrot roots from sludge treatment underscore the severity of contamination compared to fast-food salads (Zhou et al. 2020). Manure application is implicated in increasing ARGs incidence in the vegetable phyllosphere, likely due to soil-borne ARGs dispersing into the air and depositing on leaf surfaces. Bacteria in soil can attach to plant roots, forming biofilms and potentially transferring carried ARGs to indigenous endophytic bacteria, which depends on flagella movement and transpiration of plants (Afzal et al. 2019). A recent study found fluorescently labelled *E. coli* carrying ARGs can enter plants from the rhizosphere and travel to the phyllosphere via vascular bundles (Xu et al. 2021). However, there's no evidence yet of free extracellular ARGs transfer into plant tissue from the environment.

Impact of biosolid application on the transmission of ABs, ARGs and MGEs

In this study, bioaccumulation or bioconcentration factors were found to increase with higher exposure concentrations in soil (Fig. S6c), similar to the findings of Mohy-u-Din et al. (2023). Azanu et al. (2016) noted that higher bioconcentration factor values indicate greater antibiotic accumulation in plants, posing a higher risk to human health. Various factors, including biotic and abiotic, influence antibiotic uptake and absorption by plants. Both barley and carrot showed higher uptake and translocation of antibiotics, indicating dependence on the transpiration stream of plant vascular tissue (Pullagurala et al. 2018). The translocation factor generally hovered around 1 for azithromycin and amoxicillin in crops under different treatment conditions, with significant differences observed between crop species and treatment types (Pan et al. 2014). Antibiotic accumulation and distribution in crops are affected by species type and growth stage. Additionally, the hydrophilic properties of compounds play a significant role in facilitating their high translocation through the xylem (Wu et al. 2016). Uptake and translocation of antibiotics vary between species; for instance, radish exhibited higher uptake and translocation of norfloxacin and sulfamethoxazole compared to Chinese cabbage at the same dosage (Wang et al. 2016). In the present study, the BCF and TLF analyzed to assess ARGs enrichment and transfer within vegetables, showed higher ARGs enrichment in roots than in leaves, aligning with previous findings on the role of roots in ARGs transfer from soil to plants (Wang et al. 2021). Microbes act as carriers for ARGs in plants, primarily in the rhizosphere, emphasizing the crucial role of roots in ARGs transfer in the soil-vegetable system (Cordovez et al. 2019).

Health risk analysis

The human health risk was assessed by taking into consideration the HQ, as well as the legislated values for corresponding ABs given by standard authorities. The outcome revealed that HQ depends on the ABs dosage. CIP was the AB found at the greatest concentration in the spinach and carrot edible parts, and thus the one with the greatest HQ and HI in D1S and D2S treated scenarios, indicating that the intake of fertilized lettuce

thus poses a greater human health risk specifically children (Matamoros et al. 2022). The EDI of antibiotics from consuming edible vegetables remained below the ADI doses, except for ciprofloxacin (CIP), which exceeded the minimum inhibitory concentration. The estimated daily intake of ciprofloxacin (CIP) was found to be higher in children, indicating the potential promotion of emergence and selection of resistant commensal bacteria due to daily CIP intake (Table 3). This poses particular concern for young children, as their intestinal microflora is in a critical development stage. Additionally, early exposure to broad-spectrum antibiotics before age two has been linked to a 16% increase in childhood obesity (Baron et al. 2020). Repeated disruption of the gut ecosystem by antibiotic residues in young children may impact gut bacteria development and increase the risk of childhood obesity and allergic diseases (Ben et al. 2022). However, the EDI of ARGs from consuming sludge-fertilized vegetables was notably higher compared to those treated with commercial organic fertilizer. This raises concerns about the spread of ARBs through the food chain. At the same time, studies have shown that antibiotic consumption increases ARGs abundance in fish, animal and human gut microbiomes (Saenz et al. 2019; Gasparrini et al. 2019). However, there is limited research on the impact of ARGs-contaminated food on human gut microbiomes. Quantifying the risk of ARGs transmission through the food chain remains a significant challenge that requires further investigation.

Conclusion

The study concludes that sludge application increases ABs residues, ARGs, and MGEs in soil, varying with sludge type and potential translocation within vegetables. ABs (ciprofloxacin and chloramphenicol) and ARGs (Sul1, intl1, and IS26) were notably elevated throughout the sludge-amended system. The prevalence of intl1 and IS26 in sludge-treated vegetables suggested increased horizontal gene transfer potential. Nearly all MAREB exhibited resistance to beta-lactam antibiotics, with *Bacillus* sp. strains E1R, E7R, and E18L, *Stenotrophomonas* sp. strain E9R, *Exiguobacterium* sp. strains E13L and E21L resistant to multiple drugs (>69% MARI). The strong correlation between the abundance of ARGs, MGEs, and high MARI, as well as the high motility, aggregation, and biofilm production ability of MAREB highlighted their collective contribution to antibiotic resistance. Furthermore, MAREB's survival in high concentrations of vancomycin and clarithromycin underscores the urgency of addressing AMR dissemination, emphasizing the need for effective strategies to combat its spread and preserve the efficacy of antibiotics. The observed convergent evolution adaptation of environmental bacteria to ABs, HMs, and endophytic conditions suggests their resilience and versatility. Additionally, the identification of EBs as both plant-associated and human pathogens, with a subset solely pathogenic to humans, underscores the need for comprehensive risk assessment and mitigation strategies in agricultural practices to safeguard both environmental and human health. The risk assessment for antibiotics indicated that the risk increased in the order of BS < D1S < D2S, with children being particularly vulnerable to risk upon consuming vegetables. Therefore, it is crucial to monitor soil antibiotic levels to mitigate significant long-term health risks. Consequently, the feasibility of biosolid application should be evaluated in terms of antibiotics and ARGs in the future, and the abundance of ARGs should be considered in the regulatory standards for biosolids applied in agriculture. Implementing appropriate regulatory measures and advancing existing technologies will aid in addressing this emerging contaminant.

Supplementary Information

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Supplementary Material 1.

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Availability of data and materials
All the data in the manuscript are given in manuscript file in the form of main and supplementary file.

Declarations

Ethics approval and consent to participate
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Competing interests
The authors declared that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

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References

Abdellah YAY, Luo YS, Sun SS, Yang X, Ji HY, Wang RL (2023) Phytochemical and underlying mechanism of *Mikania micrantha* Kunth on antibiotic resistance genes, and pathogenic microbes during chicken manure composting. Bioresour Technol 367:128241

Afzal I, Shinwari ZK, Sikandar S, Shahzad S (2019) Plant beneficial endophytic bacteria: mechanisms, diversity, host range and genetic determinants. Microbiol Res 221:36–49

Ahmed MBM, Rajapaksha AU, Lim JE, Vu NT, Kim IS, Kang HM, Lee SS, Ok YS (2015) Distribution and accumulative pattern of tetracyclines and sulfonamides in edible vegetables of cucumber, tomato, and lettuce. J Agric Food Chem 63(2):398–405

Al Mamun S, Saha S, Ferdush J, Tusher TR, Islam MS (2022) Organic amendments for crop production, phosphorus bioavailability and heavy metal immobilisation: a review. Crop Pasture Sci. https://doi.org/10.1071/CP21726

Albalasmeh A, Gharaibeh MA, Mohawesh O, Alajlouni M, Quzaih M, Masad M, El Hanandeh A (2020) Characterization and artificial neural networks modelling of methylene blue adsorption of biochar derived from agricultural residues: effect of biomass type, pyrolysis temperature, particle size. J Saudi Chem Soc 24(11):811–823

Aleynova OA, Kiselev KV (2023) Interaction of plants and endophytic microorganisms: molecular aspects, biological functions, community composition, and practical applications. Plants 12(4):714

Aminov RI, Garrigues-Jeanjean N, Mackie R (2001) Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Appl Environ Microbiol 67:22–32

Argudín MA, Cariou N, Salandre O, Le Guennec J, Nemeghaire S, Butaye P (2013) Genotyping and antimicrobial resistance of *Staphylococcus aureus* isolates from diseased turkeys. Avian Pathol 42(6):572–580

Azanu D, Mortey C, Darko G, Weisser JJ, Styrishave B, Abaidoo RC (2016) Uptake of antibiotics from irrigation water by plants. Chemosphere 157:107–114

Baron R, Taye M, Besseling-Van Der Vaart I, Ujčič-Voortman J, Szajewska H, Seidell JC, Verhoeff A (2020) The relationship of prenatal and infant antibiotic exposure with childhood overweight and obesity: a systematic review. J Dev Orig Health Dis 11(4):335–349

Barraud O, Baclet MC, Denis F, Ploy MC (2010) Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. J Antimicrob Chemother 65(8):1642–1645

Barreiro A, Cela-Dablanca R, Nebot C, Rodríguez-López L, Santás-Miguel V, Arias-Estévez M, Sanjurjo MF, Delgado AN, Rodriguez EA (2022) Occurrence of nine antibiotics in different kinds of sewage sludge, soils, corn and grapes after sludge spreading. Span J Soil Sci 12:10741

Belani N, Kandya A, Kumar V, Varshney L, Patel D, Ganguly C (2023) Sewage sludge—a latent biogold. In: Yadav KD, Jariwala ND, Kumar A, Sinha A (eds) Invitation to national level conference on sustainable waste management practices. Springer Nature Singapore, Singapore, pp 167–174

Ben Y, Hu M, Zhong F, Du E, Li Y, Zhang H, Andrews CB, Zheng C (2022) Human daily dietary intakes of antibiotic residues: dominant sources and health risks. Environ Res 212:113387

Bhattacharyya A, Haldar A, Bhattacharyya M, Ghosh A (2019) Anthropogenic influence shapes the distribution of antibiotic resistant bacteria (ARB) in the sediment of Sundarban estuary in India. Sci Total Environ 647:1626–1639

Bhattacharjee AS, Phan D, Zheng C, Ashworth D, Schmidt M, Men Y, Ferreira JF, Muir G, Hasan NA, Ibekwe AM (2024) Dissemination of antibiotic resistance genes through soil-plant-earthworm continuum in the food production environment. Environ Int 183:108374

Blake GR (1965) Bulk density. Methods of soil analysis: Part 1 physical and mineralogical properties, including statistics of measurement and sampling, 9:374–390

Bressan W, Borges MT (2004) Delivery methods for introducing endophytic bacteria into maize. BioControl 49(3):315–322

Carroll AC, Wong A (2018) Plasmid persistence: costs, benefits, and the plasmid paradox. Can J Microbiol 64(5):293–304

Carvalho IT, Santos L (2016) Antibiotics in the aquatic environments: a review of the European scenario. Environ Int 94:736–757

Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, Colomb-Cotinat M, Kretzschmar ME, Devleesschauwer B, Cecchini M, Ouakrim DA (2019) Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect Dis 19(1):56–66

Center for Disease Control and Prevention (2017) Antibiotic use in the united states progress and opportunities. Center for Disease Control and Prevention. p. 1–40. https://www.cdc.gov/antibiotic-use/stewardship-report/pdf/stewardship-report.pdf. Accessed 29 Apr 2024

Chebotar VK, Chizhevskaya EP, Baganova ME, Keleinikova OV, Yuzikhin OS, Zaplatkin AN, Khonina OV, Kostitsin RD, Lapenko NG (2022) Endophytes from halotolerant plants aimed to overcome salinity and draught. Plants 11(21):2992

Cherian T, Ragavendran C, Vijayan S, Kurien S, Peijnenburg WJ (2023) A review on the fate, human health and environmental impacts, as well as regulation of antibiotics used in aquaculture. Environmental Advances, p 100411

Christou A, Papadavid G, Dalias P, Fotopoulos V, Michael C, Bayona JM, Pina B, Kassinos-Fatta D (2019) Ranking of crop plants according to their potential to uptake and accumulate contaminants of emerging concern. Environ Res 170:422–432

Colomer-Lluch M, Jofre J, Muniesa M (2014) Quinolone resistance genes (qnrA and qnrS) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes. J Antimicrob Chemother 69(5):1265–1274

Cordovez V, Dini-Andreote F, Carrión VJ, Raaijmakers JM (2019) Ecology and evolution of plant microbiomes. Annu Rev Microbiol 73:69–88

Council E (1986) Council directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. Waste Manag Clean Technol

Cycoń M, Mrozik A, Piotrowska-Seget Z (2019) Antibiotics in the soil environment—degradation and their impact on microbial activity and diversity. Front Microbiol 10:412419

ECDC (2009) The bacterial challenge: time to react, European Centre for Disease Prevention and Control. https://www.ecdc.europa.eu/en/publications-data/ecdcemea-joint-technical-report-bacterial-challenge-time-react. Accessed 29 Apr 2024

Ehalt Macedo H, Lehner B, Nicell J, Grill G, Li J, Limtong A, Shakya R (2022) Distribution and characteristics of wastewater treatment plants within the global river network. Earth Syst Sci Data 14(2):559–577

Enne VI, Livermore DM, Stephens P, Hall LM (2001) Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. Lancet 357(9265):1325–1328

European Centre for Disease Prevention and Control (2020) Antimicrobial resistance in the EU/EEA (EARS-Net)—Annual Epidemiological Report 2020, ECDC. https://www.ecdc.europa.eu/en/publications-data/antimicrobial-resistance-eueea-ears-net-annual-epidemiological-report-2020. Accessed 29 Apr 2024

Galié S, García-Gutiérrez C, Miguélez EM, Villar CJ, Lombó F (2018) Biofilms in the food industry: health aspects and control methods. Front Microbiol 9:315815

Gao FZ, He LY, He LX, Zou HY, Zhang M, Wu DL, Liu YS, Shi YJ, Bai H, Ying GG (2020) Untreated swine wastes changed antibiotic resistance and microbial community in the soils and impacted abundances of antibiotic resistance genes in the vegetables. Sci Total Environ 741:140482

Garder JL, Moorman TB, Soupir ML (2014) Transport and persistence of tylosin-resistant Enterococci, erm genes, and tylosin in soil and drainage water from fields receiving swine manure. J Environ Qual 43:1484–1493

Gasparrini AJ, Wang B, Sun X, Kennedy EA, Hernandez-Leyva A, Ndao IM, Tarr PI, Warner BB, Dantas G (2019) Persistent metagenomic signatures

of early-life hospitalization and antibiotic treatment in the infant gut microbiota and resistome. Nat Microbiol 4(12):2285–2297
Goryluk-Salmonowicz A, Myczka AW, Popowska M (2023) Antibiotic-and metal-resistant endophytes inhabit *Armeria maritima* hyperaccumulator. Plant Soil 495:1–20
Gudda F, Odinga ES, Tang L, Waigi MG, Wang J, Abdalmegeed D, Gao Y (2023) Tetracyclines uptake from irrigation water by vegetables: accumulation and antimicrobial resistance risks. Environ Pollut 338:122696
Gupta K, Chatterjee C, Gupta B (2012) Isolation and characterization of heavy metal tolerant Gram-positive bacteria with bioremedial properties from municipal waste rich soil of Kestopur canal (Kolkata), West Bengal, India. Biologia 67:827–836
Gupta S, Sreekrishnan TR, Ahammad SZ (2022) Effects of heavy metals on the development and proliferation of antibiotic resistance in urban sewage treatment plants. Environ Pollut 308:119649
Gworek B, Kijeńska M, Wrzosek J, Graniewska M (2021) Pharmaceuticals in the soil and plant environment: a review. Water Air Soil Pollut 232(4):145
Hawrami KA, Crout NM, Shaw G, Bailey EH (2020) Assessment of potentially toxic elements in vegetables cultivated in urban and peri-urban sites in the Kurdistan region of Iraq and implications for human health. Environ Geochem Health 42:1359–1385
Hembach N, Bierbaum G, Schreiber C, Schwartz T (2022) Facultative pathogenic bacteria and antibiotic resistance genes in swine livestock manure and clinical wastewater: a molecular biology comparison. Environ Pollut 313:120128
Hu J, Shi J, Chang H, Li D, Yang M, Kamagata Y (2008) Phenotyping and genotyping of antibiotic-resistant *Escherichia coli* isolated from a natural river basin. Environ Sci Technol 42(9):3415–3420
ISTA N (2006) ISTA method validation for seed testing. The International Seed Testing Association, Bassersdorf, Switzerland
Jackson ML (1958) Soil chemical analysis. Prentice Hall. Inc., Englewood Cliffs. 498:183–204
Jackson ML (1973) Soil chemical analysis. Pentice Hall of India Pvt. Ltd., New Delhi. 498:151–154
Jacob C, Velásquez AC, Josh NA, Settles M, He SY, Melotto M (2021) Dual transcriptomic analysis reveals metabolic changes associated with differential persistence of human pathogenic bacteria in leaves of *Arabidopsis* and lettuce. G3 11(12):jkab331
Karmakar R, Bindiya S, Hariprasad P (2019) Convergent evolution in bacteria from multiple origins under antibiotic and heavy metal stress, and endophytic conditions of host plant. Sci Total Environ 650:858–867
Kläui A, Bütikofer U, Naskova J, Wagner E, Marti E (2024) Fresh produce as a reservoir of antimicrobial resistance genes: a case study of Switzerland. Sci Total Environ 907:167671
Laxminarayan R, Matsoso P, Pant S, Brower C, Røttingen JA, Klugman K, Davies S (2016) Access to effective antimicrobials: a worldwide challenge. Lancet 387(10014):168–175
Łepecka A, Zielińska D, Szymański P, Buras I, Kołożyn-Krajewska D (2022) Assessment of the microbiological quality of ready-to-eat salads—are there any reasons for concern about public health? Int J Environ Res Public Health 19(3):1582
Li S, Ondon BS, Ho SH, Zhou Q, Li F (2023) Drinking water sources as hotspots of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs): occurrence, spread, and mitigation strategies. J Water Process Eng 53:103907
Li Y, Li R, Hou J, Sun X, Wang Y, Li L (2024) Mobile genetic elements affect the dissemination of antibiotic resistance genes (ARGs) of clinical importance in the environment. Environ Res 243:117801
Liebert CA, Wireman J, Smith T, Summers AO (1997) Phylogeny of mercury resistance (mer) operons of gram-negative bacteria isolated from the fecal flora of primates. Appl Environ Microbiol 63(3):1066–1076
Lindsay WL, Norvell W (1978) Development of a DTPA soil test for zinc, iron, manganese, and copper. Soil Sci Soc Am J 42:421–428
Lu L, He Y, Peng C, Wen X, Ye Y, Ren D, Tang Y, Zhu D (2022) Dispersal of antibiotic resistance genes in an agricultural influenced multi-branch river network. Sci Total Environ 830:154739
Margaryan AA, Panosyan HH, Birkeland NK, Trchounian AH (2013) Heavy metal accumulation and the expression of the copA and nikA genes in *Bacillus subtilis* AG4 isolated from the Sotk Gold Mine in Armenia. Biolog J Armenia 3(65):51–57
Martens E, Demain AL (2017) The antibiotic resistance crisis, with a focus on the United States. J Antibiot 70(5):520–526
Matamoros V, Casas ME, Mansilla S, Tadić Đ, Cañameras N, Carazo N, Portugal J, Pina B, Diez S, Bayona JM (2022) Occurrence of antibiotics in Lettuce (*Lactuca sativa* L.) and Radish (*Raphanus sativus* L.) following organic soil fertilisation under plot-scale conditions: crop and human health implications. J Hazard Mater 436:129044
Mohy-u-Din N, Farhan M, Wahid A, Ciric L, Sharif F (2023) Human health risk estimation of antibiotics transferred from wastewater and soil to crops. Environ Sci Pollut Res 30(8):20601–20614
Moudgil P, Bedi JS, Aulakh RS, Gill JPS, Kumar A (2019) Validation of HPLC multiresidue method for determination of fluoroquinolones, tetracycline, sulphonamides and chloramphenicol residues in bovine milk. Food Anal Methods 12:338–346
Murray CJ, Ikuta KS, Sharara F, Swetschinski L, Aguilar GR, Gray A, Han C, Bisignano C, Rao P, Wool E, Johnson SC (2022) Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet 399(10325):629–655
Nahim-Granados S, Quon H, Polo-López MI, Oller I, Agüera A, Jiang S (2024) Assessment of antibiotic-resistant infection risks associated with reclaimed wastewater irrigation in intensive tomato cultivation. Water Res 254:121437
Nelson LB, Heidel H (1952) Soil analysis methods as used in the Iowa state college soil testing laboratory
Nieuwenhuize J, Poley-Vos CH, van den Akker AH, Van Delft W (1991) Comparison of microwave and conventional extraction techniques for the determination of metals in soil, sediment and sludge samples by atomic spectrometry. Analyst 116:347–351
Nurpermatasari A, Harahap U, Siagian P (2018) Identification gene mutations rpoB cause of multidrug resistance tuberculosis in Haji Adam Malik hospital. Asian J Pharm Clin Res 11:155–158
Nyashanu PN, Shafodino FS, Mwapagha LM (2023) Determining the potential human health risks posed by heavy metals present in municipal sewage sludge from a wastewater treatment plant. Sci Afr 20:e01735
Olsen SR, Kemper WD, Van Schaik JC (1965) Self-diffusion coefficients of phosphorus in soil measured by transient and steady-state methods. Soil Sci Soc Am J 29:154–158
Pan M, Chu LM (2017) Transfer of antibiotics from wastewater or animal manure to soil and edible crops. Environ Pollut 231:829–836
Pan M, Wong CK, Chu LM (2014) Distribution of antibiotics in wastewater-irrigated soils and their accumulation in vegetable crops in the Pearl River Delta, Southern China. J Agric Food Chem 62(46):11062–11069
Pan J, Zheng N, An Q, Li Y, Sun S, Wang S, Ji Y, Li N (2024) The effects of cadmium-copper stress on the accumulation of antibiotic-resistance genes in soil and pakchoi leaves. Soil Biol Biochem 191:109362
Patra M, Pandey B, Dubey SK (2024) Prevalence of diverse antimicrobial resistance genes and bacteria in sewage treatment plant derived sludge environment. FEMS Microbes 5:xtae004
Percival SL, Woods E, Nutekpor M, Bowler P, Radford A, Cochrane C (2008) Prevalence of silver resistance in bacteria isolated from diabetic foot ulcers and efficacy of silver-containing wound dressings. Ostomy Wound Manage 54(3):30–40
Piper CS (1945) Soil and plant analysis. J Phys Chem 49:45–45
Pullagurala VLR, Rawat S, Adisa IO, Hernandez-Viezcas JA, Peralta-Videa JR, Gardea-Torresdey JL (2018) Plant uptake and translocation of contaminants of emerging concern in soil. Sci Total Environ 636:1585–1596
Rathinavelu S, Uluseker C, Sonkar V, Thatikonda S, Nambi IM, Kreft JU (2024) Mapping the scarcity of data on antibiotics in natural and engineered water environments across India. Front Antibiot 3:1337261
Roosa S, Wattiez R, Prygiel E, Lesven L, Billon G, Gillan DC (2014) Bacterial metal resistance genes and metal bioavailability in contaminated sediments. Environ Pollut 189:143–151
Sáenz JS, Marques TV, Barone RSC, Cyrino JEP, Kublik S, Nesme J, Schloter M, Rath S, Vestergaard G (2019) Oral administration of antibiotics increased the potential mobility of bacterial resistance genes in the gut of the fish *Piaractus mesopotamicus*. Microbiome 7:1–14
Salam MA, Al-Amin MY, Salam MT, Pawar JS, Akhter N, Rabaan AA, Alqumber MA (2023) Antimicrobial resistance: a growing serious threat for global public health. Healthcare 11(13):1946

Sandhu R, Dahiya S, Sayal P (2016) Evaluation of multiple antibiotic resistance (MAR) index and Doxycycline susceptibility of Acinetobacter species among inpatients. Indian J Microbiol Res 3(3):299
Scaria J, Anupama KV, Nidheesh PV (2021) Tetracyclines in the environment: an overview on the occurrence, fate, toxicity, detection, removal methods, and sludge management. Sci Total Environ 771:145291
Sharma B, Shukla S, Rattan R, Fatima M, Goel M, Bhat M, Dutta S, Ranjan RK, Sharma M (2022) Antimicrobial agents based on metal complexes: present situation and future prospects. Int J Biomater 2022:6819080
Sharma M, Yadav A, Mandal MK, Pandey S, Pal S, Chaudhuri H, Chakrabarti S, Dubey KK (2022) Wastewater treatment and sludge management strategies for environmental sustainability. In: Stefanakis A, Nikolaou I (eds) Circular economy and sustainability. Elsevier, Amsterdam, pp 97–112
Smith CJ, Nedwell DB, Dong LF, Osborn AM (2006) Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. Environ Microbiol 8(5):804–815
Sriram A, Kalanxhi E, Kapoor G, Craig J, Balasubramanian R, Brar S, Criscuolo N, Hamilton A, Klein E, Tseng K, Boeckel T (2021) State of the world's antibiotics 2021: a global analysis of antimicrobial resistance and its drivers. Center for Disease Dynamics, Economics & Policy, Washington, pp 1–115
Stedtfeld RD, Guo X, Stedtfeld TM, Sheng H, Williams MR, Hauschild K, Gunturu S, Tift L, Wang F, Howe A, Chai B (2018) Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements. FEMS Microbiol Ecol 94(9):p.fiy130
Subramaniam G, Girish M (2020) Antibiotic resistance—a cause for reemergence of infections. Indian J Pediatr 87(11):937–944
Sun F, Xu Z, Fan L (2021) Response of heavy metal and antibiotic resistance genes and related microorganisms to different heavy metals in activated sludge. J Environ Manag 300:113754
Szymańska S, Deja-Sikora E, Sikora M, Niedojadło K, Hrynkiewicz K (2024) Colonization of *Raphanus sativus* by human pathogenic microorganisms. Front Microbiol 15:1296372
Tamura K, Stecher G, Kumar S (2021) MEGA11: molecular evolutionary genetics analysis version 11. Mol Biol Evol 38(7):3022–3027
Tan MF, Li HQ, Yang Q, Zhang FF, Tan J, Zeng YB, Wei QP, Huang JN, Wu CC, Li N, Kang ZF (2023) Prevalence and antimicrobial resistance profile of bacterial pathogens isolated from poultry in Jiangxi Province, China from 2020 to 2022. Poult Sci 102(8):102830
Turek A, Wieczorek K, Wolf WM (2019) Digestion procedure and determination of heavy metals in sewage sludge—an analytical problem. Sustainability 11(6):1753
Urbaniak M, Baran A, Giebułtowicz J, Bednarek A, Serwecińska L (2024) The occurrence of heavy metals and antimicrobials in sewage sludge and their predicted risk to soil—is there anything to fear? Sci Total Environ 912:168856
Vaithyanathan VK, Cabana H (2021) Integrated biotechnology management of biosolids: sustainable ways to produce value—added products. Front Water 3:729679
Vilas Boas D, Castro J, Araújo D, Nóbrega FL, Keevil CW, Azevedo NF, Vieira MJ, Almeida C (2024) The role of flagellum and flagellum-based motility on *Salmonella* enteritidis and *Escherichia coli* biofilm formation. Microorganisms 12(2):232
Walkley A, Black IA (1934) An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci 37:29–38
Wang J, Lin H, Sun W, Xia Y, Ma J, Fu J, Zhang Z, Wu H, Qian M (2016) Variations in the fate and biological effects of sulfamethoxazole, norfloxacin and doxycycline in different vegetable–soil systems following manure application. J Hazard Mater 304:49–57
Wang C, Hu R, Strong PJ, Zhuang W, Huang W, Luo Z, Yan Q, He Z, Shu L (2021) Prevalence of antibiotic resistance genes and bacterial pathogens along the soil–mangrove root continuum. J Hazard Mater 408:124985
Wang L, Nie L, Liu Y, Hu L, Zhou A, Wang D, Xu X, Guo J (2024) Antimicrobial resistance and molecular characterization of *Salmonella* Rissen isolated in China during 2008–2019. Infect Drug Resist 17:969–976
Wang Y, Wang Y, Shao T, Wang R, Dong Z, Xing B (2024b) Antibiotics and microplastics in manure and surrounding soil of farms in the Loess Plateau: occurrence and correlation. J Hazard Mater 465:133434
Wu JJ, Ko WC, Tsai SH, Yan JJ (2007) Prevalence of plasmid-mediated quinolone resistance determinants QnrA, QnrB, and QnrS among clinical isolates of *Enterobacter cloacae* in a Taiwanese hospital. Antimicrob Agents Chemother 51(4):1223–1227
Wu CF, Xu XM, Zhu Q, Deng MC, Feng L, Peng J, Yuan JP, Wang JH (2014a) An effective method for the detoxification of cyanide-rich wastewater by *Bacillus* sp. CN-22. Appl Microbiol Biotechnol 98:3801–3807
Wu XL, Xiang L, Yan QY, Jiang YN, Li YW, Huang XP, Li H, Cai QY, Mo CH (2014b) Distribution and risk assessment of quinolone antibiotics in the soils from organic vegetable farms of a subtropical city, Southern China. Sci Total Environ 487:399–406
Wu Z, Wang F, Liu S, Du Y, Li F, Du R, Wen D, Zhao J (2016) Comparative responses to silicon and selenium in relation to cadmium uptake, compartmentation in roots, and xylem transport in flowering Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* var. *utilis*) under cadmium stress. Environ Exp Bot 131:173–180
Wu J, Wang J, Li Z, Guo S, Li K, Xu P, Ok YS, Jones DL, Zou J (2023) Antibiotics and antibiotic resistance genes in agricultural soils: a systematic analysis. Crit Rev Environ Sci Technol 53(7):847–864
Xiao R, Huang D, Du L, Song B, Yin L, Chen Y, Gao L, Li R, Huang H, Zeng G (2023) Antibiotic resistance in soil-plant systems: a review of the source, dissemination, influence factors, and potential exposure risks. Sci Total Environ 869:161855
Xu H, Chen Z, Huang R, Cui Y, Li Q, Zhao Y, Wang X, Mao D, Luo Y, Ren H (2021) Antibiotic resistance gene-carrying plasmid spreads into the plant endophytic bacteria using soil bacteria as carriers. Environ Sci Technol 55(15):10462–10470
Yan Q, Zhong Z, Li X, Cao Z, Zheng X, Feng G (2024) Characterization of heavy metal, antibiotic pollution, and their resistance genes in paddy with secondary municipal-treated wastewater irrigation. Water Res 252:121208
Yang QE, Agouri SR, Tyrrell JM, Walsh TR (2018) Heavy metal resistance genes are associated with bla NDM-1-and bla CTX-M-15-carrying *Enterobacteriaceae*. Antimicrob Agents Chemother 62(5):10–1128
Zhang RM, Liao MN, Wu JE, Lu XQ, Tan HZ, Sun J, Liao XP, Liu YH (2022) Metagenomic insights into the influence of mobile genetic elements on ARGs along typical wastewater treatment system on pig farms in China. Sci Total Environ 839:156313
Zhang Y, Cheng D, Xie J, Hu Q, Xie J, Shi X (2023) Long-term field application of manure induces deep selection of antibiotic resistomes in leaf endophytes of Chinese cabbage. Sci Total Environ 882:163334
Zhao B, Park K, Kondo D, Wada H, Nakada N, Nishimura F, Ihara M, Tanaka H (2024) Comparison on removal performance of virus, antibiotic-resistant bacteria, cell-associated and cell-free antibiotic resistance genes, and indicator chemicals by ozone in the filtrated secondary effluent of a sewage treatment plant. J Hazard Mater 465:133347
Zheng X, Zhong Z, Xu Y, Lin X, Cao Z, Yan Q (2023) Response of heavy-metal and antibiotic resistance genes and their related microbe in rice paddy irrigated with treated municipal wastewaters. Sci Total Environ 896:165249
Zhou SYD, Wei MY, Giles M, Neilson R, Yang XR (2020) Prevalence of antibiotic resistome in ready-to-eat salad. Front Public Health 8:513102

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