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EVALUATION OF *ENTEROCOCCUS DURANS* E1T1 ISOLATED FROM INFANT FECES AS A PROMISING PROBIOTIC FOR CADMIUM DETOXIFICATION

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ABSTRACT

The purpose of the current study was to evaluate the ability of some metal-resistant lactic acid bacteria (LAB) to remove cadmium in order to highlight the importance of probiotic LAB in the detoxification of these pollutants at the level of the digestive tract or in fermented foods. Infant feces samples were used to isolate Cd-resistant LAB, they were screened using the spread plate technique on agar media after being supplemented with cadmium at an initial concentration of 50 mg.L^{-1}. In this research, out of 106 isolates tested for Cd-resistance, 11 isolates were selected based on their high capacity to grow in presence of cadmium. After which they were tested for their ability to resist different heavy metals and for probiotic properties. The study was completed using the isolate E1T1 identified as *Enterococcus durans* based on 16S rRNA gene sequence, which showed the highest cadmium (MIC 600 mg.L^{-1}) and other heavy metals resistance capacity and better probiotic properties. The selected isolate presented strong adhesion ability to epithelial cells, good antibacterial activity, and was non-hemolytic. Furthermore, it had excellent cadmium removal efficiencies, with rates of 65.94% and 28.78% after 96 hours in living and dead cells, respectively. *E. durans* E1T1 had also high cadmium accumulation efficiencies, with an estimated value of 11.47 mg Cd.g^{-1} of cells (wet weight). According to the findings from the study, the Cd-resistant *E. durans* E1T1 may be employed as a promising probiotic isolate for removing cadmium.

Keywords: Probiotic; *Enterococcus durans*; Cadmium; Detoxification; Bioaccumulation

INTRODUCTION

Several toxic substances can be found in food and water, and their abundance depends on various factors such as the type of food, its origin, and how it is processed. Some of the most common toxic substances found in food and water include mycotoxins, pesticide residues and heavy metals. These pollutants has deleterious effects on human and animal body systems. Mycotoxins represented by aflatoxins were reported to disturb some endocrine functions of the reproductive organs, which could adversely affect productivity (**Amin *et al.*, 2019**). Pesticides are also responsible of many reproductive disorders (**Fu *et al.*, 2021**). Heavy metals represented by arsenic, cadmium, chromium, lead, and mercury are of the priority metals that are of great public health significance. They are all systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure (**Tchounwou *et al.*, 2012**). One of the major hazardous heavy metals is cadmium (Cd), a non-essential trace metal and a highly toxic substance to almost all living organisms. Due to several anthropogenic activities, its concentration in the environment is rising at an alarming rate, which raises the risk of Cd human exposure (**Ali and Khan, 2019**). According to several scientists, exposure to Cd in humans and other mammals' leads easily to its accumulation in organs such as testicles, liver, kidneys, lungs, etc. causing various diseases **(Thévenod and Lee, 2013; Jaishankar *et al.*, 2014; Kapahi and Sachdeva, 2019).**
Lactic acid bacteria (LAB) have been known to detoxify, absorb, and even accumulate heavy metals, according to several authors **(Feord, 2002; Varma *et al.*, 2010; Kinoshita *et al.*, 2013**). LAB are common microorganisms present in the human gastrointestinal tract (GIT) and are considered as safe probiotics with various beneficial effects (**Gupta *et al.*, 2018**). The beneficial effects of probiotic bacteria were reported by many researchers (**Castro et *al.*, 2016; Sánchez *et al.*, 2017; Zakaria, *et al.*, 2019; Divyashree *et al.*, 2021).** Probiotics can control intestinal inflammatory disease, inhibit pathogenic infections by the production of antimicrobial compounds, inhibit the adhesion of pathogens to the surface of the intestinal and vaginal epithelial cells and stimulate the immune system via interactions with immune cells. Furthermore, **Chen *et al.* (2022)** have shown that one of the useful properties of probiotic bacteria is their capacity to bind different targets including heavy metals. They also revealed their possible role for detoxification of heavy metals and eliminating them through feces. According to **Djurasevic *et al.* (2017)** probiotics actively contribute to the elimination of heavy metals by different mechanisms, for instance, through binding to their bacterial cell wall. Similarly, **George *et al.* (2021)** assessed the ability of LABs (*lactobacilli*, *enterococci*, and *Weissella* spp.) to remove potentially harmful lead, cadmium and aluminium *in vitro* by metal biosorption or bioaccumulation to decrease heavy metal contamination in aqueous media.
Based on the above information, the aim of our study is to evaluate *in vitro* the probiotic potential of the high Cd-resistant bacterium *Enterococcus durans* E1T1 isolated from infant feces, to prove its Cd removal and bioaccumulation ability and to highlight the different mechanisms by which E1T1 can remove Cd from culture medium.

MATERIAL AND METHODS

Isolation and identification of LAB from infant feces

The LAB used in this work were isolated from 2-3 months breast fed infant's feces, according to the method described by **Adetoye *et al.* (2018)** with minor modifications. One g of each sample was added to 9 mL of sterile distilled water and after vortexing, a series of decimal dilutions was performed, then; the appropriate dilutions were plated out on MRS agar and incubated for 24 h at 37°C in aerobic conditions. Morphologically distinct colonies were picked from each plate and sub-cultured to obtain pure cultures. Gram-positive and catalase-negative, isolates were preserved in 20% glycerol in MRS stock at -20 °C until further use.
Among the obtained isolates, eleven were selected for a secondary screening (E2S, E1T1, E2AT7, E1S5T, E1S2M2, E1K2R1, E1A2M2, E2AT6, E2S7T, E1C1, E1K1R1), they were identified based on 16S rRNA sequencing or MALDI-TOF-MS (CIP-Collection of Institut Pasteur, Institut Pasteur, France).

Cadmium stock solution

The cadmium stock solution (1000 mg.L^{-1}) was prepared by dissolving cadmium chloride (CdCl2) (SIGMA-ALDRICH, Germany) in distilled water. Further, the stock was sterilized by autoclaving and serially diluted with distilled water to prepare working concentrations from 10 to 800 mg.L^{-1} (**Bhakta *et al.*, 2012; Kinoshita *et al*., 2013).**

Screening of cadmium-resistant LAB

A total of 106 Gram-positive and catalase-negative bacteria isolated from infant's feces were screened for heavy metal resistance against cadmium (Cd). Isolated LAB were inoculated in MRS broth medium containing 50 mg Cd.L^{-1} then incubated with agitation at 37°C for 5 days. The cell concentration of the screened isolates was determined by absorbance at 600 nm (OD_{600}) using a UV–vis spectrophotometer (**Guo *et al*., 2010; Kinoshita *et al*., 2013)**. The specific growth rates of the different isolates were obtained using the following formula:

$\mu = 1 / OD_0 \times (OD_t - OD_0) / (T_t - T_0)$ **(Mathivanan and Rajaram, 2014).**

Where OD_0 is the optical density at initial time, OD_t is the optical density at time t, and T_t and T_0 represent corresponding times (h).

Cadmium MIC determination and co-resistance to other heavy metals

The Cd tolerance of the eleven selected isolates was determined by the minimum inhibitory concentration (MIC) approach **(Zhai *et al*., 2015)**. Sterile Cd(II) solutions were added into the MRS broth to a final concentration ranging from 10 to 800 mg.L^{-1}. Then a volume of 2% (v/v) of inoculum, adjusted to 10^9 CFU.mL^{-1} was inoculated into MRS broth containing the above mentioned concentrations of cadmium. MRS without Cd was used as control and incubated for 24 h at 37°C. The lowest Cd(II) concentration that greatly inhibits the LAB growth was considered the MIC **(Pakdel *et al*., 2019).**

All the selected cadmium-resistant isolates were tested for their ability to resist different metals, the MICs of Pb(II), Fe(II), Cr(VI) and Hg(II) were determined. A concentration of 10 mg.L^{-1} was used as initial concentration that was gradually increased of 50 mg.L^{-1} each time until MIC was reached **(Benmalek *et al*., 2014; Lin *et al*., 2016).**

Acid and bile tolerance

The acid-tolerance of the screened isolates was investigated following the method described by **Ahmed *et al.* (2017)** and **Yi *et al.* (2017)**. Briefly, bacterial cells from a 20 h culture were harvested by centrifugation at 6000 rpm for 10min and washed twice with phosphate buffer saline (PBS), pH 7.4, before being resuspended (10^9 CFU.mL^{-1}) into the acidified PBS adjusted to pH 2, pH 3, pH 4 and control pH 6.4 and incubated at 37°C for up to 24 h. The viable cells were counted at 0 h, 2 h, 4 h and after 24 h. For bile tolerance, the LAB (10^9 CFU.mL^{-1}) were incubated at 37°C for 24 h in PBS with 0.3% and 1% (w/v) oxgall powder and PBS without bile was used as control. Aliquots were drawn at intervals of 0 h, 2 h and 4 h, 8 h and 24 h, serially diluted and plated onto MRS agar at 37°C for 48 h. Viability was examined using the plate count method in triplicate and expressed as mean ± SD. The survival rate was calculated as follows:

Survival rate % = $\frac{N_1}{N_0} \times 100$ % **(Archer and Halami, 2015)**, where

N_1 is the total viable count of each isolate after treatment with acid or bile and N_0 is the total viable count of each isolate before treatment.

Survival in simulated human intestinal fluid and sodium chloride

Based on the highest acid and bile tolerance, survival of the selected isolate E1T1 under simulated human GIT conditions was tested as described by **Kaewnopparat *et al.* (2013) and Menconi *et al.* (2013)**. Briefly, fresh cultures were collected by centrifugation at 6000 rpm for 5 minutes, washed twice with PBS, and then resuspended (10^9 CFU.mL^{-1}) in the following tested solutions. For the test of resistance to simulated human intestinal fluid (SIF) bacterial cells were resuspended in a solution prepared with 9 g.L^{-1} of NaCl, 10 g.L^{-1} of pancreatin,10 g.L^{-1} of trypsin and 3 g.L^{-1} of bile salts (pH 6.4 and pH 8). For NaCl resistance, bacterial cells were resuspended in PBS, pH 6.4, with 3.5% and 6.5% of added NaCl. Resistance of the tested isolate was assessed in terms of viable colony count on MRS agar after the treatment at 0 h and after 1 h, 3 h, and 24 h of incubation. The survival rate was calculated according to the described above formula.

Antibacterial activity

The agar-well diffusion method was used to test the ability of the selected isolate to inhibit some indicator bacteria, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumonia* ATCC 700603, *Pseudomonas aeruginosa* (UHC, Constantine, Algeria) and some clinically isolated bacteria *Klebsiella* spp, *Salmonella* sp. and *Escherichia coli* (Bekioua. A. Medical analysis laboratory, Jijel, Algeria). With a few minor modifications, the antibacterial activity was conducted as described by **Giri *et al.* (2019)**. Hence, indicator strain (10^7 CFU.mL^{-1}) was seeded on nutrient agar. Wells were bored and inoculated with 50 µL of the cells free supernatant CFS (pH 3.95) or neutralized CFS (pH 6.8) obtained by centrifugation of an overnight culture at 6000 rpm for 10 min, and filtered through Millipore filter (0.22 µm), then left to diffuse for 2 h at 4°C. The plates were incubated at 37°C for 24h and observed for zone of inhibition.

Bacterial adhesion assay

The adhesion capacity of the selected isolate to poultry epithelial cells was assessed based on the method described by **Ouled-Haddar *et al.* (2012)**. The cell pellet from an overnight culture of E1T1 was resuspended to approximately 1×10^8 cells.mL^{-1} in PBS. Epithelial cell sample collected from poultry ileum was washed with sterilized PBS (pH 7.2). It was held in PBS at 4°C for 30 min and then washed three times with PBS. One mL of the bacterial suspension was mixed with 1 mL of the cell suspension of epithelial cells. The mixture was incubated at 37°C for 30 min. The adhesion was observed using a light microscope (magnification fold, x100) after staining with 0.5% crystal violet for 5 min **(Ouled-Haddar *et al*., 2012; Idoui, 2014)**.

Hemolytic activity

The hemolytic activity is the ability of the tested strain to lyse red blood cells, leading to the release of hemoglobin. E1T1 was incubated on blood agar for 24 h to 48 h at 37°C **(Pieniz *et al*., 2014; Li *et al*., 2018).** The strain was considered hemolytic if it produces green-hued halo (α-hemolysis) or a clear halo around colonies (β hemolysis).

Antibiotic resistance

Resistance of E1T1 to the following antibiotics ampicillin (10 µg), amoxicillin (10 µg), penicillin (10 µg); gentamicin (10 µg), tetracyclin (30 µg), streptomycin (10 µg) and ciprofloxacin (5 µg) was tested by the disk diffusion method (**Adetoye *et al*., 2018; Mulaw *et al*., 2019).**

Cadmium removing capacity

All experiments for Cd(II) removal were performed in 250 mL Erlenmeyer flasks. Two percent of overnight cultures were cultivated in MRS broth and after incubation at 37ºC for 24 h, the culture was centrifuged (6000 rpm/20 min) then cells were washed three times with sterile distilled water. Cell samples (10^9 CFU.mL^{-1}) were used to inoculate 100 mL of MRS broth containing an initial concentration of Cd(II) of 50 mg.L^{-1}, the culture was incubated at 37°C with shaking at 100 for 96 h according to **Akhter *et al.* (2017)**. In order to highlight the role of any others factors on Cd(II) reduction, three types of control sets were run in parallel to the test solution: (A) control flask with MRS broth devoid of Cd(II), (B) MRS broth containing Cd(II) inoculated with (10^9 CFU.mL^{-1}) of autoclaved cells and (C) flask with un-inoculated MRS broth containing Cd(II). During incubation (t_0, t_{24}, t_{48}, t_{72}, and t_{96} h), aliquots of samples (10 ml) from all the flasks were centrifuged (6000 rpm/20 min) and supernatants thus obtained were used for the estimation of residual Cd(II) concentration by flame atomic absorption spectrophotometry (Shimadzu AA-6200) **(Bhattacharya and Gupta, 2013; Zhai *et al*., 2015).** Bacterial growth was measured via OD at 600 nm. The reduction rate was calculated using the following formula according to **Akhter *et al.* (2017)**:

Reduction rate $\mathbf{R\% = [(C_0\text{-}C_t) / C_0] \times 100}$, where

C_0 and C_t are the initial Cd concentration and the residual Cd concentration after removal in time t, respectively.

Bioaccumulation capacity of E1T1 isolate

This test was carried out as described by **Akhter *et al.* (2017)** with some modifications. Two percent (v/v) of cell samples (10^9 CFU.mL^{-1}), obtained by harvesting and processing the cells as described above, were mixed with 100 mL of sterile cadmium solution at 50 mg.L^{-1}, then incubated on a shaking incubator at 150 rpm at 37ºC for 96 h. The effect of contact time and initial metal concentration (10, 50, 100, 200 and 300 mg.L^{-1}) on cadmium bioaccumulation was systematically tested. Aliquot were taken out at prearranged time intervals (0, 24, 48, 72 and 96 h) and centrifuged (6000 rpm/20 min) after repeated washing with distilled water. The collected pellet was firstly dried at 80ºC until constant weight and then the dried cells were weighed out. Secondly, the metal content was determined after cell lysis through acid dissolution of collected pellet as described by **Nithya *et al.* (2011)** and **Aslam *et al.* (2020)**, the pellets were treated with HNO_3 (1%) for 24 h at 4°C. The bacterial lysates were centrifuged (6000 rpm/10 min) after filtration through Millipore filters (0.22 µm), and supernatant was used for spectrophotometric determination of intracellular concentration of Cd(II). The amount of accumulated metal in mg Cd.g^{-1} of the bacterial biomass was calculated by the equation:

$$\mathbf{qe = C_i .V/M}$$

Where,

qe: the amount of metal accumulated in mg.g^{-1} of bacterial biomass at equilibrium,

Ci: Intracellular concentration of metal ion in mg.L^{-1}.

V: solution volume in liters

M: the weight of biomass in grams.
Besides, growth of the selected strain in absence and presence of 10, 50, 100, 200 and 300 mg.L^{-1} Cd was estimated by measuring the optical density at 660 nm at different time intervals.

Statistical analysis

All experiments were carried out in triplicate and all data are represented as the mean ± the standard error of the mean (SEM) except for removal and bioaccumulation tests which were done in duplicate. The obtained data were analysed by analysis of variance (ANOVA) and Student's test using Excel stat 2016. A $p \leq 0.05$ was considered statistically significant.

RESULTS

Isolation and identification of LAB isolates

A hundred and six isolates have been isolated in this study, all of them have shown resistance to cadmium (50 mg.L^{-1}) for 96 h, only eleven Cd-resistant bacteria with higher specific growth rates were selected for further analysis, they were identified using 16S rRNA sequencing or MALDI-TOF-MS as *Enterococcus durans* (E2S, E1T1, E2AT7, E1S5T), *Enterococcus faecium* (E1S2M2, E1K2R1), *Enterococcus avium* (E1A2M2), *Levilactobacillus brevis* (E2AT6), *Lactobacillus paracasei* (E2S7T), *Lactobacillus plantarum* (E1C1) and *Leuconostoc lactis* (E1K1R1).

Cd-resistant ability of LAB isolates

As shown in figure 1, five isolates, *E. durans* (E1T1, E2AT7), *L. brevis* (E2AT6), *L. paracasei* (E2S7T) and *L. plantarum* (E1C1) showed a high resistance to cadmium and did not differ significantly ($p>0.05$) in growth rates in presence of the metal compared with the controls. Although, significant ($p<0.05$) growth inhibition were observed with the remaining isolates compared with the controls (absence of metal).

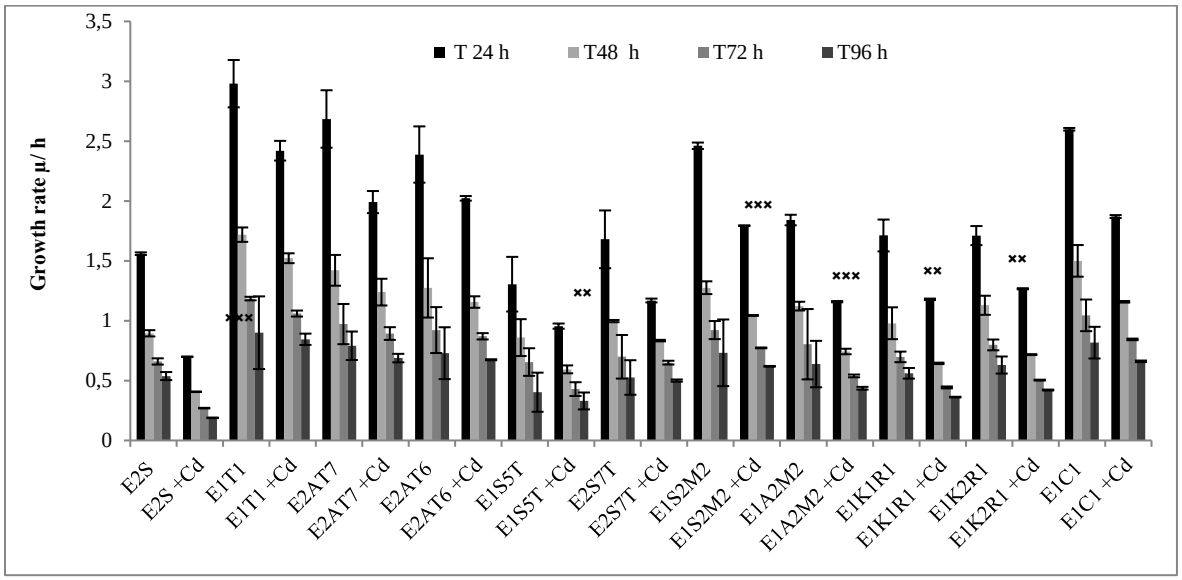


Figure 1 Growth rate (± SE) of eleven selected lactic acid bacteria isolates in MRS broth in absence and in presence of cadmium (initial concentration 50 mg. L^{-1}). ($p < 0.05$) indicates a statistically significant difference between the strains with and without metal (*:$p < 0.05$; *:$p < 0.01$;***:$p < 0.001$)

MIC and co-resistance to heavy metals

Cd-resistant bacteria showed high resistance for cadmium with MICs ranging from 300 to 600 mg.L^{-1}. As shown in Table 1, the isolates E1T1, E2AT6, E1C1and E1S2M2 were the most resistant to cadmium with MIC values > 450 mg.L^{-1}. However, the other isolates were less resistant showing MIC values between 250 and 350 mg.L^{-1}. All the Cd-resistant bacteria were tested for their co-resistance to Pb, Cr, Fe and Hg. The obtained results, summarized in Table 1, indicated that the MICs determined for different metals are very high. As for cadmium, the isolates E1T1, E2AT6, E1C1 and E1S2M2 were the most resistant with MICs ranging from 400 to 600 mg.L^{-1} for Pb(II), 700 to >800 mg.L^{-1} for Cr(VI) and 450 to 650 mg.L^{-1} for Fe (II). In the case of mercury, it was very important to note that the MIC was also significant with values ranging from 250 to 350 mg.L^{-1}.

Table 1 MIC values of cadmium-resistant bacteria and heavy metal co-resistance

	MIC (mg.L^{-1})				
Bacteria	Cd(II)	Pb(II)	Cr(VI)	Hg(II)	Fe(II)
Enterococcus durans E2S	350	200	650	150	300
Enterococcus durans E1T1	600	600	>800	350	650
Enterococcus avium E1A2M2	450	300	550	50	150
Levilactobacillus brevis E2AT6	450	500	750	250	550
Lactobacillus plantarum E1C1	550	500	>800	350	500
Lactobacillus paracasei E2S7T	350	350	450	50	150
Enterococcus faecium E1S2M2	550	400	700	250	450
Enterococcus durans E2AT7	400	350	600	100	200
Enterococcus durans E1S5T	350	200	550	150	300
Enterococcus faecium E1K2R1	350	300	600	150	200
Leuconostoc lactis E1K1R1	400	300	550	100	300

Survival under acidic, bile, SIF and NaCl conditions

All tested Cd-resistant LAB were selected as probiotics based on their ability to survive at strongly acid conditions including pH 2, varying bile salt levels up to 1% concentration, SIF conditions and NaCl concentrations with high survival rates (data not shown), however, three isolates namely E1S5T, E2AT7 and E2S showed a lower survival rate. The isolate *E. durans* E1T1 was selected for further investigations because it showed the highest specific growth rates in the presence of Cd, the highest MIC values for the different heavy metals (Cd, Pb, Cr, Hg and Fe) and the highest survival rate under conditions that simulated the human GIT. It appears from the presented results that the acid tolerance profile of the selected isolate (figure 2A) showed no significant decrease on cell numbers at pH 4 and 3 with high survival rates of (95.63% and 92.51%) after 2h and (85.93% and 75.33%) after 4h, respectively. However, at pH 2, the isolate displayed high survival rates

(81.17%) after 2 h with a significant ($p<0.05$) decrease (61.09%) after 4h when compared to cells before treatment, it was also noticed an important survival rate even after 24h of incubation; 49.01%, 32.52% and 24.01% at pH 4, 3 and 2, respectively. Similarly, *E. durans* E1T1 survived with bile salts (figure 2B) were it showed no significant change in survival rates of exposure to 0.1, 0.3 and 1% bile salts after 2 h (98.15%, 84.85% and 76.52%, respectively) and 4h (93.26%, 74.83% and 65.86%, respectively). In contrast, a significant ($p<0.05$) decrease was observed after exposure over 24h to bile salts with a progressive reduction of the cell number to about 62.61%, 31.37% and 7.52%, respectively, for each concentration. Concerning the survival of E1T1 in SIF and high NaCl concentrations, it is shown in figure 2C and 2D, respectively. When exposed to SIF at either pH 6.8 or 8, *E. durans* E1T1 was able to survive with high survival rate (more than 50%) compared to the control after 1 and 3 h of incubation. However, it showed also a significant ($p<0.05$) decrease, 49.81% and 37.52%, respectively, after 24 h. It was the same case with NaCl exposure at 3.5% and 6.5% where the isolate was also able to tolerate high osmotic concentrations of NaCl with survival rates of 75.53 at 3.5% and 59.12 at 6.5% after 3h of incubation. While after 24 h of incubation, the survival rate decreased below 50% (about 47.52% and 30.3%, respectively).

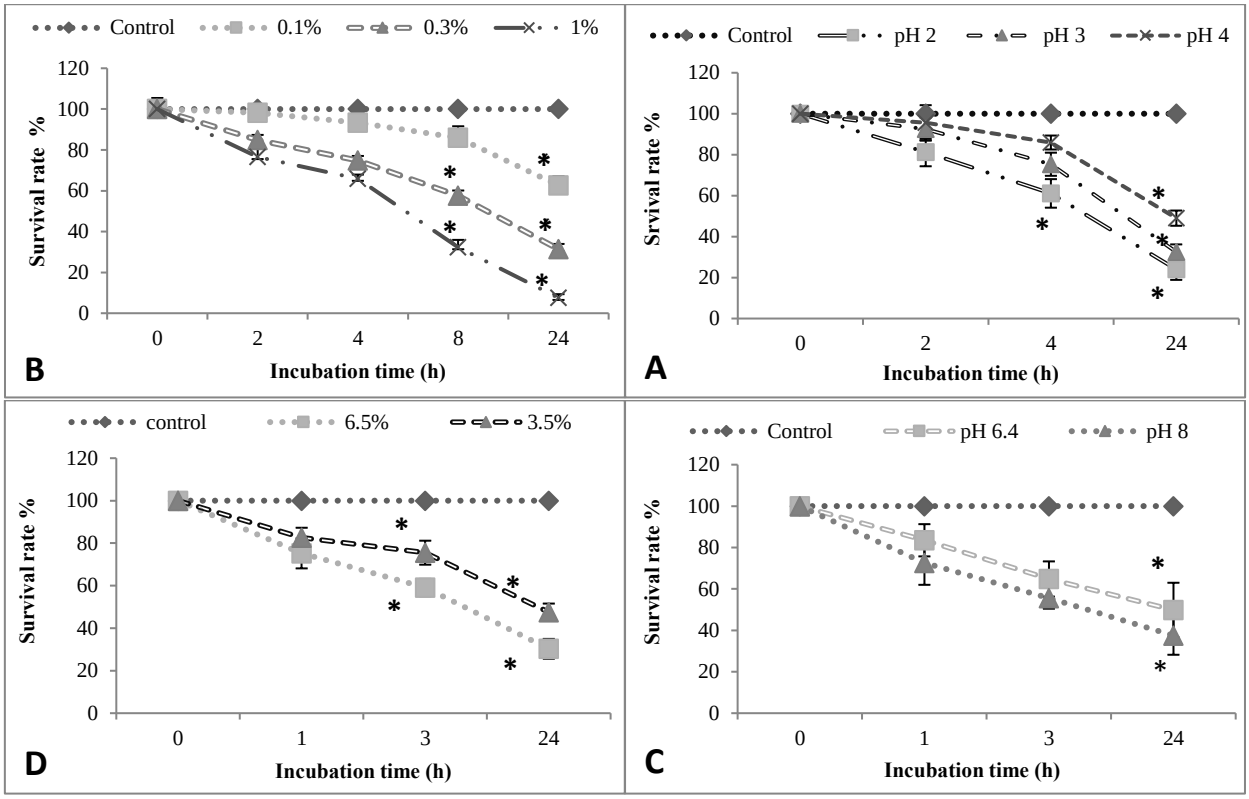


Figure 2 Effect of simulated human GIT conditions on the survival of E1T1: **A** acid conditions, **B** presence of bile salts, **C** simulated intestinal fluid and **D** presence of NaCl (*: $p < 0.05$ compared with before treatment).

Antibacterial activity of the isolate *E. durans* E1T1

One of the important characteristics of probiotic is the ability to produce substances having antibacterial activity such as bacteriocins, organic acids and hydrogen peroxide to inhibit the growth of pathogenic bacteria **(Divyashree et al., 2021).** The results in table 2 show that *E. durans* E1T1 presents varying diameters of inhibition zones toward indicator bacteria using the cell-free supernatant (CFS). With the clinical isolates *Klebsiella* spp, *Salmonella* sp. and *E. coli* it showed lower diameters of inhibition zone, however, the tested strain exhibited a remarkable inhibition zones against *E. coli* ATCC 25922, *K. pneumonia* ATCC 700603, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC. The same table shows the inhibition zone diameters of *E. durans* after neutralizing the pH of CFS to 6.8, we detected a slight reduction in antimicrobial activity with ATCC strains *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 and almost a complete loss of inhibition zone with clinical isolates (*Klebsiella* spp, *Salmonella* sp. and *E. coli*). These results indicated that some released antimicrobial compounds contributed greatly to the inhibitory effect of isolate *E. durans* E1T1.

Table 2 Mean zone of inhibition (mm ± SE) of the cell-free supernatant (CFS) and neutralized CFS of *Enterococcus durans* against ATCC and clinical isolates.

	ATCC strains				Clinical isolates		
Test strains	*E. coli* ATCC25922	*S. aureus* ATCC25923	*K. pneumonia* ATCC700603	*P. aeruginosa* ATCC27853	*Klebsiella* spp	*Salmonella* sp.	*E. coli*
CFS	20.3± 0.57	18.6±1.16	19±1	18.3±0.57	9 ±1.41	8.5±0.7	11.2±0.4
Neutralized CFS	16.5±0,7	14.5±0.7	15.5±0.7	14±1.41	3±0.7	3.5±0.7	00±00

Bacterial adhesion assay

One of the most targeted properties for the selection of probiotics is their ability to adhere to host's intestinal mucosa. The bacterial isolate of LAB is considered as a ''positive'' adherent isolate if more than 15 cells adhered on one epithelial cell (≥ 15 LAB cells/epithelial cell). As presented in figure 3, a high ability of *E. durans* E1T1 isolate to adhere to poultry epithelial cell is shown compared to the control.

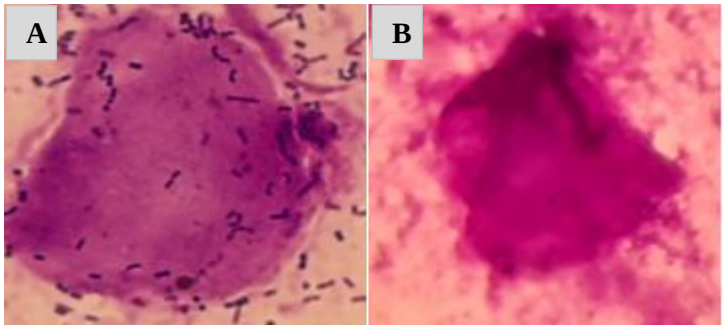


Figure 3 A: Adherence of isolate *E. durans* E1T1 to poultry epithelial cells. B: Control (epithelial cell without *E. durans* E1T1)

Hemolytic activity

The tested isolate *E. durans* E1T1 did not exhibit any hemolytic effect (γ-hemolysis) after 48 h of incubation on blood agar plates (data not shown), which supports the safety of this isolate.

Antibiotic resistance

The antibiotic susceptibility test of *E. durans* E1T1 showed sensitivity to ampicillin, amoxicillin, penicillin, streptomycin and tetracyclin. Nevertheless, the isolate presented resistance to ciprofloxacin and gentamicin (Table 3).

Table 3 Mean values (mm ± SE) of growth inhibition zones of the antibiotic susceptibility profile of probiotic *Enterococcus durans* E1T1.

	Inhibition zone (mm)						
Antibiotic	Ampicillin	Amoxicillin	Tetracyclin	Penicillin	Gentamicin	Ciprofloxacin	Streptomycin
E1T1	33.3 ± 1.52^{S}	22 ± 1^{S}	17 ± 1^{I}	16.6 ± 0.57^{I}	00^{R}	00^{R}	16.3 ± 0.57^{I}

Zone of inhibition (diameter in mm) for each antibiotic was measured and expressed as susceptible, S (≥21 mm); intermediate, I (16–20 mm); and resistance, R (≤15 mm) **(Mulaw *et al*., 2019).**

Cadmium removal capacity

Cadmium removal capacity results displayed by *E. durans* E1T1 are shown in Figure 4. Results showed that the growth rate of *E. durans* E1T1 was accompanied by a removal of Cd(II) in case of living cells. The growth profile showed an initial rapid increase ($p<0.001$) in cell density to reach 3.625± 0.077 after 24 h. At the same time, the efficiency of the bacterial isolate to remove Cd(II) was noteworthy with highly significant increase ($p<0.001$), where it reached 30.21% in the first 24 h of incubation followed by a slightly slower rate with increasing time each 24h and no significant increase in removal rate (65.94%) after 96 h of incubation was noticed.

In separate group of tests, the intracellular quantity of Cd and the effect of abiotic factors (oxidation and photo-oxidation) on metal removal rate were found negligible, no significant change was marked (less than 10%). Whereas in set inoculated with dead E1T1 cells, the removal rate of Cd(II) was highly significant ($p<0.001$) after 24 h reaching 20.28% with no significant change after 96 h of incubation (28.78%).

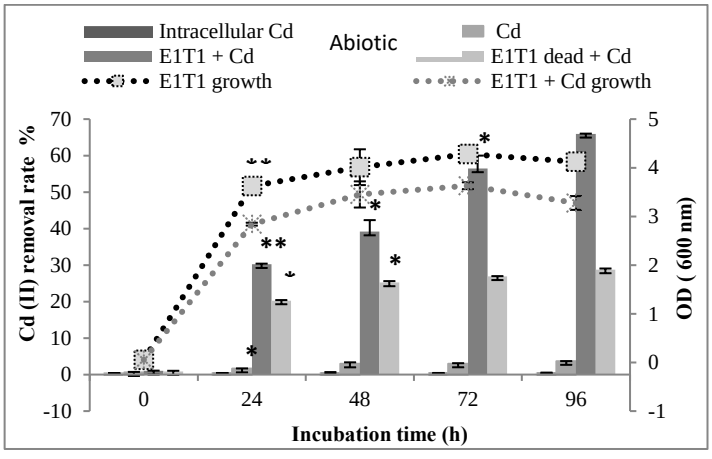
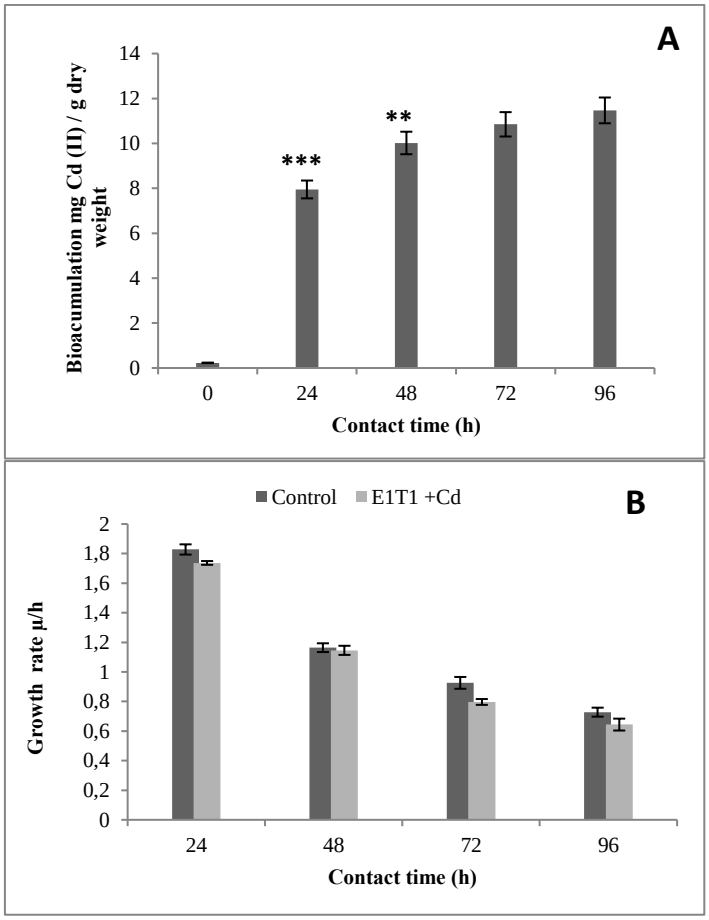


Figure 4 Growth and Cd(II) removal rate by living and dead cells with the effect of abiotic factors and the intracellular quantity of Cd in *Enterococcus durans* E1T1 cells on metal removal rate with initial Cd(II) concentration of 50 mg. L^{-1}. Values are presented as mean ±SE. (*:$p < 0.05$; *:$p < 0.01$;***:$p < 0.001$)

Bioaccumulation capacity of the selected isolate

The cadmium accumulation capacity of *E. durans* E1T1 was examined at different contact times (metal concentration 50 $mg.L^{-1}$) and different metal concentrations (incubation time 96 h). As shown in figure 5A, the quantity of bioaccumulated Cd(II) by the tested isolate increased with the increase of contact time, especially during the first 24h where it showed very highly significant increase ($p<0.001$) from 0.23 to 7.95 mg $Cd.g^{-1}$ dry weight compared with cells before treatment, where the growth rate (figure 5B) is not affected by the presence of metal ions (50 $mg.L^{-1}$ of Cd); no significant decrease was observed compared with the control sample (1.737 h^{-1} and 1.828 h^{-1}, respectively). It was clear that the bioaccumulation of metal ions increased slowly as time progressed where the maximum uptake efficiency and the growth rate after 96 h were (11.47 mg $Cd.g^{-1}$ dry cells and 0.644 h^{-1}, respectively). The effect of Cd(II) concentrations on Cd bioaccumulation capacity by *E. durans* E1T1 was examined at different concentrations ranging from 10 to 300 $mg.L^{-1}$. As revealed in figure 5C, a highly significant increase ($p<0.001$) in accumulation of the metal ion with the rise of initial Cd(II) concentration was recorded, and it reached a maximum of 14.11mg $Cd.g^{-1}$ dry cells in presence of 200 $mg.L^{-1}$ of the metal ion, while a significant decrease ($p<0.01$) was observed when the cadmium concentration was 300 $mg.L^{-1}$. This is parallel to a highly significant ($p<0.001$) decrease in growth rate (figure 5D) due to the increasing concentration of the metal, where they reached 11.29 mg $Cd.g^{-1}$ dry cells and 0.115 h^{-1}, respectively.



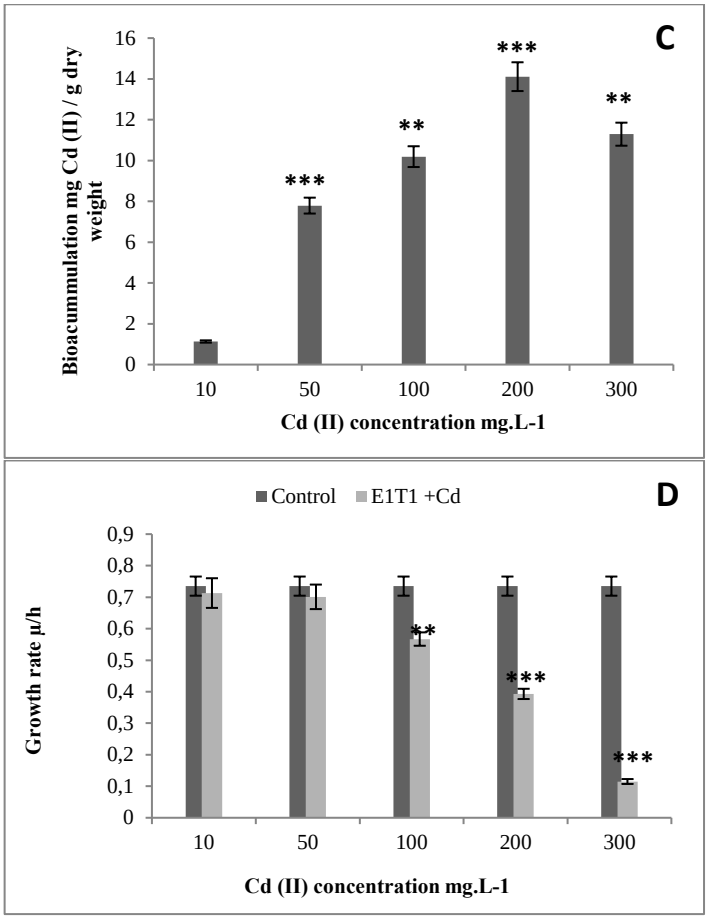


Figure 5 Effect of incubation time (A) and metal ion concentration (C) on cadmium accumulation efficiency by the *E. durans* E1T1. Growth rate of selected isolate in absence and in presence of cadmium; (B) with 50 mg. L^{-1} of Cd (II) and increasing time, (D) with increasing concentration of metal after 96 h. Values are expressed as mean ±SE. (*: $p < 0.05$; *: $p < 0.01$;***: $p < 0.001$)

DISCUSSION

Many species of LAB have been isolated from different environments with promising applications in bioremediation approaches for the removal of toxic heavy metals **(Zhai *et al*., 2016).** Reports concerning the *in vitro* characteristics of enterococci are relatively scarce, in comparison with the abundant information on lactobacilli. In recent years, promising LAB strains were isolated including members of the genera *Lactobacillus*, *Bifidobacterium* and *Enterococcus*, which can improve immunity, inhibit the adherence of some pathogens, display anticancer capacity and have the potential to remove heavy metal **(Hasan *et al*., 2022; Shu *et al*., 2021).**

In the current study, LAB isolates were obtained using MRS medium supplemented with cadmium at an initial concentration of 50 $mg.L^{-1}$. Among the hundred and six Cd-resistant LAB isolated from infant faeces, eleven Cd-resistant isolates were further tested for their tolerance to four different heavy metals Pb(II), Fe(II), Cr(VI) and Hg(II). The isolate E1T1 which was identified as *E. durans* showed the highest ability to grow in MRS broth supplemented with 50 mg/L of cadmium (growth rate 2.42 h^{-1} compared with the control 2.98 h^{-1}). It also showed the highest growth rate in presence of four other toxic heavy metals. In the report of **Mathivanan and Rajaram (2014)**, the researchers isolated seventeen Cd resistant strains grown under cadmium stress (50 and 100 $mg.L^{-1}$), they were termed highly cadmium-resistant bacteria (HCRB). The HCRB growth rate varied with the cadmium concentration. As the added cadmium concentration increased, the growth rates of bacterial cultures decreased.

The MIC reflects the bacterial metal tolerance or the ability of an organism to survive in an environment with high concentration of metals or to accumulate high concentration of metal without dying **(Dobrowolski *et al*., 2017**). In this study, the MICs results showed the effect of increasing concentrations (10 to 1000 $mg.L^{-1}$) of different toxic metals on the viability and the growth of the tested isolates. As the concentration of metal increased the number of metal tolerating cells decreased. The MICs values were different for each isolate, which can be due to the various bacterial structures, membrane variety and various functional groups as reported by **Pakadel *et al.* (2019)**. According to **Bhakta *et al.* (2012)**, no relationship between the bacterial resistance for heavy metals, the removal efficiencies and their bioaccumulation abilities for these metals is noticed, in contrast to the opinion of **Zhai *et al*. (2015)** and **Mustapha and Halimoon (2015)** who reported that bacterial strains known as metal-resistant are considered useful tools in removal and bioaccumulation of heavy metals only when the MIC value is higher than 100 $mg.L^{-1}$. All of the hundred and six isolates were considered resistant to the tested metals. The selected isolate *E. durans* E1T1 was highly resistant to heavy metals; especially to Cd (600 $mg.L^{-1}$). The order of toxicity of heavy metals to *E. durans* E1T1 was Cr > Fe > Cd > Pb >Hg. *E. durans* E1T1 was selected as a highly Cd-resistant LAB, despite the fact that previous studies indicated that Cd(II) is poisonous for bacterial cells and can prevent cellular metabolism and finally cause cell death **(Zhai *et al*., 2015**).

Concerning the probiotic properties, several authors reported that LAB exert a good health effect on the host as probiotics (**Harzallah and Belhadj, 2013; Ouled Haddar *et al*., 2016; Sifour *et al*., 2012; Yerlikaya and Akbulut, 2020**). According to **Amund (2016**), the bacteria need to resist the harsh conditions of the GIT. The low pH of the stomach and bile salts in the upper intestine are known as effective barriers for the entry of bacteria into the human gastric transit. In the present study, it was observed that *E. durans* E1T1 have the highest tolerance to survive in pH 3 and 4 compared with the control pH 6.4 and the others tested isolates. Moreover, it appears that its survival rate is significant at pH 2 after 2h of exposure; the bacterium remained alive for more than 24 h despite the decrease in cell number. Similarly, the same behavior was observed in all tested concentrations of bile salts (from 0.1% to 1%). SIF had also no effect on *E. durans* E1T1 survival rate even after 24 hours. Furthermore, *E. durans* E1T1 survived in 3.5% or 6.5% of NaCl, this test gave an indication of the osmotolerance level of the strain. According to **Menconi *et al*. (2013),** bacterial cells cultured in a high salt concentration could have a loss of turgor pressure, which would then affect their physiology, enzyme activity, water activity, and metabolism. These findings are consistent with those of **Pieniz *et al*. (2014)** who found that, *E. durans* LAB18s survived in all tested times at pH 3 and pH 4, with bile salts and in the SIF. However, the LAB did not survive at pH 2, with no viable cell counts even in the first hour; this contradicts our results where *E. durans* E1T1 resists to pH 2 for more than 24 h.

The antibacterial activity of LAB may be due to the production of organic acids, hydrogen peroxide, or bacteriocins **(Ahmadova *et al*., 2013; Ren *et al*., 2022; Sifour *et al*., 2014).** This property may well increase the safety and the quality of fermented products, it can also prevent the growth of pathogenic and spoilage bacteria. According to **Yerlikaya and Akbulut (2020),** the formed zones of inhibition to which the diameter is lower than 6 mm including the disc diameter were evaluated as 'small inhibition zones' and regarded as negative, whereas the zones larger than 6–18 mm were evaluated as 'large inhibition zones', regarded as positive. Therefore, *E. durans* E1T1 presented an evident antagonism against indicator and pathogenic bacteria, which indicate its ability to produce substances having antibacterial activity. On the same context, **Pieniz *et al.* (2014)** found that probiotic strain *E. durans* LAB18s showed a broad spectrum of antimicrobial activity, inhibiting *Listeria monocytogenes*, *E. coli*, *Salmonella typhimurium*, *P. aeruginosa* and *Aeromonas hydrophila* and all of the tested indicator microorganisms. This result is strongly supported by the interpretation of **Yerlikaya and Akbulut (2020)** who found that numerous *Enterococcus* strains including *E. faecalis*, *E. durans* and *E. faecium* have the ability to produce bacteriocins that have an inhibitory effect against *Clostridium botulinum*, *Staphylococcus aureus*, *Vibrio cholera*, and *Clostridium perfringens*.

Adherence to intestinal mucosae and hemolytic activity are considered as important criterions for probiotic selection **(Castro *et al*., 2016; Wang *et al*., 2016). Idoui (2014)** detected a large difference in the level of adhesion within the same species of lactobacilli strains. Furthermore, he considered that the ability of probiotic strains to adhere to epithelial cells and intestinal mucosal influences bacterial retention time in the intestine and the functional activity of bacteria. The selected isolate *E. durans* E1T1 is of human fecal origin and it exhibited a good ability to adhere to epithelial cells. In a similar respect, **Archer *et al.* (2015)** concluded that isolates of human fecal origin displayed a better adhesion in comparison to those obtained from dairy products. On the other hand, our results on hemolytic activity were in agreement with those obtained by **Pieniz *et al*. (2014)** who found that the isolate *E. durans* LAB18s did not exhibit any effect (γ-hemolysis); green area (α-hemolysis), and/or inhibition zone (β-hemolysis) after 48 h of incubation in blood agar plates. It is very important to qualify a bacterial strain as a probiotic candidate, assessing its susceptibility or resistance to a range of antibiotics **(Li *et al*., 2018)**. The antibiotic resistance assays of the isolate *E. durans* E1T1 showed sensitivity to ampicillin, amoxicillin, penicillin, streptomycin and tetracycline. However, the strain exhibited gentamicin and ciprofloxacin resistance. According to **Kaewnopparat *et al*. (2013).** Probiotic strains' Resistance to antibiotic may be an advantage in the case of co-administrations, but not all lactobacilli have intrinsic resistance.

Concerning the metal removal assay, the living biomass was found to remove high concentrations of cadmium compared to the dead cells; this could be due to the capacity of the living cells to remove cadmium by two mechanisms, one is "metabolism-dependent" namely bioaccumulation, and the second is "metabolism-independent" called biosorption. This supposition can be supported by the results reported by many authors (**Akhter *et al*., 2017; Bhakta *et al*., 2012; Giri *et al*., 2019; Lin *et al*., 2016**). The capacity of living or inactive (non-living) biomass of LAB strains to adsorb heavy metals (metabolism-independent) or to accumulate them (passive uptake) depends totally on the physicochemical characteristics of the cell wall, through the existence of different functional groups, known as

efficient metal chelators such as thiol groups of cysteine (R-SH), metallothioneins,–OH groups, COO–C=O groups, and –NH groups **(Zhai *et al.*, 2016)**. In the same context, **Kenney and Fein (2011)** revealed that the adsorption mechanism in LAB strains is performed via the fixation of metal ions to the extracellular polysaccharides (EPS) of the cell surface. Similarly, **Venkatesh *et al.* (2016)** found that the metal adsorption capacity of EPS produced by *E. faecalis* is excellent for Cd(II), where it reached 334.4 ± 1.4 mg/g. On the contrary, in order to compare the ability of dead and living cells of *Lactobacillus* strains to adsorb cadmium, **Pakdel *et al.* (2019)** reported that the biosorption of Cd(II) by dead cells (55.82% ± 0.83) was higher than that of living cells (42.19% ± 0.22) and interpreted their results by the fact that dead cells treated by heat (100ºC) released their intracellular proteins on cell surface and lead to an increase of the number of binding sites.

The intracellular bioaccumulation of cadmium by living biomass (metabolism-dependent) or ‘‘active uptake’’ which occurs only in living cells, requires an active metabolism and energy to transport the metal ions across the cell membrane into the cells, this mechanism is investigated in this study. The result reflects a high bioaccumulation capacity of *E. durans* E1T1 for cadmium. The effect of incubation time on Cd(II) accumulation was also studied, it increased with increasing the contact time even up to 96 h of incubation, and this can be explained by the rapid increase in cell density to reach its maximum which was not affected by the presence of cadmium at a concentration of 50 $mg.L^{-1}$, far from its MIC (**Zhang *et al.*, 2019**). It is clear from the obtained results that the bioaccumulation capacity of *E. durans* E1T1 gradually increases with the increasing of initial Cd(II) concentration from 10 to 200 $mg.L^{-1}$, which may be due to the existence of electrostatic interactions of these cationic metals with the negatively charged density on bacterial cell surface (**Akhter *et al.*, 2017)**, in addition to their bridging between negatively charged functional groups of biomolecules like nucleic acids, proteins, carbohydrates or lipids of the bacterial cell wall. However, accumulating ability decreased with the decrease in bacterial growth when the concentration of Cd(II) was higher than 300 $mg.L^{-1}$. These results are strongly supported by the interpretation of **Ahemad and Malik (2011) and Akhter *et al.* (2017)** which explained that this decrease might be due to the saturation of the cells with metal ions then accumulation of metabolite products which lead to reduce bacterial growth or due to increased toxicity of metal at high concentration close to the MIC.

CONCLUSION

Based on the above discussion, the results clearly revealed that the metal-resistant E1T1 isolate identified as *E. durans* showed a remarkable tolerance in presence of different heavy metals and an outstanding removing and bioaccumulation capacity for cadmium. Moreover, probiotic traits, metal-resistance, Cd-removal, Cd-bioaccumulation, antibiotic-resistance, adhesion and -no hemolysis criteria implied that *E. durans* E1T1could be a potential agent for the *in vivo* detoxification of cadmium, however, further *in vivo* studies on the exposure of *E. durans* E1T1 to this toxic metal are needed to confirm these findings.

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Conflict of Interest Authors declare that they have no conflict of interest.

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