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Hazardous pollutants in agricultural soil and environment

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Hazardous pollutants in agricultural soil and environment

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Editorial: Hazardous pollutants in agricultural soil and environment

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Editorial on the Research Topic
Hazardous pollutants in agricultural soil and environment

Food security is a cornerstone of global health, human wellbeing, and economic stability. Ensuring the availability, accessibility, and quality of food is fundamental for sustaining populations worldwide, but this critical need is threatened by contaminants that diminish agricultural productivity and compromise food security. These pollutants, including heavy metals and metalloids, persistent organic pollutants, pesticides, and emerging threats like microplastics and nanoplastics, not only disrupt agricultural productivity but also pose significant risks to environmental sustainability, and human health.

The detrimental effects of soil contamination on agricultural outputs are multifaceted. Heavy metals such as lead and cadmium can severely hinder plant growth, reduce crop yields, and compromise the nutritional quality of the food produced (Kumar et al., 2021; Madhav et al., 2024). Arsenic on the other hand is a toxic metalloid that has been reported as the major determinant for decreasing grain yield besides causing straighthead disease in rice (Kumar et al., 2022). Similarly, the widespread use of chemical pesticides and herbicides, while aiming to enhance crop protection, often leads to long-term soil degradation and loss of biodiversity, further diminishing the soil's natural capacity to support agriculture. The infiltration of these toxic substances into soils, a direct consequence of industrial activities, agricultural practices, and inadequate waste management, leads to a cascade that disrupts the delicate balance of nutrients and microbial life critical for plant growth and soil health (Swain, 2024). This xenobiotic contamination undermines the nutritional value of crops and raises the chances of bioaccumulation of harmful chemicals in the human body. This can potentially lead to chronic health conditions in humans, including cancers, neurological disorders, and developmental issues in children. The challenge is further compounded by the emerging threat of microplastics, whose long-term ecological and health impacts are only beginning to be understood. Therefore, both, food safety and security are compromised due to the presence of toxic contaminates in agricultural soil.

Addressing the challenges posed by soil pollution to food security requires a concerted effort to implement sustainable agricultural practices, innovative remediation technologies, and robust policies aimed at protecting soil health, thereby ensuring the production of safe, nutritious food and safeguarding public health for future generations (Goel et al., 2021). In this direction, the current Research Topic highlights the problems associated with hazardous pollutants in agricultural soil and recent advancements to tackle their remediation. It further aims to spotlight the critical issues surrounding soil pollution and showcase recent breakthroughs in pollution remediation for agricultural soil. This Research Topic includes 13 original research articles and one review article.

Chang et al. demonstrate the hazards posed by the commonly used fungicide (dimethomorph) and pesticide (imidacloprid) in Taiwanese vineyards. Through orthogonal approaches of microbial population analysis, the authors reveal alteration in soil bacterial composition leading to an inhibitory effect on soil metabolism. Notably, imidacloprid is banned by the European Union due to its toxicity for bees and wild pollinators (https://www.science.org/content/article/european-union-expands-ban-three-neonicotinoid-pesticides). Reiß et al. observed the similar results by studying the microbial composition of honeybee (*Apis mellifera*) cuticles. Both studies conclude that the use of fungicides alone or in combination with other pesticides can alter microbiome composition. Similar impacts of xenobiotics on microbial diversity have been reported in this Research Topic by Gangola et al. through 16S rDNA-based metagenomic analysis of pesticide-contaminated soil.

An appropriate combination of xenobiotics and organic farming approaches can mitigate this loss of microbial diversity and maintain the bioremediation potential of the soil microbiota. This idea is supported by the observations reported by Colautti et al., where the authors compared the organically vs. conventionally managed vineyards. Emerging solutions, such as the use of bio-inoculants and leguminous plants for the remediation of heavy metal-contaminated soils, offer promise for restoring soil health and reducing pollutant bioavailability. The work of Zheng et al., for example, provides evidence supporting the efficacy of such bioremediation strategies in dealing with cadmium and lead contamination. Additionally, research into soil amendment techniques, including composting and the use of chelators like EDTA, as reported by Xu et al. and Kamal et al., respectively, suggests practical methods for improving soil conditions and mitigating pollutant impacts. Kumar et al., further emphasized the role of exogenously applied brassinosteroids in phytoremediation projects by ameliorating heavy metal stress. Avatsingh et al., highlighted the concerns on the presence of antibiotic-resistant bacteria in polluted irrigation-purpose wastewaters. Debbarma et al., explored the potential of microbial bioformulation (monoculture *Pseudomonas aeruginosa* strain PE10) for e-waste bio-recycling in agricultural soil ecosystems, thus managing the e-waste crisis in agricultural soil through sustainable ways.

Conclusively, this Research Topic confers useful updates and advancements about the existence of hazardous pollutants in the agricultural fields. The collaborative efforts of researchers, farmers, policymakers, and the broader public are imperative in this endeavor, highlighting the collective responsibility to preserve the foundation of food security for generations to come. We have a firm believe that these studies, along with sustainable agricultural practices and policy reforms, will be useful in combating soil pollution, ensuring the sustainable future of agriculture, and protecting public health.

Author contributions

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Rhizospheric bacteria: the key to sustainable heavy metal detoxification strategies

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The increasing rate of industrialization, anthropogenic, and geological activities have expedited the release of heavy metals (HMs) at higher concentration in environment. HM contamination resulting due to its persistent nature, injudicious use poses a potential threat by causing metal toxicities in humans and animals as well as severe damage to aquatic organisms. Bioremediation is an emerging and reliable solution for mitigation of these contaminants using rhizospheric microorganisms in an environmentally safe manner. The strategies are based on exploiting microbial metabolism and various approaches developed by plant growth promoting bacteria (PGPB) to minimize the toxicity concentration of HM at optimum levels for the environmental clean-up. Rhizospheric bacteria are employed for significant growth of plants in soil contaminated with HM. Exploitation of bacteria possessing plant-beneficial traits as well as metal detoxifying property is an economical and promising approach for bioremediation of HM. Microbial cells exhibit different mechanisms of HM resistance such as active transport, extra cellular barrier, extracellular and intracellular sequestration, and reduction of HM. Tolerance of HM in microorganisms may be chromosomal or plasmid originated. Proteins such as *MerT* and *MerA* of *mer* operon and *czcCBA*, *ArsR*, *ArsA*, *ArsD*, *ArsB*, and *ArsC* genes are responsible for metal detoxification in bacterial cell. This review gives insights about the potential of rhizospheric bacteria in HM removal from various polluted areas. In addition, it also gives deep insights about different mechanism of action expressed by microorganisms for HM detoxification. The dual-purpose use of biological agent as plant growth enhancement and remediation of HM contaminated site is the most significant future prospect of this article.

KEYWORDS

toxicity, heavy metals, detoxification, rhizospheric, bioremediation

1. Introduction

The term "heavy metals (HMs)" represents a unique group of metals and metalloids existing naturally with high density and atomic weight. Among several HMs contamination of arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), and mercury (Hg) in environment are considered as highly toxic and are found in terrestrial, aerial, and aquatic eco-systems more than their threshold values (World Health Organization [WHO], 2010; Agency for Toxic Substances and Disease Registry [ATSDR], 2015). Commonly such metals are called as "toxic HMs" or "most problematic HMs" (Rahman and Singh, 2019). HMs are important for the growth of organisms at their optimum desirable concentrations (WHO: 0.001–3 mg/L), however at higher concentrations, these can lead to biotoxicity and have detrimental consequences on human and environmental health (Musa et al., 2013; Gupta et al., 2016). HMs are major industrial effluents, which may subsequently get accumulated in different ecosystems leading to immense threat to the various agro-ecosystems (Cheung and Gu, 2007). HM pollution has now become a crucial matter of environmental concern worldwide due to its non-biodegradability and bioaccumulation in nature (Gautam et al., 2014). Exposure of HMs exhibited severe consequences in humans (like inflammatory, respiratory, and cardiovascular diseases), animals (Engwa et al., 2019), plants (reduced growth rate, photosynthesis, and yield) (Asati et al., 2016), microorganisms (metabolism, growth, and morphology) (Ayangbenro and Babalola, 2017) and aquatic lives (death and reproduction) (Pandey and Madhuri, 2014). Worldwide, there are almost 5 million contaminated sites of soil with HMs concentration exceeding the regulatory levels (Li et al., 2019). Biogeochemical cycles on disruption leads to deposition of HM and other contaminants into aquatic and terrestrial environment like, combustion of fossil fuels, mining, nuclear power plants, industrial effluents, sludges, preservatives including organometallic compounds, dust from smelters, waste from brewery, and distillery units (Zhang et al., 2013; Gangola et al., 2023a). Apart from being toxic in nature, few HMs are also reported as essential micronutrients for efficient plant growth. HMs may also function as cofactor of several important enzymes, required for metabolism of hydrogen, involved in methane biogenesis and acetogenesis.

Metal contamination of food and water has been reported to result into several births related defects like cancer, lesion of skin, impairment of liver and kidney functions, and many more. Millions of people of Argentina, Taiwan, Bangladesh, India, Poland, China, Hungry, Japan, Belgium, North Mexico, Chile, and Mongolia are suffering from health-related issues mentioned above due to metal contamination of ground water (Tseng et al., 2005). Lead, cadmium and mercury have been given second, third and seventh rank, respectively, due to their highly toxic and widespread nature. A huge number of superfund sites were found to be HM polluted (Peters, 1999). HMs are present in different ecosystem naturally or due to anthropogenic activities like smelting of metal ores, fuel and energy production, sludge dumps, mine tailings, agricultural activities, and gas exhaust (Raskin et al., 1994). HMs impose more intensive challenge because of its recalcitrant nature and occurrence in a form that cannot undergo complete degradation but can only be complexed with some compounds or only its chemical form can be altered. Several parameters such as physical, chemical, and ecological characteristics of the polluted sites should be checked for successful achievement of bioremediation (Gu, 2021). Physicochemical and biological methods are used for remediation of sites contaminated with HMs, both of which have their own pros and cons while use of microorganisms as a biological tool is the emerging technology for degradation and remediation of pollutants at contaminated sites (Gu, 2018; Ansari et al., 2023). Microbial bioremediation is a simple, economic, ecofriendly, inexpensive, and efficient approach done for removal and detoxification of toxic pollutants using native microorganisms (Kumar Mishra, 2017; Spain et al., 2021). Removal of pollutant from any contaminated sites by employing microbial system could be achieved maximally by knowing the toxic concentration of pollutant and maintenance energy required by the microbial system (Gao and Gu, 2021). Several bioremediation methods are reported for the degradation of more than 50 pollutants, but very little innovative approach was found (Gu, 2016). Mechanisms involved mainly were biosorption, bio-oxidation and bio mineralization (Jin et al., 2018). Microbial population associated with plant roots are efficient in remediation of polluted soils and promote plant growth by several direct and indirect mechanisms such as siderophore production, phytohormone production, phosphate solubilization, biological N_2 fixation, antibiotic production, synthesis of lytic enzymes, etc. (Goswami et al., 2016; El-Meihy et al., 2019; Joshi et al., 2023a). Exploitation of plant growth promoting bacteria (PGPB) having potential of metal detoxification as well as multiple plant growth promoting traits are promising metal bioremediation tool. Catabolic efficiency as well as production of bio surfactant and enzymes by microbes is a novel approach to enhance their remediation efficacy (Le et al., 2017). Plant growth promoting rhizobacteria (PGPR) has shown improvement in metal immobilization or mobilization in HM contaminated soils and plant biomass as they are metal resistant and phytoremediation enhancing agents (Ma et al., 2016). Removal of environmental contaminants that cause ecological imbalance is a global concern. Considering this important, this review addresses microbes as a tool for bioremediation of toxic HMs from different contaminated systems with emphasis on the involved mechanism.

2. Environmental presence of heavy metal

Heavy metals are present in the environment as a result of both naturally occurring pedogenetic processes and human activities such as mining, smelting, electroplating, pesticide use, release of biosolids and phosphate fertilizer (Dixit et al., 2015). HM concentrations are also influenced by weathering of minerals, erosion, volcanic activity, atmospheric deposition, and other natural sources (Fulekar et al., 2009; Sabiha-Javied et al., 2009). Unfortunately, human activity has the potential to interfere with the natural geochemical cycle of metals, causing HMs to accumulate in soil and water. When HM concentrations reach permissible levels, this can pose a risk to human health, as well as to the health of plants, animals, and aquatic life (D'amore et al., 2005). Due to excessive production from anthropogenic and natural sources, movement from mines to areas where humans are more

exposed, industrial waste discharge, and increasing bioavailability, HMs end up as pollutants in soil and water. As is a metal that can be found in biosolids, insecticides, ore mining, smelting, and wood preservatives. Electroplating, phosphate fertilizers, plastic stabilizers, paints, and pigments are sources of Cd. Fly ash, steel manufacturing, and tanneries all include Cr. Biosolids, fertilizers, ore mining, pesticides, and smelting are sources of copper. Hg is present in medical waste, coal combustion, and the mining of silver. Ni can be found in surgical equipment, wastewater, culinary appliances, and automotive batteries. Pb was observed in the aerial emissions produced by the burning of used batteries, insecticides, and herbicides.

According to Lombi and Gerzabek (1998), the equation can be used to depict how HMs are distributed/mass balance in the soil's surroundings:

$$M_{total} = (M_p + M_a + M_f + M_{ag} + M_{ow} + M_{ip}) - (M_{cr} + M_l) \quad (1)$$

In the equation, the following variables are included: M for HM, *p* for parent material, *a* for atmospheric deposition, *f* for fertilizer source, *ag* for agrochemical source, *ow* for organic waste source, *ip* for inorganic pollutant, *cr* for crop removal, and *l* for losses due to leaching, volatilization, and other processes.

2.1. The bioavailability of metals present in soil

Metals in soil occur in both available and non-available forms to a microorganism (Sposito, 2000) which is directly related to positive and negatively charged salts of the metal. Various factors influencing availability of HMs in soil includes temperature, cation exchange capacity (CEC), redox potential, pH, aeration capacity, organic matter content, microbial activity in the rhizosphere, clay minerals, water quantity, root exudates, hydrous metal oxides, climate, and metal chemical properties (Roane and Pepper, 2000; Fischerová et al., 2006). Lasat (2002) showed bioavailability of HMs to be affected by some bacterial traits such as acidification, synthesis of chelating agents, and altering the redox potential. Metals exist in soluble cationic form under aerobic and oxidized conditions whereas found as insoluble carbonate or sulfides under anaerobic and reduced environment. Moreover, bioavailability of metals in free ionic form increases at acidic pH whereas reduced at high pH because of precipitation. Availability of HMs in soils is observed to be highest for zinc followed by copper, cadmium, and nickel. Metals with high CEC are observed to have reduced toxicity even at their higher concentrations (Roane and Pepper, 2000). Several reports on availability of HMs and their uptake by plants can be essential for predicting the effect of HMs on growth of plant under stressed conditions and population of rhizospheric microbes, as well as assessment of execution of bioremediation techniques for remediation of metal stressed soils. HMs are recalcitrant which affect and alters their toxicity over time. High concentration of HMs negatively affects physiology of microbes whereas also used as important micronutrient for their growth (Ahemad, 2012). Bioavailability of metals decides the possibility of interaction between metal species and bacteria that can occur either to neutralize their toxic effects or to fulfill their metabolic needs. Mostly, naturally existing microbial biomass are most effective and participate actively in detoxification mechanism, but still they are not well characterized or known. This scientific gap is due to lack of knowledge in cultivation of such unculturable microorganisms and continuous dynamic changes in their traits essential for adaptation to the existing environment. Bacteria in rhizospheric region affect HM speciation which further leads to changes in bioavailability of metals. Furthermore, they prevent phytotoxicity in plants by converting bioavailable form of HMs to their non-bioavailable forms in soils (Jing et al., 2007). Bioavailability of metal varies from species to species. Speciation of metals and the resulting bioavailability decides the overall toxicity as well as physiological effects of a metal on biological systems (Knotek-Smith et al., 2003). Nutrient status of the soil also affects the bioavailability of HMs. Several *in situ* experiments rely on the enrichment of the existing microbial population by adding nutrients such as carbon, nitrogen, phosphorus, etc., called bioaugmentation. Most of the degradation processes are oxidative in which microorganisms harness energy through electron exchange. The availability of the oxygen is the major kinetic obstacle to the aerobic microbes due to low solubility in water.

3. Heavy metals and ecotoxicity

According to Gao and Gu (2021) at a particular concentration of pollutant or below its toxicity limit, microorganisms require lower energy for their maintenance, isolation, enrichment, and their efficient utilization for biodegradation. HMs are categorized according to their reactivity, effect on biological systems and their target sites. Some of them have vital role (such as zinc, iron, copper, magnesium, and calcium) in biological system whereas others are also reported to have carcinogenic and cytotoxic effects (like mercury, lead, and cadmium). Rapid industrialization, urbanization as well as mining activities have altered biogeochemical cycling which further raised accumulation of HMs in terrestrial, atmosphere, and aquatic eco-system. Such changes may severely affect the biotic communities present on those sites. Various reports showed HM stress on higher plants like cadmium, lead, and mercury inhibiting chlorophyll biosynthesis, respiration as well as oxidative stress which may further cause cellular damage, disturbs ionic balance of the cell, and toxicity in higher organisms (Mithoefer et al., 2004; Han F. X. et al., 2006; Han S. H. et al., 2006). Toxic HMs contaminates groundwater and biota and have negative effects on health of human beings. It is essential to examine the distribution and concentration of toxic HMs in riverine ecosystems (Islam et al., 2018). Major sources of contamination in aquatic system include domestic sewage, industrial effluents, agricultural run-off, and mining operations (Zhuang et al., 2013). Water contamination with HMs is a sensitive environmental issue which adversely affects surface water, ground water, plants, human, animal's health (Rezania et al., 2016), aquatic organisms (Abrar et al., 2011) as well as alters histopathological tissues of aquatic organisms (Ahmed et al., 2014). HM contaminated water bodies are a severe concern worldwide because of biomagnifications, environmental

TABLE 1 Impact of HMs on plant growth and their toxicological effects on microbes.

Heavy metal	Distribution	Essential/Non-essential	Role in plants	Toxicity effect on plants	Toxic effect on microbes	References
Cu	Lakes, earth's crust, rivers, and oceans	Essential	Photosynthesis; synthesis of ATP and CO_2; essential part of some proteins like cytochrome oxidase and plastocyanin; cofactor for several enzymes like superoxide dismutase, dioxygenase and ascorbate oxidase.	Cu causes cytotoxic effects, induces stress, reduced plant growth, generates reactive oxygen species, chlorosis in leaf.	Inhibition of several enzyme activities and cellular functions.	Chatterjee et al., 2006; Adrees et al., 2015; Habiba et al., 2015; Fashola et al., 2016; Sayqal and Ahmed, 2021
Cd	Soil, sedimentary rocks, and water	Non-essential		Cd toxicity can cause reduced availability of essential elements such as iron, calcium, phosphorus, and magnesium; stunted growth, browning of roots, chlorosis; several cytotoxic effects; decrease in nitrogen fixation.	Impairment of different proteins, DNA and RNA; interference with transcription process and cell division.	Balestrasse et al., 2003; Guo et al., 2008; Asgher et al., 2015; Fashola et al., 2016; Khan et al., 2016; Sayqal and Ahmed, 2021
Zn	Surface water, soil, and rock	Essential	Plant growth and cell metabolism; gene expression, activation of enzyme, gene regulation, protein synthesis; cofactor in metabolic pathways of different biomolecules; reproductive development.	Zn toxicity may lead to inhibition of growth and several plant metabolic functions; restricts root and shoot growth, chlorosis; interfere with uptake of other important elements like copper and manganese.	Decline in biomass as well as growth.	Foy et al., 1978; Fontes and Cox, 1998; Cakmak, 2000; Malik, 2004; Dhankhar et al., 2012; Sayqal and Ahmed, 2021
As	Soil and volcanic eruption	Non-essential		As(V) is analog to PO_4^{3+} therefore competes with uptake of PO_4^{3+} which further have negative effects on ATP production, oxidative phosphorylation and transport system; arsenic toxicity may cause growth inhibition, low yield, free radical and ROS formation, protein activity inhibition, deficiency of other essential elements.	Inhibition of enzyme activities.	Tripathi et al., 2007; Gunes et al., 2009; Sankarammal et al., 2014; Kumar et al., 2015; Anjum et al., 2016; Sayqal and Ahmed, 2021.
Ni	Air, soil, sediments, and water	Essential	Essential part of different metalloenzymes such as urease, hydrogenases, superoxide dismutases, methyl coenzyme M reductase, RNase-A, dehydrogenases, acetyl Co-A synthase.	High concentration of nickel can cause chlorosis, necrosis, and wilting; impairment of photosynthesis, sugar transport, and water balance; negative effects on balance of nutrients and ATPase activity leading to impaired functions of cell membrane.	Negative effects on cell membrane, oxidative stress, and deactivation of various enzymes.	Nakazawa et al., 2004; Sethy and Ghosh, 2013; Fashola et al., 2016; Sayqal and Ahmed, 2021

(Continued)

TABLE 1 (Continued)

Heavy metal	Distribution	Essential/Non-essential	Role in plants	Toxicity effect on plants	Toxic effect on microbes	References
Cr	All environments	Non-essential		Cr toxicity can result in chlorosis, growth inhibition, and low synthesis of photosynthetic pigments; low uptake of important elements like iron, phosphorus, calcium, magnesium, potassium; inhibition of ETC (electron transport chain) photophosphorylation, and some enzyme activities; disorganization of chloroplasts.	Inhibition of growth, oxygen uptake; extension of lag phase.	Cervantes et al., 2001; Peralta-Videa et al., 2009; Vikram et al., 2011; Ahemad, 2015; Sayqal and Ahmed, 2021
Pb	Soil	Non-essential		Lead accumulation may cause several deleterious direct and indirect effects on physiology, morphology and biochemical functions of plants; negative effects on membrane permeability, enzyme activities, nutrition, growth hormones water uptake, ATP synthesis, lipid peroxidation; synthesis of ROS in large amount leading to damage of DNA.	DNA and protein denaturation, transcription termination and stop enzymatic regulation.	Sethy and Ghosh, 2013; Fashola et al., 2016; Sayqal and Ahmed, 2021
Mn	Earth's crust	Essential	As cofactor, in photosynthesis and form metalloproteins.	Mn toxicity can reduce efficiency of photosynthesis; cause necrosis, cracks in root, chlorosis, and brown coloring of leaf, stem and petiole.	Negative effects on metabolic functions and respiration.	Bachman and Miller, 1995; Foy et al., 1995; Kitao et al., 1997; Amorim et al., 2018
Hg	Water, soil, and air	Non-essential		Toxic amount can result in visible injuries and physiological issues in plants; synthesis of reactive oxygen species and inhibition of mitochondrial activity; negative effects on cellular metabolism in plants; interferes with dark and light reactions of photosynthesis.	Impairment of cell membrane, deactivation of proteins and enzymes.	Cargnelutti et al., 2006; Zhou et al., 2007; Fashola et al., 2016.

persistence, bioaccumulation, and toxicity (Rajaei et al., 2012). Contamination of riverine sediments by HMs may cause ecological risk to organisms present in benthic region (Pacle Decena et al., 2018). Soil with more inputs of fertilizers is enriched with HMs. Bioavailability of HMs varies according to physicochemical properties and metal speciation of soil which is further essential for plant uptake. Urban areas soil has been reported with high amount of lead, out of which only up to 85% is bio accessible (Mackay et al., 2013). Non-essential HMs are reported to be hazardous and highly toxic to plants, humans, and animals even at minimum concentrations (Mahboob et al., 2014). Also, few essential HMs may increase risk of toxicity if used at increased concentrations as listed in **Table 1**. Metals that are toxic, persistent, and bio accumulative are more harmful (DeForest et al., 2007). Several HMs have been referred as teratogenic, mutagenic, and carcinogenic. Cadmium with potential of bioaccumulation and high toxicity resulted into population decline of freshwater mussels (Ngo, 2007).

4. Challenges and possible solution for heavy metal detoxification

Undoubtedly, the combination of ecological analysis and chemical data can offer greater understanding of the environmental condition of ecosystems and the transformations that have occurred (Gu, 2014). HMs cannot be subjected to complete degradation, i.e., modification in the nuclear structure does not occur, thus they can only undergo change in their oxidation states which in turn changes their chemical behavior (Sobariu et al., 2017). Change in oxidation may lead to various consequences like solubility of metal increase in water that can be further easily removed by leaching or may decrease its solubility by which it becomes less available for decontamination, may get converted to less toxic forms or can be removed from polluted sites via volatilization (Garbisu and Alkorta, 2003).

The root of plants provides aeration to the soil and influences the distribution of rhizospheric microbes through soil. The root system is also capable of penetrating the impermeable zones of soils and draw the soluble forms of the organic contaminants. A significant symbiotic relationship between the plant root system and rhizospheric microbes is observed and this relationship has long been exploited for rhizo-remediation (Gangola et al., 2022a). Successful rhizo-remediation is dependent upon several factors such as: root and rhizosphere colonization by microbes, formation of various useful metabolites by the plants, survival, and ecological interactions with other organisms. This novel approach can also be called phytoremediation. The employment of leguminous plants for bioremediation has seen a rise in the recent times due to immense bioremediation ability and capability of biological nitrogen fixation as well as root nodule formation (Pastor et al., 2003; Kamaludeen and Ramasamy, 2008; Gangola et al., 2022b). Rhizospheric microbes have immense potential of nutrient cycling, restoration of soil structure, decontamination of various contaminants, pest management, and enhanced plant growth (Gangola et al., 2023b). Therefore, soil microbes can improve the bioremediation potential of plants and in turn lower the phytotoxicity of the soil contaminants. In symbiotic associations between microbes and plants, plant make carbon source available to the associated microbes which in turn helps in reduction of phytotoxicity of the soil contaminants. In addition, some non-specific associations are also observed in between microbes and plants where metabolic processes of plant stimulate the microbial growth, which in turn degrade the soil pollutants due to their inherent metabolic activity. Plants roots are capable of releasing root exudates and increasing the metal ion solubility. These biochemical processes increase the bioremediation potential of rhizospheric microbes associated with the plant roots and thus is of significance for remediation of heavy-metal pollution soils (Gangola et al., 2021, 2022c). This association also accounts

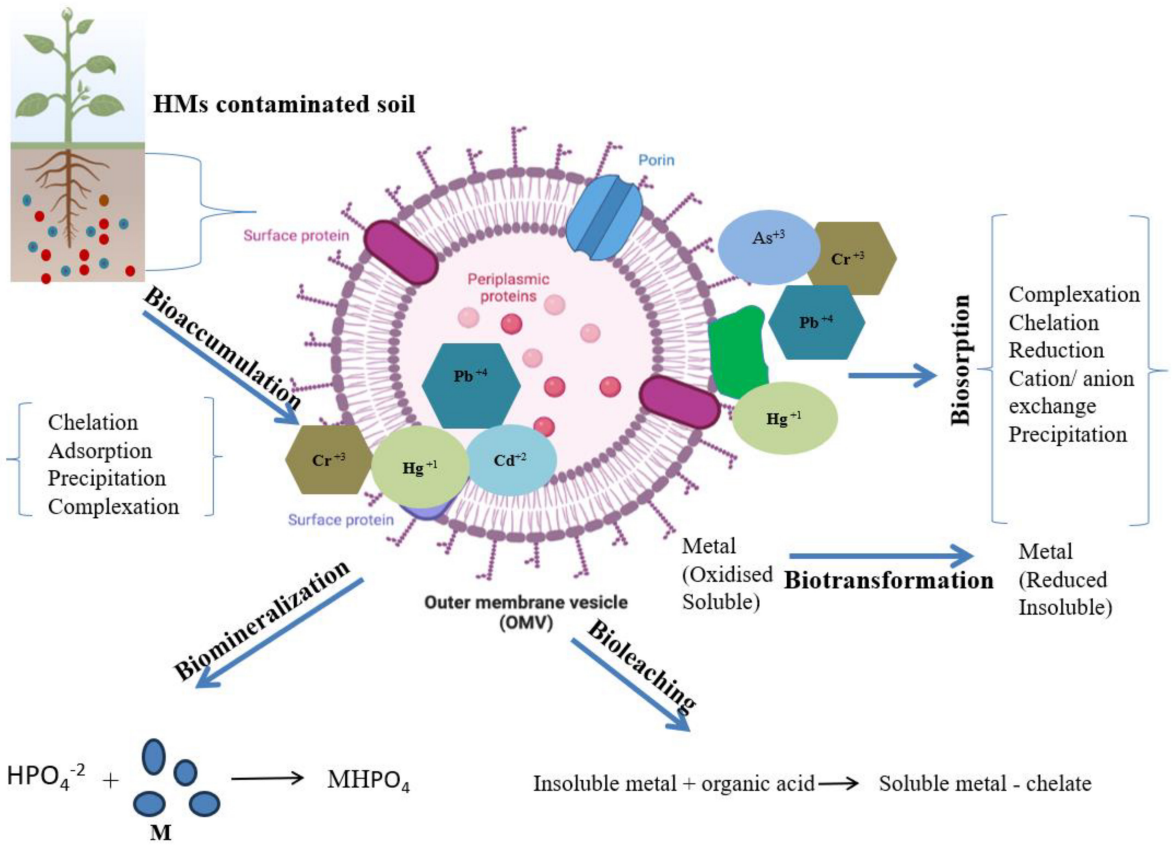

FIGURE 1
Different strategies such as: bioleaching, biomineralization, biotransformation, biosorption, and bioaccumulation by microbial system for the removal or transformation of toxic HMs from contaminated sites.

TABLE 2 Heavy metal mediated toxicological effects and microorganisms involved in the process of removing them from soil.

Heavy metal	Toxicological effects	Microorganisms involved	Process of removing heavy metal	References
Cd, Pb	Cancer, toxicity to different biological system such as skeletal, reproductive, urinary, cardiovascular, respiratory, and nervous system	*Pseudomonas aeruginosa* BS2	Di-rhamnolipid based soil washing	Juwarkar et al., 2007
Cd, Zn, As	Stomach pain, nausea and vomiting, gastrointestinal and kidney dysfunction, nervous system disorders, cancer	*Pseudomonas aeruginosa* LFM 634	Soil washing and chemical precipitation with the help of rhamnolipid	Lopes et al., 2021
Cd, Co, Pb, Zn, Ni	Low levels of energy, dysfunctioning of brain, damage lungs, kidney, and liver, disturbed blood, dermatitis and ulcers, increases the occurrence of risk to lung, nasal, sinus, anemia, dizziness, nausea, vomiting, diarrhea, cancer	*Bacillus subtilis* A21	Soil washing with the help of lipopeptide	Singh and Cameotra, 2013
Cu, Zn	Abdominal pain, diarrhea, nausea, vomiting, blue/green colored faces, fatigue, anemia, and dizziness	*Pseudomonas aeruginosa* ATCC 9027	Soil washing in presence of rhamnolipid	Mulligan et al., 2001
As, Cu, Zn, Pb	Dysfunctioning of kidney, nervous system failure, lesions on skin, vascular damage, weakened immunity congenital disorder and cancer	*Pseudomonas aeruginosa*	Soil flushing in presence of rhamnolipid (JBR-425)	Mulligan, 2009
Pb, Ni, Cd, Ba, Zn	Nausea, vomiting, diarrhea, headache, cough, shortness of breath, anemia, cancer	*Pseudomonas aeruginosa*	Adsorption by rhamnolipid	Elouzi et al., 2012
Cu	Nausea and vomiting	*Pseudomonas aeruginosa* MTCC2297	Soil washing in presence of rhamnolipid	Venkatesh and Vedaraman, 2012
Cu, Zn	Abdominal pain, diarrhea, nausea, vomiting, blue/green colored faces, fatigue, anemia, and dizziness	*Torulopsis bombicola* ATCC 22214	Soil washing in presence of sophorolipid	Mulligan et al., 2001
Cr, Cd, Pb, Zn, Cu	Dermatitis, ulcers, lung disease risk increase, sinus, anemia, and dizziness, pulmonary sensitization, cancer	*Candida bombicola*	Sophorolipid	Ahuekwe et al., 2016
Cd, Pb	Gastrointestinal disorders, dysfunctioning of kidney, nervous system disorders, weakened immune system, congenital defects, cancer	*Starmerella bombicola* CGMCC 1576	Sophorolipid dependent soil washing	Qi et al., 2018
Zn	Abdominal pain, nausea, and vomiting	*Cryptococcus* sp. VITGBN2	Sophorolipid based immobilization	Basak and Das, 2014
As	Gastrointestinal disorders, dysfunctioning of kidney, nervous system disorders, weakened immune system, cutaneous rash congenital defects, cancer	*Candida bombicola*	Soil washing with the help of sophorolipid (SL18)	Arab and Mulligan, 2018
Zn, Cu	Stomach pain, nausea, and vomiting	*Candida tropicalis* UCP 0996	Soil washing with the help of lipopeptide	da Rocha Junior et al., 2019
Hg, Pb, Mn, Cd	Gastrointestinal disorders, dysfunctioning of kidney, nervous system disorders, weakened immune system, cutaneous rash congenital defects, cancer, damage of lungs and liver, cancer	*Bacillus* sp. MSI 54	Co-precipitation	Ravindran et al., 2020
Pb, Cd, Cr	Eczema and skin ulcers that are typically painless, lung disease risk increases, sinus, nasal, anemia, dizziness, cancer	*Bacillus cereus*	Soil washing with the help of lipopeptide	Ayangbenro and Babalola, 2020
Cu, Zn	Stomach pain, nausea, and vomiting	*Bacillus subtilis* ATCC 21332	Surfactin based soil washing	Mulligan et al., 2001
Cu, Zn, Cr, Cd	Eczema and skin ulcers that are typically painless, lung disease risk increases, sinus, nasal, anemia, dizziness, cancer	*Bacillus* sp. HIP3	Metal chelation by surfactin	Md Badrul Hisham et al., 2019
Zn, Cu	Stomach pain, nausea, and vomiting	*Candida lipolytica* UCP 0988	Precipitation-dissolution	Rufino et al., 2012

(Continued)

TABLE 2 (Continued)

Heavy metal	Toxicological effects	Microorganisms involved	Process of removing heavy metal	References
Fe, Zn	Conjunctivitis, choroiditis, and retinitis, stomach pain, nausea, and vomiting	*Candida lipolytica* UCP 0995	Soil washing	Luna et al., 2016
Cu, Cr	Abdominal pain, nausea, vomiting, irritation in the respiratory tract, pulmonary sensitization, lung disease risk increases, nasal, sinus cancer	*Rahnella* sp. RM	Soil washing	Govarthanan et al., 2017
Al, Pb, Zn, Cd, Fe, Cu, Mn	Immune system disorder, oxidative stress, mutagenic to DNA, inflammation, protein denaturation, inhibition of metabolic enzymes, apoptosis, dysplasia, increase lung risk, liver and kidney damage, cancer	*Citrobacter freundii* MG812314.1	Precipitation	Gomaa and El-Meihy, 2019
Pb, Mn, Cu, Zn, Cd, As	Low levels of energy, dysfunctioning of brain, damage lungs, kidney, and liver, disturb composition of blood, dermatitis, and ulcers, increases the occurrence of risk to lung, nasal, sinus, anemia, dizziness, nausea, vomiting, diarrhea, cancer	*Burkholderia* sp. Z-90	Combined bioleaching and flocculation	Yang et al., 2018
Pb, Cd	Dysfunctioning of brain, damage lung, kidney, and liver, change the blood composition, eczema, and skin ulcers that are typically painless cancer	*Bacillus circulans*	Co-precipitation	Das et al., 2009
Cr	Irritation in the respiratory, pulmonary sensitization, hypersensitivity	*Bacillus* sp.	Reduction	Gnanamani et al., 2010
Cd, Pb	Dysfunctioning of brain, damage lungs, kidney, and liver, can change blood composition, eczema and skin ulcers that are typically painless, cancer	*Pseudomonas* sp. LKS06	Biosorption	Huang and Liu, 2013

for effective phytoremediation due to potential of microbes to influence bioavailability and solubility of the HM. PGPRs boost up the growth and development as well as improve the metal tolerance by decontaminating the toxic HMs (Tassi et al., 2008).

4.1. Bioremediation

Bioremediation offers transformation, degradation, or detoxification of hazardous compounds, organic or inorganic waste by natural biological activity of bacteria, fungi, or plants in the environment (Gu and Pan, 2006; Gu and Wang, 2013; Siddiquee et al., 2015; Gu, 2021). This process is less expensive; low technology based and can be done on site as well as it can be improved by addition of nutrient, an electron acceptor, potent microbial population, pH, soil type, and temperature (Vidali, 2001; Gu, 2019). Moreover, alteration of environmental parameters to provide optimum conditions for microbial activity and growth allow bioremediation to be more effective and proceed at a faster rate (Gu, 2003; Su, 2014). Microbes with ability to remediate when applied to the contaminated site results into transformation of toxic compounds via several metabolic reactions (Siddiquee et al., 2015). Efficient biodegradation and remediation of pollutants at contaminated sites can be achieved by the knowledge of microbial biochemical reactions along with its engineering and management (Gu, 2021; Joshi et al., 2023b). Several approaches for bioremediation include bioreactor, biosorption, biostimulation, bioventing, bio filters, composting, bioaugmentation, and land farming. Several reports have demonstrated efficient elimination of HMs or their conversion to benign or less toxic forms using potent microbes (Qazilbash, 2004). Potential of microbes of exploiting available nitrogen or carbon source for their growth and survival is used in bioremediation and thus bacteria utilizes the contaminants as source of nutrient (Tang et al., 2019). Soil microbes in rhizospheric zone are considered as efficient degraders to overcome HM stress. Other than these plants also possess several detoxifying strategies like synthesis of thiols with high ability to take up HMs. MacNaughton et al. (1999) showed bioremediation as a significant technology to detoxify ground water, soils, and coastal water bodies. Interaction of microbes with HMs can occur via different mechanisms as shown in **Figure 1**, such as biomineralization, biotransformation, bioleaching, biosorption, etc., which further helps in metal bioremediation (Pande et al., 2022). Several investigations as listed in **Table 2**, revealed that bacterial communities belonging to genus *Escherichia, Bacillus*, and *Mycobacterium* isolated from various contaminated sites are found efficient for the removal of HM (Cd, Cr, and Cu) (Jiang et al., 2015). Similarly, they can be remediated by fungal genera, such as *Pleurotus, Acremonium,* and *Fusarium*. Li et al. (2019) discovered microbial consortia with high remediation potential of HMs. Composting and Immobilization are used to enhance the rate of remediation by increasing the activity of microorganisms (Poulsen and Bester, 2010; Chen et al., 2015). Rhizobium-legume symbiotic association is a significant bioremediation method for

metal contaminated soil and have been exploited for removal of arsenic from soils. Mandal et al. (2011) have reported isolation of arsenate (2.8 mM arsenate) resistant symbiotic bacteria *Rhizobium* strain (VMA301) from roots of *Vigna mungo*. This resistant strain showed efficient N_2 fixation as well as helped in decontamination of arsenic containing soil. However, a delayed nodulation and reduced nitrogenase activity was also observed in arsenic treated plants. Arsenic was found to accumulate in roots at higher rate than in the root nodules. Although a lot of research has been done on environmental pollutant degradation but still waiting for the need of efficient biodegradation technology (Gu, 2016).

4.2. Mechanisms utilized in heavy metal bioremediation

Biosorption by microorganisms remediates the contaminated sites or HMs through binding with extracellular polymers or adsorption on the cell surface (Gangola et al., 2018). The outer cell shield of microbes carries active groups of compounds which provides sorption ability and further helps in binding of metals. Such linking occurs due to negative potential of active groups present on outer membrane and cationic metal ions. Biosorption is a reversible process which makes it essential for both recovery [for non-toxic and essential HMs such as gold (Au), Zinc (Zn), and copper (Cu)] and removal of HMs. Various studies showed binding of Cr^{3+}, Cd^{2+}, Pb^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, and Ni^{2+} to microbial biomass by sorption at pH between 5 and 7 and get liberated at low pH whereas few metal ions like Ag^{2+} and Au^{2+} stay bound to biosorbents even at low pH (Kisielowska et al., 2010). Microorganisms are found as rapid adsorbers of HM ions for example *Bacillus* sp. showed 60% sorption of its Cu^{2+} at pH 7.2, at initial phase and reached to adsorption equilibrium within 10 min (He and Tebo, 1998). Several studies demonstrated good binding affinity of microbes via biosorption such as *Streptomyces rimosus* for iron and lead (Sahmoune, 2019), *Staphylococcus hominis* strain AMB-2 for cadmium and lead (Rahman et al., 2019), *Cronobacter muytjensii* KSCAS2 for multiple HMs (Saranya et al., 2018), and *Staphylococcus aureus* biofilms for U(IV) remediation (Shukla et al., 2020).

Bioleaching process mainly involves complexation, biological dissolution, bio-oxidation (Jin et al., 2018), or conversion of sparingly soluble metal compounds into easily soluble forms which can be removed easily (Kisielowska et al., 2010). Various microorganisms known as potent metal mobilizers produces low molecular weight organic acids such as tartaric acid, oxalic acid, gluconic acid, malic acid, citric acid, and butyric acid for the solubilization of HMs from the insoluble ores and exudation of complexion agents that can easily dissolve HMs from soil particles comprising HM minerals. Application of *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans* in metal leaching is proven to be effective because of their biochemical attributes as well as high resistance to temperature and pH (Błaszczyk, 2007). Few studies showed that rate of cadmium leaching promoted by providing more nutrients to microbes for increased production of organic acids (Jin et al., 2018). Furthermore, a few genera such as *Citrobacter* synthesized free inorganic phosphate with high potential to trap toxic metal ions and forming insoluble metal phosphates (Marchenko et al., 2015). Some efficient strains of bacteria like *Bacillus licheniformis* and *Corynebacterium* sp. are reported to perform oxidation-reduction reactions and convert the valance of HMs leading to alter their toxicity and mobility (Gavrilescu, 2004). Acidophiles are mainly involved in bioleaching (Srichandan et al., 2014). Arsenic removal by bioleaching using individual and mixed culture of *A. ferrooxidans* and *A. thiooxidans* was reported by Zhang and Gu (2007). *Bioprecipitation* and *biocrystallization* of HM compounds by microbes results in conversion of metal into form which is less toxic. This occurs mainly due to enzymatic activities (Sklodowska, 2000). Transformation of toxic metal by microbes may occur by several reactions like demethylation, methylation, oxidation, and reduction. For example, Gram-positive bacteria obtained from tannery sewers, transformed highly toxic chromium (VI) to its less toxic form chromium (III) (Kisielowska et al., 2010). *Bioaccumulation* is a toxic kinetic process in which active metabolism leads to HM uptake of contaminants inside the organism when the rate of absorption of contaminant exceeds its rate of loss (Chojnacka, 2010). Microbes with bioaccumulation potential should have high tolerance to variety of contaminants as well as, superior bio transformational ability to make it non-toxic in nature (Mishra and Malik, 2013). This is not considered as much valuable approach as after certain amount of accumulation it can exert toxic effects to cells. Studies showed removal of HM to be more effective by bioaccumulation as compared to biosorption (Henriques et al., 2015). In an investigation, biomasses of *Bacillus megaterium* and *Bacillus circulans* demonstrated efficient removal of toxic form of chromium (VI) at much high rate (32–34.5 mg g^{-1} dw) by bioaccumulation as compared to biosorption (15.7–39.9 mg g^{-1} dw) (Srinath et al., 2002). Bioaccumulation potential was shown by *Pseudomonas putida* 62 BN for cadmium and *Bacillus cereus* M116 for nickel (II) (Rani and Goel, 2009; Naskar et al., 2020). Microbial transformation results in reduced toxicity and vitalization of HMs. Some reports showed detoxification of arsenic (III) by *Acinetobacter* sp. as well as *Micrococcus* sp. and chromium (VI) by *Bacillus* sp. SFC 500-1E (Tayang and Songachan, 2021). Some of the key bacterial HMs resistant mechanisms are explained.

4.2.1. Extracellular barrier

The cell wall, plasma membrane, and capsule act as an extracellular barrier to stop metal ions from entering the cell (El-Helow et al., 2000). Through ionizable groups like amino, carboxyl, hydroxyl and phosphate on their cell wall or capsule, bacteria can bind metal ions. According to Pardo et al. (2003), this adsorption is a passive process that can even take place in dead bacterial cells. The two phases involved in the accumulation of metal ions by living cells are gradual active transport into the cytoplasm and non-specific initial adsorption by the cell wall (McEldowney, 2000).

Acinetobacter sp. (Irawati et al., 2015), *Marinobacter* sp. (Bhaskar and Bhosle, 2006), *Klebsiella* sp. (Mohan et al., 2019), and *Enterobacter cloacae* (Iyer et al., 2005) are just a few of the bacteria whose capsules have been shown to accumulate metal ions primarily through carboxyl groups of their polysaccharides. Exopolysaccharide (EPS) production by copper-tolerant *Pseudomonas aeruginosa* strains is twice as high as that of copper-sensitive strains (Kazy et al., 2002). Metal ions, however,

can also stop bacteria from producing EPS. Multiple copper-tolerant but deficient in the synthesis of EPS gellan *Sphingomonas paucimobilis* mutants were found by Richau et al. (1997). The authors hypothesized that because EPS synthesis is a very energy-intensive process, the mutants' higher copper tolerance was caused by a slower growth rate and the use of stored energy for defense against metal stress.

4.2.2. Active transport

The majority of HM resistance methods used by bacteria, sometimes referred to as efflux, export metal ions from cells. On chromosomes (Franke et al., 2001; Lee et al., 2007) and plasmids (Nies, 2000), efflux system genetic factors can be discovered. The magnesium transport system allows the entry of cobalt, cadmium, nickel, zinc, and manganese ions to the *Ralstonia metallidurans*, whereas some metal ions can enter the cell through the essential element uptake systems such as chromate which is transported via the sulfate transport system (Gonzalez Henao and Ghneim-Herrera, 2021). Through ATP hydrolysis or an electrochemical gradient (Mathivanan et al., 2021), metal ions are exported from the cell. Proteins from three families make up efflux systems: P-type ATPases, CDF (cation diffusion facilitator), and RND (resistance, nodulation, cell division). Gram-negative bacteria's P-type ATPases and CDF proteins move particular substrates across the plasma membrane and into the periplasm. It is important to note that CDF proteins specifically interact with divalent metal ions (Zn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Fe^{2+}), whereas P-type ATPases primarily transfer metal ions that have a high affinity for sulfhydryl groups (Cu^{+}/Ag^{+}, $Zn^{2+}/Cd^{2+}/Pb^{2+}$). RND proteins assemble into transport complexes that carry cations from the periplasm across the plasma membrane (Nies, 2003).

The efflux system that allows the multi-resistant bacterium *R. metallidurans* CH34 to tolerate copper, cobalt, and zinc ions is encoded by the Czc operon. The system makes use of an electrochemical gradient and is made up of the *CzcCB2A* efflux complex, which also functions as a cation-proton antiporter and includes the subunits *CzcC, CzcB,* and RND-protein *CzcA*. While *CzcA* might contribute to some degree of HM resistance, *CzcC* and *CzcB* are necessary for the efflux system to operate properly (Nies, 2000). With CPx-type ATPases discovered in bacteria like *Enterococcus hirae* (*CopA* and *CopB*), *Streptococcus mutans* (Vats and Lee, 2001), and *Escherichia coli* (Rensing et al., 1999), the P-type ATPase family comprises transporters for mono- and divalent metal cations. In bacteria like *Staphylococcus aureus* and *P. putida*, P-type ATPases called *CadA* and *ZntA* play a role in cadmium tolerance (Oger et al., 2003; Lee et al., 2007).

Certain bacteria can use other mechanisms in addition to efflux systems to resist HMs (Saxena et al., 2002). For instance, the *P. putida* strain S4 transports copper ions from the cytoplasm and sequesters them in the periplasm using an ATPase efflux mechanism. Another illustration is the *ars* system, which has 3–5 genes and is found in both Gram-positive and Gram-negative bacteria. The *ArsA/ArsB* ATPase pump and the *ArsC* reductase are both encoded by the *ars* operon. According to Mukhopadhyay et al. (2002), in the first stage, cytoplasmic *ArsC* arsenate reductase enzymatically converts arsenate to arsenite, which is then exported by the efflux system through the plasma membrane.

4.2.3. Intracellular sequestration

Through a procedure known as intracellular sequestration, diverse substances in cell cytoplasm combine metal ions. Two kinds of eukaryotic metal-binding peptides—metallothioneins and phytochelatins—are high in cysteine residues and bind metal ions via sulfhydryl groups (Pinto et al., 2003). For metallothionein synthesis, which is triggered by cadmium and zinc ions, *Synechococcus* sp. encodes two genes, *smtA* and *smtB* (Ybarra and Webb, 1999). Low-molecular-weight proteins with a high cysteine content enable a cadmium-tolerant strain of *P. putida* to sequester copper, cadmium, and zinc ions intracellularly. According to Ivanova et al. (2002), some marine gamma-proteobacteria synthesize phytochelatin-like low-molecular-weight proteins that are cadmium-inducible. Glutathione is used by *Rhizobium leguminosarum* cells to sequester cadmium ions intracellularly (Lima et al., 2006).

4.2.4. Extracellular sequestration

Metal ion accumulation in the periplasm or outer membrane of microbial cell, or their fusion as insoluble compounds, is referred to as extracellular sequestration. For instance, copper-resistant strains of *Pseudomonas syringae* produce *CopA, CopB,* and *CopC,* which bind copper ions and cause copper to build up in the periplasm or outer membrane, resulting in blue bacterial colonies (Cha and Cooksey, 1991). Similar to how copper-tolerant *Pseudomonas pickettii* US321 builds up copper ions in the periplasm or outer membrane, resistant strains likely store copper as a complex and transport it into the cytoplasm, while sensitive strains likely build up copper in a toxic free ionic form that damages cells (Gilotra and Srivastava, 1997). *Pseudomonas stutzeri* AG259, which was isolated from the soil of a silver mine, accumulates silver ions as sulfide complexes on the cell surface or in elemental form in the periplasm due to plasmid-encoded resistance to high concentrations of silver ions in the medium (Haefeli et al., 1984; Slawson et al., 1992; Klaus et al., 1999). Some bacteria release metal ions from the cytoplasm into the periplasm, where they are then trapped. The periplasmic protein *SilE* of the *Salmonella* sp. strain selectively binds silver ions, which are then exported by the ATPase pumps *SilCBA* and *SilP* (Silver, 2003).

4.2.5. Reduction of HM ions

Smirnova (2005) claims that a variety of HM ions, such as chromate, molybdate, and vanadate, are reduced by bacteria from various biological niches. Some bacteria can use metals and metalloids as electron acceptors or donors to produce energy. During anaerobic respiration, bacteria can use oxidized metals as terminal electron acceptors, and enzymatic reduction of metal ions can result in the creation of less hazardous forms of HMs including mercury and chromium (Barkay et al., 2003; Viti et al., 2003).

The *mer*-operon, which imparts tolerance to mercury by encoding proteins like *MerT* and *MerA*, is one of the best-studied mechanisms for metal detoxification (Brown et al., 2002). Divalent mercury ions can enter the cell by the *MerT* transport protein and are then converted to elemental mercury by the intracellular *MerA* reductase.

According to Nies (2003), some plasmids or transposons that can be shared by different bacteria through horizontal gene transfer (HGT) contain genetic determinants of HM tolerance.

Natural transformations occurring frequently, the isolation of plasmid- and transposons-bearing bacteria from a variety of environments, and the acquisition of new traits by autochthonous microbiota after exposure to plasmid-bearing bacteria are all evidence supporting the role of HGT in bacterial evolution under changing environmental conditions (Coombs and Barkay, 2005).

Bacterial plasmids were where metal resistance mechanisms were first discovered (Summers and Silver, 1972). The *Ars* operons on the chromosomes of *E. coli*, *P. aeruginosa*, and *Bacillus subtilis,* which structurally mimic plasmid-borne genetic determinants, are examples of metal resistance systems that are similar to those on plasmids (Mukhopadhyay et al., 2002). However, hazardous metal resistance genes are carried on plasmids while important metal ion homeostasis genes are typically found on chromosomes (Bruins et al., 2000; Cervantes et al., 2001).

The *mer* operon, independent of its position on the chromosome or plasmid, is a well-studied metal tolerance system that is found in diverse types of bacteria, according to Narita et al. (2003) and Reniero et al. (1998). *merA, merT, merP*, and *merR* make up the majority of the *mer* operon, while other genes like *merB, merC, merD, merE, merF,* and *merG* may also be present. According to Große et al. (2004), the *czc* operon on the PMOL30 plasmid of *R. metallidurans* CH34 encodes resistance to cadmium, zinc, and cobalt ions through the *czcCBA* genes as well as regulatory, promotor, and unknown functional genes *czcN* and *czcI*. According to Mergeay et al. (2003), *R. metallidurans* CH34 also harbors the PMOL28 mega plasmid, which encodes resistance to Co^{2+}, Ni^{2+}, and chromate as well as tolerance genes for Tl^{+} and Hg^{2+} and contains several previously unidentified metal resistance genes. *ArsR, ArsA, ArsD, ArsB*, and *ArsC* are just a few of the proteins that are regulated by *ars* operon and involved in arsenate tolerance, found in many different bacterial groups (Mukhopadhyay et al., 2002). Both Gram-positive (Oger et al., 2003) and Gram-negative (Lee et al., 2007) bacteria have the *cop* operon, which confers tolerance to copper ions. The *cop* operon and the cadmium tolerance *cad* system may differ in structure and location, as observed in various bacterial groups, such as *E. hirae* and *Pseudomonas* spp. (Bruins et al., 2000).

4.2.6. Microbially induced carbonate precipitation

Researchers have proposed microbially induced carbonate precipitation (MICP) as a viable approach for environmentally friendly and sustainable bioremediation of metal contaminants (Dhami et al., 2013; Zhang et al., 2018). This mechanism, which involves the secretion of urease by microorganisms, offers a simple and controllable method to rapidly generate substantial amounts of carbonates. When urea is hydrolyzed by urease, CO_2 and NH_3 are produced, which subsequently react in the solution to produce ammonium, bicarbonate, and hydroxide. As a result, the pH increases (becomes more alkaline), and carbonate ions are formed. Under appropriate conditions of sufficient ionic activity and the presence of divalent cations, carbonate ions can precipitate out of the solution.

According to Yin et al. (2021), cadmium (Cd) and strontium (Sr), along with other metals and radionuclides, can undergo precipitation and create insoluble carbonate minerals of their own by following same pathway. Alternatively, these metals can be co-precipitated with calcium carbonate if the calcifying microorganisms are able to sustain in HM contaminated environment. Certain HM ions, such as Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, and Sr^{2+}, which have ion radii similar to that of Ca^{2+} can be integrated into the crystal lattice of $CaCO_3$ through isomorphic substitution of Ca^{2+} or by penetrating crystal interstices or defects (Kim et al., 2021). As a result, the HMs transform from soluble state of ions into insoluble state, effectively prevent their release back into the environment. The wide-ranging application of MICP and its capacity to sequester HMs establish it as a feasible *in situ* remediation technique for HMs polluted sites. Additionally, its resilience to changes in redox potential in the surrounding environment contributes to its high effectiveness and long-term stability in bioremediation (Lauchnor et al., 2013).

4.3. Other microbial detoxification mechanism

Microorganisms survive via different mechanisms in the presence of HMs to resist metal toxicity as shown in **Figure 2**. Some commonly used strategies employed by microbes include extrusion, exopolysaccharide secretion, biotransformation, metallothionein as well as enzyme synthesis (Dixit et al., 2015). Furthermore, major mechanisms to resist HMs by microorganism involves several procedures like ion exchange, metal efflux pumps, metal oxidation, electrostatic interaction, methylation, redox process, metal-organic complexion, precipitation, exclusion by permeability barrier, metal ligand degradation, surface complexation, demethylation, metal sequestration, and bio surfactant production. Detoxification of metals by microbes can also be done by extracellular chemical precipitation, volatilization, and valence conversion (Ramasamy et al., 2006; Yang et al., 2015). Microbes have negatively charged groups on their cell surface like sulfonate, hydroxyl, alcohol, phosphoryl, carboxyl, thioether, sulfhydryl, amine, ester, and thiol implicated in metal adsorption (Gavrilescu, 2004). Few studies reported ability of bacteria to accumulate metals intracellularly such as sequestration of zinc, cadmium, and copper ions by cadmium-tolerant *E. coli*, *P. putida* strain (Hussain et al., 2022), and *R. leguminosarum* cells (Pereira et al., 2006).

Accumulation of metal ions extracellularly occurs by complexation as insoluble compounds or via cellular components in the periplasm. Several species belonging to *Geobacter* and *Desulfuromonas* are efficient to convert extremely hazardous metals to their benign forms. Some studies showing extracellular sequestration of HMs includes removal of copper ions by copper-resistant *P. syringae*, zinc ions by *Synechocystis* PCC 6803 strain, reduction of lethal forms of Manganese (IV), Uranium (VI), and Chromium (VI) to their non-toxic forms Manganese (II), Uranium (IV), and Chromium (III) by application of *Geobacter metallireducens,* a strict anaerobe (Igiri et al., 2018). Removal of cadmium (Cd) ions by *Klebsiella planticola* and *P. aeruginosa* as well as lead by the *Vibrio harveyi* strain via precipitation has been stated by several researchers (Sharma et al., 2000; Wang et al., 2002; Mire et al., 2004). Non-viable cells of *Brevibacterium* sp., *P. putida*, and *Bacillus* sp., showed effective biosorption of numerous HM ions (Green-Ruiz, 2006). Methylation by microbes play a crucial role in remediation of HMs. Methylated substances are frequently explosive; for example, *Escherichia* spp., *Pseudomonas* spp., *Clostridium*

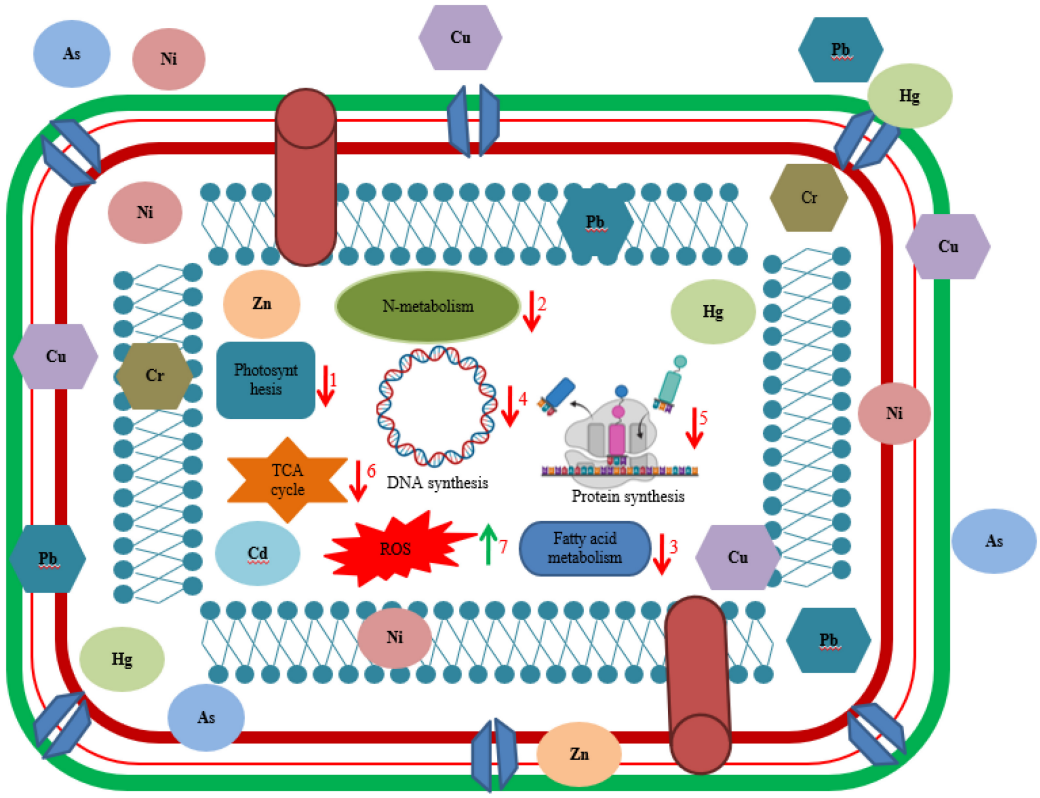

FIGURE 2
Illustration of interaction of living cell with heavy metals. Different biochemical dysfunctioning of cell occurred due to toxicity of heavy metal such as (1) decrease in rate of photosynthesis by inhibition of photosynthetic enzymes and chlorophyll biosynthesis, (2) significant decrease in nitrogen metabolism, (3) decreased rate of fatty acid metabolism, (4) disruption of DNA synthesis, (5) inhibition of protein synthesis, (6) negatively affecting key enzymes in TCA cycle, and (7) overproduction of reactive oxygen species (ROS).

spp., and *Bacillus* spp., can bio methylate mercury (II) to gaseous methyl mercury. In polluted topsoil, bio methylation of arsenic, lead, and selenium to gaseous arsines, dimethyl lead and dimethyl selenide was observed, respectively (Ramasamy et al., 2006).

5. Bioremediation agents based on PGPB

Bioaccumulation and biosorptive abilities of metal-resistant bacteria are used to remediate HM-contaminated environments (Kim et al., 2015). Several properties of bacteria such as, high surface-to-volume ratios, wide distribution, capacity to thrive in controlled environments, active chemisorption sites, size, and resistance to environmental conditions contribute to their robust biosorption potentiality (Srivastava et al., 2015; Mosa et al., 2016).

Bacteria and fungi are also reported to produces organic acids as a natural chelating agent for HMs (Seneviratne et al., 2017). Gluconic, acetic, oxalic, and malic acids are the most commonly reported organic acids for HM solubilization (Ullah et al., 2015; Gube, 2016). Out of single, consortium or immobilized forms of bacterial culture consortiums are observed to be more stable, metabolically superior, survive longer for metal biosorption and suited more for field use (Gu and Berry, 1991; Kader et al., 2007). Some studies demonstrating remediation of HM using bacteria includes removal of chromium (Cr) by consortia of *Acinetobacter* sp. and *Arthrobacter* sp. (De et al., 2008), cadmium, lead, and chromium removal in tannery effluent by mixed culture of *B. megaterium*, *Penicillium* sp., *B. subtilis*, and *Aspergillus niger* (Abioye et al., 2018), Pb removal by *Micrococcus luteus* (Puyen et al., 2012), removal of Cr^{6+}, Cu, and Ni utilizing zeolite immobilized *Desulfovibrio desulfuricans* (Kim et al., 2015).

Biofilms are also proven as effective bioremediating agent, biological stabilizer as well as possess high tolerance against toxic compounds at lethal quantities. Biofilms act as biosorbents or secrete exo-polymeric substances having surfactant or emulsifier properties for remediation of HMs (El-Masry et al., 2004). Several issues faced due to metallic stress includes reduced soil microbial activity as well as crop output (Ahemad, 2012), oxidative stress, modification, and loss of protein functionality leading to growth impairment, browning of roots, photosystems inactivation, and chlorosis in plants (Shaw et al., 2004; Göhre and Paszkowski, 2006; Seth et al., 2008). PGPB are well known to have adapted mechanisms for removal of metal pollutants like oxidation–reduction, biosorption, complexation, and precipitation (Muñoz et al., 2006; Singh et al., 2010). The bacterial response to a specific HM in the cleanup of metal-contaminated locations is of significant use (Hemambika et al., 2011). Various studies

reported use of PGPB as bioremediating agents such as remediation of copper toxicity by *Azospirillum lipoferum* (UAP154 and UAP40), *R. leguminosarum* CPMex46 and *Pseudomonas fluorescens* Avm, in alfalfa *Medicago sativa* seeds (Carrillo-Castañeda et al., 2005); nickel toxicity by *Microbacterium arabinogalactanolyticum, Sphingomonas macrogoltabidus*, and *Microbacterium liquefaciens* in *Alyssum murale* plants (Abou-Shanab et al., 2003). Furthermore, another study showed that hydroxamate siderophores produced by PGPB strains chelates HMs present in rhizospheric region of plants leading to inhibition of free radical formation and prevention of oxidative damage to plants (Dimkpa et al., 2009). Tripathi et al. (2005) reported growth promotion as well as inhibition of lead and cadmium toxicity by *P. putida* in *Phaseolus vulgaris*. Similarly, inoculation with *Pseudomonas* sp. Ps29C and *B. megaterium* Bm4C showed plant growth promotion and low nickel toxicity in *Brassica juncea* (Rajkumar and Freitas, 2008). Barzanti et al. (2007) found that bacteria aided plant growth under nickel stress, which is consistent with previous observations. In all, these investigations clearly demonstrated the potential of PGPB to improve plant biomass under HM stress. As a result, using metal detoxifying PGPB in conjunction with other plant growth-promoting activities can significantly improve the efficiency of the entire remediation process (Glick, 2012).

Rhizoremediation involves crucial role of rhizomicrobial population in detoxification process of HM in contaminated soils (Kuiper et al., 2004). These microbes exhibit high metabolic activity around the roots of plants. Bacterial population dominating HMs stressed sites includes *Pseudomonas, Rhizobia, Arthrobacter*, and *Bacillus* (Pires et al., 2017). One of the most well-known symbiotic associations found between legume and rhizobia is capable to remediate HM toxicity and improve the condition of contaminated soils (Checcucci et al., 2017). They have the potential to transform a wide range of metals and alter metal dissolution, toxicity, speciation, mobility, and degradation in soil (Gadd, 2010). Several reviews are available on metal–microbe's interaction by Giller et al. (2009), Khan (2005), Gadd (2010), and Kong and Glick (2017). Interaction between microorganisms and metals in the rhizosphere is highly specific and influenced by physico-chemical properties of soil, soil type, metabolic activity, concentration, type of metal species, and microbial diversity.

6. Conclusion

Globalization and the technological improvement is achieving its height day-by-day for the convenience to the humankind. But its use is not done at an optimal level or judicially by the generation due to which its toxic components accumulating exponentially in the environment are affecting human health as well as agroecosystems by causing toxicity. Bioremediation using indigenous microorganisms from contaminated sites seems prudent, for exploiting their true potential. Several strategies including, oxidation, reduction, condensation, hydrolysis, isomerization, chelation, precipitation, complexation, immobilization, adsorption, bioaccumulation, and production of biosurfactant are expressed by microbial system for the removal or transformation of toxic HM into their non-toxic state in the contaminated sites. Preventive measures must be taken to avoid use of metal containing pesticides in agriculture and proper monitoring must be done before releasing metal containing effluents into water bodies. For future prospects, the use of genetically modified microorganisms could remove HM efficiently from the contaminated sites. Hence need to develop some other biological based techniques which efficiently work at contaminated sites and transformation of research from laboratory level to field or industrial level is also required.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effect of different levels of EDTA on phytoextraction of heavy metal and growth of *Brassica juncea* L.

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Heavy metal pollution of soil is a major concern due to its non-biodegradable nature, bioaccumulation, and persistence in the environment. To explore the probable function of EDTA in ameliorating heavy metal toxicity and achieve the sustainable development goal (SDG), *Brassica juncea* L. seedlings were treated with different concentrations of EDTA (0, 1.0, 2.0, 3.0, and 4.0 mM Kg^{-1}) in heavy metal-polluted soil. Plant samples were collected 60 days after sowing; photosynthetic pigments, H_2O_2, monoaldehyde (MDA), antioxidant enzymes, and ascorbic acid content, as well as plant biomass, were estimated in plants. Soil and plant samples were also examined for the concentrations of Cd, Cr, Pb, and Hg. Moreover, values of the phytoremediation factor were utilized to assess the accumulation capacity of heavy metals by *B. juncea* under EDTA treatments. In the absence of EDTA, *B. juncea* seedlings accrued heavy metals in their roots and shoots in a concentration-dependent manner. However, the highest biomass of plants (roots and shoots) was recorded with the application of 2 mM kg^{-1} EDTA. Moreover, high levels (above 3 mM kg^{-1}) of EDTA concentration have reduced the biomass of plants (roots and shoots), photosynthetic area, and chlorophyll content. The effect of EDTA levels on photosynthetic pigments (chlorophyll a and b) revealed that with an increment in EDTA concentration, accumulation of heavy metals was also increased in the plant, subsequently decreasing the chlorophyll a and b concentration in the plant. TLF was found to be in the order Pb> Hg> Zn> and >Ni, while TF was found to be in the order Hg>Zn>Ni>Pb, and the best dose was 3 mM kg^{-1} EDTA for Hg and 4 mM kg^{-1} for Pb, Ni, and Zn. Furthermore, hyperaccumulation of heavy metals enhanced the generation of hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\bullet-}$), and lipid peroxidation. It also interrupts mechanisms of the antioxidant defense system. Furthermore, heavy metal stress reduced plant growth, biomass, and chlorophyll (chl) content. These findings suggest that the exogenous addition of EDTA to the heavy metal-treated seedlings increases the bioavailability of heavy metals for phytoextraction and decreases heavy metal-induced oxidative injuries by restricting heavy metal uptake and components of their antioxidant defense systems.

KEYWORDS

heavy metals, EDTA, oxidative stress, phytoremediation, *Brassica juncea*, SDG

1. Introduction

In the recent past, rapid industrialization and enhanced urbanization have given rise to an amplified level of heavy metal (HM) contamination in the ecosystem, which has arisen as a global concern (Saleem et al., 2018). HMs contamination of soil is a key concern due to their nonbiodegradable nature, bioaccumulation, and perseverance in the ecosystem (Din et al., 2020). HMs contamination has augmented in the soil as well as in the water because of the release of HMs encompassing effluents from several industries, namely alloying, electroplating, metallurgy, paints, tanneries, textile dyes, and timber processing (Ganesh et al., 2009; Gill et al., 2016). Various toxic HMs existing in diverse oxidation states, such as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb), and zinc (Zn); radioactive elements, namely uranium and strontium; along with organic compounds like trinitrotoluene, 1,3,5-trinitro-1,3,5-hexahydrotriazine; petroleum hydrocarbons (benzene, toluene, xylene, etc.), all are hard to eliminate from the ecosystem due to their nonbiodegradable nature, and they become acutely toxic if their concentration exceeds a certain threshold. Due to their hydrophilic nature and prevalent mobility, HMs can simply enter the rhizospheric region of plants, be transported to the shoot part of plants, and become a serious risk for living organisms, including humans, by food-chain transfer (Singh and Singh, 2017). Human exposure to HMs comes frequently via different food crops, which accounts for approximately 89% of the total intake, whereas the remaining 11% arises via skin contact and breathing of polluted dust. These HMs may commonly react with biological systems via losing one or more electrons and forming metal cations which have affinity to the nucleophilic sites of vital macromolecules. Several acute and chronic toxic effects of heavy metals affect different body organs. Birth defects, cancer, gastrointestinal, immune system and kidney dysfunction, nervous system disorders, skin lesions, and vascular damage are examples of heavy metals toxic effects (Balali-Mood et al., 2021; Mitra et al., 2022). Various studies have proved that surplus quantities of HMs contamination negatively influence the physiology and phenotype of some plants. The general phenotypic signs of HM-induced stress are chlorosis, epinasty of leaves, disturbance in tube growth along with pollen germination, necrosis, and stunted plant growth. HMs reduces nutrient uptake, disturbs chlorophyll (chl) content, abolishes the ultrastructural mechanisms of the chloroplast, and changes nitrogen and sulfur metabolism, thereby damaging the photosynthetic process and hindering the metabolism of plants (Benavides et al., 2005; Singh et al., 2022). As few heavy metals are redox-inactive metals, they can indirectly produce more reactive oxygen species (ROS), which contain singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and the hydroxyl radical ($OH^{\bullet}$).

The phytoremediation method utilizes plants (with or without the associated microorganism) for the removal of notorious contaminants. It has been extensively accepted and applied in the last few years. As this methodology is economical, sustainable, eco-friendly, and unintrusive, it is anticipated to play a vital role concerning industrial scale if executed with proper consideration (type of pollutant, composition of waste generated, seasonal variation, and diversity of plants to be utilized) (Lin et al., 2002; Yang et al., 2017). This method eradicates HMs by taking benefit of several plants ability to absorb and accrue metals and congregating them within the plant biomass. The perspective of this type of remediation technique is to decrease the concentration of HMs from polluted soil so that these plants can be utilized favorably for agriculture, horticulture, forestry, grazing, etc. Several plant species are capable of developing several approaches to combat HMs toxicity and reduce hostile effects by evading toxicity via metal-binding on the cell walls, averting transport across cell membranes, active efflux, compartmentalization and excretion methods as well as by internal metal chelation (Singh and Singh, 2017).

At present, several researchers are focused on the supportive treatments for phytoremediation by utilizing genetic engineering, sorbents, phytohormones, microbiota, microalgae or nanoparticles. In future, purification of soils on an industrial scale will most likely be possible through genetically modified organisms. However, there is a substantial risk of gene transfer from transgenic plants or microorganisms to the environment. Engineering bioremediation offers few operative solutions in the form of the use of various organic substances (e.g., sewage sludge, sorbents, enzymatic and microbial preparations or nanoparticles). Moreover, few research related to new techniques such as *in situ* solar driven technology make use of vascular plants to accumulate and translocate metals from root to shoot. Harvesting the plant shoots can permanently remove these contaminants from environment (Jadia and Fulekar, 2009; Mocek-Płociniak et al., 2023).

Plants have a coordinated and multifaceted antioxidant defense system to sustain reactive oxygen species (ROS) at a steady-state level by rummaging the generated ROS to cope with oxidative stress (Gill and Tuteja, 2010; Ma et al., 2017; Ashraf et al., 2021). The defense system, containing enzymes namely ascorbate peroxidase (APOX), catalase (CAT), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPOX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), guaicol peroxidase (POD) and superoxide dismutase (SOD) has an imperious part in rummaging the produced ROS because of metal toxicity (Gill and Tuteja, 2010). Numerous studies have reported different functions of several antioxidant enzymes in diverse species of plants under HM stress conditions (Gill et al., 2015; Kanwar et al., 2015; Handa et al., 2018). Non-enzymatic antioxidants such as ascorbic acid and glutathione tocopherols also function in a harmonized way with various enzymatic antioxidants to counterbalance HM-induced ROS. For example, ascorbic acid and glutathione levels were enhanced under heavy metal stress conditions (Ashger et al., 2018; Ulhassan et al., 2019). These antioxidant enzymes diminish oxidative damage encouraged by ROS. Cellular macromolecules, *viz.* proteins, lipids, and nucleic acids, are oxidized through the elevated level of ROS (Sigfridsson et al., 2004; Hasanuzzaman et al., 2012). These enzymes act as quenchers of lipid and ROS radicals (Noctor and Foyer, 1998; Smirnoff, 2000; Hollander-Czytko et al., 2005). *B. juncea* is a fast-growing crop with medicinal and oil-yielding properties. It produces a high amount of biomass and has a robust and well-studied antioxidant defense system. However, heavy metal contamination results in a noteworthy loss of yield (Bhuiyan et al., 2011; Shekhawat et al., 2012).

Hyperaccumulation of these heavy metals can be encouraged with the addition of chemical alteration, such as ethylene diamine tetraacetic acid (EDTA), as a plant substrate to formulate a soluble or insoluble target metal, *viz.* Pb (Christopher et al., 1998). EDTA

generally act as chemical chelator for eradicating toxic HMs from contaminated soil systems, predominantly where there is less metal bioavailability (Hernandez-Allica et al., 2007). EDTA function in the uptake of HMs and reducing its toxicity has already been documented in some plants (Dipu et al., 2012; Shahid et al., 2014; Khan et al., 2019). Moreover, different metals' solubility is augmented by EDTA in the soil system, which enhances their uptake, bioavailability, and translocation from the rhizospheric region to the shoots in most vascular plants (Farid et al., 2015). EDTA has been utilized in plentiful experiments with diverse species of the Brassicaceae family as a metal chelator for monitoring a range of biochemical and physiological parameters. Contrariwise, Evangelou et al. (2007) stated that Cd accumulation is inhibited by EDTA in *Nicotiana tabacum* L. plants. Correspondingly, this chelating agent was also detected to alter Cd uptake by conquering Cd toxicity in several plant species, namely *Beta vulgaris* L., *Oryza sativa* L., *Phaseolus vulgaris* L., and *Vigna unguiculata* (L.). Moreover, several studies have reported that EDTA improves the antioxidant defense mechanism and growth of plants under HMs stress conditions (Hardiman and Jacoby, 1984; Greger and Lindberg, 1986; Weihong et al., 2009; Xu et al., 2010; Agbadah et al., 2016). Therefore, the aim of this study was to investigate the possible role of EDTA in mitigating HMs toxicity and its effect plant health. In view of this, we examined the effect of different EDTA levels on metal accumulation, physiological parameters, and the antioxidant defense mechanism of *B. juncea* in heavy metal contaminated soil.

2. Materials and methods

2.1. Plant material, physicochemical analysis, and experimental conditions

The collection of soil was done from a commercial horticulture field in Cairo, Egypt. Soil was oven-dried at 35°C for 4 days and filtered through a 6 mm mesh. The physico-chemical properties of soil were assayed as per the method described by Merkl et al. (2005). The soil had the properties of pH (7.4), Electrical conductivity (1.13 dsm^{-1}), total nitrogen (0.09%), total phosphorus (0.78%), organic carbon 0.487% and Zn 2.7, Fe 708.45, Mn 44.7, Ni 0.5 $mg\,kg^{-1}$ dw, respectively. Afterwards, 5 different gradients of EDTA (0, 1, 2, 3, and 4 $mM\,kg^{-1}$) were prepared by adding the respective weight of the EDTA salt (292.2 g EDTA to 1 kg of water to prepare 1 $M\,kg^{-1}$) to distilled water to prepare the different gradients and applied to the soil. Moreover, wastewater from the industry was collected in a propylene container from El Tebin industrial area, Egypt. The quantity of heavy metals present in the chemical wastewater was analyzed by inductive coupled plasma mass spectrometer (ICPMS). The composition of heavy metals were 12.36 Pb, 10.64 Ni, 30.45 Zn and 6.5 Hg mgL^{-1} and their physicochemical parameters were pH 6.2 and chemical oxygen demand (COD) 1917, biological oxygen demand (BOD) 196, Cl 1895, Ca 824.5 and Mg 586.2 mgL^{-1}. The tap water had the properties of pH 6.9, total dissolved solids 145, total hardness 240, calcium hardness 106, dissolved oxygen 3.6, chloride ion 83, alkalinity 110, Na 25 and K 6 mgL^{-1}.

Seeds of *B. juncea,* cultivar Balady were obtained from the commercial market, in Cairo, Egypt for conducting *in situ* pot experiments. 5 seedlings were maintained in each pot and the experiment was executed using a completely randomized design (CRD) with three replications. Seeds of *B. juncea* were sown in pots sized 15 × 15 cm, with 15 kg of garden soil in each pot and different amendments of heavy metals. Irrigation of each pot was done with a constant quantity of 5 L of industrial wastewater per day at the same time for 90 days. The implications of water as well as industrial wastewater levels were constant across all treatment pots.

2.1.1. Plant sampling

Plant samples were collected after 60 DAS (days after sowing) from each pot and washed repeatedly using tap water to eliminate unwanted debris for the estimation of photosynthetic pigments. Soil and plant samples were again collected at 90 DAS to examine heavy metal concentrations of Cd, Cr, Pb, and Hg, as well as plant biomass.

2.2. Measurement of heavy metal content transfer factor and translocation factor

For heavy metal analysis in plants, 1 g of plant samples was dried out and finely grinded in an electric grinder, then digested in HNO_3:$HClO_4$ (3:1, v/v) at 80°C. Metal concentrations (Pb, Ni, Zn, and Hg) in plant samples were analyzed by means of an Inductively-Coupled Plasma Mass Spectrometer, Perkin Elmer Corporation (ICP Optima 3,300 RL). For metals, a standard reference material (E-Merck, Germany) was utilized for calibration and quality assurance for each analytical investigation. The detection limits of Hg, Pb, Ni, Zn, and Zn were 0.01, 0.1, 0.5, and 2.0 µg/L, respectively. Replication analysis ($n = 5$) was done to measure the precision of the analytical techniques. Triplicate analysis for each metal varied by not more than 5%. The treatments of EDTA were adjusted to different values in contrast with the controls and repeated three times (Al Mahmud et al., 2019).

$$TF = \left[\begin{array}{l} \left(\text{Metal concentration} \left(\text{root} + \text{shoot} \right), \text{mg kg}^{-1} \right) / \\ \left(\text{Metal concentration of soil}, \text{mg kg}^{-1} \right) \end{array} \right]$$

$$TLF = \left[\begin{array}{l} \left(\text{Metal concentration in the shoots}, \text{mg kg}^{-1} \right) / \\ \left(\text{Metal concentration in the roots}, \text{mg kg}^{-1} \right) \end{array} \right]$$

2.3. Estimation of photosynthetic pigments content

Leaf chlorophyll content was estimated as per the procedure described by Hiscox and Israelstam (1979). 0.05 g of leaf tissue from each treatment was weighed and chopped in a test tube containing 10 mL of dimethyl sulfoxide (DMSO) and incubated for 3 h at 60°C. The absorbance was analyzed through a spectrophotometer at 645 and 663 nm. The chlorophyll content was calculated using the following formula:

$$\text{Chlorophyll a} = \frac{[12.7\text{x } A_{663} - 2.69 \text{ x } A_{645}] \text{ x V}}{1000 \text{ x W}}$$

$$\text{Chlorophyll b} = \frac{[22.9\text{x } A_{645} - 4.68 \text{ x } A_{663}] \text{ x} V}{1000 \text{ x W}}$$

Where:
A = Absorbance at specific wave length,
V = Final volume of solution,
W = fresh weight of tissue.

2.4. Estimation of H_2O_2 and monoaldehyde (MDA) content

Estimation of H_2O_2 content was done through the method given by Velikova et al. (2000). The fresh leaf tissues were homogenized in 1.5 ml of tri-chloroacetic acid (0.1%) and centrifuged at 4°C for 15 min at 12,000 rpm. 0.4 ml of supernatant was supplemented with equal volume of 10 mM PPB (Potassium Phosphate Buffer) and 0.8 mL of potassium iodide (KI, 1 M). Absorbance of reaction mixture was observed at 390 nm. Concentration of H_2O_2 was examined by preparing standard curve of H_2O_2.

The MDA level was examined via Heath and Packer (1968) method. One gram of fresh leaf tissue was extracted in 3 ml of tri-chloroacetic acid (0.1%) and centrifuged at 4°C at 13, 000 rpm for 10 min. Afterwards, 4 ml of thiobarbituric acid (0.5%) in 20% tri-chloroacetic acid was further added in supernatant. The mixture was placed in a water bath for 30 min at 95°C and immediately cooled by keeping it on an ice bath for reaction termination. Reaction mixture absorbance was monitored at 532 and 600 nm. MDA content was analyzed by taking the difference in absorbance using extinction coefficient, i.e., 155/mm/cm.

2.5. Estimation of antioxidant enzymes and ascorbic acid

The enzymatic activity of SOD (EC 1.15.1.1) was evaluated according to the procedure given by Beauchamp and Fridovich (1971). Reaction mixture containing 75 µM NBT, 50 mM potassium phosphate buffer with 2 µM riboflavin, 100 µM EDTA, 13 mM DL-methionine, and 15 µl of enzyme extract was used for estimation of enzymatic activity of SOD. The absorbance was measured at 560 nm. The reaction mixture was illuminated for 30 min at 25°C. Enzymatic activity (1 unit) was revealed as the quantity of enzymes essential for 50% inhibition at 25°C of NBT reduction.

Ascorbic acid was analyzed through the Roe and Kuether (1943) method. 0.1 g of activated charcoal and 4 ml double distilled water was added in the mixture containing 0.5 ml plant extract, and 0.5 ml TCA (50%). The mixture was filtered through Whatman filter paper #1. 0.4 ml of 2, 4-dinitrophenylhydrazine (DNPH) reagent was added to 1 mL of filtrate and incubated for 3 h at 37°C. Afterwards, 1.6 mL of chilled H_2SO_4 (65%) was supplemented to mixture and incubated at room temperature for 30 min. The absorbance was recorded at 520 nm.

2.6. Statistical analysis

All data is represented as mean value ($n = 3$) ± standard deviation (SD). To study the significance at $p < 0.05$ of the given data, analysis of variance (ANOVA) with LSD *post hoc* tests were done to examine substantial differences through SPSS version 17.0 software.

3. Results

A statistical examination of the data revealed that the interaction of heavy metals, EDTA, and Brassica species had a substantial consequence on root as well as shoot biomass. The obtained data indicated that the maximum plant biomass attained for root and shoot was 35 g and 65 g, respectively, with a 2 mM kg^{-1} EDTA application. Furthermore, the maximum concentration of EDTA (3 and 4 mM kg^{-1}) given to *B. juncea*'s shoot and root leads to a modest decrease in plant biomass, as depicted in Figure 1. Though there were no signs of rot or chlorosis in the plants, this divulged that *B. juncea* has virtuous tolerance for heavy metals (Pb, Ni, Hg, and Zn,) and EDTA.

3.1. Effect of EDTA on heavy metal concentration in soil and plant samples

The comparative study of dissimilar concentrations of EDTA divulged that absorption of heavy metals via the roots of *B. juncea* was as follows: Zn > PbNi>Hg, as depicted in Figure 2A, while in shoot Pb > Zn > Ni > Hg (Figure 2B). The phytoremediation effects

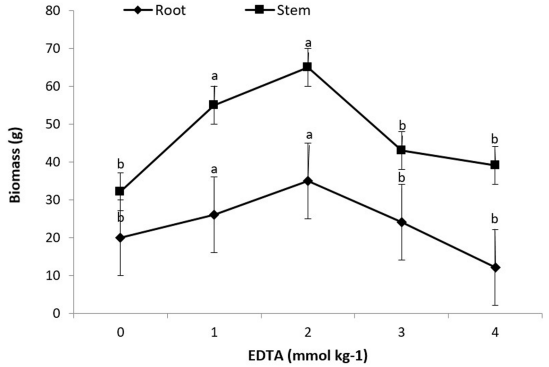

FIGURE 1
Effect of different EDTA concentration on plant biomass. The vertical bars represent means ± S.D. (n = 3). Bars with different letters are significantly different ($p \leq 0.05$).

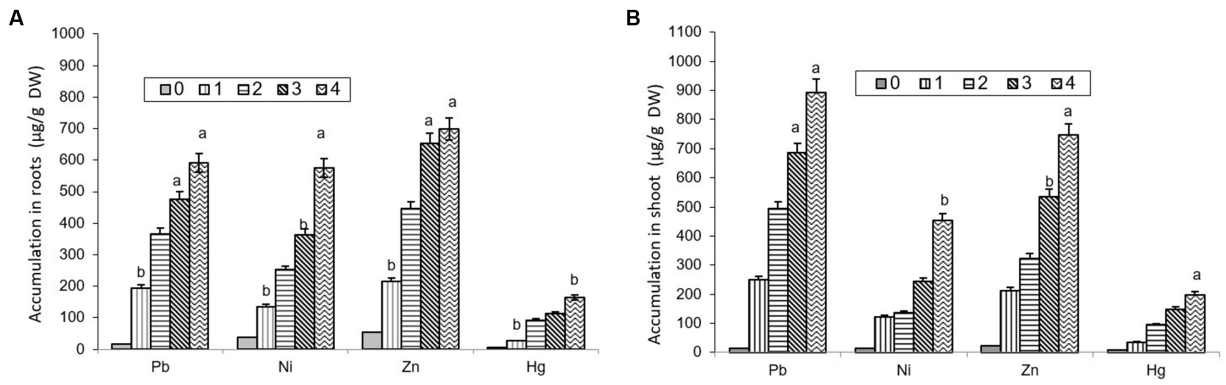

FIGURE 2
Depiction of heavy metal accumulation in roots **(A)** and shoot **(B)** under different EDTA concentration. The vertical bars represent means ± S.D. (n = 3). Bars with different letters are significantly different ($p \leq 0.05$).

of water in plants on different elements Pb, Ni, Zn, and Hg were 0.11, 0.33, 0.30, and 0.60 µg/g DW, respectively. It was observed that the effect differs depending on the constituents. The effect of phytoremediation on different elements (Pb, Ni, Zn, and Hg) in the shoot region was changing at different values, but it was identified that at 100, the values were at their maximum and were obtained as 894, 454, 748, and 198 µg/g DW. The procedure was also applied to roots, and the consequences on various elements Pb, Ni, Zn, and Hg were 592, 575, 698, and 165 ug/g DW respectively, but the values were depicted least at a value of 0. The treatment containing high levels of EDTA has encouraged Zn and Pb uptake capacity in the shoot part of *B. juncea*. Heavy metal concentrations in soil have increased by increasing the level of EDTA, as shown in Figure 3. Due to EDTA's high concentration, plants have absorbed and deposited more heavy metals in the soil.

The translocation factor (TLF) and transfer factor (TF) in plants were deliberated to predict the heavy metal accretion rate in *B. juncea* under different treatments (Table 1). TF and TLF values in EDTA applications are amplified by enhancing the EDTA levels. The highest TF in Pb was recorded at 1.19 and 1.63 µg/g DW for Zn, with an implication of 4 mM/kg EDTA. Moreover, the highest TF was recorded as 1.26 in the case of Ni with no supplementation of EDTA. Furthermore, a TF value of 3.91 µg/g DW was the maximum in Hg with the application of 3 mM/kg EDTA. While the highest TLF values in Pb and Zn were recorded at 1.51 and 1.07 µg/g DW with supplementation of 4 mM/kg EDTA, In the case of Ni and Hg, the TLF values were recorded as 0.90 and 1.33 µg/g DW in 1 mM/kg EDTA concentration, respectively. On average (EDTA applications only), TLF was found to be in the order Pb > Hg > Zn > and > Ni (Figure 4), while TF was examined in the order Hg > Zn > Ni > Pb.

3.2. Photosynthetic pigment determination

The impact of various EDTA concentrations on photosynthetic pigments (chlorophyll a and b) is shown in Figure 5. Maximum chlorophyll content was recorded at 5.12 in 1 mM EDTA concentration, followed by 3.45 in 0 mM concentration. These data clearly indicate that as EDTA levels increased, heavy metal accumulation subsequently decreased chlorophyll a and b content in plants.

3.3. H_2O_2 and monoaldehyde (MDA) content activity

Brassica sp. seedlings exposed to EDTA revealed that maximum levels of oxidative stress were due to an increase in H_2O_2 production. A severe rise in H_2O_2 level of 161.64% in 3 mM Kg $^{-1}$ EDTA-treated seedlings was revealed, contrary to control seedlings (0 mM EDTA). However, a sharp decline in H_2O_2 content of 111.58% was examined in seedlings grown with the application of 1 mM Kg^{-1} EDTA. These results clearly signify the effect of EDTA treatments on plant health under oxidative stress. Similarly, *Brassica* seedlings grown with EDTA showed a steep increment in the MDA content (640.19%), contrary to the control (0 mm EDTA). As EDTA concentration increases from 1 to 4 mM Kg^{-1}, the lipid peroxidation level in leaves also increases (from 232.35 to 640.19%) (Figure 4).

3.4. SOD and ascorbic acid activity

Exogenous EDTA supplementation in heavy metal affected plants raised SOD activity in contrast to plants without EDTA supplementation. As the EDTA concentration increased from 0 to 4 mM/kg, the activity of SOD also increased from 19.6 to 49.7 U min^{-1} mg^{-1} protein. Moreover, non-enzymatic antioxidants like ascorbic acid were correspondingly examined and found to be increased in seedlings under heavy metal stress conditions. A tremendous increment of 11.94% in ascorbic acid content was monitored in seedlings under heavy metal stress with the application of 1 mM Kg^{-1} EDTA, contrary to control, which was further promoted by 23.88, 29.59, and 112.83% with 2, 3, and 4 mM Kg^{-1} EDTA treatment (Figure 4).

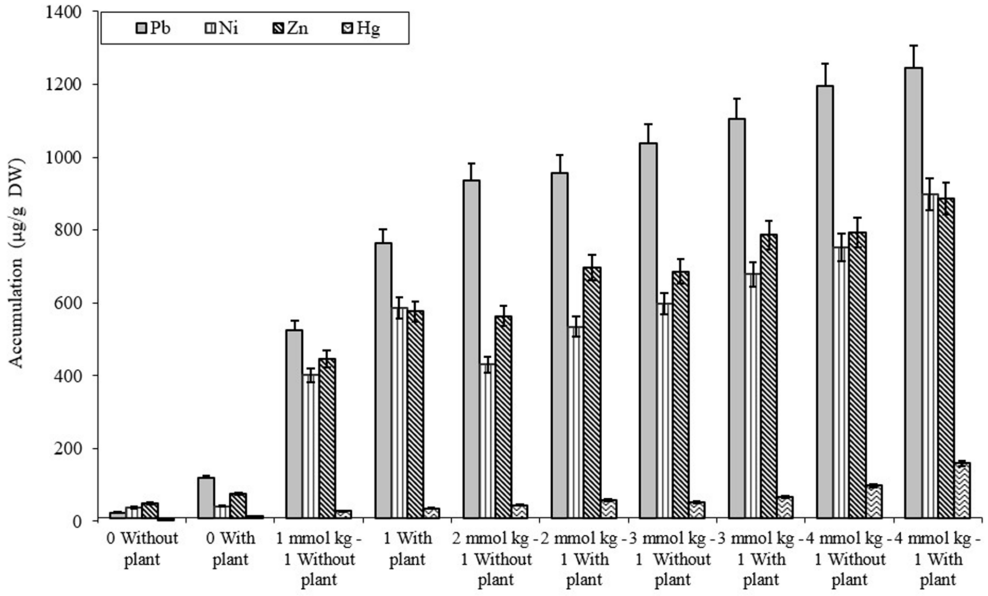

FIGURE 3
Illustration of heavy metal accumulation in soil under different EDTA concentration. The vertical bars represent means ± S.D. (n = 3).

TABLE 1 Transfer factors and translocation factors for different EDTA concentration.

EDTA Level (mM kg^{-1})	Transfer factor				Translocation factor			
	Pb	Ni	Zn	Hg	Pb	Ni	Zn	Hg
0	0.25	1.26	1.00	1.07	0.76	0.36	0.43	1.29
1	0.58	0.44	0.74	1.75	1.28	0.90	0.99	1.33
2	0.90	0.73	1.10	3.17	1.35	0.54	0.73	1.03
3	1.05	0.90	1.51	3.91	1.44	0.67	0.82	1.32
4	1.19	1.15	1.63	2.28	1.51	0.79	1.07	1.20

4. Discussion

HMs uptake through plant rhizosphere and its transportation into the shoots of *B. juncea* plants were enhanced in parallel with stress intensity when HMs was implemented in the medium. HMs mobilization in the rhizosphere, uptake by plant roots, translocation from roots to aerial parts of plants, and heavy metal ion sequestration and compartmentation in plant tissues are some of the steps utilized by plants to extract HMs. Enhanced HMs concentrations result in reductions in elongation, biomass, and seedling growth. A statistical study revealed that HM, EDTA, and the interaction of HM × EDTA with plant species had a substantial effect on both root and shoot length. In the current investigation, heavy metal stress substantially affected the biomass of *B. juncea* seedlings, as observed by a decrease in the fresh and dry weights of the seedlings. Similarly, Qadir et al. (2004) studied *B. juncea* sps. For the efficacy of phytoextraction and found a decrease in shoot length of *B. juncea* subjected to Cd (0.0–2.0 mM). Furthermore, plants imperiled to heavy metal stress have detrimental effects on numerous metabolic cycles because of ROS generation, resulting in reduced plant yield, production, and biomass. Heavy metal toxicity has also been revealed to decrease biomass in *Solanum lycopersicum*, *B. juncea*, *S. seban*, and *S. melongena* (Mahmud et al., 2017; Singh and Prasad, 2019; Din et al., 2020). Zheng et al. (2010) affirmed that application of Cd to the growth medium leads to increased Cd accretion in the root, in contrast to the shoot, which was validated by a greater reduction in root growth compared to shoot growth. Implementing EDTA in the soil decreases free Cd^{2+} ions around the rhizosphere and thereby reduces plant metal uptake. Moreover, Jiang et al. (2003) illustrated that EDTA perhaps decreases the solubility and bioavailability of HMs in the soil system and consequently reduces plant uptake.

Moreover, strong metal chelators like EDTA are known to have a significant impact on chemical speciation, which in turn affects soil solution phase mobility, solubility, and bioavailability as well as root absorption and accumulation of metals. The current study indicated that high-concentration EDTA treatment has encouraged Zn and Pb uptake capability in the shoot part of *B. juncea*. This is confirmed by a previous study that observed the application of EDTA to the soil, which results in heavy metals being phytoextracted and moved from the rhizospheric region to the plant's harvestable above-ground components (Meers et al., 2008; Singh and Singh, 2017). Several experiments have been done by utilizing soil that was artificially enriched with heavy metals, which may result in high phytoextraction effects (Zhuang et al., 2007) due to the greater accessibility of heavy metals in artificially enriched soils. However, Wu et al. (2004) also concluded that Cu and Pb concentrations were also increased by EDTA application in shoots of *B. juncea*.

The impact of EDTA was examined on the uptake, leaching, and mobilization of heavy metals, along with the effects of EDTA inoculations on *B. juncea*. The most effective EDTA dose was 4 mM EDTA kg^{-1} in soil, where Ni, Pb, and Zn were significantly higher in

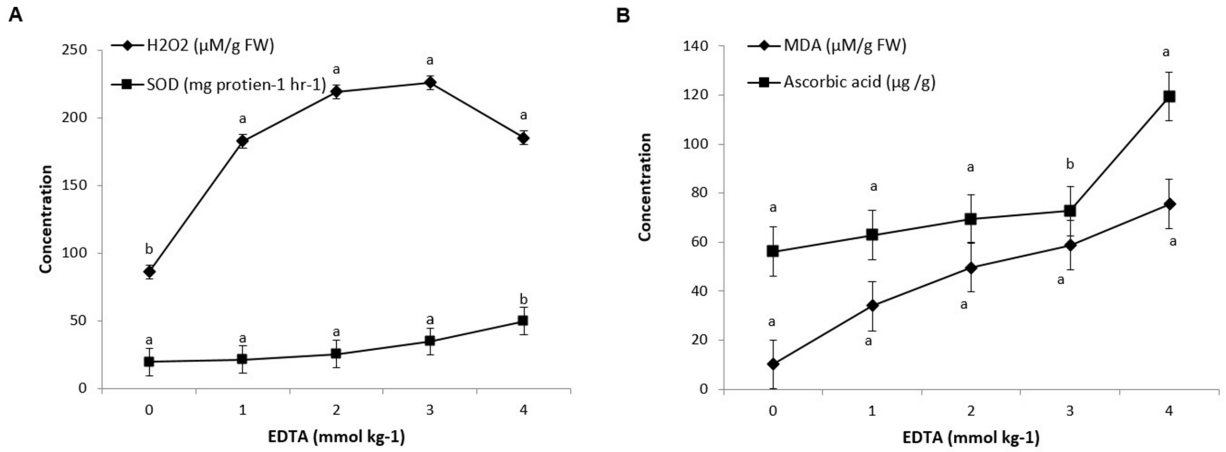

FIGURE 4
Antioxidant enzyme **(A)** and MDA, ascorbic acid **(B)** activity in *Brassica juncea* seedlings induced by implication of different EDTA concentration under heavy metal stress condition. The vertical bars represent means ± S.D. (n = 3). Bars with different letters are significantly different ($p \leq 0.05$).

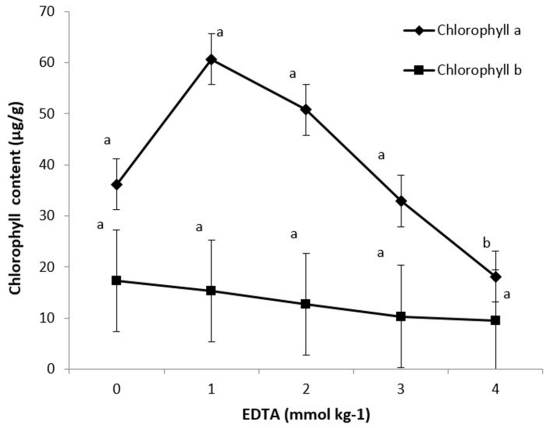

FIGURE 5
Effect of different EDTA concentration on photosynthetic pigments (Chlorophyll *a* and *b*). The vertical bars represent means ± S.D. (n = 3). Bars with different letters are significantly different ($p \leq 0.05$).

the shoot biomass, and a 3 mM kg $^{-1}$ concentration for Hg that was maximum in the shoot biomass, contrary to the control. Implications of EDTA on soil-induced Hg, Ni, Pb, and Zn bioavailability, which consequently stimulated phytoaccumulation and facilitated phytoextraction. Conversely, enhanced Cd and Pb bioavailability also reduced plant growth. The exact mechanism by which EDTA increases metal absorption is still being investigated.

The efficacy of phytoextraction is associated with both plant dry matter generation and heavy metal content. The best plant species for cleaning up a polluted area should be capable of producing the driest matter while tolerating and accumulating the target toxins (Wu et al., 2004; Clemens, 2006; Singh et al., 2017). Heavy metal concentrations by roots and shoot parts are unveiled in Figure 5, depicting less accretion of heavy metals in roots and a relatively high amount of heavy metals translocated to the shoot of *B. juncea.* Moreover, as per Brunetti et al. (2011), who studied *B. napus* plants in polluted soil, the accrual of the several metals under investigation (Cd, Cr, Cu, Ni, Pb, and Zn) was more prominent in shoots than in roots, as is characteristic of accumulator species. Zaier et al. (2010) analyzed the effect of EDTA on *B. napus* to eliminate metals from soils amended with sludge in HM removal, and the study disclosed an improvement in shoot metal accumulation. Furthermore, inoculation with EDTA has also encouraged the accretion of all heavy metals in the roots and shoot parts of *B. juncea*. Grcman et al. (2001) reported high metal (Pb, Zn, and Cd) accumulation performance in *B. rapa*. Moreover, for the disposal of phytoremediation plants with HMs, a variety of techniques including heat treatment, extraction treatment, microbiological treatment, compression landfilling, and nanomaterial synthesis can be applied. Each disposal technique has a unique operation procedure and set of technical requirements. HMs can move and change throughout various disposal procedures. Some techniques of disposal and usage may produce byproducts (Liu and Tran, 2021).

Heavy metal stress is commonly associated with oxidative stress and altered metabolism, such as alteration of chlorophyll biosynthesis, enzymatic activity, and pigment content (Szollosi et al., 2009). Heavy metal-induced oxidative stress also damages chlorophyll. Therefore, chlorosis of leaves is a common deleterious outcome of heavy metal stress. Plants have evolved multiple adaptation mechanisms to protect themselves from heavy metal stress. Disproportionate generation of ROS has deleterious effects on various cellular components, which affect cellular integrity and result in cell death (Askari et al., 2021). Heavy metal stress has damaging consequences for plant biomass and augments ROS production, which hinders the plant's function (Qureshi et al., 2020). Several plant sps. Can be reconnoitered as phytoremediators to diminish the contrary effects of heavy metals via the modulation of enzymatic and non-enzymatic antioxidants. The current study demonstrated the efficacy of *B. juncea* in the presence of EDTA in ameliorating heavy metal-induced oxidative damage in *B. juncea* seedlings via the production of enzymatic and non-enzymatic antioxidant molecules. The decrease in biomass in seedlings in the presence of heavy metals could be attributed to overproduction of ROS and reduced nutrient and water uptake. Our results are in agreement with Niakan and Kaghazloo (2016) and Farid et al. (2017) who stated that chlorophyll content in *Helianthus annuus* L. and *B. napus* plants increase in the presence of EDTA.

Moreover, SOD enzyme activity in *B. juncea* showed a significant increase with different EDTA concentrations since the increment in EDTA level upsurges the heavy metal concentrations in different plant parts. The enhanced SOD activity was concomitant with reduced $O^{2\bullet-}$ content as the conversion of $O^{2\bullet-}$ to H_2O_2 is controlled by SOD. These findings support the earlier work carried out with diverse plant types (Shaw and Mueller, 2009). SODs are a group of enzymes that accelerate the dissociation of superoxide radicals into H_2O_2. Several studies have reported the repressive effect of lipid peroxidation in leaf and root tissues under heavy metal stress (Demirbas et al., 2005; Charriau et al., 2016). Malondialdehyde-derived toxic compounds are produced downstream of ROS to mediate metal stress-induced oxidative damage in several crops. Our results clearly signify the consequences of EDTA as well as heavy metals on plant membrane oxidative damage. The non-enzymatic antioxidants, *viz.,* ascorbic acid, are important redox buffering mediators in cells, which regularize oxidative stress by quenching ROS and conserving the redox status of the cell (Noctor et al., 2018). Additionally, ascorbic acid is also engaged in governing numerous plant developmental functions such as cell division and differentiation, homeostasis, pollen growth, phytohormones, etc. (Potter et al., 2012). The findings of the current study showed an elevated level of ascorbic acid in EDTA-treated seedlings under heavy metal stress, which was analogous to the results observed in *O. sativa*, *B. napus,* and *Z. mays* under heavy metal stress (Chen et al., 2017; Ulhassan et al., 2019; Adhikari et al., 2020; Mocek-Płociniak et al., 2023).

5. Conclusion

Our study suggests that heavy metal stress significantly reduced the growth, biomass, and photosynthetic pigments of *B. juncea*. It also changes the antioxidative machinery of the plant system due to the overgeneration of ROS and lipid peroxidation. Furthermore, the effects of EDTA on the heavy metal-treated seedlings reduced heavy metal accumulation and upregulated the various nonenzymatic metabolites and antioxidant enzyme activity, which scavenged the toxic ROS (H_2O_2 and $O^{2\bullet-}$) from the plant. The most effective EDTA dose was 4 mM EDTA kg − 1 in soil, where Ni, Pb, and Zn were found to be significantly higher in the shoot biomass, and a 3 mM kg −1 concentration for Hg that was maximum in the shoot biomass. Thus, EDTA can be a potent candidate for conferring heavy metal stress, but the underlying molecular mechanism of heavy metal stress should be further elucidated.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

MK, KP, and FK: conceptualization, investigation, and manuscript writing. MK, FK, RS, and OH: data collection and analysis. KP, SB, JS, and MQ: manuscript reviewing and editing. KP, SB, and JS: manuscript finalization and submission. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Prevalence of antibiotic-resistant Gram-negative bacteria having extended-spectrum β-lactamase phenotypes in polluted irrigation-purpose wastewaters from Indian agro-ecosystems

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Antibiotic resistance in bacteria has emerged as a serious public health threat worldwide. Aquatic environments including irrigation-purpose wastewaters facilitate the emergence and transmission of antibiotic-resistant bacteria and antibiotic resistance genes leading to detrimental effects on human health and environment sustainability. Considering the paramount threat of ever-increasing antibiotic resistance to human health, there is an urgent need for continuous environmental monitoring of antibiotic-resistant bacteria and antibiotic resistance genes in wastewater being used for irrigation in Indian agro-ecosystems. In this study, the prevalence of antibiotic resistance in Gram-negative bacteria isolated from irrigation-purpose wastewater samples from Sirmaur and Solan districts of Himachal Pradesh was determined. Bacterial isolates of genera *Escherichia, Enterobacter, Hafnia, Shigella, Citrobacter*, and *Klebsiella* obtained from 11 different geographical locations were found to exhibit resistance against ampicillin, amoxyclav, cefotaxime, co-trimoxazole, tobramycin, cefpodoxime and ceftazidime. However, all the isolates were sensitive to aminoglycoside antibiotic gentamicin. *Enterobacter* spp. and *Escherichia coli* showed predominance among all the isolates. Multidrug-resistance phenotype was observed with isolate AUK-06 (*Enterobacter* sp.) which exhibited resistant to five antibiotics. Isolate AUK-02 and AUK-09, both *E. coli* strains showed resistant phenotypes to four antibiotics each. Phenotypic detection revealed that six isolates were positive for extended-spectrum β-lactamases which includes two isolates from *Enterobacter* spp. and *E. coli* each and one each from *Shigella* sp. and *Citrobacter* sp. Overall, the findings revealed the occurrence of antibiotic resistant and ESBL-positive bacterial isolates in wastewaters utilized for irrigation purpose in the study area and necessitate continuous monitoring and precautionary interventions. The outcomes of the study would be of significant clinical, epidemiological, and agro-environmental importance in designing effective wastewater management and environmental pollution control strategies.

KEYWORDS

wastewater, antibiotics, pollution, antibiotic-resistant bacteria, *Enterobacterales*, extended-spectrum beta-lactamases, agro-ecosystems

1. Introduction

Antibiotics are important anti-infective agents which have been used since the 20th century for the treatment of human infections (Hutchings et al., 2019; Walesch et al., 2023). The β-lactam antibiotics are clinically important antimicrobial medicines and have remained the first-line chemotherapeutic intervention against Gram-positive and Gram-negative bacteria since the 1950s (Hutchings et al., 2019; Soliman et al., 2023). Bacterial resistance to β-lactams has increased substantially in past few decades (Jani et al., 2021; Zaatout et al., 2021; Mutuku et al., 2022; Tan et al., 2023). However, their irrational, injudicious, and excessive use is on steady rise which not only worsen the issue of antibiotic resistance but also resulted in their accumulation in the environment as micro-pollutant (Du and Liu, 2012; Fazaludeen Koya et al., 2022; Gitter et al., 2023). The emergence of antibiotic resistance has threatened the effective treatment of microbial infections (Sanz-García et al., 2023). According to World Health Organization, the majority of pathogenic Gram-negative bacteria, especially those of the family *Enterobacteriaceae,* are included in the critical-priority group and are represented by multidrug resistant (MDR) bacteria commonly encountered in healthcare settings (Janda and Abbott, 2021; Dantas Palmeira et al., 2022). Further, antibiotic resistance is recognized as one of the top ten threats to public health globally (World Health Organization, 2023). In 2019, an estimated 4·95 million deaths were attributed to antibiotic resistance in bacterial pathogens such as *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Streptococcus pneumoniae* (Antimicrobial Resistance Collaborators, 2022).

Among various mechanisms by which bacteria acquired antibiotic resistance, the production of β-lactamases (EC 3.5.2.6) which cleaves β-lactam antibiotics, is considered the most significant from clinical perspective (Sawa et al., 2020; Castanheira et al., 2021; Noster et al., 2021; Zaatout et al., 2021). These enzymes are categorized into four classes according to their amino acid sequence and catalytic mechanisms (Bush, 2023). Class A β-lactamases are represented by narrow-spectrum β-lactamases (TEM-1, TEM-2, and SHV-1), extended-spectrum β-lactamases also called ESBLs (CTX-M, SHV-2, VEB-1) and serine carbapenemases which includes KPC-1 and SME-1 (Bush and Bradford, 2020; Castanheira et al., 2021; Bush, 2023; Cho et al., 2023). ESBLs are highly diverse, clinically important, and constitutively expressed antibiotic-degradative enzymes capable of hydrolyzing penicillins, cephalosporins (first-, second-, and third-generations), and monobactams (Livermore, 2008; Castanheira et al., 2021; Cho et al., 2023). ESBLs have been reported from several members of *Enterobacterales*, predominantly from genera *Escherichia, Enterobacter, Citrobacter,* and *Klebsiella* (Janda and Abbott, 2021; Cho et al., 2023). Worldwide, the most prevalent ESBL groups are CTX-M-1, CTXM-2, CTX-M-8, CTX-M-9, and CTX-M-25 with $bla_{CTX\text{-}M\text{-}15}$ genotype being the predominant (Bush and Bradford, 2020; Castanheira et al., 2021; Bush, 2023; Cho et al., 2023). The ESBL-encoding genes are usually carried by mobile genetic elements *viz.* plasmids, insertion sequences, integrons, integrative conjugative elements, mobile integrative conjugative elements, transposons and prophages, which have facilitated their wide dissemination to other bacterial species through transformation, conjugation, and transduction (Partridge et al., 2018; Castanheira et al., 2021; Zaatout et al., 2021).

Wastewaters from municipal corporations, hospitals, pharmaceutical companies, animal husbandry, and poultry farms contain a diverse array of antibiotic residues, antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs) (Leiva et al., 2021; Uluseker et al., 2021; Larsson and Flach, 2022; Gitter et al., 2023). The combination of all these factors in aquatic environments allows rapid genetic exchange of ARGs from pathogenic to non-pathogenic bacterial strains leading to their genetic evolution and subsequent dissemination to human communities and animals (Liguori et al., 2022; Matthiessen et al., 2022; Muntean et al., 2022; Mutuku et al., 2022). Overuse and misuse of antibiotics have resulted in the widespread occurrence of ARB in surface water of rivers, irrigation-wastewater, soil, meat products, vegetables, etc., which possess the risk of dissemination in vulnerable human populations such as children, elderly, and immunocompromised persons (Uluseker et al., 2021; Zaatout et al., 2021; Gitter et al., 2023). The infections caused by multidrug-resistant bacteria can cause high morbidity and mortality, higher treatment costs and longer hospitalization duration. Over the years, the use of treated wastewater for agricultural purposes have gained widespread acceptance as an alternative irrigation method to relieve pressure on freshwater resources, to increase agricultural production, and to reduce the need for chemical fertilizers (Leiva et al., 2021; Minhas et al., 2022). However, this practice has led to continuous buildup of antibiotic residues in agro-ecosystems. ARB enter the aquatic environments from human and animal waste, fecal matter and hospital discharge where these are able to proliferate due to the availability of the nutrients, antibiotic residues, and inappropriate treatment and disinfection practices. Transmission of ARB from wastewaters into agro-ecosystems represents a serious ecological and public health concern and requires immediate interventions (Bhagat et al., 2020; Helmecke et al., 2020; Tucker et al., 2022). The occurrence of ARGs *viz. bla* ($bla_{CTX\text{-}M}$, bla_{TEM}), *tet* (*tet*O, *tet*Q, *tet*W), *sul* (*sul*1, *sul*2), and *ermB* has been reported from ARB present in the riverine systems, municipal wastewater treatment plants, pharmaceutical industries effluents, and irrigated soils (Karkman et al., 2018; Grenni, 2022; Cho et al., 2023; Shin et al., 2023). Therefore, there is an urgent need for environmental monitoring of ARB and ARGs in aquatic environments for limiting the transmission of antibiotic resistance.

The prevalence and dissemination of ARB carrying ESBLs genes has been reported from diverse agro-ecosystems worldwide. ESBLs genotypes $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}14}$ were found in *E. coli*, *K. pneumoniae*, *E. hormaechei*, and *C. freundii* from Tunisian farm irrigation water samples (Ben Said et al., 2015). Similarly, agricultural-purpose irrigation water also found to harbor ESBL-positive *E. coli* strain exhibiting $bla_{CTX\text{-}M\text{-}55}$, $bla_{CTX\text{-}M\text{-}65}$ and $bla_{CTX\text{-}M\text{-}15}$ genotypes (Montero et al., 2021). The predominant prevalence of $bla_{CTX\text{-}M,}$ bla_{CMY} and bla_{SHV} in water samples was also reported from Nepal and Canada (Subramanya et al., 2021; Anderson et al., 2023). India being the largest consumer of antibiotics and other antimicrobials, witnessed a significant increase in the prevalence of ARB and ESBL-positive *Enterobacterales* in recent years (Farooqui et al., 2018; Jani et al., 2021; Fazaludeen Koya et al., 2022).

Escherichia coli and other Gram-negative bacteria resistant to β-lactams, flouroquinolones, tetracyclines and other classes of antibiotics have been reported from wastewaters, river waters and wastewater treatment plants from different states of India along with co-prevalence of ESBL genotypes and other Class A β-lactamases including $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-152}}$, $bla_{\text{CTX-M-205}}$, bla_{SHV} and bla_{TEM} (Bajaj et al., 2015; Hanna et al., 2020). Environmental monitoring revealed the presence of bacteria resistant to multiple antibiotics as well as $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV} and other β-lactamase genotypes in water of river Ganga, Gomti, Yamuna and Hindon (Chaturvedi et al., 2020, 2021). The scientific data on the prevalence of antibiotic-resistant Gram-negative bacteria from irrigation-purpose wastewaters are either limited or lacking from Himachal Pradesh. Therefore, this study was aimed to determine the antibacterial susceptibility profiles of Gram-negative bacteria against β-lactams and other classes of antibiotics and the occurrence of MDR and ESBL phenotypes in these isolates from irrigation-purpose wastewaters utilized in different agro-ecosystems of lower Himalayan regions within Himachal Pradesh from Northern India.

2. Materials and methods

2.1. Study area

The present study was carried out from January, 2022 to July, 2022 in Sirmaur and Solan districts located in the outer Himalayas (Shivalik range) of Himachal Pradesh, India (Figure 1).

2.2. Wastewater sample collection

The irrigation-purpose wastewater samples (50 mL) in triplicate were collected (from January, 2022 to July, 2022) in sterile polystyrene screw-capped test tubes from each sampling site as mentioned in Figure 1 and Table 1, kept in ice and immediately transported to the laboratory for bacteriological analysis under aseptic conditions. All necessary safety guidelines and precautions were followed during the sample collection, transportation, analysis and disposal steps.

2.3. Isolation and characterization of gram-negative bacteria

Gram-negative bacteria were isolated from wastewater samples on Eosin Methylene Blue agar (HiMedia, India). About 100 µL of wastewater sample was spread on selective agar plates in triplicate and incubated at 35–37°C for 24 h as per the methods described earlier (Chaturvedi et al., 2021; Sivaraman et al., 2021). Morphologically distinct colonies based on appearance, color size, margins, texture, etc. were picked and streaked on selective agar plates for bacterial identification using Gram staining kit (HiMedia, India) and biochemical tests kits (HiMedia, India) according to Bergey's Manual of Systematic Bacteriology/Determinative Bacteriology, and manufacturer's instructions.

2.4. Antibiotic susceptibility testing

Bacterial isolates were evaluated for antibiotic susceptibility profile by Kirby-Bauer disk diffusion method according to standard published methods and Clinical Laboratory Standards Institute (CLSI) guidelines (Drieux et al. 2008; CLSI 2018, 2020). *Escherichia coli* ATCC 25922 (HiMedia, India) was used as a control. The antibiotics and antibiotics/inhibitor combination were obtained from HiMedia, Mumbai (India). Antimicrobial agents belonging to class penicillins, cephalosporins, aminoglycosides, folate pathway antagonists, and β-lactam/inhibitor combinations were tested. The following antibiotics were evaluated: ampicillin (AMP; 10 µg), amoxyclav (AMC; 30 µg), cefotaxime (CTX; 30 µg), co-trimoxazole (COT; 25 µg), gentamicin (GEN; 10 µg), tobramycin (TOB; 10 µg), cefpodoxime (CPD; 10 µg), ceftazidime (CAZ; 30 µg), cefpodoxime/clavulanic acid (CCL; 10/5 µg), ceftazidime/clavulanic acid (CAC; 30/10 µg) and cefotaxime/clavulanic acid (CEC; 30/10 µg). Bacterial isolates were cultured in Mueller-Hinton broth at 35–37°C and the turbidity was adjusted using 0.5 McFarland standard prior to swabbing on Mueller-Hinton agar plates. The antibiotic hexadiscs were placed on agar surface aseptically, followed by incubation at 35–37°C for 16–18 h. The diameter of the zones of inhibition around the disks was recorded to the nearest mm using Antibiotic Zonescale (HiMedia, India). The zones of inhibition diameters were compared with the Performance Standards for Antimicrobial Testing, 2020, Table 2A of M100 document as published by CLSI in order to classify the bacterial isolates either resistant, intermediate, or susceptible to test antibiotics (Drieux et al., 2008); Tan et al., 2023). Bacterial isolate exhibiting resistance against at least three different antibiotics classes was categorized as multidrug-resistant (Magiorakos et al., 2012). The Multiple Antibiotic Resistance (MAR) index was calculated according the formula (Krumperman, 1983) which is as follows: MAR index = number of antibiotics to which an isolate showed resistance/total number of antibiotics evaluated for susceptibility teesting.

2.5. Phenotypic detection of extended-spectrum β-lactamases

Bacterial isolates were tested for ESBLs production by phenotypic confirmatory disk diffusion test (PCDDT) using ESBLs identification kit (HiMedia, India) as per the manufacturer's instructions. Bacterial isolates were cultured in Mueller-Hinton broth at 35–37°C and the turbidity was adjusted using 0.5 McFarland standard prior to swabbing on Mueller-Hinton agar plates. The disks containing cefotaxime (30 µg) and cefotaxime/clavulanic acid (30/10 µg) were aseptically placed on agar plate containing test bacterial culture at least 24 mm apart and followed by incubation at 35–37°C for 16–18 h. The diameter of the zones of inhibition around the disks was recorded to the nearest mm using Antibiotic Zonescale (HiMedia, India). An increase in the zone of inhibition by ≥5 mm with cefotaxime/clavulanic acid disk was considered ESBL positive result according to the CLSI criteria.

2.6. 16S rRNA gene sequencing

Selected bacterial isolates were subjected to 16S rRNA gene sequencing from Chromus Biotech Pvt. Ltd., India using primer set 27F 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R: 5′-GGTTACCTTGTTACGACTT-3′ (Palkova et al., 2021). PCR products were sequenced using BigDye terminator cycle sequencing kit (V3.1) in an ABI Prism 3,730 Genetic Analyzer (Applied Biosystems,

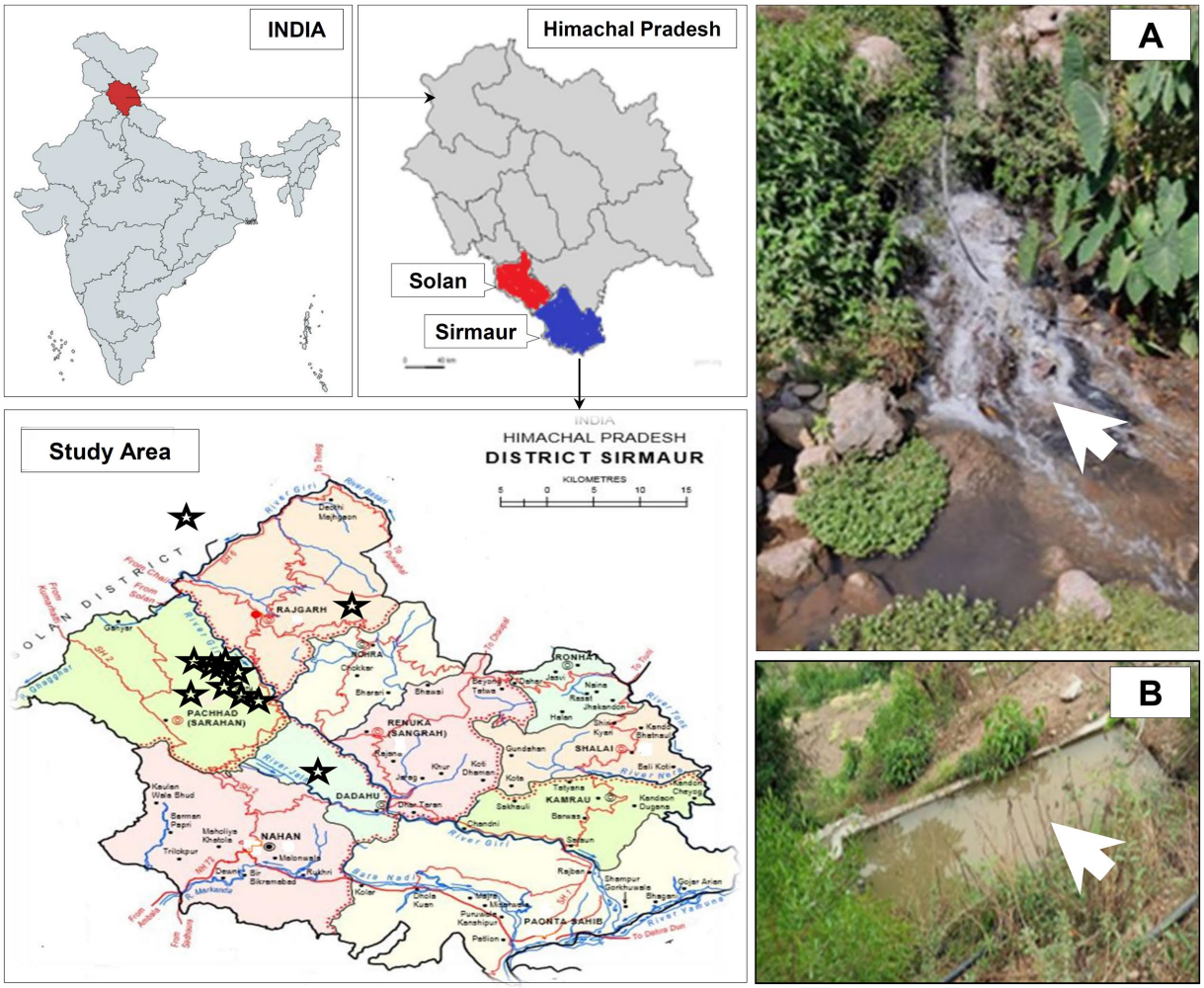


FIGURE 1
Geographical location of the study area and sampling sites in Sirmaur and Solan districts of Himachal Pradesh, India. The sampling sites are indicated by star symbols. The maps were prepared using https://www.mapchart.net/india.html and https://gadm.org/maps/. Panel **(A, B)** represents two of the sampling sites from where the wastewater samples were collected during the study.

TABLE 1 List of sampling sites, geographical locations and bacteria isolated from irrigation-purpose wastewaters from lower Himalayan agro-ecosystems located in Himachal Pradesh, India.

S.No.	Sampling site	Tehsil and District	Sample Source	Gram's reaction	Isolate's Code
1.	Baru Sahib	Pachhad, Sirmaur	Irrigation-wastewater	Gram-negative	AUK-01 AUK-02
2.	Kakli	Pachhad, Sirmaur	Irrigation-wastewater	Gram-negative	AUK-03
3.	Lana Machher	Pachhad, Sirmaur	Irrigation-wastewater	Gram-negative	AUK-04
4.	Kheri	Pachhad, Sirmaur	Irrigation-wastewater	Gram-negative	AUK-05
5.	Solan City	Solan, Solan	Irrigation-wastewater	Gram-negative	AUK-06
6.	Dadahu	Dadahu, Sirmaur	Irrigation-wastewater	Gram-negative	AUK-07
7.	Rajgarh Town	Rajgarh, Sirmaur	Irrigation-wastewater	Gram-negative	AUK-08
8.	Soda Dhayari	Pachhad, Sirmaur	Irrigation-wastewater	Gram-negative	AUK-09
9.	Bagroti	Pachhad, Sirmaur	Irrigation-wastewater	Gram-negative	AUK-10
10.	Lana Bhalta	Pachhad, Sirmaur	Irrigation-wastewater	Gram-negative	AUK-11
11.	Neri Nawan	Pachhad, Sirmaur	Irrigation-wastewater	Gram-negative	AUK-12

TABLE 2 Antibiotic susceptibility testing and resistance phenotypes of Gram-negative bacteria isolated from irrigation-purpose wastewaters from lower Himalayan agro-ecosystems located in Himachal Pradesh, India.

S. No.	Isolate	Genera/species	Antibiotic resistance phenotypes			ESBL phenotype
			Resistant	Intermediate	Sensitive	
1.	AUK-01	*Shigella* sp.	AMP, AMC	CPD	CTX, CPD, GEN, TOB, CAZ	+
2.	AUK-02	*Escherichia coli*	AMP, AMC, CTX, CAZ	CPD	COT, GEN, TOB	+
3.	AUK-03	*Hafnia* sp.	AMP, CTX, CAZ	AMC	COT, GEN, TOB, CPD	–
4.	AUK-04	*Citrobacter* sp.	AMP, AMC	–	CTX, COT, GEN, TOB, CPD, CAZ	+
5.	AUK-05	*Enterobacter* sp.	AMP, AMC, CTX	–	TOB, COT, GEN, CPD, CAZ,	–
6.	AUK-06	*Enterobacter* sp.	AMP, AMC, CTX, COT, TOB	CAZ	GEN, CPD	+
7.	AUK-07	*Enterobacter* sp.	AMP, AMC	CTX	COT, GEN, TOB, CPD, CAZ	–
8.	AUK-08	*Enterobacter* sp.	AMP, AMC, CTX	–	COT, GEN, TOB, CPD, CAZ	+
9.	AUK-09	*E. coli*	AMP, AMC, CTX, CAZ	–	COT, GEN, TOB, CPD	+
10.	AUK-10	*E. coli*	AMP, AMC	–	CTX, COT, GEN, TOB, CPD, CAZ	–
11.	AUK-11	*Klebsiella* sp.	AMP, AMC	–	CTX, COT, GEN, TOB, CPD, CAZ	–
12.	AUK-12	*Enterobacter* sp.	AMP, AMC	–	CTX, COT, GEN, TOB, CPD, CAZ	–

(+) indicate ESBLs positive result, (–) indicates ESBL negative result.
ESBL, Extended-spectrum β-lactamase; AMP, Ampicillin; AMC, Amoxyclav; CTX, Cefotaxime; COT, Co-trimoxazole; GEN, Gentamicin; TOB, Tobramycin; CPD, Cefpodoxime; CAZ, Ceftazidime.

United States) The nucleotide sequences were subjected to a similarity search using the BLAST algorithm at National Center for Biotechnology Information (NCBI) GenBank Database[1] and the sequences showing the highest scores were retrieved for further analysis. The 16S rRNA gene sequences of isolates AUK-01, AUK-02, AUK-03 and AUK-04 were deposited in NCBI GenBank database under accession numbers ON968448, ON968449, ON968450, and ON968451, respectively.

2.7. Phylogenetic analysis

The 16S rRNA gene sequences were imported to MEGA X v10.2.5 software and phylogenetic trees were constructed on the aligned datasets using the neighbor-joining method (Saitou and Nei, 1987; Tamura et al., 2007). A multiple sequence alignment was performed using the CLUSTAL W program (Thompson et al., 1994) and the data converted to PHYLIP format. All positions containing gaps and missing data were eliminated from the data set (complete deletion option) and branches containing more than 50% gaps were also removed. One sequence from each group was selected as a representative operational taxonomic unit. The phylogenetic tree was constructed by taking the sequences of bacterial strains along with their ten closest type strain matches available in the NCBI database using neighbor joining method with 1,000 bootstrapped replications to estimate evolutionary distance between all pairs of sequences simultaneously.

2.8. Statistical analysis

Experiments were performed in triplicates and data were expressed as mean ± standard deviation. Results were analyzed using Microsoft Excel and Graphpad Prism (Dotmatics).

3. Results

Wastewaters used for irrigation purpose were collected from 10 different geographical locations in Sirmaur district and one sampling site from Solan district of Himachal Pradesh, India (Table 1). The bacteria growing on selective medium were characterized on the basis of colony features, Gram reaction and biochemical parameters (Supplementary File 1). A total of 12 Gram-negative bacterial isolates were isolated from Baru Sahib (2), Kakli (1), Lana Machher (1), Kheri (1), Solan city (1), Dadahu (1), Rajgarh town (1), Soda Dhayari (1), Bagroti (1), Lana Bhalta (1) and Neri Nawan (1) as shown in Table 1. These isolates were subjected to antibiotic susceptibility testing and detection of ESBL phenotype. The characterization details of the bacterial isolates are provided in Supplementary File 1.

The antibiotic susceptibility testing data are shown in Table 2 and as heatmap in Figure 2. Isolate AUK-06 showed resistant phenotype against five antibiotics *viz.* ampicillin, amoxicillin/clavulanic acid, cefotaxime, co-trimoxozole and tobramycin (Figure 3). It was

1 http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn

susceptible to other antibiotics except ceftazidime where it exhibited intermediate phenotype. AUK-02, AUK-03 and AUK-09 were resistant to four antimicrobials (Table 2 and Figure 2). On the other hand, isolates AUK-04, AUK-10, AUK-11 and AUK-12 were susceptible to all the tested antimicrobials except ampicillin and amoxicillin/clavulanic acid combination. AUK-06 was the only isolate that displayed MDR phenotype as it exhibited resistance to at least three classes of antibiotics *viz.*, β-lactams (ampicillin and amoxyclav), aminoglycosides (tobramycin), and cephalosporins (cefotaxime and ceftazidime). As shown in Figure 2, all the bacterial isolates (n = 12) were resistant to ampicillin. In contrast, gentamicin exhibited antimicrobial action against all the isolates, as none were resistant to this antibiotic. In terms of susceptibility toward each of the tested antibiotic, all of the bacterial isolates were resistant to ampicillin whereas 11 were resistant to amoxyclav and six to cefotaxime. Other tested antibiotics were also able to inhibit the bacteria growth in disk diffusion assays as depicted in Figure 2 and Table 2.

Phenotypic determination assay for ESBLs production revealed that six bacterial isolates namely AUK-01, AUK-02, AUK-04, AUK-06, AUK-08 and AUK-09 were ESBL-positive which represents half of the total isolates (Table 2). Among these, two isolates were *Enterobacter* spp., two were *E. coli* and one each was related to *Shigella* sp. and *Citrobacter* sp. Isolate AUK-06 (*Enterobacter* sp.) which was prevalent in wastewater sample of Solan city had exhibited both MDR and ESBL phenotypes. MAR index values for bacterial isolates ranged from 0.20 ± 0.07 to 0.75 (Figure 4A). The highest MAR index was exhibited by isolate AUK-06 (*Enterobacter* sp.) followed by isolate AUK-02 (0.58 ± 0.07) and AUK-03 (0.45 ± 0.07). The lowest MAR index was associated with isolates AUK-10, AUK-11 and AUK-12 which correlates with their low antibiotic resistance. In terms of percentage of bacterial isolates having a MAR index value of >0.25 was 66.7% whereas only 33.3% isolates had MAR index ≤0.25 (Figure 4B). These data indicated that the bacterial isolates originated from high antibiotic contamination aquatic environments.

Morphological, biochemical, and 16S rRNA gene sequence-based molecular characterization of Gram-negative bacterial isolates showed the dominant prevalence of *Enterobacter* sp. (41.3%) and *E. coli* (25%) as depicted in Figure 5. On the other hand, prevalence of *Klebsiella* sp., *Citrobacter koseri*, *Hafnia paralvei,* and *Shigella* sp. was low, i.e., 8.33% in wastewater samples. Four bacterial isolates, AUK-01, AUK-02, AUK-03, and AUK-04, were characterized by 16S rRNA gene sequencing and were phylogenetically related to *Shigella* sp., *Escherichia coli*, *Hafnia paralvei,* and *Citrobacter koseri*, respectively (Figure 6). The 16S rRNA gene sequences of these isolates were deposited in the NCBI GenBank database with the following accession numbers: ON968448, ON968449, ON968450, and ON968451. Further, the phylogenetic trees were constructed to assess the evolutionary

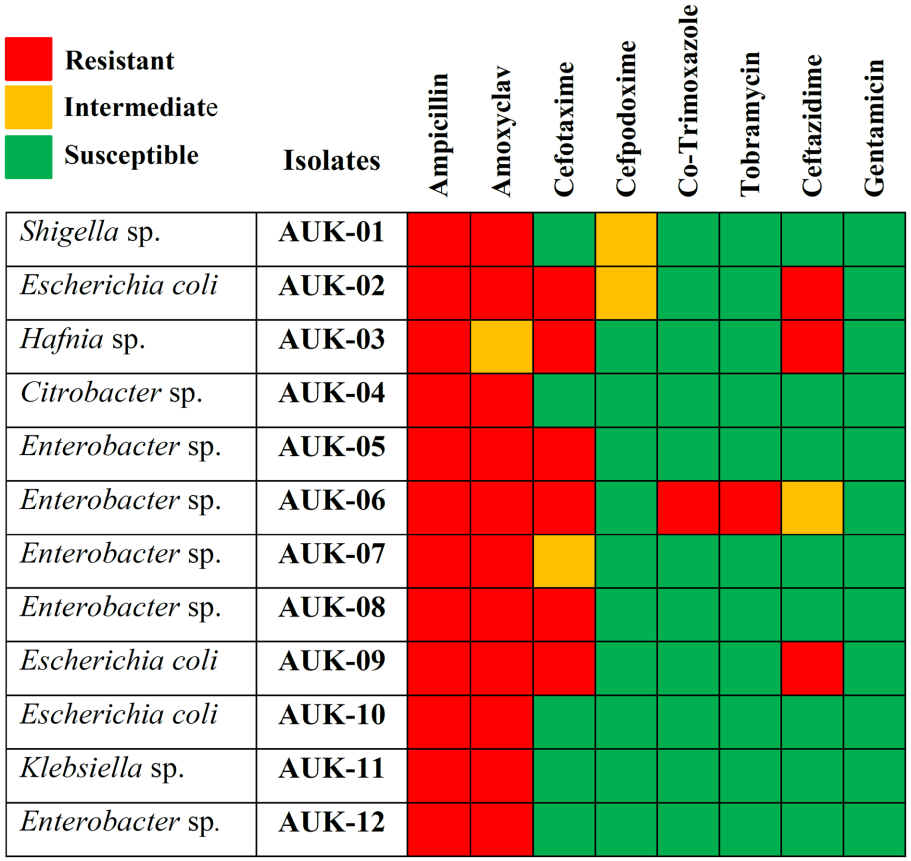
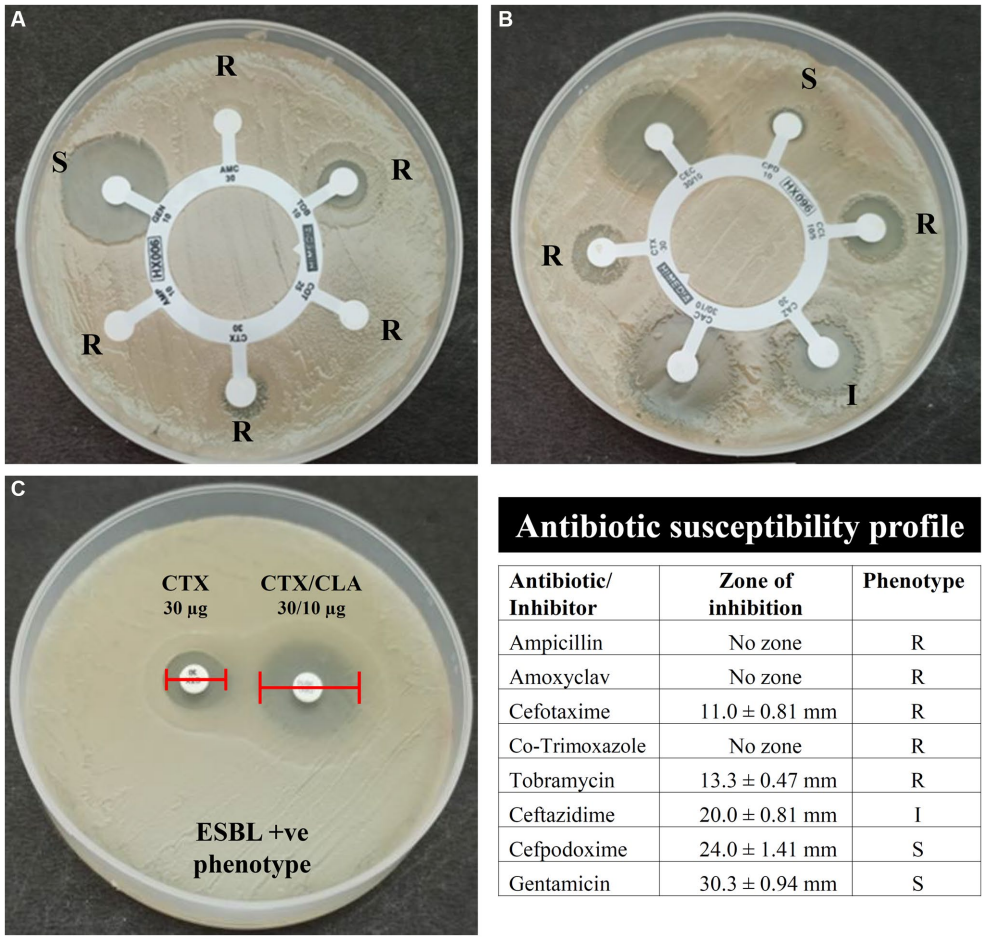


FIGURE 2
Antibiotic susceptibility heatmap of Gram-negative bacterial isolates as determined by Kirby-Bauer disk diffusion method and interpreted according to CLSI breakpoints. Rows represent bacterial isolates and column represents antimicrobials tested. Red-colored blocks indicate resistance; green blocks indicate susceptible and orange blocks represent intermediate action of the antimicrobial agents.



Antibiotic susceptibility profile

Antibiotic/ Inhibitor	Zone of inhibition	Phenotype
Ampicillin	No zone	R
Amoxyclav	No zone	R
Cefotaxime	11.0 ± 0.81 mm	R
Co-Trimoxazole	No zone	R
Tobramycin	13.3 ± 0.47 mm	R
Ceftazidime	20.0 ± 0.81 mm	I
Cefpodoxime	24.0 ± 1.41 mm	S
Gentamicin	30.3 ± 0.94 mm	S

FIGURE 3
Antibiotic susceptibility and ESBL phenotype of bacterial isolates AUK-06 (*Enterobacter* sp.) as determined by the Kirby-Bauer disk diffusion method. Here, **(A)** indicates susceptibility of isolate AUK-06 toward ampicillin (AMP), amoxyclav (AMC), cefotaxime (CTX), co-trimoxazole (COT), gentamicin (GEN), and tobramycin (TOB); panel **(B)** represent cefpodoxime (CPD), cefpodoxime/CLA (CCL), ceftazidime (CAZ), ceftazidime/CLA (CAC), cefotaxime (CTX), and cefotaxime/CLA (CEC); Phenotypic detection of ESBL production by PCDDT is depicted in **(C)**. The antibiotic susceptibility profile of isolate AUK-06 is shown in tabulated form according to the interpretation criteria mentioned in M100 document, CLSI.

relatedness among the bacterial isolates and their nearest neighbors available in the database (Figure 6).

4. Discussion

Wastewater contains high nutrient contents and hence found re-usability in agricultural irrigation systems since it provides organic carbon, nutrients (nitrogen, phosphorus, potassium), and inorganic micronutrients to the crop plants (Alcalde-Sanz and Gawlik, 2017; Minhas et al., 2022). The untreated wastewaters are frequently utilized for irrigation purposes in regions where rain shortfall are common or groundwater availability is limited which also include several states of India, including Himachal Pradesh. Their use in agricultural practices, however represent a serious health risk due to its inherent nature of carrying ARB and ARGs (Wang et al., 2020; Tucker et al., 2022). The antibiotic contents present in wastewaters facilitate mutation and genetic modifications in ARB thus making these multi-drug resistant and difficult to treat under clinical settings (Leiva et al., 2021; Uluseker et al., 2021; Mutuku et al., 2022). In the present study, 12 bacterial isolates belonging to six genera were identified in irrigation-purpose wastewater samples on the basis of morphological, biochemical, and molecular methods from 11 different geographical locations in Sirmaur and Solan districts of Himachal Pradesh. These bacteria belonged to *E. coli*, *Enterobacter* sp., *Hafnia* sp., *Shigella* sp., *Citrobacter* sp., and *Klebsiella* sp. *Enterobacter* spp. and *E. coli* were found to be most dominant among 12 bacterial isolates, respectively. The antibiograms of these isolates against penicillins (ampicillin and amoxyclav), aminoglycosides (gentamicin and tobramycin), sulfonamides (co-trimoxazole), and third-generation cephalosporins (cefotaxime, cefpodoxime, and ceftazidime) indicated resistance against all antibiotics except gentamicin. All isolates were resistant to ampicillin. In contrast, gentamicin exhibited antimicrobial action against all the isolates as all were susceptible to this antibiotic. Further, 11 isolates were resistant to amoxyclav whereas six of the isolates displayed resistance to cefotaxime. These findings are supported by previous research reports in which widespread resistance against similar antibiotic classes was reported in bacteria from *Enterobacteriaceae*. A high frequency of resistance to ciprofloxacin, tetracycline, cefoxitin, amoxicillin/clavulanic acid, cefotaxime, and aztreonam was reported

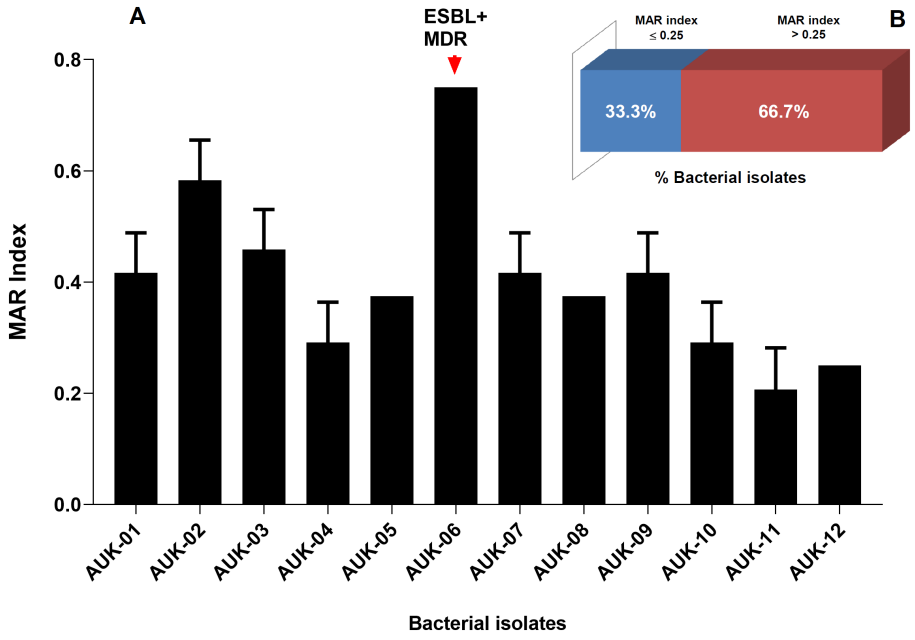


FIGURE 4
Multiple Antibiotic Resistance (MAR) index of bacteria isolated from irrigation-purpose wastewater samples from lower Himalayan agro-ecosystems located in Himachal Pradesh, India. MAR index was calculated according to the formula described by Krumperman (1983). Here, **(A)** represent the MAR indices of bacterial isolates and **(B)** represent the percentage of bacterial isolates having a MAR index ≤0.25 or > 0.25. Data represents mean ± SD of three independent experiments.

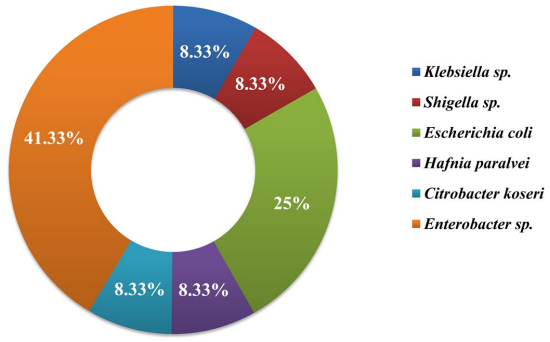


FIGURE 5
Genera-wise distribution (%) of bacteria ($n = 12$) isolated from irrigation-purpose wastewaters from lower Himalayan agro-ecosystems located in Himachal Pradesh, India.

from water samples of the Mondego River in Portugal (Amador et al., 2015). Schmiege et al. (2021); Zagui et al. (2020); Veloo et al. (2022) also observed MDR and ESBL-producing ARB in urban wastewaters. MAR index values for 12 isolates ranged between 0.21–0.75. Ali et al. (2021) found the MAR index value to range between 0.2 and 0.32 in wastewaters from Delhi-NCR region. The MAR index value >0.2 suggest high pollution load and antibiotic exposure in a specific sampling site (Krumperman, 1983; Ali et al., 2021).

Five isolates (AUK-05, AUK-06, AUK-07, AUK-08, and AUK-12) were identified to be *Enterobacter* spp. which showed their predominant prevalence in irrigation-purpose wastewater samples of the sampling sites. AUK-6 which was obtained from wastewater sample of Solan City was the sole MDR isolate of our study and it also showed ESBL phenotype. Similar to our findings, Ben Said et al. (2015) found the presence of the multidrug-resistant ESBL-producing *Enterobacter hormaechei* in irrigation water samples from Tunisia. In this study, 50% of bacterial isolates showed ESBL phenotypes, as confirmed by cefotaxime and cefotaxime/clavulanic acid combination-based qualitative methods. Among these isolates, AUK-01, AUK-02, AUK-04, AUK-06, AUK-08, and AUK-09 were ESBL producers. AUK-02 and AUK-09 were identified as *E. coli*, whereas AUK-06 and AUK-08 were related to *Enterobacter* spp. Further, *Shigella* sp. (AUK-01) and *Citrobacter* sp. (AUK-04) also displayed the ESBL phenotype. Ali et al. (2021) found 106 ESBL-positive isolates (24.3%) out of 436 bacterial isolates isolated from Hauz Khas lake, Ghazipur slaughterhouse, Jasola wastewater treatment plant, and Lodhi garden pond in the Delhi-NCR region. Among these isolates, 42–78% also exhibited MDR phenotypes, depending upon the location. Similarly, Singh et al. (2021) found ESBL-producing strains of *E. coli* ($n = 34$) and *K. pneumoniae* ($n = 39$) with a prevalence of $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, and $bla_{CTX\text{-}M\text{-}15}$ genotypes from Manali, Kullu, and Baddi in Himachal Pradesh. In previous studies, antibiotic-resistant strains of *E. coli, Enterobacter, Klebsiella, Pseudomonas, Citrobacter, Hafnia, Acinetobacter, Shigella,* and *Aeromonas* have been reported from different regions of India (Lamba and Ahammad, 2017; Chaturvedi et al., 2020, 2021). The prevalence of the ESBL phenotype in 58% of *E. coli* isolates in irrigation water samples from several provinces of Ecuador was observed (Montero et al., 2021). Similarly, 33% Gram-negative bacterial isolates exhibited MDR phenotypes and ESBL genotypes (bla_{KPC}, bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$) from hospital sewage and urban wastewater in Brazil (Zagui et al., 2020). Igbinosa et al. (2023) reported that 85.9% of *E. coli* strains isolated from agricultural farms (irrigation water, soil, manure) were resistant to various antimicrobial classes and exhibited MDR phenotype as well ESBL

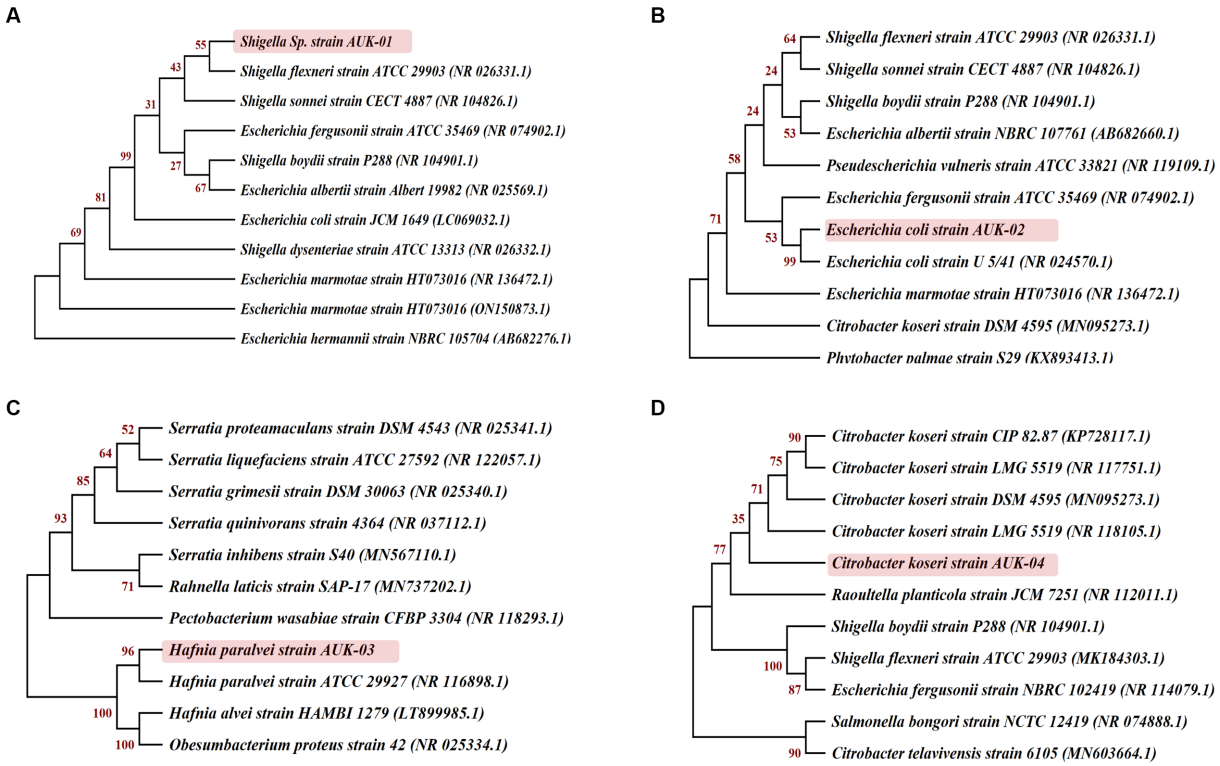


FIGURE 6
Phylogenetic relationships of selected bacterial isolates obtained from irrigation-purpose wastewater samples were inferred on the basis of 16S rRNA gene sequences by the neighbor-joining method using MEGA X software (version 10.2.5). Bootstrap values expressed as a percentage of 1,000 re-samplings are shown at the nodes. Here **(A)**, **(B)**, **(C)** and **(D)** represent the isolates AUK-01, AUK-02, AUK-03, and AUK-04, respectively.

genotypes which might be due to the use of untreated water for irrigation purposes. Tiwari et al. (2023) showed the presence of β-lactamase (bla_{GES}, bla_{MOX} and bla_{TEM}) producing *Enterobacter* spp., *Aeromonas* spp., and *Klebsiella* sp. and *E. coli* in untreated municipal wastewater from Helsinki, Finland.

There are two ways that antibiotics can infiltrate agricultural ecosystems: firstly, by fertilizing with animal manures, biosolids, sewage sludge, and sediments that include antibiotics, and secondly, by irrigating with reclaimed water that has been contaminated with antibiotics from sewage treatment plants, wastewater, surface water, or groundwater since these sources are regularly contaminated with antibiotics (Du and Liu, 2012; Leiva et al., 2021; Minhas et al., 2022). In aquatic ecosystems, pathogenic, commensal, and environmental bacteria serve as reservoirs of antibiotic-resistance genes, mobile genetic elements, and bacteriophages, with consequent dissemination through conjugation, transformation, and transduction (von Wintersdorff et al., 2016). Utilization of wastewater for agricultural irrigation may assist in mobilizing clinically relevant antibiotic resistance genes (resistome) from non-pathogenic commensal bacteria to pathogenic strains, thus leading to the spread of resistance. The origin of bla_{CTX-M} genes in *Enterobacteriaceae* can be traced to the environmental *Kluyvera* sp. via HGT (Canton and Coque, 2006). Therefore, efficient treatment technologies are essentially required for the removal of microbial pathogens, antibiotic residues and heavy metal hazards from wastewaters (Tucker et al., 2022). Regular monitoring and surveillance of antibiotic residues and bacteria resistant to antibiotics in wastewaters, rivers, ponds and wastewater treatment plants, and effluent treatment sites should be prioritized and included in regulatory action plans.

5. Conclusion

Wastewaters are being used in agricultural irrigation in several water-scarce countries due to their organic and inorganic nutrients, however this wastewater-based irrigation practices also present serious public health and environmental sustainability concerns. Our findings also confirmed the prevalence of antibiotic resistant Gram-negative bacteria resistant to multiple antibiotics and exhibiting the ESBL phenotype in irrigation-purpose wastewater samples from Himachal Pradesh. Therefore, there is an urgent need for an integrated approach focusing on environmental monitoring, identifying key hotspots of antibiotic resistance, early and rapid detection, continuous surveillance, stringent regulatory guidelines and improved agri-environmental risk assessment models before their use in the agro-ecosystems for ensuring public health and environmental safety.

Data availability statement

The datasets presented in this study are deposited in the NCBI repository, accession numbers ON968448, ON968449, ON968450, ON968451: https://www.ncbi.nlm.nih.gov/nuccore/ON968448, https://www.ncbi.nlm.nih.gov/nuccore/ON968449, https://www.ncbi.nlm.nih.

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Author contributions

AA, ShiS, and SK: conducted the experiments, and manuscript preparation. YA, SheS, DJ, PC, KP, MK, and NS: editing, review, and finalization of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Effects of compost as a soil amendment on bacterial community diversity in saline–alkali soil

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Introduction: Soil salinization poses a worldwide challenge that hampers agricultural productivity.

Methods: Employing high-throughput sequencing technology, we conducted an investigation to examine the impact of compost on the diversity of bacterial communities in saline soils. Our study focused on exploring the diversity of bacterial communities in the inter-root soil of plants following composting and the subsequent addition of compost to saline soils.

Results: Compared to the initial composting stage, Alpha diversity results showed a greater diversity of bacteria during the rot stage. The germination index reaches 90% and the compost reaches maturity. The main bacterial genera in compost maturation stage are *Flavobacterium*, *Saccharomonospora*, *Luteimonas* and *Streptomyces*. Proteobacteria, Firmicutes, and Actinobacteria were the dominant phyla in the soil after the addition of compost. The application of compost has increased the abundance of Actinobacteria and Chloroflexi by 7.6 and 6.6%, respectively, but decreased the abundance of Firmicutes from 25.12 to 18.77%. Redundancy analysis revealed that soil factors pH, solid urease, organic matter, and total nitrogen were closely related to bacterial communities.

Discussion: The addition of compost effectively reduced soil pH and increased soil enzyme activity and organic matter content. An analysis of this study provides theoretical support for compost's use as a saline soil amendment.

KEYWORDS

composting, saline-alkali stress, high-throughput sequencing, soil bacterial community, physical and chemical properties, soil amendment

Introduction

Saline land refers to soil with high levels of salinity and alkalinity, which are harmful to plant growth and agricultural production (Yin et al., 2022). Soil saline–alkalinization is a global problem, especially in coastal and semiarid areas. Approximately 1 billion hectares of salinized soil cover the world's arable land, which is increasing at a rate of 2% a year (He et al., 2020; Cui et al., 2021). The main characteristic of saline land is the high level of salinity and alkalinity in the soil, which severely affects plant growth. When salts accumulate to a certain level in the soil, they can poison the roots of plants and affect their absorption of nutrients and water, eventually

leading to slow growth or even death (Ali et al., 2017). Saline soils may also decrease soil quality, biodiversity, water content, and oxygen content, thereby affecting agricultural production and the ecological environment (Setia et al., 2013). Three key attributes of saline soils that impede plant growth and diminish agricultural productivity encompass elevated salinity levels, deterioration of soil structure, and nutrient depletion (Liu et al., 2021). Hence, enhancing the condition of saline land is deemed crucial not only for safeguarding the ecological environment and agricultural productivity, but also as an imperative approach to augment agricultural efficacy and enhance the quality of human life. Solving the problem of soil salinization is also significant for protecting the ecological environment, promoting agricultural development, and improving human quality of life (Chen et al., 2021; Yang et al., 2022).

Composting is an effective biological method to improve soil texture and crop yields (Shen et al., 2019). Waste is converted into humus material using microorganisms, which can be transformed into fertilizer and soil conditioner through organic waste treatment and compost production (Qayyum et al., 2017). Using green organic amendments (compost products, such as plant and animal fertilizers, sludge, and food and agricultural wastes) to repair soils can improve their fertility. These soil amendments significantly improve the biological properties and soil organic matter (SOM) of saline–alkali soil (Chavez-Garcia and Siebe, 2019). Research has demonstrated that the application of biochar compost to saline soils can enhance various physicochemical attributes, including soil organic matter (SOM) and nutrient content, consequently fostering plant growth and augmenting crop yields (Zheng et al., 2018; Liang et al., 2021). Aiad et al. (2021) found that restoration of saline soils using soil amendments (i.e., compost and zeolite) significantly reduced soil salinity and increased wheat and corn yields by 16.0 and 35.0%, respectively. A recent study has shown that by increasing the addition rate of cow manure compost, corn yields can be increased by 6.0–28.4% (Li et al., 2022). Nevertheless, there remains a need for further investigation to comprehensively elucidate the effects of compost amendment on the microbial community structure in saline-alkali soil, as well as to establish a clearer understanding of the intricate relationship between microbial communities and environmental factors.

As a key component of soil ecosystems, microorganisms are widely considered important indicators of soil quality (Joergensen and Wichern, 2018). Soil microorganisms play a crucial role in the processes of material and energy cycling, soil structure maintenance, and soil microecological balance within the soil. During composting, microorganisms facilitate the decomposition, transformation, and synthesis of organic matter (Wang S. P. et al., 2022). Composting microorganisms mainly include bacteria, fungi, protozoa, and viruses (Duan et al., 2022). These microorganisms are mainly obtained from the starting composting material and recruited from the composting environment (Wei et al., 2018). With the decomposition of organic materials at each stage, various biochemical indicators in the composting system keep changing, and different microorganisms alternately dominate, independently or in cooperation with other microorganisms, to decompose and transform organic materials and promote compost decomposition (Singh and Nain, 2014). Zhao et al. (2022) found that Oceanobacillus, Bacillus, Pseudogracilibacillus, and Nocardiopsis were mainly present in sheep manure compost; At different stages, bacterial communities differed in abundance. Additionally, Neher et al. (2013) discovered that composting microorganisms exhibit associations with various materials, methods, and stages employed in the composting process. Furthermore, it has been documented that microbial communities exhibit responses to various external environmental stimuli, including temperature fluctuations, compost composition, and carbon-to-nitrogen ratios (C/N; Chen et al., 2019). However, the predominant focus of previous research has been on the analysis of changes in microbial community dynamics during the composting process. There has been a limited number of investigations that have explored the effects of compost application with different materials on the microbial composition in saline soils, along with its correlation with environmental factors. The primary aim of this study was to investigate the influence of compost application as a soil amendment on the composition and functionality of microbial communities in saline soils.

The utilization of high-throughput sequencing (HTS) technology has proven to be an efficient method for detecting alterations within microbial communities. This technology is extensively employed in the examination of intricate microbial communities inhabiting soil across various habitats. Therefore, in this study, we performed composting and potting experiments and hypothesized that compost addition could improve saline soils by changing the soil microenvironment. The aim of this study was to examine the influence of different levels of saline-alkali soil on bacterial communities throughout the composting process, employing high-throughput sequencing. Moreover, the primary objective of this study was to evaluate the efficacy of compost application in augmenting plant growth and ameliorating soil quality. Additionally, the study aimed to investigate the impact of compost application on the composition of bacterial communities in saline soils, along with their correlation with environmental factors.

Materials and methods

Materials

Saline soils collected from Zhenlai County, Baicheng City, Jilin Province (122°47′–124°04′E, 45°28′–46°18′N), were prepared by Jiangsu Guoxin Xielian Energy Co. Using a 2-mm sieve, the saline soils were mixed evenly after air-drying. The experimental crop was radish (Fengguang Generation), and the experiment began in the laboratory in January 2022. Sheep manure and corn straw were purchased separately from the market. Saline soils had the following initial properties: total nitrogen (TN): 1.39 g kg^{-1}, total carbon: 7.02 g kg^{-1}, SOM: 2.84 g kg^{-1}, pH: 10.74, and electrical conductivity (EC): 1.36 dS m^{-1} (Table 1).

Aerobic composting procedure

The reactor for composting was a 75-cm × 55-cm × 60-cm polypropylene plastic box with a total reactor volume of 247.5 L. During composting, porous sieve plates were placed 10 cm from the bottom of the plastic box. Aeration was maintained throughout the composting process at 0.2 L/(L min). Three different proportions of saline soil were used in the composting process (MA: 0.8 kg, MB: 4 kg, and MC: 8 kg) mixed mechanically with straw and sheep manure. The total mass and volume of saline soil, sheep manure

TABLE 1 Basic properties of raw materials.

Composting materials	*w* (C)/%	*w* (N)/%	C/N	Moisture (%)	pH
Sheep dung	25.60 ± 0.86	2.07 ± 0.10	12.36 ± 0.36	10.43 ± 070	8.30 ± 0.48
Rice straw	41.37 ± 0.90	1.60 ± 0.05	25.91 ± 0.31	10.40 ± 0.59	6.8 ± 0.22
Saline-alkali soil	7.02 ± 0.22	1.39 ± 0.15	5.11 ± 0.61	6.49 ± 0.37	10.74 ± 0.04

Values are presented as mean ± standard deviation ($n = 3$).

and straw in each reactor were 32 kg and 240 L. We mixed sheep manure with corn straw in a ratio of 1:2 by dry weight, with a C/N of approximately 25. Composting was adjusted at 60% soil moisture content (SMC; Wu et al., 2017). The material was turned and mixed well at regular intervals (days 0, 2, and 5 and then weekly). Table 1 shows the properties of the raw materials. The composting process was divided into four stages based on temperature variations: (S) warming period (day 0), (G) high-temperature period (days 1–5), (J) cooling period (days 6–10), and (F) decaying period (days 11–30). A portion of the soil samples was stored at 4°C for physicochemical analysis, while the other portion was stored at −80°C to facilitate microbial analysis.

Pot experiments

Pot experiments were conducted using control (CK) and three treatments (MA, MB, and MC compost addition), with four replicates per treatment, obtaining a total of 16 pots. Each pot, measuring 19 cm in diameter and 17 cm in depth, was filled with 0.8 kg of substrate and subsequently planted with a total of 20 radishes. The substrate consisted of saline soil and compost (1,3). For CK, no compost was added to the saline soil; for AP, MA compost product was added; for BP, MB compost product was added; and for CP, MC compost product was added. The pots were irrigated by hand until they reached 60% of their water holding capacity. In addition, roots are collected from potted plants, soil is stripped, and ~1 mm of soil is left around the roots. Subsequently, ~1 mm of soil was washed away in the PBS buffer, and 4 composite samples were prepared from each group of treated soil. The collected soil samples were subsequently partitioned into two segments: one segment was preserved at a temperature of 4°C for physicochemical analysis, while the other segment was preserved at a temperature of −80°C for microbial analysis.

Physicochemical parameter analysis

The pH of all fresh soil samples was determined by air-drying, grinding, and passing them through a 2-mm sieve; temperature (T); EC; and SMC, TN, sodium (Na), solid catalase (SCAT), and SOM contents. A fully automatic Kjeldahl nitrogen tester (Foss, NKY6120, Germany) was used to determine the TN content of the soil. The pH and electrical conductivity (EC) were determined by employing a pH electrode (HM-25G, TOA DKK, Japan) and an EC indicator (SG3, Mettler Toledo, United States), respectively. The measurement of SOM content was conducted through the utilization of the muffle furnace scorch method, while the determination of soil potassium (K) and sodium (Na) contents was carried out using a flame photometer (FP640; Li et al., 2021). Soil enzyme activity was determined using a soil enzyme kit. In the conducted seed germination experiments, a water extract was utilized. Specifically, a total of 10 radish seeds were evenly distributed onto a filter paper and subsequently subjected to incubation in darkness at a temperature of 20°C for a duration of 48 h. Three replicates of each compost sample were individually analyzed, with each treatment being assessed through the quantification of germinated seeds and the measurement of root length. The germination index (GI) was calculated as follows:

$$\mathrm{GI}(\%) = \frac{\text{Seeds germinated in the extract}(\%) \times \text{Root length of treatment}}{\text{Seeds germinated in the extract}(\%) \times \cdot \text{Root length of control}} \times 100$$

Microbial community analysis

DNA extraction and detection

The Power Soil DNA extraction kit (MoBio Laboratories, Inc., Carlsbad, CA, United States) was employed to extract the complete genetic DNA from compost and potted soil samples. Compost and potting soil samples in triplicate and then combined. The quality and quantity of extracted DNA was assessed using the NanoDrop 1000 spectrophotometer (Thermal Sciences, Wilmington, DE, United States). The polymerase chain reaction (PCR) was conducted using TransGen AP221-02: TransStart Fastpfu DNA polymerase (20 μL) along with 0.8 μL (5 μM) of both forward and reverse primers, 10 ng of template, 4 μL of buffer (5×), 2 μL of deoxynucleoside triphosphates (2.5 mM), 0.4 μL of polymerase, and 0.2 μL of bovine serum albumin. After detection on 2% (w/v) agarose gel, the final amplicon was quantified using the AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, United States). Using the Yili MiSeq platform, the same amount of purified amplicon was collected at Alway Gene (Beijing, China) for follow-up sequencing. The region of the bacterial 16S rRNA gene, specifically 338F_806R, was amplified using primers 338F (5-ACTCCTACGGGAGGCAGCAG-3) and 806R (5-GGACTACHVGGGTWTCTAAT-3; Xu et al., 2020). The processes of PCR amplification, library preparation and detection, and computer sequencing analysis were conducted by Shanghai Meiji Biomedical Technology Co., Ltd. The Illumina sequences were deposited in the NCBI Sequence Read Archive (accession numbers SRR24001333–SRR24001347 for the rhizosphere soil bacteria of potted 16S rRNA gene data and SRR24031951–SRR24031993 for the compost rhizosphere soil 16S rRNA gene data).

Bioinformatics analysis

After performing sample splitting of PE reads acquired through Illumina sequencing, the data was optimized by subjecting it to quality control (QC) splicing. This involved initially conducting QC procedures and subsequently filtering the double-ended reads based on sequencing quality. Additionally, the splicing process was carried out by considering the overlapping relationship between the double-ended reads. The optimized data underwent processing utilizing sequence noise reduction techniques, such as DADA2/Deblur, in order to acquire representative sequences and abundance information for amplicon sequence variants (ASVs). Using ASV representative sequences and abundance information, various statistical and visual analyses can be conducted, encompassing species taxonomy, community diversity, species dissimilarity, correlation, phylogenetic, and functional prediction analyses.

Statistical analysis

For the multivariate statistical analysis (correlation analysis), SPSS version 19.0 was used. A significance threshold of 0.05 was used to determine statistical significance. Data were collected in four replicates and subjected to analysis of variance (ANOVA). Principal component analysis (PCA) was employed to evaluate variations in bacterial and fungal communities, as well as their transformations throughout the composting process. The application of redundancy analysis (RDA) unveiled the correlation between environmental factors and the manifold alterations in bacterial community composition (Chen et al., 2020). The R heat map package (version 3.3.1) was employed for the production of heat maps. The sample distance matrix was subjected to clustering and analysis, with the R language employed for constructing the dendrogram. Majorbio I-Sanger was used for all bioinformatics analyses.

Results and discussion

Evolution of physicochemical properties during composting

The temperature, pH, soil moisture content (SMC), and germination rates of the compost were assessed at various time points. It was observed that temperature played a crucial role in influencing the decomposition of organic matter and microbial activity throughout the composting process. Figure 1A illustrates that the temperature fluctuations were consistent across the three treatment groups, which underwent four distinct stages of composting: warming, high-temperature, cooling, and decaying periods (Ren et al., 2018). There was a significant difference in the rate of temperature change among the three treatments. The temperatures of MA and MB rose to a peak of around 55C within a span of 2 to 3 days, surpassing the rate of warming observed in MC. This observation implies that the rate at which organic constituents degrade increases as compost maturity increases, possibly due to the enhancement of microbial community diversity during the composting process (Kato and Miura, 2008). The higher the content of saline-alkali soil, the slower the temperature rise of compost. As the composting carbon source was consumed, the composting temperature decreased until a stabilization period was reached around day 12. The trend in pH changes was similar for all composting processes (Figure 1B). However, the higher the content of saline-alkali soil, the greater the pH change during composting. The pH started to increase significantly on day 2, possibly due to ammonia emission and organic acid depletion; this discovery aligns with the research conducted by Mei et al. (2021). On day 8, a decline in pH was observed as a result of microbial activity leading to the generation of small-molecule organic acids that rapidly decompose within the composted material. After day 18, the pH eventually leveled off as the compost reached the decomposition stage, forming large amounts of humus, followed by slight fluctuations.

SMC was also monitored at different times. MA had the highest average SMC of 69%, followed by MB with 62%, and the overall SMC reached >50% (Figure 1C). In the high-temperature stage, the microbial metabolism led to a large amount of water evaporation. MA, MB, and MC decreased by 22.15, 12.32, and 10.10%, respectively. These changes indicated that some microorganisms in different proportions of saline–alkali soil play an important role in fermentation, similar to a previous study (Guo et al., 2015). The maturity and phytotoxicity of the compost were assessed using the germination index (GI). Upon reaching a GI of 90%, the compost demonstrated the loss of phytotoxicity and achieved maturity (Figure 1D). In the early stages of composting, the glycemic index (GI) of each group exhibited a relatively low value, which could be potentially ascribed to the existence of NH3 and fatty acids generated through the swift breakdown of organic matter (Jiang et al., 2018).

Bacterial community profiles in composting systems

The modulation of composting process and product quality is significantly influenced by alterations in bacterial communities, as demonstrated by Wang X. H. et al. (2022). Figure 2A illustrates the noteworthy temporal dynamics observed in the genus-level bacterial community composition across different treatments throughout the composting progression. In the warming period of composting (S), *Cellvibrio*, *Pedobacter*, *Devosia*, *Planococcus*, *Planococcus*, and *Microbacterium* were the dominant genera. The findings of this study align with the outcomes of a prior investigation pertaining to the fluctuations in bacterial community dynamics throughout the composting process of distilled grain waste (Wang et al., 2022a). As composting proceeded into the high-temperature period, *Pedobacter*, *Pseudomonas*, and *Cellvibrio*, belonging to Bacteroidetes and Proteobacteria phyla, increased greatly and became the dominant genera. During the progression of composting into the thermophilic phase (MA-G, MB-G, and MC-G), there was a significant increase in the abundance of the Pedobacter genus, which emerged as the predominant genera, constituting 25.2, 22, and 17.56%, respectively, of the overall bacterial community (Figures 2A,C,E). In MA-G, MB-G, and MC-G, compared with the warming period of composting, Actinobacteriota abundance decreased significantly. During the cooling phase, there was a notable similarity in the bacterial community structure observed among

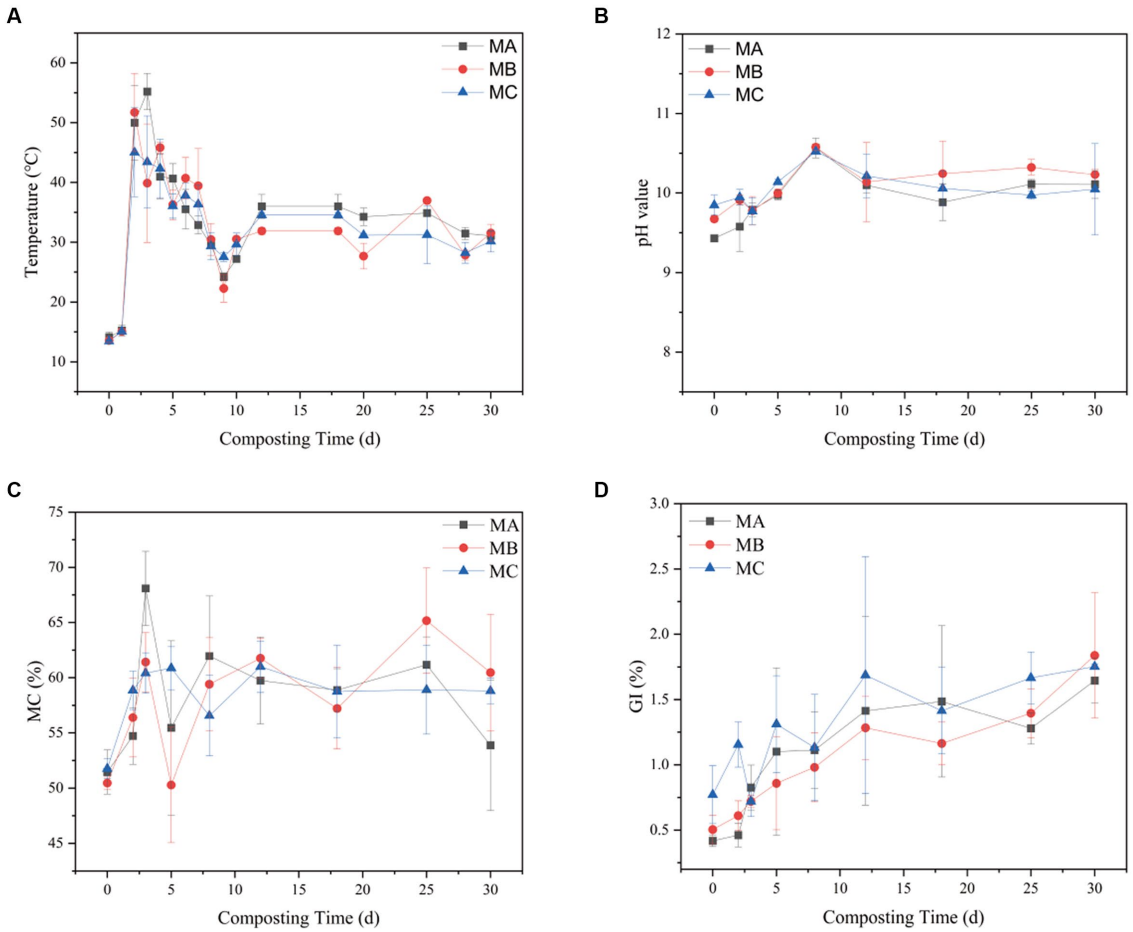

FIGURE 1
Changes in **(A)** temperature, **(B)** pH, **(C)** SMC, and **(D)** GI during composting in the three treatments.

MA, MB, and MC; however, the abundance was different. In comparison to the thermophilic phase, it was observed that the relative abundance of Pedobacter decreased from 25.2 to 8.32% in MA and from 22.06 to 6.86% in MB. Furthermore, there was a notable increase in the prevalence of Bacillus in the composts, with percentages rising from 2.79 to 8.44% in MA and from 2.70 to 8.06% in MB (Figures 2A,C). The findings of this study exhibited a resemblance to those of a prior investigation (Wang S. et al., 2020), indicating that microbial community succession is related to compost temperature. Proteobacteria, Bacteroidetes, and Actinobacteria are the predominant phyla of the three groups. Prior research has similarly documented that these bacteria exhibit the highest prevalence in saline-alkali soil (Wang S. et al., 2020; Nan et al., 2022). Intriguingly, during the decay phase, there was a notable decrease in the abundance of Bacteroidetes and Actinobacteria, while the presence of Proteobacteria exhibited a significant increase. These findings suggest that the development of fully mature compost has a discernible impact on the progression of bacterial communities. Proteobacteria assume a significant function in the process of nutrient recycling (nitrogen and carbon) and decomposition of organic matter (Wan et al., 2021). The increase in Proteobacteria increased the nutrient level in saline–alkali soil and decreased soil alkalinity. Furthermore, Proteobacteria, a group of thermophilic heterotrophic bacteria, have the ability to augment the process of cellulose degradation in composting (Zhang et al., 2020). Among them, *Flavobacterium*, *Saccharomonospora*, *Luteimonas*, and *Streptomyces* mainly appeared in the decaying period. These bacteria exhibited a positive influence on plant growth and demonstrated the ability to suppress the proliferation of soil-borne diseases, aligning with prior research findings (Andric et al., 2020; Ding et al., 2021), indicating that three different compost treatments have finally reached maturity. Hence, the agricultural suitability of the products derived from all three composting processes is evident.

By calculating the alpha-diversity index, the ACE indices of the three compost treatments at different stages (warming, high-temperature, cooling, and decaying periods) were compared. ANOVA revealed significant changes in bacterial alpha-diversity at different stages of composting (Figures 2B,D,F). The bacterial community diversity trend was similar among the three treatments at the mature stage of compost, and the bacterial community diversity at the rot stage was higher than that at the initial stage of compost. The findings of this study indicate that the process of composting resulted in a significant increase in the bacterial

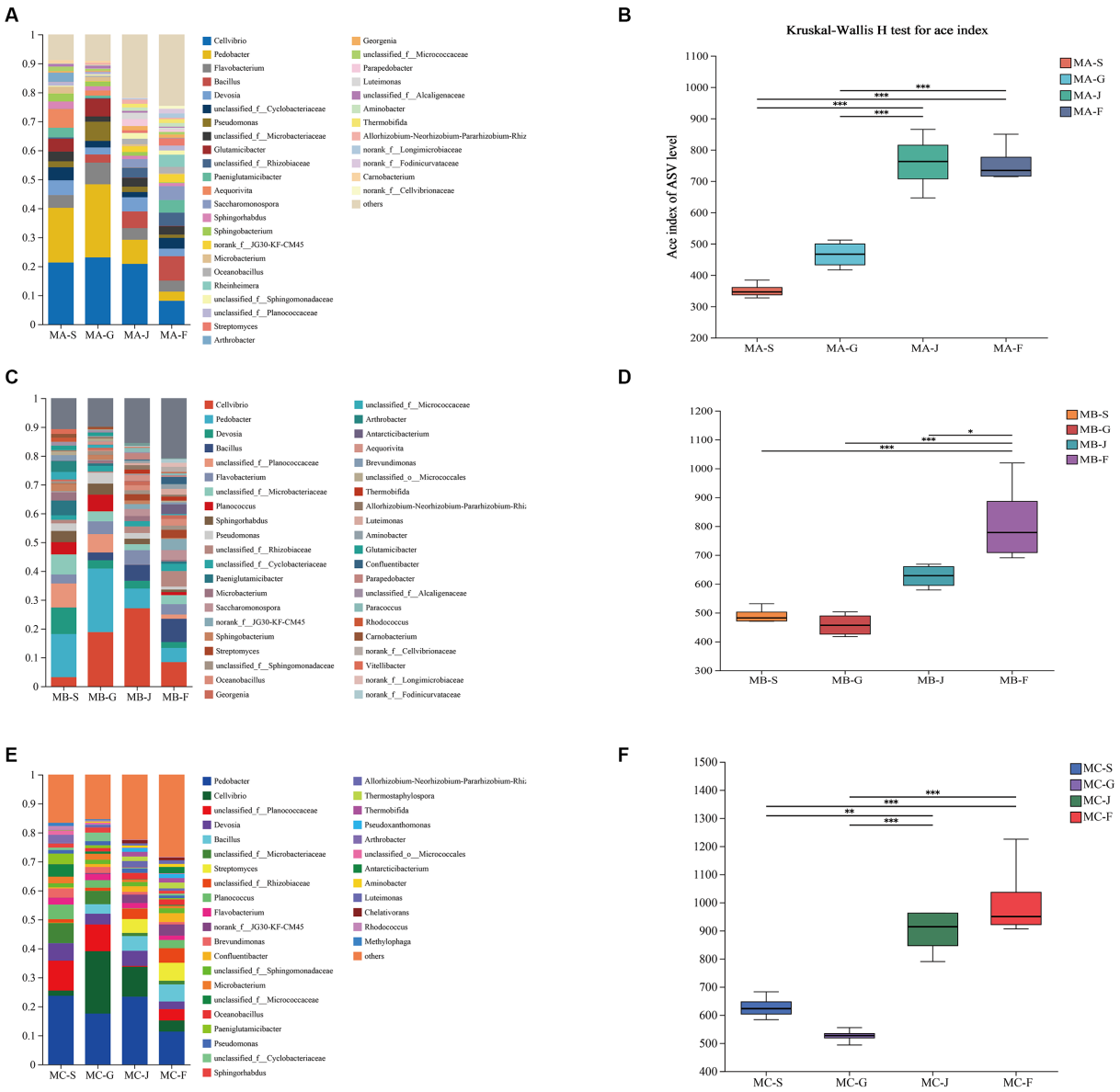

FIGURE 2
Evolution of bacterial community characteristics in different composting stages of the three treatments. **(A,C,E)** Relative abundance of bacteria at the genus level. Horizontal/vertical coordinates are sample names, vertical/vertical coordinates are the proportion of species in that sample, different colored bars represent different species, and the length of the bar represents the size of the proportion of that species. **(B,D,F)** Bacterial α-diversity. Significant differences between different samples mark the two groups with significant differences ($0.01 < *p \leq 0.05$, $0.001 < **p \leq 0.01$, $***p \leq 0.001$).

diversity of saline-alkali soil-straw-sheep manure compost. This increase in bacterial diversity is considered advantageous for the enhancement of saline-alkali soil quality (Shi et al., 2020; Wu et al., 2021). In the high-temperature period, the alpha-diversity index of MA, MB, and MC was lower than that of other periods, probably because the metabolism of thermophilic microorganisms was enhanced with the increase in temperature, resulting in the decreased diversity of other bacteria (Zhang et al., 2021). Hence, the genus species exhibited no notable disparity across various composting stages; however, the variability in genus abundance varied among distinct composting processes.

Effects of compost products on the bacterial community structure in saline–alkali soil

The diversity of bacterial communities is of utmost importance in soil ecosystems, as it is crucial for understanding the structural characteristics of microbial communities (Zhou et al., 2020). The current investigation observed a significant influence of compost products on the bacterial community's composition and diversity in the rhizosphere soil of plants (Figure 2). Examination at the phylum level indicated that Proteobacteria, Firmicutes,

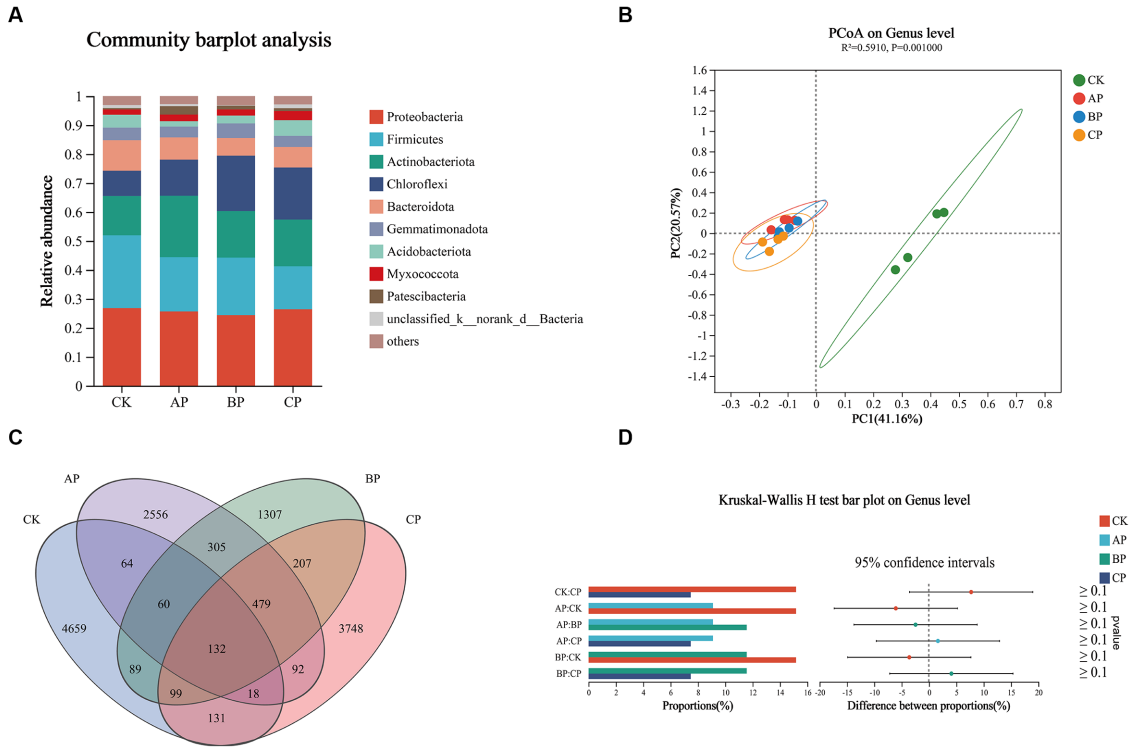

FIGURE 3
Effects of composting product on the bacterial community in saline–alkali soil plant with pot. **(A)** Relative abundance of bacteria at the phylum level. **(B)** PCA of bacterial community composition based on Bray–Curtis similarity. **(C)** Venn diagram showing the shared bacterial ASVs. **(D)** Analysis of species differences based on the Kruskal–Wallis H test. The horizontal coordinate of the bar graph on the left shows the percentage of *Bacillus* abundance in each group, and the ordinate shows the group type compared in pairs. The figure on the right shows the proportion of differences in species abundance.

Actinobacteria, and Chloroflexi were the dominant phyla in the rhizosphere soil (Figure 3A), aligning with previous studies and suggesting a substantial increase in these bacterial taxa in saline-alkali soil (Wang et al., 2022b; Yuan et al., 2023). Moreover, in these saline-alkali soils, Proteobacteria exhibit a significant prevalence, constituting 12.81, 25.62, 24.39, and 26.38% of the bacterial community in the control, MA, MB, and MC groups, respectively. Prior research has demonstrated that Proteobacteria possess the capability to degrade diverse macromolecules and facilitate the cycling of carbon, nitrogen, sulfur, and other essential substances. Additionally, they play a crucial role in mitigating abiotic stress through nitrogen fixation and promoting plant growth (Bruto et al., 2014). Therefore, Proteobacteria can be applied in agricultural production as biological control agents in soil–plant ecosystems (Hu et al., 2022). In the current investigation, the application of compost resulted in a notable increase in the abundance of Actinobacteriota and Chloroflexi, while concurrently causing a significant decrease in the abundance of Firmicutes when compared to the control group. Actinobacteriota exhibit a pivotal function in the process of global soil decomposition and carbon cycling, which demonstrates an inverse relationship with salt stress. Consequently, an augmentation in carbon cycling is anticipated to alleviate the detrimental effects of salt stress (Upchurch et al., 2018). Chloroflexi also positively affected plant growth by promoting nitrogen absorption (Hua et al., 2021). Furthermore, it has been observed that the saline-alkali soil of Songnen Plain in northeast China harbors a substantial population of Firmicutes. Both Firmicutes and Proteobacteria have been recognized as bacterial taxa with the ability to withstand and mitigate saline-alkali stress (Wang Y. et al., 2020).

The effects of different proportions of compost products mixed with saline–alkali soil on microbial community diversity in plant rhizosphere soil were studied via PCA based on the bacterial level (Figure 3B). The findings indicated that the bacterial communities in the rhizosphere soil of the three treatments exhibited a similar distribution along the axis, suggesting that the application of varying proportions of compost products had minimal impact on the bacterial communities (Wu et al., 2017). The bacterial ASV numbers in CK, AP, BP, and CP were 4,659, 2,556, 1,307, and 3,748, respectively (Figure 3C). There was a significant disparity in the quantity of bacterial ASVs observed in saline-alkali soil with compost products compared to the control group. The examination of discrepancies between the groups revealed a notable distinction in the genus Bacillus between the control group and the other three groups at the genus level (Figure 3D). According to Khan et al. (2019), a prior investigation demonstrated the significant involvement of Bacillus in the metabolic processes of carbohydrates within the soil environment, as well as its ability to secrete plant hormones, thereby influencing the growth and development of plants. These findings suggest that the introduction of compost products alters the composition of the microbial community

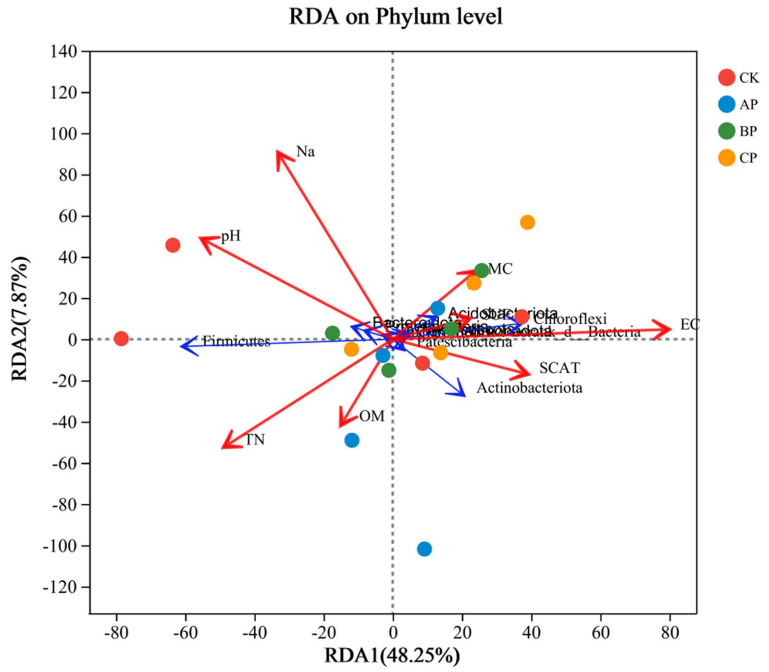

FIGURE 4
RDA of soil chemical properties and relative abundance of bacterial taxa. Red arrows represent the environmental factors (soil properties), and blue arrows represent the top 10 bacterial phyla in terms of relative abundance. TN, total nitrogen; OM, organic matter; EC, electrical conductivity; MC, moisture content; SCAT, solid catalase; SUE, solid urease.

in soil, potentially leading to the presence of beneficial bacteria that can mitigate saline-alkali stress and enhance soil nutrient levels.

Correlation among bacterial communities with physicochemical factors

Canonical correspondence analysis (CCA) was employed to investigate the correlation between the diversity of bacterial communities and the physicochemical characteristics of soil in the inter-rhizosphere soil of plants subsequent to the application of compost products in saline soils refer to Figure 4. The application of compost exhibited a significant and positive correlation with soil total nitrogen (TN), organic matter (OM), soil moisture content (SMC), electrical conductivity (EC), solid urease (SUE), and soil catalase (SCAT). Conversely, it displayed a negative correlation with soil sodium (NA) and pH. In general, soil pH, TN, SCAT, and EC were identified as the primary factors influencing the composition and diversity of the bacterial community. The salinity of saline soils leads to soil nutrient deficiency and affects plant growth. Compost application alleviated soil salinity and pH (Supplementary Figure S1A). The abundance of Proteobacteria, Firmicutes, Actinobacteriota, and Bacteroidota exhibited a significant correlation with soil physicochemical properties. Furthermore, Actinomycetes and Chloroflexi demonstrated a positive association with electrical conductivity (EC) and catalase, while displaying a negative relationship with pH. The study revealed that the association between the relative abundance of Chloroflexi and EC was not statistically significant. However, a positive correlation was observed between Chloroflexi abundance and pH and TN, while a negative correlation was observed with urease levels. This phylum, characterized by its thick-walled structure, exhibits the ability to generate budding spores as a defense mechanism against detrimental external factors, showcasing a high resistance to stress (Dai et al., 2019). The findings of this study indicate a positive correlation between Proteobacteria and Bacteroidetes with pH and Na, while also revealing a negative correlation with organic matter, which aligns with the results of a previous investigation (Lauber et al., 2009). Bacteroidetes prefer high pH environments for survival, and pH has been considered as a major factor in the formation of bacterial communities (Ganzert et al., 2014). Consequently, the reduction in the abundance of Bacteroidetes can be primarily attributed to the alteration in pH subsequent to the application of compost. This implies that alterations in microbial communities within the soil have the potential to enhance soil characteristics, diminish sodium content in saline-alkali soil, and foster plant growth (Supplementary Figure S1D). The findings of this study further substantiate the hypothesis that the addition of compost to saline soils can enhance soil nutrient levels. The various alterations made to the soil, particularly the changes in pH resulting from compost application, significantly influenced the diversity of the bacterial community in saline soil (Naeem et al., 2018).

Soil enzymes play a crucial role in assessing soil fertility and are actively engaged in the cycling and transformation of soil nutrients, thereby serving as indirect indicators of microbial activity (Jing et al., 2019). The imposition of salinity stress has been found to considerably diminish the activities of soil urease and catalase, while concurrently exerting a detrimental impact on soil protease activity (Azadi and Raiesi, 2021). In the current investigation, the application of compost exhibited a significant enhancement in soil catalase and urease activities, as well as alterations in the abundance of soil bacterial communities

when compared to the control group (CK). These findings suggest a substantial increase in soil enzyme activity due to the application of compost (Supplementary Figures S1E,F). This was caused by the interaction between the plant itself and its microorganisms. The augmentation of enzyme activity further substantiates the efficacy of compost product application in ameliorating saline soil conditions (Wang Y. M. et al., 2022). Therefore, cultivating these dominant flora in saline soils and inoculating them into plant roots may improve the microbial diversity in the soil. These microorganisms release some active enzymes, promote the synthesis of organic matter and humus, and release some growth-promoting factors, among other biochemical reactions (Ye et al., 2009), thereby improving the plant growth environment and enhancing saline soil nutrients. The application of this intervention resulted in an improved availability of soil nutrients and enhanced physicochemical properties. Additionally, it led to an increase in both the diversity and activity of soil bacteria, consequently altering the composition of soil bacterial communities. Notably, the presence of Proteobacteria, Firmicutes, Actinobacteriota, and Bacteroidota was found to positively impact the quality of saline-alkali soil.

Conclusion

This study showed that composting with different proportions of raw materials produced mature end-products and altered the bacterial community succession. This study additionally examined the impacts of incorporating compost as a soil amendment on the fertility and microbial diversity of saline soils. The findings from 16S RNA amplicon sequencing revealed that Proteobacteria, Firmicutes, Actinobacteria, and Chloroflexi were the prevailing bacterial taxa in saline soils. RDA results indicated that pH, TN, organic matter, and soil enzymes were the key parameters affecting the bacterial community. These findings may provide new ideas for compost as a bioamendment for saline soils.

Data availability statement

The Illumina sequences were deposited in the NCBI Sequence Read Archive (accession numbers SRR24001333–SRR24001347 for the rhizosphere soil bacteria of potted 16S rRNA gene data and SRR24031951– SRR24031993 for the compost rhizosphere soil 16S rRNA gene data).

Author contributions

DX: Writing – original draft. XY: Methodology, Writing – review & editing. JinC: Methodology, Visualization, Writing – review & editing. XL: Formal analysis, Methodology, Software, Writing – review & editing. JiaC: Conceptualization, Supervision, Writing – review & editing. JL: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Enhancing remediation potential of heavy metal contaminated soils through synergistic application of microbial inoculants and legumes

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Soil microorganisms play a crucial role in remediating contaminated soils in modern ecosystems. However, the potential of combining microorganisms with legumes to enhance the remediation of heavy metal-contaminated soils remains unexplored. To investigate this, we isolated and purified a highly efficient cadmium and lead-tolerant strain. Through soil-cultivated pot experiments with two leguminous plants (*Robinia pseudoacacia* L. and *Sophora xanthantha*), we studied the effects of applying this microbial agent on plant nutrient uptake of soil nutrients, heavy metal accumulation, and the dynamics of heavy metal content. Additionally, we examined the response characteristics of inter-root microbial and bacterial communities. The results demonstrated that microorganisms screened from heavy metal-contaminated soil environments exhibited strong survival and adaptability in heavy metal solutions. The use of the *Serratia marcescens* WZ14 strain-phytoremediation significantly increased the soil's ammonium nitrogen (AN) and organic carbon (OC) contents compared to monoculture. In addition, the lead (Pb) and cadmium (Cd) contents of the soil significantly decreased after combined remediation than those of the soil before potting. However, the remediation effects on Pb- and Cd-contaminated soils differed between the two legumes following the *Serratia marcescens* WZ14 inoculation. The combined restoration altered the composition of the plant inter-rhizosphere bacterial community, with the increase in the relative abundance of both Proteobacteria and Firmicutes. Overall, the combined remediation using the tolerant strain WZ14 with legumes proved advantageous. It effectively reduced the heavy metal content of the soil, minimized the risk of heavy metal migration, and enhanced heavy metal uptake, accumulation, and translocation in the legumes of *S. xanthantha* and *R. pseudoacacia*. Additionally, it improved the adaptability and resistance of both legumes, leading to an overall improvement in the soil's environmental quality. These studies can offer primary data and technical support for remediating and treating Cd and Pb in soils, as well as rehabilitating mining sites.

KEYWORDS

co-remediation, soil contamination, high-throughput sequencing, bacterial community structure, legume

1. Introduction

The issue of heavy metal pollution in soil, driven by unnatural factors from rapid industrialization, is increasingly severe and requires urgent solutions. Soil, as the most abundant and diverse ecosystem on Earth, plays a crucial role in reflecting soil health and function through the dynamics of soil quality, surface vegetation, and microbial communities in complex environments (Jiang et al., 2016). Mine remediation using microorganisms has been vastly studied over recent decade for remediation and ecological systems restoration at various mine sites (Xiao et al., 2023).

The unnatural uptake of heavy metals during mining is the primary cause of soil heavy metal contamination (Rachelle et al., 2018; Wang et al., 2022). Heavy metals (HMs) contamination has led to severe environmental issues, including soil nutrient losses, sharp reductions in soil microbial diversity, and hindered plant growths (Liu et al., 2019; Gonalves et al., 2020; Shuaib et al., 2021). Unlike organic pollutants, heavy metal pollution is characterized by difficult degradation, hidden, long-term, and high toxicity, and it is difficult to achieve the intended effect of complete removal in the short term in the remediation of soil heavy metal pollution (Luo et al., 2019). As traditional remediation techniques like physical and chemical methods are increasingly limited in addressing soil heavy metal pollution, phytoremediation has gained significant attention as an alternative due to its ecological, economic, and sustainable advantages (Fatima et al., 2016; Saxena et al., 2019; Xiao L. et al., 2021), emerging as one of the most promising remediation approaches. In addition, plants play a vital role in improving soil quality and optimizing the soil microbial community (Schloter et al., 2018; Beiyuan et al., 2021). Various microorganisms living in the rhizosphere, can have beneficial effects on plant growth, and health and increase plant biomass production (Evlat et al., 2023). Therefore, investigating changes in soil nutrients and microbial community structure during phytoremediation is crucial for successful ecological restoration.

Most studies on phytoremediation for soil heavy metal remediation, especially on phytoextraction, have primarily focused on utilizing super-enriched plants (HMH) to extract soil heavy metals (Duan et al., 2020; Atikur-Rahman et al., 2022). Although HMH has demonstrated favorable remediation results, certain studies have presented its limitations, such as slow growth and shallow root systems, which hinder its ability to reach deeper soil layers and extract heavy metals to a treatable level (Słomka et al., 2012). In addition, Wood et al. (2016) observed that non-HMH species often extracted more heavy metals than HMH when measuring the net number of metals extracted per plant, with the number of extracted heavy metals closely correlated to plant biomass. Although HMH remains valuable in practical phytoremediation, non-HMH species with high biomass may represent a more suitable option for developing efficient phytoextractors in the future.

Leguminosae, with 172 genera, 1,485 species, and 153 varieties in China (Hei et al., 2019), are widely distributed throughout the country and hold significance in soil improvement and ecological restoration (Dary et al., 2010; Cai et al., 2015). Legumes have exhibited excellent tolerance and effectiveness in heavy metal remediation, with some species exhibiting remediation capabilities comparable to HMH due to their robust root biomass (Shi et al., 2012; Guo and Chi, 2017; Zeng, 2017). The advantages of legumes in this regard include: (i) their strong root biomass can produce abundant secretions that confer resistance to heavy metal pollution stress (Pereira et al., 2006), with the dissolution of insoluble heavy metals in the soil by the organic acids released from the roots, which can enhance plant uptake of soil heavy metals. (ii) Legume roots contain abundant rhizobia, effectively improving soil quality (Maynaud et al., 2013). These traits enable legumes to enhance, maintain, and develop stable soil systems. Therefore, employing legumes in phytoremediation holds significant potential for soil heavy metal remediation, contributing to the future refinement of plant species screening and phytoextraction in ecological restoration.

In order to improve phytoextraction efficiency, the inoculation of characteristic microorganisms into plant roots is a common strategy (Abhilash et al., 2012; Sessitsch et al., 2013). The identification and development of new, effective PGPR strains would be very efficient, providing several beneficial activities such as improved nutrient uptake, improved stress tolerance, enhanced plant growth, and resistance to fungal or bacterial pathogens (Xiao C. Q. et al., 2021). The phytoremediation coupled with Pb-resistant phosphate-solubilizing bacteria effectively improved the efficiency of Pb bioremediation. For example, The inoculation of soil with strain LA greatly promoted the growth of ryegrass and sonchus, increased the concentration of bioavailable P and Pb in plants, and decreased the bioavailability of Pb in the soil (Guo et al., 2021). In the "biotrophic bacteria-plant" mechanism, on the one hand, "bacteria" generally refers to inter-root biotrophic bacteria that can promote plant growth or improve soil quality, and the microorganisms promote plant growth through the production of iron carriers, phytohormones, organic acids and functional enzymes to enhance the remediation of heavy metals in the soil; On the other hand, some microorganisms themselves have the ability to dissolve and activate heavy metals, which reduces the content of heavy metals in the soil, increases the base of heavy metals that can be absorbed by the soil, and improves the possibility of plant uptake of heavy metals in the soil, so that the total amount of heavy metals in the soil is reduced to a harmless level (Ni et al., 2019). Several studies have demonstrated the microorganisms can convert Cr (VI) in soil into Cr (III) or directly adsorb it in their bodies by means of bioreduction and biosorption, while plants uptake and accumulate Cr in tissues, thus reducing the total Cr content in the soil (Wu et al., 2014). Li (2021) inoculated *Variovorax paradoxus* DE5 into *Celosia argentea* plants and found that DE5 was able to significantly promote the growth of *Celosia argentea* plants and enhance the uptake of soil cadmium by *Celosia argentea*. The results of the pot test showed that the application of *Lactobacillus casei* at 105 cfu·mL^{-1} reduced the pH of the soil, increased the soil enzyme activity, promoted the growth and development of cabbage mustard, and facilitated the remediation efficiency of cabbage mustard on Cd and Zn composite contaminated soil (Liu, 2022). It has been shown that the application of organic acid-secreting endophytic bacteria effectively increased the conversion of insoluble Pb into the effective state of Pb, increased the content of soil heavy metal Pb in the effective state, and significantly improved the enrichment efficiency of oilseed rape for soil heavy metal Pb (Ma Y. et al., 2022). Typically, the candidate microorganisms inoculated into plants originate from internal plant tissues or root secretions and function as "probiotics," directly or indirectly influencing plant growth (Li et al., 2017). However, such microorganisms often exhibit weak resistance against external pollution stress (Zhang et al., 2011). Conversely, microorganisms exposed to and surviving in heavy metal-contaminated soil over extended periods may possess higher tolerance and resistance (Ibiang

et al., 2020). Therefore, understanding the source of microorganisms, their impact on heavy metal morphology, and their probiotic effects on plants prove beneficial for soil heavy metal remediation efforts.

Our study addressed these limitations through a comprehensive approach. We first screened lead (Pb)- and cadmium (Cd)-tolerant microorganisms (resistant bacteria) for inoculum in experiments, using long-term heavy metal-contaminated soil as substrate. In addition, the heavy metal environment was simulated through indoor resistance growth experiments. Concurrently, outdoor pot experiments were conducted to investigate the impact of legumes (*Sophora xanthantha* and *Robinia pseudoacacia* L.) and resistant bacteria-legume combinations on the remediation of heavy metal-contaminated soil. The objective of this study was (a) to determine whether tolerant bacterial inoculum would enhance the effectiveness of legume-based remediation, (b) to examine whether tolerant bacterial inoculum would lead to plant improvement in soil physicochemical properties and reduction in heavy metal content, and (c) to investigate key species of contaminated soil bacterial communities using high-throughput sequencing techniques. These studies have provided essential data and technical support for the utilization of legumes in the remediation of Cd and Pb in soils and the rehabilitation of mining sites.

2. Materials and methods

2.1. Study area overview and experimental design

The lead-zinc-silver mine is situated in Zhijiadi, Gaojiazhuang Township, south of Lingqiu County, Shanxi Province (39.21°N, 114.12°E). The region experiences a temperate semi-arid continental climate, with an average annual temperature of 7°C and an average annual rainfall of 421 mm. The mine, commissioned in 2003, is expected to have a lifespan of 20 years or more. Long-term mining activities have led to long-term heavy metal pollution in the soil. In order to address this issue, soil samples were collected from three different functional types: mine center, mine wasteland, and tailings. These soils were used for screening heavy metal Pb and Cd tolerant microorganisms (Figure 1). The experiment involved soils contaminated with two heavy metal complexes, Pb and Cd. Sterilized soil was uniformly sprayed with a solution of 400 mg/L Pb^{2+} (Pb $(NO_3)_2$) and 60 mg/L Cd^{2+} ($CdCl_2$). After thorough mixing, the soil was left for 15 days for potting experiments.

2.1.1. Resilient growth experiment

Bacteria and fungi were isolated from soil samples using 10-fold serial dilutions plated onto heavy metal-free nutrient agar and potato dextrose agar solid media, respectively. The plates were then incubated for 3 days at 28°C. Once colonies covered two-thirds of the plates, colonies indicating different morphological colors were selected and purified. Subsequently, solid medium plates containing a Pb^{2+} concentration of 100 mg/L and a Cd^{2+} concentration of 40 mg/L were prepared. The purified strains were plated on these plates using the "trilinear" method. The screening of strains that grew well on plates containing heavy metals for resistance growth experiments was conducted to identify strains capable of thriving in environments contaminated with lead and cadmium while reducing unnecessary testing efforts.

The viability of the screened strains was quantitatively assessed through resistance growth experiments. Luria broth (LB) cultures with varying concentrations of Pb and Cd (suitable for bacterial and fungal colonization) were prepared. The Pb concentrations in the LB cultures were set at 400, 600, 800, 1,000, 1,500, and 2,000 mg/L, achieved by

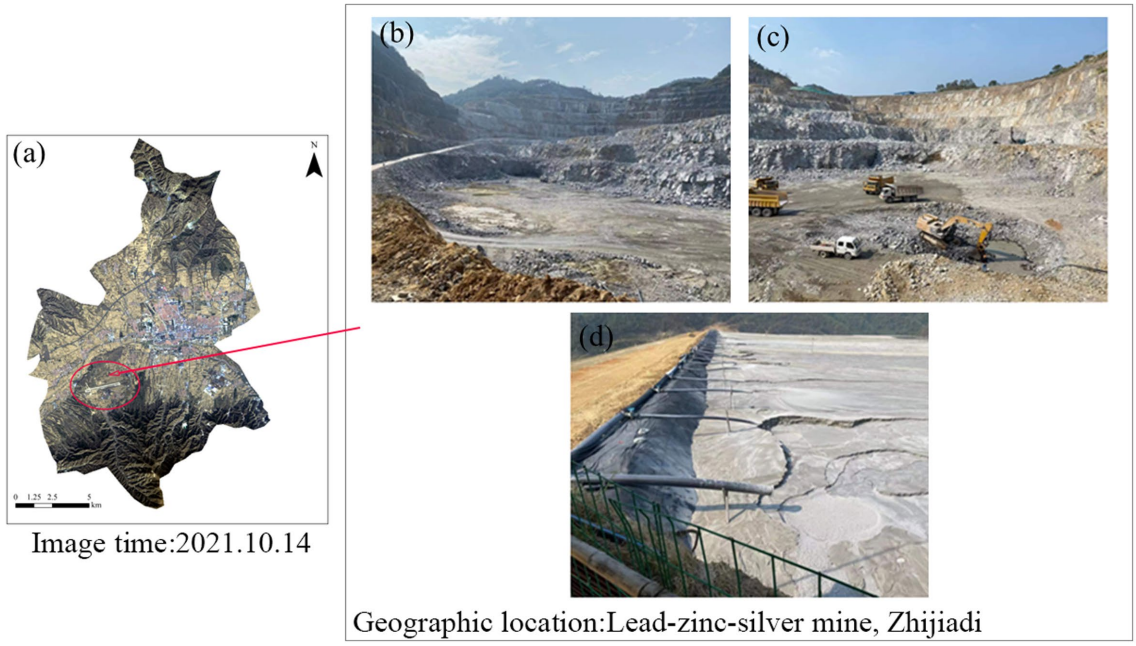

FIGURE 1
Collection of three different functional types of soils **(a)**, including mine center soils **(b)**, mine wasteland soils **(c)**, and tailings soils **(d)**.

replacing pure water with different concentrations of Pb $(NO_3)_2$ solution. Similarly, the Cd^{2+} concentrations in LB cultures were maintained at 50, 100, 150, 200, 250, and 300 mg/L. Well-grown colonies (WZ13, WZ14, WG20, and S22) with a diameter of 1 mm were added to 500 mL conical flasks containing 400 mL LB culture medium with varying Pb and Cd concentrations. Control flasks without colonies were also included. The flasks were then incubated on a shaker (speed: 180 rpm, temperature: 25°C ± 0.3) for 2 days. During this period, absorbance values were measured at 12, 24, 36, and 48 h for each treatment, providing insights into the strain's ability to survive in an environment contaminated with lead and cadmium (Fang et al., 2012).

2.1.2. Pot experiment

Based on the results of resistance growth and soil cultivation experiments, a promising resistant strain was selected for investigating the remediation effects of soil Pb and Cd through potting experiments. The seeds of the test plants were provided by the State-owned Qiaotou Forest Farm, Wengniute Banner, Chifeng City, Inner Mongolia. Seeds were soaked in pure water for 12 h (Zheng et al., 2021). After filtering the water and allowing the seeds to dry on the surface, the obtained strain was disinfected by immersing them in a 5% sodium hypochlorite solution for 10 min (Zhuang et al., 2021). After washing the seeds with pure water until they were odorless, 5 to 7 seeds were placed in seedling cups for 1 week. Seedlings of similar height and growth were carefully selected for transplantation into plastic pots (15 cm in diameter, 20 cm in height, containing 2.0 kg of soil mixture; Bao, 2000). Once the seedling roots stabilized, the prepared WZ14 inoculum was inoculated into the plant roots, creating a combination of tolerant bacteria-plant treatment. Seedlings inoculated with sterile culture served as the control treatment. Each treatment was replicated three times and consistently watered and randomized to maintain a standardized regimen. After 3 months, plant and soil samples were collected for analysis.

2.2. Sample collection and analysis

After a 3-month growth period, all plants and soil were harvested. Plant samples were collected by separating roots, stems, and leaves, which were then washed, dried, and crushed. Soil samples were obtained by shaking the inter-root soil attached to plant roots. The selected soil samples were divided into two parts and stored. One portion of the soil was stored in a 4°C ice box and brought back to the laboratory for further analysis. The second portion of the soil samples was air-dried to determine the physical and chemical properties of the soil.

The pH of the soil solution and the soil's physicochemical properties were analyzed following Bao (2000) and Lou et al. (2019) standard method. Potentiometric measurements determined the pH, volumetric potassium dichromate analysis determined soil organic matter, and the elemental analyzer determined total soil nitrogen. The molybdenum-antimony anti-colorimetric method was employed for the determination of total soil phosphorus, while total soil potassium was measured using the NaOH alkali fusion-flame photometric method. Soil alkaline nitrogen was determined via the alkaline diffusion method, and effective phosphorus was analyzed using the molybdenum-antimony anti-colorimetric UV spectrophotometric method. Fast-acting potassium was determined by ammonium acetate extraction followed by flame analysis. The effective phosphorus content was quantified using molybdenum antimony anti-colorimetric UV spectrophotometry. The total amount of heavy metal elements in soil samples was analyzed through soil digestion using the electric hot plate digestion method. Additionally, the effective state content of soil heavy metals was extracted using the hydrochloric acid leaching method (HJ804-2016). Both analyses were conducted using an Optima 5300 DV inductively coupled plasma emission spectrometer test (ICP-AES, PerkinElmer, United States).

1. Measurement of total heavy metals in soil: Soil samples were air-dried and sifted through a 100-mesh sieve. Then, 0.1 g of the soil sample was put in a 25 mL PTFE beaker, lightly moistened with Milli-Qultrapure water and heated at a low temperature of 180°C with 5 mL of concentrated hydrochloric acid for 20 min (evaporated to about 3 mL) to eliminate sulfur compounds present in the sample. Subsequently, 3 mL of concentrated nitric acid, 3 mL of hydrofluoric acid, and 1 mL of perchloric acid were added. The beaker was then heated on a heating plate with a lid to a temperature of 280°C for approximately 1.5 h until a clear solid was produced. Afterward, 19 mL of ultrapure water and 1 mL each of concentrated hydrochloric acid and concentrated nitric acid (concentrated hydrochloric acid: concentrated nitric acid = 3:1) were added to the beaker. Then, the mixture was transferred to a 25 mL volumetric flask, and the volume was determined. Ultimate, taking 5 mL of the solution that has passed through a 0.45 μm aqueous filter membrane and place it in a 10 mL centrifuge tube. For testing.
2. Determination of the effective state content of soil heavy metals: The soil samples were air-dried and passed through a 100-mesh sieve, and 5 g of soil samples were placed in a 15 mL centrifuge tube. Then 0.1 mol-L^{-1} of dilute hydrochloric acid was added to the centrifuge tube, the lid was tightly closed, and the tube was placed on a shaker (speed: 200 rpm, temperature: 20°C ± 2°C) for 4 h to allow the reaction to complete, and the tube was removed from the shaker. Ultimate, after being left standing for 24 h, take 5 mL of the solution that has passed through a 0.45 μm aqueous filter membrane and transfer it to a 10 mL centrifuge tube for testing.

The method for determining heavy metals in different parts of the plant was identical to the one described above for total soil heavy metals.

2.3. Soil DNA extraction, PCR amplification, and high-throughput gene sequencing

The PowerSoil DNA Isolation Kit (MO BIO, CA, United States) was utilized to extract DNA from all soil samples. In order to minimize the impact of soil heterogeneity on test results and avoid biases from single DNA extraction or low DNA content in samples, each soil sample underwent multiple DNA extracts for subsequent analysis. The purity and concentration of the DNA were assessed using a NanoDrop-2000 spectrophotometer (Thermo-Scientific, DE, United States) and agarose gel electrophoresis (Li et al., 2020). For soil DNA amplification, specific

primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) targeting the V3–V4 region of bacterial 16S rRNA were employed (Ma et al., 2020). The PCR amplification steps were as follows: the predenaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 30s, 55°C for 30 s, and 72°C for 45 s, were performed with a final extension at 72°C for 5 min. The total volume of the PCR reaction was 25 μL. After PCR amplification, the constructed libraries were analyzed through Qubit and qPCR before being sequenced on an Illumina Miseq system (Guangzhou Kidio Technology Services). The raw data obtained from sequencing was processed by trimming, filtering, and splicing to obtain valid data for subsequent analysis. The optimized sequences, showing >97% similarity, were clustered into operational taxonomic units (OTUs). The taxonomic identities of the bacteria were determined using RDP software and Silva schemes (Wang et al., 2007; Quast et al., 2012).

For further experiments, the nucleotide sequence of WZ14 and the ITS sequence of the potting soil have been uploaded to the NCBI database under the accession numbers OR492361 and PRJNA1012100, respectively.

2.4. Data analysis

Data were compiled using Microsoft Excel 2019 software, and statistical analyses were conducted using one-way analysis of variance (ANOVA) with SPSS 20.0 (IBM, United States) for total soil heavy metals, effective soil heavy metal status, and soil physicochemical properties. Statistical significance was accepted at $p < 0.05$. To address soil microbial diversity and richness variations, Simpson and Shannon diversity indices, as well as Chao1 and ACE richness indices, were calculated using Mothur and analyzed for alpha diversity. The data were plotted using the Origin 2019 software, and the significance of differences was determined using the DUNCAN method ($\alpha = 0.05$). Additionally, heat maps were generated to illustrate the correlation between soil physicochemical factors and soil heavy metals. All bioinformatics analyses were performed using the Omicsmart online analysis platform developed by Guangzhou Kidio Technology Services.

3. Results and analysis

3.1. Resistance growth and soil culture trials based on microbial screening

In this study, we isolated a total of 70 strains from the mine center, mine wasteland, and tailings. A Pb^{2+} concentration of 100 mg·kg^{-1} and a Cd^{2+} concentration of 40 mg·kg^{-1} were used as screening thresholds for microbial adaptation to heavy metal environments. Among the strains tested, 26 exhibited the ability to tolerate heavy metals. Following a qualitative evaluation, we selected four strains with excellent lead and cadmium tolerance, which were named WZ14, WZ13, S22, and WG20. These selected strains were further subjected to resistance growth experiments.

The four strains exhibited varying growth responses to cultures with different Pb^{2+} and Cd^{2+} concentrations (Figures 2, 3). Each strain displayed distinct sensitivity levels to the same heavy metal. Notably, WZ14 exhibited the most favorable adaptation to different Pb^{2+} concentrations without showing a peak in the growth curve for each concentration. It differed from other strains in cultures with different Pb^{2+} concentrations. WZ13 and S22 demonstrated weak acclimation in Cd^{2+} cultures, with both strains exhibiting growth of no more than 0.5 cfu·mL^{-1} in various Cd^{2+} concentrations, significantly lower than that of WZ14 and WG20. Comparatively, WZ14 displayed a higher growth curve than WG20 at the same Cd^{2+} concentration. In addition, its curve gradually shifted downward as Cd^{2+} concentration increased, suggesting the suitability of WZ14 for survival in a low-concentration Cd-contaminated environment. The results from the resistance growth experiment indicated that WZ14 exhibited robust growth and excellent resistance in both Cd- and Pb-contaminated environments, outperforming other strains.

WZ14 exhibited the highest resistance and most effective reduction in heavy metal content than other three strains, leading to its selection for the subsequent tests.

3.2. Influence of applied microbial inoculants on nutrient uptake and accumulation of Pb and Cd by plants

Robinia pseudoacacia monoculture, WZ14-*R. pseudoacacia*, *S. xanthantha* monoculture, and WZ14-*S. xanthantha* significantly increased soil pH compared to the soil background (BJZ; Table 1). However, when WZ14 was applied to *R. pseudoacacia* and *S. xanthantha*, it slightly reduced the soil pH compared to plant monocultures. All four treatments significantly increased the organic matter content (OC) of the soil compared to BJZ. WZ14-*R. pseudoacacia* and WZ14-*S. xanthantha* showed a significant difference, with an increase of 14.36% and 18.29%, respectively, compared to no strain application. For soil alkaline nitrogen content, all treatments significantly reduced it compared to BJZ. *Robinia pseudoacacia* and *S. xanthantha* significantly increased soil alkaline nitrogen content by 79.66% and 61.04%, respectively, after applying the WZ14 strain compared to no strain application. All four treatments significantly reduced the soil's effective phosphorus content compared to BJZ, with *R. pseudoacacia* having the lowest soil effective phosphorus content significantly after the application of the WZ14 strain. The strain-legume combinations significantly reduced soil fast-acting potassium content compared to plant monocultures with WZ14-*R. pseudoacacia* showing the most significant reduction. Regarding allotropic nutrients, compared to plant monocultures, both *R. pseudoacacia* and *S. xanthantha* showed increased soil allotropic nitrogen, phosphorus, and potassium content after applying the WZ14 strain, indicating a higher potential for allotropic nutrients to transform into effective nutrients that can be easily absorbed by plants. However, the application of WZ14 also caused irregular changes in soil effective nutrient content, influenced by both the decomposition of insoluble nutrients and the absorption capacity of surface plants.

After phytoremediation and combined remediation, the soil Pb and Cd contents were significantly lower than the soil heavy metal contents before potting (Figure 4; $p < 0.05$). However, the effectiveness of the two legumes in remediating Pb- and Cd-contaminated soil differed after the application of the WZ14 strain. Compared to the single plant treatments, the combined WZ14-*R. pseudoacacia* and WZ14-*S. xanthantha* combinations reduced total soil Pb by 13.33% and 19.45%, respectively, and reduced the effective state of soil Pb by 29.63% and 18.11%,

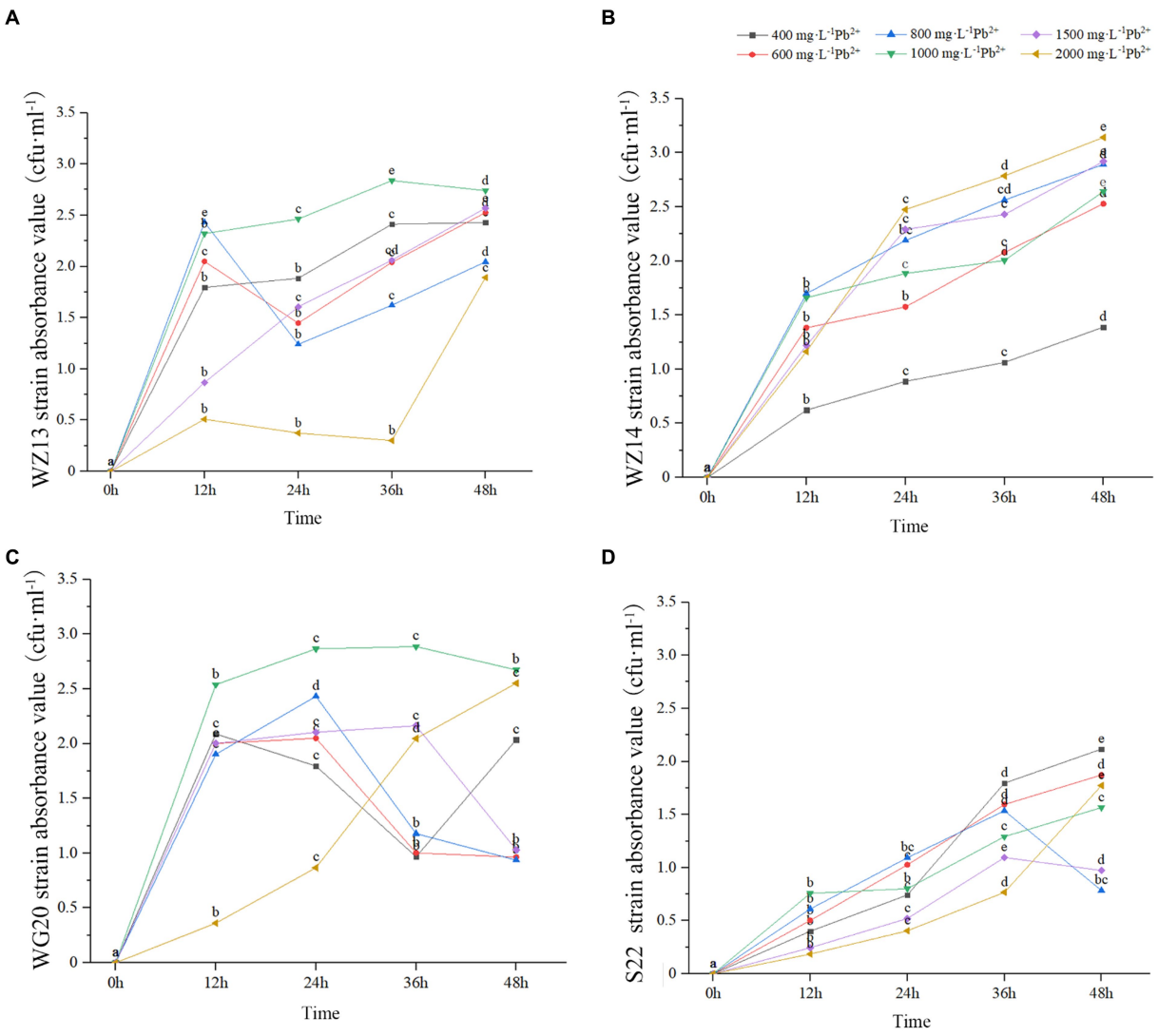

FIGURE 2
Growth of four strains in medium with different concentrations of Pb^{2+}. Different lowercase letters indicate significant differences between concentrations ($p < 0.05$). WZ13 strain **(A)**; WZ14 strain **(B)**; WG20 strain **(C)**; S22 strain **(D)**.

respectively. In addition, the WZ14-*R. pseudoacacia* combination treatment significantly reduced the soil Cd total and Cd active state content by 20.37% and 90.72%, respectively, compared to the soil heavy metal content before potting (Figure 4; $p < 0.05$). The WZ14-*S. xanthantha* combination treatment significantly reduced the soil Cd total and Cd active state content by 55.72% and 82.71%, respectively. Regarding the reduction of soil Cd effective state, the plant monoculture treatments of *R. pseudoacacia* and *S. xanthantha* showed a reduction of 91.51% and 83.14% in soil Cd effective state, respectively, compared to the soil background values before potting. The WZ14-legume combinations were more effective than single-crop treatments in reducing soil Cd levels but were weaker than single-crop treatments in effectively remediating soil Cd status.

The Pb and Cd contents in the roots of *S. xanthantha* were significantly higher than in the CK (plant monoculture treatment) and *R. pseudoacacia* treatments under bacterial addition. In contrast, the Pb content in the stem of the WZ14-*R. pseudoacacia* treatment was significantly higher than in other treatments (Figure 5, $p < 0.05$). For *R. pseudoacacia* plants, Pb content in roots, stems, and leaves was generally low, ranging from 12.37 to 26.07, 3.01 to 7.35, and 1.24 to 3.29 $mg \cdot kg^{-1}$, respectively. However, when combined with WZ14, the Pb content significantly increased in WZ14-*R. pseudoacacia* roots by 110.8% and in stems and leaves by 144.2% and 165.3%, respectively, compared to *R. pseudoacacia* monoculture (Figure 5, $p < 0.05$). In the case of Cd content, there was no significant difference in the stems of the two plants under the strain application treatment, and both Pb and Cd content in *R. pseudoacacia* roots were significantly lower than in *S. xanthantha* under monoculture ($p < 0.05$). Cd content was higher in *S. xanthantha* plants, and under the strain application treatment, Cd content in roots, stems, and leaves increased significantly by 130.79%, 88.64%, and 159.13%, respectively, compared to CK ($p < 0.05$). Pb content in *S. xanthantha* stems and leaves also increased significantly by 124.9% and 157.8%, respectively, compared to CK ($p < 0.05$). In comparing the uptake capacity of Pb

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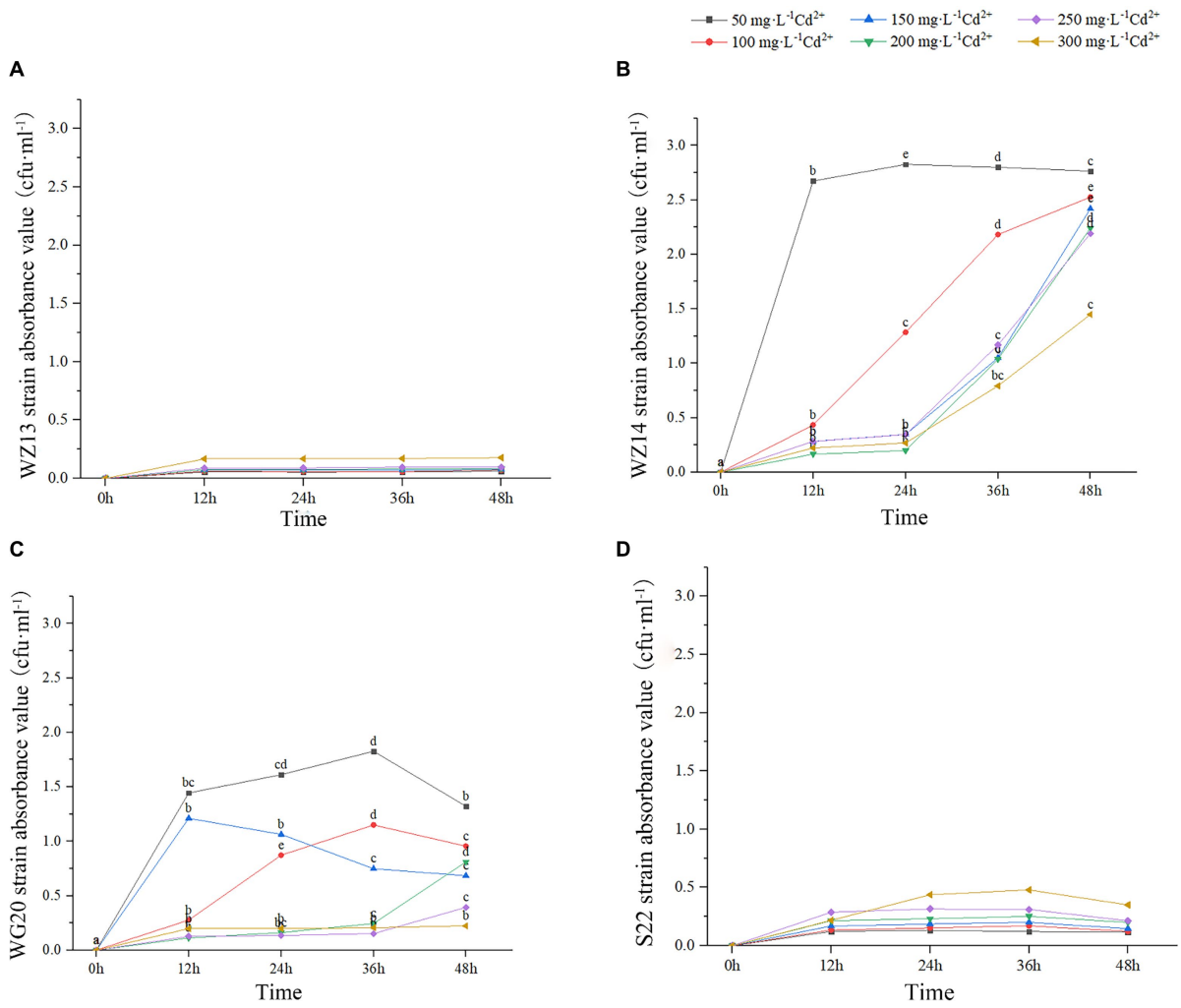

FIGURE 3
Growth of four strains in medium with different concentrations of Cd^{2+}. Different lowercase letters indicate significant differences between concentrations ($p < 0.05$). WZ13 strain **(A)**; WZ14 strain **(B)**; WG20 strain **(C)**; S22 strain **(D)**.

TABLE 1 **Effects of different treatments on soil physicochemical properties.**

Soil physicochemical	BJZ	*Robinia pseudoacaca*	WZ14-*R. pseudoacacia*	*Sophora xanthantha*	WZ14-*S. xanthantha*
pH	6.90 ± 0.08a	6.98 ± 0.02b	6.95 ± 0.02b	6.99 ± 0.02b	6.96 ± 0.02b
OC (mg/kg)	11.54 ± 0.50a	12.88 ± 0.43ab	14.73 ± 0.27c	13.83 ± 0.72bc	16.36 ± 0.86d
TN (g/kg)	2.31 ± 0.03e	2.09 ± 0.02d	1.74 ± 0.01a	1.96 ± 0.01c	1.87 ± 0.02b
TP (g/kg)	0.87 ± 0.03b	0.83 ± 0.03ab	0.79 ± 0.02a	0.80 ± 0.01a	0.78 ± 0.03a
TK (g/kg)	23.14 ± 0.31a	25.65 ± 0.27d	24.80 ± 0.30c	26.30 ± 0.25d	23.85 ± 0.31b
AN (g/kg)	203.46 ± 1.69d	74.05 ± 2.33a	133.04 ± 3.54c	84.98 ± 2.30b	136.85 ± 1.56c
AP (mg/kg)	114.50 ± 0.43d	96.38 ± 0.05c	87.48 ± 0.14b	86.94 ± 5.62b	71.37 ± 0.07a
AK (mg/kg)	247.90 ± 2.84e	153.90 ± 3.49c	117.90 ± 4.03a	163.84 ± 3.57d	132.71 ± 3.50b

BJZ represents soil physicochemical property values before the pot experiment. Different lowercase letters indicate significant differences between treatments ($P < 0.05$).

and Cd, it was observed that the Pb and Cd contents in roots and leaves were significantly higher in the combined WZ14-*S. xanthantha* treatment than in the combined WZ14-*R. pseudoacacia* treatment. However, in stems, the Pb content was lower in the combined WZ14-*S. xanthantha* treatment than in WZ14-*R. pseudoacacia*, and there was no significant difference in the Cd content between the two ($p > 0.05$). This pattern was consistent with the single plant treatment, indicating that *S. xanthantha* (except for the stem) had a better capacity for soil Pb and Cd uptake than *R. pseudoacacia* (except for the stem).

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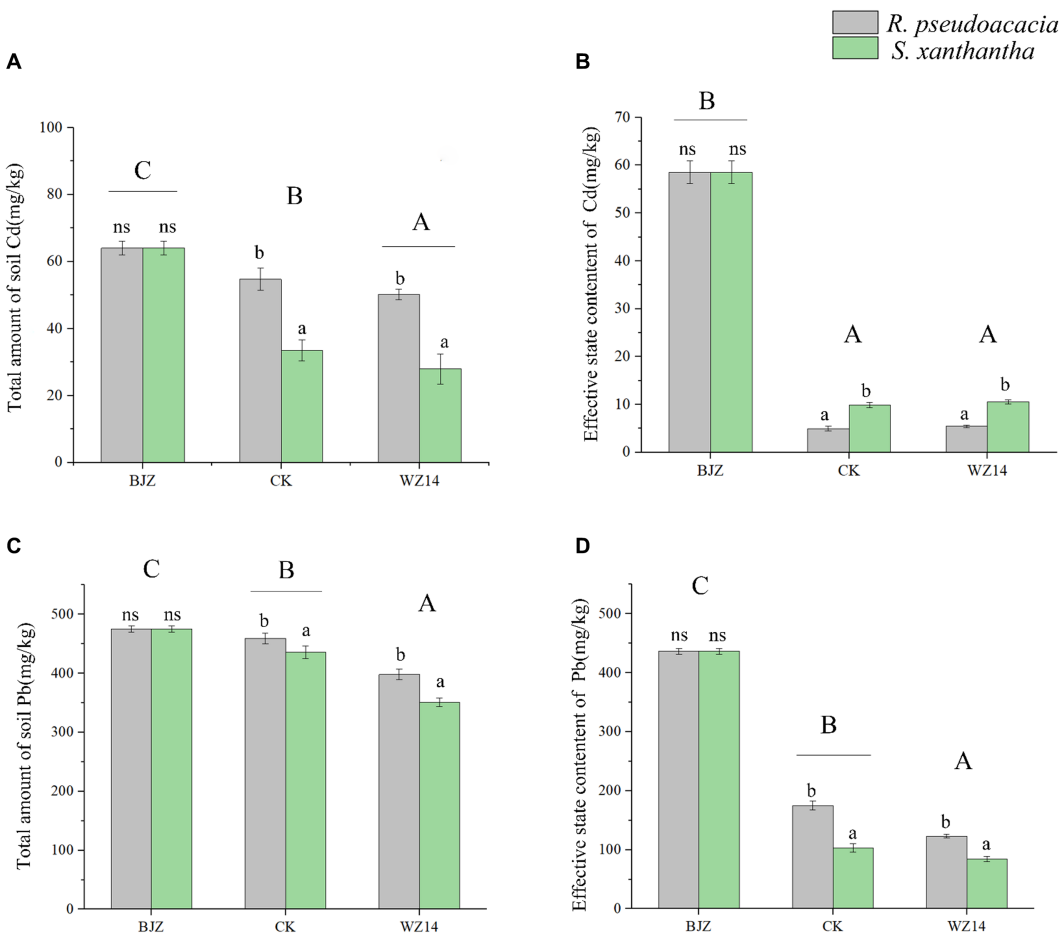

FIGURE 4
Effect of different treatments on total soil heavy metals Cd **(A)** and Pb **(C)** and effective state content of Cd **(B)** and Pb **(D)**. BJZ, background value of soil before pot experiment; CK, no application of microbial agent; WZ14, the application of WZ14 microbial inoculum. Different lowercase letters indicate significant differences between different plants in the same treatment ($p < 0.05$). Different uppercase letters indicate significant differences between treatments ($p < 0.05$). ns: indicates no significant differences between different plants in the same treatment ($p < 0.05$).

3.3. Impact of the added bacterial agent on the inter-rhizosphere soil bacterial community

Overall, the addition of the WZ14 strain had varying effects on soil bacterial alpha diversity in *R. pseudoacacia* and *S. xanthantha* (Table 2). In *R. pseudoacacia*, the presence of the WZ14 strain increased the abundance and diversity of soil microbial communities compared to the plant treatment without the WZ14 strain. However, in *S. xanthantha* soil, the addition of the WZ14 strain significantly reduced the abundance of OTUs and the diversity of bacterial communities, with the Chao1 index and Shannon index decreasing by 9.86% and 6.45%, respectively, compared to *S. xanthantha* monoculture.

At the phylum level (Figure 6A), the dominant phyla of the bacterial community in the soil samples were *Proteobacteria*, *Bacteroidetes*, *Patescibacteria*, *Chloroflexi*, and *Acidobacteria*, each representing the relative abundance of more than 5% of the total soil bacterial community in the upper soil, accounting for 64.48% to 72.61%. After inoculation with WZ14, the relative abundances of *Proteobacteria* and *Firmicutes* in the soil of *R. pseudoacacia* and *S. xanthantha* significantly increased after inoculation with WZ14 compared to the treatment without microbial inoculants. Specifically, the relative abundance of *Proteobacteria* in the soil of *R. pseudoacacia* increased from 32.76% to 37.42% and in *S. xanthantha* from 20.92% to 29.78%, while *Firmicutes* increased from 2.60% to 5.35% in *R. pseudoacacia* and from 3.52% to 6.88% in *S. xanthantha*. Additionally, WZ14 inoculation significantly increased the relative abundance of *Bacteroidetes* from 10.28% to 17.91% ($p < 0.05$) and reduced the relative abundance of *Patescibacteria* from 13.51% to 8.01% in *R. pseudoacacia* and from 20.49% to 10.18% in *S. xanthantha* soils, respectively. These findings suggested that WZ14 inoculation may play a vital role in improving plant quality and remediating heavy metal contamination by increasing the abundance of dominant bacterial communities, particularly the relative abundance of *Proteobacteria*, *Bacteroidetes,* and *Firmicutes*.

At the genus level (Figure 6B), the dominant genera in the soil samples were *Flavisolibacter*, *Sphingomonas*, *Bacillus*, *Streptomyces*, and *Paenibacillus*. The addition of WZ14 significantly increased the relative abundance of the dominant bacterial genera (total relative abundance of the five most abundant genera). Specifically, the relative abundances of *Flavisolibacter*, *Sphingomonas*, and *Bacillus* increased from 1.79%, 5.19% and 1.98% to 7.02%, 7.57%, and 5.03%, respectively, after the addition of WZ14. However, the relative abundance of *Paenibacillus* in the soil was significantly

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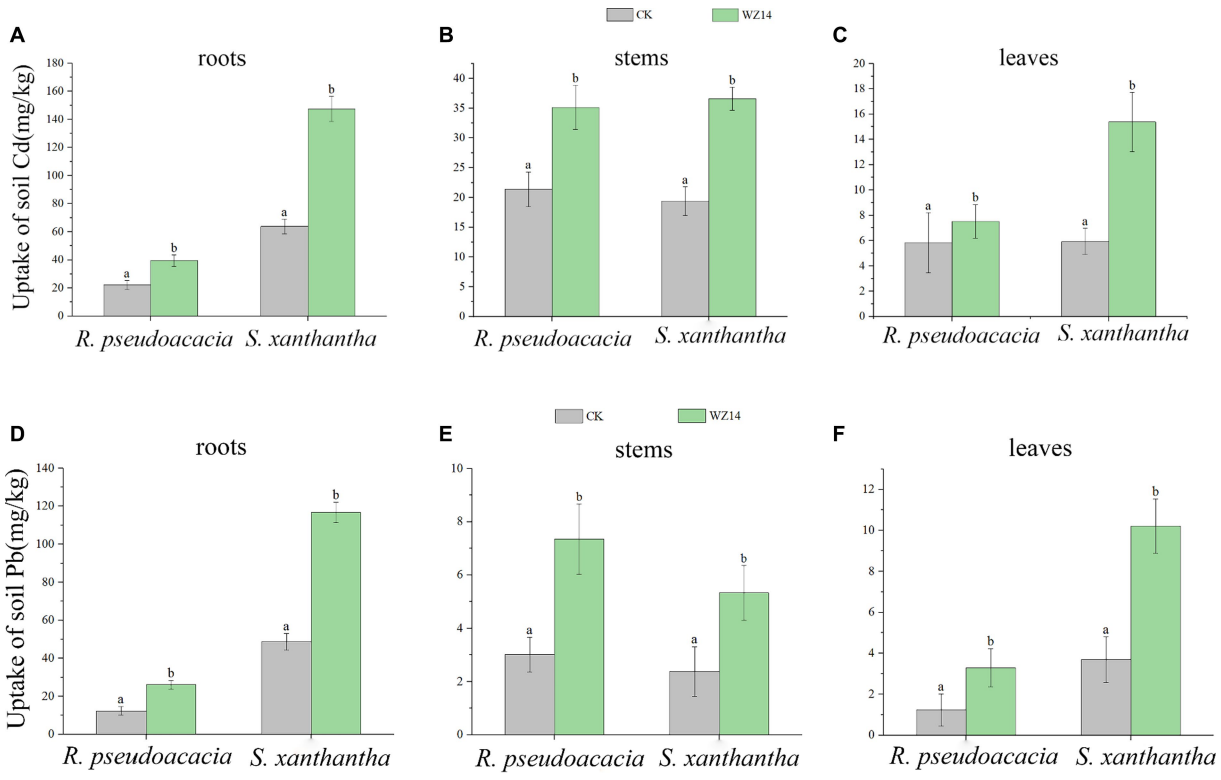

FIGURE 5
Uptake of soil heavy metals Cd **(A–C)** and Pb **(D–F)** by roots, stems, and leaves of *Robinia pseudoacacia L.* and *Sophora japonica*. Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

TABLE 2 **Difference of alpha diversity index of soil bacteria under different treatments.**

Different treatments	Abundance index		Diversity index		Sequencing depth Index
	ACE index	Chao1 index	Simpson index	Shannon index	Coverage
Robinia pseudoacacia	3096.85 ± 12c	2919.08 ± 22c	0.991 ± 0.001a	8.725 ± 0.013b	0.991
WZ14-*R. pseudoacacia*	3130.05 ± 8d	2943.37 ± 11d	0.990 ± 0.001a	8.766 ± 0.009d	0.988
Sophora xanthantha	2881.66 ± 10b	2826.08 ± 9b	0.988 ± 0.001a	8.745 ± 0.021c	0.994
WZ14-*S. xanthantha*	2601.58 ± 9a	2547.43 ± 13a	0.986 ± 0.02a	8.181 ± 0.019a	0.986

Different lowercase letters indicate significant differences between treatments ($P < 0.05$).

reduced. Similar trends were observed in the abundance of *Bacillus* and *Streptomyces* in *R. pseudoacacia* soils, with relative abundances increasing from 1.43% and 1.82% to 2.96% and 2.71%, respectively, after WZ14 application. However, the application of WZ14 did not significantly change the abundance of *Sphingomonas* and *Flavisolibacter* in *R. pseudoacacia* soils.

3.4. Correlation between inter-root bacterial communities and soil environmental factors

Spearman's correlation analysis was used to clarify the relationship between soil bacterial communities and soil physicochemical factors in *R. pseudoacacia* and *S. xanthantha* after WZ14 inoculant application at the phylum and genus levels.

According to Spearman's correlation heat map (Figure 7), *Verrucomicrobia* exhibited significant positive correlations with soil pH, AK, AP, and TN while showing a significant negative correlation with soil solution AN content. On the other hand, *Firmicutes* showed a significant negative correlation with soil pH and AK. Additionally, *Bacteroidota* showed significant positive correlations with OC and AN and negative correlations with soil pH, AP, AK, TN, and TK in *S. xanthantha* soils. *Acidobacteriota*, in the same soils, demonstrated significant negative correlations with OC and AN while positively correlating with AK. At the genus level, environmental factors appeared to mainly affect *Bacillus*, *Steroidobacter*, and *Paenibacillus* in *R. pseudoacacia*. For example, *Bacillus* showed significantly negative correlations with soil pH, AP, AK, and TN, but positive correlations with OC and AN. TP exhibited a significant positive correlation with *Steroidobacter*, and pH showed a negative correlation with *Paenibacillus*. *Flavisolibacter* in *S. xanthantha* soils showed highly

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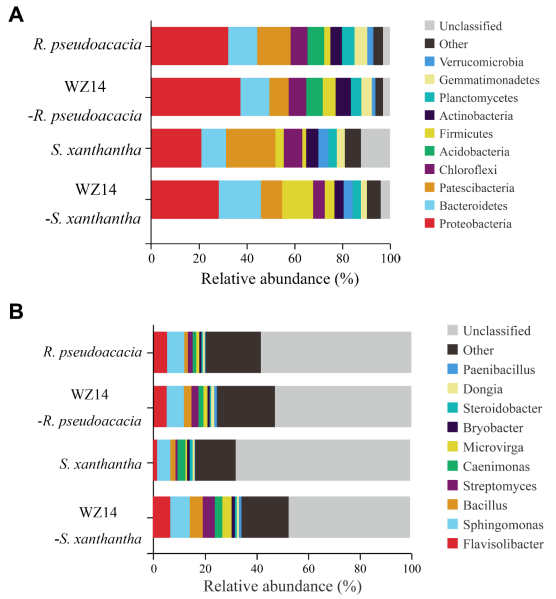

FIGURE 6
Effects of different treatments on the relative abundance of major phyla **(A)** and genera **(B)** of soil bacterial communities.

significant negative correlations with AK and TK while exhibiting positive correlations with OC and AN. *Microvirga* showed significant negative correlations with AK and TK and positive correlations with OC and AN. *Steroidobacter* indicated significant negative correlations with pH, AK, TN, and TK and positive correlations with AN. This study identified that soil Pb content was significantly and positively correlated with *Verrucomicrobia*, but negatively correlated with *Bacteroidota*. The effective state of soil Cd was significantly and negatively correlated with *Gemmatimonadetes*, while the total soil Cd was significantly and negatively correlated with *Bacteroidota*.

4. Discussion

4.1. Effect of soil physicochemical properties under combined remediation

Soil physicochemical properties are fundamental indicators used to characterize soil nutrients, which in turn determine the overall fertility level of the soil. The presence of volatile nutrients, susceptible to environmental influences, directly reflects soil quality (Glick, 2010). In this context, microorganisms act as essential "regulators" of geobiochemical processes, playing a vital role in maintaining soil vitality and ecological functions (Liu et al., 2023).

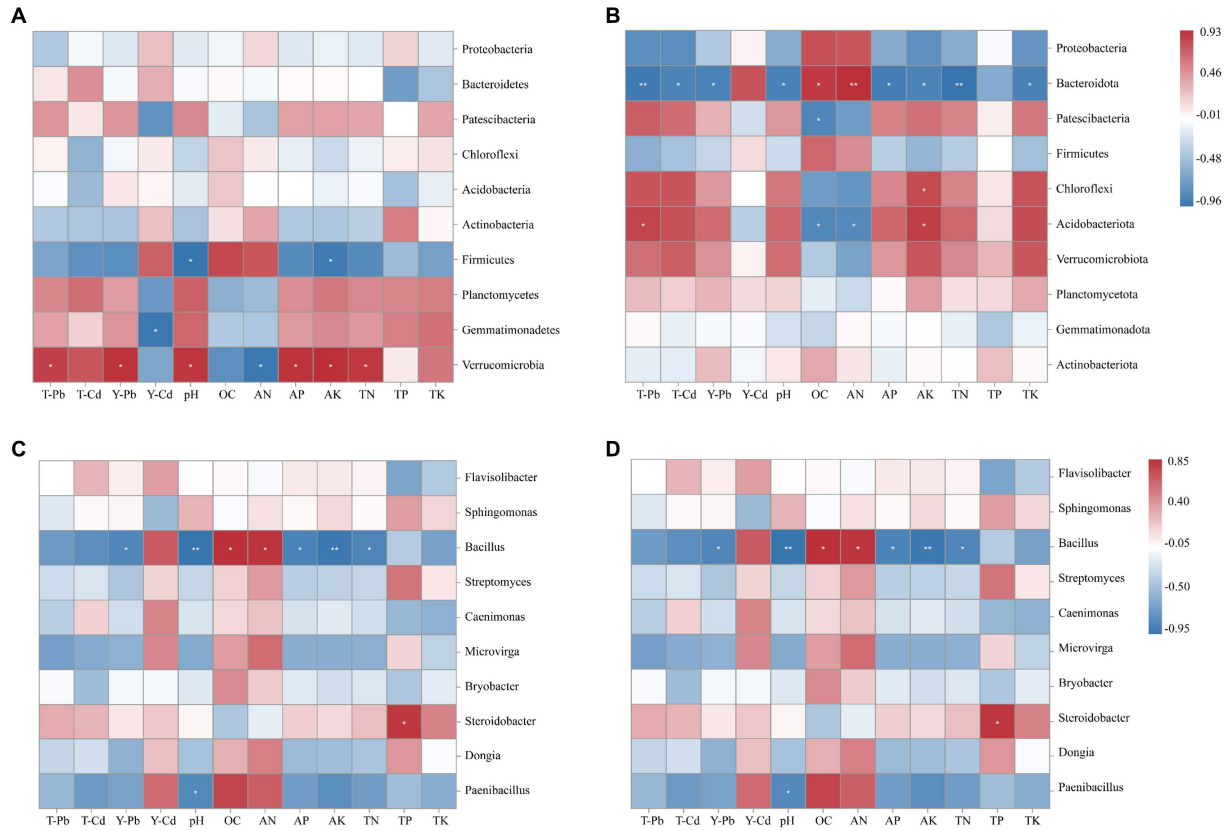

FIGURE 7
Spearman analysis between soil physicochemical properties in *Robinia pseudoacacia* **(A,C)** and *Sophora xanthantha* **(B,D)** and soil bacterial community at phylum and genus levels. Color represents R value; * indicates $p < 0.05$; ** indicates $0.05 \leq p \leq 0.01$; *** indicates $p < 0.01$.

The total soil nutrient content, including total N and total P, and fast-acting nutrients demonstrated a decrease but without significant difference, consistent with the findings of Hussain et al. (2018). Soil nutrient loss is directly related to heavy metal contamination, with higher contamination levels leading to more significant loss of soil nitrogen, phosphorus, potassium, and fast-acting nutrients. Moreover, in this study, the observed changes in soil phosphorus content after applying mycorrhizal fungi can be attributed to the enhancement of plant nutrient uptake by these fungi. This, in turn, promoted plant growth and optimized the plant's ability to remediate heavy metals through phosphorus solubilization (Table 1; Guo et al., 2013; Hu et al., 2019). Soil pH changes can directly affect the effective levels of soil nutrients, microbial activities, and the toxic effects of heavy metal ions. Under Cd and Pb contamination, both monoculture and combined remediation improved the soil organic carbon levels to some extent, with the combined system of inoculated bacteria and legumes showing the best results. However, propagating plants under heavy metal contamination reduced the alkaline-dissolved nitrogen levels of the soil. The inoculation of bacteria in plant monocultures inhibited the declining impact of plants on soil alkaline digestion of nitrogen. It also mitigated the decline in soil alkaline-dissolved nitrogen, consistent with the findings of Cui (2019) and Hussain et al. (2018). This may be attributed to the ability of inoculation treatment to enhance the accumulation of soil carbon and nitrogen nutrient to varying degrees, promote plant root growth, and improve the antagonistic ability of plants.

4.2. Effect of soil Pb and Cd uptake under combined remediation

Heavy metals in soil exist in various forms. The exchangeable form of heavy metals in soil is highly mobile and easily absorbed by plants, unlike the organic, carbonate, and ferro-manganese oxidation forms, which are less readily absorbed. Determination of the effective state content of heavy metals is an effective way to determine the extent of heavy metal contamination and to predict the impact of heavy metals on ecosystems (Zhou et al., 2017). Therefore, the primary objective of remediation is to reduce the amount of active heavy metals in the soil, which in turn affects their crop uptake (Wang et al., 2020). Numerous studies have demonstrated that the interaction between plant and microorganisms can enhance plant biomass and improve heavy metal tolerance, facilitating the absorption, fixation, and reduction of heavy metal concentrations in the soil. This process reduces the toxic effects of heavy metals (Khan et al., 2017; Fu et al., 2022; Zheng et al., 2022a). In this study, the combined treatment with WZ14 was more effective than individual plant treatments. The roots of *R. pseudoacacia* and *S. xanthantha* showed significantly higher levels of Pb and Cd than stems and leaves, which were the primary sites of soil Pb and Cd uptake. This difference was due to the limited translocation capacity of non-enriched plants, leading to the accumulation of Pb and Cd in their roots. These findings aligned with previous research (Sun and Mao, 2015).

Microorganisms in the soil play a crucial role in both the formation of soil humus and the mineralization of organic matter, the uptake and accumulation of soil Pb and Cd by *R. pseudoacacia* and *S. xanthantha* were strongly influenced by soil properties and fertility. In this study, the addition of WZ14 bacterial agent increased the OC content of soils occupied by *R. pseudoacacia* and *S. xanthantha*. Additionally, it enhanced the metabolic processes of inter-root microorganisms and their byproducts. These changes influenced the migration and release mechanisms of Pb and Cd, leading to reduced toxicity of heavy metals in the soil.

4.3. Correlation analysis of soil environmental factors and soil bacterial communities

Strong correlations exist between plant and soil microbial communities, with plant root secretions and residues influencing the function and structure of soil microbial communities (Bian et al., 2018). In this study, the addition of WZ14 bacterial agent had contrasting effects on soil bacterial communities of *R. pseudoacacia* and *S. xanthantha*. Although it enriched the richness and diversity of soil bacterial communities in *R. pseudoacacia*, it reduced the richness and diversity in *S. xanthantha*. These changes could be due to short-term responses of bacterial communities to environmental and pollution changes, reflecting their microenvironmental conditions (Yao et al., 2019). Although the exogenous soil microorganism WZ14 partially promoted the development of heavy metal-tolerant microorganisms, it also intensified competition among soil microorganisms. Consequently, less adaptable microorganisms struggled to cope with environmental changes and experienced a decline in abundance and diversity (Zhang et al., 2020).

The majority of bacterial communities in this study showed a significant negative correlation with soil physicochemical factors, possibly due to the ability to decompose and utilize soil nutrients for energy, either for themselves or plants (Yao et al., 2022). The results of this study revealed that Proteobacteria had the highest relative abundance in both monoculture and grafted-plant combination models, indicating the prevalence of Proteobacteria in heavy metal-contaminated soils and their tolerance to high levels of Cd and Pb contamination (Gupta et al., 2017). Proteobacteria emerged as the dominant phylum in the heavy metal-contaminated soil of the mining area and positively contributed to improving soil contamination status. However, its relative abundance was negatively correlated with the overall soil quality (Yu et al., 2020; Ma J. D. et al., 2022). In contrast, our study discovered the highest abundance of Proteobacteria in the soil of *R. pseudoacacia* and *S. xanthantha* when the heavy metal contamination level decreased after the application of the microbial inoculant. This discrepancy may arise from the specific focus of our experiment on a combined Pb and Cd contamination pattern, resulting in different responses of bacterial communities to Pb and Cd under heavy metal-contaminated conditions.

5. Conclusion

Microorganisms residing in heavy metal environments for extended periods have developed a notable tolerance and resistance to heavy metal ions. In order to address heavy metal contamination in soil, one of the effective approaches can be exploring heavy

metal-resistant bacteria for their capacity to absorb and accumulate heavy metals (Zheng et al., 2022b). In this study, we successfully isolated and purified an efficient Cd- and Pb-tolerant strain from the contaminated soil of lead-zinc-silver mine in Zhijiadi, Shanxi Province. This strain exhibited robust acclimation in high Pb^{2+} concentration cultures (2,000 mg/L and 1,500 mg/L), with peak values ranging from 2.92 cfu·mL^{-1} to 3.14 cfu·mL^{-1}. Additionally, it demonstrated strong viability and adaptability in Cd^{2+} cultures at varying concentration gradients, showing growth even at concentrations exceeding 100 mg/L, with growth ranging from 1.45 cfu·mL^{-1} to 2.77 cfu·mL^{-1}. Both *R. pseudoacacia* and *S. xanthantha* are suitable for cultivation in heavy metal-contaminated soils, as they can absorb and translocate heavy metals, such as Pb and Cd, resulting in an improved soil microenvironment. Combined with crop-applied WZ14 inoculants, the remediation efficacy was enhanced, resulting in increased soil AN and OC levels and decreased total Pb and active heavy metals compared to monoculture. Furthermore, the application of WZ14 significantly facilitated the uptake of Cd by *S. xanthantha*, particularly in the roots, where the Cd uptake reached 147.44 mg/kg after microbial inoculant. This represented a substantial increase of 130.79% compared to monoculture, signifying the root's significant role in the remediation of soil Cd contamination by *S. xanthantha*. The community structure of soil microorganisms in heavy metal-contaminated areas exhibited significant differences between planting and combined remediation treatments, resulting in notable changes in microbial abundance and diversity. The combination of *R. pseudoacacia* monoculture with WZ14-*R. pseudoacacia* proved to be particularly effective in enhancing microbial diversity, as the WZ14-*R. pseudoacacia* soils displayed the highest abundance and diversity of microorganisms compared to other treatments. In heavy metal-contaminated soils, the dominant bacterial phyla were identified as *Proteobacteria*, *Bacteroidetes*, *Patescibacteria*, *Chloroflexi*, and *Acidobacteria*. The inoculation with WZ14 significantly increased the relative abundance of *Proteobacteria* and *Firmicutes*. Soil microorganisms have been proven crucial in remediating heavy metal-contaminated soils, with combined mycorrhizal agents and plant remediation proving highly promising for reducing heavy metal content and enhancing the soil environment.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI—OR492361, PRJNA1012100.

Author contributions

KZ: Conceptualization, Data curation, Writing – original draft. ZL: Investigation, Software, Writing – review & editing. CL: Formal Analysis, Methodology, Supervision, Writing – review & editing. JL: Methodology, Supervision, Writing – review & editing. JZ: Funding acquisition, Project administration, Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Brassinosteroids as promoters of seedling growth and antioxidant activity under heavy metal zinc stress in mung bean (*Vigna radiata L.*)

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The escalation of harmful pollutants, including heavy metals, due to industrialization and urbanization has become a global concern. To mitigate the negative impacts of heavy metal stress on germination and early plant development, growth regulators have been employed. This study aimed to evaluate the response of mung bean (*Vigna radiata L.*) to zinc stress in the presence of brassinosteroids, focusing on seedling growth and antioxidant potential. Mung bean seedlings were treated with three concentrations of 24-epibrassinolide (EBL) (0.1, 0.2, and 0.4 PPM) with or without zinc. Results demonstrated that the application of brassinosteroids, combined with zinc stress, significantly enhanced germination percentage (about 47.06, 63.64, and 120%), speed of germination (about 39.13, 50, and 100%), seedling growth (about 38% in case of treatment combined 0.4 PPM 24-EBL and 1.5mM $ZnSO_4$) and seedling vigor index (204% in case of treatment combined 0.4 PPM 24-EBL and 1.5mM $ZnSO_4$) compared to zinc-treated seedlings alone after 24 h. The activities of antioxidative enzymes (catalase, ascorbate peroxidase, polyphenol oxidase, and peroxidase) and total soluble protein content decreased, while lipid peroxidation and proline content exhibited a significant increase ($p \leq 0.05$) when compared to the control. However, the negative effects induced by heavy metal stress on these parameters were significantly mitigated by EBL application. Notably, the most effective concentration of EBL in overcoming zinc stress was found to be 0.4 PPM. These findings underscore the potential of exogenously applied brassinosteroids as a valuable tool in phytoremediation projects by ameliorating heavy metal stress.

KEYWORDS

brassinosteroids, EBL, mung bean, antioxidative enzymes, zinc, heavy metal

1. Introduction

Contamination of soil and water by heavy metals poses a significant concern and is primarily a result of human activities such as mining, sewage sludge disposal and increased emissions from vehicles and industries (Zorpas et al., 2021). Heavy metals, which generally fall under the category of transition metals, consist of various chemical elements. While some lighter heavy metals like

Zn, Co, Cu, Ni, Mn, Mo, and Fe are essential, the majority of them are non-essential. However, all heavy metals can potentially be hazardous depending on their bioavailability levels and the susceptibility of the exposed organism. The detrimental effects of heavy metals extend beyond humans and animals, affecting a wide range of species, including plants. Excessive exposure to heavy metals leads to reduced biomass, leaf chlorosis, delayed root development and morphological abnormalities in plants, ultimately resulting in plant death (Patel et al., 2021). Plants generate reactive oxygen species at different sites within their respiratory and photosynthetic electron transport chains in response to phytotoxic effects. This process induces oxidative stress in cellular systems. Plants have developed a highly efficient antioxidative defense mechanism, encompassing low-molecular-weight antioxidants, to counteract the accumulation of these reactive molecules.

Various approaches, including conventional plant breeding and modern genome editing, have been employed at different times to confer stress resistance in plants. However, due to the advantages and disadvantages associated with these methods, a singular viable solution to address this problem has yet to be discovered. Traditional breeding and marker-assisted selection are cost-effective and widely recognized methods for achieving desirable outcomes (Sharma et al., 2021). Nonetheless, they are time-consuming and face challenges such as reproductive barriers and limited genetic variability. Modern techniques like genetic engineering and genome editing offer potential solutions for stress resistance in plants, but they encounter significant obstacles related to ethical concerns, including germplasm cross-contamination, health risks associated with consumption and public acceptance (Ahmad et al., 2021; Harfouche et al., 2021). In this context, researchers are compelled to explore environmentally friendly techniques that can yield desired results in a shorter timeframe.

Plant growth regulators, specifically brassinosteroids (BRs), naturally occur in trace amounts in all plants and have been extensively studied for their agricultural significance in promoting the growth and yield of various crops. Previous research has also demonstrated the ability of BRs to modulate plant responses to oxidative stress induced by different abiotic stresses (Sharma et al., 2013; Sami et al., 2022; Yaqoob et al., 2022). The potential use of BRs in agriculture lies in their capacity to enhance crop productivity while mitigating abiotic stress. While numerous studies have examined the combined effects of plant hormones on crop performance in normal conditions, limited attention has been paid to the influence of BRs during heavy metal stress.

Mung bean (*Vigna radiata L.*), commonly referred to as green gram, holds a significant position as a traditional crop. It distinguishes itself from other pulses due to its elevated nutritional value. The seeds of *V. radiata* boast a considerable protein content, with dried green gram seeds containing 24.7% protein, 0.6% fat, 0.9% fiber, and 3.7% ash (Maddi and Tayde, 2022). Notably, the mung bean crop forms a symbiotic relationship with specific bacteria, enabling N2 fixation within root nodules to fulfill the plant's nitrogen requirements. V. radiata finds cultivation across various regions in India, predominantly as a kharif crop. However, in regions with mild winters, like the southern parts, it is grown as a rabi crop. An annual rainfall ranging from 60 to 90 cm proves favorable for this crop. While adaptable to different soil types, mung beans thrive in medium loamy soils. Given the crop's significance, the current research delves into the interaction between brassinosteroids and zinc, aiming to comprehend their combined impact on seedling growth and the antioxidative defense system during mung bean germination. This study sheds light on the stress-relieving qualities of BRs in this specific context.

2. Materials and methods

2.1. Chemicals

The chemicals used in this study were procured from M/s Sigma-Aldrich Chemical Co., USA, SRL, Hi-Media Laboratories, Qualigens.

2.2. Plant material

Mung bean seeds obtained from the local market were subjected to a washing process using distilled water and then surface sterilized with 0.1% $HgCl_2$ for 5 min. Following sterilization, the seeds were rinsed multiple times with sterile distilled water and allowed to imbibe in distilled water for a period of 12 h. For each treatment and control condition, six petri dishes with a diameter of 15 cm were prepared by placing two layers of Whatman No. 1 filter papers. In each Petri dish, ten healthy seeds of similar size were carefully selected and sown.

To create the experimental conditions, different concentrations of 24-EBL alone, $ZnSO_4$ alone and a combination of both were prepared and added to the respective Petri plates, with each solution measuring 20 mL (Table 1). The Petri dishes were then placed in a dark room at a temperature of 25 ± 2°C. The different concentrations of 24-EBL (0.1, 0.2 and 0.4 PPM) were labeled as T1, T2 and T3, respectively. The concentrations of $ZnSO_4$ (0.5, 1.0 and 1.5 mM) were labeled as T4, T5 and T6, respectively. The combinations of both compounds were labeled as T7 (T1 + T4), T8 (T2 + T5) and T9 (T3 + T6), respectively.

2.3. Germination and seedling length

The germination percentages and speed of germination were assessed by recording seed germination counts after 24 h (Soares et al., 2020). Radicle development was considered as the indicator of germination. Additionally, the length of seedlings was measured at 24, 72, and 96 h to calculate the seedling vigor index as per the method of Zhao (2016), with the control group serving as the reference for comparison. The following formula was used to

TABLE 1 Details of experimental treatments.

S. No.	Treatments	Descriptions
1	Control	Double distilled Water
2	T1	0.1 PPM 24-EBL
3	T2	0.2 PPM 24-EBL
4	T3	0.4 PPM 24-EBL
5	T4	0.5 mM $ZnSO_4$
6	T5	1.0 mM $ZnSO_4$
7	T6	1.5 mM $ZnSO_4$
8	T7	0.1 PPM 24-EBL + 0.5 mM $ZnSO_4$
9	T8	0.2 PPM 24-EBL + 1.0 mM $ZnSO_4$
10	T9	0.4 PPM 24-EBL + 1.5 mM $ZnSO_4$

calculate the seed germination percentage, speed of germination and seedling vigor index (SVI):

$$\text{Germination percentages} = \frac{\text{No.of seed germinated}}{\text{Total no.of seed}} \times 100$$

$$\text{Speed of germination} = \Sigma \frac{\text{Nt}}{\text{t.}}$$

Where,
Nt = total number of seed germination in time
t = day of germination

$$\text{Seedling vigor index} = \text{Germination percentage} \times \text{Seedling length (cm)}$$

2.4. Extraction of enzymes

For the analysis of various enzyme activities and other parameters, fresh seedlings were collected at different time points: 2, 4 and 6 days after germination. Three biological replicates and three technical replicates were used. Enzymes such as catalase (CAT) and ascorbate peroxidase (APX) were extracted from germinated seeds (1 g) by homogenizing them in 10 mL of extraction buffer (50 mM potassium phosphate buffer, pH 7.0) using a chilled mortar and pestle. The resulting homogenate was then subjected to centrifugation at 10,000 × *g* for 15 min at 4°C and the supernatant was collected for the determination of CAT and APX activities.

The extraction procedure for peroxidase (POX) and polyphenol oxidase (PPX) enzymes was similar to that used for CAT and APX, with the exception of using a different buffer (Sharma et al., 2013). In this case, a 0.1 M, pH 6.8 buffer was utilized instead of the 50 mM, pH 7.0 buffer used previously.

2.5. Quantification of catalase activity

A modified method of the Aebi (1984) was employed to determine the catalase (EC 1.11.1.6) activity. A final reaction volume of 3 mL was achieved by combining 1.4 mL of 50 mM potassium phosphate buffer having pH 7.0, 1.5 mL of 12.5 mM H_2O_2 and 0.1 mL of enzyme extract in order to assess CAT activity. By adding H_2O_2, the reaction was initiated, while a blank sample was concurrently run without the inclusion of enzyme extract. The decrease in H_2O_2 concentration was monitored by measuring the absorbance reduction at 240 nm at 30 s intervals over a 3 min period. This measurement was performed using a double beam UV–VIS spectrophotometer (SPECORD® PLUS 250, Analytik Jena, Germany). One unit (U) of CAT activity was defined as the amount of enzyme catalyzing the decomposition of 1 µmol of H_2O_2 per minute at 240 nm, using an extinction coefficient of 0.036 cm^2 μmol^{-1} for H_2O_2 absorbance at 240 nm. The results were expressed as units (U) of CAT activity per gram of fresh weight (U g^{-1} FW).

2.6. Quantification of ascorbate peroxidase activity

The activity of ascorbate peroxidase (EC 1.11.1.11) was determined using a modified method based on Kumar et al. (2021) protocol. An assay mixture of 3.0 mL was prepared, which included 1.0 mL of 50 mM potassium phosphate buffer (pH 7.0), 1.0 mL of 39 mM H_2O_2, 0.8 mL of 0.5 mM ascorbic acid and 0.2 mL of the enzyme extract. The initiation of the reaction occurred by adding hydrogen peroxide, while a blank sample was concurrently prepared in the absence of the enzyme extract. Over a period of 3 min at 30 s intervals, the rate of ascorbate oxidation at 290 nm was monitored to measure the activity of APX. This measurement was conducted using a double beam UV–VIS spectrophotometer (SPECORD® PLUS 250, Analytik Jena, Germany). One unit of APX activity was defined as the amount required to decompose 1 µmol ascorbic acid oxidized min^{-1} calculated from the extinction coefficient of 2.6 mM^{-1} cm^{-1}. The results were expressed as U g^{-1} FW.

2.7. Quantification of POX activity

The peroxidase (EC 1.11.1.7) activity was assessed using the method described by Kumar et al. (2021). In this assay, the reaction mixture consisted of o-dianisidine (2.4 µmol), H_2O_2 (20 µmol), crude extract (0.05–0.5 mg protein) and 0.05 M citrate buffer (pH 4.8), resulting in a final volume of 3 mL. A blank was prepared by excluding H_2O_2 from the incubation mixture. By measuring the absorbance at 430 nm at 15 s intervals, the enzyme activity was determined. The results were reported as U g^{-1} FW.

2.8. Quantification of PPO activity

Polyphenol oxidase (EC 1.14.18.1) activity was determined using the method described by Sharma et al. (2013). In this assay, 1 mL of enzyme extract was mixed with 2.5 mL of 0.1 M phosphate buffer (pH 6.8) and 0.1 mL of 0.01 M pyrogallol. Following the preparation of the mixture, it was incubated at 4°C for 5 min. To stop the reaction, 1.0 mL of 2.5 N H_2SO_4 was added. In the blank sample, enzyme deactivation was achieved by adding 2.5 N H_2SO_4. The measurement of the absorbance at 420 nm was used to determine the amount of purpurogallin formed. One unit (U) of polyphenol oxidase activity corresponds to a 0.01 increase in absorbance at 420 nm per minute caused by the enzyme. The enzyme activity was expressed in U g^{-1} FW.

2.9. Estimation of malondialdehyde

Following the method outlined by El-Moshaty et al. (1993), the estimation of malondialdehyde (MDA) was conducted. Tissues weighing two grams from seedlings on the 2nd, 4th and 6th day were homogenized with 3 mL of 0.2 M citrate–phosphate buffer (pH 6.4) in a chilled mortar and pestle. The homogenate was then centrifuged at 10,000 × *g* for 30 min and the resulting supernatant was used for MDA content estimation. To conduct the estimation, 1 mL of the supernatant was combined with an equal volume of MDA reagent, which consisted of 20% trichloroacetic acid in 5% thiobarbituric acid. The mixture was placed in a water bath set at 95°C and incubated for a duration of 40 min. Following the incubation, the mixture was promptly cooled on ice for 15 min. Subsequently, the mixture was subjected to centrifugation at 10,000 × *g* for 30 min. The absorbance of the resulting supernatant was then measured at 520 and 600 nm. The absorbance at 600 nm was subtracted from the

absorbance at 520 nm to eliminate non-specific absorbance. Using the extinction coefficient of 155 mM^{-1} cm^{-1}, the content of malondialdehyde was calculated. The results were expressed in nanomoles of MDA per gram of fresh weight (nmoles g^{-1} FW).

2.10. Estimation of soluble proteins

The estimation of soluble proteins was performed following the method described by Lowry et al. (1951). Fresh leaves weighing one gram were homogenized in 5 mL of phosphate buffer (0.1 M, pH 7.0) using a mortar and pestle. The resulting homogenate was then centrifuged at 10,000 × *g* for 20 min and the supernatant was collected. To 1.0 mL of the extracted solution, 1 mL of 20% trichloroacetic acid (TCA) was added. After allowing it to stand for half an hour, the mixture was centrifuged at 5,000 × *g* for 10 min. The resulting pellet was washed twice with acetone, followed by centrifugation as before and the supernatant was discarded. The pellet was then dissolved in 5 mL of 0.1 N NaOH and used for protein estimation. For protein estimation, 1.0 mL of the protein solution obtained above was mixed with 5 mL of freshly prepared alkaline copper sulfate reagent and thoroughly mixed. A blank was prepared using 1 mL of 0.1 N NaOH in place of the protein sample. After incubating for 10 min at room temperature, 0.5 mL of 1 N Folin-Ciocalteau reagent was added and left in the dark for 30 min. The absorbance of the resulting blue color was measured at 660 nm against the blank. The amount of soluble proteins was calculated in mg g^{-1} FW using a standard plot of bovine serum albumin ranging from 0 to 150 µg.

2.11. Estimation of proline content

The estimation of proline content was conducted following the method outlined by Bates et al. (1973). Proline was extracted using sulphosalicylic acid. In the extract, an equal volume of glacial acetic acid and ninhydrin solutions were added. The sample was then heated at 100°C and subsequently cooled. After cooling, 5 mL of toluene was added to the sample. The absorbance of the toluene layer was measured at 528 nm using a spectrophotometer (SPECORD® PLUS 250, Analytik Jena, Germany).

2.12. Statistical analysis

Factorial CRD was used to examine the data for the two factors. SAS (Version 9.3, SAS Institute Inc., Cary, NC, USA) was used to compare treatments and number of days for critical difference (CD) at the 5% level of significance. The mean and standard error were calculated and every analysis was done in triplicate.

3. Results and discussion

3.1. Effect of BR and Zn on germination percentage and speed of germination

The impact of BR and Zn on mung bean's germination percentage and speed of germination varied across different treatment levels, as depicted in Figure 1. There were no significant differences observed in the germination percentage and speed among treatments T1, T2, T3 and the control group. However, an increase in the level of $ZnSO_4$ (from 0.5 mM to 1.5 mM $ZnSO_4$) resulted in a decrease in both germination percentage and germination speed. Conversely, treatments T7 to T9 exhibited an increment in both parameters compared to the $ZnSO_4$ levels alone. Specifically, treatment T7 showed a 39.13% increase in germination speed and a 47.06% increase in germination percentage compared to treatment T4. Similarly, treatment T8 displayed increments of approximately 50 and 63.64%, respectively as compared to treatment T5. Treatment T9 exhibited the maximum improvement, with a 100% increase in germination speed and a 120% increase in germination percentage compared to treatment T6. The outcomes highlight the intricate interplay between BR and Zn concentrations in influencing mung bean germination. While $ZnSO_4$ at higher concentrations appeared to suppress germination, the inclusion of BR counteracted these inhibitory effects, leading to improved germination attributes. This suggests a potential synergistic effect between BR and lower Zn concentrations. Numerous studies have demonstrated that BR treatment significantly ($p < 0.05$) enhances seed germination percentage and germination speed. An instance of this can be seen in the study conducted by Basit et al. (2021), where they discovered that EBL treatment had a significant positive impact on the germination characteristics of rice. Likewise, Basit et al. (2022b) observed improved seed germination percentage and other related parameters in soybean seeds treated with EBL when exposed to chromium toxicity.

3.2. Effect of BR and Zn on seedling growth and seedling vigor index

The effect of BR and Zn on seedling growth and seedling vigor index (SVI) is presented in Table 2. A significant reduction in seedling length and SVI was observed with an increase in heavy metal concentration (from 0.5 mM to 1.5 mM $ZnSO_4$) compared to the control condition after 24 h. Similar trends were observed for seedling length and SVI after 72 and 96 h. These results strongly suggest that elevated $ZnSO_4$ concentrations have a substantial inhibitory effect on seedling growth and vigor, potentially hindering the initial stages of seedling development. In contrast, individual BR treatments (T1 to T3) did not yield statistically significant differences in terms of seedling length and SVI after 24, 72, and 96 h. This suggests that, within the scope of this study, BR application alone did not lead to observable effects on seedling growth and vigor. This could imply that BR alone might not be a sole determinant of seedling development and that its influence might be subject to interactions with other factors. Conversely, the results take an intriguing turn when considering combined BR and Zn treatments (T7 to T9). These combined treatments showcased a clear positive impact, leading to enhanced seedling length and SVI compared to treatments with Zn alone (T4 to T6) across all time intervals. This phenomenon suggests a potential synergistic effect between BR and Zn in promoting seedling growth and vigor. This observation aligns with the complex interplay of plant hormones and essential nutrients in influencing plant physiology. The maximum improvement was observed in treatment T9, with approximately 38% increase in seedling length and approximately 204% increase in SVI compared to treatment T6. These findings align with

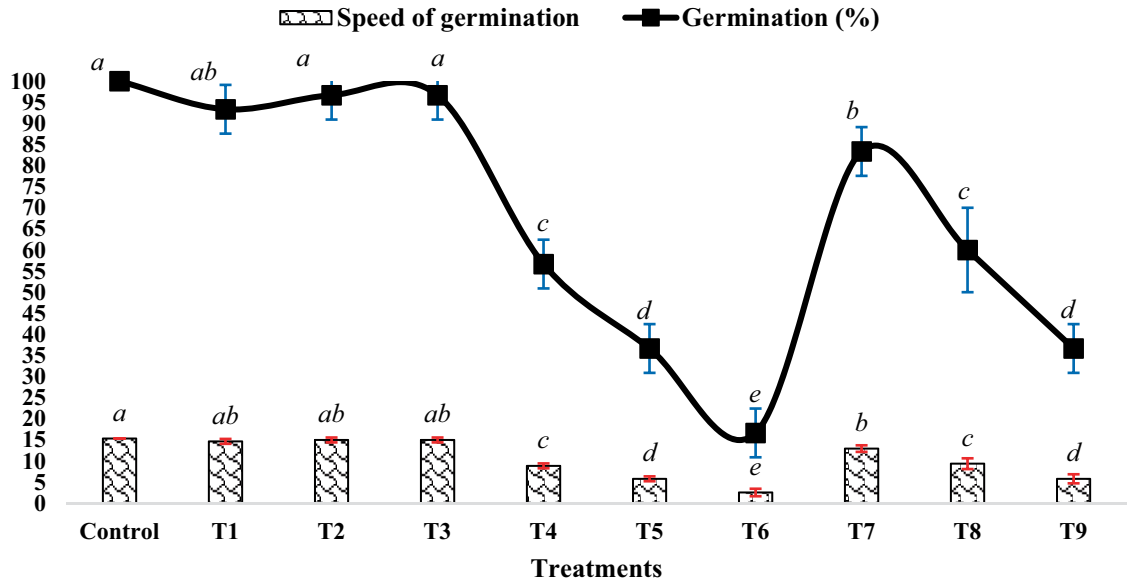

FIGURE 1
Effect of BR with or without $ZnSO_4$ on germination percentage and speed of germination after 24 h of treatment. The shown data are the means of three replications, and the error bars are the standard errors of the means. Alphabets on line and each column indicate the level of significance of at $p < 0.05$. (Where, T1–0.1 PPM 24-EBL; T2–0.2 PPM 24-EBL; T3–0.4 PPM 24-EBL; T4–0.5 mM $ZnSO_4$; T5–1.0 mM $ZnSO_4$; T6–1.5 mM $ZnSO_4$; T7–0.1 PPM 24-EBL + 0.5 mM $ZnSO_4$; T8–0.2 PPM 24-EBL + 1.0 mM $ZnSO_4$; T9–0.4 PPM 24-EBL + 1.5 mM $ZnSO_4$).

TABLE 2 Effect of BR with or without ZnSO4 on seedling length and seedling vigor index after 24, 72, and 96 h.

	Seedling length (cm)			Seedling vigor index (SVI)		
	24 h	72 h	96 h	24 h	72 h	96 h
Control	4.1 ± 0.16^{cd}	8.7 ± 0.32^{cd}	12.5 ± 0.35^{cde}	396 ± 10.30^{ab}	870 ± 32.47^{bc}	$1{,}250 \pm 34.70^{bc}$
T1	4.6 ± 0.20^{abc}	9.2 ± 0.17^{bc}	12.9 ± 0.44^{bc}	446 ± 44.06^{ab}	920 ± 16.59^{ab}	$1{,}290 \pm 44.19^{ab}$
T2	4.9 ± 0.10^{ab}	9.6 ± 0.19^{ab}	13.4 ± 0.40^{ab}	490 ± 9.72^{ab}	960 ± 19.04^{ab}	$1{,}340 \pm 39.86^{ab}$
T3	5.1 ± 0.20^{a}	10.2 ± 0.30^{a}	14.0 ± 0.24^{a}	510 ± 20.01^{a}	$1{,}020 \pm 30.01^{a}$	$1{,}400 \pm 23.98^{a}$
T4	3.8 ± 0.06^{de}	7.4 ± 0.18^{e}	11.9 ± 0.53^{e}	215 ± 18.98^{de}	447 ± 10.97^{de}	714 ± 31.54^{d}
T5	3.3 ± 0.09^{ef}	6.4 ± 0.17^{f}	11.1 ± 0.12^{f}	121 ± 16.32^{ef}	256 ± 6.92^{f}	444 ± 4.80^{e}
T6	2.9 ± 0.07^{f}	5.9 ± 0.20^{f}	10.4 ± 0.36^{g}	48 ± 16.09^{f}	98 ± 31.74^{g}	175 ± 64.64^{f}
T7	4.6 ± 0.09^{abc}	9.0 ± 0.18^{bc}	13.0 ± 0.12^{bc}	383 ± 19.07^{bc}	780 ± 50.84^{c}	$1{,}127 \pm 82.42^{c}$
T8	4.3 ± 0.07^{bcd}	8.6 ± 0.38^{cd}	12.6 ± 0.35^{cd}	258 ± 39.04^{cd}	545 ± 59.96^{d}	798 ± 81.02^{d}
T9	4.0 ± 0.15^{cd}	8.2 ± 0.27^{d}	12.1 ± 0.26^{de}	146 ± 19.97^{def}	327 ± 74.03^{ef}	485 ± 127.34^{e}

Values represent the mean ± SE. Alphabets in superscript indicate the level of significance of at $p < 0.05$; Where, T1–0.1 PPM 24-EBL; T2–0.2 PPM 24-EBL; T3–0.4 PPM 24-EBL; T4–0.5 mM ZnSO4; T5–1.0 mM ZnSO4; T6–1.5 mM ZnSO4; T7–0.1 PPM 24-EBL + 0.5 mM ZnSO4; T8–0.2 PPM 24-EBL + 1.0 mM ZnSO4; T9–0.4 PPM 24-EBL + 1.5 mM ZnSO4.

the results reported by Basit et al. (2022a), who observed that rice under chromium stress exhibited decreased seedling length and vigor index, but supplementation with BR externally enhanced both parameters. Similarly, Srivastava et al. (2011) demonstrated that the application of BR can stimulate seed germination parameters in mung bean.

3.3. Effect of BR and Zn on CAT activity

The catalase activity in seedlings of mung bean seeds treated with BR was consistently higher compared to the control group. Specifically, among all the treatments of BR alone (T1 to T3), mung bean seedlings exhibited the highest catalase activity at a higher concentration of BR (Treatment T3), which was at 0.4 PPM, across all stages of seedling growth. This trend underscores the concentration-dependent impact of BR on catalase activity, highlighting its potential role as an inducer of antioxidative responses. When mung bean seeds were exposed to varying concentrations of zinc sulfate (Treatments T4 to T6), their catalase activity in seedlings decreased. The reduction in catalase activity suggests a potential interference of $ZnSO_4$ with the normal functioning of antioxidative pathways, which could be attributed to the intricate interactions between heavy metals and plant defense mechanisms. However, when different concentrations of BR were combined with zinc (Treatments T7 to T9), the catalase activity was higher than that observed when zinc sulfate was present alone. This observation is depicted in Figure 2, where the catalase activity in the treatments with

both BR and zinc was higher compared to the treatments with zinc sulfate alone. The results indicate that the treatment with BR stimulated catalase activity, leading to enhanced antioxidative metabolism in the germinated mung bean seedlings. On the other hand, the reduction in CAT activity observed in the presence of zinc sulfate alone suggests that zinc sulfate suppresses the antioxidative metabolism of the seedlings. The findings from previous research studies conducted on other crops, such as those by Arora et al. (2010) and Tadaiesky et al. (2021), have shown a similar effect of brassinosteroids. These studies observed that the application of brassinosteroids resulted in increased CAT activity. In the current study, when BR was applied in combination with zinc sulfate, the catalase activity was higher compared to the presence of zinc alone. This suggests that BR had a mitigating effect on the adverse impact of zinc, further supporting the notion that BR can alleviate the negative effects of zinc on antioxidative metabolism.

3.4. Effect of BR and Zn on APX activity

The activity of ascorbate peroxidase gradually increased under the influence of BR alone (Treatments T1 to T3). This observed trend suggests that BR application might serve as a positive modulator of APX activity, potentially enhancing the antioxidative capacity of mung bean seedlings. This aligns with the established role of BR in promoting various stress responses, including antioxidative defenses, in plants (Kaur et al., 2022; Manghwar et al., 2022). In stark contrast, the response to varying concentrations of zinc sulfate (Treatments T4 to T6) presents an opposing trend. The APX activity decreased as the concentration of zinc sulfate increased. This observation indicates that higher concentrations of zinc sulfate could potentially inhibit the normal functioning of APX, thereby diminishing the seedlings' antioxidative capabilities. The inverse relationship between zinc concentration and APX activity suggests a potential vulnerability of antioxidative pathways to the inhibitory effects of elevated zinc levels. This response was consistent across Day 2, Day 4 and Day 6. The data presented in Figure 3 demonstrates that the decline in APX activity was more pronounced with higher concentrations of zinc sulfate. However, when the combination of BR and zinc was used (Treatments T7 to T9), APX exhibited higher activity compared to the activity observed in the presence of zinc sulfate alone (Treatments T4 to T6). The observed results suggest that the application of both BR and zinc sulfate had a relieving effect on seedlings subjected to zinc metal stress by influencing antioxidative metabolism. The activity of APX is crucial in detoxifying ROS and mitigating oxidative stress. APX plays a significant role in the reduction of hydrogen peroxide to water through the ascorbate-glutathione cycle (Hasanuzzaman et al., 2019). Therefore, the higher APX activity observed in the treatments with both BR and zinc sulfate indicates that they played a role in ameliorating oxidative stress and reducing the detrimental effects of zinc metal stress on the seedlings.

3.5. Effect of BR and Zn on POX

The peroxidase activity in the mung bean increased notably compared to the control when exposed to different concentrations of BR treatments (T1 to T3), except for treatment T2 on Day 2 (Figure 4). Conversely, when different concentrations of zinc were used alone (Treatments T4 to T6), zinc exhibited an inhibitory effect on POX activity, as depicted in Figure 4. However, when BR was applied in combination with zinc (Treatments T7 to T9), the activity of POX was enhanced compared to the presence of zinc alone. This indicates the stress-alleviating effect of BR. Similar to CAT, POX also plays a role in breaking down H_2O_2 into water and oxygen. The results of POX activity in mung bean demonstrated an increase in enzyme activity after the application of BR, either alone or in combination with zinc, at different concentrations. Indeed, other researchers, such as Arora et al. (2010) and Soares et al. (2020), have also reported an increase in POX activity in response to the application of brassinosteroids. POX is involved in the synthesis of lignin and other phenolic polymers. The enhancement in POX activity may serve as a defense mechanism for

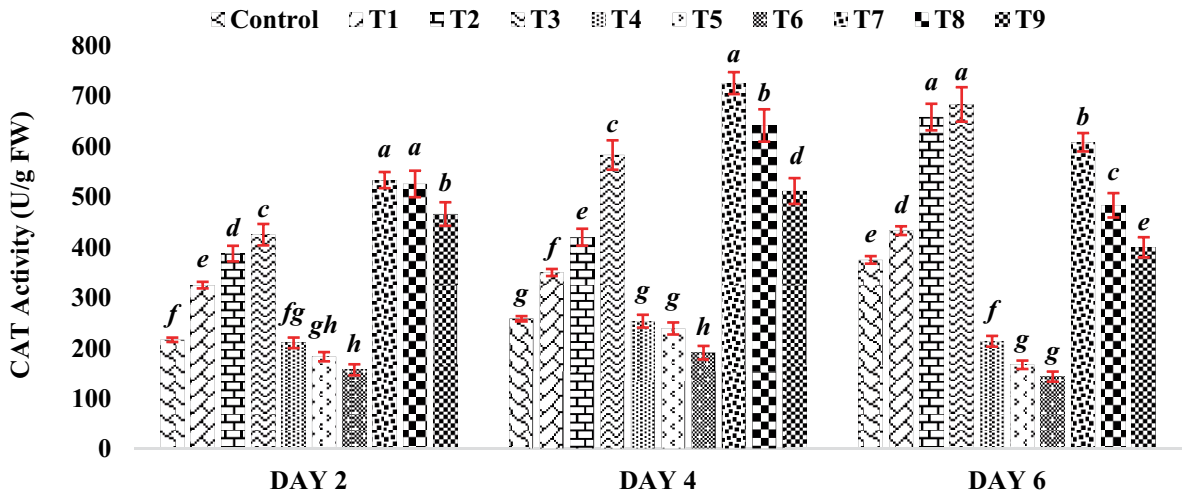

FIGURE 2
Effect of BR with or without $ZnSo_4$ on catalase activity. The shown data are the means of three replications, and the error bars are the standard errors of the means. Alphabets in each column indicate the level of significance of at $p < 0.05$. (Where, T1–0.1 PPM 24-EBL; T2–0.2 PPM 24-EBL; T3–0.4 PPM 24-EBL; T4–0.5 mM $ZnSO_4$; T5–1.0 mM $ZnSO_4$; T6–1.5 mM $ZnSO_4$; T7–0.1 PPM 24-EBL + 0.5 mM $ZnSO_4$; T8–0.2 PPM 24-EBL + 1.0 mM $ZnSO_4$; T9–0.4 PPM 24-EBL + 1.5 mM $ZnSO_4$).

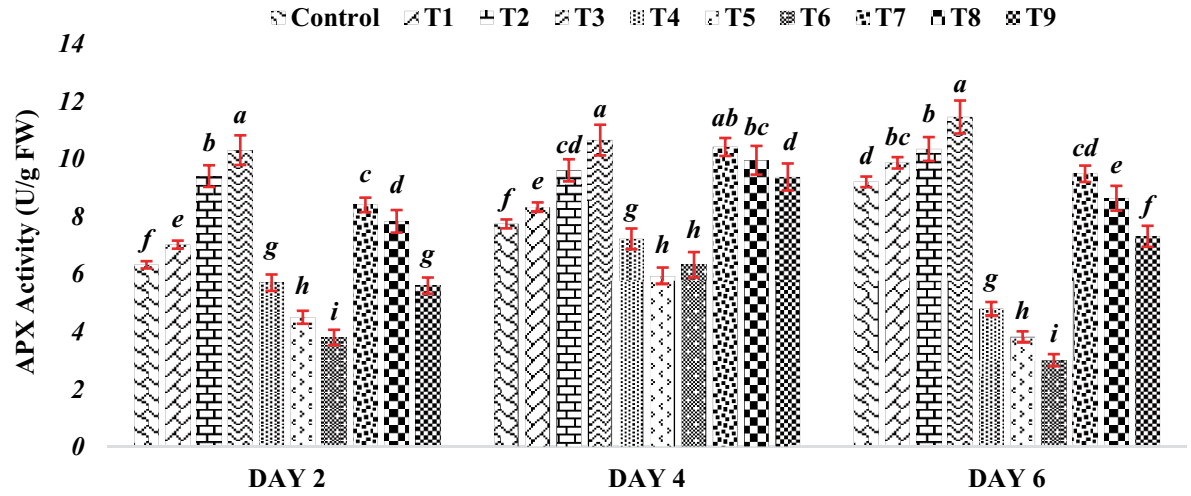

FIGURE 3
Effect of BR with or without $ZnSO_4$ on APX activity. The shown data are the means of three replications, and the error bars are the standard errors of the means. Alphabets in each column indicate the level of significance of at $p < 0.05$. (Where, T1–0.1 PPM 24-EBL; T2–0.2 PPM 24-EBL; T3–0.4 PPM 24-EBL; T4–0.5 mM $ZnSO_4$; T5–1.0 mM $ZnSO_4$; T6–1.5 mM $ZnSO_4$; T7–0.1 PPM 24-EBL + 0.5 mM $ZnSO_4$; T8–0.2 PPM 24-EBL + 1.0 mM $ZnSO_4$; T9–0.4 PPM 24-EBL + 1.5 mM $ZnSO_4$).

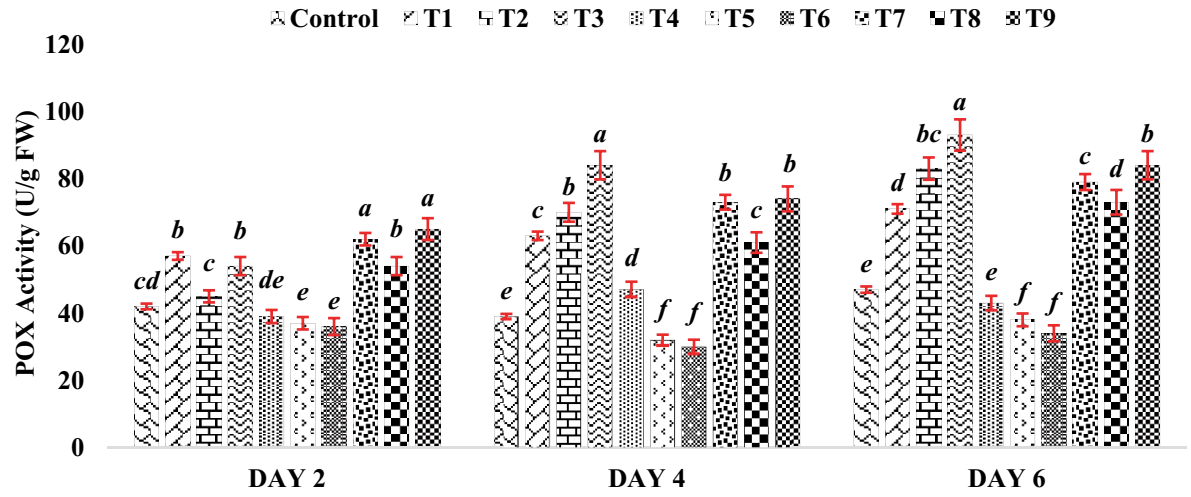

FIGURE 4
Effect of BR with or without $ZnSO_4$ on POX activity. The shown data are the means of three replications, and the error bars are the standard errors of the means. Alphabets in each column indicate the level of significance of at $p < 0.05$. (Where, T1–0.1 PPM 24-EBL; T2–0.2 PPM 24-EBL; T3–0.4 PPM 24-EBL; T4–0.5 mM $ZnSO_4$; T5–1.0 mM $ZnSO_4$; T6–1.5 mM $ZnSO_4$; T7–0.1 PPM 24-EBL + 0.5 mM $ZnSO_4$; T8–0.2 PPM 24-EBL + 1.0 mM $ZnSO_4$; T9–0.4 PPM 24-EBL + 1.5 mM $ZnSO_4$).

cells against harmful concentrations of hydroperoxides, thereby protecting cellular components such as proteins and lipids from oxidation. This notion aligns with the findings of Beauchamp and Fridovich (1971), who suggested that increased POX activity helps safeguard cellular components against oxidation.

3.6. Effect of BR and Zn on PPO activity

In this experiment, the activity of PPO increased as the concentration of BR increased (Treatments T1 to T3) on day 2, 4 and 6 of seedling growth (Figure 5). Among all the BR treatments, the highest PPO activity was observed at a concentration of 0.4 PPM (Treatment T3). However, when mung bean seeds were treated with zinc alone (Treatments T4 to T6), the seedlings showed an inhibitory effect on PPO activity throughout the sampling (Day 2, 4 and 6), as shown in Figure 5. Interestingly, when mung bean seeds were treated with both BR and zinc sulfate (Treatments T7 to T9), the PPO activity was substantially higher compared to the presence of zinc alone (Treatments T4 to T6). This suggests that BR plays a protective role against heavy metal stress, as it enhances PPO activity and counteracts the inhibitory effect of zinc on PPO activity, indicating a potential mechanism for mitigating the negative effects of heavy metals on mung bean seedlings. The results of this experiment indicate that both

BR alone and in combination with different concentrations of zinc metal were effective in enhancing the activity of polyphenol oxidase compared to the control group. This finding is consistent with the observations made by Asghari and Rezaei-Rad (2018), who reported an increase in PPO activity in table grape when treated with BRs. These findings suggest that BRs have a positive impact on PPO activity and may contribute to the improvement of physiological processes related to polyphenol metabolism in plants.

3.7. Effect of BR and Zn on lipid peroxidation

Oxidative stress can lead to lipid peroxidation of the cell membrane, resulting in the production of malondialdehyde. However, in this experiment, it was observed that the content of MDA reduced in germinated mung beans treated with BR (Table 3). The decline in MDA content was more pronounced when BR was applied in higher concentrations (Treatments T1 to T3). This decline in MDA level indicated that BR treatment caused a decrease in lipid peroxidation, implying that BR has antioxidative properties. Conversely, treatment with varying concentrations of zinc metal alone (Treatments T4 to T6) caused an increase in MDA content, indicating an increase in lipid peroxidation. The highest MDA content was observed in treatment T6, which suggests that higher concentrations of zinc induced greater lipid peroxidation. When mung bean seeds were treated with a combination of Zn and BR (Treatments T7 to T9), the content of MDA initially decreased. However, with an increase in the concentration of both Zn and BR, the MDA content increased. This indicates that the combined treatment initially mitigated lipid peroxidation, but at higher concentrations of Zn and BR, there might have been a reversal, resulting in an increase in lipid peroxidation. In summary, the results suggest that BR treatment reduced lipid peroxidation (as evident from the decline in MDA content), while zinc treatment increased lipid peroxidation. The combined treatment of Zn and BR showed a complex effect on MDA content, with an initial decrease followed by a subsequent increase at higher concentrations of both Zn and BR. Previous research by Arora et al. (2010) supports the observation that the level of MDA decreases with an increase in the concentration of BRs in the presence of zinc stress in *Zea mays*.

This decrease in MDA content can be attributed to the enhanced activities of antioxidative enzymes. This suggests that BRs may serve as secondary messengers, triggering the induction of the antioxidant defense system in stressed plants. Consequently, BRs effectively scavenge reactive oxygen species in plants experiencing stress. This aligns with the proposed role of BRs in activating the antioxidant defense system and mitigating oxidative stress in plants under challenging conditions, as suggested by Hu et al. (2021) and Kretynin et al. (2021).

3.8. Effect of BR and Zn on total soluble proteins content

The level of total soluble proteins in mung bean seedlings was influenced by the application of BRs (Table 3). It was observed that there was an increase in the soluble protein content, indicating that the BR responses were dependent on protein synthesis. This finding is in line with the suggestion made by Siddiqi and Husen (2021), who proposed that BRs can modulate protein synthesis and contribute to changes in the level of soluble proteins. The increase in soluble protein content suggests that BRs may play a role in regulating protein metabolism and synthesis in mung bean seedlings, potentially

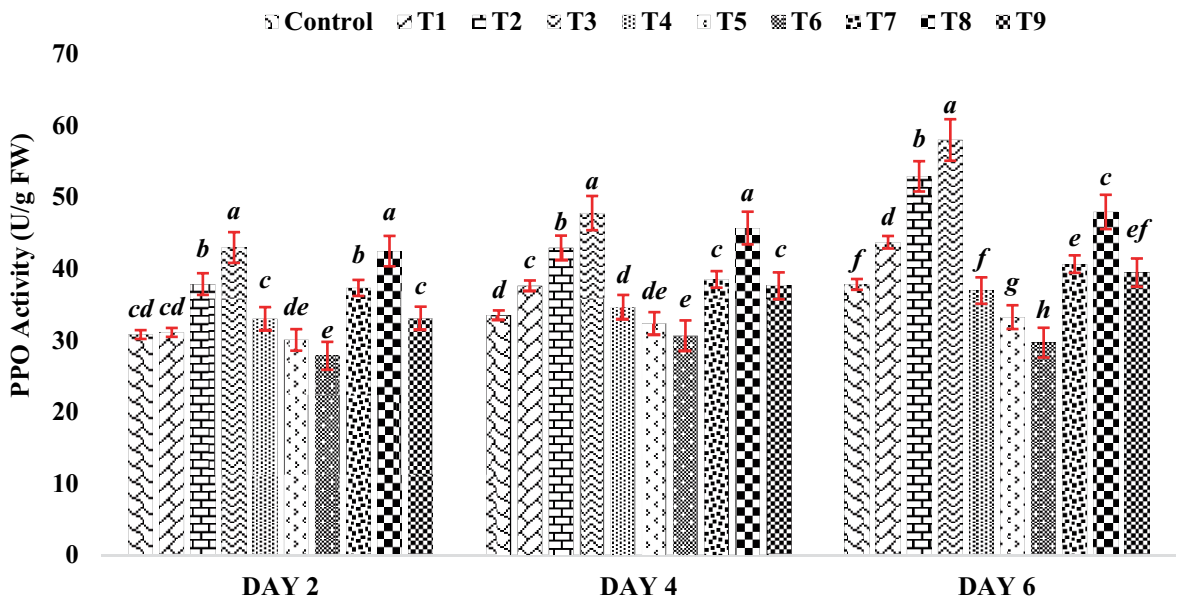

FIGURE 5
Effect of BR with or without $ZnSO_4$ on PPO activity. The shown data are the means of three replications, and the error bars are the standard errors of the means. Alphabets in each column indicate the level of significance of at $p < 0.05$. (Where, T1–0.1 PPM 24-EBL; T2–0.2 PPM 24-EBL; T3–0.4 PPM 24-EBL; T4–0.5 mM $ZnSO_4$; T5–1.0 mM $ZnSO_4$; T6–1.5 mM $ZnSO_4$; T7–0.1 PPM 24-EBL + 0.5 mM $ZnSO_4$; T8–0.2 PPM 24-EBL + 1.0 mM $ZnSO_4$; T9–0.4 PPM 24-EBL + 1.5 mM $ZnSO_4$).

TABLE 3 Effect of BR with or without $ZnSO_4$ on MDA, total soluble protein and proline content.

	MDA content (nmoles/g FW)			Total soluble protein (mg/g FW)			Proline content (μg/g FW)		
	Day 2	Day 4	Day 6	Day 2	Day 4	Day 6	Day 2	Day 4	Day 6
Control	13.10 ± 0.07^{de}	17.40 ± 0.16^{ab}	15.70 ± 0.05^{b}	45.79 ± 0.42^{fg}	76.83 ± 2.69^{de}	88.67 ± 1.55^{de}	5.12 ± 0.22^{gh}	6.22 ± 0.07^{f}	7.35 ± 0.22^{g}
T1	12.87 ± 0.24^{e}	16.80 ± 0.34^{bc}	15.40 ± 0.33^{b}	68.00 ± 1.15^{c}	83.97 ± 1.05^{c}	94.51 ± 1.61^{c}	4.64 ± 0.17^{h}	6.73 ± 0.13^{ef}	7.87 ± 0.03^{fg}
T2	11.21 ± 0.56^{f}	15.60 ± 0.43^{de}	15.20 ± 0.63^{b}	79.56 ± 1.50^{b}	98.55 ± 0.27^{b}	107.99 ± 3.47^{b}	5.53 ± 0.04^{g}	7.60 ± 0.21^{d}	8.71 ± 0.13^{e}
T3	9.83 ± 0.43^{g}	14.80 ± 0.37^{e}	15.10 ± 0.54^{b}	93.77 ± 3.02^{a}	114.78 ± 2.47^{a}	134.97 ± 3.14^{a}	6.79 ± 0.28^{ef}	8.85 ± 0.36^{bc}	9.94 ± 0.04^{bcd}
T4	14.40 ± 0.72^{bc}	17.10 ± 0.36^{bc}	14.60 ± 0.07^{b}	53.54 ± 1.29^{de}	81.61 ± 0.73^{cd}	93.84 ± 2.43^{cd}	6.21 ± 0.26^{f}	7.33 ± 0.21^{de}	8.50 ± 0.33^{ef}
T5	15.20 ± 0.09^{ab}	17.25 ± 0.40^{abc}	15.30 ± 0.44^{b}	49.75 ± 0.93^{ef}	72.58 ± 1.75^{e}	83.80 ± 2.24^{ef}	7.43 ± 0.29^{de}	8.58 ± 0.29^{c}	9.75 ± 0.04^{cd}
T6	16.30 ± 0.10^{a}	18.24 ± 0.13^{a}	16.90 ± 0.72^{a}	42.70 ± 0.72^{g}	66.51 ± 0.24^{f}	78.52 ± 1.28^{fg}	8.11 ± 0.20^{bc}	9.29 ± 0.39^{b}	10.50 ± 0.28^{b}
T7	14.60 ± 0.29^{bc}	16.90 ± 0.58^{bc}	15.40 ± 0.43^{b}	58.81 ± 2.04^{d}	72.63 ± 2.14^{e}	77.48 ± 1.93^{g}	7.56 ± 0.16^{cd}	8.67 ± 0.03^{bc}	9.54 ± 0.35^{d}
T8	14.00 ± 0.16^{cd}	16.20 ± 0.61^{cd}	15.00 ± 0.58^{b}	67.78 ± 0.85^{c}	86.71 ± 3.80^{c}	95.77 ± 3.85^{c}	8.26 ± 0.22^{b}	9.24 ± 0.02^{bc}	10.39 ± 0.37^{bc}
T9	14.40 ± 0.50^{bc}	16.80 ± 0.03^{bc}	15.30 ± 0.30^{b}	72.92 ± 1.30^{c}	97.59 ± 1.84^{b}	130.63 ± 2.94^{a}	8.99 ± 0.31^{a}	10.12 ± 0.39^{a}	11.28 ± 0.39^{a}

Values represent the mean ± SE. Alphabets in superscript indicate the level of significance of at $p < 0.05$; Where, T1–0.1 PPM 24-EBL; T2–0.2 PPM 24-EBL; T3–0.4 PPM 24-EBL; T4–0.5 mM ZnSO4; T5–1.0 mM ZnSO4; T6–1.5 mM ZnSO4; T7–0.1 PPM 24-EBL + 0.5 mM ZnSO4; T8–0.2 PPM 24-EBL + 1.0 mM ZnSO4; T9–0.4 PPM 24-EBL + 1.5 mM ZnSO4.

contributing to the overall response to BR treatment. In the presence of zinc stress alone (Treatments T4 to T6), the content of soluble protein in germinating mung seedlings decreased with increasing concentrations of zinc metal. However, when BR concentrations were applied together with the zinc concentrations (Treatments T7 to T9), there was a gradual increase in the content of soluble protein. These observations suggest that BR alleviated the zinc stress in germinating mung seeds. It can be inferred from these findings that BRs have the ability to enhance protein content even under normal conditions in plants, as reported by Arora et al. (2010) and Sharma et al. (2013).

3.9. Effect of BR and Zn on proline content

In this experiment, it was observed that mung bean seedlings accumulated proline content in response to zinc stress and the supplementation of BRs further increased the proline content (Table 3). Proline is known to accumulate in plants as a response to abiotic stresses, including zinc stress. The accumulation of proline serves as a protective mechanism for plants under stress conditions. Proline acts as a compatible solute, helping to maintain osmotic balance and stabilizing cellular structures and enzymes. It plays a crucial role in alleviating stress on enzymes and cellular structures by acting as a scavenger of reactive oxygen species and protecting macromolecules from oxidative damage. The higher proline content observed in mung bean seedlings treated with BRs suggests that BRs enhance the plant's ability to cope with zinc stress by promoting proline accumulation. Indeed, the role of proline as an organic nitrogen store during recovery from stress has been proposed (El Moukhtari et al., 2020). In the context of BRs, their induction of aluminum stress tolerance in mung bean was associated with increased levels of free proline (Ali et al., 2008). Additionally, Li et al. (2022) demonstrated that brassinosteroid-induced cadmium stress tolerance in grape seedlings was accompanied by stimulated proline accumulation. These studies further support the notion that proline accumulation is a common response mechanism induced by BRs under various stress conditions, including zinc stress in mung bean seedlings. The enhanced proline levels observed in the present study suggest that BRs play a crucial role in promoting stress tolerance by modulating proline metabolism.

4. Conclusion

To sum up, the utilization of BR treatments holds promise in enhancing seed germination, seedling growth and other biochemical activities in mung beans under heavy metal stress. The positive effects observed on seed germination and seedling growth following BR treatments are likely attributed to an increase in total soluble protein and proline content. The application of brassinosteroids further augmented the activity of antioxidative enzymes in response to Zn stress. Consequently, it is plausible that the reinforced antioxidant system played a significant role in conferring resistance to Zn stress in mung bean seedlings. These findings suggest that employing BRs as a novel approach could enhance seedling growth in the presence of Zn stress and prove beneficial for phytoremediation initiatives.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

NK: Data curation, Investigation, Writing – original draft. VS: Data curation, Investigation, Writing – original draft. GK: Data curation, Investigation, Writing – original draft. CL: Formal analysis, Writing – review & editing. HD: Writing – review & editing, Formal analysis. KP: Writing – review & editing. FK: Writing – review & editing. VG: Writing – review & editing. MK: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Unraveling the functional instability of bacterial consortia in crude oil degradation via integrated co-occurrence networks

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Introduction: Soil ecosystems are threatened by crude oil contamination, requiring effective microbial remediation. However, our understanding of the key microbial taxa within the community, their interactions impacting crude oil degradation, and the stability of microbial functionality in oil degradation remain limited.

Methods: To better understand these key points, we enriched a crude oil-degrading bacterial consortium generation 1 (G1) from contaminated soil and conducted three successive transfer passages (G2, G3, and G4). Integrated Co-occurrence Networks method was used to analyze microbial species correlation with crude oil components across G1-G4.

Results and discussion: In this study, G1 achieved a total petroleum hydrocarbon (TPH) degradation rate of 32.29% within 10 days. Through three successive transfer passages, G2-G4 consortia were established, resulting in a gradual decrease in TPH degradation to 23.14% at the same time. Specifically, saturated hydrocarbon degradation rates ranged from 18.32% to 14.17% among G1-G4, and only G1 exhibited significant aromatic hydrocarbon degradation (15.59%). Functional annotation based on PICRUSt2 and FAPROTAX showed that functional potential of hydrocarbons degradation diminished across generations. These results demonstrated the functional instability of the bacterial consortium in crude oil degradation. The relative abundance of the *Dietzia* genus showed the highest positive correlation with the degradation efficiency of TPH and saturated hydrocarbons (19.48, 18.38, $p < 0.05$, respectively), *Bacillus* genus demonstrated the highest positive correlation (21.94, $p < 0.05$) with the efficiency of aromatic hydrocarbon degradation. The key scores of *Dietzia* genus decreased in successive generations. A significant positive correlation (16.56, $p < 0.05$) was observed between the *Bacillus* and *Mycetocola* genera exclusively in the G1 generation. The decline in crude oil degradation function during transfers was closely related to changes in the relative abundance of key genera such as *Dietzia* and *Bacillus* as well as their interactions with other genera including *Mycetocola* genus. Our study identified key bacterial genera involved in crude oil remediation microbiome construction, providing a theoretical basis for the next step in the construction of the oil pollution remediation microbiome.

KEYWORDS

***Dietzia* genus, *Bacillus* genus, Co-occurrence networks, *Mycetocola* genus, functional potential, functional decline**

1. Introduction

Crude oil is a mixture of liquid hydrocarbons encompassing aliphatic, aromatics, resins, and asphaltenes (Varjani, 2017). When crude oil spills into the environment, it presents a potential hazard to both the ecosystem and biotic entities (Tang et al., 2011; Wang et al., 2021). Biological methods are more environment-friendly and cost-effective compared to physical and chemical methods (Vidonish et al., 2016; Johnson and Affam, 2018; Zhou et al., 2019). Microbial consortia are preferred over single microorganisms, because they possess distinct strengths in terms of degradation efficiency and adaptability to intricate environmental conditions (Abbasian et al., 2015; Gurav et al., 2017; Cui et al., 2020).

The constructing approaches of bacterial consortia include bottom-up and top-down (Lawson et al., 2019). Bottom-up is a random combination of single bacteria, which is also a common method to build crude oil degradation consortium. For example, several researchers constructed hydrocarbon–degradation consortia by combining biosurfactant–producing strains and crude oil–degrading strains (Lee et al., 2018; Chen et al., 2020; Cui et al., 2020; Dai et al., 2020), non–alkane–consuming and alkane degrader (Hu et al., 2020). The approach is full of randomness based on the complementary functions. By contrast, the top-down approach enriched specific function consortium by applying specific environmental pressure on the natural consortium (Lee et al., 2013). However, due to varying complexities of pollutants, functionality stability from top-down approach varies across successive generations. For microbial communities involved in degrading single-component contaminants, their degradation functionalities remain unaffected by the process of successive generations (Feng et al., 2019; Guo et al., 2021; Yin et al., 2021). However, for microbial communities engaged in degrading mixed pollutants, the stability of their degradation potential diminishes across generations (Chen et al., 2019; Gao et al., 2022; Lewin et al., 2022). Petroleum constitutes a complex mixture, adopting the top-down approach for selecting degradative microbial communities may lead to functional instability. To acquire microbial communities capable of crude oil degradation, it is vital to uncover the factors contributing to instability within top-down populations and identify crucial stabilizing species.

Keystone taxa were not numerically dominant in the communities (Banerjee et al., 2018), they had an effect on the structure and function of the consortium (Berry and Widder, 2014). By constructing species interaction networks, researchers have investigated microbial communities in groundwater and soil contaminated with hydrocarbons. They have identified key species exhibiting distinct traits: an elevated affinity for carbon sources, an abundance of genes related to hydrocarbon degradation (Ma et al., 2021), and the ability to produce metabolites that nourish other microorganisms (Geng et al., 2022; Jia et al., 2023). Recently, co–occurrence network analyses using high–throughput metagenomic data have been developed to identify key species and syntrophic relationships in microbial consortia. Involved measures include Pearson correlation, Spearman correlation, Bray–Curtis dissimilarity, Mutual information, and GBLM. Compared with Spearman and Pearson correlations, GBLM can mitigate spurious correlations among non–independent measurements as the increase in one relative abundance must be accompanied by a compositional decrease in another (Faust et al., 2012; Mac Aogain et al., 2021). Mac Aogain et al. (2021) integrated these five similarity measures to study disease-related microorganisms. To find key species and better understand the interrelationships within the microbial consortium, it would be beneficial to generate co–occurrence networks by merging the five similarity measures.

The objectives of this study were as follows: (1) enrich the crude oil–degrading bacterial consortia; (2) identify the key genera present and syntrophic relationships in the consortia; and (3) analyze the functional stability of the consortia through successive acclimation.

2. Materials and methods

2.1. Chemicals and reagents

Aerobic hydrocarbon degradation medium (AHDM), which was used for enriching crude oil–degrading bacterial populations, contains 10 g crude oil, 10 g $(NH_4)_2SO_4$, 1.1 g KCl, 1.1 g NaCl, 1.97 g Na_2HPO_4, 0.22 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, and 0.5 mL TES per liter. Trace element solution (TES) contains 0.56 g $FeSO_4$, 0.17 g $MnSO_4$, 0.25 g $CuSO_4$, 0.24 g $CaCl_2$, and 0.29 g $ZnSO_4$, per liter of the solution. Petroleum was obtained from the Xinjiang oil field in China. All other chemicals and reagents used were of analytical grade.

2.2. Enrichment and successive transfer culture of crude oil–degrading bacterial consortia

Soil samples polluted with crude oil were collected near the Luliang oil field in Xinjiang, China. The soil samples showed that approximately 70% of the crude oil degraded after 2–3 months in a natural environment. To enrich the crude oil–degrading bacterial consortia, 5 g of soil was cultivated for 0.5 h at 200 rpm in a 100 mL flask containing 45 mL 0.9% NaCl under aerobic conditions, at 37°C, then allowed to stand for 1 h to obtain the leachate. The leachate was transferred to the shake flasks containing 100 mL AHDM and the culture was generation 1 (G1) culture. AHDM without any bacteria was used as the control. The AHDM was autoclaved at 121°C and 30 min before the cultivation experiment. The G1 cultures was incubated on an electrical shaker at 150 rpm and 37°C for 10d. At the end of cultivation, G1 culture was continuously transferred (10% v: v) into fresh 100 mL AHDM every 10 days. The subsequent culture generations were referred to by their consecutive batch culture numbers (G2, G3, and G4).

2.3. Separation of saturates, aromatics, resins, and asphaltenes

TPH content was assessed using a gravimetric method (Capelli et al., 2001). Briefly, residual oil from the culture (100 mL) was recovered by adding 40 mL carbon tetrachloride. The lower organic phase was filtered and dehydrated with anhydrous sodium sulfate. The TPH content was quantified gravimetrically, after solvent evaporation.

The residual oil was extracted and divided into saturates, aromatics, resins, and asphaltenes, based on the Chinese National Standard SY/T 5119–2016. Briefly, n-hexane was gradually added to the residual oil. The oil sample was left to stand for more than 12 h to

fully precipitate the asphaltenes and measured. The filtrate was then subjected to chromatographic separation using a silica gel-neutral alumina column. Successive elution was performed using distinct organic solvents to sequentially recover the saturated hydrocarbon, aromatic hydrocarbon, and resin fractions. Saturated hydrocarbons were eluted with n-hexane, while a mixture of dichloromethane and n-hexane was employed for eluting aromatic hydrocarbons. Resin components were separated using anhydrous ethanol and chloroform as eluents. Following complete solvent evaporation, the weights of these three fractions were individually recorded.

2.4. DNA extraction and 16S rRNA gene-based community analysis

The medium was filtered through a sterile 0.45 μm organic membrane filter (Tianjin Jinteng experimental equipment Co. Ltd., Tianjin, China). Filtered membrane was utilized for DNA extraction. DNA extraction, PCR amplification, and sequencing were performed by Guangdong Magigene Biotechnology Co., Ltd. (Guangzhou, China). The V4 and V5 hypervariable regions of microbial 16S rRNA were amplified using the primers 515F (5′–GTGCCAGCMGCCG CGGTAA–3′) and 909R (5′–CCCCGYCAATTCMTTTRAGT–3′). The constructed libraries were pooled and sequenced using 250PE (paired–end) sequencing on an Illumina novaseq platform (Illumina, USA). Analysis of bacterial consortium composition and diversity was performed in triplicate. Demultiplexing, quality filtering, clustering into Amplicon-sequence variant (ASV), and construction of the ASV table were performed in QIIME2, v.2020.2 (Bolyen et al., 2019). The analysis yielded 1,503,255 high–quality reads (94.7% of reads averaged ≥ Q30 scores) distributed across 12 samples, with the minimum and maximum number of reads per sample being 88,513 and 171,446, respectively. The deblurring algorithm was used to construct the ASV table (Amir et al., 2017). The final ASV abundance table was then rarefied at 88513 sequences per sample through the alpha-rarefaction subcommand in QIIME2. Rarefaction curves based on observed ASV and the Good's coverage index were used to evaluate the adequacy of the sampling depth, which was established at 88513 quality-filtered reads per sample. The feature classifier script implemented in QIIME2 was employed for the taxonomic assessment using the SILVA reference database, v. 138 (Quast et al., 2013). Alpha diversity was assessed using diversity (Shannon) and evenness indices. Beta diversity was assessed by computing the Bray–Curtis distance between the samples and by Principal coordinate analysis (PCoA). Core OTUs within G1-G4 were discerned based on specific criteria: OTUs with high frequency, present in over 80% of samples, and abundant OTUs with relative abundances exceeding 0.2% across the entirety of samples (Wu et al., 2019). The neighbor-joining phylogenetic tree of the 16S rRNA gene was constructed using MEGA11 software, employing representative sequences of highly abundant bacteria (relative abundances exceeding 1%). The resulting tree was then visualized using the Interactive Tree of Life (iTOL) platform.[1] In this study, the potential functions of microbial consortia within G1-G4 were predicted by using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2, v2.5.2) and Functional Annotation of Prokaryotic Taxa (FAPROTAX, v1.2.4).

1 https://itol.embl.de/

2.5. Co-occurrence analysis of microbial interaction

Weighted co–occurrence analysis with an ensemble of similarity measures (Bray–Curtis, Pearson, Spearman, and MI) and regression techniques (GBLM) (Faust et al., 2012) were used to generate microbial association networks (Mac Aogain et al., 2021).

Specifically, the input file containing the relative abundance of genera in G1–G4 was calculated by five diverse measures and obtained relationships (positive and negative) between genera in G1–G4. The Gephi and Cytoscape software was used to analyze Betweenness Centrality, Closeness Centrality, and Degree of nodes. Following the result of Banerjee et al. (2018), keystone species have the characteristics of low Betweenness Centrality, high Closeness Centrality, and high Degree. To obtain the scores of the genera of the keystone species, the three indices were homogenized and used Eq. 1:

$$\text{Key} - \text{scores} = \text{Degree} + \text{Closeness Centrality} - \text{Betweeness Centrality} \quad (1)$$

2.6. Statistical analysis

An input file, which contained the relative abundance of all genera in G1–G4 and the degradation efficiency of TPH, saturates, and aromatics, calculated the relationship between the relative abundance of genera and the degradation efficiency of TPH, saturates, and aromatics by five diversity measures. Alpha diversity in different samples was compared using the Kruskal–Wallis pairwise test (Liddicoat et al., 2020). We used Permutational multivariate analysis of variance (PERMANOVA) to investigate the effects of time and four transfers on each component of beta diversity. Network metrics such as node degree, stress centrality, and betweenness centrality were calculated and visualized using Cytoscape. Statistical analysis and visualization of bacterial consortium and alpha and beta diversities were performed using the R packages reshape2, ggplot2, dplyr, VennDiagram, igraph, and vegan. The efficiency of crude oil degradation was assessed using Eq. 2

$$\text{Degradation efficiency} = \frac{\text{Residual content of (control} - \text{treat)}}{\text{Residual content of control}} \quad (2)$$

Data are expressed as mean ± standard deviation (SD) of three replicates. The Wilcoxon-Mann–Whitney test and Kruskal–Wallis test were performed for statistical analysis of values in different groups to determine the presence of significant differences.

3. Results

3.1. Crude oil biodegradation across four successive transfers

In this study, we enriched a crude oil-degrading bacterial consortium generation 1 (G1) from contaminated soil, achieving a TPH degradation rate of 32.29%. The crude oil degradation capacity dramatically decreased with G1 being continuously transferred. In comparison to G1, G4 consortium only achieved a crude oil

degradation rate of 23.14% within the same incubation time (Figure 1A). The saturates hydrocarbons were degraded at 18.72, 22.89, 17.82, and 14.16% by bacterial consortia G1–G4 (Figure 1B), this indicates a decline in the degradation capacity of saturated hydrocarbons after G2. While the G1 consortium was capable of degrading aromatics, G2, G3, and G4 consortia exhibited negligible degradation of aromatics (Figure 1C), suggesting a loss in aromatics degradation capacity after G1. The degradation of resins and asphaltenes showed no significant difference between the bio-treatment and control groups (Supplementary Figures S1A,B). These results illustrate that TPH degradation efficiency declined during successive transfer cultures.

3.2. Dynamics of bacterial community composition

The rarefaction curve analysis indicated that the sequencing depth in our study was adequate to capture the diversity of bacterial communities in each sample, ensuring representative coverage (Supplementary Figure S2A). Furthermore, statistical analysis using the Kruskal-Wallis test revealed no significant differences in the Shannon diversity index and evenness index among the G1-G4 consortia ($p > 0.1$; Supplementary Figures S2B,C). When assessing beta diversity based on the Bray-Curtis distance, PERMANOVA analysis demonstrated no significant difference ($p > 0.1$) in bacterial community structure between G1-G4 consortia (Supplementary Figure S2D).

Fourteen shared OTUs were observed in the four generations, while 24, 10, 9, and 16 unique OTUs were found, respectively, in G1, G2, G3, and G4 (Figure 2A). There were 5 OTUs in 14 shared OTUs identified as the regional core OTUs, accounting for 20.0 to 51.6% of the bacterial abundance of four generations (Figure 2B). All the 59 unique OTUs were classified to 5 phyla, including Actinobacteriota, Bacteroidota, Chloroflexi, Firmicutes, and Proteobacteria (Figure 2C). Compared to another generations, the unique OTUs of G1 were predominantly concentrated in Firmicutes and Proteobacteria. Those of G2 were mainly centered around Bacteroidota, and G4 primarily exhibited a concentration of Actinobacteriota.

In Figure 2D, the representative sequences of highly abundant bacteria (relative abundances exceeding 1%) in this study were selected to construct the phylogenetic tree. We found that the bacterial consortia of G1-G4 had the characteristic of high diversity. In G1, dominant OTUs were primarily concentrated in the *Bacillus*, *Dietzia*, *Novosphingobium*, *Xanthomonas* genus, and the Pseudomonadales order with *Acinetobacter* and *Pseudomonas* genera. In G2, predominant OTUs were mainly centered around *Pseudomonas*, *Dietzia*, *Novosphingobium*, and *Paenibacillus*. For G3, dominant OTUs were primarily found in *Novosphingobium*, *Sporosarcina* genus, and the Enterobacterales order. In G4, predominant OTUs were focused on the *Luteibacter* genus and the Enterobacterales order.

The analysis of the bacterial community revealed that Proteobacteria (59.35% ± 17.64%), Firmicutes (25.29% ± 9.18%), and Actinobacteriota (14.25% ± 8.63%) were the predominant phyla in G1-G4 consortia (Figure 2E). The dominant genera exhibited variations among the consortia. In G1, prevalent genera included *Pseudomonas* (18%), *Bacillus* (27.9%), *Dietzia* (20%), and *Novosphingobium* (8.27%). However, in G4, the dominant genera shifted to *Serratia* (22%), *Luteibacter* (18.9%), *Sporosarcina* (6.97%), *Bacillus* (4.77%), *Pseudomonas* (7.93%), and *Novosphingobium* (6.89%) (Figure 2F). Statistical analysis using the Wilcoxon-Mann–Whitney test indicated significant differences in the relative abundance of certain genera between G4 and the earlier generations. Specifically, the relative abundance of *Pseudomonas* in G4 was significantly lower than in G1 and G2, while *Bacillus* in G3 and G4 showed significantly lower abundance compared to G1 and G2. *Dietzia* in G4 exhibited a significantly lower abundance compared to G1-G3, and *Novosphingobium* in G4 showed a significantly lower abundance than in G3. By contrast, *Serratia* and *Luteibacter* in G4 had significantly higher abundance than in G3, and *Sporosarcina* in G4 showed significantly higher abundance than in G1 (Figure 2F).

3.3. Functional prediction analysis by FAPROTAX and PICRUSt2

FAPROTAX and PICRUSt2 software were be used to predict the function of bacterial communities. FAPROTAX was used to predict

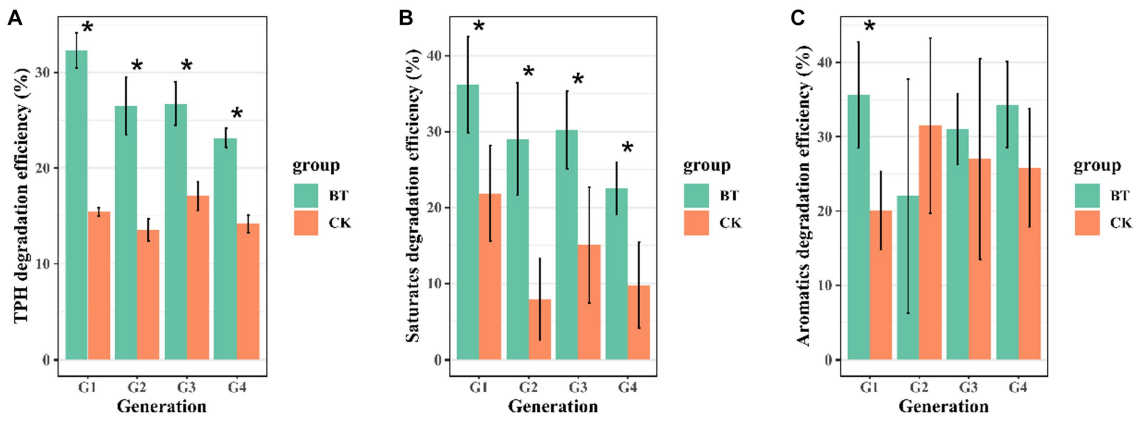

FIGURE 1
Degradation efficiency of crude oil and its two fractions in four successive transfers: **(A)** TPH degradation efficiency, **(B)** saturates degradation efficiency, and **(C)** aromatics degradation efficiency. The abbreviations used are CK for the control treatment and BT for the bacterial consortium treatment. The significance level is indicated by asterisks (*), with $p < 0.05$.

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the biochemical cycle of environmental samples. To display potential function of crude-oil degradation of bacterial communities, C transformation functions were promoted, including chemoheterotrophic, aerobic chemoheterotrophic, and aromatic_compound degradation processes (Figure 3). Our findings demonstrated that as successive generations progress, the aerobic chemoheterotrophic process of G1 and G3 became significantly more active in G4. The aromatic degradation processes of G1 exhibited pronounced activation in G2, G3, and G4. Notably, no significant differences in chemoorganotrophic were observed across G1 to G4.

Alkanes primarily undergo intricate transformations facilitated by specific enzymatic systems such as alkane hydroxylases, ethanol dehydrogenases, acetaldehyde dehydrogenases, and acetyl-CoA. These processes culminate in their eventual entry into the fatty acid beta-oxidation pathway, leading to their complete oxidation into carbon dioxide and water (Rojo, 2009). The degradation of polycyclic aromatic hydrocarbons (PAHs) involves a series of sequential reactions including hydroxylation, dehydrogenation, isomerization, and ring cleavage. This intricate sequence results in the generation of intermediates that become integrated into the tricarboxylic acid (TCA) cycle. Subsequently, these intermediates undergo enzymatic conversions orchestrated by microorganisms, ultimately leading to the production of carbon dioxide and water. Notably, the ring cleavage process constitutes a pivotal step in the degradation of PAHs, involving two distinct pathways (Varjani, 2017): (1) ortho-cleavage catalyzed by catechol 1,2-dioxygenase (C12O) at ortho positions, and (2) meta-cleavage catalyzed by catechol 2,3-dioxygenase (C23O) at meta positions. Utilizing the PICRUSt2 software, we acquired the abundance of genes encoding these enzymes. As illustrated in the Figure 4, the relative abundance of the alkane hydroxylase gene (K00496) in G1 notably surpassed that in G3 and G4, while both G2 and G3 exceed G4. The acetaldehyde dehydrogenase gene (K00001) in G1 and G3 also exhibited significantly higher abundance than in G4. Furthermore, the acetyl-CoA gene (K01897), along with the C12O gene (K03381) and C23O gene (K07104) involved in aromatic hydrocarbon metabolism, exhibits significantly higher abundance in G1, G2, and G3 compared to G4. Notably, the ethanol dehydrogenase gene (K00128) displays negligible variation between G1 and G4. Consequently, the trend observed across G1 to G4 generations unveils a marked decline in the relative prevalence of genes linked to alkane and aromatic hydrocarbon metabolism, excluding the ethanol dehydrogenase gene.

3.4. Functional decline of bacterial consortia

Based on key-score values and the relationship between genera, we constructed Figure 5. *Pseudomonas* exhibited the highest key-score

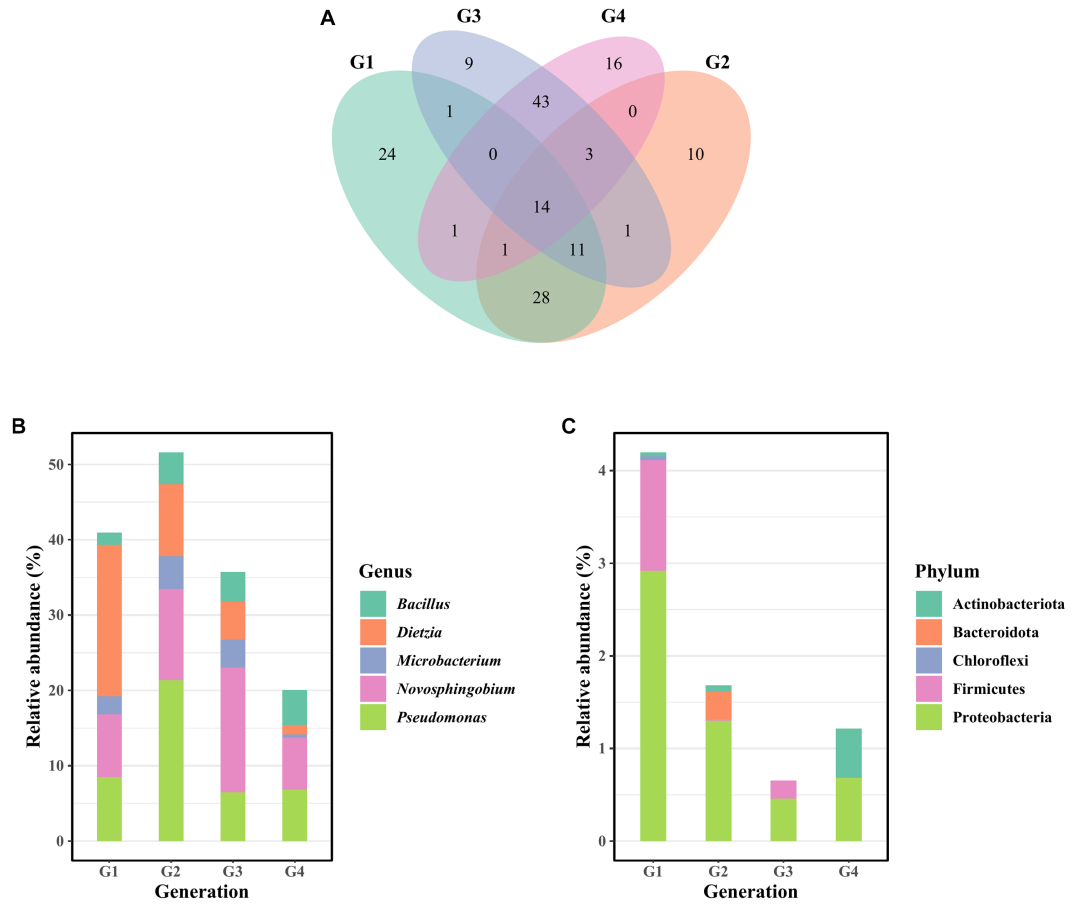

FIGURE 2 (Continued)

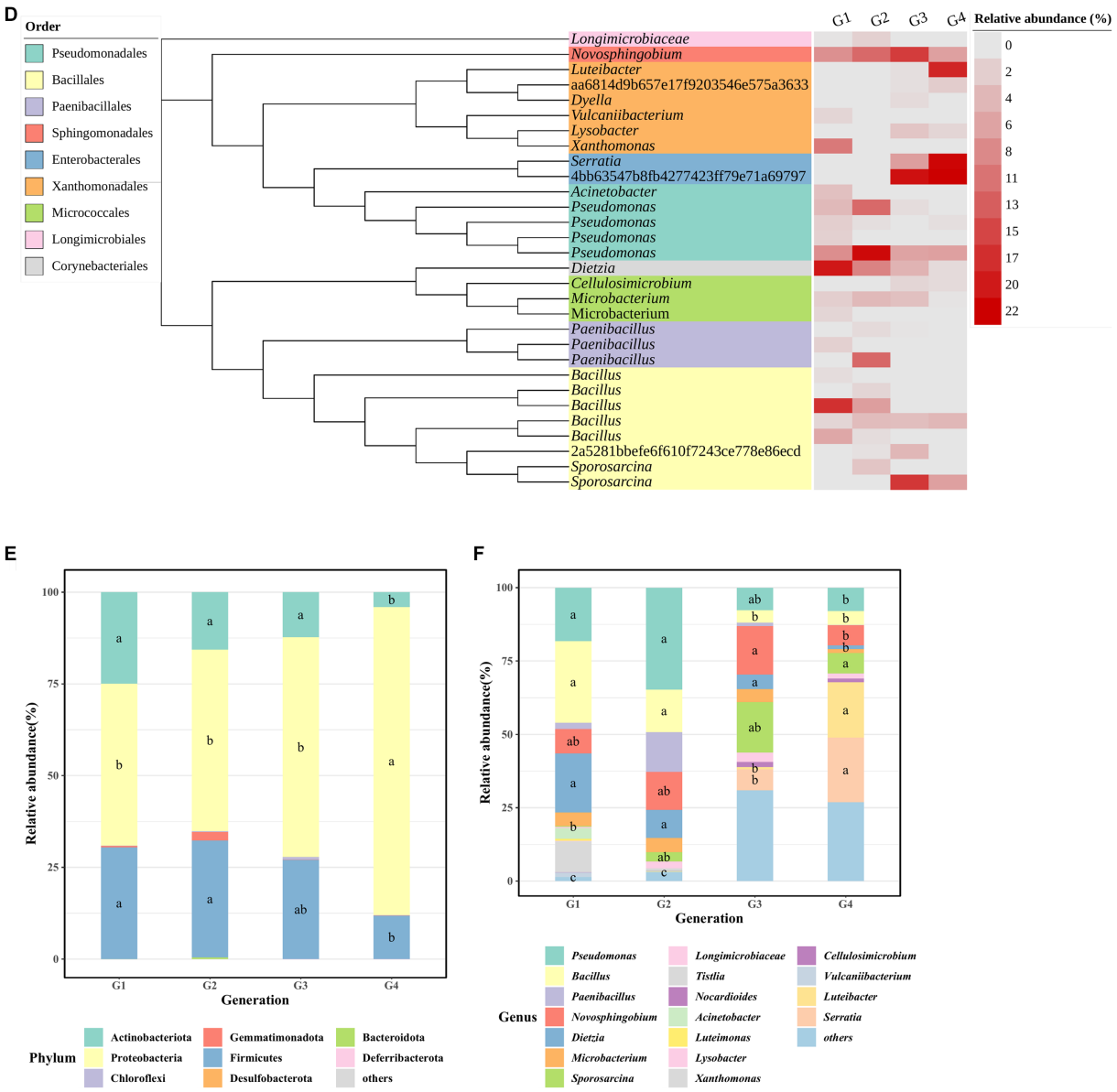

FIGURE 2
Bacterial taxonomic composition. **(A)** The Venn plot showing the shared and unique OTUs between G1–G4. **(B)** Core OTUs composition of four generations at the genus level. **(C)** Unique OTUs composition of four generations at the phylum level. **(D)** Taxonomic dendrogram showing the highly abundant bacteria of G1–G4. Color ranges identify order within the tree. Heatmap gradient represent the relative abundance of each OTU in G1-G4. **(E)** The relative abundance of bacterial phylum in the samples. **(F)** The relative abundance of the 10 most represent bacterial genera in the samples. Within each column, means with different letters are significantly different at $p<0.05$.

in G1, followed by *Arthrobacter* in G2, *Taonella* in G3, and *Lysobacter* in G4 (Figures 5A–D). Their relative abundances were 18, 0.21, 0.03, and 1.77%, respectively (Supplementary Table S1). These findings indicate that the presence of key functional species in the consortium is not solely dependent on their relative abundance. Alternatively, important functional species can exist as either dominant populations with high relative abundance or non-dominant populations with low relative abundance.

Table 1 showed the correlation between the genus of top key scores in G1-G4 and the degradation efficiency of TPH, saturates, and aromatics. The correlations ranged from 15.42 to −9.54 for TPH, 11.91 to −9.79 for saturates, and 8.03 to −5.96 for aromatics. These results suggest a decline in the degradation capacity of TPH, saturated hydrocarbons, and aromatic hydrocarbons by the dominant species in the consortia. In Table 2, the highest positive correlation with TPH and saturated hydrocarbons degradation was observed for the *Dietzia* genus, with correlation values of 19.48 and 18.38, respectively ($p < 0.05$). Similarly, the *Bacillus* genus showed the highest positive correlation with aromatic hydrocarbons degradation, with a correlation value of 16.56 ($p < 0.05$). Conversely, in Table 3, *Serratia* exhibited the highest negative correlation with TPH and saturated hydrocarbon degradation, with correlation values of −19.38 and − 15.78, respectively ($p < 0.05$). Additionally, *Psychrobacter* showed the highest negative correlation with aromatic

hydrocarbon degradation, with a correlation value of −8.92 ($p < 0.05$). These results emphasize the important roles of the *Dietzia* and *Bacillus* genera in the degradation of TPH, saturated hydrocarbons, and aromatic hydrocarbons, while highlighting the negative influence of *Serratia* and *Psychrobacter* on the degradation process.

The key scores of the *Dietzia* genus decreased from 3.62E-02 to 2.58E-02 during successive transfer cultures (Table 4). In contrast, the key scores of the *Serratia* and *Psychrobacter* genera increased from 0 to 2.87E-02 and 3.72E-02, respectively, after G2 (Table 4). The decline in the degradation of TPH and saturated hydrocarbons can be ascribed to the gradual weakening of the relationship between *Dietzia* and other genera in the consortia, as well as the progressive strengthening of the relationship between *Serratia* and *Psychrobacter* with other genera. These changes in inter-genera relationships within the consortia have significant implications for the overall degradation efficiency of TPH and saturated hydrocarbons.

The *Bacillus* genus, exhibiting the highest positive correlation (16.56) with aromatic hydrocarbon degradation, exhibit positive correlation with *Mycetocola* genus only in G1 (Figures 5A–D). This correlation pattern aligns with the observed degradation pattern of aromatic hydrocarbons during successive transfer cultures, suggesting that the relationship between these two genera plays a role in influencing the degradation of aromatic hydrocarbons.

4. Discussion

In environmental bioremediation, microbial consortia are more effective in pollutant degradation compared to single microorganisms (Gurav et al., 2017). Unlike the stochastic nature of the bottom-up approach, the top-down method facilitates the acquisition of degradation microbial consortia with heightened environmental competitiveness. Nevertheless, microbial consortia enriched through the top-down approach necessitate multiple generations for practical application. In this study, we employed the top-down approach to cultivate a stable and efficient microbial consortium for crude oil degradation. However, degradation efficiency and related functional potential diminish across generations. To obtain more steadfast and competitively adept microbial consortia, a thorough understanding of instability reasons through microbial interactions becomes imperative. To explore microbial interactions, researchers often employ correlation analysis or build regression linear models to construct co-occurrence networks (Matchado et al., 2021). In order to obtain more reliable species relationships, a common approach is to integrate multiple analysis methods, which has been extensively applied in plant research (Duran et al., 2018; Zhang et al., 2018) and soil microbiome study (Mandakovic et al., 2018). However, when studying species relationships in petroleum-degrading microbial communities, current practices have been limited to utilizing a single method to construct co-occurrence networks (Alvarez-Barragan et al., 2022; Xiao et al., 2022; Zhou et al., 2023). To infer more robust species interactions in the investigation of petroleum-degrading microbial communities, we adopt a method similar to that proposed by Mac Aogain et al. (2021), integrating five different correlation analysis methods to build a co-occurrence network. This integration approach alleviated the occurrence of spurious correlations among species.

During successive transfers, the abundance of the most critical genera in each generation did not exhibit the highest values, contrary to the conventional belief that species importance within consortia is solely dictated by their relative abundance. In fact, rare microorganisms have been found to exert a significant positive impact on the degradation capacity of consortia, as demonstrated by Delgado-Baquerizo et al. (2016). The *Desulfosporosinus* genus, despite its scant representation (0.006%) among the total 16S rRNA genes in the microbial community of peatlands, exhibits exceptional efficiency in sulfate reduction (Pester et al., 2010). These findings underscore the crucial role of rare species in providing the necessary genetic resources for the intricate degradation processes that occur within consortia. The constrained proliferation of these rare species is likely attributed to specific environmental conditions that impose unfavorable growth circumstances, as proposed by Jousset et al. (2017).

The strongest positive correlation was observed between the degradation rates of total petroleum hydrocarbons (TPH) and saturated hydrocarbons and the abundance of the *Dietzia* genus. Numerous studies have reported the hydrocarbon degradation capacity of the *Dietzia* genus (Wang et al., 2011; Venil et al., 2021). Comparative genomic analysis has revealed that the genome of this

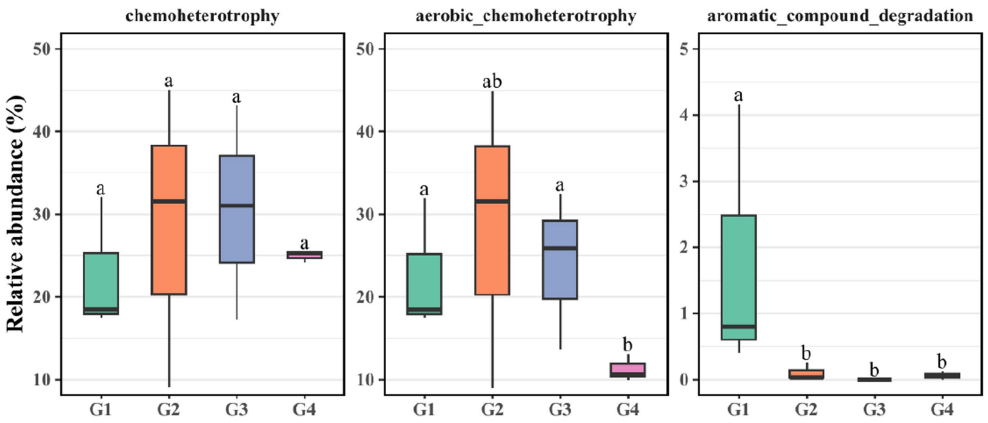

FIGURE 3
FAPROTAX analysis was utilized to evaluate the relative abundance of carbon transformation within G1–G4.

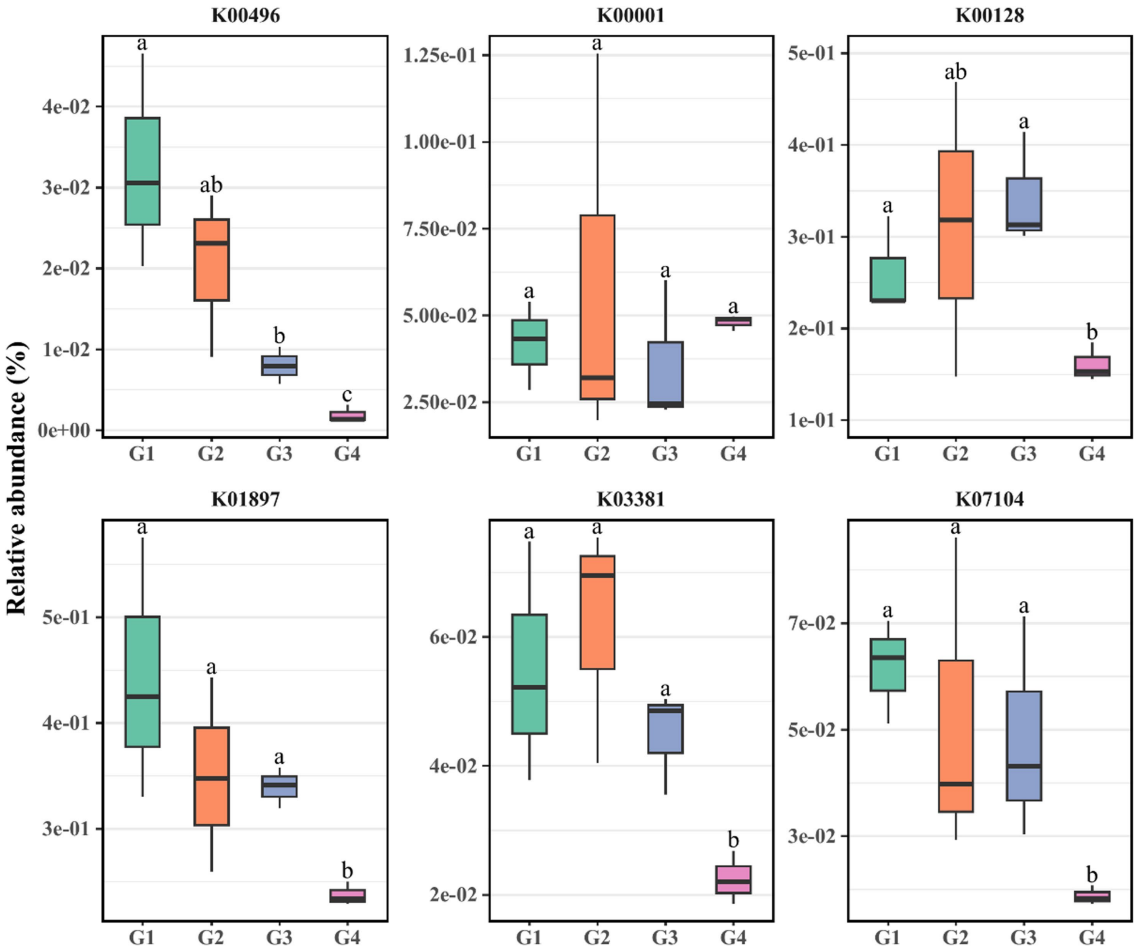

FIGURE 4
PICRUSt2 analysis was utilized to evaluate the relative abundance of hydrocarbon-degrading genes within G1–G4. K00496, *alkB*: alkane 1-monooxygenase [EC: 1.14.15.3]; K00001, *adh*: alcohol dehydrogenase [EC: 1.1.1.1]; K00128: aldehyde dehydrogenase (NAD+) [EC: 1.2.1.3]; K01897, *fadD*: long-chain acyl-CoA synthetase [EC: 6.2.1.3]; K03381, *catA*: catechol 1,2-dioxygenase [EC: 1.13.11.1]; K07104, *catE*: catechol 2,3-dioxygenase [EC: 1.13.11.2].

genus contains a higher number of genes associated with lipid transport, metabolism, secondary metabolic production, synthesis, transport, and metabolism compared to other bacterial genomes (Fang et al., 2021). Lipid transport and metabolism play crucial roles in the degradation of components related to crude oil, while certain secondary metabolites, such as rhamnolipids, contribute to the emulsification of crude oil (Wang et al., 2014; Liu et al., 2018). Our study revealed an intriguing finding: the key scores of the *Dietzia* genus showed a gradual decline during successive transfers, indicating a weakening relationship between *Dietzia* and other genera in the consortium. However, when the *Dietzia* genus was co-cultured with other genera, it resulted in enhanced degradation of alkanes (Hu et al., 2020). This suggests that the declining relationship between *Dietzia* and other genera influences the efficiency of crude oil degradation. In conclusion, the relative abundance of the *Dietzia* genus in the consortia and its interaction with other genera play a significant role in the degradation of total oil and saturated hydrocarbons.

The highest positive correlation was observed between the degradation rate of aromatic hydrocarbons and the abundance of the *Bacillus* genus, which is well-known for its capability to degrade aromatic hydrocarbons (Das and Mukherjee, 2007; Eskandari et al., 2017; Ghorbannezhad et al., 2022). Interestingly, our analysis revealed a significant positive correlation between the *Bacillus* genus and the *Mycetocola* genus, but only in the G1 generation of the bacterial consortia. This correlation aligns with the observed pattern of aromatic hydrocarbon degradation during successive transfers. *Mycetocola* genus has been reported to counteract the toxic effects of tolaasin I produced by the *Pseudomonas* genus, which inhibits the growth of both Gram-negative bacteria like *Escherichia coli* and Gram-positive bacteria like *Bacillus subtilis* (Rainey et al., 1991; Hermenau et al., 2020; Castaldi et al., 2022). Notably, in the G1-G4 bacterial consortia, the *Pseudomonas* genus was consistently present with a relative abundance above 7.93%. Therefore, we speculate that the lack of *Mycetocola* genus in the G2–G4 consortia could have resulted in the persistence of the toxic effect of tolaasin I, thereby limiting the degradation of aromatic hydrocarbons by the *Bacillus* genus. In summary, our findings suggest that the abundance of the *Bacillus* genus is positively correlated with the degradation of aromatic hydrocarbons. Furthermore, the presence of the *Mycetocola* genus appears to be crucial in alleviating the toxic effects of tolaasin I and facilitating the degradation of aromatic hydrocarbons by the *Bacillus* genus.

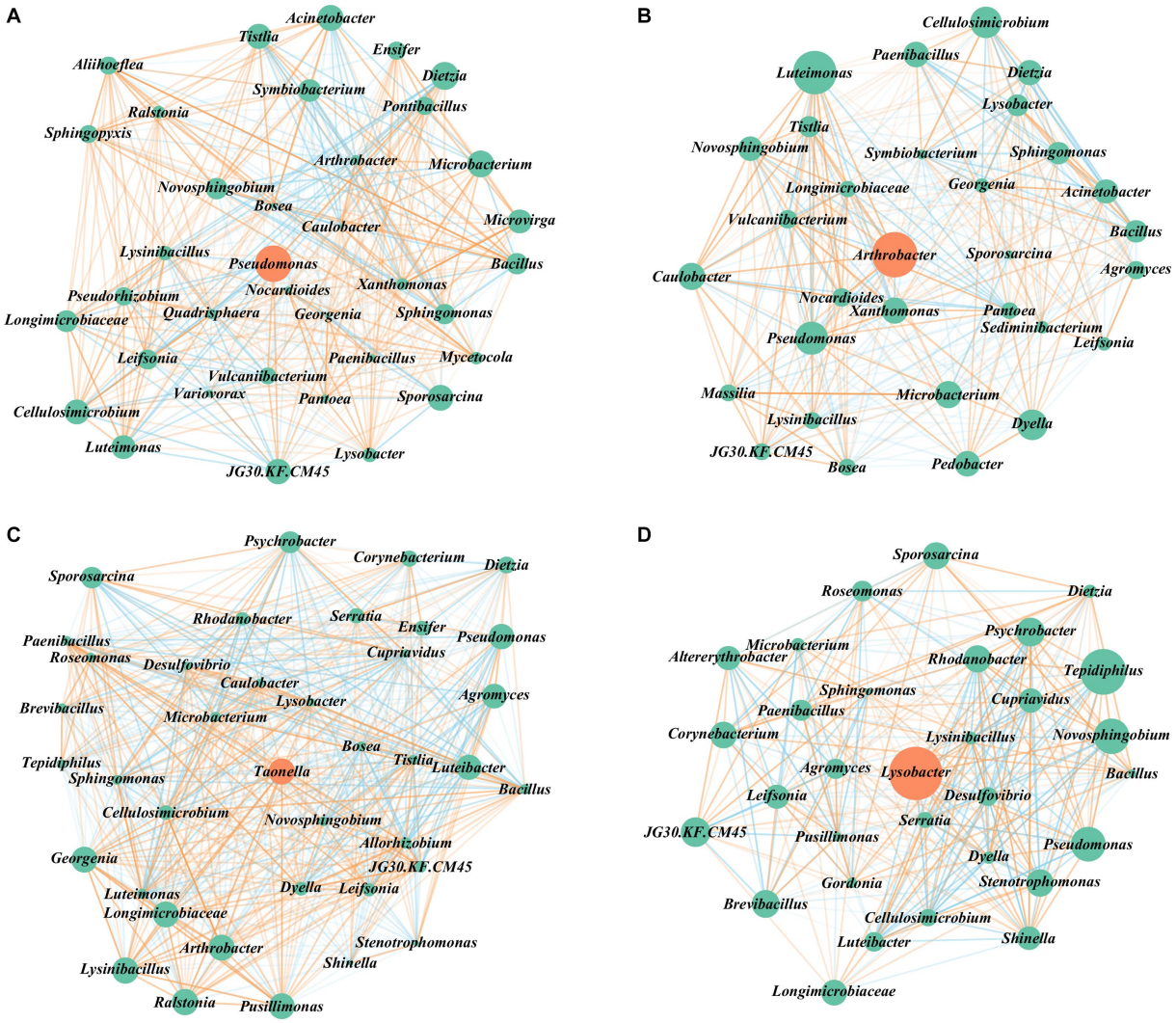

FIGURE 5
Co−occurrence analysis of bacterial network along successive transfer cultures. Interactions between microbes (nodes) are represented by connecting lines (edges), and the key−scores are reflected by node size. **(A–D)** Visualization of positive and negative interactions between all taxa on G1, G2, G3, G4. Interactions between microbes are classified as red if the sign of the edge weights between them is positive (positive correlation) and vice versa, the intensity of the colors reflected the strength of the correlations.

TABLE 1 The relationship between the degradation of TPH, saturates, aromatics, and the genus of top key-scores.

Genus	The correlation coefficient with TPH degradation	The correlation coefficient with saturates degradation	The correlation coefficient with aromatics degradation
Pseudomonas	15.42*	11.91*	8.03*
Arthrobacter	4.02*	4.36*	3.10*
Taonella	−1.29*	−3.76*	−2.86*
Lysobacter	−9.54*	−9.79*	−5.96*

The significance level is indicated by asterisks (*), with $p < 0.05$.

During successive transfers, we observed that the degradation rates of total petroleum hydrocarbons (TPH) and saturated hydrocarbons exhibited the strongest negative correlation with the relative abundance of the *Serratia* genus. Similarly, the degradation rates of aromatic hydrocarbons showed the highest negative correlation with the relative abundance of the *Psychrobacter* genus. It is noteworthy that both the *Serratia* and *Psychrobacter* genera have been reported to possess crude oil degradation capabilities (Fagbemi and Sanusi, 2017; Lasek et al., 2017; Semai et al., 2021). By analyzing the relationships between the *Serratia* and *Psychrobacter* genera and other bacterial species, we noticed a gradual increase in the key scores of these genera within the bacterial consortia during successive transfers. This suggests a strengthening association between these genera and other members of the consortium. Considering the degradation capabilities of *Serratia* and *Psychrobacter* genera, it is reasonable to speculate that the

TABLE 2 The strongest positive relationship between the degradation of TPH, saturates, aromatics, and the genera.

Genus	The correlation coefficient with TPH degradation	Genus	The correlation coefficient with saturates degradation	Genus	The correlation coefficient with aromatics degradation
Dietzia	19.48*	*Dietzia*	18.38*	*Bacillus*	21.94*
Bacillus	18.67*	*Bacillus*	14.50*	*Dietzia*	17.10*
Pseudomonas	15.42*	*Novosphingobium*	13.30*	*Xanthomonas*	16.93*
Vulcaniibacterium	12.41*	*Pseudomonas*	11.91*	*Pseudorhizobium*	14.42*
Xanthomonas	10.96*	*Vulcaniibacterium*	9.45*	*Acinetobacter*	12.63*
Variovorax	8.79*	*Variovorax*	9.35*	*Luteimonas*	12.25*
Acinetobacter	8.74*	*Luteimonas*	9.17*	*Vulcaniibacterium*	11.97*
Luteimonas	8.70*	*Symbiobacterium*	8.64*	*Mycetocola*	10.88*
Pseudorhizobium	7.78*	*Microbacterium*	6.91*	*Aliihoeflea*	10.84*
Sphingomonas	7.69*	*Tistlia*	6.24*	*Sphingopyxis*	10.84*

The significance level is indicated by asterisks (*), with $p < 0.05$.

TABLE 3 The strongest negative relationship between the degradation of TPH, saturates, aromatics, and the genera.

Genus	The correlation coefficient with TPH degradation	Genus	The correlation coefficient with saturates degradation	Genus	The correlation coefficient with aromatics degradation
Serratia	−19.38*	*Serratia*	−15.78*	*Psychrobacter*	−8.92*
Luteibacter	−16.42*	*Luteibacter*	−13.7*	*Corynebacterium*	−8.27*
Novosphingobium	−12.86*	*Microvirga*	−10.36*	*Serratia*	−8.2*
Desulfovibrio	−11.81*	*Altererythrobacter*	−10.16*	*Luteibacter*	−7.28*
Altererythrobacter	−11.08*	*Dyella*	−10.14*	*Agromyces*	−7.2*
Psychrobacter	−10.95*	*Desulfovibrio*	−10.09*	*Desulfovibrio*	−7.16*
Gordonia	−9.79*	*Lysobacter*	−9.79*	*Cellulosimicrobium*	−6.98*
Lysobacter	−9.54*	*Gordonia*	−9.66*	*Leifsonia*	−6.52*
Cellulosimicrobium	−9.35*	*Pusillimonas*	−9.14*	*Brevibacillus*	−6.37*
Shinella	−9.06*	*Psychrobacter*	−8.39*	*Tepidiphilus*	−6.19*

The significance level is indicated by asterisks (*), with $p < 0.05$.

TABLE 4 Betweenness Centrality, Closeness Centrality, Degree, and key−score of *Dietzia*, *Serratia*, and *Psychrobacter* at G1−G4.

Genus	Generation	Betweenness Centrality	Closeness Centrality	Degree	Key−score
Dietzia	G1	1.08E-02	2.49E-02	2.21E-02	3.62E-02
	G2	2.48E-02	3.05E-02	2.93E-02	3.50E-02
	G3	1.74E-02	2.35E-02	2.25E-02	2.86E-02
	G4	4.36E-02	3.39E-02	3.55E-02	2.58E-02
Serratia	G1	0	0	0	0
	G2	0	0	0	0
	G3	1.89E-02	2.35E-02	2.25E-02	2.71E-02
	G4	4.08E-02	3.39E-02	3.55–02	2.87E-02
Psychrobacter	G1	0	0	0	0
	G2	0	0	0	0
	G3	1.64E-02	2.45E-02	2.42E-02	3.23E-02
	G4	2.98E-02	3.30E-02	3.40E-02	3.72E-02

competition among microbial species within the consortium contributed to a decline in the overall crude oil degradation capacity (Abtahi et al., 2020).

By employing network analysis methods, we can provide insights into the underlying causes of the functional decline in crude oil degradation. Nonetheless, to substantiate our research outcomes, it is

imperative to conduct subsequent experimental studies focusing on the pertinent microorganisms and genes. These additional investigations will contribute to a more comprehensive and robust understanding of the complex interactions within the microbial community and their implications for crude oil degradation.

5. Conclusion

The relevant functional potential and degradation capacity of the bacterial consortium for crude oil gradually declined during the successive transfers. To investigate the factors influencing the degradation function of the bacterial consortia, we employed the 16S rRNA amplification technique and conducted bioinformatics statistical analysis. Our results revealed that the relative abundance of key genera within the bacterial consortia was not the sole determinant of their importance. The relative abundance of the *Dietzia* genus and its interactions with other genera emerged as critical factors influencing the degradation of TPH and saturated hydrocarbons. Furthermore, the decreasing relative abundance of the *Bacillus* genus and its interaction with the *Mycetocola* genus were found to impact the degradation of aromatic hydrocarbons. These findings highlight the intricate and dynamic nature of microbial interactions within crude oil degradation processes, underscoring the importance of gaining a comprehensive understanding of microbial community dynamics for the development of effective bioremediation strategies.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number (s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA997000.

Author contributions

PL: Conceptualization, Methodology, Writing – original draft. XL: Writing – original draft. RS: Conceptualization, Writing – review & editing. YW: Methodology, Writing – review & editing. SH: Methodology, Writing – review & editing. YZ: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1270916/full#supplementary-material

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Organic vs. conventional: impact of cultivation treatments on the soil microbiota in the vineyard

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The aim of this study was to compare the effects of two vineyard management practices on the soil and its associated microbiota. The experiments were conducted in two adjacent plots, one completely organically managed and the other conventionally managed in terms of phytosanitary treatments but fertilized with organic amendments. The chemical soil analyses were correlated to the prokaryotic and fungal communities, which were studied using the metabarcoding technique. The main difference between the two treatments was a significantly higher amount of Cu in the organic managed vineyard soil, while conventional managed soil presented higher concentration of Na and Mg and was also associated with higher pH values. Despite these differences, no significant diversities were observed on soil biodiversity and microbial composition considering alpha and beta diversity metrics. However, the percentages of some phyla analyzed individually differed significantly between the two managements. Analyzing the metabolisms of these phyla, it was discovered an increment of species correlated to soils with higher organic matter content or land not used for agricultural purposes in the organic treated soil. The findings indicate that, despite the use of copper-based phytosanitary products, there was no degradation and loss of biodiversity in the organic soil microbial population compared to conventional management with the same type of fertilization, and the observed microbial population was more similar to that of natural soils.

KEYWORDS

microbiota, soil, vineyard, organic, wine, environment

1. Introduction

Plants and soil microbiota interact in a mutualistic way, influencing each other. Plants produce root exudates that act as signal molecules to the bacteria in the rhizosphere, and each microorganism interacts with the soil microbiome, influencing plant health and productivity (Chaparro et al., 2012). Up to 10^4 species of prokaryotes can be present in soil with high organic content, at concentrations of up to 10^{10} cells per cm^3 (Torsvik et al., 2002). These microbial species, particularly bacteria, are the primary source of biodiversity in the soil ecosystem, where they participate in a variety of functions and balances, including nutrient cycling and plant health. However, biodiversity is linked to a number of factors, including ecosystem stability (Maherali and Klironomos, 2007; Jing et al., 2015). A stable ecosystem is distinguished by high genetic variability, which enables resistance to environmental changes (Yang et al., 2018). Several factors, many of which are related to agricultural activities, can disrupt this balance by altering the microbial community (Allison and Martiny, 2009). For example, increased nutrient

availability due to fertilization can alter species composition (Zhao et al., 2020), whereas different agricultural management systems influence the carbon activity of microbial biomass (García-Orenes et al., 2010). Furthermore, the use of synthetic fungicides and pesticides used in conventional agriculture has a significant impact on the fungal and bacterial populations of the soil (Sigler and Turco, 2003; Rivera-Becerril et al., 2017). The loss of some species specialized in unique biochemical processes, such as nitrogen fixation or toxic compounds degradation, can result in nutrient loss and toxins accumulation. Fortunately, for the most important functions, the soil microbiota can compensate for this loss through functional redundancy, resulting in a stable environment with high buffering capacities (Pan et al., 2014). Organic farming is one possible strategy for reducing the impact of agricultural practices on soil microbiota, as evidenced by numerous studies that found positive effects when this management was implemented. For example, it has been reported that during the transition from conventional to organic farming, the number of plant species increases (Gabriel et al., 2006), and this recovery occurs quickly (Jonason et al., 2011). Organic farming has been shown also to have a more complex bacterial network than conventional agriculture. This is a determining factor because not only the number of present taxa, but also their interconnection, influences global characteristics (Banerjee et al., 2019). When compared to mineral (i.e., inorganic) fertilizers, the use of compost in organic farming improves soil quality by activating different microbial groups, increasing functional diversity, and increasing levels of microbial organic carbon, nitrogen, and biomass (Chaudhry et al., 2012). The greater presence of phospholipid fatty acids and phospholipid ether lipids, as well as the association of a higher biomass with regard to fungi, provide additional evidence on the efficacy of manure-based fertilization in organic farming on microbial biomass and its diversity indices (Esperschütz et al., 2007). Further genomic studies have confirmed the ability of organic farming to increase wealth, reduce uniformity and dispersion, and modify the structure of the soil microbiota thanks to organic fertilizers (Hartmann et al., 2015). Organic soil management can thus increase bacterial activity and culturable bacterial counts (Reilly et al., 2013). However, copper accumulation in the soil is a factor that must be considered in organic farming (Jez et al., 2023). In facts, especially in orchards and vineyards, there is a widespread use of copper compounds used as fungicides to combat many plant diseases (Pietrzak and McPhail, 2004). After carrying out their action, copper residues are washed away from the plant and end up accumulating in the soil, up to very high levels, which in some cases reached 1,500 mg kg^{-1} (Flores-Vèlez et al., 1996).

Several studies have shown that the use of copper-based products for an extended period of time can lead to an accumulation of this potentially toxic metal in the soil, particularly on the surface (Arias et al., 2004; Lamichhane et al., 2018), with values ranging from 100 to 3,200 mg/kg (Mirlean et al., 2007). This issue is exacerbated in vineyard cultivation, which makes extensive use of Cu-based compounds, resulting toxic to plants at high concentrations. Although some studies showed that phytotoxicity and the negative effects on the microbiota are limited (Ruyters et al., 2013), the interaction between plants and bacteria in the rhizosphere, which can increase the tolerance to copper and detoxify the soil is critical in this case (Brunetto et al., 2016). For these reasons, it is important to understand the significance of soil bacteria in sustainable and low-input cultivation systems. In fact, in addition to the need to select specific plant characteristics for organic farming (Van Bueren et al., 2002), the role of these microorganisms in maintaining soil fertility and biocontrol of pathogenic microorganisms to limit pesticides use is critical (Hacquard et al., 2017). As a result, it is becoming increasingly important to understand and monitor the microbiota present in the rhizosphere, especially given the importance of mycorrhizal colonization for plant development and health, which compensates for a lower control of pathogens with pesticides and increases the nutrients availability in these low input systems (Johansson et al., 2004). The knowledge and characterization of the soil microbiota is even more important in the wine-growing sector, as it has been demonstrated that the microbiota is a unique and characteristic biomarker for each vineyard, influencing the quality of the obtained wine both indirectly, acting on the physiology and health of the vine, and directly, acting as the main source of indigenous fermentative bacteria. Indeed, it has been reported that the microbial biodiversity associated with a specific vineyard plays a key role in plant growth, grape quality and the winemaking process (Gilbert et al., 2014; Belda et al., 2020). Also, the association between the microbial metabolic profiles of wine based on grape origin, underlines the importance of fungal community of vineyard soil in wine characterization (Liu et al., 2020).

The aim of this study is to focus on the effect of the phytosanitary treatments on the soil microbiota of organically vs. conventional managed vineyards, in particular paying attention to the possible effects of copper, the main component of organic phytosanitary treatments, to verify any positive or negative effects on vineyard soil microbial biodiversity. As far as fertilization is concerning, only few differences are applied. In fact, the fertilization of this conventional management was made up of only organic manures, thus making soil organic inputs very similar to the biological vineyards, reducing to zero level the chemicals. The chemical composition of the soil was also analyzed, to verify the possible accumulation of compounds such as Cu, and to evaluate their contribution in shaping the prokaryotic and fungal communities. The sampling was also done taking into account the distance from the roots of the plant, because the root exudates are reported to substantially influence the bacterial communities present in their vicinity compared to those present in the bulk soil (Philippot et al., 2013).

2. Materials and methods

2.1. Sampling

This study was conducted in a restricted geographical area to minimize pedoclimatic influences such as altitude, temperature, rainfall, humidity, wind, sun exposure, temperature excursion, soil slope or latitude and longitude (Bokulich et al., 2014). The Collio region is in fact a very heterogeneous hilly geographical area, both in terms of climate and soil composition, a characteristic that makes this region famous worldwide for the production of Collio D.O.C. wines (designation of controlled origin), characterized by a high qualitative and sensorial differentiation despite being produced in the same area. The sites of the two analyzed managements were therefore chosen after a careful selection to maintain all the environmental factors in the exact same conditions, condition that was not possible to maintain in other vineyards even in immediately adjacent areas Furthermore,

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the work was set up to give a real and concrete response to local producers, with the actual cultivation conditions implemented. Therefore, given the impossibility of standardizing conditions as would happen in a greenhouse experiment on which it would have been possible to apply other experimental designs, and given the fact that analyzing multiple plots located in places distant from each other would only increase the background noise leading to the observation of possible differences not induced by the treatment but by environmental conditions and soil composition, we implemented sampling with a clumped segregation design (Hurlbert, 1984).

Twelve sampling points were considered between these two adjacent vineyards of cultivar Merlot. Six sampling points concerned a vineyard managed with organic cultivation methods and six concerned a vineyard managed with conventional methods, both maintained for 10 years with the same agronomic practices. The vineyard was located within the Collio DOC area in Corno di Rosazzo (Italy), and sampling carried out during October 2020. Sampling was conducted considering the entire portion of the vineyard, equally sampling the plants present at the beginning, centre, and end of the rows. The different management protocols of these vineyards, including planting period, phytosanitary treatments, fertilization and agronomic practices, and sampling GPS coordinates are summarized in Table 1. For each sampling point, soil aliquots were obtained with the aid of a mini excavator at a depth of 50 cm, perpendicularly to the stem of the vine and intercepting the root system and the rhizosphere zone at three different distances from the plant roots: on the plant root (R), from the rhizosphere soil (VR), and from bulk soil without the presence of roots (GEN). The microbiological analyzes were carried out on soil kept moist at 5°C (for 24 h), while the chemical analyzes were carried out on a portion of dried and sieved soil.

TABLE 1 Summary of samples and their agronomic management differences between the conventional and organic vineyards in the sampling year.

	Organic	Conventional
Sample GPS coordinates	**C1** 46°00′25.937″ N 13°24′49.275″ E	**C7** 46°00′26.290″ N 13°24′44.260″ E
	C2 46°00′25.540″ N 13°24′49.398″ E	**C8** 46°00′25.113″ N 13°24′41.020″ E
	C3 46°00′25.183″ N 13°24′49. 483″ E	**C9** 46°00′24.176″ N 13°24′37.263″ E
	C4 46°00′27.471″ N 13°24′45.282″ E	**C10** 46°00′25.667″ N 13°24′44.340″ E
	C5 46°00′26.641″ N 13°24′43.630″ E	**C11** 46°00′25.500″ N 13°24′42.801″ E
	C6 46°00′27.240″ N 13°24′40.966″ E	**C12** 46°00′25.330″ N 13°24′40.984″ E
Vineyard history		
Vineyard age (years)	72	24
Years of organic management	10	/
Differences in phytosanitary treatments		
Cu usage	3.75 kg ha^{-1} year	1.85 kg ha^{-1} year
Other products	*Products approved for organic farming*: Natural pyrethrum, orange essential oil, seaweed extract, amino acids	*Chemical synthesis products*: acetamiprid, metrafenone, phosphite, mandipropamid, metiram, fluxapyroxad, fenbuconazole, zoxamine, oxathiapiprolin
Green manure		
Management	Alternate rows (Mix Brassicaceae, legume, grasses)	None
Fertilizations		
Products	Organic mix (1.5 t ha^{-1} year^{-1}) Manure (2.0 t ha^{-1} year^{-1})	Organic mix (1.5 t ha^{-1} year^{-1}) Manure (1.0 t ha^{-1} year^{-1})

2.2. Soil chemical analysis

For chemical analyses, 0.5 kg of the collected soil, air-dried for 48 h, were homogenized by sieving excluding particles with a diameter > 2 mm. For pH measurement, 10 g of soil were dissolved in 25 mL of deionized H_2O and stirred for 15 min. After 30 min of rest, the values were measured using a pH-meter with glass-combined electrode (Basic 20, Crison Instruments, Spain).

Pseudo-total macro-, micro-and toxic elements were measured using the USEPA 3052 (USEPA, 1995) mineralization method and inductively coupled plasma atomic emission spectroscopy, ICP-AES (Agilent 5800). In brief, 0.5 g of soil was digested in a microwave oven using 10 mL of concentrated nitric acid. The digest solution was filtrated (<0.2 μm) and measured, after dilution and addition of scandium (Sc) as internal standard.

Total organic carbon and total nitrogen (Corg and Ntot) were determined by automated thermal analyses where carbon is converted to CO_2 by flash combustion at 1080°C (MicroCube, Elementar). Carbonates were previously removed from 10 mg of soils by treatment with HCl in silver capsules, then calculating C/N ratios.

Carbonates have been measured volumetrically with a Scheibler calcimeter (Austrian Standards International, 1999. Önorm 1,084: Chemical analyses of soils—Determination of carbonate. Vienna, Austria.).

Soil microbial biomass C and N (Biomass-C and Biomass N) were measured by the fumigation-extraction method (Vance et al., 1987) on preincubated moist soils (25°C for 5 to 7 days). Briefly, three portions of moist soil, each containing 25 g oven dry soil, were fumigated with ethanol-free chloroform for 24 h. After the removal of chloroform, soil samples were transferred to 250 mL plastic bottles and extracted with 100 mL 0.5 M potassium sulfate (1:4 soil to solution ratio). A set of non-fumigated soils were extracted similarly. Organic C and N in the soil extracts were measured by liquid TOC-TN (Shimadzu VCPN). Soil microbial biomass C (Biomass-C) was calculated from: Biomass C = (extractable C in the fumigated sample) minus (extractable C in the unfumigated sample) divided by 0.45 (K_{EC}). Biomass N was calculated from: Biomass N = (extractable N in the fumigated sample) minus (extractable N in the unfumigated sample) divided by 0.53 (K_{EN}) (Joergensen, 1996).

Basal respiration was calculated from the CO_2 trapped during incubation minus the blank (no soil) and divided by the incubation time

(Anderson and Domsch, 1993). On the obtained data, the statistical analysis was carried out through R v4.1.2. After verifying the variance with F test and normality by Shapiro–Wilk, the significance was evaluated by T test, considering significant results with value of $p < 0.05$.

2.3. DNA sequencing

For DNA extraction, 10 g of soil sample added with 10 mL of sterile ultrapure water were homogenized in a sterile stomacher bag to remove coarse debris, centrifuged at 12000× *g* for 10 min to remove the water, and then dried using a vacuum concentrator (Concentrator 5301, Eppendorf, Germany). Genomic DNA was extracted from 0.25 g of dry medium using the DNeasy Power Soil Kit (QIAGEN, Germany). The quantity and quality of the purified DNA were determined by spectrophotometry at 260 nm using a NanoDrop 2000c (ThermoFisher Scientific, USA). For DNA metabarcoding analysis, 16S rRNA gene for bacteria and ITS for fungi were used as target regions. Libraries were prepared with the KAPA Hyper Plus Kit (Roche, Swiss). For the amplification of the V3-V4 region of the 16S the pair of primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHV GGGTATCTAATCC-3′) were used (Hugerth et al., 2020), while for the amplification of the ITS1 region the primers pair ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCT GCGTTCTTCATCGATGC-3′) were used (Op De Beeck et al., 2014). The samples were then sequenced in paired-end mode, with a length of 300 bp with the MiSeq platform (Illumina, USA). The obtained sequences were published on Sequence Read Archive (SRA) from the National Center for Biotechnology Information (NCBI) with the bioproject number PRJNA946685.

2.4. Bioinformatic analysis

After a preliminary quality check of the raw reads carried out through FastQC v0.11.9 (Andrews, 2010), the subsequent bioinformatics analyzes were conducted in the Qiime2 Release 2021.11 environment (Bolyen et al., 2019). The primers were trimmed using Cutadapt v3.5 (Bolyen et al., 2019), and through DADA2 v2020.11.0 (Callahan et al., 2016) the reads were re-checked for their quality, filtered and denoised. The pre-trained SILVA (Quast et al., 2013) v.138 99% OTUs full-length sequences Naïve Bayes classifier was used for the taxonomic analysis of the 16S reads, while for the analysis of the ITS reads the Unite (Nilsson et al., 2019) v8.3 dynamic 2021-05-10 database with all eukaryotic species was used for training the Naïve Bayes classifier. From the elaborations obtained, the reads belonging only to bacteria and fungi, classified at least at the phylum level, and deprived of singletons were extrapolated. The subsequent statistical analyzes were conducted in R v4.1.2 environment using the Supplementary Table 1 as input metadata, taking in account the different treatment (Treatment), the distance from the plant roots (Site) and their interaction (SiteGroup). The rarefaction curves on species richness were calculated using ggrare from ranacapa package (Kandlikar et al., 2018). After rarefaction, on the samples implemented through the phyloseq package, the Alpha-diversity analysis was carried out. Significant effects were tested using a pairwise Kruskal-Wallis H test considering significant results at value of $p < 0.05$. Using the vegan package (Oksanen et al., 2013), a PcoA was used to visualize the distribution pattern of the microbial and fungal populations. Through the adonis function of the vegan package, a PERmutational Multivariate Analysis of Variance was also carried out, making 4,999 permutations on the rarefied ASVs in order to study the effect of the Treatment and of the Site. To understand how microbial populations correlated with environmental data, a distance-based redundancy analysis (dbRDA) was performed using the vegan capscale function. The reconstruction of metabolic functions was made with FAPROTAX v 1.2.4 (Louca et al., 2016). The graphical representations of the results were plotted in R using the ggplot2 library (Wickham, 2016).

3. Results

3.1. Environmental data

The average values and the significance of each environmental variable, compared in relation to the Organic and Conventional treatments were reported in Table 2. As regards the content of microelements, it was possible to detect significant differences at a statistical level between the two treatments only for Cu. This element was present at a higher concentration ($p < 0.01$) in the Organic treatment, with an average value of 146.10 ± 34.97 μg/g, compared to the Conventional treatment which showed an average value of 79.46 ± 18.46 μg/g. Furthermore, analyzing the microelements it was observed a significantly higher concentration ($p < 0.05$) of Mg (5030.80 ± 548.83 μg/g for Organic; 5769.67 ± 550.06 μg/g for Conventional), and Na (135, 47 ± 20.88 μg/g for Organic; 189.97 ± 41.67 μg/g for Conventional) in the Conventional treatment. Although the average concentrations of Ca, K, P, S and Al were generally higher in the Conventional treatment, their difference was not statistically significant. Considering the potentially toxic elements, only Pb was significantly higher ($p < 0.05$) in the Organic treatment (24.33 ± 4.22 μg/g) compared to Conventional (19.01 ± 1.00 μg/g). Regarding other parameters, $CaCO_3$ showed a significant difference ($p < 0.01$) between the two vineyards, with higher values for Conventional (46.03 ± 3.89 μg/g) than for Organic (37.29 ± 4.55 μg/g). These results also correlated with significantly different ($p < 0.05$) pH values, that resulted higher for the Conventional (7.95 ± 0.12) in comparison to Organic (7.73 ± 0.23) soil. Through the Principal Component Analysis (PCA) reported in Figure 1, it was possible to represent these results, visualizing how the different sampling points were arranged based on chemical parameters which were statistically different between the two treatments ($p < 0.05$). In fact, it was possible to observe a clear separation between the Conventional soil samples, which were more associated with higher values of Na, Mg, $CaCO_3$ and pH, compared to the Organic soil samples, associated with higher concentrations of Cu and Pb.

3.2. Sequencing results

Through the sequencing process, 4,413,339 reads were obtained from the sequencing of 16S regions for bacterial population, while 4,442,942 raw reads were obtained from the sequencing of the ITS region of the fungi population, that following the denoising operation carried out via DADA2, resulted in 2,482,232 reads for the 16S and 2,411,200 reads for the ITS. After the removal of the reads unclassified at least at the phylum level and the reads present with a frequency $n < 2$ (singletons), 17,292 features with a total frequency of 1,884,598 for the 16S, and 2,519 features with a total frequency of 998,124 for ITS were

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TABLE 2 Soil chemical properties.

Variable	Organic		Conventional		Value of *p*
	Mean	SD	Mean	SD	
General parameters					
pH	7.82	0.02	7.97	0.05	*
$CaCO_3$	37.29	4.55	46.03	3.89	**
Basal resp.	18.45	3.02	17.09	2.97	
Corg	1.46	0.34	1.17	0.34	
Ntot	0.14	0.03	0.15	0.04	
Biomass C	203.35	62.66	249.51	15.91	
Biomass N	62.08	13.71	68.05	34.18	
Macro-elements					
Ca	17985.48	8825.15	26401.93	3694.74	
Mg	5030.80	548.83	5769.67	550.06	*
Al	26061.67	1618.31	27945.43	1769.84	
Na	135.47	20.88	189.97	41.67	*
K	4017.17	479.04	5127.00	1286.82	
P	480.04	158.13	546.52	220.07	
S	202.99	32.92	279.66	105.44	
Micro-elements					
Cu	146.10	34.97	79.46	18.46	**
Zn	89.17	4.62	92.71	6.93	
B	15.82	2.59	18.72	4.93	
Mn	1615.63	349.65	1275.34	184.23	
Fe	33813.08	2460.70	33626.38	1046.14	
Toxic elements					
Co	15.89	2.69	14.82	1.15	
Cr	58.77	5.66	64.08	9.56	
Ni	78.67	5.79	82.22	4.39	
Pb	24.33	4.22	19.01	1.00	*
Cd	0.92	0.08	0.92	0.04	

Statistical analysis (*T* test) is reported (*$p < 0.05$, **$p < 0.01$). Total carbonates $CaCO_3$ were expressed as g kg^{-1}; Basal resp. as mg CO_2 g^{-1} soil h^{-1}; Corg and Ntot as g kg^{-1}; Biomass C and Biomass N as μg g^{-1}; Macro- Micro-and Toxic elements (Ca, Mg, Al, Na, K, P, S, Cu, Zn, B, Mn, Fe, Co, Cr, Ni, Pb, Cd) as μg g^{-1} of soil.

observed. From the plot of the rarefaction curves reported in Supplementary Figure 1, it can be seen how the sequencing effort was sufficient to analyze the biodiversity of the samples, as all the curves reached the plateau.

3.3. Alpha diversity

The alpha diversity between samples was investigated using the Species Richness and Shannon's H indexes. Considering the differences brought by the SiteGroup using the Kruskal Wallis test for bacterial community, no significant differences were identified either for the Species Richness (Figure 2A) or for the Shannon index (Figure 2B). Furthermore, since there were no significant differences within the two Treatments according to the Site, it was possible to the Kruskal Wallis test for bacterial community, no significant differences were identified either for the Species Richness (Figure 2A) or for the Shannon index (Figure 2B). Furthermore, since there were no significant differences within the two Treatments according to the Site, it was possible to compare the alpha diversity indices overall between the samples belonging to the Conventional and Organic treatment. Analyzing the species richness, i.e., evaluating only the number of species, the value of this index was higher in the Conventional treatment (1577.56 ± 26.93) than in the Organic treatment (1523.67 ± 262.61). Similarly, accounting both for abundance and evenness of the taxa, the Conventional treatment showed values of the Shannon index (6.80 ± 0.12) higher than the Organic treatment (6.77 ± 0.20). However, also in this case, analyzing the samples based on the two treatments using the Wilcoxon test, there were no significant differences ($p > 0.05$; Figures 2C,D).

The same dynamics were observed for the fungi population. In fact, no significant differences were found for alpha diversity in relation to the SiteGroup both by evaluating the species richness and the Shannon index (Figures 3A,B). Also in this case, since there were no significant differences made by the Site within the Treatments, it was possible to compare the Conventional and Organic treatments as a whole. In this case, the Organic treatment showed higher values of species richness (218.28 ± 69.48) and Shannon index (69.48 ± 0.61) in comparison to the Conventional treatment (201.56 ± 3.58 for species richness, 63.71 ± 0.62 for Shannon's H). However, even in this case, it was not possible to detect significant differences using the Wilcoxon test ($p > 0.05$) (Figures 3C,D).

3.4. Beta diversity

Differences in the microbial communities among the two treatments were assessed using the Bray–Curtis dissimilarity. As regards the bacterial population, from the PcoA reported in Figure 4 it was possible to observe the results brought to light by PERMANOVA on the significance of the considered variables. As reported in Supplementary Table 2, Treatment ($p < 0.01$) and Site ($p < 0.05$) resulted significant in shaping the prokaryotic community, while the result of the interaction between the two (SiteGroup) was not significant. However, given the low values of R^2 the reconstructed model was not very effective in explaining the variance (as can be seen, Treatment explained ~8.8% of the variance and Site ~8.6%, and the total variance explained by all considered factors was ~23.7%). Also with regard to ITS whose PcoA is reported in Figure 5, the PERMANOVA results once again highlighted the significance of the Treatment ($p < 0.01$) in the shaping of the fungal community. However, even in this case, as can be seen in Supplementary Table 3, although significant, given the low R^2 value of the model, the Treatment explained only ~9.0% of the variance.

3.5. Relations of soil microbiota with soil chemical properties

To analyze the relationship between soil chemical properties and prokaryotic and fungal communities, a variance partitioning analysis and dbRDA were performed on a reduced model in order to avoid variables correlation, considering only the factors Cu, pH and Na. The variance partitioning analysis showed that these three parameters together explained 7.6% of the variance for prokaryotic and 7.7% of the variance for fungi population (Supplementary Table 4). The dbRDA

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(Supplementary Table 5) highlighted a significant role (value of $p<0.01$) of Cu in shaping both the bacterial and fungal communities. pH also contributed significantly to both communities, however more significantly in the fungal (value of $p<0.01$) than in the prokaryotic community (value of $p<0.05$). Conversely, the contribution of Na was not significant (value of $p>0.05$). In Figure 6 the results obtained from the dbRDA for the 16S (A) and ITS (B) were graphically represented. It was possible to note how Cu and pH effected the composition between the two treatments, with a clear separation especially in fungal community.

3.6. Taxonomic composition

Through to the conducted analyses, it was also possible to verify how the considered microbial populations were influenced from the two treatments. From Figure 7, representing relative percentages of the 10 major phyla, it was possible to observe the structure of the prokaryotic populations of the samples. Overall, *Acidobacteriota* was the phylum present in higher percentages with a mean relative abundance of 25.89%, followed by *Proteobacteriota* with a mean relative abundance of 22.20%, representing combined almost half of the identified phyla. By analyzing the data reported in Supplementary Table 6, it was possible to find significant differences for 15 phyla. In fact, in the same most representative groups, it was possible to observe a significantly higher presence of *Proteobacteria* in the conventional treatment, while a higher level of *Acidobacteriota* characterized the organic treatment. Other major groups that increased in conventional treatment included *Bacteroidota* and *Planctomycetota*. Considering the phyla at lower percentages, conventional treatment was characterized by a significantly higher presence of *Patescibacteria*, *Elusimicrobiota*, *Sumerlaeota*, *Fibrobacterota*, WS2, and *Deinococcota*, while the organic treatment showed higher percentages of *Methylomirabilota*, NB1-j, *Desulfobacterota*, and MBNT15.

Considering the fungal population, as can be seen from the Figure 8, it was observed a different distribution in comparison to the prokaryotic one. In this case, in fact, the phylum *Ascomycota* present with an average relative abundance of 55.34%, alone constituted more than half of the phyla present, while the second most present phylum was *Basidiomycota* with a median relative abundance of 19.98%. In addition, the presence of fungal species, which constitute the majority of some samples but are not present at significant levels in the other samples, was observed. For example, the sample C4R taken near the root system of the plant, was characterized by the presence of a fungus belonging to the genus *Olpidium* (62.46%), a zoosporic pathogen of plants, animals, fungi, and oomycetes. Also, samples taken at point C7 were characterized by the high presence of fungi belonging to the genus *Leucoglossum* constituting 51.49% of the reads in the sample C7VR, 15.67% in C7R, and 7.06% in C7GEN. Other examples were in

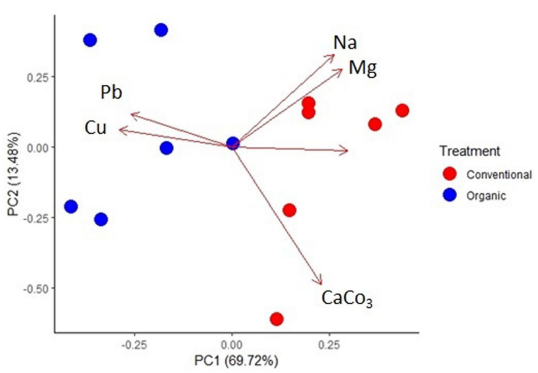

FIGURE 1
PCA analysis representing the relation between chemical parameters significantly different between the two treatments, in relation to the different sampling points.

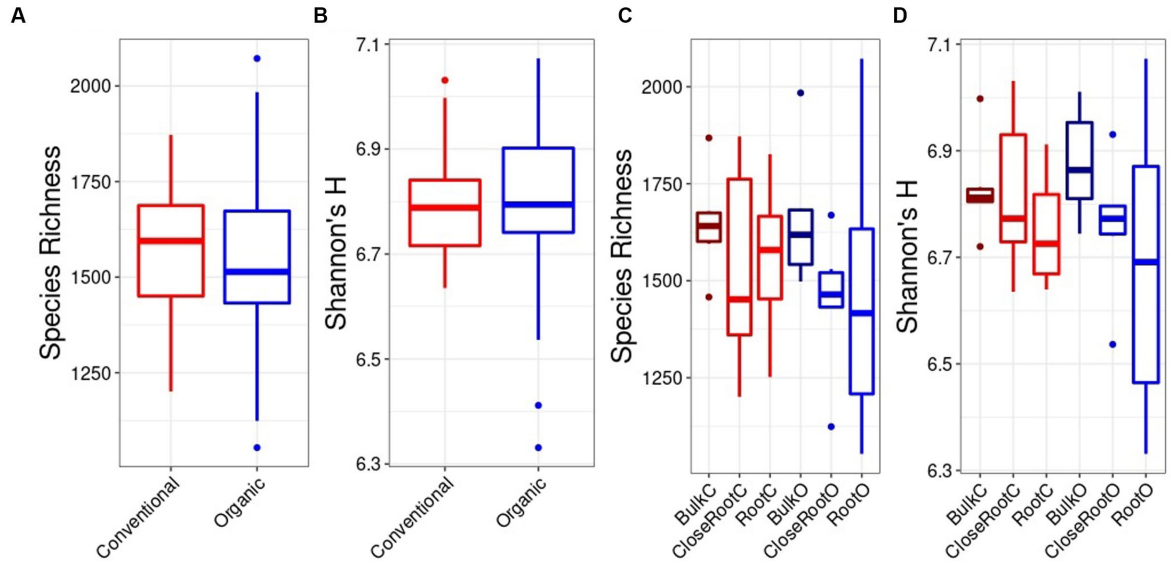

FIGURE 2
Bacteria alpha diversity evaluated in relation to the SiteGroup (Species Richness **A**, Shannon's H **B**) and Treatment (Species Richness **C**, Shannon's H **D**) represented as boxplots.

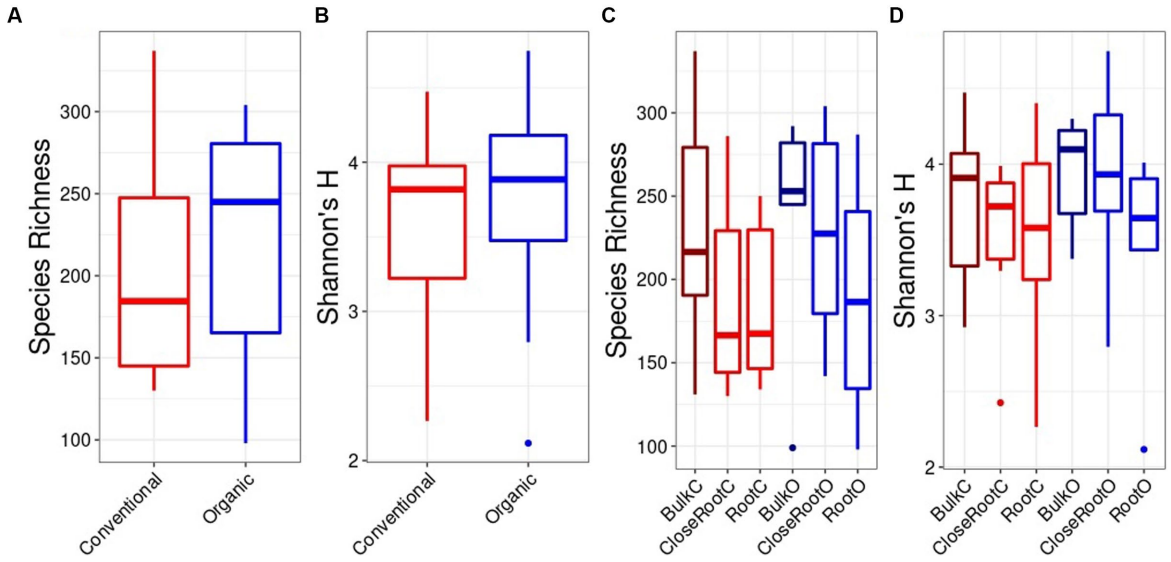

FIGURE 3
Fungi alpha diversity evaluated in relation to the SiteGroup (Species Richness **A**, Shannon's H **B**) and Treatment (Species Richness **C**, Shannon's H **D**) represented as boxplots.

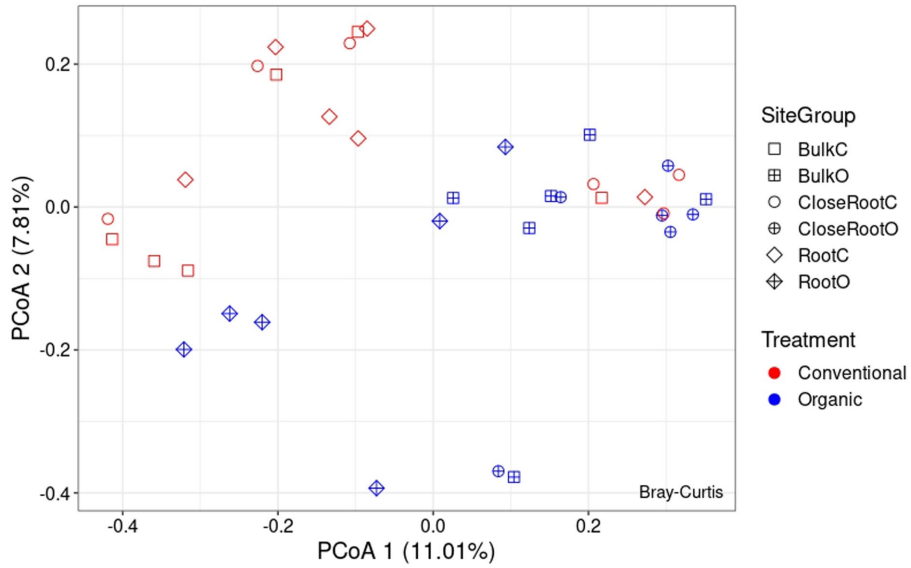

FIGURE 4
Principal Coordinate Analysis (PcoA) based on Bray–Curtis dissimilarity in prokaryotic community.

sample C3R highly characterized by the presence of *Armillaria borealis* (24.12%), the presence of *Psathyrella* spp. in samples C2VR (39.16%) and C2GEN (12.93%), *Phaeomonillea chlamydospora* in sample C9R (19.58%), and *Alfaria* spp. in sample C12VR (25.64%).

Due to such a high internal variability between samples, the cases in which the differences were significant were few (Supplementary Table 7). The main difference concerned the *Ascomycota*, which were present in higher percentages in the conventional treatment (57.85%) than in the organic treatment (48.28%). The second significant difference concerned the *Entorrhizomycota*, in this case present in higher percentages in the organic treatment (0.09%) than in the conventional treatment (0.02%).

3.7. Metabolic function differences

Using the FAPROTAX database, it was possible to analyze the metabolic and ecologically relevant functions of the prokaryotic clades (Figure 9). It can be seen that the main functions found were mainly related to chemoheterotrophy metabolisms, identified in significantly higher percentages in the conventional treatment, and specifically aerobic chemoheterotrophy. Other functions significantly more present in the conventional treatment were nitrite respiration, ureolysis, aromatic compound degradation, and fermentation. On the contrary, the organic

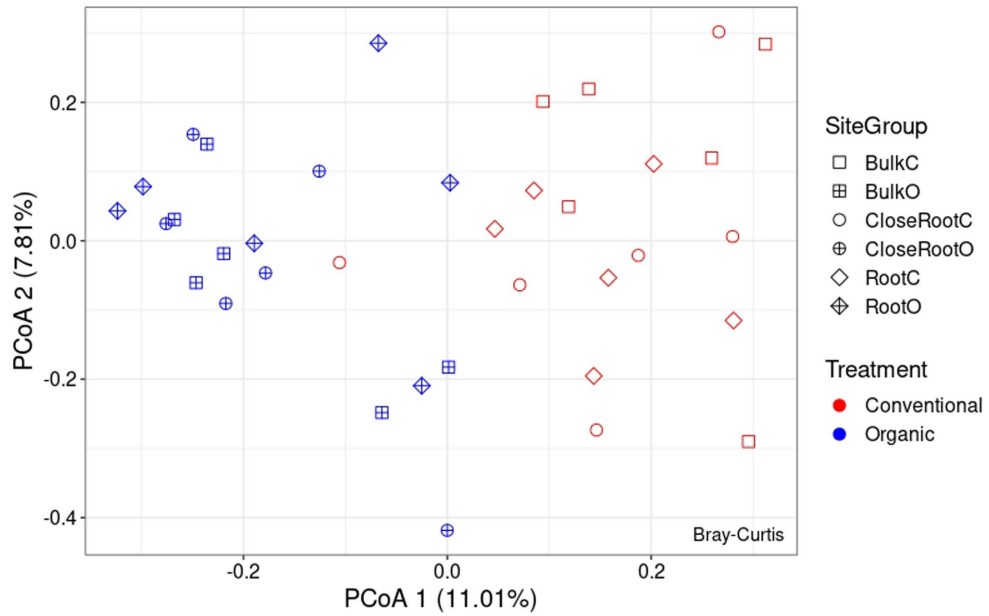

FIGURE 5
Principal Coordinate Analysis (PcoA) based on Bray–Curtis dissimilarity in fungal community.

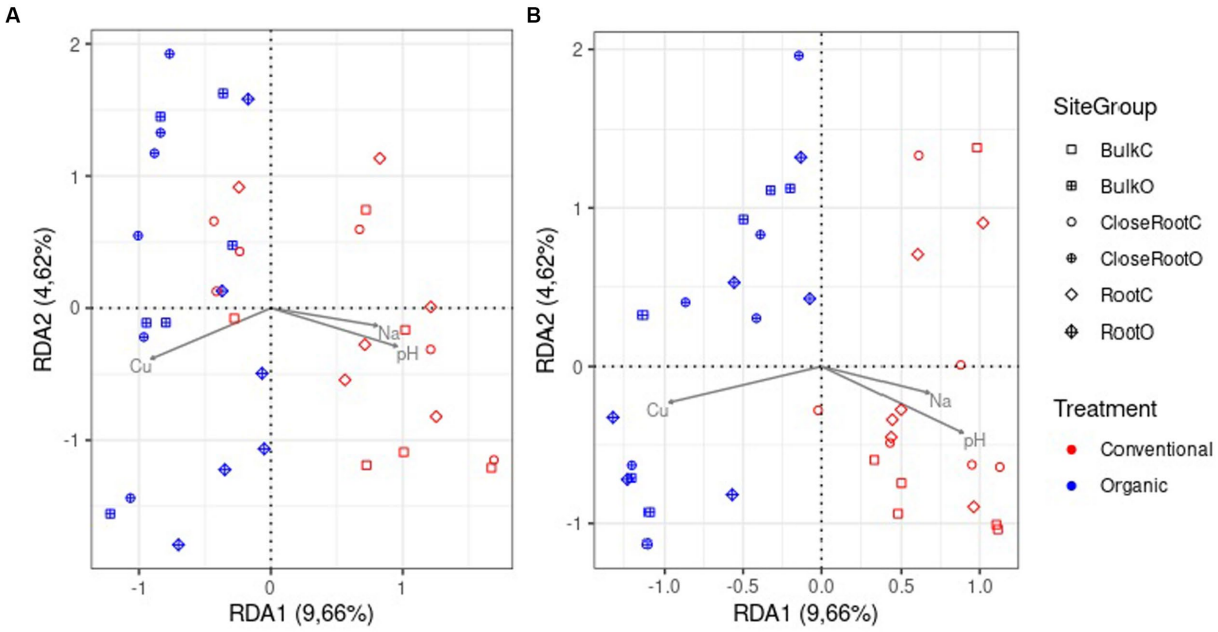

FIGURE 6
Distance-based redundancy analysis (dbRDA) based on Bray–Curtis dissimilarity in community composition for prokaryotic **(A)** and fungi **(B)** communities.

treatment was significantly characterized by nitrate reduction and nitrogen fixation.

4. Discussion and conclusion

The soil chemical parameters revealed significant differences between the two treatments for some of the considered parameters. Higher concentrations of Cu were measured in the organic managed soil, as would be expected given the higher use of copper-based compounds as phytosanitary treatment. In fact, this compound is one of the most important active ingredients of fungicide products used in organic agriculture, and its use can lead to its accumulation in the soil (Pellegrini et al., 2021; Tamm et al., 2022; Jez et al., 2023). Surprisingly, the Organic treatment was also linked to a higher concentration of Pb in the soil. In the literature, an increase in Pb

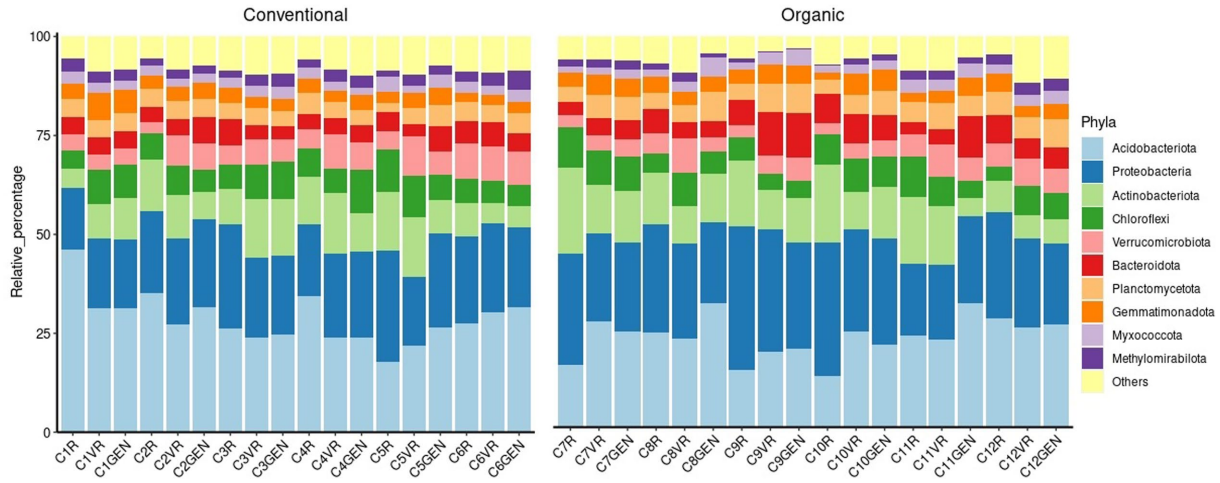

FIGURE 7
Prokaryotic composition at phylum level.

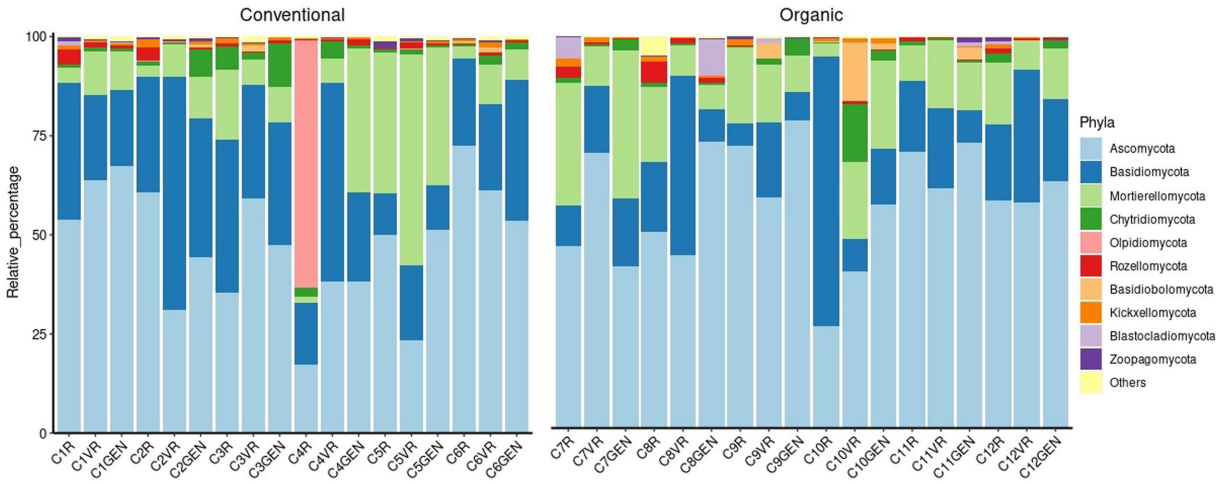

FIGURE 8
Fungi composition at phylum level.

concentration due to the use of livestock manure (Kumar et al., 2013) or of some phytosanitary treatments (Merry et al., 1983) has been reported. However, the same organic manure was used in both treatments, and the employed phytosanitary treatments did not contain Pb. So, in our opinion, given the low levels when compared to the average levels of natural soil contamination (Bindler et al., 1999) and other comparable agricultural sites (Beone et al., 2018), their minimum differences, albeit statistically significant, can be attributed to minimal differences in the soil rather than from the different treatments performed. It should also be noted that Pb, along with Cu and all the other potentially toxic compounds, were found to be well below the Italian legislation threshold, which sets contamination levels for agricultural soils at 200 mg/kg for Cu and 100 mg/kg for Pb (Gazzetta Ufficiale della Repubblica Italiana, 2019, Art. 3). Even the other elements regulated by Italian law such as Zn and Co, Cr, Ni and Cd, which did not show significant differences between the two treatments, were found to be well below the legislative thresholds, indicating a general good condition of the soil. Instead, differences in Na and Mg concentrations were discovered. These compounds were present at higher levels in conventional treatment, a phenomenon also observed in other studies (Zarraonaindia et al., 2020). This difference can be ascribed to the use of mineral fertilizers in previous years, whose contribution may have increased their soil concentration (Nemera et al., 2018). This observation can also be extended to the other analyzed macroelements, which had higher average concentrations in conventional treatment but were not statistically different (Pogrzeba et al., 2018). Another significant difference found in the soil between treatments was the higher pH values in the conventional treatment. This observation was also related to the higher $CaCO_3$ content in the conventional soil, which could be the cause (Magdoff and Bartlett, 1985) and to higher Corg (about +25%) albeit not statistically significant. In contrast, no significant differences were found for bioindicators such as basal respiration, biomass C, biomass N, and factors such as Corg and Norg. The company's care to

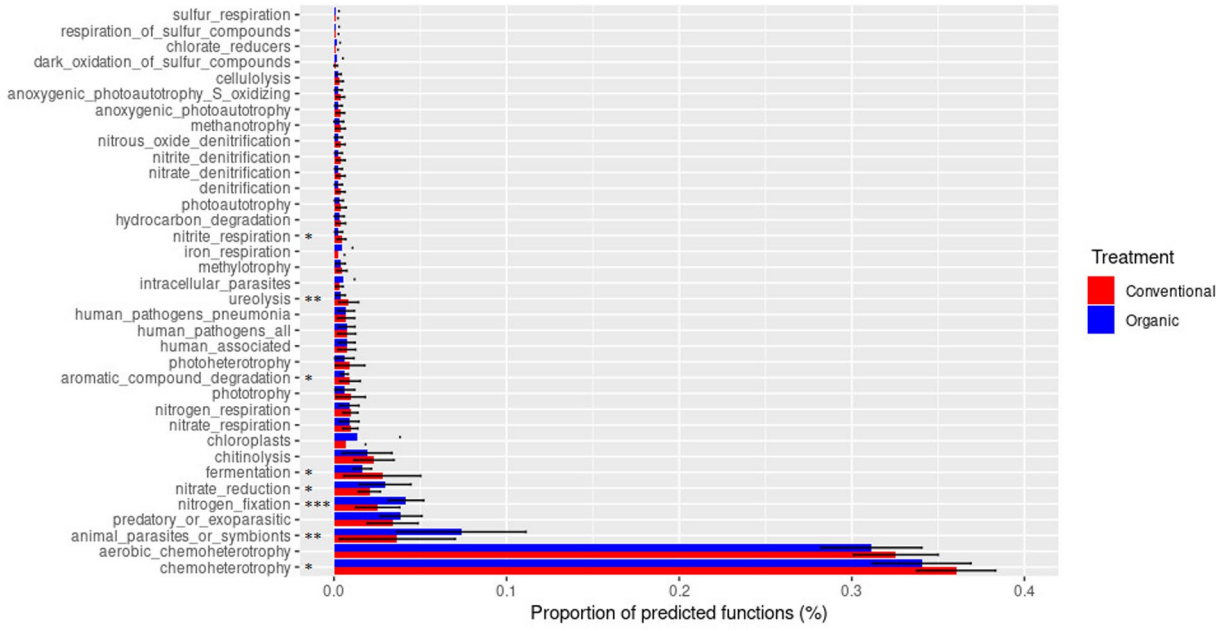

FIGURE 9
Metabolic and ecologically relevant functions of prokaryotic clades reconstructed through the FAPROTAX database. Statistical analysis (T test) made on the percentage of the predicted function is reported (*$p < 0.05$, **$p < 0.01$ ***$p < 0.001$).

soil agronomic practices to either conventional or organic vineyards can be traced back to the scarcity of differences found in the chemical–physical characteristics of the organic soil compared to the soil cultivated with conventional agronomic methods, in contrast to the widespread differences reported in the bibliography (Angelova et al., 2013). Indeed, as described in the materials and methods section, for the last 10 years the conventional vineyard has been fertilized with products allowed for organic farming, potentially reducing the differences between the two vineyards in particular regarding the soil organic matter. The following metagenomic analysis took into account the significantly different soil parameters between the two treatments, given the ability of factors such as Cu (Fagnano et al., 2020), Pb (Liu et al., 2007), Mg (Nicolitch et al., 2019), Na (Yan et al., 2015), and pH values (Zhou et al., 2017) to modulate the microbial population of the soil. There were no statistically significant differences in the number of bacterial and fungal species between the two treatments or the sampling point based on distance from the root (SiteGroup). This implied that any chemical–physical difference between the treatments had no effect on species richness. In particular, it can be deduced that the higher concentration of Cu due to the organic agronomic treatments had no significant impact on biodiversity levels, especially on the fungal one, which is particularly sensitive to the levels of this element (Bonanomi et al., 2016). However, as observed in the beta diversity analysis, the PERMANOVA results revealed differences in the composition of the microbial populations, particularly in relation to the treatment, while also in this case the SiteGroup was not significant. Although it had a significant impact on the shaping of microbial communities, the treatment could only explain a small amount of variation observed between samples, as seen in the reconstructed representations via PCoA. This result could be explained by the samples' low variance due to the high conservation of the microbial groups present. In fact, all of the samples revealed a core of conserved species, which had previously been discovered in other similar studies on the soil of Italian vineyards (Coller et al., 2019). However, despite a general uniformity of the terrain, significant differences in the relative percentages of some phyla were observed, possibly indicating differences between the two treatments. In terms of the prokaryotic community, the most significant difference was observed in *Proteobacteria*, the main phylum of the conventional treatment, and *Acidobacteriota*, the main phylum of the organic treatment. This observation was consistent with other studies in the literature, which show that *Proteobacteria* are more associated with tilled agricultural soils, whereas *Acidobacteriota* are associated with forested soils and lower pH values (Kim et al., 2021). The ratio between these two indicator microorganisms can therefore suggest that the biological management has resulted in a condition more similar to that of a natural environment than conventional management methods. Other studies reported a positive correlation between *Proteobacteria* and *Patescibacteria*, and a negative correlation between *Methylomirabilota* and the level of mineralization of soil organic carbon (Dong et al., 2021). This phenomenon was also observed in this study, with a significantly higher presence of *Proteobacteria* and *Patescibacteria* in the conventional treatment, and a significantly higher presence of *Methylomirabilota* in the organic treatment, implying a possible correlation between the greater availability of organic carbon that had not yet been mineralized in the organic treatment. Differences have also been discovered for *Planctomycetota*, which are reported to be sensitive to the type of fertilization used (Buckley et al., 2006), and in the phylum NB1-J, whose functions are not yet clear, but the frequency of which has been reported to decrease in cultivated soil (Maretto et al., 2022). *Desulfobacterota* (as well as the related candidate phylum MBNT15) were found in higher concentrations in the organic treatment. Given their type of metabolism linked to the presence of organic matter, this

could indicate a higher presence of the latter mentioned into the soil (Vipindas et al., 2022). Noteworthy was the exclusive presence of *Deinococcus* in the conventional treatment. In particular, this group of extremophile microorganisms (Slade and Radman, 2011) was discovered to be dependent on soil aeration (Ren et al., 2021). *Elusimicrobiota* were found at higher percentages in conventional, and their presence was reported to be related to higher $CaCO_3$ concentrations (Constancias et al., 2015). In terms of fungi, there was a significant decrease in *Ascomycota* (which remained the main group) in the organic treatment, compared to a strong increase in *Basidiomycota*, which was not statistically significant. The increase of *Basidiomycota* in the organic treatment related to the application of organic fertilizer corresponds to what several authors reported (Dong et al., 2021; Xu et al., 2022). Another significant difference was identified with regard to the *Entorrhizomycota*, fungi related to the root system (Bauer et al., 2015) which were found to be more prevalent in the organic treatment in this test. Based on these findings, it was possible to conclude that there were no substantial differences in the core microbiota phyla ratio between organic management with low use of copper-based phytosanitary products and conventional management with fertilization using products permitted in organic farming, albeit with differences. In particular, no dysbiosis or reduction in biodiversity was observed due to the higher concentration of Cu in the soil, which was one of the main differences of the soil between the two samples studied (Mackie et al., 2013).

The FAPROTAX database analysis also revealed a conservation of bacterial functions between the two treatments, albeit with differences for some of them. Through this program that associates bacterial taxa with metabolic or ecologically relevant functions, which has been reported as valid in the literature for the analysis of soil functions (Sansupa et al., 2021), it was in fact possible to investigate any differences between the two treatments. The most notable distinction has been identified in terms of nitrogen fixation, which was more prevalent in the organic treatment. This could be explained in part by the larger use of organic fertilizer (Shi et al., 2021), but it could also be explained by the planting of legumes as green manure (Toda and Uchida, 2017; Table 1). Another difference found in agreement with other studies was the presence of a minor ureolysis function in the organic treatment (Fernández-Calviño et al., 2010). Other differences were discovered in nitrite respiration, fermentation, nitrate reduction, and chemoheterotrophy, which may indicate a difference in the availability of metabolic substrates and oxygen in the soil.

In conclusion, despite the phytosanitary treatments in organic vineyards caused more Cu accumulation in the soil than conventional management, it resulted not responsible for negative changes in microbial populations or the loss of biodiversity. In fact, when two soils were compared with the only significant difference being the phytosanitary protocols, the treatment was not identified as being responsible for high levels of induced variability. The concern that looms over the use of copper-based phytosanitary products about their accumulation and associated decrease in environmental biodiversity (Karimi et al., 2021), was not supported by these results. This suggests that with the doses applied in these treatments, Cu employment is compatible with the vineyard management without causing an impact on the soil microbiota, resulting in dysbiosis that could have a negative effect on vine's health and productivity. These results could also be attributable to the use of organic fertilizers, in particular manure, that itself contains a great microbial biodiversity. Nonetheless, differences in the proportions of microbial populations can be used as indexes to confirm the treatment's efficacy. Indeed, in the organic treatment, species more akin to non-cultivated environments have been promoted, and the merits could be attributable to the beneficial practices of this agricultural management approach.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

EC, LI, and MCi: conceptualization. EC, LI, MCo, and MCi: resources. AC, LI, MCo, and MCi: methodology. LI and MCi: supervision. AC and MCo: formal analysis, data curation, and writing – original draft. AC, MCi, and LI: visualization. LI: writing – draft revision. MCi and LI: writing – review and editing. LI and MCi: funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1242267/full#supplementary-material

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Exploring microbial diversity responses in agricultural fields: a comparative analysis under pesticide stress and non-stress conditions

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Exposure to pesticides changes the microbial community structure in contaminated agricultural fields. To analyze the changes in the native microbial composition qRT-PCR, a metagenomic study was conducted. The qRT-PCR results exhibited that the uncontaminated soil has a higher copy number of 16S rDNA relative to the soil contaminated with pesticide. Metagenome analysis interprets that uncontaminated soil is enriched with proteobacteria in comparison with pesticide-contaminated soil. However, the presence of Actinobacteria, Firmicutes, and Bacteroides was found to be dominant in the pesticide-spiked soil. Additionally, the presence of new phyla such as Chloroflexi, Planctomycetes, and Verrucomicrobia was noted in the pesticide-spiked soil, while Acidobacteria and Crenarchaeota were observed to be extinct. These findings highlight that exposure to pesticides on soil significantly impacts the biological composition of the soil. The abundance of microbial composition under pesticide stress could be of better use for the treatment of biodegradation and bioremediation of pesticides in contaminated environments.

KEYWORDS

pesticides, biodegradation, metagenomics, agriculture, environment

Introduction

The application of agrochemicals including fertilizers and pesticides has become an essential component of agriculture (Malla et al., 2018). Consumption of pesticides is tremendously increasing globally in order to fulfill the food demand of the growing population (FAO, 2021; Gangola et al., 2023a). The global consumption of pesticides was 4.2 metric tons in 2021 and is expected to reach 4.4 million metric tons by 2026, with a 0.5% increase every year (PMO, 2022). Based on the consumption of pesticides, China is at the top, followed by the United States, Brazil, and Argentina. Pesticides can travel from the soil surface to a water reservoir or groundwater, and their

fate depends on the environmental conditions, such as adsorption to the matrix/soil sediment, transport with water, chemical transformation, and formation of recalcitrant metabolites (Gonzalez-Rodriguez et al., 2011). Although the formation of pesticides is examined under standard rules and systems, some are very recalcitrant in nature, becoming a threat to the ecosystem and polluting water bodies (Sjerps et al., 2019).

Agricultural and natural habitats are regularly contaminated by anthropogenic activity (Jeffries et al., 2018). The application of pesticides on agricultural fields released into the environment and reaching the soil surface, different water sources, and underground water is of major concern for environmental sustainability and human health (Aldas-Vargas et al., 2022). Transformation of pesticides in the environment depends on environmental conditions and physical, chemical, and biological degradation mechanisms (Gangola et al., 2023b). Microorganisms play a significant contribution in nutrient recycling, enhance crop growth and nutritional quality, and are vital components of our living environment. Therefore, it is necessary to check the negative impact of pesticides on beneficial microbial populations and their surrounding environment (Gangola et al., 2022a; Bhatt et al., 2023). The implementation of a microbial system for the degradation of xenobiotic compounds from a contaminated environment is the most favorable approach for the sustainable environment and human health (Saibu et al., 2020; Doolotkeldieva et al., 2021). The complete dissolution of pollutants from contaminated sites depends on several factors such as concentration, chemical structure, temperature, pH, soil microbial community composition, and their activity (Kowalczyk et al., 2015). Due to the variation in season, geographical location, and environmental conditions, the distribution of pesticides is uneven and affects microbial composition significantly (Verma et al., 2013; Raj et al., 2023). The process of biodegradation may vary from one ecosystem to another and depends more on the microbial composition as agricultural soil is rich and active in microbial composition as compared with the oligotrophic environment. Hence, monitoring pesticide biodegradation mechanism by structural and functional attributes of native microflora under different environmental conditions and their environmental fate is important as an indicator and for better understanding of the study (Fenner et al., 2013). Microorganism-mediated pesticide mineralization involves several chemical reactions such as oxidation–reduction, dehalogenation, hydrolysis, dehydrogenation, dealkylation, methylation, conjugation, and ring cleavage (Cycoń et al., 2017). Additionally, the development of a novel approach is crucial to describe microbial diversity and give in-depth knowledge of microbial responses to pesticide exposure (Gangola et al., 2022b,c).

Pesticide concentration, residue, and metabolites are the traditional indicators that are not applicable for several pesticides in an anaerobic environment. Moreover, these indicators are unable to differentiate between biotic and abiotic pesticide biodegradation processes (Aldas-Vargas et al., 2022). Therefore, the use of advanced research tools is important to monitor genes involved in the biodegradation of pesticides or identify the microorganisms in the contaminated environment. The cultivation-dependent approach only allows to cultivate a small proportion of total microorganisms and restricts their accessibility for research study (Schloss and Handelsman, 2006). However, in cultivation-independent methods, the study relies on DNA sequencing of the environmental sample to examine the complete study of microbial community structure, biomass composition, nutritional status, and physiological stress response for a particular environment (Su et al., 2012; Costa et al., 2020). The introduction of advanced technology such as metagenomics and metabolomics is under development and has shown their promising application in characterizing pesticide effects on soil biomass (Hou et al., 2015; Jeffries et al., 2018; Malla et al., 2022).

From the development of next-generation sequencing (NGS), the researchers preferably work on targeted and non-targeted genes to explore more information using some advanced techniques such as metagenomics and metatranscriptomics. These molecular tools have enough potential to extract the entire microbial composition and their metabolic potential without having any prior knowledge (Zhou et al., 2015). The metagenomics-based approach extends several new ways of opportunities to explore the dominant pesticide-degrading genes and their distribution in different microbial genera both in culturable and in non-culturable microorganisms within a complex environment (Fang et al., 2018). Aldas-Vargas et al. (2022) used a metagenomic approach to monitor the biodegradation of pesticides. Through implementing metagenomics, the genes *atzABCDEF* responsible for atrazine biodegradation were identified in agricultural soil (Malla et al., 2022) and the rhizospheric region of different trees (Aguiar et al., 2020). The metagenomic approach was successfully used to study seasonal variation in microbial communities and pesticide biodegrading genes linked to metabolic pathways from different aquatic environments such as freshwater and marine sediments (Fang et al., 2014). These water bodies were contaminated with 10 pesticides, namely, atrazine, carbendazim, chlorothalonil, isoproturon, linuron, metamitron, nicosulfuron, 2,4-dichlorophenoxyacetic acid (2,4-D), organophosphates, and pyrethroid (Fang et al., 2014). After metagenomic analysis of the activated sludge sample, a total of 68 subtypes of pesticide-degrading genes were identified, and out of them, *dhn* gene (encode dehydrogenase and degrade metamitron) was found to be dominant. Pesticide contaminates the metagenomic analysis of soil sample, revealing that as the concentration of pesticide increases in the soil, the expression and number of pesticide-biodegrading genes also increases and are mostly peroxidase, monooxydase, and cytochrome P450 (Russell et al., 2021). Hence, these studies confirmed that high-throughput techniques have enough potential to examine the microbial community and their different powerful pesticide-degrading genes under complex environments. Very limited study has been conducted on the comparative microbial community analysis of pesticide-contaminated and non-contaminated soil. Therefore, this study aimed to analyze and differentiate the microbial community of pesticide-contaminated and non-contaminated agricultural soil.

Materials and methods

Soil samples were collected from two different agricultural fields in Gularbhoj (29.0918°N, 79.3156°E), Uttarakhand, India. One field was contaminated with pesticides, and the other was uncontaminated. The rice crop was grown in both fields during the Kharif season. The soil sample was collected using stainless steel auger. The 10 cm of soil at the top was discarded, and the next 5 cm of soil was collected from both the contaminated and uncontaminated sites. Both the soil samples were labeled and stored in a deep freezer at −20°C. The soil sample taken from uncontaminated sites acts as a control for this study. Pesticide residues such as chlorpyrifos, cypermethrin fipronil, and imidacloprid were majorly found in the pesticide-contaminated soil. Soil DNA was extracted from both soil samples (500 mg) using the HiPurATM Soil DNA Purification Kit. The purity was checked using a NanoDrop spectrophotometer at wavelengths of 260 and 280 nm with a concentration of 50 ng/L (Jeffries et al., 2018; Malla et al., 2022).

The highly variable region (V3–V4) of the 16SrRNA gene in the soil bacterial community was targeted using the Illumina MiSeq platform. The primers used for amplification were V3: 341 F (5′ CCTACGGGAGGCAGCAG 3′) and V4: 806 R (5′ GGACTACHVGGGTWTCTAAT 3′). Paired-end reads obtained from sequencing were subjected to several quality control checks, including score distribution, base quality, average base content, and GC distribution. The FLASH program (Magoč and Salzberg, 2011) was utilized to merge the paired-end reads. High-quality reads ranging from ∼350 to 450 base pairs were obtained by applying multiple filters. The UCHIME algorithm was employed to identify and remove chimeric sequences. Subsequent analysis of the data was performed using the QIIME program (version 1.9.1) (Caporaso et al., 2010). The pre-processed reads were pooled and clustered into operational taxonomic units (OTUs) at a 97% similarity threshold using the UCLUST program. Representative sequences for each OTU were chosen by aligning the sequences against the Greengene database *via* PyNAST (DeSantis et al., 2006; Jeffries et al., 2018). Taxonomic classification was conducted using the RDP classifier against the SILVA 16S rRNA gene database (Joshi et al., 2021). Obtained reads and OTUs from both samples were classified into bacterial phylum and genera (Khati et al., 2019). Statistical Analysis of Metagenome Package (STAMP) was used for additional statistical analysis and heatmap visualization, while UPGMA clustering was employed to generate dendrograms (Parks et al., 2014). Alpha diversity was assessed by the Shannon index using the QIIME program (version 1.9.1) (Chaudhary et al., 2021; Joshi et al., 2021).

qRT-PCR analysis

The 16S rDNA extracted from the soil was subjected to qRT-PCR analysis using the iCycler iQTM Multicolor instrument (Bio-Rad Laboratory, Hercules, CA, USA) and SYBR green dye (Kumar et al., 2019). The qRT-PCR amplification employed a pair of universal primers, specifically primer 1 (5′-CCTACGGGAGGCAGCAG-3′) and primer 2 (5′-ATTACCGCGGCTGCTGG-3′).

Results

Real-time PCR analysis

In both the soil samples' native soil bacterial community, their abundance was observed using high-throughput sequencing and qRT-PCR analysis. After qRT-PCR analysis, it was observed that pesticide-contaminated soil had less copy number of 16S rDNA than uncontaminated soil, i.e., 1.96×10^8 and 5.25×10^8, respectively, per gram of soil.

Comparative microbial diversity analysis

Comparative analysis for efficient functional microbiome and taxonomic community composition under pesticide stress and non-stress conditions was performed with the help of a high-throughput metagenomic approach.

Total reads in the pesticide-contaminated (2G) and non-contaminated (2GC) soil samples were 562,416 and 873,083, respectively. Furthermore, the reads were classified at the phylum and genus levels, i.e., for contaminated soil, the reads were 562,416 and 716, while for non-contaminated soil, the reads were 873,083 and 725. During the study, only the top 8 dominant phyla and genera were selected for comparative analysis. At the genus level, the unclassified category comprised of most abundant genera in both the 2G (20.35%) and 2GC (18.52%) soil samples. In the 2G soil sample, the second most dominant genus was *Clostridium* (8.30%), subsequently followed by *Nocardioides* (3.41%), *Bellilinea* (3.14%), *Anaerolinea* (2.75%), *Longilinea* (2.48%), *Caldilinea* (2.33%), and *Phycicoccus* (2.21%). In 2GC soil sample, the second most abundant genus was *Candidatus Koribacter* (5.89%), subsequently followed by *Bacillus* (5.06%), *Candidatus Solibacter* (5.00%), *Clostridium* (3.74%), *Conexibacter* (1.94%), *Streptomyces* (1.90%), and *Edaphobacter* (1.87%) (Figure 1).

Additionally, the comparative study at the phylum level exhibited the prominent existence of Firmicutes (specifically Clostridium), Actinobacteria (Nocardioides and Phycicoccus), and Chloroflexi (Bellilinea, Anaerolinea, Longilinea, and Caldilinea) in the 2G soil sample (Supplementary Figure). However, in the 2GC soil sample, the dominant phylum was Acidobacteria (specifically *Candida tuskoribacter* and *Candida tussolibacter*), followed by Firmicutes (*Bacillus and Clostridium*), Actinobacteria (*Conexibacter and Streptomyces*), and Proteobacteria (*Edaphobacter*). The phyla Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Planctomycetes were consistently present in both soil samples. Two unique phyla, Chloroflexi and Nitrospira, were found exclusively in the 2G soil sample. With the exception of *Clostridium*, the genus-level composition of the microbial communities exhibited variations between the two soil samples.

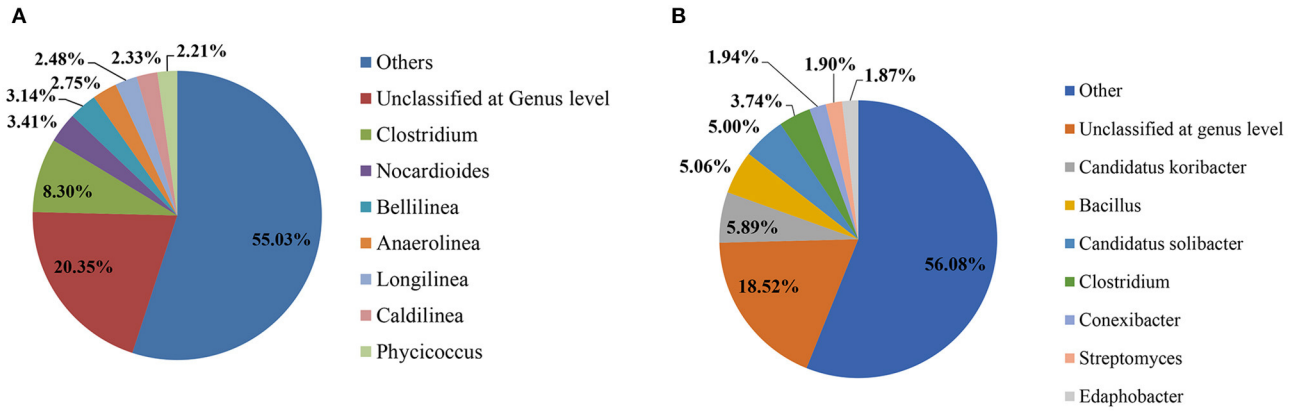

FIGURE 1
Pie chart provides a comparative analysis of the impact of pesticides on the soil microbial community at the genus level. **(A)** Depicts the soil sample contaminated with pesticides (2G), while **(B)** represents the soil sample without any pesticide contamination (2GC).

Alpha diversity

The genus-level relative abundance of the top 25 classified operational taxonomic units (OTUs) was investigated. In the 2G soil sample, the Shannon species diversity index was high (3.198). Genotypically, a total of 1,627 species were identified, whereas in the 2GC soil sample (control), the Shannon species diversity index was 2.739, and 1,850 species were identified genotypically (Figure 2).

In the 2G soil sample, the population of *Clostridium* was larger, whereas in the 2GC soil sample, the population of *Bacillus* was dominant. Unclassified bacteria at the genus level were predominantly found in both soils, but their abundance decreased in the 2GC soil sample. Genera from the phyla Actinobacteria (*Nocardioides*) and Firmicutes (*Clostridium and Oenococcus*) were evenly distributed in both soil samples. Abundant genera in the 2G soil sample included *Clostridium* (10.6%), *Nocardioides* (4.3%), *Bellilinea* (4.0%), *Anaerolinea* (3.5%), *Longilinea* (3.2%), and *Phycicoccus* (2.8%). The remaining microbial population in the 2G soil sample was distinct from that in the 2GC soil sample.

Hierarchal clustering

The heat map generated by hierarchical clustering exhibited the number of OTUs per sample. The color intensity on the heat map corresponds to the relative abundance of an OTU within a sample (Figure 3). In the heat map generated by hierarchical clustering, the pesticide-contaminated soil sample (2G) exhibited significant prevalence of the following classes: Clostridia, Betaproteobacteria, Ignavibacteria, Gemmatimonadetes, Dehalococcoides, Caldineae, and Thermoprotei.

Discussion

Several microbial communities have been studied previously in response to pesticide contamination (Floch et al., 2011; Zabaloy et al., 2012); however, fewer studies have utilized metagenomics for this purpose. Gangola et al. (2021) isolated a bacterial strain *Bacillus cereus* 2D from the same sampling sites, i.e., Gularbhoj, and found a highly efficient strain to tolerate higher concentrations of pesticides and a high rate of degradation, and they evaluated the expression of protein profiling under pesticide stress. The decrease in the copy number of 16S rDNA genes in the pesticide-contaminated soil indicated exposure of the pesticide to the native microbial flora, leading to inhibition of metabolic activity and growth. Application of real-time PCR was also employed by several researchers for the investigation of gene expression, microbial abundance, and functional and taxonomic gene expressions (Rastogi and Sani, 2011; Gangola et al., 2018; Kumar et al., 2019; Joshi et al., 2021). Yale et al. (2017) used quantitative PCR to assess and quantify the expression of pesticide-degrading genes (trzN, atzB, and atzA). Bacteria play an important role in balancing the ecosystem, nutrient composition, and cycling, but exposure to pesticides suppresses the microbial community significantly in agricultural fields (Onwona-Kwakye et al., 2020). Although the development of molecular techniques (qPCR) is quite laborious and difficult, these techniques give more insights into and better monitoring of pesticide-degrading genes.

Several hidden facts have been resolved since the development of emerging explorative techniques such as metagenomics. However, though it requires extensive data analysis, it justifies its potential in monitoring more than one pesticide compound at a time and analyzing their effects on the microbial community at different levels, such as class, phylum, genus, and species levels (Hou et al., 2015; Aldas-Vargas et al., 2022). The decrease in reads in the pesticide-treated soil (2G) is supported by various findings that clearly indicate the toxic nature of the pesticide (Onwona-Kwakye et al., 2020; Bhatt et al., 2021; Gangola et al., 2022d). The reduction in read numbers in the 2G soil sample may be attributed to the excessive and repeated use of pesticides in agricultural practices, ultimately resulting in the decline in the microbial population. Regular application of different pesticides decreases the microbial communities such as *Aeromonas, Bordetella, Comamonas, Enterobacter,* and *Staphylococcus*. Apart

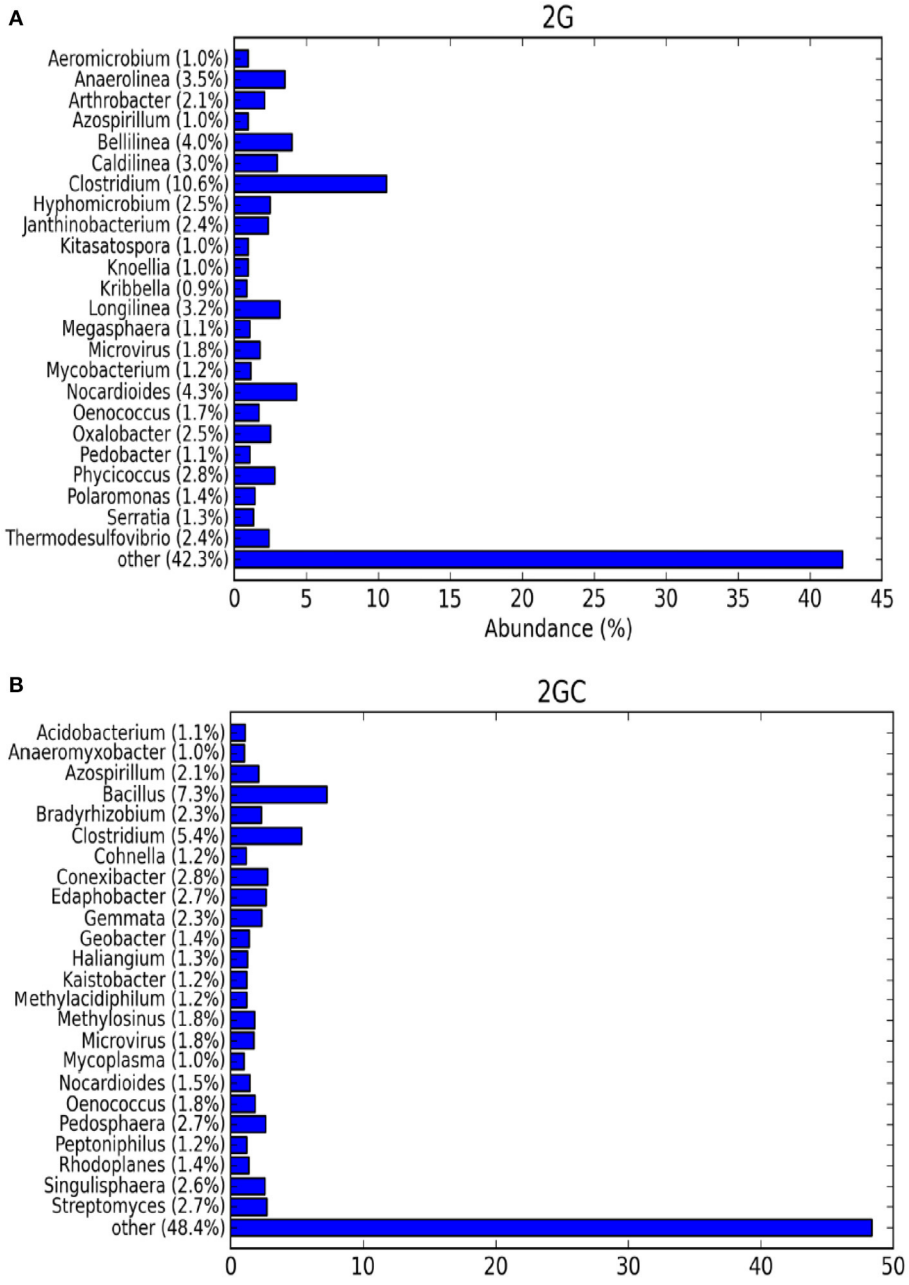

FIGURE 2
The bar graph exhibits alpha diversity of bacterial communities. The top 25 OTUs, taxonomically classified at genus level of **(A)** pesticide contaminated (2G) and **(B)** non-contaminated (2GC) soil samples.

from that, exposure to pesticides may decrease the degradation rate of organic pollutants, microbial homeostasis, and plant growth and protection from pathogens (Hayward et al., 2010; Khalifa et al., 2016; Hamidou Soumana et al., 2017; Pereira et al., 2017). Kantachote et al. (2001) and Rodríguez et al. (2020) have demonstrated the negative impact of pesticides on soil microorganisms on agar plates.

The large portion covered by others in the pie chart represents those microorganisms presenting their existence in a very minor proportion compared with others present dominantly, while the dominance of the unclassified category in both the soil samples represents those microorganisms that are still not identified and classified or are novel. In the 2G soil sample, *Clostridium* is the dominant genus followed by *Nocardioides*, *Bellilinea*, *Anaerolinea, Longilinea*, *Caldilinea*, and *Phycicoccus*. *Clostridium* has already been reported for its pesticide degradation potential such as Alachlor, chlorpropham, DDT, and lindane and is capable of surviving in pesticide-contaminated soil (Alipour et al., 2018). The presence of atrazine and chloro-s-trazine-degrading enzyme TrzN was identified in *Nocardioides* sp. and utilizes atrazine as the sole carbon and nitrogen source from contaminated soil (Topp, 2001; Ortiz-Hernández et al., 2013). Although *Bellilinea* sp. and

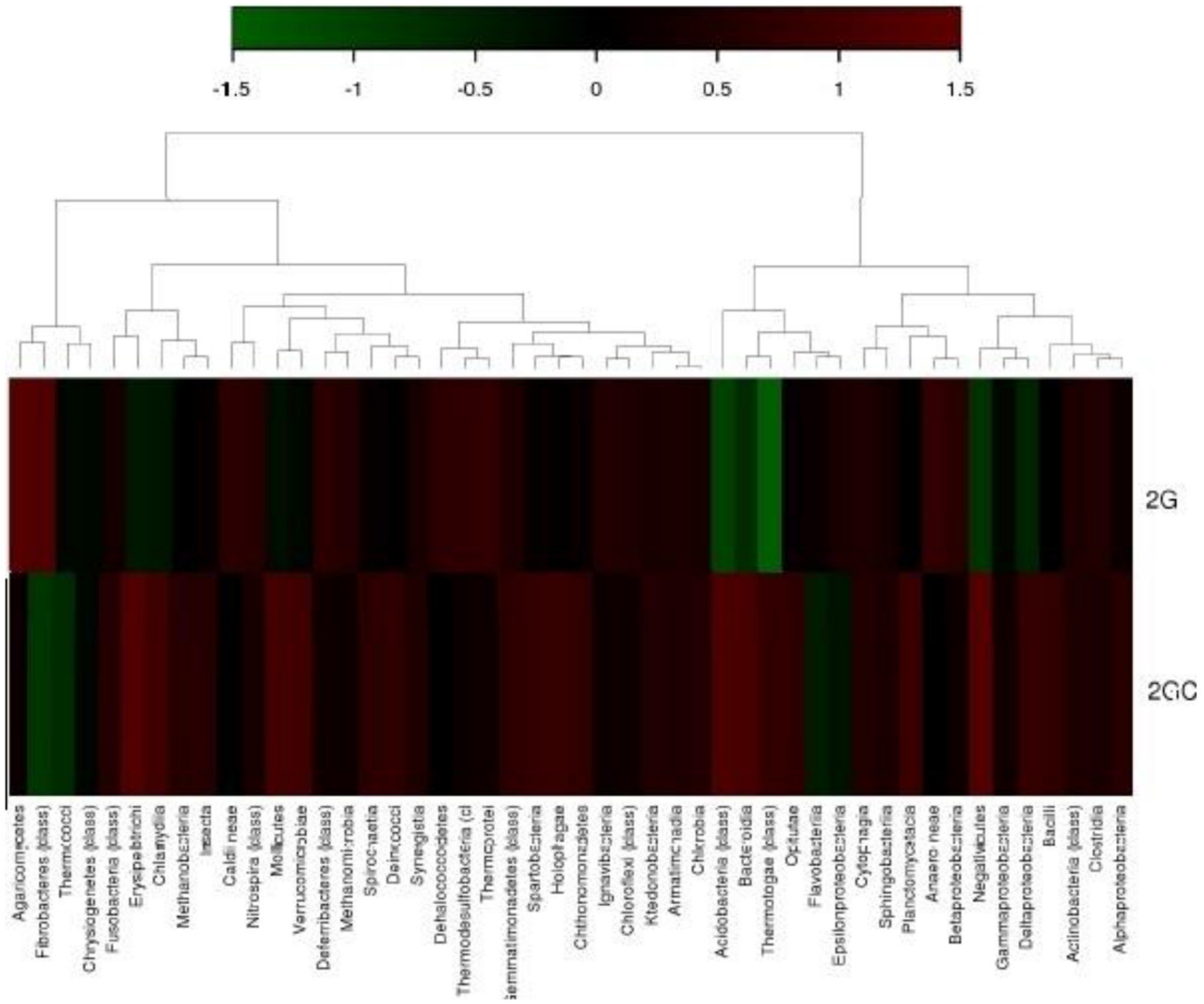

FIGURE 3
Hierarchical clustering illustrates the total count of operational taxonomic units (OTUs), according to the number of hits and % hits against the Greengene database. The heatmap indicates the abundance of each class within every sample. The color gradient from deep brown (+1) to dark green (−1) represents the distribution of OTUs, ranging from higher to lower abundance.

Longilinea sp. have not been reported for pesticide biodegradation, in previous report, *Bellilinea* sp. showed their potential to degrade a carcinogenic xenobiotic compound, i.e., 2-methyl-naphthalene, while *Longilinea* sp. was characterized for propionate degradation (Yamada et al., 2007; Musat et al., 2009; Rodríguez et al., 2020). The role of *Anaerolineae* has been reported to degrade and minimize the concentration of different xenobiotic hydrocarbons under wastewater (Yamada et al., 2007). In soil conditions, the initial application of chlorpyrifos suppresses the expression of genes and metabolic action of the native microbial community; after 2 weeks of the application, the suppressed microbial community reverts back to the level of control (non-contaminated soil) (Fang et al., 2008). Hence, it concluded that due to the different environmental fate of the pesticides after their applications, the native microbial community gets a chance to recover or can adapt to the conditions by expressing pesticide-degrading genes and metabolic potential.

Our research reveals variations in microbial communities and population sizes, signifying the repeated use of pesticides in agricultural fields. This leads to the replacement of older populations with new ones over time. As a consequence, the number of pesticide-sensitive species declines while pesticide-tolerant species survive and proliferate (Gangola et al., 2021). Our findings indicate that pesticides have a toxic impact on specific species within the same phylum, while others are capable of utilizing pesticides as a source of carbon and energy, enabling their prolonged survival. The increased population sizes of Proteobacteria, Actinobacteria, Firmicutes, and Chloroflexi point to their active growth and proliferation in pesticide-contaminated soil. The positive association between these present microbes in consortia may enhance their degradation abilities under pesticide stress (Yilmaz et al., 2022). Furthermore, our study demonstrates that microbial communities with a high metabolic potential for

pesticide degradation are more abundant in soils where pesticides persist for an extended period or are regularly used over time. Comparing the two soil types, we observed that the abundance of highly metabolically active communities was higher in rapidly degrading soil, indicating the superior functional capacity of these microbes in terms of nutrient cycling. Consequently, we can infer that the microbial community in 2GC soil exhibits sensitivity to pesticides, whereas the microbial community in 2G soil displays resistance to them (Gangola et al., 2021). At the phylum level, our research indicates that these microbes utilize pesticides as a source of carbon and energy for their growth and development. The toxic nature of pesticides leads to the absence of certain bacterial populations in the treated samples as compared with the control sample (Jeffries et al., 2018). Notably, the higher richness of Firmicutes, Actinobacteria, and Chloroflexi in the treated soil suggests their active involvement in pesticide-contaminated soil.

As observed in the alpha diversity analysis, the Shannon species diversity index (3,198) was maximum for the 2G soil sample, while in the 2GC soil sample, the Shannon species diversity index was minimum (2.739). This indicates that more the Shannon index value, the more the diversity in species. Hence, it could be analyzed easily that the more diversity in the system, the more the system stable. Although the total number of species (1,850) present in 2GC soil is more as compared with the 2G (1,627), in the context of species diversity, 2G soil sample is more dynamic. The possible reason is that pesticides create selective pressure on the microbial community to adapt and acclimatize under stress.

In the hierarchical map, the different classes of microbial communities were analyzed in a comparative manner between 2G and 2GC soil samples. More intense color signifies the dominance of the native microbial class. A hierarchical map was used by Parks et al. (2014) and Jeffries et al. (2018) to analyze the relative abundance of microbial functions in soil samples contaminated with organophosphorus pesticides. Previous reports also marked similar communities for pesticide degradation in different studies. Parks et al. (2014) and Jeffries et al. (2018) employed the hierarchical map approach, utilizing UPGMA clustering, to investigate the comparative prevalence of microbial function in soil samples contaminated with organophosphorus pesticides.

Conclusion

The application of indigenous microorganisms present in contaminated environments is a more reliable approach for the biodegradation of agricultural pesticides. Under pesticide stress, the bacterial communities altered their genetic pool and developed as efficient mutant strains for the utilization of pesticides as a source of carbon and energy. Monitoring of microbial communities is important because they play a crucial role in the regulation of biogeochemical cycles and soil health, enhancing crop productivity, and maintaining a sustainable environment. Additionally, by conducting metagenomic studies of contaminated soil samples, it becomes possible to identify various types of microbial populations present and the interaction between them. This knowledge is indispensable for comprehending the natural biodegradation of pesticides in environmental settings. Looking ahead, exploring the activity of bacterial isolates against other xenobiotic compounds and delving into the mechanisms of gene regulation involved would be valuable avenues for research and potential advancements in the field.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found in the NCBI repository under accession numbers: SAMN37722849 and SAMN37722850.

Author contributions

SG: Conceptualization, Data curation, Formal analysis, Writing—original draft. SJ: Conceptualization, Formal analysis, Writing—review and editing. GB: Formal analysis, Investigation, Methodology, Writing—review and editing. GP: Writing—review and editing, Formal analysis. KP: Formal analysis, Validation, Writing—review and editing. NB: Formal analysis, Validation, Writing—review and editing. RR: Validation, Writing—review and editing. AS: Formal analysis, Supervision, Validation, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1271129/full#supplementary-material

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Fungicides and insecticides can alter the microbial community on the cuticle of honey bees

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Honey bees are crucial for our ecosystems as pollinators, but the intensive use of plant protection products (PPPs) in agriculture poses a risk for them. PPPs do not only affect target organisms but also affect non-targets, such as the honey bee *Apis mellifera* and their microbiome. This study is the first of its kind, aiming to characterize the effect of PPPs on the microbiome of the cuticle of honey bees. We chose PPPs, which have frequently been detected in bee bread, and studied their effects on the cuticular microbial community and function of the bees. The effects of the fungicide Difcor® (difenoconazole), the insecticide Steward® (indoxacarb), the combination of both (mix A) and the fungicide Cantus® Gold (boscalid and dimoxystrobin), the insecticide Mospilan® (acetamiprid), and the combination of both (mix B) were tested. Bacterial 16S rRNA gene and fungal transcribed spacer region gene-based amplicon sequencing and quantification of gene copy numbers were carried out after nucleic acid extraction from the cuticle of honey bees. The treatment with Steward® significantly affected fungal community composition and function. The fungal gene copy numbers were lower on the cuticle of bees treated with Difcor®, Steward®, and PPP mix A in comparison with the controls. However, bacterial and fungal gene copy numbers were increased in bees treated with Cantus® Gold, Mospilan®, or PPP mix B compared to the controls. The bacterial cuticular community composition of bees treated with Cantus® Gold, Mospilan®, and PPP mix B differed significantly from the control. In addition, Mospilan® on its own significantly changed the bacterial functional community composition. Cantus® Gold significantly affected fungal gene copy numbers, community, and functional composition. Our results demonstrate that PPPs show adverse effects on the cuticular microbiome of honey bees and suggest that PPP mixtures can cause stronger effects on the cuticular community than a PPP alone. The cuticular community composition was more diverse after the PPP mix treatments. This may have far-reaching consequences for the health of honey bees.

KEYWORDS

pesticides, bee, cuticular microbiome, fungi, bacteria, plant protection products, neonicotinoids

1. Introduction

A significant decline in pollinators has been observed in the past decade, despite animal pollination being one of the most important ecosystem services (Potts et al., 2010). One of the main drivers that could lead to this pollinator decline is the intensive use of plant protection products (PPPs) in agriculture (Kluser and Peduzzi, 2007; Goulson et al., 2015). For the investigation of adverse side effects of PPPs on beneficial insects, the honey bee *Apis mellifera* is an excellent model organism because of its rich behavioral repertoire and the large diversity of methods for investigating their behavior (Scheiner et al., 2013).

Among the different PPP groups, insecticides are the best-studied class as they often show negative effects on beneficial insects (Galvan et al., 2006; El Hassani et al., 2008; Shi et al., 2019). Fungicides are usually not sufficiently investigated since no harmful impact on insects is assumed (Zubrod et al., 2019; Schuhmann et al., 2022). Nevertheless, some fungicides can synergistically interact with insecticides or prolong their undesirable effects on the health of pollinators (Wernecke and Castle, 2020). This study tested combinations of PPPs frequently detected in bee bread (Rosenkranz et al., 2019). The insecticide Steward® was combined with the fungicide Difcor®, and the insecticide Mospilan® was applied together with the fungicide Cantus® Gold (Table 1). Both combinations have been used in agriculture and are consumed by bees as they are applied to mass-flowering crops (Holzschuh et al., 2013).

Indoxacarb is the active ingredient of the insecticide Steward® and acts as a sodium channel modulator, leading to a quick inhibition of feeding in pest insects (Börner et al., 2009). Acetamiprid (active ingredient of the insecticide Mospilan®) is a neonicotinoid that acts as an agonist on nicotinic acetylcholine receptors, resulting in constant ion flow and neurotoxic effects (Börner et al., 2009). Several negative side effects of the isolated application of both insecticides on beneficial insects have already been demonstrated (Galvan et al., 2006; Laurino et al., 2011; Shi et al., 2020). In contrast to these insecticides, the fungicides difenoconazole (an active ingredient of Difcor®) and boscalid or dimoxystrobin (active ingredients of Cantus® Gold) appear to have small or no effects on beneficial insects when applied on their own (Wernecke et al., 2019; Almasri et al., 2020).

Bees are often exposed to a mixture of several PPPs. This can result from tank mixtures, spraying sequences, the combined use of seed and spray treatment, or bees foraging sequentially at different flowers (Thompson et al., 2014). Thus, various active substances are present in bee bread (Rosenkranz et al., 2019). It has been shown that certain insecticide–fungicide combinations can lead to synergistic negative effects (Schuhmann et al., 2022), which makes it important to study more intensively the interaction of insecticides and fungicides in insects.

PPPs affect not only pest organisms but also non-targets such as bees and microorganisms in the environment. Honey bees come in contact with a wide variety of microbiomes, namely pollen (Gilliam et al., 1989; Martinson et al., 2011), nectar (Fridman et al., 2012; Alvarez-Pérez and Herrera, 2013), bee bread (Sinpoo et al., 2017), and plants' surfaces (Keller et al., 2021). Nevertheless, little is known about the cuticular microbiome of bees and its effects on the health and performance of honey bees. In contrast, the gut microbiome is well studied since it plays a central role in metabolism, growth, development, protection against pathogens, and immune defense (Zheng et al., 2017; Kwong et al., 2017a).

The core gut microbiome of honey bees is dominated by nine bacterial species clusters, which account for 95 to 99.9% of the bacteria in almost all individuals (Babendreier et al., 2007; Engel et al., 2012; Moran et al., 2012; Sabree et al., 2012; Corby-Harris et al., 2014). Five core bacterial species clusters and four rarer species clusters form the dominant cluster of bacteria found in honey bees (Kwong et al., 2017b; Raymann and Moran, 2018). The four core bacteria are *Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus* Firm-4, *Lactobacillus* Firm-5, and *Bifidobacterium asteroids* (Babendreier et al., 2007; Martinson et al., 2011; Kwong and Moran, 2013).

The gut mycobiome of most worker bees is dominated by the members of the genus *Saccharomyces*, whereas the intestines of forager bees and queens were colonized by various fungal taxa, including *Zygosaccharomyces*, *Candida,* and *Ascomycota*. Katsnelson (2015) pointed out that the gut microbiome is not the only microbial community that is important for bees. For example, bees have close contact with bacteria on the inner walls of the hive (Anderson et al., 2013).

The gut of the honey bee and whole-body extracts appeared to have the same dominant microbiomes but vary in relative abundance and composition (Mattila et al., 2012; Ribière et al., 2019). Aizenberg-Gershtein et al. (2013) analyzed the cuticular bacterial community composition. The cuticular bacterial microbiome is colonized by the bacterial classes *Gammaproteobacteria*, *Actinobacteria*, *Bacilli,* and *Alphaproteobacteria*. *Arsenophonus* represented the most dominant genus within *Gammaproteobacteria*. *Arsenophonus* represents an extensive cluster of symbiotic bacteria in insects (Nováková et al., 2009), which has already been recovered in the gut of honey bees (Babendreier et al., 2007). Saccà and Lodesani (2020) identified and isolated bacteria (*Apilactobacillus kunkeei*, *Bacillus thuringiensis*, and *Acetobacteraceae*) from the cuticle of honey bees, which might function as a natural antagonist of the external parasitic mite *Varroa destructor*.

TABLE 1 Overview of the plant protection products (PPPs) used (FMC Agricultural Solutions, 2020, 2021; BASF SE, 2021; Fungicide Resistance Action Committee, 2021; Insecticide Resistance Action Committee, 2021; PLANTAN GmbH, 2021).

	PPP	Authorization holder	Active ingredient	Group	Area of application
Mix A	Difcor®	Globachem NV, Sint-Truiden, Belgium	Difenoconazole	SBI* fungicide	Fruit growing
	Steward®	Cheminova Deutschland GmbH & Co. KG, Stade, Germany	Indoxacarb	Oxadiazine	Fruit growing
Mix B	Cantus® Gold	BASF SE, Ludwigshafen, Germany	Boscalid + Dimoxystrobin	Succinate-dehydrogenase inhibitor + quinone outside inhibitors	Rapeseed cultivation
	Mospilan®	Nisso Chemical Europe GmbH, Düsseldorf, Germany	Acetamiprid	Neonicotinoid	Rapeseed cultivation

*Sterol biosynthesis inhibitors.

The study herein aimed to analyze (i) the effects of single neonicotinoids and fungicides; (ii) the effects of a mixture of a non-neonicotinoid (Steward®) with an SBI fungicide (Difcor®) (mix A, Table 1), and (iii) the effects of a combination of a neonicotinoid (Mospilan®) and a non-SBI fungicide (Cantus® Gold) (mix B, Table 1) on the bacterial and fungal communities of honey bees. The quantity, composition, and function of the different microbes were investigated. We hypothesize that fungal taxa and, to a lesser extent, bacterial taxa will be reduced in abundance, and some taxa might even be sensitive to PPPs. On the other hand, we expect that other microbes will gain an advantage due to the inactivation of competitors and the addition of PPPs as substrates.

2. Methods

2.1. Treatment of honey bees

Honey bee workers (*A. mellifera carnica*) were collected randomly from a hive in the departmental apiary at the University of Würzburg.

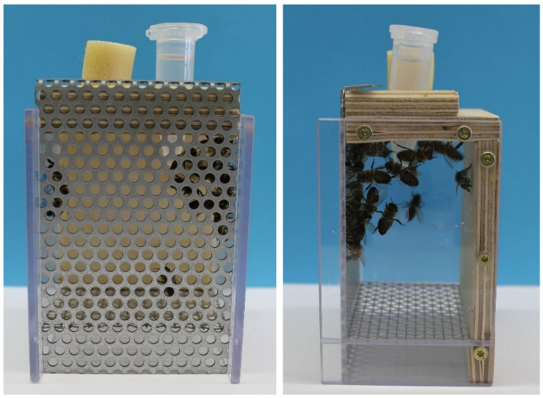

FIGURE 1
Experimental setup of the bee's treatment. Experimental setup of *Apis mellifera* nursing bees in a cage system. The feeding solution is provided via a prepared 2mL cup with holes at the bottom. Picture A. Schuhmann.

For each treatment, five cages were prepared on five consecutive days. Each cage contained 30 honey bees. The cages were maintained in an incubator (30°C, 50% humidity), and the honey bees received the treatment solutions for 1 week. The feeding solutions were provided via prepared 2 mL cups (Figure 1). The amount of food per cage was adapted to the number of individuals so that the bees could eat *ad libitum*. The 2 mL cups were replaced every day to guarantee a controlled food supply.

The feeding solutions were calculated based on residue levels found in the field and consisted of 30% sugar water with the addition of PPPs (McArt et al., 2017; Lüken and von der Ohe, 2018; El-Nahhal, 2020; Friedle et al., 2021). For reasons of data availability, we relied on residue values from pollen for the calculation of Steward® and Difcor® and on residue values from honey for Mospilan® and Cantus® Gold.

Using the intake of honey or pollen per bee per day (Rortais et al., 2005), it was calculated how much active ingredient a honey bee would consume per day based on the selected residues. It was assumed that a caged honey bee consumes 60 µL (Hesselbach and Scheiner, 2019) of feeding solution per day. Therefore, the feeding concentration was adjusted so that a honey bee would ingest the corresponding calculated active ingredient concentration in these 60 µL (Table 2).

We used the water-soluble formulation of PPPs to mimic field conditions (Cox and Surgan, 2006). The honey bees were either treated with the insecticide alone, the fungicide alone, a mixture of both, or with a control solution (30% sugar water). All four treatments belonged to one experimental series (Table 2). Each treatment group had its own control ("control A" belongs to Difcor®, Steward®, and PPP mix A treatments; "control B" belongs to Cantus® Gold, Mospilan®, and PPP mix B treatments). The concentrations of the single PPPs were maintained in the mix A and B treatments. After the 1-week treatment, the bees were frozen in liquid nitrogen and stored at −80°C.

2.2. Cuticle preparation and DNA extraction

Dissection and preparation of cuticles were performed under frozen conditions. Antennae, legs, and wings were removed (but not

TABLE 2 Overview of plant protection product (PPP) treatments.

PPP treatments	Residue [µg/kg]	PPP concentration in the feeding solution [µg/L]	Average of PPP consumed per day per bee in a cage [µg]
Difcor®	48 (in pollen) (46)	9	0.000576
Steward®	557 (in pollen) (43)	100	0.006
Mix A (Difcor® + Steward®)	48 (in pollen) (46) 557 (in pollen) (43)	9 + 100	0.000576 + 0.006
Control A		0	0
Cantus® Gold	5 (in honey) (44)	10	0.0008
Mospilan®	72.5 (in honey) (45)	200	0.012
Mix B (Cantus® Gold+ Mospilan®)	5 (in honey) (44) 72.5 (in honey) (45)	10 + 200	0.0008 + 0.012
Control B		0	0

Doses and their corresponding residue values in the field based on which the concentrations were calculated are indicated. Feeding solution consists of 30% sugar water and the addition of PPP.

discarded). Afterward, the inner organs (i.e., brain, muscles, gut, and sting apparatus) were carefully removed from the head capsule, the thorax, and the abdomen (Ribière et al., 2019; Subotic et al., 2019). The prepared cuticles and outer body parts underwent DNA isolation. Cuticles from four individuals were pooled for one sample. DNA extraction was performed using the Quick-DNA™ Fecal/Soil Microbe Microprep Kit according to the manufacturer's protocol (Zymo Research Europe GmbH, Freiburg im Breisgau, Germany). All DNA extracts were stored at −20°C until further use. Five independent bee replicates per treatment were analyzed.

2.3. Quantitative PCR of bees cuticular DNA extracts

The quantitative PCR (qPCR) was performed to quantify the gene copy numbers of the bacterial 16S rRNA gene with the primer sets BAC341f (5′-CCTACGGGNGGCWGCAG-3′) and BAC758R (5′-GACTACHVGGGTATCTAAKCC-3′) (Klindworth et al., 2013) and of the fungal internal transcribed spacer (ITS) DNA regions with the primer sets fITS7 (5′-GTGAATCATCGAATCTTTG-3′) (Ihrmark et al., 2012) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1994). Each independent replicate was quantified in three technical triplicates in 96-well plates using the CFX96™ Real-Time System C1000™ Thermal Cycler (Bio-Rad Laboratories GmbH, Feldkirchen, Germany). Fungal ITS and bacterial 16S rRNA gene-base qPCR were performed in 20 µL reaction mixtures containing 1 µL of DNA template, 0.3 µM each primer, 1x PCR-Enhancer (Biozym Scientific GmbH, Oldendorf, Germany), 1x iTaq Universal SYBR Green Supermix (Bio-Rad, Munich, Germany), and nuclease-free water (Sigma-Aldrich, St. Louis, MO, USA). Nuclease-free master mix blanks were run as negative controls. Reaction conditions for 16S qPCR involved an initial 3-min denaturation at 95°C, followed by 40 cycles of 5 s of denaturation at 95°C, annealing at 52°C for bacteria and 52.7°C for fungi over 30 s, respectively, and elongation at 60°C for 30 s. The final elongation step was at 72°C for 10 min. Gene copy numbers were calculated as previously described by Lasota et al. (2019) by comparing PCR-cycle threshold (CT) values to a standard curve of triplicate 10-fold dilutions of genomic DNA (gDNA) extracted from a known concentration of *Escherichia coli* K12 (DSM 423) and *Fusarium solani* (DSM 1164) by employing the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit according to the manufacturer's instructions (Zymo Research Europe GmbH). The genomic DNA concentration per PCR reaction of *E. coli* and *F. solani* standard ranged from 1×10^9 to 5×10^3 and 6.51×10^6 to 65.1 gene copies.

2.4. Amplicon sequencing of the cuticular microbiome

Cuticular DNA samples were further analyzed by amplicon sequencing. The 16S rRNA gene and ITS DNA region were amplified with the same primer sets used for the qPCR analysis to create amplicon sequencing libraries for each of the 40 bees' cuticular DNA samples. Inline barcodes and Illumina sequencing adapters were added to the amplicon sequence libraries using the Nextera CT Index Kit (Illumina, San Diego, CA, USA) and MiSeq Reagent Kit v3 600 cycles (Illumina) according to the manufacturer's instructions. PCR products for library preparation were purified by AMPure XP beads (Beckman Coulter, Brea, CA, USA). The sequencing of libraries was performed by 300-bp paired-end sequencing on an Illumina MiSeq platform (Illumina MiSeq V3; Illumina) based on a standard protocol from the manufacturer. Amplicon sequencing library preparation, sequencing, and sequence quality checks were carried out by LGC Genomics GmbH (Berlin, Germany).

2.5. Bioinformatics

Raw data pre-processing with demultiplexing, sorting, adapter trimming, and merging reads was congregated using Illumina bcl2fastq conversion software v2.20 and BBMerge v34.48 (DOE Joint Genome Institute, 2022). The sequence quality of the reads was controlled with the FastQC software, version 0.11.8 (Babraham Bioinformatics, 2020). Sequence pre-processing and Operational Taxonomic Units (OTUs) picking from amplicons were conducted using Mothur 1.35.1 (Schloss et al., 2009). Sequences were aligned against the 16S Mothur-Silva SEED r119 reference alignment depending on their Phred quality score over 33 (Whelan et al., 2019). Filtering short alignments and reducing sequencing errors were conducted by pre-clustering, where a maximum of one base mismatch per 100 bases within a cluster was allowed. Chimeras were eliminated with the UCHIME algorithm (Edgar et al., 2011). Afterward, taxonomical classification of the sequences against the Silva reference classification was conducted, and sequences of other domains of life were removed for OTU picking. OTUs were selected and assigned to a taxonomic level by clustering at the 97% identity level (Edgar, 2018; Nilsson et al., 2019; Kõljalg et al., 2020). Thereby, OTU tables for DNA samples were constituted.

Ecological and metabolic functions of detected bacterial OTUs were predicted using the functional annotation tool of the prokaryotic taxa v.1.1 (FAPROTAX) database (Louca et al., 2016). The functions of each prokaryotic taxon were annotated using the literature on cultivable strains. The FungalTraits database (Põlme et al., 2021), a specific functional prediction tool, was used to taxonomically parse fungal genera by ecological guild independent of the sequencing method. Bacterial and fungal function count tables for each DNA sample were generated. Sequence counts of OTUs for fungi ranged from 919 to 345,516 and from 3,131 to 204,067 for bacteria.

2.6. Statistics

Statistical analyses were performed after OTUs were taxonomically summarized at the genus level. The normal distribution of each dataset was tested via OriginPro 2022 (OriginLab Corporation, Northampton, MA, USA) by the Shapiro–Wilk test ($p < 0.05$). Rarefaction analysis as well as the estimation of alpha diversity (OTU richness, Shannon index, Simpson index, and Pielou's Evenness) and OTU richness estimators [bias-corrected Chao1 and an abundance-based coverage estimator (ACE)] were performed for cuticular DNA samples in RStudio (Version 2022.02.1, RStudio, Inc., Boston, MA, USA) and the package vegan 2.5–7 (Oksanen et al., 2022; R: The R Project for Statistical Computing, 2022).

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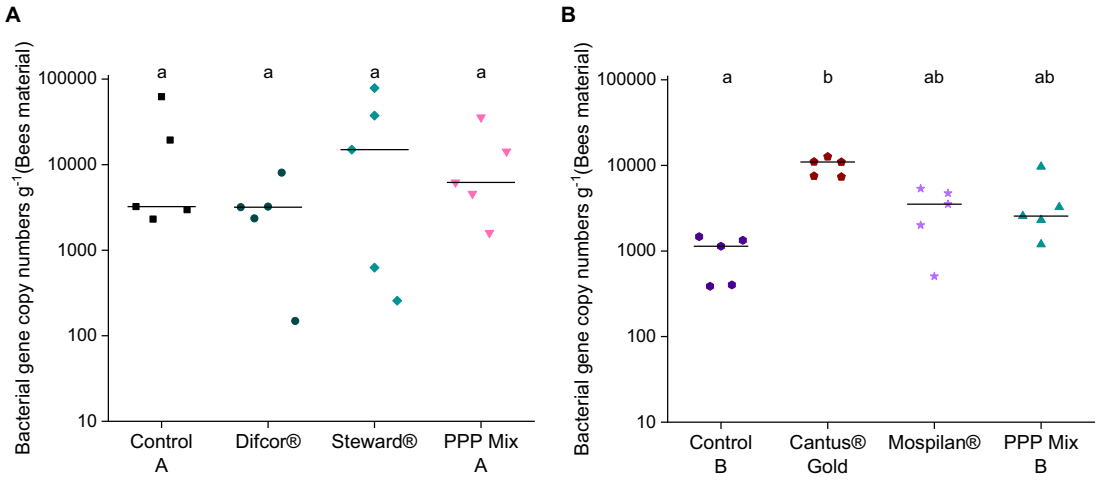

FIGURE 2
Bacterial gene copy **(A,B)** numbers after PPP treatment. PPP Difcor®, Steward®, or the combination of both (mix A) **(A)** and PPP Cantus® Gold, Mospilan® and the combination of both (mix B) **(B)** treatments ($n = 5$). Different letters indicate statistically significant differences according to the Dunn test ($p = 0.05$). If the letters are the same, treatments were not significant to each other, and if they were different, treatments were significantly different. The fungal gene copy numbers are shown in Supplementary Figure 1.

Alpha diversity indices were tested for normal distribution by Shapiro–Wilk test ($p < 0.05$). Significant effects ($p < 0.05$) on cuticular alpha diversity indices for each PPP treatment were calculated either by one-way ANOVA with a post-hoc adjusted Tukey test, if data were normally distributed, or Kruskal–Wallis ANOVA with a post-hoc Dunn test, if data were not normally distributed, using OriginPro (Version 2022. OriginLab Corporation). The same statistical procedure was used to analyze the effects of PPP treatments on bacterial and fungal gene copy numbers on the cuticles. Permutation multivariate analysis of variance (NPMANOVA) based on Bray–Curtis similarity was performed using the software PAST 2.17c (Hammer et al., 2001) to analyze the differences between the different PPP treatments on the cuticular microbial communities and functions. The results were visualized by OriginPro (Version 2022. OriginLab Corporation). Noll et al. (2005) explained that relative abundances were calculated for each sample and visualized with OriginPro (Version 2022. OriginLab Corporation). Significantly distinctive cuticular bacterial and fungal genera of the PPP treatments were identified using indicator species analysis conducted using the "multipatt" function in the indicspecies package (de Cáceres and Legendre, 2009), which calculates indicator values with the "r.g." function.

3. Results

3.1. PPP treatment significantly altered bacterial and fungal gene copy numbers

Our data showed that fungicides can significantly affect the bacterial and fungal gene copy numbers on the cuticle of honey bees. The fungicide Difcor® significantly reduced fungal gene copy numbers compared to control A ($p = 0.04246$), whereas neither the insecticide Steward® nor the mixture of Difcor® and Steward® affected fungal gene copy numbers (Supplementary Figure 1A).

Bacterial gene copy numbers were not affected by the fungicide Difcor®, the insecticide Steward®, or PPP mix A treatments (Figure 2A). In contrast, the fungicide Cantus® Gold significantly increased the bacterial gene copy numbers compared to control B ($p = 0.00167$) (Figure 2B). The insecticide Mospilan® and the PPP mix B had no significant effect on bacterial gene copy numbers compared to the control B (Figure 2B). PPP mix B treatment increased fungal gene copy numbers in all treatment groups (Supplementary Figure 1B). The highest fungal gene copy numbers could be found in the fungicide Cantus® Gold treatment, which differed significantly from the control B ($p = 0.01528$) and the insecticide Mospilan®-treated group ($p = 0.01815$) (Supplementary Figure 1B).

3.2. PPP treatments showed different effects on bacterial and fungal community compositions

The data indicate that the insecticide and fungicide treatments affect the fungal community composition. The fungal community composition was significantly altered by the insecticide Steward® treatment and differed significantly from control A ($p = 0.0157$). The fungicide Cantus® Gold significantly altered fungal community composition ($p = 0.0239$) in comparison with control B. Cantus® Gold, Mospilan®, and PPP mix B treatments significantly changed bacterial community composition in all treatments compared to control B (Figure 3; Supplementary Table 1).

In contrast, the insecticide Mospilan® and PPP mix B treatments did not lead to any alterations in the fungal community composition compared to the Cantus® Gold treatment and control B (Supplementary Table 1). The fungicide Difcor®, insecticide Steward®, and PPP mix A treatments did not significantly change the bacterial community composition (Figure 3; Supplementary Table 2). Bacterial and fungal OTU richness were not affected by the Difcor®, Steward®, and PPP mix A treatments [Dunn's test ($p = 0.05$)] or Cantus® Gold, Mospilan®, and PPP mix B treatments [Tukey test ($p = 0.05$); Figure 3].

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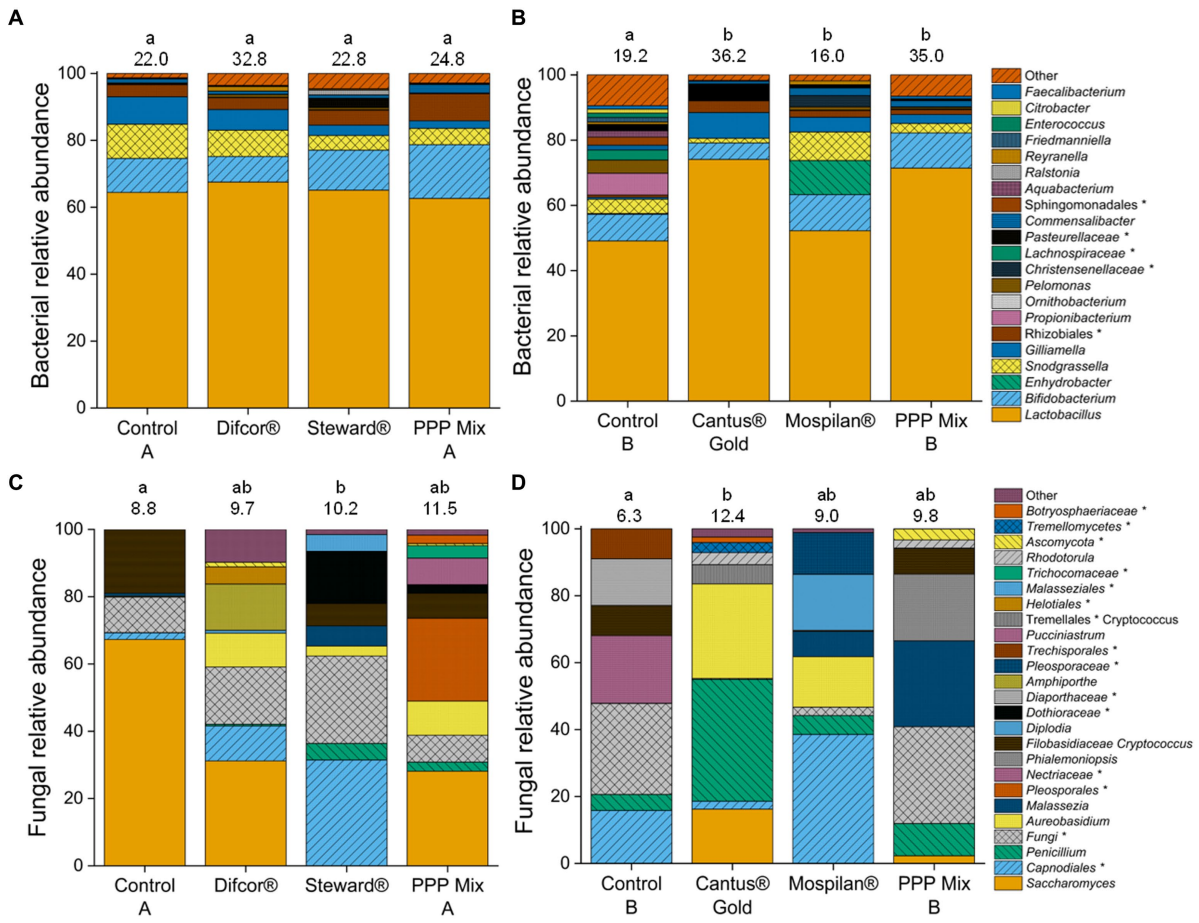

FIGURE 3
Relative sequence read abundance of the bacteria **(A,B)** and fungi **(C,D)** after PPP treatment. PPP Difcor®, Steward®, or the combination of both (mix A) **(A,C)** and PPP Cantus® Gold, Mospilan® and the combination of both (mix B) **(B,D)** treatments ($n = 5$). Bacterial species with a relative abundance of <1% were summarized as other. Complete bacterial abundance graphs without a summary of the low abundance can be found in Supplementary Figure 2. Fungal species with a relative abundance of <2% were summarized as other. Fully fungal abundance graphs without a summary of the low abundance can be found in different letters indicating statistically significant differences according to one-way non-parametric multivariate analysis (p=0.05). If the letters are the same, treatments were not significantly different to each other, and if they were different, treatments were significantly different. The numbers above the bars reflect the respective OTU richness. Unclassified members of the taxon are marked with *.

3.3. PPP treatments showed different effects on bacterial and fungal community functions

According to our data, insecticides and fungicides had an impact on fungal and bacterial functional composition. The insecticide Steward® treatment significantly impacted the fungal functional composition compared to control A ($p = 0.0154$) (Figure 4A). Furthermore, the fungicide Cantus® Gold treatment significantly altered fungal functional community composition in comparison with control B ($p = 0.0255$) (Figure 4B).

Plant pathogenic fungi had increased sequence read abundance in the fungicide Difcor® (19%) and insecticide Steward® (22%)-treated bees in comparison with control A (<1%) (Figure 4A). Whereas sequence read abundance of plant pathogenic fungi was reduced in the treatment's fungicide Cantus® Gold (2%), and PPP mix B (20%) or not altered in the insecticide Mospilan® (30%) treatment in comparison with control B (34%) (Figure 4B). The fungicide Difcor®-treated bees' cuticular fungal microbiome was highly associated with the fungal genus *Amphiporthe* (Table 3), and the fungicide Cantus® Gold-treated bees' cuticular fungal microbiome was associated with the genus *Saccharomyces*. The insecticides Steward® and PPP mix A, and control A, as well as the insecticides Mospilan®, PPP mix B, and control B, were not associated with any fungal indicator species. Fungicide Difcor® and PPP mix A did not differ in the fungal functional community composition compared to control A and the insecticide Steward® treatment (Figure 4A). The same could be observed for the insecticide Mospilan® and PPP mix B in comparison with control B and the Cantus® Gold treatment (Figure 4A).

In contrast to the fungal community composition, only the insecticide Mospilan® treatment led to significant differences in the bacterial functional composition compared to control B ($p = 0.0165$) (Supplementary Figure 4B). Indicator analysis of Difcor®, Steward®, and PPP mix A treatments showed that *Commensalibacter* was highly associated with the bacterial community of the PPP mix A treatment (Table 3). The fungicide Difcor® treatment, the insecticide Steward® treatment, and control A did not bear any bacterial indicators. The cuticular microbiome of Cantus® Gold-treated bees was significantly

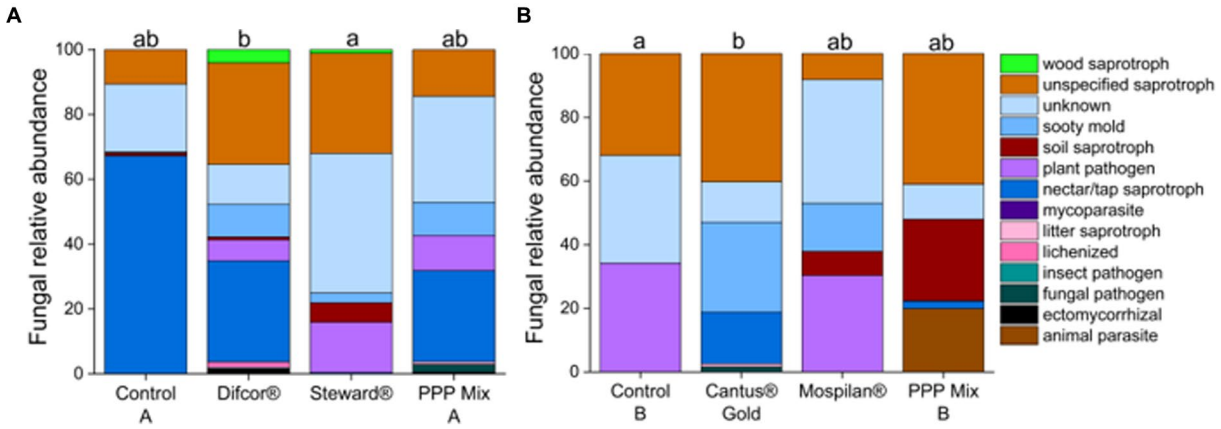

FIGURE 4
Fungal functional composition on genus level after PPP treatment. PPP Difcor®, Steward®, or the combination of both (mix A) **(A)** and PPP Cantus® Gold, Mospilan® and the combination of both (mix B) **(B)** treatments ($n = 5$). Different letters indicate statistically significant differences according to one-way non-parametric multivariate analysis ($p = 0.05$). If the letters are the same, treatments were not significant to each other, and if they were different, treatments were significantly different. Supplementary Tables 3, 4 provide which fungal genera were assigned to which functional traits.

TABLE 3 Indicator species analysis of cuticular bacterial and fungal community members after PPP treatment.

	PPP treatment	Stat	Value of *p*	Significance	Family	Genus	Function/bee location
Bacteria	Mix A	0.703	0.0059	**	*Acetobacteraceae*	*Commensalibacter*	Nitrate and nitrite respiration; Bee gut
	Cantus® Gold	0.480	0.0314	*	*Lactobacillales**	*Lactobacillales**	NA; Bee gut
		0.399	0.0046	**	*Flavobacteriaceae*	*Ornithobacterium*	Aerobic chemoheterotrophy
	Mix B	0.739	0.0054	**	*Orbaceae*	*Frischella*	NA; Bee gut
	Mospilan®	0.659	0.0077	**	*Neisseriaceae*	*Snodgrassella*	NA; Bee gut
Fungi	Difcor®	0.447	0.0088	**	*Gnomoniaceae*	*Amphiporthe*	Unspecified saprotroph
	Cantus® Gold	0.558	0.0352	*	*Saccharomycetaceae*	*Saccharomyces*	Nectar/tap saprotroph

The Stat value and significance of each species and its function are shown, ordered after the Stat-value. Unclassified members of the taxon are marked with *. Significant levels: '**' 0.01 '*' 0.05 '.' 0.1. NA: No function was assigned.

associated with unclassified members of the order *Lactobacillales*. Furthermore, a weaker association with *Ornithobacterium* could be observed (Table 3). The cuticular bacterial microbiome of Mospilan®-treated bees' high associations with the genus *Snodgrassela* was observed. The cuticular bacterial microbiome of the combined treatment with PPP mix B was highly associated with the genus *Frischella* (Table 3). The fungicide Difcor®, insecticide Steward®, and PPP mix A treatments compared to control A (Supplementary Figure 4A), as well as the fungicide Cantus® Gold and PPP mix B treatments, did not affect the bacterial functional community composition compared to control B and the insecticide Mospilan® treatment (Supplementary Figure 4B).

4. Discussion

Tremendous effects on the fungal microbiome could be observed for all treatments. Moreover, single pesticide treatments such as the fungicide Difcor® significantly reduced the fungal gene copy numbers (Supplementary Figure 1B). The insecticide Steward® led to significant alterations in the fungal community composition and function (Figures 3B; Supplementary Figure 4A).

Fungicide Cantus® Gold, insecticide Mospilan®, and PPP mix B treatments increased bacterial and fungal gene copy numbers (Figures 2C,D; Supplementary Figure 1B), and all treatments significantly altered the bacterial community composition (Figure 3C; Supplementary Table 1). The insecticide Mospilan® led to significant changes in the bacterial functional composition (Supplementary Figure 4B). Fungicide Cantus® Gold had tremendous effects on the fungal cuticular community and functional composition (Figure 3D; Supplementary Figure 4B).

The fungicide Difcor® and insecticide Steward® treatments significantly impacted the cuticular bacterial community composition (Figure 3A; Supplementary Table 1A), while the bacterial gene copy numbers and bacterial community functions were unaffected (Figure 2A). The genus *Commensalibacter* was identified as an indicator taxa of the cuticular bacterial microbiome after PPP mix A treatment (Table 3). Members of the genus *Commensalibacter* were previously described as a core member of the honey bees' gut microbiome (Martinson et al., 2011; Kwong and Moran, 2016) and as an essential part of the honey bees' microbial ecosystem (Wu et al., 2022).

The fungicide Difcor®, insecticide Steward®, and PPP mix A treatments caused a reduction of the fungal gene copy numbers

compared to the control A. Moreover, the fungicide Difcor®, insecticide Steward®, and PPP mix A treatments led to a higher diversity of the cuticular fungal community composition (Figure 3B). Similar results could be observed for the intestine microbiome of bees after treatment with pesticides (Syromyatnikov et al., 2020). Similarly, the fungicide Difcor® significantly reduced the fungal gene copy numbers (Supplementary Figure 1B), and members of the genus *Amphiporthe* were significantly associated with this treatment. However, a recent study found that members of the same family, *Valsaceae,* were described as sensitive toward difenoconazole (Silva-Campos et al., 2022), indicating an ambivalent biocide response in this family. Furthermore, Steward® significantly changed cuticular fungal community composition and function (Figures 3B, 4A; Supplementary Table 2). *Saccharomyces* was not found in the insecticide Steward®-treated groups, indicating that members of *Saccharomyces* were highly sensitive to the insecticide Steward®. However, *Saccharomyces* was the most abundant genus in control A (Figure 3). Unclassified members of the genera *Dothioraceae* and *Capnodiales* were the most abundant species in the Steward®-treated cuticular fungal microbiomes (Figure 3B). Members of the genus *Dothioraceae* were already found in the guts of nectar-collecting *Apis cerana* (Basukriadi et al., 2010). *Capnodiales* was previously described as increasing abundance of chlorothalonil-based fungicides in field-relevant level-treated hives (Steffan et al., 2017). Even though the active ingredient differs from the insecticide Steward®, this result indicates that *Capnodiales* benefits from the treatment with PPP either directly by the inactivation of competitors or their predators and/or indirectly by microbial metabolites or degradation products released from PPP-sensitive species. Nectar/tap saprotrophs were two-thirds the most abundant group in control A. Nectar/tap saprotrophic fungi were reduced in the fungicide Difcor®, insecticide Steward®, and PPP mix A treatments compared to control A (Figure 4A). Those were reduced by less than 1% in the insecticide Steward® treatment, indicating that this insecticide alone already caused this reduction of nectar/tap saprotrophic fungi. The active ingredient of Steward® is indoxacarb; the insecticidal activity occurs by blocking the sodium channels within the nervous system of insects (Wing et al., 2000). Even though mycorrhizal fungi are described as playing an important role in balancing salinity within the environment and the use of sodium for signaling, no voltage-gated sodium channels have been found for fungi (Scharnagl et al., 2017). Thereby, we are the first to describe this non-target effect of indoxacarb on nectar/tap saprotrophic fungi. To date, honey analyses have not shown any negative effects of the use of any of these fungicides. However, based on our study, existing data and set-ups should be revisited in detail if they do affect honey quality.

Interestingly, plant pathogens gained abundance in the cuticular fungal community of the fungicide Difcor® and insecticide Steward®-treated bees in comparison with control A (Figure 4A). Moreover, sooty mold fungi were increased in the cuticular fungal community of fungicide Difcor®, insecticide Steward®, and PPP mix A-treated bees. Those fungi are reported to show resistance against difenoconazole (Difcor®) (Yang et al., 2019). Moreover, those were described as showing cross-resistance even for fungicides with a different mode of action (Yang et al., 2019), which might be the reason for their high sequence read abundances in the insecticide Steward®-treated bees (Figure 4A). Similar to the cuticular fungal community composition, the diversity of functional composition was increased due to the fungicide Difcor®, insecticide Steward®, and PPP mix A treatments, which were already observed for the intestinal microbiome of bees (Silva-Campos et al., 2022).

The fungicide Cantus® Gold, insecticide Mospilan®, and PPP mix B treatments shifted the bacterial and fungal cuticular community composition. Fungicide Cantus® Gold significantly increased bacterial and fungal gene copy numbers (Figures 2C,D). Fungicide Cantus® Gold, insecticide Mospilan®, and PPP mix B treatments significantly changed cuticular bacterial community composition (Figure 3C; Supplementary Tables 1, 2). Although the composition of the bacterial community was altered by the treatments with the fungicide Cantus® Gold, the insecticide Mospilan®, and the PPP mixture B, only the insecticide Mospilan® showed a significant change in the functional composition of the cuticular bacteria (Supplementary Figure 4B). Alberoni et al. (2021) observed a significant decrease in the neonicotinoid-treated groups and a compirsed functionality of the gut microbiome of bees. This is in line with our results for the bacterial functional community composition after treatment with the insecticide Mospilan.

The indicator analysis for fungicide Cantus® Gold, insecticide Mospilan®, and PPP mix B-treated bees revealed indicator species for all treatments. For example, *Snodgrassella* was significantly associated with the insecticide Mospilan®-treated bees (Table 3), while *Frischella* was significantly associated with the PPP mix B. Both genera are dominant intestinal bacteria of bees (Babendreier et al., 2007). *Ornithobacterium* and unclassified members of the order *Lactobacillales* were significantly associated with the fungicide Cantus® Gold treatment. *Lactobacillales* are also known members of the bee's gut microbiome (Babendreier et al., 2007; Martinson et al., 2011; Kwong and Moran, 2013). As gut microbiota are specialized to an ecological niche, it is likely to find those in another hive niche of its host species (Anderson et al., 2011). Furthermore, it was shown that grooming plays a role in implementing the bees' gut microbiome (Powell et al., 2014). Thereby, the grooming processes of bees could also lead to the distribution of gut-associated bacteria on the cuticular.

The fungicide Cantus® Gold treatment significantly altered the cuticular fungal community composition of the bees compared to control B (Figure 3D; Supplementary Table 1). The insecticide Mospilan® and PPP mix B did not significantly differ from control B or the fungicide Cantus® Gold treatment. The fungal functional cuticular community composition of the fungicide Cantus® Gold treatment differed significantly from control B (Figure 4B). The relative sequence read abundance of sooty mold and nectar/tap saprotrophic fungi was significantly increased compared to the insecticide Mospilan® and PPP mix B treatments. Moreover, plant pathogenic fungi were significantly reduced by the fungicide Cantus® Gold from one-third to less than 1% (Figure 4B). It is known that fungicide treatment alters the hive fungal community composition by introducing residues from pollen or bees (Sammataro et al., 2012). Yoder et al. (2013) described that the mixture of boscalid and pyraclostrobin did alter the fungal community of bee bread. Pyraclostrobin is a strobilurin and belongs to the same chemical group as dimoxystrobin, which forms together with boscalid, the active ingredient of the fungicide Cantus® Gold. We already described that the in-hive microbiome is closely connected to the bees' microbiome. Therefore, it is likely that this is also the case for fungi. *Saccharomyces* was identified as an indicator of the fungicide Cantus® Gold-treated cuticular fungal microbiomes (Table 3) and is described as nectar/tap saprotrophic. Hnátová et al. (2003) described that mutants of *Saccharomyces* show resistance against strobilurin fungicides. In our experiments, we did not analyze the effects of the fungicides, the insecticides, or their combinations on honey bee health or honey production. Based on

studies by Degrandi-Hoffman et al. (2015), it can be assumed that the fungicide boscalid, which was also applied in our study, can increase pathogens such as deformed wing virus or black queen cell virus. Furthermore, Pettis et al. (2013) demonstrated the effect of boscalid ingestions on the probability of Nosema infections. Similar to fungicides, neonicotinoids can affect honey bee health. Harwood and Dolezal (2020) showed negative effects on hemocyte differentiation and function following neonicotinoid application. Brandt et al. (2016) demonstrated that neonicotinoids can reduce hemocyte density, encapsulation response, and antimicrobial activity in honey bee. In order to investigate the further effects of PPPs on bee health, experiments using a different experimental design compared to our study should be performed.

5. Conclusion

Our results have demonstrated for the first time that both insecticides and fungicides can have adverse effects on the microbiome on the cuticle of honey bees, which has to date been completely neglected when investigating the side effects of PPPs. The cuticle microbiome may serve important functions as a barrier against harmful microbes. A change in the composition of the microbiome may have severe effects on honey bee health, which might only become apparent long after the collection or consumption of the respective insecticides or fungicides. The insecticide Steward® with the active substance indoxacarb and the fungicide Cantus® Gold with the active substances boscalid and dimoxystrobin are the most frequent residues in beebread, altering the fungal community composition of honey bee cuticles significantly. The neonicotinoid Mospilan® with the active substance acetamiprid significantly affected bacterial functional community composition. Mixtures of fungicides and insecticides could enhance the side effects of single substances, which have rarely been observed because fungicides are generally believed to be harmless to bees and other pollinators. In particular, fungal cuticular community composition was affected, showing a phylogenetic diversification due to the PPP mix treatments and an increase in pathogenic fungi on the bees' cuticle. Our results urge more studies on side effects on honey bees and other bees caused by the interaction of insecticides and fungicides and demonstrate that the microbiome of the cuticle is a promising site for investigation because it is susceptible to the actions of PPPs.

Data availability statement

The datasets presented in this study can be found in online repositories. The microbiome's bacterial 16S rRNA and fungal ITS gene sequences were deposited in the NCBI nucleotide sequence databases (https://www.ncbi.nlm.nih.gov/) under accession PRJNA880009.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because our protocols comply with standard welfare practice in our field. The experiment involved honey bee from an apiary dedicated to research.

Author contributions

FR: Funding acquisition, Investigation, Supervision, Validation, Writing – original draft, Data curation, Formal analysis, Methodology, Visualization. AS: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. LS: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing, Software, Validation, Visualization. MT: Writing – review & editing, Conceptualization. RS: Conceptualization, Writing – review & editing, Funding acquisition, Project administration, Resources, Supervision. MN: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Investigation, Validation, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1271498/full#supplementary-material

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Co-exposure of dimethomorph and imidacloprid: effects on soil bacterial communities in vineyard soil

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In Taiwan, the pesticides dimethomorph and imidacloprid are recommended for pest control in vineyards. Therefore, tank-mixing of these two pesticides is usually a routine practice before application. This study analyzed the influence of vineyard soil microbial flora under the recommended and high dosages (100 times the recommended dosage) of dimethomorph and imidacloprid. Individual and combined applications of pesticides were also tested through batches of soil incubation experiments. Four treatments—control (C), dimethomorph (DT), imidacloprid (IM), and mixed application of dimethomorph and imidacloprid (ID)—were used in the experimental design. From the soil metabolism, no significant reaction was observed after 2 months in the recommended dosage group, regardless of whether the pesticides were being applied individually or combined. For the high dosage, imidacloprid showed a higher effect than the co-exposure treatments, showing a possible prolonged effect after its repetitive application. From PCoA analysis, pesticide treatments altered the soil ecology after 2 months, and the effect of imidacloprid can be explicitly observed at high dosages. At the phylum level, *Acidobacteria* can indicate pesticide application around the recommended dosage. It was inhibited by ID on day 7 and was augmented by all pesticides on day 63. The effect of the recommended dosage of pesticide mixtures after 2 months of incubation was revealed in the minor families *Gemmataceae* and *Pirellulaceae*, while the high dosage treatments affected both the core and the minor families. Our findings verified the changes in the composition of microbial communities upon pesticide application, which would affect carbon, nitrogen, sulfur, phosphorous cycles, and contaminant removal ability within the vineyard.

KEYWORDS

morpholine fungicide, neonicotinoid insecticide, non-target organisms, relative microbial abundance, ecological functions

1. Introduction

In the process of agricultural production, to save labor and effort, different pesticides can be mixed before application. In Taiwan, dimethomorph and imidacloprid are both recommended in grape production (Ministry of Agriculture, 2018), and the mixed application of the two pesticides is often found. In addition, to effectively control pests and diseases such as thrips and downy mildew, fungicides and insecticides can be applied weekly

in the vineyard during the growing season until the required pre-harvest intervals are due (Pers. Commun.).

The neonicotinoid systemic insecticide imidacloprid {1- [(6-chloropyridin- 3- yl) methyl]- N- nitro- 4, 5- dihydroimidazol- 2- amine} has relatively high water solubility (0.61 gL^{-1}) and is therefore considered to have high groundwater pollution potential (Flores-CéSpedes et al., 2012). It acts on the central nervous system of insects such as thrips, termites, and fleas (Magalhaes et al., 2009). It is widely used in agriculture production (Gervais et al., 2010; Wang et al., 2023). However, imidacloprid can persist without sunlight, with a half-life of 3 years (Bonmatin et al., 2015).

Dimethomorph is a cinnamic acid derivative and is a member of the morpholine chemical family. The fungicide inhibits fungal cell wall synthesis, leading to the death of fungal cells. In a study by Liang et al. (2011), dimethomorph had an estimated half-life of 11.5–18.5 days following a first-order kinetic degradation formulation. However, due to its high hydrolytic stability, dimethomorph can remain stable for up to 5 years without exposure to sunlight.

Many studies have shown that the mixed application of pesticides has adverse effects on soil-borne organisms. A meta-study examined 394 studies and concluded that negative effects, such as lower reproduction rates, higher mortality rates, or changes in behaviors, were found in 70.5% of the 2,842 tested parameters in soil invertebrates (Gunstone et al., 2021). It also revealed a reduction in microbial diversity and abundance after pesticide application in paddy rice fields (Onwona-Kwakye et al., 2020). A global-scale geostatic study found the highest pesticide mixture contents in orchard and grape cropping systems (Tang and Maggi, 2021). The co-exposure of pesticides such as dimethomorph and imidacloprid is a critical issue, especially in grape vineyards.

Agricultural practices in the field causing pesticide exposure in soils may disturb the sensitive balance of microflora, affecting the soil's nutrient cycles and fertility (Prashar and Shah, 2016). The changes in microbial communities and activities have often been observed as indicators for the degree of effect on various agricultural inputs, such as pesticides (Gangola et al., 2021, 2022b). A study assessed bacterial communities to utilize the sensitive nature of some bacteria as biomarkers of heavy metal contamination (De La Rosa-Acosta et al., 2015). Burns et al. (2013) measured soil enzyme activities to indicate microbial diversity and soil quality. Gianfreda and Rao (2008) observed the changes in soil microbial activity and biomass induced by pesticides. Microorganisms may be an excellent indicator of soil health change, quality improvement, or soil degradation (Mahdi et al., 2017). The study aims to investigate the effect of dimethomorph and imidacloprid applied at the recommended application rate and high dosages individually and as a mixture on soil microflora in the vineyard soil.

2. Material and methods

2.1. Soil sampling and soil physicochemical properties

Soil samples were taken from the top layer (0–20 cm) at bare land areas in a vineyard located at Taichung District Agricultural Research and Extension Station, Taiwan (24°00'04.0"N 120°32'04.7"E). The soil was collected by shovels after the grapes were harvested for 2 weeks. Plant protection products or fertilizers were added during this period. The soil was sieved (2 mm) and air-dried at room temperature for a week before the study.

The soil properties were determined as described in brief: air-dried soil samples were analyzed for pH and electrical conductivity (EC) at the soil-to-water ratio of 1:2 (Multi 9620 IDS, W.T.W., Germany). Soil-available nitrogen was determined using the Kjeldahl method (Bremner, 1960). The soil organic matter and soil texture were determined using the dry combustion method (Davies, 1974) and the hydrometer method (Bouyoucos, 1936), respectively. Soil available phosphorus was determined using the Bray-1 method at a wavelength of 650 nm (Thermo Scientific GENESYS™ 30 Visible Spectrophotometer). Soil exchangeable potassium was determined using flame atomic adsorption spectrophotometry (ICP-AES; PerkinElmer Avio200, Waltham, MA, USA).

The pH of the soil was 6.03, and the organic matter content of loamy sand was 19.1%. Before the treatments, the available N, P, and K were 257.8, 7.81, and 146.5 mg kg^{-1}, respectively.

2.2. Pesticide treatments and analysis

The pesticide products of 28.8% SL formulation of imidacloprid (Great Victory Chemical Industry Co., LTD) and 50% SC formulation of dimethomorph (Chia Tai Enterprise Co., LTD) were each diluted and applied at the recommended rate (Ministry of Agriculture, 2018) and at 100 times the recommended rate into each pot of 1 kg of bare soil. A ratio of 100 times the recommended dosage was chosen to represent the heavy and repetitive application scenario in the grape vineyard.

The water content of the bare soil was adjusted to 60% of the moisture content. The pots were pre-incubated for 2 weeks in darkness at 30 ± 2°C before pesticide applications to restore soil microcosms. The pesticide treatments included C (control soils), IM (0.0369 mg kg^{-1} and 3.69 mg kg^{-1}), DT (0.0769 mg kg^{-1} and 7.69 mg kg^{-1}), and ID (a combined mixture of IM 0.0369 mg kg^{-1} with DT 0.0769 mg kg^{-1} and IM 3.69 mg kg^{-1} with DT 7.69 mg kg^{-1}). Each treatment had three replicates, giving a total of 24 containers. The control treatment was added with the same amount of deionized water to replace the pesticide application. Compensation for water loss during incubation for all treatments was done every 2 days with the addition of deionized water.

We conducted the experiments in two batches: high dosage (12 pots) and recommended dosage (12 pots). The pots were kept in a lab space with ambient room temperature and air moisture levels, so the variance between two batches of experiments and different sampling dates was expected.

2.3. Soil bacterial community-level physiological profiling analysis

Approximately 10 g of soil samples were taken from each pot at 0, 7, 14, 28, 56, and 63 days after pesticide treatments to determine the bacterial CLPP using the Biolog EcoPlate™

system (Biolog Inc., CA, USA). Before the analysis, 1 g of soil was serially diluted to 10^{-3} using a phosphate-buffered saline solution. Moreover, 130 μl of the above soil solution was transferred into each well of the Biolog EcoPlate to incubate it at 25°C for 72 h. Optical densities were observed at each 24-h interval at 590 nm and 750 nm, respectively (Classen et al., 2003). The average well-color development (AWCD), substrate richness (S), evenness (E), and Shannon diversity index (H') were calculated following the previous study (Zak et al., 1994).

2.4. Soil DNA extraction and next-generation sequencing

Soil microbial DNA extraction was performed using DNA Power Soil extraction kits (MO BIO Laboratories, Inc.) and stored at −20°C. DNA samples were sent to a company for NGS analysis (Genomics BioSci & Tech Ltd., Taiwan). The procedures were described in brief as follows: PCR amplifying the V3-V4 region of 16S rDNA using the primer set 341F-805R with the KAPA High-Fidelity PCR kit (KAPA BIOSYSTEMS) was performed. The products were purified using the QIAquick Gel Extraction Kit (QIAGEN). Afterward, the sequence libraries were generated using the Truseq nano DNA Library Prep Kit (Illumina, USA) and sequenced on an Illumina Miseq platform. Primer sequences were trimmed using the Cutadapt program and merged using FLASH software (v1.2.11). Mothur (v1.39.5) was used for picking operational taxonomic units (OTUs) with 97% identity. The OTU table was produced using UCHIME (v4.2) software.

2.5. Statistical analyses

The analysis of variance using a general linear model (multivariate) was performed at a significance level of p-value of < 0.05 by the least significant different *post-hoc* test (IBM SPSS Statistic version 25). Weighted variants of the UniFrac matrix were calculated and then visualized in the principal coordinates analysis (PCoA) using QIIME software (Bolyen et al., 2019).

3. Results and discussion

3.1. CLPP profiles and OTUs in the soils

The CLPP profiles were analyzed using the AWCD and Shannon diversity indices from EcoPlate™ results (Figures 1, 2). It is worth noting that this study's pot incubation was conducted in a lab space with ambient room temperature and air moisture level, so the variance was observed among different batches of experiments (recommended dosage group and high dosage group) and different sampling dates.

At the recommended dosage (Figure 1A), the AWCD level decreased upon co-exposure to dimethomorph and imidacloprid on day 7; however, on day 63, all treatments, including the combined treatment, were not significantly different from the control. A slight enhancement of soil metabolism was observed for dimethomorph and imidacloprid treatment on day 7; however, it was neither significant nor prolonged. This shows that, although the considerable effect on soil metabolism from co-exposure to pesticides may occur right after their application under the recommended dosage, soil metabolic activity could be restored within 2 months.

For the high dosage, imidacloprid slightly decreased average soil metabolism throughout the incubation period, showing its possibility of a prolonged effect (data not shown). However, the dimethomorph treatments did not alter soil metabolism during the 2 months of the incubation period (Figure 1B). As for the combined treatments of the two pesticides, inhibition of soil metabolism was observed on days 0, 14, and 28 but not on days 56 and 63. The results indicated that, under repetitive or high dosages, no significant or prolonged inhibition of soil metabolic activity was observed in the combined application either. We presumed that this could be due to the fact that dimethomorph can be more easily utilized by some soil microbes (Zhang et al., 2020) than imidacloprid or that the utilization of organic material released from dying fungal species was happening in the soil matrix (Katayama and Kuraishi, 1978) so that the effect of imidacloprid was concealed. A more pronounced decline of microbial metabolism by imidacloprid's separate applications under high dosage was recorded in our experiments rather than in the combined pesticides' applications.

A study reported that soil respiration was impeded by 100 mg kg^{-1} of dimethomorph but not by 1 or 10 mg kg^{-1} of that (Wang et al., 2017). In our study, 0.0769 mg kg^{-1} dimethomorph slightly increased AWCD in the short term, but within 2 months, it returned to the untreated state. Taking 100 times the recommended dose of dimethomorph could increase AWCD index on some sampling days, potentially obscuring the effects of other pesticides on soil.

Another study examined the soils treated with 50 mg kg^{-1} imidacloprid and found that the AWCD index was lower than the control (Garg et al., 2021). It was also reported that 1 mg kg^{-1} and 10 mg kg^{-1} of imidacloprid significantly decreased soil AWCD index (Cycoń et al., 2013). In our study, 0.0369 mg kg^{-1} of the imidacloprid did not significantly alter the soil metabolic activity, while 3.69 mg kg^{-1} of the imidacloprid could slightly decrease it. This shows that the effect of imidacloprid on soil metabolism could follow a dose-dependent pattern.

Fluctuations of bacterial diversity were observed at the recommended dosage for all treatments (Figure 2A); however, no significant difference was recorded from day 14 to day 63. A high dosage of dimethomorph increased bacterial diversity on day 28, while for imidacloprid, an inhibition was slightly shown on day 7 (Figure 2B). Nevertheless, regardless of whether the pesticide was administered at the recommended or high dosage, the influence of its individual and combined application was recovered within 2 months.

From NGS data (Table 1), an increase in OTU values can be observed from the combined treatments both at the recommended dosage and high dosage on day 7. However, the individual application of high-dose pesticides decreased the number of OTUs, but only in the short term. The application of dimethomorph and imidacloprid did not impose a significant difference after 2 months

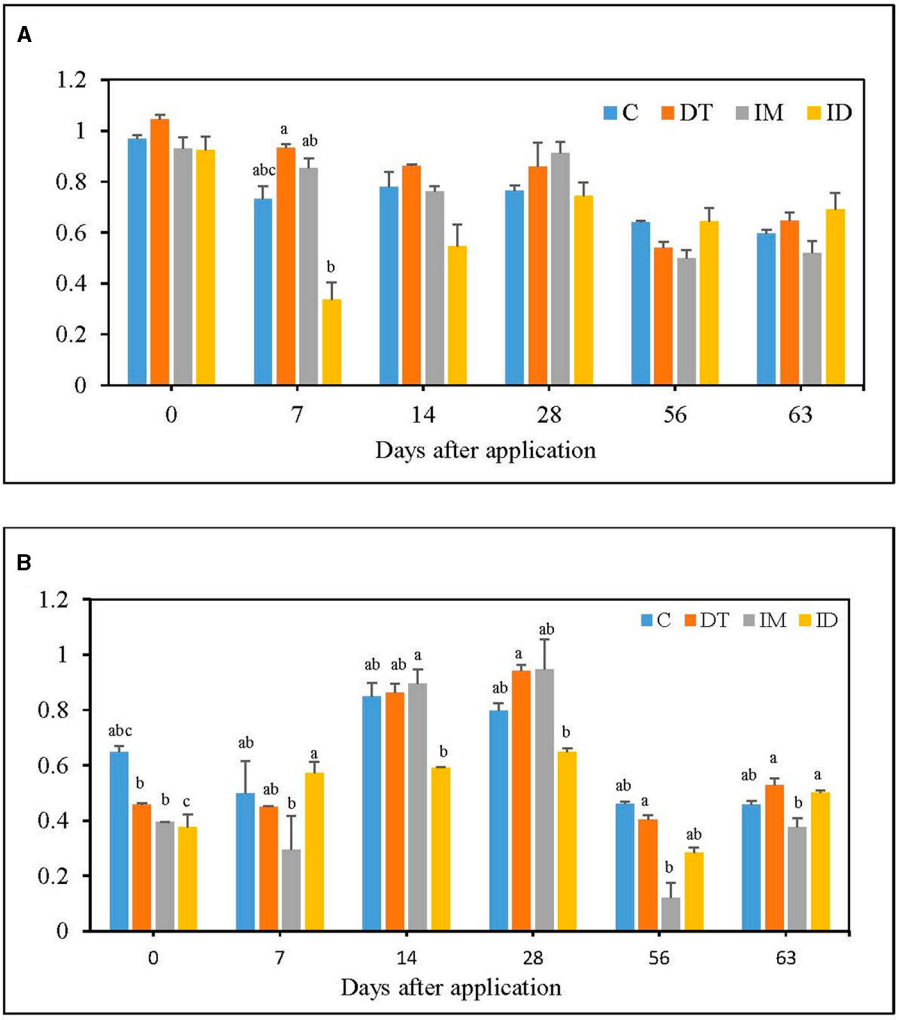

FIGURE 1
AWCD of EcoPlate **(A)** at the recommended dosage; **(B)** at 100 times the recommended dosage. The letters on the bars denote the difference in mean at a *p*-value of 0.05 in the LSD test.

of examining the OTUs, which were in good accordance with the findings obtained from EcoPlates™.

3.2. PCoA analysis of individual and co-exposure to pesticides on soil microbial taxa

To further evaluate the effect of individual and co-exposure to pesticides on soil bacterial communities, the similarity matrix of the bacterial species and abundance from different treatments were calculated and then visualized using PCoA dendrograms in Figures 3, 4.

At the recommended dosage (Figure 3), the control and individual treatments were both distant from the combined treatment ID on days 7 and 63. On day 7, the combined treatments were in a separate group from the individual treatments, while on day 63, the individual treatments of pesticides could be grouped with the combined treatments. The result indicates that, once treated with pesticides, regardless of whether it is a separate application or a combined application, the soil ecology would be divergent from the control after 2 months. However, under the recommended dosage, the more significant effect of combined treatments was only observed in the short term.

At high dosages (Figure 4), the control treatments on days 7 and 63 were distant from the pesticide treatments. It was observed that the combined treatments had exerted a more significant effect on the shifting of the bacterial ecology in soils throughout the incubation period since ID-7 and ID-63 were in unique groups distant from other treatments. In addition, the individual treatment of imidacloprid made a distinct cluster on day 63, showing a prolonged effect on the soil bacterial ecology.

Although studies have reported the shift in microbial metabolism and diversity under dimethomorph and imidacloprid separate applications (Cycoń et al., 2013; Wang et al., 2017), the effect of co-exposure to these two pesticides has not been reported. Our research found no evident long-term effect if examined only in

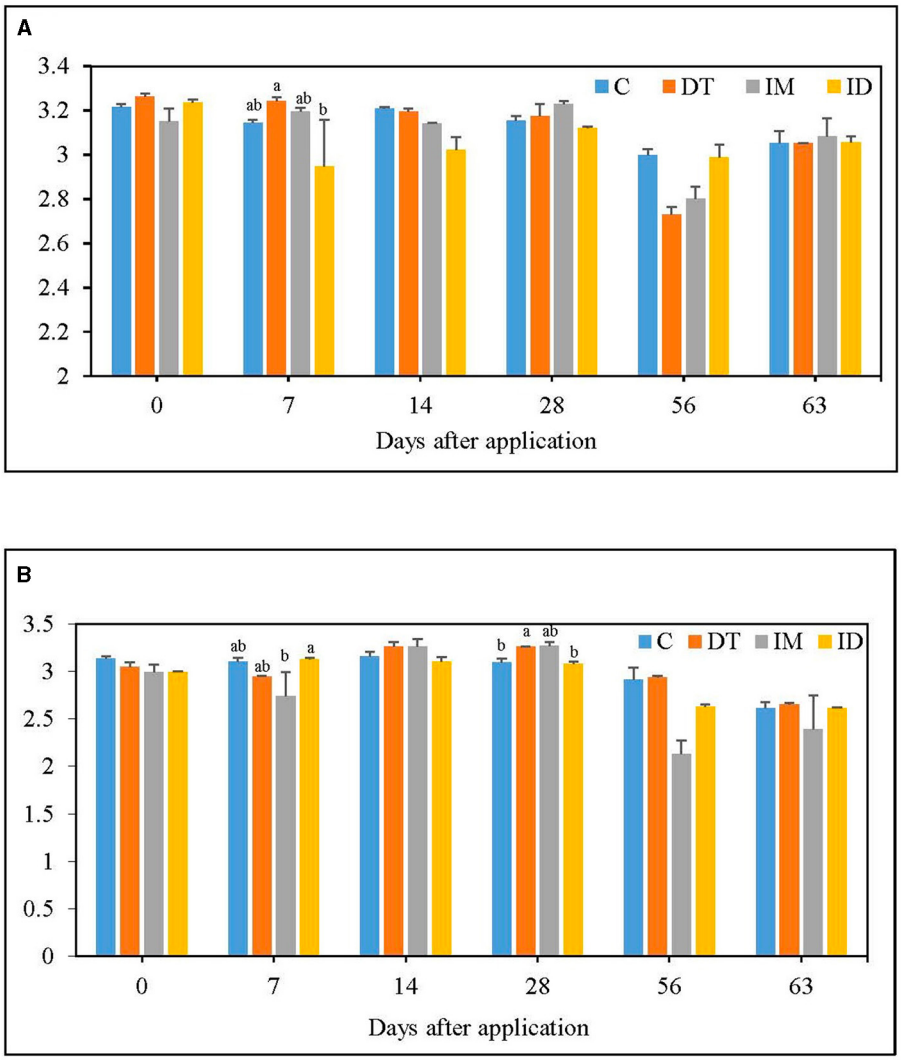

FIGURE 2
H index of EcoPlate **(A)** at the recommended dosage; **(B)** at 100 times the recommended dosage. The letters on the bars denote the difference in mean at a *p*-value of 0.05 in the LSD test.

soil metabolic activity, as elucidated in Section 2.1. If we consider both bacterial species and abundance data from NGS results, the possible long-term effect of pesticide application was revealed, whether in separate or combined application treatments.

In the following sections, we will examine the detailed change in the bacteria species, both at the phylum level and family level, to investigate the possible ecological effect of two pesticide applications.

3.3. Pesticide treatments on the bacteria phyla

Previous studies on the microbial biosphere have usually set 0.1 or 0.01% as the relative abundance threshold for rare taxa (Galand et al., 2009; Anderson et al., 2015) and 1% for abundant taxa within a sample (Galand et al., 2009; Liu et al., 2012). In this study, we focused on the examination of abundant taxa with a relative abundance >1%.

The core phyla in the bare vineyard soil before and after imidacloprid and dimethomorph treatments were *Acidobacteria*, *Actinobacteria*, and *Proteobacteria* (Figures 5, 6). These three phyla accounted for 11.5, 31.3, and 24.0%, respectively, of the control treatments on day 7 (Figure 5A). It was previously reported that *Actinobacteria* and *Proteobacteria* phyla were the key taxa in two soil biomes in Brazil (Lupatini et al., 2014). *Acidobacteria* and *Proteobacteria* were also proposed as bacterial indicators for land-use change (Kim et al., 2021). Our findings are well-fitted with these previous studies.

Among the recommended dosage treatments (Figure 5), *Proteobacteria* were not altered significantly by the pesticide treatments. As for *Acidobacteria*, they were significantly decreased by the co-exposure of dimethomorph and imidacloprid on day 7 and was significantly increased by all pesticide treatments on day 63 (Figure 5). A previous study reported a shift in *Proteobacteria*

TABLE 1 OTUs of treatments C, DT, IM, and ID on days 7 and 63.

	Recommended dosage				High dosage			
	Day 7		Day 63		Day 7		Day 63	
C	10,281.0 ± 1,466.9	b	21,140.3 ± 2,068.0	a	20,221.7 ± 1,714.6	ab	27,057.3 ± 723.4	a
DT	12,521.7 ± 814.1	b	17,570.3 ± 2,898.6	a	17,483.3 ± 940.3	b	23,930.3 ± 7,418.5	a
IM	15,308.3 ± 3,876.4	ab	16,240.3 ± 3,031.0	a	17,283.0 ± 2,764.1	b	30,994.7 ± 4,786.1	a
ID	18,529.0 ± 3,735.2	a	17,532.3 ± 1,803.8	a	23,681.3 ± 1,789.4	a	30,628.0 ± 1,212.5	a

Letters denote the difference in mean at a p-value of < 0.05 in the LSD test.

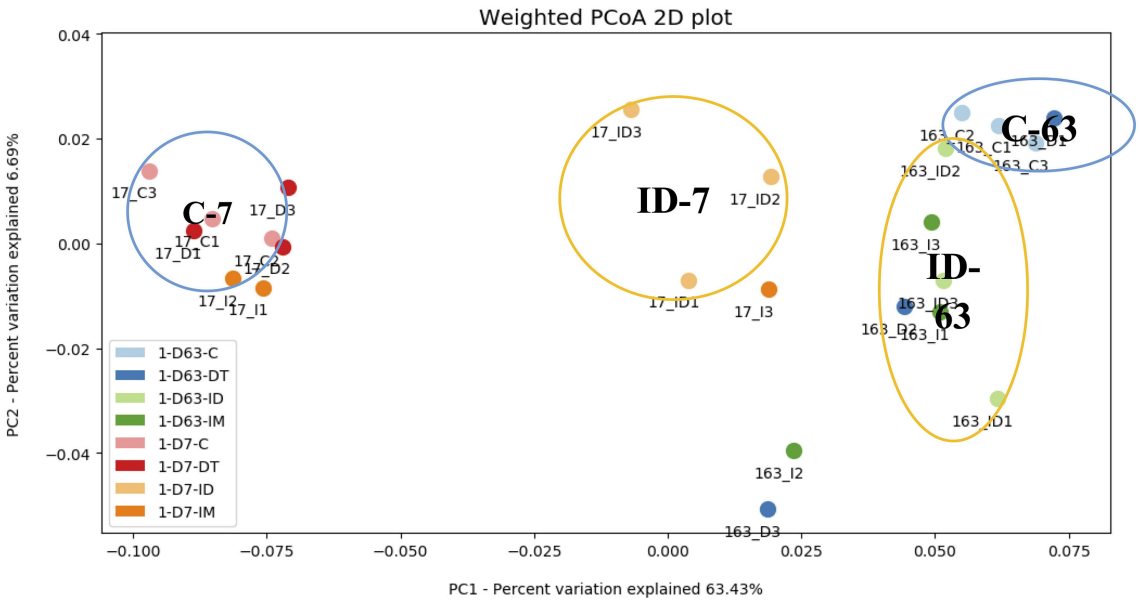

FIGURE 3
PCoA dendrogram of treatments C, DT, IM, and ID at the recommended dosage.

and *Acidobacteria* by the change of soil pH and heavy metal pesticides (Kim et al., 2021). *Acidobacteria* were also reported to be augmented in fungicide-tebuconazole-contaminated soil (Baćmaga et al., 2022). In this study, we found that *Acidobacteria* were relatively sensitive to the effect of pesticides among these three abundant taxa when pesticide concentrations were around the recommended dosage. The phylum would be augmented once treated with dimethomorph and imidacloprid, either separately or combined.

Under high-dosage treatments (Figure 6), the minority soil species exhibited a more significant change than the core phyla, as no significant differences were recorded in the latter. *Gemmatimonadetes* was inhibited by ID on day 7; however, on day 63, it was significantly augmented by imidacloprid (Figure 6B). It was also found that the recommended dosage of imidacloprid had augmented it on day 7 (Figure 5A). We assumed that some members of *Gemmatimonadetes* might be able to utilize imidacloprid or its metabolites at a suitable concentration, although this has not been reported. *Gemmatimonadetes* comprise roughly 2% of soil bacterial communities, yet little is known of their ecology. The type strain of *Gemmatimonadetes* was *G. aurantiaca* strain T-27, a polyphosphate-accumulating isolate from wastewater (DeBruyn et al., 2011). Members of *Gemmatimonadetes* may probably serve as indicators upon imidacloprid application.

Latescibacteria can be inhibited by the high dosage of dimethomorph application but not by the co-exposure of pesticides on day 7 (Figure 6A). *Latescibacteria* was reported to be found in an aquifer contaminated with hydrocarbon and chlorinated solvents with protein-, lipid-, and polysaccharide-degradation abilities (Farag et al., 2017). The inhibition of *Latescibacteria* by the high dose of dimethomorph suggested a shift in soil functional genomics upon a high amount of dimethomorph application in the short term. Nevertheless, the co-exposure of pesticides also compensated for their ecological effect on soils.

The above results regarding the bacteria phylum indicated that the effect of co-exposure to pesticides would not be clearly observed compared with separate applications. For example, in Figure 5B, the boost of *Gemmatimonadetes* could only be observed in imidacloprid treatment, whereas in Figure 6A, the inhibition of *Latescibacteria* could only be observed in dimethomorph treatment. There were reports on the utilization of pesticides and their metabolites (Gangola et al., 2018a,b, 2022a,c, 2023; Bhatt et al., 2023) and on the fact that the microbes killed by biocides may

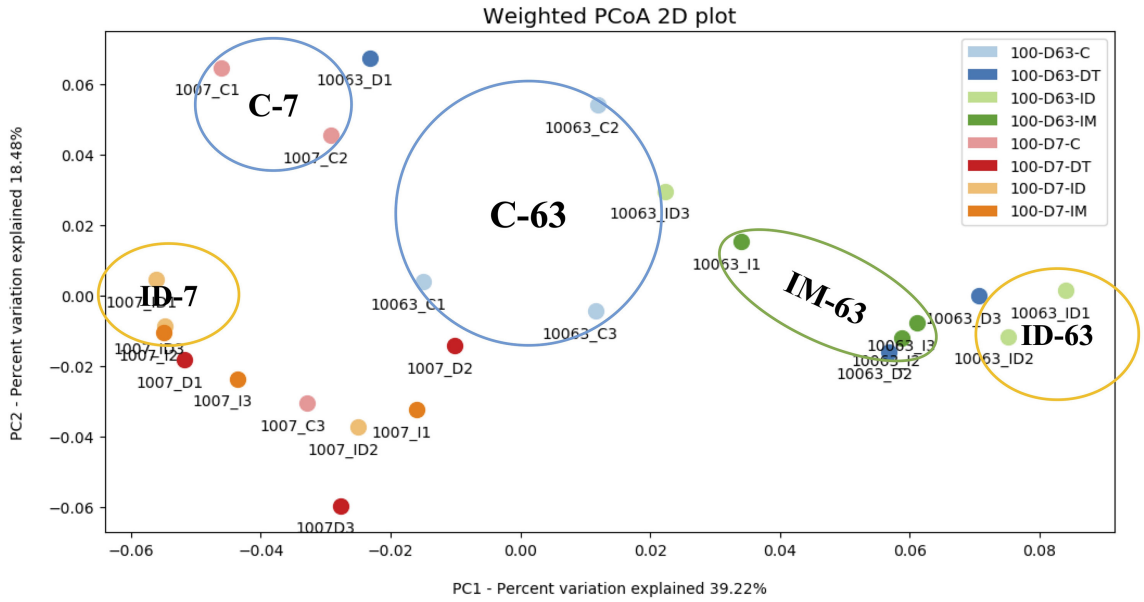

FIGURE 4
PCoA dendrogram of treatments C, DT, IM, and ID at high dosage.

become carbon and nitrogen sources of surviving microbes (Ullah and Dijkstra, 2019).

3.4. Pesticide treatments on the bacteria families at the recommended dosage

The core family members of the bare vineyard soil before and after the recommended dosage of pesticide applications were *Bacillaceae*, *Gaiellaceae*, *Nocardioidaceae*, and *Streptomycetaceae* (Figures 7, 8). Except for *Streptomycetaceae*, significant changes upon pesticide application were observed, but only on day 7.

On day 7, the relative abundance of *Bacillaceae* was significantly decreased by the individual application of dimethomorph and imidacloprid but not by the co-exposure of these two pesticides (Figure 7). This family comprises a large and diverse group of heterotrophic bacteria. It is known to participate in the carbon, nitrogen, sulfur, and phosphorous cycles in natural habitats (Mandic-Mulec et al., 2015). It was also reported that some bacteria belonging to this family had imidacloprid and other pesticide degradation abilities (Sabourmoghaddam et al., 2015; Gangola et al., 2021, 2023). Therefore, the degradation of pesticides in soils could also be impeded.

The bacterial family *Gaiellaceae* was significantly increased in DT and ID soil on day 7 (Figure 7). There is only one known strain, *Gaiella occulta*, in this family, which shows various abilities, including reducing soil nitrate (Albuquerque and da Costa, 2014). The augmentation in this family suggests that dimethomorph may enhance some ecological functions related to *Gaiellaceae*.

The relative abundance of the bacterial family *Nocardioidaceae* was significantly higher in the individual application of imidacloprid and in the co-exposure of dimethomorph and imidacloprid. Some members of *Nocardioidaceae* were active in the degradation of recalcitrant chemicals, such as phenols and nitrophenolic compounds, or toxic environmental pollutants and derivatives (Rosenberg et al., 2014; Tóth and Borsodi, 2014). The increase in the *Nocardioidaceae* abundance suggested the degradation of imidacloprid immediately after their application. A shift in pesticide degradation bacteria may also be observed from *Bacillaceae* to *Nocardioidaceae* at the recommended dosage.

Although the abundance change in the bacterial family can be observed among the core taxa on day 7, the above results show that if the recommended dosage was followed, the application of dimethomorph and imidacloprid, or the combined treatment, did not alter soil core bacterial families for more than 2 months or induce long-term reaction in the relevant carbon, nitrogen, sulfur, and phosphorous cycles, as well as the recalcitrant chemical degradative abilities related to those core families.

As for those bacterial families with lower abundances, *Chthoniobacteraceae*, *Gemmataceae*, and *Pirellulaceae* were significantly decreased by the co-exposure of pesticides but not the individual applications on day 7. After 2 months, we still observed a decline in the co-exposure treatment in *Gemmataceae*, while for *Pirellulaceae*, all the pesticide treatments would inhibit the abundance. These two families all belong to the *Planctomycetes* phylum. The *Planctomycetes* phylum is a unique heterotrophic free-living bacteria with a large genome and large genes, which might be responsive to the production of bioactive molecules (Wiegand et al., 2018).

Gemmataceae inhabit a wide variety of freshwater and terrestrial environments. Some of the members can utilize and degrade polysaccharides, chitin, and biopolymers, demonstrating their pronounced hydrolytic capabilities (Kulichevskaya et al., 2020). After 2 months, the decline in its abundance suggests that,

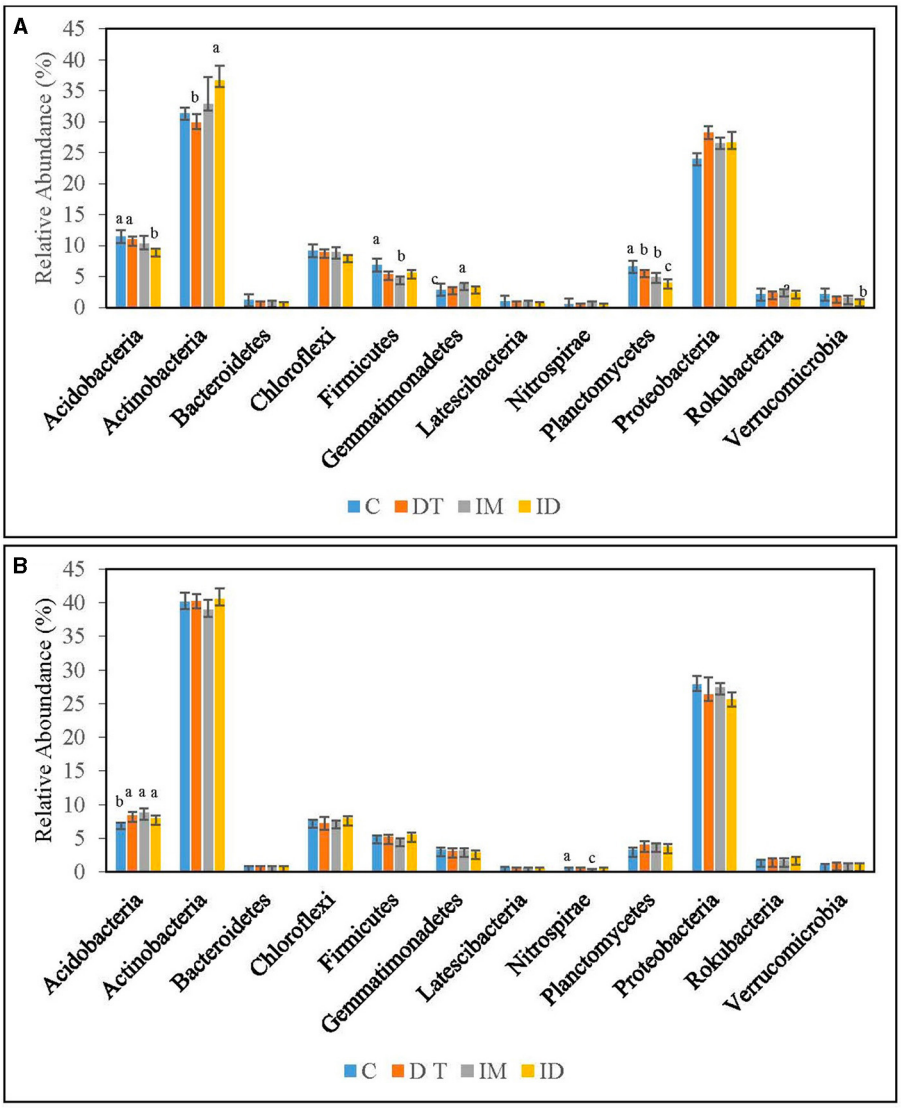

FIGURE 5
Bacterial phyla of treatments C, DT, IM, and ID at the recommended dosage on **(A)** days 7 and **(B)** 63. The letters on the bars denote the difference in mean at a *p*-value of 0.05 in the LSD test.

even under the pesticide-recommended dosage treatments, the soil hydrolytic pathways toward those recalcitrant chemicals may still be impeded, and the co-exposure of pesticides may augment the effect.

As for the family *Pirellulaceae*, they can generally be found in fresh and marine water environments (Kulichevskaya et al., 2022). They were reported to be an ammonia-oxidizing bacteria, thus participating in the nitrogen cycle (de Celis et al., 2020). A previous study also showed that one species belonging to *Pirellulaceae* could be found in hexavalent chromium-contaminated garden soil, and after enrichment, the microbial consortia from the garden soil showed 99% of the chromium removal ability (Singh et al., 2022). After 2 months, the decline of its abundance suggests that pesticide application at the recommended dosage may also impede the nitrogen cycle and some contaminant removal abilities in soils.

3.5. Pesticide treatments on the bacteria families at high dosage

The core families of the bare vineyard soil before and after dimethomorph and imidacloprid treatments were *Gemmatimonadaceae*, *Nitrosommonadaceae*, *Nocardioidaceae*, *Phodanobacteraceae*, *Sphingomonadaceae*, and *Xanthobacteriaceae* throughout the incubation period (Figures 9, 10). Although the shift in the core families for the two batches of experiments (recommended dosage and high dosage) was observed, it should be noted that the room temperature and air moisture level also contributed to the difference, as described in Section 2.2.

Among the core families, the relative abundance of the bacterial family *Nocardioidaceae* was significantly augmented by the co-exposure of imidacloprid and dimethomorph treatment on day 7 (Figure 9) and was significantly inhibited by day 63 (Figure 10).

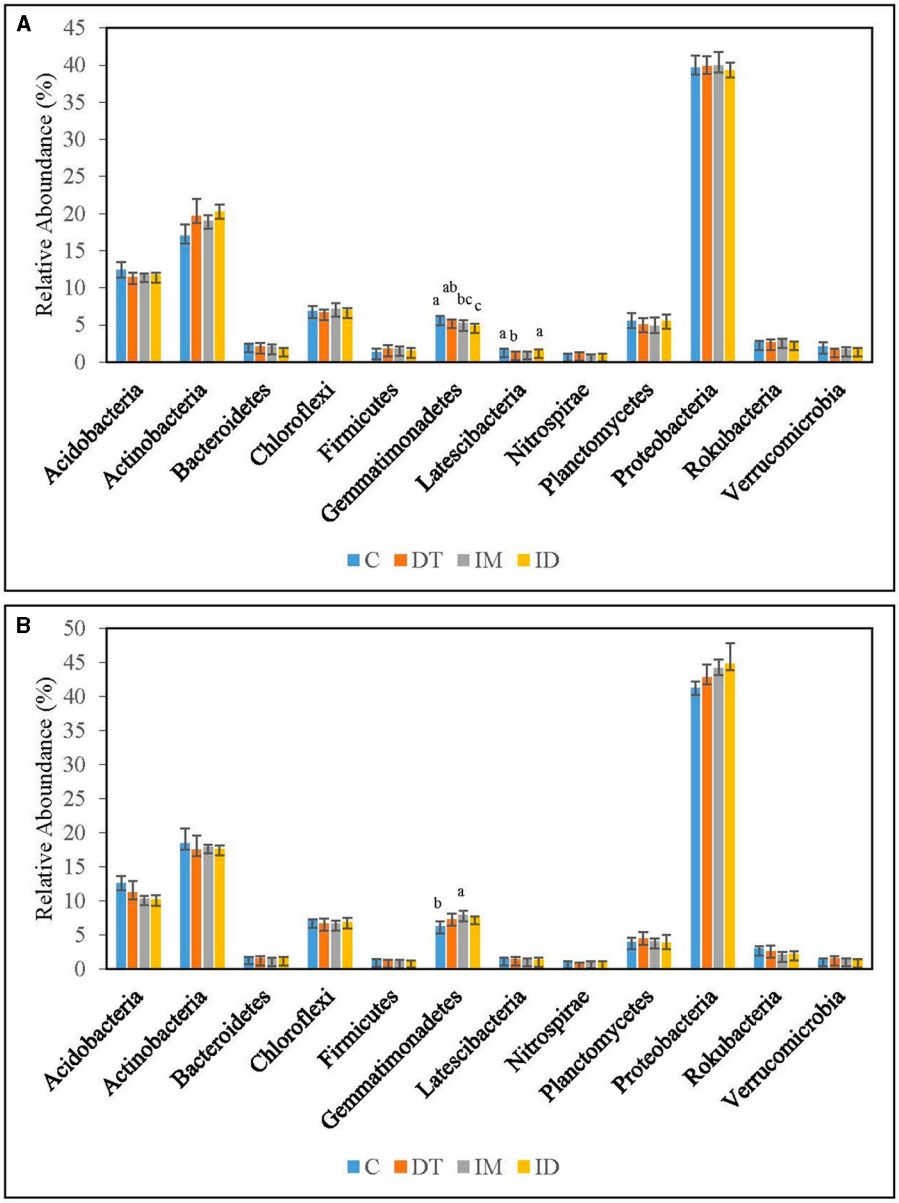

FIGURE 6
Bacterial phyla of treatments C, DT, IM, and ID at high dosage on **(A)** days 7 and **(B)** 63. The letters on the bars denote the difference in mean at a *p*-value of 0.05 in the LSD test.

This confirms that some members of *Nocardioidaceae* may utilize these pesticides, as we assumed in Section 3.4, but after 2 months, the lower substrate level may also limit their population.

Core family *Gemmatimonadaceae* was decreased by all pesticide treatments on day 7 (Figure 9); however, on day 63 (Figure 10), it was increased, especially by imidacloprid. *Gemmatimonadaceae* has only one type strain, *G. aurantiaca*, which grows slowly in activated sludge equipped with phosphate removal abilities (Hanada and Sekiguchi, 2014). It was also reported to have nitrate- and vanadium-reducing abilities (Jia et al., 2019; Fei et al., 2022). Other studies have reported that this family responded positively to nitrogen supply (Yuan et al., 2017). Relevant mechanisms related to *Gemmatimonadaceae* should be studied to reveal the effect of imidacloprid application.

Several species belonging to *Sphingomonadaceae* were reported to degrade xenobiotic and recalcitrant aromatic compounds of natural or anthropogenic origin (Glaeser and Kämpfer, 2014). This family was inhibited at the beginning of the imidacloprid application (Figure 9) but was augmented after 2 months (Figure 10).

Many species in *Xanthobacteraceae* were reported to be equipped with the nitrogen-fixing ability or the ability to grow on hydrocarbon substrates (Oren, 2014). The population was slightly augmented by imidacloprid and was slightly inhibited by dimethomorph after 2 months (Figure 10).

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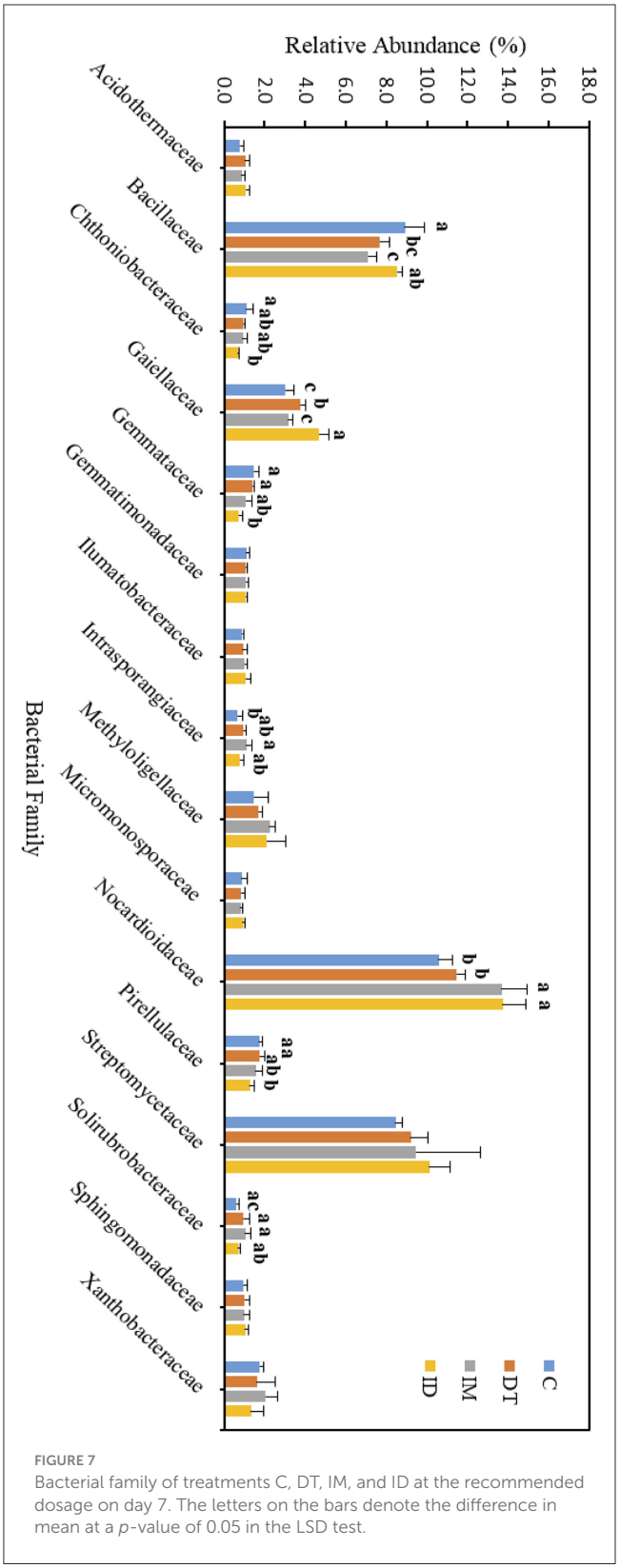

FIGURE 7
Bacterial family of treatments C, DT, IM, and ID at the recommended dosage on day 7. The letters on the bars denote the difference in mean at a *p*-value of 0.05 in the LSD test.

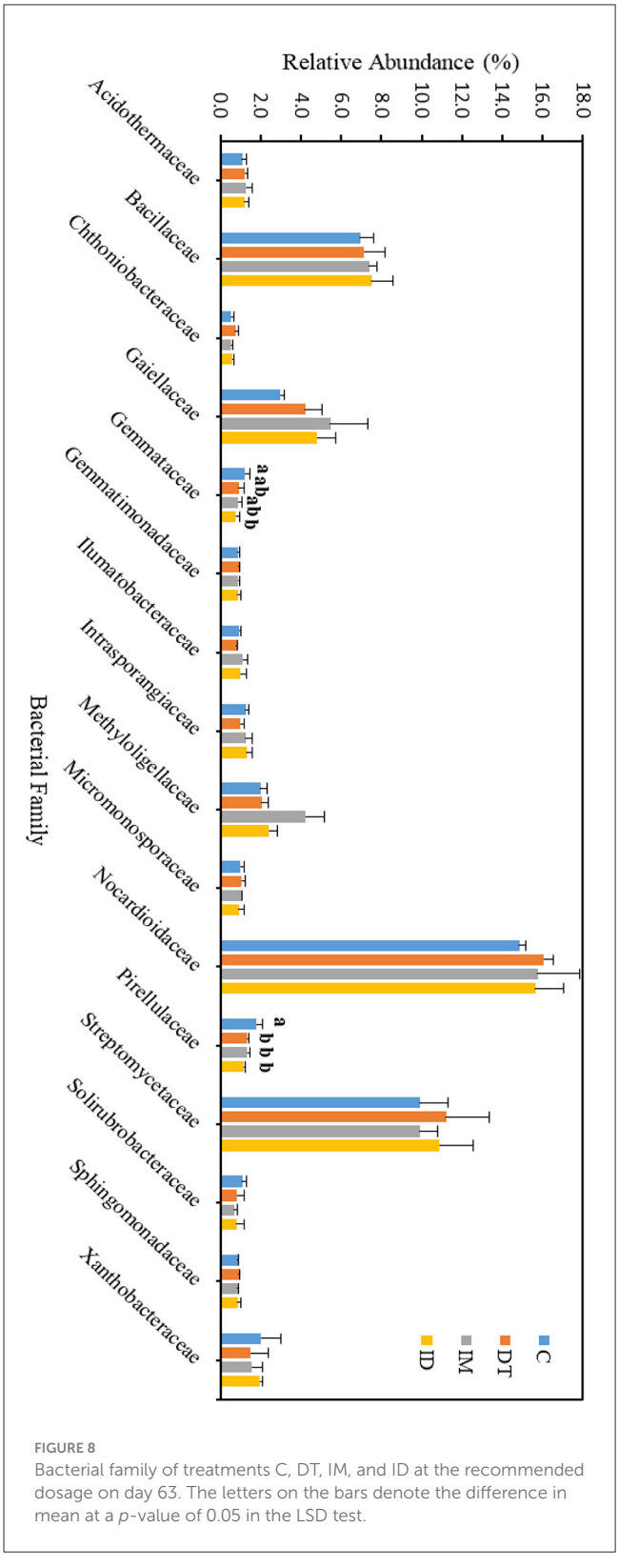

FIGURE 8
Bacterial family of treatments C, DT, IM, and ID at the recommended dosage on day 63. The letters on the bars denote the difference in mean at a *p*-value of 0.05 in the LSD test.

As for the lower abundance of bacterial families, the application of imidacloprid significantly increased *Geminicoccaceae*, while treatment ID significantly increased *Micromonosporaceae* on day 7 (Figure 9). However, on day 63, the two bacterial families were lower than the control (Figure 10). Some species belonging to the *Geminicoccaceae* family can have carbon dioxide fixation ability as well as starch and chitin degradation ability (Proença et al., 2018). These bacteria were also proposed to be sulfur-oxidizing bacteria (Vavourakis et al., 2019). The function of *Micromonosporaceae* in soil ecosystems is not well known. It was also reported that chitin

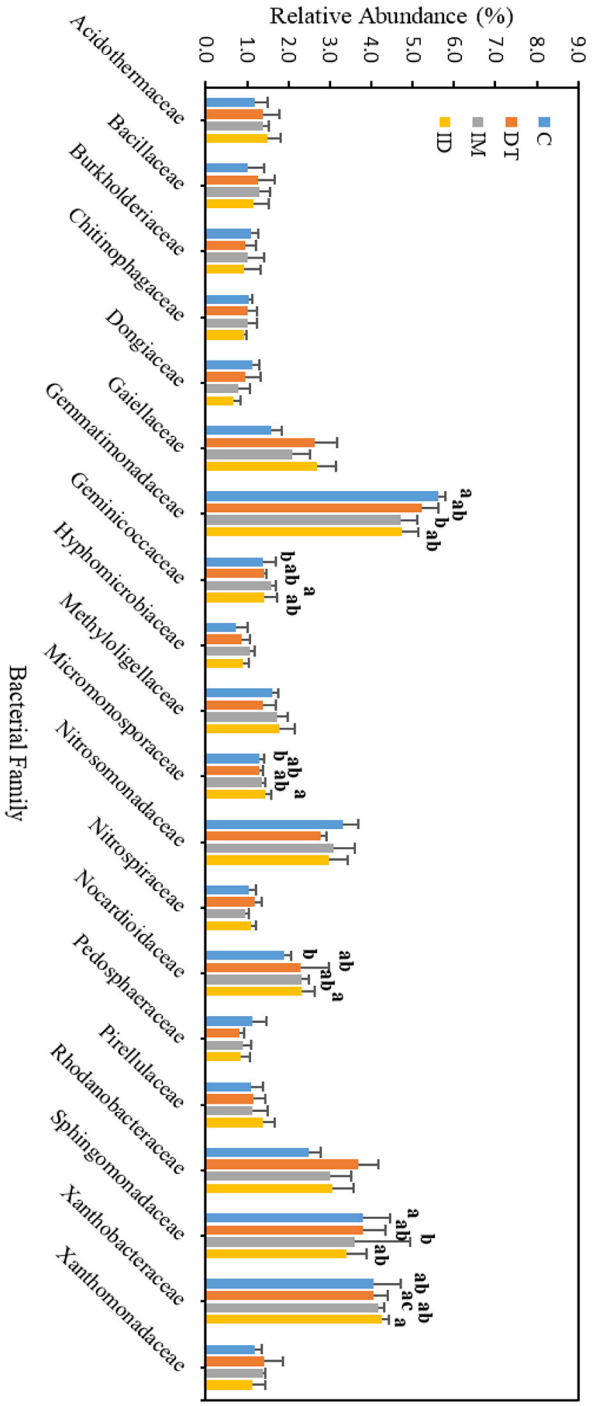

FIGURE 9
Bacterial family of treatments C, DT, IM, and ID at high dosages on day 7. The letters on the bars denote the difference in mean at a *p*-value of 0.05 in the LSD test.

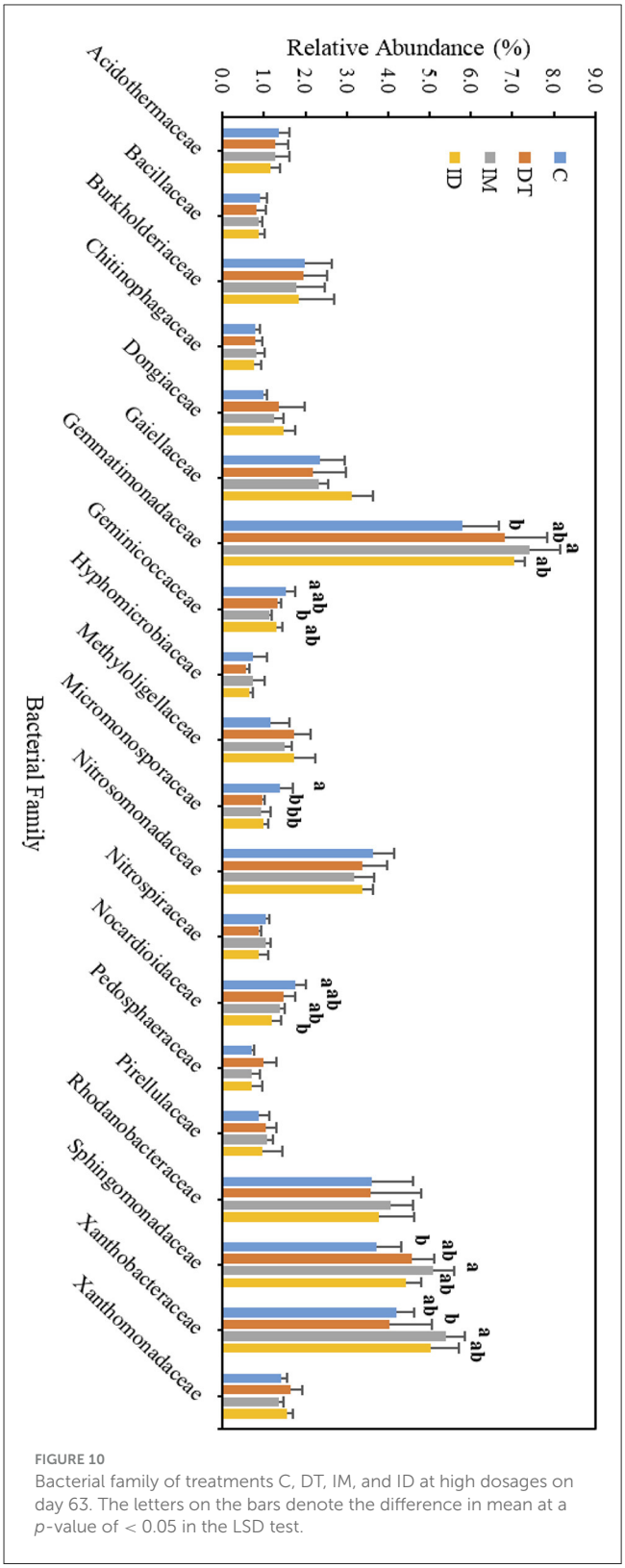

FIGURE 10
Bacterial family of treatments C, DT, IM, and ID at high dosages on day 63. The letters on the bars denote the difference in mean at a *p*-value of < 0.05 in the LSD test.

had been used as a carbon source to isolate strains (Trujillo et al., 2014).

The above results showed that a high dosage or repeated application of imidacloprid can have a more significant effect on soil biological functions, including exotic aromatic carbon removal and the cycling of carbon, nitrogen, phosphate, and sulfur in the short term. However, the effect of pesticide application, regardless of whether it is a combined application or individual application, would be noticeable at the family level over a 2-month period.

4. Conclusion

In this present study, both EcoPlate™ and NGS data were used to analyze the effects of dimethomorph and imidacloprid on soil microbes and metabolic activity. Under the recommended dosage, the soil metabolic activity would have shown transient changes if we had examined soil metabolic activity only. Under repeated or high dosages, imidacloprid has a more significant inhibitory effect on soil metabolism, an impact that may be masked by the effects of dimethomorph. However, when bacterial species and abundance are considered jointly in the analysis, even at the recommended dosage, this leads to a divergence in overall soil ecology from the control group after 2 months, as shown in the PCA dendrogram—regardless of whether the pesticides are applied individually or in combination.

Even when considering species data, the effects of co-exposure to pesticides can still be hidden in some species. For example, the effect of imidacloprid on *Gemmatimonadetes* was immediately noticeable by the end of the incubation period. However, when dimethomorph was present, the adverse effects of imidacloprid were alleviated. We assume that the co-exposure of dimethomorph may provide the metabolites or the dead fungi as the carbon source in the soil; thus, the effect of individual applications of imidacloprid may be concealed. A similar compensation effect provided by imidacloprid to dimethomorph could also be observed. Nevertheless, further studies are urgently required for the combined application of pesticides with various metabolites and mechanisms to further reveal the possible mechanisms and ecological impact.

The results on bacterial families affected by pesticide applications in bare soil reflect changes in indigenous bacterial composition in the field. Changes in the composition of microbial communities would, in turn, affect the cycles of carbon, nitrogen, sulfur, and phosphorous, as well as contaminant removal ability within the vineyard. The pesticides used in the vineyard can threaten the natural environment by promoting the accumulation and migration of toxic substances in the ecosystem through weekly applications.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

JC and W-CC conceived and designed the experiments. JC conducted the experiments, analyzed the data, and drafted the article. F-TS modified the experiments and analyzed the data. W-AL guided JC in some experiments. W-CC analyzed the data and wrote the article. C-SL helped to revise the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1249167/full#supplementary-material

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Comparative e-waste plastics biodegradation efficacy of monoculture *Pseudomonas aeruginosa* strain PE10 and bacterial consortium under *in situ* condition

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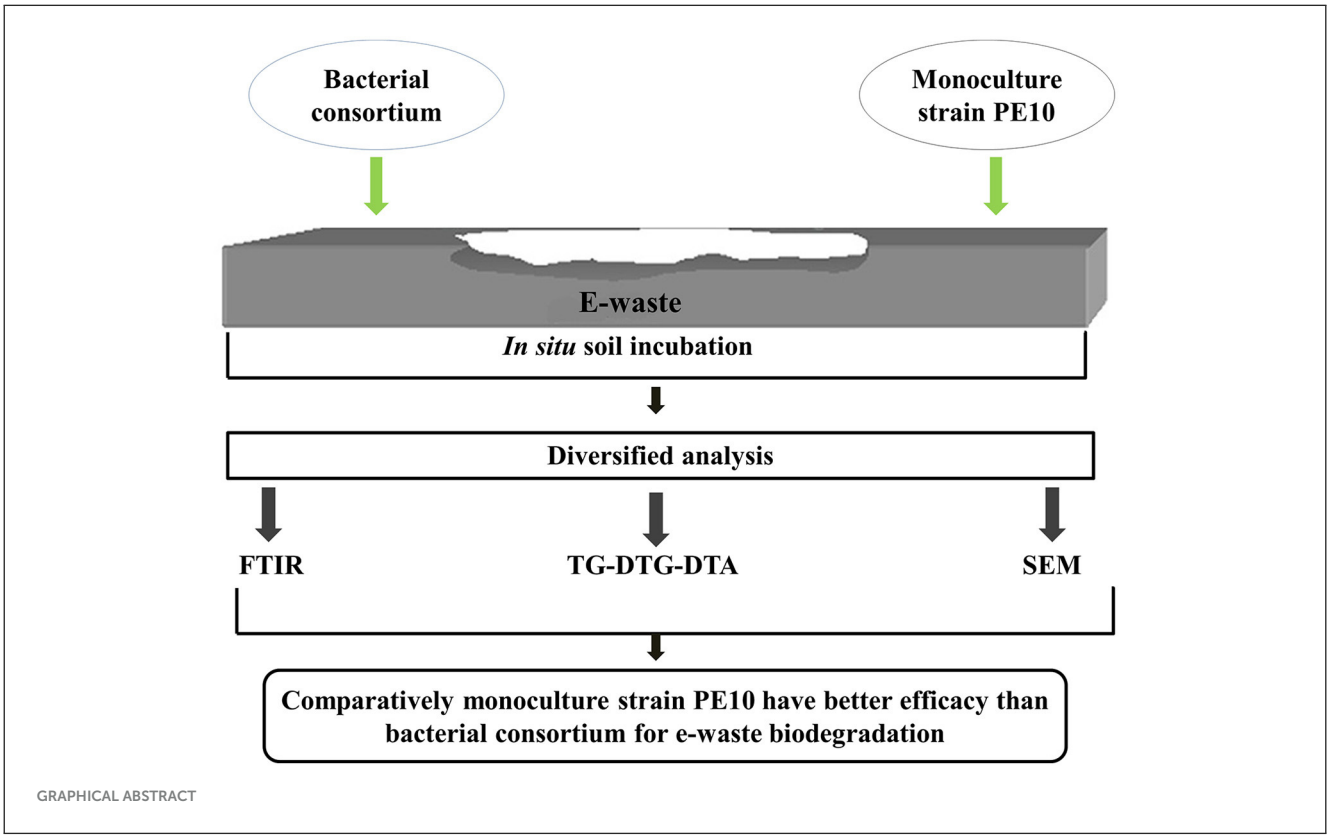
A significant amount of electronic obsoletes or electronic waste (e-waste) is being generated globally each year; of these, ~20% of obsolete electronic items have plastic components. Current remediation practices for e-waste have several setbacks due to its negative impact on the environment, agro-ecosystem, and human health. Therefore, comparative biodegradation studies of e-waste plastics by monoculture *Pseudomonas aeruginosa* strain PE10 and bacterial consortium consisting of *Achromobacter insolitus* strain PE2 (MF943156), *Acinetobacter nosocomialis* strain PE5 (MF943157), *Pseudomonas lalkuanensis* PE8 (CP043311), and *Stenotrophomonas pavanii* strain PE15 (MF943160) were carried out *in situ*. Biological treatment of e-waste with these candidates in soil ecosystems has been analyzed through diversified analytical techniques such as Fourier transform infrared spectroscopy (FTIR), thermogravimetric-derivative thermogravimetry-differential thermal analysis (TG-DTG-DTA), and scanning electron microscopy (SEM). Both *P. aeruginosa* strain PE10 and the bacterial consortium have a tremendous ability to accelerate the biodegradation process in the natural environment. However, FTIR analysis implied that the monoculture had better efficacy than the consortium, and it was consistent until the incubation period used for the study. Some polymeric bonds such as ν C=C and δ C-H were completely removed, and ν C=C ring stretching, ν_{asym} C–O–C, ν_{sym} C–H, etc. were introduced by strain PE10. Furthermore, thermal analysis results validated the structural deterioration of e-waste as the treated samples showed nearly two-fold weight loss (W_L; 6.8%) than the untreated control (3.1%) at comparatively lower temperatures. SEM images provided the details of surface disintegrations. Conclusively, individual monoculture *P. aeruginosa* strain PE10 could be explored for e-waste bio-recycling in agricultural soil ecosystems thereby reducing the cost, time, and management of bioformulation in addition to hazardous pollutant reduction.

KEYWORDS

e-waste, consortium, monoculture, biodegradation, bio-recycling



1 Introduction

Electronic waste (e-waste) possesses various hazardous and non-hazardous substances, and thus, it is a complex waste stream. Therefore, the challenge involved in the appropriate management of e-waste is crucial to sustaining our ecosystem, livelihood, and environment. The sustainable approach comprises a challenging task to the digital societies which would further necessitate organized efforts to deal with e-waste. Existing conventional practices have failed to manage these huge electronic obsoletes sustainably, and therefore, the waste is growing exponentially around the world (Forti et al., 2020). Based on chemical composition, e-waste contains mainly metals (60%), plastics and their blends (30%), and other harmful materials (10%) (Gaidajis et al., 2010). However, this composition is so complex that it varies with different electronic items of different categories. Moreover, different types of thermoplastics such as polyvinyl chloride (PVC), polyurethane (PU), polystyrene (PS), high-impact polystyrene (HIPS), and acrylonitrile–butadiene–styrene (ABS) are present in e-waste (Mohan et al., 2016; Sekhar et al., 2016; Debbarma et al., 2018). Hazardous substances such as various additives (organic and inorganic) and fillers are used in plastics to enhance the material properties (Morf et al., 2007; Erickson and Kaley, 2011).

All these materials of e-waste release very harmful gases and inert chemicals, viz., CFCs, di-oxides, and furans, when they are incinerated. These compounds are potentially carcinogenic to humans, and thus, it is a serious concern. Furthermore, the landfilling of e-waste can deliver these dangerous materials to the groundwater, and they accumulate as leachates. During the recycling processes, harmful particles containing flame retardants and heavy metals are also discharged into the atmosphere (Kiddee et al., 2013). Therefore, the accumulation of e-waste in the environment is a major issue of the current era, and the management of this waste is thus a daunting task that needs to be tackled in an eco-friendly manner. Traditional methods because of their disadvantages have fuelled the use of biological tools to recover the precious metals present in e-waste and to promote the studies of bioleaching and biodegradation processes.

In the past decades, microbial leaching and bio-hydrometallurgical techniques have been exploited for the recovery of base and precious metal ions from e-waste (Brandl et al., 2001; Shah et al., 2015). Therefore, remediation of other toxic materials such as plastics present in e-waste is incomplete. Literature focusing on the direct biodegradation of complex e-waste is also rare. Recently, a group of researchers addressed the problem and studied the biodegradation of e-plastic. Potential isolates, viz., *Alcaligenes* sp., *Enterobacter* sp., *Citrobacter sedlakii* and *Brevundimonas diminuta,* and *Pseudomonas* and *Bacillus* strains are found to be able to degrade high-impact polystyrene (HIPS) present in e-plastic (Mohan et al., 2016; Sekhar et al., 2016). Zhu et al. (2021) studied the biodegradation of e-plastics, namely, polyurethane (PU), polystyrene (PS), and acrylonitrile–butadiene–styrene (ABS) present in e-waste by a wax moth's (Galleria mellonella) gut microbes, namely, *Enterococcus* and *Enterobacter*, respectively. However, in all those cases, the biodegradation studies were targeted only for a single polymer at a time. Moreover, previous studies have not carried out *in situ* biodegradation experiments; therefore, in a previous study by the author group, five new potential e-waste degrading bacteria were identified which were originally isolated from polluted soil

and found to be very promising for bioremediation (Debbarma et al., 2018; Thorat et al., 2020). In the above context, the present study is conducted to compare the efficacy of monoculture *Pseudomonas aeruginosa* strain PE10 with the bacterial consortium for e-waste biodegradation under *in situ* conditions. Therefore, this investigation would further unravel the anomaly between the use of monoculture and consortium for effective large-scale biodegradation of synthetic polymeric e-waste and their exploration in waste management. This study may have important implications for e-waste bio-recycling and sustainable ways to tackle the e-waste crisis at present and in the coming decades.

2 Materials and methods

2.1 Materials

Randomly discarded computer keyboards were collected, and plastic materials mainly keycaps were sorted out from the rest of the wastes, viz., printed circuit boards (PCBs), metals, glasses, and wires. Thereafter, sorted e-waste plastics are grounded under "Wiley® Mill" and sieved (∼5 mm) through to collect e-waste granules. Then, e-waste granules are washed with 70% EtOH for 15 min. and dried at 50 ± 1°C (dry oven) for 1 h before using those e-waste granules as primary carbon sources. Soapstone (HiMedia, India) used as carrier material for bioformulation consists of talcum powder; steatite; talc, fine powder; and hydrous magnesium silicate.

2.2 Characterization of e-waste by FTIR spectroscopy

Prior to conducting the experiment, milled e-waste regarded as pure e-waste was subjected to an analysis of its chemical composition for characterization using a Fourier transform infrared spectroscopy (FTIR) spectrophotometer (Perkin Elmer version 10.03.06). The characteristics of FTIR absorbance are illustrated as wave numbers (cm^{-1}) in the range of 4,000–450 cm^{-1}.

2.3 *Pseudomonas aeruginosa* strain PE10 and bacterial consortium

The cultures of *P. aeruginosa* strain PE10 (NCBI accession no. MF943159) and consortium comprising of *Achromobacter insolitus* strain PE2 (MF943156), *Acinetobacter nosocomialis* strain PE5 (MF943157), *Pseudomonas lalkuanensis* PE8 (CP043311), and *Stenotrophomonas pavanii* strain PE15 (MF943160) were revived from 50% glycerol stocks (stored in −80°C at Departmental Culture Collection, Department of Microbiology, College of Basic Sciences and Humanities, GBPUAT, Pantnagar) by inoculating into 5.0 ml nutrient broth test tubes and incubated at pH (7 ± 0.2) and temperature (35 ± 1°C) for 24 h. Furthermore, aliquots of 500 µl overnight culture were used to inoculate into 10 ml nutrient broth and incubated for another 4 h at ambient growth conditions until an optical density (OD) of 0.6 was attained at 600 nm (OD_{600}) to obtain mid-log phase active culture. All the used cultures in this study were originally isolated from the Net House Experimental Pit, Pantnagar, and from a dump yard of the Century Pulp and Paper Mill, Lalkuan, Uttarakhand, India (Debbarma et al., 2018; Thorat et al., 2020).

2.4 Preparation of bioformulations and shelf-life determination

The active consortium (800 ml) was divided into 16 parts, 50 ml each in centrifuge tubes and spun at 5,000 rpm for 10 min to separate the cells using a Sigma 3–16K centrifuge. Later, the supernatant was partially decanted, and then the tubes were vortexed for 15 min. Then, 5 g soapstone was weighed and added properly to each tube with pellets under sterile conditions. The tubes were vortexed again for a homogenous mixing of talc with the bacterial suspension. With a sterile spatula, the mixture was then emptied into a glass dish and kept at room temperature (28 ± 1°C) aseptically for drying the mixture. Later, the viability of bacterial strains in the formulation was ascertained according to a previous study (Goel et al., 2015) (Supplementary Text 1).

2.5 *In situ* efficacy studies

2.5.1 In situ incubation of e-waste

Topsoil was dug from the Crop Research Center (CRC) at Pantnagar, India, and half-filled into 60 cm × 30 cm × 30 cm (length × width × depth) experimental net house pits. First, the soil was mashed manually and 15 g of e-waste granules were mixed with mashed soil for the treatments. Then, 200 g of prepared active bioformulation of the consortium was added to the soil of the treatment pits which was then incubated under natural conditions. Furthermore, autoclaved distilled water was sprinkled at regular intervals of 2–3 days to maintain the moisture content of the soil. Pit cleaning and aeration conditions were maintained by shoveling the soil at regular intervals of 15 days. Keeping the point of shelf-life of consortium in bioformulation, an active bioformulation was added in the pit at regular intervals of 60 days. The biodegradation study of the treatments, i.e., (a) soil + e-waste + monoculture *P. aeruginosa* strain PE10 and (b) soil + e-waste + consortium, was performed with respective positive (soil + e-waste) control. The study was carried out for a period of 6 months.

2.5.2 Recovery of biodegraded samples

The treated e-waste samples from the soil pits (i.e., positive and treatment pits) were carefully recovered after 3 and 6 months of incubation and collected in sterile Whirl-Pak™ sample bags with the help of trowel/khurpi and sieved after the incubation period. The biodegraded samples were washed and surface-sterilized with 70% EtOH for 10 min and subsequently vortexed vigorously followed by drying at 50 ± 1°C (dry oven) for 1 h to evaporate leftover liquid residues. After washing with EtOH, the collected e-waste samples were added to 15 ml centrifuge tubes containing 10 ml of millipore water, and the tubes were then centrifuged at 5,000 rpm at 4°C for 10 min to remove the remaining soil particles and microbial biomass. Finally, the supernatant was carefully

removed, and the leftover water was evaporated by placing the residues in an oven at 50 ± 1°C (dry oven) for 24 h.

2.5.3 Comparative analysis of treated samples

2.5.3.1 FTIR spectroscopy

The standards and programming of the spectrophotometer were maintained as same as mentioned in Section 2.2, wherein ν and δ are used to represent the stretching and bending vibrations, respectively.

2.5.3.2 Simultaneous TG-DTG-DTA

The study of simultaneous thermogravimetric-derivative thermogravimetry-differential thermal analysis (TG-DTG-DTA) was performed for e-waste treated with monoculture *P. aeruginosa* strain PE10 and bacterial consortium using untreated e-waste control as a reference. This experiment was carried out to compare the thermal stability of biodegraded e-waste on an EXSTAR (SII 6300 EXSTAR) thermal analyzer under a nitrogen atmosphere at 200 ml/min programmed at 35°C to 800°C temperature range with a heating rate of 5°C/min on a platinum sample pan.

2.5.3.3 SEM

Scanning electron microscopy (SEM) was performed to study the surface morphology of both treated samples and positive control samples. For this, the samples were thoroughly washed with 70% EtOH for 10 min and dried properly using a desiccator for 24 h under vacuum. Later, the samples were metalized with gold particles and observed under SEM (JEOL JSM-6610 LV) at 8.00 kV EHT with a magnification of 400×.

3 Results and discussion

3.1 Structural characterization of e-waste

The analytical results of the e-waste FTIR spectrum corresponding to their polymers are summarized in Figure 1 (Supplementary Table 1). During the investigation, symmetrical and asymmetrical absorptions were observed for some bonds, and they are symbolized by "$_{asym}$" and "$_{sym}$," respectively. Pure e-waste has shown the common characteristic wave numbers (KBr, cm^{-1}) of O–H (3,390.49), $_{asym}$ C–H (2,922.8), C=O (1,755.21), C=C (1,645.87), C–H (1,402.48), C–O (1,155.8), $_{sym}$ C–O–C (1,069.02), and C–Cl (759.38), respectively.

The interpretation of the results identified typical spectra of base polymers which are acrylonitrile–butadiene–styrene (ABS) and high-impact polystyrene (HIPS) as well as some minerals. The FTIR spectrum of pure e-waste has shown evidence of the presence of acrylonitrile at a wavelength of 2,922.8 cm^{-1} corresponding to C-H bonds. Acrylonitrile can be present in PC/ABS blends due to the carbonyl (C=O) peak of the polycarbonate functional group observed at 1,755.21 cm^{-1} (Arnold et al., 2010). The absorbance bands observed at 1,645.87 and 1,402.48 cm^{-1} correspond to the benzene rings from the HIPS and C-H bonds which were used as reference peaks for the butadiene peak, respectively (Sekhar et al., 2016). Characteristic peaks related to the para aryloxy group and fillers at 1,155.8 and 1,069.02 cm^{-1}, respectively, were also found (Vazquez and Barbosa, 2016). The peak at 759.38 cm^{-1} is characteristic of the presence of an aromatic ring or substituted phenyl ring. The presence of hydroxyl groups is identified by the peak which absorbs at 3,390.49 cm^{-1} wave number (Tiganis et al., 2002).

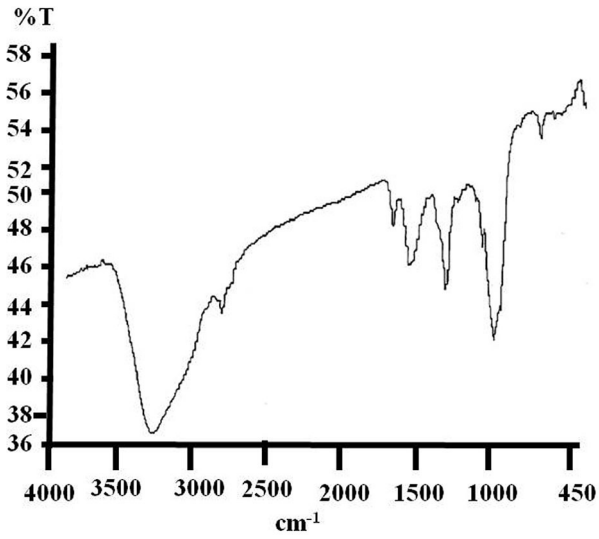


FIGURE 1
Characteristic FTIR spectrum of pure e-waste showing the absorbtion peaks corresponds to its base polymers.

Therefore, in addition to ABS and HIPS, this absorbance indicated the possible presence of polymers such as polystyrene (PS), styrene-acrylonitrile (SAN), polycarbonate (PC), blends of polycarbonate (PC)/ABS, and blends of HIPS/poly(p-phenylene oxide) (PPO) as discussed by the abovementioned research groups. These varieties of polymers were found to be present in e-plastics which have good properties such as high-temperature resistance, mechanical strength, chemical stability, flame retardancy, rigidity, impact strength, and creep resistance (Beigbeder et al., 2013).

3.2 Bioformulations and its viability

Viability observations of monoculture and consortium confirm that cells present in the formulation were active even after 70 days of storage. The growth of both monoculture and consortium was slightly variable after 2 days of storage in terms of CFU ml^{-1}, and a percentage (%) survival decrease was calculated. At 70 days, a percentage survival decrease rate for bacterial monoculture and the consortium was 4.84 and 3.88%, respectively, which suggests that bioformulations have considerable shelf-life longevity that allows the formulation to be used as a suitable carrier with active monoculture strain and consortium for *in situ* efficacy experimentation (Table 1).

The selection of the type of formulation developed and carriers used is dependent on the nature of active cells and the factors related to the site of application. Many other bioformulations have been reported in previous studies such as *Pseudomonas fluorescence*, *Rhizobacteria*, *Bacillus*, and *Pseudomonas oryzae* for the treatment of damping-off of cotton seeds and enhancement

TABLE 1 Viability of bio-formulations under ambient conditions during storage period.

Bacterial agents	Dilution factor	CFU/ml at subsequent time intervals (days)							
		2nd	4th	11th	18th	25th	40th	55th	70th
Consortium	10^7	284 (±2)	283 (±2)	279 (±2)	280 (±2)	278 (±2)	275 (±2)	274 (±2)	273 (±2)
% survival decrease		0%	0.36%	1.77%	1.41%	2.12%	3.17%	3.53%	3.88%
Monoculture strain PE10	10^7	165 (±2)	165 (±2)	164 (±2)	161 (±2)	163 (±2)	160 (±2)	159 (±2)	157 (±2)
% survival decrease		0%	0%	0.60%	2.42%	1.21%	3.00%	3.63%	4.84%

Each value is the mean of three replicates. Values in (±) indicate standard error.

of induced systemic resistance, as a fertilizer and plant growth promoters (Bharathi et al., 2004; Mishra and Arora, 2016).

3.3 Efficacy analysis through diversified analytical techniques

During the soil incubation period under natural conditions, biodegraded samples from each experimental pit were recovered at 3- and 6-month intervals and subjected to qualitative analysis with reference to untreated control, respectively. Diversified analytical techniques, viz., FTIR, TG-DTG-DTA, and SEM analysis, were exploited for spectral, thermal, and morphological changes, respectively.

3.3.1 FTIR spectra of biodegraded e-waste

FTIR spectra for the relative functional potential of used monoculture and bacterial consortium toward the degradation of e-waste have been reflected through the changes in the wave numbers (cm^{-1}) as well as the addition and deletion of functional groups and chemical bonds in the structure compared to untreated control. The changes in the chemical structural compositions of biodegraded e-waste are shown in Figures 2, 3 and Supplementary Tables 2, 3. Analysis of biodegraded samples has revealed variable peaks corresponding to diverse bond stretching and bending vibrations.

Untreated e-waste samples recovered from a soil bed after 3 months illustrated the wave numbers (cm^{-1}) of ν OH (3,391.65), ν_{asym} C–H (2,923.58), δ C=O (1,730.09), ν C=C (1,641.54), δ C–H (1,386.95), ν C–O (1,117.49), ν C–Cl (758.79), and δ =C–H (701.06; Figure 2A and Supplementary Table 2). During this period of incubation under natural conditions, few changes in chemical structure have been noticed comparing the characteristic wave numbers of pure e-waste, i.e., the absolute deletion of ν_{sym} C–O–C peak and formation of a completely new peak that corresponds to δ =C–H (701.06 cm^{-1}) bond. These changes may be attributed to the environmental conditions (temperature, light, heat, pressure, etc.) and also due to the unsterilized soil used in the study (Raghuwanshi et al., 2018). Comparison of e-waste exposed under bacterial consortium has shown remarkable changes in FTIR spectra such as the introduction of a new group aromatic δ =C–H corresponding to 618.41 cm^{-1}. Total removal of ν_{asym} C–H, δ C=O, ν C–O, ν_{sym} C–O–C, and ν C–Cl from the structural composition directly reflects the action and efficacy of consortium for degradation of e-waste under soil ecosystem (Figure 2B). The effect of biodegradation on e-waste by monoculture *P. aeruginosa* strain PE10 is clearly seen in Figure 2C. This bacterium could degrade the e-waste just as the used consortium. The FTIR spectrum of PE10-treated e-waste elucidated four new peak characteristic to δ N–H (1,600.96 cm^{-1}), ν C=C (1,450.99–1,492.80 cm^{-1}) ring stretching, ν_{asym} C–O–C (1,215.11 cm^{-1}), and ν_{sym} C–H (928.74 cm^{-1}), respectively, as compared to the consortium-treated samples and the untreated control. The absolute removal of ν C=C, δ C–H, and ν C–O (Supplementary Table 2) groups from the polymeric backbone suggested that these changes are clearly attributed to the effect of strain PE10. Therefore, the 3-month *in situ* treatment result evidently suggests that both *P. aeruginosa* strain PE10 and bacterial consortium have remarkable efficacy for e-waste degradation under identical situations.

Furthermore, the final sample was recovered and collected from the soil bed after the completion of the incubation period, i.e., 6 months. FTIR absorptions of the untreated control samples showed additional peaks of ν C=C ring stretching and δ =C–H corresponding to the wave numbers (cm^{-1}) of 1,452.18–1,502.65 and 669.26–701.42 cm^{-1}, respectively. This sample also showed complete deletion of ν C=C and δ C–H functional groups as compared to pure e-waste (Figure 3A and Supplementary Table 3). A similar trend of changes in the spectrum was also observed after the 3-month soil incubation which could be attributed to environmental factors. However, the bacterial consortium-treated samples have depicted more significant degradation as the complete removal of functional groups such as δ C=O, ν C–O, and ν_{sym} C–O–C was more prominent for the consortium used in this study (Figure 3B). Nevertheless, a significant shift in the absorption frequencies such as the addition of δ N-H (1,600.69 cm^{-1}) and ν_{asym} C–O–C (1,222.24 cm^{-1}) was also observed in the samples treated with a consortium. Biodegradation with the consortium brought about significant shifts in the fingerprint region of the IR spectrum between 1,700 and 950 cm^{-1} of treated e-waste as compared to the control. Though, exposure of monoculture *P. aeruginosa* strain PE10 has induced remarkable changes in the spectra reflecting the complete degradation of δ C=O and ν C–O bonds from the structure of polymeric backbone and additional new absorption frequencies of ν N–H, δ N–H, and ν_{asym} C–O–C, i.e., at 2,402.35, 1,601.03, and 1,216.43 cm^{-1}, respectively, were also observed (Figure 3C). Furthermore, reducing in the wave numbers of ν O–H, ν_{asym} C–H, ν C=C ring stretching and δ =C–H to 3,023.17, 2,852.67–2,924.19, 1,451.97–1,492.72, and 667.13–700.43 cm^{-1}, respectively, were attributed by this culture unlikely to consortium and control. This result indicates that monoculture *P. aeruginosa* strain PE10 was rather more consistent in efficacy toward the progressive biodegradation of e-waste than

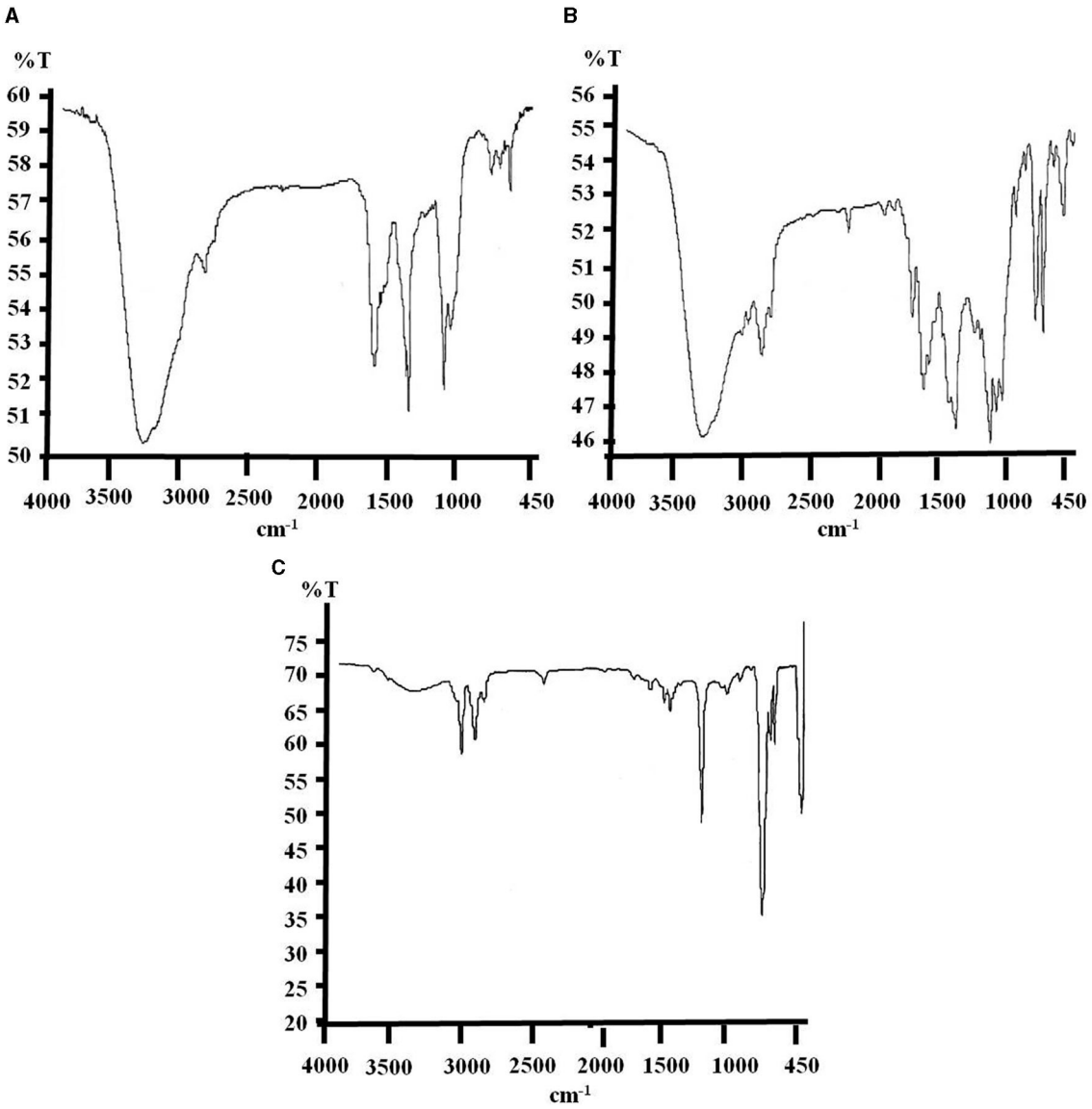


FIGURE 2
FTIR spectra of biodegraded e-waste samples, where spectrum **(A)** represent untreated control showing the absorption peaks with minor differences from pure e-waste spectrum due to environmental factors, after 3 months of soil incubation and spectrum **(B, C)** correspond to consortium and strain PE10 treated samples, respectively depicting significant changes and differences in absorption peaks at the same time of incubation period.

the consortium. However, the bacterial consortium has shown advancement in its efficacy following the preceding sample analysis as the incubation period extends. Conclusively, comparative results of FTIR spectra analysis have clearly revealed that both bacterial consortium and monoculture *P. aeruginosa* strain PE10 have the potential to accelerate the biodegradation of e-waste under natural conditions. Therefore, to acquire further evidence on the organic degradation of the samples, simultaneous TG-DTG-DTA analysis was performed.

3.3.2 Comparative thermal analysis

Biodegradation of e-waste particularly its organic fraction would reduce its thermal stability due to the composition changes after *in situ* treatment. Thermogravimetric analysis accurately determines the percentage weight loss (% W_L) of the samples in accordance with programmed temperature and time interval conditions. Thermograms of the treated samples are portrayed in Table 2 and Supplementary Figures 1, 2 with reference to untreated control.

The thermal analysis of 3-month samples indicated that the TG onset temperature 339°C with 7.2% weight loss (W_L) of untreated soil control was much higher than bacterial consortium (321°C with 10.0% W_L) and strain PE10 (300°C with 6.9% W_L), respectively (Table 3). The percentage weight loss during TG onset clearly reveals the biodegradation of e-waste by the consortium, where the treated samples showed 10.0% W_L as compared with the untreated control which exhibited a weight loss of 7.2%, whereas

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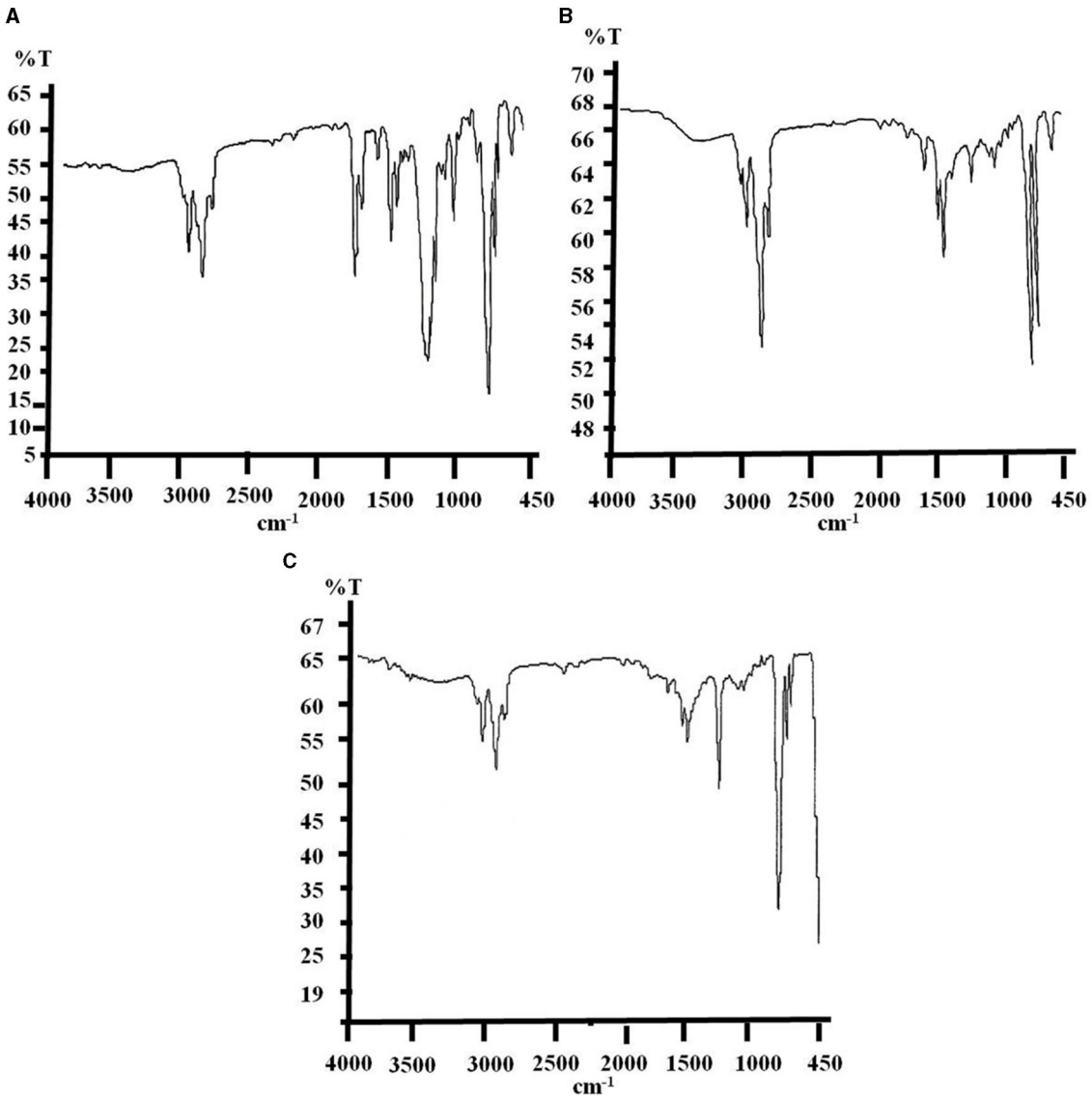

FIGURE 3
Final FTIR spectra of biodegraded e-waste samples after 6 months of soil incubation, where spectrum **(A)** portraying for untreated control with some changes in absorption peaks from last 3 months sample and spectrum **(B, C)** represents consortium and strain PE10 treated samples, respectively illustrating tremendous alterations in absorption peaks viz. removal, addition and shifting of wave numbers (cm^{-1}) of peaks signifying the comparative biodegradation efficacy.

TABLE 2 **Simultaneous thermal analysis of biodegraded e-waste under *in situ* conditions by bacterial consortium and strain PE10 with reference to the control after 3 months of soil incubations.**

Samples	% W_L		DTG		DTA	
	TG onset	TG endset	T_{max} (°C)	Rate (mg/°C) $\times 10^{-3}$	T_{max} (°C)	ΔH_f (kcal/mole) $\times 10^{-3}$
Untreated control	7.2 (339°C)	96.0 (507°C)	419	15.8	422	3.8
Consortium treated e-waste	10.0 (321°C)	89.9 (660°C)	411	17.4	411	4.9
PE10 treated e-waste	6.9 (300°C)	84.4 (471°C)	409	24.7	412	5.1

TABLE 3 Simultaneous thermal analysis of biodegraded e-waste under *in situ* conditions by bacterial consortium and strain PE10 with reference to the control after 6 months of soil incubation.

Samples	% W_L		DTG		DTA	
	TG onset	TG endset	T_{max} (°C)	Rate (mg/°C) $\times 10^{-3}$	T_{max} (°C)	ΔH_f (kcal/mole) $\times 10^{-3}$
Untreated control	3.1 (300°C)	93.9 (418°C)	419	11.9	427	4.5
Consortium treated e-waste	6.8 (300°C)	90.1 (419°C)	412	18.5	418	5.3
PE10 treated e-waste	6.3 (300°C)	80.0 (432°C)	401	22.7	410	6.3

P. aeruginosa strain PE10-treated sample has shown 6.9% W_L which was lower than both the untreated and consortium-treated samples; however, remarkably, reduced TG onset temperature shows the potential efficacy of this bacterium within this period of incubation. Moreover, the DTG peak of *P. aeruginosa* strain PE10 was observed at the lowest temperature of 409°C with the highest decomposition rate of 24.7 × 10^{-3} mg/°C among other samples, and the DTA peak was detected at 412°C with heat of fusion (ΔH_f) 5.1 × 10^{-3} kcal/mole compared with other treated and untreated samples as seen in Table 3 and Supplementary Figure 1. Nonetheless, as the decomposition of the sample progresses under thermal influences, bacterial consortium-treated e-waste sample also showed considerable lower temperatures of the DTG peak at 411°C with rate of decomposition at 17.4 × 10^{-3} mg/°C and the DTA peak at 411°C with ΔH_f 4.9 × 10^{-3} kcal/mole in comparison with the control, where sample decomposition rate was at the lowest at 15.8 × 10^{-3} mg/°C with an elevated temperature of 419°C and the heat of fusion (ΔH_f) was 3.8 × 10^{-3} kcal/mole at 422°C. Therefore, these findings were at par with FTIR results and thus further validated the significant efficacy of *P. aeruginosa* strain PE10 and bacterial consortium for e-waste biodegradation within an undistinguishable *in situ* environment.

Furthermore, to establish the progressive nature of e-waste biodegradation by both treatments over a long period of incubation, thermal analysis of 6-month samples was characterized with reference to untreated control samples. As the soil incubation period was over, it was observed that all three samples have shown TG onset at 300°C with a significant amount of weight loss at this temperature, i.e., 3.1%, 6.8%, and 6.3% W_L for untreated control, consortium, and *P. aeruginosa* strain PE10, respectively (Table 2 and Supplementary Figures 2a–c). The decomposition of the treated samples at particular heat was remarkable as the percentage W_L was at least twice that of the control. In addition, the TG onset temperature requirement of 6-month samples was very much at minimum compared with TG onset temperatures of 3-month samples which suggested that the structural backbone of the polymers was disintegrated and the composition of the samples shattered by the influence of consortium to become brittle. Thus, the thermal stability of the biodegraded samples and TG onset temperature were reduced after the incubation period. Furthermore, these results are supported by DTG and DTA peak analyses, where the consortium-treated samples have shown considerably lower temperatures of DTG peak at 412°C with a rate of decomposition at 18.5 × 10^{-3} mg/°C, and the DTA peak was observed at 418°C with the heat of fusion (ΔH_f) 5.3 × 10^{-3} kcal/mole (Supplementary Figure 2b). Comparatively, *P. aeruginosa* strain PE10-treated samples elucidated the lowest temperature of 401°C with the rate of decomposition 22.7 × 10^{-3} mg/°C along with DTA peak at 410°C showing the energy required for heat of fusion (ΔH_f) 6.3 × 10^{-3} kcal/mole (Supplementary Figure 2c).

Conclusively, thermal analysis has clearly revealed the efficacy of bacterial consortium and *P. aeruginosa* strain PE10 which was determinately responsible for the progressive decomposition of biodegraded samples with much higher decomposition rate and increased weight loss at comparatively lower temperatures than the respective control after the long period of incubation. However, as seen during FTIR analysis, monoculture *P. aeruginosa* strain PE10 has proven to be consistent in efficacy, whereas the consortium was advancing in its efficacy as the incubation duration progressed. Changes in the thermal profiles of treated e-waste samples might be due to the action of bacterial enzymes with the functional groups present in the polymers, which, subsequently causes the alterations in chemical structure of the polymeric backbone as the result substantiated FTIR spectra. Thus, it was clear that both the consortium and *P. aeruginosa* strain PE10 could utilize e-waste polymer as their carbon and energy source when treated. Furthermore, the development of various DTG and DTA peaks was previously found and documented in the case of high-density polyethylene (HDPE) and low-density polyethylene (LDPE), polycarbonate, non-poronized and poronized LDPE (Kapri et al., 2010), epoxies and their silicone blends, and epoxy and cold-mix epoxy (CME) during the biodegradation studies (Shikha et al., 2015).

3.3.3 SEM observations of recovered samples

Based on the comparative results obtained from FTIR and thermal analyses, it was confirmed that the treated e-waste was evidently degraded by the used consortium and monoculture strain PE10. Therefore, additional SEM micrographs were taken for conclusive evidence of e-waste biodegradation by the used bacterial agents under *in situ* conditions. Comparative analysis of the efficacy of *P. aeruginosa* strain PE10 and bacterial consortium on e-waste surface morphology at 3 and 6 months of soil incubation was apparently confirmed through SEM analysis.

During the incubation period, changes in e-waste surface morphology by the *P. aeruginosa* strain PE10 and the bacterial consortium were analyzed by taking untreated control samples as reference. The control samples from 3- and 6-month

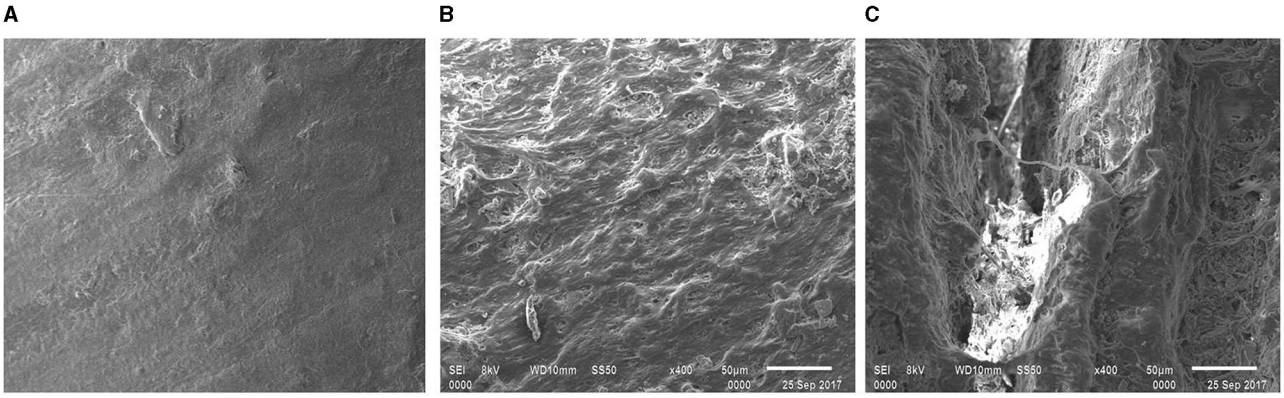

FIGURE 4
Comparative SEM micrograph of the e-waste recovered from untreated control **(A)**, bacterial consortium **(B)**, and strain PE10 **(C)** treated soils, respectively after 3 months of incubation. Scale bar = 50 µm; magnification = 400×.

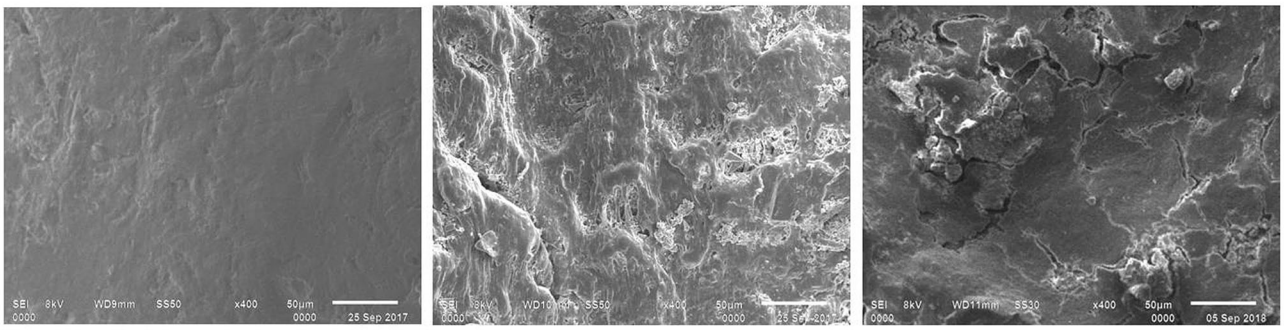

FIGURE 5
Comparative surface morphology of the e-waste recovered from untreated control **(A)**, bacterial consortium **(B)**, and strain PE10 **(C)** treated soils, respectively after 6 months of incubation. Scale bar = 50 µm; magnification = 400×.

incubation revealed comparatively smooth and homogenous surface morphologies (Figures 4, 5A). However, the 3-month SEM image obtained from the treated (consortium and strain PE10) e-waste was visibly distinguishable from the untreated samples as the fissures and crumbles on the e-waste surface were extensive, showing major attributes, viz., well-resolved distortions, cracks, and formation of tiny cavities (Figures 4B, C). Furthermore, in the case of *P. aeruginosa* strain PE10-treated e-waste, the occurrence of fissures, heterogeneous morphology, fractures, and widened cracks was found to be remarkably predominant in comparison with the bacterial consortium.

Similarly, the surface resolutions, porosities, roughness, and cracks have been seen in the e-waste samples treated with the consortium and strain PE10 after 6 months of soil incubation (Figures 5B, C), whereas the control was comparable with that of the 3-month sample (Figure 5A). These heterogeneous surface morphologies on the surface of treated e-waste were obviously imparted after the exposure of bacterial agents which further substantiate the results of FTIR and thermal analyses. Thus, the SEM micrographs revealed the intensive surface deterioration of treated e-waste after soil incubation under natural conditions over successive periods of time. Similar biodegradation studies utilized SEM micrographs as a tool to provide evidence for the deterioration of the plastic film due to the action of the plastic degrading enzymes which demonstrate cavities and grooves formed on the plastic film, which directly reflected the extent of microbial colonization and degradation (Yoshida et al., 2016).

All the above analyses revealed that both the *P. aeruginosa* strain PE10 and the bacterial consortium have shown the capacity to degrade e-waste under *in situ* conditions with different contrasts. Therefore, further investigations such as proteogenomic study, degradation pathway prediction, and bacterial community analysis in the soil pit of the bacterial strains used may reveal more novel insights into the overall mechanism of e-waste biodegradation.

4 Conclusion

From this study, it can be concluded that both *P. aeruginosa* strain PE10 and bacterial consortium can potentially degrade e-waste under *in situ* conditions with their different levels of efficacy. The FTIR analysis of the biodegraded samples clearly proved that strain PE10 is as efficient as the bacterial consortium for the biodegradation of e-waste. It is also speculated that monoculture

had consistency in its efficacy throughout the experimentation period, unlike the consortium which rather perpetuates its efficacy with the progression of incubation time.

Thermal analysis and SEM images of degraded samples further strongly substantiated these findings where monoculture-treated samples have shown thermal decaying at lowest temperatures with maximum decomposition rate and extensive surface disintegrations, respectively, indicating the surface bacterial colonization and degradation. Furthermore, this investigation also provided the details of physico-chemical nature of polymeric e-waste used in this study. Therefore, this is a sincere effort to resolve the anomaly between monoculture and consortium for eco-friendly management and bio-recycling of e-waste. Hence, it is proposed that monoculture *P. aeruginosa* strain PE10 can be used singly for large-scale biological management of e-waste sustainably in the near future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

PD: Data curation, Formal analysis, Investigation, Writing—original draft, Resources. DS: Formal analysis, Investigation, Methodology, Writing—review & editing. SK: Writing—review & editing, Software. MZ: Formal analysis, Data curation, Writing—review & editing. RG: Conceptualization, Resources, Supervision, Writing—review & editing, Validation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1277186/full#supplementary-material

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Differences in soil bacterial community structure during the remediation of Cd-polluted cotton fields by biochar and biofertilizer in Xinjiang, China

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Introduction: Heavy metal pollution is a major worldwide environmental problem. Many remediation techniques have been developed, these techniques have different performance in different environments.

Methods: In this study, soil sampling was conducted in multiple cotton fields in Xinjiang, China, and found that cadmium (Cd) was the most abundant soil heavy metal. Then, to find the most suitable technique for the remediation of Cd pollution in cotton fields, a two-year study was conducted to explore the effects of cotton straw-derived biochar (BC, 3%) and *Bacillus*-based biofertilizer (BF, 1.5%) on cotton Cd uptake and transport and soil microbial community structure under Cd exposure conditions (soil Cd contents: 1, 2, and 4 mg·kg^{-1}).

Results: The results showed that the bioaccumulation coefficients (Cd content of cotton organs / soil available Cd content) of cotton roots, stems, leaves, and buds/bolls reduced by 15.93%, 14.41%, 23.53%, and 20.68%, respectively after the application of BC, and reduced by 16.83%, 17.15%, 22.21%, and 26.25%, respectively after the application of BF, compared with the control (no BC and BF). Besides, the application of BC and BF reduced the transport of Cd from soil to root system, and enhanced the diversity of soil bacterial communities (dominant species: *Alphaproteobacteria* and *Actinobacteria*) and the metabolic functions related to amino acid synthesis. It was worth noting that the differential species for BF group *vs* BC group including *Alphaproteobacteria*, *Gemmatimonadetes*, *Bacilli*, and *Vicinamibacteria* were associated with the enrichment and transport of Cd, especially the transport of Cd from cotton roots to stems.

Discussion: Therefore, the application of BC and BF changed the soil bacterial diversity in Cd-polluted cotton field, and then promoted the transport of Cd in cotton, ultimately improving soil quality. This study will provide a reference for the selection of soil heavy metal pollution remediation techniques in Xinjiang, China.

KEYWORDS

soil heavy metal contamination, bacterial diversity, cadmium enrichment, bioremediation, cotton

1 Introduction

Soil heavy metal pollution remediation is a hot topic worldwide. In 2018, China issued the *Standard for Risk Control of Arable Soil Pollution* (GB 15618-2018), a standard for the evaluation of heavy metal pollution in arable soil in China. Studies have shown that the content of heavy metal in arable soil in Xinjiang gradually increases (Zheng et al., 2018; Abudureheman et al., 2021). For example, Ma et al. (2021) reported that the contents of Cr, Cu, Zn, As, and Cd in the arable soil in Altay, Xinjiang exceeded the average values of Xinjiang, and the average Cd content was as high as 0.20 mg·kg^{-1}, which seriously affected crop growth and threatened human health. Zheng et al. (2018) reported that the contents of Zn, As, Pb, Cr, and Cu were very high in the soil of Bole, Changji, and Kashgar in Xinjiang.

Soil microorganisms play a very important role in soil biogeochemical cycles and ecosystem functions, and have been widely used to assess soil health (Song J. W. et al., 2022). Under heavy metal pollution, soil microbial ribosomes, RNA polymerase, DNA polymerase, RNA and protein processing, and carbon sequestration are inhibited to varying degrees. Besides, the higher the content of heavy metals, the more obvious the inhibition is (Ma et al., 2021). The genetic basis of microbial resistance to heavy metals includes energy-dependent efflux (ATPase, RND, CDF family), enzymatic detoxification (redox and demobilization), and cell fixation and uptake (Senthil Kumar et al., 2023). Besides, soil bacterial communities such as *Acinetobacter*, *Citrobacter*, and *Pseudomonas* resist heavy metal stresses through enzymatic detoxification or transforming heavy metals into nontoxic forms by intracellular/extracellular binding (Wu et al., 2021). Therefore, the study of soil microbial diversity, structure, and function is of great significance for the remediation of heavy metal pollution.

Biochar produced by high-temperature anaerobic pyrolysis of manure, plant straw, wood, etc. has been widely used to reduce the bioavailability of soil heavy metals (Lin et al., 2022; Wan et al., 2022). The rich pore structure and large specific surface area of biochar provide a good habitat for soil microorganisms, and its rich nutrients such as organic matter, nitrogen, and phosphorus stimulate soil microbial growth and metabolism (Liu et al., 2021). Studies have shown that biochar increases not only the relative abundance of microorganisms associated with the carbon-nitrogen cycle (Actinobacteria, and *Pseudomonas*), but also increases the bioavailability of chemically bonded phosphates in soil. Besides, it also plays a great role in the fixation of free metal ions (Ahmad et al., 2017). Some components in biochar, such as water-soluble nutrients, affect soil microbial activity, and increase soil nutrient content and organic carbon mineralization, which ultimately promotes bacterial growth and activity and increases soil bacterial community diversity (*Proteobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Actinobacteria*, *Nitrospirae*, and *Patescibacteria*; Wang B. H. et al., 2020; Sun T. et al., 2023). Biofertilizer, as another material for the remediation of soil heavy metal pollution, can increase the diversity of beneficial bacteria in the soil, and these beneficial bacteria can promote the growth and development of plants and the adsorption of soil pollutants such as heavy metals. In addition, the application of biofertilizer can also promote the secretion of metabolites of plants, and reduce the morbidity of plants, ultimately increasing soil quality, crop yield and quality (Wei et al., 2023). Recent studies have shown that inoculating the soil with *Bacillus* can not only increase crop yield and resistance to external abiotic stresses, but also reduce the bioavailability of inorganic and organic pollutants such as heavy metals (Han et al., 2018), plant diseases (Sun Y. et al., 2023), and polycyclic aromatic hydrocarbons (Song L. et al., 2022) in the soil. Dong et al. (2019) also found that the application of *Bacillus*-rich biofertilizer significantly increased the total saponin content of *Panax notoginseng* roots by 51.49%, and promoted the shoot and root biomass accumulation. It can be seen that the application of biofertilizer with *Bacillus* as the dominant bacterium not only has a positive effect on crop growth and soil quality, but also reduces the risk of environmental pollution and the transportation and accumulation of pollutants in the food chain (Wang et al., 2019).

It can be seen that the use of biochar and biofertilizer in the remediation of heavy metal pollution in arable soil is feasible. Biochar and biofertilizer can not only reduce the content of available Cd in the soil, but also improve the living environment of soil microorganisms and soil quality (soil pH, nutrients, and physical characteristics; Zhu Y. et al., 2022). Our previous study results showed that biochar and biofertilizer had different effects on soil available Cd content, soil physicochemical properties, and cotton Cd uptake (Zhu Y. Q. et al., 2022). To further explore the reasons behind this from the perspective of soil microorganisms, in this study, the effects of cotton straw-derived biochar (BC, 3%) and *Bacillus*-based biofertilizer (BF, 1.5%) on cotton Cd migration and transformation and soil microbial community structure were investigated under Cd exposure conditions (soil Cd contents: 1, 2, and 4 mg·kg^{-1}). This study hypothesized that the application of BC and BF might reduce the bioavailability and migration of soil Cd by adjusting soil bacterial community structure. The objectives of this study were to clarify: (1) the most abundant heavy metal in the cotton fields in Xinjiang, (2) the effects of BC and BF application on the content of Cd in cotton organs and the quality of Cd-polluted soil (soil available Cd content, soil bacterial community and diversity), and (3) the reasons for the difference in the effects of BC and BF on soil bacterial community structure based on the function prediction of dominant species. This study will provide a reference for the selection of soil heavy metal pollution remediation technologies and the improvement of arable soil quality in arid areas.

2 Materials and methods

2.1 Study site

Xinjiang is located in northwest China (34°25′–49°10′N, 73°40′–96°23′E), with an arid climate. The average annual precipitation was 150 mm, and the average annual temperature was 33°C. The favorable climate and advanced cotton planting technology have made Xinjiang the largest cotton production base in China. According to statistics, Xinjiang's cotton planting area accounted for 60%–70% of the total arable land in Xinjiang, and most cotton fields had been continuously cropped for 10–15 years. In this study, soil sampling was carried out in eight regions of Xinjiang, including Changji, Shihezi, Bole, Kuitun, Shawan, Korla, Aksu, and Kashgar, according to the ranking of cotton planting area and cotton yield (Zheng et al., 2018).

2.1.1 Soil sampling

Continuous cotton cropping is very common in Xinjiang. In this study, 60 cotton fields (>33.3 × 103 m^2) that had been continuously cropped for more than 10 years were selected

(Supplementary Figure S1) for soil sampling (0~20 cm soil layer) from September to October 2020. In each cotton field, five points were selected along the diagonals, and three soil samples were collected from each point. The soil samples of a cotton field were mixed and divided into three equal parts. Finally, a total of 180 soil samples were collected (1 kg per sample). After removing impurities such as stones and plant roots, the soil samples were brought back to the laboratory to be air-dried and sieved for the determination of soil heavy metal content.

2.1.2 Determination of soil physical and chemical properties and soil heavy metal content

The contents of As, Cd, Cr, Cu, Ni, and Pb in the soil samples were determined by graphite furnace atomic absorption spectrophotometer (Z2000, Hitachi, Tokyo, Japan), after digesting the samples in concentrated nitric acid, concentrated hydrochloric acid, and hydrofluoric acid (Zhu Y. et al., 2022).

The soil heavy metal survey results showed that soil Cd content exceeded the background value the most. To test the impacts of Cd, $CdCl_2{\cdot}5H_2O$ was mixed with soil to prepare soils with different Cd concentrations (1 (H1), 2 (H2), 4 (H3) $mg{\cdot}kg^{-1}$), and then outdoor pot (40 cm in height, 25 cm in diameter) experiment was conducted at the Experimental Station of Agricultural College, Shihezi University, Xinjiang (44°18′42.37″N, 86°03′20.72″E). H1, H2, and H3 were about 4, 8, and 16 times the average soil Cd content in Xinjiang. After 60 days, the following tests were carried out.

2.1.3 Materials

The preparation method of BC was as follows: Cotton straw was crushed in a muffle furnace under hypoxia condition at 450°C for 6 h. After cooling to room temperature in the muffle furnace, the straw were ground and sieved through a 0.15 mm sieve. The conversion rate from cotton straw to BC was 37.5%. The BF used in this study was purchased, and the physicochemical properties were determined according to the national standard of China (GB 20287-2006; Supplementary Table S1).

2.2 Experimental design

This experiment had 12 groups totally (Table 1), and each treatment had five replicates. Fifteen cotton seeds (variety Xinluzao 53, a widely cultivated variety in Xinjiang) were sown in a ceramic pot in April 2019/2020, and 5–6 plants were retained when the true leaves were fully unfolded. A total of 345 $kg{\cdot}hm^{-2}$ of urea (N), 555 $kg{\cdot}hm^{-2}$ of compound fertilizer (N-P_5O_2-K_2O, 17–17-17), and 4.8 $kg{\cdot}hm^{-2}$ of potassium polyacrylate (K_2O) were applied during the whole growth period. All phosphorus and potassium fertilizers and half of the urea were applied before sowing, and the rest urea was applied after the bud stage. After 120 days of culture, cotton organs (roots, stems, leaves, and buds/bolls) were collected, dried in an oven at 105°C, to determine dry matter yield and Cd content. At the same time, soil samples were collected from the pots using wooden shovel. The samples of each group were mixed. About 100 g was used for the determination of soil Cd content and available Cd content after air-drying and sieving, and the rest was used for bacterial diversity analysis. The sampling methods and tests were consistent in 2019 and 2020.

TABLE 1 Experimental design.

Treatments	Cd ($mg{\cdot}kg^{-1}$)	Biochar (%)	Biofertilizer (%)
H0T (Control)	0.25	0	0
H0B	0.25	3%	0
H0J	0.25	0	1.5%
H1T	1	0	0
H1B	1	3%	0
H1J	1	0	1.5%
H2T	2	0	0
H2B	2	3%	0
H2J	2	0	1.5%
H3T	4	0	0
H3B	4	3%	0
H3J	4	0	1.5%

T, no modifiers; B, 3% biochar was applied; J, 1.5% biofertilizer was applied; H0, no Cd; H1, 1 $mg{\cdot}kg^{-1}$ of Cd was applied; H2, 2 $mg{\cdot}kg^{-1}$ of Cd was applied; H3, 4 $mg{\cdot}kg^{-1}$ of Cd was applied.

The translocation factor (TF) and bioaccumulation coefficient (BCF) of Cd were used to characterize the migration and uptake of Cd in cotton, respectively (Amin et al., 2023).

$$TF = \frac{\text{Root / Stem / Leaf / Boll Cd content}}{\text{Soil / Root / Stem / Leave Cd content}}$$

$$\text{Bioaccumulation coefficient}\left(BCF\right) = \frac{\text{Cd content in a plant organ}}{\text{Cd content in soil}}$$

The available Cd content in soil and the Cd content in cotton organs are shown in Supplementary Tables S2, S3 (Yu et al., 2022).

2.3 Parameters and measurement methods

2.3.1 Determination of Cd content in cotton organs

The available Cd in the Cd-polluted soils was extracted by diethylenetriaminopentaacetic acid (DTPA; Zheng et al., 2018). To determine the Cd content in cotton roots, stems, leaves, and buds/bolls, 0.5 g of dried sample of each cotton organ was digested with the mixture of nitric acid and perchloric acid (2:1, v/v) under sealed condition, followed by the determination with a graphite furnace atomic absorption spectrophotometer (Z2000, Hitachi, Tokyo, Japan; Yang et al., 2016).

2.3.2 Determination of soil bacterial community diversity

Based on the 16S rRNA gene, high throughput sequencing was performed to determine soil bacterial diversity. The DNA extraction procedure was as follows: phosphoric acid buffer (pH: 8.0) and Tris(hydroxymethyl)methyl aminomethane (pH: 8.0) were mixed with soil sample (0.5 g). Then, the mixture was broken using a disruptor (Fastprep-24, United States), and centrifuged to obtain the

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TABLE 2 Descriptive statistics of soil heavy metal content in cotton filed in Xinjiang, China (mg·kg^{-1}).

Heavy metal Statistics analysis	As	Cd	Cr	Cu	Ni	Pb
Mean	6.64	0.24	37.60	19.24	13.80	15.20
Coefficient of variation	0.31	0.44	0.39	0.35	0.36	0.42
Minimum	1.34	0.01	11.77	8.95	3.46	5.99
Maximum	12.38	0.47	92.39	78.67	27.73	33.36
Standard deviation	2.09	0.10	14.64	12.45	4.92	6.42
Median	6.65	0.25	36.45	15.35	13.49	14.34
Soil background values in Xinjiang	11.20	0.12	49.30	26.70	26.60	19.40
Threshold values in the *Standard for Risk Control of Arable Soil Pollution* (GB 15618-2018)	25	0.60	250	100	60	170
Percentage of soil samples with heavy metal content exceeding the background value	3.33%	88.33%	23.33%	15.00%	1.67%	25.00%

supernatant. After that, the sample was extracted with reagents PCI (phenol: chloroform: isoamyl alcohol = 25: 24: 1) and CI (chloroform: isoamyl alcohol = 24: 1), followed by a centrifugation. The obtained precipitate was dissolved in Tris-EDTA buffer solution (pH: 8.0) to obtain the DNA solution.

The DNA solution was purified and sent to Shanghai Paysenno Co., Ltd. for high-throughput sequencing. The paired-end sequencing of bacterial DNA fragments was carried out on the illumina Mi Seq 300PE platform, and the obtained sequences were subjected to de-priming, filtering, denoising, splicing, and dechimerism using the DA-DA2 method. The sequences obtained were the representative sequences of operational taxonomic units (OTUs). Subsequently, sequence analysis was conducted using the QIIME2 software package, specifically employing the qiime dada2 denoise-single, qiime feature-table summarize, and qiime feature-table tabulate-seqs tools. Finally, the diversity indices (Chao1, Coverage, Simpson, and Shannon indices) of soil bacteria were calculated by IIME 2 software (Puga et al., 2015; Kiran and Prasad, 2019). Fisher's exact test was carried out to detect species with abundance difference between groups, and hypothesis testing was carried out to evaluate the significance of observed differences (Romanowski et al., 2023). Bacterial diversity analysis was performed under the soil Cd content 4 mg·kg^{-1} (H0T, H3T, H3B, and H3J).

2.4 Data analysis

Before data analysis, normal distribution was tested using the Kolmogorov–Smirnov (K-S) test using SPSS 20.0 software (SPSS Inc., Chicago, United States). Data were expressed as mean ± standard error. Duncan test was performed to test the significance of differences in bioaccumulation coefficients and translocation factor between groups using SPSS 20.0 software (SPSS Inc., Chicago, United States; $p < 0.05$). The maximum, minimum, average, standard error, median, and variability of each element content in the soil samples were statistically analyzed using Excel software version 2016 and SPSS software version 23.0. Figures were drawn using Origin software version 8.0 (Origin Lab, Massachusetts, United States), and layout was completed using Adobe Illustrator CS6 (Adobe, United States).

3 Results

3.1 The contents of soil heavy metals in cotton fields in Xinjiang

The Cd content in soil was twice the background value in Xinjiang. Among the heavy metals, Cd had the highest coefficient of variation (43.72%), followed by Pb (42.34%), Cr (38.95%), Ni (35.65%), Cu (35.05%), and As (31.27%; Table 2).

3.2 The translocation factor and bioaccumulation coefficient of Cd

During the two-year culture period, with the increase of exogenous Cd concentration, the Cd content in cotton roots, stems, leaves, and buds also increased. For example, the content of Cd in cotton roots in the H1T, H2T, and H3T groups increased by 11.01% ($p > 0.05$), 29.46% ($p < 0.05$), and 45.24% ($p < 0.05$), respectively compared with that in the H0T group. The BC treatment reduced the Cd content in cotton organs. For example, the content of Cd in cotton roots in the H0B, H1B, H2B, and H3B groups decreased by 20.54% ($p < 0.05$), 12.87% ($p > 0.05$), 17.47% ($p > 0.05$), and 18.24% ($p < 0.05$), respectively compared with the H0T, H1T, H2T, and H3T group. The BF treatment also reduced the Cd content in various organs of cotton. For example, the Cd content in cotton roots in the H0J, H1J, H2J, and H3J groups decreased by 23.81% ($p < 0.05$), 12.60% ($p > 0.05$), 15.86% ($p > 0.05$), and 17.01% ($p < 0.05$), respectively compared with the H0T, H1T, H2T, and H3T group (Supplementary Tables S2, S3). The BCF of Cd of cotton roots was higher than that in other organs in all groups, but the BC and BF treatments reduced the BCF of each organ. In 2019 (2020), the BCF of

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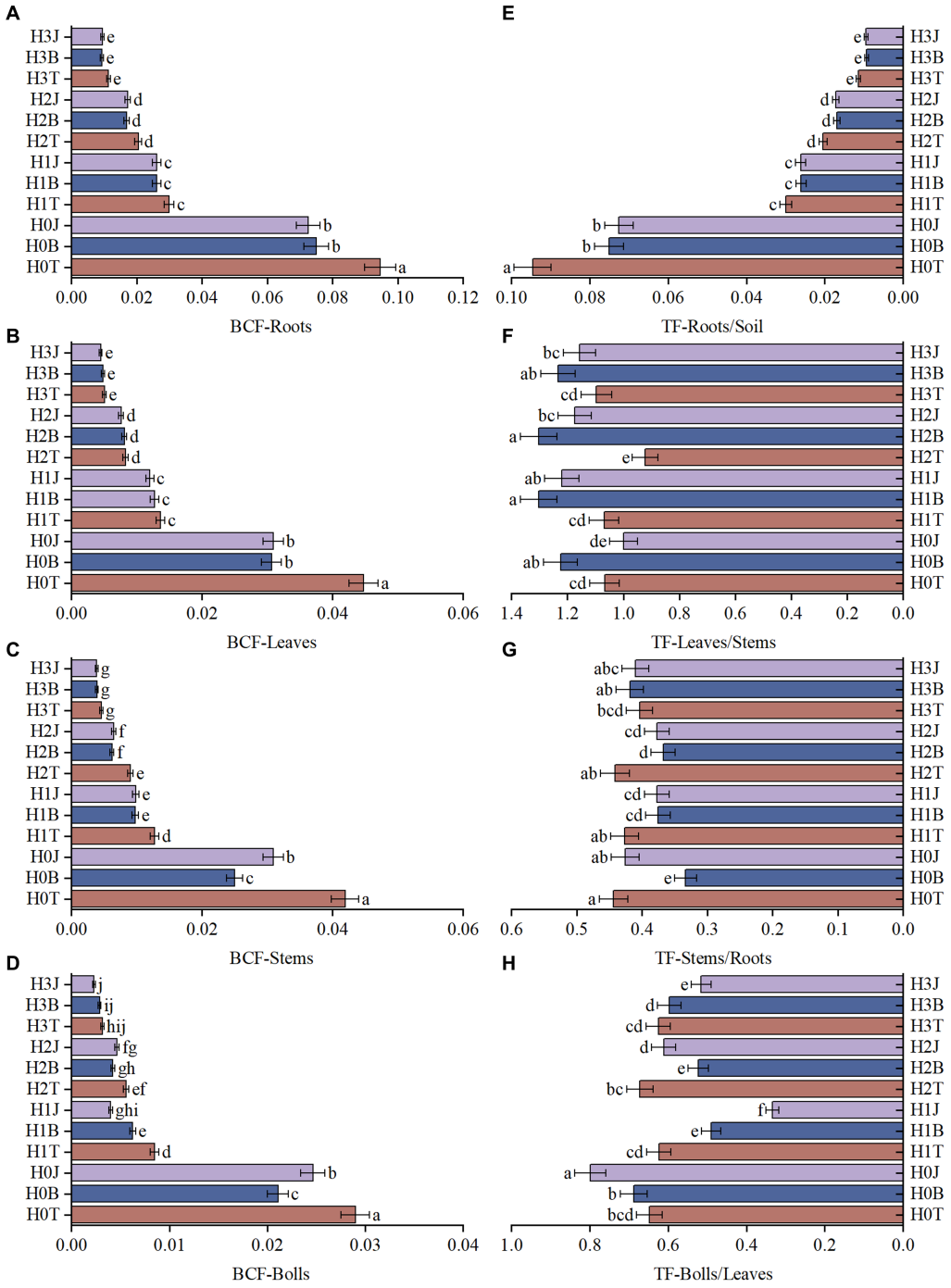

FIGURE 1
Bioaccumulation coefcients (BCFs) and Translocation factor (TF) of Cd in cotton organs (2019). **(A)** BCF-Roots; **(B)** BCF-Leaves; **(C)** BCF-Stems; **(D)** BCF-Bolls; **(E)** TF-Roots/Soil; **(F)** TF-Leaves/Stems; **(G)** TF-Stems/Roots; **(H)** TF-Bolls/Leaves. Diferent lowercase letters indicae signifcant diference between groups at $p < 0.05$. T, no modifiers; B, 3% biochar was applied; J, 1.5% biofertilizer was applied; H0, no Cd; H1, 1 mg·kg^{-1} of Cd was applied; H2, 2 mg·kg^{-1} of Cd was applied; H3, 4 mg·kg^{-1} of Cd was applied. The same below.

roots, leaves, stems, and buds/bolls in the H0B group reduced by 20.64% (14.28%), 31.53% (30.39%), 40.34% (10.51%), and 27.28% (25.09%), respectively ($p < 0.05$) compared with those in the H0T group (Figures 1A–D). The BCF of roots, leaves, stems, and buds/bolls in the H0J group reduced by 23.27% (14.72%), 30.96% (29.81%), 26.33% (17.61%), and 14.94% (26.27%), respectively ($p < 0.05$), compared with those in the H0T group (Supplementary Figures S2A,B).

The BC and BF treatments reduced the transport of soil Cd to cotton roots. In 2019 and 2020, the TF-Roots/Soil in the H0B group reduced by 20.64 and 14.28%, respectively ($p < 0.05$), and the TF-Roots/Soil in the H0J group reduced by 23.27 and 14.71%, respectively ($p < 0.05$), compared with that in the H0T group. However, the BC and BF treatments increased the transport of stem Cd to cotton leaves. In 2019 and 2020, the TF-Leaves/Stems in the H1B group reduced by 21.89 and 14.19%,

respectively ($p < 0.05$; Figures 1E–H), and the TF-Leaves/Stems in the H1J group reduced by 14.06 and 14.32%, respectively ($p < 0.05$), compared with that in the H1T group (Supplementary Figures S2E–H).

3.3 Soil bacterial α-diversity and β-diversity

In 2019 (Figure 2), the Shannon index in the H3T group reduced by 4.84% compared with that in the H0T group ($p > 0.05$). The analysis of coverage index showed that the sequencing coverage of each sample was above 97.97%, reflecting the reliability of the sequencing results. The Simpson's diversity index in the H3B group increased by 15.38% ($p < 0.05$), the Chao 1 index reduced by 3.71% ($p < 0.05$), and the Shannon index increased by 8.44% ($p > 0.05$), compared with those in the H3T group. The Simpson's diversity index in the H3J group increased by 303.9% ($p < 0.05$), the Chao 1 index reduced by 3.73% ($p < 0.05$), and the Shannon index increased by 50.41% ($p > 0.05$), compared with those in the H3T group.

In 2020 (Figure 3), there was no significant difference in the Chao 1 and coverage indices between groups. The Simpson index in the H3T group reduced by 50.91% ($p < 0.05$) compared with that in the H0T group. The Shannon index in the H3B group reduced by 12.18%, and the Simpson index in the H3B and H3J group increased by 298.84% and 734.52%, respectively ($p < 0.05$), compared with those in the H3T group.

The soil microbial β-diversity was similar in 2019 and 2020 (Supplementary Figure S3). The distribution of the β-diversity in the H3B and H3J groups was close in Supplementary Figures S3A,B, indicating that the effects of BC and BF treatments on soil microbial β-diversity were similar. However, the distribution of the soil microbial β-diversity in the H0T and H3T groups were far away, indicating that the addition of Cd greatly affected the soil bacterial community structure.

3.4 Changes in relative abundance of soil bacteria at the class level

The Cd, BC, and BF treatments had significant effects on the relative abundance of soil bacteria in 2019 and 2020. In 2019, *Alphaproteobacteria* (5.14%–18.41%), *Gammaproteobacteria* (2.24–27.50%), *Subgroup_6* (4.48%–15.25%), *Blastocatellia_Subgroup_4*

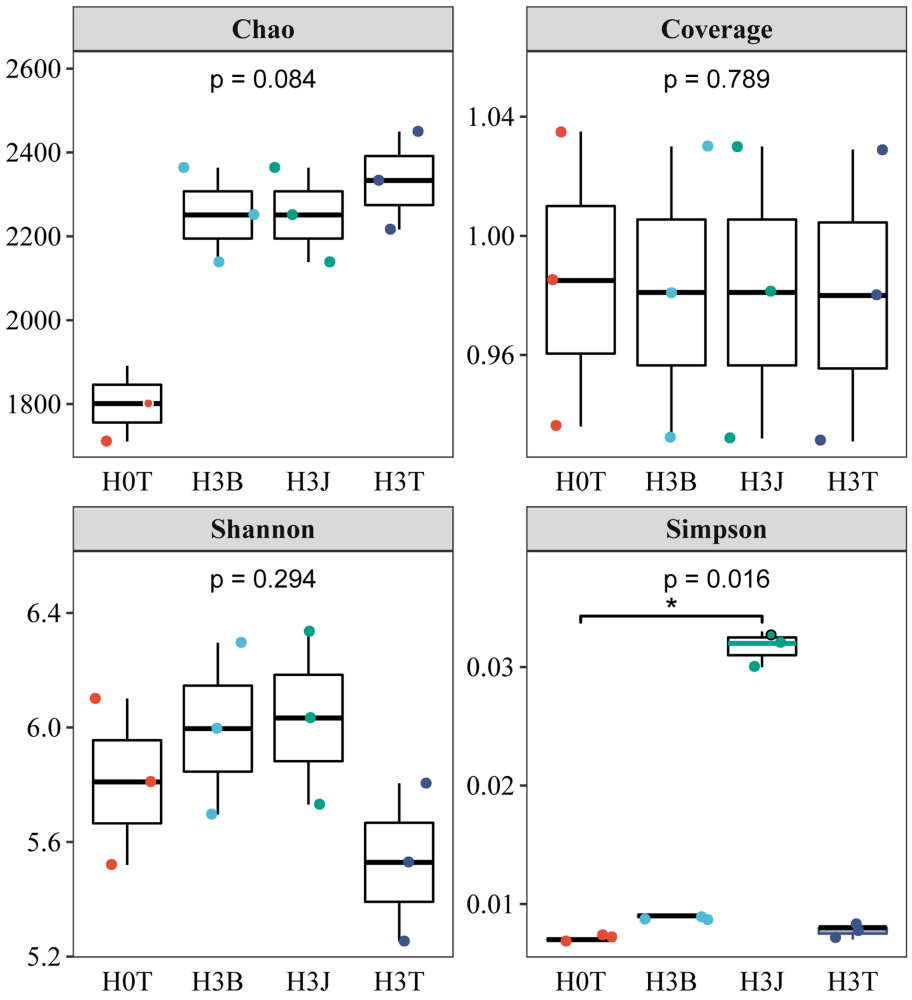

FIGURE 2
Effect of biochar (B) and biofertilizer (J) on microbial α-diversity in Cd contaminated soil (2019).

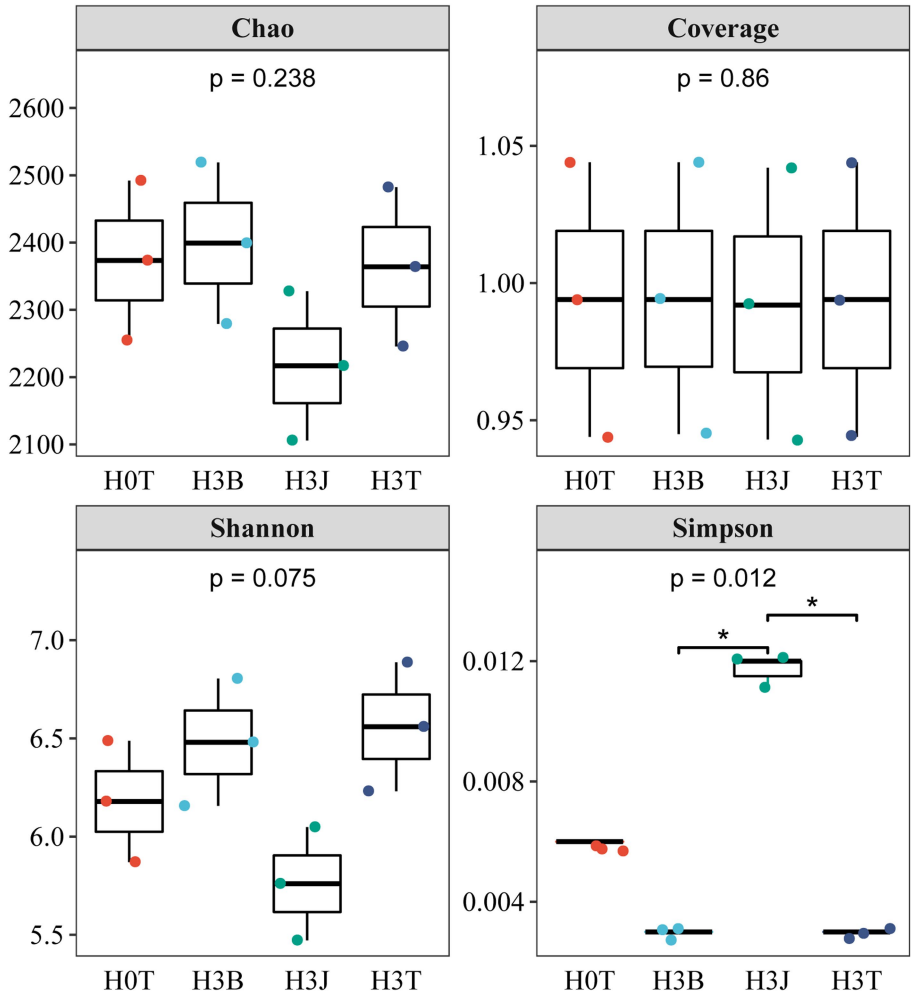

FIGURE 3
Effects of biochar (B) and biofertilizer (J) on microbial α-diversity in Cd contaminated soil (2020).

(5.90%–15.23%), *Gemmatimonadetes* (4.44%–12.07%), *Bacteroidia* (1.08%–7.70%), *Actinobacteria* (2.46%–9.13%), *Anaerolineae* (2.16%–8.99%), and *Chloroflexia* (1.33%–10.81%) were the dominant bacteria in the groups. The ternary phase diagram showed that the soil bacterial diversity varied among different samples (Figures 4A,B). *Actinobacteria* (9.13%) and *Gemmatimonadetes* (5.86%) were the dominant bacteria in the H3T group, and *Blastocatellia_Subgroup_4* (15.23% and 12.94%) and *Gemmatimonadetes* (8.88 and 12.07%) were the dominant bacteria in the H3B and H3J groups (Figure 4A). Besides, it was found that the relative abundance of *Blastocatellia_ Subgroup_4* and *Gemmatimonadetes* in the H3B group increased by 9.33 and 3.03%, respectively, while that of *Alphaproteobacteria* and *Gammaproteobacteria* reduced by 2.83% and 17.42%, respectively, compared with those in the H3T group. The relative abundance of *Blastocatellia_ Subgroup_ 4* and *Gemmatimonadetes* in the H3J group increased by 7.04% and 6.22%, respectively, while that of *Alphaproteobacteria* and *Gammaproteobacteria* decreased by 5.44% and 17.18%, respectively, compared with those in the H3T group. The relative abundance of *Gemmatimonadetes* in the H3J group increased by 3.19% compared with that in the H3B group (Figure 4C).

In 2020, *Alphaproteobacteria* (13.42%–15.93%), *Actinobacteria* (8.36%–16.88%), *Bacilli* (3.92%–17.97%), *Vicinamibacteria* (3.26%–12.54%), *Gammaproteobacteria* (6.18%–11.73%), *Chloroflexia* (6.08%–8.80%), *Thermoleophilia* (2.11%–5.98%), *Blastocatellia* (1.86%–5.70%), and *Bacteroidia* (2.57%–5.51%) were the dominant bacteria in the groups. The ternary phase diagram (Figure 4B) showed that *Vicinamibacteria*, *Thermoleophilia*, and *Bacilli* were the dominant bacteria in the H3T, H3J, and H3B group, respectively. The relative abundance of *Alphaproteobacteria*, *Actinobacteria*, and *Bacilli* in the H3B group increased by 2.51%, 3.74%, and 11.85%, respectively, while that of *Vicinamibacteria* decreased by 9.27%, compared with those in the H3T group. The relative abundance of *Alphaproteobacteria*, *Actinobacteria*, and *Chloroflexia* in the H3J group increased by 2.14%, 3.49%, and 2.72%, respectively, while that of *Bacilli* and *Gammaproteobacteria* decreased by 2.19% and 0.86%, respectively, compared with those in the H3T group. Besides, the relative abundance of *Vicinamibacteria* in the H3J group increased by 7.21% compared with that in the H3B group (Figure 4D).

In 2019, *Alphaproteobacteria* and *Gemmatimonadetes* were the differential bacteria for the H3J group *vs* H3B group (Supplementary Figure S4A). In 2020, *Bacilli* and *Vicinamibacteria*

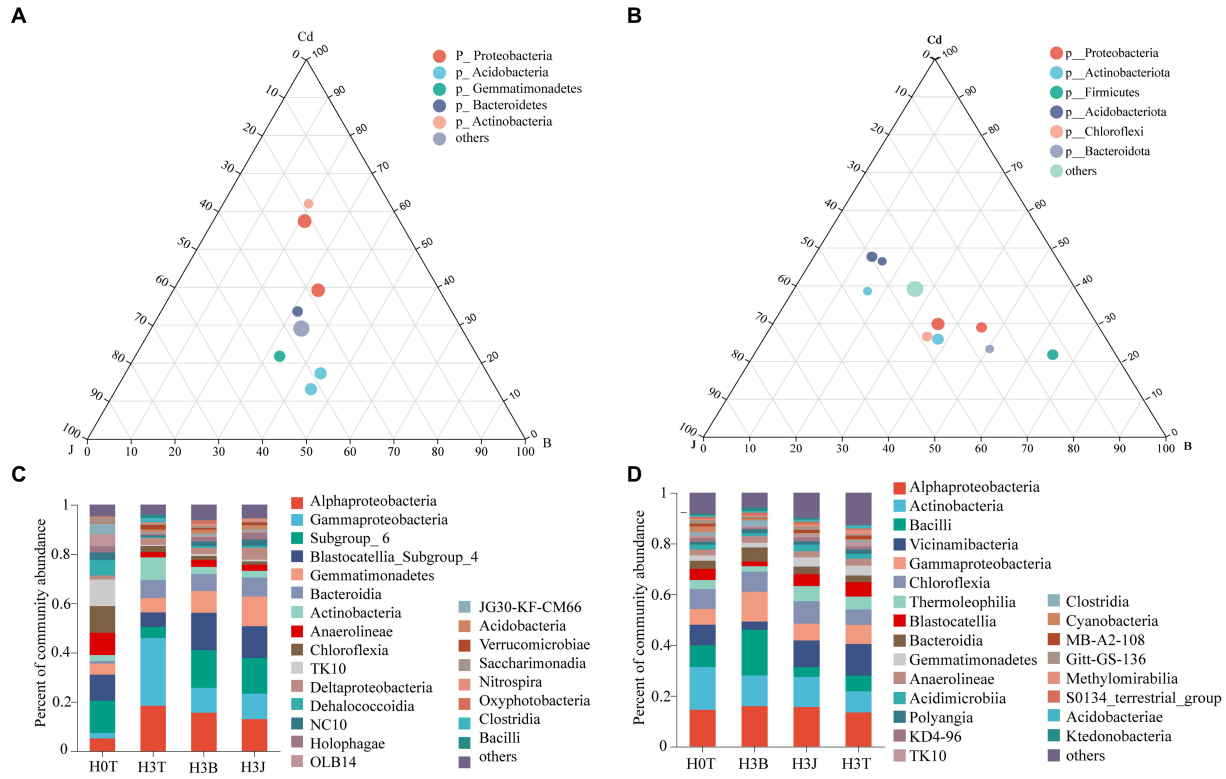

FIGURE 4
Efects of biochar [B] and biofertilizer [J] on bacterial diversity in Cd contaminated soil (2019 and 2020). **(A)** Ternary phase diagram of 2019; **(B)** Ternary phase diagram of 2020; **(C)** Percent of community abundance in 2019; **(D)** Percent of community abundance in 2020.

were the differential bacteria for the H3J group *vs* H3B group (Supplementary Figure S4B).

3.5 Prediction of soil bacterial functions

In 2019 (Figure 5A), the Energy production and metabolism, Amino acid transport and metabolism, and General function prediction only were the main soil bacterial functions, and the Amino acid transport and metabolism and General function prediction only in the H3T group were enhanced compared with those in the H0T group, indicating that the addition of exogenous Cd led to the enhancement of the two functions. Besides, the Replication, recombination and repair was also enhanced in the H3B and H3J group compared with that in the H3T group. Similar results were obtained in 2020 (Figure 5B).

3.6 Correlation analysis between dominant species and soil/cotton Cd content

The heatmap showed that TF-Leaves/Stems was positively correlated with the abundance of *Gammaproteobacteria*, *Chloroflexia*, *Anaerolineae*, *Actinobacteria*, *Gemmatimonadetes*, *Bacilli*, and *Bacteroidia* ($p < 0.01$; Figure 6A). TF-Bolls/Leaves was positively correlated with Chao 1, Shannon, and Coverage index ($p < 0.01$), but negatively correlated with Simpson index ($p < 0.01$). Chao 1 index was positively correlated with Cd-Stems, Cd-Leaves, and Cd-Bolls ($p < 0.01$), and Simpson index was negatively correlated with Cd-Bolls, BCF-Roots, BCF-Bolls, TF-Roots/Soil, and TF-Bolls/Leaves ($p < 0.01$; Figure 6A). The bacterial community structure in the H3T, H3B, and H3J groups were similar, and the effects of H3T, H3B, and H3J treatments on BCF-Bolls, BCF-Leaves, BCF-Stems, BCF-Roots, and TF-Roots/Soil were similar (Figure 6B).

4 Discussion

In 2020, the average contents of Pb, Cd, Hg, and Cu in Xinjiang's cotton fields were 1.04, 5.73, 2.22, and 1.14 times the threshold values in the *Standard for Risk Control of Arable Soil Pollution* (GB 15618-2018), respectively, while the average contents of Cr, As, and Ni were lower than the threshold values. Previous study has found that Pb, Cd, As, and Cu pollution hotspots are widely distributed in southwest China, the North China Plain, the Yangtze River Basin, the Yangtze River Delta, and the Pearl River Delta (Shi et al., 2023). Besides, a survey of 341 arable soil samples in Shaanxi Province in northwest China found that both Cd and Pb accumulated in large quantities in the soil, and the contents of Cd, Hg, and Zn increased with the increase of cropping years (Jing et al., 2023). This study results suggest that the heavy metal pollution of arable soil in the study area deserves attentions. Among heavy metal polluted cotton fields (with heavy metal content exceeding the background value in Xinjiang), Cd polluted cotton fields had the highest proportion (88.33%; Table 2). In terms of the coefficient of variation, the variations of soil As, Cd, Cr, Cu, Ni, and Pb were all medium, among which, the coefficient of

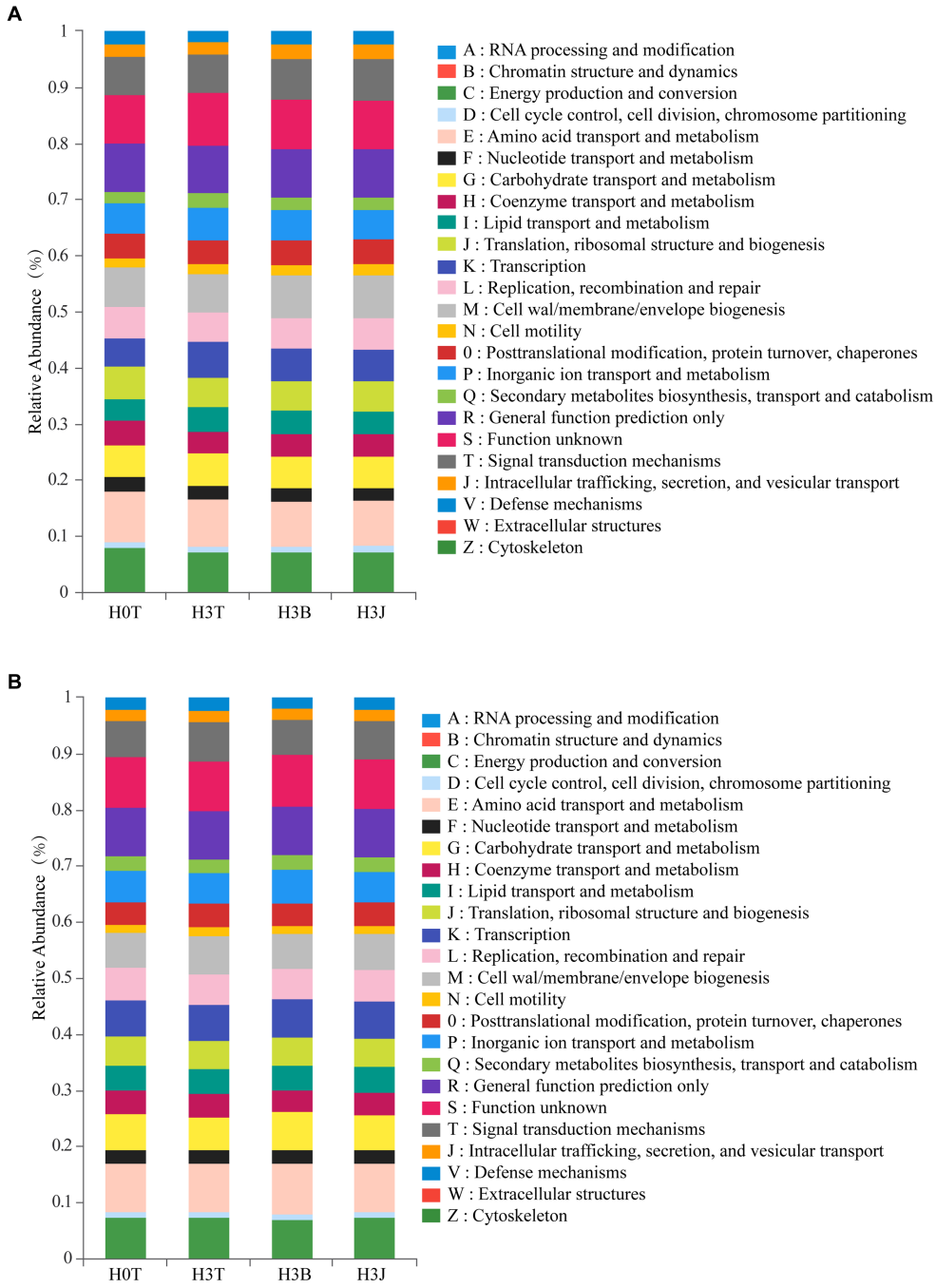

FIGURE 5
Prediction of soil bacterial functions based on PICRUSt2 in 2019 **(A)** and 2020 **(B)**.

variation of Cd and Pb were greater than that of other heavy metals. In addition, the dispersion degree of Cd and Pb was high (Table 2), indicating that their contents varied greatly in different cotton fields. This also indicates that the heavy metal content in the study area is affected by random factors such as industrial activities, excessive application of fertilizers, and agricultural irrigation, and Cd is the most abundant heavy metal in the cotton fields of Xinjiang (Table 2; Huang et al., 2021; Yuan et al., 2021).

The TF and BCF are the two main parameters for evaluating the uptake and accumulation of heavy metals in plants. If TF and BCF are greater than 1, it indicates the uptake of heavy metals by plants; If TF and BCF are less than 1, it indicates the exclusion of heavy metals (Ahmad et al., 2017). In this study, the TF and BCF of Cd in cotton roots, stem, leaves, and buds/bolls were less than 1, and the TF and BCF of Cd in roots were greater than those in other organs (Figure 1; Supplementary Figure S2). However, Li et al. (2012) reported that the BCF of the vegetative organs, aerial parts, and whole plants of three cotton varieties were greater than 1. This difference may be due to: 1) differences in cotton varieties lead to different enrichment and transport of Cd in cotton organs; and 2) differences in soil pH lead to

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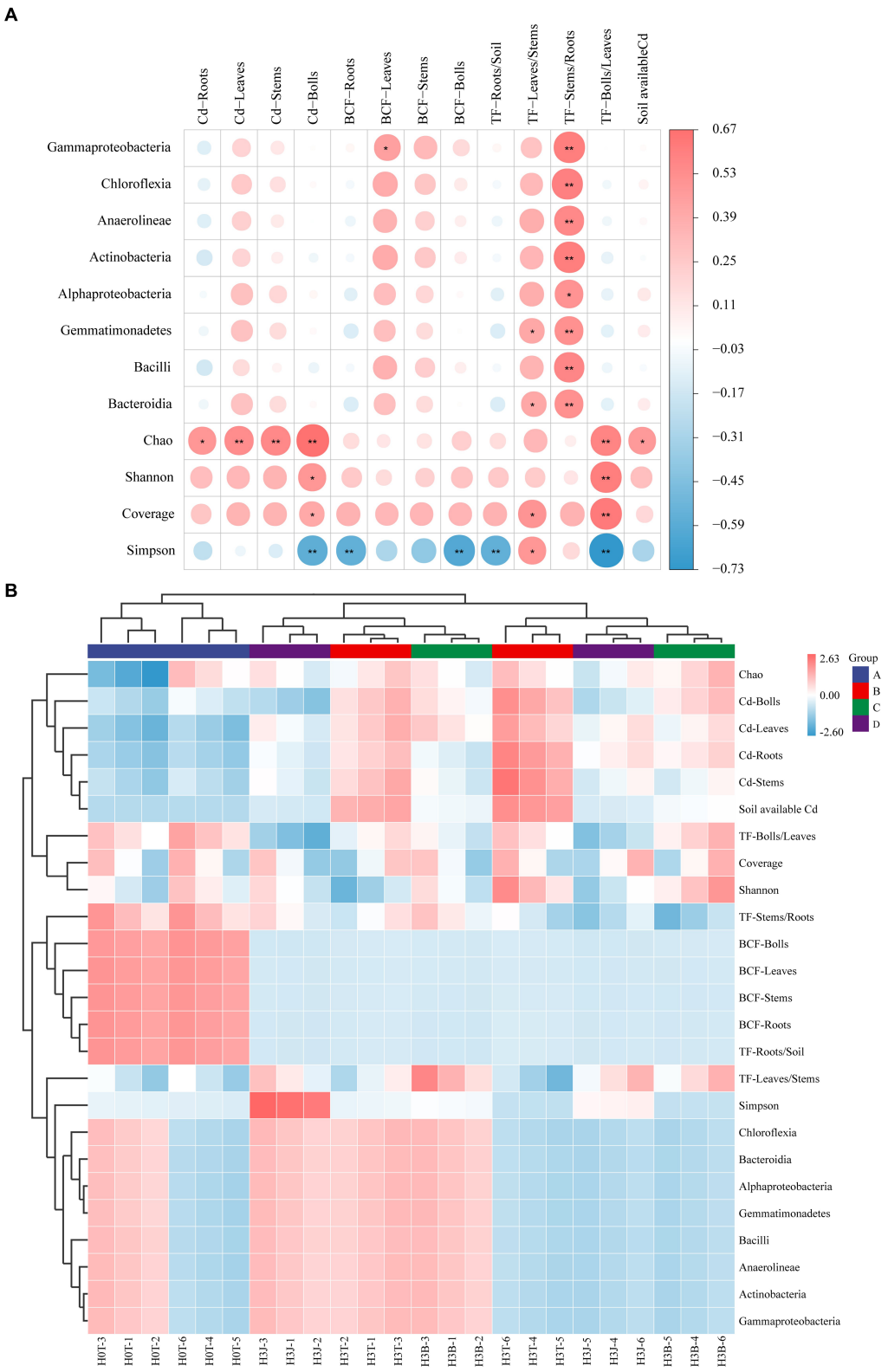

FIGURE 6
Correlation analysis between soil/cotton Cd enrichment and transport and soil bacterial community. **(A)** Correlation analysis; **(B)** Heatmap analysis.

different bioavailability and migration of Cd in soil (the soil is weakly alkaline in this study, while the soil is acidic in the study of Li et al. (2012) and Nawab et al. (2016). It has been reported that the TFs and BCFs of heavy metals of corn grains are different under the treatments of biochar derived from different raw materials (For BCF, *Ganoderma lucidum* substrate derived biochar treatment (0.0651) < mushroom

substrate derived biochar treatment (0.0817) < *Hericium* substrate derived biochar treatment (0.0742); For TF, mushroom substrate derived biochar treatment (0.204) < *Ganoderma lucidum* substrate derived biochar treatment (0.211) < *Hericium* substrate derived biochar treatment (0.222; Li et al., 2012). In this study, the application of BC and BF increased the transport of Cd from stems to leaves at different Cd levels. This indicates that in addition to cotton roots, cotton leaves also have strong Cd enrichment and transport capacities, which may be related to the bioavailability of Cd in the soil and soil physicochemical properties (Noli and Tsamos, 2016). Studies have shown that the content of Cd in wheat grains is significantly reduced by 26.13%–46.43% compared with the control after the application of 1.25% rice straw biochar, vegetable peel biochar, corn straw biochar, and rice husk biochar, and the TF and BCF of Cd are also significantly reduced (Song J. W. et al., 2022; Amin et al., 2023; Sun T. et al., 2023). In this study, the application of BC and BF significantly reduced the content of available Cd in soil (Supplementary Table S2), the absorption of Cd by various organs of cotton (Supplementary Table S3), the BCF of Cd in cotton (Figures 1A–D; Supplementary Figures S2A,B), and increased the transport of Cd from roots to stems (1, 2, 4 $mg \cdot kg^{-1}$ Cd levels). The difference in TF may be due to the different raw materials of biochar, the different heavy metal absorption capacity and the different heavy metal tolerance in different crops (Amin et al., 2023).

The application of heavy metal-tolerant bacteria and biofertilizer can reduce the bioavailability of soil heavy metals and reduce the absorption of heavy metals by plants. Abdelkrim et al. (2020) showed that the total heavy metal content of the soil treated with heavy metal-tolerant bacteria (PGPR) was less than that of the soil without PGPR. These scholars provided the following reasons: (1) the PGPR converted Cd and Pb into bioavailable forms in the rhizosphere, which enhanced the absorption of heavy metals by alfalfa. (2) The PGPR released degrading enzymes, organic acids, and metal chelates (such as siderophores) into the rhizosphere, enhancing heavy metal uptake and accumulation in alfalfa roots (Abdelkrim et al., 2020). This study obtained similar results, that is, the application of BF reduced the content of available Cd in soil and the content of Cd in cotton organs (Figure 1; Supplementary Figure S2; Supplementary Tables S2, S3). This may be due to the abundant organic matter in BF, as well as differences in bacterial species and their tolerances (Sahar et al., 2019). In this study, the effects of BC and BF on soil available Cd content and cotton Cd enrichment and transport were inconsistent (Figure 1; Supplementary Figure S2; Supplementary Tables S2, S3), and the performance of BF in reducing cotton BCF was superior to that of BC (Figures 1A,B). This may be related to the differences in soil bacterial community composition and metabolites caused by the application of BC and BF and the differences in the physical and chemical properties of BC and BF (Ma et al., 2022; Zhu Y. Q. et al., 2022). In addition, the application of BC and BF increased the abundance of Amino acid transport and metabolism and Replication, recombination and repair metabolism-related bacteria in the soil. This promotes cotton growth and improves cotton resistance to heavy metal stress (Hasnain et al., 2023).

Bacteria have mechanisms that resist heavy metal stresses, such as biological detoxification, efflux, and cellular resistance to oxidative stress. Besides, bacteria also play a key role in the redox reactions, methylation, and demethylation of heavy metals, and the formation of organometallic complexes (Kou et al., 2023). Wang Z. et al. (2020) found that *Actinobacteria* and *Chloroflexia* were the dominant species in Cd-polluted soils, and soil Cd and Pb contents were positively correlated with the abundance of *Actinomarinales* ($p < 0.001$), *Pedomicrobium* ($p < 0.05$), *Xanthobacteraceae* ($p < 0.001$), and *Alphaproteobacteria* ($p < 0.001$; Kou et al., 2023). Sun Y. et al. (2023) reported that after applying rice husk powder derived biochar into Cd-polluted soil, the relative abundance of *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Actinobacteria*, *Planctomycetes* and *Chloroflexi* were increased by 28.69%–33.36%, 17.94%–20.19%, 7.10%–9.09%, 9.06%–11.69%, 5.02%–6.98%, 3.32%–6.14% and 2.69%–5.28%. Jin et al. (2021) reported that *Proteobacteria*, *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Planctomycetes* were the dominant species in the soil after the application of biofertilizer. It can be seen that the changes in soil microbial community structure in previous studies are different from those in this study. This may be due to differences in soil pH, biochar and biofertilizer dosages, and agricultural managements (such as fertilizer application rates and irrigation volumes; Sun T. et al., 2023). In this study, in the 2 years, *Alphaproteobacteria*, *Gammaproteobacteria*, *Blastocatellia*, and *Gemmatimonadetes* were the dominant species under BC and BF treatments (Figure 4). This indicates that the above taxa have a high resistance to Cd stress. Study has shown that long-term heavy metal pollution leads to changes in soil microbial community structure and increases in the relative abundance of heavy metal-tolerant microorganisms and soil microbial diversity (Han et al., 2020). This may be due to that heavy metal-resistant microorganisms have multiple heavy metal oxidase genes involved in heavy metal fixation and resistance. These microorganisms participate in nitrogen nitrification and denitrification, which provides nitrogen for plants and improves the living environment of microorganisms (Han et al., 2020; Cheng et al., 2023). In addition, the pore-rich structure and abundant carbon and nitrogen of BC provide favorable conditions for the growth and reproduction of bacteria, and the large number of bacteria and rich nutrients in BF can increase the diversity of soil bacteria that fix and adsorb soil heavy metals (Figures 2, 3; Ahamad et al., 2023; Wei et al., 2023; Yu et al., 2023). It was also found that there was a positive correlation between soil dominant bacteria and TF-Stems/Roots ($p < 0.05$). This indicates that the dominant species under BC and BF treatments promote the transport of Cd from cotton roots to stems (Figure 6A).

This study found that *Alphaproteobacteria*, *Gemmatimonadetes*, *Bacilli*, and *Vicinamibacteria* were differential species in H3B *vs* H3T. Many studies have shown that *Alphaproteobacteria*, *Gemmatimonadetes*, and *Bacilli* are the dominant species under heavy metal stress conditions. These species play an important role in resisting exogenous Cd stress and reducing the toxicity of heavy metals to plants (Czarny et al., 2020; Qi et al., 2022; Sun et al., 2022). *Gemmatimonadetes* can indicate heavy metal pollution in arable soil as their relative abundance increases significantly with the increase of pollutant concentration in the soil (Niepceron et al., 2013). *Vicinamibacteria* abundance is negatively correlated with soil available Cd content and positively correlated with soil total phosphorus content. The anionic groups on *Vicinamibacteria* cell wall such as hydroxyl, carboxyl, amino, and amide groups can bind with heavy metals to reduce their bioavailability (Yu et al., 2023). This study found that BC and BF treatments had a certain effect on the diversity, relative

abundance, and function of soil bacterial communities, which ultimately improved the quality of Cd-polluted soil. This further confirms that the diversity, function, and relative abundance of soil bacteria could be used as evaluation indicators for soil quality (Tang et al., 2019). It was worth noting that BF treatment had a better effect on reducing the enrichment of Cd in cotton than BC treatment.

5 Conclusion

In this study, Cd was the most abundant heavy metal in the cotton fields of Xinjiang. Both biochar and biofertilizer could reduce the transport of Cd from soil to roots and the enrichment of Cd in cotton organs. However, biofertilizer was superior to biochar in reducing Cd transport and enrichment. The dominant bacteria in soil (*Gammaproteobacteria*, *Chloroflexia*, *Anaerolineae*, *Actinobacteria*, *Alphaproteobacteria*, *Gemmatimonadetes*, *Bacilli*, and *Bacteroidia*) were significantly associated with the transport of Cd from cotton roots to stems under biochar and biofertilizer treatments. Besides, biochar and biofertilizer treatments increased soil bacterial diversity, and reduced the transport of Cd from soil to the aboveground organs of cotton. Biochar treatment mainly increased the relative abundance of *Gemmatimonadetes*, which in turn reduced the bioavailability of soil Cd and the enrichment of Cd in various organs of cotton. Biofertilizer treatment mainly regulated the abundance of *Alphaproteobacteria*, *Gemmatimonadetes*, *Bacilli*, and *Vicinamibacteria* to enhance the metabolic function Replication, recombination and repair, to reduce the enrichment of Cd in cotton. This study deepens our understanding of the remediation of soil Cd pollution by BC and BF from the perspective of soil microbial community, and provides a reference for the selection of soil heavy metal pollution remediation technique in Xinjiang, China.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

YZ: Conceptualization, Data curation, Formal asnalysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. MA: Writing – review & editing. TA: Writing – review & editing. HW: Resources, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1288526/full#supplementary-material

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The accumulation of active ingredients of *Polygonatum cyrtonema* Hua is associated with soil characteristics and bacterial community

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Introduction: With the increasing demand for health products derived from Polygonati rhizoma (PR), people begin to artificially plant *Polygonatum cyrtonema* Hua (*P. cyrtonema*) in the middle and lower reaches of the Yangtze River. To promote *P. cyrtonema* cultivation and increase farmers' income, efforts are needed to understand the ways to obtain high-quality PR under artificial cultivation conditions.

Methods: Rhizomes of artificial planting *P. cyrtonema* and rhizosphere soils were collected across five regions in Zhejiang Province, China. Subsequently, the contents of the main active ingredients of *P. cyrtonema* and soil properties were analyzed, and both rhizosphere and endophytic bacteria of *P. cyrtonema* were detected by 16S rDNA sequencing. The relationship between the active ingredients and soil properties, and the dominant bacteria were investigated by correlation analysis.

Results: The content of active ingredients of *P. cyrtonema* from the five regions varied significantly, especially polysaccharides and saponins. High-throughput sequencing demonstrated that Proteobacteria was the dominant bacterial phylum in all samples, and *Burkholderia-Caballeronia-Paraburkholderia* was the main endophytic bacterial genus in rhizome. In addition, the bacterial diversity and richness of rhizosphere soil samples were higher than those of rhizome samples. Soil physicochemical properties and enzyme activities were significantly different across regions, leading to notable variations in the community structures of rhizosphere and endophytic bacteria. Redundancy analysis (RDA) displayed that pH and urease (UE) were the major factors altering shifting rhizosphere bacteria community structure. Moreover, the composition and diversity of rhizome endophytic bacteria were principally affected by both soil physicochemical properties and soil enzyme activities. Soil properties and bacteria from rhizosphere soil and rhizome had a considerable impact on certain active ingredients in *P. cyrtonema* under artificial cultivation conditions after Pearson correlation analysis. Polysaccharides were significantly correlated with nutrient-rich soil and endophytic bacteria, such as *Burkholderia-Caballeronia-Paraburkholderia*, *Pseudomonas*, *Ralstonia*, and *Bacillus*. However, flavonoids were associated with nutrient-poor soil. Saponins were positively correlated with OM and available phosphorous (AP) and were significantly negatively affected by rhizosphere bacterial communities.

Conclusion: The study demonstrated that bacterial microorganisms were involved in the accumulation of active ingredients of *P. cyrtonema* together with soil physicochemical properties and enzyme activities, which provided a theoretical basis for the scientific and effective artificial cultivation of high-quality *P. cyrtonema*.

KEYWORDS

active ingredient, soil physicochemical properties, soil enzyme activities, bacterial community, correlation analysis, *Polygonatum cyrtonema* Hua

1 Introduction

Polygonati rhizoma (PR), a traditional homology of medicine and food in China, is the rhizome of several perennial *Polygonatum* species in the family of *Liliaceae*. There are more than 60 species globally, mainly distributed among the north temperate zone and the north subtropical zone (Tian and Zhao, 2007). Only *Polygonatum sibiricum* Red., *Polygonatum cyrtonema* Hua, and *Polygonatum kingianum* Coll. et Hemsl., were introduced in Chinese State Pharmacopeia (2020). Among these species, *P. cyrtonema* is mainly distributed in the middle and lower reaches of the Yangtze River, including Zhejiang, Anhui, and Jiangxi Provinces. The main chemical components of *P. cyrtonema* include polysaccharides, flavonoids, steroidal saponins, lignans, alkaloids, and anthraquinones (Zhang et al., 2019), among which the first three are the main active ingredients (Chen et al., 2019). Modern pharmacological research demonstrated *P. cyrtonema* had a variety of physiological functions, including anti-tumor, anti-bacterial, hypoglycemic, hypolipidemic, anti-aging, antioxidant, immunomodulatory, and other physiological activities (Yu et al., 2008; Liu J. Y. et al., 2019; Chen et al., 2021; Wang F. F. et al., 2022; Wang Q. L. et al., 2022; Zhang et al., 2023). With a further understanding of the pharmacological effects of *P. cyrtonema* and the improvement of healthcare awareness, the demand for health products derived from *P. cyrtonema* is increasing. Recently, the market gap of *P. cyrtonema* has become increasingly prominent, and the price of *P. cyrtonema* medicinal materials has risen from 2.51 dollars·kg^{-1} (2010) to 8.37–9.77 dollars·kg^{-1}, resulting in a rapid decrease in wild *P. cyrtonema* resources. As a result, people begin to artificially plant *P. cyrtonema* to meet market demand. Discovering ways to obtain high-quality *P. cyrtonema* under artificial cultivation conditions will promote the sustainable development of the *P. cyrtonema* essence industry.

Soil is a site where plant rhizosphere bacteria can settle and exchange material energy. Plants interact with the soil and its microorganisms through rhizosphere at all stages of growth, thereby altering the soil's physicochemical composition and enzyme activity (Wu and Liu, 2022). Soil fertility and plant health are greatly influenced by the microbial population, and soil is thought to play a crucial role in the composition of microorganisms (Diao et al., 2022). Rhizosphere soil microorganisms can improve the ability of Chinese medicinal plants to adapt to the environment, and also increase the content of their active ingredients, thereby affecting the formation of medicinal plants (Guo et al., 2017). Li Q. L. et al. (2023) found that rhizosphere microbiota structure changed dynamically at different growth stages of *Epimedium sagittatum*, and rhizosphere microbes, along with soil physicochemical properties and enzyme activities, participated in the synthesis and accumulation of effective ingredients. The fertilization of fields with decomposed hot pepper stalks improved the quality of *P. kingianum*, changed the structure of rhizosphere bacterial community, and enriched beneficial microorganisms (Wang et al., 2023). However, there are few related studies on the relationship between *P. cyrtonema* and rhizosphere microorganisms.

Endophyte is a microorganism that inhabits plant tissues and takes host plant metabolites as nutrients (Chen et al., 2023). Endophytic bacteria also play a crucial role in plant growth and secondary metabolism (Cui et al., 2022). Wang et al. (2002) demonstrated that the main endophytic Colletotrichum gloeosporioides of *Artemisia annua* increased the amount of artemisinin in *Artemisia annua* L. hairy root culture. Li et al. (2019) isolated two strains of endophytic fungi promoted the accumulation of saponin content in the tissue culture of *P. polyphylla* var. *Yunnanensis*. It was reported that the dominant endophytic fungi like *Setophoma* and *Arbuscular* mycorrhizal in *P. sibiricum* rhizome might be important microbial communities affecting the biosynthesis of the terpene and alkaloid (Fang et al., 2021). Cai et al. (2021) found that the relationship between endophytic microorganisms and active ingredients content was complex, and genera of endophytic bacteria related with polysaccharides, saponins, flavonoids, 5-Hydroxymethylfurfural were identified in *P. cyrtonema* through correlation analysis. Some characteristic endophytic bacteria derived from different origins were significantly correlated with active ingredients of *Eucommiae cortex* (Liang et al., 2023).

Microorganisms and environmental factors are significant contributors to the development of the quality of Chinese herbs as well as their growth and active ingredient accumulation. However, such factors related with the quality of *P. cyrtonema* under artificial cultivation conditions are not yet clear. Therefore, this study analyzed the characteristics of both rhizosphere and endophytic bacteria of *P. cyrtonema* from five regions of Zhejiang Province by 16S rDNA sequencing technology and performed correlation analysis with their active ingredients to identify microorganisms and environmental factors associated with the quality of *P. cyrtonema* under artificial cultivation conditions. The results will provide a theoretical basis for the scientific and effective artificial cultivation of high-quality *P. cyrtonema*.

2 Results

2.1 Soil physicochemical properties and enzyme activity

The physicochemical properties and enzyme activities of rhizosphere soil in the five plots were significant differences (Table 1). The range of rhizosphere soil pH of *P. cyrtonema* was 4.52–6.81 under

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TABLE 1 Statistical table of physicochemical properties and enzyme activities in rhizosphere soil.

Name	Yuhang	Qiantang	Tiantai	Wuyi	Jingning
pH	4.86 ± 0.04c	6.81 ± 0.07a	4.93 ± 0.14c	4.52 ± 0.03d	5.09 ± 0.06b
OM (g/kg)	20.29 ± 1.04c	28.45 ± 0.85ab	27.48 ± 1.40b	30.91 ± 2.24a	28.00 ± 0.90b
AN (mg/kg)	110.27 ± 1.17c	100.10 ± 5.58d	146.95 ± 3.42b	96.83 ± 3.38d	170.94 ± 1.75a
AP (mg/kg)	7.66 ± 0.04b	5.21 ± 0.41d	5.89 ± 0.15c	4.62 ± 0.23d	21.95 ± 0.51a
AK (mg/kg)	175.03 ± 2.39b	67.79 ± 1.15e	162.61 ± 2.85c	88.96 ± 4.48d	272.71 ± 3.38a
ACP (nmol/h/g)	1410.17 ± 16.92b	1320.32 ± 65.20c	1075.13 ± 23.79e	1812.05 ± 36.15a	1224.14 ± 17.42d
UE (μg/d/g)	365.42 ± 20.95d	1368.09 ± 31.26a	402.58 ± 9.46 cd	430.8 ± 19.18bc	467.27 ± 20.78b
SC (mg/d/g)	8.37 ± 0.40c	12.57 ± 0.31b	5.47 ± 0.21d	13.40 ± 0.11a	5.73 ± 0.20d

pH, hydrogen ion concentration; OM, organic matter; AN, alkali-hydrolyzable nitrogen; AP, available phosphorous; AK, available potassium; ACP, acid phosphatase; UE, urease; SC, sucrase. Means with different lower case letters within a row are significant differences ($P < 0.05$).

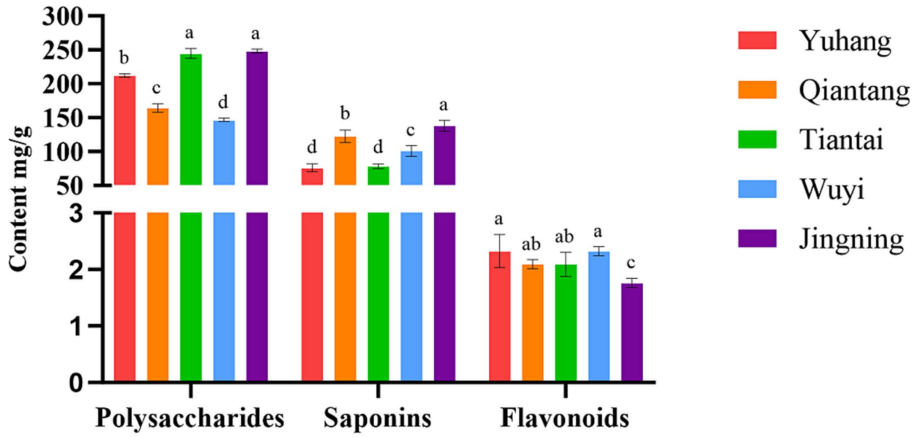

FIGURE 1
Active ingredient content of Polygonatum cyrtonema in different regions. Means with different lower case letters within a row are significant differences ($P < 0.05$).

different regions, the highest pH (6.81 ± 0.07) recorded on rhizosphere soil was in Qiantang. Organic matter (30.91 ± 2.24 g/kg) was highest in Wuyi. Alkali-hydrolyzable nitrogen (170.94 ± 1.75 mg/kg), available phosphorous (21.95 ± 0.51 mg/kg) and available potassium (272.71 ± 3.38 mg/kg) were significantly high in Jingning. Acid phosphatase (1812.05 ± 36.15 nmol/h/g) and sucrase (13.4 ± 0.11 mg/d/g) in Wuyi were significantly higher than the other four regions. Urease (1368.09 ± 31.26 μg/d/g) was significantly high in Qiantang.

2.2 Quantitative analysis of active ingredients in *Polygonatum cyrtonema*

The main active ingredients in rhizomes of *P. cyrtonema* from the five plots were detected, and the results were presented in Figure 1. The content of polysaccharides and saponins varied greatly in different regions. The highest concentration of polysaccharides was observed in the plots Tiantai and Jingning, and the lowest polysaccharides were found in Wuyi. The content of saponins was relatively high in the plot Jingning, followed by the plot of Qiantang, Wuyi, Tiantai, and Yuhang. The concentration of flavonoids in Jingning was the lowest, and there was no significant difference in the other four plots. These indicated different patterns of variation in the content of active ingredients of *P. cyrtonema* under different artificial cultivation conditions.

2.3 Analysis of 16S rDNA amplicon sequencing data

2.3.1 Analysis of the OTUs, alpha-diversity, and beta-diversity of *Polygonatum cyrtonema*

Each sample was analyzed by 16S rDNA amplicon sequencing and the data were summarized. The sequences were clustered into OUTs (100% similarity), and the good coverage of rhizosphere soil or endophytic bacteria included more than 99% (Table 2), respectively. Additionally, along with an increase in the amount of sequencing, the rarefaction curves of all the samples tended to be smooth (Figure 2). Such results indicated that the data amount of sequencing was gradually reasonable and comprehensively reflected the microbial community composition. The Venn diagram was constructed to assess the number of unique and common OTUs across all samples. The number of unique rhizosphere soil bacterial OTUs was 52, 198, 62, 35, and 61 in Yuhang, Qiantang, Tiantai, Wuyi, and Jingning, respectively (Figure 3A). Among them, the number of unique OTUs in Qiantang was the highest, indicating that there were more endemic microbial

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TABLE 2 Alpha diversity of rhizosphere soil microorganisms and rhizome microorganisms.

Sample name		EffectiveTags	OTU	Shannon	Simpson	Chao1	Coverage (%)
Rhizosphere soil bacteria	Yuhang	70,529 ± 2,012a	1,150 ± 286b	9.25 ± 0.51b	0.997 ± 0.00b	1164.63 ± 269.63b	99.87
	Qiantang	62,587 ± 550c	2,624 ± 172a	10.59 ± 0.01a	0.999 ± 0.00a	2683.42 ± 193.61a	99.63
	Tiantai	70,842 ± 2,208b	2,620 ± 124a	10.62 ± 0.07a	0.999 ± 0.00a	2697.15 ± 151.05a	99.42
	Wuyi	62,965 ± 941c	2,194 ± 92a	10.32 ± 0.14a	0.999 ± 0.00a	2232.61 ± 93.40a	99.67
	Jingning	72,109 ± 2,364ab	2,282 ± 484a	10.39 ± 0.30a	0.999 ± 0.00a	2322.81 ± 501.63a	99.72
Rhizome endophytic bacteria	Yuhang	77,051 ± 1,598a	177 ± 19b	3.38 ± 0.22c	0.811 ± 0.14b	184.03 ± 22.26b	99.95
	Qiantang	67,288 ± 9,621a	196 ± 56b	4.95 ± 0.20a	0.960 ± 0.03ab	196.77 ± 56.90b	99.98
	Tiantai	67,843 ± 11,937a	315 ± 76a	4.50 ± 0.70ab	0.860 ± 0.00ab	250.29 ± 75.81a	99.95
	Wuyi	70,527 ± 15,913a	138 ± 30b	3.54 ± 0.93bc	0.770 ± 0.13b	143.08 ± 26.40b	99.97
	Jingning	72,509 ± 6,631a	173 ± 48b	4.91 ± 0.19a	0.960 ± 0.3a	174.54 ± 49.84b	99.98

Means with different lower case letters within a row are significant differences ($P < 0.05$).

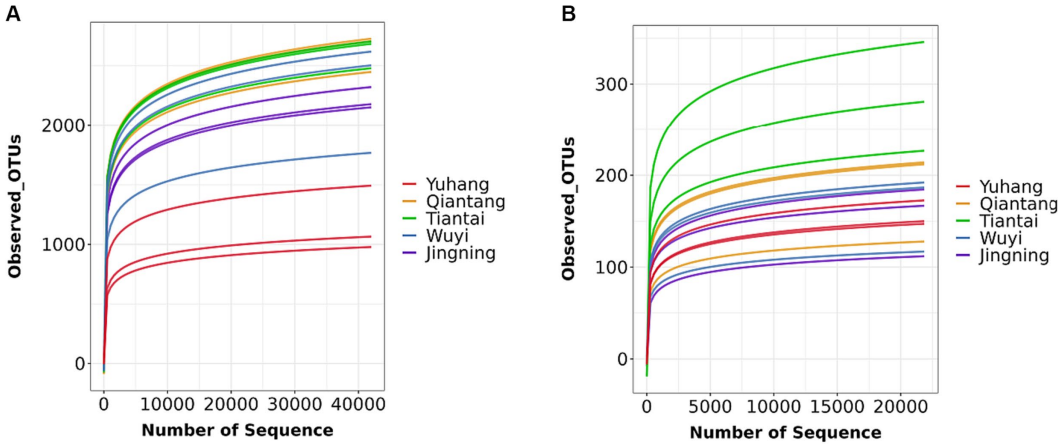

FIGURE 2
Rhizosphere soil bacteria **(A)** and endophytic bacteria **(B)** rarefaction curves.

species. The number of unique endophytic bacterial OTUs accounted for 22, 8, 114, 12, and 27, in Yuhang, Qiantang, Tiantai, Wuyi, and Jingning, respectively (Figure 3B). Tiantai had the highest number of OUTs, and Qiantang had the fewest. In addition, the proportion of common rhizosphere soil bacterial OTUs and endophytic bacterial OTUs was 20.50% and 9.50%, respectively, which indicated that the composition of bacteria in different regions was significantly different.

There were differences between rhizosphere and endophytic bacteria diversity. Alpha diversity analysis of microorganisms in rhizosphere soil and rhizome of *P. cyrtonema* was shown in Table 2. The mean OTU abundance, Chao1index, Shannon index, and Simpson index showed that the diversity of the bacterial community in rhizosphere soil was lowest in Yuhang. At the same time, other regions showed no significant difference in the alpha diversity of rhizosphere soil. In addition, the alpha diversity significantly varied along with the sampling plots of rhizomes. The Chao1 index of endophytic bacteria was the highest in Tiantai, while Shannon and Simpson indexes were higher in Qiantang and Jingning. The alpha diversity also demonstrated that the diversity of the bacterial community in rhizosphere soil was higher than that in rhizome. The spatial location map of the samples was obtained by the NMDS method to analyze their β diversity. The results showed that there was little difference between the within-group samples in both rhizosphere soil bacterial and endophytic bacterial community composition (Figures 3C,D).

2.3.2 Comparison of species composition of *Polygonatum cyrtonema* bacterial communities in different regions

The rhizosphere soil bacterial composition in different plots was similar at the phylum level. Proteobacteria, Acidobacteriota, Actinobacteriota, Planctomycetota, and Chloroflexi were the most abundant phylum of the *P. cyrtonema* rhizosphere soil (Figure 4A). For the endophytic bacteria, Proteobacteria was the major component of each bacterial community (Figure 4B). The endophytic bacterial composition of rhizomes in Qiantang and Wuyi was similar at the phylum level, while the composition in the other three plots was different (Figure 4B). The top 20 most abundant bacterial genera of rhizosphere soil and endophytic bacteria were selected for further analysis. The rhizosphere soil bacterial composition of Yuhang, Tiantai, and Wuyi was similar but was different in the dominant genus (Figure 4C). The main dominant bacteria of Yuhang was

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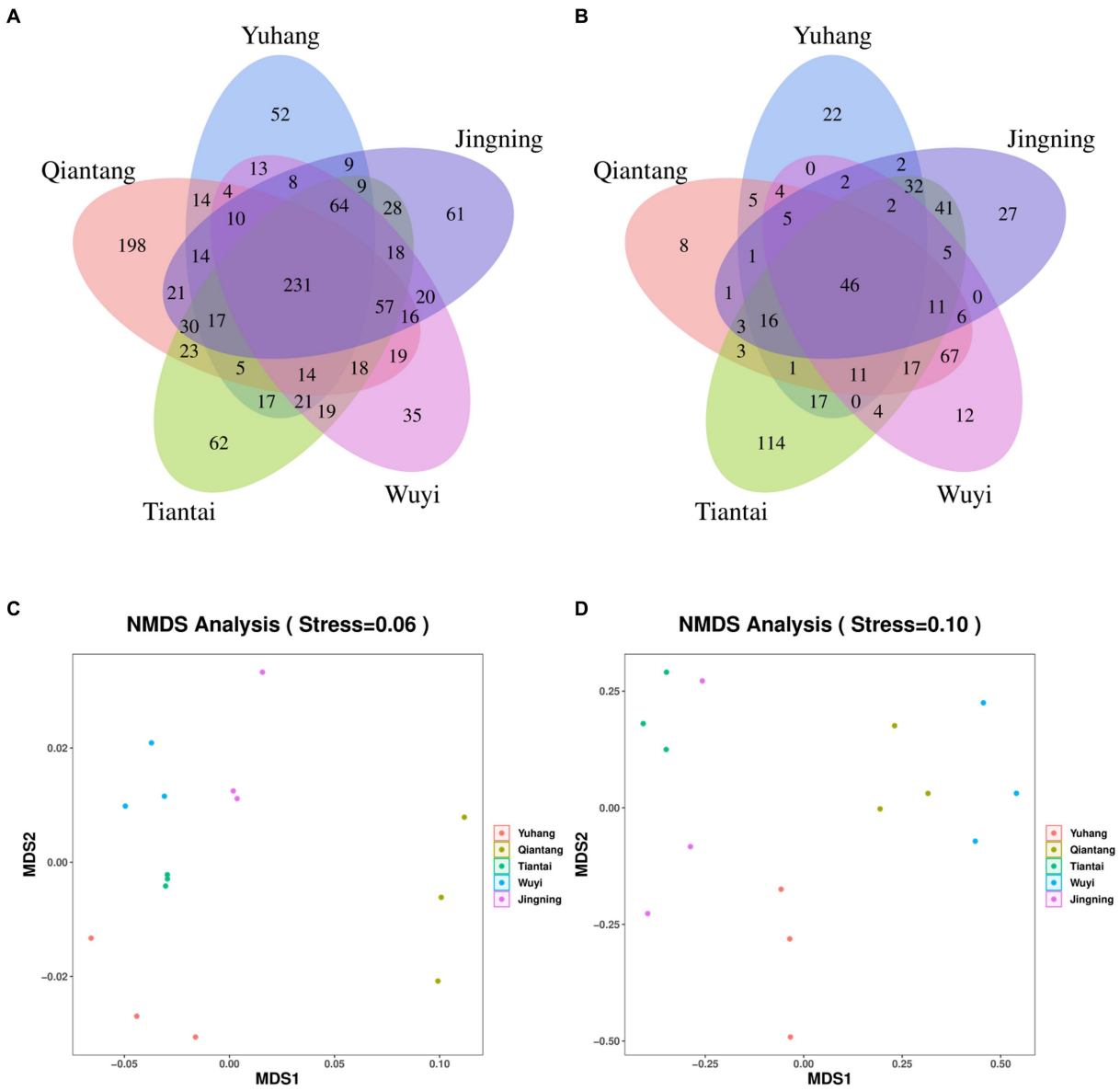

FIGURE 3
Venn diagrams of rhizosphere soil bacteria **(A)** and endophytic bacteria **(B)** in different regions; Analysis of Beta-bacterial diversity in rhizosphere soil **(C)** and rhizome **(D)** of *Polygonatum cyrtonema* in different origins by NMDS method.

*Gemmataceae*_unclassified, while *Subgroup*_2_unclassified were the abundant bacteria of Tiantai and Wuyi. In addition, *Vicinamibacteraceae*_unclassified and *Burkholderia-Caballeronia-Paraburkholderia* were the dominant bacteria in rhizosphere soil of Qiantang and Jingning, respectively (Figure 4C). The endophytic bacterial composition of Qiantang and Wuyi was highly similar, whereas their composition of other plots differed (Figure 4D). Additionally, the genus with the highest abundance in rhizome was *Burkholderia-Caballeronia-Paraburkholderia*, accounting for 73.03%, 16.12%, 69.94%, 15.94%, and 61.13% of the total sequences in Yuhang, Qiantang, Tiantai, Wuyi, and Jingning, respectively.

Spearman heatmap analysis of rhizosphere soil differential bacteria showed that 69 pairs of bacteria exhibited significant positive correlations and 36 pairs of bacteria displayed significant negative correlations (Figure 5A). There was a significant positive correlation among 30 pairs of endophytic bacteria and a significant negative correlation among 24 pairs of endophytic bacteria in rhizome (Figure 5B).

2.4 Relationship between soil physicochemical properties, soil enzyme activity, bacterial community

Redundancy analysis (RDA) was used to shed light on the influence of variation of soil enzyme activities, and soil physicochemical properties (explanatory variables) on the microbial community (response variables). The rhizosphere soil bacteria RDA analysis showed that the RDA1 was 78.10%, and the RDA2 was 7.04%, which could better reflect the relationship between soil enzyme

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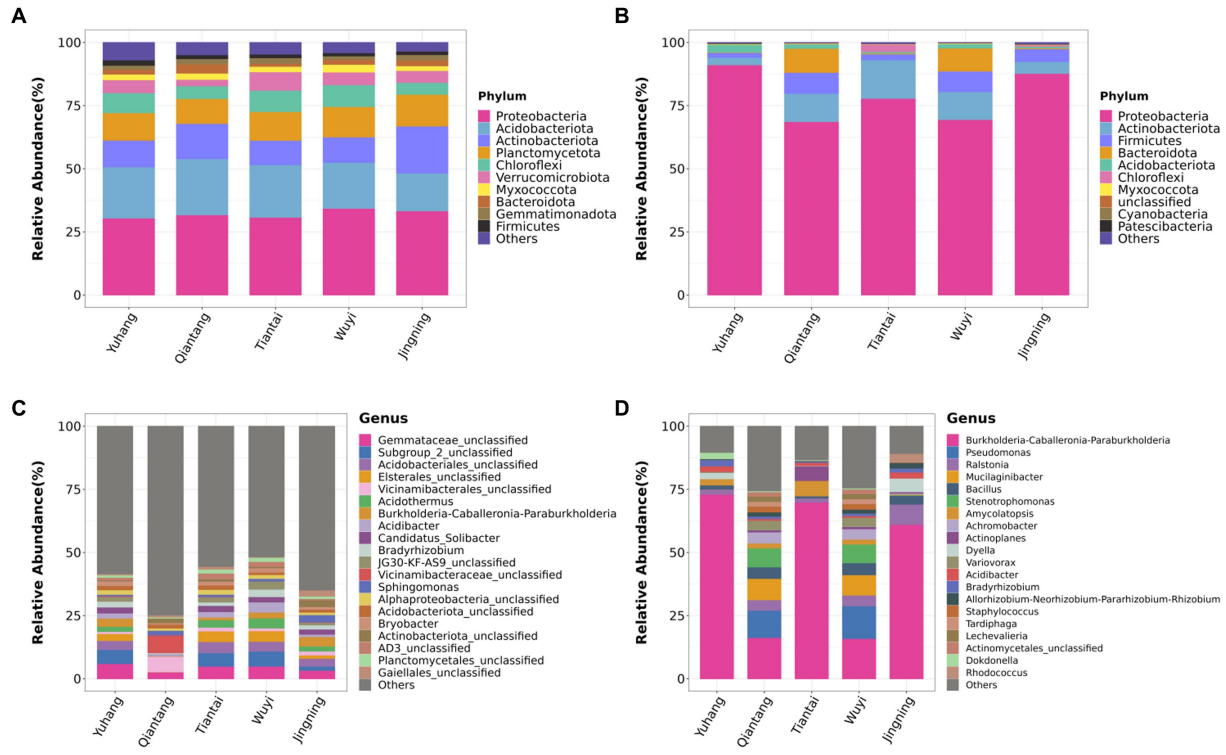

FIGURE 4
Phylum composition of rhizosphere soil bacteria **(A)** and endophytic bacteria in rhizome **(B)**; Genus composition of rhizosphere soil bacteria **(C)** and endophytic bacteria in rhizome **(D)**.

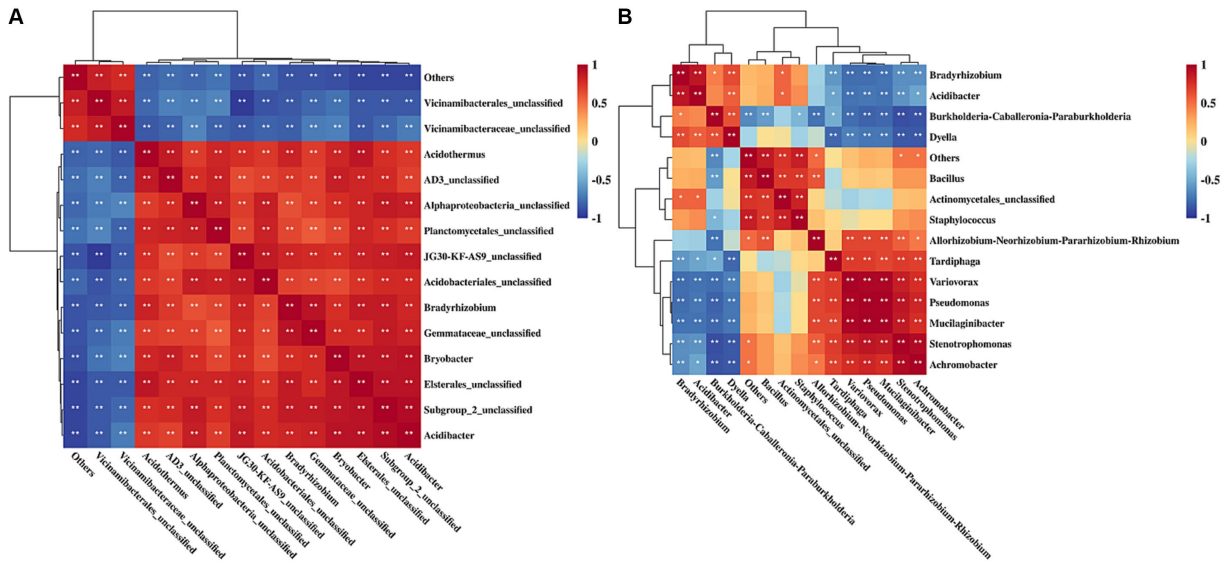

FIGURE 5
Correlation analysis of rhizosphere soil bacteria **(A)** and endophytic bacteria **(B)** of *Polygonatum cyrtonema*. $^{**}p < 0.01$; $^{*}p < 0.05$.

activities, soil physicochemical properties, and rhizosphere soil bacterial community (Figure 6A). *Gemmataceae*_unclassified and *Acidobacteriales*_unclassified had a certain correlation with AK, AN, and ACP, and were negatively correlated with pH, UE, and OM. *Subgroup*_2_unclassified and *Elsterales*_unclassified had a certain correlation with ACP, SC, and AK, and were negatively correlated with pH, UE, and OM. *Vicinamibacterales*_unclassified was highly correlated with pH and UE and had a certain correlation with SC, and OM. The rhizome endophytic bacteria RDA analysis showed that the RDA1 was 69.73%, and the RDA2 was 2.11% (Figure 6B).

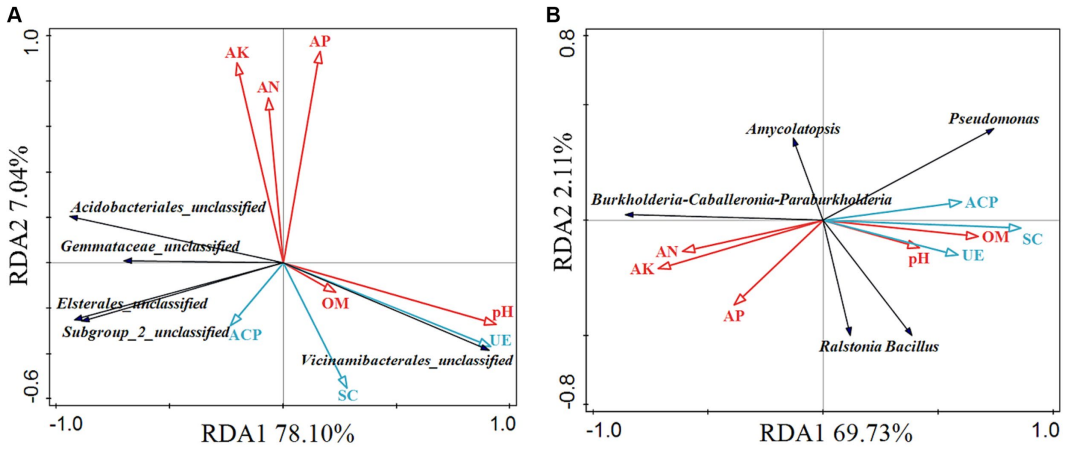

FIGURE 6
Redundancy analysis (RDA) of rhizosphere soil bacteria **(A)** and endophytic bacteria in rhizome **(B)**. The red arrow and the blue arrow represent the relative position of physicochemical properties and enzyme activity on the horizontal plane. The black arrow represents the species distribution at the genus level, and the longer the arrow, the greater the impact of the species in the sample. Where the angle between the arrow and the sort axis is different, the correlation strength is also different. The smaller the angle, the greater the correlation, and, the longer the length of the arrow, the greater the effect of the environmental factor. pH, hydrogen ion concentration; OM, organic matter; AN, alkali-hydrolyzable nitrogen; AP, available phosphorous; AK, available potassium; ACP, acid phosphatase; UE, urease; SC, sucrase.

Burkholderia-Caballeronia-Paraburkholderia was related to AK, AN, and AP, and negatively correlated with pH, OM, UE, ACP, and SC. *Ralstonia* and *Bacillus* had a certain correlation with pH, OM, AP, UE, ACP, and SC. *Pseudomonas* were related to pH, OM, UE, SC, and ACP, and were negatively correlated with AN, AP, and AK. By comparison, *Amycolatopsis* was not much related to each factor. Pearson correlation analysis revealed that polysaccharides were positively correlated with AN, AP, AK, and *Burkholderia-Caballeronia-Paraburkholderia*, and were negatively correlated with SC, ACP, *Ralstonia*, *Pseudomonas*, and *Bacillus* (Table 3). The correlation analysis revealed that saponins were negatively correlated with four rhizosphere soil bacterium (*Gemmataceae*_unclassified, *Subgroup*_2_unclassified, *Acidobacteriales*_unclassified, and *Elsterales*_unclassified), and were positively correlated with OM and AP. Besides, flavonoids were negatively correlated with AN, AP, and AK.

3 Discussion

The quality of medicinal plants was closely related to the ecological factors in growing locations, and the active ingredients and biopotency reflected their quality (Liu Y. J. et al., 2019). Microorganisms directly or indirectly participated in the growth and development, metabolism, and active ingredient accumulation of medicinal plants in a variety of ways (He et al., 2020). The active ingredients of artificially planted *P. cyrtonema* in different regions varied greatly, especially polysaccharides and saponins (Figure 1). It was reported that polysaccharides (7.48%~15.23%) and saponins (1.82%~6.49%) contents of *P. sibiricum* from different origins were significantly different (Qian et al., 2022). He et al. (2022) found that the different growth environments led to differences in polysaccharide contents of *P. sibiricum*, with comparatively higher levels of polysaccharides produced in Yunnan and Hebei Province. Polysaccharides contents of the five regions were more than 14.68% (Figure 1), which was more than twice the amount specified in State Pharmacopeia (2020), indicating that artificial planting was beneficial for the accumulation of such active ingredients. Jiao et al. (2016) also reported that all the new rhizome polysaccharides of *Polygonatum* Mill. were increased and met the Chinese Pharmacopoeia standards limits after the artificial planting in *Polygonatum* Planting Base of Buchang Pharma Group, Lueyang County, Shaanxi Province. Considering the effect of the environment on the active ingredients of *P. cyrtonema*, it was feasible that artificial cultivation might provide suitable growth conditions to accumulate active ingredients, especially polysaccharides.

High-throughput sequencing showed that rhizosphere and endophytic bacteria of different regions were abundant, and the number and species of rhizosphere bacteria were higher than those of endophytic bacteria (Table 2). Wang Y. B. et al. (2022) found that the diversity of bacteria in *Cinnamomum camphora* (L.) Presl rhizosphere soil was significantly higher than that of endophytic bacteria in plant organs such as camphor roots. At the phyla level, rhizosphere bacteria groups of *P. cyrtonema* were dominated by Proteobacteria, Acidobacteria, Actinobacteriota, Planctomycetota, and Chloroflexi (Figure 4A). Both Proteobacteria and Chloroflexi were also the major bacteria identified in rhizosphere soil of *P. kingianum* (Wang et al., 2023). The dominant phylum of endophytic bacteria groups was Proteobacteria (Figure 4B), which was also identified in the endophytic bacteria community of *P. cyrtonema* from Xinhua, Hunan Province (Cai et al., 2020). At the genus level, most of the bacteria in rhizosphere soil were unclassified, indicating that rhizosphere soil samples contained a large number of unknown microbial species (Figure 4C), which was also reported in the study of *Cynanchum bungei* Decne (Li et al., 2022), *Asarum heterotropoides* F. Schmidt var. *mandshuricum* (Maxim.) Kitag (Yu et al., 2022), and *Phyllostachys edulis* (Yuan et al., 2023). These studies showed that the most of rhizosphere bacteria at the genus level were not identified and studies of rhizosphere bacteria might be useful to improve our knowledge of their behavior and effects on the accumulation of active ingredients in medicinal plants. *Burkholderia-Caballeronia-Paraburkholderia* was the dominant genus of endophytic bacteria and also demonstrated relatively high

TABLE 3 Pearson correlation analysis of active ingredients.

Impact factor		Polysaccharides		Saponins		Flavonoids	
		CC	P	CC	P	CC	P
Soil physicochemical properties	pH	−0.257	0.354	0.442	0.099	−0.237	0.396
	OM	−0.354	0.195	0.520*	0.047	−0.160	0.570
	AN	0.890**	0.000	0.293	0.289	−0.700**	0.004
	AP	0.620*	0.014	0.613*	0.015	−0.687**	0.005
	AK	0.860**	0.000	0.205	0.464	−0.537**	0.039
Soil enzyme activities	SC	−0.990**	0.000	0.112	0.692	0.456	0.088
	UE	−0.465	0.081	0.456	0.088	−0.099	0.724
	ACP	−0.806**	0.000	−0.018	0.948	0.509	0.053
Rhizosphere soil bacteria	*Gemmataceae*_unclassified	0.140	0.618	−0.743**	0.002	0.505	0.055
	*Subgroup_2*_unclassified	0.034	0.904	−0.751**	0.001	0.482	0.069
	*Acidobacteriales*_unclassified	0.428	0.112	−0.562*	0.029	0.157	0.575
	*Elsterales*_unclassified	0.052	0.853	−0.652**	0.008	0.334	0.224
	*Vicinamibacterales*_unclassified	−0.380	0.163	0.428	0.112	−0.133	0.636
Endophytic bacteria in rhizome	*Burkholderia-Caballeronia-Paraburkholderia*	0.953**	0.000	−0.288	0.415	−0.333	0.225
	Ralstonia	−0.600**	0.018	0.177	0.528	0.051	0.856
	Pseudomonas	−0.566*	0.031	0.236	0.396	0.078	0.781
	Bacillus	−0.853**	0.000	0.159	0.572	0.358	0.190
	Amycolatopsis	−0.022	0.938	−0.280	0.311	0.246	0.377

*Significant correlation ($P < 0.05$);
**Extremely significant correlation ($P < 0.01$).

abundance in rhizosphere soil (Yuhang, 3.24%; Qiantang, 0.17%; Tiantai, 1.07%; Wuyi, 2.32%; Jingning, 3.73%; Figure 4D). It seemed that endophytic bacteria might transmit via either a horizontal pathway (i.e., obtained from the vicinal environment) or vertically (i.e., gained directly from the parent) (Shade et al., 2017), due to its abundance in rhizosphere soil (Figure 6B). The results indicated that the relationship between rhizosphere soil bacteria genera was mainly mutualistic and symbiotic, while the correlation between endophytic bacteria genera was not as close as that between rhizosphere soil bacteria genera (Figure 5). According to research by Xie et al., most of the rhizosphere bacterial genera of *Angelica sinensis* at various growth stages exhibited a positive association, suggesting that rhizosphere soil bacteria were more likely to coexist in a mutually beneficial symbiotic relationship (Xie et al., 2023).

Soil environmental factors and soil enzyme activity were important indicators of the natural environment, which determined the composition of soil microbial communities (Gong et al., 2020; Ma et al., 2021). RDA analysis showed that both rhizosphere soil bacteria (*Vicinamibacterales*_unclassified) and endophytic bacteria (*Pseudomonas*, *Bacillus*, and *Ralstonia*) were associated with pH (Figure 6). Jiang et al. (2023) found that pH remarkably affected the bacterial community assembly in the tobacco rhizosphere. Soil enzyme activity also correlated with bacterial community structure, the rhizosphere bacteria *Vicinamibacterales*_unclassified had a significant positive correlation with UE, while the other four rhizosphere bacterium were negatively correlated with UE (Figure 6A). According to the research of *Angelica sinensis* by Xie et al. (2023), there was a significant positive correlation between UE and *Bacteroides* and *Chaetomium* in the rhizosphere, and UE was negatively correlated with other bacteria, such as *Marseillia*. The soil enzyme activity was negatively correlated with the endophytic bacteria *Burkholderia-Caballeronia-Paraburkholderia* and correlated with *Pseudomonas*, *Bacillus*, and *Ralstonia* (Figure 6B). Besides, bacterial communities were influenced by AN, AP, and AK, with endophytic bacteria being more susceptible to soil properties than rhizosphere bacteria (Figure 6), which was consistent with the results by Li J. Q. et al. (2023). However, the AN, AP, and AK were the main driving forces of *P. kingianum* rhizosphere bacterial community structure by chemical fertilizer treatment (Wang et al., 2023), possibly due to the higher AN, AP, and AK content in the cultivation of *P. kingianum* compared to our research. Moreover, Liu et al. (2022) found that rhizosphere bacteria of *Lycium barbarum* fruit positively correlated with soil pH, monthly average atmospheric humidity and monthly average soil humidity. All of these suggested that soil characteristics played important roles in the selection of plant microbiomes (Gupta et al., 2021). Therefore, soil physicochemical properties and enzyme activities of rhizosphere soil microorganisms, which was also demonstrated in *Stellera chamaejasme* L. (Cheng et al., 2022).

For medicinal plants, the ecological environment factors of their origin were closely related to the quality and authenticity of Chinese medicinal plants (Li Q. L. et al., 2023). The Pearson correlation analysis showed that the active ingredients were greatly affected by bacterial communities and soil properties of *P. cyrtonema*. Polysaccharide was strongly correlated with soil physicochemical properties, enzyme activity, and endophytic bacteria (Table 3). The significant positive correlation between polysaccharides and AN and

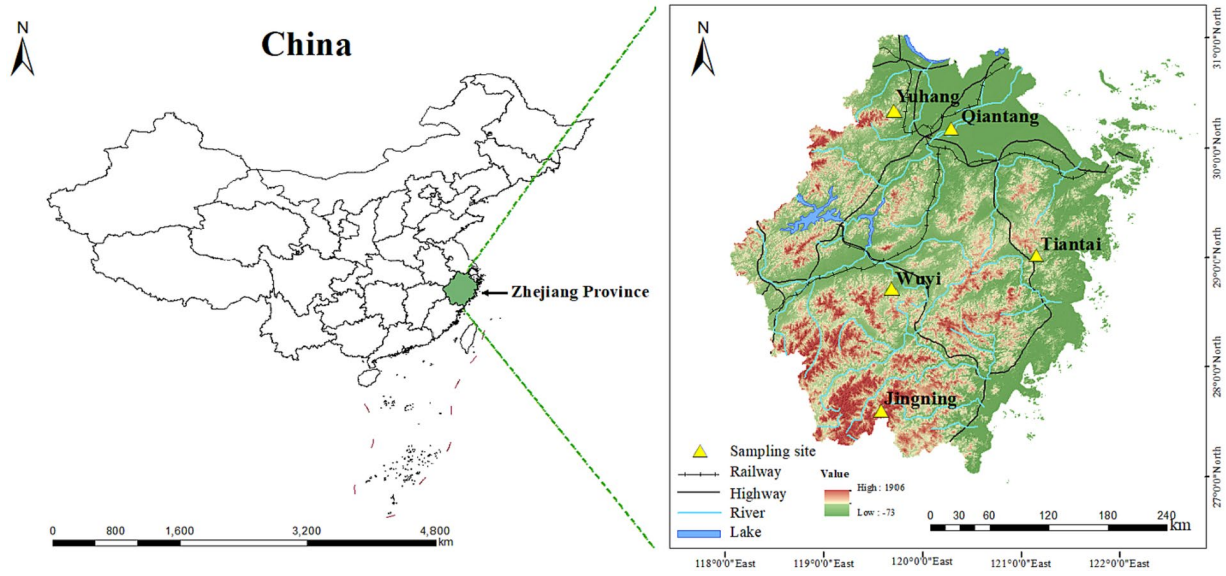

FIGURE 7
Map of sampling sites in Zhejiang, China. The map was plotted by Arcgis (Version 10.8, Esri, United States).

AP was also reported in *Linze Jujube* (Tian et al., 2023). In the present study, *Burkholderia-Caballeronia-Paraburkholderia* was positively correlated with polysaccharide content, while most of the dominant bacterial groups were negatively correlated with polysaccharide content, which might be related to the metabolic consumption of endophytic bacteria based on polysaccharides and other carbohydrates (Cai et al., 2020, 2021). Saponins were significantly positively correlated with OM and AP, and negatively correlated with four rhizosphere bacterial communities (Table 3). The saponins of ginseng under three matrix combinations were significantly positively correlated with soil AP (Fang et al., 2022). There was a positive correlation between saikosaponin and OM content of *Bupleurum chinense* in different habitats (Liu et al., 2020). Studies showed that the soybean field soil bacterial α-diversity was decreased with four different saponin treatments (Nakayasu et al., 2021). In addition, our results showed that flavonoids were negatively affected by soil physicochemical properties (Table 3). Studies displayed that the content of total *Epimedium koreanum* flavonoids was negatively related to AN (Liu et al., 2021). Li et al. (2017) found that the AK had an inhibitory effect on the flavonoid content of *Caulis Spatholobi*. It was reported that abiotic stress such as temperature, salt, and UV radiation could enhance the total flavone synthesis in *Sarcandra glabra* (Thunb) Nakai (Su and Zhou, 2009). Therefore, nutrient deficiencies might also promote *Polygonatum* flavonoids accumulation. Consequently, *P. cyrtonema* from Jingning showed the highest content of polysaccharides and saponins (Figure 1), because Jingning plot had a higher level of AN, AP, and AK and a higher abundance of *Burkholderia-Caballeronia-Paraburkholderia* (Table 1; Figure 4D). On the contrary, Wuyi had the lowest polysaccharides concentration and a relatively high flavonoids concentration than other regions, possibly due to its poor quality of the soil nutrients (Table 1; Figure 1). Our study demonstrated that bacterial microorganisms were involved in the accumulation of active ingredients of *P. cyrtonema* together with soil physicochemical properties and enzyme activities, which provided a theoretical basis for the scientific and effective artificial cultivation of high-quality *P. cyrtonema*.

4 Materials and methods

4.1 Material collection and preparation

Polygonatum cyrtonema originated from Huangshan City, Anhui Province was planted in Yuhang, Qiantang, Tiantai, Wuyi, and Jingning in Zhejiang Province in November 2019, respectively. All the samples were collected in November 2022 (Figure 7). Five representative plants were collected from each region as sampling plants and were divided into two parts. After being thoroughly cleaned and sanitized, a part of rhizomes was divided into three subsamples and refrigerated at −80°C for high-throughput sequencing of endophytic bacteria. The other parts were used to determine the content of active ingredients after drying at 55°C and passed through a 60 mesh sieve. The rhizosphere soil (at 2 mm to rhizomes, and at 5–10 cm depth from the soil surface) of each region was collected by sterile brush and mixed as a sample. Soil samples were transported to the laboratory in an icebox, and each sample was further divided into two subsamples. One subsample of rhizosphere soil was stored at −80°C for high-throughput sequencing of rhizosphere bacteria, and the other was used for physicochemical properties and enzyme activities analysis after naturally drying and passed through a 2 mm sieve. The sample names of rhizomes and rhizosphere soil corresponded one by one.

4.2 Soil physicochemical properties and enzyme activity analysis

Soil physicochemical properties were determined according to the methods in "Laboratory Analysis Methods for Soil Investigation"

(Zhang and Gong, 2012), including pH value (laboratory pH meter), organic matter (the external heating method of concentrated sulfuric acid and potassium dichromate), alkali-hydrolyzable nitrogen (alkali hydrolysis diffusion method), available phosphorus (sodium bicarbonate leaching molybdenum-antimony anti-colorimetric method), and available potassium (ammonium acetate leaching flame photometer method). Soil urease, soil saccharase, and soil acid phosphatase were measured by soil enzyme kits (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), and a Multimode reader (Synergy HTX, BioTek Instruments, Inc., United States).

4.3 Quantification of active ingredients in *Polygonatum cyrtonema*

The anthrone-sulfuric acid method was used to determine the polysaccharides content (State Pharmacopeia, 2020). The content of total flavonoids was determined according to Chen et al. (2012). The total saponins were measured as described by You et al. (2010).

4.4 Bacterial community profiling by 16S rDNA amplicon sequencing

DNA from rhizosphere soil and rhizome samples was extracted using the DNA Kit (D4015-02, Omega, Inc., United States) according to the manufacturer's instructions. The reagent designed to uncover DNA from trace amounts of the sample was effective for the preparation of DNA of most bacteria. Nuclear-free water was used for blank. The total DNA was eluted in 50 μL of Elution buffer and stored at −80°C until analysis by LC-Bio Technology Co., Ltd., Hang Zhou, Zhejiang Province, China (Zhang Q. Q. et al., 2018; Zhang X. Y. et al., 2018; Chen et al., 2019). PCR amplification of 16S rDNA high variable V3/V4 region of bacteria was performed using 341F (5′-CCTACG GGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTA TCTAATCC-3′) primers (Xiong et al., 2012; Sundberg et al., 2013; Xu et al., 2016). All the amplicons were sequenced using Illumina NovaSeq PE250 (PE250, CA, United States) high-throughput sequencing technology, and bioinformatics analysis of sequences was performed using software such as QIIME2 (Bolyen et al., 2019).

4.5 Data processing and analysis

After sequencing, paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (Magoc and Salzberg, 2011). Quality filtering on the raw reads was performed under specific filtering conditions to obtain high-quality clean tags according to the fqtrim (Version 0.94, CCB, United States). Chimeric sequences were filtered using Vsearch (Rognes et al., 2016). After dereplication using DADA2 (Callahan et al., 2016), a feature table and feature sequence were obtained. Alpha diversity and beta diversity were calculated by normalizing to the same sequences randomly. Then according to the SILVA (Quast et al., 2013) classifier, feature abundance was normalized using the relative abundance of each sample. Alpha diversity was applied in analyzing the complexity of species diversity for each sample through 5 indices, including Chao1, Observed species, Goods coverage, Shannon, and Simpson, which were calculated with QIIME2. Beta diversity was calculated by QIIME2, and the graphs were drawn by the R package (R Core Team, 2019). Blast (Boratyn et al., 2019) was used for sequence alignment, and the feature sequences were annotated with the SILVA database for each representative sequence. Other diagrams were implemented using the R package.

The data of soil physicochemical properties, soil enzyme activities, and active ingredients were checked by IBM SPSS Statistics 25.0 (SPSS Inc., Chicago, IL, United States) to test whether they met the normal distribution, and then conducted a differential analysis. The content of active ingredients was plotted by GraphPad Prism (Version 9.5, GraphPad, United States). Redundancy analysis (Desarbo et al., 2016) in Canoco5.0 (Version 5.0, Microcomputer Power, United States) was used to explore the correlation among rhizomes endophytic bacteria or rhizosphere soil bacteria, soil physicochemical parameters, and enzyme activities (Cheng et al., 2022). A Pearson correlation analysis (Pearson, 1980s) with the SPSS 25.0 Software was used to study the relationship between active ingredients of *P. cyrtonema* rhizomes and rhizomes endophytic bacterial community index, rhizosphere soil bacterial community index, soil physicochemical parameters, and enzyme activities.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI—PRJNA1071871.

Author contributions

QZ: Writing – original draft, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization. YC: Conceptualization, Investigation, Writing – original draft. LZ: Formal analysis, Writing – original draft. ML: Methodology, Visualization, Writing – original draft. LY: Investigation, Methodology, Writing – original draft. DW: Supervision, Validation, Writing – review & editing. QJ: Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1347204/full#supplementary-material

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