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Physiological response, microbial diversity characterization, and endophytic bacteria isolation of duckweed under cadmium stress

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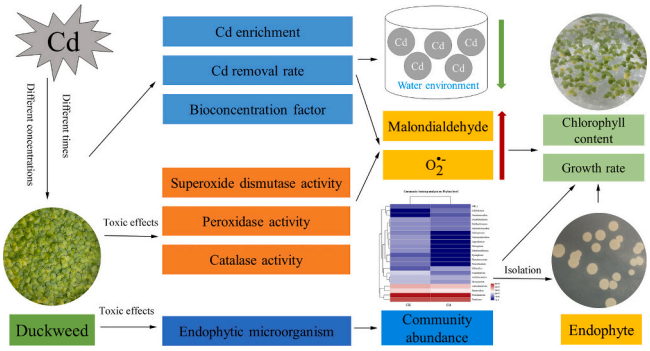
HIGHLIGHTS

- The damage to duckweed deepened with increasing time of cadmium treatment.
- Up to 90 % removal of cadmium in the culture medium.
- Cadmium treatment affected the diversity of endophytic community of duckweed.
- Endophyte can improve the growth of duckweed under cadmium stress.

GRAPHICAL ABSTRACT



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ABSTRACT

Duckweed is a cadmium (Cd) hyperaccumulator. However, its enrichment characteristics and physiological responses to Cd have not been systematically studied. The physiological responses, enrichment characteristics, diversity of endophytic bacterial communities, and isolation of Cd-resistant endophytes in duckweed (*Lemna minor* 0014) were studied for different durations and Cd concentrations. The results indicated that peroxidase (POD) and catalase (CAT) activities decreased while superoxide dismutase activity first increased and then decreased with increasing Cd stress duration. POD activities, CAT activities, and $O_2^{\bullet-}$ increased as Cd concentrations increased. Malondialdehyde content and Cd accumulation in duckweed increased with increasing concentrations and time. This endophytic diversity study identified 488 operational taxonomic units, with the dominant groups being Proteobacteria, Firmicutes, and Actinobacteria. *Paenibacillus* sp. Y11, a strain tolerant to high concentrations of Cd and capable of significantly promoting duckweed growth, was isolated from the plant. Our study revealed the effects of heavy metals on aquatic plants, providing a theoretical basis for the application of duckweed in water pollution.

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1. Introduction

With continuous industrial development, pollution from heavy metals, including copper, lead, and cadmium (Cd), has become increasingly prominent (Khan et al., 2022). Cd-contaminated wastewater degrades water quality and thus water cannot be directly used for potable water (via desalination) and industrial applications (Panagopoulos and Giannika, 2022a; Panagopoulos and Giannika, 2022b). In addition, Cd in the environment is highly toxic with chronic presence, affecting normal plant growth, photosynthesis, respiration, antioxidation, and destroying photosynthetic pigments, thereby leading to plant damage and even death (Genchi et al., 2020; Zhu et al., 2021). It can also accumulate in crop seeds gradually increasing in toxicity with the progressing food chain, eventually being ingested by the highest consumer in the food chain, humans (Khan et al., 2022). Cadmium can affect the reproductive system and the physiological functions of muscles and bones in animals (da Silva et al., 2021; Järup and Akesson, 2009). Currently, physical, chemical, and biological methods are used to address Cd pollution, with biological methods receiving considerable attention (Wang and Chao, 2020). Biological methods, including phytoremediation and microbial remediation technologies or a combination of both, are important tools for the environmental remediation of water (Genchi et al., 2020). However, phytoremediation's efficiency is limited due to the low biomass and slow growth of hyperaccumulators, as well as the low metal bioavailability (Rajkumar et al., 2009). Therefore, it is necessary to investigate possibilities to promote plant growth and heavy metal accumulation in phytoremediation.

Researchers have recently proposed using plant endophytes (PEs) to assist phytoremediation (Wang et al., 2021c). PEs are widely present microorganisms in all tissues of healthy plants either during a certain period of the life cycle or even the entire life cycle and interact with the plants to establish a stable and complex mutually beneficial symbiotic relationship through a series of physicochemical interactions (Puri et al., 2020; Sturz et al., 2000; Tian et al., 2016). Endophytes boost plant tolerance to heavy metals by creating metal-chelating chemicals, assisting in the activation of antioxidant enzymes, and lowering lipid peroxidation. And eliminate the toxic effects of heavy metals by promoting the growth of host plants through plant growth hormones, such as indole-3-acetic acid, to reduce the heavy metal content in plant tissues (Liu et al., 2019; Nagata et al., 2015; Rocha et al., 2016; Ruan et al., 2016). Endophytic bacteria isolated from plants with certain properties such as those mentioned earlier can, to a certain extent, promote their nutritional growth and improve resistance to heavy metals and enrichment capacity when they are reintroduced into host plants or inoculated into other plants (Adediran et al., 2015). The endophytic bacterium *Acrocalymma vagum* isolated from *Ilex chinensis* significantly promoted the biomass and root structure of *Medicago sativa* and *Ammopiptanthus mongolicus* under Cd stress and improved the accumulation of Cd in plant tissues (Hou et al., 2019). Another endophytic bacterium, *Aspergillus violaceofuscus*, isolated from Cd-hyperenriched Chilli plants, alleviated Cd stress in okra through physiological and biochemical modulation (Aziz et al., 2022). In addition, studies have shown that microbial communities are all critical regulators during phytoremediation (Liu et al., 2022a). Cd stress affects the microbial diversity of plants by altering the community abundance of their endophytes (Du et al., 2021). Therefore, it is important to study the effect of Cd stress on plant endophyte communities' diversity and identify potentially dominant populations.

Duckweed is an aquatic floating plant with a rapid growth rate (Its biomass increased 1-fold every 16–24 h), small size, and environmental adaptability and has been proven to be an excellent Cd hyperaccumulator (The Cd content in the aerial parts of the plants reaching 100 mg/kg is considered the standard for Cd hyperaccumulator plants) (Küpper and Leitenmaier, 2013; Yang et al., 2021a; Zheng et al., 2022). In a study on phytoremediation technology to mitigate Cd pollution, aquatic plants such as *Microsorum pteropus* and *Eichhornia crassipes* removed up to half of the Cd content in one week, while in a study on duckweed, *Lemna* and *Landolti* removed 70 % of the Cd in water (Ekperusi et al., 2019; Khan et al., 2020; Lan et al., 2019; Wang et al., 2022; Zhou et al., 2020). In a previous study, an excellent strain (*Lemna minor* 0014) was obtained through screening and removed 82.50 % of Cd after 7 d of 10 mg/L Cd treatment, with a Cd enrichment of 2834.3 mg/kg, which was significantly higher than the standard for Cd-hyperenriched plants (Zheng et al., 2022). The effects of Cd have been studied previously, primarily in duckweed plants themselves; however, the effects of Cd on the endophytic community structure of duckweed have not been reported.

Therefore, the objectives of this study were as follows: (1) To systematically investigate the enrichment characteristics and physiological responses of duckweed under Cd stress. (2) To analyze the diversity of endophytic bacteria of duckweed in Cd stress and determine the dominant populations. (3) To set screening conditions according to the dominant populations, and isolate endophytic bacteria with high Cd tolerance and promote the growth of host plants. The growth rate, antioxidant enzyme activity, malondialdehyde (MDA) and chlorophyll content, observation of free radical O_2^- tissue staining, and determination of Cd enrichment and removal rate in *L. minor* 0014 were analyzed after applying different concentrations of Cd^{2+} treatment for 1–7 d to duckweed as experimental materials. Further studies on the effect of Cd on endophytic bacteria in *L. minor* 0014 and isolation of Cd-tolerant strains from the plant will not only improve its adaptability in polluted water bodies but also provide new insights for better utilization of combined *L. minor*-endophytic bacteria in environmental remediation.

2. Materials and methods

2.1. Materials and experimental design

A duckweed strain, *L. minor* 0014, preserved in the duckweed germplasm resource bank, was used for this study. The duckweed was precultured in the Hoagland medium and expanded with 1/5 Hoagland medium after attaining satisfactory growth. The culture temperature, light–dark ratio, humidity, and light intensity were set at 25 °C, 16:8 h, 75 %, and 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 7 d, respectively. According to related studies and our previous work, we set different Cd concentrations to treat the duckweed (Wang et al., 2022; Wang et al., 2021d; Xu et al., 2018; Zheng et al., 2022). The duckweed was treated with concentration gradients of 0, 0.1, 0.3, 0.5, 0.7, 1.0, 2.0, 3.0, 4.0, and 5.0 mg/L Cd^{2+} for 1, 3, 5, and 7 d. Each treatment biology was repeated three times.

2.2. Determination of growth rate and chlorophyll content

The duckweed was salvaged, dried, and its fresh weight was measured. The growth rate (GR) was calculated according to changes in fresh weight and treatment duration (Kruger et al., 2019). The chlorophyll content of duckweed was also measured (Liu et al., 2021).

2.3. Antioxidant enzyme activity assay, MDA content assay, and tissue staining

Duckweed (0.1 g) was frozen in liquid nitrogen and ground on ice after adding 1 mL of extract. The mixture was centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was removed and kits (Solarbio, Beijing, China) were used to detect superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POD; EC 1.11.1.7), and catalase (CAT; EC 1.11.1.6) activity. MDA content was determined using the thiobarbituric acid method (Draper et al., 1993). The duckweed tissues were stained with Nitro blue tetrazolium chloride (NBT) (Jambunathan, 2010).

2.4. Determination of Cd content

The treated duckweed was rinsed with running tap and deionized water, dried in an oven, and ground. A sample (0.1 g) of the duckweed powder was weighed into a digestion tube, digested for 4 h after adding 4 mL of HNO_3, diluted to 50 mL with deionized water, and the Cd content was determined using the flame atomic absorption method (NovaAA 400P; Analytik Jena, Germany).

2.5. Analysis of microbial diversity in duckweed

Duckweed was treated with no Cd^{2+} or 0.1 mg/L Cd^{2+} for 15 days. The experiment was performed in triplicate. Duckweed samples were selected (1 to 3 g), snap frozen in liquid nitrogen, stored in a refrigerator at −80 °C as standby, and sent to Shanghai Majorbio Bio-Pharm Technology Co. Ltd. for microbial diversity analysis. Each treatment biology was repeated three times.

2.6. Isolation of endophytic bacteria from duckweed

The aseptically treated duckweed samples were well ground, allowed to stand for 15 min, and the supernatant was diluted in sterile water in gradients of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}; 200 μL of the dilution was added to LB medium for coating. After standing for 30 min, the plates were placed in a dark incubator at 28 °C for 72 h. The endophytic bacterial species were isolated from the treatment based on the initial judgment of morphology, color, and other characteristics of the colonies, and the colonies were purified by selecting them according to the conventional method and stored in a refrigerator at −80 °C with glycerol for backup.

2.7. Identification of endophytic bacterial species in duckweed

Endophytic bacterial species in duckweed were identified using 16S rDNA sequence analysis, and their DNA was extracted using the Tennenbacter genome extraction kit. PCR amplification of the conserved sequences of the target strains was performed using the universal bacterial primers 27F: AGAGTTTGATCCTGGCTCAG and 1492R: TACGGCTACCTTGTTACGACTT. The PCR products were sent to Shanghai Majorbio Bio-Pharm Technology for sequencing. The sequencing results were BLAST searched against the NCBI database, and the sequences of strains with 99 % or greater similarity were downloaded and used to construct a phylogenetic tree together with the sequences measured in this experiment. After comparison with Clustal X2, all sequences were cropped to the same first and last sequences, and the output results were used to construct a phylogenetic tree using MEGA 7 to analyze the affinity between endophytes and determine the endophyte species.

2.8. Determination of growth index of replanted endophytes in duckweed

The endophytes were inoculated into duckweed, with four biological replications, and the duckweed samples were collected after 7 d of treatment with no Cd^{2+} (nCd) or 1 mg/L Cd^{2+} (+Cd) added to the culture solution. Biomass was collected for the relevant measurements. Each treatment biology was repeated four times.

2.9. Data analysis

Graph Pad Prism 6.02 was used for plotting, and one-way ANOVA was used to compare the differences between the experimental and control groups in SPSS 20.0. All data are reported as the mean ± standard error of the mean of three replicates per sample. $p < 0.05$ was considered statistically significant. Network analysis was performed using R (version 3.3.1), and the co-occurrence network was visualized in Cytoscape 3.7.1 (https://cytoscape.org/) based on the Fruchterman Reingold algorithms. Network topological properties (average degree, average clustering coefficient, clustering coefficient, and modularity) were calculated for both the observed and random networks using Cytoscape 3.7.1.

3. Results

3.1. Effect of Cd on duckweed growth

With increasing Cd concentrations, the duckweed growth rate decreased (Table 1); however, the duckweed could grow under 0–5.0 mg/L Cd stress treatment. A concentration of 0.1–0.3 mg/L Cd did not significantly inhibit duckweed growth, but 0.5–5.0 mg/L Cd did. Cd treatment of 0–0.3 mg/L for 1 d produced the fastest growth. The growth rate of duckweed was approximately 0.5 g/d in 0.5–5.0 mg/L treatment for 1 d. The growth rate was approximately 0.5 g/d under 0–0.3 mg/L stress for 3–7 d, and close to 0.2 g/d under 0.5–5.0 mg/L stress.

With increasing Cd concentrations, the chlorophyll content of duckweed exhibited a downward trend (Table 1). With increasing duration of Cd stress, the chlorophyll content of duckweed first decreased and then increased (decreased by approximately 0.5–1.5 mg/L for 3 d, and increased to approximately 4.0–5.0 mg/L for 5–7 d). The chlorophyll content of duckweed was the highest under 0.1–0.3 mg/L Cd stress for 7 d.

3.2. Effects of Cd on antioxidant enzyme activity, MDA content, and tissue staining of duckweed

Cd stress causes oxidative stress in duckweed, generating and accumulating reactive oxygen species (ROS). The antioxidant defense system is activated and antioxidant enzymes, including SOD, POD, and CAT, play a role in scavenging the ROS (Farooq et al., 2020). Fig. 2a–c illustrates the antioxidant enzyme activities of *L. minor* 0014. The POD activity of *L. minor* 0014 gradually decreased with increasing stress duration. At 1 d and 7 d of treatment, the POD activity tended to increase and then decrease with increasing Cd concentration, peaking at 0.7–1.0 mg/L. At 3 d and 5 d of treatment, POD activity increased with increasing Cd concentration. At 3 d of stress, POD activity was lowest at 0.3 mg/L Cd, 163.3 U/g. At 5 d of stress, POD activity was the lowest at 0.1 mg/L Cd (130.7 U/g) (Fig. 1a). The SOD activity of *L. minor* 0014 tended to increase and then decrease with increasing stress duration, with the highest SOD activity (446.6 U/g) observed at 3 d. The lowest SOD activity of 8.7 U/g was achieved at 5 d with 0.1 mg/L Cd (Fig. 1b). The CAT activity of *L. minor* 0014 gradually decreased with prolonged stress. From 1 to 5 d of stress, CAT activity first increased and then decreased with increasing Cd concentration, reaching a peak at 1.0 mg/L (Fig. 1c).

The ROS produced by plants can cause lipid peroxidation and produce aldehydes, including MDA, which can cross-link with DNA and membrane proteins to increase cell membrane permeability and cause cell damage. The MDA content reflects the degree of lipid peroxidation in plants. The MDA content in duckweed increased with increasing Cd stress, as observed at 5–7 d of stress in Fig. 1d. At 3 d of stress, the lowest MDA content was 0.27 μmol/g at 0.3 mg/L Cd and the highest MDA content was 0.82 μmol/g at 2.0 mg/L Cd (Fig. 1d). Superoxide anion (O_2^-) is a ROS, which can reduce NBT to a blue substance insoluble in water. By staining the leaf tissue with NBT, the O_2^- produced by duckweed was observed to determine the degree of damage. As illustrated in Fig. 1e, the blue fraction appearing in the leaves increased with increasing Cd concentrations, indicating that the damage increased in severity as the O_2^- produced by duckweed increased with increasing Cd concentration.

3.3. Effects of Cd on the enrichment and removal rate of duckweed

The Cd content in duckweed can directly reflect its level of enrichment by duckweed. Cd enrichment in *L. minor* 0014 increased

Table 1
Growth rate and chlorophyll content of *L. minor* 0014 under different Cd concentrations and time.

Cd (mg/L)	Growth rate (g/d)				Chlorophyll content (mg/g)			
	1 d	3 d	5 d	7 d	1 d	3 d	5 d	7 d
0	0.91 ± 0.11	0.52 ± 0.02	0.39 ± 0.06	0.67 ± 0.26	1.47 ± 0.12	0.94 ± 0.16	2.96 ± 0.05	2.93 ± 0.18
0.1	1.22 ± 0.07	0.47 ± 0.02	0.47 ± 0.03	0.46 ± 0.22	1.19 ± 0.01	0.84 ± 0.21	2.33 ± 0.74	2.72 ± 0.15
0.3	0.86 ± 0.23	0.40 ± 0.08	0.38 ± 0.05	0.37 ± 0.07*	1.34 ± 0.18	0.72 ± 0.17	2.69 ± 0.70	2.86 ± 0.91
0.5	0.30 ± 0.43**	0.18 ± 0.15**	0.26 ± 0.08**	0.26 ± 0.03**	1.16 ± 0.22*	0.92 ± 0.19	2.50 ± 0.70	2.39 ± 0.29
0.7	0.67 ± 0.05	0.30 ± 0.11*	0.30 ± 0.06*	0.30 ± 0.03**	0.82 ± 0.39**	0.74 ± 0.08	2.58 ± 0.86	2.72 ± 0.20
1	0.52 ± 0.11*	0.16 ± 0.13**	0.37 ± 0.02	0.39 ± 1.03*	0.84 ± 0.09**	1.00 ± 0.18	2.31 ± 0.53	2.82 ± 0.30
2	0.51 ± 0.11*	0.15 ± 0.05**	0.15 ± 0.04**	0.24 ± 2.11**	0.89 ± 0.05**	0.72 ± 0.18	2.07 ± 0.46	2.64 ± 0.61
3	0.42 ± 0.07**	0.17 ± 0.08**	0.05 ± 0.01**	0.25 ± 3.12**	1.27 ± 0.08	0.49 ± 0.10**	2.46 ± 0.33	2.76 ± 0.06
4	0.64 ± 0.06	0.21 ± 0.01**	0.10 ± 0.01**	0.22 ± 4.02**	0.90 ± 0.12**	0.34 ± 0.03**	2.21 ± 0.61	2.26 ± 0.12*
5	0.28 ± 0.09**	0.12 ± 0.04**	0.07 ± 0.05**	0.13 ± 5.05**	0.80 ± 0.19**	0.29 ± 0.06**	2.09 ± 0.64	2.20 ± 0.17*

Values are means ± standard error (n = 3) (*: $p < 0.05$, **: $p < 0.01$).

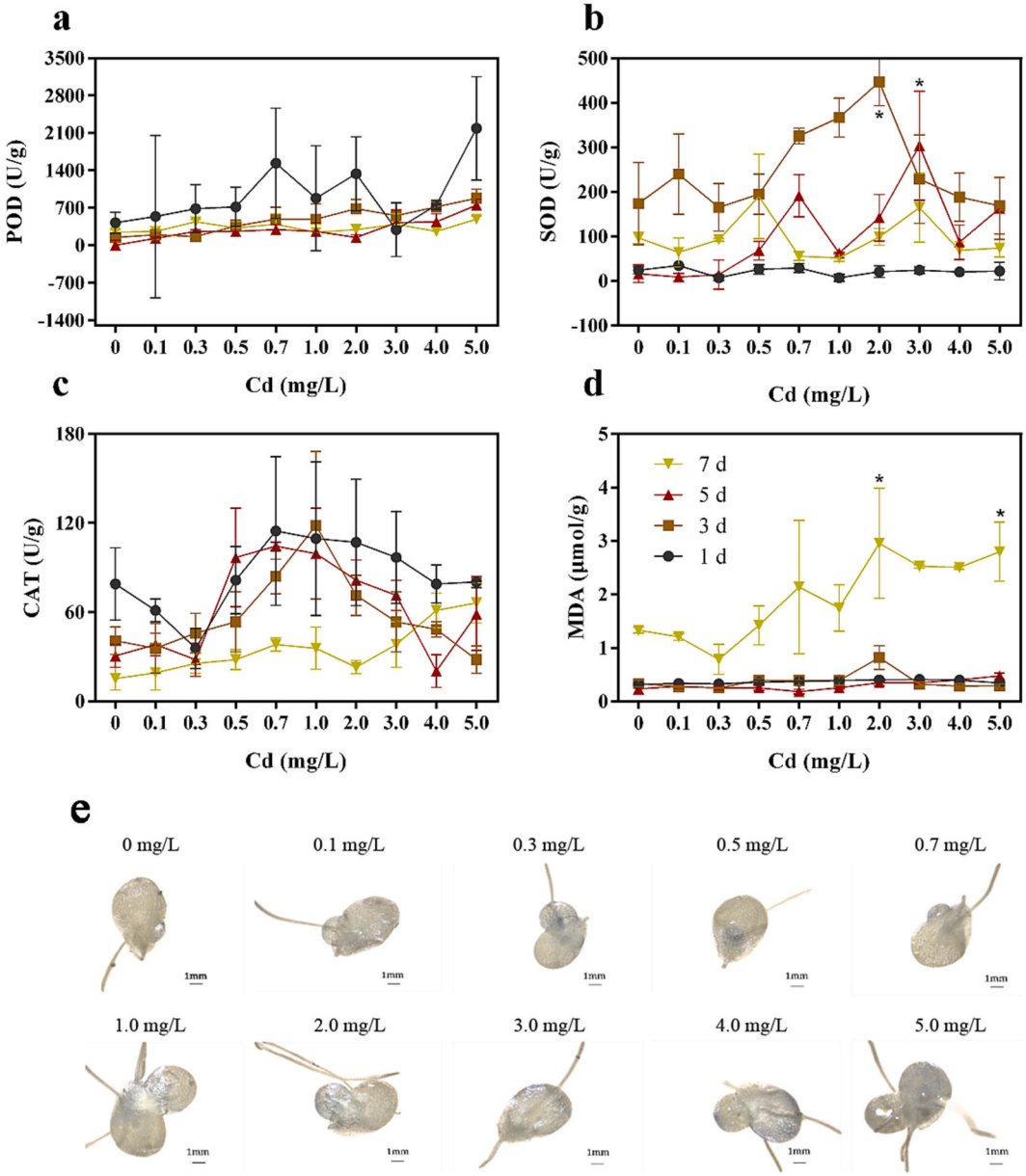

Fig. 1. Antioxidant activity and oxidative damage of *L. minor* 0014 under different Cd concentrations and durations. (a) POD activity, (b) SOD activity, (c) CAT activity, and (d) MDA content; (e) O_2^- tissue staining of *L. minor* 0014 under different Cd concentrations. Bars indicate mean ± SEM ($n = 3$) ($p < 0.05$).

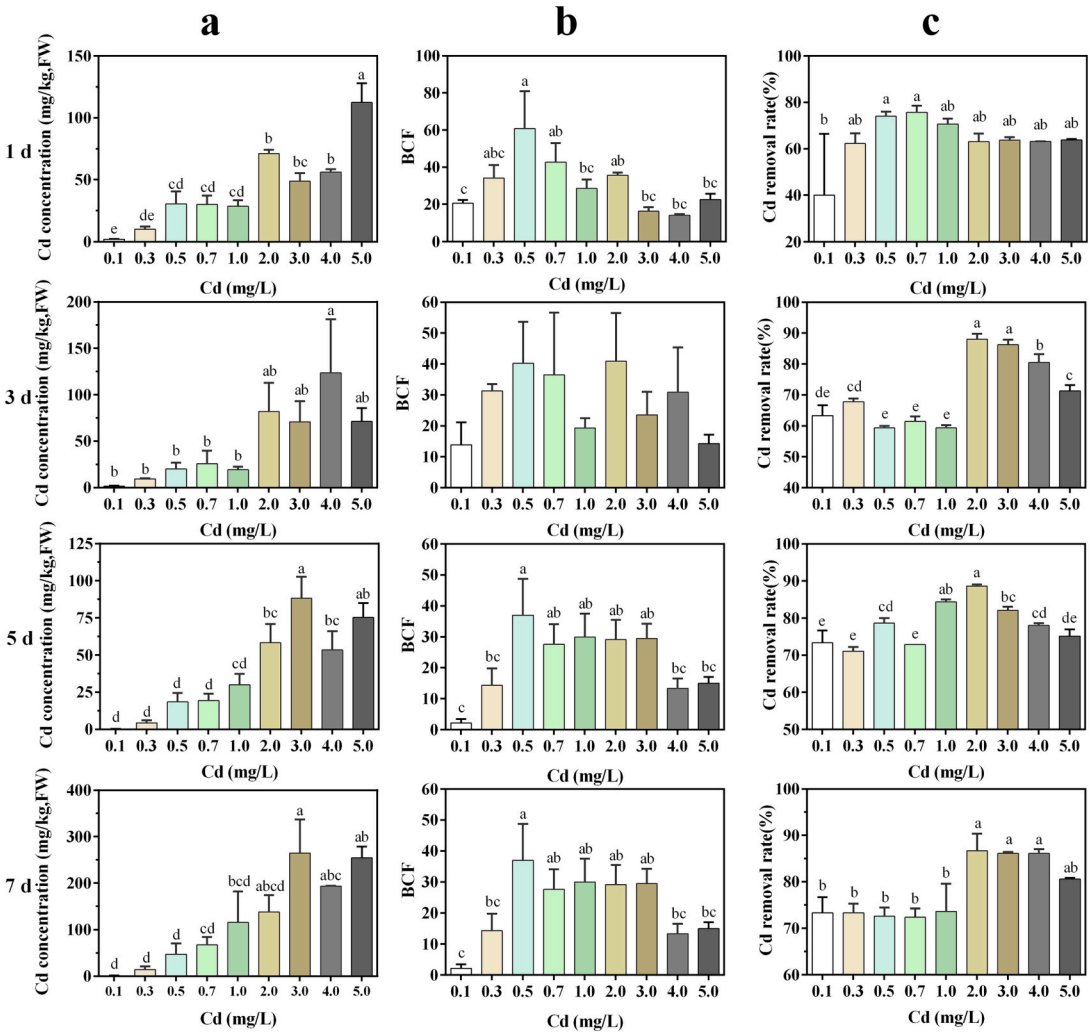

Fig. 2. The Cd enrichment and removal rate of *L. minor* 0014 under different Cd concentrations and durations. (a) Cd content, (b) BCF, and (c) Cd removal rate. The lowercase letters and corresponding error bars indicate significant differences. Bars indicate mean ± SEM ($n = 3$) ($p < 0.05$).

significantly with increasing Cd stress duration and concentration. After 7 d of 1.0–5.0 mg/L Cd stress, the Cd enrichment in *L. minor* 0014 exceeded 100 mg/kg and reached the standard for hyperaccumulator plants, with a maximum value of 264.6 mg/kg (Fig. 2a).

The bioconcentration factor (BCF) is an important index for evaluating the enrichment capacity of plants, calculated by dividing the concentration of heavy metals in the plant by that in the environment. With the increase in Cd concentration, the BCF exhibited a trend of first increasing (at 0.1–0.5 mg/L Cd stress) and then decreasing (at 0.5–5.0 mg/L Cd stress). The highest BCF of *L. minor* 0014 reached 115.97 after 7 d of 1.0 mg/L Cd stress, indicating its great application potential in treating Cd-contaminated water (Fig. 2b).

The effect of Cd removal from water can be expressed directly in terms of the removal rate. As illustrated in Fig. 2c, the Cd removal rate generally increased with increasing treatment duration. After 1 d of stress, the removal rate of 0.3–5.0 mg/L Cd reached 62–76 %, except for 0.1 mg/L Cd (40 %), indicating that *L. minor* 0014 can achieve satisfactory Cd removal in a short period. After 3 d of 0.1–1.0 mg/L Cd treatment, *L. minor* 0014 removed 60–68 % of Cd, and the removal rate increased to higher than 70 % after 7 d, indicating its excellent removal effect even at low concentrations. The Cd removal rate of *L. minor* 0014 reached 70–90 % after 5–7 d of 0.1–5.0 mg/L Cd treatment, indicating that the aquatic plant can be used to remove heavy metal pollution from water with a satisfactory removal effect.

3.4. Effect of Cd on endophytic bacteria of duckweed

3.4.1. High-throughput sequencing results

Using high-throughput sequencing analysis, a total of 387,094 valid sequences with 145,803,731 bases were sequenced among the different treatments, and the sequence numbers of each sample ranged from 59,081 to 631,159, with an average sequence length of 376 bp. 22 phyla, 43 classes, 101 orders, 180 families, 302 genera, 405 species, and 488 operational taxonomic units (OTUs) were detected in the six sample groups. The coverage of all samples exceeded 98 %, indicating that the sequencing results truly reflected the species and abundance of microbial communities under different treatments (Fig. S1).

3.4.2. Diversity analysis of the microbial community in duckweed under Cd treatment

Alpha diversity refers to the diversity within a specific area or ecosystem. A higher diversity index indicates a higher abundance and diversity of microbial communities. Commonly used metrics include the Chao, Shannon, ACE, and Simpson indices. The Shannon and Simpson indices reflect the diversity of species among communities, and their high values indicate high and low diversity of species in the community, respectively. The Chao and Ace indices reflect community richness, and large values indicate a higher total number of species and a higher number of species in the sample, respectively (Jaiswal et al., 2018; Li et al., 2022). A high coverage index in this study indicated that the

sequencing results accurately reflected the real situation and species richness of the samples (Fig. S1). The abundance and diversity of microbial communities under different treatments were determined, and the results revealed that the abundance and diversity of endophytic bacteria in duckweed decreased under Cd treatment. The Chao and Ace indices were lower for the Cd treatment group than for the control group, indicating that Cd treatment reduced the abundance of endophytic bacteria in duckweed. The Shannon index of the Cd-treated group was smaller than that of the control group, indicating that Cd treatment reduced the diversity of endophytic bacteria in duckweed. Similarly, the Simpson index of the Cd-treated group was higher than that of the control group, further indicating that Cd treatment reduced the diversity of endophytic bacteria in duckweed (Table S1).

3.4.3. Distribution of microbial community OTUs under Cd treatment

To explore the effect of Cd treatment on the endophytic community of duckweed, 16S rRNA sequences were used to study the endophytic community of duckweed samples under different conditions, and the variability and percentage of the OTU composition of the microbial community among different treatments are presented in Fig. 3. Total 488 OTUs for species classification were obtained based on a similarity level of ≥97 %, and the OTUs specific to the microbial communities of the CK and Cd groups were observed to be 210 and 80, respectively (Fig. 3a). Different OTUs have different percentages (Fig. 3b). The results revealed differences in the microbial community composition of duckweed under Cd treatment.

(a) Venn diagram of the OUT distribution of different taxa; (b) Microbial community occupancy at the OTU level. CK denotes blank control and Cd denotes the Cd-treated group.

3.4.4. Microbial community composition structure under Cd treatment

Fig. 4a illustrates the differences in microbial distribution at the phylum level, with all samples having similar dominant flora but slightly different abundances. Proteobacteria, Firmicutes, Actinobacteria, and other dominant bacteria were commonly observed, with Proteobacteria being the dominant group. The abundance of Proteobacteria was higher in the Cd treatment than in the control (Fig. 4a). At the family taxonomic level, differences existed in the endophytic bacteria of duckweed under the Cd treatment (Fig. 4b), and the three families with the highest abundance of endophytic bacterial communities in the control were Comamonadaceae, Paenibacillaceae, and Caulobacteraceae. The three families with the highest abundances of endophytic bacterial communities in the Cd-treated group were Comamonadaceae, Paenibacillaceae, and Enterobacteriaceae. The abundance of Enterobacteriaceae was higher in the Cd-treated group than that in the control group. These results indicated that Cd stress affects the abundance of endophytic bacterial communities in duckweed.

Microbial composition analysis revealed 22 phyla, including Proteobacteria, in duckweed samples. The abundances of Proteobacteria, Firmicutes, and Actinobacteria were high both in the control and Cd-treated groups (Fig. 4c). This further confirmed that endophytic bacteria of duckweed can exist under Cd stress and that they are affected by it.

(a) Percentage of community abundance at the phylum level; (b) Percentage of community abundance at the family level. CK denotes blank control and Cd denotes the Cd-treated group. (c) Heat map at the level of different sample gates. The variation in abundance of different species in the sample is presented by the color gradient of the color blocks. The values represented by the color gradient are shown on the right side of the graph, with red and blue representing gates of high and low abundance, respectively. (d) Analysis of differences in bacterial community structure (PLS-DA analysis). The scales of the X and Y axes are relative distances. Red and blue indicate the control and Cd treatment groups, respectively. (e) Species correlation network diagram. The top 50 species in total abundance at the genus level were selected, and the correlation coefficients such as Spearman's rank between species were calculated to reflect the correlation between species. Species with $p < 0.05$ are shown by default in the figure. The size of nodes in the figure indicates the species abundance size and different colors indicate species of different phyla. The red and green connecting lines indicate positive and negative correlations, respectively. The higher the number of lines, the closer the association between the species and other species.

3.4.5. Effect of Cd treatment on the microbial community structure of duckweed

Partial least squares discriminant analysis (PLS-DA) was used to determine any significant differences between groups by performing a similarity analysis between the sample groups. The results revealed that the points on the graph of the duckweed samples for Cd treatment and control group samples had a large separation (Fig. 4d), indicating a significant difference between the inter-root bacterial community in untreated duckweed and under different treatment conditions, which can differentiate between the control and Cd-stressed flora.

3.4.6. Network analysis of microorganisms in duckweed under Cd treatment

Co-occurrence network diagrams mainly reflect the co-occurrence relationships of species in environmental samples and highlight the similarities and differences between the samples. Single-factor network analysis refers to the construction of species correlation network diagrams by calculating the correlations between species and is used to

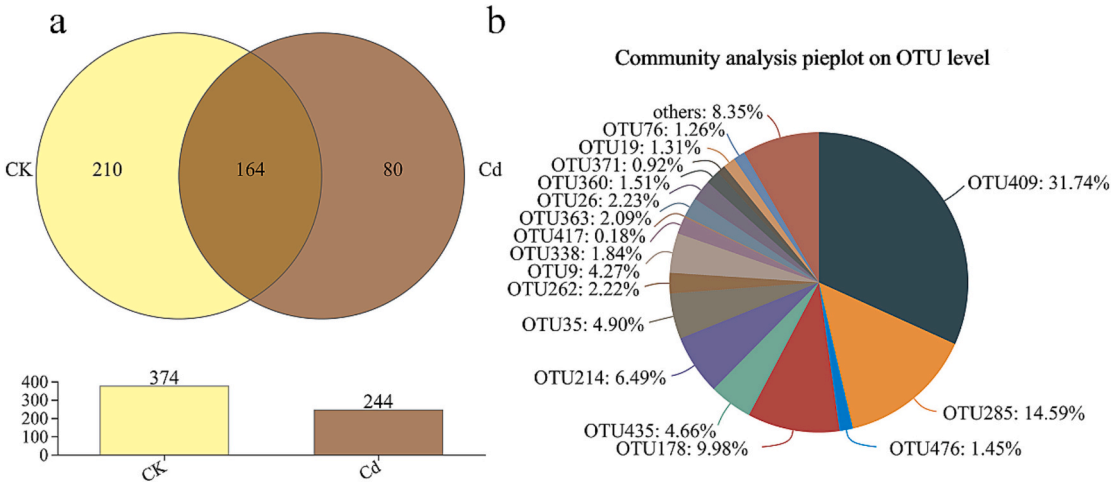

Fig. 3. Distribution of microbial community OTUs under Cd treatment.

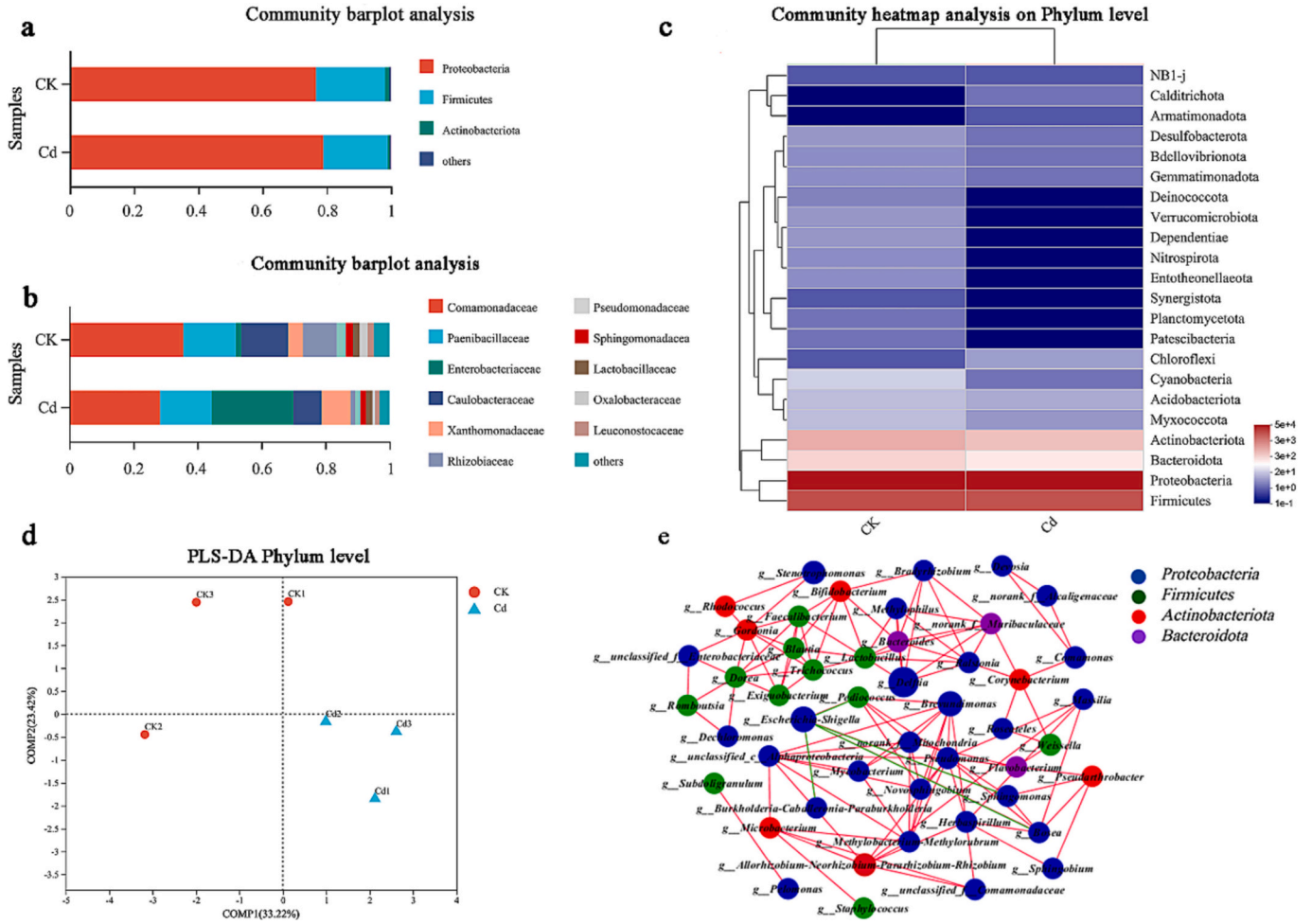

Fig. 4. Analysis of endophytic microbial communities of duckweed under Cd treatment.

reflect the interactions between species in a sample (Perez De Souza et al., 2020). The co-occurrence network analysis revealed that 32 bacterial genera with an abundance >50 were significantly correlated with Cd concentration after Cd treatment ($p < 0.05$; Fig. S1). For example, the relative abundance of *Paenibacillus* was correlated with Cd concentration. *Delftia* not only exhibited a correlation with Cd stress but also had the highest abundance (34,225.66). Species co-occurrence network analysis revealed that the four bacterial genera associated with *Escherichia-shigella* were negatively correlated, while all other bacteria were positively correlated with each other (Fig. 4e).

3.5. Isolation and identification of endophytic bacteria in duckweed

A total of 22 endophytic strains were isolated from the *L. minor* 0014 samples and were numbered from Y1 to Y22. Fig. S3 illustrates the partial isolation of the isolated endophyte phenotypes.

3.5.1. Effect of Cd on the growth of endophytic bacteria

Endophytic bacteria were isolated after treatment with low concentrations of Cd^{2+} (10 mg/L). The results demonstrated that strains Y2, Y3, Y7, and Y11 can grow at low concentrations of Cd (Table S2). Further, the endophytic bacteria were treated with high concentrations of Cd, and all four strains grew at high Cd concentrations (Fig. 5a). Different bacterial strains had different response times to Cd; for example, Y3 biomass increased significantly only after 72 h of Cd treatment, whereas that of Y2 increased significantly after 24 h. Y11 at 48 h was biomass was significantly higher than other strains. Therefore, Y11 was selected for further systematic Cd tolerance experiments. The biomass of strain Y11 gradually decreased with increasing Cd concentration (Fig. 5b).

(a) Growth of different strains under 80 mg/L cadmium treatment; (b) Growth of Y11 under different Cd concentration treatments; (c) Growth indicators of endophytic bacteria back planted in duckweed; (d) 16S rDNA amplification results of endophyte Y11; (e) Phylogenetic tree of 16S rDNA sequence of endophyte Y11. M is the 2 Kb marker and Y11 is the amplification product of strain Y11. nCd: no Cd^{2+} added; +Cd: 1 mg/L Cd^{2+} added. Bars indicate mean ± SEM ($n = 4$). Asterisks indicate significant differences between treatment and control groups (*: $p < 0.05$, **: $p < 0.01$).

3.5.2. Effect of endophytic bacteria replantation on the growth index of duckweed

The Cd-tolerant endophytes were further replanted into a liquid medium containing the same amount of duckweed without the addition of Cd^{2+} (nCd) or with added 1 mg/L Cd^{2+} (+Cd) to observe their effect on the growth index of duckweed. According to the results, strain Y7 significantly inhibited the growth of duckweed when the endophytic bacteria were added back to the duckweed without Cd. Y11 significantly increased the fresh weight of the duckweed, whereas the other strains did not affect the fresh weight of the duckweed. Y11 also significantly increased the duckweed root length (Fig. 5c). When the endophytic bacteria were added back to the Cd-supplemented duckweed, Y11 significantly increased the fresh weight and root length of the duckweed. Additionally, Y3 significantly increased the fresh weight of duckweed under Cd stress. The significant decrease in chlorophyll content in Y3 and Y11 with and without Cd addition could be attributed to the high

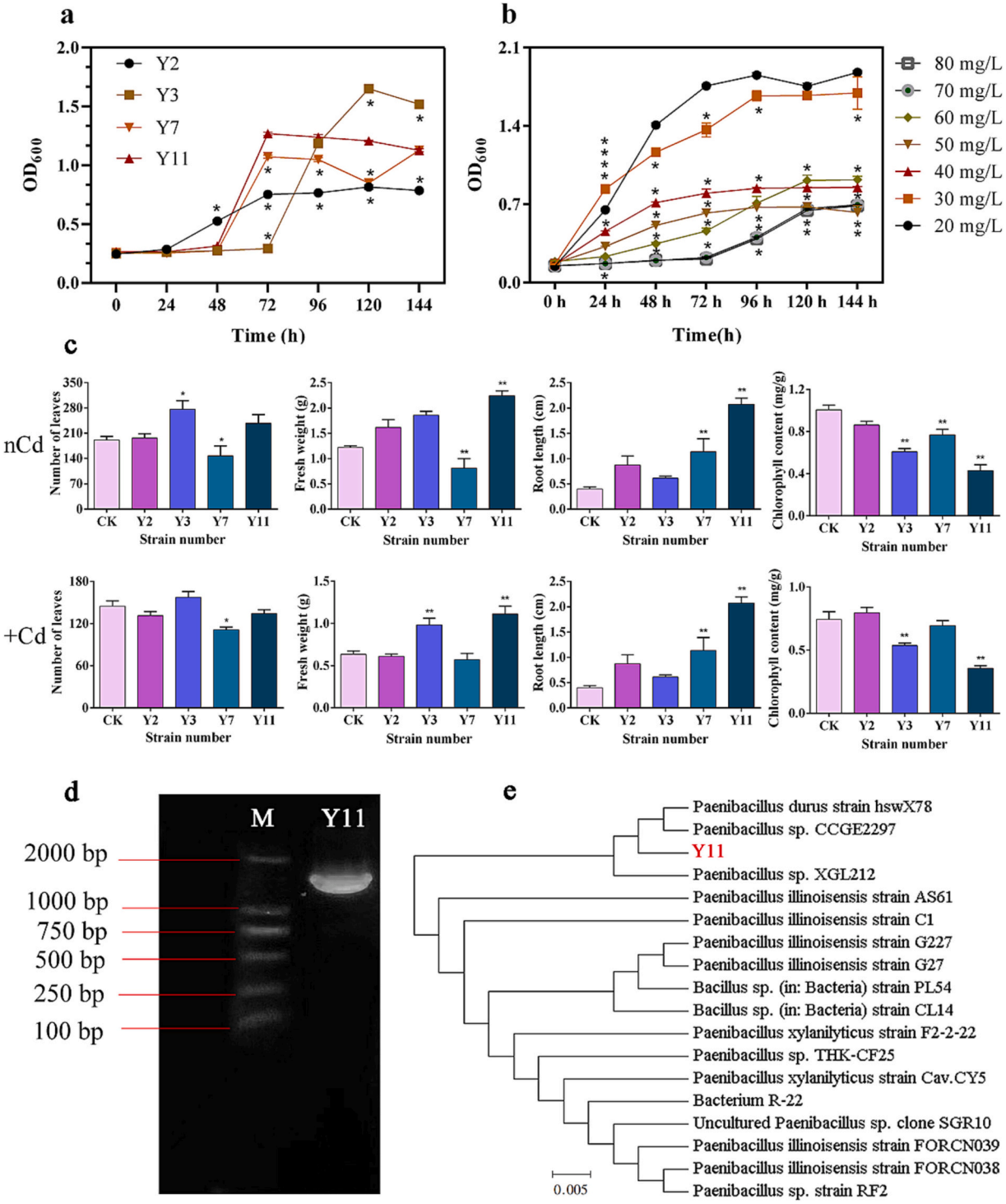

Fig. 5. Related growth indicators and identification of endophytic species of duckweed.

biomass of the strain competing with duckweed for nutrients (Fig. 5c).

3.5.3. *Analysis of 16S rDNA sequences of endophytic bacterium Y11*

The 16S rDNA sequence of the endophytic bacterium Y11 was amplified to 1455 bp (Fig. 5d). The genetic distance between the 16S rDNA sequence of endophyte Y11 and the full 16S rDNA sequence of the 17 bacterial strains with the highest homology was calculated, and a phylogenetic tree was constructed based on the genetic distance (Fig. 5e). As illustrated in Fig. 8b, the endophytic bacteria exhibited close homology with several strains of *Paenibacillus*. These results indicate that the isolated strain was *Paenibacillus* sp. Y11.

4. Discussion

As an abiotic stressor, Cd can stimulate oxidative stress in plants, induce lipid peroxidation, and increase the accumulation of ROS, leading to oxidative damage (Farooq et al., 2020). In addition, Cd can cause slow growth, decreased chlorophyll content, and impaired photosynthesis rates in plants (Jambunathan, 2010). Antioxidant enzymes in plants, which are important components of the antioxidant defense

system, can help scavenge ROS, including SOD, CAT, and POD, thus reducing plant damage (Yang et al., 2021b). In a study on the Cd hyperaccumulator *Pterocypsela laciniata*, POD activity increased with increasing Cd concentrations, whereas CAT activity increased and then decreased, which is consistent with the results of this study (Zhong et al., 2019). SOD is the first line in the antioxidant defense system and is capable of reacting with O_2^- to form H_2O_2 and O_2 (Alfonso-Prieto et al., 2009; Farooq et al., 2020). In the aquatic plant *Bacopa monnieri L.*, SOD activity first increased and then decreased with prolonged Cd stress, which is consistent with the results of this study (Singh et al., 2006). These results indicate that the antioxidant enzyme system of duckweed plays an important role in coping with Cd-induced oxidative stress.

Cd pollution has always been an important topic in the field of heavy metal pollution, and phytoremediation technology has been gradually applied to control Cd pollution (Genchi et al., 2020). Four submerged plants, *Hydrilla verticillata*, *Myriophyllum verticillatum*, *Ceratophyllum demersum*, and *Potamogeton crispus*, were enriched by 27.89, 15.28, 22.54, and 32.74 mg/kg Cd, respectively (Wang et al., 2018). Plants that are submerged for long durations have root system degradation, require high water quality requirements, and are currently mainly used in the management of eutrophication (Wu et al., 2021). In contrast, duckweed floats on the water surface and has a strong adaptability to water quality and a well-developed root system that can actively absorb heavy metals from the water (Ekperusi et al., 2019). The wetland plant *Polygonum hydropiper* L. enriched <60 mg/kg of Cd (Ge et al., 2020). Another aquatic plant, *Canna indica L*, exhibited the highest Cd enrichment at 58.69 mg/kg (Solanki et al., 2018). In this study, *L. minor* 0014 enriched up to 264.60 mg/kg Cd, which is noticeably higher.

The Cd concentration in the environment has a significant influence on its accumulation in plants (Yu et al., 2021). BCF not only accurately reflects the enrichment ability of plants but also reduces the possible impact of concentration (Khan et al., 2020). The BCF of the emergent plant *Nasturtium officinale R.Br* is 2.38–5.98 (Jin et al., 2015), whereas that of *Satureja hortensis* L. is only approximately 0.4 (Azizollahi et al., 2019). In addition to the Cd concentration, the duration of treatment may affect the enrichment effect of the plant. Generally, the longer the treatment duration, the greater the enrichment of heavy metals as the plants maintain normal growth (Ge et al., 2020). The experimental period of this study was 7 d, which is generally considered the shortest experimental period, and the enrichment effect of plants in a short period could be judged by the 7 d treatment. *Chlorophytum comosum* exhibited a BCF of <100 after 50 d of treatment (Wang et al., 2021a). *Polygonum hydropiper* L. was treated for 30 d, and the highest BCF was 111.69 (Ge et al., 2020). The BCF of *L. minor* 0014 reached a maximum of 115.97 after only 7 d, indicating that duckweed can enrich a large amount of Cd in a short period. Therefore, duckweed is an excellent material for the remediation of Cd-polluted water.

In addition to plant enrichment, the removal rate can be obtained by measuring the amount of heavy metals in the environment before and after the experiment, which is a visual indicator (Bora et al., 2020). *Juncaceae*, *Eichhornia crassipes*, and *Oenanthe javanica* were mixed and treated with 5 mg/L Cd for 7 d, with a removal rate of 68.14 % (Chen et al., 2021). Seven plants, including *Thalia dealbata*, *Juncaceae effusus*, *Lythrum salicaria*, *Schoenoplectus tabernaemontani*, *Iris sibirica*, *Pontederia cordata*, and *Sagittaria sagittifolia*, were combined in groups of three under 0.41–3.87 mg/L Cd treatment conditions for 75 d, and the final removal rate reached 73.85–85.52 % (Wang et al., 2021b). The maximum Cd removal rate of the hyperaccumulator *Pseudacorus Iris* was only 4.53 % (Xu et al., 2022). In another study on duckweed, *Landoltia punctata* also achieved a maximum removal of 75 % with 0.5 mg/L Cd treatment for 7 d (Wang et al., 2022). In this study, *L. minor* 0014 was treated with 0.1–5.0 mg/L Cd for 5–7 d and achieved 70–90 % removal within a short treatment time with satisfactory effects.

Endophytic microbes live asymptomatically inside their hosts throughout the different stages of their life cycle and play crucial roles in the growth, development, fitness, and diversification of plants. The endophyte associations range from mutualism to pathogenicity. These microbes help the host combat a diverse array of biotic and abiotic stressful conditions (Rana et al., 2020). Proteobacteria are the most tolerant microorganisms of heavy metals. High concentrations of heavy metals and toxic substances are sources of energy and nutrients for these microorganisms; therefore, they can promote the growth of duckweed by tolerating or using the pollutants (Drzewiecka, 2016). Proteobacteria encode multiple heavy metal oxidase genes involved in heavy metal resistance, thus providing the possibility of phytoremediation by duckweed (Altimira et al., 2012). Firmicutes, a symbiotic bacterium, play an important role in trophic turnover, and Cd exposure leads to a significant increase in the relative abundance of Firmicutes (Moron et al., 2019; Tinkov et al., 2018). In addition, heavy metal resistance genes have been found in the plasmids of endophytic bacteria of this phylum (Lanza et al., 2015). Firmicutes and Proteobacteria were also found as the dominant phyla in the soil of the mining area, due to their heavy mtals tolerant gene groups (Zhao et al., 2019). Liu et al. (2022b) found that following Firmicutes and Proteobacteria, the phyla were dominated by Actinobacteria, which could decompose a variety of organic compounds and use different carbohydrates as a source of energy. In this study, the species and abundances of Proteobacteria and Firmicutes were higher in the Cd treatment group, with Proteobacteria being the most dominant phylum under all treatment conditions, followed by Firmicutes. In addition, compared to the control group, the abundance of Proteobacteria was higher in the Cd-treated group. Proteobacteria may play an important role in Cd resistance. These results suggest that the resistance of duckweed to Cd was improved by changing the abundance of endophytic bacterial communities under heavy metal stress.

Few studies on the isolation of endophytic bacteria from duckweed have been reported, and the reported growth-promoting strains of duckweed are mainly rhizosphere growth-promoting strains, whereas few microbial strains are available for screening plant growth from endophytic bacteria (Ishizawa et al., 2017). Endophytic bacteria have strong colonization and targeting abilities. When planted back into the host plant, they can enter the plant through the root system to achieve stable colonization and perform related functions. Therefore, endophytic bacteria hold greater advantages than rhizosphere microorganisms (Saha et al., 2016). Previous studies have shown that many *Paenibacillus* species can promote crop growth directly via biological nitrogen fixation, phosphate solubilization, production of the phytohormone indole-3-acetic acid, and release of siderophores that enable iron acquisition (Grady et al., 2016). Microbiome analyses have revealed enrichment in the genus *Paenibacillus* under different adverse conditions, which is often accompanied by improved growth conditions (Langendries and Goormachtig, 2021). *Paenibacillus* sp. Y11 isolated in this study not only had high Cd tolerance but also significantly promoted plant growth, probably because heavy metal ions entered the cells for chelation through the transporter protein on the surface of the bacterium, reducing the toxic effects of the heavy metals on plants. The results showed that the strain provided the possibility for the future plant microbial joint remediation of environmental pollution.

5. Conclusions

In this study, high Cd concentrations significantly inhibited duckweed growth and were accompanied by a decrease in chlorophyll content. The POD activity and CAT activity decreased with increasing stress time, while SOD activity increased and then decreased, and MDA content increased. In terms of stress concentration, the higher the concentration (0.1–5.0 mg/L Cd), the higher the POD and MDA content, the more O_2^- accumulated, and the more serious the damage to *L. minor* 0014. In addition, lower Cd concentration (0.1–0.5 mg/L) had little effect on *L. minor* 0014, and high concentration (0.7–5.0 mg/L) had stronger growth inhibitory effect on *L. minor* 0014. The cadmium content in duckweed increased up to 264.6 mg/kg as the cadmium stress duration and concentration increased, whereas the cadmium removal

rate increased up to 70–90 %. 0.1–1.0 mg/L Cd treatment for 3 days, *L. minor* 0014 removed 60–68 % of Cd, and the removal rate reached >70 % after 7 days, indicating that *L. minor* 0014 also had a good removal effect for low concentration of Cd. A total of 488 OTUs were identified by high-throughput sequencing of endophytic bacteria of duckweed, and the dominant groups were Proteobacteria, Firmicutes, and Actinobacteria. The Cd treatment also affected the diversity of duckweed microbial communities. The endophytic bacteria were isolated and 22 strains were obtained, four of which were tolerant to 80 mg/L Cd. The isolated Cd-tolerant endophytic strain *Paenibacillus* sp. Y11 significantly increased duckweed biomass. Our results indicate that colonization of endophytes can help plants effectively respond to Cd stress, laying the foundation for future joint plant-microbe remediation of heavy metal pollution.

CRediT authorship contribution statement

Xiao Yang: Investigation, Methodology, Writing – original draft. **Ai-Juan Tan:** Supervision, Methodology. **Meng-Meng Zheng:** Writing – original draft. **Dan Feng:** Investigation, Writing – original draft. **Kang Mao:** Methodology. **Gui-Li Yang:** Supervision, Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166056.

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