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Effect of Pretreatments on bioremoval of metals and subsequent exposure to simulated gastrointestinal conditions

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ORIGINAL ARTICLE

Abstract

Water contamination with heavy metals is increased due to environmental contaminants. Arsenic, cadmium, mercury, and lead are well-known toxic heavy metals for humans. *Lactobacillus acidophilus* is an ideal absorbent for the removal of metals from drinking water. In this study, the ability of treated and untreated *L. acidophilus* ATCC 4356 to remove four heavy metals, simultaneously, from multi-metallic contaminated water in 24 h was investigated. In addition, the stability of the bacteria–metal complexes was evaluated in simulated gastrointestinal tract conditions. According to the results, untreated *L. acidophilus* could remove 99.01% and 92.35% of mercury and lead in water, respectively (initial concentration of 700 $\mu g.L^{-1}$; inoculum size of 2.6×10^{12} $CFU.mL^{-1}$; pH 4; 37°C; 24 h), whereas removal of arsenic and cadmium, under the same conditions, was 91.28% and 61.91% by heat and NaOH treated cells, respectively. In the digest condition, the complexes of bacteria-metal were reversible and the bond stability of untreated bacteria–Hg complexes was stronger than other complexes. The results suggest that treated or untreated *L. acidophilus* ATCC 4356 cells have the potential to adsorb heavy metals in contaminated water.

Keywords: Adsorption; bioremediation; bioremoval; heavy metals; lactic acid bacteria

Introduction

The water pollution by heavy metals is a concern for human and wildlife health. Heavy metals are non-degradable contaminants that are toxic to animals, plants, and humans. Lead (Pb), cadmium (Cd), arsenic (As), and mercury (Hg), ubiquitous dangerous metals in water, have hazardous effects on various living organisms. The mining processes, pesticides and fertilizers, petrol production, and industrial wastewater are the main sources of heavy metal contamination (Pandey and Madhuri 2014, Asati, Pichhode *et al*., 2016, Lentini, Zanoli *et al*., 2017).

Effects of Hg on the human body depend on various factors, including the chemical form of Hg (elemental, organic, and inorganic), the pathway of exposure (foods, dental products, water, and atmosphere), and the duration of exposure to Hg (Park and Zheng 2012). Hg2+ is soluble in lipids and can easily penetrate human cells. In addition, Hg2+ may cause neurological human disorders (Carocci, Rovito *et al*., 2014).

Various levels of exposure to Cd may cause teratogenic effects, hypertension, renal dysfunction, hepatic injuries, and lung damage (Sharma, Rawal *et al*., 2015). Exposure to a high level of Pb can cause hematopoietic, renal, reproductive, and central nervous systems disorders in the human body (Assi, Hezmee *et al*., 2016). Transfer of As to food chains may lead to cell poisoning through metabolic enzyme dysfunction and hence cause multisystem organ failure and death. Long-time exposures to As may cause various types of cancers of the bladder, lung, kidney, and liver (Medina-Pizzali, Damián-Bastidas *et al*., 2019). These four heavy metals are chosen as the preference control of heavy metals (Li, Ma *et al*., 2014). The World Health Organization (WHO) has set acceptable doses of Hg, Pb, As, and Cd in water at 6, 10, 10, and 3 $\mu g.L^{-1}$, respectively (Water and Organization, 2006).

Different strategies exist for heavy metal removal in water. For instance, ion exchange, membrane filtration, chemical precipitation, adsorption, and nanotechnology treatments (Bianchi, Biancalani *et al*., 2020). The limitations of these methods include low selectivity, high cost, high energy consumption, and releasing toxic by-products. Therefore, a low-cost and highly efficient method for heavy metal removal is preferred, such as the biological methods include using the dead and viable microorganisms. Applying these microorganisms for heavy metal removal is a cheap method and often useful for human health (Hussein, Hassan *et al*., 2011). Many studies have reported that lactic acid bacteria (LAB) can decrease the bio-accessibility of toxic metals through surface-binding between the bacterial cells and metals (Bhakta, Ohnishi *et al*., 2012, Musawi, Johari *et al*., 2019, Mirmahdi, Zoghi *et al*., 2021). Most LABs are introduced as probiotics. Probiotics are defined as living microorganisms that could create health profits such as treatment of urinary infection, diarrhea, and lactose intolerance in the host (Amiri, Teymorlouei *et al*., 2021, Sohrabpour, Rezazadeh Bari *et al*., 2021, Amiri, Mokarram *et al*., 2022). *Lactobacillus* (*L.*) *acidophilus* ATCC 4356 is a probiotic and is prevalent in food products. Different Lactobacillus strains were evaluated regarding their potential for heavy metals removal (Moghari, Razavi *et al*., 2015, Ryan, Hutchings *et al*., 2020, Taghizadeh Moghaddam, Javadi *et al*., 2020).

A previous work reported the influence of *L. plantarum* CCFM8610 on Cd toxicity reduction (Zhai, Yin *et al*., 2015). They also demonstrated that this microorganism can remarkably prevent Cd adsorption in the mice' intestines, which in turn reduces the accumulation of Cd in tissues and reduces the histopathology of Cd-induced tissue damage (Zhai, Wang *et al*., 2014). The major bacterial cells' mechanism of biosorption is the reaction of ion exchange of teichoic acid and peptidoglycan and ligands with N^{3-} and O^{2-} (Monachese, Burton *et al*., 2012, Hadiani, Khosravi-Darani *et al*., 2018). Different factors can affect bioremoval, such as initial heavy metal concentration, pH, temperature, biomass concentration, presence of different heavy metal ions in solution, and exposure time (Afraz, Younesi *et al*., 2020). Bacterial cell pretreatments can enhance the biosorption of heavy metals (Göksungur, Üren *et al*., 2005). Zoghi *et al*., (2021) have reported that heat and NaOH pretreatments can enhance the biosorption of patulin by *L. acidophilus* (Zoghi, Khosravi-Darani *et al*., 2021).

Several studies have been done on removing one or two types of heavy metals from water or foodstuff using microorganisms (Göksungur, Üren *et al*., 2005, Zhai, Wang *et al*., 2014, Zhai, Yin *et al*., 2015, Hadiani, Khosravi-Darani *et al*., 2018, Afraz, Younesi *et al*., 2020). In most cases, polluted drinking water contains different kinds of heavy metals. Therefore, it is necessary to investigate regarding simultaneous removal of heavy metals in multi-metallic contaminated drinking water. In this research teams' previous work, we used *Saccharomyces cerevisiae* ATCC 9763 for biosorption of heavy metals (As, Pb, Hg, and Cd) from drinking water (Mirmahdi, Mofid *et al*., 2022). In this study, *L. acidophilus* ATCC 4356 was used to remove these metals due to the ability of this bacterium in biosorption of heavy metals. Additionally, based on our knowledge, there are limited studies regarding the effect of different pretreatments on the *Lactobacillus* bacteria biosorption of heavy metals in multi-metallic solutions. So, in this study, the effect of NaOH and heat pretreatments of *L. acidophilus* on four heavy metals (As, Pb, Hg, and Cd) adsorption was investigated. Moreover, the stability of pretreated and untreated *L. acidophilus*–metal complexes under simulated gastrointestinal tract (GIT) conditions was evaluated.

Materials and Methods

Probiotic bacterium and master culture preparation

The *L. acidophilus* ATCC 4356 was supplied from Tak Gene Zist (Tehran, Iran) in freeze-dried form. First, bacteria cells were cultured in de Man Rosa and Sharp (MRS) broth. The incubation condition of the culture was 37°C for 24 h (aerobic condition). Then, the cell cultures were centrifuged at 5000 × *g* for 15 min and washed with deionized water. New seed culture was provided for each series of biosorption from the master culture. For preparing seed culture, 5 mL of master culture was added to 50 mL MRS broth (pH 6.2 ± 0.2) and incubated for 24 h at 37°C and 75 rpm. For counting seed culture serial dilution method was applied (Sieuwerts, De Bok *et al*., 2008, Amiri, Mokarram *et al*., 2022).

Solutions and reagents

Culture components and analytical reagent chemicals were obtained from Merck (Darmstadt, Germany) except for Standard solutions of As (1000 $mg.L^{-1}$ in

0.1 M HNO_3), which were purchased from Panreac (Panreac Quimica SA, Spain, Barcelona). Working solutions were made in deionized water. All glass containers were soaked in 15% v/v HNO_3 for 24 h and then washed with deodorized water for removing microbial contamination before working. In addition, all glass containers were autoclaved before starting the experiments.

L. acidophilus pretreatment

For heat pretreatment, bacterial cells were autoclaved at 121°C for 20 min. For NaOH pretreatment, bacterial cells were mixed with 0.1 M NaOH and then incubated for 1 h at 37°C. Treated bacteria were centrifuged (9000 × *g*, 15 min) and washed with deionized water. Washing was repeated 3 times (Wang, Wang *et al*., 2015). Pretreated bacterial cells were used for biosorption of multi-metallic (Hg, As, Pb, and Cd) water.

Heavy metals adsorption by L. acidophilus from water

A volume of 6.2 mL sterile deionized water was mixed with 700 µL of each metal; Pb (10 ppm in HCl 10%), Cd (10 ppm in HCl 10%), As (10 ppm in HCl 10%), and Hg (10 ppm in HCl 2%). pH was adjusted to 4 by HCl and 0.1M NaOH using a pH meter. Then, 1 mL (2.6×10^{12} CFU.mL^{-1}) of *L. acidophilus* (treated or untreated) was added to the solution and incubated for 24 h, 75 rpm, and 37°C. Next, all samples were passed from simulated GIT conditions for examining the bond stability of *L. acidophilus*–heavy metal complexes (Afraz, Younesi *et al*., 2020).

Preparation of gastrointestinal juices

Bile salts, Pepsin, and pancreatin were provided from Sigma-Aldrich, Darmstadt, Germany. For making simulated gastric condition, pepsin with a final concentration of 3 g.L^{-1} was added to 0.5% (w/v) sterile sodium chloride solution. Then, pH was adjusted to 2 using 30% HCl. For simulated small intestine juices, bile salt with a final concentration of 1.5 g.L^{-1} and pancreatin with a final concentration of 1 g.L^{-1} were added to a 0.5% (w/v) sterile sodium chloride solution. pH was adjusted to 8.0 using 1 M NaOH. Small intestine and gastric juices were sterilized using a membrane filter (Nalge Co., 0.45 µm, Rochester, USA, NY). Freshly prepared reagents/juices are prepared (Yin, Wu *et al*., 2018).

Assessment of bond stability between L. acidophilus and heavy metals in simulated GIT condition

A volume of 10 mL of each metal-bacteria solution (after 24 h of biosorption) was added to 40 mL of simulated gastric juice then for 10 s stirred by vortex (Vortex-Genie 2, Scientific Industries, Bohemia, USA NY). Next, this solution was incubated for 2 h at 37°C. A volume of 40 mL of this solution was sampled for heavy metals analysis. For simulated gastric conditions, 10 mL of gastric solution was inserted into 50 mL of the simulated intestinal juice and incubated for 2 h at 37°C. This solution was shaken manually every 10 min. Then sampling for heavy metals analysis was repeated (Yin, Wu *et al*., 2018).

Analysis of heavy metals by inductively coupled plasma-mass spectroscopy (ICP-MS)

In this study, inductively coupled plasma-mass spectroscopy (Agilent 7500, Agilent Technologies, USA) was used for measuring the concentration of heavy metals. Parameters of Plasma were: (1) Radio Frequency (RF) generator power: 1200W, (2) Nebulizing argon flow: 0.8 L.min^{-1}, (3) Resonance RF frequency: 24 MHz, (4) Plasma gas flow rate: 12.2 L.min^{-1} and (5) Auxiliary gas flow rate: 0.8 L.min^{-1}. The analyzer limit of quantitation (LOQ) for Pb and As was 1 µg.L^{-1} and for Cd and Hg were 5 µg.L^{-1}. Additionally, the analyzer limit of detection (LOD) for Pb and As was 3.3 µg.L^{-1} and for Cd and Hg was 1.7 µg.L^{-1}. Measurements for each sample were done in triplicate.

Isotherm model studies

Isotherm models of heavy metals biosorption were studied using four isotherm models of Freundlich, Langmuir, Tempkin, and Dubinin–Radushkevich (D–R). The models' parameters were calculated according to a previous study (Chen, Zhang *et al*., 2015). The regression coefficient values (R^2) and the sum of error squares (ERRSQ) describe the best model of heavy metal biosorption by the untreated *L. acidophilus* ATCC 4356. Untreated *L. acidophilus* was examined at a specific concentration (10^{12} CFU.mL^{-1}), at different metal initial concentrations (20, 40, 60, 80, and 100 µg.L^{-1}) for each heavy metal adsorption, and after 24 h exposure time in order to measure its capacity. All experiments were performed in triplicate.

Freundlich equation is stated as Equation 1(Freundlich 1906):

$$Q_e = K_F \times C_e^{1/n_F} \quad (1)$$

where Q_e (µg.mg^{-1}) is the amount of each heavy metal per unit weight of adsorbent in adsorbing equilibrium, K_F and n_F are Freundlich constants relating to the adsorption capacity, C_e (µg.L^{-1}) is the equilibrium concentration of each heavy metal in water. The K_F and n_F are determined from the linear plot of ln Q_e versus ln C_e.

Langmuir equation is according to the Equation 2 (Langmuir 1918):

$$Q_e = Q_{max} [K_L C_e / (1 + K_L C_e)] \quad (2)$$

where Q_{max} (μg.mg^{-1}) is the maximum amount of heavy metals adsorption in high C_e and K_L (L.μg^{-1}) is the Langmuir constant relating to the free energy of the binding sites. $C_e.Q_e^{-1}$ versus C_e can demonstrate a straight line with a slope of $1.Q_{max}^{-1}$ and intercept of 1. $(K_L Q_{max})^{-1}$.

The Tempkin model is defined by the Equation 3 (Tempkin and Pyzhev 1940):

$$Q_e = \beta \ln\alpha + \beta \ln C_e ; \quad \beta = RT/K_T \quad (3)$$

where α (L.g^{-1}) and K_T (J/mol) are Tempkin constants relating to the heat of adsorption. R is the universal gas constant (8.314 J.mol^{-1}.K^{-1}) and T (K) is the absolute temperature. A plot of the Q_e versus ln *Ce* shows the constants from the intercept and slope.

The D–R model is described by the equation 4 (Dubinin 1960):

$$Ln\ Q_e = \ln Q_{max} + K_D \varepsilon^2 ; \quad \varepsilon = RT \ln(1+ 1/C_e) \quad (4)$$

where K_D (mol^2.KJ^{-2}) is D–R isotherm constant. Plotting ln Q_e versus ε^2 demonstrates a straight line with an intercept of ln Q_{max} and the slope of K_D.

Statistical analysis

All experiments in this study were accomplished in triplicates and the data are demonstrated as mean ± standard deviation (X ± SD). Data processing was done using Statistical Package for the Social Sciences (SPSS) Statistics version 22.0 (SPSS Institute, USA Chicago, IL). One-way Analysis of Variance (ANOVA) was foliated to estimate P-value and confidence levels. Moreover, the Tukey–Kramer test was applied for the identification of statistical differences. P-values < 0.05 for all data were considered.

Results and Discussion

Effect of L. acidophilus pretreatment on heavy metal removal

Pretreating Lactobacilli cells affects the bioremoval process. This method leads to denaturation of proteins, alteration in charge distribution, and changes in the hydrophobic surface arrangement on bacterial surfaces, and therefore improves the adsorption (Zoghi, Massoud *et al*., 2021). Heat treatment of *Lactobacillus* strains increases bioremoval as the cell surface alters to facilitate adsorption. It is stated that the heating process would develop binding sites via more hydrophobic bindings for heavy metals (Perczak, Goliński *et al*., 2018). The cell wall of *Lactobacillus* includes layers of teichoic acids, peptidoglycans, polysaccharides, and proteins. The structure and thickness of these layers may be reduced and their pore size may be increased in heat treatments. The glycosidic links in polysaccharides are broken by Maillard reaction (between peptides and polysaccharides in heat treatments). Additionally, proteins will denature from heat treatments (Teodorowicz, Van Neerven *et al*., 2017). Lactobacilli alkaline pretreatments are able to eliminate the coating compounds on the surface and so the availability of the binding sites will change, the acidic groups will be neutralized and the whole cells electronegativity of the cell surface will alter (Wang, Wang *et al*., 2015). The bindings increase after the cell wall's degradation and it will improve by increasing the amount of peptidoglycans (Zoghi, Massoud *et al*., 2021).

According to previous investigations, the heavy metal adsorption mechanism includes the ion exchange in cell walls' teichoic acid and peptidoglycan or the ligand formation (Zoghi, Khosravi-Darani *et al*., 2014). *L. acidophilus* cell wall involves a thick layer of peptidoglycan, exopolysaccharides, and teichoic acid. Hydroxyl, amide, phosphate, and carboxyl are the crucial functional groups that can create negative charges on the cell wall, which lead to heavy metal cation adsorption (Wang, Wang *et al*., 2015, Chen, Pan *et al*., 2016). In addition, the presence of S-layer proteins in the *L. acidophilus* cell wall was reported to interact with heavy metals, which causes the filling of the cell surface with anionic compounds to absorb the cationic metal ions (Gerbino, Mobili *et al*., 2011). It is reported that a bag-like structure around the bacterial cell wall, containing covalently-linked polypeptide and polysaccharide chains, plays a key role in heavy metal ion adsorption (Hadiani, Khosravi-Darani *et al*., 2019). Based on studies on the chemical properties of bacterial cell walls, hydrophobic cell walls (due to the presence of proteins) and cell walls containing electron-donating groups are capable of binding high amounts of metals (Daisley, Monachese *et al*., 2019). Therefore, this phenomenon depends on the capacity of the *L. acidophilus* strain and the heavy metal electronegativity.

Concentrations of all heavy metals (Hg, Pb, Cd, and As) decreased after 24 h of viable or pretreated *L. acidophilus* ATCC 4356 exposures, and the results are shown in Figure 1. Figure 1a demonstrated that most content of Hg was removed using untreated bacteria followed by NaOH-treated and heat-treated cells. Untreated *L. acidophilus* showed a strong capacity to remove Hg from the multimetallic water within 24 h compared with other heavy metals. This result appeared to be similar to those by Li *et al*., (2020) and Massoud *et al*., (2020) (Li, Ming *et al*., 2020, Massoud, Khosravi-Darani *et al*., 2020). The best potential of untreated cells for removing Hg can be explained by the selective and sometimes competitive ability of live *L. acidophilus* for Hg removal in the presence of other metals.

As shown in Figure 1b, untreated *L. acidophilus* adsorbed the highest Pb content from the water after 24 h followed by heat- and NaOH-treated bacterial cells. As mentioned above, heat treatment can increase hydrophobicity of the cell surface. Based on previous research, less hydrophobicity leads to more adsorption of Pb using Lactobacilli strains (Kirillova, Danilushkina *et al*., 2017), in accordance with our results. On the other hand, untreated *L. acidophilus* was not very useful in adsorbing Cd within 24 h (Figure 1c). Similarly, Massoud *et al*., (2020) reported that the optimum Pb adsorption yield in milk using *L. acidophilus* was 80% on the 4th day, and the initial ions concentration of 100 $\mu g.L^{-1}$. In contrast, Afraz *et al*., (2020) reported the tendency of *L. acidophilus* for removing metal ion was in order: $Cd^{2+} > Pb^{2+}$. This difference can be explained by the difference between strain-specific, experimental conditions (pH, temperature, time), inoculum size, and heavy metal content.

Based on Figure 1c, the highest amount of Cd was adsorbed by NaOH-treated followed by heat treated and untreated. This result is in accordance with the results of Zoghi *et al*., (2021), which reported the highest amount of patulin removal from apple juice by NaOH-treated *L. acidophilus*. Pretreatment of *Lactobacillus* bacteria using NaOH removes compounds that coat porous surfaces; so, accessibility of potential binding sites is increased, which can lead to neutralization of chemical groups and improving the electronegativity of the cell surface (Wang, Wang *et al*., 2015). Additionally, NaOH treatments may cause cell wall degradation, especially protein groups, and provide more binding sites for Cd adsorption (Khosravi-Darani, Zoghi *et al*., 2020). In contrast, it is reported that heat treatment increases Cd removal by LAB strains, because of increasing the accessibility of metal-binding sites on the bacterial surface (Göksungur, Üren *et al*., 2005). On the other hand, heat pretreatments could stabilize soluble proteins of the cell wall on the cell surface, which can lead to competition with surface binding sites (Zoghi, Khosravi-Darani *et al*., 2014).

According to Figure 1d, heat-treated bacteria removed the highest As concentration followed by NaOH-treated and untreated *L. acidophilus.* Similarly, Elsanhoty *et al*., (2014) showed that heat-pretreated *L. rhamnosus* could adsorb more aflatoxin M_1 in yogurt (Elsanhoty, Salam *et al*., 2014). The heat pretreatment could cause protein denaturation in the cell wall of LAB strains and more sites will be provided to bind to the metals (Maham, Karami-Osboo *et al*., 2013). Prasad *et al*., (2013) reported that the As (III) and As (V) ions adsorption by *Arthrobacter sp.* biomass was exothermic and spontaneous (Prasad, Ramanathan *et al*., 2013). They also indicated the role of some functional groups (-OH, -NH, and -C=O) in the As ion adsorption process.

Overall, lactobacilli strains show great variety in cell surface structure and can modify their surface properties in response to environmental changes such as pretreatments. So, they indicate a different of the removal process of different kinds of heavy metal. Therefore, the best results included untreated *L. acidophilus* for removing 99.01% Hg and 92.35% Pb from the multi-metallic water after 24 h. Additionally, heat- and NaOH-treated *L. acidophilus* could remove 91.28% As and 61.91% Cd, respectively (Figure 1). Results allow the conclusion that heavy metal binding to *L. acidophilus* depends strongly on the metal ion and bacterial cell wall structure.

Stability of L. acidophilus–heavy metal complexes in simulated GIT conditions

Probiotics application, such as *Lactobacillus* strains, in heavy metals elimination in foodstuffs relates to their complex stability in the GIT. Lactobacilli cells are likely to adhere to intestine cells and the degree of adhesion is very strain-specific. Most *Lactobacillus* strains miss intestinal adhesion after binding to heavy metals. Thus, the *Lactobacillus*–heavy metal complex is rapidly passed and excreted (Zoghi, Massoud *et al*., 2021). It is important that these bonds do not dissociate in our digestive tract (Hsu, Yi *et al*., 2018). Many studies have been conducted to evaluate the stability of the bond. It is stated that the bindings mainly relate to the cell wall composition, environmental conditions, and bacterial strains (Zoghi, Khosravi-Darani *et al*., 2014). The binding stability of heavy metals and *L. acidophilus* in GIT has a major role in examining the efficiency of adsorption. Heavy metals concentration after 4 h of exposure to simulated GIT conditions is illustrated in Figure 1. Based on Figure 1a, some Hg ions were released from all strain forms (treated or untreated) after simulated gastric conditions, and this phenomenon continued in simulated intestinal condition. Generally, the bond stability of untreated *L. acidophilus*–Hg complexes was stronger than other complexes under simulated GIT conditions.

According to Figure 1b, a high concentration of Pb (78.4% and 62.4%) was released after exposure to simulated gastric conditions, except for NaOH–treated *L. acidophilus* (26.2%), but after the simulated intestinal condition, biosorption is enhanced compared to gastric juice. Therefore, the NaOH–treated *L. acidophilus*–Pb complexes showed the best stability under simulated GIT conditions, in comparison to other complexes. Similarly, as illustrated in Figure 1c, some amount of Cd (53.9% and 33.1%) was released after exposure to simulated gastric conditions, except for untreated *L. acidophilus* cells. Adsorption of Cd ions is enhanced after simulated intestinal conditions, compared to gastric juice. In this case, untreated *L. acidophilus*–Cd complexes were more stable, because the adsorption process was continued under simulated GIT conditions, and none of the Cd ions were released into the environment.

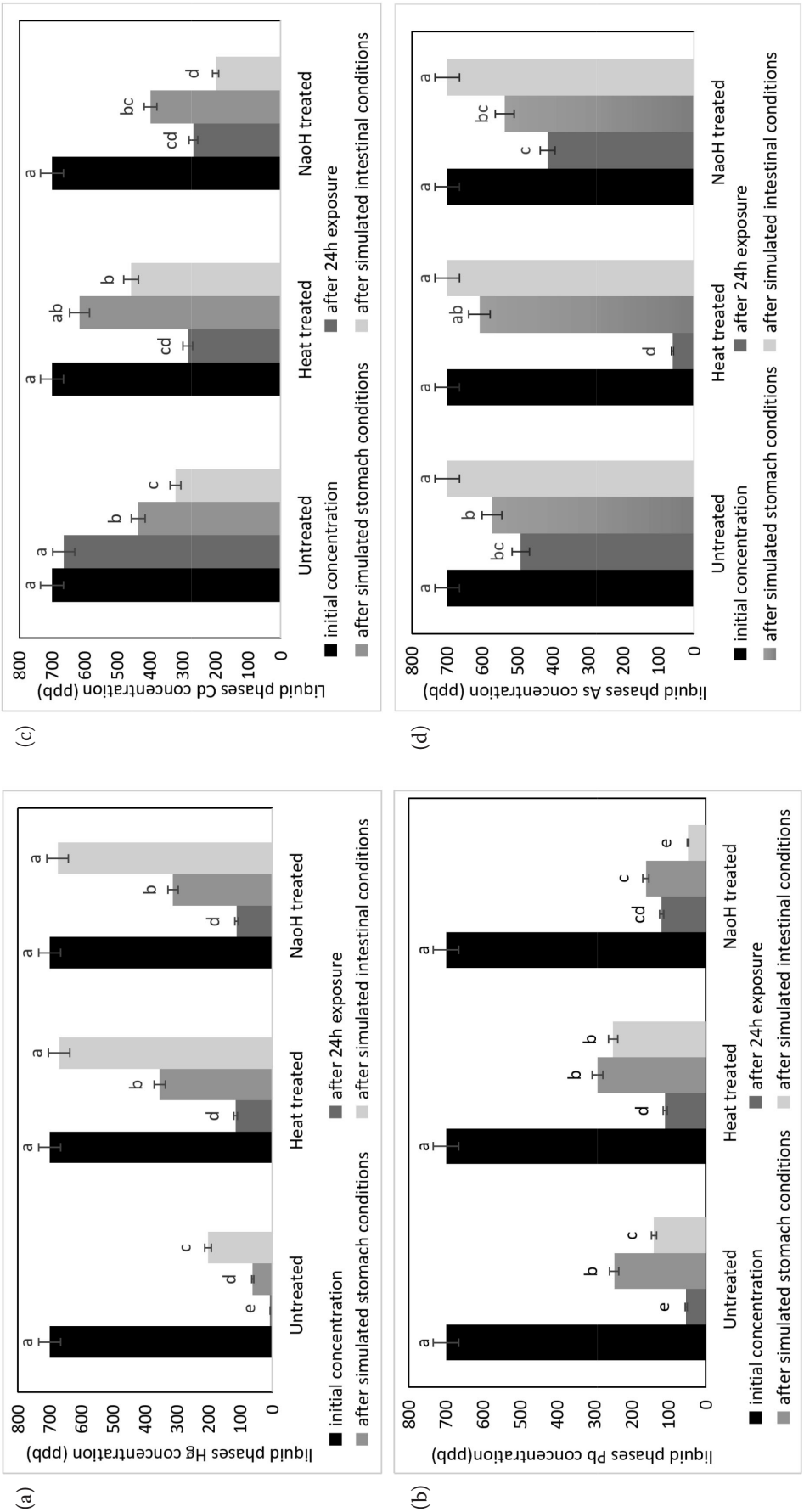

Figure 1. Concentration of four heavy metals (a) mercury (Hg); (b) lead (Pb); (c) cadmium (Cd); (d) arsenic (As)) after adsorption by untreated and pretreated (heat–, and NaOH-treated) *Lactobacillus acidophilus* ATCC 4356 in contaminated multi-metallic water after 24 h at 37°C and after 2 h of exposure to simulated gastric juice as well as 2 h of exposure to simulated small intestinal condition. Standard deviation calculated with 95% confidence.

As shown in Figure d, although heat-treated *L. acidophilus* was successful in adsorption of a high amount of As ions (91.28%) after 24 h, none of the bacterial strains (treated or untreated) could retain any As binding after 4 h exposure to simulated GIT conditions. Similarly, Petruzzi *et al*., (2016) assessed the stability of *Saccharomyces cerevisiae* W13-ochratoxin A complex under simulated GIT conditions and stated that approximately 70% of the toxin was released after 6 h exposure to salivary and simulated GIT juices (Petruzzi, Corbo *et al*., 2016). In addition, Mirmahdi *et al*., (2022) reported that bonds of heavy metal-*Saccharomyces cerevisiae* complexes in digestion conditions were reversible.

It could be concluded from the results that the heavy metal adsorption using *L. acidophilus* ATCC 4356 was partially reversible under simulated GIT conditions. This binding reversibility might happen due to the occurrence of chemical and physical adsorptions of heavy) metal ions, simultaneously. Furthermore, the binding reversibility could suggest the significance of non-covalent electrostatic bonds, such as Van der Waals and hydrogen bonds (Zoghi, Khosravi-Darani *et al*., 2021). Similar results have been observed by Le and Yang, (2019). They found that Cd-*Pediococcus pentosaceus* complexes were not stable after simulated GIT conditions and 44.7%–46.8% of Cd ions were released into the solution. Additionally, Zhao *et al.,* (2015) observed zearalenone removal by *L. plantarum* and the process was partially reversible (Zhao, Jin *et al*., 2015). In contrast, it is reported by Elsanhoty *et al*., (2014), that aflatoxin M1-*L. rhamnosus* TISTR 541 and aflatoxin M1-*L. plantarum* EMCC complexes were stable under simulated GIT conditions (Elsanhoty, Salam *et al*., 2014). This disagreement could be due to the different strains of bacteria, the structure of the cell wall, the concentration of bacterial cells, and the amount of toxin compounds. The reversibility of heavy metals binding to LAB strains has been confirmed by several investigations (Pan, Ge *et al*., 2006, Tunali, Akar *et al*., 2006, Teemu, Seppo *et al*., 2008). Fochesato *et al*., (2019) stated that the dynamics of toxin adsorption and desorption by *Lactobacillus* strains were significantly influenced by the salivary environment. Therefore, the information about the adsorption dynamics of heavy metal with a *Lactobacillus* strain will help to anticipate it at each point of the GIT (Fochesato, Cuello *et al*., 2019).

Biosorption isotherm of heavy metals

For designing the adsorption processes, the biosorption isotherms are crucial because the adsorption mechanism is usually achieved by equation parameters (Zoghi, Khosravi-Darani *et al*., 2021). In this research, four isotherm models (Freundlich, Langmuir, Tempkin, and D–R) were utilized for predicting the adsorption model of heavy metal removal by untreated *L. acidophilus* ATCC 4356. The parameters of isotherm models are illustrated in Table 1 and the

Table 1. Parameters of isotherm models (Freundlich[a], Langmuir[b], Tempkin[c], and Dubinin-Radushkevich[d]) for adsorbing heavy metals (cadmium, mercury, lead, and arsenic) by *Lactobacillus acidophilus* ATCC 4356[e]

Initial concentration of metals (µg.L^{-1})	Cadmium						Mercury						Lead						Arsenic					
	C_e	Q_e	$C_e.Q_e^{-1}$	ln C_e	ln Q_e	ε^2	C_e	Q_e	$C_e.Q_e^{-1}$	ln C_e	ln Q_e	ε^2	C_e	Q_e	$C_e.Q_e^{-1}$	ln C_e	ln Q_e	ε^2	C_e	Q_e	$C_e.Q_e^{-1}$	ln C_e	ln Q_e	ε^2
20	13.6	6.4	2.03	2.59	1.88	33440.24	12.6	7.4	1.70	2.53	2.00	38745.64	12	8	1.50	2.48	2.07	42558.56	10.6	9.4	1.12	2.36	2.24	53986.53
40	23.4	16.8	1.38	3.14	2.82	11632.53	22	18	1.22	3.09	2.89	13125.66	18	22	0.81	2.89	3.09	19418.31	18	22	0.81	2.89	3.09	19418.31
60	27	33	0.81	3.29	3.49	8785.64	27	33	0.81	3.29	3.49	8785.64	22.8	37.2	0.61	3.12	3.61	12239.53	21.6	38.4	0.56	3.07	3.64	13605.26
80	28	52	0.53	2.33	3.95	8179.80	28	52	0.53	3.33	3.95	8179.80	24	56	0.42	3.17	4.02	11069.59	24	56	0.42	3.17	4.02	11069.59
100	29	71	0.40	3.36	4.26	7634.53	29	71	0.40	3.36	4.26	7634.53	24.5	75.5	0.32	3.19	4.32	10631.12	24.5	75.5	0.32	3.19	4.32	10631.12

[a] Freundlich equation: $Q_e = K_F \times C_e^{1/n}$; [b] Langmuir equation: $Q_e = Q_{max}[K_L C_e / (1 + K_L C_e)]$; [c] Tempkin equation: $Q_e = \beta \ln\alpha + \beta \ln C_e$; $\beta = RT/K_T$; [d] Dubinin-Radushkevich equation: $\ln Q_e = \ln Q_{max} + K_D \varepsilon^2$; $\varepsilon = RT \ln(1+ 1/C_e)$
[e] *L. acidophilus* concentration was 10^{12} CFU.mL^{-1}
Standard deviation calculated with 95% confidence

Table 2. The regression coefficient values (R^2) and line equations of isotherm models (Freundlich[a], Langmuir[b], Tempkin[c], and Dubinin-Radushkevich[d]) for adsorbing heavy metals (cadmium, mercury, lead, and arsenic) by *Lactobacillus acidophilus* ATCC 4356

Isotherm model	Cadmium		Mercury		Lead		Arsenic	
	R^2	Line equation	R^2	Line equation	R^2	Line equation	R^2	Line equation
Freundlich	0.858	$\ln Q_e = 2.813 \ln C_e - 5.548$	0.874	$\ln Q_e = 2.477 \ln C_e - 4.400$	0.949	$\ln Q_e = 2.889 \ln C_e - 5.164$	0.949	$\ln Q_e = 2.292 \ln C_e - 3.285$
Langmuir	0.918	$C_e.Q_e^{-1} = -0.104\ C_e + 3.524$	0.912	$C_e.Q_e^{-1} = -0.077\ C_e + 2.754$	0.965	$C_e.Q_e^{-1} = -0.087\ C_e + 2.510$	0.984	$C_e.Q_e^{-1} = -0.054\ C_e + 1.739$
Tempkin	0.047	$Q_e = 12.45 \ln C_e - 0.85$	0.654	$Q_e = 59.83 \ln C_e - 150.64$	0.753	$Q_e = 77.348 \ln C_e - 190.43$	0.734	$Q_e = 65.80 \ln C_e - 152.93$
Dubinin-Radushkevich	0.793	$\ln Q_e = -8^{-5} + 4.35\ \varepsilon^2$	0.801	$\ln Q_e = -6^{-5} + 4.24\ \varepsilon^2$	0.900	$\ln Q_e = -6^{-5} + 4.61\ \varepsilon^2$	0.841	$\ln Q_e = -4^{-5} + 4.35\ \varepsilon^2$

R^2 values and line equations of isotherm models are shown in Table 2. Based on the R^2 values, the Langmuir isotherm model exhibited the best fit for all heavy metals (Hg, Cd, Pb, and As) adsorption by untreated *L. acidophilus* ATCC 4356 cells.

The Langmuir isotherm demonstrates the adsorption of a monolayer on a surface, with a limited number of similar sites. The Langmuir model coefficients consist of real meanings; thus, it is the most commonly applied model (Langmuir 1918). This proves that the heavy metal adsorption by untreated *L. acidophilus* cells was based on monolayer adsorption. Additionally, since the Langmuir equation presumes that the adsorbent surface is homogeneous (Hasr Moradi Kargar and Hadizadeh Shirazi 2020). It can be concluded that heavy metal binding was due to the homogeneous distribution of binding sites on the bacterial cell wall. This finding is in accordance with the results of previous studies (Hasr Moradi Kargar and Hadizadeh Shirazi 2020, Massoud, Khosravi-Darani *et al*., 2020, Massoud, Khosravi-Darani *et al*., 2020), which showed that Langmuir isotherm included a better fit than the Freundlich model to predict adsorption of Pb, Hg and Cd by lactobacilli strains. In addition, Ameen *et al*., (2020) suggested the contribution of both electrostatic reaction and complex formation together with the high affinity of Cd and Pb binding to the bacterial cells (Ameen, Hamdan *et al*., 2020). Results from Tables 1 and 2 showed that Freundlich, Tempkin, and D–R models seem to be inappropriate for predicting the adsorption of heavy metals according to the lowest R^2. The Freundlich isotherm indicates that the adsorption occurred on a heterogeneous surface (Freundlich 1906). The Tempkin model is based on the linear reduction of the heat of adsorption of all layer molecules (Tempkin and Pyzhev 1940). The D–R model demonstrates the adsorption mechanism using Gaussian energy distribution on a heterogeneous surface (Dubinin 1960).

Conclusions

In this study, the removal of four toxic heavy metals from multi-metallic water was performed simultaneously, using *L. acidophilus* ATCC 4356. The biosorption of As and Cd was increased by heat- and NaOH-pretreated *L. acidophilus*, respectively; but untreated bacterial cells were more effective for removing Hg and Pb. In general, the heavy metal adsorption by *L. acidophilus* ATCC 4356 was partially reversible through simulated GIT juices. In specific, the binding of untreated *L. acidophilus*-Hg/Cd and NaOH–treated *L. acidophilus*–Pb complexes under simulated GIT conditions were more stable compared with others. The results from the isotherm studies showed that the heavy metal adsorption by untreated *L. acidophilus* ATCC 4356 obeys the Langmuir isotherm model. There are still more studies required regarding using other *Lactobacilli* strains and a combination of applied pretreatments for the elimination of toxic heavy metals in water. Further investigations should focus on controlling the release of heavy metals through the GIT of animals and considering factors affecting the binding reversibility. Moreover, Fourier transform infrared analysis should be applied to evaluate the possible binding sites and probable functional groups of treated and untreated *L. acidophilus* strains for heavy metal adsorption.

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