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Original article

# Lead biosorption efficiency of *Levilactobacillus brevis* MZ384011 and *Levilactobacillus brevis* MW362779: A response surface based approach

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ABSTRACT

Lead (Pb) is a substantial contaminant in the environment and a potent toxin for living organisms. Current study describes probiotic characteristics of Pb-biosorbing lactic acid bacteria (LAB), and response surface methodology (RSM) based optimization of physical conditions for maximum Pb biosorption. A total of 18 LAB, isolated from carnivore feces (n = 8) and human breast milk (n = 9), along with one reference strain *Lactobacillus acidophilus* ATCC4356 were included in the study. Pb biosorption was strain specific. Eight strains, demonstrating ≥ 70 % lead biosorption, were selected for further testing. The lactobacillus-Pb complex was found to be stable and strains had a negative surface charge. The strains displayed good probiotic properties with the survival rate of 71–90 % in simulated gastric environment, 36–69 % in intestinal condition (1.8 % bile salts) and 55–72 % hydrophobicity. On the basis of excellent probiotic ability, *Levilactobacillus brevis* MZ384011 and *Levilactobacillus brevis* MW362779 were selected for optimization of physical conditions of Pb biosorption through RSM. Maximum biosorption was observed at pH 6 in 60 min at a cell density of 1 g/L. *L. brevis* MZ384011 and *L. brevis* MW362779 are recommended for experimentation on Pb toxicity amelioration and safety evaluation in *in-vivo* setting.


## 1. Introduction

Heavy metals (HM) are the major source of toxicity in humans, wildlife and plants and are contaminating global food, water supply and soil (Banwo et al., 2021; Rahbari et al., 2021). Lead (Pb) is a ubiquitous contaminant and was ranked 2nd most dangerous substance by the US Agency for Toxic Substance and Disease Registry (ATSDR) (Kenny et al., 2020; Kermanshahi et al., 2020; Banwo et al., 2021). Industries concerned with mining, smelting, manufacturing batteries, paints, pigments, leaded gasoline and ceramics, emit significant quantities of Pb (Sedighi et al., 2012; Tchounwou et al., 2012; Oves et al., 2013). In human and animals, horizontal exposure to Pb leads to hepatic, renal, neurologic, hematological, pulmonary, cardiovascular dysfunctions, and reproductive disorders (Kirillova et al., 2017; Boskabady et al., 2018; Olawoyin et al., 2018; Reuben, 2018). Pb stays in bones and brain for years while in bloodstream, its biological half-life is 30–35 days (Maret, 2017; Rodríguez and Mandalunis, 2018). Moreover, previous studies suggested that Pb toxicity affects the antioxidative enzyme activities resulting in production of free radicals in cells (Bhakta et al., 2012; Zhai et al., 2013; Kumar et al., 2017; Ojekunle et al., 2017).

Hazard Quotient (HQ) is the risk to a human receptor for exposure to a contaminant via a single pathway. The sum of HQs for all pathways with similar hazardous effects is called a Hazard Index (HI). HI<1.0 is acceptable while HQ<0.2 is acceptable for a specific pathway. In Pakistan, adults had a mean hazard index of 0.37, whereas children had a mean hazard index of 3.23 for Pb exposure. High levels of Pb were found in the soil of connecting roads of

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Lahore, Pakistan. As a result of roadside soil contamination, children were found to be at a significant risk of Pb poisoning (Ahmad et al., 2019b; Ahmad et al., 2020). Hazard index was recorded in children of roadside (4.3) and residential area (2.26) schools of Lahore, Pakistan (Rehman et al., 2020). Removal and purification techniques are very expensive and cannot be applied on a large scale in underdeveloped countries (Daisley et al., 2019). Currently, there is no effective and reliable method of treating Pb poisoning. Chelation therapy is ineffective in the majority of cases and can have a variety of negative effects (Yi et al., 2017).

Previous research provides the information about the HM biosorption potential of various microorganisms including probiotics (Monachese et al., 2012; Aryal and Liakopoulou-Kyriakides, 2015; Limcharoensuk et al., 2015; Huet and Puchooa, 2017). Probiotics are living microbes which when administrated in sufficient concentration, have a significant positive impact on the health of hosts (FAO/WHO, 2001). Heavy metal biosorption by lactic acid bacteria (LAB) based probiotics, including, *Lactobacillus rhamnosus, L. casei*, L. fermentum, L. plantarum, *L. acidophilus* and *L. bulgaricus* has been reported (Sedighi et al., 2012; Yin et al., 2016; Ahmed et al., 2018; Ameen et al., 2020; Kermanshahi et al., 2020). Microbial surfaces have physicochemical interactions with heavy metals that enhance bioaccumulation process by forming complexes, ion exchange, chelation, microprecipitation and adsorption (Monachese et al., 2012; Mrvčić et al., 2012; Zoghi et al., 2014). These interactions are due to existence of anions like carboxyl, hydroxyl, phosphoryl, amino and amide groups present on bacterial surfaces (Passot et al., 2015; Li et al., 2017) which serve as principal sites for attachment of HM cations (Mishra et al., 2013).

In extract, the LAB have become an attractive option for detoxification because of their specificity, minimal cost, environmental friendly nature, and GRAS (Generally Recognized As Safe) status (Zanjani et al., 2017). Some authors suggested that it could be an operational solution of Pb poisoning in the environment and *in-vivo* system. However, the bio-detoxification and protective efficiencies of LAB are source, strain and species specific (Pop et al., 2022) and requires data on strains from diverse resources. To the best of our knowledge, no research has been conducted on Pb biosorption using probiotics from Pakistan. Present study is designed to determine Pb biosorption potential of locally isolated LAB strains. The effect of pH, exposure time and bacterial concentration has been investigated and RSM based approach was used to optimize the physical conditions of Pb biosorption. Selection of Pb biosorbant lactobacilli, for use in food industry or *in vivo* system, requires experimental data on reliability of effectiveness and their safety towards mammalian system (Bhattacharya, 2019; Duan et al., 2020), therefore, along with surface characteristics, the probiotic potential and safe nature of selected LAB have also been figured out.

## 2. Materials and methods

### 2.1. Growth media and culture conditions

LAB strains used in current study were previously isolated from carnivore feces and human breast milk samples and taken from a repository of laboratory (Microbiology and Immunology Laboratory, Institute of Zoology, University of the Punjab, Lahore). These strains were refreshed in De Man, Rogosa and Sharpe (MRS) broth and incubated at 37 °C for 24 h under anaerobic conditions. *L. acidophilus* ATCC4356 was used as a reference strain. For preparation of cell suspension, the strains were inoculated in MRS broth and incubated at 37 °C for 24 h. Bacterial pellet was collected after centrifugation at 8000 × *g* for 20 min and washed thrice with phosphate buffer saline (PBS). Washed cell pellets were resuspended in PBS to attain absorbance ($OD_{600}$) 1 ± 0.02 corresponding to the cell count of 1x $10^9$ CFU/ml.

### 2.2. Pb resistance

Pb resistance of LAB strains was determined in terms of minimum inhibitory concentration (MIC) following Bhakta et al. (2012) with slight modification. Lead nitrate stock solution was prepared in MRS broth to make final concentrations ranging from 50 to 1000 ppm with a difference of 50 ppm. Briefly, in a microtiter plate, MRS broth (50 µl) without lead nitrate was added in 1$^{st}$and last well, while equal volume of MRS broth containing different concentrations of lead nitrate were added in well no. 2–11. Later on, bacterial suspension of LAB was added from well 1–11, while equal volume of PBS was added in the last well (12th well). Well number 1 and 12 served as positive and negative control, respectively. The same procedure was followed for all LAB strains. Plates were placed in anaerobic jar and incubated at 37 °C for 24 h. The minimum quantity of lead nitrate which inhibited the visible bacterial growth was considered as MIC.

### 2.3. Pb biosorption assay

Pb biosorption assay was performed following Pakdel et al. (2019) with minor changing. Freshly prepared LAB cultures were inoculated (wet weight 1 g/L) in MRS broth (5 ml) containing lead nitrate (10 mg/L) and incubated at 37 °C anaerobically for 1 h. Uninocculated MRS broth containing lead nitrate was processed similarly as negative control. Later on, cell- free supernatants (CFS) were separated following centrifugation at 8000 × *g* for 20 min and processed for estimation of residual Pb by flame atomic absorption spectrophotometry (AAS), (ThermoUnicam-SOLAAR) using acid digestion. For acid digestion, conc. $HNO_3$ (4 ml) and conc. HCl (1 ml) were added in 5 ml of CFS. Samples were heated on a hotplate until digestion completed and filtered with Whatman filter no. 1. Filtrates were diluted with distilled water and stored at 4 °C until subjected to AAS. Calculations were based on calibration curve that was prepared by using the absorbance value of standard solutions with different concentrations of Pb ranging from 2 to 20 ppm. Pb biosorption was calculated using following formula:

$$\text{Pb biosorption } (\%) = (C_i - C_f / C_i)\ 100$$

where, $C_i$ and Cf represent initial and final Pb concentration in control and test samples, respectively.

### 2.4. Pb bacterial binding stability

Pb bacterial binding stability was determined by measuring the quantity of Pb detached after three washes with milli Q water (Topcu and Bulat, 2010; Kumar et al., 2017). LAB were incubated in MRS broth supplemented with $Pb(NO_3)_2$ for 1 h. Bacterial cells were pelleted out by centrifugation at 8000 × *g* for 20 min. The pellet of one tube was kept as control while the pellet of the other tube was washed thrice MQ-water. Bound quantity of Pb in pellets (with and without washing) was determined by AAS after acid digestion and calculated by subtracting the value of the washed pellet from the value of the unwashed pellet.

### 2.5. Zeta potential

Surface charge of selected lactobacilli was estimated by calculating zeta potential (ζ) following Kirillova et al. (2017). Briefly, cells were harvested from 24 h incubated culture by centrifugation at 8000 × *g* for 20 min at 4 °C and washed thrice with phosphate buffer saline (PBS). Cells were resuspended in PBS to obtain optical

density $OD_{600}$ of 1 ± 0.02. Cell suspension was divided in aliquots and pH of the aliquots was adjusted to 2–10 (with a difference of 2 units) with NaOH and HCl. Zeta potential was measured for each sample in triplicate using Zetasizer (Zetasizer nano; ZS-90, Malvern, UK). Calculated electrophoretic mobility was converted to zeta potential by using Helmholtz– Smoluchowski's approximation.

### 2.6. Probiotic characteristics of LAB

#### 2.6.1. Tolerance to simulated gastric juice

Tolerance to simulated gastric conditions was determined using the method of Wang et al. (2012). Fresh cell suspension was centrifuged at 8000 × *g* for 20 min at 5 °C and pelleted cells were resuspended in 10 ml simulated gastric juice (5g/L of NaCl and 3g/L of pepsin). The final pH was adjusted to 3 with HCl and incubated for 3 h. Growth in MRS at pH 6.5 was used as negative control. Bacterial suspension was spread on MRS agar plates and log CFU was calculated after overnight incubation at 37 °C through plate count method (Sutherland et al., 1996). Results were analyzed in terms of survival rate (%).

Survival rate (%) = C-T/C 100

where, T is log CFU in test samples and C is log CFU in control samples.

#### 2.6.2. Bile salt tolerance

Bile salt tolerance was determined following the procedure of García-Ruiz et al. (2014). Eight LAB isolates were cultured in MRS broth containing 0.3 %, 1 % and 1.8 % of bile (w/v) (Sigma-Aldrich, Germany) and incubated for 1 h at 37 °C. Control for each sample was prepared without bile salt. The growth of strains was estimated by comparing absorbance at $OD_{600}$ (Francois et al., 2005). Bile salt tolerance was calculated as mentioned in section 2.6.1.

#### 2.6.3. Hydrophobicity

Hydrophobicity of metal resistant strains was estimated by the attachment of microbes to hydrocarbons by using the method of Yadav et al. (2016). Bacterial pellet from overnight culture was collected after centrifugation at 8000 × *g* for 20 min, washed thrice and resuspended in the PBS buffer to achieve initial absorbance (A0) 0.5 ± 0.02 at $OD_{(600)}$. Equal volume of cell suspension and hydrocarbon (xylene) were vortexed for 2 min. Sample solution was placed in a separating funnel and kept for 2 h for phase separation at 37 °C. Lower aqueous phase was collected and absorbance (A1) at 600 nm was recorded. Hydrophobicity (%) was calculated as follows:

Hydrophobicity (%) = (Ai- Af/Ai) ×100

where, $A_i$ is the initial absorbance while $A_f$ is the final absorbance.

### 2.7. Safety profiles of selected LAB

#### 2.7.1. Hemolytic activity

Hemolytic activity of selected strains was evaluated following the procedure of Pieniz et al. (2014). Overnight incubated LAB strains were streaked on tryptic soy agar medium supplemented with 7 % v/v sheep blood and incubated at 37 °C for 48 h. Appearance of greenish tint or clear zone around the bacterial growth was checked to identify α and β hemolysis, respectively.

#### 2.7.2. Dnase activity

DNase enzyme (deoxyribonuclease) production by LAB strains was evaluated by using the method of Yadav et al. (2016). Refreshed strains were streaked on DNase agar medium and incubated for 24–48 h at 37 °C. DNase activity was considered positive for the presence of clear and pinkish zones around colonies.

#### 2.7.3. Antibiotic susceptibility

Antibiotic resistance assay for selected isolates was conducted following Kirby-Bauer disc diffusion method (Chen et al., 2020). Studied antibiotics were amoxicillin (30 μg), ceftriaxone (30 μg), clindamycin (2 μg), ciprofloxacin (5 μg), clarithromycin (15 μg), doripenem (10 μg), gentamicin (10 μg) penicillin G (10 units), Tetracycline (30 μg) and trimethoprim (25 μg). Briefly, 24 h activated LAB culture (100 μl) was spread on MRS agar medium and allowed to dry. Antibiotic discs were placed on medium plates and were incubated at 37 °C for 24 h under anaerobic conditions. Diameter of the clear zone (mm) around the antibiotic discs was measured to categorize the strains as resistant, intermediate or susceptible.

### 2.8. Effect of physical parameters on Pb removal and their optimization

Effect of different physical parameters *viz*., pH (2–6), bacterial density (0.5, 1 and 1.5 g/L wet weight) and time of incubation (5, 15, 30, 60, 90, 180 and 240 min) on Pb removal was determined as mentioned above.

Response surface methodology (RSM) was used to optimize three independent factors including pH, bacterial density (g/L) and incubation time (min) for Pb removal. In this study, tests were conducted according to the central composite design (CCD). Levels of independent variables in CCD are presented in Table 1. The entire design consisted of 18 combinations and was carried out in random order (Table 5). Multiple regression equation was used to analyze the data. Response (Pb biosorption) was calculated using following equation:

$$R = \beta + \sum_{i=0}^{n} \beta iXi + \sum_{i=0}^{n} \beta iiXi + \sum_{i\neq i}^{n} \beta ijXiXj$$

where, R is the response (Pb biosorption), β is the intercept, n is the number of parameters evaluated. Linear, quadratic, and interaction model coefficients are denoted by the symbols βi, βii and βij respectively. The low and high range of the independent parameters is represented by the letters Xi and Xj.

### 2.9. Statistical analysis

The Pb binding stability was assessed using the paired sample *t* test. One-way analysis of variance (ANOVA) followed by Tukey Post hoc test was used to analyze the other data in SPSS statistics software (version 25). Differences at $p \leq 0.05$ were considered statistically significant. RSM was used to determine optimal biosorption conditions of three independent variables in Design Expert software (version 12).

## 3. Results

### 3.1. Pb resistance for LAB

Pb resistance of 17 locally isolated LAB strains and one reference strain (*L. acidophilus* ATCC4356) was measured in terms of minimal inhibitory concentration of Pb that could inhibit visual growth of strains, while 50–1000 ppm of lead nitrate was used in the test system. The MIC of strains varied from 250

**Table 1**
List of physical parameters and their levels for optimization of Pb biosorption used in central composite design (CCD).

| Symbol | Physical factors | Units | low range | high range |
|---|---|---|---|---|
| A | pH | - | 2 | 6 |
| B | Bacterial concentration | g/L | 0.15 | 1.5 |
| C | Incubation time | min | 5 | 115 |

to > 1000 ppm. Isolates could be separated into four categories on the basis of their survival at different Pb concentrations *viz*; I (≥900 ppm), II (≥700 ppm), III (≥500) and IV (⟨500). In total, 6 isolates including reference strain displayed ≥ 900 ppm MIC while three strains exhibited < 500 MIC value (Table 2).

### 3.2. Pb biosorption profile of LAB

Pb biosorption of LAB strains was estimated through atomic absorption and calculated by measuring remaining amount of Pb in spent broth following LAB growth in MRS containing 10 mg/L of lead nitrate. The 70 % biosorption was set as minimum criteria for selection of potent Pb removing strain. The biosorption was categorized as high (≥80 %), medium (<80 and ≥ 75) and low (<75 and ≥ 70). The biosorption ability among strains varied significantly ($F_{(d.f\ 17;\alpha\ 0.05)}$ = 32.345; $p$ = 0.000).The values of biosorption ranged from 37.14 ± 1.34 to 83.18 ± 0.76 %. *L. brevis* MZ384011 and *L. brevis* MW362779 displayed highest biosorption (83.18 ± 0.76 % and 81.41 ± 0.64 %, respectively) and were ranked as best isolates. Two isolates (*L. brevis* MW365351 and *L. brevis* MZ061639) presented < 80 and ≥ 75 % biosorption while three strains (*L. brevis* MW084366, *L.brevis* MT950194 and *L. brevis* MW450245) presented in the range of < 75 and ≥ 70 Pb biosorption. The reference strain (*L. acidophilus* ATCC4356), could remove 71.32 ± 0.92 % Pb (Table 2). The performance of other strains was poor. Moreover, Pearson correlation analysis between Pb resistance and Pb biosorption revealed direct correlation between the two parameters, r = 0.847 and $p$ < 0.01 (Fig. S1). Seven LAB strains and reference strain depicting ≥ 70 % Pb biosorption were selected for further studies.

### 3.3. Pb bacterial binding stability

Data regarding amount of bound Pb with bacterial cells, grown in lead nitrate supplemented broth, indicated that selected LAB strains and reference strain (*L. acidophilus* ATCC4356) present stability of lactobacilli-Pb complex. The values of Pb biosorption, obtained with AAS, before and after washing with MQ water were not significantly different from each other (Fig. 1).

### 3.4. Zeta potential

Zeta potential as a function of bacterial surface charge was calculated with the help of zetasizer, which revealed that selected strains possess negative charge at all pH levels used in test system (pH 2–10). However, the zeta potential was found pH dependent and varied significantly among the strains. Maximum surface charge was recorded at pH 6 which did not change significantly with further increase in pH ($p$ > 0.05). Direct correlation was observed between pH and negative surface charge upto pH 6, r = 0.962 and $p$ < 0.000 (Fig. S2). *L. brevis* MZ384011 displayed the highest negative surface charge followed by *L. brevis* MW362779 and *L. brevis* MZ061639 which exhibited the value of −31.84 and −28.62, respectively at pH 6.Three strains (*L. brevis* MW450245, *L. brevis* MT950194, and *L. brevis* MW365351) had similar surface charge of around −20, *L. brevis* MW084366 and reference strain displayed lowest surface charge of −12 at same pH (Fig. 2).

### 3.5. Probiotic characteristics of selected LAB isolates

#### 3.5.1. Survival in simulated gastro-intestinal tract (GIT) conditions

In GIT, the strains are expected to tolerate both acidic and alkaline environment, therefore survival was checked in gastric and intestinal simulated conditions. All selected LAB strains exhibited

**Table 2**
Lead resistance (minimal inhibitory concentration) and lead biosorption potential of LAB isolated from different sources

| Source | Strain | GenBank Acessionnumber | MIC (ppm) | $Pb^{2+}$ biosorption(%) | | |
|---|---|---|---|---|---|---|
| Human breast milk | *Levilactobacillus brevis* | MW084366 | 800 | 70.81 | ± | $0.92^{def}$ |
| | *Levilactobacillus brevis* | MW084653 | 600 | 55.41 | ± | $1.33^{i}$ |
| | *Levilactobacillus brevis* | MW084946 | 450 | 43.83 | ± | $0.67^{j}$ |
| | *Levilactobacillus brevis* | MW362785 | 350 | 42.87 | ± | $0.93^{j}$ |
| | *Levilactobacillus brevis* | MW362788 | 550 | 65.25 | ± | $1.45^{g}$ |
| | *Levilactobacillus brevis* | MW362789 | 550 | 67.35 | ± | $0.72^{fg}$ |
| | *Levilactobacillus brevis* | MW362779 | ≥1000 | 81.41 | ± | $0.64^{ab}$ |
| | *Weissella confusa* | MW051433 | 250 | 37.14 | ± | $1.34^{k}$ |
| | *Limosilactobacillus fermentum* | MW084960 | 500 | 57.62 | ± | $0.33^{hi}$ |
| Carnivore feces | *Levilactobacillus brevis* | MT950194 | 850 | 73.81 | ± | $1.18^{cd}$ |
| | *Levilactobacillus brevis* | MW365351 | 900 | 75.52 | ± | $0.61^{c}$ |
| | *Levilactobacillus brevis* | MZ400640 | 700 | 59.27 | ± | $0.86^{h}$ |
| | *Levilactobacillus brevis* | MZ384002 | 500 | 45.36 | ± | $1.13^{j}$ |
| | *Levilactobacillus brevis* | MZ040404 | 900 | 68.73 | ± | $0.53^{efg}$ |
| | *Levilactobacillus brevis* | MZ384011 | ≥1000 | 83.18 | ± | $0.76^{a}$ |
| | *Levilactobacillus brevis* | MW450245 | 850 | 72.68 | ± | $0.43^{cd}$ |
| | *Levilactobacillus brevis* | MZ061639 | ≥1000 | 79.16 | ± | $1.22^{b}$ |
| Reference strain | *Lactobacillus acidophilus* | ATCC4356 | ≥1000 | 71.32 | ± | $0.92^{de}$ |

Data are represented as mean ± SEM of three replicates. Analysis was made by using one way ANOVA followed by Tukey test. Means within the same column followed by different superscript letters are significantly different ($p$ < 0.05).

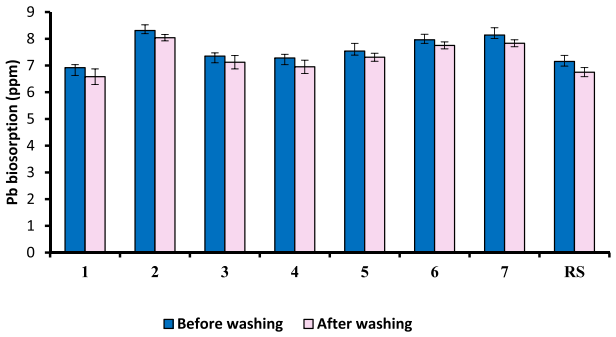


**Fig. 1.** Lead bacterial binding stability of selected lactic acid bacteria. Values are presented as mean ± SEM of three replicates. Analysis was made by using paired sample "*t*" test.

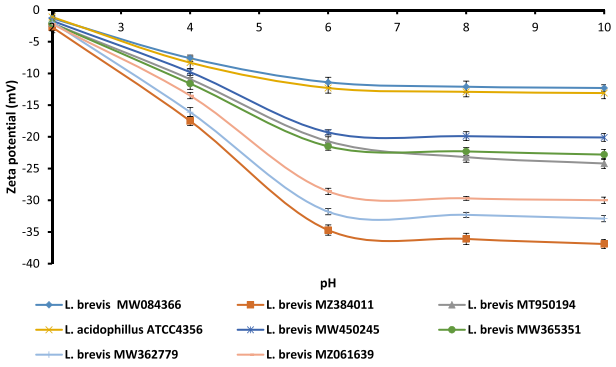


**Fig. 2.** Zeta potentials (ζ) of efficient Pb biosorbent strains at different pH. Data are expressed as mean ± SEM of three replicates.

tolerance towards simulated gastric juice that was evidenced by survival rate of ≥ 70 %. However, the differences in gastric environment tolerance among strains were statistically significant, $F_{(d.f\ 7;\alpha\ 0.05)}$ = 27.988; $p$ = 0.000 (Table 3). The survival rate ranged from 71.67 to 90.33 % after 3 h incubation in gastric conditions. *L. brevis* MZ384011 displayed highest resistance (90.33 ± 0.3) while *L. brevis* MW450245, *L. brevis* MZ061639) and reference strain (*L. acidophilus* ATCC4356) presented ≥ 80 % and < 90 % survival rate. Other isolates (*L. brevis* MW08466, *L. brevis* MT950194, *L. brevis* MW365351 and *L. brevis* MW362779) showed tolerance between ≥ 70 % and < 75 %.

Similarly, the tolerance to intestinal environment was checked by using three concentrations of bile salt (0.3, 1 % and 1.8 %) in simulated intestinal secretion. Performance (survival) of strains at aforementioned bile salt concentrations was statistically significant ($F_{(d.f\ 7;\alpha\ 0.05)}$ = 16.895; $p$ = 0.000, $F_{(d.f\ 7;\alpha\ 0.05)}$ = 14.844-; $p$ = 0.000 and $F_{(d.f\ 7;\alpha\ 0.05)}$ = 47.884; $p$ = 0.000, respectively). Bacterial growth decreased with increase in bile salt concentration that ranged 65–83 %, 56–77 % and 36–70 % in the presence of 0.3 %, 1 % and 1.8 % bile salt, respectively (Table 3). *L. brevis* MZ384011 presented highest growth in the presence of all salt concentrations.

### 3.5.2. Hydrophobicity

Following Rajoka et al. (2017), 40 % hydrophobicity was decided as the minimum criteria to declare the probiotic potential of strains. The hydrophobicity was confirmed by bacterial adherence to hydrocarbons, the xylene. The strains presented significantly different values of hydrophobicity ($F_{(d.f\ 7;\alpha\ 0.05)}$ = 20.671; $p$ = 0.000) that ranged from 56 % to 72 %. Highest hydrophobicity (72.61 ± 0.6 %) was displayed by *L. brevis* MZ384011 while *L. brevis* MZ061639, *L. brevis* MW365351, *L. brevis* MW08466 and reference strain showed 60–67 % hydrophobicity. Other strains exhibited hydrophobicity between 55 and 58 % (Table 3).

### 3.6. Safety assessment

The potential probiotic strains are expected to be safe and non-pathogenic towards mammalian system. Absence of hemolytic activity towards mammalian erythrocyte, DNase production and transferable antibiotic resistance are commonly used for *in vitro* safety evaluation of probotic strains (Yadav et al., 2016; Das et al., 2020; Yasmin et al., 2020).

### 3.6.1. Hemolytic and DNase activity

Strains included in the study did not display hemolytic or DNase activity, as evidenced by the absence of a greenish hue or clear zone on sheep blood agar plates, as well as lack of pinkish or clear zones on DNase agar plates (Data not shown).

### 3.6.2. Antibiotic susceptibility

The selected strains were resistant against ciprofloxacin, ceftriaxone, clindamycin, and trimethoprim (Table 4). *L. brevis* MT950194 showed resistance against all antibiotics except penicillin G. Conversely, all the strains were susceptible to amoxicillin and penicillin G except *L. brevis* MT950194. All the strains displayed resistance towards tetracycline except *L. acidophilus* ATCC 4356. Similarly, the strains were found resistant to gentamicin except *L. brevis* MZ061639. The responses against clarithromycin and doripenem were strain specific, ranging from susceptible to resistant.

### 3.7. Effect of physical parameters on Pb biosorption and their optimization using RSM

Physical parameters *viz;* pH, inoculum size and incubation period are known to influence the biosorption property of the strains. The pH values higher than 6 were not included in the experiment

**Table 3**
Probiotic potential of selected Pb biosorbing lactic acid bacteria: the survival in gastrointestinal conditions and hydrophobicity.

| Strain | Survival rate (%) under simulated gastric juice | Growth (%) in the presence of bile | | | Hydrophobicity (%) |
|---|---|---|---|---|---|
| | | 0.3 % | 1 % | 1.8 % | |
| *L. brevis* MW08466 | 72.67 ± 1.4$^{c}$ | 79.12 ± 1.7$^{a,b}$ | 65.92 ± 1.3$^{bc}$ | 55.48 ± 0.9$^{c}$ | 63.32 ± 1.1$^{bc}$ |
| *L. brevis* MZ384011 | 90.33 ± 0.3$^{a}$ | 83.19 ± 0.6$^{a}$ | 77.34 ± 0.7$^{a}$ | 69.57 ± 1.7$^{a}$ | 72.61 ± 0.6$^{a}$ |
| *L. brevis* MT950194 | 74.18 ± 0.6$^{c}$ | 78.67 ± 0.7$^{a,b}$ | 53.67 ± 1.6$^{c}$ | 49.37 ± 0.8$^{d}$ | 56.83 ± 0.4$^{d}$ |
| *L. brevis* MW450245 | 82.52 ± 1.4$^{b}$ | 73.48 ± 1.2$^{bc}$ | 55.83 ± 0.9$^{cd}$ | 43.86 ± 1.2$^{e}$ | 55.67 ± 0.8$^{d}$ |
| *L. brevis* MW365351 | 73.39 ± 1.7$^{c}$ | 65.39 ± 0.9$^{d}$ | 55.65 ± 1.3$^{cd}$ | 39.42 ± 0.3$^{ef}$ | 62.52 ± 1.3$^{bc}$ |
| *L. brevis* MZ061639 | 83.79 ± 1.2$^{b}$ | 69.26 ± 1.4$^{cd}$ | 56.37 ± 0.6$^{cd}$ | 36.17 ± 1.2$^{f}$ | 60.46 ± 0.5$^{cd}$ |
| *L. brevis* MW362779 | 71.67 ± 0.7$^{c}$ | 78.48 ± 0.8$^{a,b}$ | 71.22 ± 0.3$^{ab}$ | 61.25 ± 0.5$^{b}$ | 58.91 ± 1.5$^{cd}$ |
| *L. acidophilus* ATCC4356 | 80.21 ± 0.9$^{b}$ | 82.57 ± 1.3$^{a}$ | 66.18 ± 0.6$^{abc}$ | 53.51 ± 1.4$^{cd}$ | 67.14 ± 0.7$^{b}$ |

Values are represented as mean ± SEM of three replicates. Analysis was made by using one way ANOVA followed by Tukey post hoc test. Mean values in the same column, separated by different superscript letters are significantly different ($p$ < 0.05).

**Table 4**
Antibiotic resistance profile of selected Pb biosorbing lactic acid bacteria.

| Strains | Zone of Inhibition (diameter in mm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | AMX(30 μg) | CTX(30 μg) | CDM(2 μg) | CIP(5 μg) | CTM(15 μg) | DRP(10 μg) | GTM(10 μg) | PCG(10 U) | TTC(30 μg) | TMP(25 μg) |
| *L. brevis* MW08466 | S | I | I | R | S | S | R | I | I | R |
| *L. brevis* MZ384011 | S | R | R | R | S | I | S | I | I | R |
| *L. brevis* MT950194 | R | R | R | R | R | I | R | S | R | R |
| *L. brevis* MW450245 | I | R | I | R | R | I | R | S | R | R |
| *L. brevis* MW365351 | S | I | R | R | S | R | I | I | R | I |
| *L. brevis* MZ061639 | S | I | R | R | S | I | S | S | I | I |
| *L. brevis* MW362779 | S | R | R | R | R | I | R | S | I | R |
| *L. acidophilus* ATCC4356 | S | R | R | R | S | S | R | S | S | R |

Abbreviations are: susceptible (S), intermediate (I), resistant (R), amoxicillin (AMX), ceftriaxon (CTX), clindamycin (CDM), ciprofloxacin (CIP), clarithromycin (CTM), doripenem (DRP), gentamicin (GTM) penicillin G (PCG), Tetracycline (TTC) and trimethoprim (TMP). For each antibiotic, the zone of inhibition (mm) was interpreted as follows: S $\geq$ 21 mm, I = 16–20 mm and R $\leq$ 15 mm (Chen et al. 2020).

**Table 5**
Central composite design for Pb biosorption.

| Run | Independent variables[a] | | | Pb removal (%) | |
|---|---|---|---|---|---|
| | A | B | C | S1[b] | S2[c] |
| 1 | 4 | 1 | 60 | 78.48 | 69.48 |
| 2 | 6 | 1 | 60 | 83.24 | 81.45 |
| 3 | 2 | 0.5 | 115 | 49.13 | 49.33 |
| 4 | 2 | 0.5 | 5 | 43.56 | 31.18 |
| 5 | 4 | 0.15 | 60 | 51.67 | 54.51 |
| 6 | 6 | 1.5 | 5 | 59.11 | 66.54 |
| 7 | 2 | 1.5 | 5 | 47.42 | 46.27 |
| 8 | 4 | 1 | 60 | 69.87 | 73.87 |
| 9 | 4 | 1 | 60 | 72.34 | 72.34 |
| 10 | 6 | 0.5 | 5 | 55.13 | 51.43 |
| 11 | 6 | 1.5 | 115 | 85.47 | 83.47 |
| 12 | 4 | 1 | 60 | 76.37 | 74.36 |
| 13 | 2 | 1.5 | 115 | 66.29 | 65.55 |
| 14 | 6 | 0.5 | 115 | 74.51 | 78.36 |
| 15 | 4 | 1 | 60 | 74.62 | 71.68 |
| 16 | 4 | 1 | 115 | 74.17 | 79.23 |
| 17 | 4 | 1 | 60 | 73.32 | 70.12 |
| 18 | 4 | 1.5 | 60 | 77.33 | 79.33 |

[a] A (pH), B (Inoculum size, g/L) and C (Incubation period, min).
[b] S1 (*L. brevis* MZ384011).
[c] S2 (*L. brevis* MW362779).

to avoid precipitation and conversion of metal into hydroxides (Halttunen et al., 2007; Topcu and Bulat, 2010; Pakdel et al., 2019). Direct correlation was found in pH and Pb biosorption (r = 0.931, $p$ = 0.000) while amount of biosorbed Pb varied significantly among strains at pH 6 ($F_{(d.f\ 7;\alpha\ 0.05)}$ = 20.557; $p$ = 0.000). Maximum biosorption ability of selected isolates ranged from 70 % to 83 % at pH 6. The performance of *L. brevis* MZ384011 and *L. brevis* MW362779 was the best at all pH levels used in the study ($p$ = 0.000). *L. brevis* MZ384011 presented 25 % biosorption at pH 2, that increased to > 80 % at pH 6 (Fig. 3a). Similarly Pb biosorption was also associated with inoculum size with the highest biosorption (78 % to 91 %) recorded at 1.5 g/L. The biosorption increased 1.7 fold by increasing inoculum size from 0.5 to 1.0 g/L while, further increase in inoculum size resulted in 2–20 % increase in biosorption ability of 5 strains (Fig. 3b). Based on these findings, moderate level of bacterial concentration (1 g/L) was selected in further experiments. Effect of different incubation periods (5, 15, 30, 60, 90, 180, and 240 min) on Pb biosorption was investigated. Pb removal activity could be recorded just after 5 min of incubation; it remained correlated with incubation time up to 60 min. However, the quantity of Pb removed did not vary significantly with further increase in incubation period ($p$ > 0.05). After 5 min of incubation the mean overall Pb biosorption was 20–27 % that increased 59–69 % following 60 min incubation period ($p \leq$ 0.000). *L. brevis* MZ384011 and *L. brevis* MW362779 presented highest biosorption of 83 % and 82 %, respectively after one hour of incubation (Fig. 3c).

RSM based optimization analysis of three physical parameters for two LAB strains with the maximum Pb biosorption capacity (>80 %) was conducted. Pb removal (%) for *L. brevis* MZ384011 (S1) and *L. brevis* MW362779 (S2) for three independent variables *viz*., pH, bacterial concentration (g/L) and incubation time (min) is given in Table 5. Data were analyzed by ANOVA and multiple regression analysis. Results of ANOVA and Lack of Fit are summarized in Table 6. The obtained results were statistically significant and had acceptable determination coefficient, $R^2$ = 0.9806 for S1 and $R^2$ = 0.9906 for S2 for Pb removal (Table 7; Fig. S3). All linear model terms (A, B and C) were statistically significant ($p$ < 0.05) for both strains. Interactive model terms (AC and BC) for S1 and (AB and BC) for S2 were significant ($p$ < 0.05). Moreover, quadratic model terms ($B^2$ and $C^2$) for S1 and ($A^2$, $B^2$ and $C^2$) for S2 were also significant ($p$ < 0.05). Lack of fit using $p$ values was found to be insignificant ($p$ > 0.05) for both strains. A coefficient of variance (CV) was 2.04 % and 1.65 % for S1 and S2, respectively, which indicate the reliability of data. Second-order polynomial equations for Pb removal by S1 and S2 are given below:

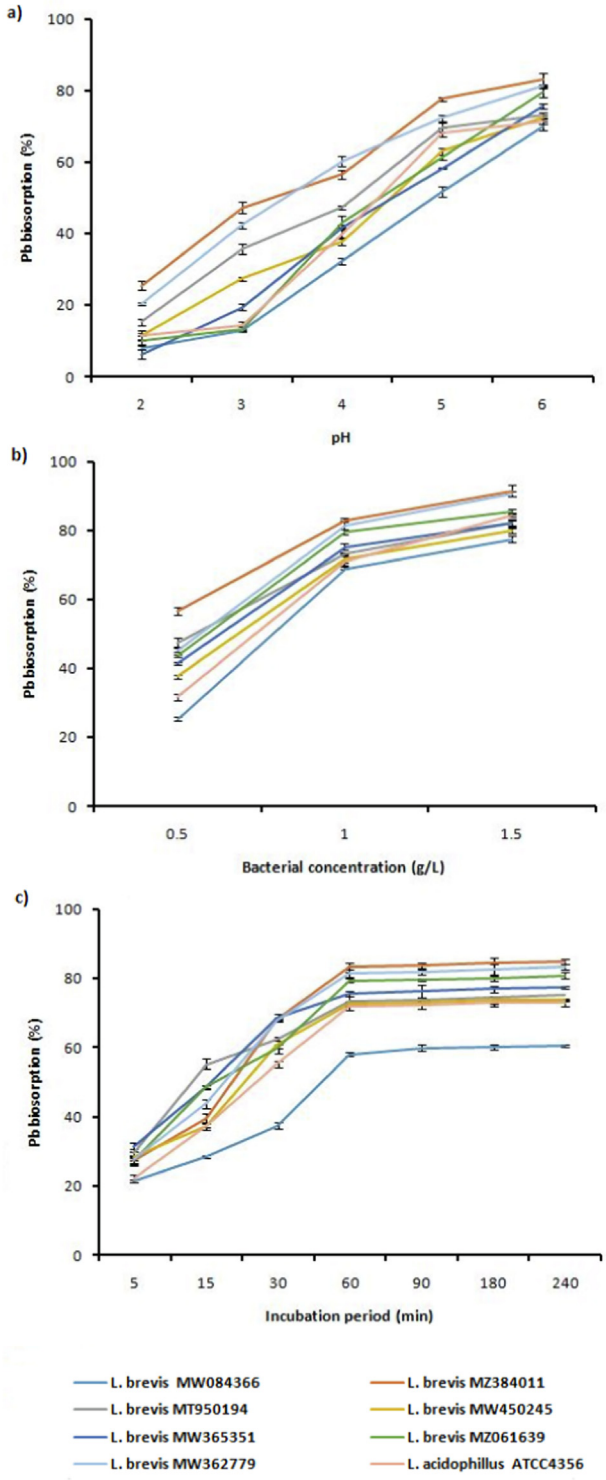


**Fig. 3.** Effect of different physical parameters on Pb biosorption by selected isolates. Values are presented as mean ± SEM with the initial concentration of 10 mg/L lead nitrate. **a:** Effect of pH, **b:** effect of bacterial concentration (g/L) and **c:** effect of incubation period (min).

$$R_{(S1)} = +8.63 + 0.5434A + 0.3177B - 0.5550C - 0.0667AB + 0.1384AC + 0.1484BC - 0.0500A^2 - 0.3015B^2 - 0.5731C^2$$

$$R_{(S2)} = +8.52 + 0.7483A + 0.4456B - 0.6809C - 0.1201AB - 0.0017AC - 0.1135BC - 0.3559A^2 - 0.1402B^2 - 0.4123C^2$$

Optimum range for independent factors for Pb removal by *L. brevis* MZ384011 and *L. brevis* MW362779 are presented in Fig. 4 (a-c) and Fig. 4 (d-f), respectively. Response surface plots for interaction of pH (A) and bacterial concentration (B) with fixed incubation time at its central point (60 min) on Pb biosorption are presented in Fig. 4 (a and d) These 3D response surface plots indicate that Pb removal was directly correlated with pH and bacterial concentration for both strains ($p < 0.05$). However, their interaction (AB) had a significant effect on the removal process of S2 only. The results further showed that maximum biosorption was achieved at pH 6 and removal efficiency improved with rise in bacterial concentration. Combined effect of pH (A) and incubation time (C) with fixed bacterial concentration (1 g/L) on Pb removal efficiency is given in Fig. 4 (b and e). Significant effect of interaction of AC was found for S1 only (Table 4). Synergistic effect of bacterial concentration (B) and incubation time (C) with fixed pH (4) on Pb removal efficiency is given in Fig. 4 (c and f). BC interaction had a significant effect on removal efficiency for both strains ($p < 0.05$).

### 3.7.1. Verification of RSM model

This step was carried out for the verification of the optimum values for physical parameters generated by using the data of the CCD under RSM analysis. The optimization condition were pH 6, bacterial concentration 1 g/L and incubation time 60 min for maximum predicted Pb removal for S1 (83.18 %) and S2 (79.39 %). However, using the same physical parameters, the observed Pb biosorption values were 83.34 % and 81.21 % for S1 and S2, respectively, which were close to predicted values (Table 7).

## 4. Discussion

To the best of our information, not a single report is available on exploitation of locally isolated LAB strains on Pb removal from Pakistan. Current study describes the Pb removal efficiency of locally isolated LAB strains and RSM based optimization of physical conditions for their best performance. Presumptive metals removing LAB are expected to survive in Pb contaminated environment. Previously, the Pb removing strains were isolated by different researchers on the basis of their survival in the presence of 100–2000 ppm of Pb (Bhakta et al., 2012; Oves et al., 2013; Li et al., 2017; Yi et al., 2017; Ahmed et al., 2018; Pakdel et al., 2019). In the current study, initial selection of LAB was performed on the basis of their survival in the presence of 50–1000 ppm Pb. The strains having MIC $\geq$ 250 ppm of Pb were included in the study. Microbes develop resistance after being exposed to a highly polluted environment and utilize a number of mechanisms to ensure their survival in this environment (Jarosławiecka and Piotrowska-Seget, 2014). Microbes adopt metabolism dependent and independent strategies, to combat the negative effects of HM. Biosorption, the binding of heavy metal with bacterial surface via membrane binding protein (MBP), is one of these mechanisms.

Our strains presented 37 to 83 % biosorption, while two strains (*L. brevis* MZ384011 and *L. brevis* MW362779) displayed outstanding biosorption potential ($\geq$80 %), while five strains (*L. brevis* MW084366, *L. brevis* MT950194, *L. brevis* MW365351 *L. brevis* MW450245, *L. brevis* MZ061639) and reference strain (*L. acidophilus* ATCC4356) had $\geq$ 70 % Pb binding ability. Yin et al. (2016) studied biosorption capacity of 25 LAB strains and mentioned that only four strains achieved around 30 % Pb removal. Conversely, Li et al. (2017) reported that *L. bulgaricus* KLDS1.0207 has 83 % Pb binding ability. The variation in Pb biosorption capacity

**Table 6**
ANOVA analysis using CCD for Pb removal by selected strains.

| Strain | Model parameters[a] | Sum of squares | df | Mean square | F value | *p* value |
|---|---|---|---|---|---|---|
| *L. brevis* MZ384011 (S1) | Model | 11.19 | 9 | 1.24 | 44.86 | < 0.0001 |
| | A | 2.47 | 1 | 2.47 | 89.17 | < 0.0001 |
| | B | 1.04 | 1 | 1.04 | 37.4 | 0.0003 |
| | C | 2.58 | 1 | 2.58 | 93.04 | < 0.0001 |
| | AB | 0.0356 | 1 | 0.0356 | 1.28 | 0.2899 |
| | AC | 0.1533 | 1 | 0.1533 | 5.53 | 0.0465 |
| | BC | 0.1762 | 1 | 0.1762 | 6.36 | 0.0357 |
| | $A^2$ | 0.0039 | 1 | 0.0039 | 0.1421 | 0.716 |
| | $B^2$ | 0.6375 | 1 | 0.6375 | 23.01 | 0.0014 |
| | $C^2$ | 0.5166 | 1 | 0.5166 | 18.64 | 0.0026 |
| | Residual | 0.2217 | 8 | 0.0277 | | |
| | Lack of Fit | 0.066 | 3 | 0.022 | 0.7064 | 0.5881 |
| | Pure Error | 0.1557 | 5 | 0.0311 | | |
| | Cor Total | 11.42 | 17 | | | |
| *L. brevis* MW362779 (S2) | Model | 15.15 | 9 | 1.68 | 93.86 | < 0.0001 |
| | A | 4.69 | 1 | 4.69 | 261.28 | < 0.0001 |
| | B | 2.04 | 1 | 2.04 | 113.68 | < 0.0001 |
| | C | 3.88 | 1 | 3.88 | 216.33 | < 0.0001 |
| | AB | 0.1153 | 1 | 0.1153 | 6.43 | 0.0349 |
| | AC | 0 | 1 | 0 | 0.0012 | 0.9728 |
| | BC | 0.103 | 1 | 0.103 | 5.75 | 0.0434 |
| | $A^2$ | 0.1992 | 1 | 92 | 11.11 | 0.0103 |
| | $B^2$ | 0.1379 | 1 | 0.1379 | 7.69 | 0.0242 |
| | $C^2$ | 0.2674 | 1 | 0.2674 | 14.91 | 0.0048 |
| | Residual | 0.1435 | 8 | 0.0179 | | |
| | Lack of Fit | 0.0767 | 3 | 0.0256 | 1.91 | 0.2455 |
| | Pure Error | 0.0668 | 5 | 0.0134 | | |
| | Cor Total | 15.29 | 17 | | | |

[a] A, B and C are the main effects of pH, bacterial concentration and Incubation period respectively, AB, AC and BC are their interactive effects while $A^2$, $B^2$ and $C^2$ indicate quadratic effect of independent variables. Lack of Fit i.e., the fitness of model.

**Table 7**
Regression analysis and confirmation of CCD based optimized levels of independent variables for Pb removal by selected strains.

| Strain[a] | $R^2$ | Adj-$R^2$ | Pred-$R^2$ | Adeq. precision | Std. Dev. | Mean | C.V (%) | Pb removal (%)[b] | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Predicted | Observed |
| S1 | 0.980 | 0.959 | 0.846 | 22.828 | 0.13 | 8.10 | 1.65 | 83.18 | 83.34 |
| S2 | 0.990 | 0.980 | 0.894 | 37.566 | 0.17 | 8.17 | 2.07 | 79.39 | 81.21 |

[a] S1 (*L. brevis* MZ384011), S2 (*L. brevis* MW362779).
[b] The expected and observed values of Pb removal were confirmed at pH 6, inoculum size 1 g/L and incubation period 60 min.

points towards strain specificity (Bhakta et al., 2012; Dobrowolski et al., 2017; Pakdel et al., 2019).

The ionic strength was determined by estimating the quantity of Pb before and after repeated washing of cells incubated in the presence of Pb. The selected strains exhibited least desorption and amount of Pb before and after washing did not change considerably ($p \geq 0.05$), suggesting that the lactobacilli-Pb complex was quite stable and selected isolates qualify for further investigation as a tool of bioremediation. The findings are in accordance with other authors who demonstrated that LAB forms a stable complex with Cd and Pb (Topcu and Bulat, 2010; Yin et al., 2016; Kumar et al., 2017).

Microorganisms have a net negative charge on their surface which is probably related to $COO^-$ and $HSO_3^-$ groups on their cell walls. The bioremediation ability of the strains has been linked with electrostatic cell surface properties of LAB (Kirillova et al., 2017). We subsequently determined electrostatic charge on the strains by measuring the electrophoretic mobility, in zetasizer. Our findings are in agreement with Dertli et al. (2015), the zeta potential ($\zeta$) was found electronegative for selected LAB strains at all pH levels confirming negative surface charge and presence of anionic compounds on the surfaces of lactobacilli. The negative charge could be due to the presence of anionic substances such as phosphate groups in lipoteichoic acids, acidic polysaccharides, and proteins on the bacterial cell surface. Kirillova et al. (2017) also reported negative zeta potential for all the lactobacillus strains. However, zeta potential varied significantly between strains. On the basis of zeta potential the strains are arranged as *L. brevis* MZ384011 > *L. brevis* MW362779 > *L. brevis* MZ061639 > *L. brevis* MW365351 = *L. brevis* MT950194 = *L. brevis* MW450245 > *L. acidophilus* ATCC4356 = *L. brevis* MW084366.

The high biosorbent LAB may also have prophylactic or therapeutic application in metal detoxification of animals. Therefore, the probiotic characteristics of potent strains were determined. The survival in the gastro-intestinal environment, tolerance of bile salt, hydrophobicity and safety towards the mammalian system were selected as important probiotic characteristics. Our Pb resistant strains remained viable in acidic environment while four strains (*L. brevis* MZ384011, *L. brevis* MZ061639, *L. brevis* MW450245 and *L. acidophilus* ATCC4356) exhibited $\geq$ 80 % survival at pH 3. Adherence to epithelial cells of intestine is associated with bile tolerance of LAB (Foley et al., 2021). All the selected strains displayed bile salt tolerance. Although the growth was inversely proportional to bile concentration, the strains presented 36–69 % survival at highest bile salt concentration (1.8 %) used in the study. Our results are consistent with the findings of Fuochi et al. (2015), Shehata et al. (2016) and Munir et al. (2022). Bhakta et al. (2012) used 4000 ppm bile concentration for determining the probiotic

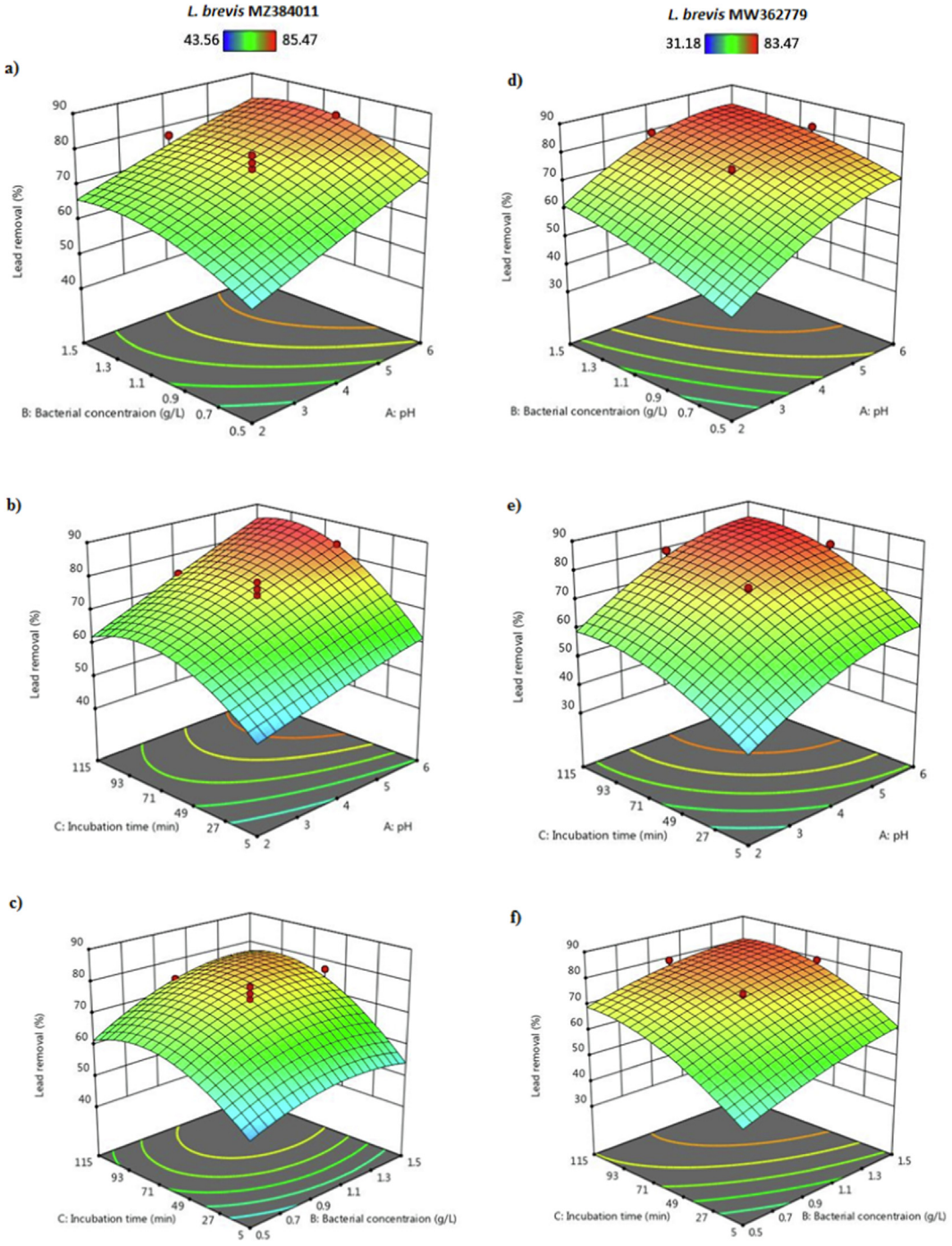


**Fig. 4.** The response surface 3D plots for effect of independent variables on Pb removal by *L. brevis* MZ384011 (a-c) and *L. brevis* MW362779 (d-f), respectively. **a & d:** Effect of pH and bacterial concentration on lead removal at 60 min. **b & e:** Effect of pH and incubation time on Pb removal by using bacterial concentration of 1 g/L. **c & f:** Effect of bacterial concentration and incubation time on Pb removal at pH 6.

potential of Pb resistant strains. Bile salt levels are typically between 0.2 and 0.5 percent however, higher concentrations have also been tested. Survival rate of 80 % in 1 % bile salt conc. is declared as selection criterion for probiotics (Turchi et al., 2013). In the current study, much higher bile salt conc. (1.8 % equivalent to 18000 ppm) was used. High tolerance capacities to acid and bile salt suggest that the selected strains could thrive in the gastrointestinal environment by mitigating the adverse effects of acid and bile salt.

Selection of potential probiotic also depends upon adherence property to human intestinal cells, which could be directly connective with the hydrophobic nature of the isolates. Adherence property to hydrocarbons also indicates ability of strains to attach with intestinal cells. Hydrophobicity > 40 % is considered as selection criteria for probiotics (Rajoka et al., 2017; Javed et al., 2022). All the selected isolates presented > 50 % hydrophobicity, while highest hydrophobicity (72 %) was recorded for *L. brevis* MZ384011. The hydrophobicity (%) was greater than the value of previously reported Pb resistant *Lactobacillus* species (Duary et al., 2011) while results are consistent with the findings of other authors (Li et al., 2015; Pringsulaka et al., 2015; Dlamini et al., 2019).

Despite the fact that LAB have a long history of being "safe to use", safety assessment is mandatory for proclamation of probiotic potential of new isolates (Pino et al., 2019). Hemolytic activity towards mammalian erythrocytes and DNase production are used as gold standards of safety evaluation. The selected strains satisfied the fundamental probiotic criteria since none of them displayed hemolysin or DNAse production indicating that could be assumed safe for *in vivo* usage. Some authors believe that development of antibiotic resistance in LAB is of safety concern that may result in horizontal transfer of antibiotic resistance (Nallala et al., 2017). Conversely, others claim that intrinsic antibiotic resistance aid in survival of probiotic strain survival during antibiotic treatment (Gueimonde et al., 2013). All of the isolates included in study were susceptible to amoxicillin and penicillin G, indicating their safe nature. Our findings are consistent with Gueimonde et al. (2013), who reported that LAB strains are often susceptible to antibiotics that target cell wall. However, all the strains were resistant to ciprofloxacin, ceftriaxone, clindamycin, and trimethoprim. Further investigation is needed to confirm the intrinsic nature of LAB for resistance.

Biosorption is a complex process and depends on various factors *viz*., pH, incubation period and inoculum size etc. The pH is among the most important factors that significantly influence biosorption process since it changes the metal accessible binding sites (Ahmad et al., 2019a). Higher pH values than 6 were not included in the experiment to circumvent precipitation of metal into metal hydroxides (Halttunen et al., 2007; Topcu and Bulat, 2010; Pakdel et al., 2019). All isolates included in study, presented pH-dependent biosorption, with maximum activity at pH 6. In contrast, *Klebsiella* sp. 3S1 has been reported to show highest (89 %) Pb removal at pH 5 (Muñoz et al., 2015). Our findings are consistent with the findings of (Dai et al., 2019), who mentioned that *L. brevis* and *L. plantarum* PTCC 1896 show direct correlation in pH and biosorption from pH 2.0 to 7.0. The competition between protons and HM ions for negatively charged binding sites leads to low biosorption at acidic pH (Zoghi et al., 2014). Secondly, the pH also influences the charge on the microbial surface as evidenced in experiments of zeta potential. The strains possessing the highest surface charge at pH $\geq$ 5 could also be the reason for maximum biosorption at pH 5–7.

Regarding the influence of the incubation period, Pb biosorption progressively increased with the passage of time until reaching a balancing point at 1 h. Consistent with Pakdel et al. (2019), the removal of Pb started in 5 min of incubation. In contrast, Elsanhoty et al. (2016) determined rise in Pb biosorption occurred with increasing the treatment duration and maximum removal was observed at 300 min. The variation in results is probably due to strain specificity. Moreover, high level of Pb removal in less time also points to high superiority of our strains. Similarly, Pb biosorption was also found to be reliant on biomass concentration, since raising the bacterial density from 0.5 g/L to 1.5 g/L enhanced Pb removal, which can easily be explained by an increase in the number of accessible binding sites (Zoghi et al., 2014; Elsanhoty et al., 2016; Daisley et al., 2019).

The RSM was employed to identify and confirm the influence and interaction of important factors that control Pb removal. This method not only helps in optimizing different physical conditions for greatest biosorption efficiency but also give information about positive or negative interaction between physical factors. Two LAB strains (*L. brevis*, MZ384011 and *L. brevis* MW362779) were selected in RSM based optimization on the basis of maximum Pb removal efficiency. Effect of three independent variables *viz*., (A) pH, (B) bacterial concentration (g/L) and (C) incubation time (min) was determined. In the current study, optimal conditions were pH 6, bacterial concentration 1 g/L and incubation time 60 min for maximum Pb removal efficiencies (82.64 % for S1 and 78.21 % for S2). The evaluation of metal-binding ability employing *L. acidophilus* (Afraz et al., 2020) revealed 87 % Pb removal at pH. The response surface plots show that increasing the pH from 4 to 6 promotes metal ion biosorption. It's plausible that the high concentration of hydrogen ions prevents metal cation removal, leading them to preferentially attach with binding sites on bacterial cells (Hadiani et al., 2018). Conversely, the decrease in H + ions at a high pH generates negative charge on absorbent that primarily biosorb HM ions. Khanniri et al. (2021) optimized physical conditions using chitosan, *Bifidobacterium longum* and *Saccharomyces cerevisiae* for Pb removal and reported 97 % Pb biosorption after 180 min of contact time. The discrepancies in binding capacity of the strains to metal cations at different time periods appears to be related to the variations in functional groups on the cell surface, which indicates strain specificity. The appraisal of Pb and Cd removal efficiency using *Vibrio alginolyticus* PBR1 exhibited 82 % Pb and 59 % Cd biosorption with 2 g/L biomass concentration (Parmar et al., 2020). The increased metal biosorption efficacy due to subsequent increase in biomass density is justified by the presence of more accessible binding sites for metal cations.

## 5. Conclusion

In conclusion, LAB could be employed as a cheap biosorbent for Pb removal. Pb resistance and biosorption are strain specific attributes as different strains showed variation for MIC and Pb removal rates. The high biosorption capacity of our strains indicate their superiority over previously mentioned isolates, these isolates may later be exploited for Pb detoxification in animals and reducing Pb levels in the environment. The exceptional stability of the Pb-LAB-complex and presence of electronegative charge on the cell surface give important insights for effective Pb biosorption through LAB. According to RSM data, maximum Pb biosorption could be achieved at pH 6 with biomass of 1 g/L in 60 min. Highest removal at pH 6 indicate that our strains could survive in the intestine and efficiently sequester Pb ions. Moreover, this study suggests that studied strains have tolerance to GIT conditions and adherence ability which make them potent candidates for *in vivo* removal studies. Further work targeting, influence of expression of gene involved in Pb resistance and removal are required to attest the full spectrum of strains.

## 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.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2022.103547.

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