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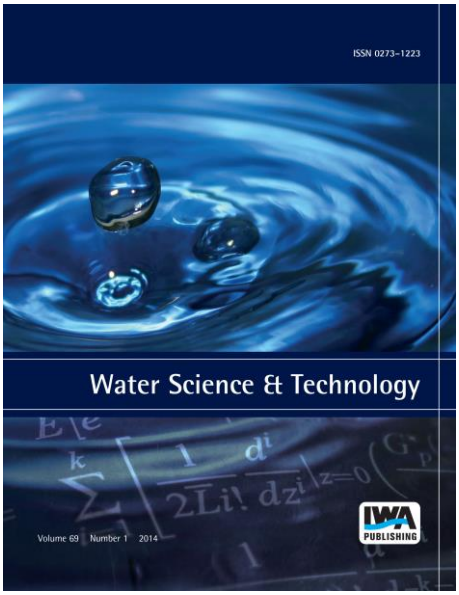

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Application of lactic acid bacteria in removing heavy metals and aflatoxin B1 from contaminated water

Rafaat M. Elsanhoty, I. A. Al-Turki and Mohamed Fawzy Ramadan

ABSTRACT

In this study selected lactic acid bacteria (LAB, *Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus plantrium* and *Streptococcus thermophiles*) and probiotic bacteria (*Bifidobacterium angulatum*) were tested for their ability in removing heavy metals (HM) including cadmium (Cd), lead (Pb) and arsenic (As) as well as aflatoxin B1 (AFB1) from contaminated water. The biosorption parameters (pH, bacterial concentration, contact time and temperature) of removal using individual as well as mixed LAB and probiotic bacteria were studied. Removal of HM and AFB1 depended on the strain, wherein the process was strongly pH-dependent with high removal ability at a pH close to neutral. The increase in bacterial concentration enhanced the removal of Cd, Pb and As. Also, increasing of contact time and temperature increased the ability of LAB to remove HM. The effect of contact time on Cd removal was slightly different when freshly cultured cells were used. The removal of Cd, Pb and As decreased with the increase in the initial metal concentration. The most effective HM removers were *Lactobacillus acidophilus* and *Bifidobacterium angulatum*. The system was found to be adequate for concentrations of HM under investigation. At the end of the operation, the concentration of HM reached the level allowed by the World Health Organization regulations.

Key words | arsenic, cadmium, *Lactobacillus plantarum*, lead, mycotoxin

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INTRODUCTION

The problems of environmental pollution have become more critical with the increasing human population and associated rapid industrialization. The water pollutants cause undesirable changes which in turn affect the ecological balance of the environment. Heavy metals (HM) are non-degradable compounds that may exist in different inorganic and organic forms. When discharged into the water, HM represent a serious threat to the human health. Also, accumulation of HM in marine ecosystems is of global importance due to their adverse impact on human health. Some HM such as Fe, Cu and Zn are essential trace elements but others such as Cd, Pb, Hg and As are toxic even in traces (Munoz-Olivas & Camara 2001; Kheradmand *et al.* 2006; Halttunen 2007; Zoghi *et al.* 2014).

Long-term exposure to arsenic (As) in drinking water is reported to cause skin lesions as well as lung, bladder and kidney cancer (WHO 2001). Arsenic in groundwater is mainly found in two oxidation states, +5 (As(V)) and +3 (As(III)). When conventional water purification methods (coagulation–precipitation, adsorption, ion exchange and membrane filtration) are used, oxidation of As(III) to As(V) often occurs (Wang & Mulligan 2006). Because of its toxicity, WHO (2004) has set a provisional value of 10 μg/L for As in drinking water. Cadmium (Cd) is a toxic heavy metal that can contribute to a variety of adverse health effects. Food and tobacco smoking are the main sources of Cd exposure (Satarug *et al.* 2011; Zhai *et al.* 2015). The Joint FAO/WHO Expert Committee on Food Additives has set a tolerable weekly intake of Cd at 7 mg/kg body weight (FAO/WHO 2006; Zhai *et al.* 2015). Based on human studies involving chronic exposures, the Integrated Risk Information System (IRIS) of the United States Environmental Protection Agency set the threshold of Cd reference dose at 0.5 mg/(kg·d) in water and 1 mg/(kg·d) in food (IRIS 2012; Zhai *et al.* 2015). To date, no specific treatment for Cd poisoning has been confirmed (Nordberg *et al.* 2011), and novel strategies against Cd toxicity need to be developed. Lead (Pb) is a long-lasting environmental pollutant that is responsible for causing various dysfunctions, central and peripheral nervous system

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damages, memory deterioration and diminishing intellectual capacity for children (Jarup 2003). Pb accumulation occurs in the bones and teeth of adults (90%) and children (70%) (Halttunen 2007). Aflatoxins (AFs) are a group of carcinogenic mycotoxins causing various acute or chronic intoxications and liver cancer (Williams *et al.* 2004). Toxicological studies showed that among four naturally occurring AFs (AFB1, AFB2, AFG1 and AFG2) AFB1 is the most common and toxic one (Zoghi *et al.* 2014).

Water and food are the key routes for HM contamination in the biological systems. Aquatic animals, especially fish, are the major sources of HM contamination in the human body (Cheng & Gobas 2007). Therefore, removal of HM from water should be the priority measure to control the problem of bioaccumulation. Different methods for removal of HM from water including flocculation, precipitation, ion exchange and membrane filtration were reported. However, these methods are claimed to have disadvantages such as incomplete HM removal, expensive equipment, monitoring system requirements, high reagent or energy requirements, and generation of toxic sludge or other wastes that require disposal (Zouboulis *et al.* 2004; Halttunen *et al.* 2007a, 2007b; Zoghi *et al.* 2014).

Removal of HM using algal, fungal and bacterial biomass was introduced as an inexpensive and novel method. Mechanisms involved in HM biosorption include complex formation, ion exchange, adsorption, chelation and microprecipitation (Halttunen 2007; Zoghi *et al.* 2014). Lactic acid bacteria (LAB) and bifidobacteria are generally recognized as safe and the most known probiotic microorganisms for reducing biocontamination (Davis *et al.* 2003; Mehta & Gaur 2005). LAB comprise species from genera *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Axelsson 2004). LAB are also often used with the genus *Bifidobacterium*, although they have unique sugar fermentation pathways and are phylogenetically unrelated. Specific strains of LAB are used to ferment food and beverages because of their useful properties or beneficial health effects.

LAB have been reported to remove HM (Halttunen *et al.* 2007a, 2007b) and mycotoxins (Haskard *et al.* 2001; Turbic *et al.* 2002) from water *in vitro*. The removal of HM and mycotoxins from water using LAB has been observed to be strain dependent (Haskard *et al.* 2001; Halttunen *et al.* 2007a, 2007b; Nybom *et al.* 2007). Therefore, combinations of bacteria may be required to efficiently remove different contaminants. HM and AFB1 have been reported to passively bind to the bacterial surface by electrostatic and hydrophobic interactions (Haskard *et al.* 2000; Lahtinen *et al.* 2004; Halttunen *et al.* 2007a, 2007b). Cd and Pb removal occurred rapidly at the bacterial surface, by an ion exchange mechanism (Ibrahim *et al.* 2006; Halttunen *et al.* 2007a, 2007b). Because LAB have negative surface charge, they are optimal for cation binding. However, the removal of negatively charged As species from water may be more complicated. Arsenic removal by *Lactobacillus* strains in native and chemically modified forms was studied (Halttunen *et al.* 2007a, 2007b), where the removal depended on pH and As(V) concentration. In addition, aminated *L. casei* was observed to remove As(V) but not As(III) from water. Zhai *et al.* (2015) screened 33 LAB strains for their potential probiotic capacity against Cd toxicity. *L. rhamnosus* and *L. plantarum* showed good binding ability and Cd tolerance. In addition, evidence that probiotic bacteria and LAB could bind AFB1 was reported (Elsanhoty *et al.* 2013).

The application of the biofiltration methods in the purification of contaminated water was reported in the literature. This study was designed to assess the removal of model toxins (Cd, Pb, As and AFB1) from aqueous solution by individual strains of LAB and their combinations.

MATERIALS AND METHODS

Materials

Chemicals

The HM salts used in this work were lead(II) nitrate $Pb(NO_3)_2$, (Fluka Chemie GmbH, Buchs, Switzerland), cadmium nitrate $Cd(NO_3)_2$, (Fluka Chemie GmbH, Buchs, Switzerland) and sodium arsenate dibasic heptahydrate $Na_2HAsO_4.7H_2O$ (Sigma-Aldrich, Germany). Hydrochloric acid and sodium hydroxide for adjusting pH were obtained from Sigma-Aldrich (Germany).

Microbiological media, bacterial strains and preparation of cultured bacteria

Man Rogosa Sharp broth media (MRS broth) for subculturing and MRS agar for slant culture were obtained from Oxoid (England). In this work, three strains of LAB and two strains of probiotic bacteria were used (Table 1). The strains were selected based on their use in the food industry and on the available information regarding their effects on mutagens. *Lactobacillus rhamnosus* TISTR 541 and *Lactobacillus plantarum* were obtained from Galilaeus Oy (Kriaana, Finland).

Table 1 | Incubation conditions of bacterial strains

Strain	Oxygen requirement
Lactobacillus acidophilus ATCC 20552	Anaerobic
Lactobacillus rhamnosus TISTR 541	Aerobic
Bifidobacterium angulatum DSMZ 20098	Anaerobic
Lactobacillus plantrium	Aerobic
Streptococcus thermophiles	Aerobic

Bifidobacterium angulatum DSMZ 20098 was obtained from Leibniz Institute (Germany) in lyophilized state. *Lactobacillus acidophilus* ATCC 20552 and *Streptococcus thermophilus* were obtained in MRS agar media from Egyptian Microbial Culture Collection at Cairo Microbiological Resources Centre (Cairo MIRCEN, Faculty of Agriculture, Ain Shams University, Egypt). Each strain was maintained in the MRS medium and appropriate proportions were used for the experiment. Standard sterile techniques were used for inoculation of cultures. All glassware and the medium for the microorganism were sterilized autoclaved at 15 lb/in^2 pressure and 121 °C for 20 min.

Methods

Preparation of individual HM solutions

The different HM concentrations were prepared according to Haskard *et al.* (2001) by dissolving $Pb(NO_3)_2$ and $Cd(NO_3)_2$ in double distilled water to get metal concentrations of 5, 10, 15, 20, 30 and 40 mg/L. A stock solution of 1,000 mg/L was prepared and other concentrations were prepared from it. AFB1 was purchased from Sigma (St Louis, MO, USA) and dissolved in phosphate buffered saline (PBS) according to the methods described by Haskard *et al.* (2001).

Preparation of binary metal solutions

Pb, Cd and As binary metal solutions were prepared using 10 mg/L each and mixed in equal proportions. Prior to experiment all the glassware was treated with 0.1 M HCl before and after the removing experiments to avoid binding of HM to it.

Biosorbent preparation

One litre of MRS media was prepared according to the Oxoid manual (http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0359&org=82&c=UK&lang=EN) in a Duran bottle. The pH for the medium was adjusted accordingly; then the medium was sterilized at 15 lb/in^2 pressure and 121 °C for 30 min and allowed to cool. For activation of the stains a loop full of bacterial culture was taken and streaked on the agar plate to obtain more colonies. They were later transferred to MRS broth and grown for subculture. Sterilized culture medium (100 mL) was transferred to a 250 mL Erlenmeyer flask. The medium was allowed to cool and then the microbial solution was inoculated into the medium in a laminar air flow chamber. The aerobic strains were inoculated in an orbital shaker (Lab Line Orbit Environ-Shaker, Germany) at 250 rpm at 37 °C for 48 h and the anaerobic strains were incubated in a BBL anaerobic jar (Becton Dickinson Microbiology Systems, Sparks, MD, USA) provided with a disposable BBL gas generating pack (CO_2 system envelopes, Oxoid, West Heidelberg, Victoria, Canada) for 48 h at 37 °C, to obtain the biomass. Mixed cultures were prepared by adding equal amounts of individual cultures. The cells were harvested from the medium by centrifugation at 9,000 rpm for 10 min. The supernatant was discarded and the cells were re-suspended in PBS prepared in purified water for washing and again centrifuged as mentioned above to make sure that no medium remained on the cell surface. The strains in lyophilized form were suspended to obtain suspensions of lyophilized bacteria (2 g) to be used in the experiment. The biomass was heat killed in a conventional hot air oven at 60 °C for 24 h. This biomass was used in experiments. Both the biomasses (microbial cells) were added in equal amounts for experiments with mixed culture.

Biosorption experiment

The different concentrations of washed and freshly cultured biomass (pure/mixed cultures) were combined with 100 mL of metal solution in 250 mL Erlenmeyer flasks. The flasks were placed on a shaker with a constant speed of 300 rpm and left to equilibrate. Samples were collected at predefined time intervals and centrifuged as above and the amount of metal in the supernatant was determined. Biosorption experiments were carried out according to Halttunen *et al.* (2007a, 2007b). After the growth of microorganisms in media, the biomass was harvested from the medium by centrifugation at 9,000 rpm for 10 min. The supernatant was discarded and the cells were re-suspended in PBS for washing and again centrifuged as above to make sure that no medium remained on the cell surface. The bacterial suspension was spiked with the same solvent containing either one of the HM or AFB1 to give a final bacterial concentration of 1 g/L,

2 g/L or 10^9 cells/mL. When necessary, the pH of the suspension was adjusted to a desired value with dilute HNO_3 or NaOH. Samples were incubated at 22 °C or 37 °C from 5 min to 24 h. After incubation, bacteria were separated from the supernatant by centrifugation and a sample from the supernatant was taken for either metal or AFB1 analysis. The samples for HM analyses were preserved by addition of a small volume of concentrated ultrapure HNO_3 (Fluka Chemie GmbH, Buchs, Switzerland). All experiments were performed at least in duplicate, and both positive controls (solvent substituted for bacteria) and negative controls (solvent substituted for toxin) were included. The biomass was heat killed in a conventional hot air oven at 60 °C for 24 h. This biomass was used for the sorption experiments. Biosorption studies were done using biomass as a function of various parameters such as pH, biomass concentration, temperature, contact time and initial metal concentration.

Effect of contact time, pH, bacterial concentration and temperature

To study the effect of different conditions including pH, biomass concentration, temperature, contact time, initial metal concentration and the metal removal, the experiments were performed as described above. In each experiment, one of the conditions was changed while others were kept constant. The experiment variables included contact time (30–300 min), pH (1–7), bacterial concentration (0.5–2 g/L), the final bacterial concentration of 1 g/L and 2 g/L, wherein the total count of LAB was 10^9 cells/g from fresh and lyophilized bacteria before the experiment and after counting by flow cytometry (Virta *et al.* 1998), respectively and temperature was 25–50 °C. The results from HM removal studies with different HM concentrations were fitted to a theoretical model which enabled calculation of specific descriptive parameters. In this work, the HM removal ability of different LAB strains was evaluated using a Langmuir isotherm (Davis *et al.* 2003).

Desorption of bound HM

The bacterial pellets, after ultrapure water, 0.1 mM and 1.0 mM EDTA, were used to determine the desorption of bound NM by washing with 1.5 mM and 15 mM HNO_3 and 1.5 mM NaOH. The experiment was performed as described above. The conditions were pH 5, metal concentration 50 mg/L, and contact time 10 min for Cd and Pb, while for As conditions were pH 7, metal concentration 0.5 mg/L and contact time 5 min. After the first binding experiment the bacterial pellet was separated from the supernatant by centrifugation and re-suspended to an equal volume of one of the desorbents tested and incubated at room temperature for 10 min. For HM analysis, the suspension was centrifuged and a sample from the supernatant was taken for metal analysis. Desorption treatment was repeated three times.

Resorption of HM

The reusability of biomass used in HM binding was assessed using the methods described by Halttunen *et al.* (2007b). The experiment was performed as described above at pH 5 (metal concentration 100 mg/L and incubation time 10 min). The bacterial pellet was suspended in 10 mM EDTA or 15 mM HNO_3 for desorption of the bound HM. After desorption, the pellet was washed twice with ultrapure water to remove residual EDTA/HNO_3 and used again in metal binding. This cycle was repeated three times. The characterization of the components responsible for removal of HM by microorganisms under investigation was studied according to Rosenborg *et al.* (1980).

Surface properties of LAB

Hydrophobicity

According to Halttunen *et al.* (2008) the hydrophobicity of *L. rhamnosus*, *L. acidophilus, Bifidobacterium angulatum*, *Streptococcus thermophilus* and *L. plantrium* was characterized using the microbial adhesion to hydrocarbons (MATH) test. The lyophilized cells were washed twice with 10 mmol/L PBS, pH 7.4, and resuspended in the same buffer to an absorbance ($\lambda = 600$ nm) of about 0.25 in order to standardize the number of bacteria (10^7 to 10^8 CFU/mL). After addition of an equal volume of *p*-xylene or *n*-hexadecane, the two-phase system was thoroughly mixed by vortexing for 1 min. The aqueous phase was removed after 1 h incubation at room temperature and its absorbance at 600 nm was measured. Affinity to hydrocarbons (hydrophobicity) was reported as the adhesion percentage according to the formula: $[(A_0 - A)/A_0] \times 100$, where A_0 and A are the absorbance before and after extraction with organic solvents, respectively. Hydrophobicity was calculated from three replicates as the percentage of decrease in the absorbance of the original bacterial suspension due to partitioning of cells into the hydrocarbon layer.

Auto/co-aggregation

Bacterial suspensions of *L. rhamnosus*, *L. acidophilus* and *L. plantrium* and their combination were prepared as described for MATH test. The bacterial suspensions were incubated in aliquots at room temperature without agitation and the absorbance ($\lambda = 600$ nm) of bacterial suspensions was measured for determination of auto/co-aggregation at 0, 1, 2, 3 and 4 h. Auto/co-aggregation percentage was expressed as an aggregation index $= [(A_0) - (A_t)/(A_0)] \times 100$, where A_0 represents the A_{600} of the bacterial suspension at time 0 min and at the A_{600} of the bacterial suspension after incubation of 2 h. For comparison of auto- and co-aggregation data, a predicted value for co-aggregation was calculated by summing up the auto-aggregation indexes of each strain, multiplied by the proportional bacterial concentration of each strain in the combination.

Effect of biomass concentration, temperature, contact time and initial metal concentration

Biomass was centrifuged at 9,000 rpm and different weights of the biomass ranging from 0.5 to 3 mg/mL were dispersed in solutions containing the 10 mg/L metal concentration. The solutions were adjusted to the optimum pH in which maximum biosorption of the metal ion occurred. Flasks were left for equilibration. The solutions were centrifuged at 9,000 rpm and the metal ion concentrations were determined using the procedures described before. The optimum biomass concentration with optimum pH was used to monitor the temperature effect on biosorption. Experiments were carried out at different temperatures from 10–50 °C for each culture and kept on a rotary shaker at 240 rpm. The samples were allowed to attain equilibrium. The samples were collected at regular intervals as above and analyzed for metal concentration. The cell pellet was dispersed in metal solution of 10 mg/L concentration with a working volume of 100 mL. The experiment was carried out at the optimum pH system. Flasks were allowed to attain equilibrium on a rotary shaker at 240 rpm and samples were collected at regular time intervals. Centrifugation at 9,000 rpm was done and the supernatant was analyzed for the residual metal content. The biosorption experiments were conducted by taking different initial HM concentrations by fixing all the parameters. Metal solutions were prepared as described before. With increase in metal concentration (5 to 30 mg/L) percentage biosorption was observed. All experiments were repeated at least twice.

Measurement of HM and AFB1

Cd and Pb concentrations were determined with atomic absorption spectrometry (Atomic Absorption spectrophotometry, 6800 Shimadzu, Japan) either by flame or graphite furnace method depending on the metal concentration. In each analysis, samples spiked with Cd and Pb were used as quality control samples. Arsenic was determined by atomic absorption spectroscopic method according to Halttunen *et al.* (2007a, 2007b). AFB1 was analyzed using high-performance liquid chromatography (HPLC) according to Lee *et al.* (2005) and Meriluoto *et al.* (2005). The standard for AFB1 was obtained from Sigma (St Louis, MO, USA). The HPLC was carried out using an HP gradient system, fitted with an HP1100 pump, an HP 1100 fluorescence detector (detector wavelength was set at 360 nm for excitation, and at 440 nm for emission) and a D-2500 integration system. Twenty five microlitres of the samples were injected into a C_{18} column (25 cm × 4.6 mm ID; Merck, Darmstadt, Germany) at a flow rate of 1 mL/min.

The percentage of toxin/HM removed (bound by bacteria) was calculated as $100 \times [(C_0 - C_1)/C_0]$, where C_0 and C_1 are the initial concentration of toxin and residual concentration of toxin after removal, respectively. For isolation of bacterial cell that absorbed the HM and AFB1, a filter syringe of diameter 25 mm diameter and 0.22 μm pore (Nalgene™ Syringe Filters) was used.

Effect of bacteria and their combination on the removal of HM and AFB1

To study the effect of LAB and their combination on the removal of HM and AFB1, the microorganisms and their combinations were prepared according to the methods described before, but the biosorption conditions were pH 7, the bacterial concentration was 2 g/L, the contact time was 300 min and the temperature was 40 °C. At the end of the experiment, HM and AFB1 were determined. The combination of the microorganisms (1:1) was prepared from the biomass as mentioned before, using the designed filter (Figure 1).

Statistics analysis

Statistical analysis was performed using the SPSS version 11.0 to calculate the paired *t*-test or analysis of variance (ANOVA). When ANOVA was used, either Tukey's HSD (honest

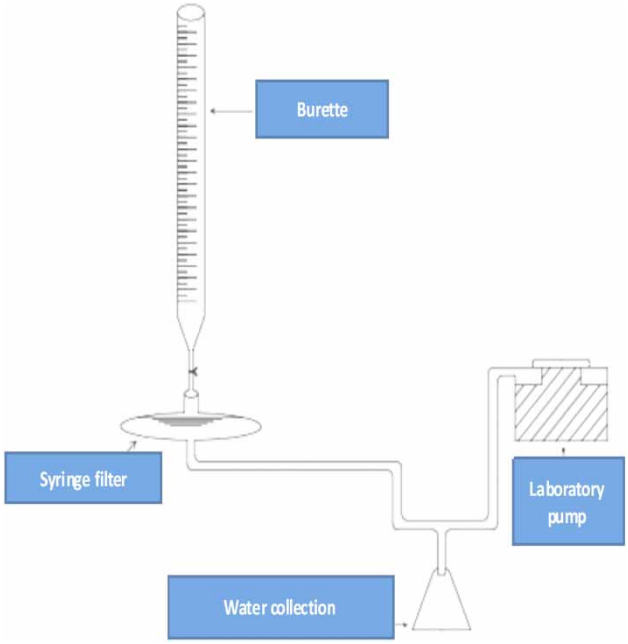

Figure 1 | The design of the biofilter used in the experiment.

significant difference) or the Tamhane test was used to test differences, depending on the homogeneity of variances.

RESULT AND DISCUSSION

Effect of pH on HM removal

Figure 2 presents the effect of pH on the removal of HM under study. Data showed that the removal is strongly pH-dependent with the highest removal potential at a pH close to neutral. There are similar effects on HM removal with all studied strains. Increasing pH caused almost linear increase in removal wherein the highest removal of Cd (62.9%), Pb (71.1%) and As (74.9%) was achieved at pH 7. The effect of pH may be result from competition for negatively charged binding sites between HM cations and protons (H^+).

LAB can bind to cationic HM and the binding depends on the microbial strain and pH. Very low removal level is typically observed at pH below 2–3, while at pH above 3 a sharp increase in removal occurs wherein the maximum removal was reached at pH 4–6. The effect of pH is a result of competition for negatively charged binding sites between cationic metals and protons (Halttunen 2007; Zoghi *et al.* 2014). Hansen *et al.* (2006) reported that optimal pH for As(V) removal with *Lessonia nigrescens* was acidic, and significant removal of As(V) was observed throughout the pH range 2.5–6.5. Lower removal was also reported for *Rhizopus oryzae* (Mcafee *et al.* 2001). There are only a few other reports available where As(V) removal by different microbes from water has been studied. Seki *et al.* (2005) observed low As(V) removal with a yeast biomass, amino and imidazol groups being the most probable binding sites. As(III) removal was reported to occur by a different mechanism to that of As(V) removal. Immobilized biomass of blue green algae, *Scytonema*, was reported to remove As(III) effectively from water (Prasad *et al.* 2006).

Beveridge & Murray (1980) and Doyle *et al.* (1980) explained that mechanisms such as complex formation, ion exchange, adsorption, chelation and microprecipitation can be involved in HM biosorption. The dependence on pH indicated that ion exchange is probably responsible for the observed Cd and Pb removal. The observed drop in pH during the incubation, which is probably a result of proton replacement by HM ions, supports this conclusion. Involvement of anionic surface groups in HM binding has been reported for the Gram-positive bacterium *Bacillus subtilis*. The extraction of the teichoic acid moieties (phosphodiester groups), and reduction of the number of free carboxyl groups, reduced the cation uptake by *B. subtilis* cell walls. Some cationic metals, e.g. copper, prefer binding to neutral amino groups, whereas some, such as lead, form negatively charged complexes in water that can interact electrostatically with positively charged amino groups (Beveridge & Murray 1980). In most cases, amino groups have been shown to interfere with the interactions between cationic metals and anionic surface groups on microbes (Doyle *et al.* 1980). Cd and Pb binding of *L. fermentum* ME3 and *B. longum* 46 was reduced when the negative charge of carboxyl and phosphoryl groups was neutralized by chemical modification. This indicated that both groups have a significant role in binding of these metals, and they may be the sites where ion exchange occurs.

Effect of bacterial concentration on HM removal

Figure 3 presents the effect of bacterial concentration on the HM removal from artificially contaminated water samples. As can be noted, the increase in the bacterial concentration enhanced the removal of Cd, Pb and As. The strong HM removals at pH 7 and high bacterial concentration were observed especially with *L. plantarum* as single strain as well as the combination of *Bifidobacterium angulatum*,

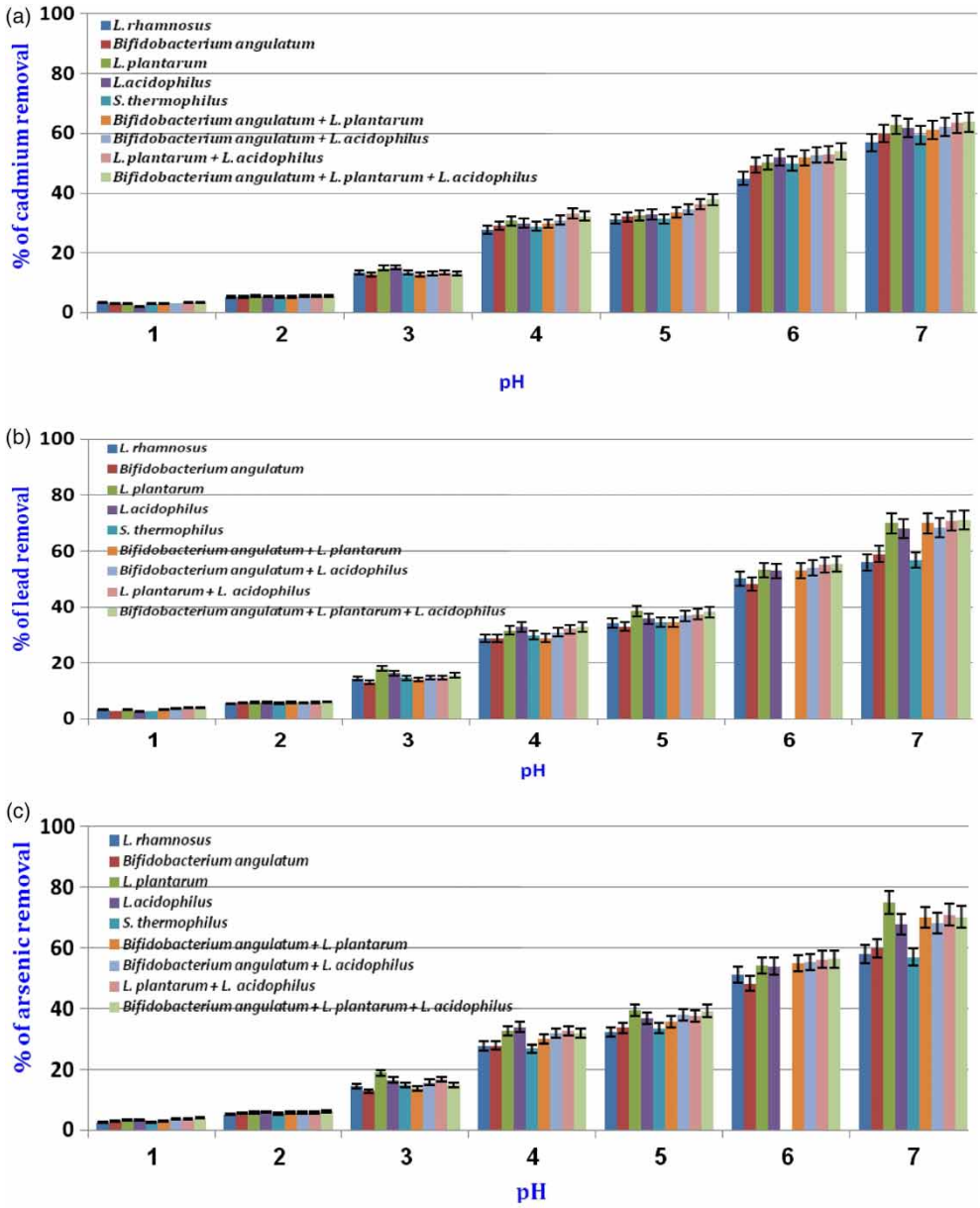

Figure 2 | Effect of pH on the percentage of (a) cadmium, (b) lead, and (c) arsenic removal by different bacterial strains.

L. plantarum and *L. acidophilus*. These could be a result of a high number of available phosphate groups on the bacterial surface. The obtained results were similar to the results obtained by Davis *et al.* (2003). The preference for certain metals over others has been reported for other microorganisms (Davis *et al.* 2003; Halttunen *et al.* 2007b; Teemu *et al.* 2008). From the obtained results, we noted that removal of Pb was higher than Cd. This may have resulted from bigger ionic radius of Pb compared with Cd (Davis *et al.* 2003). The results also demonstrated that there was an increase in the HM removal with the increase in the biomass concentration of lyophilized and freshly cultured strains. This may be explained by a higher biomass/metal ratio (i.e. higher number of binding sites available). The effect of biomass concentration has been observed in other studies wherein similar results were reported (Esposito *et al.* 2002; Ngwenya *et al.* 2003). Puranik & Paknikar (1999), Halttunen *et al.* (2007b) and Teemu *et al.* (2008) observed also the reduction of HM removal at high biomass concentrations. This may result from sorption of metals to dissolved organic acids that interfere with sorption to bacterial surface structures or formation of cell aggregates

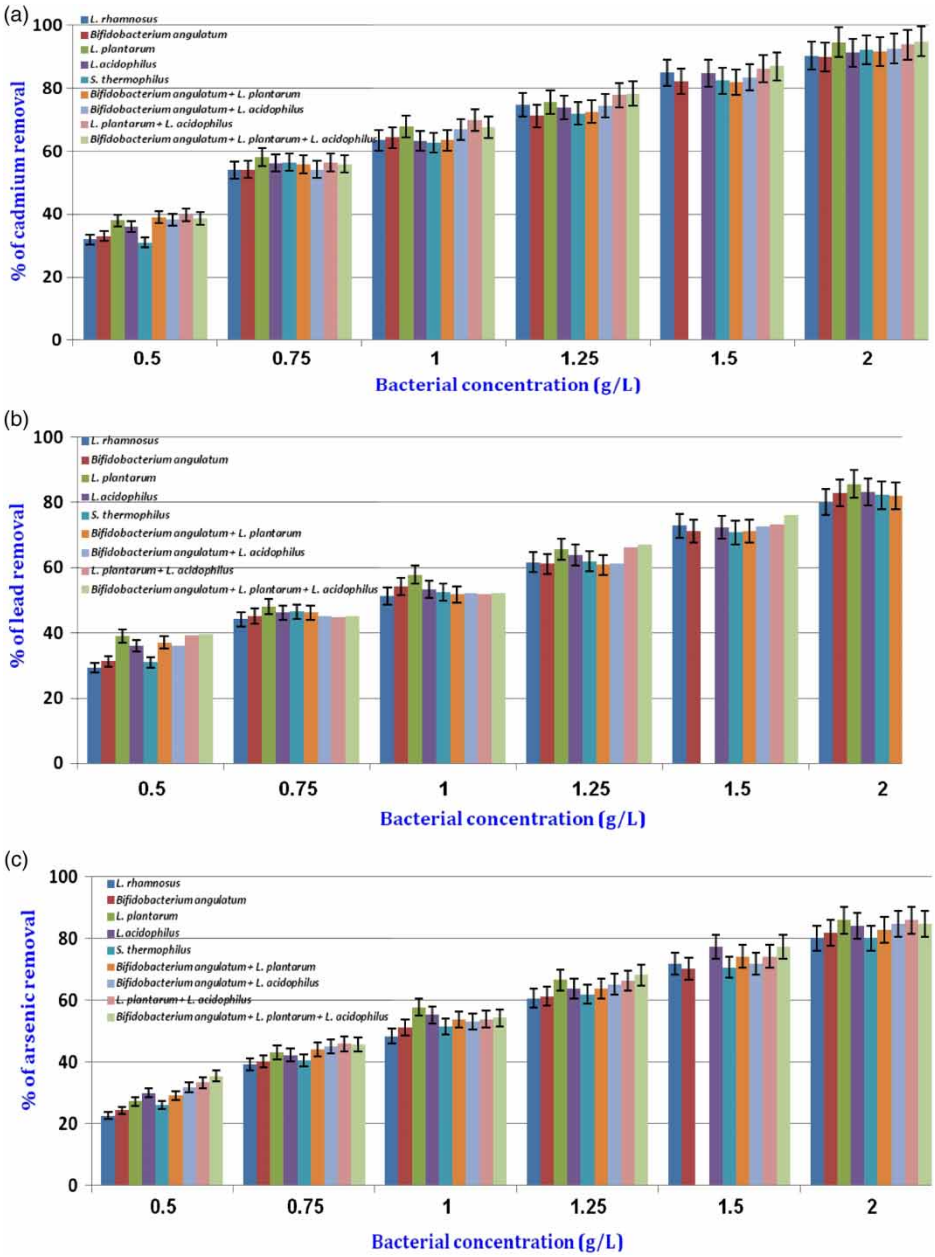

Figure 3 | Effect of bacterial concentration (g/L) on the percentage of (a) cadmium, (b) lead, and (c) arsenic removal.

that reduce the surface area available for binding (Harvey & Leckey 1985; Zoghi *et al.* 2014). The binding of HM increased rapidly when lyophilized cells were used.

It was reported that Cd and Pb binding to *L. fermentum* and *B. longum* was reduced when the negative charge of carboxyl and phosphoryl groups was neutralized by chemical modification. Both groups have a significant role in binding of Cd and Pb, and they may be the sites where ion exchange occurs (Zoghi *et al.* 2014).

Effect of contact time on HM removal

Figure 4 presents the effect of contact time (min) on HM removal. Data showed that there were increases in the ability of LAB strains to remove HM by increasing of contact time. There were similar effects on Cd, Pb and As removal with all strains. Increasing of contact time caused an almost linear increase in the removal, wherein the highest binding of Cd (86.8%), Pb (85.5%) and As (85.7%) was

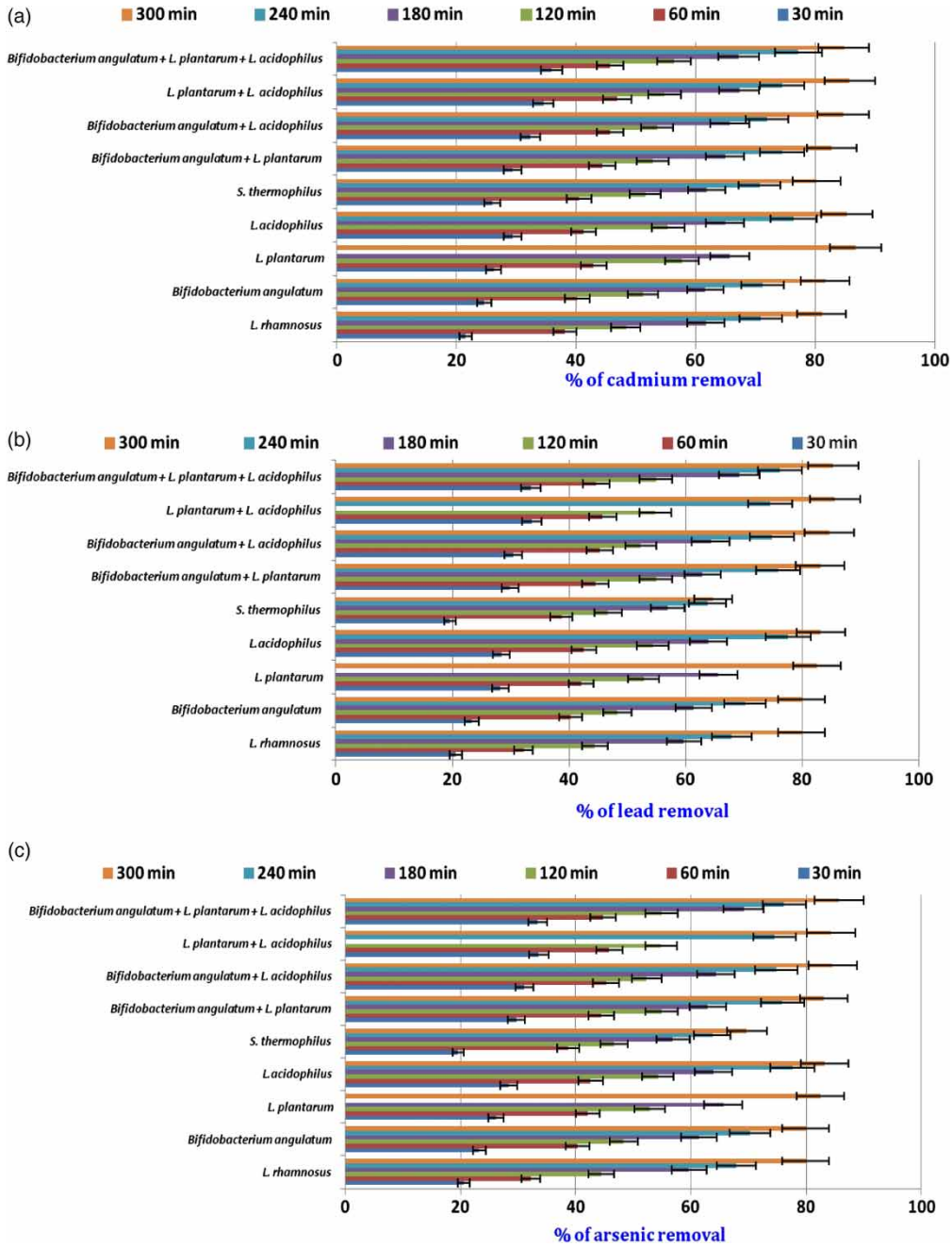

Figure 4 | Effect of contact time (min) on the percentage of (a) cadmium, (b) lead and (c) arsenic removal by different bacterial strains.

achieved at the end of contact time (300 min). The obtained results disagree with the results obtained by Halttunen *et al.* (2007b) and Teemu *et al.* (2008) who found that the incubation time had no effect on the removal of Cd. They also mentioned that the effect of contact time on Cd removal was slightly different when freshly cultured cells were used wherein prolonged incubation reduced the removal. The obtained results were in agreement with the results of Selatnia *et al.* (2004) and Pan *et al.* (2006).

Effect of temperature on HM removal

Figure 5 presents the effect of temperature on the HM removal from contaminated water samples. It could be

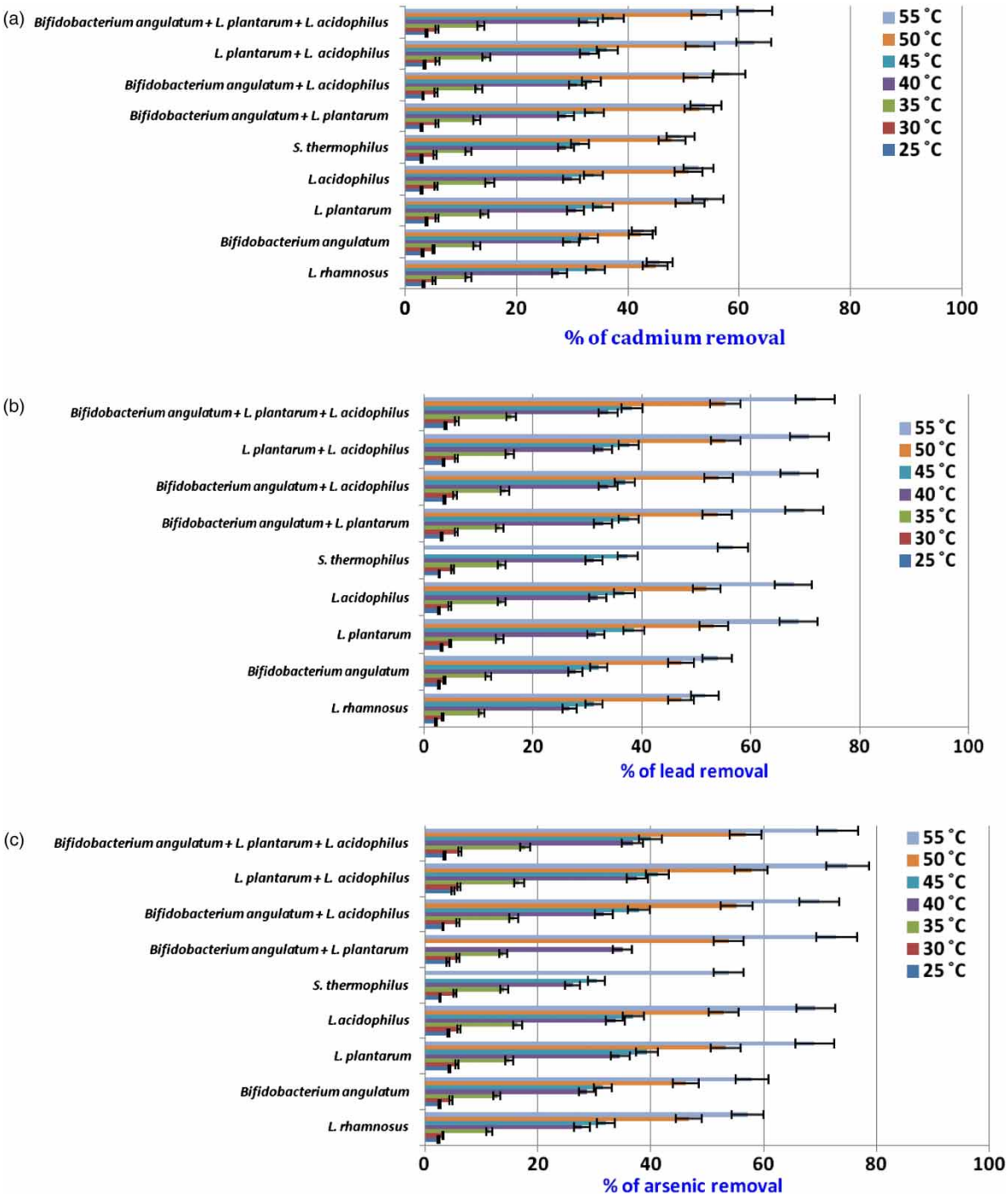

Figure 5 | Effect of temperature (°C) on the percentage of (a) cadmium, (b) lead and (c) arsenic removal by different bacterial strains.

noted that higher temperature enhanced the removal of HM. The results indicated that there was increase in the removal of HM by increasing of temperature. The obtained results were in agreement with Cho & Kim (2003) who found that Cd removal by fresh biomass increased significantly when the incubation temperature was raised. This may have resulted from active transport of Cd into the bacterial cells. Accumulation of Cd by an energy-dependent mechanism has been reported to take place in *L. plantarum* (Hao *et al.* 1999). Our results indicated that

the microorganisms under investigation were able to remove As from contaminated water. As(V) removal from water by different microbes has been studied. The data indicated the involvement of electrostatic interactions between anionic As species and the cationic surface groups of the biomass. Native, non-viable *Penicillium chrysogenum* biomass was reported to remove As under conditions similar to this study whereas the positively charged amino groups were probably responsible for the binding of anionic As species. In addition, the effect of temperature on the removal of Cd and Pb by *L. rhamnosus* was small (Halttunen 2007). Heat treatment increased Cd removal in most cases; the enhancement of removal by heat treatment results from the increased availability of metal binding sites on the bacterial surface (Göksungur *et al.* 2005; Zoghi *et al.* 2014).

Effect of bacteria and their combination on the removal of HM and AFB1

The data in Table 2 describe the removal of HM and AFB1 by LAB and their combination. The removal of Cd and Pb was high, in the range 28.3–59.9% and 42.6–72.6%, respectively. The removal of As was 22.7–49.8% while the removal of AFB1 was low ranging from 8.9 to 21.6%. The removal of HM and AFB1 in this study depended on the strain. The obtained results indicated that the binding of HM and AFB1 by LAB and bifidobacteria developed rapidly in the aqueous solution. Similar observations have been reported earlier for other bacteria such as *Bacillus subtilis* and *Pseudomonas putida* (Fein *et al.* 1997; Pardo *et al.* 2003) and by the combination of *B. subtilis* and *P. aeruginosa* (Davis *et al.* 2003; Mehta & Gaur 2005; Romera *et al.* 2006; Halttunen *et al.* 2007b; Teemu *et al.* 2008; Tarangini 2009), wherein the quick absorption and uptake depended on the binding that occurred passively to the surface of bacteria rather than by accumulation inside the cell and the microbial biomass. The removal ability for HM and AFB1 of the LAB was varied. The removal process depended on the strains, the mixing between the strains, and the growth phase. The maximum HM removal capacities in this study may be attributed to differences in the quantity of functional groups involved in HM binding and the functional group in the cell wall of the strains.

Table 2 | Ability of bacteria under study to remove Cd, Pb, As and AFB1 from artificially contaminated aqueous solutions*

	% Removal			
Strain(s)	**Cd**	**Pb**	**As**	**AFB1**
S. thermophilus	25.7	40.5	38.3	14.9
L. rhamnosus	29.5	43.2	42.7	19.56
Bifidobacterium angulatum	28.3	42.6	47.6	23.6
L. plantarum	59.9	45.9	44.8	19.9
L. acidophilus	65.5	72.6	49.8	18.6
Bifidobacterium angulatum + *L. plantarum*	46.8	46.8	46.7	16.7
Bifidobacterium angulatum + *L. acidophilus*	52.6	45.6	38.2	19.4
L. plantarum + *L. acidophilus*	44.2	45.2	31.9	18.5
Bifidobacterium angulatum + *L. plantarum* + *L. acidophilus*	48.1	47.6	39.9	21.6

*Average of three replicates at pH 7 and 37 °C.

Previous reports have shown that the biosorption of Cd by microorganisms varies from study to study due to the different binding conditions (Ibrahim *et al.* 2006; Halttunen *et al.* 2007a, 2007b; Zhai *et al.* 2015). Halttunen *et al.* (2008) found that lyophilized *Propionibacterium freudenreichii shermanii* had the highest removal percentage (49.1%). Two phase kinetics were observed in Cd and Pb removal, wherein initial rapid binding was followed by slow and nearly constant removal lasting for hours. The first phase was the sorption of Pb to the outermost structures of the lipopolysaccharide layer, and the second slow phase was slow diffusion of Pb further into the lipopolysaccharide layer (Harvey & Leckey 1985; Zoghi *et al.* 2014).

Physical adsorption to the bacterial surface appears to be the main mechanism for HM and AFB1 removal (Haskard *et al.* 2001) but it is only partly responsible for microcystin-LR removal (Meriluoto *et al.* 2005; Nybom *et al.* 2007). Therefore, a lower number of available binding sites or smaller surface area may explain the lower predicted removal with combinations. Auto-/co-aggregation of bacteria would reduce the total surface area of the biomass. In fact, the observed co-aggregation index was practically identical to the predicted value. Therefore, the co-aggregation of bacteria cannot explain the lower predicted removal of model toxins.

Adhesion to *n*-hexadecane and *p*-xylene as affected by bacteria and their combinations

Table 3 presents the adhesion percentages of *L. rhamnosus, L. acidophilus, Bifidobacterium angulatum*, *S. thermophilus* and *L. plantrium* as well as their combinations to *n*-hexadecane and *p*-xylene. In general, the adhesion percentage of *p*-xylene was higher than *n*-hexadecane. High adhesion

Table 3 | Adhesion percentages of bacteria under study to *n*-hexadecane and *p*-xylene

Strain(s)	Adhesion percentages	
	n-hexadecane	*p*-xylene
Bifidobacterium angulatum	29.8	34.8
L. plantarum	49.7	77.9
L. acidophilus	45.9	75.6
L. rhamnosus	44.3	65.8
S. thermophilus	44.6	57.6
Bifidobacterium angulatum + *L. plantarum*	39.9	68.8
Bifidobacterium angulatum + *L. acidophilus*	45.0	79.0
L. plantarum + *L. acidophilus*	46.2	74.0
Bifidobacterium angulatum + *L. plantarum* + *L. acidophilus*	44.9	75.6

*Average of three replicates at pH 7 and 37° C

percentages to *p*-xylene were observed with *L. plantarum*, *L. acidophilus*, *L. rhamnosus* and in combination, respectively. Linkage to *n*-hexadecane of *L. plantarum*, *L. acidophilus* and their combination was observed to be lower, ranging between 44.0 and 49.7%. The adhesion of *Bifidobacterium angulatum* was below this range (29.8%). The obtained results were similar with the results obtained by Tobin *et al.* (1990) and Halttunen *et al.* (2007a, 2007b) who found that the chemical modifications of carboxyl and phosphoryl groups were observed to reduce Cd, Pb, and As removal by *Aspergillus niger*, *Rhizopus arrhizus* and LAB. Earlier results reported the involvement of electrostatic interactions between HM and LAB (Halttunen *et al.* 2007a, 2007b). Hydrophobic interactions have also been reported to be behind the auto-aggregation of bifidobacteria (Canzi *et al.* 2005).

Haskard *et al.* (2000) mentioned that the lack of correlation between the removal of HM and AFB1 was probably a result of different binding/elimination mechanisms and strain-specific properties. It seems that AFB1 is mainly bound to surface carbohydrates and proteins by hydrophobic interactions (Lahtinen *et al.* 2004), whereas in HM removal, ion exchange to surface constituents seems to be involved (Halttunen *et al.* 2007a, 2007b). Physical adsorption to the bacterial surface appears to be the main mechanism for HM (Halttunen *et al.* 2007a, 2007b; 2008) and AFB1 (Haskard *et al.* 2001) removal.

The surface chemistry of Gram-negative (*Shewanella putrefaciens*) and Gram-positive bacteria (*Bacillus subtilis* and *Bacillus licheniformis*) was reported (Ngwenya *et al.* 2003). They found that surface densities of carboxyl, phosphoryl and hydroxyl/amine groups were higher in Gram-positive strains. A twofold difference was also observed in the quantity of phosphoryl and hydroxyl/amine groups between the two Gram-positive *Bacillus* species. Surface densities and deprotonation constants of functional groups of *B. subtilis* have been established to vary depending on the growth phase. Daughney *et al.* (2001) reported that surface densities and deprotonation constants of carboxyl, phosphoryl and amine groups decreased as the growth phase of biomass moved from the exponential to the stationary and the sporulated phase. Supporting these results, the decrease of Cd removal when older cells of *Pseudomonas aeruginosa* were used was reported by Chang *et al.* (1997). However, the opposite was observed when Pb removal was studied with the same biomass. Fein *et al.* (1997) and Ngwenya *et al.* (2003) reported that stability constants of a given metal vary with functional groups. Therefore, the observed inter-biomass differences in maximum HM removal capacities in this work may be attributed to differences in the quantity of functional groups involved in HM removal.

CONCLUSION

Our results demonstrated that the LAB and bifidobacteria under investigation have been able to remove HM and AFB1 efficiently and rapidly from contaminated water. It is hypothesized that the removal starts by electrostatic interactions between HM cations and anionic functional groups. Depending on the obtained results, LAB and bifidobacteria might be used for production of commercial bio-filters to purify water contaminated with HM and AF.

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