

Navigating challenges and innovations in antimicrobial resistance, environmental microbiology, and industrial solutions

Edited by

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Navigating challenges and innovations in antimicrobial resistance, environmental microbiology, and industrial solutions

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Editorial: Navigating challenges and innovations in antimicrobial resistance, environmental microbiology, and industrial solutions (ARTEMIS)

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Editorial on the Research Topic

[Navigating challenges and innovations in antimicrobial resistance, environmental microbiology, and industrial solutions \(ARTEMIS\)](#)

The silent pandemic of Antimicrobial Resistance (AMR) continues to escalate, posing one of the most significant threats to global health, food security, and development in the 21st century. The complex web of factors driving its emergence and spread transcends clinical settings, deeply embedding itself within our environment and industrial practices. It is from this understanding that our Research Topic, “*Navigating Challenges and Innovations in Antimicrobial Resistance: Environmental Microbiology, and Industrial Solutions*,” was born.

This Research Topic is proud to be organized in collaboration with the 2-day National Conference on ARTEMIS 2024, held on the 21st and 22nd of March, 2024, at the Institute of Advanced Research in Gandhinagar, India. This pivotal event, sponsored by the Gujarat State Biotechnology Mission (GSBTM), the Science and Engineering Research Board (SERB), and Frontiers, brought together the next generation of scientists to address this global challenge. The conference was a resounding success, hosting 140 participants and featuring vibrant scientific exchange through 27 oral and 46 poster presentations from postgraduate and doctoral students. This Research Topic of articles was conceived to create a lasting, peer-reviewed platform for the ideas and research discussed, uniting researchers to explore the intersections of these critical fields and fostering a holistic approach to understanding and combating AMR. The diverse and innovative articles presented here are a testament to that collaborative vision.

The contributions to this Research Topic highlight three interconnected pillars: the direct challenge of AMR, the crucial role of environmental microbiology, and the development of sustainable industrial and agricultural solutions.

Several articles tackle the challenge of AMR head-on, focusing on improved detection and novel therapeutic strategies. The development of rapid and accurate diagnostics is paramount to effective surveillance and treatment. Addressing this, the work by [Zhu et al.](#) provides a valuable new tool with their evaluation of a two-dimensional PCR method for the rapid detection of hypervirulent *Klebsiella pneumoniae*, a notoriously difficult pathogen. Complementing this, [Liang et al.](#) developed three simple and cost-effective assays to detect the activity of the AAC(6′)-Ib-cr enzyme, which confers resistance to multiple antibiotics, offering a practical solution for resource-limited settings. Beyond detection, the search for alternatives to conventional antibiotics is urgent. [Wang et al.](#) present a promising approach by displaying a prophage lysin on the surface of *Bacillus subtilis* spores, creating a potent antibacterial agent against the swine pathogen *Streptococcus suis*. In a similar vein, [Liu et al.](#) explore the natural world for solutions, providing a preliminary but encouraging look into the antimicrobial and antioxidant potential of *Magnolia* essential oil, a potential source for novel bioactive compounds.

The environment is a critical reservoir and conduit for the spread of resistance genes and resistant organisms. Several of our authors have focused on harnessing the power of microorganisms to remediate and protect our ecosystems. Recognizing that contaminated waterways are hotspots for AMR, [Patel et al.](#) demonstrate an innovative solution using a synergistic system of plants and bacteria in floating treatment beds to efficiently remove emerging contaminants from polluted river water. Directly addressing pharmaceutical pollution, [Yang et al.](#) isolated and characterized bacteria capable of efficiently degrading the antibiotic neomycin, offering a bioremediation strategy for contaminated wastewater and soil. The impact of industrial pollution is tackled by [Zheng et al.](#), who characterized a bacterium from activated sludge capable of degrading N,N-dimethylformamide, a widely used industrial solvent, showcasing the power of microbes in waste treatment.

Finally, this Research Topic underscores the immense potential of microbial solutions in industrial and agricultural contexts, which are key to a sustainable future. In agriculture, [Du et al.](#) show how thermophilic microbial agents can accelerate the composting of pig manure and spent mushroom substrate, transforming agricultural waste into a valuable resource. Shifting from waste management to crop protection, [Kamath et al.](#) explore the use of siderophores produced by bacteria to defend mung bean plants against fungal disease, presenting a biological alternative to chemical fungicides that can inadvertently drive resistance.

Together, these articles paint a hopeful picture. They showcase a dynamic research landscape where innovative diagnostics, nature-inspired antimicrobials, and powerful bioremediation strategies are being developed. The findings collected here reinforce the necessity of a “One Health” perspective, where the intricate links between human, animal, and environmental health are not just acknowledged but are central to our research endeavors. We extend

our sincere gratitude to all the authors who contributed their valuable research and to the reviewers whose diligent efforts upheld the quality of this work. It is our hope that this Research Topic, born from the energy and intellect of the ARTEMIS 2024 conference, will stimulate further discussion, collaboration, and innovation, moving us closer to a world where the threat of antimicrobial resistance is effectively contained.

Author contributions

AS: Funding acquisition, Conceptualization, Resources, Writing – review & editing, Writing – original draft, Supervision. DG: Writing – review & editing. CJ: Writing – review & editing.

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Characterization and genome sequence of N,N-dimethylformamide degradation in *Paracoccus sulfuroxidans* DM175A1-1 isolated from activated sludge of wastewater

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N,N-dimethylformamide (DMF) is an organic solvent with stable chemical properties and high boiling point. Based on its good solubility, DMF is widely used in synthetic textile, leather, electronics, pharmaceutical and pesticide industries. However, the DMF pollutes the environment and does harm to human liver function, kidney function, and nerve function. Herein, an efficient DMF-degrading strain, DM175A1-1, was isolated and identified as *Paracoccus sulfuroxidans*. This strain can use DMF as the sole source of carbon and nitrogen. Whole-genome sequencing of strain DM175A1-1 revealed that it has a 3.99-Mbp chromosome a 120-kbp plasmid1 and a 40-kbp plasmid2. The chromosome specifically harbors the *dmfA1* and *dmfA2* essential for the initial steps of DMF degradation. And it also carries the some part of genes facilitating subsequent methylotrophic metabolism and glutathione-dependent pathway. Through further DMF tolerance degradation experiments, DM175A1-1 can tolerate DMF concentrations up to 10,000 mg/L, whereas the majority of *Paracoccus* strains could only show degradation activity below 1,000 mg/L. And the efficiency of organic nitrogen conversion to NH₃-N in DMF can reach 99.0% when the hydraulic retention time (HRT) is controlled at 5 days. Meanwhile, it showed a significant degradation effect at a pharmaceutical enterprise in Zhejiang Province with high concentration of DMF wastewater. This study provides a new strain *Paracoccus sulfuroxidans* DM175A1-1 which shows a significant influence on DMF degradation, and reveals the characterization on its DMF degradation. It lays the foundation for the application of biological method in the efficient degradation of DMF in industrial wastewater.

KEYWORDS

N,N-dimethylformamide, *Paracoccus sulfuroxidans*, wastewater treatment, efficient degradation, biological method

Introduction

N,N-dimethylformamide (DMF) is a common multifunctional solvent that is widely used in pesticide, pharmaceutical, petrochemical, and other production industries (Ding and Jiao, 2012; Bromley-Challenor et al., 2000; Nisha et al., 2015). In recent years, with the expansion of the scale of chemical production, DMF has aggravated the pollution of the environment with the increase of industrial wastewater (Chen et al., 2016). DMF can enter the human body through breathing and skin contact, which has greater harm to human liver function, kidney function, and nerve function (Wang et al., 2014; Li and Zeng, 2019). At present, there are physical, chemical, and biological methods for treating DMF wastewater, and biological processes have been applied in industrial wastewater treatment because of their advantages of low cost and low pollution (Das et al., 2006; Sun et al., 2008; Dziewit et al., 2010). When the concentration of pollutants in wastewater is high, it will influence the growth and degradation effect of microorganisms (Astals et al., 2015). At present, the treatment of the DMF wastewater is mainly relying on activated sludge. However, the activated sludge could only tolerant about 200 mg/L DMF concentration compared to the 2,000 mg/L DMF concentration of factory wastewater (Zhou et al., 2018; Astals et al., 2015). The low degradation rate of DMF will cause the exceedance of total nitrogen released, which increase the difficulty in subsequent treatment of nitrogen content. Hence, it needs to be domesticated and isolated from the environment to get some strains that can degrade DMF efficiently, which is essential for practical industrial production applications.

Several bacteria that degrade DMF have been isolated and identified, such as *Alcaligenes* (Hasegawa et al., 1997), *Paracoccus* (Nisha et al., 2015; Doronina et al., 1998; Czarnecki et al., 2017), *Pseudomonas* (Ghisalba et al., 1985), *Methylobacterium* (Doronina et al., 2000), *Mycobacterium* (Urakami et al., 1990), *Ochrobactrum* (Veeranagouda et al., 2006), etc. *Paracoccus* is the main genus for degrading DMF, and there are several *Paracoccus* strains were reported that have the capability of degrading DMF effectively (Zhou et al., 2018; Astals et al., 2015). Swaroop et al. (2009) found that the *Paracoccus* sp. strain DMF bacteria would briefly accumulate dimethylamine and methylamine during the growth process using DMF, which would eventually be converted into ammonia and carbon dioxide and degrade DMF.

In previous study, they found that some of the strains *Paracoccus aminophilus* JCM 7686 and *Methylobacterium* sp. Strain DM1 contained enzymes that could degrade DMF, named DMFase (Dziewit et al., 2010; Lu et al., 2019). In contrast, others did not include this enzyme, so they speculated that there might be two pathways of DMF degradation. The critical enzyme in one of the pathways was DMFase, which degraded DMF to dimethylamine (DMA) and formic acid (Dziewit et al., 2010). Subsequently, DMA forms methylamine and formaldehyde by the action of dimethyl dehydrogenase. Then methylamine will form ammonia and formaldehyde by the activity of methylamine dehydrogenase (Ghisalba et al., 1985; Veeranagouda et al., 2006). Another pathway of DMF degradation is achieved through repeated oxidative demethylation of DMF, which is speculated to be possessed by some *Pseudomonas* species (Hans-Peter et al., 2010). The two methyl groups on N are removed by methyl formamide oxidase and formamide oxidase, respectively, to form formamide, which is further hydrolyzed by formamidase to produce

ammonia and formic acid (Ghisalba et al., 1985). The DMF degradation mechanisms of different genera may differ significantly, and there are relatively few studies on DMF degradation mechanisms in terms of genes. Therefore, it is essential to identify the DMF degradation mechanisms of *Paracoccus sulfuroxidans*.

In this study, we isolated four efficient DMF degrading strains by acclimatization, which were able to utilize DMF as the sole carbon and nitrogen source. Among them, DM175A1-1 showed the best degradation effect, and the whole gene sequencing data of this strain were analyzed to elucidate its relevant genes and degradation pathways responsible for DMF degradation. The DM175A1-1 can achieve efficient ammonification of DMF, which could solve the difficulty of converting organic nitrogen to ammonia nitrogen during the degradation then, the nitrogen removal can be achieved in combination with nitrification or denitrification processes subsequently. Finally, in order to enhance the tolerance of the high DMF concentration about DM175A1-1, we further investigated its degradation ability by tolerance test.

Materials and methods

Culture medium

The inorganic salt medium with DMF as the sole source of carbon and nitrogen (1.2 g/L KH_2PO_4 , 6.8 g/L K_2HPO_4 , 0.5 g/L NaCl, 0.1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.1 g/L CaCl_2 , 0.1 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.006 g/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.006 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.007 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0001 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.0124 g/L H_3BO_3 , 0.00001 g/L Vitamin B1, pH 6.0) was used to domesticate the isolates. LB medium as the seed solution medium for strains. DMF1 medium as the purification medium for strains (additional 0.2 g/L DMF and 0.2 g/L dimethylamine were added on top of the ingredients in LB medium).

Isolation and identification of domesticated strains

Fresh sludge of 10 mL from each pharmaceutical plant was inoculated into a triangular flask containing 100 mL of inorganic salt medium (DMF concentration of 1,000 mg/L) and incubated in an incubator at 30°C with 130 r/min oscillation, during which the total nitrogen (TN) and $\text{NH}_3\text{-N}$ values in the system were sampled every one day. The culture was incubated until the ammonification rate of TN reached more than 90% (ammonification refers to the conversion of organic nitrogen in TN into $\text{NH}_3\text{-N}$), then transferred to fresh inorganic salt medium (DMF concentration of 2,000 mg/L) at 10% inoculum, monitored the TN and $\text{NH}_3\text{-N}$ values in the system and continued to incubate. The DMF concentration was gradually increased to 5,000 mg/L by transferring five times consecutively.

One millilitre of bacterial broth from the culture system with good growth condition of the strain was taken for high-throughput sequencing. At the same time, the above bacteria were at a dilution of 10^{-2} to 10^{-8} spread onto a solid medium containing 1,000 mg/L DMF and incubated at 30°C for 5 days single colonies were picked into DMF1 medium, incubated at 30°C for 3 days, and then purified by scribing, and the single purified strains were identified by DNA sequencing. The morphology of the bacterium was observed by light

microscopy and cultured with Petri dishes. The isolated and purified strains were subjected to Gram staining and physiological and biochemical characterization experiments, and the identification results of 16S rDNA were used as the species name.

Genome sequencing and genetic information analysis

The version of MEGA was MEGA X. The evolutionary using the Neighbor-Joining method, Kimura 2-parameter method, Numbers at branch points indicate bootstrap percentages (based on 1,000 replications). Only values above 50% are shown. Bar, 0.01 substitutions per nucleotide position. The 16S rRNA gene sequence of *Tropicibacter naphthalenivorans* CECT 7648 was used as the outgroup.

Genomic DNA was extracted using the Wizard genomic DNA purification kit (product no. A1125; Promega). Whole-genome sequencing was performed on the Illumina MiSeq and PacBio sequencing platforms. Functional gene predictions and annotations were performed using GeneMarkS,¹ the Rapid Annotation Subsystem Technology (RAST) database, BLAST,² and UniProt.³ Insertion elements were predicted using Isfinder.

Degradation of the DMF

The strains were amplified and cultured in LB medium until the OD600 reached about 1.5, centrifuged at 6,000 r/min for 3 min, the supernatant was discarded, and the pellet was used further. The pellet was then resuspend with sterile distilled water to prepare the seed solution. The feed water was an inorganic salt medium containing 5,000 mg/L DMF, adjusted pH = 6.0–7.0, which was artificially configured wastewater. The inorganic salt medium was added to the strain treatment tank, adjusted pH = 6.0–7.0, and various sub-liquids were inoculated in the strain treatment tank according to 20% inoculum, and granular activated carbon was added as the strain carrier to enrich and retain the strain. The hydraulic retention time (HRT) of the influent water in the strain treatment tank was controlled to be 3 days, dissolved oxygen (DO) >2.0 mg/L, and water temperature 30–35°C. The primary water quality data in the system were monitored daily, such as COD, NH₃-N, and TN.

Statistical analysis

Every group of the experiments repeated for 4 times, $n=4$. Data were expressed as the mean $\pm$ standard error of the mean (SEM), visualized using GraphPad Prism (8.3.0), one-way analysis of variance (ANOVA) of SPSS (version 25.0), and Dunnett's multiple comparison test for statistical analysis, with significant differences between groups ($p < 0.05$).

Results and discussion

The acclimatization and isolation of the strains

Fresh sludge was used for acclimatization, in which the strains were cultured in an inorganic medium with a DMF concentration of 1,000 mg/L for 1 week, the NH₃-N in the system increased significantly to about 160 mg/L, and the TN ammonification rate reached more than 90%. Four strains of bacteria with DMF degradation effect were obtained by acclimatization culture.

As shown in the results of Figure 1 and Table 1, in the group system of *P. sulfuroxidans* DM175A1-1, both NH₃-N and TN showed a slowly increasing trend in the first 6 days, and the proportion of NH₃-N to TN was basically above 95%; as shown in Table 1, after the 7th day, both NH₃-N and TN data in the treated system were stable at about 780 mg/L, and the proportion of NH₃ to TN was at above 98%. In the system of the *A. lusatiense* 3-1-1, after the 7th day, the NH₃-N data in the treatment system were stable at about 575 mg/L, the TN data were about 780 mg/L, and the proportion of NH₃-N to TN was maintained at about 74%.

In the *G. flavus* 2-CZ-5 experimental system, after the 7th day, the NH₃-N data in the treated system were stable at about 473 mg/L, the TN data at about 780 mg/L, and the proportion of NH₃-N to TN was maintained at about 60%. in the *S. paucimobilis* 3-3-10 experimental system, after the 7th day, the NH₃-N data in the treated system were stable at about 575 mg/L, the TN data at about 780 mg/L, and the proportion of NH₃-N to TN was maintained at about 60%. The NH₃-N data were stable at about 572 mg/L, and the TN data were about 780 mg/L, and the proportion of NH₃-N to TN was maintained at about 73%.

The experimental results of DMF degradation by single bacteria showed that in the four single bacteria experimental systems, all strains could take advantage of DMF effectively and their OD600 is over 1.50, pH in all four systems increased significantly to more than 8.0, and a certain amount of acid should be added daily to adjust pH to about 6. The DMF degradation effect of *P. sulfuroxidans* DM175A1-1 was better than the other three monocultures, and the DMF degradation rate reached more than 90%.

Identification of the strains

After 3 days incubation on LB Petri dishes, the colony morphology was round and raised. The colony surface was smooth and moist. The edge was neat and beige in color, Gram negative, and the physiological and biochemical characteristics were initially identified as *Paracoccus*. 16S rDNA sequence comparison, the similarity between strain DM175A1-1 and *Paracoccus sulfuroxidans* CGMCC 1.5364T was 99%, so the phylogenetic tree was constructed by MEGA software, and strain DM175A1-1 was also closely clustered with *Paracoccus sulfuroxidans* CGMCC 1.5364T. As shown in Figure 2, the phylogenetic tree was constructed by MEGA software, and strain DM175A1-1 was also closely clustered with *Paracoccus sulfuroxidans* CGMCC 1.5364T, so it was identified as *Paracoccus sulfuroxidans* strain.

¹ <http://exon.gatech.edu/GeneMark>

² <https://www.ncbi.nlm.nih.gov/BLAST>

³ www.uniprot.org

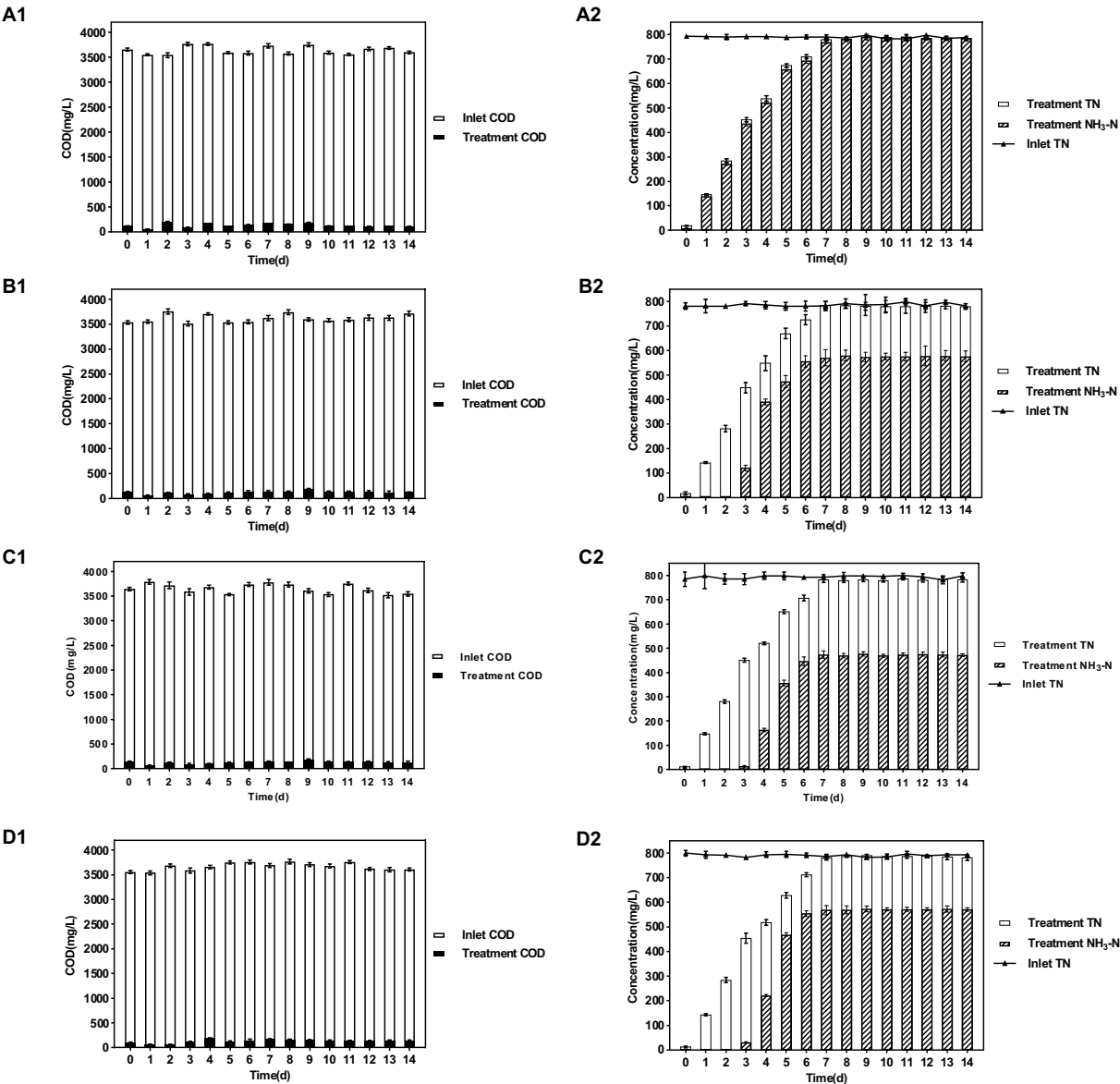


FIGURE 1
Treatment effect of 4 strains on various indicators of wastewater. (A–D) Represent *P. sulfuroxidans* DM175A1-1, *A. lusatiense* 3-1-1, *G. flavus* 2-CZ-5, *S. paucimobilis* 3-3-10, respectively. The number 1 after alphabet represents the COD treatment effect of the strain, number 2 represents the TN treatment effect of the strain and the ammonification effect. Values were displayed as the mean $\pm$ SEM, $n = 4$; the difference between groups was statistically significant ($p < 0.05$).

TABLE 1 Treatment effect of 4 strains on various indicators of wastewater.

Index	<i>P. sulfuroxidans</i> DM175A1-1	<i>A. lusatiense</i> 3-1-1	<i>G. flavus</i> 2-CZ-5	<i>S. paucimobilis</i> 3-3-10
Inlet COD (mg/L)	3,639	3,616	3,655	3,665
Treatment COD (mg/L)	130	139	139	146
Removal rate of COD	96.4%	96.2%	96.2%	96.0%
Inlet TN (mg/L)	790	786	793	791
Treatment TN (mg/L)	786	781	782	786
Treatment NH ₃ -N (mg/L)	786	577	473	571
Ammonification rate	100%	73.8%	60.4%	72.7%

Values were displayed as the mean $\pm$ SEM, $n = 4$; the difference between groups was statistically significant ($p < 0.05$).

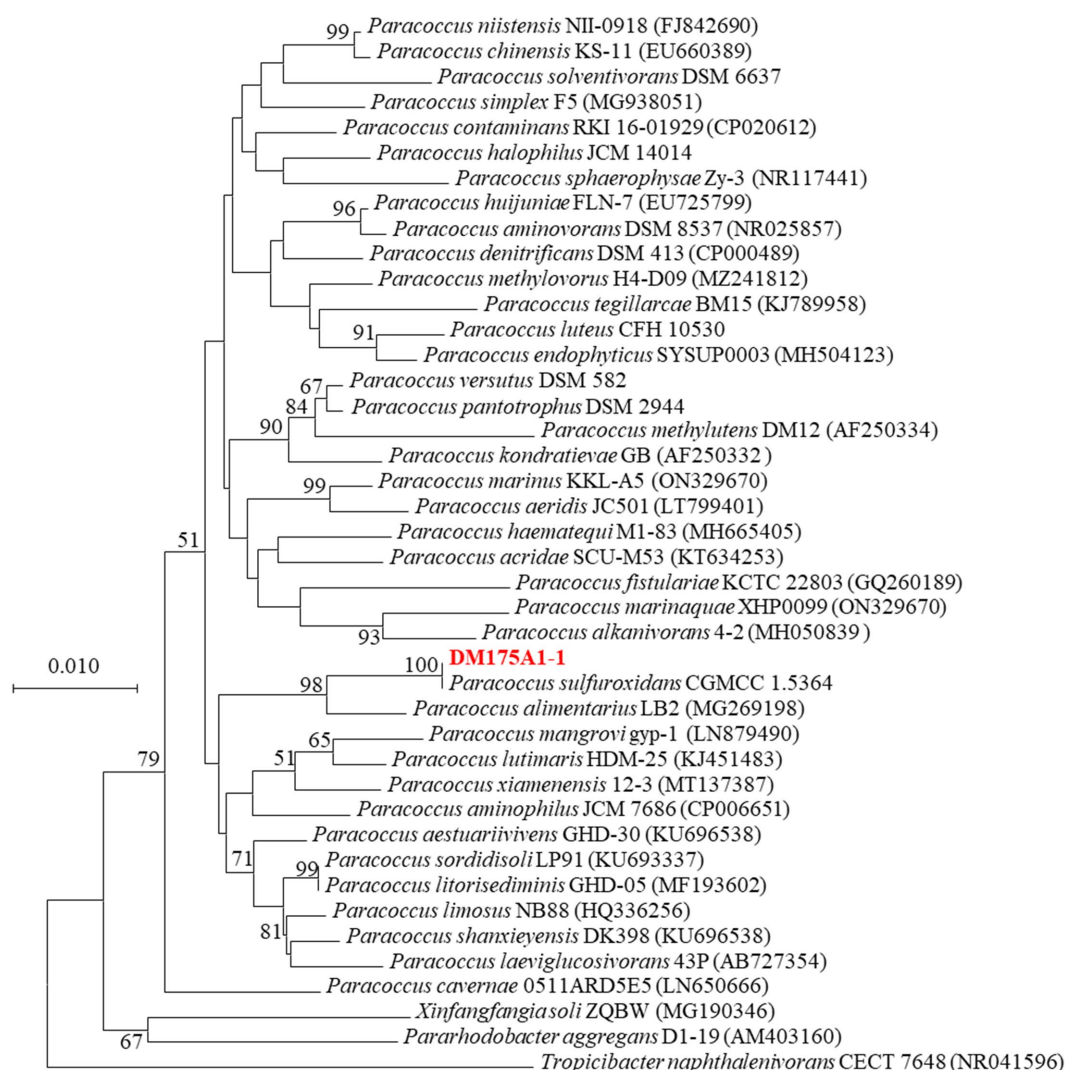


FIGURE 2
Phylogenetic tree of strain *P. sulfuroxidans* DM175A1-1.

Multireplicon genome of strain DM175A1-1

Whole-genome sequencing was performed, and analysis of the data revealed that the genome of strain DM175A1-1 has a multireplicon structure, with a single circular chromosome and two plasmids (Figure 3). The circular chromosome is 3,993,573 bp in size; the plasmid1 is 123,272 bp in size; and the plasmid2 is 46,324 bp in size. The whole length of the genome sequence is 4,163,169, with a G+C content of 64.40%. After the analysis of the function, the CDS results showed that the Gene number is 4,139, and the gene total length is 3,800,551 bp, with a coding percentage of 91.3% (Table 2).

We submitted the genome of the DM175A1-1 to the blast server. *P. aminovorans* JCM 7685, which has a complete DMF degradation pathway, carries two genes, *dmfA1* and *dmfA2*, responsible for encoding the DMFase that initially degrades DMF to DMA and MA (Czarnecki and Bartosik, 2019). The DM175A1-1 also has the *dmfA1* and *dmfA2* genes encoding the DMFase, as shown in Figure 3, so the initial pathway of DM175A1-1 to degrade DMF to DMA and MA may be the same as that of the *P. aminovorans* JCM 7685. Then

P. aminovorans JCM 7685 degrades DMA to formate by the NMG pathway, in which key genes in the NMG pathway are not found in DM175A1-1, so it was hypothesised that other DMA degradation pathways may exist in DM175A1-1 (Czarnecki and Bartosik, 2019). The MA degradation pathway has been reported in *Paracoccus* spp. *P. denitrificans* Pd 1222, MA is degraded to formaldehyde by the action of MA dehydrogenase encoded by *mauA* and *mauB*, and *P. denitrificans* Pd 1222 oxidises formaldehyde to formate by a glutathione-dependent pathway requiring three enzymes, namely S-(hydroxymethyl) glutathione synthetase (*gfa*), S-(hydroxymethyl) glutathione synthase, S (hydroxymethyl) glutathione dehydrogenase (*flhA*) and S-formylglutathione hydrolase (*fghA*) (Czarnecki and Bartosik, 2019; Lu et al., 2019). As shown in Figure 3, the chromosome of DM175A1-1 carries the *mau* gene cluster encoding formaldehyde dehydrogenase, where the cluster contains *mauA* and *mauB*, and three genes in the pathway for further breakdown of formaldehyde to formate, as shown in the Figure 3, *flhA*, *fghA* and *gfa*, respectively. Therefore, the product of the initial degradation of DM175A1-1, MA, can be converted to formaldehyde through the MA dehydrogenase encoded by *mauA* and

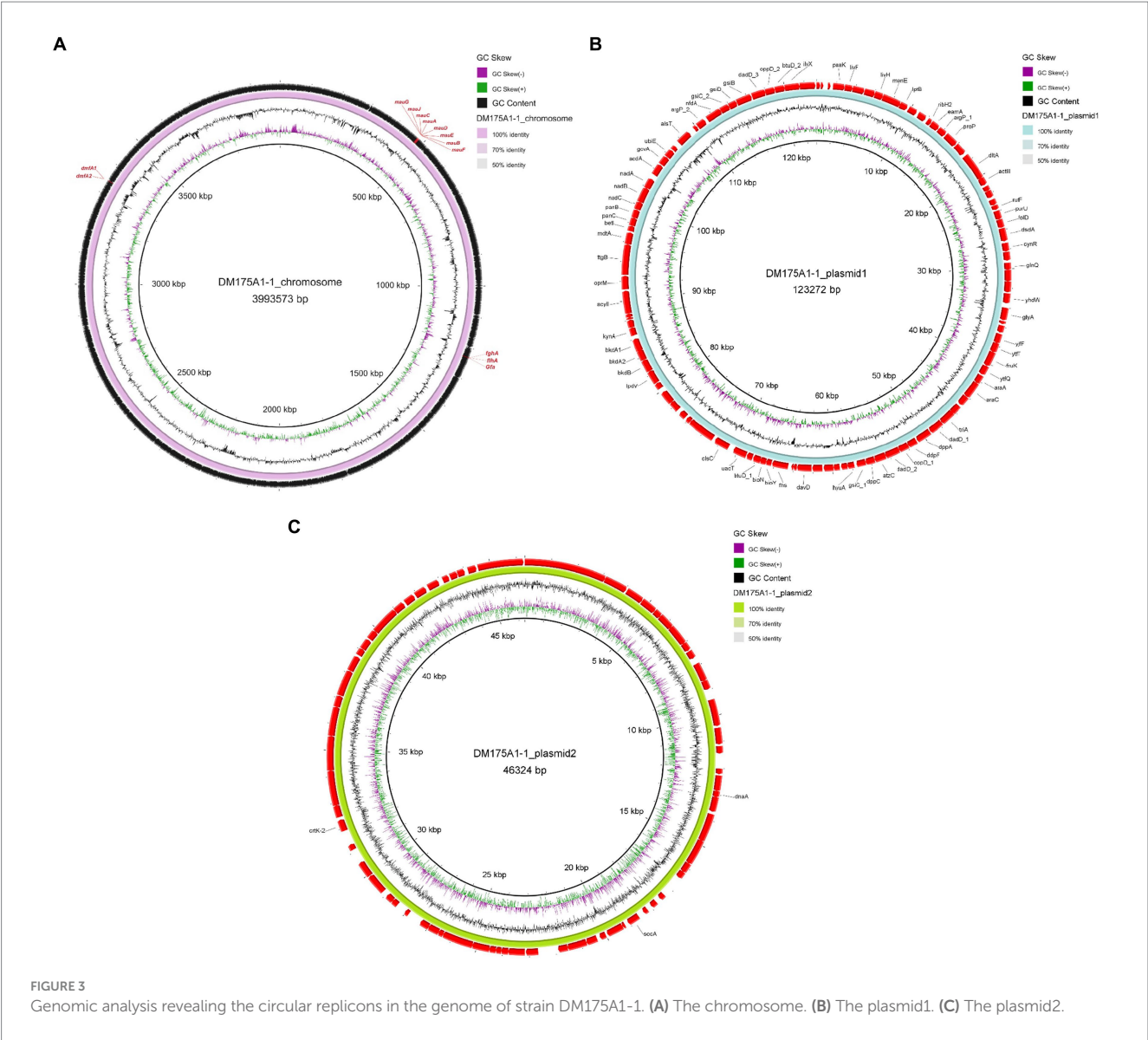


TABLE 2 Gene information statistics.

Sample	DM175A1-1
Gene number	4,139
Gene total length (bp)	3,800,551
Gene average length (bp)	918
Gene density (gene number/Kb)	0.994
GC content in gene region (%)	64.9
Gene/geonme (%)	91.3
Intergenetic region length (bp)	362,618
GC content in intergenetic region (%)	58.8
Intergenetic length/genome (%)	8.71

mauB, which in turn is oxidised to formate through the glutathione-dependent pathway. As DM175A1-1 contains some of the genes in the DMF degradation pathway that have been studied, but also lacks some

of the genes in the degradation pathway, new degradation pathways may exist in DM175A1-1 and are the next direction for further research.

Tolerance test of the *Paracoccus sulfuroxidans* DM175A1-1

To further verify the practical effect of strain DM175A1-1, the DMF concentration in the influent water was increased to 10,000 mg/L for the experiment. The experimental procedure was the same as 1.4.2. The system without the strain was set as the blank group, and the system with the activated sludge (MLVSS of 6,000 mg/L) was set as the control group, and the treatment effects of HRT 3 days and 5 days were investigated. The purpose was to check the tolerance of the strain *P. sulfuroxidans* DM175A1-1 to DMF and compare it with the treatment effect of activated sludge.

The results are shown in Figure 4 and Table 3. When the influent DMF concentration reached 10,000 mg/L, the activated sludge group

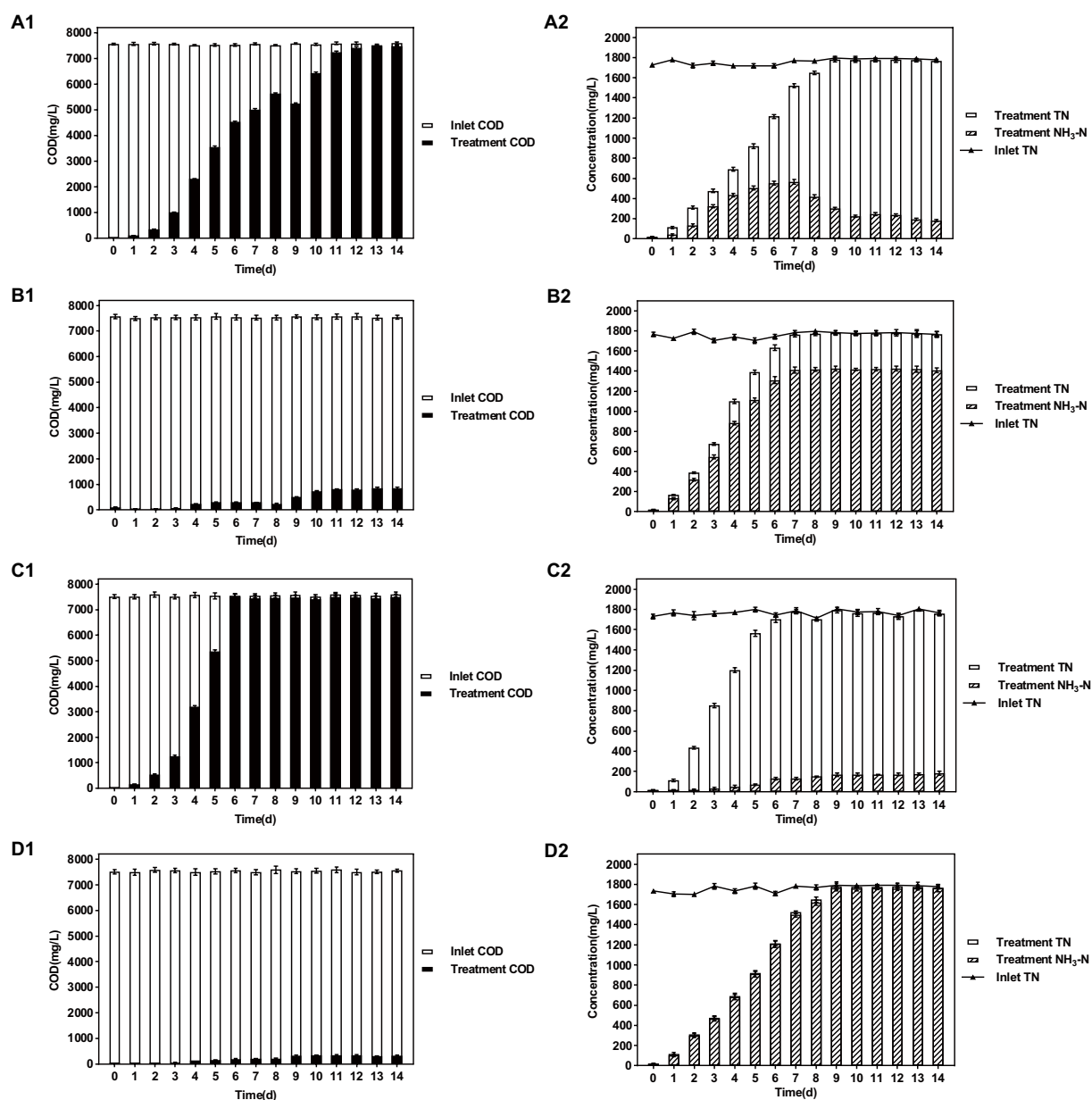


FIGURE 4

Effect of *P. sulfuroxidans* DM175A1-1 on the treatment of high concentration DMF. (A–D) Space Team (HRT 3 days), Action Pollution Team (HRT 3 days), *P. sulfuroxidans* DM175A1-1 Team (HRT 3 days), *P. sulfuroxidans* DM175A1-1 Teams (HRT 5 days), 1,2 represents the COD treatment effect of the strain, the TN treatment effect of the strain, and the ammonification effect. Values were displayed as the mean $\pm$ SEM, $n = 4$; the difference between groups was statistically significant ($p < 0.05$).

decreased in the late ammonification period, probably due to poisoning, and the final ammonification rate was as low as 14.6%. In contrast, the *P. sulfuroxidans* DM175A1-1 strain treatment group (groups C and D) had apparent growth and better activity in the treatment system, with specific higher COD removal capacity and ammonification capacity, and when the HRT was 3 days, the COD removal rate was 91.0%, and ammonification rate was 80.0%; when the HRT was extended to 5 days, the COD removal rate continued to rise to 91.0% and When the HRT was extended to 5 days, the COD removal rate continued to increase to 91.0%, and the ammonification rate reached 99.0%. In conclusion, *P. sulfuroxidans* DM175A1-1 can tolerate DMF concentrations up to 10,000 mg/L, and the conversion efficiency

of organic nitrogen to $\text{NH}_3\text{-N}$ in DMF can reach 99.0% when the HRT is controlled at 5 days.

The treatment of the wastewater based on *Paracoccus sulfuroxidans* DM175A1-1

The wastewater came from a pharmaceutical enterprise in Zhejiang, with a DMF content of about 1.2% (w/v), a water volume of 60 t/d, and wastewater quality, as shown in Table 4. In order to reduce the introduction of nitrogenous substances from the strain medium into the wastewater, the centrifuges are not provided in the

factory, so the strain was fermented with 1/5 concentration of LB medium and was pitched when the fermentation reached about 1.5 OD600 value. The wastewater was supplemented with KH_2PO_4 0.5 g/L, and H_2SO_4 was used to adjust the pH of the wastewater = 6.0.

TABLE 3 Effect of *P. sulfuroxidans* DM175A1-1 on the treatment of high concentration DMF.

Index	A	B	C	D
Inlet COD (mg/L)	7,555	7,550	7,547	7,542
Treatment COD (mg/L)	7,468	6,695	680	308
Removal rate of COD	1.2%	11.3%	91.0%	95.9%
Inlet TN (mg/L)	1,768	1,786	1,781	1,785
Treatment TN (mg/L)	1,758	1,758	1,775	1,758
Treatment $\text{NH}_3\text{-N}$ (mg/L)	182	257	1,420	1,740
Ammonification rate	9.6%	14.6%	80.0%	99.0%

Values were displayed as the mean $\pm$ SEM, $n=4$; the difference between groups was statistically significant ($p<0.05$).

The device is used to handle the wastewater, a filler layer is set at 1/3 of the distance from the bottom of the device, and the filler is preferably granular activated carbon, with a dosage of 10–13% (the volume ratio of filler to the device). The aerobic process was adopted, controlling HRT about 5 days, $\text{DO}>2\text{ mg/L}$, water temperature $30\text{--}33^\circ\text{C}$, and 20% of strain injection. During the strain treatment, the pH of the strain treatment pool will rise to 9.0, and H_2SO_4 needs to be added to consistently control the pH of the strain treatment pool = 6.0–7.0 (optimal 6.0). When the treatment effect is not good, additional carbon sources such as glucose 2–3 g/L can be supplemented or supplemented strains. The primary water quality data in the system were monitored daily.

The results are shown in Figure 5 and Table 5. *P. sulfuroxidans* DM175A1-1 strain treated wastewater with DMF content up to 1.2% and TN about 2,000 mg/L well, and the COD removal rate was nearly 95%, which could basically achieve mineralization; DMF removal rate reached 100%, and combined with COD data, it was presumed that the remaining material was the intermediate product

TABLE 4 Basic water quality of high concentration DMF wastewater.

COD (mg/L)	$\text{NH}_3\text{-N}$ (mg/L)	TP (mg/L)	$\text{NO}_2\text{-N}$ (mg/L)	$\text{NO}_3\text{-N}$ (mg/L)	TN (mg/L)	pH	TDS
10,000	125	0	0	0	2,000	10	0.03%

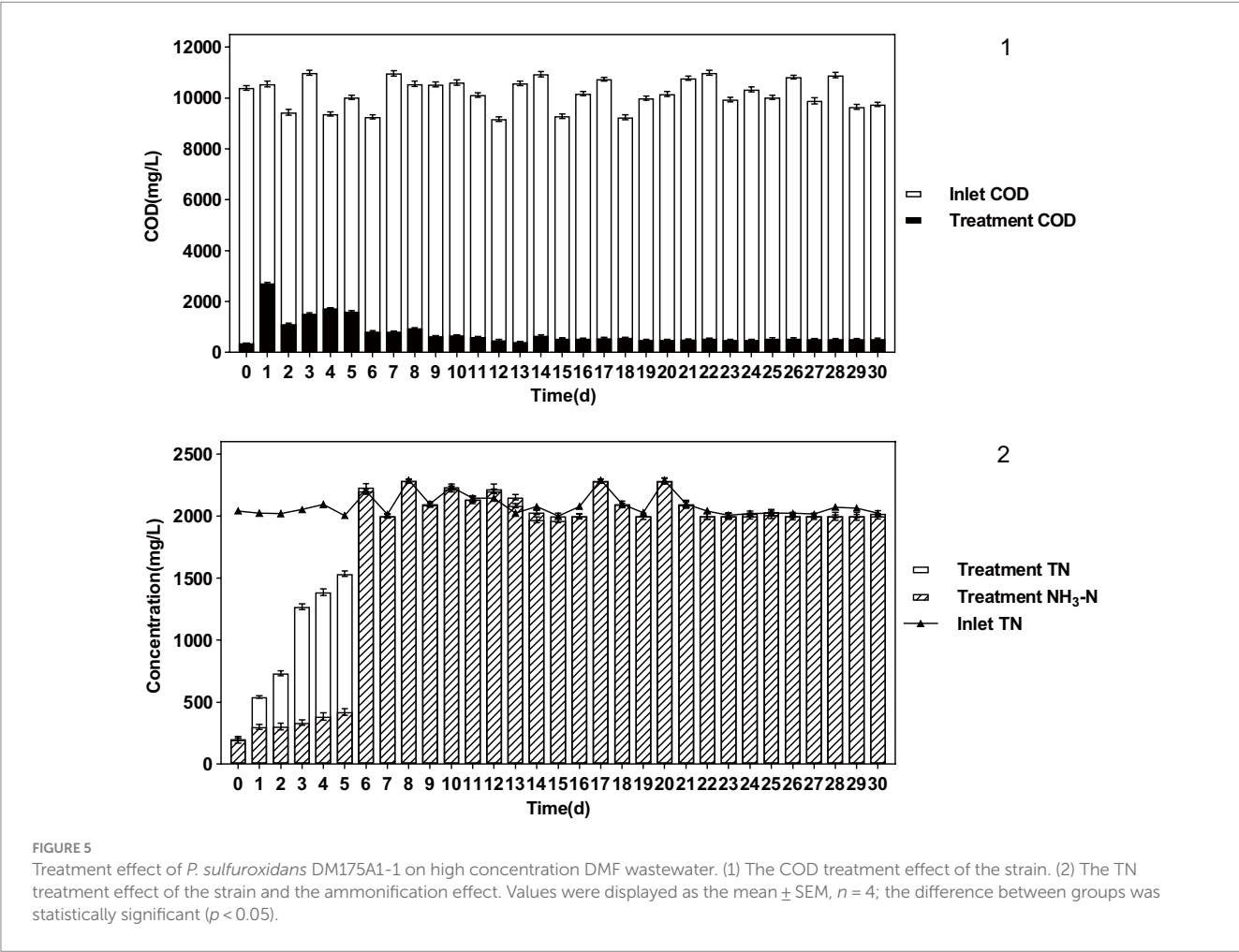


TABLE 5 Treatment effect of *P. sulfuroxidans* DM175A1-1 on high concentration DMF wastewater.

No.	Index	Data
1	Inlet COD (mg/L)	10,232
	Treatment COD (mg/L)	531
	Removal rate of COD	94.81%
2	Inlet DMF (mg/L)	11,700
	Treatment COD (mg/L)	0
	Removal rate of COD	100%
3	Inlet TN (mg/L)	2,079
	Treatment TN (mg/L)	2,058
	Treatment NH ₃ -N (mg/L)	2,056
	Ammonification rate	99.89%

Values were displayed as the mean ± SEM, *n* = 4; the difference between groups was statistically significant (*p* < 0.05).

in the process of DMF degradation; TN Basically, all of the TN was converted into NH₃-N, and the ammonification rate reached 99.89%.

Conclusion

In this study, the DM175A1-1 was identified as *Paracoccus sulfuroxidans*, which were growing with DMF as the only carbon and nitrogen source. Whole-genome sequencing of strain DM175A1-1 revealed that it has a 3.99-Mbp chromosome a 120-kbp plasmid1 and a 40-kbp plasmid2. Through the comparison of the genome to others, we found that the chromosome has the key genes in the initial phase of methylotrophy pathways in the genus *Paracoccus*. Then after the further DMF tolerance degradation experiments, DM175A1-1 can tolerate DMF concentration up to 10,000 mg/L, and the efficiency of organic nitrogen conversion to NH₃-N in DMF can reach 99.0% when the HRT is controlled at 5 days. Meanwhile, the application experiments were conducted for a pharmaceutical enterprise in Zhejiang Province with a high concentration of DMF wastewater, and the wastewater treatment effect was good. The prevailing treatment methods for DMF-containing wastewater are typically biochemical, which are frequently costly and environmentally benign. At present, there are few applications of microbial degradation in industry. This study has successfully demonstrated the degradation of DMF in enterprise applications, providing insights and support for future research. As the specific degradation pathway of this strain has not yet been fully elucidated, future research will focus on elucidating the mechanism and mechanism. This will further deepen the connection between the genome and degradation products, and determine the subsequent degradation mechanism and pathway.

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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

GZ: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. YS: Writing – review & editing, Resources, Investigation. WZ: Writing – review & editing, Resources, Investigation. XL: Writing – review & editing, Resources, Investigation. LS: Writing – review & editing, Resources, Investigation. ZS: Writing – review & editing, Resources, Investigation. DZ: Writing – review & editing, Resources. KW: Writing – review & editing, Validation, Investigation. ZM: Writing – review & editing.

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Conflict of interest

YS and WZ were employed by Trend Biotech Co., Ltd. XL and LS were employed by Zhoushan Sewerage Treatment Co., Ltd. The remaining 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.2024.1419461/full#supplementary-material>

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Fortifying plant fortresses: siderophores in defense against *Cercospora* leaf spot disease in *Vigna radiata* L.

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Siderophores, specialized iron-chelating molecules produced by *Bacillus amyloliquefaciens* D5, were investigated for their role in enhancing plant defense mechanisms against *Cercospora canescens* in mung bean (*Vigna radiata* L.). Siderophores were extracted and purified using Amberlite XAD-4 and applied to plants at concentrations of 5, 10, and 15 µg/mL, followed by pathogen inoculation. The treatments significantly influenced enzymatic activities and defense-related gene expression. On Day 6, peroxidase (POD) activity reached its highest value of 0.563 in the SP15 (siderophore + pathogen at 15 µg/mL) treatment, with S15 (siderophore-only at 15 µg/mL) showing a lower but significant increase of 0.453, while control groups remained unchanged. Polyphenol oxidase (PPO) activity peaked in SP15 (0.10 U/mL), followed by S15 (0.08 U/mL), highlighting the role of these treatments in enhancing stress responses. Chitinase activity was significantly elevated in SP15 on Day 6, with a sustained response through Day 8, while no significant change was observed in the control group. Total phenolic content was highest in SP15 (100 µg/mL), showing a ramified immune response whereas S15 recorded 80 µg/mL, significantly above the control. Gene expression analysis further demonstrated the effectiveness of siderophore and siderophore + pathogen treatments. Catalase expression was upregulated by 21.1-fold in siderophore-only treatment and amplified to 25.9-fold in SP15. Epoxide hydrolase (EH) gene expression increased by 77.3-fold in S15 and further synergized to over 90-fold in SP15. Similarly, PR10 expression showed moderate upregulation in S15 and significantly higher levels in SP15, reflecting enhanced pathogen defense. Calmodulin (CAL) gene expression was moderately regulated in S15 but significantly amplified in SP15. These findings underscore the dual role of siderophores in nutrient acquisition and as potent elicitors of plant defenses, highlighting their potential as bio-stimulants. Field trials are essential to validate these results under natural conditions and optimize their use in agriculture.

KEYWORDS

siderophore, plant growth-promoting rhizobacteria, leaf spot disease, plant defense, induced systemic resistance

Introduction

In the quest for sustainable agricultural practices, researchers continually explore novel strategies to enhance plant growth and defense against pathogens. Among the myriad of approaches, harnessing the potential of plant growth-promoting bacteria (PGPB) has garnered significant attention due to their ability to enhance nutrient availability, suppress pathogens, and induce systemic resistance, making them a cornerstone of sustainable agricultural practices (Ha-Tran et al., 2021). One of the key mechanisms by which PGPR stimulate plant growth is through

the solubilization of mineral nutrients, such as phosphorus and iron, making them more accessible to plants. PGPR produce siderophores, small molecules that chelate iron, making it available to plants in environments where it is limited (Goswami et al., 2016; Vocciante et al., 2022). PGPR also contribute to plant growth promotion through the production of phytohormones, such as auxins, cytokinins, and gibberellins, which regulate various aspects of plant growth and development. These hormones stimulate root elongation, enhance nutrient uptake, and promote the formation of lateral roots, leading to increased root surface area and improved nutrient acquisition efficiency (Santoyo et al., 2021; Jeyanthi and Kanimozhi, 2018; Prasad et al., 2019). Additionally, PGPR-mediated induction of systemic resistance pathways primes plants to defend against pathogen attacks, enhancing their resilience to biotic stresses. Furthermore, PGPR can suppress plant pathogens directly through mechanisms such as competition for nutrients and space, antibiosis, and the induction of systemic resistance in plants. By colonizing the rhizosphere and outcompeting deleterious microorganisms for resources, PGPR produce signaling molecules, including volatile organic compounds and quorum-sensing molecules as well as secondary metabolites which modulate plant gene expression and systemic defense responses. In response, plants secrete specific compounds, such as flavonoids and phenolic acids, that attract beneficial microorganisms to the rhizosphere and enhance their colonization. *Bacillus amyloliquefaciens*, a ubiquitous soil bacterium, has emerged as a promising candidate owing to its multifaceted beneficial effects on plant health. These include enhancing nutrient uptake, producing antimicrobial compounds, inducing systemic resistance pathways, and secreting siderophores that chelate iron, thus improving plant resilience against pathogens (Wu et al., 2024; Márquez et al., 2020).

As a prolific producer of bioactive compounds, this Gram-positive bacterium exerts beneficial effects on plant health through various mechanisms. *B. amyloliquefaciens* enhances plant defense mechanisms against phytopathogens through the production of antimicrobial compounds and the induction of systemic resistance pathways. Notably, the secretion of siderophores, small iron-chelating molecules, plays a pivotal role in the interaction between *B. amyloliquefaciens* and plants. In a study reported by Dimopoulou et al., 2021 the siderophore bacillibactin, produced by *Bacillus amyloliquefaciens* MBI600, under iron-limiting conditions, and its biocontrol activity against both bacterial and fungal pathogens was explored. The findings demonstrate that bacillibactin contributes to antimicrobial activity by inhibiting the growth of *Pseudomonas syringae* pv. *tomato* and enhancing antifungal activity. Additionally, the biosynthesis of bacillibactin is tightly regulated with genes related to microbial competition and fitness, further supporting its role in biocontrol. In comparison, the isolate *Bacillus amyloliquefaciens* D5, identified in our study, also produces significant amounts of siderophores, contributing to its biocontrol properties and potential of inducing systemic resistance in plants against pathogen invaders. Induced systemic resistance (ISR) is triggered by plant growth-promoting rhizobacteria (PGPR) and other microorganisms, primarily through jasmonate- or ethylene-sensitive pathways. This mechanism, which helps plants resist various pathogens, is activated by PGPR strains like *Pseudomonas*, *Bacillus*, and *Trichoderma*. ISR operates similarly to pathogen-induced defense mechanisms but involves complex microbial signals and determinants that remain poorly understood. Future research aims to explore the molecular regulation of ISR by addressing specific questions, such as which microbial signals most effectively trigger resistance and how these signals interact with plant hormonal pathways. Investigating the

temporal dynamics of ISR activation and identifying genetic determinants in both plants and microbes that enhance resistance could guide the development of tailored biocontrol strategies (Pradhan et al., 2023; Luo et al., 2022).

Siderophores are essential for iron acquisition in environments where this vital micronutrient is limited. However, beyond their role in iron uptake, siderophores exhibit diverse functions in plant-microbe interactions, including plant growth promotion and defense mechanisms facilitates iron acquisition from the rhizosphere, thereby alleviating iron deficiency stress in plants (Ghazy and El-Nahrawy, 2021).

Iron, an essential micronutrient for various physiological processes, is often limited in alkaline and calcareous soils, adversely impacting plant growth and development. By solubilizing iron through chelation, siderophores enhance its availability for plant uptake, consequently ameliorating iron deficiency-induced chlorosis and promoting overall plant vigor. Furthermore, siderophores play a pivotal role in modulating the rhizosphere microbiome and enhancing plant resistance against phytopathogens. The competitive advantage conferred by siderophore-producing bacteria enables them to outcompete deleterious microbes for iron, thereby suppressing their proliferation and pathogenicity (Aznar and Dellagi, 2015). Moreover, siderophores exhibit direct antagonistic effects against phytopathogens by sequestering iron essential for their growth and virulence, thus impeding their pathogenicity. Beyond their role in iron acquisition and pathogen suppression, siderophores serve as signaling molecules mediating plant-microbe communication and eliciting systemic resistance responses in plants. Through intricate signaling pathways, siderophores prime plants to mount a more robust defense response upon subsequent pathogen encounters, thereby enhancing their resistance to biotic stresses. Moreover, siderophore-mediated induction of systemic resistance pathways stimulates the production of secondary metabolites and phytohormones associated with plant defense, further fortifying plants against pathogen invasion. This study aims to investigate the role of siderophores extracted from *Bacillus amyloliquefaciens* D5 as elicitors of plant defense mechanisms and enhancers of resilience under biotic stress conditions. It focuses on evaluating their ability to activate defense enzymes, regulate gene expression, and influence protein expression associated with systemic resistance pathways in *Vigna radiata*. By elucidating these biochemical and molecular responses, the research highlights the multifaceted potential of *Bacillus amyloliquefaciens*-derived siderophores in suppressing phytopathogens through rhizosphere modulation, and inducing systemic resistance. These findings highlight the significance of siderophores as eco-friendly elicitors and bio-stimulants, offering sustainable and effective strategies for agricultural biotechnology.

Materials and methods

Bacterial and fungal strain

In this study, *Bacillus amyloliquefaciens* D5, previously isolated from a soil sample collected from the Vagadkhol area, was used. The strain was identified through 16S rRNA sequencing, and its taxonomic position was confirmed using a phylogenetic tree constructed with closely related sequences. The culture has been submitted to GenBank with the accession number OQ536061, providing a validated reference for its identity.

The fungal strain used was *Cercospora canescens* MTCC no. 10835. The culture was routinely maintained on Potato Dextrose Agar.

Extraction and purification of siderophore

Siderophore production by *Bacillus amyloliquefaciens* D5 was carried out in an iron-depleted succinic acid medium containing potassium phosphate, magnesium sulfate, ammonium sulfate, and succinic acid. Cultures were inoculated at an initial density of 10⁶ CFU/mL and incubated at 28 ± 2°C with continuous agitation (120 rpm) for 24–48 h. Optimization of production conditions, including pH, temperature, and iron concentration, was performed prior to purification. Following incubation, the culture supernatant was harvested by centrifugation (5,000 rpm, 15 min), and siderophore content was quantified using the Chrome Azurol S (CAS) assay. The CAS-positive supernatant was concentrated using a rotary vacuum evaporator at 50°C (pH 6.0). Purification was performed using an Amberlite XAD-400 resin column prepared and activated as described by Sayyed et al. (2006) and Nithyapriya et al. (2021). The supernatant was loaded onto the column at a flow rate of 1–2 mL/min until column saturation, indicated by browning. The column was sequentially washed with distilled water, and siderophores were eluted using ethyl acetate as a solvent (Budzikiewicz, 1993). Filtrate, water wash, and eluted fractions were collected and screened for siderophore activity using the CAS assay.

Siderophore induced biochemical enzyme activity

The biochemical enzyme activity induced by siderophores was evaluated using *Vigna radiata* (mung bean) seedlings. Plants were grown in pots under controlled environmental conditions and pre-treated with varying concentrations of *Bacillus amyloliquefaciens* D5-derived siderophores (5 µg/mL, 10 µg/mL, and 15 µg/mL) to assess dose-dependent effects on enzyme activity. Foliar treatment of siderophore solution at different concentrations was applied to each plant. Control treatments included untreated plants (no siderophore application) and pathogen-only treatments, where plants were inoculated with *Cercospora canescens* without prior siderophore treatment. Siderophore application occurred 24 h before pathogen inoculation. Enzyme activity was monitored from Day 0 to Day 8 post-inoculation. The inoculum was prepared by suspending *C. canescens* spores in a sterile solution, with a final concentration of 10⁶ spores/mL used for pathogen inoculation. The treatment groups were clearly defined as follows:

Treatments	Description
C – Control	Untreated plant
CP – Control + Pathogen	Pathogen Inoculation
S5-Siderophore (5 µg)	Siderophore only (5 µg)
SP5 – Siderophore (5 µg) + Pathogen	Siderophore (5 µg) challenge inoculated with Pathogen
S10– Siderophore (10 µg)	Siderophore only (10 µg)
SP10- Siderophore (10 µg) + Pathogen	Siderophore (10 µg) challenge inoculated with Pathogen
Siderophore (15 µg)	Siderophore only (15 µg)
SP15- Siderophore (15 µg) + Pathogen	Siderophore (15 µg) challenge inoculated with Pathogen

Each treatment group consisted of 10 plants, and all experiments were conducted in triplicate to ensure statistical validity. This experimental design enabled the assessment of the effect of siderophore pre-treatment on plant defense enzyme activity in response to pathogen infection.

Peroxidase activity

Peroxidase activity was measured as the oxidation of guaiacol using hydrogen peroxide as substrate at 470 nm as described by Hammerschmidt et al. (1982). One gram of plant tissue was homogenized in 2 mL of 0.1 M phosphate buffer (pH 7.0) at 4°C. The homogenate was centrifuged; the supernatant collected was used as enzyme source. The reaction mixture consisted of 0.5 mL of enzyme extract, 1.5 mL of 0.05 M pyrogallol, 1.5 mL of 1% H₂O₂ was incubated at room temperature. Changes in the absorbance at 470 nm were recorded at 30s interval for 3 min. Activity was expressed as the increase in absorbance at 470 nm/min/g of fresh tissue.

Polyphenol oxidase activity

Polyphenol oxidase (PPO) activity was determined by Am (1954) using catechol as substrate. Formation of yellow colored product benzoquinone by the oxidation of catechol as substrate was measured at 495 nm. Reaction mixture consisted of 0.5 mL of enzyme extract, 1.5 mL of 0.1 M Sodium phosphate buffer (pH 6.5); to start the reaction 200 µL of 0.01 M catechol was added. Enzyme activity was expressed as units/mL.

Chitinase activity

The modified method of Shimahara and Takiguchi (1988) was followed to prepare colloidal chitin. 10 g of chitin flakes from shrimp cells was mixed with 200 mL of concentrated HCL with continuous stirring at chilled temperature overnight. The mixture was then filtered through muslin cloth and dropped into 600 mL of chilled ethanol with rapid stirring on ice. The colloidal chitin was then collected and centrifuged at 8000 g for 30 min at 4°C. The pellet was then washed with distilled water till the pH was neutral. The filtrate was again filtered with whatman filter paper 1.0 and washed until the washing solution was neutral. The colloidal chitin was then stored at 4°C (Joe and Sarojini, 2017; Urja Pandya et al., 2014). Homogenize plant tissues using 0.1 M sodium citrate buffer, pH 5.0, centrifuge the homogenate at 13,000 rpm for 20 min, and collect the supernatant. As per Boller and Mauch (1988), colorimetric chitinase assay was carried out using colloidal chitin as substrate. 0.4 mL of supernatant was mixed with 10 µL of 1 M sodium acetate buffer (pH 4.0) to that 10 mg of colloidal chitin was added incubate the reaction mixture at 37°C for 2 h. Centrifuge the mixture to terminate the reaction at 8000 rpm for 5 min. 0.5 mL of supernatant was taken in a fresh tube to that 50 µL of 1 M phosphate buffer; pH 7.1 was added and incubated with 2 mL of dimethyl amino benzaldehyde (DMAB) for 220 min at 37°C. The absorbance was measured at 585 nm; the enzyme activity was expressed as Units/mL.

Phenylalanine ammonia lyase activity

One gram of plant tissue was homogenized with 3 mL of chilled 0.1 M sodium borate buffer (pH 7.0) containing 1.4 mM β -mercaptoethanol and 0.1 g of polyvinylpyrrolidone (PVPP). The enzyme extract was filtered and centrifuged at 13,000 rpm for 20 min. 0.4 mL of supernatant was incubated with 0.5 mL of 0.1 M borate buffer (pH 8.8) and add 0.5 mL of 12 mM L-phenylalanine for 30 min at 30°C. Phenylalanine ammonia lyase activity was measured based on trans-cinnamic acid formation at 290 nm using L phenylalanine as substrate. The enzyme activity was expressed as Units/mL (Dickerson et al., 1984).

Total phenols

Total phenols were estimated according to Zieslin and Ben-Zaken (1993). One gram of homogenized tissues of plant seedlings were macerated in 80% of methanol and agitated for 15 min at 70°C. In 1 mL of methanolic extract, 5 mL of distilled water was added to that 250 μ L of 1 N Folin-Ciocalteu's phenol Reagent (FCR) was mixed and kept the reaction mixture at 25°C. Measurement of blue color developed was read at 650 nm.

SDS PAGE

Leaf proteins were extracted using an extraction buffer containing 1% SDS, 0.1 M Tris-Cl (pH 6.8), 2 mM EDTA- Na_2 , 20 mM DTT, and 2 mM PMSF. The homogenate was transferred to tubes and centrifuged at 15,000 rpm for 5 min. The supernatant was precipitated using equal volumes of 20% TCA-acetone. Leaf tissue (1 g) was homogenized in 4 mL of 10% (w/v) TCA-acetone, kept on ice for 5 min, and centrifuged again at 15,000 g for 5 min. The supernatant was discarded, and the pellet was washed with acetone until colorless. The pellet was dried and dissolved in SDS buffer (0.5% SDS, 50 mM Tris-Cl pH 6.8, 20 mM DTT) (Niu et al., 2018). Protein content was determined using the Bradford method (Bradford, 1976). The protein was stored at -80°C until further use. Pellets were dissolved in a buffer containing 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol, 0.25% bromophenol blue, and 63 mM Tris-HCl (pH 6.8), and heated at 95°C for 10 min. Twenty micrograms of protein from different treatments were mixed with 5 μ L of sample buffer in a microfuge tube and boiled for 10 min at 95°C. Samples containing equal amounts of protein were loaded into the wells of polyacrylamide gels (Bio-Rad System). Broad-range molecular weight markers (Bangalore Genei, India) were used, and electrophoresis was carried out at a constant voltage of 75 volts for 2 h. The gels were stained with 0.2% Coomassie Brilliant Blue (R250) solution.

Native PAGE

The isoform profile of PO and PPO were examined by native PAGE (Laemmli, 1970). For all treatments, plants were pre-inoculated with siderophores 24 h before pathogen inoculation. Plant samples for biochemical enzyme assays (PO and PPO activity) were collected on Day 6 post-pathogen inoculation, which corresponds to the peak of enzyme activity. Plant samples were collected at the 6th day of pathogen challenge for PO and PPO, respectively, from 4 treatments

C- Control, P – Pathogen, S- siderophore (50 μ g) and siderophore (50 μ g) + pathogen. The protein extract was prepared by homogenizing 1 g of plant sample in 2 mL of 0.1 M sodium phosphate buffer pH 7.0 and centrifuged at 13,000 g for 25 min at 4°C. After electrophoresis, PO isoforms were visualized by soaking the gels in staining solution containing 0.05% benzidine and 0.03% H_2O_2 in acetate buffer (20 mM, pH 4.2) (Nadolny and Sequeira, 1980). For assessing PPO isoforms profile, the gels were equilibrated for 30 min in 0.1% p-phenylenediamine followed by addition of 10 mM catechol in the same buffer (Jayaraman et al., 1987).

Pre-treatment of plants for molecular analysis

Foliar treatment of 50 μ g/mL of purified siderophore was given to the mung bean plants prior to *Cercospora canescens* inoculation. The fungal inoculation was done at concentration of 10^6 spores/mL for inoculation. Four treatments as *C. canescens* only, Siderophore only, Siderophore with *C. canescens* and Control (untreated) were maintained. Plants (3 plants from each replication) were uprooted at 6 days post-inoculation (dpi) and used for further PCR analysis.

Reverse transcription PCR

Total RNA was isolated from the treatments manually by Trizol C method. The cDNA synthesis was carried out using the Mastercycler X40 thermo cycler by Eppendorf and Quantbio qscript cDNA synthesis kit which included nuclease free water, qScript reaction mixture and qScript RT (Reverse transcriptase). 5 μ L of RNA sample was added to the mixture at concentration of 1 μ g and the total reaction mixture was of 20 μ L. The primers for various defense-related genes were designed using the sequences of mung bean defense genes available in the NCBI database (Dubey et al., 2018). The gene-specific primers (Table 1) were used for quantitative PCR analysis. The actin gene was used as the housekeeping gene for the normalization of the expression data (Dubey et al., 2018; Azeem et al., 2021; Nivya and Shah, 2024).

Quantitative real time PCR

The real-time PCR was conducted using the StepOnePlus Real-Time PCR System (Applied Biosystems). Each reaction included 10 μ L of RealQ Flex 2X Master Mix, 0.4 μ L each of forward and reverse primers (Primer A and Primer B), 0.3 μ L of ROX dye, and 7.9 μ L of PCR grade water, with 1 μ L of cDNA template added to initiate the PCR process. The ROX dye was diluted from a stock concentration of 200 μ M to 300 nM by a 1:10 dilution in PCR grade water, resulting in a final volume of 5 μ L per 50 μ L of ROX dilution. The PCR conditions for PR10, epoxide hydrolase, calmodulin and catalase genes were: initial denaturation at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s, extension at 62°C for 20 s, and plate reading at 62°C. For the calmodulin gene, the conditions were: initial denaturation at 95°C for 4 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and plate reading at 62°C. Each treatment sample was run in triplicate. The relative expression ratios of the

TABLE 1 List of primers for PCR analysis.

Genes	Accession number	Primers	Amplicon size
PR-10	AY792956.1	F-GACGAGGCAAACCTTGGGATA	217
		R-CAGCCTTGAAAAGTGCATCA	
Epoxide hydrolase	HQ316148.1	F-AACTGGGGGCTCAACTACT	243
		R-TCCTCTGCAGCTTCTTGGTT	
Catalase	D13557.1	F-AGTTCCTCCCATACCTCCTGCT	219
		R-GAGAACGGTCAGCCTGAGAC	
Calmodulin	DQ778070.1	F-AACAAGGAGGTCGTGGTGTC	300
		R-ATGCCGATCACAACAACA	
Actin	AF143208.1	F-TCGTGTGGCTCCTGAAGAAC	230
		R-AGATTGCATGTGGAAGGGCA	

defense genes were normalized using actin as a reference gene. The crossing point (C(T)) values of each sample were compared to a control sample (mock-inoculated) to determine relative expression (Patil et al., 2021; El-Maraghy et al., 2020).

Statistical analysis

For each of the investigated biochemical parameters from control and treated samples, three separate replication sets were conducted. All the experimental measurement values were expressed as means of three measurements ± standard error. The significance of the differences between the mean values of control and treatments were evaluated using ANOVA and *post hoc* test Dunnett's Multiple Range Test and Tukey's test using GraphPad Prism 5 software.

Results and discussion

Siderophore induced biochemical enzyme activity

The effects of siderophore treatments on biochemical enzyme activities and their role in plant defense against *C. canescens* were assessed.

Total phenols increased in all treatments except the control (untreated), with the highest values observed in the S15 and SP15 treatments (80 and 100 µg/mL, respectively) on Day 6. The SP15 treatment exhibited a synergistic effect of siderophore and pathogen, leading to significantly enhanced phenol production, likely due to a combination of pathogen-induced and siderophore-mimicked immune responses. The peroxidase activity was highest in SP15 (0.563 units on Day 6), reflecting an induced defense response against *C. canescens* invasion. Siderophore treatments, particularly at higher concentrations (S10, S15), resulted in significant increases in peroxidase activity, while the control remained unchanged, as peroxidase is constitutively active under normal conditions.

Polyphenol oxidase (PPO) activity was highest in SP15 and S10 (0.1 and 0.08 U/mL, respectively), with peak levels observed on Day 6 and Day 8. This increase suggests that PPO plays a key role in the plant's defense mechanism against stressors like *C. canescens*, with siderophores enhancing the availability of substrates for PPO activity. Phenylalanine ammonia lyase (PAL) activity peaked in the SP15 group

(100 U/mL) on Day 2, and increased progressively in the S10 and S15 treatments. PAL activity, which is vital for synthesizing antimicrobial compounds, was upregulated in response to both the siderophore and pathogen, indicating an enhanced defense response.

Chitinase activity was highest in the combined treatment (Siderophore + *C. canescens*) on Day 4, with sustained elevation observed through Day 8. This suggests a synergistic interaction between siderophores and the pathogen, enhancing chitinase-mediated defense mechanisms and also indicate a potential crosstalk of defense pathways activated by both siderophore and pathogens. Overall, these results demonstrate that siderophores, both individually and in combination with pathogens, can induce significant defense-related biochemical changes in plants, with higher concentrations showing more pronounced effects. This study supports the potential of siderophore applications for enhancing plant resistance to pathogens. Similar findings were reported by Ashajyothi et al. (2023), where purified hydroxamate siderophores from *Pseudomonas putida* B25 induced increased peroxidase, PPO, and phenol levels in response to *Magnaporthe oryzae* infection in rice (Figures 1–5).

SDS PAGE for protein expression analysis

The protein banding patterns was studied in various treatments treated with only siderophores and in presence of pathogen along with positive control (untreated plant) and negative control (pathogen only). The result revealed the presence of 80 KDa; 32-46KDa; 25KDa, 22KDa bands in Control, 80KDa, 46-50KDa; 32-46KDa; 25KDa, 22KDa, 17KDa bands in Pathogen treatment, Siderophore – 46-50KDa; 32-46KDa; 25KDa, 22KDa, 17KDa and Siderophore + Pathogen– 46-50KDa; 32-46KDa; 25KDa, 22KDa, 17KDa. Additional bands were observed in pathogen treated plants at 45-50Kda and 17Kda (Figure 6). Similar banding patterns to pathogen were observed in siderophore treated plants indicating some proteins may be broadly induced in response to different elicitors as part of a general stress response mechanism in plants. These stress-responsive proteins may be commonly detected in samples treated with siderophores or pathogens, contributing to the observed similarity in protein bands. The response to siderophore treatment may involve cross-talk between defense mechanisms triggered by pathogen recognition and nutrient signaling pathways activated in response to siderophores (Devi et al., 2024a, b). This cross-talk could lead to convergence in protein expression patterns observed in

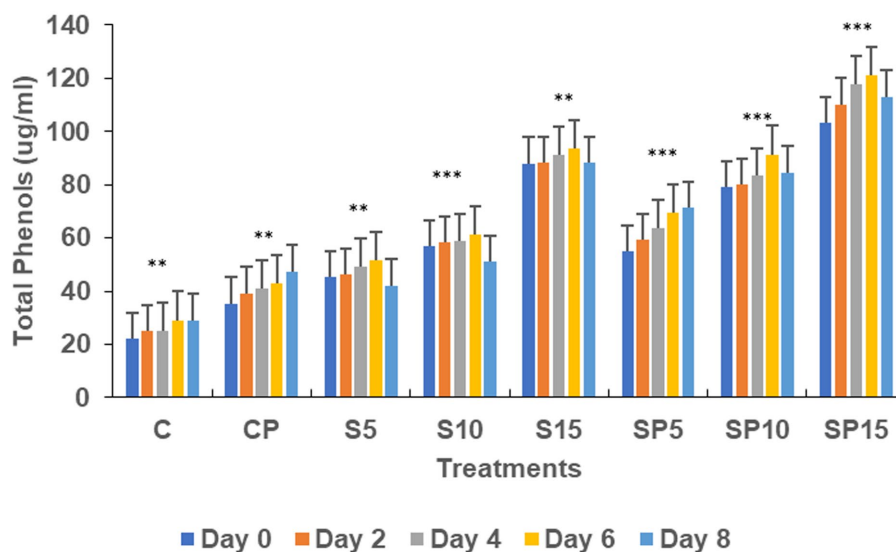


FIGURE 1

Total phenols under various treatments. Statistical analysis was determined using ANOVA followed by Dunnett's Multiple Range Test. C, Control; S, Siderophore (5, 10, 15 μ g); SP, Siderophore + Pathogen (5, 10, 15 μ g). The asterisks indicate significant differences relative to the Control group (C): ns = $p > 0.05$, $p \leq 0.05$, $p \leq 0.01$, $*p \leq 0.001$.

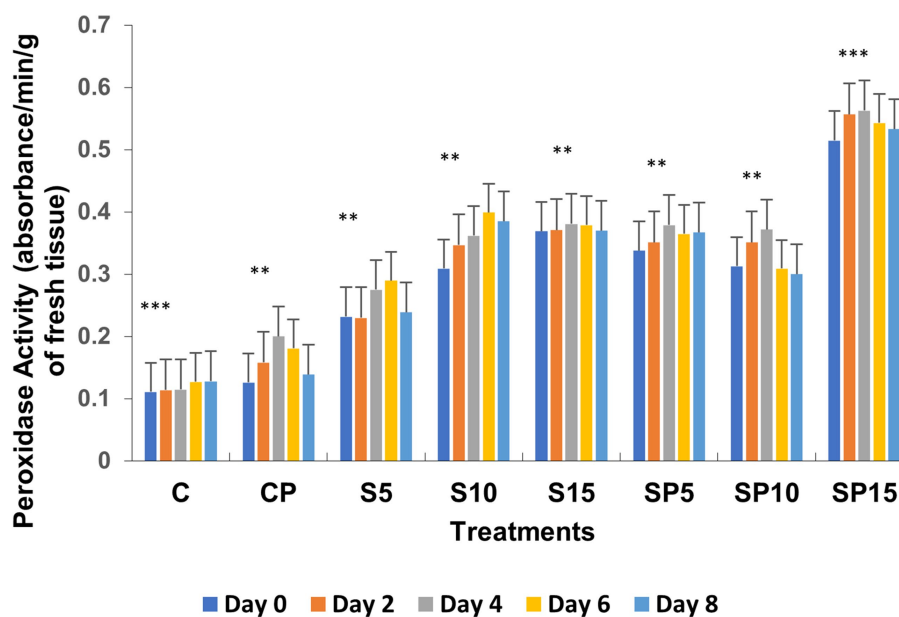


FIGURE 2

Peroxidase activity under various treatments: C, Control; S, Siderophore (5, 10, 15 μ g); SP, Siderophore + Pathogen (5, 10, 15 μ g). Statistical significance was determined using ANOVA followed by Dunnett's Multiple Range Test. The asterisks represent comparisons relative to the Control group (C): ns = $p > 0.05$, $p \leq 0.05$, $p \leq 0.01$, $*p \leq 0.001$.

siderophore-treated and pathogen-treated sample. Similar banding pattern was observed in siderophore + pathogen treatment indicating that siderophores and pathogens have synergistic effects on plant responses, leading to similar changes in protein expression or modification. The combination of siderophores and pathogens may potentiate certain defense mechanisms, resulting in overlapping banding patterns compared to individual treatments. Siderophores and pathogens may activate overlapping signaling pathways in plants.

These pathways could converge on shared downstream targets, leading to similar changes in protein expression or modification in response to siderophore + pathogen treatment as observed in individual treatments. The SDS-PAGE analysis of protein in biocontrol agents treated plant showed eight proteins with molecular weight of 14, 23, 30, 35, 45, 50, 60, and 98 kDa. In healthy plants only six proteins (excepting 14 and 98 kDa) appeared in biocontrol agents treated plant two additional new proteins appeared (Ganeshamoorthi et al., 2008).

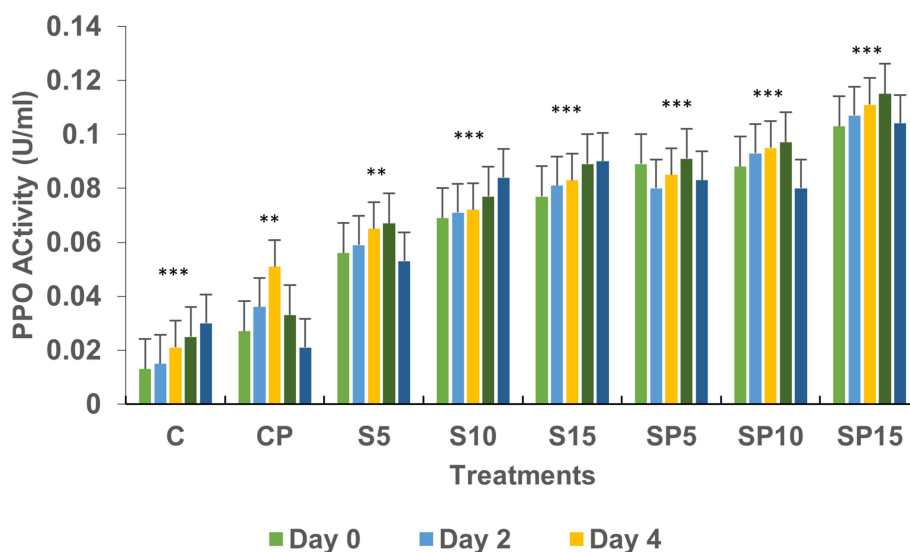


FIGURE 3

Polyphenol oxidase activity under various treatments: C, Control; S, Siderophore (5, 10, 15 μ g); SP, Siderophore + Pathogen (5, 10, 15 μ g). Statistical significance was determined using ANOVA followed by Dunnett's Multiple Range Test. Asterisks represent comparisons relative to the Control group (C): ns = $p > 0.05$, $p \leq 0.05$, $p \leq 0.01$, $*p \leq 0.001$.

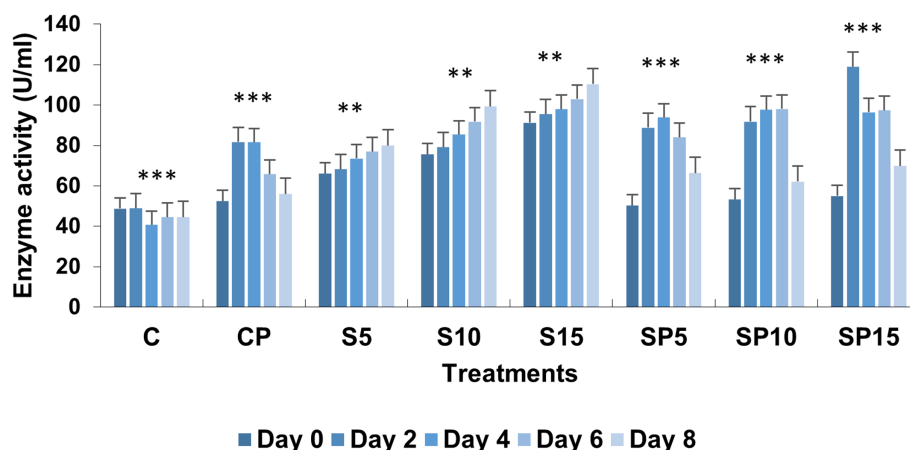


FIGURE 4

Phenylalanine ammonia lyase activity under various treatments: C, Control; S, Siderophore (5, 10, 15 μ g); SP, Siderophore + Pathogen (5, 10, 15 μ g). Statistical significance was determined using ANOVA followed by Dunnett's Multiple Range Test. Asterisks indicate comparisons relative to the Control group (C): ns = $p > 0.05$, $p \leq 0.05$, $p \leq 0.01$, $*p \leq 0.001$.

Native PAGE

The Peroxidase (PO) and Polyphenol Oxidase (PPO) isoforms were studied for the treatments Control, Pathogen, Siderophore (50 μ g) and Siderophore + Pathogen. Similar isoform banding was observed in the treatments that is PPO isoform 1, PPO isoform 2 and PPO isoform 3. Some isoforms of PPO may be constitutively expressed in plants where they are present under normal, untreated conditions (control). These isoforms may play roles in various physiological processes, such as defense against herbivores or pathogens, or in the oxidation of phenolic compounds involved in plant development.

Pathogen infection, siderophore treatment, or their combination may induce changes in PPO expression levels or activity as part of the plant defense responses. However, these treatments may not necessarily result in the expression of entirely new isoforms of PPO. Instead, they may modulate the expression levels or post-translational modifications of existing isoforms. PO isoforms 1, 2 were expressed in all treatments but PO isoform 3 was only found in control treatment as each peroxidase isoform may have a distinct role in responding to different types of stress. The treatment applied may only activate two of the isoforms observed in the control because they are more directly involved in responding to the specific stressor introduced by the

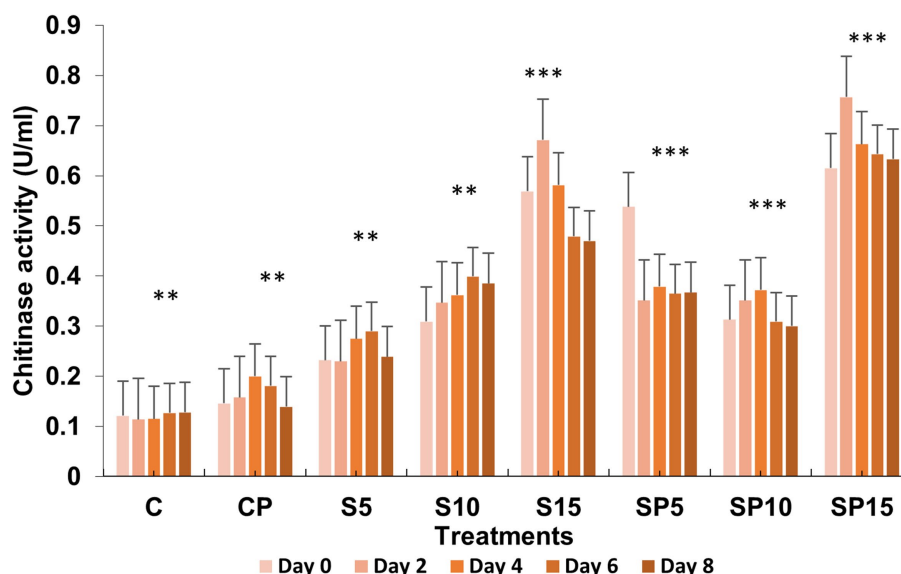


FIGURE 5

Chitinase activity under various treatments: C, Control; S, Siderophore (5, 10, 15 μ g); SP, Siderophore + Pathogen (5, 10, 15 μ g). Statistical significance was determined using ANOVA followed by Dunnett's Multiple Range Test. Asterisks denote comparisons relative to the Control group (C): ns = $p > 0.05$, $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$.

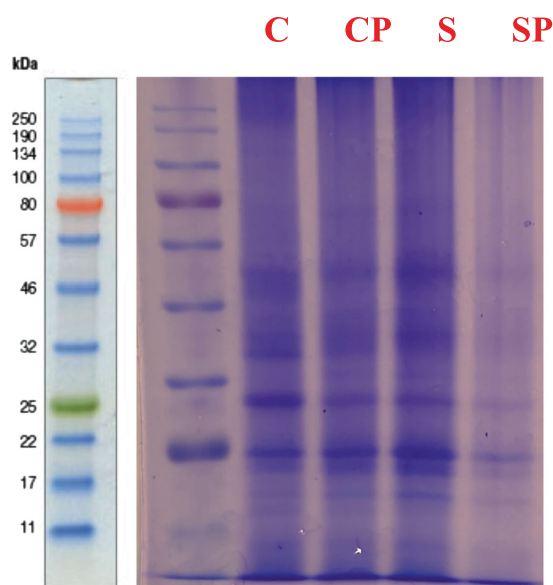


FIGURE 6

SDS PAGE analysis of proteins under various treatments; C, Control; P, Pathogen only; S, siderophore only; SP, siderophore + pathogen.

treatment, while the third isoform may be less relevant to the treatment-induced stress response (Figure 7). The native gel electrophoresis of enzyme extracts from biocontrol agent-treated plants revealed that the combination treatments of biocontrol agents led to higher induction of peroxidase (PO) and polyphenol oxidase (PPO) isoforms. Specifically, the combination of *Pf1* + *Py15* + *Bs16*, *Pf1* + *Bs16*, and *Pf1* + *Py15* resulted in stronger induction of both PO

and PPO isoforms. In contrast, the inoculated control treatment showed lesser induction of PO isoforms, while the healthy and inoculated control treatments exhibited mild induction of PPO isoforms with lower intensity. This suggests that the combination of the three biocontrol agents is more effective in enhancing enzyme activity and isoform expression in mulberry plants (Ganeshamoorthi et al., 2008).

Quantitative PCR

Catalase-mediated detoxification of ROS helps plants to limit the spread of pathogens by preventing oxidative damage to host cells. Additionally, the production of ROS by the plant in response to pathogen recognition can act as a signaling molecule to activate defense pathways and reinforce the plant immune response (Dubey et al., 2018). In the absence of any treatment or stressor, the *CAT* gene expression remains at baseline levels. This indicates that under normal conditions, the plant maintains a steady-state level of *CAT* expression necessary for basic cellular functions. The upregulation of *CAT* gene expression by approximately 4.4-fold in response to pathogen treatment suggests a significant induction of the antioxidant defense system. Pathogen infection often triggers the production of ROS as part of the plant defense response, leading to oxidative stress. The substantial upregulation of catalase gene expression by approximately 21.1-fold in response to siderophore treatment indicates a strong activation of the antioxidant defense mechanism. Siderophores are iron-chelating compounds that can induce oxidative stress in plants due to their ability to generate ROS as byproducts. The significant increase in *CAT* expression suggests that plants respond robustly to siderophore-induced oxidative stress by enhancing ROS detoxification capacity through

catalase-mediated pathways. The synergistic effect observed in the combined treatment of siderophore and pathogen, resulting in a further upregulation of catalase gene expression by approximately 25.9-fold, suggests an amplified activation of catalase expression. Lipid peroxides are generated as by-products of oxidative stress, particularly under conditions of pathogen infection, drought, or high light intensity. Epoxide hydrolases participate in the metabolism of lipid peroxides, facilitating their conversion into less

reactive and cytotoxic compounds. This process helps to alleviate oxidative damage and maintain cellular homeostasis under stress conditions (Tripathi et al., 2023).

The expression of the *EH* gene is highly overexpressed in response to pathogen treatment as part of the plant defense response to pathogen infection. The expression of the *EH* gene is moderately upregulated approximately 77.3 times in response to siderophore treatment compared to the control condition while not as pronounced as the response to pathogen treatment, this increase indicates a significant induction of gene expression in response to siderophore application, possibly as a protective and adaptive response to siderophore-induced stress.

The combined treatment of siderophore and pathogen leads to a very high expression or upregulation of *EH* gene as compared to control condition. This synergistic effect suggests an intensified response to the combined stressors resulting in a more pronounced induction of gene expression compared to either treatment alone. Following an inducing treatment, plant resistance may manifest in three ways: (i) defenses are activated immediately with no further change after pathogen challenge, (ii) initial defenses are amplified or diversified upon pathogen challenge, or (iii) defenses remain dormant until the pathogen triggers their activation (Walters et al., 2005).

Calcium/calmodulin-dependent protein kinases (CCaMKs) and other calmodulin-binding proteins phosphorylate and activate transcription factors such as WRKY and MYB, which regulate the expression of defense-related genes (Yuan et al., 2022). Calmodulin-dependent protein kinases (CDPKs) directly phosphorylate and activate enzymes involved in defense responses, such as pathogenesis-related (PR) proteins, defense-related enzymes, and antimicrobial compound. Calmodulin participates in the regulation of cell wall modifications and modulates the activity of enzymes involved in the synthesis and remodeling of cell wall components, such as callose synthases, pectin methylesterases, and xyloglucan endotransglucosylases/hydrolases (XTHs), which contribute to the strengthening of cell walls and physical barriers against invading pathogens (Wang et al., 2021). The *CAL* gene was moderately regulated in pathogen treatment as compared to other treatments. The higher gene expression was observed in siderophore pathogen treatment followed by siderophore treatment. The higher gene expression in siderophore + pathogen again attributes to the synergistic effect of both elicitors. This creates an amplified response to the presence of combined elicitors. The siderophore has the ability

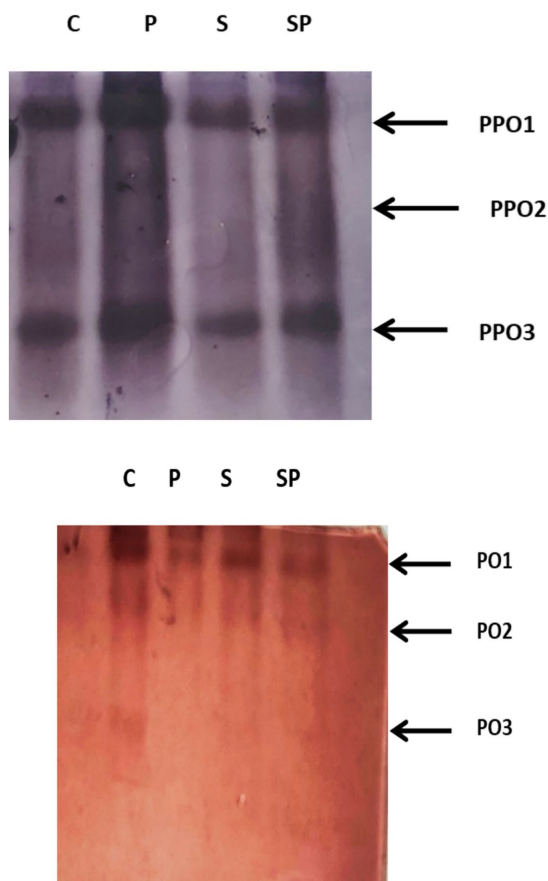


FIGURE 7
PO (Peroxidase) and PPO (Polyphenol Oxidase) isoform staining. C, Control; P, Pathogen only; S, siderophore only; SP, siderophore + pathogen.

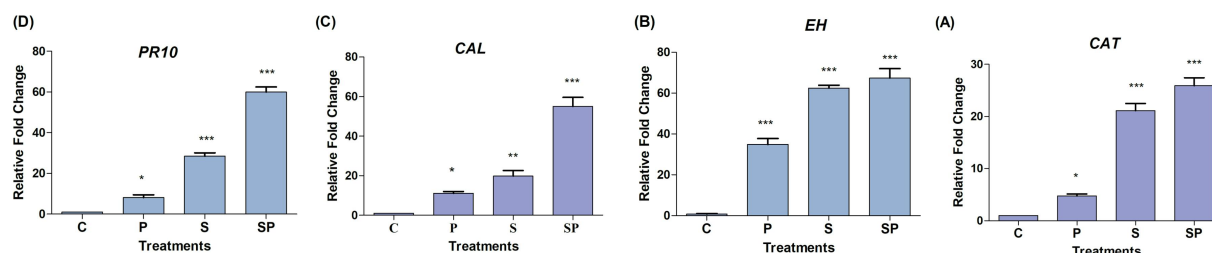


FIGURE 8
Relative fold change under various treatments: Control (C), Siderophore (S), Pathogen (P), and Siderophore + Pathogen (SP). Statistical analysis was performed using one-way ANOVA followed by Tukey's Test to compare treatment groups against the control. ns indicates no significant difference ($p > 0.05$), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

to induce upregulation of *CAL* gene owing to its adaptive and protective response toward pathogen attack.

PR-10 proteins are a subset of the pathogenesis-related (PR) protein family, which comprises several groups of proteins that are associated with plant defense mechanisms against pathogens (Dos Santos and Franco, 2023). The *PR10* gene was upregulated in both siderophore and siderophore + pathogen treatments owing to a more heightened response to pathogen invasion and enhanced protection to the plant. The pathogen treatment shows moderate levels of *PR10* expression as plants may prioritize responses to different elicitors based on the perceived severity and nature of the stress. While pathogen infection is a potent trigger of defense responses, siderophore treatment or combined stress may elicit additional or stress signals that further enhance gene expression. Gene expression patterns can vary over time, with different genes being induced or repressed at different stages of the plant's response to stress. The effects of chemical elicitors salicylic acid (SA) and jasmonic acid (JA) on expression of defense genes *PR 10*, epoxide hydrolase (EH), catalase and calmodulin with infection by *Rhizoctonia solani* were analyzed using qPCR at 1–4 days post inoculation/application (dpi) in highly susceptible (HS; Ratna) and moderately resistant (MR; HUM1) varieties of mungbean suggesting the upregulation of these genes in both the varieties (Tripathi et al., 2023; Figure 8).

Conclusion

In conclusion, the results of this study confirm that siderophores produced by *Bacillus amyloliquefaciens* enhance plant resistance through the activation of key defense mechanisms. Our findings demonstrate that siderophore treatments significantly increase peroxidase (POD), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), and chitinase activities, indicating a strong induction of plant immune responses. These enzyme activities, along with changes in gene expression, such as upregulation of catalase and epoxide hydrolase, suggest that siderophores activate antioxidant defense systems and alleviate oxidative stress, further supporting their role in boosting plant immunity against pathogens. Notably, the synergistic effects observed in plants treated with both siderophores and pathogens highlight the potential of siderophores to amplify plant defense responses, making them a promising tool for crop protection.

This study suggests that siderophore-producing bacteria could serve as an eco-friendly alternative to chemical fungicides and also that siderophores alone act as elicitor of defense responses and are potential biostimulants, reducing their use in agriculture while improving crop resilience. However, limitations such as the controlled experimental setting and the need for field validation must be addressed before broad application. Future research should focus on conducting field trials to assess the effectiveness of siderophore-based treatments under natural conditions and explore their broader

applications across various crops and environmental scenarios. Additionally, optimizing siderophore production, improving downstream processing, and exploring genetic engineering techniques could facilitate the commercialization of these bio-based solutions for sustainable agriculture.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: <https://www.ncbi.nlm.nih.gov>, accession number OQ536061.

Author contributions

AK: Writing – original draft. AbS: Project administration, Resources, Methodology, Writing – review & editing. ArS: Supervision, Writing – review & editing. PP: Supervision, Writing – review & editing. DP: Formal analysis, 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

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1492139/full#supplementary-material>

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Efficient degradation of neomycin by *Bacillus velezensis* and *Cupriavidus basilensis* isolated from mangrove soil and pharmaceutical wastewater

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Neomycin, an aminoglycoside antibiotic, is widely utilized for veterinary medicine in disease prevention. Biodegradation is a key pathway for the removal of neomycin from the environment. To date, only the white-rot fungus *Trametes versicolor* and the ericoid mycorrhizal fungus *Rhizoscyphus ericae* have been documented to efficiently degrade neomycin. However, no bacterial species with neomycin-degrading capabilities have been reported, underscoring a significant gap in microbial research related to neomycin remediation. In this study, *Cupriavidus basilensis* and *Bacillus velezensis* were isolated from pharmaceutical wastewater and neomycin-free mangrove soil through enrichment culture and gradual acclimatization, respectively. These isolates demonstrated neomycin degradation rates of 46.4 and 37.6% in 96 h with 100 mg·L⁻¹ neomycin as the sole carbon source. *Cupriavidus basilensis* achieved a degradation rate of 50.83% with ammonium sulfate supplementation, while *Bacillus velezensis* exhibited a superior degradation efficiency of 58.44% with soluble starch. Our findings offer valuable insights into the microbial degradation of neomycin. Two neomycin-degrading bacteria were isolated for the first time. Both species degraded neomycin as the sole carbon source or under co-metabolic conditions within 4 days. Microorganisms from neomycin-free environments adapted to neomycin stress and outperformed those from contaminated sources. This challenges the assumption that antibiotic-degrading microorganisms mainly originate from polluted environments. The findings expand the diversity of known neomycin-degrading microorganisms and demonstrate their potential for removing refractory neomycin from pharmaceutical wastewater.

KEYWORDS

Bacillus velezensis, *Cupriavidus basilensis*, neomycin-free soil, neomycin, biodegradation

1 Introduction

Antibiotics are widely utilized as antimicrobial agents in both human healthcare and veterinary medicine (Yang et al., 2021). Global antibiotic consumption increased by 16.3% from 2016 to 2023, with projections indicating a potential 52.3% increase by 2030 (Klein et al., 2024). The misuse and overuse of antibiotics have fueled the emergence of multidrug-resistant pathogens, posing significant threats to public and animal health (Patangia et al., 2022).

Antibiotic pollution primarily originates from pharmaceutical wastewater, aquaculture, livestock farming, and landfill leachate (Anh et al., 2021). Neomycin (Neo), an aminoglycoside antibiotic, is extensively used in livestock and poultry production (Low et al., 2021). The expansion of farming practices has further escalated the demand for veterinary antibiotics (Guo et al., 2021; Shao et al., 2021; Li S. et al., 2024). Besides agricultural runoff, pharmaceutical manufacturing is a major contributor to aminoglycoside antibiotic pollution (Tang et al., 2020). The extraction processes of antibiotics release substantial amounts of contaminated wastewater into natural ecosystems, exacerbating antibiotic resistance and promoting the spread of resistance genes (Han et al., 2024). Antibiotic concentrations in pharmaceutical effluents can reach $\text{mg}\cdot\text{L}^{-1}$ levels (Sim et al., 2011; Li K. et al., 2024). In response, the World Health Organization (WHO) issued its first guidelines on pharmaceutical antibiotic pollution, advocating preventive measures across municipal systems, manufacturing, healthcare, and agri-food sectors (WHO, 2024).

The thermal stability and resistance to acidic and alkaline conditions of aminoglycoside antibiotics render conventional removal methods ineffective (Nian et al., 2023). In contrast, microorganisms produce specific enzymes such as aminoglycoside acetyltransferases, phosphotransferases, and nucleotidyltransferases that deactivate aminoglycosides, thereby enabling microbial survival in environments with high antibiotic concentrations (Li et al., 2012; Liu et al., 2017; Cox et al., 2018; Stenholm et al., 2022). Moreover, microorganisms capable of mineralizing aminoglycosides have been identified in various ecosystems (Zeng et al., 2019), which has spurred interest in the microbial degradation of these compounds (Apreja et al., 2022). Most research has focused on the degradation of gentamicin and kanamycin. For example, *Stenotrophomonas maltophilia* and *Pseudomonas* sp. have been shown to degrade streptomycin (Fenton et al., 1973; Demars et al., 2024); *AMQD4* bacterial consortia can degrade gentamicin (Liu et al., 2017); and *Aquamicrobium* sp. I-A can degrade kanamycin (Chen et al., 2023). These microorganisms are typically isolated from antibiotic-contaminated soils, water sources, or pharmaceutical wastewater. The feasibility of isolating Neo-degrading microorganisms has also been proposed. However, Neo presents unique challenges due to its stable aminocyclitol ring, which confers strong resistance to microbial enzymatic cleavage (Obszynski et al., 2022). Only *Basidiomycetes* have been reported to biodegrade Neo (Stenholm et al., 2022), but their long growth cycle (up to 12 days) results in low degradation efficiency.

It is widely accepted that microorganisms exposed to antibiotic pollution are more likely to develop antibiotic-degrading capabilities. However, recent studies have identified antibiotic-degrading microorganisms in environments devoid of antibiotic pollution, challenging this conventional view. For instance, *E. coli* and *Cellulomonas* sp., isolated from antibiotic-free soils, were found to survive using aminoglycoside antibiotics as their sole carbon source (Dantas et al., 2008; Bello González et al., 2016). Similarly, *Stenotrophomonas maltophilia* isolated from antibiotic-free soils demonstrated efficient degradation of streptomycin (Bello González et al., 2016; Reis et al., 2020). While horizontal gene transfer (HGT) of resistance genes has been proposed as a mechanism for this phenomenon (Khmelevtsova et al., 2020; Liu et al., 2022), evidence suggests that antibiotic degradation may not always depend on HGT. In a study by Zhang et al., a strain capable of utilizing Neo as its sole carbon source was isolated from soil without prior exposure to

Neo. Although *aph3'* and *aac* (3)-Ia aminoglycoside resistance genes were detected in the soil, these genes were absent in the isolated strain itself (Zhang and Dick, 2014). This finding indicates that microbial antibiotic degradation capabilities can exist independently of HGT mechanisms. Consequently, it highlights the potential to isolate highly efficient antibiotic-degrading microorganisms from microbial reservoirs such as soil that have not been exposed to antibiotics. Based on this, we aimed to isolate Neo-degrading bacteria from both pharmaceutical wastewater with long-term exposure to high concentrations of Neo and from soil that had never been exposed to Neo, to evaluate their biodegradation capabilities and degradation characteristics.

2 Materials and methods

2.1 Chemicals and medium

Neo (purity $\geq 97\%$) used in this study was procured from Yichang Sanxia Pharmaceutical Co., Ltd. Glucose was sourced from Tianjin Kaitong Chemical Reagent Co., Ltd. Soluble starch, ammonium citrate, ammonium sulfate, and defatted soy flour were obtained from China National Pharmaceutical (Group) Shanghai Chemical Reagent Company. Peptone was supplied by Biosharp. The Luria-Bertani (LB) medium, utilized for enrichment, comprised $5\text{ g}\cdot\text{L}^{-1}$ NaCl, $10\text{ g}\cdot\text{L}^{-1}$ yeast extract, and $10\text{ g}\cdot\text{L}^{-1}$ peptone, adjusted to a pH of 7.2. Domestication and degradation experiments were conducted using a modified M9 medium, which lacked carbon and nitrogen sources, to screen for Neo-efficient degrading strains. The composition of the modified M9 medium included $0.45\text{ g}\cdot\text{L}^{-1}$ KH_2PO_4 , $0.1\text{ g}\cdot\text{L}^{-1}$ $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, and $1.79\text{ g}\cdot\text{L}^{-1}$ K_2HPO_4 . To prepare LB solid medium, 2.0% (w/v) agar powder was added to the LB medium. All media were sterilized prior to use.

2.2 Determination of Neo concentration

The Neo concentration in soil and wastewater samples was analyzed using a Thermo Scientific Dionex U3000 High-Performance Liquid Chromatography (HPLC) system equipped with an ELSD 6000 Evaporative Light Scattering Detector. Separation was achieved on an Apollo C18 column ($4.6\text{ mm} \times 250\text{ mm}$, $5\text{ }\mu\text{m}$ particle size) at a column temperature of 25°C . The mobile phase consisted of a mixture of 2.0% trifluoroacetic acid and 0.1% heptafluorobutyric acid (20:1, v/v), filtered through a $0.22\text{ }\mu\text{m}$ membrane. Each sample injection volume was set to $20\text{ }\mu\text{L}$, and the flow rate was maintained at $0.6\text{ mL}\cdot\text{min}^{-1}$. The evaporative light-scattering detector was configured with an evaporation temperature of 110°C and a gas flow rate of $3.0\text{ mL}\cdot\text{min}^{-1}$. The gain value was set to 1 DT, and the impaction mode was turned off. The total run time for each analysis was 20 min.

For liquid samples, Neo extraction involved centrifuging at $9,040\times g$ for 10 min. The supernatant was then mixed with Na_2EDTA -McIlvaine buffer to chelate metal ions (He et al., 2021). For soil samples, 20 mL of extraction solution (methanol: water = 1:1, v/v) was added to sieved soil, followed by ultrasonication for 30 min and centrifugation at $9040\times g$ for 10 min. The resulting supernatants were filtered through a $0.22\text{ }\mu\text{m}$ membrane and stored at -20°C for further analysis (Yu et al., 2019; He et al., 2021). The linear regression equation

for Neo standard solutions was determined as $y = 0.3282x - 14.49$ (Figure 1A), where y represents peak area and x represents Neo concentration. The chromatographic profile of Neo exhibited consistent retention times at 8.645 ± 0.05 min (Figure 1B).

The degradation rate was calculated as follows:

$$\text{Degradation rate} = \frac{\text{initial Neo concentration} - \text{residual Neo concentration}}{\text{initial Neo concentration}} \times 100\%$$

2.3 Sample sources and preparation

Shenzhen Longgang District (N22.48894, E114.58263), situated within the city of Shenzhen, is a critical region for mangrove distribution. The Shenzhen Bay Mangrove Wetland serves as an essential ecological conservation area. Previous studies have indicated that many mangrove wetlands in Shenzhen face significant ecological risks due to contamination from various pollutants (Lingyun et al., 2019). Soil samples were collected from three distinct locations, excluding the topsoil layer and sampling at depths between 5 and 20 cm. The collected soil samples were air-dried for 7 days, subsequently ground into a fine powder, and stored in sterile sealed bags at temperatures below -20°C until further analysis. HPLC analysis revealed no detectable Neo residues in the soil samples (Figure 1C). Wastewater samples were obtained from a pharmaceutical factory in Hubei Province, China, which manufactures Neo through a fermentation-based process. Biochemical hydrolysis effectively eliminates microorganisms that cannot tolerate antibiotics, rendering the treated wastewater suitable for this study.

2.4 Enrichment culture of neo-tolerant microorganisms

The LB medium supplemented with $100 \text{ mg}\cdot\text{L}^{-1}$ Neo was used to enrich the isolates. This concentration of Neo mimics the high levels typically encountered in pharmaceutical wastewater treatment processes. Initially, 1 mL of pharmaceutical wastewater or 1 g of soil was suspended in 9 mL of sterile distilled water and mixed thoroughly for 1 min. The suspension was then serially diluted 10^6 -fold using sterile distilled water. Subsequently, a 10% (v/v) inoculum of the

diluted suspension was added to 100 mL of sterile LB medium containing $100 \text{ mg}\cdot\text{L}^{-1}$ Neo. Cultures were incubated at 35°C with shaking at 220 rpm for 7 days until an optical density (OD_{600}) value of 1.000 was achieved. These cultures served as the inoculum for subsequent domestication steps.

2.5 Domestication of neo-tolerant microorganisms

To acquire strains capable of utilizing Neo as the sole carbon source, 10 mL of the enriched bacterial suspension was inoculated into 90 mL of optimized M9 medium supplemented with $50 \text{ mg}\cdot\text{L}^{-1}$ Neo. Cultures were incubated at 35°C with shaking at 220 rpm for 7 days to complete the first domestication cycle. For subsequent cycles, 10 mL of the previous culture was transferred to 90 mL of fresh optimized M9 medium, with Neo concentrations incrementally increased by $50 \text{ mg}\cdot\text{L}^{-1}$ per cycle, reaching $200 \text{ mg}\cdot\text{L}^{-1}$ by the fourth cycle. OD_{600} values were measured following each acclimation period.

2.6 Isolation, purification, and 16S rRNA identification of neo-degrading strains

The final domesticated culture was serially diluted 10^3 -fold with sterile distilled water and plated onto LB agar plates. The cultures were incubated at 35°C for 48 h. Distinct colonies based on morphology were isolated using the streak plate method for further purification. This purification process was repeated three times to ensure pure cultures. The purified strains were then inoculated onto fresh LB agar plates and incubated at 35°C for 18 h. Crystal violet staining and bacterial morphology were examined under a biological microscope (MODEL-ECLIPSE-E200, Nikon Corporation, Shanghai, China). Genomic DNA was extracted from the purified strains using the MGIEasy Bacterial DNA Extraction Kit (MD01T-96, MGI, Wuhan, China). The 16S rRNA gene was amplified using universal primers 27F and 1492R (Yu et al., 2013). PCR amplification conditions included an initial denaturation at 96°C for 5 min, followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were verified by agarose gel electrophoresis and subsequently sent to BGI (Beijing Genomics Institute) for sequencing. The obtained sequences were analyzed using the BLAST tool (<https://www.ncbi.nlm.nih.gov/>) for species identification. A phylogenetic tree

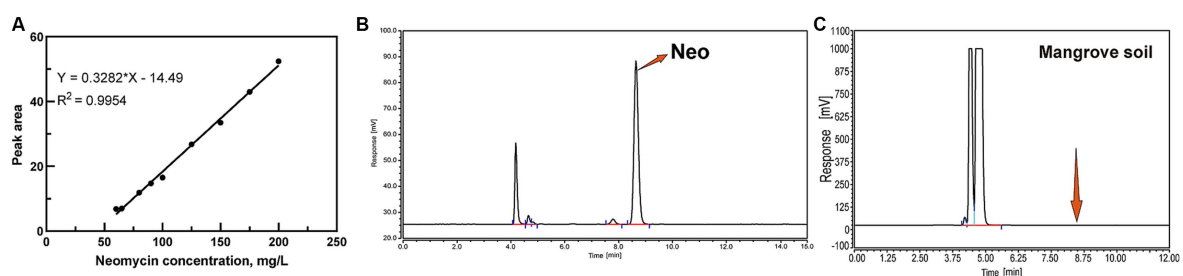


FIGURE 1
HPLC analysis of Neo. (A) Standard curve for Neo; (B) Chromatogram of Neo; (C) Neo content in Mangrove soil.

was constructed using the Neighbor-Joining method in MEGA 11.0 software. The nucleotide sequences have been deposited in the GenBank database (SH1: [SUB14883397](#) and RS2: [SUB14885154](#)).

2.7 Neo biodegradation ability of strains SH1 and RS2

The purified cultures of strain SH1 and strain RS2 were grown in LB medium until they reached the logarithmic growth phase. Cells were harvested and washed three times with 0.9% NaCl solution. The OD₆₀₀ values of the cell suspensions was adjusted to 1.000 by diluting with fresh LB medium, and measurements were conducted using a SpectraMaxiD3 Microplate Reader (Shanghai Minggujia Electronic Technology Co., Ltd.). Subsequently, the bacterial suspensions were inoculated at a 10% inoculum size into modified M9 medium containing 100 mg·L⁻¹ Neo, with an initial pH of 7.2, for biodegradation assays (Yin et al., 2020). This Neo concentration was selected based on preliminary studies, as concentrations exceeding 100 mg·L⁻¹ significantly inhibited bacterial growth during strain domestication (Supplementary Table S1). Samples were collected every 24 h to monitor Neo residuals and OD₆₀₀ values (Li et al., 2021). A control group with the same Neo concentration in M9 medium was established to minimize potential external influences.

2.8 Study of Neo biodegradation characteristics

To optimize the cultivation conditions for the Neo-degrading strains SH1 and RS2, we systematically evaluated the effects of varying substrate concentrations (100, 500, and 1,000 mg·L⁻¹), nitrogen sources (ammonium sulfate, peptone, and defatted soy flour), and carbon sources (citric acid ammonium, glucose, and soluble starch) on degradation efficiency. Each condition was tested in triplicate with a blank control group included. Bacterial suspensions were collected at multiple time points over a 96 h incubation period, and OD₆₀₀ values were measured to assess bacterial growth. After sample processing, Neo concentrations were quantified using HPLC, and degradation rates were subsequently calculated.

2.8.1 Effect of substrate concentration on growth and degradation rates of strains SH1 and RS2

Neo solutions with initial concentrations of 100, 500, and 1,000 mg·L⁻¹ were added to M9 medium adjusted to pH 7.2. A 10% (v/v) inoculum of the bacterial suspension was introduced into the medium, and the cultures were incubated at 35°C for 96 h. Uninoculated samples served as controls. Each condition was tested in triplicate. After the 96 h incubation period, OD₆₀₀ values and residual Neo concentrations were measured.

2.8.2 Effect of nitrogen sources on growth and degradation rates of strains SH1 and RS2

Neo solutions at a concentration of 100 mg·L⁻¹ were added to the M9 medium, which was adjusted to pH 7.2. Ammonium sulfate, peptone, and defatted soy flour were incorporated into the medium at concentrations of 0.1 g·L⁻¹ each. A 10% (v/v) inoculum of the bacterial suspension was introduced into the medium, and the cultures were

incubated at 35°C for 96 h. Uninoculated samples served as controls. Each condition was tested in triplicate. Following the 96 h incubation period, OD₆₀₀ values and residual Neo concentrations were measured.

2.8.3 Effect of carbon sources on growth and degradation rates of strains SH1 and RS2

A 100 mg·L⁻¹ Neo solution was added to the M9 medium adjusted to pH 7.2. Ammonium citrate, glucose, and soluble starch were each added at a concentration of 0.1 g·L⁻¹. A 10% (v/v) inoculum of the bacterial suspension was introduced into the cultures, which were then incubated at 35°C for 96 h. Uninoculated samples served as controls. Each condition was tested in triplicate. After the 96 h incubation period, OD₆₀₀ values and residual Neo concentrations were measured to evaluate the impact of different carbon sources on bacterial growth and Neo degradation efficiency.

3 Results and discussion

3.1 Isolation, purification, and 16S rRNA identification of neo-degrading strains

Through enrichment culture and gradual domestication using Neo-contaminated pharmaceutical wastewater and Neo-free mangrove soil samples, two strains capable of utilizing Neo as the sole carbon source were isolated. These strains were designated as SH1 and RS2. The strains were purified, stained with crystal violet, and characterized based on their morphological features. Strain SH1 was identified as a Gram-negative short rod with small, smooth, raised colonies, while strain RS2 was identified as a Gram-positive rod with larger, wrinkled, raised colonies (Figure 2).

Strains SH1 and RS2 were identified as *Cupriavidus basilensis* and *Bacillus velezensis*, respectively (Table 1). Molecular biological analysis revealed that PCR-amplified DNA fragments from strains SH1 and RS2 exhibited consistent single-band patterns, with lengths of approximately 1,360 bp and 1,410 bp, respectively (Figure 3). Sequencing confirmed full-length gene sequences of 1,368 bp and 1,413 bp for strain SH1 and strain RS2, respectively, corroborating the electrophoresis results (Figure 3A). BLAST comparisons of the 16S rRNA sequencing results showed 100% sequence similarity for both strains. Based on these findings, a phylogenetic tree was constructed (Figures 3B,C).

3.2 Neo biodegradation ability of strains SH1 and RS2

Strains SH1 and RS2 demonstrated 96-h Neo degradation rates of 37.67 and 46.49%, respectively, when Neo was used as the sole carbon source at a concentration of 100 mg·L⁻¹ (Figure 4). Previous studies have shown that several *Cupriavidus* species can effectively degrade ochratoxin A (Ferenczi et al., 2014), bisphenol A (Fischer et al., 2010), and assist in copper bioremediation (Kugler et al., 2022). Similarly, some *Bacillus* species have been identified as capable of degrading sulfamethoxazole (Lin et al., 2024) and tetracycline (Al-Dhabi et al., 2021). This study is the first to report that *Cupriavidus basilensis* and *Bacillus velezensis* can degrade Neo. Most antibiotic-degrading strains are isolated from

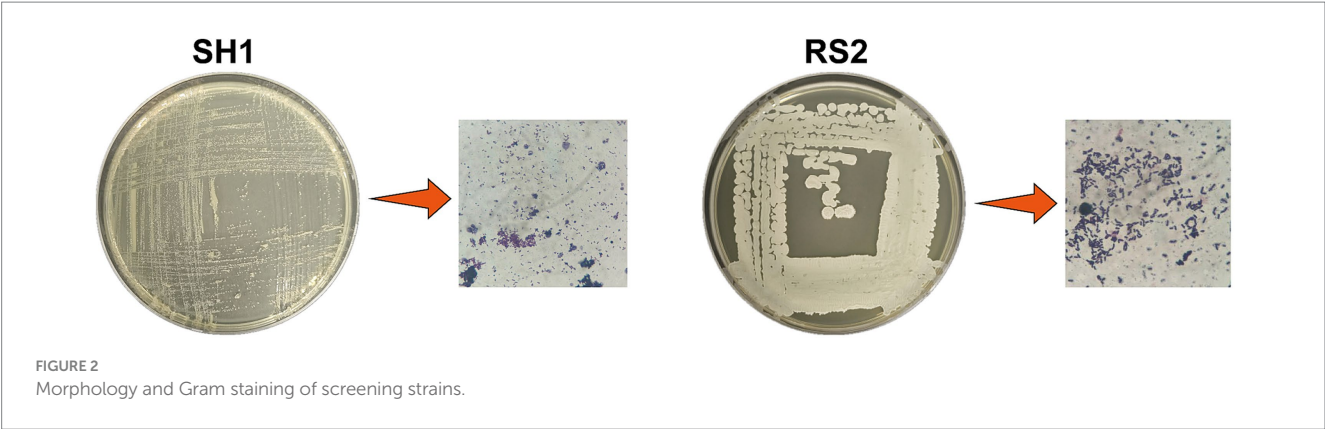
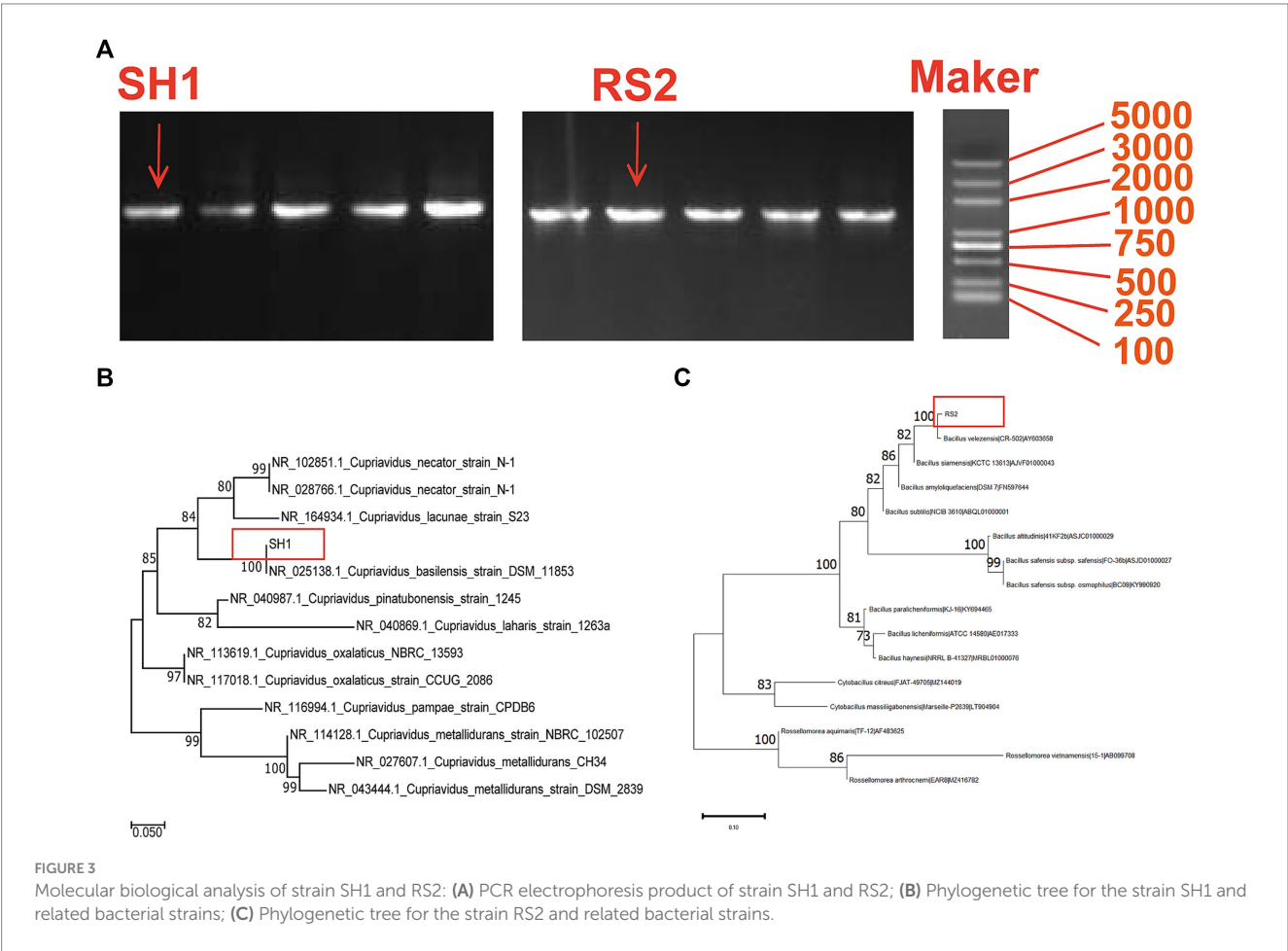


TABLE 1 Homology comparison of 16S rRNA gene sequences of screened strains.

Strains number	The closest species		Identity %	Genus submission
	Latin name	Accession No.		
SH1	<i>Cupriavidus basilensis</i>	CP062804.1	100	SUB14883397
RS2	<i>Bacillus velezensis</i>	CP054714.1	100	SUB14885154



antibiotic-contaminated environments such as wastewater, solid waste, and soils (Table 2). Wang et al. isolated sulfadiazine-degrading bacteria from antibiotic-contaminated soils and found that sulfadiazine resistance genes played a key role in the degradation process (Wang et al., 2024). Additionally, Rodríguez-Verdugo et al. evolved *Escherichia coli* B under heat stress (42.2°C) over 2000

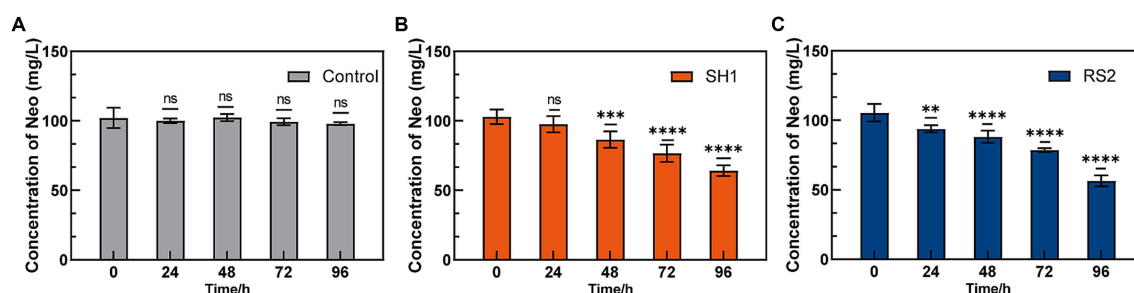


FIGURE 4
Neo degradation ability of enriched strains. (A) Control; (B) SH1; (C) RS2.

TABLE 2 Antibiotics from different sources degrade microorganisms.

Antibiotic	Microorganisms	Sample source	References
Streptomycin	<i>Stenotrophomonas maltophilia</i>	Soil-dwelling	Fenton et al. (1973)
	<i>Pseudomonad bacterium</i>	Estrogen-containing urban soil	Demars et al. (2024)
Gentamicin	AMQD4	Gentamicin-containing sludge	Liu et al. (2017)
	<i>Aspergillus terreus</i> FZC3	Gentamicin produces solid waste and wastewater	Liu et al. (2016)
Kanamycin	Domestication strain DSM, <i>Aquamicrobium</i> sp. I-A	Antibiotic contaminated soil and Wastewater	Chen et al. (2023)
Tetracycline	<i>Arthrobacter nicotianae</i> OTC-16	Activated sludge from pharmaceutical Wastewater treatment plants	Shi et al. (2021)
	<i>Pseudomonas</i> sp. DX-21	Sludge of a long-operating SBR	Yang et al. (2024)
Erythromycin	<i>Paracoccus versutus</i> W7	Sewage sludge for municipal sewage treatment	Ren et al. (2023)
Chloramphenicol	<i>Klebsiella</i> sp. YB1	Earthworm gut content	Tan et al. (2023)
Sulfamethoxazole	<i>Proteus mirabilis</i> sp. ZXY4	Sludge from sewage treatment plants	Yan et al. (2023)

generations in an environment free of rifampicin (Rodríguez-Verdugo et al., 2013), resulting in spontaneous mutations in the *rpoB* gene, which conferred rifampicin resistance. This suggests that gene mutations leading to antibiotic resistance can occur even in environments without antibiotics, depending on the mutation background and environmental conditions. In this study, we have discovered and isolated neo-degrading bacteria from environments free of Neo. Specifically, *Bacillus velezensis* was isolated from Neo-free soil, while *Cupriavidus basilensis* was isolated from Neo-pharmaceutical wastewater, both exhibiting Neo degradation abilities. The degradation ability of these strains may be linked to Neo resistance genes.

3.3 Effects of different culture conditions on Neo biodegradation by SH1 and RS2

When Neo served as the sole carbon source, strains SH1 and RS2 exhibited differential degradation efficiencies over 96 h at concentrations ranging from 100 to 1,000 mg·L⁻¹ (Figures 5A,B). As the Neo concentration increased, both the degradation rate and bacterial growth were progressively inhibited. Specifically, at an initial Neo concentration of 100 mg·L⁻¹, optimal degradation rates and growth conditions were observed for strains SH1 and RS2, with degradation rates reaching 35.83 and 43.54%, respectively, and OD₆₀₀ values attaining 0.235 and 0.302. These findings suggest that the initial

substrate concentration significantly affects the efficiency of Neo biodegradation. The high degradation rate at 100 mg·L⁻¹ can likely be attributed to enhanced bacterial growth and the secretion of functional enzymes involved in biotransformation (Sánchez-San Martín et al., 2024). Conversely, at higher initial concentrations of 500 mg·L⁻¹ and 1,000 mg·L⁻¹, metabolic overload may have occurred, resulting in the accumulation of metabolites or by-products that inhibited bacterial growth and reduced degradation efficiency (Chen et al., 2022).

In the optimized M9 medium containing 100 mg·L⁻¹ Neo, we investigated the effects of different nitrogen sources—ammonium sulfate, peptone, and defatted soy flour—on Neo degradation by strains SH1 and RS2 (Figures 5C,D). Compared to the control, defatted soy flour significantly inhibited Neo degradation in both strains, resulting in degradation rates of only 9.54% for SH1 and 9.14% for RS2. The underlying mechanism of this inhibition warrants further investigation. In contrast, ammonium sulfate enhanced the Neo degradation ability of both strains, achieving the highest degradation rates of 50.83% for SH1 and 50.71% for RS2. When bacteria utilize inorganic nitrogen sources such as ammonium sulfate for metabolism, they require additional carbon sources (Hargreaves, 2006), which may explain the observed increase in Neo degradation rates. This finding is consistent with previous studies on sulfonamide biodegradation, where sodium nitrate and ammonium chloride were shown to promote biodegradation (Vijayaraghavan et al., 2021). Unlike glucose, Neo served as the sole carbon source in our study,

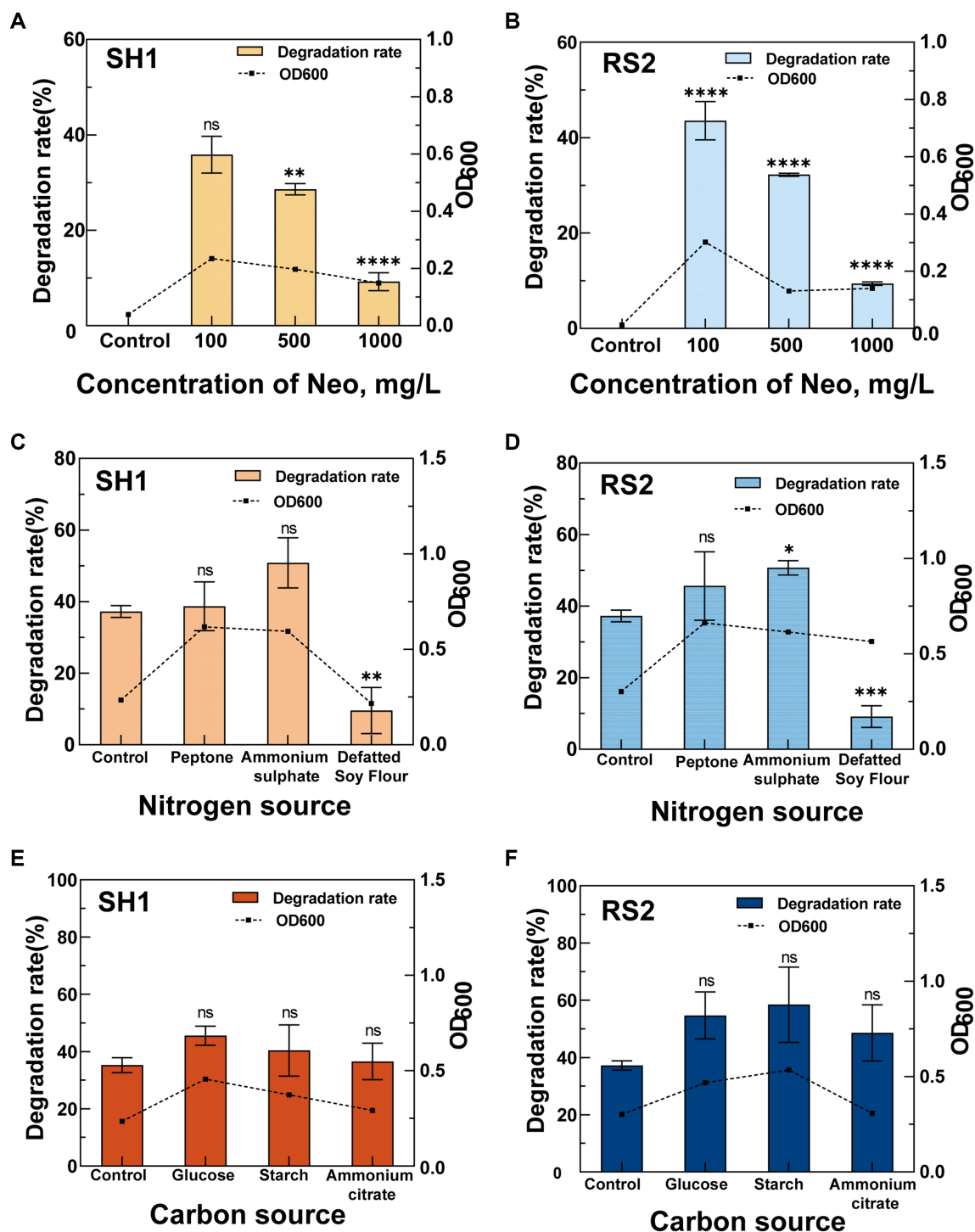


FIGURE 5

Effects of different conditions on the Neo degradation rate of strains SH1 and RS2. (A,B) Concentration of Neo; (C,D) Nitrogen source; (E,F) Carbon source.

suggesting that inorganic nitrogen might enhance carbon metabolism similarly.

The effects of different carbon sources—ammonium citrate, glucose, and soluble starch—on the Neo degradation ability of the two strains at a 100 mg·L⁻¹ Neo concentration (Figures 5E,F) were investigated. Compared to the control group (without additional carbon sources), all three carbon sources significantly increased the degradation

rates to varying extents, indicating that these carbon sources can enhance Neo removal by the bacteria (Leng et al., 2016; He et al., 2021). Specifically, strain SH1 exhibited the highest Neo degradation rate of 45.53% when supplemented with glucose. Strain RS2 also showed enhanced growth and Neo degradation ability in the presence of glucose and soluble starch, achieving degradation rates of 54.69 and 58.44%, respectively. The addition of appropriate co-substrates can

activate functional enzymes (Zhang et al., 2020) and accelerate the biodegradation of recalcitrant organic compounds (Barnhill et al., 2010).

4 Conclusion

In this study, *Cupriavidus basilensis* and *Bacillus velezensis* were isolated from Neo pharmaceutical wastewater and from soil that had not been exposed to Neo. These isolates demonstrated the ability to utilize Neo as the sole carbon source. The degradation experiments revealed that both *Cupriavidus basilensis* and *Bacillus velezensis* exhibited significant Neo degradation capabilities. Specifically, *Cupriavidus basilensis* achieved a maximum degradation rate of 50.83% for 100 mg·L⁻¹ Neo within 96 h when ammonium sulfate was used as the nitrogen source. Meanwhile, *Bacillus velezensis* reached a maximum degradation rate of 58.43% under the same Neo concentration when soluble starch served as the additional carbon source.

The results demonstrate that substrate concentration significantly influences the efficiency of Neo degradation, with appropriate co-substrates promoting bacterial growth and enhancing Neo biodegradation. The identification of *Cupriavidus basilensis* and *Bacillus velezensis* as Neo-degrading strains expands the known range of microorganisms capable of degrading Neo. These findings suggest their potential utility in the bioremediation of Neo-contaminated soils and wastewater. However, further studies are required to evaluate their performance under actual environmental conditions.

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

QY: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. WH: Data curation, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. XY: Data curation, Formal analysis, Investigation, Software, Writing – review & editing. QD: Formal analysis, Investigation, Writing – review & editing. JL: Formal analysis, Writing – review & editing. BC: Conceptualization, Methodology, Visualization, Writing – review & editing. TD: Methodology, Visualization, Writing – review & editing.

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Conflict of interest

XY was employed by Yichang Humanwell Pharmaceutical Co., Ltd.

The remaining 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.2025.1544888/full#supplementary-material>

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Synergistic removal of emerging contaminants using bacterial augmented floating treatment bed system (FTBs) of *Typha latifolia* and *Canna indica* for rejuvenation of polluted river water

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Introduction: Floating Treatment Bed systems (FTBs) provide an effective approach to remove pollutants from the rivers. These systems consist of aquatic plants anchored on mats, which support the growth of microbial communities. Such a synergy between plants and microbes in FTBs plays a pivotal role to improve efficacy of river restoration strategies.

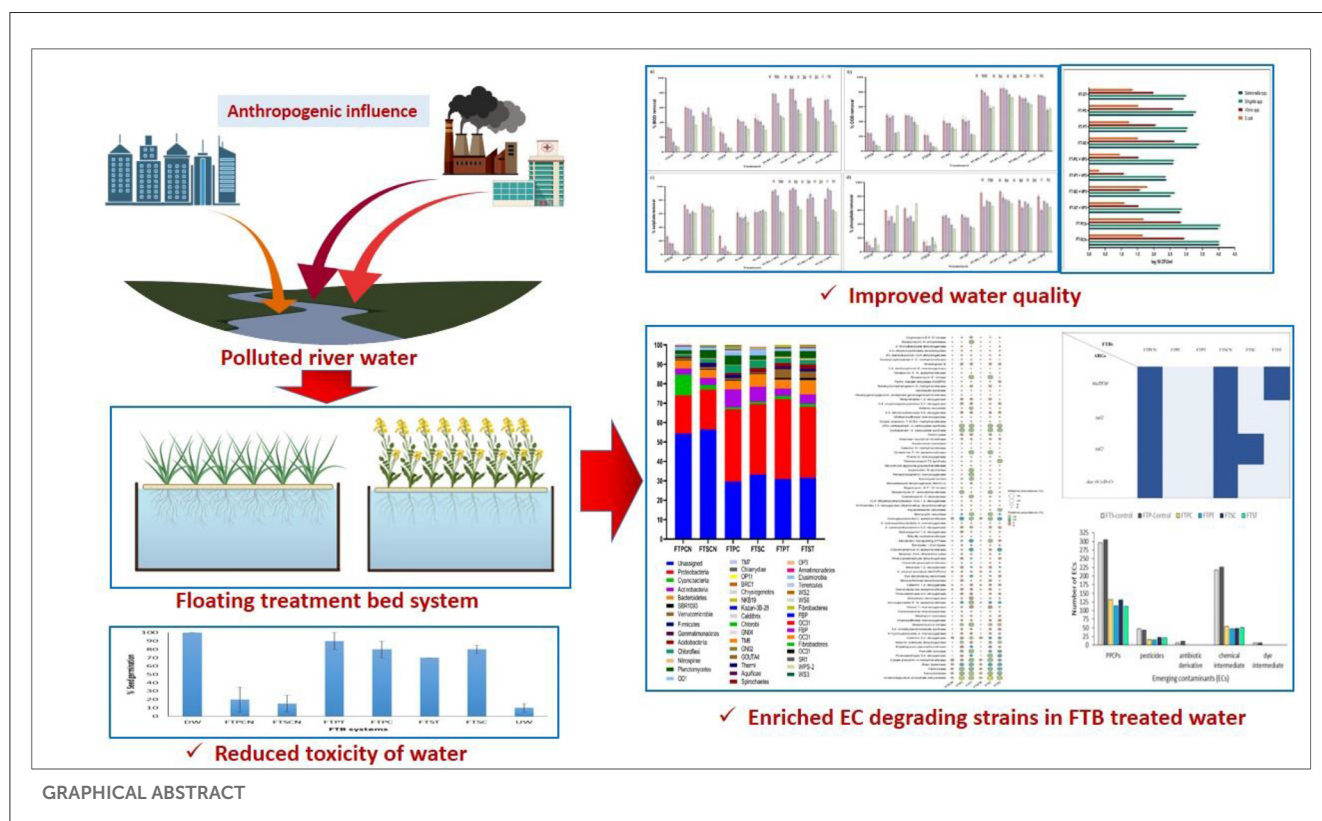
Methodology: The effectiveness of the FTBs was evaluated for the rejuvenation of polluted water from the Mini River in Gujarat, India. These systems consisted of wetland plants, either *Typha latifolia* or *Canna indica*, which were augmented with the bacterial consortium VP3. Furthermore, the 16S *rRNA* gene amplicon sequencing approach identified the dominant bacterial communities and relative microbial community shifts within the FTBs. The presence of emerging contaminants, antimicrobial resistance genes, and pathogenic bacterial species in the untreated river water was evaluated, along with their reduction following treatment through FTBs. This analysis yielded important insights into the microbial dynamics governing the reduction of these contaminants.

Results and discussion: The bacterial augmented FTBs consisting wet plants achieved reduction of 57%, 70%, 74%, and 80% in biochemical oxygen demand (BOD), chemical oxygen demand (COD), total phosphate, and sulfate, respectively. Moreover, the 16S *rRNA* gene amplicon sequencing identified *Proteobacteria* as the dominant phylum, with *Pseudomonas* species and *Hydrogenophaga* species being the most abundant genera in FTBs containing *T. latifolia* and *C. indica*, respectively. The functional gene prediction indicated presence of various xenobiotic degrading genes too. Non-targeted LC-HRMS analysis of treated water demonstrated complete elimination of antibiotic derivatives and dye intermediates, along with the partial removal of pharmaceutical and personal care products (PPCPs) and chemical intermediates. Additionally, the abundance of probable pathogenic bacteria and dominant antibiotic resistance genes was significantly reduced upon treatment. The phytotoxicity analysis of the treated water supported the outcomes. The studies

on removal of emerging contaminants in the polluted river ecosystem has been relatively less explored, highlighting novelty and future possible applications of the plant-microbial augmented FTBs in rejuvenation of polluted rivers.

KEYWORDS

antibiotic resistance genes, bacterial community, emerging contaminants, floating treatment bed systems, pollutant removal, river water treatment



1 Introduction

The rivers are facing an unprecedented crisis as a result of rapid industrialization and anthropogenic activities globally (Gomes et al., 2023; Bandyopadhyay and De, 2017). Starting from the mighty Amazon to the Ganges, these crucial waterways that have nurtured life for centuries are now in the danger (Gomes et al., 2023; Su et al., 2023; Richards et al., 2023). This crisis has damaged the aquatic ecosystems and also poses health risks to billions of people depended on these waterways (Madhav et al., 2020).

These rivers are directly exposed to varieties of anthropogenic pollutants originating from industrial effluents, agricultural runoff, domestic sewage, and disposal of solid waste. Consequently, the number of severely polluted rivers in India is increasing at an alarming rate, posing substantial health risks to communities residing along their banks. The presence of highly toxic, carcinogenic, and mutagenic substances in these water bodies underscores the urgent need for eco-friendly, cost-effective, and sustainable mitigation strategies to address river pollution.

Biological treatment technologies have emerged as promising solutions to mitigate river pollution effectively (Singh et al., 2020). Amid, traditional phytoremediation techniques, such as constructed wetlands have shown superior pollutant removal efficiency and cost-effectiveness. However, their widespread adoption is hindered by significant land area they require. In contrast, floating treatment bed system have gained prominence as viable alternatives for restoring eutrophic rivers and lakes due to their demonstrated effectiveness and ease of management (Tao et al., 2020; Yadav and Goyal, 2022). Studies have highlighted its potential as an affordable technology for restoring polluted rivers, primarily due to its minimal land requirement and lower operational costs compared to other treatment methods (Yadav and Goyal, 2022; Asghar et al., 2022; Fahid et al., 2020; Tao et al., 2020).

The Floating Treatment Bed system (FTBs) represent a practical approach to enhance pollutant removal in rivers (Nandy et al., 2022; Liu et al., 2016; Samal and Trivedi, 2020; Huang et al., 2020; Yao et al., 2021). FTBs typically consist of aquatic plants and a specialized mat structure that supports an enhanced microbial population. This synergistic combination of plants and microbes

plays a crucial role in improving the efficacy of river restoration strategies (McCorquodale-Bauer et al., 2023). However, despite their advantages, the application of FTBs for removing emerging contaminants from polluted river water remains underexplored. Rivers contaminated by diverse waste sources often carry emerging contaminants, posing challenges for effective river restoration (Srivastava et al., 2017).

Antibiotic resistance has emerged as a critical environmental and public health concern, affecting ecosystems, human health, as well as animal husbandry (Koch et al., 2021; Hernando-Amado et al., 2019). The origins of antibiotic resistance are multifaceted and complex, influenced by factors such as overuse and improper disposal of antibiotics in both human medicine and agriculture (Kinney and Heuvel, 2020; Bengtsson-Palme et al., 2018). Ávila et al. (2021) demonstrated the effective removal of antibiotics and antibiotic resistance genes (ARGs) from urban wastewater using vertical subsurface flow constructed wetlands, highlighting the potential of phytoremediation techniques in mitigating antibiotic resistance in the aquatic environments. Similarly, Bano et al. (2023) successfully treated amoxicillin-contaminated water using an improved FTB augmented with antibiotic-degrading bacteria, showcasing the capability of microbial technologies to address antibiotic pollution in water bodies.

The current study employed two native wetland plants, *Typha latifolia* (*T. latifolia*) and *Canna indica* (*C. indica*), cultivated on floating beds to investigate their efficacy in improving river water quality. The study monitored changes in physico-chemical parameters, such as biological oxygen demand (BOD), chemical oxygen demand (COD), total phosphate and sulfate along with pathogenic bacterial counts as indicators of water quality improvement. Additionally, 16S *rRNA* gene amplicon sequencing approach was used to identify the predominant bacterial communities within the FTBs, while the Shannon-Wiener diversity index characterized microbial community structures. The occurrence of antibiotics and corresponding ARGs was assessed in all FTBs at the end of the operational period, providing insights into the dynamics of microbial populations involved in antibiotic removal. This comprehensive approach lays a foundation for enhancing the overall quality of polluted urban rivers, offering valuable insights into the potential of FTBs as sustainable solutions for mitigating anthropogenic pollution and improving overall health of the invaluable riverine ecosystems.

2 Materials and methods

2.1 Collection and characterization of water from polluted river

The water was collected from the Mini river, Vadodara, Gujarat, India (22°23'35" N, 73°05'49" E). The Mini river receives pollutants from various sources, i.e., industrial, household as well as agricultural runoff. Samples were filtered using 0.45 µm nylon filter paper (PALL Corporation, USA) prior to analysis, and all the mentioned parameters were evaluated in triplicates. The parameters such as pH, EC, TDS, total nitrogen and dissolved oxygen (DO) were analyzed using HQ440D multi-meter (HACH, USA). The samples were analyzed for chemical

TABLE 1 Source of enriched bacterial consortia; where, GIDC stands for Gujarat Industrial Development Corporation.

Consortia	Source	Location
VP1	Wastewater polluted site	GIDC, Vatva, Gujarat
VP2	Waste disposal site	Sankarda, Gujarat
VP3	Wastewater polluted site	GIDC, Nandesari, Gujarat
VP4	Mini river	Anagadh, Gujarat
VP5	Mahi river	Sindhrot, Gujarat

oxygen demand (COD), biochemical oxygen demand (BOD₅), total phosphate, and sulfate were estimated using closed reflux spectrophotometric method, titrimetric method, stannous chloride method, and turbidimetric method respectively as per American Public Health Association (APHA, 2017) for the characterization of the river water quality based on the Indian Standard Water Quality Guidelines (BIS, 2012).

2.2 Enrichment of bacterial consortium from soil sediment of industrially polluted sites

The bacterial consortia for bioremediation of the polluted Mini river water were developed following enrichment culture technique using polluted soil and sediments collected from the Mini river as shown in Table 1. A total of five bacterial consortia were developed and enriched in the polluted water of the Mini river supplemented with Bushnell-Hass medium (BHM) along with co-substrates (0.01% w/v glucose and 0.01% w/v yeast extract) at 37°C. The active cultures were sub-cultured on every 5th day. After 50 subcultures the enriched bacterial consortia were evaluated for treatment of the polluted water of the Mini river by analyzing their treatment efficiencies for COD and BOD removal as described in the standard methods for water and wastewater analysis (APHA, 2017).

2.3 Development of small-scale floating treatment bed systems

Specifically, six parallel laboratory scale FTBs were developed using plastic tanks (60 cm × 40 cm × 22.5 cm). The effective depth of the device was about 20 cm, and the total volume was 10 L. The FTBs were consisted of either of the two floating frameworks; (1) Styrofoam sheet or (2) Polyvinyl chloride pipe-frames (Supplementary Figure S1a). Each of the FTB developed with Styrofoam sheet has equally spaced eight PVC holders with the diameter of 6 cm. All the holders contain coconut coir as a floating bed material. Whereas, FTB developed with PVC pipe-frames have fiber mat with coconut coir as support (Supplementary Figure S1b). Two potential macrophytes, *T. latifolia* and *C. indica* originated from polluted zone of the Mini river, Gujarat, India were collected for the study. Both the plants were acclimatized in polluted river

water for 21 days before actual experiments in FTBs. Each FTB contains total of 24 plant saplings. Detailed description of each FTB is enlisted in Table 2. The bacterial consortium, VP3 was enriched using the samples collected from the polluted source to capture the indigenous bacteria capable of removal of emerging contaminants. VP3 augmentation was performed for VP3-augmented FTBs taking 1L active culture of VP3 consortium. The consortium VP3 for augmentation was prepared using Bushnell Haas Medium (BHM) without the addition of external BOD. The inoculum was applied at a concentration of 10^7 cells/mL, ensuring an effective microbial load for bioaugmentation. The developed FTBs were operated at different HRTs, mainly 1 day, 2 day, 3 day, 5 day, and 10 day.

2.4 Performance evaluation of FTB

The quality of FTB treated polluted water was determined using various physico-chemical parameters, according to the standard methods for water and wastewater analysis (APHA, 2017) as described in the Section 2.1.

Pathogenic indicator bacterial populations of each FTB were determined using membrane filtration techniques (MFT) using different selective media such as Hi-Chrome M-TEC for *Escherichia coli*, deoxycholate citrate agar for *Salmonella* species and *Shigella* species, and TCBS media for *Vibrio cholerae*. All media for the study were purchased from Hi-Media, India. All samples were serially diluted for enumeration of bacterial pathogens using 0.9% (w/v) sterile normal saline. The samples were filtered using S-Pak white gridded filter membranes (Merck Millipore, Germany). The filter membrane was aseptically removed and placed over the agar surface of the respective agar plate. All the plates were incubated at 37°C for 24 h. Standard viable counting was performed to evaluate the colony-forming units (CFU/mL) representing the pathogens.

2.5 Determination of emerging contaminants removal by FTB from polluted river water

Removal of emerging contaminants from polluted water of the Mini river by FTB were analyzed based on the previously reported methods with few modifications (Liu et al., 2021; Zhou et al., 2012). The untreated and treated river water samples were subjected to solid-phase extraction (SPE) using Oasis HLB cartridges with specifications of 60 μ m particle size, 80 Å pore size, 500 mg sorbent weight and 6 cc barrel size (Waters, Milford, MA, USA). Cartridges were pre-conditioned with 10 mL methanol, followed by 15 mL ultrapure water (3 times $\times$ 5 mL). Later, the untreated and treated river water samples were passed through the pre-conditioned SPE cartridges and elution was carried out using 10 mL methanol in the Waters Extraction Manifold (Waters, Milford, MA, USA). The extracts were concentrated and re-constituted in 10 mL ACN: H₂O (9:1) and filtered through 0.2 μ m filter before subjecting to LC-HRMS analysis. The extracted sample (10 μ L) was injected using the in-built auto-sampler into a Thermo Scientific™ Hypersil GOLD™ C18 column [100 mm (l) $\times$ 2.1 mm (i.d.), 1.9 μ m particle size] maintained at 30 °C at a flow rate of 0.3 mL/min.

TABLE 2 Floating treatment bed (FTB) systems used for the study; where, first two letters FT, Floating bed treatment; P, Polystyrene frame; S, styrofoam frame; CN, control without plant; CI, *C. indica*; TL, *T. latifolia*.

Name of FTB	Floating mat	Floating matrix	Plant used
FT-P-CN	Polystyrene pipes and nylon mesh	Coconut coir	No plant (control)
FT-S-CN	Styrofoam sheet and plastic pots	Coconut coir	No plant (control)
FT-P-CI	Polystyrene pipes and nylon mesh	Coconut coir	<i>C. indica</i>
FT-P-TL	Polystyrene pipes and nylon mesh	Coconut coir	<i>T. latifolia</i>
FT-S-CI	Styrofoam sheet and plastic pots	Coconut coir	<i>C. indica</i>
FT-S-TL	Styrofoam sheet and plastic pots	Coconut coir	<i>T. latifolia</i>

The mobile phase comprised of eluent A (0.1% formic acid in ultrapure water) and eluent B (acetonitrile with 0.1% formic acid). Subsequently, elution and detection of ECs were performed using Thermo Scientific™ Vanquish™ (liquid chromatography) coupled with Orbitrap Exploris™ 240 mass spectrometer (Thermo Fisher Scientific, USA). The mass spectrometry was conducted with a heated electrospray ionization (HESI) source operating in positive and negative ionization modes and data analysis was conducted using Thermo Scientific™ Xcalibur™ 4.4 software.

2.6 Microbial community analysis

All the VP3-augmented FTBs and control FTBs (FT-P-CN, FT-S-CN) were dismantled and the plant roots and matrix materials were vigorously mixed with water samples of respective FTBs to acquire complete microbial community of the system. The metagenomic DNA was extracted by filtering 1,500 mL sample through using 0.22 μ m DURAPORE PVDF membrane filter (Merck Millipore, Germany). The filter pads were transferred aseptically to the extraction of the metagenomic DNA using XpressDNA Soil Kit (MagGenome, India) according to the manufacturer's protocol. The experiments were conducted in triplicates. The extracted metagenomic DNA quantity and quality was checked using Nanodrop 2000 (Thermo Fisher Scientific, USA) and 0.8% w/v agarose gel electrophoresis, respectively. Pooled metagenomic DNA of each VP3-augmented FTBs were outsourced for high-throughput amplicon sequencing of the V3–V4 regions of the 16S *rRNA* gene using Illumina NextSeq 2000 platform (MedGenome, India). All of the 16S *rRNA* gene sequence data obtained for the study was submitted to NCBI under Project ID: 1170026 Biosample: All of the 16S *rRNA* gene sequence data obtained for the study was submitted to NCBI under Project ID: 1170026 Biosample: SAMN44091849 (Sample VP3), SAMN44089104 (Sample FT-S-TL+VP3), SAMN44089103 (Sample FT-S-CI+VP3), SAMN44089102 (Sample FT-P-TL+VP3), SAMN44089101

(Sample FT-P-CI+VP3), SAMN44089100 (Sample FT-S-CN), and SAMN44089099 (Sample FT-P-CN).

Multiplexed paired-end reads (2×300 bp) generated on the Illumina platform were analyzed using The Quantitative Insights into Microbial Ecology 2 (QIIME 2) program version 2022.8 (Bolyen et al., 2019). The “demux summarize” plugin was employed to assess the read quality, followed by quality filtering, denoising to amplicon sequence variants (ASVs), and chimeric sequence removal via “dada2 denoise-paired” plugin, where reads with $Q < 30$ were removed (Callahan et al., 2016). Feature-IDs were mapped to sequences from the representative sequence file and feature-table obtained after “q2-dada2.” Taxonomy classification was performed with the “feature classifier” (classify-sklearn) plugin leveraging a naive Bayesian classifier trained on the latest version (2022.10) of the Greengenes database (McDonald et al., 2012). The classified taxonomies were visualized using the “taxa barplot” plugin in QIIME 2-view.

The functional metabolic capability of the VP3-augmented FTB was predicted using PICRUSt2 software (Douglas et al., 2020) according to the denoising algorithms that enables phenotypic predictions via functional Enzyme Commission (EC) number (Kanehisa and Goto, 2000).

2.7 Effect of FTBs on antibiotic resistance profiles of Mini river water

Each metagenome was evaluated for the presence of four antibiotic resistance genes (ARGs): *sul1* and *sul2* (resistant to sulphonamides), *blaTEM* (resistant to beta-lactams), and *aac(6)-Ib-Cr* (resistant to aminoglycosides). These ARGs were previously identified as abundant in the Mini river (Patel et al., 2024), and thus were selected for evaluating the antibiotic resistance in Mini river water treated by VP3-augmented FTBs along with control FTBs. The Supplementary Table S1 contains a list of the ARG-specific primers used during the investigation. The primer sequences were obtained from the previous studies (Park et al., 2006; Pei et al., 2006; Adegoke et al., 2020). The polymerase chain reaction (PCR) was conducted in triplicates along with no template control using SureCycler 8800 Thermocycler (Agilent Technologies, USA). 1.2% w/v Agarose gel electrophoresis was performed to determine the presence of ARGs. The PCR program was carried out as follows: initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 45 s and extension at 72°C for 60 s; and final extension at 72°C for 5 min.

The Invitrogen™ PureLink™ Quick Gel Extraction Kit (Thermo Fisher Scientific, USA) was used to extract the PCR products for cloning. Subsequently, the purified DNA was cloned using the Mighty TA-Cloning Kit (Takara, Japan) according to the manufacturer's instructions. Cloned plasmids were isolated using Invitrogen™ PureLink™ Quick Plasmid Miniprep Kit (Thermo Fisher Scientific, USA). Quantification of cloned plasmids was performed using NanoDrop 2000 (Thermo Fisher Scientific, USA). Log dilution of cloned plasmids was used in RT-qPCR to prepare standard curves for each primer pair. Prior to use for final RT-qPCR analysis, each pair of primers was validated against each standard for product size and annealing temperature confirmation using normal PCR amplification and 1.2% agarose gel

electrophoresis. Quantification of targeted ARGs was performed using the Stratagene Mx3005P (Agilent Technologies, USA) in triplicate experiments as per the user instructions. The RT-PCR analysis was conducted using PowerUp™ SYBR® Green MasterMix (Thermo Fisher Scientific, USA). The RT-PCR program was carried out as follows: initial denaturation at 95°C for 3 min; followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s; and final extension at 72°C for 5 min.

2.8 Phytotoxicity analysis

Phytotoxicity of FTBs treated and untreated Mini river water was performed using *Vigna radiata* plant seeds following the procedure described by Rane et al. (2014). In brief, 10 healthy *V. radiata* seeds were kept in 20 mL FTBs treated and untreated samples at an ambient temperature, where 20 mL distilled water served as a control. The seed germination percentage was calculated after 3 days using following equation according to Ghosh et al. (2023):

$$\text{Seed Germination Percentage (SG\%)} =$$

$$(\text{Number of germinated seeds} / \text{Total number of seeds}) \times 100$$

2.9 Statistical analysis

Statistical analysis was performed for all the dataset using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). One-way ANOVA was used for the data analysis. Post-hoc analysis was conducted using Tukey's multiple comparison test to determine significant differences between the groups. A value of $p < 0.05$ was considered statistically significant.

3 Results

3.1 River water characterization

The overall quality of the Mini river water is mentioned in Table 3. Designated best use water quality criteria suggested by the Central Pollution Control Board (CPCB) (2018) of India (<https://cpcb.nic.in>) reveals that the obtained values for BOD and DO were Below-E. It means that the water is not suitable for drinking, outdoor bathing, fisheries or irrigation purposes. Moreover, the values of COD, sulfate and phosphate are also above the acceptance limits of general standards set by CPCB of India for industrial discharge.

3.2 Performance evaluation of the enriched bacterial consortia

The degradation potential of the enriched bacterial consortia was evaluated based on the removal efficiencies for COD, BOD, sulfate, and phosphate. In total, 5 consortia were established (Table 1), of which VP3 performed best and was therefore used for

TABLE 3 Water quality assessment of the Mini river with reference to the standard Indian water quality criteria.

Water quality parameters	Mini river water	Water quality status as per IS:10500*	General industrial discharge limits (CPCB**)
pH	8.62 ± 0.04	C	5.5–9.0
Temperature (°C)	27.5 ± 0.00	-	Not ≤ 5°C from AT
DO (mg/L)	1.55 ± 0.78	Below-E	-
COD (mg/L)	898.4 ± 25.35	-	250
BOD (mg/L)	35.27 ± 0.42	Below-E	30
Sulfate (mg/L)	872 ± 0.06	-	10
Phosphate (mg/L)	119.38 ± 1.98	-	5
Total Nitrogen (mg/L)	0.29 ± 0.02	-	0.3

*Indian Standard for drinking water specification. **Central Pollution Control Board of India. C represents drinking water source after conventional treatment and disinfection, whereas Below-E represents not suitable for drinking, bathing or irrigation.

bio-augmentation. The bacterial consortium VP3 demonstrated the following removal efficiencies (as shown in Table 4): COD (60.4 ± 4.14%), BOD (72.45 ± 0.1%), sulfate (57 ± 4.22%), and phosphate (75.89 ± 0.59%).

To characterize the microbial community of VP3, metagenomic DNA was extracted and sequenced. QIIME-2 analysis was performed to reveal the microbial community composition of VP3 (Figure 1A). The dominant phyla in VP3 were *Proteobacteria*, followed by *Firmicutes*, and *Bacteroidetes*. Where, the abundance of multiple genera namely *Pseudomonas*, *Hydrogenophaga*, *Brevibacillus*, *Brevundimonas*, *Aquicola*, *Delftia*, *Methylibium*, *Acidovorax*, and *Sphingomonas* belonging to different class of phylum *Proteobacteria* were obtained in consortium VP3.

3.3 Performance evaluation of the floating treatment bed systems

The biological oxygen demand (BOD) and chemical oxygen demand (COD) are generally used to evaluate the organic and other pollutants in the waterbodies and their quality. In this study, VP3-augmented Floating Treatment Bed systems (FTBs) demonstrated significant performance, achieving over 57 ± 1.65% reduction in BOD (Figure 2A) and approximately 70 ± 1.34% reduction of COD (Figure 2B) in 3-day Hydraulic Retention Time (HRT). The performance of floating treatment beds (FTBs) was improved in presence of macrophytes, *T. latifolia* and *C. indica*. These plants achieved better remediation compared to the non-vegetated FTB controls (FT-P-CN and FT-S-CN), i.e., approximately 40 ± 1.65% BOD removal and 37 ± 1.67% COD removal at a 3-day hydraulic retention time (HRT). This highlights the role of macrophytes in improving the purification efficiency of FTBs. Moreover, sulfate and phosphate contributes significantly in the eutrophication process, generation of algal blooms, and oxygen depletion in the aquatic ecosystems. All vegetated floating treatment beds (FTBs),

including FT-P-TL, FT-P-CI, FT-S-TL, and FT-S-CI, achieved over 60 ± 2.5% sulfate removal efficiency. Among these, VP3-augmented FTBs demonstrated the highest efficiency, with sulfate removal exceeding 80 ± 2.04% (Figure 2C). A similar pattern was observed for phosphate removal, with vegetated FTBs achieving an average efficiency of 51 ± 0.42% and the highest removal of 74 ± 0.6% recorded in VP3-augmented *T. latifolia*-planted FTBs (Figure 2D, Table S3).

3.4 Assessment of FTBs for the mitigation of the probable pathogens

The effectiveness of VP3-augmented Floating Treatment Bed systems (FTBs) for the removal of microbial pathogenic strains was assessed using the selective plate count method. The control FTBs, which did not incorporate plants or the VP3 consortium, exhibited nearly twice as many pathogens compared to the experimental setups (Figure 3). Specifically, *Salmonella* and *Shigella* were found in the highest concentrations in the FT-P-CN (control FTB without plants and VP3) and were least prevalent in the FTBs containing VP3 and *T. latifolia* (FT-P-TL). Similarly, the counts for *Vibrio* species and *E. coli* were highest in FT-P-CN and FT-S-CN (control FTBs). The lowest pathogen counts were observed in the FTBs containing both VP3 and *T. latifolia*. This indicates that the combination of *T. latifolia* and VP3 is highly effective in reducing pathogen levels.

3.5 Evaluation of the microbial status of polluted Mini river water after FTB treatment

The microbial community structure of floating treatment beds (FTBs) was analyzed to assess the impact of the VP3 consortium on microbial composition. The predominant microbial phyla in VP3-augmented FTBs included *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Planctomycetes*, demonstrating notable differences compared to control FTBs. The dominance of the *Proteobacteria* highlighted the significant influence of VP3 on the overall performance of the FTBs. In particular, the relative abundance of *Actinobacteria* increased to 7.2%, 6.4%, 3.1%, and 4.4% in FT-P-TL+VP3, FT-P-CI+VP3, FT-S-TL+VP3, and FT-S-CI+VP3, respectively, compared to 2.1% and 1.7% in the control FTBs FT-S-CN and FT-P-CN (Figure 1B). This phylum, previously associated with the bioremediation of heavy metals, antibiotics, and pesticides, exhibited enhanced abundance in VP3-augmented FTBs (Behera and Das, 2023; Antezana et al., 2023; Alvarez et al., 2017).

Additionally, increased relative abundances of *Acidobacteria*, *Chloroflexi*, and *Firmicutes* were observed in FT-P-CI+VP3 and FT-S-CI+VP3 compared to the controls. The phylum *Verrucomicrobia* was notably enriched in FT-P-TL (3.8%) and FT-S-TL (2.7%), suggesting plant-specific microbial community shifts. The *Proteobacteria* are known for their role in the bioremediation of xenobiotic pollutants, remained the most dominant phylum (Jokhakar et al., 2022; Srivastava and Verma,

TABLE 4 Analysis of % removal efficiencies (±SD) regarding the potential of enriched bacterial consortia after 5days; where, COD, Chemical Oxygen Demand; BOD, Biochemical Oxygen Demand.

	VP1	VP2	VP3	VP4	VP5
COD	31.82 ± 9.88	23.43 ± 4.21	60.40 ± 4.13	44.55 ± 9.16	19.01 ± 2.57
BOD	63 ± 4.17	61.5 ± 2.69	72.45 ± 0.1	47.29 ± 0.51	55.02 ± 3.43
Sulfate	45.93 ± 1.18	37.19 ± 2.06	57.9 ± 4.22	41.06 ± 1.79	21.97 ± 3.59
Phosphate	70.9 ± 1.25	57.82 ± 1.08	75.89 ± 0.59	71.98 ± 1.04	53.27 ± 2.94

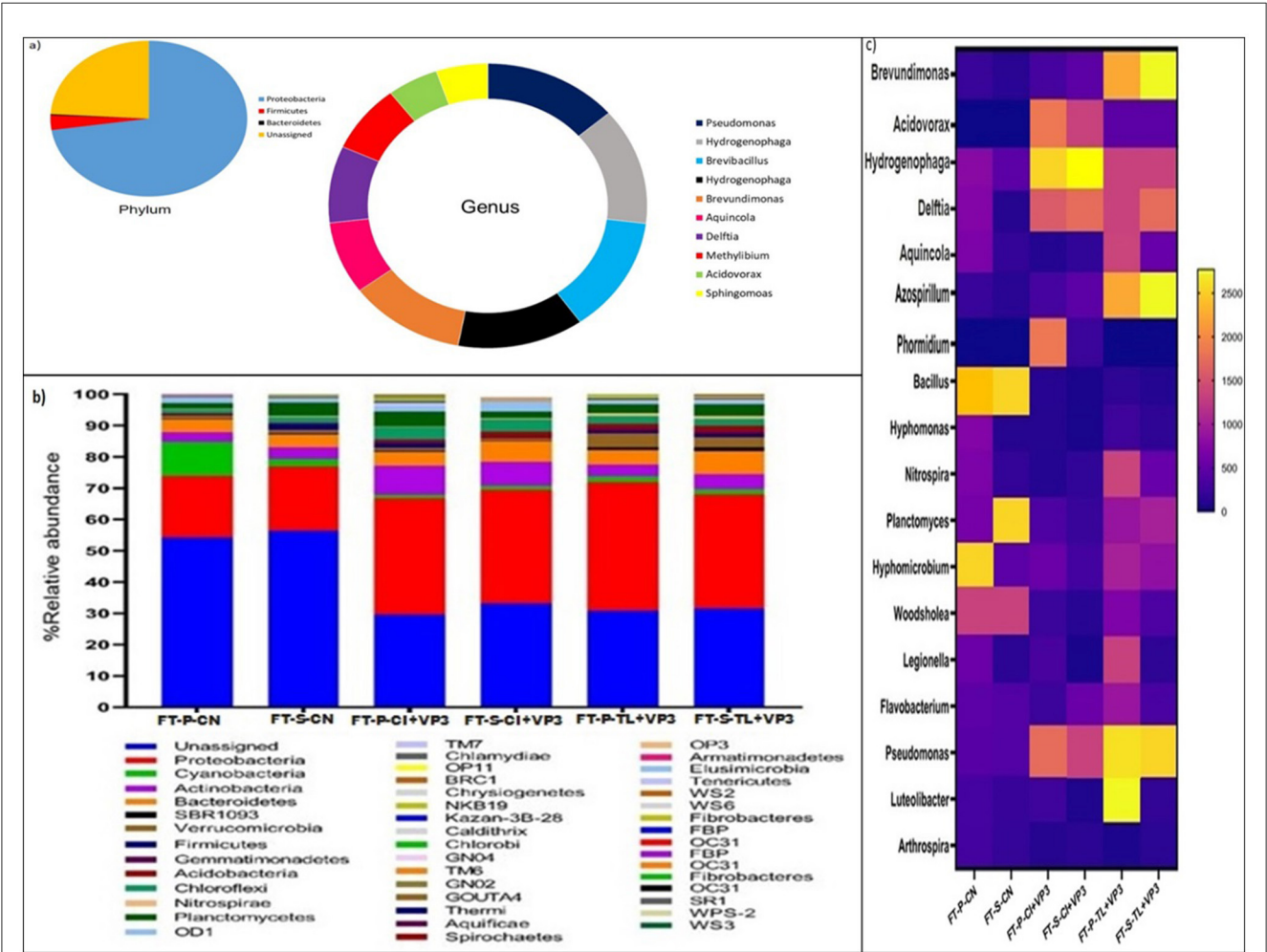


FIGURE 1 (A) Microbial community composition of the developed consortium VP3. Pie chart represents the phylum level microbial community and donut chart represents abundant genera of the consortium VP3. (B) Relative abundance of bacterial community at phylum level in VP3-augmented FTBs and FTB controls. FT, Floating bed treatment; P, Polystyrene frame; S, styrofoam frame; CN, control without plant; CI, *C. indica*; TL, *T. latifolia*. (C) Relative abundance of bacterial community at genus level in VP3-augmented FTBs and FTB controls. FT, Floating bed treatment; P, Polystyrene frame; S, styrofoam frame; CN, control without plant; CI, *C. indica*; TL, *T. latifolia*.

2023; Zhang et al., 2020). *Firmicutes*, along with *Proteobacteria* and *Actinobacteria*, have been reported to establish compatibility with the rhizosphere of *T. latifolia* (Pietrangelo et al., 2018). Moreover, *Proteobacteria* were predominant in FTBs with *C. indica* for tetracycline antibiotic bioremediation (Xu et al., 2023). Notably, *Verrucomicrobia*, a key phylum in submerged membrane bioreactors for sulfonamide antibiotic removal, showed increased relative abundance in FTBs planted with *T. latifolia* (Yu et al., 2018).

At the genus level, microbial shifts were evident between control FTBs (FT-P-CN, FT-S-CN) and VP3-augmented FTBs (FT-P-CI+VP3, FT-S-CI+VP3, FT-P-TL+VP3, FT-S-TL+VP3), indicating the influence of VP3 and the planted macrophytes on microbial community composition. The control FTBs were dominated by the genera *Bacillus* species and *Woodsholea* species, whereas VP3-augmented FTBs with *T. latifolia* (FT-P-TL+VP3, FT-S-TL+VP3) exhibited a shift toward dominance of *Pseudomonas* species. In contrast,

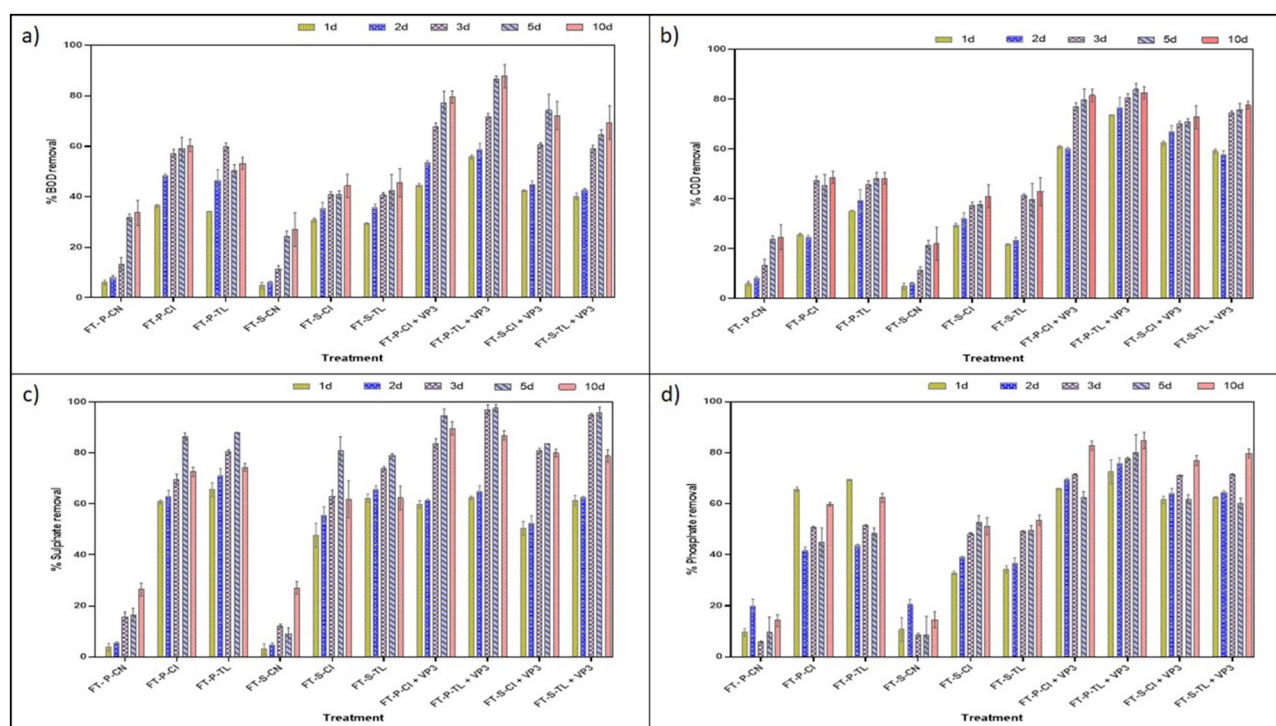


FIGURE 2

Performance evaluation of FTBs at different HRT (A) % BOD removal (B) % COD removal (C) % sulfate removal (D) % phosphate removal. FT, Floating bed treatment; P, Polystyrene frame; S, styrofoam frame; CN, control without plant; CI, *C. indica*; TL, *T. latifolia*.

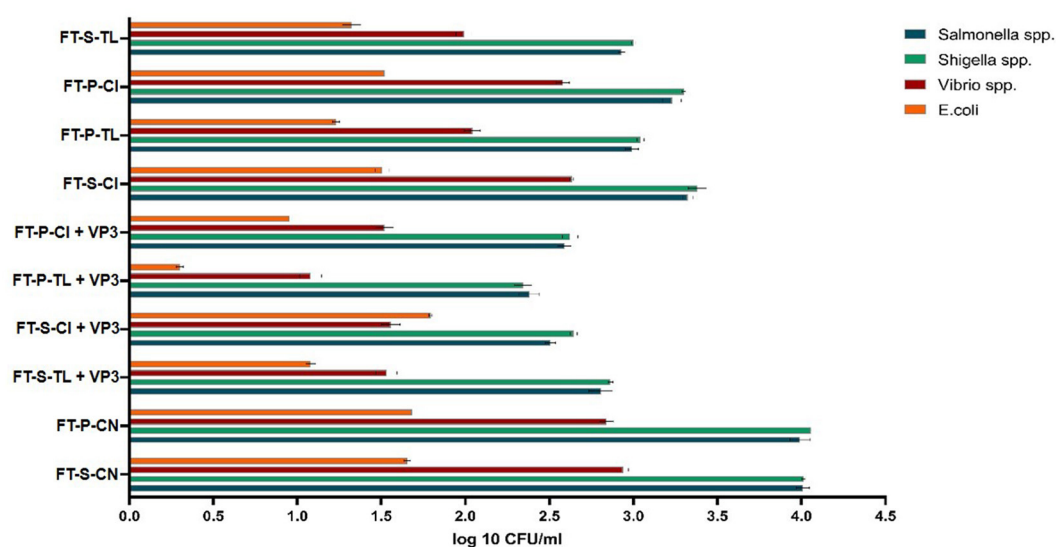


FIGURE 3

Probable pathogen abundance in Mini river water after FTB treatment. FT, Floating bed treatment; P, Polystyrene frame; S, styrofoam frame; CN, control without plant; CI, *C. indica*; TL, *T. latifolia*.

FTBs with *C. indica* (FT-P-CI+VP3, FT-S-CI+VP3) were predominantly enriched with *Hydrogenophaga* species (Figure 1C). The genus level microbial profiles in VP3-augmented FTBs closely mirrored those of the developed VP3 consortium. FTBs with *T. latifolia* were characterized by the dominance

of *Pseudomonas* species, along with notable abundances of *Brevundimonas*, *Azospirillum*, *Hydrogenophaga*, *Delftia*, *Hyphomicrobium*, and *Planctomyces*. In contrast, FTBs with *C. indica* were primarily dominated by *Hydrogenophaga*, followed by *Delftia*, *Acidovorax*, *Pseudomonas*, and *Azospirillum*, showcasing

plant-specific microbial community structures induced by the VP3 augmentation.

3.6 Functional gene annotation of the bacterial community involved in the FTB treatment

The predicted metabolic capabilities acquired from PICRUSt based on 16S profiles of the FTB-treated samples identified 80 genes associated with the degradation of various xenobiotic compounds (Figure 4). Notably, the genes involved in antibiotic degradation were more prevalent in the test FTBs (FT-P-TL+VP3, FT-S-TL+VP3, FT-P-CI+VP3, FT-S-CI+VP3) compared to the control FTBs (FT-P-CN, FT-S-CN). Among these, approximately 40 genes were linked to the degradation of a range of pollutants, including antibiotics, pesticides, aromatic hydrocarbons, plasticizers, detergents, heavy metals, and azo dyes. The abundance of these genes was higher in the test FTBs (FT-P-TL+VP3, FT-S-TL+VP3, FT-P-CI+VP3, FT-S-CI+VP3) compared to the control FTBs (FT-P-CN, FT-S-CN). Conversely, 9 genes associated with antibiotic resistance were found in higher abundance in the control FTBs (FT-P-CN and FT-S-CN) and were present in lower quantities in the test FTBs (FT-P-TL+VP3, FT-S-TL+VP3, FT-P-CI+VP3, FT-S-CI+VP3). This suggests a reduced prevalence of antibiotic resistance mechanisms in the test FTBs compared to the controls. These findings indicate that VP3-augmented FTBs have a greater potential for the removal of various emerging contaminants.

3.7 Removal of emerging contaminants by FTB treatment

Non-targeted screening using LC-HRMS revealed the presence of various pollutants in the VP3-augmented FTBs after treatment. The analysis showed that FT-P-CN contained 304 pharmaceutical and personal care chemicals (PPCPs), which were reduced to 114 in FT-P-TL+VP3 and 132 in FT-P-CI+VP3. Similarly, FT-S-CN initially had 296 PPCPs, which were decreased to 112 in FT-S-TL+VP3 and 131 in FT-S-CI+VP3. Chemical intermediates, which are often associated with anthropogenic pollution and adverse effects on aquatic ecosystems were found in quantities of 226 and 217 in FT-P-CN and FT-S-CN, respectively. These numbers were significantly reduced in the VP3-augmented FTBs, with 49 intermediates in FT-P-TL+VP3, 47 in FT-S-TL+VP3, 51 in FT-S-CI+VP3, and 54 in FT-P-CI+VP3 (Supplementary Figure S2). Pesticides, known contributors to river pollution, were present in 44 compounds in FT-P-CN and 47 in FT-S-CN. The VP3-augmented FTBs reduced pesticide concentrations to 29 in FT-P-TL+VP3, 26 in FT-P-CI+VP3, 27 in FT-S-TL+VP3, and 24 in FT-S-CI+VP3. Notably, 7–12 antibiotic derivatives and 6 dye stuff intermediates were detected in FT-P-CN and FT-S-CN, but these compounds were completely eliminated in all VP3-augmented FTBs. The detailed information about various emerging contaminants have been provided in the Supplementary Table S2.

3.8 Impact of FTBs on antibiotic resistance profiles of the Mini river water

The results showed that the majority of the abundant ARGs of untreated Mini river water were absent in the FTBs treated samples (Supplementary Table S4). Specifically, the FT-P-CI+VP3 and FT-P-TL+VP3 completely eliminated all four ARGs. Whereas, FT-S-CI+VP3 and FT-S-TL+VP3 reduced the prevalence of these ARGs after treatment. However, the control FTBs; FT-P-CN and FT-S-CN revealed presence of all four tested ARGs. It indicates role of bacterial consortium and macrophytes in reduction of ARGs.

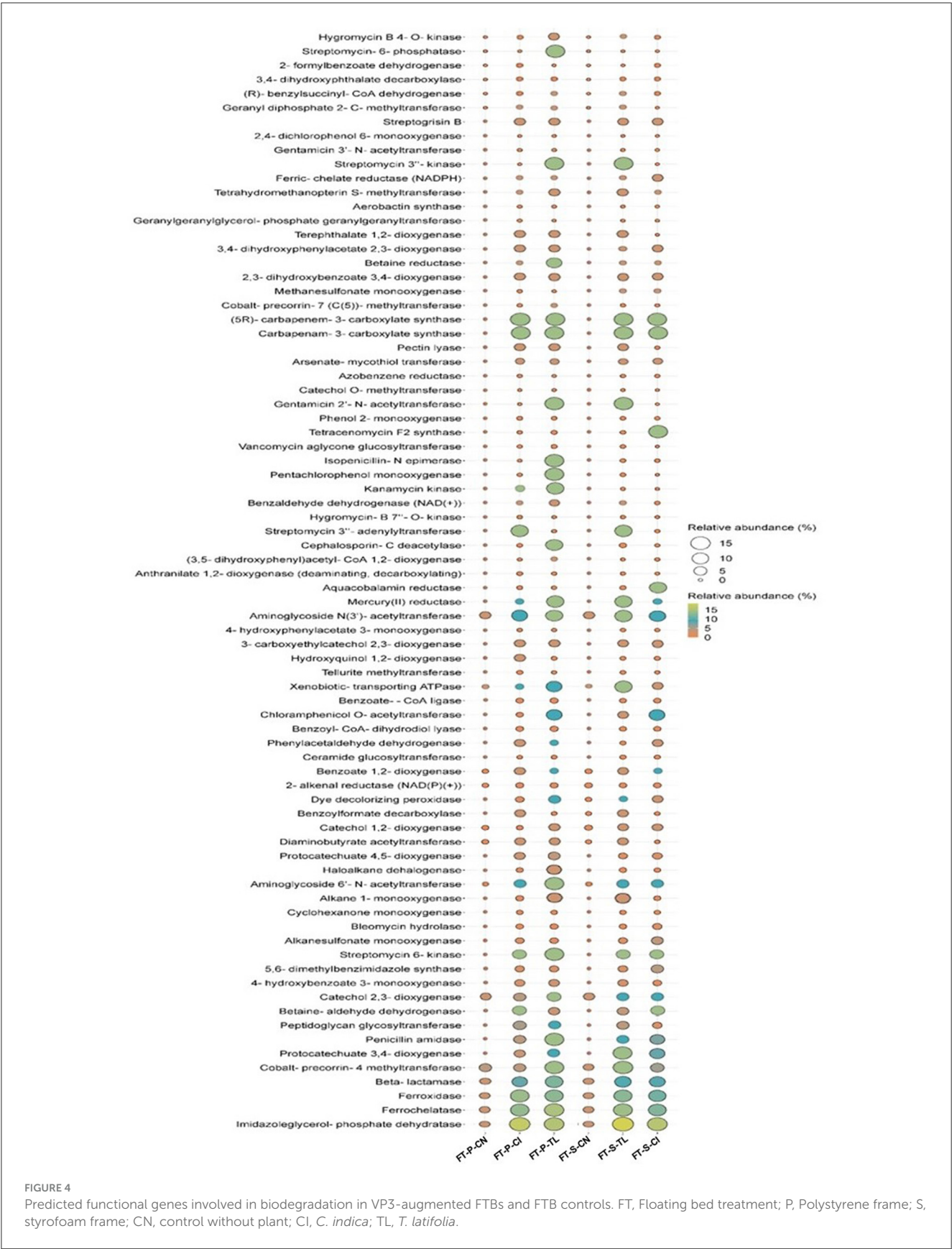
The Real-time PCR analysis of four selected antibiotic resistance genes (ARGs) confirmed a significant reduction in gene copy numbers following treatment with floating treatment beds (FTBs). The standard curves for the ARGs exhibited strong linear relationships ($R^2 > 0.925$), and the melt curves validated the absence of non-specific amplification during RT-PCR (Supplementary Figure S3). Comparative analysis revealed a significant reduction in ARG abundance in FT-P-CI and FT-P-TL compared to control FTBs, FT-P-CN and FT-S-CN (Figure 5).

The *sul1* gene, conferring resistance to sulfonamide antibiotics, was the most prevalent ARG, with initial copy numbers of 3.57×10^9 and 1.42×10^{10} copies/mL in FT-P-CN and FT-S-CN, respectively. These were substantially reduced to 4.72×10^4 , 7.55×10^4 , 1.13×10^5 , and 1.88×10^5 copies/mL in FT-P-TL, FT-P-CI, FT-S-TL, and FT-S-CI, respectively. A similar trend was observed for *sul2*, which initially had 2.74×10^9 and 4.22×10^9 copies/mL in FT-P-CN and FT-S-CN, showing a reduction of 5.0 to 5.68 log folds in FT-P-TL, FT-P-CI, and FT-S-TL.

The aminoglycoside resistance gene *aac* (6′)-Ib-Cr showed a marked decrease from 6.04×10^8 to 1.52×10^8 copies/mL in the control FTBs to 1.91×10^4 copies/mL in FT-P-CI and FT-P-TL. Similarly, the extended-spectrum beta-lactam resistance gene *bla*TEM exhibited significant reductions, with initial copy numbers of 9.99×10^8 and 4.21×10^8 in FT-P-CN and FT-S-CN dropping to 9.97×10^2 , 1.33×10^4 , 5.62×10^6 , and 6.2×10^4 copies/mL in FT-P-TL, FT-P-CI, FT-S-TL, and FT-S-CI, respectively.

3.9 Phytotoxicity evaluation of the Mini river water treated using FTBs

The toxicity of Mini river water treated by VP3-augmented FTBs was evaluated using the *Vigna radiata* seed germination assay. The results indicated that the FTB planted in a PVC frame with *T. latifolia* was the most effective in reducing toxicity, followed by the FTBs with *C. indica* in PVC and Styrofoam frames. Water treated by the VP3-augmented FTBs demonstrated a higher percentage of seed germination compared to the controls FT-P-CN and FT-S-CN (Supplementary Figure S4). However, the percentage of seed germination in the treated water was slightly lower than that observed in the positive control (distilled water). Untreated Mini river water exhibited the lowest percentage of seed germination due to the presence of various pollutants.



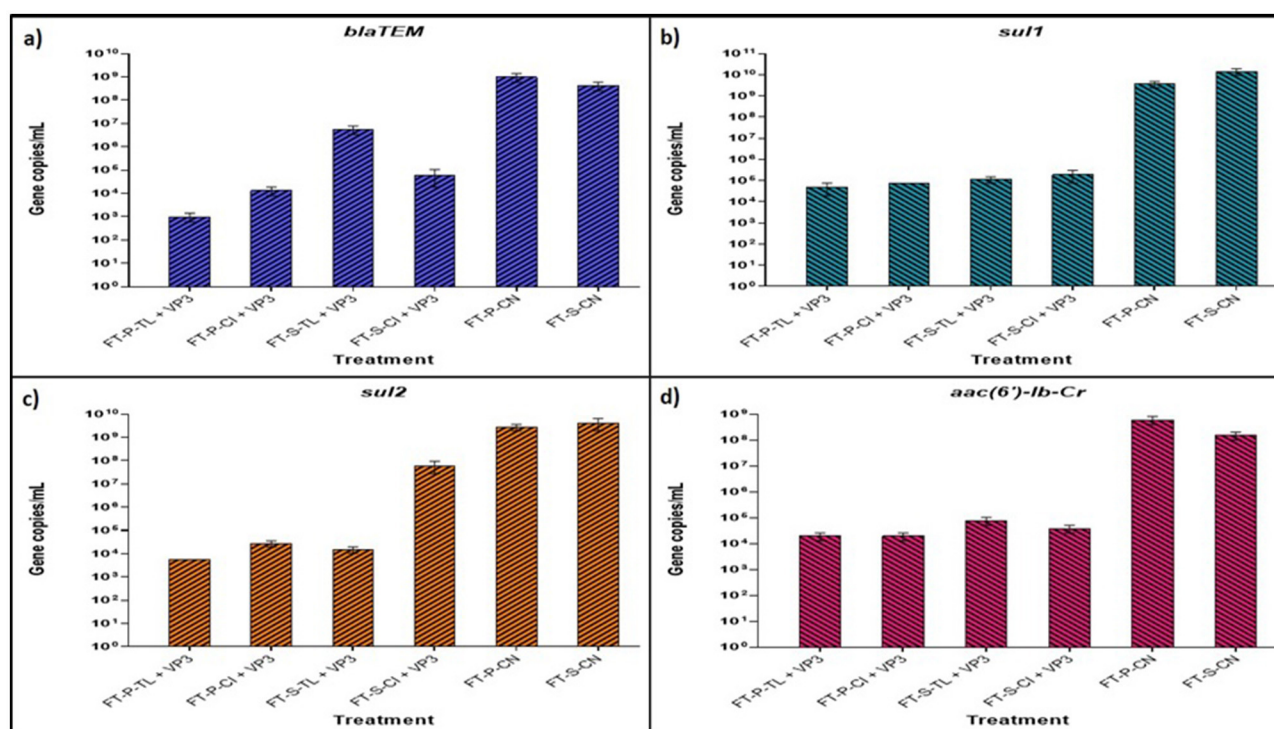


FIGURE 5

Absolute quantification of abundant ARGs using real-time qPCR of VP3-augmented FTB treated water samples (A) Absolute abundance of gene *blaTEM* (B) absolute abundance of gene *sul1* (C) absolute abundance of gene *aac(6')-Ib-Cr* (D) absolute abundance of gene *sul2*. FT represents Floating bed treatment, P stands for Polystyrene frame, S stands for styrofoam frame, CN stands for control without plant, CI stands for *C. indica*, and TL stands for *T. latifolia*.

4 Discussion

The Mini river water quality was not suitable for drinking as per the CPCB criteria. This adversely affects the riverine ecosystems and ultimately disturbs the food web. The FTBs vegetated with *T. latifolia* and *C. indica* in presence of VP3 efficiently reduced the COD and BOD of the polluted water of Mini river. This efficiency aligns with the known capabilities of floating treatments in handling organic pollutants. The VP3-augmented FTBs achieved approximately 70% COD removal within a hydraulic retention time (HRT) of 3 days. This aligns with previous studies demonstrating that microbial augmentation significantly enhances the biodegradation of organic and inorganic pollutants, achieving COD reductions of 65% to 85% depending on pollutant load and system design (Liu et al., 2021). Among the systems, FT-P-TL+VP3 achieved the highest phosphate removal of 100.1 ± 1.07 mg/L ($\sim 84.7 \pm 0.91\%$) from an initial phosphate concentration of 118.1 ± 1.53 mg/L. The phosphate uptake by macrophytes such as *C. indica*, *T. latifolia*, *Phragmites australis*, and *Iris pseudacorus* has been previously reported to reduce nutrient levels in polluted wastewater due to their robust root systems, which support microbial growth and enhance phosphate uptake (Haritash et al., 2017; Han et al., 2022). Similarly, Chand et al. (2021) reported that constructed wetlands incorporating biochar and *Typha* species achieved 73.92% sulfate removal after a 6-day retention time. In this study, the VP3-augmented FTBs demonstrated a maximum sulfate removal of over 83.6% within a 5-day HRT, indicating the

role of microbial mechanisms in bioremediation and facilitating nutrient uptake by macrophytes such as *T. latifolia* and *C. indica*. Kumar and Singh (2017) also reported that microbial consortium augmentation with *Eichhornia crassipes* enhanced sulfate removal efficiency in constructed wetlands. These findings confirm that VP3-augmented FTBs, including FT-P-TL+VP3, FT-P-CI+VP3, FT-S-TL+VP3, and FT-S-CI+VP3, exhibit superior pollutant removal potential compared to non-augmented FTBs (FT-P-CI, FT-P-TL, FT-S-CI, and FT-S-TL).

The effectiveness of VP3-augmented FTBs suggests a synergistic interaction between the bacterial consortium and the vegetated system. These findings are consistent with the earlier studies, such as Shahid et al. (2019); which reported improved water quality in FTBs vegetated with *Typha domingensis* and *Lepidagathis fusca*, augmented with a mixed bacterial culture for treatment of polluted water of Ravi river, Lahore. Where, they obtained the COD and BOD₅ decreased to 47 and 21 mg/L from initial concentration of 405 and 190 mg/L, respectively in the system consisting bacteria augmented *T. domingensis*.

The probable pathogenic enumeration reveals that the inclusion of VP3 and *T. latifolia* in FTBs significantly reduces the abundance of microbial pathogens compared to control FTBs that lack these components. The significantly lower pathogen counts in the FTBs containing both VP3 and *T. latifolia* suggest a synergistic effect, where both components work together to improve the overall efficacy of the FTBs. The presence of *T. latifolia* likely contributes to enhanced pathogen removal through

processes such as root filtration and microbial interactions, while the VP3 consortium may further assist in reducing pathogen counts through its bioremediation capabilities. This finding supports the use of VP3-augmented FTBs with macrophytes like *T. latifolia* in mitigating waterborne pathogenic outbreaks, especially in polluted waterways such as the Mini river. Earlier, [Donde et al. \(2020\)](#) emphasized the critical role of macrophytes in reducing fecal pathogenic bacteria and importance of combining local emergent and submerged macrophytes for efficient treatment. These findings uncover the potential of VP3-augmented FTBs in managing microbial contamination in water bodies.

The microbial community of VP3 exhibits greater similarity with the microbial community at similarly polluted sites in Budha Nala and Tung Dhab drain at Punjab, India ([Kumar and Saini, 2024](#)). The phyla *Proteobacteria* and *Actinomycetota* consist of variety of genera efficient for bioremediation of polluted waterways ([Joye et al., 2016](#)) as well as potential plant growth promoting microbes involved in rhizo-remediation ([Behera and Das, 2023](#); [Singh et al., 2018](#)). The phylum *Proteobacteria* comprises of genera such as *Brevundimonas* species, *Pseudomonas* species and *Desulfobacca* species reported for bioremediation of petroleum and oily sludge contaminated by total petroleum hydrocarbons, saturates, aromatics, and polar compounds ([González-Toril et al., 2023](#)). [Chen et al. \(2023\)](#) noted that a consortium dominated by *Pseudomonas* species, *Stenotrophomonas* species, and *Delftia* species exhibited high degradation potential for phenanthrene. This suggests that the microbial community within consortium VP3 has a robust capacity to metabolize a range of emerging pollutants.

To understand the potential of the FTBs, the composition of microbial communities developed during treatment is crucial. In this study, the dominant phyla *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Planctomycetes* were consistent across all FTB configurations, indicating their fundamental role in the treatment processes. The microbial community of the VP3-augmented FTBs was dominated by phylum *Proteobacteria* representing the successful augmentation of the VP3 with the macrophytes used for the study. This consistent dominance of *Proteobacteria* in all the VP3-augmented FTBs reinforces potential of VP3 consortium for degradation of various pollutants ([Jokhakar et al., 2022](#); [Srivastava and Verma, 2023](#); [Zhang et al., 2020](#)). However, the control FTBs (FT-P-CN and FT-S-CN) where no plants were introduced has relatively less abundance of the phylum *Proteobacteria*. The increase in the relative abundance of *Acidobacteria*, *Chloroflexi*, and *Firmicutes* in FTBs with *T. latifolia* and *C. indica* suggests that these plant species may enhance the growth of these microbial groups, potentially improving bioremediation efficacy. Observed presence of *Actinobacteria* aligns with its ability to remediate heavy metals and organic pollutants ([Antezana et al., 2023](#); [Alvarez et al., 2017](#)). Earlier, [Pietrangelo et al. \(2018\)](#) noted that the phyla of *Proteobacteria*, *Actinobacteria*, and *Firmicutes* involved in the foundation of the microbiota with some other phyla such as *Acidobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Verrucomicrobia* which varies according to the plant species. The presence of *Firmicutes* in association with *T. latifolia* and its compatibility with rhizosphere environments supports its role in effective bioremediation within VP3-augmented FTBs with *T. latifolia*.

Similarly, the prominence of *Proteobacteria* in FTBs with *C. indica* highlights its adaptability and efficacy in various bioremediation contexts. This combination of *Proteobacteria* with *C. indica* was earlier reported for degradation of tetracycline antibiotics from water using mycorrhizal fungi based vertical flow constructed wetlands with more than 90% removal efficiency ([Xu et al., 2023](#)). The increased abundance of *Verrucomicrobia* in FTBs planted with *T. latifolia* is compelling interest, as this phylum has been previously associated with the removal of sulfonamide antibiotics ([Yu et al., 2018](#)). This suggests that *T. latifolia* may foster conditions conducive to the growth of *Verrucomicrobia*, enhancing the overall pathogen removal efficiency of the FTBs.

These findings indicate that both plant species, *T. latifolia* and *C. indica*, play significant roles in shaping microbial community structure and enhancing the bioremediation capacity of FTBs. The synergy between plant and microbial components in FTBs discloses the potential for improved water treatment of polluted rivers. The prevalence of the genus *Pseudomonas* in FTBs with *T. latifolia* aligns with its well-documented role in biodegrading a wide range of pollutants, including antibiotics, personal care products (PPCPs), pesticides, heavy metals, and aromatic hydrocarbons ([Tang et al., 2024](#); [Pápai et al., 2023](#)). The dominance of potential plant growth promoting bacterial genus *Azospirillum* in the VP3-augmented FTBs planted with *T. latifolia* enhances the stress tolerant abilities of the plant. Earlier genus *Azospirillum* was reported to have significantly reduced the damage to roots of *Polygonum hydropiperoides* planted constructed wetlands exposed to the heavy metal contamination ([Barbosa et al., 2023](#)). The presence of the Genus *Hydrogenophaga* species in FTBs with *C. indica* is also significant, as this genus is known for its ability to degrade aromatic compounds and certain chlorinated compounds used in pesticides and PPCPs ([Borah et al., 2023](#); [Yang et al., 2023](#)). These findings unveil the impact of macrophytes on shaping microbial communities and highlight the role of specific bacterial genera in the bioremediation processes within FTB systems. The interplay between macrophytes and microbial communities are key contributors optimizing the effectiveness of pollutant removal in FTBs.

The effective degradation of xenobiotics is essential for mitigating their environmental impact and preventing the proliferation of antibiotic-resistant bacteria (ARB). The identification of approximately 40 genes related to the degradation of various pollutants including pesticides, aromatic hydrocarbons, plasticizers, detergents, heavy metals, and azo dyes, demonstrates the versatility of the test FTBs in addressing a wide spectrum of contaminants. These findings, supported by LC-HRMS analysis, confirm that VP3-augmented FTBs are highly effective in degrading critical pollutants such as antibiotics and dyes. Overall, the results underscore the strong potential of VP3-augmented FTBs to reduce diverse contaminants, making them valuable tools for improving water quality and addressing pollution challenges in river systems. The potential transfer of ARGs to pathogenic bacteria remains a significant global public health concern. However, the present study revealed a substantial reduction in ARG absolute abundances (1.84 to 6 log fold) within the VP3-augmented FTBs. The observed reduction in gene copies/mL highlights the progressive elimination of ARGs

across these systems. For instance, [Porrás-Socias et al. \(2024\)](#) reported a decrease in *sul1* abundance from 4.3×10^6 to 6.9×10^4 copies/mL in constructed wetlands planted with *Sparganium erectum*. Similarly, in this study, the VP3-augmented FTBs significantly reduced ARGs, including *sul1*, *sul2*, *blaTEM*, and *aac(6')-Ib-Cr*, through mechanisms such as plant uptake, bacterial host die-off, or sorption to organic matter ([Sabri et al., 2021](#)). The robust root systems of wetland plants like *T. latifolia* and *C. indica* play a pivotal role in this process by supporting microbial communities involved in filtration, adsorption, absorption, and biotransformation of ARGs. Additionally, macrophytes provide oxygen to microbes and act as primary filters for solid particles, enhancing microbial activity, reducing ARG accumulation, and promoting overall ARG removal ([Fang et al., 2017](#)). These findings align with [Donde et al. \(2020\)](#), who demonstrated the effectiveness of *T. latifolia* in constructed wetlands for the removal of 12 antimicrobial agents and their associated resistance genes, achieving a purification efficiency of 75% in effluents from Lake Dianchi, China. Similarly, [Ávila et al. \(2021\)](#) reported that subsurface flow constructed wetlands vegetated with *Phragmites australis* effectively removed antibiotics, including ciprofloxacin, ofloxacin, piperidic acid, and azithromycin, alongside ARGs such as *sul1* (46%–97%), *sul2* (33–97%), *ermB* (9%–99%), *qnrS* (18%–97%), and *blaTEM* (11%–98%). These studies support the current findings, emphasizing the significant role of the VP3 microbial consortium, combined with suitable macrophytes, in mitigating antibiotic resistance in river systems. This integrated bioremediation strategy highlights its potential to address complex pollution challenges in environmental remediation effectively.

The findings from the *Vigna radiata* seed germination assay suggests that the VP3-augmented FTBs effectively reduce the toxicity of Mini river water. Specifically, the FTBs planted with *T. latifolia* in PVC frames showed the highest efficiency in toxicity removal, making it the most promising option for further applications. The treated water from the VP3-augmented FTBs exhibited significantly higher seed germination rates compared to the untreated controls (FT-P-CN and FT-S-CN), although it did not fully match the germination rates seen with distilled water. These results are consistent with previous studies, such as those by [Kumar et al. \(2018\)](#), who reported that polluted Hindon river water adversely affected *Vigna radiata* seed germination, indicating poor water quality due to anthropogenic pollution. Their study concluded that diluted water was more suitable for irrigation practices. In contrast, the treated water from the VP3-augmented FTBs, particularly with *T. latifolia*, showed that it could be used directly without further dilution for agricultural purposes. Overall, the data highlighted the effectiveness of the VP3-augmented FTBs in removing toxicity from polluted water bodies, suggesting a viable approach for improving water quality and suitability for agricultural use.

5 Conclusion

The study demonstrates that the plant-microbial combination enhances the overall efficiency of floating treatment bed (FTB) systems in removing sulfate, phosphate, COD, and BOD from polluted river water. Further, it also exhibited significant

potential for pathogen elimination from contaminated water. The metagenomic analysis revealed that the VP3-augmented FTBs host a microbial community predominantly composed of the genera *Pseudomonas* species and *Hydrogenophaga* species, both belonging to the phylum *Proteobacteria*. In addition, the VP3-augmented FTBs possess a range of functional genes critical for the degradation of antibiotics, pesticides, pharmaceutical and personal care products (PPCPs), polycyclic aromatic hydrocarbons (PAHs), and other emerging contaminants (ECs). ARG detection confirmed that VP3-augmented FTBs were more efficient in reduction of ARGs, with lower ARG distribution compared to control FTBs. It could remove 7–12 antibiotic residues, 6 dye intermediates, and over 50% of pesticides, which further establishes its effectiveness in reduction of ECs in the polluted rivers. Phytotoxicity testing using *Vigna radiata* revealed that water treated by FT-P-TL and FT-P-CI exhibited improved seed germination rates, indicating enhanced environmental safety. These findings highlight the potential of FTBs, especially those enriched with bacterial consortia, as effective and sustainable solutions for removing a broad range of emerging pollutants under environmental conditions. The study underscores the advantages of FTBs over intensive treatment approaches for improving water quality in polluted river systems.

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

VP: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. SS: Data curation, Formal analysis, Investigation, Writing – original draft. CD: Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing. BK: Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. DM: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, 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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Surface display of Lys0859, a *Streptococcus suis* prophage lysin, on *Bacillus subtilis* spores and its antibacterial activity against *Streptococcus suis*

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Introduction: *Streptococcus suis*, an important zoonotic and opportunistic pathogen in pigs, brings huge economic losses to the pig-raising industry and infects humans with diseases. Phage lysin is regarded as a promising substitute for antibiotics due to its ability to quickly and efficiently kill bacteria without easily developing resistance. However, their clinical applications have been hindered by inherent instability under environmental stressors.

Methods: We constructed *B. subtilis* spores displaying bacteriophage lysin Lys0859 using spore coat protein CotG as an anchoring motif. Environmental tolerance was evaluated through thermal (37–95°C), pH (1.0–8.0), and enzymatic challenges, while antibacterial efficacy against *S. suis* was assessed using agar diffusion assays and murine infection models with systemic bacterial load quantification.

Results: The spore-display system enhanced environmental resistance of Lys0859 while preserving its bactericidal efficacy. *In vitro* assays demonstrated 1×10^6 CFU rBS^{CotG-0859} spores exhibited equivalent bactericidal activity to 39.11 µg free Lys0859 against *S. suis*. *In vivo*, spore treatment reduced *S. suis* SC19 colonization by 0.47–1.96 log units ($p < 0.05$) across all tissues compared with PBS controls.

Discussion: This study achieved functional display of prophage lysin Lys0859 on *B. subtilis* spores through CotG anchoring, demonstrating potent *in vitro* anti-streptococcal activity. Crucially, this strategy streamlined bioproduction by eliminating purification demands and lowering costs, lays the foundation for the clinical application of prophage lysin.

KEYWORDS

Streptococcus suis prophage lysin, *Bacillus subtilis*, CotG, spore surface display, *Streptococcus suis* infection

1 Introduction

Streptococcus suis (*S. suis*) is an important zoonotic pathogen responsible for infecting both humans and a wide range of animal species. *S. suis* can spread and infect through contact with infected animals or contaminated animal products, causing serious threats to the pig industry (Wang et al., 2022) and public health security worldwide (Feng et al., 2010).

In China, there were two large-scale human infections of *S. suis* in 1998 and 2005, resulting in 53 deaths, which attracted great social attention (Normile, 2005; Tang et al., 2006). So far, 29 serotypes of *S. suis* have been identified based on capsular polysaccharide, among which serotype 2 is the serotype with the highest clinical isolation rate and is considered to be one of the most virulent serotypes (Goyette-Desjardins et al., 2014; Liu et al., 2019; Okura et al., 2016). *S. suis* mainly inhabits the upper respiratory tract of pigs, especially the tonsils and nasal cavities, as well as the digestive and genital tracts (Goyette-Desjardins et al., 2014). After humans or pigs are infected with the virulent strains of *S. suis*, they often exhibit meningitis, septicemia, arthritis, endocarditis, pneumonia, and other diseases (Gottschalk and Segura, 2000; Segura et al., 2017). Like most other bacterial infections, antibiotic therapies remain one of the most effective methods for *S. suis* infection. However, an increasing number of studies have reported that the prolonged use or misuse of antibiotics can lead to environmental pollution, antibiotic residues, and the production of multidrug-resistant bacteria (Brown and Wright, 2016; Zhang et al., 2018; Hall et al., 2020). Therefore, there is an urgent need to find a novel antibacterial drugs or antibiotic alternatives to combat the infection of *S. suis*.

Phage lysin is a cell wall hydrolase encoded by bacteriophage, which can rapidly destroy the cell wall structure by degrading peptidoglycans in the cell wall upon contact with the host cells, and release newly assembled bacteriophages (Young et al., 2000). Compared with traditional antibiotics, bacterial resistance to lysin is difficult to develop, and lysin can specifically lyse target pathogens without disturbing commensal flora (Wang Z. et al., 2019). Therefore, lysin as a promising antibacterial agent has been studied for various biomedical applications, and its effectiveness has been conformed in multiple animal model of infected with resistant bacteria (Gilmer et al., 2013; Eichenseher et al., 2022). However, lysin is sensitive to multiple environmental factors, such as temperature, pH, and proteases, which affect their bioactivity and thus limit the bactericidal effect in clinical practice. Therefore, there is an emergent search for a method to enhance the resistance of lysin to cope with environmental stresses. *B. subtilis* spore surface display is considered one of the most effective ways to deliver heterologous proteins with high biological activity and stability (Iwanicki et al., 2014). The United States Food and Drug Administration (FDA) has classified *B. subtilis* as a Generally Recognized as Safe (GRAS) strain. It can be used as a food additive in food preparations for both humans and animals, and is also applicable in the treatment of gastrointestinal diseases (Cutting et al., 2009). *B. subtilis* spores offer several advantages in anchoring heterologous proteins: (1) *B. subtilis* spores exhibit greater adaptability to extreme environments, including nutrient deprivation, drastic temperature and pH fluctuations, ultraviolet irradiation, and toxic substances (Ullah et al., 2024). (2) Heterologous proteins anchored on the spore surface do not need to cross the cell membrane. This not only avoids protein misfolding but also ensures the structure and biological activity of the exogenous proteins (Guo et al., 2018). (3) Enzymes displayed on the spore surface generally possess good reusability and stability (Guo et al., 2018). For instance, the p75 protein was displayed on the surface of *B. subtilis* spores using spore coat protein CotG as an anchoring motif, and the peptidoglycan hydrolase activity, stability, and the antibacterial activity of the spore-displayed p75 protein were significantly enhanced (Kang et al., 2020). However, we have hardly found any reports on the display of prophage lysin on the spore surface of *B. subtilis*.

In this study, the prophage lysin Lys0859 from *S. suis* SS0859 was successfully exhibited on the spore surface by employing the outer coat protein G (CotG) of *B. subtilis* spores as the anchor protein. Then, we evaluated the *in vitro* resistance of Lys0859 displayed on the spore surface of recombinant bacteria against environmental assaults, as well as antimicrobial spectrum of the recombinant bacteria *in vitro*. Furthermore, we explored the protective effect of recombinant bacteria on *S. suis* SC19 infection by murine model of systemic *S. suis* infection.

2 Materials and methods

2.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in [Supplementary Table S1](#), and plasmids and primers are listed in [Supplementary Table S2](#). The *streptococci* strains were cultured in tryptic soy broth (TSB) (BD Biosciences, MD, United States) supplemented with 5% (v/v) fetal bovine serum (Solarbio, Beijing, China) or TSB plates containing 1.5% (w/v) agar and 5% (v/v) fetal bovine serum at 37°C. The other strains were cultured in Luria-Bertani (LB) broth or LB plates containing 1.5% (w/v) agar at 37°C. *B. subtilis* sporulation was cultured in Difco sporulation medium (DSM), as previously described (Mascarenhas et al., 2002).

2.2 Construction of CotG-0859 fusion protein expression plasmid

To display phage lysin 0859 (Lys0859), which was previously discovered in our laboratory (Li et al., 2023), on the surface of *B. subtilis* 168 spores, we constructed a CotG-0859 fusion. The CotG gene was amplified with primers CotG-F/CotG-R from the *B. subtilis* 168 chromosomes. The Lys0859 gene from *S. suis* 0859 was amplified using primers 0859-F/0859-R by PCR. Then, the CotG and 0859 genes were constructed into a CotG-0859 fusion by overlap-extension PCR, digested with *Bam*H I and *Eco*R I (Takara, China), and ligated into pDG364 vector to obtain the recombinant plasmids pDG364-CotG-0859. Meanwhile, pCold-CotG-0859 (CotG-F/CotG-R, 0859-F/0859-R1, *Bam*H I/*Hind* III) and pCold-0859 (0859-F1/0859-R1, *Bam*H I/*Hind* III) were constructed using the same methods. Recombinant plasmids were transformed into *E. coli* DH5 α and the positive colonies were identified by colony-PCR.

2.3 Construction of recombinant *Bacillus subtilis* 168

The obtained pDG364-CotG-0859 recombinant plasmid was linearized by *Kpn* I (Takara, China) enzyme digestion, and transformed into the amylase E (*AmyE*) gene of the competent *B. subtilis* genome by the natural transformation method (Spizizen, 1958). Then, the clones generated after the integration of the target gene at the *AmyE* locus of *B. subtilis* were selected on LB agar plates supplemented with 5 μ g/mL chloramphenicol. Used LB agar plates containing 1% starch to screen positive strains with amylase gene deletion. Extracted recombinant bacterial genomic DNA and used *AmyE*-F/*AmyE*-R, G8-F/G8-R, G8-F/*AmyE*-R, and *AmyE*-F/G8-R for PCR identification, respectively.

2.4 Spore preparation

The sporulation of wild-type *B. subtilis* 168 (WT) and recombinant *B. subtilis* were induced at 37°C for 48 h using Difco Sporulation Medium (DSM). Spore purification was performed as previously described, with some modifications. The spores were collected by centrifugation 8,000 g for 10 min at 4°C, and washed with 0.5 M NaCl. Resuspend the spores in Tris-HCl (50 mmol/L, pH 7.2) containing a final concentration of 50 µg/mL of lysozyme and incubate at 37°C in a water bath for 1 h. Then, the spores were harvested by centrifugation and washed with 1M NaCl, 1M KCl, and distilled water (three times), respectively. After incubation at 65°C for 1 h, pure spores were harvested and resuspended in sterile PBS, and stored at -40°C.

2.5 Expression of fusion proteins in *Bacillus subtilis* and SDS-PAGE and western blot analysis

The purified spores were incubated with SDS-DTT extraction buffer at 70°C for 30 min to extract spore coat proteins. To confirm that the surface display of Lys0859 on the spore coat, the extracted spore coat proteins was subjected to SDS-PAGE. Subsequently, one portion was stained with Coomassie Brilliant Blue, while the other portion was transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with PBST containing 5% skim milk for 2 h. After three washes with PBST, incubates with Lys0859 antiserum (1:5,000 in PBST) for 2 h. Then, the membrane was incubated with HRP-conjugated Goat anti-Mouse IgG second antibody (1:5,000 in PBST) for 1 h at room temperature and visualized by ECL detection.

2.6 Immunofluorescence microscopy

To detect Lys0859 on the surface of spores, 1 mL of purified spore solution was harvested after 48 h of *B. subtilis* induction and fixed on a glass slide, and blocked with 5% BSA for 2 h. Anti-Lys0859 antibody (1:400 in PBST) was added and incubated for 2 h at room temperature. Then, FITC labeled goat anti-mouse IgG antibody (1:400 in PBST) was added and the spores were observed with a fluorescence microscope (Olympus, Japan).

2.7 The growth curves and sporulation rate of recombinant *Bacillus subtilis*

To investigate whether the insertion of exogenous proteins affects the activity of the strain, we determined the growth curves and sporulation rate of *B. subtilis* 168 and rBS^{CotG-0859}. *B. subtilis* 168 and rBS^{CotG-0859} were inoculated into LB medium or DSM sporulation medium (final concentration 1×10^4 CFU/mL) respectively, and incubated at 37°C with shaking (180 rpm). Samples were collected every 2 h to prepare 10-fold serial dilutions and spread onto LB agar plates and cultured at 37°C for 12 h before bacterial counting. For spore counting, the spore suspensions were treated at 85°C for 5 min, and then 10-fold serial dilutions were prepared to place on LB agar

plates and cultured at 37°C for 12 h before counting. The sporulation rate was calculated with the following equation: Sporulation rate = number of spores/ number of vegetative cells.

2.8 *In vitro* resistance assay of recombinant spores

The rBS^{CotG-0859} spores (1×10^7 CFU) were separately resuspended into 1 mL of sterile PBS with different pH values (1, 2, 3, 4, 5, 6, 7, or 8), or simulated gastric fluid (SGF, HCl, pH 1.2) containing 10 g/L of pepsin in 0.85% NaCl solution, or simulated intestinal fluid (SIF, NaOH, pH 6.8) containing 10 g/L of trypsin in 0.05 M KH₂PO₄ solution, and incubated at 37°C. At predetermined time points, the samples were centrifuged at 6,000 g for 5 min at 4°C, and bacterial cells were washed twice with sterile PBS and resuspended in 100 µL of PBS. Next, bacteriostatic activity of rBS^{CotG-0859} spores (1×10^7 CFU) against *S. suis* SC19 was tested by the agar-well diffusion assays. The plates were incubated at 37°C for 12 h, and the diameter of inhibition zone was measured with vernier caliper.

One milliliter of rBS^{CotG-0859} spores (1×10^7 CFU) were separately placed in water baths at different temperatures (37°C, 45°C, 55°C, 65°C, 75°C, 85°C, or 95°C) for 30 min. Bacterial cells were then collected by centrifugation (6,000 g, 5 min, 4°C), washed twice and resuspended in 100 µL sterile PBS. The bacteriostatic activity was measured as described above.

2.9 Genetic stability of recombinant spores

To assess the genetic stability of recombinant bacteria, the rBS^{CotG-0859} were serially passaged on LB agar plates up to 10 times. A single bacterial colony was picked and inoculated into 1 mL of LB medium (contains 5 µg/mL chloramphenicol) and incubated overnight at 37°C with shaking (180 rpm). PCR identification of rBS^{CotG-0859} clone was performed using 168-F/168-R and G8-F/G8-R primers, respectively. The rBS^{CotG-0859} (the 1st to 10th generation colonies) were induced to sporulate in DSM for 48 h at 37°C and purified them. The antibacterial activity of rBS^{CotG-0859} (the 1st to 10th generation) against *S. suis* SC19 by agar diffusion assay. All plates were cultured at 37°C for 12 h before observing the inhibition zone, and the diameter of the inhibition zone was measured using a vernier caliper. The presence of a clear zone indicated antagonistic activity.

2.10 *In vitro* antibacterial activity of recombinant spores

The *in vitro* antagonistic activity of rBS^{CotG-0859} spores was tested using the agar well-diffusion method against common pathogens. The bacterial lawns were prepared by mixing 10 mL of TSB medium containing 5% fetal bovine serum (FBS) with bacterial culture suspension (1×10^6 CFU/mL) and then poured onto a sterile plate covered with LB agar and Oxford cups, wells of 8 mm diameter were made on TSB agar after removing the cups. The rBS^{CotG-0859} spores and different dosages of Lys0859 in a total volume of 100 µL were then added separately into the well on the agar plate. After incubating at 37°C for 12 h, and measuring the diameter of inhibition zone. The diameter of the inhibition zone

(mm) was the average of three independent experiments and presented in the form of a bar graph. The five known doses of Lys0859 and their corresponding inhibition zone sizes were displayed on the x-y scatter plot, respectively. Thereafter, the best-fit linear regression equation (LRE) was plotted based on the linear trend line. After inhibition zone diameter ($rBS^{CotG-0859}$) were fitted in the LRE, the equivalent dose of lysis enzyme activity of the recombinant bacteria was ultimately obtained.

2.11 *In vivo* study of bactericidal activity of recombinant spores

A model of *S. suis* SC19 infection mouse was established using 4-week-old specific pathogen-free (SPF) female ICR mice purchased from the Experimental Animal Center of Huazhong Agricultural University, Wuhan, China. Mice were randomly divided into 4 groups ($n = 6$): (i) control; (ii) SC19 + PBS group; (iii) SC19 + BS168; (iiii) SC19 + $rBS^{CotG-0859}$. On the first experimental day, all mice were injected intraperitoneally with 100 μ L of SC19 (6×10^7 CFU/mouse) in the SC19 + PBS group, SC19 + BS168 group, and SC19 + $rBS^{CotG-0859}$ group. On days 1, 2, 3 and 4 following SC19 infection, mice in the SC19 + $rBS^{CotG-0859}$ group and SC19 + BS168 group separately received 200 μ L (2×10^7 CFU/mouse) of $rBS^{CotG-0859}$ spores and BS168 spores via gavage administration. In contrast, the mice in the control group and the SC19 + PBS group were administered the same volume of sterile PBS. Fresh fecal samples were collected daily following SC19 infection and directly resuspended in sterile PBS. The number of $rBS^{CotG-0859}$ in mice feces from the SC19 + $rBS^{CotG-0859}$ group was then determined by spreading a series of 10-fold dilutions on LB agar plates containing 10 μ g/mL chloramphenicol. The general health of all mice was monitored on a regular daily throughout the experiment. On the 7th day after SC19 infection, all mice were sacrificed by cervical vertebra dislocation, the blood and the major organs (heart, liver, spleen, lung, kidney, and brain) were collected and fixed with 4% paraformaldehyde. Then, the remaining organ tissues were weighed and homogenized. The number of SC19 in the heart, liver, spleen, lungs, kidneys, brain, and blood were determined by spreading a series of 10-fold dilutions on TSA plates.

To further study the prophylactic efficacy of $rBS^{CotG-0859}$ spores against SC19 infection, another independent experiment was conducted using four-week-old female ICR mice (SPF). Twenty-four mice were randomly divided into four groups ($n = 6$): control group, PBS + SC19 group, BS168 + SC19 group and $rBS^{CotG-0859}$ + SC19 group. On days 1 to 7, each mouse in the $rBS^{CotG-0859}$ + SC19 group received 200 μ L (2×10^7 CFU/mouse) of $rBS^{CotG-0859}$ spores via gavage once a day. At the same time, mice in the BS168 + SC19 group received an equal amount of BS168 spores, while intragastric gavages with 200 μ L of sterile PBS to mice of the control group and PBS + SC19 group. On day 7, all mice in the PBS + SC19 group, BS168 + SC19 group, and $rBS^{CotG-0859}$ + SC19 group were injected intraperitoneally with 200 μ L of SC19 (6×10^7 CFU/mouse). Fresh fecal samples were collected daily following SC19 infection and directly resuspended in sterile PBS. The number of $rBS^{CotG-0859}$ in feces of mice from the $rBS^{CotG-0859}$ + SC19 group was determined referring to the method described in the $rBS^{CotG-0859}$ therapeutic trial. Throughout the study, the health of the mice was monitored on a regular daily. On day 12, all mice were sacrificed by cervical vertebra dislocation, the blood and the major organs (heart, liver, spleen, lung, kidney, and brain) were collected and fixed with 4%

paraformaldehyde. Then, the remaining organ tissues were weighed and homogenized, and the number of SC19 in the heart, liver, spleen, lungs, kidneys, brain, and blood was measured using TSA plates.

All animal experiments were performed with the approval of the Scientific Ethic Committee of Huazhong Agricultural University (no. HZAUMO-2024-0075).

2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.3.0 (GraphPad Software, San Diego, CA, United States). Data were expressed as mean $\pm$ standard deviation (SD), and analysis comparisons were carried out using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

3 Results

3.1 Construction of recombinant plasmids and bacterial strains

To validate whether Lys0859 displayed on the spore surface enhances antibacterial activity or tolerance, we constructed a recombinant *B. subtilis* strain ($rBS^{CotG-0859}$) expressing Lys0859 on its spore surface. Briefly, the exogenous fusion gene CotG-0859 was inserted into the amylase gene of *B. subtilis* through homologous recombination, resulting in the inactivation of the amylase gene (Figure 1a). Therefore, recombinant bacteria ($rBS^{CotG-0859}$) could not hydrolyze starch compared to the wild-type (Figure 1b). To further verify whether CotG-0859 was successfully inserted into the *AmyE* gene, PCR were performed using 4 sets primers. The results of agarose-gel electrophoresis showed that the bands amplified from the genome of $rBS^{CotG-0859}$ were 4,448 bp, 1,544 bp, 3,831 bp, and 2,161 bp from left to right, which was consistent with the expected size (Figure 1c). We used Coomassie blue staining to identify the capsid proteins extracted from the $rBS^{CotG-0859}$ spores. A specific band with molecular weight of 51 kDa appeared in the extracts of $rBS^{CotG-0859}$ spores, but extract from wild strains (*B. subtilis* 168) did not (Figure 1d). In addition, western blot results showed that a positive hybridization band with 51 kDa was detected in the capsid proteins extracted from the $rBS^{CotG-0859}$ spores. Conversely, it was not detected in the extracts of wild strains (*B. subtilis* 168) (Figure 1e). The surface expression of the fusion protein was also confirmed by immunofluorescence (IF), with strong fluorescence was observed on $rBS^{CotG-0859}$ spores compared to wild-type spores (Figure 1f).

3.2 The growth and sporulation rate of $rBS^{CotG-0859}$ and its *in vitro* resistance assay

The growth curve and sporulation rate of *B. subtilis* 168 and $rBS^{CotG-0859}$ were determined through a standard plate-counting method. As shown in Figure 2a, the growth curve of the $rBS^{CotG-0859}$ was always consistent with the *B. subtilis* 168, indicating that the insertion of exogenous DNA did not affect the growth of the recombinant strains. In addition, the high-level expression of heterologous proteins

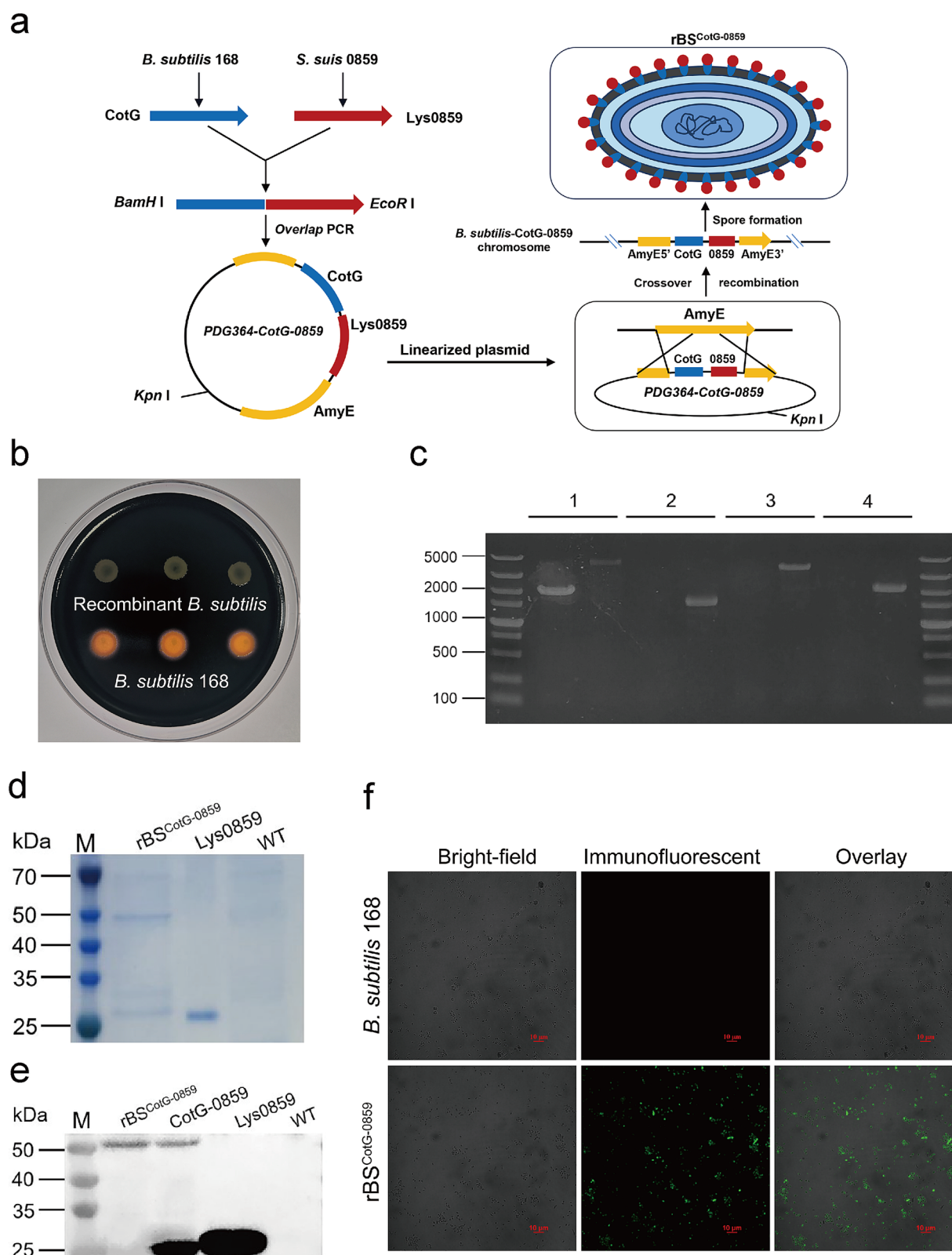


FIGURE 1

Construction of recombinant *B. subtilis* with surface display of phage lysin 0859. **(a)** The construction process of recombinant *B. subtilis* with surface display of Lys0859 on the surface (rBS^{CotG-0859}). **(b)** Starch hydrolysis test. *B. subtilis* 168 and rBS^{CotG-0859} were cultured on a LB-agar medium containing 1% starch and stained with iodine. **(c)** PCR analysis of *B. subtilis* using different primer pairs: (1) AmyE-F and AmyE-R; (2) G8-F and G8-R; (3) G8-F and AmyE-R; (4) AmyE-F and G8-R. In the results displayed for each primer pair, the left lane represents *B. subtilis* 168 and the right lane represents rBS^{CotG-0859}. **(d)** SDS-PAGE and **(e)** western blot analysis of proteins extracted from spores of the *B. subtilis* wild-type (WT) strain and rBS^{CotG-0859}; and Lys0859 or CotG-0859 proteins obtained from prokaryotic expression were used as a control; **(f)** Immunofluorescence of CotG-0859 on the spore surface at 48 h after induction of wild-type (WT) and rBS^{CotG-0859}. The scale bar represents 10 μm.

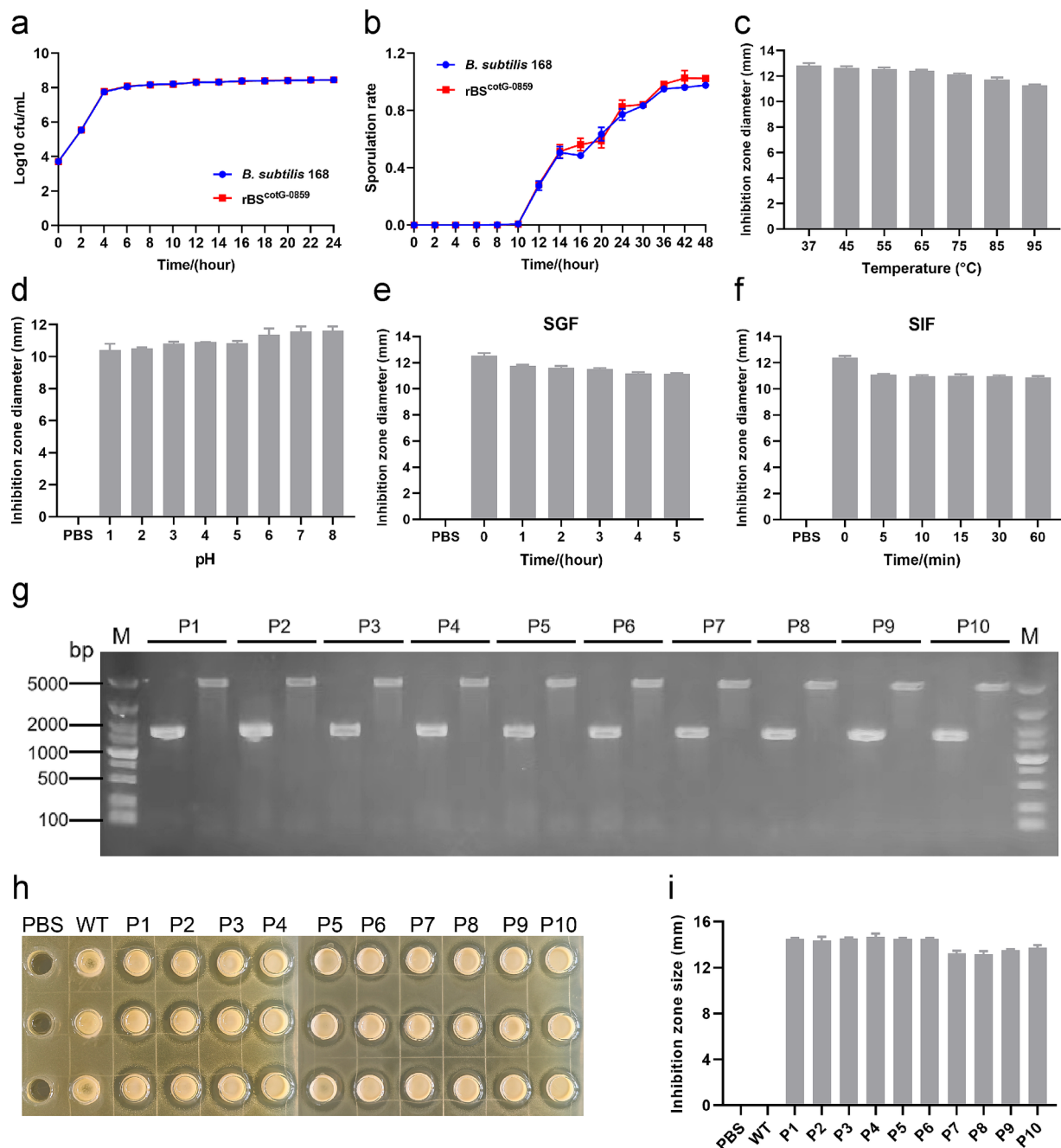


FIGURE 2

The growth and sporulation rate of *rBS^{CotG-0859}* and resistance assay *in vitro*. (a) Growth curves of *B. subtilis* 168 and *rBS^{CotG-0859}*. (b) The sporulation curves of *B. subtilis* 168 and *rBS^{CotG-0859}*. Effects of (c) different temperatures, and (d) different pH on the antibacterial activity of *rBS^{CotG-0859}* spores. The *rBS^{CotG-0859}* spore (1×10^7 CFU) was exposed to (e) SGF supplemented with gastric protease (pH 1.2) and (f) SIF containing trypsin (pH 6.8). At predetermined time points, bacteriostatic activity of *rBS^{CotG-0859}* spores (1×10^7 CFU) against *S. suis* SC19 was tested by the agar-well diffusion assays. The diameter of inhibition zone was measured after incubation for 12 h at 37°C. (g) PCR analysis of the 1st to 10th generation *rBS^{CotG-0859}* using different primer pairs: G8-F and G8-R; 168-F and 168-R. (h) The antibacterial activity of *rBS^{CotG-0859}* (the 1st to 10th generation) against *S. suis* SC19 after passage. (i) The size of each inhibition zone shown in figure H was measured using a vernier caliper, and presented as a bar chart.

did not have a significant effect on the sporulation rate of *rBS^{CotG-0859}* compared with the *B. subtilis* 168 (Figure 2b).

Moreover, we evaluated the effects of temperature, pH, SGF, and SIF on the antibacterial activity of *rBS^{CotG-0859}* spores. As shown in Figure 2c, the antibacterial activity of *rBS^{CotG-0859}* spores against *S. suis* SC19 showed a gentle downward trend with increasing temperature. Meanwhile, the antibacterial activity of *rBS^{CotG-0859}* spores showed a

declining tendency toward when the pH decreased (Figure 2d), and still maintained excellent antibacterial activity at pH 1.0. Similar experimental results were observed in both simulated gastric fluid (pepsin 1 mg/mL, pH 1.2) (Figure 2e) and simulated intestinal fluid (trypsin 1 mg/mL, pH 6.8) (Figure 2f). Furthermore, the *rBS^{CotG-0859}* were serially passaged on LB agar plates up to 10 times (Supplementary Figure S1), and identified by PCR analysis. Nucleic

acid electrophoresis showed that the bands amplified from the 1st to 10th generation rBS^{CotG-0859} genome were 1,544 bp and 4,854 bp (Figure 2g), which was consistent with the expected size. As shown in Figures 2h,i, the 1st to 10th generation rBS^{CotG-0859} spores exhibited good antibacterial activity against *S. suis* SC19.

3.3 Bactericidal activity of Lys0859 displayed by rBS^{CotG-0859} against *Streptococcus suis* SC19

Following confirmation that the Lys0859 enzyme successfully displayed on the surface of rBS^{CotG-0859} spores, we validated whether the rBS^{CotG-0859} spores exhibited bactericidal activity against *S. suis* SC19 through an agar-well diffusion assay. The purified rBS^{CotG-0859} spores were added into an 8-mm diameter well on agar plate confluent with *S. suis* SC19, and incubated at 37°C for 12 h. The well containing Lys0859 enzyme displayed an antibacterial zone, served as a positive control, while the spore of *B. subtilis* 168 has no antibacterial activity and used as a negative control (Figure 3a). The inhibition zone diameters (11.56, 11.92, 12.17, and 12.41 mm, respectively) of Lys0859 enzyme with different dosages (from low to high were 20, 30, 40, and 50 µg) were measured on a double-layer agar plate containing *S. suis*

SC19 (Figure 3c). To better understand the relationship between dose and inhibition zone, three known dosages of Lys0859 enzyme and corresponding inhibition zones were transformed into a linear regression equation, which is $y = 0.0028x + 11.035$, $R^2 = 0.9896$ (Figure 3b). After incorporating the inhibition zone diameters of rBS^{CotG-0859} spores (1×10^6 CFU) into the regression equation, the antibacterial potency against *S. suis* SC19 were equivalent to 39.11 µg Lys0859 enzyme, respectively.

3.4 Bactericidal activity of rBS^{CotG-0859} against common pathogens

Based on the excellent antibacterial effect of Lys0859 displayed by rBS^{CotG-0859} against *S. suis* SC19, we next examined whether rBS^{CotG-0859} spores could kill clinical isolates (pathogenic bacteria) of *S. suis*. Here we still use Lys0859 as a positive control for the agar well diffusion assay, and *B. subtilis* 168 spores (1×10^7 CFU) as a negative control. The results of the study demonstrated that Lys0859 exhibited a dose-dependent antibacterial effect against *streptococci*. Four dosages of Lys0859 with concentrations of 20, 30, 40, and 50 µg showed an average inhibition zone of 11.2, 12.02, 12.41, 12.66 mm (Supplementary Figure S2a), 14.15, 14.77, 14.92, 15.25 mm (Supplementary Figure S2b), 12.95, 13.3, 13.73,

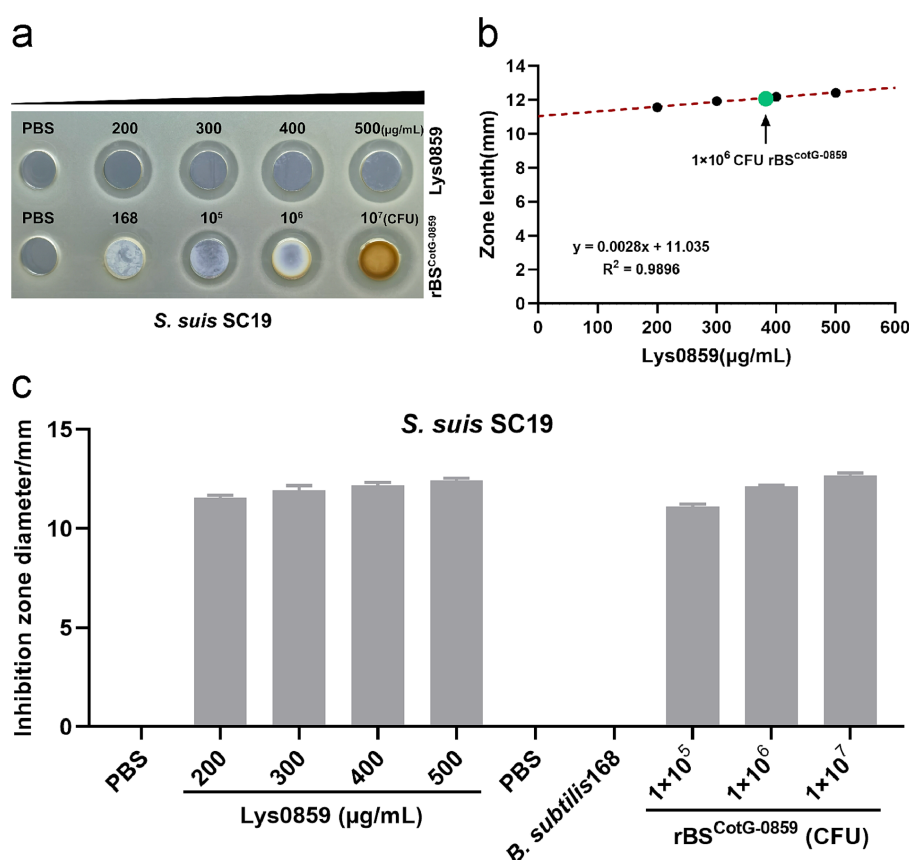


FIGURE 3

Bactericidal activity of Lys0859 displayed by rBS^{CotG-0859} against *S. suis* SC19. (a) The antibacterial efficacy of rBS^{CotG-0859} was determined by agar diffusion assay against *S. suis* SC19. (b) The regression equation was obtained based on four known dosages of Lys0859 that corresponded to their inhibition zones. Antibacterial potency equivalent to Lys0859 of rBS^{CotG-0859} spores against *S. suis* SC19 was determined. (c) The size of each inhibition zone shown in (a) was measured using a vernier caliper, and presented as a bar chart.

13.96 mm (Supplementary Figure S2c), 12.03, 12.31, 12.74, 12.92 mm (Supplementary Figure S2d), 11.22, 11.53, 11.86, 12.17 mm (Supplementary Figure S2e), 8.98, 9.25, 9.82, 9.93 mm (Supplementary Figure S2f), and 12.65, 12.98, 13.34, 13.57 mm (Supplementary Figure S2g), respectively, on the agar plate containing *S. suis* SS3, *S. suis* 18SS35, *S. suis* SS4, *S. suis* 18SS8, *S. suis* SS19, *S. suis* 1SS3 or *S. suis* 18SS75. The rBS^{CotG-0859} spores with concentrations of 1×10^6 CFU exhibited an average inhibition zone of 12.54, 14.34, 14.11 and 12.25 mm against *S. suis* SS3 (Supplementary Figure S2a), *S. suis* 18SS35 (Supplementary Figure S2b), *S. suis* SS4 (Supplementary Figure S2c) and *S. suis* 18SS8 (Supplementary Figure S2d), respectively. Additionally, the rBS^{CotG-0859} spores at a concentration of 1×10^5 CFU displayed mean inhibition zone of 11.78 mm, 10.86 mm, and 13.58 mm against *S. suis* SS19 (Supplementary Figure S2e), *S. suis* 1SS3 (Supplementary Figure S2f) and *S. suis* 18SS75 (Supplementary Figure S2g), respectively. Based on the linear regression analysis (Table 1), the bactericidal activity of 1×10^6 CFU rBS^{CotG-0859} spores against *S. suis* SS3, *S. suis* 18SS35, *S. suis* SS4 and *S. suis* 18SS8 were equivalent to 44.52 μ g, 22.14 μ g, 52.45 μ g and 26.93 μ g of Lys0859, respectively. The bactericidal activity of 1×10^5 CFU rBS^{CotG-0859} spores against *S. suis* SS19, *S. suis* 1SS3 and *S. suis* 18SS75 were equivalent to 37.44 μ g, 75.35 μ g, 49.58 μ g of Lys0859, respectively.

For *S. suis* SS15, *S. suis* SS23, *S. suis* SS30, *S. suis* 18SS23, *S. suis* 18SS29, *S. suis* 18SS91 and *S. suis* 19SS9, four different dosages of Lys0859 (20, 30, 40, and 50 μ g) exhibited an average inhibition zone of 13.86, 14.22, 14.51, 14.94 mm (Supplementary Figure S3a), 14.75, 15.11, 15.34, 15.49 mm (Supplementary Figure S3b), 13.67, 14.17, 14.35, 15.21 mm (Supplementary Figure S3c), 12.89, 13.16, 13.46, 13.89 mm (Supplementary Figure S3d), 13.87, 14.31, 14.66, 14.87 mm (Supplementary Figure S3e), 12.85, 13.22, 13.76, 14.32 mm (Supplementary Figure S3f), and 9.72, 10.38, 10.97, 11.21 mm (Supplementary Figure S3g), respectively. The rBS^{CotG-0859} spores with concentrations of 1×10^7 CFU showed an average inhibition zone of 13.57, 14.39, 12.85, 13.67, 14.43, 13.19 and 11.12 mm against *S. suis* SS15 (Supplementary Figure S3a), *S. suis* SS23 (Supplementary Figure S3b), *S. suis* SS30 (Supplementary Figure S3c), *S. suis* 18SS23 (Supplementary Figure S3d), *S. suis* 18SS29 (Supplementary Figure S3e), *S. suis* 18SS91 (Supplementary Figure S3f) and *S. suis* 19SS9 (Supplementary Figure S3g), respectively. Calculated based on the linear regression equation shown in Table 1, the bactericidal activity of 1×10^7 CFU rBS^{CotG-0859} spores against *S. suis* SS15, *S. suis* SS23, *S. suis* SS30, *S. suis* 18SS23, *S. suis* 18SS29, *S. suis* 18SS91 and *S. suis* 19SS9 were equivalent to 12.09 μ g, 30 μ g, 3.75 μ g, 44.70 μ g, 34.56 μ g, 27.7 μ g and 45.51 μ g of Lys0859, respectively.

Next, we performed another antimicrobial test on *Streptococcus agalactiae* ATCC13813, *Streptococcus agalactiae* X2 and *Streptococcus dysgalactiae* SD002. Analogous to the above experiments, different dosages of Lys0859 (20, 30, 40, and 50 μ g) exhibited an average inhibition zone of 9.95, 10.42, 10.83, 11.19 mm, 8.81, 9.46, 9.91, 10.21 mm, and 9.78, 10.42, 10.65, 10.87 mm on the agar plate containing *S. agalactiae* ATCC13813 (Supplementary Figure S4a), *S. agalactiae* X2 (Supplementary Figure S4b) or *S. dysgalactiae* SD002 (Supplementary Figure S4c), respectively. The average inhibition zones of rBS^{CotG-0859} spores (1×10^6 CFU) against *S. agalactiae* ATCC13813, *S. agalactiae* X2 and *S. dysgalactiae* SD002 were 10.21, 11.68, and 10.84 mm, respectively. The bactericidal activity of 1×10^6 CFU rBS^{CotG-0859} spores against *S. agalactiae* ATCC13813,

TABLE 1 The linear regression equation (LRE) of each pathogen showed on agar well diffusion assay.

Pathogenic bacteria	Enzyme used	LRE	R ²
<i>S. suis</i> SS4	Lys0859	$y = 0.0035x + 12.274$	0.9876
<i>S. suis</i> SS15	Lys0859	$y = 0.0035x + 13.147$	0.9945
<i>S. suis</i> SS19	Lys0859	$y = 0.0032x + 10.582$	0.9998
<i>S. suis</i> SS30	Lys0859	$y = 0.0048x + 12.67$	0.9332
<i>S. suis</i> 1SS3	Lys0859	$y = 0.0034x + 8.298$	0.9431
<i>S. suis</i> SS23	Lys0859	$y = 0.0025x + 14.315$	0.9642
<i>S. suis</i> 18SS8	Lys0859	$y = 0.0031x + 11.415$	0.9786
<i>S. suis</i> 18SS23	Lys0859	$y = 0.0033x + 12.195$	0.9875
<i>S. suis</i> 18SS29	Lys0859	$y = 0.0034x + 13.255$	0.9768
<i>S. suis</i> 18SS35	Lys0859	$y = 0.0035x + 13.565$	0.9339
<i>S. suis</i> 18SS75	Lys0859	$y = 0.0031x + 12.043$	0.9923
<i>S. suis</i> 18SS91	Lys0859	$y = 0.005x + 11.805$	0.9918
<i>S. suis</i> SS3	Lys0859	$y = 0.0048x + 10.403$	0.9302
<i>S. suis</i> 19SS9	Lys0859	$y = 0.0051x + 8.799$	0.9638
<i>S. aureus</i> ATCC43300	Lys0859	$y = 0.004x + 9.997$	0.9391
<i>S. agalactiae</i> ATCC13813	Lys0859	$y = 0.0041x + 9.152$	0.9965
<i>S. agalactiae</i> X2	Lys0859	$y = 0.0047x + 7.97$	0.9723
<i>S. dysgalactiae</i> SD002	Lys0859	$y = 0.0035x + 9.205$	0.9216

LRE represented the relationship between Lys0859 dose and inhibition zone shown on the agar plate. Lys0859, phage lysin 0859; R², coefficient of correlation.

S. agalactiae X2 and *S. dysgalactiae* SD002 was equivalent to 25.85 μ g, 57.65 μ g and 46.71 μ g of Lys0859 through linear regression analysis.

We also validated the bactericidal activity of rBS^{CotG-0859} spores against *S. aureus* ATCC43300 and found that Lys0859 with different dosages of 20, 30, 40, and 50 μ g exhibited an average inhibition zone of 10.68, 11.38, 11.55, 11.95 mm (Supplementary Figure S4d), respectively. The rBS^{CotG-0859} (1×10^7 CFU) spores displayed an average inhibition zone of 10.79 mm on the agar plate containing *S. aureus* ATCC43300. Based on the results of a simple linear regression analysis, it was determined that the bactericidal activity of 1×10^7 CFU rBS^{CotG-0859} spores against *S. aureus* ATCC43300 was found to be equivalent to 19.83 μ g of Lys0859 (Supplementary Figure S4d).

3.5 In vivo pathogen challenge test

To evaluate the antibacterial efficacy of rBS^{CotG-0859} *in vivo*, we challenged mice with the *S. suis* SC19 and treated with rBS^{CotG-0859} spores by oral gavage (Figure 4a). As shown in Figure 4b, at least 10^6 CFU/g of rBS^{CotG-0859} was detected in feces of mice at day 1–4 after post-infection. Subsequently, the amount of rBS^{CotG-0859} in mouse feces showed a progressively decreasing trend from day 5 to day 7. The treatment of rBS^{CotG-0859} spores reduced the SC19 in the heart, liver, spleen, lungs, kidneys, brain, and blood of mice by 1.14, 1.53, 1.81, 1.96, 0.97, 0.47, and 0.52 logs, respectively (Figure 4c), compared to PBS-treat group. Meanwhile, the treatment of rBS^{CotG-0859} spores decreased the SC19 in the heart, liver, spleen, lungs, kidneys, brain, and blood of mice

by 0.93, 1.50, 1.43, 1.53, 1.07, 0.69, and 0.39 logs, respectively (Figure 4c), compared with the BS168 treatment group. Histology revealed that the rBS^{CotG-0859} spores significantly relieved brain and lung inflammation and pathological damages such as inflammatory cell infiltrates, alveolar thickening, alveoli interstitial congestion, and edema in the brain and lung of infected mice (Figure 4d).

Based on the enhanced efficacy of rBS^{CotG-0859} spores in the treatment of SC19 infection, the potential of disease prevention was further explored through pre-treatment (Figure 5a). As shown in Figure 5b, the number of rBS^{CotG-0859} in mouse feces showed a gradually decreasing trend from day 8 to day 12 of the experiment, which is similar to the results of the therapeutic experiment. The prophylactic treatment of rBS^{CotG-0859} spores reduced the SC19 in the heart, liver, spleen, lungs, kidneys, brain, and blood of mice by 1.42, 1.48, 0.77, 1.09, 1.21, 0.87, and 1.05 logs respectively, compared to the group pretreated with PBS (Figure 5c). Similarly, the prophylactic

treatment of rBS^{CotG-0859} spores declined the SC19 in the heart, liver, spleen, lungs, kidneys, brain, and blood of mice by 0.92, 1.25, 1.05, 1.21, 1.16, 0.38, and 0.75 logs respectively, compared with the BS168 pretreated group (Figure 5c). The results of histological analysis further that oral administration of rBS^{CotG-0859} spores significantly reduced the severity of brain and lung injury (Figure 5d), consistent with the overall results of the therapeutic trial.

4 Discussion

S. suis is an important zoonotic pathogen that cause systemic infection in pigs as well as humans (Xia et al., 2019), which not only lead huge economic losses in the pig industry, but also pose a threat to the public health (Pei et al., 2020). Although bacterial infections are commonly treated with antibiotics, the overuse of antibiotics

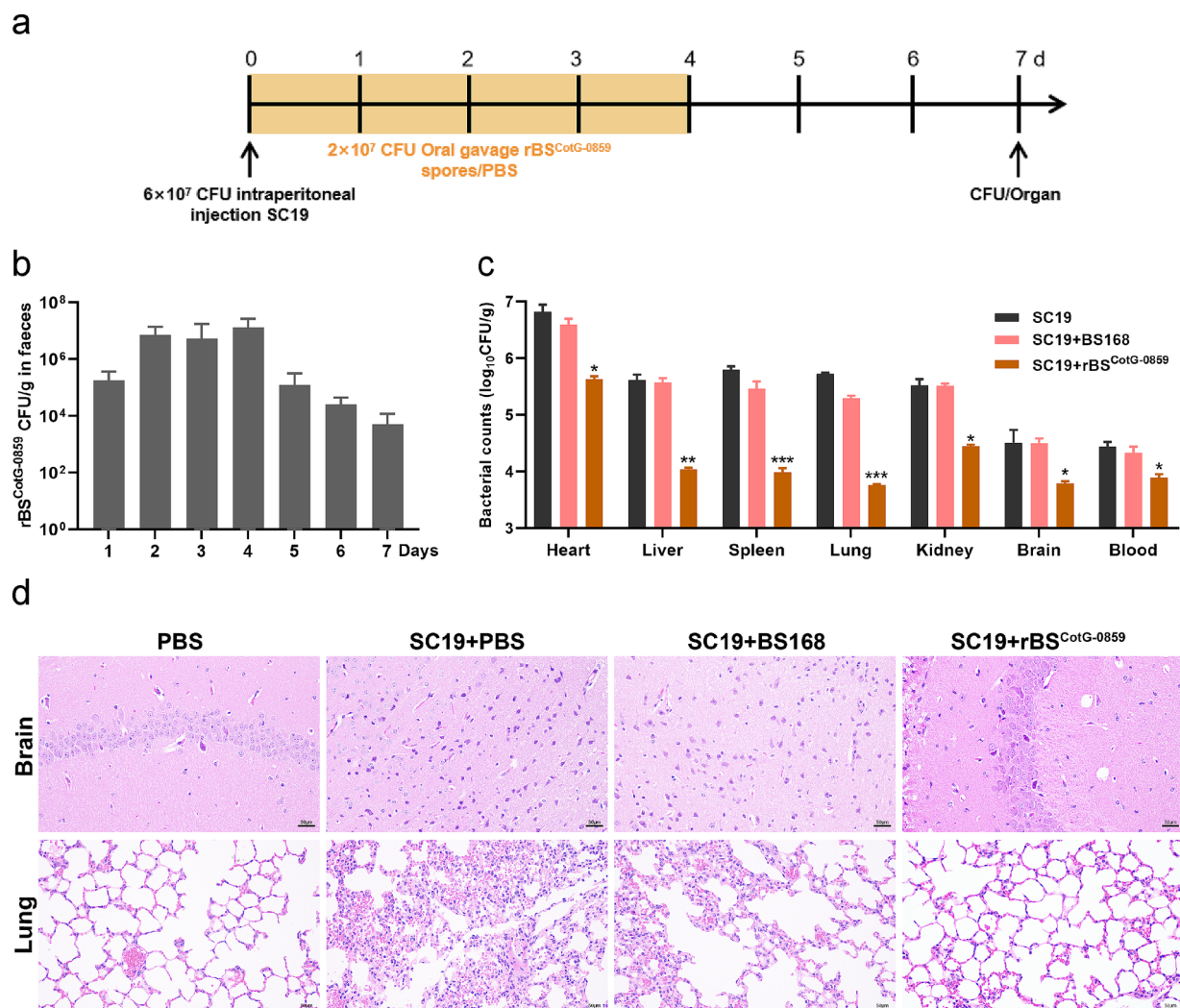


FIGURE 4

Oral rBS^{CotG-0859} spores alleviated the infection in mice with *S. suis* SC19. (a) Experimental design for treatment in this study. Orally administrated with either PBS, BS168, or rBS^{CotG-0859} spores by gavage at days 1, 2, 3, and 4 after *S. suis* SC19 infection (6×10^7 CFU/mouse), respectively. All mice were euthanized at day 7 after *S. suis* SC19 infection. (b) Bacterial count of rBS^{CotG-0859} in mouse feces. Fecal samples were collected per day after rBS^{CotG-0859} treatment and resuspended in sterile PBS (0.1 g of fecal resuspended in 1 mL of sterile PBS). Each sample performed a serial of 10-fold dilutions and spread on selective agar plates (10 μg/mL chloramphenicol) and incubated at 37°C for 12 h before bacterial counting. (c) The bacterial loads of *S. suis* SC19 in heart, liver, spleen, lungs, kidneys, brain, and blood. (d) H&E-stained brain and lung tissue sections. Scale bar: 50 μm.

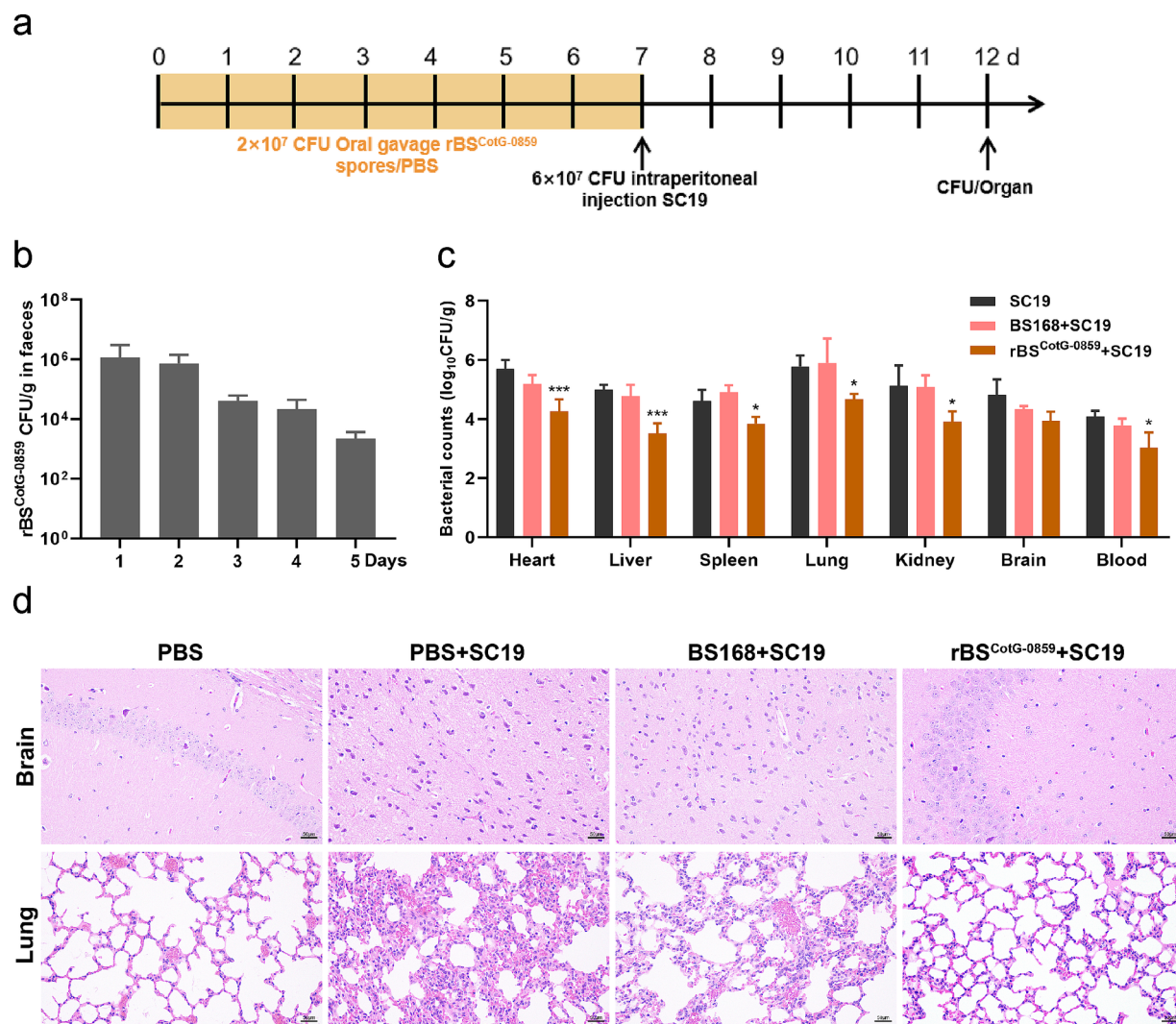


FIGURE 5

Prophylactic rBS^{CotG-0859} spores attenuated the infection in mice with *S. suis* SC19. (a) Experimental design for treatment in this study. At days 1 to 7, each mouse in the PBS + SC19 group, BS168 + SC19 group and rBS^{CotG-0859} + SC19 group were received 200 μ L (2×10^7 CFU/mouse) of sterile PBS, BS168 and rBS^{CotG-0859} spores by gavage, respectively. Then, mice in PBS + SC19 group, BS168 + SC19 group and rBS^{CotG-0859} + SC19 group were intraperitoneal injected with 200 μ L (6×10^7 CFU/mouse) of *S. suis* SC19 on day 7. On day 12, all mice were euthanized. (b) Bacterial count of rBS^{CotG-0859} in mouse faeces. (c) The bacterial loads of *S. suis* SC19 in heart, liver, spleen, lungs, kidneys, brain, and blood. (d) H&E-stained brain and lung tissue sections. Scale bar: 50 μ m.

contribute to the development of antibiotic resistance in recent years (Laxminarayan et al., 2013). Therefore, a promising alternative to antibiotics is particularly important.

An increasing number of studies demonstrated that endolysins encoded by bacteriophage is a potentially attractive method of treating bacterial infections. For example, Li et al. reported that prophage lysin Lys0859 could significantly reduce the bacterial load of *Streptococcus agalactiae* in mouse mammary glands, and also significantly improve the survival of *S. suis* mice with systemic infection (Li et al., 2023). Lood et al. also reported the novel phage lysin PlyF307 could effectively kill multidrug-resistant *Acinetobacter baumannii* in mice, thereby rescuing mice from lethal bacteremia (Lood et al., 2015). In addition, some chimeric lysins exhibited a stronger bactericidal activity (Díez-Martínez et al., 2015; Briers et al., 2014; Duan et al., 2023). However, by its very nature, lysin is formed from polypeptides or proteins by folding, which may result in the

rapid degradation and inactivation after introduced into the gastrointestinal tract. It is well known that bacillus endospores are highly resistant to many physical and chemical assaults and are able to persist in complex environment. In this study, *B. subtilis* endospores were used as a microparticle platform, introducing prophage lysin Lys0859 on the surface of the spores. It was discovered that the resistance of tethered lysin Lys0859 against environmental assaults outperformed that of the free lysin Lys0859. Similarly, the haloalkane dehalogenase DhaA displayed on *B. subtilis* spores exhibited enhanced stress resistance and activity compared to free DhaA in harsh chemical environments (Wang F. et al., 2019). Particularly, to our knowledge, Lys0859 was the first phage lysin to exhibit highly effective bactericidal activity by displaying on the surface of *B. subtilis* endospores. Furthermore, we have found that the growth rate and spore generation rate of recombinant bacteria are consistent with those of wild strains.

Although *B. subtilis* spores significantly enhanced the extreme resistance and antibacterial activity of Lys0859, stably passaged of recombinant *B. subtilis* are also crucial for clinical application from a genetic perspective. The efficacy of recombinant *B. subtilis* in exerting bactericidal effects is primarily contingent upon the presence of exogenous fusion protein on its spore surface. Nevertheless, the inadvertent loss of the target gene fragment during successive passaging may significantly compromise the antibacterial potency of the recombinant bacteria, thereby impeding its clinical utility. Hence, the stable passaging and expression of exogenous gene fragments is particularly important. The findings of this investigation demonstrate that the fusion gene remained intact and continued to demonstrate robust antibacterial or bactericidal properties even after 10 consecutive passages of the recombinant bacteria.

It has been reported that Lys0859 exhibits excellent antibacterial activity against multiple serotypes of *S. suis*, especially *S. suis* SC19 (serotype 2). In this study, we obtained consistent experimental results that support the results previously reported by Li et al. (2023). Because we have found that the recombinant spores displaying Lys0859 on the surface can effectively kill multiple streptococci, including *S. suis*, *S. agalactiae*, and *S. dysgalactiae*. More specifically, based on the agar well diffusion assay, we found that the antibacterial potency of rBS^{CotG-0859} spores with 1×10^6 CFU against *S. suis* SC19, *S. suis* SS3, *S. agalactiae* ATCC13813, and *S. dysgalactiae* SD002 were equivalent to 39.11, 44.52, 25.85, and 46.71 μ g of Lys0859, respectively. The surface display of *B. subtilis* spores not only compensates for the deficiencies of Lys0859 stress resistance, but also maintains its original bactericidal activity, which providing a feasible alternative for application of bacteriophage lyases in the clinical. Moreover, the application of rBS^{CotG-0859} may greatly reduce the widespread use of antibiotics in livestock and poultry breeding industry. More importantly, Lys0859 is a biodegradable protein that will not remain and accumulate in livestock and poultry.

In order to investigate the antibacterial activity of recombinant bacteria against *S. suis* SC19 *in vivo*, we established a mouse model of *S. suis* SC19 infection. The results showed that 4 days after treatment with rBS^{CotG-0859} spores (2×10^7 CFU), the bacterial loads had decreased 0.47 to 1.96 logs ($p < 0.05$) in all organs and blood tested for *S. suis*. Similarly, streptococcal prophage Ply30 lysin reduced the load of *S. suis* in all organs and blood of mice by 3 to 5 logs ($p < 0.01$) (Tang et al., 2015). Besides, Li et al. found that the intraperitoneal injection of 100 μ g/mouse of Lys0859 at 1 h post-infection decreased the load of *S. suis* SC19 in all organs and blood of mice by 0.57–1.39 logs (Li et al., 2023). Next, we evaluated the prophylactic efficacy of rBS^{CotG-0859} on *S. suis* SC19 infection through pretreatment. The results revealed rBS^{CotG-0859} prevention trials obtained similar results as the treatment, such as significantly reduced the load of *S. suis* in various organs. In summary, this study revealed that the efficacy of lysins anchored to the surface of rBS^{CotG-0859} spores outperform that of the free enzymes.

Although this study has demonstrated that rBS^{CotG-0859} spores can significantly reduce the bacterial load in infected tissues and improve pathological damage, immune efficacy evaluation remains a crucial missing piece in the puzzle of our research. However, the dynamic changes in the host immune response are still a key dimension for evaluating its therapeutic efficacy. Previous experiments have shown that Lys0859 can reduce the levels of TNF- α and IL-6 in mice after mastitis infection (Li et al., 2023). *B. subtilis* 168-CLE can effectively increase the levels of IgA and IgG in mice, indicating that the COE displayed on the spore surface has the ability to stimulate mucosal immunity and the production and secretion of more antigen-specific

antibodies by B cells (Tian et al., 2024). In the subsequent experimental plan, it is necessary to measure cytokine and antibody levels, analyze immune cell subsets, and study the distribution and activation of immune cells in infected tissues. By integrating these immune-related data with our existing results on tissue bacterial load and pathological changes, we can draw more comprehensive and accurate conclusions about the effectiveness and mechanism of action of our treatment.

5 Conclusion

In conclusion, in this study, we successfully displayed prophage lysin Lys0859 on the surface of *B. subtilis* spores using CotG as an anchor protein, and demonstrated excellent bactericidal activity against Streptococci *in vitro*. On the other hand, our experimental results strongly demonstrated that the surface display of *B. subtilis* spores not only significantly enhances the stress resistance of Lys0859, but also maintains the bactericidal activity of Lys0859. Most importantly, the surface display of *B. subtilis* spores not only reduces complex and time-consuming preparation and purification steps, but also reduces the costs, this lays the foundation for the clinical application of prophage lysin.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by The Scientific Ethic Committee of Huazhong Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LW: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. XD: Data curation, Methodology, Writing – review & editing. MZ: Data curation, Methodology, Writing – review & editing. HW: Writing – review & editing. XinL: Writing – review & editing. DH: Writing – review & editing. XiaL: Supervision, Writing – review & editing. PQ: Conceptualization, Funding acquisition, Project administration, 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.

Generative AI statement

The authors declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

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Magnolia essential oil: a preliminary exploration of chemical composition and its antimicrobial and antioxidant potential

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In this study, the chemical composition of Magnolia essential oil (MEO) was analyzed using gas chromatography-mass spectrometry (GC-MS). The results indicated that terpenoids were the primary constituents, with the main components being 1,8-cineole (44.87%), (+)-citronellal (6.93%), and linalool (29.1%). The antibacterial activity of MEO against four target bacteria was confirmed through inhibition zone assays, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) tests. The bacterial growth curve demonstrated that MEO significantly inhibited bacterial growth and effectively delayed the logarithmic growth phase. Mechanistic studies suggested that MEO primarily acts in the initial stages of bacterial growth by disrupting the bacterial cell membrane, leading to substantial leakage of intracellular materials, impairing metabolic activities, inducing lipid peroxidation, and enhancing oxidative stress, thereby inhibiting normal bacterial proliferation. Furthermore, MEO's antioxidant properties were evaluated through its scavenging effects on DPPH and hydroxyl radicals, as well as its ferric reducing antioxidant power (FRAP). The findings revealed that MEO exhibited the strongest scavenging activity against DPPH radicals, followed by hydroxyl radical scavenging, with the FRAP results being comparatively weaker. These results suggest that MEO not only possesses potent antibacterial effects but also exhibits notable antioxidant activity, indicating potential for broader applications.

KEYWORDS

magnoliae flos, essential oil, GC-MS, chemical composition, antibacterial, antioxidant

1 Introduction

For a long time, natural plant essential oils have been recognized for their wide-ranging biological activities, including antibacterial, antifungal, and antioxidant properties. Essential oils are complex mixtures of volatile compounds, primarily composed of terpenoids, phenols, and other bioactive constituents (Wu et al., 2020; Hadidi et al., 2020; Bouyahya et al., 2017). These compounds exhibit various therapeutic characteristics, making essential oils an attractive focus for researchers seeking alternative and effective treatments, particularly against drug-resistant pathogens (Der Torossian Torres and de la Fuente-Nunez, 2019). Rich in terpenoids, these essential oils demonstrate potent antibacterial

effects by disrupting the integrity of microbial cell walls and membranes, leading to cell lysis and ultimately resulting in bacterial death (Visan and Negut, 2024).

Magnolia essential oil (MEO), extracted from the dried flower buds of *Magnolia biondii* Pamp., *Magnolia denudata* Desr., and *Magnolia sprengeri* Pamp., is a volatile oil rich in bioactive compounds such as eugenol, α -pinene, and linalool. Traditionally, these flower buds have been used in Chinese medicine to dispel wind-cold, alleviate nasal congestion, and relieve headaches caused by cold exposure. Beyond its conventional medicinal uses, recent studies have demonstrated that MEO exhibits significant antibacterial, anti-inflammatory, and antioxidant properties, making it a promising natural agent for various applications. In the field of food preservation, MEO has been recognized for its ability to inhibit foodborne pathogens and delay spoilage by targeting bacterial cell membranes and oxidative stress pathways. Its antimicrobial efficacy, coupled with its antioxidant capacity, suggests potential for extending the shelf life of perishable foods while maintaining their quality and safety, and its volatile nature allows for application in vapor-phase antimicrobial packaging systems, offering an alternative to synthetic preservatives (Liu Y. et al., 2024). Meanwhile, in the pharmaceutical industry, MEO's anti-inflammatory and immunomodulatory effects have drawn attention for treating respiratory conditions, including rhinitis and allergic reactions. While some studies have explored its role in traditional medicine, research into its precise antibacterial mechanisms and interactions with bacterial metabolism remains insufficient. A deeper understanding of these mechanisms could pave the way for novel antimicrobial agents derived from MEO, addressing the growing concern of antibiotic resistance. By expanding the exploration of MEO beyond traditional applications, this study aims to further elucidate its potential in both food preservation and medicinal fields, contributing to the broader utilization of natural plant-based antimicrobials.

Bacteria such as *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Listeria monocytogenes* (*L. monocytogenes*), and *Salmonella typhimurium* (*S. typhimurium*) are common foodborne pathogens that can cause severe health issues when contaminating food (Oh et al., 2017). These bacteria are capable of inducing symptoms ranging from food poisoning and gastroenteritis to fever, posing significant risks, especially to vulnerable populations like the elderly, pregnant women, and children. In recent decades, the misuse and overuse of antibiotics have led to a rapid rise in antibiotic resistance, with the World Health Organization recognizing antimicrobial resistance as one of the top global public health threats (King et al., 2019). Multidrug-resistant strains of these pathogens have emerged, rendering conventional treatments increasingly ineffective and leading to higher morbidity, mortality, and economic burden worldwide. As antibiotic resistance becomes a more pressing concern, there is an urgent need to develop safe, natural antibacterial agents that are less likely to induce resistance. Essential oils derived from plants, known for their broad-spectrum antimicrobial properties, have emerged as promising alternatives (Sánchez-Hidalgo et al., 2018; Khaliullina et al., 2024). These natural substances can effectively disrupt bacterial cell structures and inhibit metabolic pathways, offering a potential solution to the growing problem of antibiotic resistance.

This study identified terpenoids as the major components of MEO via GC-MS analysis and revealed its antibacterial mechanism,

which primarily involves disrupting bacterial cell membranes, leading to metabolic dysfunction, lipid peroxidation, and oxidative stress, ultimately inhibiting bacterial growth and reproduction, while in vitro assays also confirmed its antioxidant activity. By integrating enzyme activity assays and membrane integrity tests, this study provides a more comprehensive molecular-level understanding of MEO's antibacterial effects compared to previous research, which mainly focused on its chemical composition and therapeutic applications in rhinitis. Given the rising concern over antibiotic resistance, these findings highlight MEO's potential as a natural antibacterial agent with a lower risk of resistance development, making it a promising alternative to traditional antibiotics.

2 Materials and methods

2.1 Materials and reagents

Magnolia essential oil was obtained from Jiangxi Cedar Natural Medicinal Oil Co., Ltd. in Jiangxi, China. Nutrient broth (NB) and nutrient agar (NA) purchased from Guangdong Huankai Biotechnology Co., Ltd (Guangzhou, China). *Escherichia coli* (GDMCC NO.1.1917), *Staphylococcus aureus* (GDMCC NO. 1.221), *Listeria monocytogenes* (GDMCC NO. 1.2408) and *Salmonella typhimurium* (GDMCC NO. 1.237) were obtained from Guang-dong Microbial Culture Collection Center (Guangzhou, China). Alkaline Phosphatase (AKP), 1,1-Diphenyl-2-Picrylhydrazyl Radical (DPPH), hydroxyl radicals, Ferric Ion Reducing Antioxidant Power (FRAP) and ATPase (ATP) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Malondialdehyde (MDA) and superoxide dismutase (SOD) kits were purchased from Solarbio science & technology Co., Ltd. (Beijing, China). Other chemical reagents were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Chemical composition analysis of MEO

The GC-MS analysis was conducted utilizing an Agilent 6890N-5973 gas chromatography mass spectrometer equipped with an HP-INNOWax model gas chromatography column (Li et al., 2023). In a 20 mL extraction bottle, 1 g of MEO was sealed and immersed in a 60°C water bath with magnetic stir-ring set at 500 rpm. After a 20 min equilibration period, extraction was carried out for an additional 30 min upon insertion of the extraction needle. Prior to use, the extraction needle was activated at the gas injection port for 20 min at 250°C. The temperature program included an inlet temperature of 250°C, GC interface temperature of 250°C, carrier gas flow rate of 1.5 mL/min, and a split ratio of 4:1. The temperature program was as follows: initially set at 40°C, held for 5 min, ramped at 5°C/min to 250°C, and then held for 10 min. MS conditions comprised an ion source temperature of 230°C, quadrupole temperature of 150°C, EI ionization at 70 eV, and a full scan from 35–550 m/z. Identification of compounds within MEO was accomplished using the National Institute of Standards

and Technology (NIST14) database alongside literature references. The relative content of each compound in the chromatogram was determined employing the area normalization method.

2.3 Antibacterial activity

2.3.1 Bacterial liquid incubate

The frozen strains of *E. coli*, *S. aureus*, *L. monocytogenes* and *S. typhimurium* were revived by inoculating them on NA solid medium. Single colonies were picked and transferred into NB liquid medium, where they were cultured at 37°C for 24 h (Su et al., 2023). The bacterial cultures were then stored at 4°C. Before starting the experiments, the bacterial suspensions were adjusted to a concentration of 10^8 CFU/mL using a McFar-land turbidity standard.

2.3.2 Method for assessing antimicrobial activity

The antibacterial activity of MEO against common foodborne bacteria (*E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium*) was evaluated using the filter paper disk diffusion method (El Barnossi et al., 2020). A 100 μ L bacterial suspension with a concentration of 1×10^6 CFU/mL was spread evenly on NA agar plates using a sterile spreader. Sterile filter paper disks were gently placed at the center of each plate. MEO (5 μ L) was added to the filter paper disks, and sterile water was used as a blank control. The plates were incubated at 37°C for 24 h. The inhibition zones were measured and photographed. This procedure was repeated three times for accuracy.

2.3.3 The MIC and MBC of MEO to bacteria

The MIC and MBC of MEO were determined using the microdilution method (Veiga et al., 2019). Initially, a solution of MEO in NB medium was prepared at a concentration of 60 μ L/mL, with 4% DMSO added as a cosolvent. This solution was then further diluted to concentrations of 10, 9, 8, 7, 6, 5, 4, 3, 2 and 1 μ L/mL. In a sterile 96-well plate, 100 μ L of the diluted MEO solution was added to each well from left to right, followed by the addition of 100 μ L of bacterial suspension (1×10^6 CFU/mL), gently mixing the contents. The plate was then incubated at 37°C for 24 h in a constant-temperature microbiological incubator. The bacterial growth in each well was observed and analyzed, with the lowest concentration showing no bacterial growth and no increase in optical density (OD) defined as the MIC value (Kowalska-Krochmal and Dudek-Wicher, 2021). The incubation continued for an additional 48 h, and the growth was reassessed. The lowest concentration with no bacterial growth and no increase in OD was defined as the MBC value. The experiment was repeated three times. The blank group did not contain MEO or DMSO, while the control group only included DMSO.

2.3.4 Growth curve

The determination of growth curve referred to the method of Feng et al. (2022). The effect of MEO on the growth curve of the test bacteria was assessed in a sterile 96-well plate. MEO solutions at concentrations of 0.25 MIC, 0.5 MIC, and 1 MIC, determined from preliminary experiments, were added sequentially, with 200 μ L of each solution introduced into the wells. NB medium without

MEO served as the blank control. Subsequently, 20 μ L of bacterial suspension (1×10^6 CFU/mL) was added to each well, and the plate was incubated at 37°C in a shaking water bath at 100 rpm. The OD at 600 nm was measured and recorded every 2 h to plot the growth curve. Each group was tested in triplicate, and the bacterial growth curve was represented by the average turbidity, with absorbance corresponding to the time intervals.

2.3.5 Nucleic acid and protein leakage assay

The determination of the contents is based on the method of Liu et al. (2020). In a 10 mL suspension of test bacteria (1×10^6 CFU/mL), MEO was added, using 4% DMSO as a co-solvent, to achieve a final concentration equal to the MIC. The blank group consisted of samples without MEO or DMSO, while the control group included only DMSO. The bacterial suspension was then incubated at 37°C in a shaking water bath at 100 rpm for 2 h. Following incubation, the suspension was centrifuged at 8000 rpm for 10 min to collect the supernatant. This supernatant was transferred to fresh tubes for analysis, where the optical density at 260 and 280 nm was measured to quantify the nucleic acids and proteins released from the cytoplasm.

2.3.6 Electric conductivity assay

The solution conductivity was determined according to the method of Wingfield (2014). In a 10 mL suspension of test bacteria (1×10^6 CFU/mL), MEO was added, with 4% DMSO used as a co-solvent, to achieve a final concentration corresponding to the MIC. The blank group consisted of samples without MEO or DMSO, while the control group included DMSO only. The bacterial suspension was incubated at 37°C in a shaking water bath at 100 rpm for 2 h. After incubation, the suspension was centrifuged at 8,000 rpm for 10 min to collect the supernatant, which was then transferred to fresh tubes. The conductivity of each tube's supernatant was measured using a conductivity meter.

2.3.7 Biochemical index assay

The determination of intracellular enzymes referred to previous research methods (Shi et al., 2016; Rubeena et al., 2020; Li et al., 2019). In a 10 mL suspension of test bacteria (1×10^6 CFU/mL), MEO was added, along with 4% DMSO as a co-solvent, to achieve a final concentration at the MIC. The blank group consisted of samples without MEO or DMSO, while the control group included DMSO only. The bacterial suspension was then incubated at 37°C in a shaking water bath at 100 rpm for 2 h. Following this, the suspension was centrifuged at 8,000 rpm for 10 min, discarding the supernatant and collecting the bacterial pellet. High-efficiency RIPA lysis buffer was added to the cells, and a cell disruptor was used to thoroughly lyse the cells while in an ice bath. The lysate was centrifuged again at 8,000 rpm for 10 min, and the supernatant was transferred to a new tube and kept on ice for subsequent biochemical assays. The detection steps for AKP, ATP, MDA, and SOD activities are detailed in the kit instructions.

2.3.8 Scanning electron microscope (SEM) assay

To confirm and validate the results observed under an optical microscope, we slightly modified the previously reported method of observing test bacteria using SEM (Bai et al., 2019). The bacteria (at a concentration of 10^8 CFU/mL) were treated with MEO at MIC

concentration for 2 h at 37°C. The precipitate was then centrifuged at 6,000 rpm for 10 min and washed three times with PBS. The collected cells were fixed in 2.5% glutaraldehyde at 4°C for 24 h, followed by dehydration using a series of ethanol concentrations (15%, 30%, 45%, 60%, 75%, 90%, and 100%) for 10 min each. Finally, the dehydrated samples were gold-coated and examined using SEM. A control experiment was conducted without MEO treatment.

2.4 Antioxidant activity

2.4.1 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay

To prepare the MEO solutions, anhydrous ethanol was used to create concentration gradients of 1.25, 2.5, 5, 10, 15, 20, and 40 mg/mL. A 5 mg/mL BHT solution served as the positive control. For each test, 0.5 mL of the sample solution was mixed with an equal volume of a 0.2 mM DPPH anhydrous ethanol solution. The mixture was thoroughly mixed and then placed in the dark for 30 min, with three parallel operations for each concentration. The absorbance was measured at a wavelength of 517 nm (Ashmawy et al., 2024). The experiment was repeated three times. The DPPH radical scavenging rate was calculated using the following formula (1):

$$\text{DPPH radical scavenging activity} \left[1 - \frac{(A_1 - A_0)}{A_2} \right] 100\%$$

A_0 represents the absorbance of the sample group (sample without DPPH solution), A_1 represents the absorbance of the sample group containing DPPH, and A_2 represents the absorbance of the control group (sample without DPPH solution).

2.4.2 Hydroxyl radical scavenging activity assay

To prepare the MEO solutions, anhydrous ethanol was used to create concentration gradients of 1.25, 2.5, 5, 10, 15, 20, and 40 mg/mL, with a 5 mg/mL BHT solution serving as the positive control. A 9.0 mmol/L FeSO₄ solution, a 9.0 mmol/L ethanol-salicylic acid solution, and a 3% H₂O₂ solution were prepared and stored after thorough mixing. In a test tube, 1 mL of the sample solution was taken, followed by the addition of 1 mL of the prepared FeSO₄ solution and 1 mL of the ethanol-salicylic acid solution. Finally, 1 mL of the H₂O₂ solution was added. The mixture was shaken thoroughly and incubated in a water bath at 37°C for 30 min. The absorbance was then measured at a wavelength of 510 nm (Zhu et al., 2024). This experiment was repeated three times. The hydroxyl radical scavenging rate was calculated using the following formula (2):

$$\text{Hydroxyl radical scavenging activity} \left[1 - \frac{(A_1 - A_2)}{A_0} \right] 100\%$$

A_0 represents the absorbance value of distilled water participating in the reaction instead of the sample solution. A_1 represents the absorbance value of the sample solution participating in the reaction. A_2 represents the absorbance value of the sample solution and distilled water instead of 3% H₂O₂ in the reaction.

2.4.3 Ferric ion reducing antioxidant power (FRAP) assay

To prepare the MEO solutions, anhydrous ethanol was used to create concentration gradients of 1.25, 2.5, 5, 10, 15, 20, and 40 mg/mL, with a 5 mg/mL BHT solution serving as the positive control. In a 10 mL centrifuge tube, 1 mL of the solution was centrifuged, and then 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of 1% potassium ferricyanide solution were added. The mixture was thoroughly mixed and reacted at 50°C for 20 min before being rapidly cooled. Next, 2.5 mL of 10% trichloroacetic acid solution was added, followed by centrifugation at 4,000 rpm for 15 min. The supernatant (2.5 mL) was then taken and mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride solution. After sufficient mixing, the reaction was allowed to proceed for 10 min, and the absorbance was measured at a wavelength of 700 nm. This experiment was repeated three times to ensure accuracy and reliability (Chebbac et al., 2023).

2.5 Statistical analysis

All experimental procedures were repeated three times. All data are presented as mean ± standard deviation (SD). Graphs were created using GraphPad Prism 10, and statistical analysis including one-way analysis of variance (ANOVA) and Duncan's multiple range test was conducted using SPSS 27 software. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Chemical component analysis of MEO

The chemical composition of essential oil was analyzed using GC-MS, with the results presented in Table 1. A total of 49 compounds were identified from the database search, accounting for 99.04% of the total essential oil content. The major components with higher concentrations include 1,8-cineole (44.87%), (+)-citronellal (6.93%), and linalool (29.1%). The primary constituents of MEO are terpenoids.

Based on the chemical composition obtained in Table 1 and the database search results, Figure 1 illustrates the structural formulas of monomeric compounds with a relative content greater than 0.5%.

3.2 Antibacterial activity assay

3.2.1 Determination of inhibition zone

The inhibition ability of MEO was evaluated using the disk diffusion method. As shown in Figure 2, MEO exhibited growth inhibition against *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium*. The diameters of the inhibition zones are presented in Table 2, with the inhibition zones for *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium* measured at 12.63 ± 0.35 mm, 10.94 ± 0.44 mm, 12.63 ± 0.35 mm, and 12.5 ± 0.24 mm, respectively. These results demonstrate that MEO has significant antibacterial potential.

TABLE 1 The chemical component analysis of magnolia essential (MEO).

ID	RT (min)	Compound name	CAS no.	Relative content (%)
1	8.827	α -Pinene	80-56-8	0.25
2	10.107	Camphene	79-92-5	0.07
3	11.600	β -Pinene	127-91-3	0.30
4	11.935	α -Phellandrene	99-83-2	0.17
5	12.082	β -Phellandrene	555-10-2	0.47
6	13.603	Sabinene	3387-41-5	0.81
7	13.997	α -Terpipene	99-86-5	3.72
8	14.555	Limonene	138-86-3	2.97
9	15.889	1,8-cineole	470-82-6	44.87
10	16.283	γ -Terpinene	99-85-4	0.04
11	16.459	β -(Z)-ocimene	3338-55-4	0.04
12	17.005	p-Cymene	99-87-6	0.91
13	17.323	α -Terpinolene	586-62-9	0.03
14	17.611	Octanal	124-13-0	0.01
15	18.944	6-Methyl-5-hepten-2-one	110-93-0	0.03
16	19.414	2,6-Dimethyl-5-heptenal	106-72-9	0.03
17	21.870	Cis-Linalool oxide	1365-19-1	0.02
18	22.352	3-Furaldehyde	498-60-2	0.02
19	22.570	Trans-Linalool oxide	34995-77-2	0.01
20	23.116	(+)-Citronellal	2385-77-5	6.93
21	25.031	Linalool	78-70-6	29.10
22	25.566	Isopulegol	89-79-2	0.43
23	25.930	β -Elemene	515-13-9	0.73
24	26.048	Cubebene	13744-15-5	0.06
25	26.142	Trans-Caryophyllene	87-44-5	0.03
26	26.324	Citronellyl formate	105-85-1	0.01
27	26.788	(+)-Epi-bicyclosesquiphellandrene	54324-03-7	0.02
28	27.317	Citronellyl acetate	150-84-5	0.55
29	27.640	α -Humulene	6753-98-6	0.08
30	27.910	Z-Citral	106-26-3	0.04
31	27.999	γ -Muurolene	30021-74-0	0.09
32	28.134	α -Terpineol	98-55-5	0.04
33	28.222	Geranyl formate	105-86-2	0.02
34	28.586	Germacrene-d	23986-74-5	0.44
35	28.792	α -Muurolene	10208-80-7	0.23
36	29.015	Geranial	141-27-5	0.08
37	29.415	Geranyl acetate	105-87-3	0.41
38	29.638	Citronellol	106-22-9	2.80
39	30.296	Nerol	106-25-2	0.03
40	30.372	α -Cadinene	24406-05-1	0.04
41	31.177	Calamenene	483-77-2	0.02
42	31.412	Geraniol	106-24-1	2.44
43	31.665	Geranylacetone	3796-70-1	0.02
44	33.357	(E)-2,6-Dimethylocta-3,7-diene-2,6-diol	51276-34-7	0.03
45	36.166	Elemol	639-99-6	0.20
46	37.746	Eugenol	97-53-0	0.20
47	37.858	γ -Eudesmol	1209-71-8	0.08
48	38.181	T-Muurolol	19912-62-0	0.02
49	39.021	α -Cadinol	481-34-5	0.03
	Total	–	–	99.04

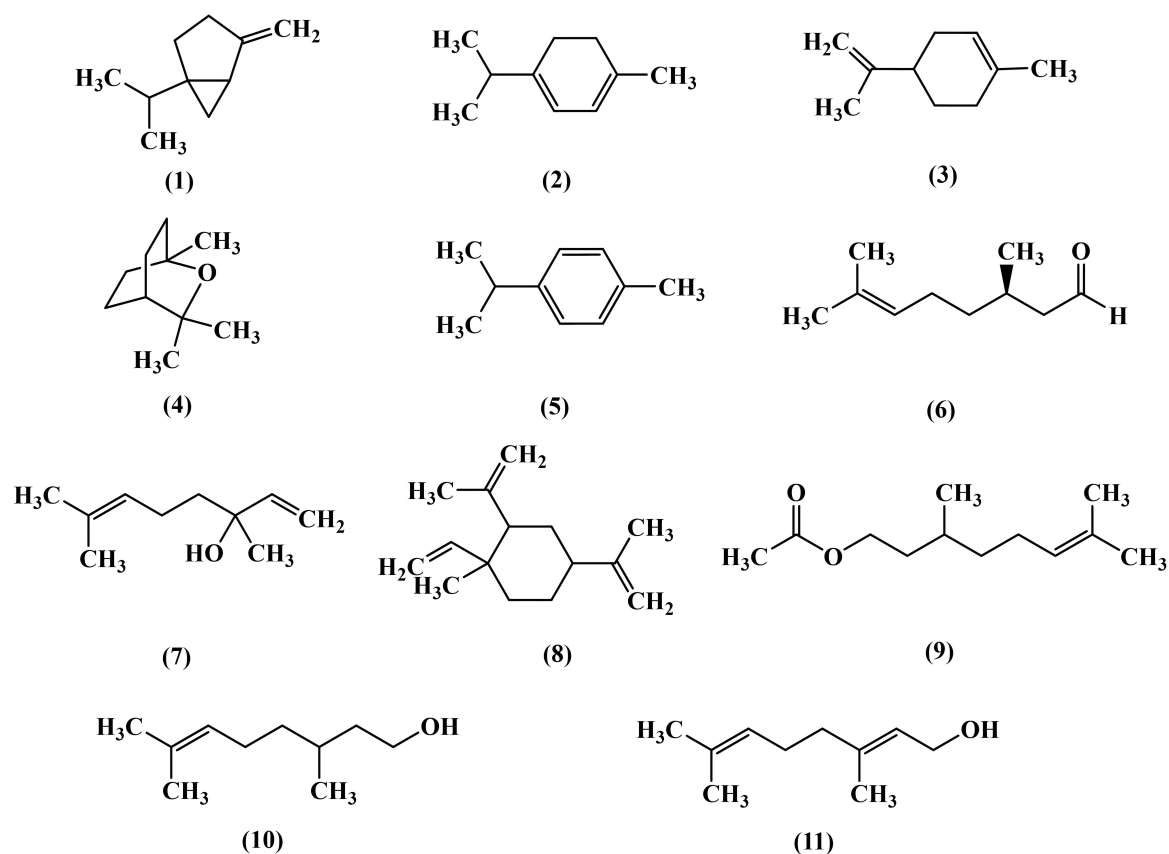


FIGURE 1
Structural formula of chemical components with content greater than 0.5% in magnolia essential oil (MEO).

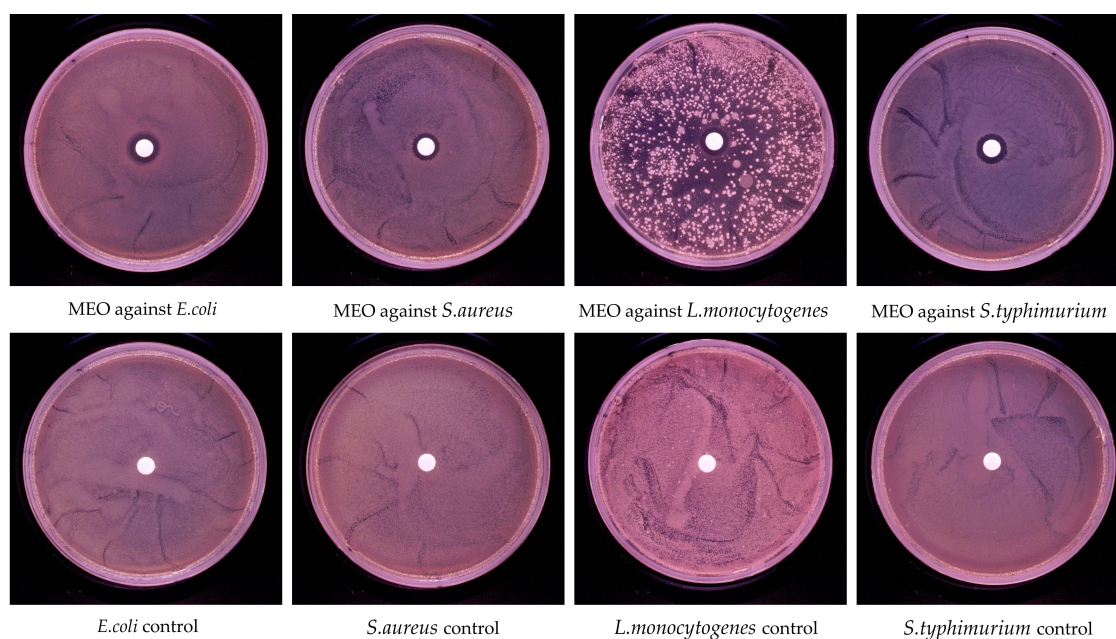


FIGURE 2
In vitro antibacterial activity of magnolia essential oil (MEO).

TABLE 2 Inhibition zone determination of magnolia essential oil (MEO) against four tested bacterial strains.

Group	Diameter of bacterial inhibition zone of different system categories (mm)			
	<i>E. coli</i>	<i>S. aureus</i>	<i>L. monocytogenes</i>	<i>S. typhimurium</i>
Control	-	-	-	-
MEO	12.63 ± 0.35 ^a	10.94 ± 0.44 ^b	11.34 ± 0.53 ^b	12.5 ± 0.24 ^a

“-” It means no bacteriostatic effect. Different letters in the same figure indicate statistically significant difference at $P < 0.05$ between different samples. Each value represents the mean of three replicates ± standard deviation.

3.2.2 The MIC and MBC of MEO to bacteria

The MIC and MBC values of MEO against four bacterial strains were determined using the broth microdilution method, with the results shown in Table 3. The MIC values for *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium* were 5, 5, 4, and 4 $\mu\text{L/mL}$, respectively. The corresponding MBC values were 5, 8, 5, and 5 $\mu\text{L/mL}$, respectively. These results indicate that MEO exhibits relatively weaker bactericidal activity against *S. aureus* compared to the other strains. In the control group, bacterial growth was normal, confirming that the addition of a certain amount of DMSO had no inhibitory effect on bacterial growth.

3.2.3 Growth curve

The effect of MEO on the growth of *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium* is shown in Figure 3. In the control group, all four bacteria rapidly entered the logarithmic growth phase. However, treatment with 0.25 MIC and 0.5 MIC of MEO effectively delayed the logarithmic growth phase for all test bacteria. At the 12 h mark, different concentrations of MEO exhibited inhibitory effects on the four bacterial strains. The optical density (OD) values at 1 MIC remained almost unchanged within 12 h, indicating that the test bacteria were highly sensitive to this concentration. Significant differences were observed between the MEO-treated groups and the control group.

3.2.4 Nucleic acid and protein leakage

The release of intracellular substances after MEO treatment was measured using UV spectrophotometry. As shown in Figure 4, after incubating the four bacterial strains with MEO for 2 h, the absorbance at 260 and 280 nm significantly increased. A statistically significant difference ($p < 0.05$) was observed between the test group and both the blank and control groups. The rise in extracellular nucleic acids and proteins suggests that MEO disrupts the permeability of the bacterial cell membranes, leading to the leakage of intracellular materials and consequently inhibiting bacterial growth and reproduction.

3.2.5 Electric conductivity

The electric conductivity of bacterial cultures was measured after incubation with MEO using a conductivity meter. As shown in Figure 5, after 2 h of incubation, the conductivity of the test group significantly increased compared to the blank group ($p < 0.05$), which aligns with the findings in Figure 4. This increase in conductivity is due to the leakage of intracellular substances such as nucleic acids and proteins, which carry charges and elevate the ionic concentration in the culture medium. In contrast, the conductivity of the control group slightly decreased compared to the blank group, likely because DMSO, a non-ionic solvent, does

TABLE 3 The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of magnolia essential oil (MEO) against four tested bacterial strains.

Bacteria	MIC ($\mu\text{L/mL}$)			MBC ($\mu\text{L/mL}$)
	Blank	Control	Test	
<i>E. coli</i>	-	-	5	5
<i>S. aureus</i>	-	-	5	8
<i>L. monocytogenes</i>	-	-	4	5
<i>S. typhimurium</i>	-	-	4	5

“-” It means no bacteriostatic effect.

not contribute to ion concentration and may reduce the number of conductive ions per unit volume.

3.2.6 AKP activity

Alkaline phosphatase enzyme is closely related to bacterial metabolic activity, membrane stability, and the synthesis and degradation of the cell wall. When the level of AKP decreases, it indicates damage to the bacterial cell membrane or wall, leading to reduced metabolic activity and slower growth. Therefore, a reduction in AKP enzyme levels can be considered a marker of bacterial damage (Zhou et al., 2022; Shi et al., 2024). As shown in Figure 6A, after 2 h of co-incubation with MEO, the AKP levels in all four test bacteria were significantly reduced. Furthermore, there was a notable difference between the test group and the blank and control groups ($p < 0.05$). This suggests that MEO has a destructive effect on the bacterial cell wall.

3.2.7 ATP activity

ATPase plays a crucial role in bacterial cells by hydrolyzing ATP into ADP, releasing energy necessary for various cellular processes. When the level of ATPase decreases, the bacteria experience multifaceted damage (Mao et al., 2024). As illustrated in Figure 6B, after 2 h of incubation with MEO, the ATPase content in all four test bacteria showed a significant reduction. Moreover, there was a noticeable difference between the experimental group and the blank and control groups ($p = 0.05$). A reduction in ATPase levels leads to insufficient energy metabolism, impaired transmembrane ion transport, membrane potential imbalance, and weakened stress responses. In severe cases, these disruptions can inhibit bacterial growth or even lead to cell death.

3.2.8 MDA activity

Malondialdehyde is one of the primary markers of lipid peroxidation in cell membranes, typically produced under oxidative stress conditions. An increase in MDA levels often

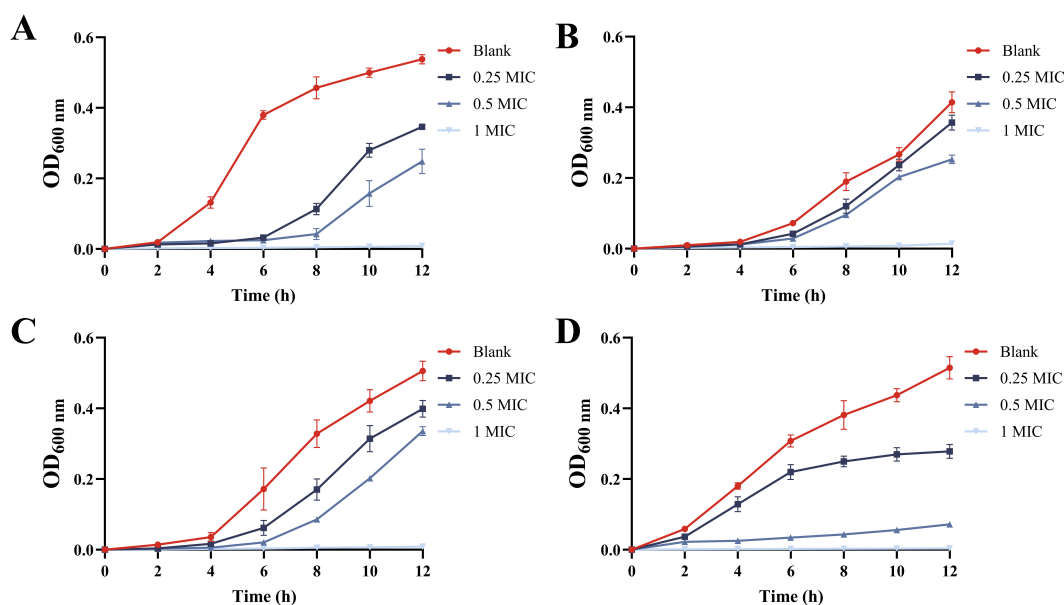


FIGURE 3

The growth curves of *E. coli* (A), *S. aureus* (B), *L. monocytogenes* (C), and *S. typhimurium* (D) were treated with different concentrations of magnolia essential oil (MEO). Each value represents the mean of three replicates $\pm$ standard deviation.

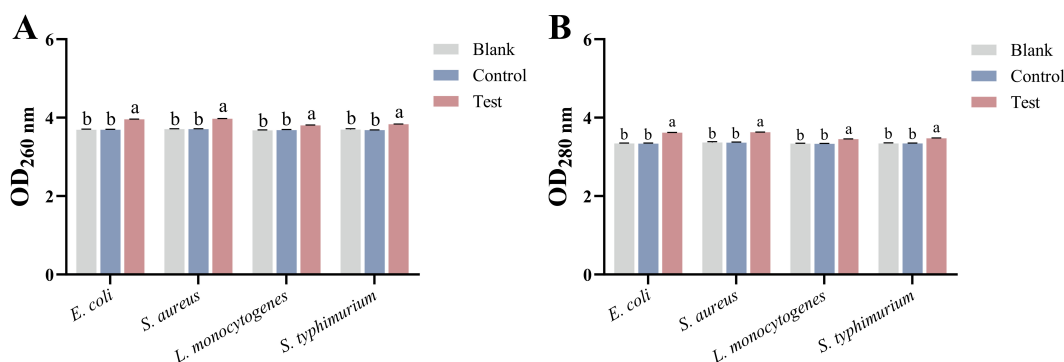


FIGURE 4

(A) Is the nucleic acid released by *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium* after treatment with different groups. (B) Is the protein released by *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium* after treatment with different groups. Different letters in the same figure indicate statistically significant difference at $P < 0.05$ between different samples. Each value represents the mean of three replicates $\pm$ standard deviation.

indicates that the bacteria have undergone oxidative damage or that their membrane structure has been compromised (Suo et al., 2022; Chen et al., 2022). As shown in Figure 7A, after 2 h of co-incubation with MEO, the MDA content in the four test bacteria significantly increased. There was a marked difference between the experimental group and the blank and control groups ($p < 0.05$). The increase in MDA levels is a sign of severe oxidative damage to bacterial cells, representing structural damage to the membrane and heightened oxidative stress, which negatively impacts bacterial growth and reproduction.

3.2.9 SOD activity

Superoxide dismutase primarily functions to eliminate superoxide anions in bacteria by catalyzing their conversion into hydrogen peroxide and oxygen, thereby protecting cells from

oxidative damage. An increase in SOD levels typically indicates that the bacteria are experiencing oxidative stress (Mohammad et al., 2014). As shown in Figure 7B, after 2 h of co-incubation with MEO, the SOD enzyme content significantly increased in the four test bacteria. There were statistically significant differences between the experimental group and the blank and control groups ($p < 0.05$). The elevated SOD levels reflect the bacterial response to oxidative stress, indicating that the bacteria are under environmental pressure, which may involve membrane damage, oxidative damage to proteins and DNA, and metabolic dysfunction.

3.2.10 Scanning electron microscope

The impact of MEO on the cellular morphology of the four test bacteria was observed using SEM. As shown in Figure 8, the control group displayed bacteria with smooth, intact surfaces,

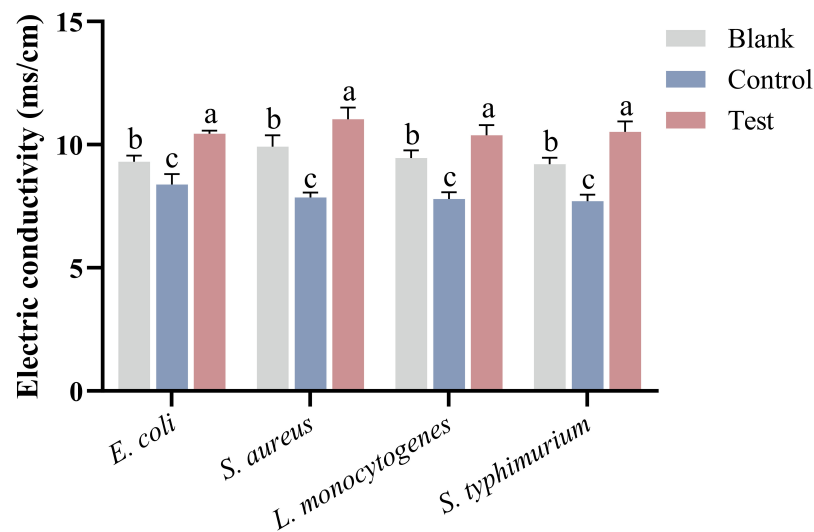


FIGURE 5

The electric conductivity of bacterial culture medium after treatment of *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium* in different groups. Different letters in the same figure indicate statistically significant difference at $P < 0.05$ between different samples. Each value represents the mean of three replicates $\pm$ standard deviation.

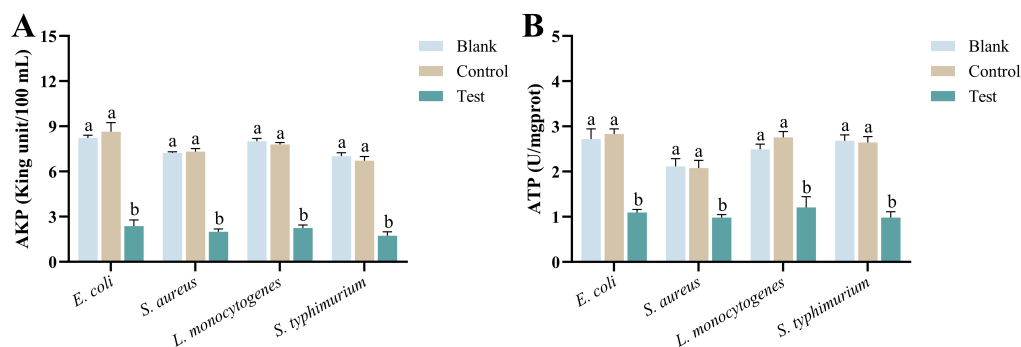


FIGURE 6

(A) Is the alkaline phosphatase (AKP) enzyme activity of *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium* in different groups. (B) Is the ATPase activity of *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium* in different groups. Different letters in the same figure indicate statistically significant difference at $P < 0.05$ between different samples. Each value represents the mean of three replicates $\pm$ standard deviation.

regular edges, full bodies, and tightly packed structures. However, after treatment with MEO at the MIC level, varying degrees of deformation were evident in all four bacteria. The bacterial surfaces became unclear, collapsed, with cell membrane indentations, ruptures, and distorted shapes. These results indicate that MEO damaged the bacterial cell wall and membrane structures. The disruption of these structures led to the leakage of intracellular contents, compromising bacterial integrity and resulting in its antibacterial effect.

3.3 Antioxidant activity

As shown in Figure 9, within the tested concentration range, the radical scavenging activity of MEO against DPPH, hydroxyl radicals, and FRAP exhibited a dose-dependent relationship. The scavenging rate increased with increasing MEO concentration, indicating that its effect was concentration-dependent. Among the

measured antioxidant capacities, MEO demonstrated the highest scavenging activity against DPPH ($IC_{50} = 7.421$ mg/mL), followed by hydroxyl radicals ($IC_{50} = 1.794$ mg/mL), while FRAP exhibited the lowest activity. This suggests that MEO possesses a stronger ability to neutralize DPPH radicals compared to its reducing power as measured by hydroxyl radical and FRAP assays.

4 Discussion

Plant-derived natural medicines have long been valued for their diverse biological activities, including antibacterial, antioxidant, anti-inflammatory, and antiviral properties (Zhang et al., 2024). Compared with traditional antibiotics, essential oils (EOs) are gaining attention due to their broad-spectrum antimicrobial efficacy and lower risk of inducing bacterial resistance (Wells, 2024). Previous studies have demonstrated that terpenoids and phenolic compounds in EOs can disrupt bacterial membranes, alter

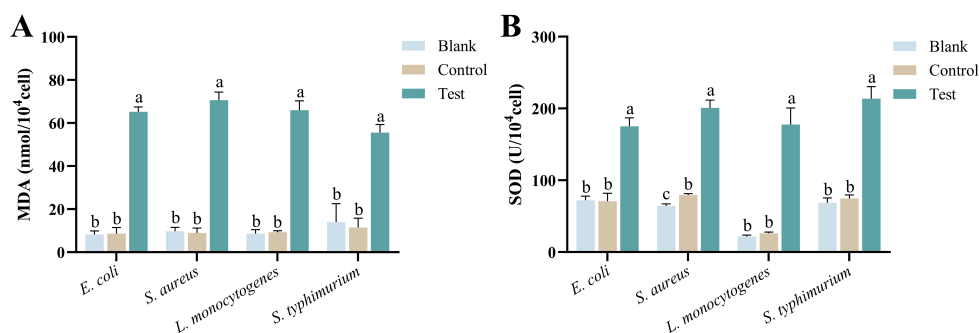


FIGURE 7

(A) Is the malondialdehyde (MDA) content of different groups of *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium*. (B) Is the superoxide dismutase (SOD) activity of different groups of *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium*. Different letters in the same figure indicate statistically significant difference at $P < 0.05$ between different samples. Each value represents the mean of three replicates $\pm$ standard deviation.

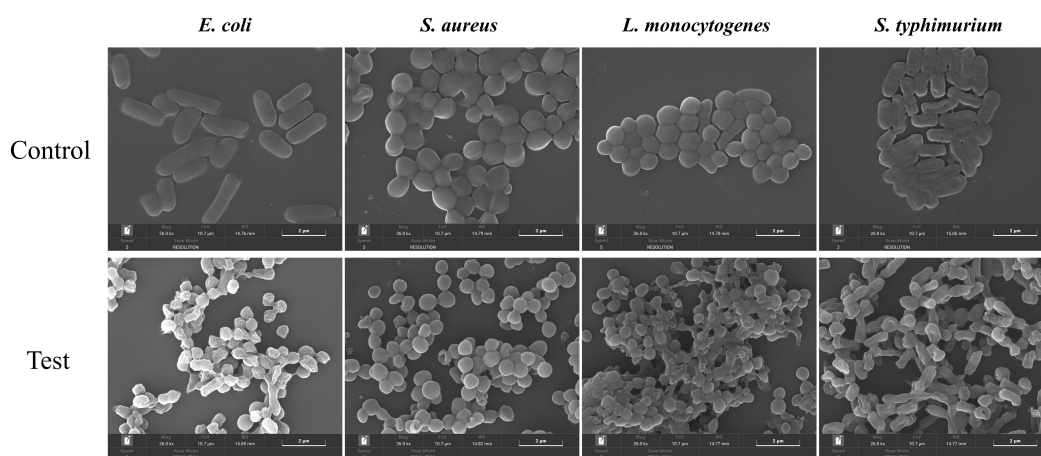


FIGURE 8

The effects of different groups of *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. typhimurium* on bacterial morphology.

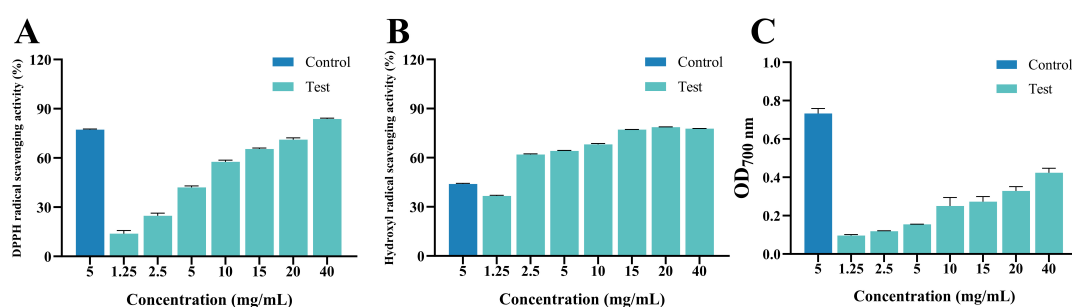


FIGURE 9

(A) Is 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) radical scavenging activity of magnolia essential oil (MEO). (B) Is hydroxyl radical scavenging activity of MEO. (C) Is Ferric Ion Reducing Antioxidant Power activity of MEO. Each value represents the mean of three replicates $\pm$ standard deviation.

ion gradients, and interfere with key metabolic pathways, ultimately leading to cell death (Visan and Negut, 2024). Among them, tea tree oil (*Melaleuca alternifolia*) and lavender oil (*Lavandula angustifolia*) are well-known for their strong antibacterial and antioxidant properties, with proven effectiveness against foodborne pathogens (Kokina et al., 2019). The present study confirms that MEO exhibits similar antibacterial activity against *Escherichia*

coli, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella typhimurium*, with its primary active components—1,8-cineole, citronellal, and linalool—playing a crucial role (György et al., 2020). Compared with other plant EOs, MEO demonstrated notable antibacterial potential against both Gram-positive and Gram-negative bacteria, suggesting that its bioactive compounds may have broader applications in food safety and medical fields.

The increasing prevalence of antibiotic-resistant bacteria has become a major global health concern, particularly in foodborne pathogens such as *E. coli*, *S. aureus*, and *L. monocytogenes* (Bajrami et al., 2024). This has driven the search for alternative antimicrobial agents that are both effective and safe. In this study, inhibition zone determination, MIC, and MBC assays confirmed the potent antibacterial effects of MEO, with results comparable to or even exceeding those of previously studied EO. Growth curve analysis further demonstrated that MEO significantly prolonged the logarithmic growth phase of bacteria, indicating a bacteriostatic effect. Moreover, MEO was found to cause substantial leakage of intracellular materials, induce lipid peroxidation, and generate oxidative stress, ultimately impairing bacterial metabolism and survival. These antibacterial mechanisms align with those reported for tea tree and cinnamon oils (Graziano et al., 2016), reinforcing the hypothesis that essential oils primarily act by disrupting bacterial membrane integrity. In addition to its antibacterial activity, MEO exhibited antioxidant properties, as evidenced by its strong scavenging effect on DPPH and hydroxyl radicals, although its ferric reducing antioxidant power (FRAP) was relatively weaker. While the antioxidant activities of tea tree and lavender oils have been widely studied, further research is needed to fully understand the antioxidative mechanisms of MEO and its potential applications in preventing oxidative deterioration in food systems.

Despite its promising antimicrobial and antioxidant properties, several challenges must be addressed before MEO can be widely applied in food preservation and healthcare. One major concern is its stability, as essential oils are highly volatile and susceptible to degradation under environmental factors such as heat, light, and oxygen exposure (Liu L. et al., 2024). Additionally, the potential cytotoxicity of MEO at high concentrations should be thoroughly evaluated to ensure its safety for human consumption. Furthermore, while essential oils have been explored as natural food preservatives, their strong aroma and hydrophobic nature may limit direct application in food products (Shaukat et al., 2023). To overcome these limitations, future research should focus on enhancing the stability and controlled release of MEO through nanoencapsulation or emulsification techniques. Additionally, investigating the synergistic effects of MEO with other natural antimicrobials, such as organic acids or plant polyphenols, may help improve its efficacy while reducing the required concentration (Timbe et al., 2021). Lastly, comprehensive toxicological studies and clinical evaluations are necessary to confirm its long-term safety and effectiveness. By addressing these challenges, MEO has the potential to become a viable natural alternative to synthetic preservatives and antibiotics, contributing to safer and more sustainable solutions in food preservation and medical applications.

5 Conclusion

In this study, the chemical constituents of MEO were analyzed by GC-MS. The highest proportion of compounds was terpenoids, and the main components with higher content were 1,8-cineole (44.87%), (+) -Citronellal (6.93%) and Linalool (29.1%). Through the determination of inhibition zone, MIC and MBC, it was preliminarily concluded that MEO had good antibacterial effect

on the four test bacteria. The growth curve shows that MEO has an inhibitory effect on the growth and reproduction of the test bacteria, and can effectively delay the logarithmic growth period of the bacteria. Through the preliminary exploration of the bacterial mechanism, it was found that MEO mainly acts on the initial stage of bacterial growth. By destroying the cell membrane, they cause a large amount of material loss in bacteria, affect bacterial metabolic activity, cause peroxidation of bacteria, stimulate their oxidative stress, and cannot maintain the normal growth and reproduction of bacteria. The scavenging ability of MEO on DPPH and hydroxyl radicals and FRAP were also determined. MEO had the best scavenging effect on DPPH, followed by hydroxyl radical scavenging effect, and FRAP had the worst effect.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

YL: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. NH: Project administration, Supervision, Writing – original draft. FM: Funding acquisition, Resources, Writing – review and editing.

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Three simple and cost-effective assays for AAC(6')-Ib-cr enzyme activity

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The enzyme AAC(6')-Ib-cr belongs to plasmid-mediated quinolone resistance (PMQR), first reported in 2006 and now widely disseminating. Here, we developed three phenotypic methods to detect AAC(6')-Ib-cr enzyme-producing *Enterobacteriaceae* (APE), two of which are proposed innovatively in this research. These tests are based on the following principles: (i) Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) can measure the mass shift of 42 Da resulting from ciprofloxacin acetylation by the AAC(6')-Ib-cr enzyme. (ii) Co-incubation of ciprofloxacin disks with APE results in inactivation of the drug activity, making it unable to inhibit the growth of the indicator organism. We named this test the quinolone inactivation method (QIM). (iii) Based on the principles of the modified Hodge test, we designed the quinolone Hodge test (QHT). Through exploration of optimal conditions for three methods, we found that MALDI-TOF MS provides the most intuitive results after 1 h of incubation. The interpretability of the QIM and QHT results was significantly improved when the indicator organism *E. coli* ATCC25922 was replaced with a quinolone-slightly-resistant isolate. However, *Proteus mirabilis* was excluded from both QIM and QHT due to its swarming motility. Next, a validation study was conducted using a prospectively collected set of 187 clinical strains, demonstrating 100% specificity (MSM: 141/141; QIM,QHT: 135/135) and 100% sensitivity (MSM: 46/46; QIM,QHT: 33/33) compared to the genotype. In a word, this study presented three simple, efficient, and cost-effective methods for detecting APE, suitable for clinical microbiology laboratories under various conditions for the prevention and control of hospital infections.

KEYWORDS

AAC(6')-Ib-cr, PMQR, quinolone, antimicrobial resistance, *Enterobacteriaceae*

Introduction

Quinolone antibiotics are chemically synthesized drugs with advantages of high blood concentrations, excellent broad-spectrum antibacterial activity, and strong tissue penetration (Ahmed and Daneshlab, 2012; Ball et al., 1998), making them a preferred agent for empirical treatment of infections (Gupta et al., 2011; Pilatz et al., 2020; Taplitz et al., 2018). They were the second most frequently prescribed antimicrobial drugs after β -lactam (Mercuro et al., 2018; Sanchez et al., 2016; Shapiro et al., 2014), but followed by serious bacterial resistance (Logan et al., 2019; Pitout et al., 2022; Zhao et al., 2024).

Researches have shown that bacterial resistance to quinolones is mainly mediated by chromosomal mechanisms, including alterations in drug action sites (DNA gyrase and topoisomerase IV gene mutations) and decreased drug accumulation capacity (loss of membrane porins and overexpression of efflux pumps) (Darby et al., 2023; Pham et al., 2019). Of greater concern were plasmid-mediated quinolone resistance (PMQR) due to their higher transmissibility (Ellabaan et al., 2021), including: Qnr protein families, which protect quinolone targets; the AAC(6′)-Ib-cr enzyme, which acetylates ciprofloxacin and norfloxacin; and efflux pumps mediated by *qepA* and *oqxAB* plasmid genes (Ruiz et al., 2012). Among them, the *aac(6′)-Ib-cr* gene is the most threatening as it provides a selective advantage in the presence of ciprofloxacin (Phan et al., 2022). Since its first report in 2006 (Robicsek et al., 2006), the prevalence of strains carrying the *aac(6′)-Ib-cr* gene has been increasing in both clinical and environmental settings. According to recent studies, this gene has been detected in various countries of Asia (Al-Khafaji et al., 2024; Kuo et al., 2024; Sohrabi et al., 2024; Zhan et al., 2021), Europe (Hrovat et al., 2019; Mounsey et al., 2021; Piccirilli et al., 2021), Africa (Al-Gallas et al., 2024; Masoud et al., 2021; Swedan et al., 2023), and the Americas (de Oliveira Alves et al., 2022; Golden et al., 2021; Logan et al., 2023) across multiple Gram-negative bacteria. The detection rates of the gene vary significantly by region and source, typically ranging from 10% to 50%. Furthermore, the distribution of the *aac(6′)-Ib-cr* gene is widespread, with its presence detected not only in clinical strains but also in animals, food, and water sources (Al-Gallas et al., 2024; Mounsey et al., 2021; Patel et al., 2024; Zahra et al., 2023).

However, unlike the *qnr*, *qepA*, and *oqxAB* screening, which can be accomplished by PCR amplification of the target genes, the detection of *aac(6′)-Ib-cr* is more complicated. This is because AAC(6′)-Ib-cr belongs to a variant of aminoglycoside acetyltransferases, and the differences between its encoding gene *aac(6′)-Ib-cr* and the wild-type *aac(6′)-Ib* exist only on just two nucleotides (Trp102Arg and Asp179Tyr) (Robicsek et al., 2006). These specific point mutations enable the acetylation of norfloxacin and ciprofloxacin at the amino nitrogen on position 7-C of the piperazine ring, thereby reducing drug activity (Vetting et al., 2008; Maurice et al., 2008). Therefore, the screening for *aac(6′)-Ib-cr* traditionally involves amplification of the target gene, followed by sequencing or restriction analysis (Pitout et al., 2008). Obviously, this is a time-consuming and expensive work. Although several studies have proposed alternative methods for *aac(6′)-Ib-cr* sequencing (Hidalgo-Grass and Strahilevitz, 2010; Wareham et al., 2010), these methods still relied on the use of large equipment not typically available in ordinary laboratories. To date, no study has proposed a method for detecting the AAC(6′)-Ib-cr enzyme based on common antimicrobial susceptibility testing consumables.

Here, we proposed three low-cost phenotypic methods of detecting the AAC(6′)-Ib-cr enzyme in *Enterobacteriaceae*. They each possess distinct characteristics and are suitable for clinical microbiology laboratories under different conditions. We aim to (i) improve existing phenotypic detection methods for AAC(6′)-Ib-cr, (ii) develop and validate QIM and QHT as reliable alternatives, and (iii) compare their performance with mass spectrometry and PCR. These methods are based on the following principles: (i) Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) is able to measure the mass transfer of 42 Da resulting

from acetylation of ciprofloxacin by the AAC(6′)-Ib-cr enzyme. Based on this, the appearance of spectrum peaks representing acetylated ciprofloxacin and its sodium or potassium adduct reveals the presence of the AAC(6′)-Ib-cr enzyme. This method was initially reported by Pardo et al. (2016), and we followed their steps with some modifications while enriching the species of the measured strains. (ii) The quinolone inactivation method (QIM), which is introduced and named for the first time in this study, is based on a principle derived from the Carbapenem Inactivation Method (CIM) (Pierce et al., 2017; van der Zwaluw et al., 2015): after co-incubation with test strains for some time, the ciprofloxacin adhered onto the disk will be inactivated by the enzyme-producing strains, thereby unable to inhibit the growth of the indicator organism. Furthermore, replacing the indicator organism *E. coli* ATCC 25922 with an isolate with only slight resistance to quinolones can greatly improve the observation of results. Because AAC(6′)-Ib-cr enzyme can easily reduce the quinolone concentration to a level where slightly resistant strains can grow, but it still cannot be lowered enough to support the growth of highly sensitive wild-type strains, as this would require nearly complete depletion of the quinolone. (iii) The last approach is based on the reduction of quinolone drug activity by the AAC(6′)-Ib-cr enzyme-producing *Enterobacteriaceae* (APE), allowing indicator organisms to grow in a curved manner along the inoculum of the tested strain toward the disk. This principle was first proposed by Hodge et al. (1978) to detect penicillinase and has subsequently been modified multiple times for detection of β -lactamases. The excellent performance of Modified Hodge Test (MHT) for screening carbapenase has been repeatedly demonstrated (Lee et al., 2001; Pasteran et al., 2016). In this study, we attempt to apply this principle to the detection of quinolone acetyltransferases, and therefore name it the quinolone Hodge Test (QHT).

Materials and methods

Bacterial isolates

- (i) Isolates genetically tested: During the first stage of this study, a total of 14 *Enterobacteriaceae* strains were included to explore the optimal conditions for the three methods. All isolates have been previously characterized at the molecular level to determine the various mechanisms of resistance to quinolone antibiotics, including *aac(6′)-Ib-cr*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, as well as the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes encoding type II topoisomerases.
- (ii) Indicator organisms: We collected four *E. coli* strains for the screening indicator organisms in QIM and QHT, including *E. coli* 28B220 with a single QRDR mutation (*gyrA*: S83L), *E. coli* B2-4 with a single QRDR mutation (*gyrA*: D87N), *E. coli* B2-5 with two QRDR mutations (*gyrA*: S83L; *parC*: S80I), and *E. coli* ATCC 25922. All of these strains were confirmed to not carry any of the aforementioned PMQR genes.
- (iii) Prospective clinical isolates: A total of 187 quinolone non-susceptible *Enterobacteriaceae* isolates representing 9 genera, including *E. coli* ($n = 104$), *K. pneumoniae* ($n = 47$), *Klebsiella*

oxytoca ($n = 1$), *P. mirabilis* ($n = 19$), *Raoultella ornithinolytica* ($n = 2$), *Serratia marcescens* ($n = 3$), *Enterobacter* spp. ($n = 3$), *Citrobacter* spp. ($n = 4$), *Morganella* spp. ($n = 3$) and *Salmonella* sp. ($n = 1$), were isolated from clinical specimens in Wenzhou Hospital of Traditional Chinese Medicine from March to June 2023. A single isolate per patient was collected. All isolates were re-identified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS; bioMérieux, Lyons, France) and utilized for methodological validation experiments of the three methods in the second stage of the study.

Antimicrobial susceptibility testing

The MICs of ciprofloxacin and levofloxacin were determined by the broth microdilution method (Nucien Pharmaceutical Co., Ltd. Guangzhou, China) and interpreted according to Clinical and Laboratory Standards Institute (CLSI)¹ guidelines.

PCR and sequencing

The test strains and indicator organisms used in this study were all characterized at the molecular level by PCR and sequencing methods to identify quinolone resistance genes, including *aac(6′)-Ib-cr*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, as well as the quinolone resistance-determining regions of *gyrA* and *parC*. Bacterial DNA was extracted by the boiling method. The amplification reagents and primer designs were obtained from Shanghai Sangon Biotech Co., Ltd (Shanghai, China). Primers for these genes are shown in the [Supplementary Table 1](#) (Kim et al., 2009; Lavilla et al., 2008; Nakano et al., 2019). Positive PCR products were sequenced by Sanger sequencing method in Shanghai Sangon Biotech Co., Ltd. The obtained DNA sequences were analyzed using the Blast program on the NCBI website² to determine the genotypes.

Exploration of optimal conditions

In the first stage of the study, we aim to explore the optimal conditions for the three methods and show the experimental steps in [Figure 1](#).

(i) Mass spectrometry method: We followed the method of [Pardo et al. \(2016\)](#) and [Oviaño et al. \(2017\)](#), and made some modifications. First, for reagent preparation, we diluted 100 ml:0.2 g ciprofloxacin injection (Nucien Pharmaceutical Co., Ltd. Guangzhou, China) with normal saline to obtain a concentration of 50 µg/mL. If the number of tests is small, the above solution could be obtained directly by immersing 5–7 ciprofloxacin disks (Kont Biology & Technology Co., Ltd. Wenzhou, China) in 2 mL normal saline. For analysis of bacteria, briefly, using a sterile inoculating loop, one colony was picked

and suspended in 100 µL of the aforementioned CIP solution in an Eppendorf tube. Then, the mixture was vortexed for 10 s and incubated at 35°C for 30 min/1 h/18 h (with the incubation time being set as the variable of this method). At the end of incubation, 1 µL of the supernatant obtained after centrifugation was dropped onto a polished steel MALDI target plate, followed by direct overlay with 1 µL of MALDI-TOF matrix [10 mg/mL α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile and 0.1% trifluoroacetic acid (Bruker Daltonics GmbH, Bremen, Germany)]. The mixture was allowed to dry together. Spectra were acquired using the flexControl 3.3 software on a MALDI-TOF MS instrument (Bruker Daltonics GmbH, Bremen, Germany), with parameter settings: positive ion linear mode, mass range of 300–600 Da, ion source 1 (IS1) at 10 kV, IS2 at 9.08 kV, lens at 3.00 kV, pulse ion extraction time of 10 ns, and laser frequency at 60 Hz. Additionally, a control experiment was conducted without the addition of any bacteria.

For data analysis, the relative molecular mass of ciprofloxacin ($C_{17}H_{18}FN_3O_3$) is 331, and the mass spectrometry peaks for $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ appeared at 332, 354, and 370 Da, respectively, as shown in [Figure 2](#). Upon acetylation by AAC(6′)-Ib-cr enzyme, N-acetylciprofloxacin ($C_{19}H_{20}FN_3O_4$) has gained an additional 42 Da, corresponding to peaks of 374, 396, and 412 Da for these three ionic adducts. Therefore, the presence of these three peaks indicating N-acetylated ciprofloxacin on the acquired spectral graph suggested that the tested bacteria were enzyme-producing strains.

(ii) Quinolone inactivation method: Briefly, using a sterile inoculation loop, 2–3 colonies of test isolate were added to a glass tube containing 1 mL of Mueller-Hinton Broth (Binhe Microbiological Reagents Co., Ltd. Hangzhou, China), followed by vortexing for 5–10 s (control test was performed without adding any bacteria). Next, a susceptibility-testing disk containing 5 µg ciprofloxacin (Kont Biology & Technology Co., Ltd. Wenzhou, China) was gently immersed at the bottom of the suspension and incubated at 35°C for 1 h/2 h/4 h (variable 1). Near the end of incubation, indicator organisms adjusted to 0.5 McFarland turbidity standard were inoculated onto Mueller-Hinton Agar (MHA; Autobio Biological Engineering Co., Ltd. Zhengzhou, China) plates by swabbing. In this study, we tested four indicator organisms (*E. coli* 28B220, B2-4, B2-5, and ATCC 25955, as previously described) as the second variable in the methodology. In addition, the antimicrobial susceptibility phenotypes and genetic characterizations of these organisms are detailed in [Table 1](#). After incubation, the disk was carefully removed from the suspension using an inoculation loop and gently placed onto freshly inoculated MHA plates. The plates were then incubated overnight at 35°C. It is worth noting that when removing the disk, one should avoid dragging it along the inner wall of the glass tube, as condensate water on the wall may dilute the concentration of antibiotics adhering to the paper disks, leading to false-positive results. In addition, the control experiment followed the same steps except that no bacteria were added during incubation, as previously described.

Next day, use a ruler to measure the diameter of the inhibition zone around each CIP disk. If the diameter of the inhibition zone in the test group was smaller than the control experiment by more than 5 mm, it was considered a positive result, i.e., the tested strain was APE. If the inhibition zone diameter was equal to or greater than the control experiment, it was considered as

¹ <http://www.clsi.org>

² <http://blast.ncbi.nlm.nih.gov/blast.cgi>

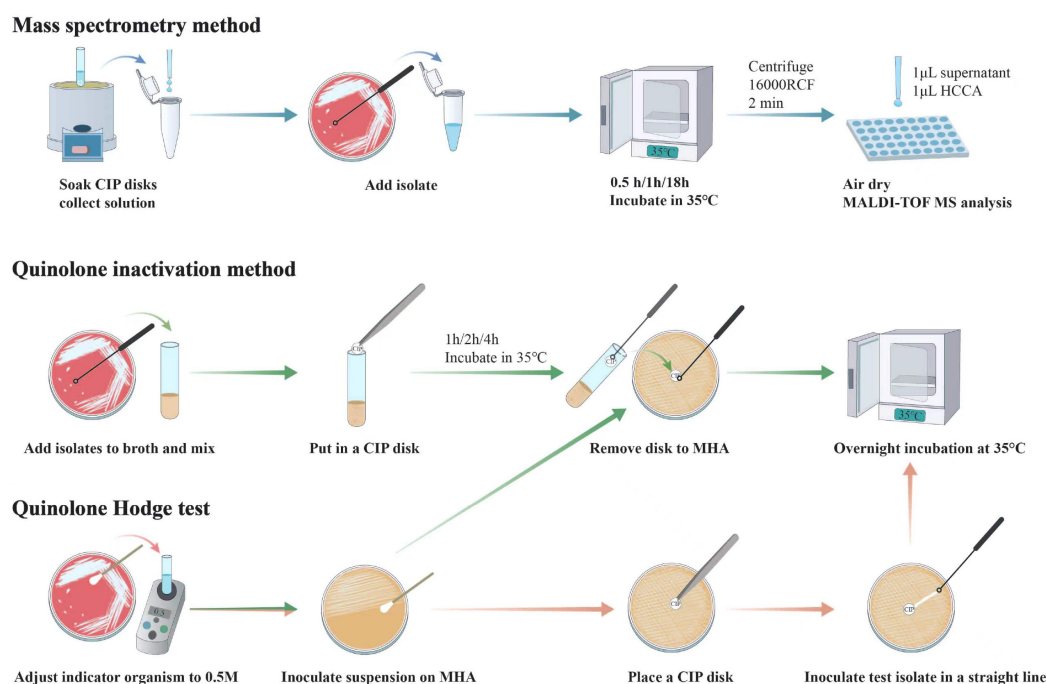


FIGURE 1

Workflows of mass spectrometry method, quinolone inactivation method and quinolone Hodge test. Each performance of mass spectrometry method and quinolone inactivation method required a concurrent control test which follows the same steps as the regular test, except that the test bacteria are not added. In addition, another variable for quinolone inactivation method and quinolone Hodge test is the four different indicator organisms, which are not annotated in the figure.

NAPE. If the value was within the range of the above two, it was considered an uncertain result. The selection of this cutoff value was based on the fact that, according to our repeated experimental experiences, conducting the test under optimal conditions resulted in most positive results (inhibition zone diameter) differing from the control experiment by more than 6 mm. Additionally, negative results always exhibited slightly larger diameters than the control experiment.

(iii) Quinolone Hodge test: First, the suspension of the indicator organisms (the four indicator organisms were the only variable for the method) was swabbed on the MHA plate as described previously. Next, a CIP disk was placed on the surface of the plate. Then, using a 10-μl loop, 1–2 monoclonal colonies of the tested bacteria were inoculated onto the plate in a straight line out from the edge of the disk, extending outward for a distance of 20–25 mm. Incubate overnight at 35°C. For the interpretation of the results, the production of AAC(6′)-Ib-cr was considered positive when the shape of inhibition zone on both sides of the tested strain was observed to bend toward the disk. Otherwise, the production of AAC(6′)-Ib-cr was considered negative when the shape of inhibition zone did not change.

Validation experiment

In the second part of the study, we evaluated the optimized procedure among the 187 blinded clinical isolates previously described. Specifically, under the optimal conditions of the three methods, 187 clinical isolates were tested by MSM, and 168

clinical isolates were tested by QIM and QHT. The prospectively collected strains were directly used for validation tests conducted every three days. Each experiment required one negative control and one positive control, which were selected from the APE and NAPE identified in the first phase of this study. It is worth mentioning that the 19 strains of *Proteus mirabilis* we collected were excluded from both QIM and QHT due to their swarming motility, which caused the entire plate to be covered and prevent observation of indicator results. Subsequently, PCR and sequencing of amplification products were performed to determine the presence of the *aac(6′)-Ib-cr* gene in each strain. Molecular characterization served as the gold standard to calculate the specificity and sensitivity of the three methods, evaluating their performance [Specificity = number of samples with negative results in both the method being evaluated and *aac(6′)-Ib-cr* gene detection / number of samples with negative results in gene detection; Sensitivity = number of samples with positive results in both the method being evaluated and gene detection / number of samples with positive results in gene detection].

Results

Antimicrobial susceptibility testing and molecular characterization of quinolone resistance genes

Antimicrobial Susceptibility Testing (AST) and the detection of quinolone resistance genes were conducted on

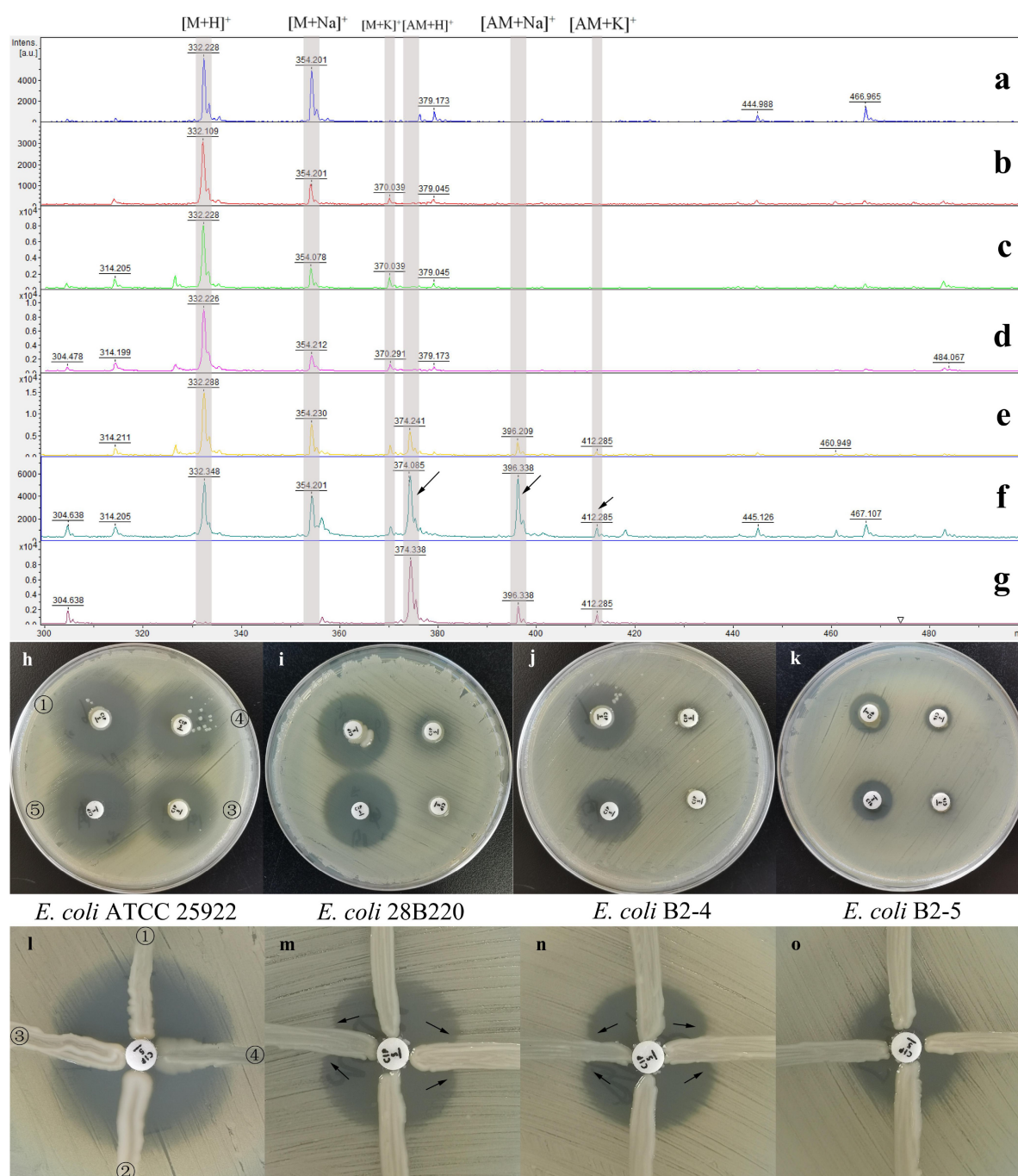


FIGURE 2

Results of the three methods under different conditions. Results of the mass spectrometry method (a–g), the quinolone inactivation method (h–k) and the quinolone Hodge test (l–o). MALDI-TOF MS spectra of ciprofloxacin after 0.5 h (b), 1 h (c), and 18 h (d) incubation with NAPE and after 0.5 h (e), 1 h (f), and 18 h (g) incubation with APE, and 1 h (a) incubation without bacteria. The spectral peaks at 322, 354, and 370 Da represent the three adducts of ciprofloxacin molecular ions: [M+H]⁺, [M+Na]⁺, and [M+K]⁺. The spectral peaks at 374, 396, and 412 Da represent the three adducts of acetylated ciprofloxacin molecular ions: [AM+H]⁺, [AM+Na]⁺, and [AM+K]⁺ (indicated by the arrows). *E. coli* ATCC 25922 (h, i), *E. coli* 28B220 [gyrA: S83L; (i, m)], *E. coli* B2-4 [gyrA: D87N; (j, n)] and *E. coli* B2-5 [gyrA: S83L, parC: S80I; (k, o)] were used as indicator organisms. Marked ①, ②, ③, ④, and ⑤ in the figures (h, i) are the results of NAPE, NAPE, APE, APE, and control test, respectively, and they correspond to the same positions on the other six plates. In figures (m, n), the curved growth of the indicator organisms on both sides of the test strains, indicated by the arrows, represents that the test strains are enzyme-producing strains.

14 *Enterobacteriaceae* isolates and 3 indicator organisms. Among these, 8 isolates carried the *aac(6')-Ib-cr* gene, 1 isolate carried wild-type *aac(6')-Ib*, and the remaining 5 isolates did not carry this gene. Additionally, the 14 isolates were found to carry various

combinations of quinolone resistance genes. The molecular characterization of the 3 indicator organisms was also re-validated. The AST results and resistance determinants for the above isolates are detailed in Table 1.

TABLE 1 Genotypes, quinolone susceptibility, and QHT results of strains involved in the first stage.

Strain (species)	MIC ^a (μg/ml)		Resistance mechanism					Mass spectrometry method			Quinolone Hodge test			
			PMQR			QRDR		Incubation time			Indicator organism (<i>E. coli</i>)			
	CIP	LVF	<i>aac(6')-lb-cr</i>	<i>qnr</i>	<i>qepA</i>	<i>gyrA</i>	<i>parC</i>	30 mins	1 h	18 h	ATCC 25922	28B220	B2-4	B2-5
Test isolate														
A2 (<i>E. coli</i>)	64	32	+	<i>qnrS</i>	–	S83L&D87N	S80I	± ^c	+ ^e	+	–	+	+	–
A3 (<i>Enterobacter hormaechei</i>)	64	16	+	<i>qnrB</i>	–	S83I	S80I	+	+	+	–	+	+	–
A141 (<i>E. coli</i>)	128	16	+	–	–	S83L&D87N	S80I	+	+	+	±	+	+	–
B24 (<i>K. pneumoniae</i>)	64	32	+	<i>qnrB</i>	–	S83I	S80I	±	+	+	–	+	+	–
B26 (<i>K. pneumoniae</i>)	16	4	+	<i>qnrB&qnrS</i>	–	wt	wt	+	+	+	–	+	+	–
A63 (<i>E. coli</i>)	> 128	32	+	–	–	S83L&D87N	S80I&E84V	±	+	+	–	+	+	–
A67 (<i>Citrobacter freundii</i>)	32	8	+	<i>qnrB</i>	–	S83T	wt	±	+	+	–	+	+	–
A113 (<i>P. mirabilis</i>)	64	16	+	–	–	S83I	S80I	±	+	+	Not applicable assay ^d			
A105 (<i>E. coli</i>)	32	32	–	–	–	S83L&D87N	S80I	–	–	–	–	–	–	–
A117 (<i>K. pneumoniae</i>)	64	64	–	<i>qnrB&qnrS</i>	–	S83I&D87G	S80I	–	–	–	–	–	–	–
A102 (<i>K. pneumoniae</i>)	32	32	–	–	–	S83L&D87N	S80I	–	–	–	–	–	–	–
6B107 (<i>E. coli</i>)	≤ 0.125	≤ 0.25	–	–	–	wt	wt	–	–	–	–	–	–	–
B1-5 (<i>E. coli</i>)	0.5	1	–	–	–	S83L	wt	–	–	–	–	–	–	–
A6 (<i>K. pneumoniae</i>)	16	32	wt	–	–	S83I	S80I	–	–	–	–	–	–	–
Indicator organism														
28B220 (<i>E. coli</i>)	0.5 (28) ^b	1	–	–	–	S83L	wt							
B2-4 (<i>E. coli</i>)	0.5 (27)	1	–	–	–	D87N	wt							
B2-5 (<i>E. coli</i>)	2 (20)	4	–	–	–	S83L	S80I							
ATCC25922 (<i>E. coli</i>)	≤ 0.125 (33)	≤ 0.25	–	–	–	wt	wt							

^aCIP, ciprofloxacin; LVF, levofloxacin; MIC, minimum inhibitory concentration; CIP MICs of ≤ 0.25 is considered susceptible, and ≥ 1 is considered resistant; LVF MICs of ≤ 0.5 is considered susceptible, and ≥ 2 is considered resistant; PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone resistance-determining regions; wt, wild type. ^bTwo values represent the MIC (outside the parentheses) and the Kirby-Bauer method results (inside the parentheses). ^c±: visually detectable positive results, but not the most readable outcomes. ^d*P. mirabilis* is excluded from QIM and QHT due to swarming motility. ^eShaded cells highlight test results under ideal conditions.

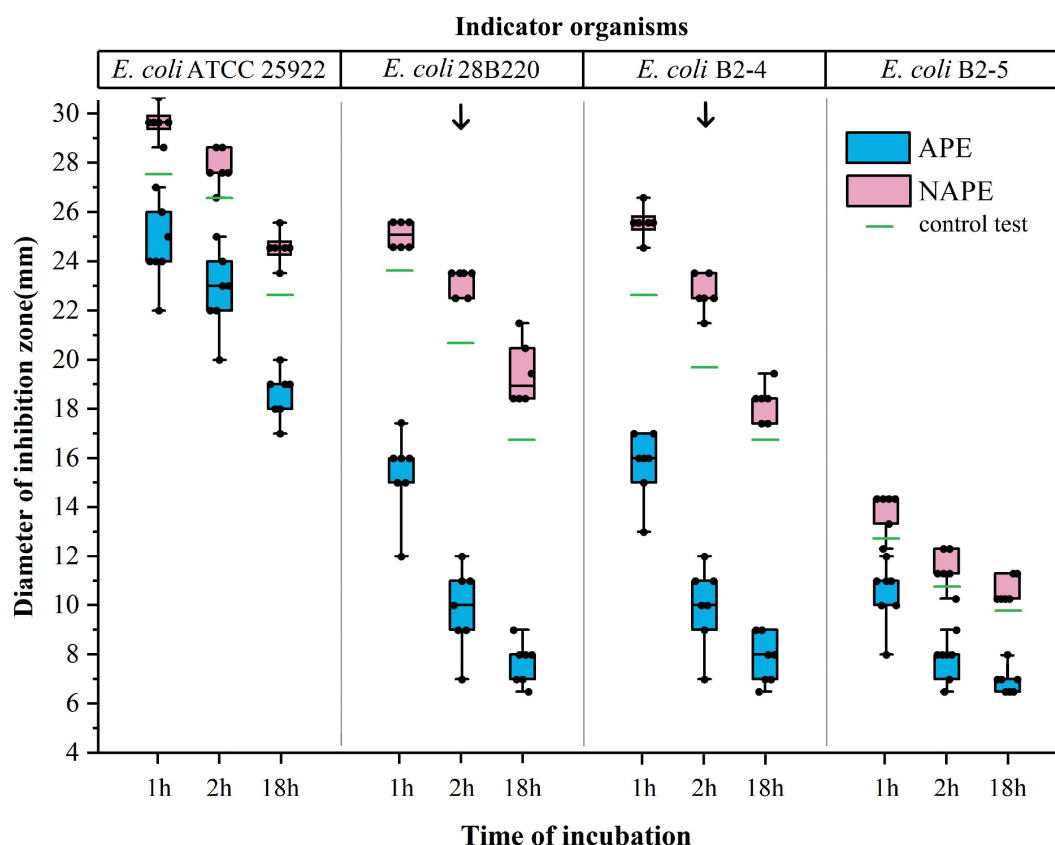


FIGURE 3

Effect of indicator organisms and incubation time on the inhibition zone diameter in the quinolone inactivation method. The x-axis represents the incubation time of the test bacterium with CIP disks, and the y-axis represents the inhibition zone diameter of four indicator organisms. Data of the blue boxes are from the results of APE, while data of the red boxes are from the results of NAPE. The green lines represent the results of the control tests. Arrows point to the test results under ideal conditions.

Mass spectrometry method

MS spectra showed the ion peaks corresponding to the ciprofloxacin molecular ions: $[M+H]^+$ at 332 Da, its sodium adduct $[M+Na]^+$ at 354 Da, and potassium adduct $[M+K]^+$ at 370 Da. The acetylation of ciprofloxacin by AAC(6')-Ib-cr resulted in a 42 Da shift, with the addition of three acetylation molecule peaks: $[AM+H]^+$ at 374 Da, $[AM+Na]^+$ at 396.35 Da and $[AM+K]^+$ at 412 Da (Figure 2).

For the evaluation of experimental variables, the incubation time significantly affects result readability. While a 30-min incubation suffices for visually detectable positive results, we found that a 1-h incubation yields the most readable outcomes, as briefly illustrated in Figures 2e–f. Upon 18 h of incubation, some positive results showed a complete disappearance of spectral peaks representing ciprofloxacin, as depicted in Figure 2g. Additionally, as expected, NAPE consistently lacked spectral peaks of the acetylated form even after 18 h of incubation. Furthermore, in the result of the control test (Figure 2a), the peak at 370 Da representing the ciprofloxacin potassium adduct was absent, whereas it appeared in the experimental groups. We believe that ciprofloxacin was dissolved in NaCl solution lacking potassium ions, which explains the absence of the 370 Da peak. Upon adding bacteria to the solution, potassium ions were partially extruded from bacterial cells

into the solution via ion pumps, thereby resulting in weak spectral peaks representing potassium adducts (370 Da and 412 Da).

Quinolone inactivation method

To explore the optimal conditions for QIM, two variables influencing the outcomes were identified: the use of indicator organism *E. coli* 28B220 (*gyrA*: S83L) or *E. coli* B2-4 (*gyrA*: D87N), and the selection of a 2-h incubation time under which all AAC(6')-Ib-cr enzyme-producing and non-producing isolates were correctly categorized based on the maximum difference in diameter of inhibition zone, as shown in Figure 3.

While studying the feasibility and accuracy of QIM, we observed in preliminary experiments that the indicator organism *E. coli* ATCC25922 was unsuitable for this method. Subsequently, we redesigned three indicator organisms and conducted comparative experiments involving the above four indicator strains in further studies. Through testing 14 isolates, we found that the difference in the inhibition zone diameters of APE and NAPE could not be effectively distinguished when either *E. coli* ATCC 25922 or *E. coli* B2-5 (*gyrA*: S83L, *parC*: S80I) was used as indicator organism, due to the inhibition zones being either too large or too small. However, when *E. coli* 28B220 or *E. coli* B2-4 was

TABLE 2 Detection of AAC(6′)-Ib-cr enzyme producing clinical isolates using the three methods in this study.

Species (No. of isolates)	MIC range (μg/ml)		No. of positive results		
	LVF	CIP	MSM	QIM	QHT
AAC(6′)-Ib-cr-producing Enterobacteriaceae					
<i>E. coli</i> (n = 12)	≥ 8	≥ 4	12	12	12
<i>K. pneumoniae</i> (n = 18)	≥ 8	≥ 4	18	18	18
<i>P. mirabilis</i> (n = 13)	4–≥ 8	≥ 4	13	ND	ND
<i>Enterobacter hormaechei</i> (n = 1)	≥ 8	≥ 4	1	1	1
<i>Citrobacter braakii</i> (n = 1)	2	≥ 4	1	1	1
<i>Citrobacter freundii</i> (n = 1)	≥ 8	≥ 4	1	1	1
non-AAC(6′)-Ib-cr enzyme producer					
<i>E. coli</i> (n = 92)	0.5–≥ 8	0.5–≥ 4	0	0	0
<i>K. pneumoniae</i> (n = 29)	1–≥ 8	0.5–≥ 4	0	0	0
<i>P. mirabilis</i> (n = 6)	1–≥ 8	1–≥ 4	0	ND	ND
<i>Salmonella</i> sp. (n = 1)	≥ 8	≥ 4	0	0	0
<i>Klebsiella oxytoca</i> (n = 1)	≥ 8	≥ 4	0	0	0
<i>Enterobacter cloacae</i> (n = 1)	≥ 8	≥ 4	0	0	0
<i>Enterobacter hormaechei</i> (n = 1)	1	1	0	0	0
<i>Raoultella ornithinolytica</i> (n = 2)	4	2	0	0	0
<i>Serratia marcescens</i> (n = 3)	0.5–1	0.25–1	0	0	0
<i>Citrobacter amalonaticus</i> (n = 1)	≥ 8	≥ 4	0	0	0
<i>Citrobacter freundii</i> (n = 1)	≥ 8	≥ 4	0	0	0
<i>Morganella morganii</i> (n = 3)	2–4	1–4	0	0	0

ND, not determined (*P. mirabilis* is excluded from QIM and QHT due to swarming motility.); MSM, mass spectrometry method; QIM, quinolone inactivation method; QHT, quinolone Hodge test.

TABLE 3 Performance evaluation of different methodologies.

Detection method	Cost per test (equipment)	TAT ^a	Time of perform test ^b	Specificity	Sensitivity	Limitations of method
MSM	< \$1.00 (MALDI-TOF MS)	< 2 h	< 0.5 h	100%	100%	MALDI-TOF MS is required in the laboratory.
QIM	< \$1.00 (Not required)	18–24 h	< 20 mins	100%	100%	This method is not applicable to <i>P. mirabilis</i> .
QHT	< \$0.10 (Not required)	18–24 h	< 10 mins	100%	100%	(i) This method is not applicable to <i>P. mirabilis</i> . (ii) The interpretation of the results is subjective.
PCR+sequencing	≥ \$10.00 ^c (PCR system and sequenator)	1–2 days	> 5 h	–	–	The cost is expensive and the equipment is complex.

^aTAT: turnaround time. ^bIncubation time is not included. ^cThe cost of testing varies in different regions.

used as indicator organism, this difference became significantly pronounced. Moreover, the maximum difference occurred with a 2-h incubation time, as shown in Figures 2h–k.

Considering that the diversity of equipment and consumable specifications may affect the outcomes of this method, we did not set specific inhibition zone diameter values for positive and negative results. Instead, differentiation between positive and negative results was determined through concurrent control testing

conducted during each trial. Values were set to ≤ 5 mm compared to the control test for positive results and ≥ the control test for negative results, as previously described. This difference was sufficient to distinguish between positive and negative results. In fact, this difference was above 6 mm for all tests under optimal conditions, as shown in Figure 3. Interestingly, the inhibition zone diameters of all NAPIs were slightly larger than those of the control test. Our explanation for it was that bacterial suspension adhered

to the lifted disk, which increased the fluid's surface tension due to bacterial particulates, resulting in greater absorption of residual antibiotics, a phenomenon that did not occur in the control test (Miller et al., 2017).

Quinolone Hodge test

We also tested the performance of the four indicator organisms in QHT. The results were similar to those in QIM. When *E. coli* ATCC 25922 was used as the indicator organism, the bending of the inhibition zone was too faint to distinguish between positive and negative results. In contrast, when using *E. coli* 28b220 or *E. coli* B2-4 instead, significantly enhanced growth of the indicator organism was observed on both sides of the APE streak, which was easily recognized by the naked eye. No such phenomenon occurred with NAPE (Figures 2l–o). Additionally, *E. coli* B2-5 was eliminated once again due to its initially small zone of inhibition.

Validation experiment

The results of PCR and sequencing of 187 clinical isolates used for method performance validation showed that a total of 46 APEs were screened in this prospective study, accounting for 24.6% (46/187), including *E. coli* (12/104), *K. pneumoniae* (18/47), *P. mirabilis* (13/19), *Enterobacter* spp. (1/3), *Citrobacter* spp. (2/4), and no APE was detected in the remaining genera (0/10). All APEs (including strains studied in the first phase) carried the *aac(6′)-Ib-cr* gene with both Trp102Arg and Asp179Tyr mutations, which means that no single-point mutation was found across all strains. This finding was consistent with earlier reports (Robicsek et al., 2006). Additionally, 7 isolates carrying the wild-type *aac(6′)-Ib* gene were detected. They exhibited identical assay results to non-AAC(6′)-Ib-cr enzyme producers in the validation study. Thus, the proportion of -cr variant among the total number of the gene was calculated as 86.8% (46/53) in this study.

Under optimal conditions, validation experiments were conducted for MSM, QIM, and QHT. The results indicated that MSM correctly identified all APEs and NAPEs with 100% specificity and sensitivity (141/141; 46/46). However, despite QIM and QHT also demonstrating 100% specificity (135/135) and sensitivity (33/33) compared to the genotype, *Proteus mirabilis* was excluded from these data. The data are detailed in Table 2.

Performance evaluation and selection guide of methods

We described the specificity, sensitivity, cost, required equipment, turnaround time, and test execution time of four APE detection methods in Table 3. And based on the experience gained from this work, we provide a step-by-step recommendation for selecting genotypic testing, MSM, QIM, or QHT based on existing resources. (i) If MALDI-TOF MS is available, MSM should be prioritized over genotypic testing. This is because the *aac(6′)-Ib-cr* gene cannot be detected by a PCR device alone without sequencing equipment. In fact, even in most hospitals of developed countries,

only PCR devices are commonly available. More importantly, Phenotypic testing provides a more accurate assessment of clinical resistance compared to genotypic testing, which only detects the presence of *aac(6′)-Ib-cr* gene without accounting for the functional expression of it. (ii) In less developed areas, QHT can be chosen as an initial screening method, followed by QIM to recheck samples with subjective controversy in the screening results.

Discussion

The resistance phenotype to quinolones is typically insufficient to distinguish between PMQR and other resistance mechanisms (Rodríguez-Martínez et al., 2016; Strahilevitz et al., 2009). As the most prevalent PMQR (Guillard et al., 2014; Jomehzadeh et al., 2022; Shrestha et al., 2023; Tayh et al., 2024), AAC(6′)-Ib-cr confers a low level of quinolone resistance in host organisms, generally below the resistance breakpoints established by CLSI (Machuca et al., 2016; Robicsek et al., 2006; Ruiz, 2019), yet it poses a significantly grave clinical threat (Redgrave et al., 2014; Tacconelli et al., 2018). Several factors substantiate its significance in this process, including its increasing prevalence (Kuo et al., 2024; Zhan et al., 2021), association with other resistance elements (Dulyayangkul et al., 2024; Raherison et al., 2017), as well as the accelerated induction of mutations in QRDRs (Liang et al., 2023; Ortiz-Padilla et al., 2020; Recacha et al., 2019). Of concern is that clinical microbiology laboratories have not yet considered these genes in antimicrobial resistance screening, which may be attributed to the current lack of reliable screening assays for AAC(6′)-Ib-cr (Strahilevitz et al., 2009). In this study, we proposed three methods for detecting the AAC(6′)-Ib-cr enzyme in *Enterobacteriaceae*, all of which demonstrated an overall performance with 100% specificity and sensitivity. Additionally, all three methods cost less than \$1 per test, and QIM and QHT did not require complex equipment and professional personnel to perform, as they were easy to operate and yield simple results. Furthermore, our experiments clearly showed that neither mutations in quinolone action target genes nor plasmid-mediated Qnr proteins would interfere with the determination of AAC(6′)-Ib-cr in these new methods. This is not difficult to explain. All tests are based on the acetylation modification activity of the AAC(6′)-Ib-cr enzyme toward ciprofloxacin, which is specific and independent among all mechanisms of resistance to quinolone (Robicsek et al., 2006; Ruiz et al., 2012).

In fact, the concept of using mass spectrometry for the detection of resistant enzymes was already commonplace and dated back to the early 2000s (Burckhardt and Zimmermann, 2011). Currently, it is mainly applied in the detection of carbapenemases (Alvarez-Buylla et al., 2013; Zhang et al., 2023). In contrast to carbapenemases, the detection of the AAC(6′)-Ib-cr enzyme by MS has an additional advantage, because the former result is explained by the complete disappearance of the spectral peaks representing ertapenem (Burckhardt and Zimmermann, 2011), which requires sufficient reaction time, while the latter result is explained by the appearance of new peaks representing the modified products, which are produced early in the reaction (Oviaño et al., 2017; Pardo et al., 2016). Therefore, although the activity of modifying enzymes is much lower than that of

hydrolyzing enzymes, the incubation time required for AAC(6′)-Ib-cr detection is still shorter than that needed for carbapenemase detection. In addition, we found that MS was extremely sensitive to CIP detection, as clear ion peaks could be obtained with a 10 µg/ml CIP solution. This was why we believed that CIP reagents could be directly obtained by immersing CIP disks in normal saline. Such adjustments offered numerous benefits: simplifying reagent preparation, reducing costs, and the fact that antibiotic disks are often easy to store for a long time. Furthermore, preliminary research experience showed us that adding the matrix solution directly before the sample droplet dries did not affect the spectral peaks (allowing co-drying). Such a research was necessary because the drying time for sample droplets without surfactants was extremely long at room temperature. Adding the matrix solution could reduce the surface tension of the liquid, similar to the effect of surfactants, and greatly shorten the waiting time for drying (Miller et al., 2017; Wu et al., 2019).

The QIM and QHT methods were proposed for the first time in this study, and our objective was to design a low-cost, as minimally instrument-free method for the detection of the plasmid-mediated quinolone resistance determinant AAC(6′)-Ib-cr in *Enterobacteriaceae*. However, in our previous study (Yunxiang et al., 2015), we found that when *E. coli* ATCC 25922 was used as an indicator organism, it was only suitable for enzymes with strong hydrolytic activity, such as carbapenemases. Its performance was unsatisfactory in detecting *aac(6′)-Ib-cr*-mediated acetyltransferase. Therefore, through the comparative experiments involving four strains of *E. coli* with different degrees of QRDR mutations, we attempted to propose new insights into indicator organisms that played a critical role in the test.

The selection of an ideal indicator organism must meet two simple criteria: (i) its minimum inhibitory concentration (MIC) of ciprofloxacin should range between 0.25 to 1 µg/ml. (ii) in the Kirby-Bauer disk diffusion susceptibility test, the indicator organism which must be non-mucoid, should exhibit a clear boundary with an inhibition zone diameter of 28–30 mm. Such organisms, which are readily obtainable from clinical samples, share a common feature of a single-point mutation (S83L or D87N) in the *gyrA* gene, resulting in low-level resistance to quinolones. However, they still exhibit good susceptibility to ciprofloxacin in the Kirby-Bauer test (Machuca et al., 2016; Pham et al., 2019). Our approaches are based on the fact that the enzyme AAC(6′)-Ib-cr produced by the test strains modifies ciprofloxacin, reducing the drug's effective concentration to such a level that the indicator organism with weak quinolone resistance is sufficient to protect itself, while the highly sensitive wild-type strain (*E. coli* ATCC 25922) remains difficult to survive (Robicsek et al., 2006). This principle is similar to that when a bacterium possesses both a *gyrA* mutation and plasmid-mediated AAC(6′)-Ib-cr enzyme, it always exhibits a higher level of resistance to quinolones (Machuca et al., 2016). We redesigned the indicator organism aiming to combine these two low-level resistance mechanisms to exhibit enhanced drug resistance effects in both QIM and QHT methods. Indeed, The combination of resistance mechanisms is also common to other classes of antibiotics. For example, the resistance of *Pseudomonas aeruginosa* to imipenem is due to a decrease in antibiotic uptake (via downregulation of OMPD2) and the production of AmpC-type β-lactamase (Livermore, 1992). Either

mechanism alone is not sufficient to cause clinically significant levels of resistance, but only their combination results in high-level resistance. Therefore, as our experimental results showed, when *E. coli* ATCC25922 was used as the indicator organism, its indication effect for AAC(6′)-Ib-cr production was not significant. However, when it was replaced by strain *E. coli* 28B220, there would be an “illusion” of high level of resistance at the junction of the two bacteria (quinolone Hodge test), similar to that shown when the two resistance mechanisms worked together. When another resistance mechanisms is added, the inherently small inhibition zone may dilute the changes caused by the AAC(6′)-Ib-cr enzyme.

In addition, it was worth noting that the *aac(6′)-Ib-cr* genes carried by all 46 strains of APE in this study exhibited identical mutations at codons 102 (Trp-Arg) and 179 (Asp-Tyr). The remaining codons showed high similarity to the wild-type *aac(6′)-Ib* gene, and no independent occurrences of the W102R or D179Y mutations were observed. This was consistent with previous studies (Robicsek et al., 2006). We believed that the high sequence homology ensures relatively consistent enzyme activities of the encoded products (Vetting et al., 2008), which was the primary reason for the high performance of these enzyme activity methods. Therefore, compared to labor-intensive, time-consuming and expensive genotype testing, direct detection of AAC(6′)-Ib-cr enzyme activity represents a more cost-effective laboratory strategy. On the other hand, we observed that CIP was completely modified after incubation with APE for up to 18 h (no 322 Da peak was shown; Figure 2g). This compelled us to reassess the AAC(6′)-Ib-cr enzyme. To our knowledge, the concept of AAC(6′)-Ib-cr as a low-level resistance mechanism is based on *in vitro* experiments (Robicsek et al., 2006), which are inherently short-term studies. Thus, its prolonged presence in the human body and its sustained impact on antibiotic efficacy warrant re-evaluation.

There are some limitations to our study. Although all three methods demonstrated 100% specificity and sensitivity, real-world variability should be acknowledged. For example, we are still unable to explain whether a single mutation of the *aac(6′)-Ib* gene (W102R or D179Y) in strains might challenge these methods, whether our methods are applicable to non-fermenting Gram-negative bacilli, whether severe porin protein loss that prevents ciprofloxacin from entering the periplasmic space could lead to false negatives, and the variability between different laboratories.

In summary, this study presents three simple, efficient, and cost-effective methods for the detection of AAC(6′)-Ib-cr in *Enterobacteriaceae*. Each method has its own advantages: MSM is a rapid and highly accurate method for AAC(6′)-Ib-cr detection, but it relies on MALDI-TOF MS. QIM and QHT offer cost-effective alternatives but are unsuitable for *P. mirabilis*. Overall, these methods can enhance routine clinical screening for PMQR mechanisms.

Data availability statement

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

Ethics statement

This study was approved by the Ethics Committee of Wenzhou Hospital of Traditional Chinese Medicine, Zhejiang, China. The research was conducted in accordance with local legislation and institutional requirements. Written informed consent was obtained from all participants.

Author contributions

SL: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing. WC: Data curation, Investigation, Methodology, Project administration, Writing – review and editing. RM: Formal Analysis, Investigation, Methodology, Writing – review and editing. MC: Data curation, Investigation, Software, Supervision, Validation, Writing – review and editing. XD: Data curation, Formal Analysis, Writing – review and editing. XJ: Resources, Writing – review and editing. WK: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Writing – review and editing.

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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.2025.1513425/full#supplementary-material>

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Thermophilic microbial agents promote the fermentation progression of spent mushroom compost and pig manure

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Livestock and poultry manure, as a significant organic resource, had an enormous annual production but a utilization rate of less than 50%. Improperly managed manure had become the primary source of agricultural non-point pollution, posing severe challenges to the ecological environment. Achieving efficient resource utilization of livestock manure was a critical step in promoting green agricultural development. Existing research indicated that microbial activity significantly influences the transfer and dissemination of antibiotic resistance genes (ARGs) and the community dynamics of human pathogenic bacteria (HPB) during pig manure composting. However, the specific mechanisms remain unclear. This study innovatively introduced two thermophilic microbial agents (TMS1 and CTMS2) into a pig manure-spent mushroom compost (SMC) aerobic composting system to systematically investigate their regulatory effects on pollutant reduction. The results showed that persistent ARGs (*ErmF*, *ErmQ*, *ErmX*, *blaR1*, *QnrA1*, *QnrA6*, *bla-F*, *QnrA2*, *QnrA5*, *Qnra4* and *bla-VIM*) primarily rely on vertical gene transfer (VGT) for dissemination, whereas easily removable ARGs (*tetX*, *tetW*, *tetG*, *tetC*, *sul1* and *sul2*) were regulated by both horizontal gene transfer (HGT) and VGT. Notably, the co-addition of thermophilic microbial agents and SMC reduced persistent ARGs by lg0.45–3.73, significantly decreased the abundances of HPB such as *Bacteroides* and *Treponema*, and reduced the enrichment of related metabolic pathways, greatly improving compost quality. In stark contrast, the control group (with only SMC and no thermophilic microbial agents) exhibited ARG proliferation. Overall, the application of thermophilic microbial agents not only extended the high temperature phase of composting by over 30% and shortened the composting cycle by 50%, but more importantly, it achieved comprehensive improvement in compost quality by selectively enriching functional microbial communities such as *Pseudomonas*. This study provides a theoretical foundation and data support for the industrial application of CTMS2 in the safe production of organic fertilizers and the synergistic control of environmental risks.

KEYWORDS

antibiotic resistance genes, aerobic fermentation, human pathogenic bacteria, spent mushroom compost, thermophilic microbial agents

1 Introduction

According to statistics, the annual amount of livestock and poultry manure resources in China reaches 4 billion tonnes, of which 40% remain untreated and unutilized, making it the primary source of agricultural non-point source pollution. Among them, pig manure accounts for 36.71% of the total output (He et al., 2021; Zhang S. et al., 2025). Pig manure, an organic complex that riched in crude protein, fiber, and hemifiber (Samanta et al., 2022), could serve as a valuable nitrogen source and slow-release fertilizer. It could enhance soil fertility and improved the physical and chemical environmental properties of soil. However, owing to various technological and process limitations, nearly 60% of pig manure resources was wasted (Wu et al., 2020), posing a great threat to environmental safety. Therefore, exploration of green treatment methods for pig manure and the development of efficient recycling systems for its utilization were urgently needed.

Current treatment methods include anaerobic digestion (AD) and aerobic fermentation (AF). While AD of livestock and poultry manure 76.5% of cellulose and 84.9% of hemicellulose were converted into methane (Ma et al., 2021; Muhammad and Birgitte, 2021), its resilience on specialized equipment and risks of secondary pollution limit practicality. In contrast, AF is simpler, cost-effective, and increasingly adopted for manure treatment (Zhao et al., 2024). Key factors influencing AF efficiency include carbon-to-nitrogen (C/N) ratios, feedstock particle size, and moisture content (Ji et al., 2022), with the optimal performance achieved at a C/N ratios of 25 and a turning frequency of twice per day (Chen et al., 2023). Agricultural wastes like spent mushroom compost (SMC)—a byproduct of mushroom cultivation with high nutrient and water-holding capacity—are widely used to adjust compost properties. For instance, adding 15% woody peat to pig manure reduces nitrogen loss by suppressing denitrifying bacteria and related functional genes (Xie et al., 2023; Wu et al., 2023).

Similarly, co-composting organic waste of different sizes, such as 5 cm corn straw (Ren et al., 2023) and 2 cm branch piles (Zhang D. et al., 2023; Jiao J. X. et al., 2023) with animal manure can reduce greenhouse gas emissions and accelerate the composting process. The co-addition of organic waste during composting has also been shown to inhibit and reduce the expression of antibiotic resistance genes (ARGs), affecting species diversity and ARG migration pathways (Zhou Y. W. et al., 2022). For example, coconut shell, bamboo (Awasthi et al., 2021), wine grape pomace (Zhang J. et al., 2023), and 5% humic acid (Shi et al., 2023) have been reported to enhance antibiotic removal from pig manure and inhibit the accumulation and spread of ARGs (Tong et al., 2022). SMC, a type of agricultural waste, with high water-holding capacity and nutrient content (Tao et al., 2022), had a production volume of 2.2×10^7 tonnes (dry weight) in China in 2020 (Guo et al., 2022). When mixed with chicken manure, it can shorten the high-temperature composting period by 2 days (Pan et al., 2023; Jia et al., 2022), promote humification, and immobilize heavy metals such as Cu, Zn, Cd, Cr, and Pb (Kong et al., 2022). This mixture also reduces emissions of ammonia (NH₃), hydrogen sulfide (H₂S), dimethyl sulfur, and dimethyl disulfide (Wei et al., 2022; Yan et al., 2020), recruits beneficial microbial communities, suppresses potential plant pathogens (Xu M. Y. et al., 2022; Wang L. et al., 2024), and significantly reduces the abundances of pathogenic fungi associated with rice blast disease (Zeng et al., 2023).

Microorganisms play an important role in the transformation of organic materials during composting, leading to significant changes in bacterial community composition (Wang Y. et al., 2024). Solid and semi-solid microbial agents, including lignocellulosic hemicellulose-degrading biological agents, thermophilic microbial agents, fungal agents, and antibiotic-degrading agents, contribute to organic matter degradation and nutrient enrichment. These microbial agents also enhance pollutant degradation, alter microbial communities, increase enzyme activity, promote fungal abundance, and immobilize heavy metals (Yin Y. N. et al., 2023; Wu et al., 2022). Additionally, they facilitate ARG removal (Chen X. J. et al., 2022; Li et al., 2022), enhance lignocellulose degradation efficiency (Bikram et al., 2021; Shangguan et al., 2022; Zhang Y. G. et al., 2023), improve methane production rates in AD systems (Bikram et al., 2020), and accelerate substance transformation (Bohrer et al., 2023). Thermophilic microbial agents have been shown to promote the decomposition of recalcitrant organic compounds in biogas residues and improve the seed germination index (Xu S. Y. et al., 2022). In this study, two thermophilic strains, *Bacillus flexus* FM and *B. cereus* KU, were screened and used as microbial agents to study their corresponding effects on promoting pig manure composting fermentation.

Recent research had addressing antibiotic residues in pig manure composting has predominantly examined the effects of non-biological and biological factors on ARGs, cadmium, human pathogenic bacteria (HPB), and other toxic substances in compost materials (Chen Z. Q. et al., 2022; Abdellah et al., 2023; Jiao J. X. et al., 2023). Under heat stress conditions, the abundances of ARGs and mobile genetic elements (MGEs) had been decreased in pig manure significantly (Sun et al., 2021; Sun et al., 2023; Zhu et al., 2023; Tang et al., 2023). The transmission and transfer of ARGs occurred through horizontal gene transfer (HGT) mechanism mediated by MGEs or through vertical gene transfer (VGT) mechanism mediated by host bacterial proliferation and functional gene enrichment (Luo et al., 2023). This study utilized thermophilic microbial agents to promote the co-composting of pig manure and SMC. The research systematically examined the dynamics of ARGs, HPB and the structural and compositional shifts in beneficial microbial communities during composting. The results provided critical theoretical and empirical support for tracking the fate of hazardous contaminants in livestock manure, while advancing the sustainable utilization of livestock and poultry manure and the development of eco-circular agriculture.

2 Materials and methods

2.1 Screening of thermophilic strains

A total of 62 culturable strains were isolated from pig manure at a farm in Tai'an city, Shandong province, China (Huang et al., 2014; Du et al., 2022). The primary selection criteria were the ability to survive at a high temperature of 60°C, along with the capacity to produce at least two of the following enzymes: cellulase (Subhojit et al., 2016; Warasirin et al., 2017), laccase (Aslam et al., 2012) and xylanase (Fatma and Filiz, 2023). Based on these criteria, *B. flexus* FM and *B. subtilis* KU were isolated. The fermentation broth of *B. flexus* FM was mixed with soybean meal in a 1:1 ratio to obtain the thermophilic microbial agents S1 (TM S1). Similarly, a composite thermophilic

microbial agent S2 (CTM S2) was prepared by mixing the fermentation broths of *B. flexus* and *B. subtilis* KU with soybean meal in a 1:1:2.

2.2 Composting experimental design and sample collection

Pig manure was collected from a breeding farm in Tai'an city, Shandong province, China. SMC and pig manure were purchased from Shandong Hengxin Biotechnology Co., Ltd., and Wenshi Pig Breeding Co., Ltd., both of which were located in Shandong province, China. The pig manure had a moisture content of 83.40%, a pH of 7.51, a total carbon content of 30.41%, and a total nitrogen content of 1.74%. The SMC had a moisture content of 62.5%, a pH of 6.39, a total carbon content of 38.57%, and a total nitrogen content of 2.50%.

Four experimental treatments were established: (A) pig manure and TM S1, (B) pig manure and SMC, (C) pig manure, SMC and TMS1, and (D) pig manure, SMC and CTMS2, each treatment was replicated three times. Physicochemical indicators, resistance gene abundance, microbial diversity, and cadmium content were measured on days 0, 1, 4, 7, and 11. Additionally, non-target metabolite indicators were assessed using LC-MS for each treatment group at 0, 4 and 11 days (the B0 sample data were the same as those of the C0 and D0 samples). Each test was performed in triplicate.

2.3 Determination of non-biological indicators

Stack and ambient temperatures were recorded three times daily and the average values were calculated. The total carbon (TC) and total nitrogen (TN) contents in the stack were analyzed using an elemental analyzer (Vario Macro Cube, Elementar, Germany). Phosphorus was determined via chromatography (Chen, 2015), while potassium content was measured using tetraphenylboron sodium mass method (Wang et al., 2016). The water content was assessed using the vacuum oven method (Yin Y. Y. et al., 2023), and the cadmium content was determined by atomic fluorescence photometry (Zhang et al., 2015). Each test was conducted also in triplicate.

2.4 DNA extraction and qPCR

DNA extraction: Genomic DNA was extracted from 100 mg of freeze-dried samples using the TIANAMP Soil DNA Kit (DP336) and eluted with low melting point solvent (DES). The quality and concentration of the extracted DNA were assessed using 1.5% (w/v) agarose gel electrophoresis and an enzyme plate instrument (BiotekELx808). High-throughput quantitative PCR (HT-qPCR) was performed using a StepOnePlus™ Real-time PCR system (Thermo Fisher Scientific) with a TB Green™ Premium Ex Taq™II (Tli RNaseH Plus) kit (Takara, Code No. RR820A). Each HT-qPCR assay was conducted in triplicate as described by Gong C. P. et al. (2024).

This study focused on six representative ARGs: (1) tetracycline resistance genes (*tetC*, *tetG*, *tetM*, *tetW* and *tetX*), (2) sulfonamide resistance genes (*sul1* and *sul2*), (3) macrolide resistance genes (*ermF*, *ErmQ* and *ermX*), (4) quinolone resistance genes (*gryA* and *qnrA*), (5) β -lactam resistance genes (*bla*-VTM and *bla*-CTX), and (6)

aminoglycoside genes [*aac* (6')-Ib-cr]. Additionally, three MGEs, named *Tn916/1545*, *int11*, and *ISC1*, were analyzed along with 16S rRNA for simultaneous quantification (Zhou et al., 2021).

2.5 Metagenomic sequencing

Total genomic DNA was extracted from the compost samples using the E.Z.N.A.® Soil DNA Kit (Omega Biotek, Norcross, GA, U.S.) according to the manufacturer's instructions. The concentration and purity of the extracted DNA were measured using a TBS-380 fluorometer and NanoDrop2000 spectrophotometer. The quality of the extracted DNA was verified by electrophoresis on a 1% agarose gel.

Metagenomic data were assembled using MEGAHIT (Li et al., 2015; <https://github.com/voutcn/megahit>, version 1.1.2), which used succinct de Bruijn graphs. Contigs with a length ≥ 300 bp were selected as the final assembly result, and these contigs were subsequently used for gene prediction and annotation.

Open reading frames (ORFs) from each assembled contig were predicted using MetaGene (Noguchi et al., 2006; <http://metagene.cb.k.u-tokyo.ac.jp/>). Predicted ORFs with a length of ≥ 100 bp were retrieved and translated into amino acid sequences using the NCBI translation table.¹ Antibiotic resistance annotation was performed using Diamond (Buchfink et al., 2015; <http://www.diamondsearch.org/index.php>, version 0.8.35) against the ARDB database² or the CARD database³ with an e-value cutoff of $1e^{-5}$.

2.6 Non-target metabolite determination

A 50 mg solid sample was added to a 2 ml centrifuge tube along with a 6 mm diameter grinding bead, and 400 μ l of extraction solution (methanol:water = 4:1, v/v) containing 0.02 mg/mL internal standard (L-2-chlorophenylalanine) was added. The samples were then ground using a Wonbio -96°C frozen tissue grinder (Shanghai Wanbo Biotechnology Co., Ltd) for 6 min at -10°C and 50 Hz followed by low-temperature ultrasonic extraction for 30 min at 5°C and 40 kHz. Afterward, the samples were incubated at -20°C for 30 min and centrifuged for 15 min at 4°C and 13,000 rpm, after which the resulting supernatant was transferred to an injection vial for LC-MS analysis.

2.7 Statistical analysis

SPSS 25.0 (IBM, Armonk, NY, USA) was used to compare differences and determine correlations among the experimental results. The histograms were generated using Origin 2024b software. Principal coordinate analysis and Procrustes analysis were performed using the Meiji Biocloud platform (Shanghai, China). Heatmap and cluster analysis were conducted with SciPy (Python) Version 1.0.0, whereas differentially abundant metabolite analyses were performed

1 <http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi?chapter=tgencodes#SG1>

2 <http://ardb.cbcb.umd.edu/>

3 <https://card.mcmaster.ca/home>

using Ropls (R packages) Version 1.6.2. The correlation coefficient between Spearman and Gephi in the network analysis was determined to be 0.9.2 ($p < 0.01$). AMOS 26.0 software was used for structural equation modeling to elucidate causal relationships between variables.

3 Results and discussion

3.1 Target ARG and MGE fate during composting process

Normalizing the copy number of ARGs to the relative abundances evaluation method of bacterial abundance changes was a more effective way for evaluating ARGs dynamics (Czekalski et al., 2015). In this study, the relative abundances indicators of 15 ARGs and three MGEs had shown that tetracycline, macrolide, sulfonamide, and quinolone-resistant ARGs dominated across all composting samples (Figure 1). Co-composting of livestock manure with plant-derived organic waste effectively reduced the relative abundances of most tetracycline ARGs (*tetM*, *tetW* and *tetX*), sulfonamide ARGs (*sul1* and *sul2*) and macrolide ARGs (*ermF*).

In this study, the addition of thermophilic microbial agents had significantly reduced the abundances of most ARGs in the windrows. Compared with traditional composting methods (Wu et al., 2023; Jiao J. X. et al., 2023), the thermophilic microbial agents extended the high-temperature phase by over 30% and shortened the compost maturation time by 50%. Moreover, compared to B0 sample, the relative abundances of the seven types of ARGs of C11 and D11 samples (*tetM*, *tetW*, *ermF*, *ermQ*, *tetC*, *gryA* and *tetX*) decreased significantly ($p < 0.05$), with values ranging from 0.45–2.98lg and 0.48–3.73lg,

respectively. Moreover, compared with those of the C11 sample (11th-day data of C sample), two types of D11 sample (11th-day data of D sample) with ARGs (*tetM* and *tetW*) presented decrease in relative abundances of 0.82lg and 0.74lg, respectively ($p < 0.05$), this indicated that CTMS2 had unique advantages over TMS1 in the degradation of some ARGs. However, compared with those in B0 sample, the two ARGs (*blaR1* and *QnrA2*) in the C11 and D11 samples increased by 0.02–0.63lg and 0.78–0.83lg, respectively, which indicated that removing some heat-resistant ARGs during composting were difficult.

In this study, *IntI1* and *Tn916/1545* emerged as the main constituents contributing to the abundances of MGEs during the composting process (Figure 1C). Compared to those of the B0 sample, the relative abundances of two types of MGEs (*ISCR1* and *Tn916/1545*) had been decreased between 0.08lg and 1.41lg in C11 and D11 samples, highlighted that the efficacy of thermophilic microbial agents in promoting the degradation of MGEs in SMC and pig manure co-composting, which was consistent with trend changes in seven ARGs (*tetM*, *tetW*, *ermF*, *ermQ*, *tetC*, *gryA* and *tetX*). Moreover, compared with that in the C11 sample, the MGE (*intI1*) in the D11 sample had been decreased by 0.28lg ($p < 0.05$), indicated that CTMS2 might have a greater advantage than TMS1 in inhibiting the rebound of MGE abundance.

3.2 Analysis of the bacterial community composition structure

The bacterial community was the main driving factor for changes in the composition and abundance of ARGs (Zhang Y. G. et al., 2023), and the differences in bacterial community structure in this study

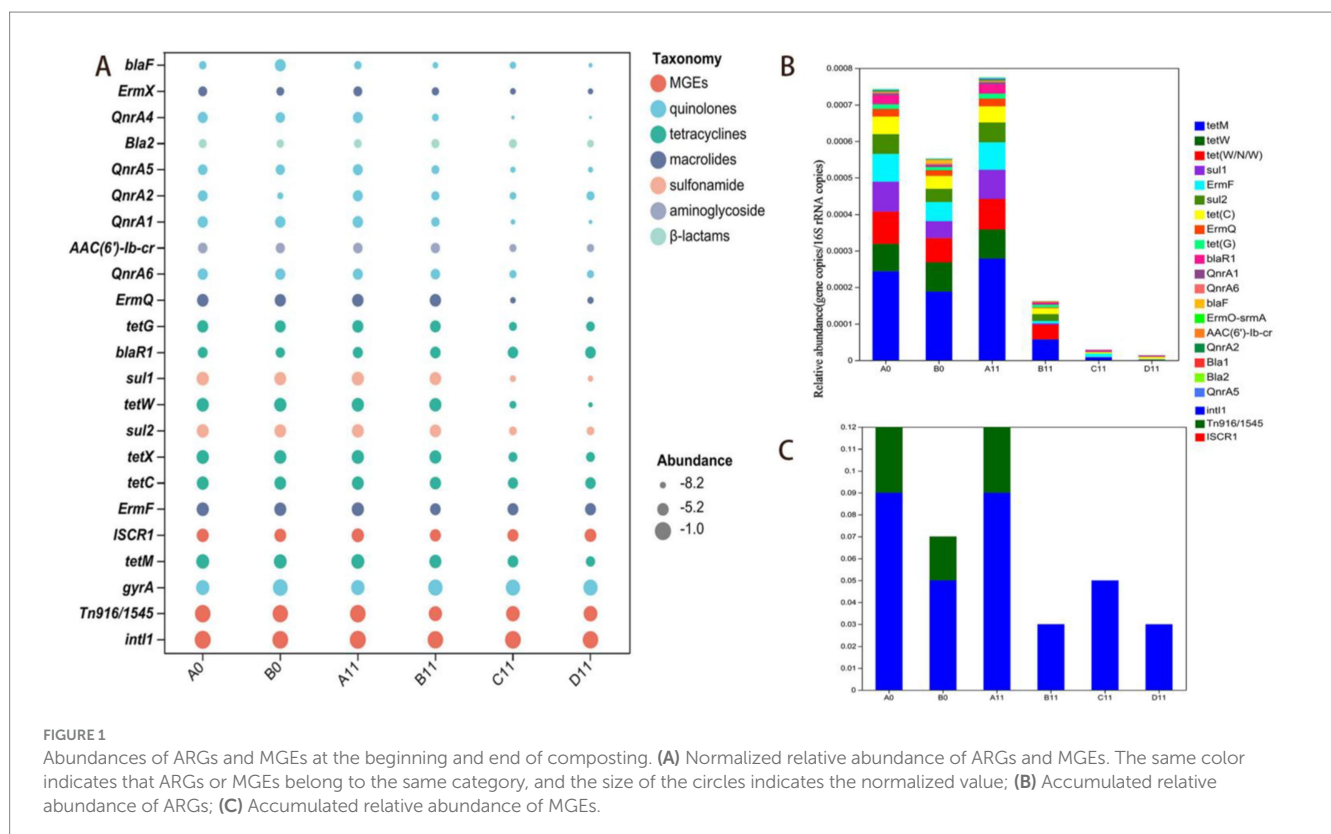


FIGURE 1

Abundances of ARGs and MGEs at the beginning and end of composting. (A) Normalized relative abundance of ARGs and MGEs. The same color indicates that ARGs or MGEs belong to the same category, and the size of the circles indicates the normalized value; (B) Accumulated relative abundance of ARGs; (C) Accumulated relative abundance of MGEs.

were using principal coordinate analysis to compared and analyzed, as detailed in the [Supplementary materials](#). The results had shown that different samples had a significant effect on the type and abundance of microbial communities in the compost ([Supplementary material S6](#)). The interpretation rate of the first and second main components for the results was 85.00%. In addition to the A0 and A11 samples, different samples were clustered together at each stage of compost, indicated that the composition of the bacterial community had an important influence on the progress of pig manure compost ([Jie et al., 2023](#)).

Figure 2 illustrates the 30 bacterial dominant phyla, classes and genera with the greatest relative abundances during pig manure composting. Among them, the horizontal distribution of the dominant

bacterial phyla in the initial stage of pig manure composting was mainly Pseudomonadota, Bacillota, Bacteroidota and Actinomycetota, accounting for more than 62.7% of the total bacterial count in each sample. Moreover, in the high-temperature thermophilic phase of pig manure composting, compared with that in the B0 sample, the abundance of Pseudomonas increased between 1.43lg and 1.37lg in the C11 and D11 samples, whereas the abundance of Bacillota increased between 0.93lg and 0.96lg. Conversely, the abundance of Bacteroidota decreased between 1.15lg and 1.24lg, whereas the abundance of Actinomycetota increased between 1.36lg and 0.89lg in the same sample. Pseudomonadota and Bacillota maintained dominance across all the samples, followed by Actinomycetota. Compared with the B11 sample, the C11 and D11 samples resulted in

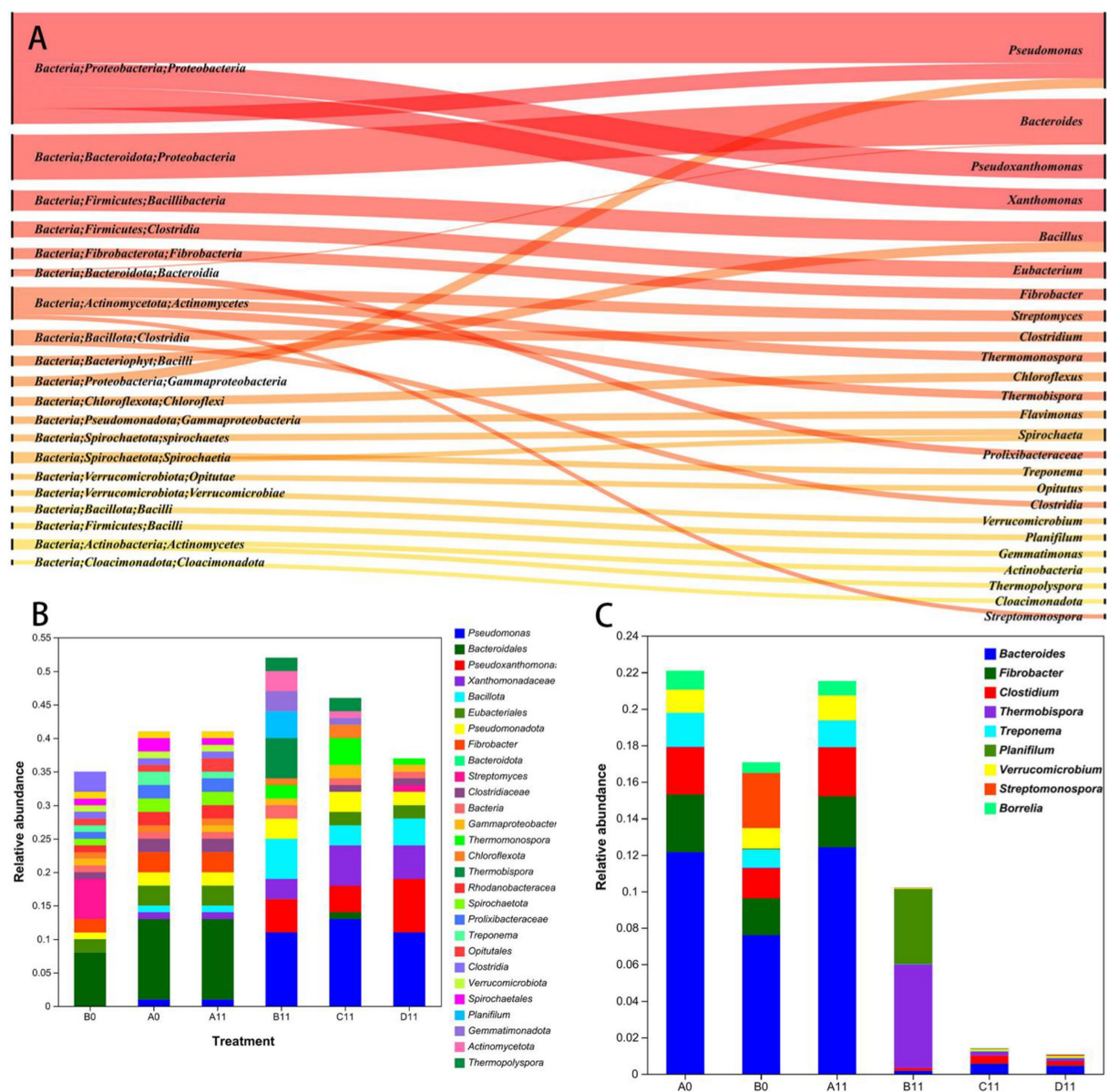


FIGURE 2

(A) Sanji diagram showing the composition of bacterial communities at the phylum, class, order, and genus levels. (B) Stacked bar chart showing the relative abundance changes in the top 30 genera of bacteria. (C) Stacked bar chart of the abundance of human pathogenic bacteria at the beginning and end of composting.

decreases of 0.60lg and 1.07lg, in Actinomycetota. Therefore, a high level of HGT mechanism might still have occurred in sample B and indirectly led to an increase in the abundances of some ARGs.

The 30 most abundant bacteria in the C11 sample were *Pseudomonas* (14.15%), *Xanthomonas* (6.48%) and *Pseudoxanthomonas* (4.87%), whereas those in the sample D11 included *Pseudomonas* (12.23%), *Pseudoxanthomonas* (8.87%) and *Xanthomonas* (5.8%). Compared with those in the B0 sample, *Bacteroides* (9.11%), *Bacillus* (0.47%) and *Actinomyces* (0.03%), significantly changed the abundances of *Bacteroides* (0.59 and 0.67%), *Bacillus* (3.65 and 3.97%), and *Actinomycetes* (0.76 and 0.25%), were observed in the C11 and D11 samples, respectively (see Figure 2B). The increase in the horizontal abundance of *Bacillus* species in the windrow might be due to the increase in the abundance of high-temperature resistant *Bacillus* (see the Supplementary material).

In this study, HPB were found to belong to 9 genera, included *Bacteroides*, *Fibrobacter*, *Clostridium*, and *Treponema*, which collectively represented 90.91% of the total HPB (Figure 2C). Compared with those in the B0 sample, the HPB abundances in the C11 and D11 samples were 0.82 lg and 0.90 lg lower, respectively ($p < 0.05$), and *Bacteroides* was the pathogenic microorganism with the greatest reduction in abundance, followed by *Treponema*. Most HPB were potential hosts of ARGs and MGEs (Imtiaz et al., 2022), therefore, HPB species and abundances were commonly used as potential measures of ARGs and MGEs. However, the B11 sample, which did not receive the addition of thermophilic microbial agents, exhibited a significant increase in the abundance of *Treponema* (see Supplementary materials). This suggests that some HPB may have undergone incomplete degradation or partial enrichment. These findings further emphasize the necessity of incorporating thermophilic microbial agents in the aerobic composting process of pig manure.

3.3 Bacterial community composition and structure lead to changes in ARG abundance

Figure 3A showed the correlation between ARGs and MGEs, one-third of the ARGs (*tetX*, *tetW*, *tetG*, *tetC*, *suI1* and *suI2*) were positively correlated with MGEs ($p < 0.05$). However, ARGs which mediated by MGEs could be easily transferred and spreaded after composting, indicated that the inhibitory effect of the HGT mechanism might play a crucial role in preventing their spread. Similarly, the remaining ARGs (*ErmF*, *ErmQ*, *ErmX*, *blaR1*, *QnrA1*, *QnrA6*, *bla-F*, *QnrA2*, *QnrA5*, *Qnra4* and *bla-VIM*) were not significantly correlated with MGEs, and were difficult to degrade during the composting process. Considered that the overall trend of abundance changes during the composting process, its degradation transfer might be mediated by other mechanisms.

Procrustes analysis had revealed that the abundances of ARGs and MGEs were significantly correlated with the bacterial community composition at the genus level ($M^2 = 0.253$, $p < 0.05$), which was consistent with the Mantel Test results ($r = 0.6062$, $p = 0.01$). Many easily removable ARGs (*tetM*, *tetW*, *tetX*, *tetG*, *tetC*, *suI1*, *suI2*, *ermF* and *ermQ*) had been found positively correlated with genus-level changes in multiple bacterial groups ($p < 0.01$). However, persistent removable ARGs correlated with the levels of only a few bacterial genera, such as *Treponema*, *Borrelia* and *Bacteroides*.

3.4 Relationships between bacterial microflora and environmental factors

In this study, redundancy analysis (RDA) was used to determine the relative contributions of environmental factors, MGEs and ARGs to the bacterial community, and the explanatory rates of the sample bacterial community were 93.32, 0.70 and 0.27%, respectively (see Supplementary materials). Among these factors, the bacterial community composition had been found to be the main environmental factor. Among all abiotic environmental factors, temperature (99.97%) and TC (99.97%) were accounted for the greatest proportion of bacterial community changes, followed by TK (75.56%). In addition, this study revealed that the changes in ARGs during composting were similar to the changes in bacterial communities and environmental factors as determined by principal coordinate analysis.

This study used structural equation modeling to explore in more detail the potential causal relationships between multiple factors and ARGs (Figure 4A). The analysis had revealed that compost characteristics had the greatest impact on compost quality ($r = 0.413$, $p < 0.01$) (Figure 4B), provided important theoretical insights for guide compost in production practices. Temperature also had a negative effect on ARGs, with a relatively high degree of influence ($\lambda = -0.834$, $p < 0.01$) (Figure 4B), which was consistent with the results of the redundancy analysis. Therefore, temperature was the main driver of changes in the ARG pedigree, the distribution of ARGs during composting might be more closely related to the biological mechanisms of microorganisms.

The organic carbon content in the windrow was significantly negatively correlated with both ARGs ($\lambda = -0.759$, $p < 0.01$) and MGEs ($\lambda = -0.538$, $p < 0.01$), mainly because of its different effects on microbial communities (Figure 4C). Organic carbon was suspected to be a key influencing factor of the host during composting ($r = 0.899$, $p < 0.01$), whereas organic carbon was significantly correlated with the quality of the compost ($r = 0.303$, $p < 0.01$). The addition of SMC that stem from agricultural waste reduced the abundances of *Bacteroides*, *Treponema* and *Borrelia*, which were carriers of persistent ARGs (*suI1*, *tetG* and *bla-VIM*) in the windrow. Moreover, compared with sample A, the addition of SMC had significantly increased the organic carbon content in the samples C11 (17.83%) and D11 (19.71%), which was also a necessary and sufficient condition for normal compost.

Among the non-biological factors, compost characteristics (TN, and C/N ratio) were found strongly correlated with ARGs ($\lambda = -0.624$, $p < 0.01$) and MGEs ($\lambda = -0.326$, $p < 0.05$) (Figure 4B), which had indirectly affected ARGs through the concentration of organic carbon. According to the RDA results, the C/N ratio had the greatest impact on ARGs. The relatively high nitrogen content and low C/N ratio in the windrow might provide more available nitrogen sources for microbial growth, increasing the TN content in the mature stage of compost (Supplementary materialsS1). Consistent with the results of this study, the difference in nitrogen content is an important environmental factor related to the relative abundance changes of ARGs and MGEs, thermophilic microbial agents were necessary for aerobic compost of pig manure, and the addition of SMC alone could not effectively reduce the abundances of ARGs in the compost.

Interestingly, on the basis of Mantel Analysis, three indicators of microbial nutritional growth (TC content, TN content and the C/N ratio) were found to be common key factors in the metabolism of

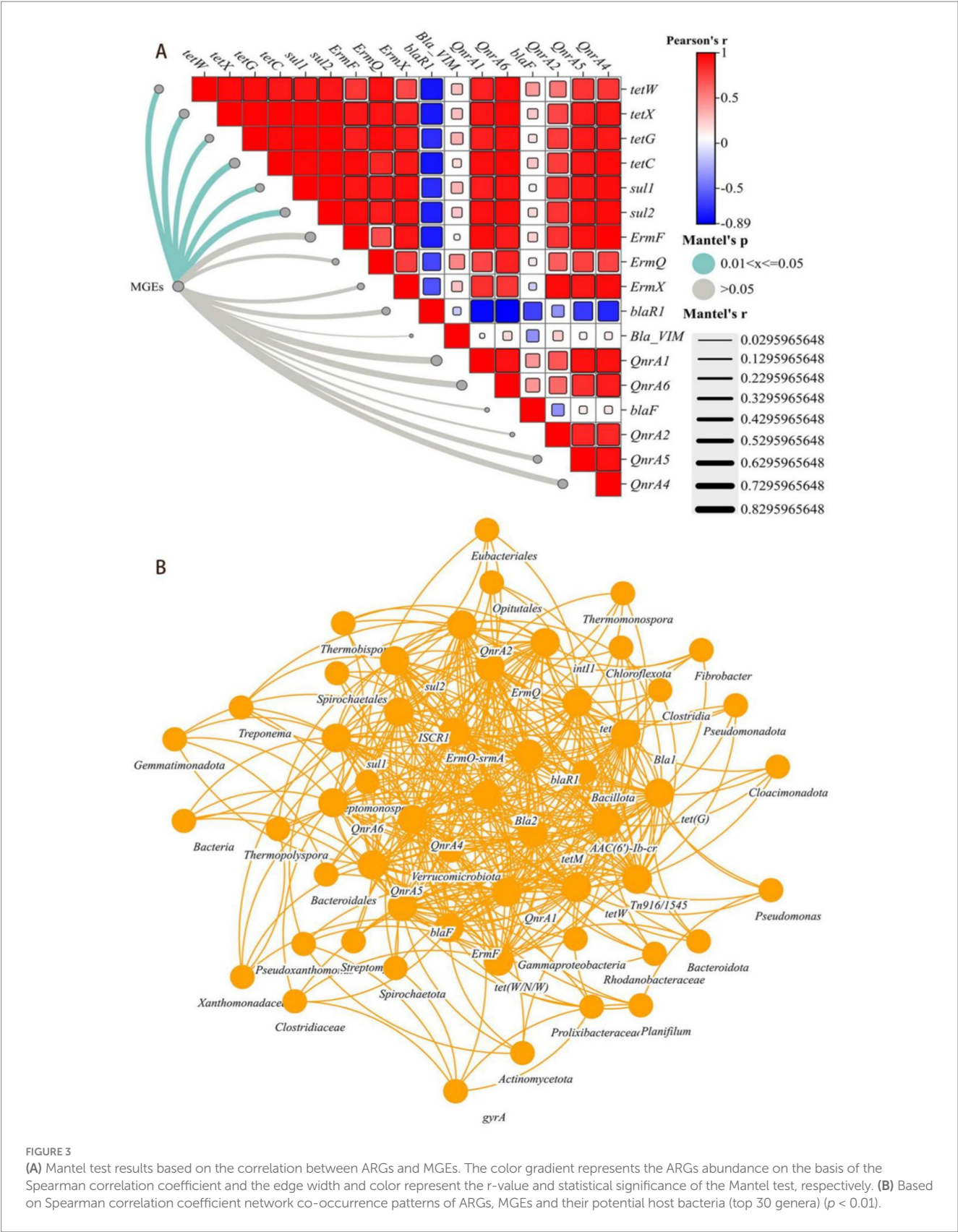


FIGURE 3 (A) Mantel test results based on the correlation between ARGs and MGEs. The color gradient represents the ARGs abundance on the basis of the Spearman correlation coefficient and the edge width and color represent the r -value and statistical significance of the Mantel test, respectively. (B) Based on Spearman correlation coefficient network co-occurrence patterns of ARGs, MGEs and their potential host bacteria (top 30 genera) ($p < 0.01$).

easily removable ARGs and persistent ARGs. The abundances of both types of ARGs was significantly correlated with temperature ($p < 0.05$) (Figure 4C). Network analysis had revealed that easily removable ARGs could be carried by many potential host bacterial communities and MGEs, whereas persistent ARGs could be carried by only a few possible host bacteria (Figure 3B).

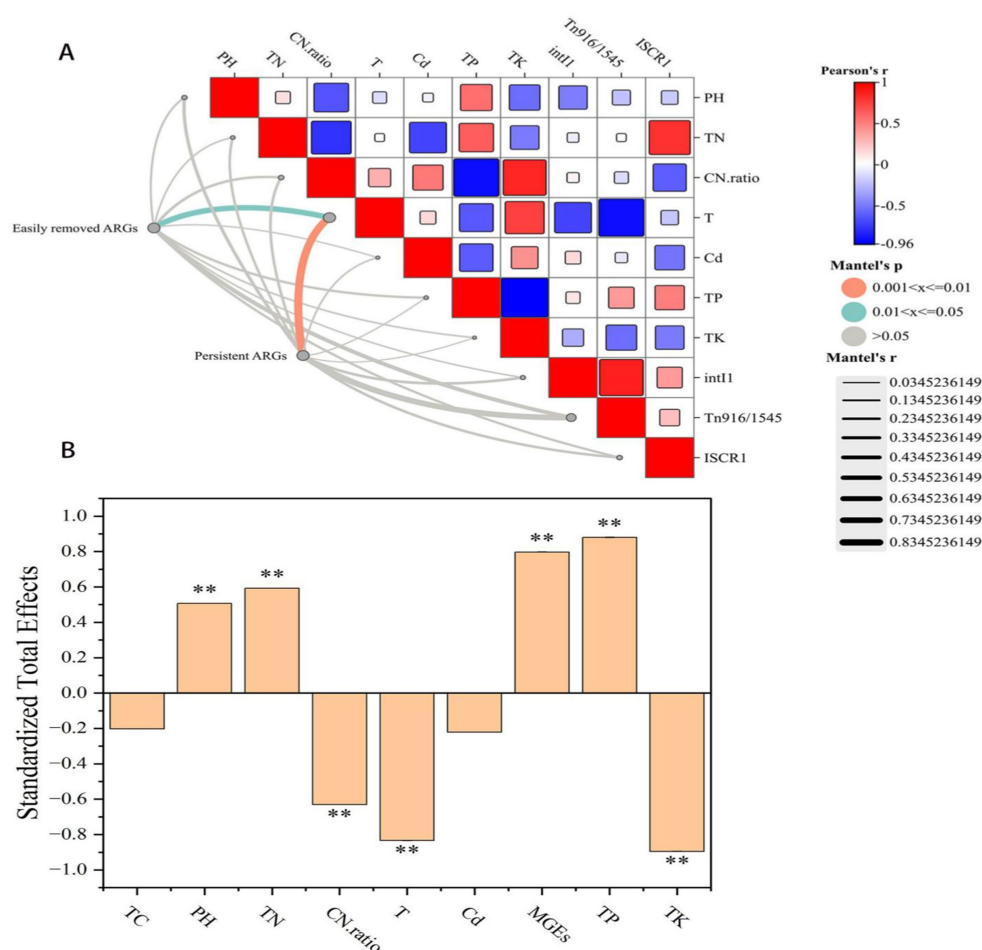


FIGURE 4

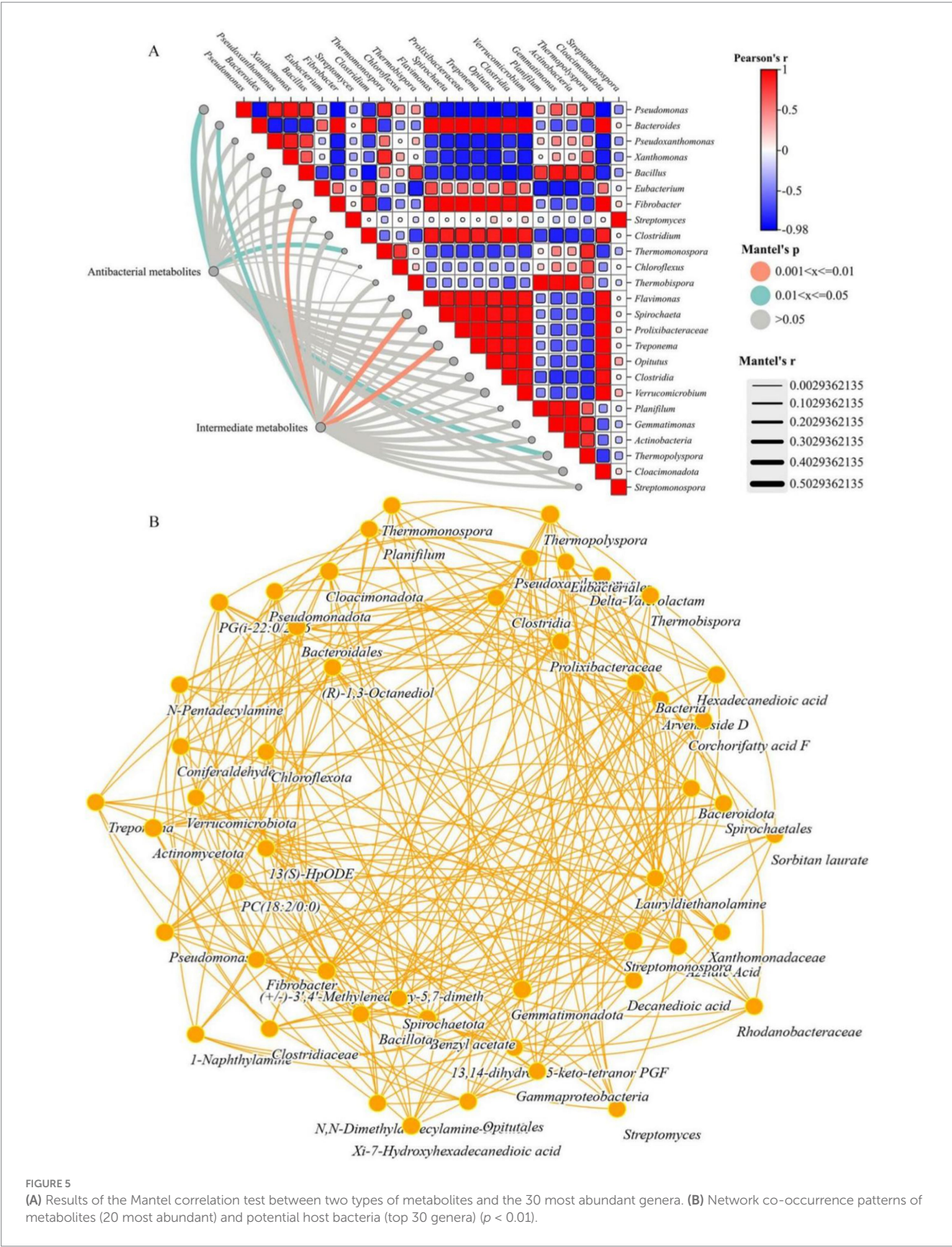
(A) Mantel correlation test results between two types of ARGs and multiple indicators (non-biological factors and MGEs). (B) Standardized total impact of selected variables of ARGs.

3.5 Bacterial microflora and metabolite analysis

Figure 5A showed the correlation between the 30 most abundant bacterial genera and the 20 most abundant metabolites in the compost. Approximately one-third of the antibacterial metabolites such as corchorifatty acid F, coniferaldehyde, 13,14-dihydro-15-keto-tetranor PGF, sorbitan laurate, and PG [i-22:0/22:6 (5Z, 7Z, 10Z, 13Z, 16Z, 1, and azelaic acid)], were associated with the induction of plant resistance metabolism (Masanobu et al., 2020; Leonard et al., 2020). These genera presented significant positive correlations with the abundances of *Pseudomonas*, *Thermomonospora*, and *Thermopolyspora* ($p < 0.05$). This enrichment also indicated that the resistance of the compost material to the growth and infection of pathogenic microorganisms increased (Supplementary materials). Research had shown that high doses of macrolide drugs inhibited the mineralization of natural 17 β -estradiol in animal manure and urine, thereby increasing the retention of 17 β -estradiol and its metabolites in free and non-extractable residue forms (He et al., 2019), and the key molecule azelaic acid in the plant biological stress response could mobilize *Arabidopsis thaliana* immunity in a concentration dependent manner (Francesca et al., 2019; Nagy et al., 2017; Finni et al., 2014). In-depth

research and exploration of the relationships between metabolic products in compost and changes in bacterial flora and related functional gene abundance had important practical significance for controlling ARGs transfer.

Microorganisms were the main influencing factors of changes in the composition and content of antimicrobial and intermediate metabolites, so studying and determining their relationships was of great practical significance. Procrustes analysis had shown that antibacterial and intermediate metabolites were significantly correlated with the bacterial community composition at the genus level ($M^2 = 0.253$, $p < 0.01$) which was consistent with the Mantel test results ($r = 0.5029$, $p = 0.01$). Therefore, the changes in bacterial community structure and abundance led to changes in the metabolites detected during the composting process. Furthermore, the co-occurrence associations between specific antibacterial metabolites, bacterial genera, and intermediate metabolites were explored to identify their potential hosts during the composting process. According to the network diagram, there were 6 antibacterial metabolites, 3 intermediate metabolites and 30 bacterial genera (Figure 3B). Differences in the distribution of potential hosts in rhizosphere soils were related to the abundance and composition of metabolites (Guo et al., 2020). Many easily removable antibacterial metals (corchorifatty acid F, coniferaldehyde, 13,14-dihydro-15-keto-tetranol



PGF, sorbitan laurate, and PG [i-22:0/22:6 (5Z, 7Z, 10Z, 13Z, 16Z, 1, and azelaic acid)] were significantly positively correlated with the abundances of multiple bacterial genera ($p < 0.05$) (Supplementary materials),

indirectly indicating that the composition and abundance of bacteria in the windrow and the inhibition of the HGT mechanism might play crucial roles in preventing its spread. Similarly, intermediate metabolites

(1-naphthylamine, benzyl acetate and xi-7-hydroxyhexadecanedioic acid) were products of antibiotics, benzene rings and pesticide metabolic intermediates. These genera were significantly positively correlated with the abundances of *Bacteroides*, *Fibrobacter*, *Treponema* and *Spirochaeta* genera ($p < 0.01$) (Supplementary material), and it was observed that they were difficult to metabolize and degrade during composting, resulting in incomplete or partial enrichment of HPB degradation.

3.6 Prediction of bacterial community function

Functional enrichment analysis was a computational method used to analyze the degree of functional pattern enrichment in gene sets or genomic data (Mahantesha et al., 2013). This information could help researchers understand the biological significance of gene sets, thereby revealing the regulatory mechanisms of biological processes, metabolic pathways, cellular components, etc., under specific conditions and providing valuable guidance for further experimental design and research (Zakrzewski et al., 2013).

Compared with sample A, the mixed composting of thermophilic microbial agents and SMC with pig manure reduced the metabolic abundance of multidrug resistance efflux pumps, the Embden-Meyerhof pathway and beta-lactam resistance pathway in mature compost piles (Figure 6A). This reduction might promote the metabolism of multiple beta-lactam antibiotics and inhibit the VGT mechanism and the HGT mechanism of ARGs from compost products to the soil environment. The transmission of ARGs was usually regulated by key regulatory genes in certain bacterial pathways.

In previous studies, 10 specific genes involved in glycolysis, multidrug-resistance efflux pumps and β -lactam resistance regulation were identified (Lin et al., 2021). As shown in Figure 6A, compared with the A11 sample, the addition of SMC was more effective than the addition of multiple drug resistant efflux pumps. Furthermore, the enrichment of pathways related to the metabolism of multiple antibiotics in β -lactam (K03585 and K02171) was relatively low, indicating that thermophilic microbial agents might inhibit the expression of related genes. Moreover, compared with those in the sample A, the enrichment of ARG-related metabolic pathways had been decreased to varying degrees by the additive of TMS1 (sample C) and CTMS2 (sample D), furthermore, the CTMS2 showed the greatest decrease in the total abundances of ARG-related genes. Therefore, in the context of co-composting SMC and thermophilic microbial agents, the combination of CTMS2 and SMC had a greater inhibitory effect on the expression of ARG-related genes, led to a deeper decrease in the abundances of ARGs (Fu et al., 2024).

Human disease-related KEGG pathway 3 significantly changed during the composting process (Figure 6B). The enrichment of clusters associated with human disease, such as β -lactam resistance, cationic antimicrobial peptide resistance, vancomycin resistance, legionellosis, alcoholic liver disease, and central carbon metabolism in cancer, tended to decrease, with the D sample showing the greatest decrease during composting. Notably, although samples C and D presented a reduced abundance of clusters related to infectious diseases, the residual levels in sample C were significantly greater than those in sample D. Therefore, on basis of the results had shown that in the HPB abundances stacking bar chart (Figure 2C), it could be find that the co-composting process of pig manure utilizing a combination

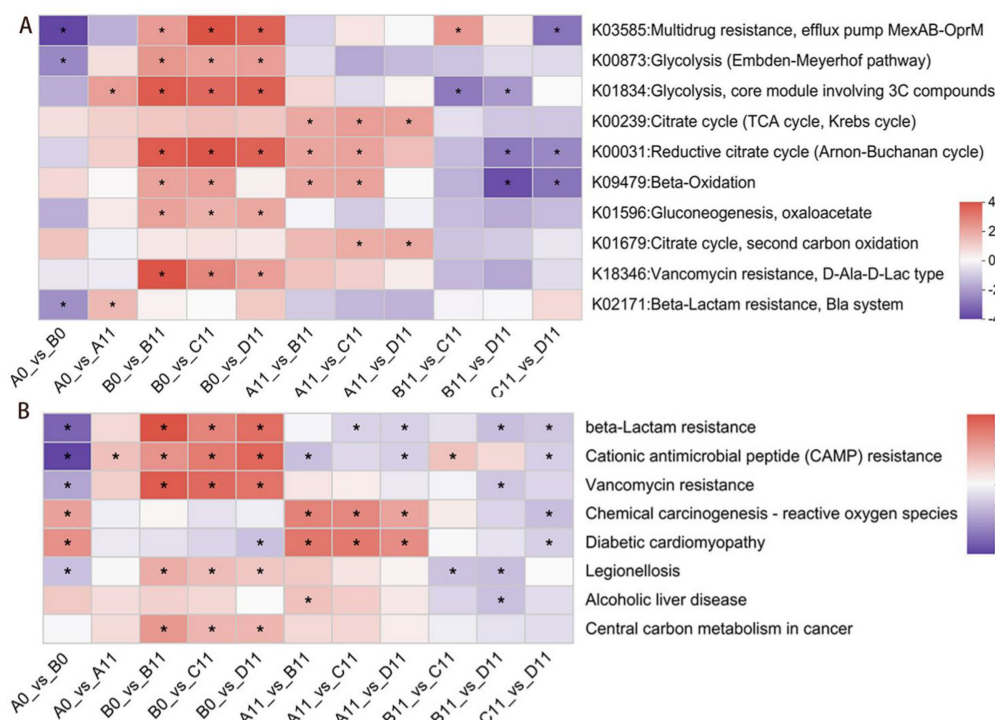


FIGURE 6

Enrichment plots of human disease-associated metabolic functions and relative abundance of genes under different treatment conditions during composting. A-KEGG enrichment map of the metabolic functions associated with ARGs. B-Human disease KEGG pathway 3 level cluster.

of CTMS2 and SMC was to yield compost products with less harmless compost products than that of sample C.

4 Conclusion

The findings of this study had indicated that the co-addition of organic waste such as SMC, which was the basis for the successful compost of pig manure during aerobic composting, and had advantages in reducing the abundances of ARGs, MGEs, and HPB. The co-composting of thermophilic microbial agents and SMC could limit the proliferation of ARG-related hosts and decrease the abundances of ARG-related metabolic pathways, regulatory genes, and human disease clusters, potentially leading to ARG attenuation. The VGT mechanism might play a key role in shaping the progressive degradation of ARGs, especially on persistent ARGs. Compared with TMS1, the CTMS2 had significantly better effects and potential in the compost process, which was convenient for storage, transportation, and easy to use, promoted rapid heating of the compost, had a long duration of high temperature, and was conducive to killing pathogenic bacteria in the compost. In addition, when site requirements were not strict, organic fertilizer could be composted separately by farmers and returned to the field or sold on a large scale, and the organic fertilizer produced had good quality and great application and promotion value.

5 Discussion

The misuse of antibiotics in livestock farming and the unregulated discharge of livestock and poultry manure (Li S. Y. et al., 2023), had caused severe environmental pollution while promoting the enrichment and transmission of ARGs to plants and animals, posing significant risks to human health (Zhang Y. et al., 2025; Wu et al., 2025). However, aerobic composting had been demonstrated to substantially reduce antibiotic residues and ARG abundance in livestock manure (Li et al., 2024; Zhao et al., 2024). Research had found that co-composting of livestock and poultry manure with plant-derived organic waste could effectively reduce the relative abundances of most tetracycline ARGs (*tetM*, *tetW*, *tetX*), sulfonamide ARGs (*sul1* and *sul2*), and macrolide ARGs (*ermF*) (Jiao J. X. et al., 2023; Fu et al., 2024). The abundance of most ARGs were significantly associated with MGEs, and reducing the abundance and suppressing HGT spread of MGEs could mitigate the spread and diffusion of ARGs (Liu et al., 2023). Bacteroidota, Proteobacteria and Actinomycetota were potential hosts associated with the enrichment and transfer of ARGs and MGEs, and were the main carriers for the transmission of ARGs and MGEs (Wang et al., 2022; He et al., 2023; Wu et al., 2024). The solid bacterial agents (TMS1 and CTMS2) inhibited the enrichment and transfer of ARGs and MGEs by altering the composition and abundance of bacteria in the windrow.

Thermophilic composting, as an innovative composting technology involving exogenous thermophilic microbial agents (Zhang Y. G. et al., 2023), induces sustained high temperatures that promoted both the degradation of extracellular ARGs (eARGs) (Jiao J. X. et al., 2023) and the release of intracellular ARGs (iARGs). Certain thermophilic microorganisms, such as *Novibacillus thermophiles*, *Bacillus thermolactis* and *Ammonibacillus agariperforans*, could accelerate cellulolytic and xylanolytic decomposition. This process

enhances microbial diversity in compost materials while facilitating the recruitment of beneficial microbes and suppressing pathogen proliferation (Youn et al., 2020; Wang et al., 2023), thereby optimizing both the composting efficiency and final product quality of livestock manure (Bang et al., 2024; Wang L. et al., 2024). Notably, Zhang et al. (2022) identified significant positive correlations between specific ARGs in swine manure (including *tetC*, *tetG*, *tetX*, *sul1* and *qnrS*) and the abundances of pathogenic microorganisms. During the compost maturation phase, the abundances of pathogenic microorganisms from *Bacteroides* and *Verrucomicrobia* genera progressively decreased, consequently reducing pathogen-mediated VGT of ARGs and significantly enhancing the quality of the organic fertilizer product. While elevated temperatures generally accelerate bacterial community succession, in contrast to conventional microbial inoculants, the TMS1 and CTMS2 that employed in this study exhibited remarkable thermophilic properties, maintaining viability even when compost temperatures exceeded 60°C. Compared to previous composting research, these novel thermophilic microbial agents demonstrated rapid temperature elevation in composting materials, extended duration of the thermophilic phase, and achievement of higher peak temperatures. These superior thermal characteristics collectively contributed to enhanced nutrient preservation, more efficient degradation of hazardous substances, and ultimately, the production of higher quality organic fertilizers.

Research had shown that, ARGs could be distributed through aerobic composting (Ren et al., 2023), anaerobic composting (Chen et al., 2023), soil mediums (Wen et al., 2024) and rivers mediums (Patel et al., 2024). ARGs had a broad range of potential hosts and they could regulate the abundances changes of ARGs by mediating the abundances of related factors such as (*tetW*, *sul1* and *Tn916/1545*) (Xiu et al., 2021; Zhou et al., 2021; Zhang D. et al., 2023). Moreover, microorganisms were the primary carriers of ARGs and MGEs, therefore, the changes in bacterial community composition and abundance led to changes in the relative abundances of ARGs and MGEs during the composting process (Wang Y. et al., 2024; Nnorom et al., 2025).

In this study, the SMC was co-composting with pig manure, the abundances of some bacterial genera which associated with organic compound degradation increased during the maturation stage, which might have led to a decrease in the abundances of potential host bacteria carrying ARGs and MGEs, at the same time, the proliferation of microbial communities associated with organic matter degradation might limit the growth of some potential hosts of ARGs (Wei et al., 2022; He et al., 2023; Wang et al., 2023). Temperature was considered the most important abiotic factor in aerobic composting processes, and changes in temperature could greatly alter the abundance and content of microbial communities and other abiotic factors (He et al., 2023; Zhao et al., 2024). Although some researchers believed that HGT is one of the important factors for the transfer of ARGs during composting, HGT might not be the main driver of ARGs distribution (Liu et al., 2023; Zhou P. Z. et al., 2022). In other words, this meant that the distribution of ARGs during composting might be more closely related to the biological mechanisms of microorganisms.

In fact, the compost properties were often considered nutrients for bacteria, as most of them were essential for microbial growth (Magid et al., 2006; Grosso et al., 2016), which was potentially related to the VGT mechanism of ARGs. Some compost characteristics could be adjusted by adding organic carbon to the compost stage, which

could affect the structural composition of bacterial communities during the composting process (Gong X. et al., 2024; Hu et al., 2023), which could affect changes in ARGs also (Ya et al., 2023; Shan et al., 2024). Guo et al. (2019) reported that differences in nitrogen content were important environmental factors which related to changes in the relative abundances of ARGs and MGEs. The TC content, TN content and C/N ratio were important environmental factors for the survival and reproduction of microorganisms in livestock and poultry manure, which might indirectly affect the abundances of ARGs and MGEs by affecting changes in the host bacterial community of ARGs (Sun et al., 2020; Kai et al., 2023; Jiao M. N. et al., 2023). Therefore, these findings suggested that the VGT mechanism might play a key role mechanism in the transfer and transformation of ARGs during the composting process in this study, especially for persistent ARGs, whereas easily removable ARGs were also regulated by the HGT mechanism.

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

HD: Writing – original draft. CL: Data curation, Writing – original draft. ML: Resources, Writing – original draft. JD: Methodology, Writing – original draft. YL: Resources, Writing – original draft. HL: Resources, Writing – original draft. XD: Writing – review & editing, Funding acquisition.

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Conflict of interest

YL was employed by Shandong Huayang Pesticide Chemical Group Co., Ltd. HL was employed by Jinan Tianding Ecological Environment Co., Ltd.

The remaining 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.2025.1575397/full#supplementary-material>

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Application and evaluation of a rapid detection method based on two-dimensional PCR technology for hypervirulent *Klebsiella pneumoniae*

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Objectives: Hypervirulent *K. pneumoniae* (hvKp) is an emerging pathogen that is more virulent than classical *K. pneumoniae* (cKp). This study aimed to develop an economical, high-throughput, and accurate two-dimensional polymerase chain reaction (2D-PCR) assay for the rapid detection of hvKp.

Materials and methods: Recombinant plasmids containing the *iucA*, *peg-344*, *rmpA2*, and *rmpA* virulence genes were constructed and used for assessing the sensitivity and specificity of the 2D-PCR. Clinical samples ($n = 105$) were collected and evaluated the performance of the 2D-PCR to comparison with conventional PCR methods.

Results: The minimum detection limit of the 2D-PCR assay for *iucA*, *peg-344*, *rmpA2*, and *rmpA* were 10^3 , 10^2 , 10^3 , and 10^3 copies/ μ L, respectively. Additionally, the concordance rates between the 2D-PCR and conventional PCR for detecting *iucA*, *peg-344*, *rmpA2*, and *rmpA* were all over 95%. The analysis revealed a sensitivity of 100.0% and a specificity of 96.2% when compared to conventional PCR.

Conclusion: A 2D-PCR-based multiplex method for virulence genes of hvKp was successfully developed, demonstrating its outstanding features of high specificity, high sensitivity, and high throughput capability. This method could be used for the rapid diagnosis of infectious diseases caused by hvKp in clinical settings.

KEYWORDS

2D-PCR, hypervirulent *Klebsiella pneumoniae*, virulence genes, diagnosis, biomarkers

Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is one of the most common gram-negative opportunistic pathogens that are responsible for a variety of infectious diseases, including urinary tract infections, bacteremia, pneumonia, and liver abscesses (Wang et al., 2020; Stojowska-Swędryńska et al., 2021). At present, *K. pneumoniae* can be divided into the following two main types: classical *K. pneumoniae* (cKp) and hypervirulent *K. pneumoniae* (hvKp) (Zhang et al., 2023). cKp strains are frequently correlated with nosocomial infections or infections in a long-term care settings, indicating that a degree of immunocompromised state is essential for inducing disease by cKp strains (Walker and Miller, 2020). hvKp differs from cKp in its clinical and phenotypic characteristics. Patients infected with hvKp, who are

typically younger and immunocompetent, present with more severe disease compared to those with cKp and originate from community settings (Choby et al., 2019). While the association of hvKp with community-acquired disease is more common (Pomakova et al., 2011), there has been an increasing number of reports suggesting the participation of hvKp isolates in healthcare-associated disease, particularly in pulmonary, ventilator, and healthcare-associated bacteremia (Zhu et al., 2021). An outbreak of carbapenem-resistant hvKp (CR-hvKp) in a hospital in China resulted in the death of five patients infected with ventilator-associated pneumonia (Gu et al., 2018). The increased transmission of hvKp strains in hospitals may raise the overall burden of this pathogen.

Currently, there is no consensus on the definition of hvKp, the accurate diagnosis and rapid identification of hvKp strains are essential for appropriate infection control measures. Traditional detection technologies, such as string tests, the mouse killing assay, and the *Galleria mellonella* infection model, cannot meet the fast and specific needs of clinical detection (Mai et al., 2023). Polymerase Chain Reaction (PCR) is an important detection technique in molecular biology, significantly enhancing the efficiency of pathogen detection (Schrader et al., 2012). Molecular diagnostic techniques, including traditional PCR, quantitative real-time polymerase chain reaction (qPCR), and other PCR assays, have been developed to identify hvKp and have shown satisfactory results. However, these methods are limited by low detection throughput and can cause nonspecific amplification (Schrader et al., 2012; Janik-Karpinska et al., 2022; Mai et al., 2023). A defining characteristic of hvKp strains is the presence of large and highly similar virulence plasmids pK2044 (224,152 bp) and pLVPK (219,385 bp), which harbor several virulence-encoding genes that confer the hypervirulent phenotype to hvKp (Chen et al., 2004; Wu et al., 2009). Therefore, the virulence genes on plasmids can be used as specific hypervirulence markers for the clinical detection of hvKp, such as *rmpA* and *rmpA2* (capsular polysaccharide (CPS)-regulating genes), *peg-344* (metabolism genes), and *iucA* (siderophore genes).

Two-dimensional polymerase chain reaction (2D-PCR) is a multiplex PCR detection method that identifies multiple target genes in the same PCR tube using base-quenched probe technology and fluorescence melting temperature (Mao et al., 2018; Zhan et al., 2020). In 2D-PCR systems, a synthesized pre-tag sequence located at the 5' end of a specific primer is utilized, and some bases in the pre-tag sequence are changed, allowing one probe to recognize multiple tags with various melting temperature (T_m) values. Therefore, this study aimed to establish a rapid molecular diagnostic method to accurately identify hvKp by detecting the virulence genes *iucA*, *peg-344*, *rmpA2*, and *rmpA*.

Materials and methods

A total of 105 nonrepetitive *K. pneumoniae* strains were collected from the clinical laboratory of Fudan University Affiliated Jinshan Hospital from January to June 2024, these isolates were identified using AUTO MS 1000 AUTOBIO (Autobio Diagnostics, Zhengzhou, China). *K. pneumoniae* strain RJF293 (Xu et al., 2021) and *K. pneumoniae* strain HS11286 (Liu et al., 2012) were used as positive control and negative control, respectively. The nucleotide sequences

of *rmpA* (633 bp), *rmpA2* (637 bp), *iucA* (1725 bp), and *peg-344* (903 bp) from the virulence plasmid pRJF293 of *K. pneumoniae* RJF293 were cloned into the pMD20-T vector (TaKaRa, Japan). The positive recombinant plasmid was subsequently transferred into *Escherichia coli* DH5 α (Tiangen Biotech, Beijing, China) and confirmed by sequencing. The gene sequences of *iucA*, *peg-344*, *rmpA2*, and *rmpA* were downloaded from the GenBank database. Subsequently, the primers were designed using Primer Premier 5 software. The sequences of the primers and probes are shown in Table 1.

DNA extraction

The genomic DNA of *K. pneumoniae* was obtained using the Tianamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Plasmid DNA of *E. coli* DH5 α was obtained by using the TianampTIANprep Mini Plasmid Kit (Tiangen Biotech, Beijing, China). Finally, approximately 80 μ L of the DNA solution was prepared to serve as a template for the DNA reaction. The extracted DNA concentration was measured with a NanoDrop™ 2000 spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE, USA) and stored at -20°C for subsequent analysis.

Traditional PCR assays for biomarkers

The virulence-associated factors *iucA*, *rmpA*, *rmpA2*, and *peg-344* were investigated using the traditional PCR method. For each reaction, 12.5 μ L Premix Taq (TaKaRa, Japan), 0.5 μ L of each primer (10 μ M), 1 μ L genomic DNA (approximately 100 ng/ μ L) and 10.5 μ L of deionized water (HPLC grade). PCR was performed under the following cycling conditions: 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min, these reactions were conducted in a thermal cycler (T-100, BioRad, USA). Each run included a positive control (*K. pneumoniae* RJF293), a negative control (*K. pneumoniae* HS11286) and a blank control (deionized water). The PCR products were analyzed by electrophoresis on a 0.8% agarose gel and visualized with UV light.

2D-PCR

The 2D-PCR assays were run on the QuantStudio 3 (Thermo Fisher, USA), with each reaction containing a total volume of 25 μ L. The FAM channel was employed for all experiments. The reaction mixture contained 2.5 μ L $10 \times$ PCR buffer (Mg^{2+} free), 1.5 μ L MgCl_2 (25 mM), 0.7 μ L dNTP Mixture (2.5 mM each), 0.5 μ L Taq Hot Start Polymerase (TaKaRa, Japan), 0.1 μ L each of labeled forward primer, 0.6 μ L reverse primer, 0.4 μ L FAM probe, 2 μ L extracted DNA template, the volume was adjusted to 25 μ L with nuclease-free water. The 2D-PCR cycling parameters were as follows: an initial heating step at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s. Following the amplification, a melting curve analysis was performed, starting at 30°C for 4 min and gradually increasing by 0.1 $^{\circ}\text{C}/\text{s}$ until reaching 80°C . The fluorescence signal was acquired every second during this step. Finally, a cooling step was carried out at 40°C for 30 s.

TABLE 1 Primers and universal probe used in this study.

Primer	Sequences (5' → 3')	Amplicons size (bp)	References
2D-PCR			
peg-344-F2	<u>CCATTACCTTGCTTATACACTTCCACAGCGAAAGAATAACCCAG</u>	160	Zhu et al. (2022) This study
Peg-344-R2	GGAAAGGACAGAAAGCCAG		
p-rmpA-F2	<u>CCATTACCAACCTTATACACTTCCACTTCAGGGAAATGGGAGGGTA</u>	252	Zhu et al. (2022) This study
p-rmpA-R2	CATTGCAGCACTGCTTGTTC		
p-rmpA2-F2	<u>CCATTACCTACCTTATACACTTCCACGTTAACTGGACTACCTCTGGTTT</u>	104	Wu et al. (2022) This study
p-rmpA2-R2	ATCCGGCTATCAACCAATACTC		
iucA-F2	<u>CCATTACCTAGCTTATACATTCCACTGTTACGGCTGAAGCGAT</u>	101	Zhu et al. (2022) This study
iucA-R2	CACGGTAGATAAGCCCGACC		
intI-P	FAM-CCATTACCAACCTTATACACTTCCAC-P		Zhu et al. (2022)
Traditional PCR			
Peg344-F	CTTGAAACTATCCCTCCAGTC	508	Luo et al. (2023)
Peg344-R	CCAGCGAAAGAATAACCCC		
rmpA-F	TTAACTGGACTACCTCTGTTCAT	332	Luo et al. (2023)
rmpA-R	AATCCTGCTGTCAACCAATACT		
rmpA2-F	ATCCTCAAGGTTGTGATTATGAC	430	Luo et al. (2023)
rmpA2-R	CCTGGAGAGTAAGCATTGTAGAAT		
iucA-F	CTCTCCCGCTCGCTATACT	239	Luo et al. (2023)
iucA-R	GCATTCCACGCT TCACTTCT		

The primer tags were underlined, and point mutations within these tags were highlighted in bold.

Results

2D-PCR specificity for identifying hvKp

The specificity of the 2D-PCR test to detect *iucA*, *peg-344*, *rmpA2*, and *rmpA* was evaluated using positive plasmid samples with concentrations of approximately 10^5 copies/ μ L. First, a single positive plasmid was used as template to confirm the specificity of the primers. The results demonstrate that the melting curves are clear, with the T_m of *iucA*, *peg-344*, *rmpA2*, and *rmpA* being approximately 43°C, 52°C, 58°C, and 64°C, respectively (Figure 1). The T_m difference between the four target genes is at least 5°C, which makes them easy to distinguish. To assess the capability of 2D-PCR in simultaneously detecting multiple target genes, a mixture of positive plasmids containing four virulence genes was utilized as a DNA template. The results showed that 2D-PCR successfully detected all four genes simultaneously. As shown in Figure 2, the 2D-PCR technique effectively detected four virulence genes simultaneously, with clearly distinguishable peaks for each target. Thus, this assay was highly specific for the detection of *iucA*, *peg-344*, *rmpA2*, and *rmpA* from hvKp.

2D-PCR sensitivity for identifying hvKp

To assess the minimum detection limit of the 2D-PCR system, four virulence plasmid samples were serially diluted across a gradient ranging from 10^7 – 10^1 copies/ μ L. As shown in Figure 3, all corresponding targets were successfully detected in samples with a concentration of 10^3 copies/ μ L. However, only the *peg-344* target

gene was detected in samples at 10^2 copies/ μ L, and no corresponding targets were identified in samples below this concentration. The lowest detectable concentrations of this method for the *iucA*, *peg-344*, *rmpA2*, and *rmpA* genes were 10^3 , 10^2 , 10^3 , and 10^3 copies/ μ L, respectively. Meanwhile, the signal for each target gradually weakened as the template concentration decreased.

Testing of clinical samples

All 105 samples confirmed strains of *K. pneumoniae* were tested using the proposed 2D-PCR system (Figure 4). In 105 samples, 2D-PCR detected 55 samples of hvKp positive (*iucA*, *peg-344*, *rmpA2*, and *rmpA*) and 50 samples of other types that were negative (Figure 5). The positive rate of *iucA*, *peg-344*, *rmpA2*, and *rmpA* were 63.8, 75.2, 63.8, and 73.3%, respectively. There are differences in the ability of 2D-PCR and conventional PCR to detect the four virulence genes, for the detection of *iucA* and *peg-344*, the concordance between both methods was 99.1%. Among these, two samples tested positive in 2D-PCR and negative in conventional PCR. For the detection of *rmpA2*, the concordance rate is 97.1%, with three samples showing positive results in 2D-PCR but negative in conventional PCR. The results for *rmpA* detection were completely consistent between the two methods. All samples that tested positive by 2D-PCR also tested positive in conventional PCR, while all inconsistent results were positive in 2D-PCR and negative in conventional PCR (Table 2). Compared with traditional PCR, the specificity of 2D-PCR is 96.2%, and the sensitivity is 100.0%.

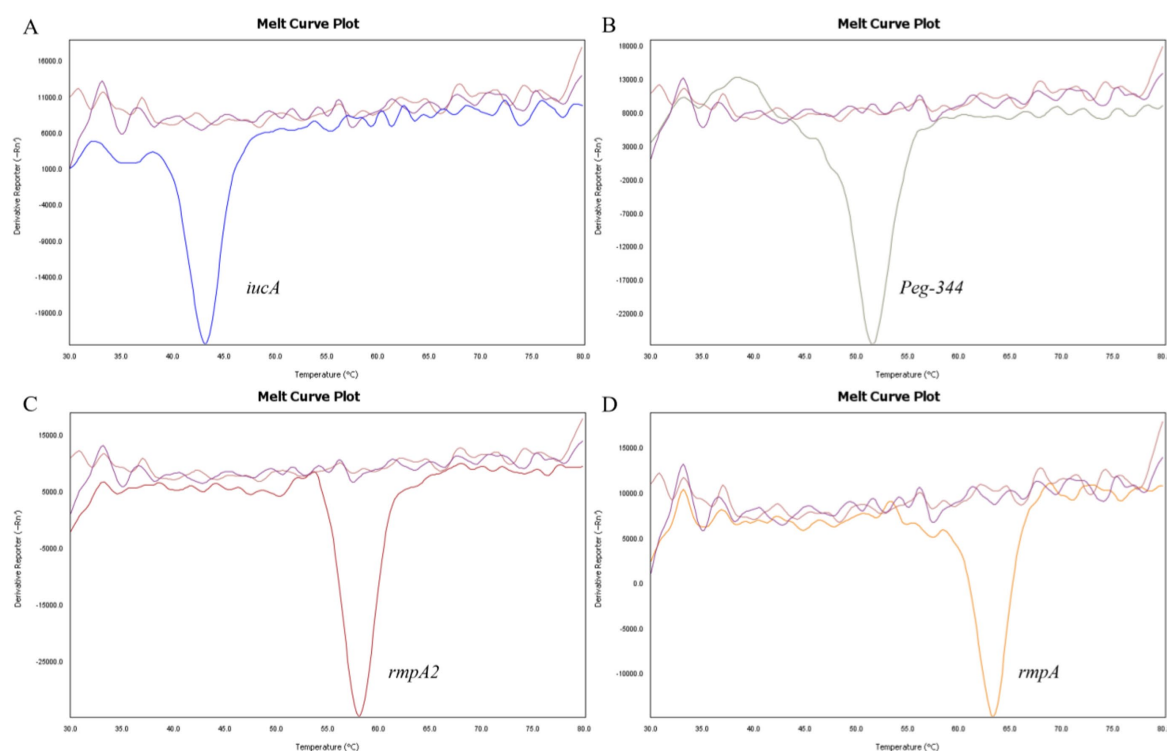


FIGURE 1

Single gene detection by 2D-PCR. (A) *iucA*, (B) *peg-344*, (C) *rmpA2*, and (D) *rmpA*. The approximate T_m values corresponding to *iucA*, *peg-344*, *rmpA2*, and *rmpA* are 43°C, 52°C, 58°C, and 64°C, respectively. The curves without dissolution peaks represent the negative control and the blank control, respectively.

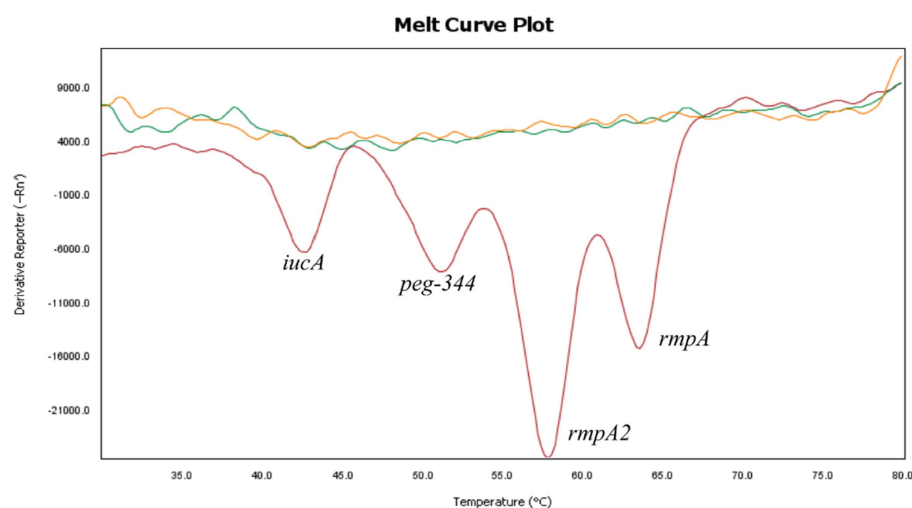


FIGURE 2

The presence of four melting valleys in the mixture of four gene plasmids confirmed the simultaneous detection of all four target genes. The curves without dissolution peaks represent the negative control and the blank control, respectively.

Discussion

HvKp is undergoing spread globally as a notorious clinical pathogen causing multiple severe infections. In the past, hvKp has been perceived as having a hypermucoviscosity phenotype, exhibiting susceptibility to antimicrobial agents, and a propensity to induce

invasive infections among healthy individuals in the community (Han et al., 2024). However, partly due to antibiotic abuse, multidrug-resistant hvKp, particularly CR-hvKp, has garnered significant attention in recent years. CR-hvKp, which is associated with high morbidity and mortality rates, poses a significant challenge to infection control and clinical treatment (Pu et al., 2023). HvKp with

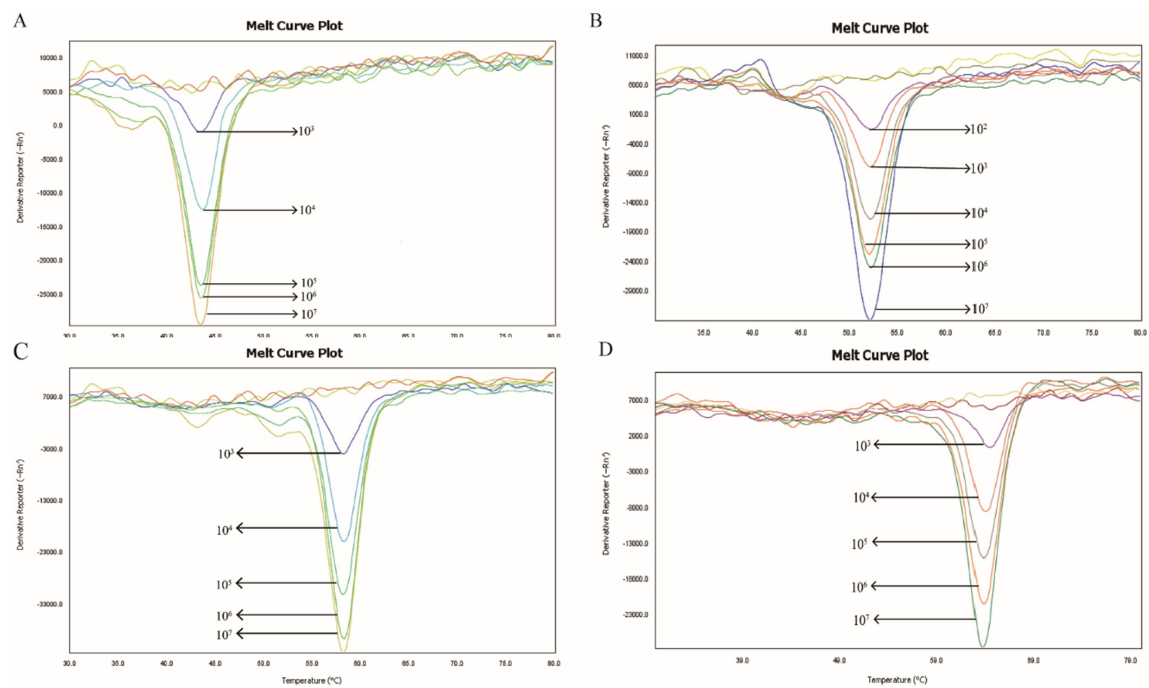


FIGURE 3
Sensitivity test of 2D-PCR. The minimum detectable concentration of (A) *iucA*, (B) *peg-344*, (C) *rmpA2* and (D) *rmpA* were 10^3 , 10^2 , 10^3 , and 10^3 copies/ μ L, respectively. The curves without dissolution peaks represent the negative control and the blank control, respectively.

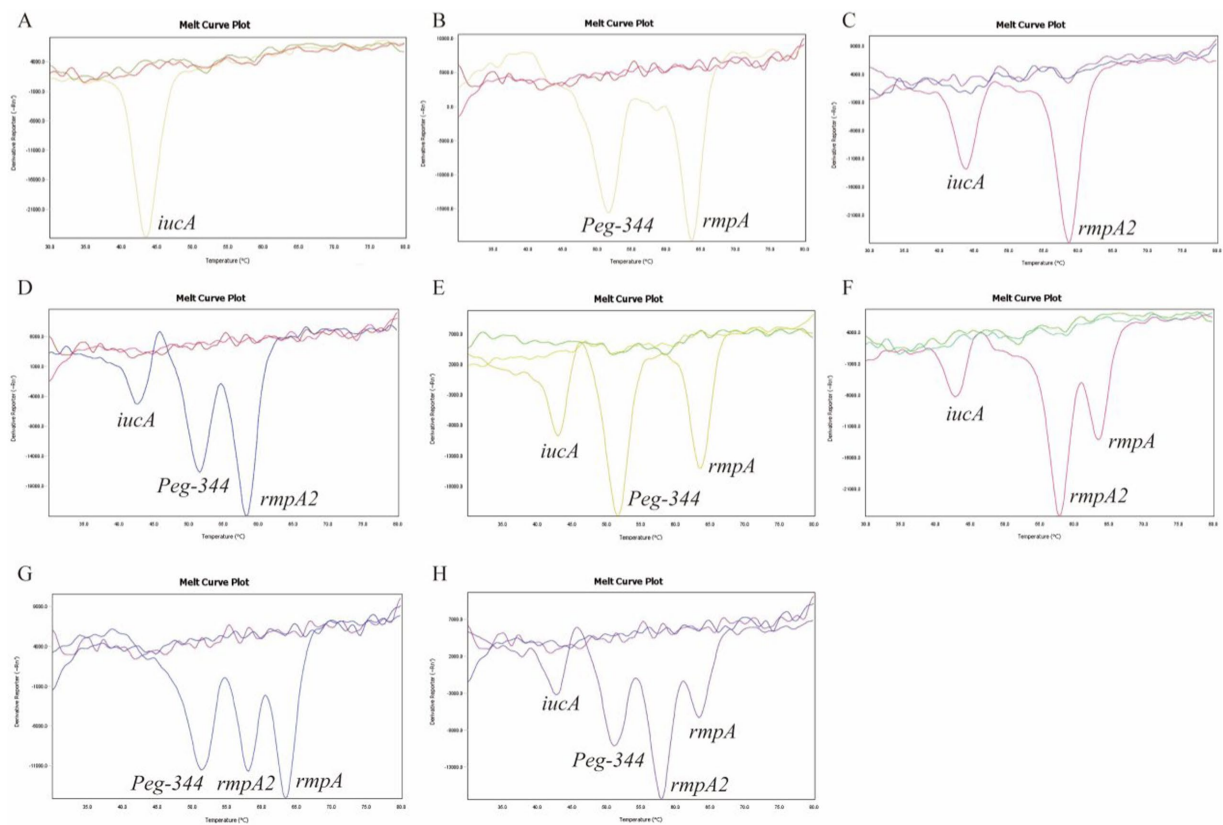


FIGURE 4
Clinical samples were examined to identify hvKp strains using 2D-PCR. The curves without dissolution peaks represent the negative control and the blank control, respectively.

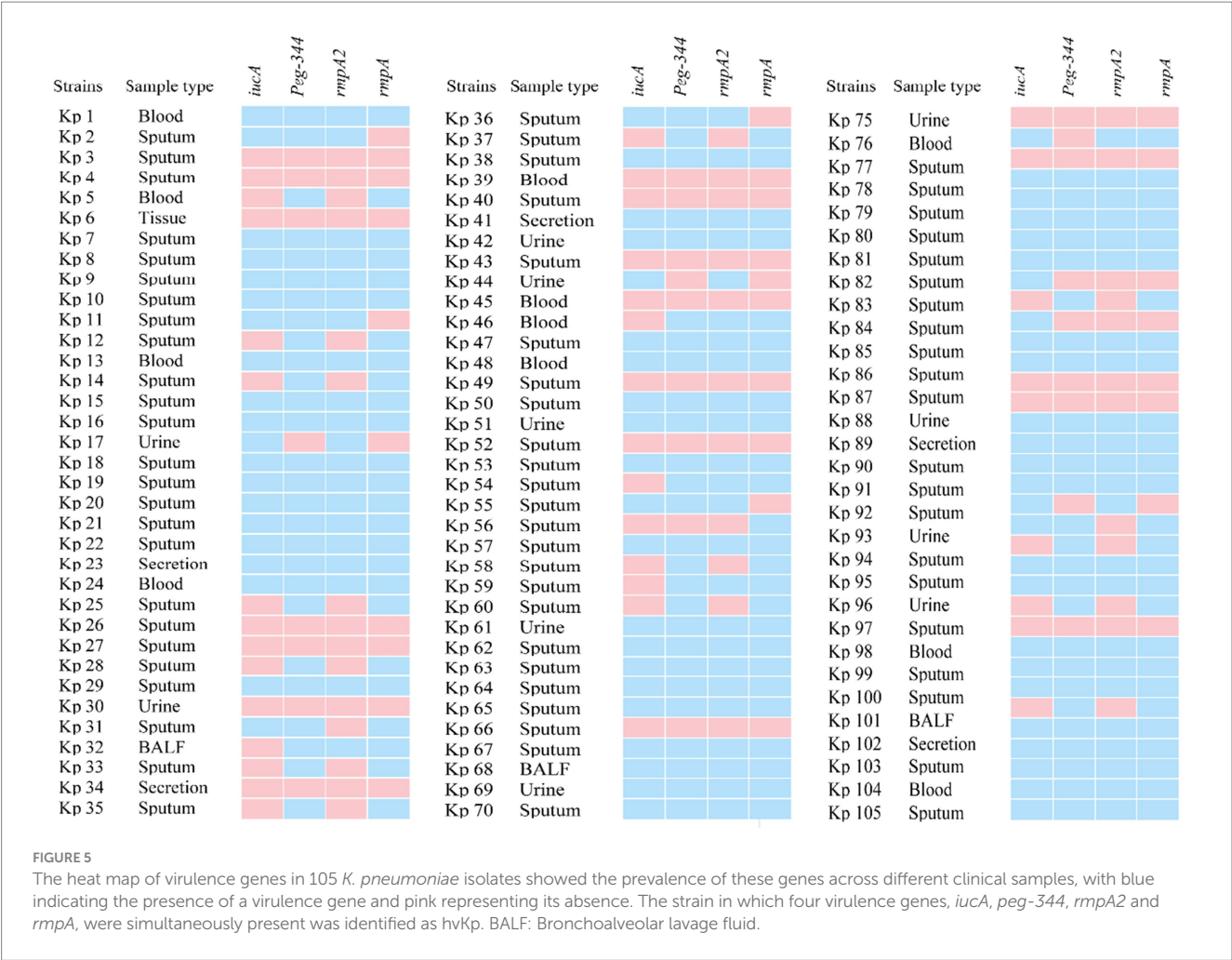


TABLE 2 Comparison of 2D-PCR and conventional PCR results.

Virulence genes	2D-PCR	Conventional PCR		Total samples	Concordance rate (%)
		Positive samples	Negative samples		
<i>iucA</i>	Positive samples	66	1	67	99.1
	Negative samples	0	38	38	
<i>peg-344</i>	Positive samples	78	1	79	99.1
	Negative samples	0	26	26	
<i>rmpA2</i>	Positive samples	64	3	67	97.1
	Negative samples	0	38	38	
<i>rmpA</i>	Positive samples	77	0	77	100.0
	Negative samples	0	28	28	

high virulence and epidemic potential is threatening human health. Improving the identification of hvKp and achieving early and accurate detection is crucial to support subsequent effective treatment among patients.

A common feature of hvKp is the combined expression of multiple virulence factors that serve as reliable biomarkers for accurately differentiating hvKp from cKp. Previous studies have established a PCR detection method for hvKp based on the combination of *peg-344*, *iroB*,

iucA, *rmpA*, and *rmpA2*, along with siderophore production (SP) exceeding 30 µg/mL, with accuracy greater than 95% (Russo et al., 2018). Cai et al. developed a multiplex q-PCR assay targeting *iroB*, *iucA*, *rmpA*, and *rmpA2* as molecular biomarkers for the rapid detection of hvKp. This assay demonstrated 100% sensitivity and 98% specificity in clinical validation studies (Cai et al., 2025). In this study, a 2D-PCR analysis of four virulence genes was conducted on 105 clinical isolates. The positive rates of the hvKP virulence genes were detected as *peg-344*,

rmpA, *iucA*, and *rmpA2* in descending order, and the detection rate of four virulence genes were more than 60%. *Peg-344* is widely distributed on the virulence plasmid of hvKp strains, and appears to be hvKp-specific (Bulger et al., 2017). Liao et al. (2020) established a LAMP detection method for hvKp based on *peg-344* as a target molecular biomarker, with an accuracy of 97%, a sensitivity of 99% and a specificity of 96%. *rmpA* and *rmpA2* are regulatory genes for polysaccharide expression in the capsule of hvKp, which decrease capsule production and virulence of strains if missing (Matono et al., 2022). Yan et al. (2023) effectively developed a recombinase-aided amplification (RAA) detection method targeting *peg-344* and *rmpA*, enabling the rapid identification of hvKp in clinical samples with both 100% sensitivity and specificity. The *iucA* genes that are responsible for producing aerobactin and salmochelin also demonstrate specificity in hvKp (Mydy et al., 2020). These biomarkers have the potential to be used as a rapid diagnostic test for differentiating hvKp from cKp. Therefore, the four virulence genes *iucA*, *peg-344*, *rmpA2*, and *rmpA* in this study can be used as accurate molecular markers for hvKP (Table 3).

Traditional PCR and multiplex PCR are often used to detect virulence genes (Compain et al., 2014; Albasha et al., 2020). The traditional PCR method has significant disadvantages in clinical diagnosis, such as long processing time, the need for electrophoresis after amplification to identify the results, and the limitation that a specific primer pair can only detect one gene type (Pan et al., 2025). Multiplex PCR has some limitations, such as false-positive results, a long processing cycle, complex workflows, and low efficiency (Dessajan and Timsit, 2024). Therefore, researchers have made significant efforts in recent years to improve and develop molecular methods for hvKp identification, such as RAA and LAMP. Compared with existing hvKp detection methods, 2D-PCR overcomes the inherent limitations of multiplex PCR by introducing Tm as the second dimension, which strictly relies on proportional relationships among fluorescence channels, probes, and detectable targets. A high-throughput closed-tube detection system has been established, significantly improving the detection throughput of single-tube reactions and reducing false positives caused by nonspecific amplification (Wang et al., 2025). Its probe design strategy allows a single probe to recognize multiple targets, greatly reducing the number of probes required. Compared with conventional probes, 2D-PCR probes do not require quenchers, significantly reducing detection costs. The experimental process is simplified, eliminating the need for subsequent operations such as electrophoresis, making it suitable for large-scale clinical screening.

2D-PCR is a closed-tube multiplex PCR technology that combines melting curve analysis with PCR and is simple and sensitive for clinical applications. 2D-PCR is considered an excellent amplification detection technology widely used for the quantification of various pathogens, assisting in the diagnosis of various diseases such as human papillomavirus (HPV) (Wu et al., 2022) and inflammatory bowel disease (Wu et al., 2024). In the present study, a 2D-PCR method was established for the detection of hvKp. To successfully detect multiple genes in a single tube while reducing the number of probes required for the detection of multiple genes, we introduced pre-labeled sequences and probes that can recognize multiple target genes in the 2D-PCR reaction system and evaluated the sensitivity and specificity of this method. The experimental results indicate that the melting temperature difference of the four virulence genes

TABLE 3 Genes, detection time, specificity, sensitivity, accuracy, and disadvantages of hvKp detection methods based on biomarkers.

Method	Genes	Detection time	Specificity (%)	Sensitivity (%)	Accuracy (%)	Advantage	Disadvantage	References
PCR ¹	<i>peg-344</i> , <i>iroB</i> , <i>iucA</i> , <i>rmpA</i> , and <i>rmpA2</i>	1 h		95		Strong specificity	Low throughput requires post-amplification analysis and specialized instruments	Russo et al. (2018)
q-PCR ²	<i>iroB</i> , <i>iucA</i> , <i>rmpA</i> , and <i>rmpA2</i>	1–2 h	98	100		High sensitivity and specificity	Low throughput	Kuypers et al. (2017); Cai et al. (2025)
LAMP ³	<i>peg-344</i>	15–60 min	96	99		No specialized equipment required High sensitivity and specificity	Prone to non-specific amplification	Cai et al. (2025)
RAA ⁴	<i>peg-344</i> , <i>rmpA</i>	10–20 min.	100	100		Rapid results without specialized equipment High sensitivity and specificity	The design requirements for primer probes are high	Yan et al. (2023)
2D-PCR ⁵	<i>iucA</i> , <i>peg-344</i> , <i>rmpA</i> , and <i>rmpA2</i>	1 h	96.2	100		High throughput High sensitivity and specificity	Primer probe labels design requirements are high	This study

¹Polymerase chain reaction.
²Quantitative real-time polymerase chain reaction.
³Loop-mediated isothermal amplification.
⁴Recombinase-aided amplification.
⁵Two-dimensional polymerase chain reaction technology.

was about 5°C, and there was no cross-reactivity. When a mixture of four virulence plasmids is used as a template, 2D-PCR can detect four virulence genes in a single closed-tube assay. Simultaneously, we found that the minimum detection limit of *iucA*, *peg-344*, *rmpA2*, and *rmpA* genes were 10³, 10², 10³, and 10³ copies/μL, respectively. The limit of detection in this study was consistent with the multiplex q-PCR method for detecting hvKP reported by Cai et al. (2025). Finally, to evaluate the clinical applicability of our 2D-PCR assay, we screened 105 clinical samples using 2D-PCR and conventional PCR. The sensitivity and specificity of the 2D-PCR assay were 100 and 96.2% compared with conventional PCR as the reference standard. The 2D-PCR method developed in this study demonstrates a high degree of concordance with traditional PCR results in clinical practice. This method can be effectively employed for the detection of clinical samples, providing a rapid diagnosis of hvKp in clinical samples.

The definition of hvKp virulence genes remains controversial, and further research and verification are necessary to determine which virulence genes should be fully defined as hvKp (Kocsis, 2023). So, this study has some limitations. First, the selection of virulence genes is not comprehensive enough. We only detected four virulence genes, this can be addressed by increasing the number of fluorescent channels and tag sequences to improve the detection throughput of 2D-PCR, it is highly possible for 2D-PCR to identify more than 30 genes simultaneously. Second, the number of clinical samples we selected is relatively small, and further expansion of the sample size to evaluate the clinical application potential of 2D-PCR comprehensively.

Conclusion

This study presents a simple, high-throughput method for the rapid identification of biomarkers for hvKp strains, enabling accurate detection of hvKp with excellent specificity and sensitivity. Additionally, 2D-PCR also shows high specificity in clinical performance analyses, meeting the needs of clinicians diagnosing hvKp. Thus, 2D-PCR is a powerful tool for early diagnosis and epidemiological surveillance of hvKp.

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.

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Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Fudan University Affiliated Jinshan Hospital (JIEC 2024-S69).

Author contributions

WZ: Data curation, Formal analysis, Methodology, Writing – original draft. YW: Methodology, Writing – original draft. XJ: Formal analysis, Writing – original draft. YZ: Data curation, Project administration, 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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