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Preliminary Study on Screening and Genetic Characterization of Lactic Acid Bacteria Strains with Cadmium, Lead, and Chromium Removal Potentials

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Abstract: Due to industrial development, heavy metal pollution has become a severe global health hazard. The bioadsorption method represented by the adsorption of lactic acid bacteria (LAB) has been widely employed. The purpose of this study is to screen LAB strains that can remove cadmium, lead, and chromium. Through the heavy metal resistance tests, four strains with significant growth inhibition rate were identified. After 16S rDNA sequencing, these resistant strains were identified by *Lactobacillus helveticus* KD-3 (Cd^{2+} removal rate 37.54 ± 0.85%), *Limosilactobacillus fermentum* B27 (Pb^{2+} removal rate 69.41 ± 0.19%), *Lacticaseibacillus rhamnosus* 7469 (Cr^{6+} removal rate 71.13 ± 0.97%), and *Lb. helveticus* K5. Three encoding genes were identified in our screen strains, namely resistance gene *czcD*, chromium resistance gene *chrA*, and lead resistance gene *pbrT*. *L.helveticus* KD-3 exhibited the best comprehensive performance. Given the diverse types of heavy metal pollution at present, the current research mainly focuses on the removal of a single heavy metal by one strain. The four strains enrich the absorption resources of LAB for heavy metals, paving a new way for the biosorption of various heavy metals in food by LABs.

Keywords: lactic acid bacteria; removal rate; 16S rDNA; heavy metal resistance gene

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1. Introduction

In recent decades, with the rapid development of urbanization and industrialization, heavy metal pollution has become a global problem. Heavy metals may enter the human body through the food chain, bringing health risks to humans. Heavy metal poisoning leads to vomiting, fatigue, and weakened immunity, and can result in various malignant tumors and chronic diseases [1]. Therefore, controlling the accumulation of heavy metals in the intestinal tract of animals has become a current research hotspot [2].

The adverse effects of heavy metals cadmium, lead, and chromium on organisms have been well documented. The toxicity of heavy metals depends on their bioavailability and their uptake by organisms. Due to the fact that heavy metals do not degrade and will continue to exist indefinitely, their excess will have harmful effects on the ecological environment [3]. Excessive intake of cadmium can cause flu-like symptoms (fever, muscle pain). Cadmium readily accumulates in invertebrates, and may also cause kidney failure in birds and mammals [4,5]. Lead has a damaging effect on the brain, especially on children. It also causes high blood pressure, anemia, and stunting of growth [6,7]. Chromium is destructive to kidneys, bones, and liver, as well as causing numbness in the extremities and mental disorders [8].

At present, the common method for reducing heavy metals is through physical and chemical methods, such as physical adsorption of activated carbon, chelation therapy with chelating agents such as EDTA, and membrane separation. However, these methods have problems in technical dependence, high cost, and operational difficulty. Worse still, they can also cause secondary environmental pollution. Simple physical and chemical methods cannot efficiently remove lead, cadmium, and chromium from the environment [9]. Compared with those methods, adopting microbial methods to remove heavy metals has the characteristics of low cost and environmental friendliness. Microbial treatment has significant advantages, such as high processing capacity, strong adaptability to the environment, low cost, and environmental friendliness. Fungi are microorganisms with the ability to remove heavy metals from nature chitin, polyphosphoric acid, and other components in of fungal cell walls can combine with heavy metals [10]. Lactic acid bacteria (LAB), as commensal microbes, play an important role in the human gut [11]. Heavy metal removal technology based on LAB has been of great concern [12]. Studies have shown that *Lactiplantibacillus* can adsorb lead and cadmium ions in vitro [13].

LAB are gram-positive bacteria that produce a variety of metabolites, including organic acids, fatty acids, bacteriocins, and exopolysaccharides (EPS) [14]. Among the metabolites, EPS can protect microorganisms from heavy metal poisoning and is exploited as an important heavy metal bioremediation tool [15]. At present, LAB that can remove cadmium includes strains of lactobacilli, weissella, enterococcus and bifidobacterium [16]. These strains that adsorb heavy metals were isolated from polluted sludge, pig and chicken manure, intestinal substances, and other medias [17,18]. Fu et al. [19] used *Lpb. plantarum* CICC21805 and *Pediococcu pentosaceus* CICC22737 as fermentation strains to remove cadmium from rice, and the cadmium removal rate of fermented rice flour reached 85.73%. Lin et al. [20] found that 2 strains (*Pseudomonas aeruginosa* and *Enterobacter cloacae*) that could scavenge various metal ions such as lead, cadmium, and copper. Xia et al. [21] proved that the adsorption efficiency of *L. reuteri* 21008 on Pb^{2+} in an aqueous solution reached 84.23%. Most research has focused on the removal of single heavy metals by LAB, but barely on multiple heavy metals.

In our previous study, 66 LAB strains were screened to adsorb heavy metal cadmium [22], and 9 cadmium-resistant strains were identified. In this study, screening tests were carried out to identify strains with the potential to remove complex heavy metals. The identified strains were analyzed at the molecular level to identify key resistance genes.

2. Materials and Methods

2.1. Microorganism and Materials

LAB strains L19, LB6, 7469, S73, KD-3, L4, 22, K5, and B27 were isolated from fermented foods and kefir grains. All strains were provided by Shaanxi University of Science and Technology [22]. All strains were inoculated in MRS broth medium and grown at 37 °C for 24 h.

Cadmium solution: 2.744 g cadmium nitrate (Cd $(NO_3)_2 \cdot 4H_2O$) (AR) is dissolved in dilute nitric acid, and diluted to 100 mL with sterile water (10 g/L), then filtered through a sterile filter membrane and stored at 4 °C. Lead solution: 1.598 g lead nitrate (Pb $(NO_3)_2$) (AR) is dissolved in 10% dilute nitric acid, and then diluted with sterile water, and filtered through a sterile filter membrane and stored at 4 °C. Chromium solution: 1.4315 g potassium dichromate (AR) is dissolved in sterile water, and then nitric acid is diluted to 100 mL. After filtering using a membrane, a 1 g/L chromium solution was produced and preserved [23].

2.2. Screening of Lead-Resistant Lactobacilli

In total, 2 levels of high-concentration lead and low-concentration lead were designed to screen lead-resistant strains. The growth of LAB in a lead ion-containing environment was characterized by the OD value, pH value, and the number of viable bacteria. The 9 strains were separated, purified, and cultured, and the lead ion solution was diluted

and added to 10 mL MRS medium to prepare 60 and 80 mg/L lead-containing medium, respectively. Lead-containing medium without adding heavy metal lead ions is used as a blank control. The cultured LAB was sampled at 0, 3, 10, 15, 18, 21, and 24 h, and the absorbance value of the bacterial liquid was measured with a full-wavelength multifunctional scanner to reflect the growth of LAB under low-concentration conditions [23,24]. Under the same conditions, the test strains were connected to a medium containing 500 mg/L lead ions to monitor the dynamic changes of pH at 0, 12, 15, and 18 h of LAB growth, and the dilution coating plate method was used to determine the high concentration of each test strain. The number of viable bacteria cultured in a lead ion environment for 24 h and the bacterial solution free of heavy metal lead ions was used as a control to judge the resistance of different types of LAB to lead ions.

The pH value was determined with a PHS-3C pH meter (Shanghai Jingke Instrument Co., Ltd., Shanghai, China).

Dilution coating plate method: after 24 h of cultivation, the bacterial solution is uniformly diluted, and 1 mL is taken with a sterile syringe, and evenly spread on the MRS agar medium. The viable bacteria are counted after 48 h.

2.3. Screening of Chromium-Resistant Lactobacilli

Screening for chromium-resistant lactobacilli, as previously described, with slight modifications [24]. Simultaneously, 9 strains were inoculated into a medium containing 300 and 500 mg/L heavy metal chromium. The medium without chromium was used as a control group. After 24 h of cultivation, the OD600 value was measured, and the inhibition rate was calculated. The effect of a high concentration of chromium on the growth of LAB was monitored.

$$\text{Inhibition rate (\%)} = 100\% \times [1 - \text{OD600 (sample)}/\text{OD600 (blank)}] \quad (1)$$

2.4. Determination of Minimum Inhibitory Concentration (MIC) of Compound Heavy Metal Resistant Lactobacilli

The three kinds of heavy metal-resistant LAB culture solution were selected according to the method in [25], and dilute it to a certain multiple. Coating 1mL LAB culture solution on the medium containing Cd^{2+} (0, 30, 50, 100, 150 mg/L), Pb^{2+} (0, 100, 500, 1000, 1500 mg/L), and Cr^{6+} (0, 100, 500, 1000, 1500 mg/L), 48 h later the viable bacteria were counted to observe the growth. The plate with colony number between 30~300 CFU and non-spread colony growth was selected to count the total number of colonies [26].

2.5. Heavy Metal Removal Performance of Resistant Strains

Atomic absorption spectroscopy (AAS) was used to test the single heavy metal removal capacity of the screened resistant strains [27]. According to the previously reported methods, the heavy metal removal performance of resistant strains has been slightly changed [22]. The initially screened resistant strains were placed in MRS medium containing 60 mg/L of Cd^{2+}, Pb^{2+}, and Cr^{6+} at an inoculum amount of 5% (v/v). The metal culture medium was used as a blank control, cultured at 37 °C for 24 h, the pH of the culture solution was measured and then centrifuged at 8000 rpm for 10 min to determine the wet weight of the bacterial sludge. Digest according to the standard procedure of the instrument (digest for 10, 15, and 25 min at 120, 150, and 190 °C, respectively), take it out after cooling, heat in a water bath at 100 °C for 30 min, dilute to 10 mL with water, take 1 mL and add 9 mL of water. After filtration with a 0.45 μm sterile filter membrane, put it into a 10 mL centrifuge tube and store the sample to be tested in a refrigerator at 4 °C. The absorbance value of each sample was measured by a flame atomic spectrophotometer, and each sample was measured in paralleled 3 times. The calculation formula for removal rate and adsorption capacity is as follows:

$$\text{Removal rate (\%)} = 100 \times (C_0 - C_1)/C_0 \quad (2)$$

$$\text{Adsorption capacity} = (C_0 - C_1)/m \times V \quad (3)$$

where C_0 (mg/L) represents the initial concentration of metal ions in the solution; C_1 (mg/L) represents the concentration of residual metal in the supernatant after adsorption; m is the wet weight of the bacteria (g); V represents the volume of the solution (L).

After all bacterial resistance and adsorption tests, the pH value and wet weight of the bacterial cells were measured, respectively, and the growth of LAB in the process of metal-binding was observed.

2.6. Resistance Gene Testing

Absolute quantitative fluorescence PCR has the advantage of accurate test results. Referring to the method reported in the past [28], five strains K5, S73, KD-3, 7469, and B27 with three types of heavy metal resistance and good adsorption properties were selected for the resistance gene detection tests.

According to the currently reported microbial heavy metal resistance gene sequence, three pairs of primers including the *czcD* gene were designed and synthesized for heavy metal cadmium, the *pbrT* gene for heavy metal lead, and the *chrA* gene for heavy metal chromium. The design results are shown in Table 1. The total DNA of the resistant strains was amplified with the above genes, and the fluorescent quantitative PCR products were designed. Absolute quantitative fluorescence PCR was used to study the causes of resistance of lactic acid bacteria.

Table 1. The primer and sequence of target gene.

Resistance Gene	Primer Name	Sequence
pbrT	pbrT-F	AGCGCGCCCAGGAGCGCAGCGTCTT
	pbrT-R	GGCTCGAAGCCGTCGAGRTA
chrA	chrA-F	TGGCTCTCGCTGTTCTTTGT
	chrA-R	TAAGTGCGACAAGGGCAACT
czcD	czcD-F	TCATCGCCGGTGCGATCATCAT
	czcD-R	TGTCATTCACGACATGAACC

According to the instructions of AceQ®qPCR SYBR® Green Master Mix (Vazyme Biotech, Co., Ltd.#Q112-02, Nanjing, China), the real-time quantitative PCR reaction was performed, using a real-time fluorescence quantitative analyzer (Triplex International Biosciences, TIB-8600, Xiamen, China). The reaction conditions are as follows: 95 °C, 5 min; 95 °C, 10 s, 60 °C, 30 s, 40 cycles. The construction of the standard product refers to the method of Liu [29]. After the reaction, the melting curve analysis of the amplified product is performed to verify the specificity of the amplified product, and a standard curve is drawn according to the C_t value and the amplification curve. At least 3 parallel samples were set for each target gene in each experiment, and the experiment was independently repeated 3 times.

2.7. Identification of Strains

With reference to the previously reported methods [22], DNA was extracted from the selected strains. 16S rDNA sequence analysis was adopted and the strain homology comparison was analyzed on NCBI website. MEGA software 6.0 was used to draw phylogenetic trees [30].

2.8. Statistical Analysis

The data from three replicated trials for each treatment are expressed as the means with standard deviation (Mean ± SD). The analysis of variance (ANOVA) is used to evaluate the significant differences among the values ($p < 0.05$). The figures and tables are drawn using the Origin 2019 software (Version 2019, USA) or Microsoft Excel (Version 2019, USA).

3. Results

3.1. Pb-Resistant Ability of LAB Strains

The lead resistance of LAB strains was evaluated by monitoring the growth of each strain under different Pb^{2+} concentrations. The results are shown in Figure 1. Compared with the control group without Pb^{2+}, the growth was barely significantly affected. The tested strains have high resistance to Pb^{2+}.

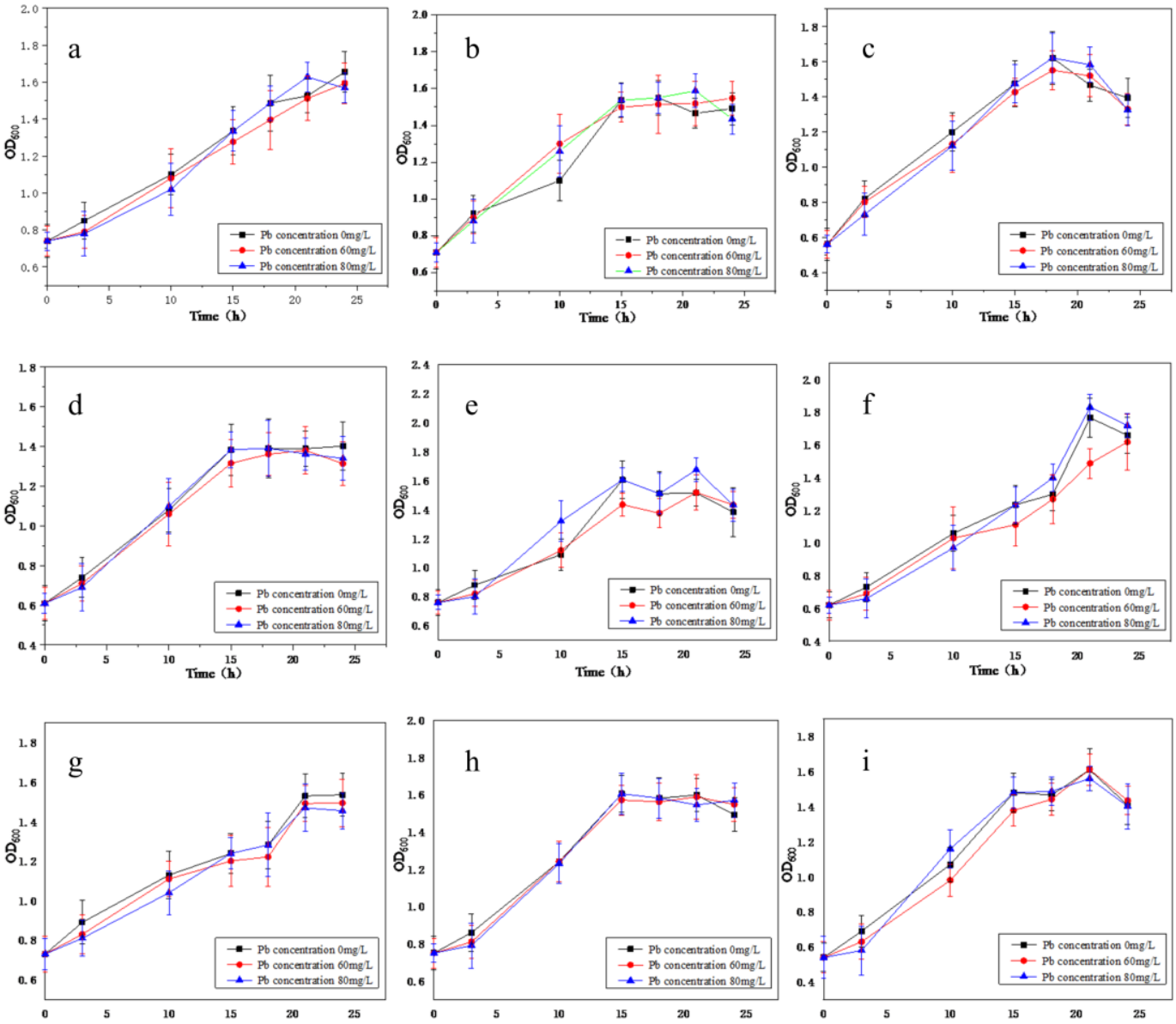


Figure 1. The effect of different Pb^{2+} concentrations on the growth of LAB, (**a**–**i**) indicated strains L19, 7469, S73, KD-3, 4, 22, K5, B27, and Lb6, respectively.

In total, 9 strains of lead-resistant LAB were transferred to solid MRS medium containing Pb^{2+} at a concentration of 500 mg/L. The evaluation of the resistance of different strains to Pb^{2+} was operated by counting the viable bacteria (Figure 2). KD-3, K5, and 22 strains were relatively resistant to Pb^{2+}. According to Bhakta et al. [31], lead-resistant strains were more likely to show better Pb^{2+} remover effectiveness, which has a high lead tolerance and might be used as a delead strain. B27 and L4 were relatively poor, but both had bacterial growth on the medium with a high concentration of lead. This indicates that

LAB has an automatic adjustment mechanism to reduce the damage of Pb^{2+} to the bacteria. All 9 strains were used for Cr^{6+} resistance screening test.

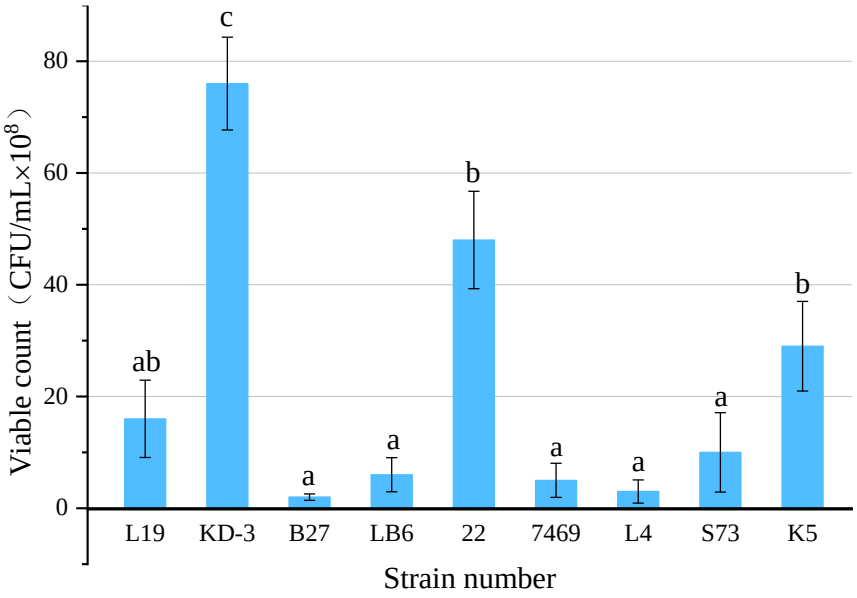


Figure 2. The effect of high concentration Pb^{2+} (500 mg/L) on LAB. Values with different letters in same bar are significantly different ($p < 0.05$).

3.2. Cr-Resistant Ability of LAB Strains

The 9 strains of LAB were tested for chromium resistance, and the results are shown in Figure 3. Except for L19, the growth of the other strains at high chromium concentration (500 mg/L) was significantly inhibited, with an inhibition rate of more than 70%. Under low Cr^{6+} concentration (300 mg/L), the resistance of each strain to chromium shows significant differences. Among them, the inhibition rates of the three strains L19, KD-3, and L4 were less than 40%, demonstrating good chromium resistance.

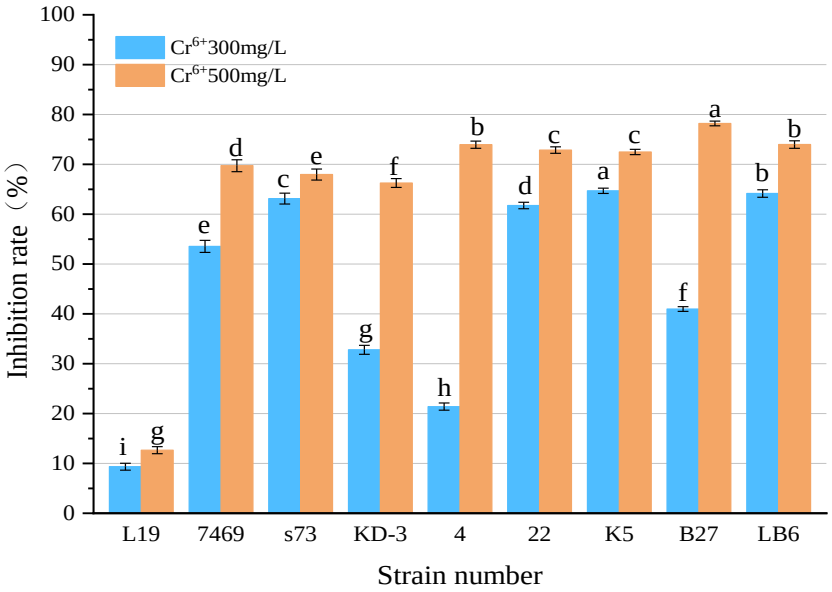


Figure 3. The effect of high concentration chromium salt on the growth of LAB. Values with different letters in same bar are significantly different ($p < 0.05$).

3.3. The MIC of Compound Heavy Metal Resistant Strains

According to the above screening results, the minimum inhibitory concentrations of the 9 tested strains under three complex metal ion environments were evaluated. The results are tabulated in Table 2. In a composite metal environment with Cr^{6+} concentrations of 1500, 150, and 1500 mg/L, only three strains K5, KD-3, and B27 can grow, while the rest strains were completely inhibited. Synthesizing the minimum inhibitory concentration, the tolerance order of the 9 strains to complex heavy metals was: KD-3 > K5 > B27 > 22 > 7469 > L19 > S73 > LB6 > L4.

Table 2. The MIC results of compound heavy metal resistant strains.

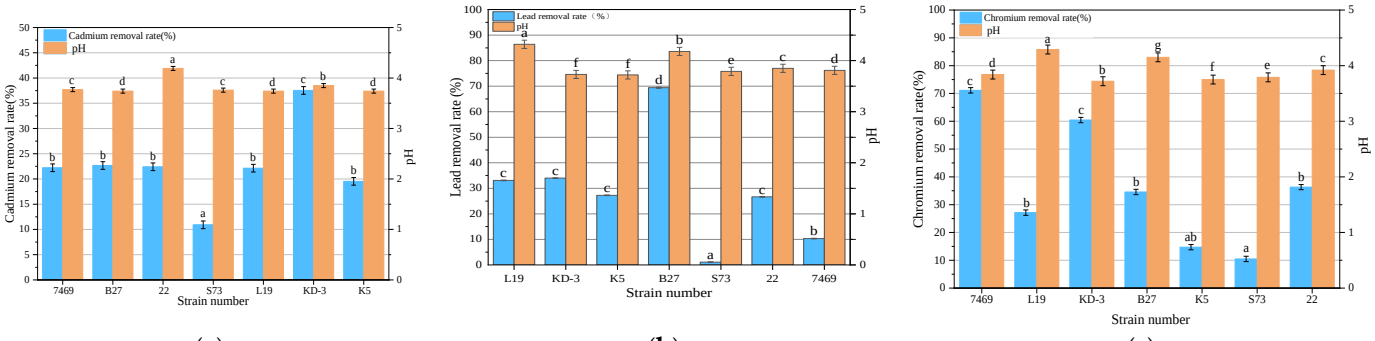
Strain Number	Heavy Metal Concentration (mg/L)					
	Pb^{2+}	0	100	500	1000	1500
	Cd^{2+}	0	30	50	100	150
	Cr^{6+}	0	100	500	1000	1500
K5		+++	+++	+++	+++	++
B27		+++	+++	++	++	+
22		+++	+++	++	+	-
L4		+++	+++	+	-	-
S73		+++	+++	+	-	-
7469		+++	+++	+++	++	-
L19		+++	+++	+	+	-
KD-3		+++	+++	+++	+++	++
LB6		+++	+++	+	-	-

"+++" means that the number of colonies is between 100–300; "++" means that the number of colonies is between 10–100; "+" means that the number of colonies is between 0–10; "-" represents the growth of aseptic colonies on the plate.

3.4. Re-Screening of Heavy Metal Removal Strains

On the basis of the results of heavy metal resistance and MIC, 7 resistant strains, including L19, 7469, S73, KD-3, 22, K5, and B27, were selected for the adsorption tests.

A metal removal rate above 18% is defined as a strain with high adsorption performance [25]. The cadmium removal rate of LAB in MRS medium was determined by atomic absorption spectroscopy. As shown in Figure 4a, strain KD-3 shows the highest cadmium adsorption effect with the rate of 37.54 ± 0.85%, and its adsorption capacity reached 159 mg/g. The strain with the lowest clearance rate was S73 (10.92 ± 0.56%), with an adsorption capacity of only 28 mg/g. The order of cadmium clearance rate is KD-3 > B27 > 22 > 7469 > L19 > K5 > S73. The pH value of the fermented broth after adsorption was used to characterize the growth of the bacteria. From Figure 4a, it can be seen that Cd^{2+} has a greater impact on the growth of the strain 22.



(**a**) (**b**) (**c**)

Figure 4. The adsorption of LAB at 60 mg/L metal concentration (**a**: Cd^{2+}; **b**: Pb^{2+}; **c**: Cr^{6+}). Different letters indicate significant differences between groups, $p < 0.05$.

According to the adsorption results of LAB on chromium ions (Figure 4b), strain 7469 has the best adsorption effect on chromium with a removal rate of 71.13 ± 0.97%, and an adsorption capacity of 342 mg/g. S73 has the worst adsorption effect with the removal rate of 10.49 ± 0.67% and an adsorption capacity of 9.76 mg/g. The comprehensive chromium removal rate ranking is 7469 > KD-3 > 22 > B27 > L19 > K5 > S73. From the results of pH measurement, it can be seen that chromium ions have a significant effect on B27 and L19.

The analysis of the effect of LAB on the adsorption of lead ions is shown in Figure 4c. The strain B27 has the best lead ion adsorption effect with a clearance rate of 69.41 ± 0.19%, and an adsorption capacity of 247 mg/g. The worst strain S73 with the clearance rate of 1.11 ± 0.09% is observed. The order of adsorption effect is B27 > KD-3 > L19 > K5 > 22 > 7469 > S73. From the pH results, it can be seen that lead ions have a significant effect on the growth of B27 and L19, while the growth of other strains is not significantly affected.

3.5. Resistance Gene Identification

3.5.1. PCR Amplification of Heavy Metal Resistance Genes

The PCR amplification was used to identify resistance genes (Figure 5).

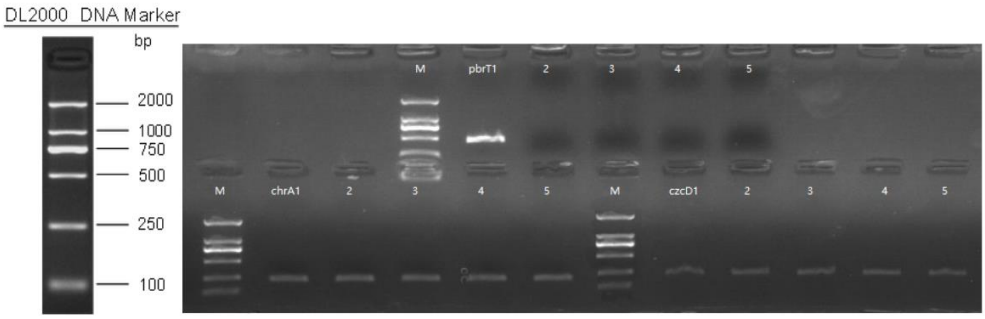


Figure 5. The PCR amplification results of three heavy metal resistance genes, 1, 2, 3, 4, and 5 were strains K5, S73, KD-3, 7469, and B27, respectively.

Figure 5 shows that strain K5 had both Cd^{2+}, Pb^{2+}, and Cr^{6+} resistance genes while strains KD-3, 7469, B27, and S73 have both Cd^{2+} and Cr^{6+} resistance genes. It is found that the heavy metal adsorption performance of LAB is not linearly related to heavy metal resistance. Zhou et al. [32] isolated *Bacillus megaterium* MDS07 from the soil of heavy metal mining areas. The results indicate that the strain is resistant to Cr^{6+}, Zn^{2+}, and Cd^{2+}. Further research has found that the strain contains *chrB*, *czcD*, and other genes, indicating that the heavy metal resistance of this strain is caused by resistance genes. Among the known Cr^{6+} resistance genes, the research on the *chrA* gene is more in-depth. The chromate transporter (ChrA) encoded by the *chrA* gene is a hydrophobic membrane protein that reduces the toxic effect of Cr^{6+} on microorganisms by actively effluxing Cr^{6+} from the cytoplasm or periplasmic space to the outside of the cell [33].

3.5.2. Real-Time PCR of Heavy Metal Resistance Genes

The linear relationship existing in the amplification reaction can be obtained by using standard products of known concentration [34].

The amplification curve shows the first exponential growth stage. During the reaction proceeds, the product growth rate slows down and the reaction enters a plateau phase. If the melting curve has multiple peaks, it proves that the reaction is non-specific or the presence of dimers [35]. From Figure 6, it can be seen that the dissolution curves of the three resistance genes shows a single peak, the amplified products are uniform, and there is no specific peak, which indicated that the results are stable and reliable. The standard curve combined with the amplification curve is shown in Figure 7.

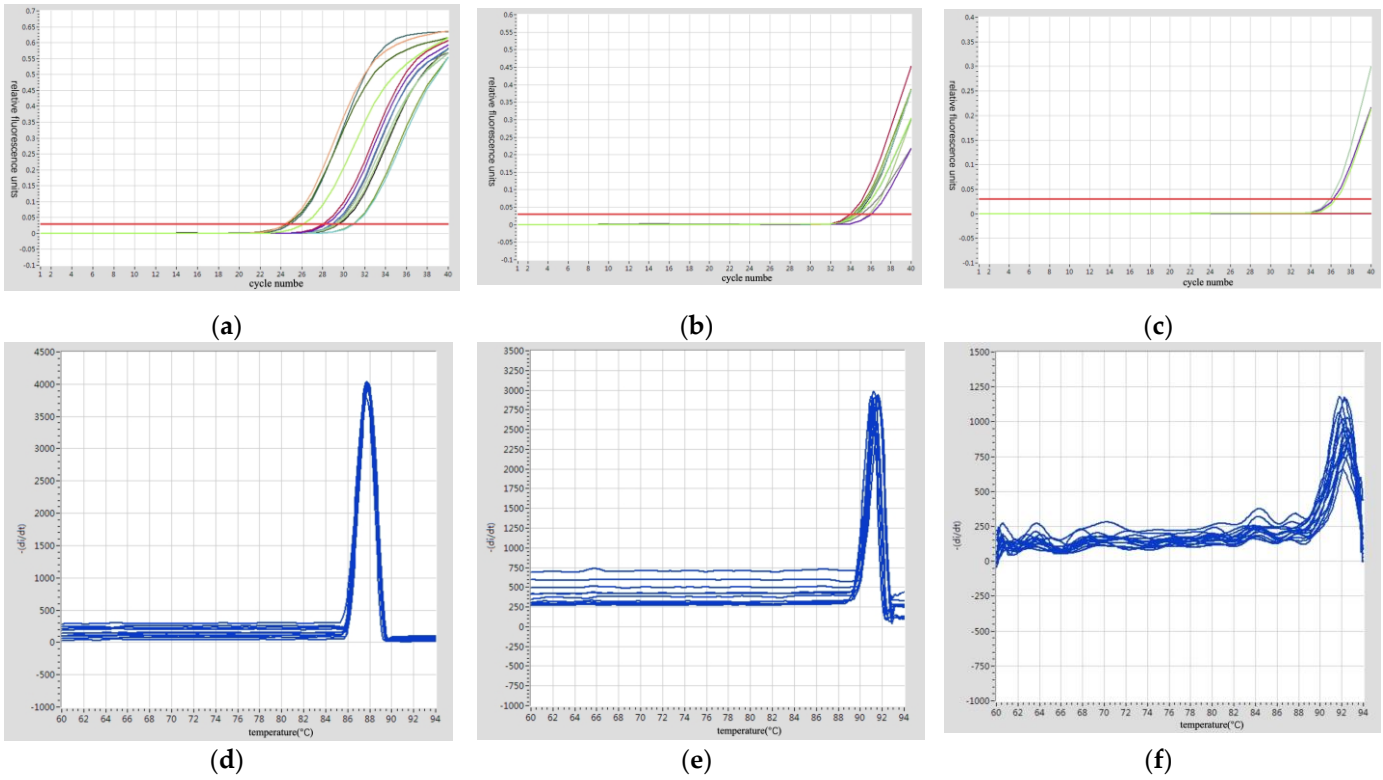


Figure 6. The amplification curve of three resistance genes (**a**: *chrA*, **b**: *czcD*, **c**: *pbrT*), and the dissolution curve of three resistance genes (**d**: *chrA*, **e**: *czcD*, **f**: *pbrT*).

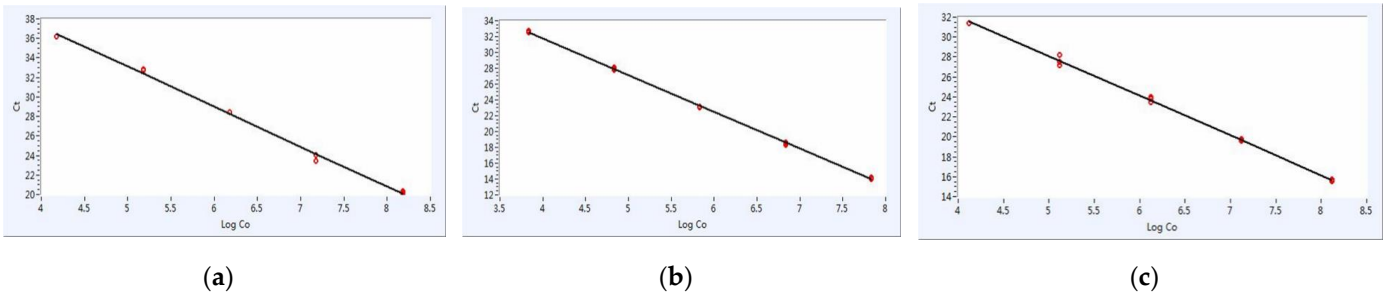


Figure 7. The standard curve of three resistance genes (**a**: *chrA*, **b**: *czcD*, **c**: *pbrT*).

3.5.3. Absolute Quantitative Detection of the Expression of Three Resistance Genes in Different Resistant Strains

Five strains of LAB with different heavy metal adsorption effects were selected for absolute quantitative detection of the expression of three resistance genes *in vivo*. The results are shown in Figure 8a. The expression of cadmium resistance gene fragment *czcD* in strain 7469 reaches 2430.74 copies/μL, which is significantly higher than that in other strains. S73 has the least expression, with an expression level of 1275.47 copies/μL. The order of cadmium resistance gene expression is 7469 > B27 > KD-3 > K5 > S73.

The expression level of chromium resistance gene fragment *chrA* in each strain is shown in Figure 8b. The expression level in strain B27 reaches 3.57×10^5 copies/μL, which is significantly higher than other strains. The expression level in strain 7469 was only 1.01×10^4 copies/μL. The expression sequence of chromium resistance genes is B27 > K5 > S73 > KD-3 > 7469.

The lead resistance gene fragment *pbrT* was only detected in strain K5, and the expression amount was 1.26×10^4 copies/μL. This gene fragment was not detected in the other strains. Based on the analysis of the adsorption of the three heavy metals by the above

strains, the strain 7469 with the best chromium adsorption performance has the lowest expression of the resistance gene, and the strain KD-3 with the best cadmium adsorption effect has the highest expression of the cadmium resistance gene. There is no necessary connection between heavy metal adsorption and heavy metal resistance.

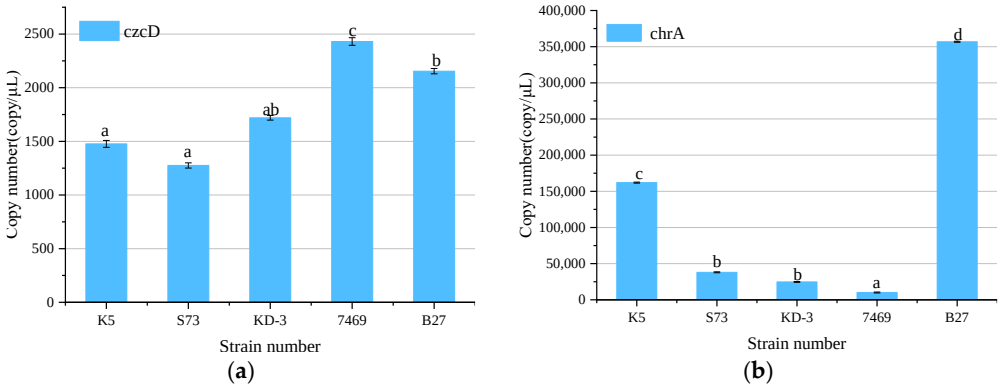


Figure 8. The expression level of $Cd^{2+}$and Cr^{6+} resistance gene in lactobacilli (**a**: Cd^{2+} and **b**: Cr^{6+}). Different letter labels indicate significant differences between groups, $p < 0.05$.

3.6. Homology Analysis of Selected LABs

The selected strain KD-3 with the best cadmium adsorption effect, strain B27 with the best lead adsorption effect, strain 7469 with the best chromium adsorption effect, and strain K5 were adopted for 16S rDNA gene sequence together with other 4 strains. The phylogenetic tree was established, as shown in Figure 9. According to the homology analysis, there are two strains were closely related to *Lb. helveticus*, one is *Lacticaseibacillus rhamnosus*, and the other is *Limosilactobacillus fermentum* (Table 3).

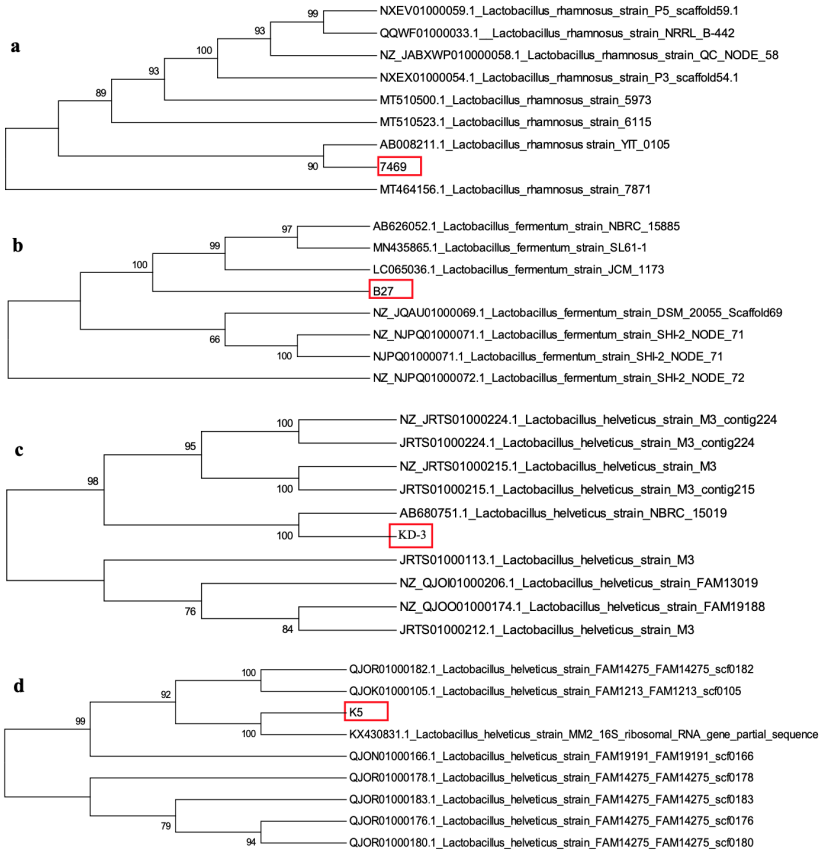


Figure 9. The phylogenetic tree of resistant strains (**a**: 7469, **b**: B27, **c**: KD-3, **d**: K5).

Table 3. Result of four LAB identification by 16S rDNA sequence analysis.

Strain Number	Lactobacilli	Similarity (%)
7469	*Lacticaseibacillus rhamnosus*	100
B27	*Limosilactobacillus fermentum*	99.25
KD-3	*Lactobacillus helveticus*	100
K5	*Lactobacillus helveticus*	99.98

4. Discussion

Heavy metals are one of the most severely polluted substances in the world. Extensive industrial activities have had a huge damaging influence on the environment and human life [36]. It is necessary and important to take immediate and comprehensive actions to eliminate such pollution [37]. In recent years, the use of microbial as adsorbent to remove metal ions has received abundant attentions. As a kind of probiotics, LAB has always been considered to be very beneficial to human health [38]. In recent years, many studies have shown that LAB can promote plant health, and effectively combine and absorb heavy metals [39]. Before being employed as adsorbents to remove heavy metal ions, LABs are screened, isolated, enhanced, and cultivated [40]. At present, most of the studies are focused on the removal of single heavy metals by LAB, but many kinds of heavy metals often exist at the same time in nature. We want to screen the LAB which can remove cadmium, lead and chromium. Our study evaluated 9 LABs with potential of cadmium-resistant in the aspects of three metal ion adsorption tests and heavy metal resistance gene detection. 4 strains with anti-cadmium gene *czcD* and anti-chromium gene *chrA* were obtained, and identified as *Lb. helveticus* KD-3, *Lacticaseibacillus rhamnosus* 7469, and *Limosilactobacillus fermentum* B27, respectively. *Lb. helveticus* K5 contained the anti-lead gene *pbrT*.

In our study, the removal rate of cadmium from MRS medium by *L. helveticus* KD-3 was 37.54 ± 0.85%, significantly higher than that observed by Zhai [41]. When the initial concentration of cadmium is 50 mg/L, the maximum removal rate of cadmium by *Lpb. plantarum* CCFM8610 is 31.34%. *L. helveticus* KD-3 has a maximum removal capacity for cadmium of 159 mg/g, which is much higher than *Bifidobacterium Longum* with a cadmium removal capacity of 54.7 mg/g [42]. The mixed *L. plantarum* and *Pediococcus pentosaceus* at a ratio of 2:1 with 3% inoculum and fermenting at 40.8 °C for 23.4 h shows an effective cadmium removal result from rice [19]. Due to the optimized fermentation process, the cadmium adsorption rate of strains increases significantly, and the removal rate of cadmium in rice flour reached 85.73%. In the future, we will continue to study the removal of cadmium by mixed fermentation of L. *helveticus* KD-3 and other bacteria. *L. rhamnosus* 7469 had the best removal effect on chromium, and the removal rate reached 71.13 ± 0.97%. Li et al. [23] isolated *L. plantarum* P1 from pickle samples in the Sichuan mining area and found that it had a high tolerance to Cr^{6+}. The adsorption rate of Cr^{6+} can reach 61%. In our study, the removal rate of chromium is higher than theirs. However, there is little research on using of LAB to remove chromium. In our study, *L. fermentum* B27 has a maximum removal rate of 69.41 ± 0.19%, and the tolerance concentration for lead ions is higher than 1500 mg/L. Its removal capacity is 247 mg/g, which is higher than that of *Lactiplantibacillus plantarum* X7021 reported by Zhang et al. with 38% chromium removal rate [43]. Bhakta et al. [31] screened *L.reuteri* Pb71-1 from the guts of heavy metal-affected fish, and the lead removal rate in MRS medium could reach 59%. Halttunen et al. [42] found that the adsorption capacity of *L.fermentum* ME3, *B.longum* 46, and *B.lactis* Bb12 for heavy metals changed with pH value, with the maximum removal capacity for cadmium and lead occurring at pH 6. The change in pH during fermentation may affect the removal capacity of LAB.

Synthesizing the minimum inhibitory concentration, the tolerance order of 9 strains to complex heavy metals is: KD-3 > K5 > B27 > 22 > 7469 > L19 > S73 > LB6 > L4. The experimental results show that the order of adsorption effect of lead ion is B27 > KD-3 > L19 > K5 > 22 > 7469 > S73. L19 has a good effect on the removal of lead, but its tolerance to lead is not very high. The results of the heavy metal removal test indicate that L19 has a

better removal effect on lead. This suggests that there is no positive correlation between the removal capacity of LAB and the tolerance to three heavy metals. Consistent with previous reports [23,41], in zhai's study, although *Lacticaseibacillus casei* CCFM30 had a good removal capacity for cadmium, its MIC for cadmium was only 50 mg/L due to the mechanisms of interaction between MIC and heavy metal adsorption.

In recent years, the research on heavy metals by LAB is mainly focused on strain screening, but there is no comprehensive and detailed theoretical system for the adsorption mechanism of LAB [24]. Some functional groups (such as -COOH and -OH) in *L. plantarum* exopolysaccharides have been found to be involved in the adsorption of Pb^{2+} [44]. Feng et al. [45] isolated *L. plantarum* 70810EPS from traditional Chinese kimchi and demonstrated that functional groups such as -OH, -NH2, and COO- were involved in the adsorption. Many studies have revealed that the surface of LAB is rich in negative electron groups and other components, which have good adsorption and removal effects on heavy metals. Zhao [46] shows that surface electrostatic interaction, complexation reaction, ion exchange, and intracellular accumulation are the main mechanisms of Pb^{2+} adsorption by *E. hirae* Qaa and *P. pentosaceus* Fe^{3}. At the same time, some macromolecule substances, such as nucleic acids, phosphates, polysaccharides and S-layer proteins, and fatty acids, are also involved in the adsorption process. The cellular components of LAB are involved in the interaction between Cd^{2+} and LAB because Cd^{2+} seriously damages the microstructure of cells. Cadmium adsorption mechanisms include extracellular complexation, ion exchange, physical adsorption (electrostatic attraction), microprecipitation (extracellular and intracellular), and intracellular diffusion [47]. In addition, extracellular polysaccharides (EPS) exist in LAB cells, and their structures play a direct role in removing toxins and heavy metals in various ways [48]. We will use Fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), energy dispersive X-ray analysis (EDX), X-ray photoelectron spectroscopy (XPS) and other methods to analyze the changes of cell morphology and surface elements before and after the adsorption of three kinds of heavy metals, and further explore and study the mechanism of bacteria adsorbing heavy metals [45,46,49–51].

At present, the microorganisms with high tolerance to heavy metals are most screened from the environment, but rarely from food. All strains in our study were screened from natural kefir and fermented foods. The in-depth study of their heavy metal adsorption characteristics and mechanisms will be beneficial for exploring their potential applications in fermented foods and probiotics. Additionally, the tolerance mechanism of LAB under heavy metal stress will be studied. The development of LAB for heavy metal adsorption is beneficial to the environment and food safety.

5. Conclusions

Through the anti-lead and chromium test of 9 strains of cadmium-resistant lactobacilli isolated in our laboratory, most of the strains have an extremely high tolerance to lead and chromium ions. The MIC test screened out strains tolerant to composite heavy metals. In the composite metal environments with Pb^{2+}, Cd^{2+}, and Cr^{6+} concentrations of 1500, 150, 1500 mg/L, respectively, only K5, KD-3, and B27 are tolerable. In the adsorption test, the lactobacilli exhibits good adsorption effects on three heavy metals at a concentration of 60 mg/L. The strains with better cadmium ion adsorption performance are KD-3, B27, and 22. The strains with better chromium ions removal performance are 7469, KD-3, and 22. The strains with better lead ion removal performance include B27, KD-3, and L19.

Five strains of lactobacilli with significantly different removal effects were selected for real-time fluorescent PCR quantitative expression test. The cadmium-resistant gene *czcD* and the chromium-resistant gene *chrA* were detected in all 5 strains, while the lead-resistant gene *pbrT* was only detected in strain K5. The identification results indicate that KD-3, 7469, B27, and strain K5 containing three resistance genes have the best adsorption effect on three heavy metals. KD-3 and K5 were identified as *Lb. helveticus*, 7469 as *Lacticaseibacillus rhamnosus*, and B27 as *Limosilactobacillus fermentum*.

Most of the current studies focus on one strain to remove single heavy metals. This study obtained strains that can simultaneously remove three types of heavy metals, and their performance is better than the results reported in the literature, providing a new way for the LABs on biosorption of multiple heavy metals in food.

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