REMOVAL OF CADMIUM, LEAD AND ARSENIC FROM WATER BY LACTIC ACID BACTERIA

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TABLE OF CONTENTS

ABSTRACT

A number of contaminants such as arsenic, cadmium and lead are released into the environment from natural and anthropogenic sources contaminating food and water. Chronic oral ingestion of arsenic, cadmium and lead is associated with adverse effects in the skin, internal organs and nervous system. In addition to conventional methods, biosorption using inactivated biomasses of algae, fungi and bacteria has been introduced as a novel method for decontamination of toxic metals from water. The aim of this work was to evaluate the applicability of lactic acid bacteria as tools for heavy metal removal from water and characterize their properties for further development of a biofilter.

The results established that in addition to removal of mycotoxins, cyanotoxins and heterocyclic amines, lactic acid bacteria have a capacity to bind cationic heavy metals, cadmium and lead. The binding was found to be dependent on the bacterial strain and pH, and occurred rapidly on the bacterial surface, but was reduced in the presence of other cationic metals. The data demonstrates that the metals were bound by electrostatic interactions to cell wall components. Transmission electron micrographs showed the presence of lead deposits on the surface of biomass used in the lead binding studies, indicating involvement of another uptake/binding mechanism. The most efficient strains bound up to 55 mg Cd and 176 mg Pb / g dry biomass. A low removal of anionic As(V) was also observed after chemical modification of the cell wall. Full desorption of bound cadmium and lead using either dilute HNO_3 or EDTA established the reversibility of binding. Removal of both metals was significantly reduced when biomass regenerated with EDTA was used. Biomass regenerated with dilute HNO_3 retained its cadmium binding capacity well, but lead binding was reduced.

The results established that the cadmium and lead binding capacity of lactic acid bacteria, and factors affecting it, are similar to what has been previously observed for other biomasses used for the same purpose. However, lactic acid bacteria have a capacity to remove other aqueous contaminants such as cyanotoxins, which may give them an additional advantage over the other alternatives. Further studies focusing on immobilization of biomass and the removal of several contaminants simultaneously using immobilized bacteria are required.

ABRREVIATIONS

AFB_1	aflatoxin B_1
AFM_1	aflatoxin M_1
As(III)	trivalent arsenic
As(V)	pentavalent arsenic
b	constant related to affinity of binding (L/mg)
COMB	combination of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bbi99/E8 and *P. freudenreichii shermanii* JS
DMA	dimethyl arsonic acid
EDTA	ethylenediamine tetraacetic acid
FAAS	flame atomic absorption spectrometry
GFAAS	graphite furnace atomic absorption spectrometry
GlcNAc	N-acetyl glucosamine
HGAAS	hydride generation atomic absorption spectrometry
IARC	International Association for Research on Cancer
IQ	intelligence quotient
JECFA	Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives
LTA	lipoteichoic acid
MC-LR	microcystin-LR
MMA	monomethyl arsinic acid
MurNAc	N-acetyl murmic acid
pI	isoelectric point
PJS	*P. freudenreichii shermanii* JS
PTWI	provisional tolerable weekly intake
q_{max}	maximum binding capacity in mg metal per g dry biomass
SPM	suspended particulate matter
TA	teichoic acid
WHO	World Health Organization
ζ	zeta potential (mV)

LIST OF ORIGINAL PUBLICATIONS

I Halttunen T., Kankaanpää P., Tahvonen R., Salminen S., Ouwehand A. (2003) Cadmium removal by lactic acid bacteria. Bioscience and Microflora 22(3):93-97

II Halttunen T., Tahvonen R., Salminen S. (2007) Rapid removal of cadmium and lead from water by specific lactic acid bacteria. International Journal of Food Microbiology 114(1):30-35

III Halttunen T., Finell M., Salminen S. (2007) Arsenic removal by native and chemically modified lactic acid bacteria. International Journal of Food Microbiology 120(1-2):173-178

IV Halttunen T., Collado M.C., El-Nezami H., Meriluoto J., Salminen S. (2007) Combining strains of lactic acid bacteria may reduce their toxin and heavy metal removal capacity. Letters in Applied Microbiology. Availabel online doi:10.1111/j.1472-765X.2007.02276.x

V Halttunen T., Salminen S., Meriluoto J., Tahvonen R. (2007) Reversible surface binding of cadmium and lead by lactic acid and bifidobacteria. Submitted

1. INTRODUCTION

Access to safe (drinking) water is essential for life and may be regarded as a basic human right. However, water may be contaminated by various microbial species (bacteria, viruses, parasites) and chemical contaminants (e.g. heavy metals, cyanotoxins, pesticides, disinfection by-products). Microbial or parasitic contamination of drinking water is a primary concern in both developing and developed countries. Large scale chemical contamination of drinking water is most frequently caused by contaminants such as fluoride, arsenic, uranium, selenium, lead, nitrate and nitrite. However, there are a number of other known contaminants causing problems on a smaller scale, and the WHO (2006) has listed over 160 microbial and chemical contaminants of drinking water.

Cadmium, lead and arsenic are toxic elements released into the environment from anthropogenic sources (Smedley and Kinniburgh, 2002; WHO, 1992 and 1995). Arsenic is also a natural contaminant of groundwater. Chronic oral exposure to even low concentrations of arsenic, cadmium and lead may lead to the development of adverse effects on the skin, internal organs and nervous system (WHO, 1992, 1995 and 2001).

Methods such as precipitation, flocculation, ion-exchange and filtration have all been conventionally used for removal of metals from water (Rautjärvi and Pöllänen, 1998). During the last few decades, removal of toxic metals using inactivated algal, fungal and bacterial biomass has been extensively studied, and introduced as an inexpensive, novel method on top of conventional methods (Volesky and Holan, 1995; Davis et al., 2003; Mehta and Gaur, 2005; Romera et al., 2006).

Lactic acid bacteria are widely used in food manufacturing for their beneficial technological properties and positive effects on health. Many of their beneficial properties are related to their capacity to adhere or bind to different targets, and one of the basic requirements for a probiotic is an ability to adhere to intestinal mucus or epithelial cells. Several lactic acid bacteria have also been reported to remove or bind toxic contaminants of food and water such as mycotoxins (El-Nezami et al., 1998a; Haskard et al., 2001; Peltonen et al., 2001), cyanotoxins (Meriluoto et al., 2005; Nybom et al., 2007) and heterocyclic amines (Turbic et al., 2002). The reported metal removal by different inactivated biomasses, and the toxin removal capacity of

lactic acid bacteria, inspired us to assess the ability of lactic acid bacteria to remove cadmium, lead and arsenic from water.

2. REVIEW OF THE LITERATURE

2.1 Heavy metals in water

Heavy metals are metallic elements having a density of over 5 g/cm^3. They are undegradable compounds that may exist in number of different inorganic and organic forms. Some heavy metals such as Fe, Cu and Zn are essential trace elements but others such as Cd and Pb have no advantageous biological function and are toxic even in very small amounts. Cd, Pb and Hg are regarded as the most toxic heavy metals. Another elemental toxicant is arsenic. Arsenic is sometimes regarded as a heavy metal although, strictly speaking, it is a metalloid.

The following text reviews some of the reports concerning Cd, Pb and As (model compounds used in the experimental part of the work) concentrations in water.

2.1.1 Cadmium (Cd)

Natural cadmium concentrations in water bodies rarely exceed the WHO guideline value of 3 µg/L (WHO, 2006) and the main sources of cadmium contamination are anthropogenic. Average cadmium concentrations from 0 to 2.6 µg/L in drinking and natural waters have been reported from different parts of the world (Al-Saleh and Al-Doush, 1998; Mohamed et al., 1998; Ryan et al., 2000; Seifert et al., 2000; Aremu et al., 2002; Rajaratnam et al., 2002; Ho et al., 2003; Rosborg et al., 2003; Barton, 2005; Tuzen et al., 2005; Virkutyte and Sillanpää, 2006; Asante et al., 2007). High aquatic cadmium concentrations are often reported in the surroundings of abandoned and active mines and metal smelters (Appleton et al., 2001; Woo and Choi, 2001; Miller et al., 2004; Lee et al., 2005a; Lee et al., 2005b), especially where non-ferrous metals have been/are produced (Fischer et al., 2003; Florea et al., 2005). Phosphate fertilizers used in agriculture may also contain high concentrations of cadmium. Acidification of soil and water may release cadmium bound to soil and sediments and thereby cause contamination. Elevated cadmium concentrations up to 57 µg/L (Al-Saleh and Al-Doush, 1998; Seifert et al., 2000; Rajaratnam et al., 2002; Barton, 2005) originating from soldered joints and zinc galvanized plumbing have been reported in tap water when first draw waters were studied. However, most of the households studied usually had acceptable cadmium levels; for example, in Germany, cadmium concentrations have been reported to exceed the WHO guideline of 3 µg/L in only 0.7 % of the samples examined (Seifert et al., 2000).

In natural waters, cadmium is distributed into three different fractions: dissolved, bound to suspended particulate matter (SPM) and sedimented. Cadmium has a strong affinity for particulate matter and sediments may contain concentrations over 100 mg/kg (Appleton et al., 2001; Woo and Choi, 2001). As a result of this, the soluble concentration of cadmium in water is generally low, although the amount of dissolved cadmium may be increased by high concentration of dissolved organic matter (mainly fulvic and humic acids) (Linnik, 2003) and low pH.

2.1.2 Lead (Pb)

The World Health Organization (WHO, 2006) has set a guideline of 10 µg/L for lead concentration in drinking water. Natural lead concentrations of water bodies are generally low, and background concentrations of <0.45—14 µg/L in groundwater have been reported (Smedley et al., 2002). However, contamination from anthropogenic sources is more common, and high lead concentrations have been found in the surroundings of metal mines and smelters (Miller et al., 2004; Florea et al., 2005; Lee et al., 2005b). Point contamination on a smaller scale also occurs as a result of industrial emission, agriculture and dumping of sewage sludge. Before the introduction of unleaded gasoline, the use of leaded gasoline was one of the major sources of lead pollution. Lead is also released into tap water from water pipes, solders and fittings of old plumbing systems (which may contain up to 50 % lead) and lead concentrations up to 5580 µg/L have been detected in tap water (Murphy, 1993; Gulson et al., 1994; Gulson et al., 1997; Seifert et al., 2000; Barton et al., 2002; Rajaratnam et al., 2002; Fertmann et al., 2004). However, these extremely high concentrations are rare, and in a German study, the WHO guideline of 10 µg/L was only exceeded in 7.7 % of the first draw water samples (Seifert et al., 2000).

Like cadmium, lead in water is distributed into three different fractions: dissolved, bound to suspended particulate matter (SPM) and sedimented. Lead has a strong affinity to particulate matter, and, therefore, it is mainly present in SPM and sediment fractions whereas the dissolved amount is low (Balls, 1988; Zarazua et al., 2006). However, lead binding to SPM and sediments is at least partly reversible and it may be released to the surrounding water under suitable conditions (Chrastný et al. 2006). The amount of dissolved lead is increased by several factors such as high dissolved organic matter (Linnik, 2003) and low pH.

2.1.3 Arsenic (As)

Arsenic is a ubiquitous metalloid occurring in the atmosphere, water, soil, rock, living organisms. It is released into the environment from both natural processes (weathering, erosion, biological and volcanic activity) and anthropogenic sources (mining, combustion of fossil fuels, agriculture and wood preservation). Although anthropogenic activities cause local problems, the most severe environmental problems due to arsenic are caused by mobilization of arsenic under natural conditions.

Arsenic in the environment may occur in several oxidation states (-3, 0, +3 and +5). In natural waters, it is mainly found as oxyanions of trivalent arsenic [As(III), arsenite] and pentavalent arsenic [As(V), arsenate] although very low concentrations of organic arsenic species, monomethylarsinic acid (MMA) and dimethylarsonic acid (DMA), have been detected. Speciation of arsenic in water is mainly determined by pH and redox-potential. Species such as $H_3As(III)O_3$ and $H_3As(V)O_4$ predominate in reducing and oxidizing conditions, respectively (Figure 1). At the normal pH of natural waters, arsenate is found in two anionic forms $H_2AsO_4^-$ and $HAsO_4^{2-}$ whereas the only abundant form of arsenite is uncharged H_3AsO_3.

The World Health Organization (WHO, 2006) has set a provisional guideline value of 10 µg/L for the arsenic concentration of drinking water. However, this limit has not been adopted by all countries, for example in India and Bangladesh the former guideline of 50 µg/L (WHO, 1984) is in use. The natural contamination of water bodies with arsenic, especially groundwater, occurs on nearly every continent. The most severe cases of arsenic contamination of groundwater affecting large areas and population are reported in Bangladesh and the State of West Bengal in India (Chowdhury et al., 2000). Other severe cases have been reported in Argentina (Nicolli et al., 1989; Smedley et al., 2002), Cambodia (Berg et al., 2007), Chile (Caceres et al., 2005; Ferreccio and Sancha, 2006), China (Sun, 2004), Hungary (Smedley and Kinniburgh, 2002), Mexico (Del Razo et al., 1990), Romania (Smedley and Kinniburgh, 2002), Taiwan (Chen et al., 1994; Smedley and Kinniburgh, 2002),

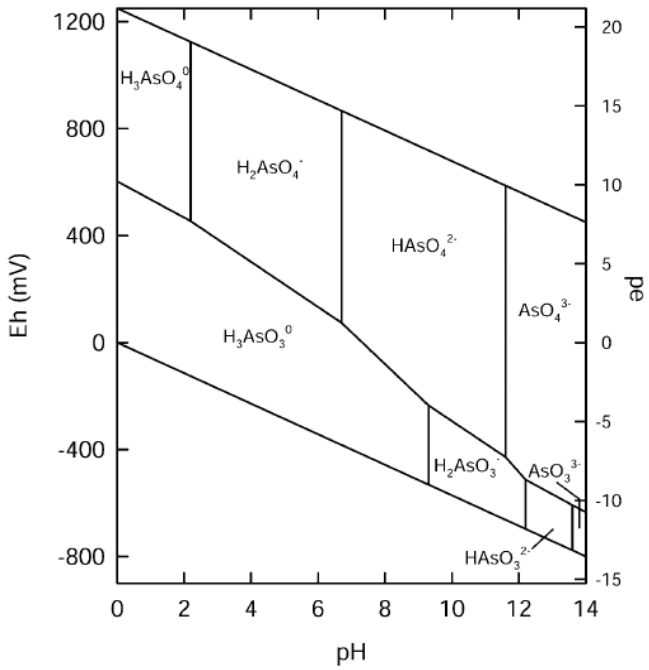

Figure 1. Redox potential (Eh) - pH diagram for aqueous As species in the system As–O_2–H_2O at 25°C and 1 bar total pressure. This figure was published in Applied Geochemistry, Vol 17, Smedley and Kinniburgh, A review of the behaviour, source and distribution of arsenic in natural waters, 517-568, Copyright Elsevier (2002).

Vietnam (Berg et al., 2001; Agusa et al., 2006) and many parts of the USA (Focazio et al., 1999; Ayotte et al., 2003). Problems also occur to a smaller extent in many other parts of the world, for example in Finland (Kurttio et al., 1999). Table 1 presents a selection of arsenic concentrations reported from different countries.

The main source of arsenic in groundwater arises due to its mobilization from arsenic-rich minerals and rocks such as iron/aluminium oxides and sulphide minerals in the aquifer, but the reasons for its mobilization from rock to groundwater are different for different geographical areas. In general, these processes may be divided into three classes based on redox-conditions: contamination under reducing, oxidizing and mixed conditions (Smedley and Kinniburgh, 2002). In Bangladesh, Taiwan, Northern China, Hungary, Romania, and Deltas of the Ganges, Red River and Mekong rivers, the primary cause of arsenic contamination is mobilization from iron oxides and iron hydro-oxides under

Table 1. Minimum and maximum total arsenic concentrations reported from ground- and riverwater samples.

	water source	min—max [As] µg/L	% over 10 µg/L	Reference
Hanoi, Vietnam	GW[a]	0.10—330	40	Agusa et al. (2006)
Hanoi, Vietnam	GW	1—3050	72	Berg et al. (2001)
Mekong river delta, Cambodia	GW	1—1610		Berg et al. (2007)
Mekong river delta, Vietnam	GW	<1—845		Berg et al. (2007)
Ghana	RW[b]	0.5—73		Asante et al. (2007)
Bangladesh	GW	0.7—640	48	Frisbie et al (2002)
Rajapur Village, India	GW	<3—1180	83	Rahman et al (2005)
West Bengal, India	GW		51	Chakraborti et al. (2003)
Bangladesh	GW	<0.25—1660		BGS and DPHE (2001)
Bangladesh	GW		57	Chakraborti et al. (2003)
La Pampa, Argentina	GW	<4—5300	95	Smedley et al (2002)
Córdoba, Argentina	GW	19—3810	82[c]	Nicolli et al. (1989)
La Pampa, Argentina	GW	<2—590		Fariás et al. (2003)
Antofagasta, Chile	Tap	33—40		Caceres et al. (2005)
Antofagasta, Chile	RW	10—3000		Queirolo et al. (2000)
Antofagasta, Chile	GW, RW	13—27000		Romero et al. (2003)
Putai, Taiwan	GW	470—897		Chen et al. (1994)
Mexico	GW	8—624	50[c]	Del Razo et al. (1990)
Romania	GW	<2—176		Smedley and Kinniburgh (2002)
Finland	GW	0—64	1	Kurttio et al. (1999)
Finland	RW	0—1.1		Niemi and Raateland (2007)

[a]groundwater, [b] river water, [c] % of samples over 50 µg As/L (former WHO guideline)

reducing conditions in the groundwater (Berg et al., 2001; BGS and DPHE, 2001; Smedley and Kinniburgh, 2002). It is typical for these areas to exhibit a large ratio of As(III)/As(V), although large variations exist. On the other hand, in Mexico, Chile and Argentina, arsenic is found in groundwater where oxidizing conditions prevail. As a result, the arsenic mainly occurs as pentavalent arsenate (Smedley et al., 2002).

Arsenic is also mobilized in large quantities as a result of mining activities such as mineral excavation, ore transportation, smelting and refining, disposal of the tailings, and waste waters around mines (Woo and Choi, 2001; Miller et al., 2004; Peplow and Edmonds, 2004; Lee et al., 2005a) both during the active operation, and also long after, the mine has been closed. For example, in Northern Chile, the opening of a gold-copper-arsenic mine raised the concentration of arsenic in a nearby river from an already high 0.4 mg/L to 1.5 mg/L (Oyarzun et al., 2006). In South-Korea, arsenic concentrations of 3—555 µg/L were reported in the surroundings of a closed gold-silver mine (Lee et al., 2005a).

2.2 Effects of cadmium, lead and arsenic on human health

This review will focus on the effects of cadmium, lead and arsenic on human health as a result of chronic exposure to relatively low concentrations.

2.2.1 Cadmium

Dietary exposure to cadmium has been reported to cause adverse health effects in the kidneys, liver, bone, peripheral vascular tissues, mammary gland, placenta, prostate, breast, pancreas and colon (Satarug and Moore 2004; Satarug et al., 2006). The kidneys are the primary target of cadmium, and approximately one third of body cadmium is stored in the kidney cortex (WHO, 1992). Renal cadmium is excreted in urine very slowly, and the half-life of cadmium in the kidney is reported to be decades (WHO, 1992). As a result, the first adverse effects caused by chronic oral exposure to cadmium are manifested in the kidneys. Exposure to higher concentrations may even lead to renal failure, but usually renal tubular dysfunction leading to proteinuria, calciuria, aminoaciduria, glucosuria and tubular necrosis is observed (WHO, 1992; Järup et al., 1998). Adverse bone effects, osteomalacia and osteoporosis, have been associated with chronic cadmium exposure in combination with adverse renal effects (Staessen et al., 1999; Alfvén et al., 2000; Nordberg et al., 2002; Järup and Alfvén, 2004). Adverse bone effects may be a consequence of hypercalciuria caused by cadmium-induced tubular dysfunction (Wu et al., 2001) and reduced activation of vitamin D_3 precursors in the kidney (Chalkley et al., 1998) reducing blood calcium concentration and increasing bone desorption. Occupational exposure to high concentrations of cadmium, mainly through the pulmonary route, has been reported to cause at least lung cancer (WHO, 1992). However, the carcinogenicity of dietary cadmium has only been reported in animal

and *in vitro* studies (Waalkes, 2000). Animal studies have also indicated the possible involvement of cadmium in adverse cardiovascular effects, mainly hypertension, but human studies have not confirmed this (WHO, 1992).

Based on the renal toxicity of cadmium, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA, 2003) has set a provisional tolerable weekly intake (PTWI) of 7 µg Cd/kg body weight/week. However, recent reports have challenged this guideline as too high, since according to a recent meta-analysis of available data, an increased concentration of beta-2-microglobulin, a biomarker for proteinuria, was detected at an exposure level comparable to a PTWI of only 3 µg Cd/kg body weight (Omarova and Phillips, 2007).

2.2.2 Lead

About 90 % of the lead body burden is stored in the bones and teeth of adults. The corresponding value for children is 70 %. Higher mobility of lead in children results from continuous growth and re/desorption of bone making children more susceptible to the adverse health effects of lead. Similar bone lead mobilization also occurs during pregnancy. Lead affects several enzymatic processes involved in heme synthesis (Barbosa et al., 2005) and elevated blood lead concentrations have been associated with reduced hemoglobin synthesis and red blood cell quantity (Schwartz et al., 1990; Factor-Litvak et al., 1999; Tripathi et al., 2001) leading, in the worst case, to anemia. Like cadmium, increased blood lead concentrations are associated with renal dysfunction, which is manifested by urinary excretion of small molecular proteins (Ehrlich et al., 1998; Factor-Litvak et al., 1999; Weaver et al., 2005). A more insidious outcome of exposure to environmental lead during early childhood has been its association with intellectual impairment (Pocock et al., 1994; Wasserman et al., 1997; Canfield et al., 2003). It has been reported that an increase in blood lead concentration from 10 to 30 µg/dL leads to a decrease in intelligence quotient (IQ) of about 1—4 points (Pocock et al., 1994; Wasserman et al., 1997). Canfield et al. (2003) reported a similar small reduction in IQ when blood lead concentrations increased from 1 to 10 µg/dL.

2.2.3 Arsenic

According to the World Health Organization (2001), there is enough evidence available to establish a relationship between inorganic arsenic in drinking water and bladder, skin, and lung cancer, as well as other skin changes such as pigmentation

changes and thickening (hyperkeratosis). Following long-term exposure, the first changes are usually observed in the skin: pigmentation changes, and then hyperkeratosis. Cancer is a late phenomenon, and usually takes more than 10 years to develop. Based on its carcinogenity, drinking-water arsenic has been classed as a group 1 human carcinogen by the IARC (2004). The symptoms and signs that arsenic causes appear to differ between individuals, population groups and geographic areas. For example, arsenic has been associated with skin and bladder cancer in Taiwan but not in the USA (Kapaj et al., 2006). Exposure to arsenic via drinking-water has been shown to cause a severe disease of blood vessels leading to gangrene in Taiwan, known as black foot disease (Tseng, 2005) but this disease has not been observed in other parts of the world. It is possible that other factors such as malnutrition, other toxic compounds in water, sunlight and smoking may work synergistically with arsenic in the development of black foot disease, skin and bladder cancer (Steinmaus et al., 2003; Rossman et al., 2004; Tseng, 2005). Indeed, some very recent work has suggested selenium deficiency as a potentiating agent in arsenic toxicity (Chen et al., 2007). There is also relatively strong evidence showing the association between arsenic exposure and hypertension and cardiovascular disease (WHO, 2001).

2.3 Biosorption of arsenic, cadmium and lead by algal, bacterial and fungal biomass

Methods such as chemical precipitation, flocculation, adsorption, ion-exhange and membrane filtration are conventionally used for removal of metals from water (Rautjärvi and Pöllänen, 1998). Although these methods are widely used, they are claimed to have several disadvantages such as incomplete metal removal, expensive equipment and monitoring system requirements, high reagent or energy requirements and generation of toxic sludge or other waste products that require disposal (Zouboulis et al., 2004).

Biosorption is a term used to generally describe the passive, energy-independent sorption of molecules onto the surface of an adsorbent. One branch of biosorption is binding of metals onto microbial biomass. The idea of using microbial biomass as a sorbent for heavy metals was discovered when more economical and effective alternatives were examined for conventional water purification methods 20-30 years ago. Once established, biosorption of metals has been applied to areas other than decontamination of water, such as enrichment of precious metals. During the last few decades, biosorption of a number of metals (e.g. Al, Au, Cd, Co, Cr, Cu,

Fe, Hg, Ni, Pb, Th, U, Zn) by a variety of biomasses including bacteria, fungi and algae have been studied (Volesky and Holan, 1995; Davis et al., 2003; Mehta and Gaur, 2005; Romera et al., 2006). Since the area of biosorption is vast, the following review will focus on biosorption of the toxic metals cadmium, lead, and arsenic.

Cadmium and lead removal by algal (Sandau et al., 1996; Feng and Aldrich, 2004; Lodeiro et al., 2004; Luo et al., 2006; Pavasant et al., 2006; Deng et al., 2007; Senthilkumar et al., 2007), fungal (Kapoor et al., 1999; Yetis et al., 2000; Yan and Viraraghavan, 2003; Arica et al., 2004; Akar and Tunali, 2005; Kiran et al., 2005; Akar and Tunali, 2006; Tunali et al., 2006) and bacterial (Puranik and Paknikar, 1999; Pardo et al., 2003; Chojnacka et al., 2005; Komy et al., 2006; Lu et al., 2006; Pan et al., 2006) biomass is a rapid process in which the bulk of the removal occurrs during the first few minutes of contact between the metal solution and the biomass. The removal is influenced by pH and very low removal is typically observed at pH's below 2-3, whereas at pH's above 3 a sharp increase in removal occurs and maximum removal is often reached at pH 4-6 (Sandau et al., 1996; Kapoor et al., 1999; Kulczycki et al., 2002; Yan and Viraraghavan, 2003; Arica et al., 2004; Feng and Aldrich, 2004; Lodeiro et al., 2004; Akar and Tunali, 2005; Kiran et al., 2005; Akar and Tunali, 2006; Apiratikul and Pavasant, 2006; Hetzer et al., 2006; Komy et al., 2006; Luo et al., 2006; Pavasant et al., 2006; Tunali et al., 2006; Deng et al., 2007; Senthilkumar et al., 2007). The effect of pH is a result of competition for negatively charged binding sites between cationic metals and protons. Other cationic metals may also compete, and a number have been reported to reduce the removal of cadmium and lead, including Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+} and Al^{3+} (Kapoor et al., 1999; Puranik and Paknikar, 1999; Arica et al., 2004; Lee et al., 2004; Alimohamadi et al., 2005; Kiran et al., 2005; Akar and Tunali, 2006; Aksu and Donmez, 2006; Apiratikul and Pavasant, 2006; Komy et al., 2006; Deng et al., 2007; Sheng et al., 2007).

Maximum cadmium and lead binding capacities have been reported to vary between different algal, fungal and bacterial genus and species, with values ranging from a few milligrams to a few hundred milligrams per gram of dry biomass (Tables 2 and 3). Inter-biomass differences may be explained by structural

Table 2. Maximum **cadmium** removal capacities (q_{max}) and affinity constants (b) reported for algal, fungal and bacterial biomass.

Biomass	pH	q_{max} [a] (mg/g)	b [b] (L/mg)	Reference
Algae				
Ascophyllum nodosum	4.9	215	0.0186	Holan et al.1993
Caulerpa lentillifera	5	4.6	0.0083	Pavasent et al. 2006
Chlorella vulgaris	4	86.6	0.042	Aksu and Dönmez 2006
Ecklonia maxima	6	88.5		Feng and Aldrich 2004
Fucus vesiculous	3.5	73	0.0021	Holan et al.1993
Laminaria japonica	4.5	136		Lee et al. 2004
Sargassum filipendula	4.5	74.2	0.046	Davis et al. 2000
Sargassum fluitans	4.5	79.8	0.046	Davis et al. 2000
Sargassum muticum	3	154	0.0025	Lodeiro et al. 2004
Sargassum natans	3.5	132	0.0209	Holan et al.1993
Sargassum vulgare	4.5	88.8	0.047	Davis et al. 2000
Sargassum vulgaris	6	118		Raize et al. 2004
Fungi				
Aspergillus niger	6	4.38		Kapoor et al. 1999
Botrytis cinerea	5	18.6	0.042	Akar and Tunali 2005
Mucor rouxii	6	20.31	0.28	Yan and Viraraghavan 2003
Phomopsis sp.	6	29.2		Saiano et al. 2005
Rhizopus arrhizus	5	30.4		Tobin et al. 1984
Saccharomyces cerevisiae		31.8	0.092	Göksungur et al. 2005
Saccharomyces cerevisiae		15.4		Chen and Wang 2007
Bacteria				
Arthrobacter	6	13.4	0.033	Pagnanelli et al. 2000
Bacillus laterosporous	7	159.5		Zouboulis et al. 2004
Bacillus licheniformis	7	142.7		Zouboulis et al. 2004
Bacillus subtilis		40.5	0.0003	Kulczycki and Ferris 2002
Citrobacter strain MCM B-181	6	43.5	0.04	Puranik and Paknikar 1999
Enterobacter sp. J1	5	46.2	0.004	Lu et al. 2006
Escherichia coli		11.2	0.002	Kulczycki and Ferris 2002
Pseudomonas aeruginosa		57.4		Chang et al 1997
Pseudomonas aeruginosa	5.1	97	0.0034	Komy et al 2006
Pseudomonas putida	6	8.0	0.15	Pardo et al. 2003

[a] Maximum binding capacity (mg metal bound/g dry biomass), [b] Coefficient related to affinity of binding (L/mg)

Table 3. Maximum **lead** removal capacities (q_{max}) and affinity constants (b) reported for algal, fungal and bacterial biomass.

Biomass	pH	q_{max}[a] (mg/g)	b[b] (L/mg)	reference
Algae				
Ascophyllum nodosum	3.5	272	0.0203	Holan and Volesky 1994
Caulerpa lentillifera	5	28.7	0.015	Pavasent et al. 2006
Chondrus crispus	3.5	195	0.0016	Holan and Volesky 1994
Cladophora fascicularis	5	227	0.077	Deng et al. 2007
Cladophora glomerata	4.5	73.5	0.216	Jalali et al. 2002
Codium taylori	3.5	376	0.026	Holan and Volesky 1994
Ecklonia maxima	6	232.6		Feng and Aldrich 2004
Fucus vesiculous	3.5	229	0.0303	Holan and Volesky 1994
Galaxaura marginata	3.5	25	0.0044	Holan and Volesky 1994
Gracilaria canaliculata	4.5	41.8	0.232	Jalali et al. 2002
Gracilaria corticata	4.5	54	0.158	Jalali et al. 2002
Laminaria japonica	4.5	348.1		Lee et al. 2004
Laminaria japonica	5.2	250.7	0.03	Luo et al. 2006
Padina gymnospora	3.5	65	0.0526	Holan and Volesky 1994
Padina pavonia	4.5	217.4	0.072	Jalali et al. 2002
Polysiphonia violacea	4.5	102	1.32	Jalali et al. 2002
Sargassum fluitans	3.5	266	0.0214	Holan and Volesky 1994
Sargassum hystrix	4.5	285	0.043	Jalali et al. 2002
Sargassum natans	3.5	253	0.0261	Holan and Volesky 1994
Sargassum natans	4.5	238	0.056	Jalali et al. 2002
Sargassum vulgare	3.5	228	0.0093	Holan and Volesky 1994
Sargassum vulgaris	6	248.6		Raize et al. 2004
Turbinaria conoides	4.5	439.4	0.048	Senthilkumar et al. 2007
Ulva lactuca	4.5	126.5	0.099	Jalali et al. 2002
Fungi				
Aspergillus flavus	5	13.5		Akar and Tunali 2006
Aspergillus niger	5	10.19		Kapoor et al. 1999
Cephalosporium aphidicola	5	92.4	0.015	Tunali et al. 2006
Mucor rouxii	6	53.75	0.27	Yan and Viraraghavan 2003
Neurospora crassa	4	43.3	0.026	Kiran et al. 2005
Phomopsis sp.	6	140.9		Saiano et al. 2005
Rhizopus arrhizus	5	56.5	0.0089	Alimohamadi et al. 2005
Rhizopus arrhizus	3.6	103.6		Tobin et al. 1984
Rhodotorula glutinis		73.5	0.02	Cho et al 2003
Saccharomyces cerevisiae		60.2	0.066	Göksungur et al. 2005
Saccharomyces cerevisiae		85.6		Chen and Wang 2007

[a] Maximum binding capacity (mg metal bound/g dry biomass), [b] Coefficient related to affinity of binding (L/mg)

Table 3. Continued.

Biomass	pH	q_{max}[a] (mg/g)	b[b] (L/mg)	Reference
Bacteria				
Bacillus cereus	6	54.8	0.06	Pan et al. 2006
Bacillus subtilis		55.95	0.001	Kulczycki and Ferris 2002
Citrobacter strain MCM B-181	4.5	58.8	0.11	Puranik and Paknikar 1999
Escherichia coli		43.5	0.0008	Kulczycki and Ferris 2002
Enterobacter sp. J1	6	50.9	0.074	Lu et al. 2006
Pseudomonas aeruginosa		110		Chang et al 1997
Pseudomonas putida	6.5	56.2	0.04	Pardo et al. 2003

[a] Maximum binding capacity (mg metal bound/g dry biomass), [b] Coefficient related to affinity of binding (L/mg)

differences in the cell walls of different biomasses. The cell surface of algae used in biosorption is mainly covered by alginate and sulfated polysaccharides (Davis et al., 2003). The outer cell wall of fungi is mainly comprised of beta-(1→3)-glucan and glycoproteins (Bowman and Free, 2006) whereas lipopolysaccharides and peptidoglycan with (lipo)teichoic acids are characteristic for gram-negative and –positive bacteria, respectively (Beveridge, 1999; Delcour et al., 1999). Concentrations of functional groups responsible for metal binding vary between biomasses (Fourest and Volesky, 1996; Yee and Fein, 2001; Ngwenya et al., 2003; Borrok et al., 2004; Chojnacka et al., 2005; Naja et al., 2005). In addition, different functional groups have different affinities for a given metal, leading to the varying removal capacities of different biomasses (Fein et al., 1997; Ngwenya et al., 2003).

Different physical and chemical treatments have been tested to increase the metal removal capacity of biomasses, and for desorption of bound metals. The results have been variable and have depended on the treatment, biomass, and metal studied (Puranik and Paknikar, 1999; Lodeiro et al., 2004; Akar and Tunali, 2005; Kiran et al., 2005; Akar and Tunali, 2006). Treatments using heat, acids, salts, EDTA, and different organic solvents have been reported to cause weight loss of biomass (Lodeiro et al., 2004; Akar and Tunali, 2006; Lodeiro et al., 2006; Senthilkumar et al., 2007). The weight loss is associated with reduced metal binding capacity (Lodeiro et al., 2006) when binding sites are destroyed, and increased metal binding capacity (Akar and Tunali, 2006) when new binding sites are generated as a result of partial cell wall degradation. Desorption experiments have established the reversibility of cadmium and lead removal by algal, fungal, and bacterial biomass. Dilute mineral acids (HCl, HNO_3, H_2SO_4), salt solutions ($CaCl_2$, NaCl, KCl, KI)

and EDTA have been tested for desorption. The best results, i.e. recovery close to 100%, have been obtained with dilute acids and EDTA (Kapoor et al., 1999; Puranik and Paknikar, 1999; Arica et al., 2004; Chojnacka et al., 2005; Lodeiro et al., 2006; Lu et al., 2006; Deng et al., 2007; Senthilkumar et al., 2007; Tuzen et al., 2007).

The resorption capacity of different biomasses after desorption of bound metal is generally lower than that of the fresh biomass. The degree of reduction in removal depends on the biomass and desorbent used, and has been reported to vary from 1 to 63 % for dilute acids, and from 6.5 to 30 % for EDTA (Puranik et al., 1995; Kapoor et al., 1999; Puranik and Paknikar, 1999; Jalali et al., 2002; Arica et al., 2004; Akar and Tunali, 2005; Chojnacka et al., 2005; Lu et al., 2006; Tunali et al., 2006; Senthilkumar et al., 2007).

For practical water purification applications, biomass is often immobilized onto a suitable carrier material. Immobilization of the biomass is important, since it improves the mechanical strength of the biomass, and especially in the case of small biomass particles, reduces resistance to fluid flow. The most frequently used technique for biomass immobilization is entrapment within a polymer matrix such as sodium alginate, agar, silica gel, polyacrylamide, and polysulfone (Veglio and Beolchini, 1997; Mehta and Gaur, 2005) but other methods such as adsorption on inert supports, covalent bonding to vector compounds and cross-linking of cells have been used (Veglio and Beolchini, 1997). Metal removal by immobilized biomass is often lower than by free biomass. For example, nickel removal by *Pseudomonas fluorescens* decreased from 145 to 37 mg/g after immobilization on agar beads (Lopez et al., 2002). The reduction in removal after immobilization depends on the matrix used, and the removal ratio of immobilized biomass/free biomass has been reported to vary from 0.92 to 0.42 for different matrices when applied to chromium binding (Bai and Abraham, 2003). This has been attributed to a reduction in the available surface area of the biomass as a result of immobilization. Experiments using column packed immobilized biomass have established that metal binding in continuous flow conditions is effective. For example, using a column containing the biomass of *Sargassum muticum* water containing less than 0.02 mg/L cadmium was produced from water initially containing 50 mg/L cadmium and concentrated water containing over 5 g/L cadmium was produced after regeneration of the column (Lodeiro et al., 2006).

Different mechanisms have been reported to be involved in cadmium and lead binding. Often an increase in the concentration of light metals such as Na^+, K^+, Mg^{2+} and Ca^{2+} and a small drop in pH is observed concomitantly with biosorption of cadmium and lead indicating ion exchange between heavy and light metals/protons (Brady and Tobin, 1995; Kapoor and Viraraghavan, 1997; Figueira et al., 2000; Romero-Gonzalez et al., 2001; Raize et al., 2004; Chojnacka et al., 2005). The amount of light metals and protons does not always correspond to the amount of metal bound (Brady and Tobin, 1995; Raize et al., 2004) indicating the involvement of other mechanisms. Raize et al. (2004) and Fourest and Volesky (1996) reported the involvement of complexation in cadmium and lead binding. Lead was also reported to form metallic precipitates on the biomass surface (Raize et al., 2004). Chemical modifications and potentiometric titrations of biomasses have revealed that carboxyl, phosphoryl, sulfonate, and amino groups are the main functional groups responsible for heavy metal binding in bacteria, algae and fungi (Beveridge and Murray, 1980; Tobin et al., 1990; Fourest and Volesky, 1996; Raize et al., 2004; Chojnacka et al., 2005).

Arsenic biosorption by algal, fungal or bacterial biomass has not been as extensively studied as the biosorption of cationic metals such as cadmium and lead. In some studies, no arsenic removal was observed and this has been attributed to the negative charge of aqueous arsenic species and the surface of the biomass studied (Tsui et al., 2006). The few papers available reporting arsenic removal show that it is a relatively rapid process. Equilibrium contact times of 20, 60, 75, 300 minutes for As(V) (Loukidou et al., 2001; Seki et al., 2005; Hansen et al., 2006; Murugesan et al., 2006) and 240 minutes for As(III) removal (Say et al., 2003) have been reported. The optimal pH of As(V) removal has been reported to be between 6 and 7 for *Penicillium purpurogenum* (Say et al., 2003), iron coated *Aspergillus niger* (Pokhrel and Viraraghavan, 2006), and methylated yeast (Seki et al., 2005) whereas a much lower optimal pH (2.5) was observed for an alga, *Lessonia nigrescens* (Hansen et al., 2006). The maximum arsenic binding capacities of 24.5, 45.2 mg As(V)/g and 35.6 mg As(III)/g have been reported for *Penicillium chrysogenum* (Loukidou et al., 2003), *Lessonia nigrescens* (Hansen et al., 2006) and *Penicillium purpurogenum* (Say et al., 2003), respectively. An increase in positively charged, and a decrease in negatively charged, surface groups have been reported to increase As(V) removal capacity (Loukidou et al., 2003; Seki et al., 2005) indicating binding occurring to positively charged surface groups probably by electrostatic interactions with the anionic arsenic species. A tentative mechanism

for As(III) removal by a cyanobacterium *Scytonema* has been reported to be complexation to sulfhydryl groups of surface proteins (Prasad et al., 2006). Therefore, it seems that biosorption of arsenic occurs by two different mechanisms depending on the oxidation state of arsenic species, i.e. ionic interactions between anionic As(V) and cationic ligands, and complex formation between chargeless As(III) and sulfhydryl groups of the biosorbent.

2.4 Lactic acid bacteria

Lactic acid bacteria are gram-positive, nonsporing, nonrespiring cocci or rods, which produce lactic acid as the major end-product of carbohydrate fermentation. Lactic acid bacteria comprise species from genera *Aerococcus Carnobacterium Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Sterptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Axelsson, 2004). The term lactic acid bacteria is also often used with the genus *Bifidobacteria* although they have unique sugar fermentation pathways and are phylogenetically unrelated. Lactic acid bacteria are natural inhabitants of humans and other animals. They live in symbiosis with the host and are found throughout the digestive system, all mucosal membranes and skin. Specific strains of lactic acid bacteria are used to ferment food and beverages because of their useful technological properties or beneficial health effects.

2.4.1 Cell wall of lactic acid bacteria

The major components of the gram-positive cell wall presented in Figure 2 are a plasma membrane, a thick layer of peptidoglycan (murein), teichoic acids, proteins, carbohydrates and sometimes S-layer proteins attached to the peptidoglycan layer.

The peptidoglycan of lactic acid bacteria is a network comprised of polymerized disaccharide N-acetyl-glucosamine-beta(1→4)-N-acetyl-muramic acid chains covalently crosslinked together by pentapeptide bridges (Figure 3). Disaccharide units of peptidoglycan are reported to have three different modifications. Acetyl groups of both N-acetyl-glucosamine (GlcNAc) and N-acetyl-muramic acid (MurNAc) may be cleaved (Logardt and Neujahr, 1975), an additional acetyl group may be added to the 6-O position of MurNAc (Delcour et al., 1999) and the C6 of MurNAc may be substituted by teichoic acids. The first three aminoacids of pentapeptide bridge attached to MurNAc are usually either L-alanine/D-glutamate/*meso*-diaminopimelic acid or L-alanine/D-glutamate/L-lysine although at least five different peptidoglycan subtypes have been identified (de Ambrosini et

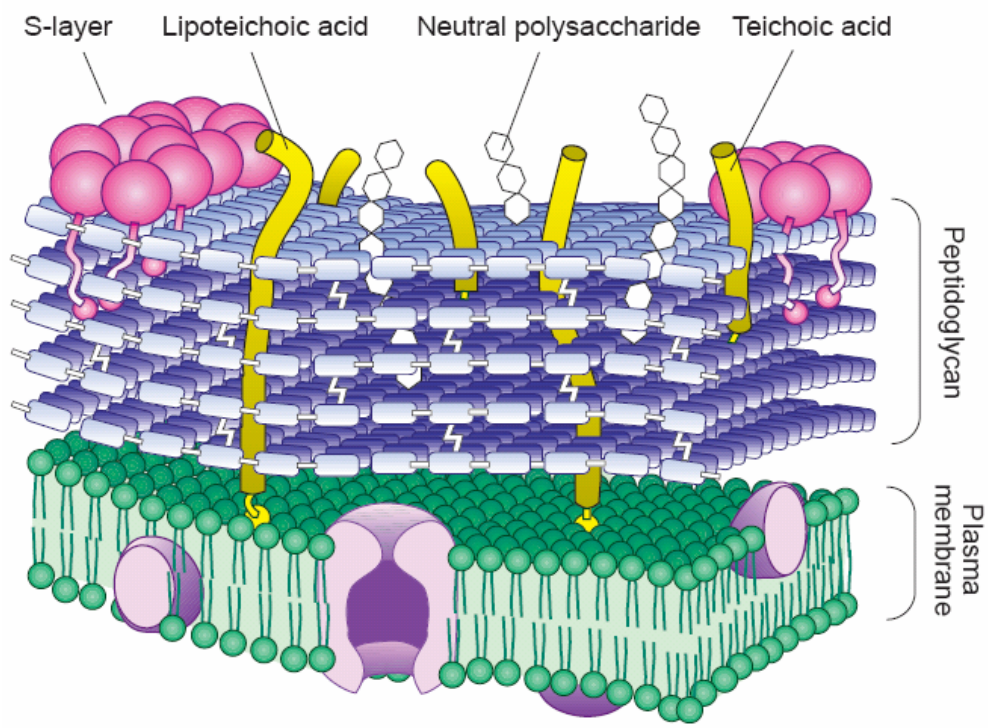

Figure 2. Cell wall of gram positive bacteria. Reprinted with kind permission of Springer Science and Business Media from: Springer and Kluwer Publisher/Antonie van Leeuwenhoek, 76, 1999, page 160, The biosynthesis and functionality of the cell-wall of lactic acid bacteria, Jean Delcour, Thierry Ferain, Marie Deghorain, Emmanuelle Palumbo & Pascal Hols, Figure 1

al., 1996; Simelyte et al., 2003). To this tripeptide, a dipeptide of two D-alanine residues is attached directly or through an interpeptide bridge. Some vancomycin resistant lactic acid bacteria are reported have a diverging aminoacid sequence of pentapeptide bridge where C-terminal D-alanine is substituted by D-lactate (*Enterococcus faecium, Pediococcus pentosaceus, Lactobacillus plantarum* and *Lactobacillus casei*) and in some cases by D-serine (*Enterococcus gallinarum*) (Billot-Klein et al., 1994; Ferain et al., 1996; Grohs et al., 2000).

Teichoic acids (TA) are anionic polymers bound to the peptidoglycan layer through a linkage unit. The structure of the linkage unit of *L. plantarum* has been reported to be glycerol-phospho-N-acetylmannosaminyl-beta(1→4)-glucosamine (Kojima et al., 1985). At least two types of TAs have been identified from lactic acid bacteria, poly(glycerolphosphate) and poly(ribitolphosphate) TAs (Archibald et al., 1961; de

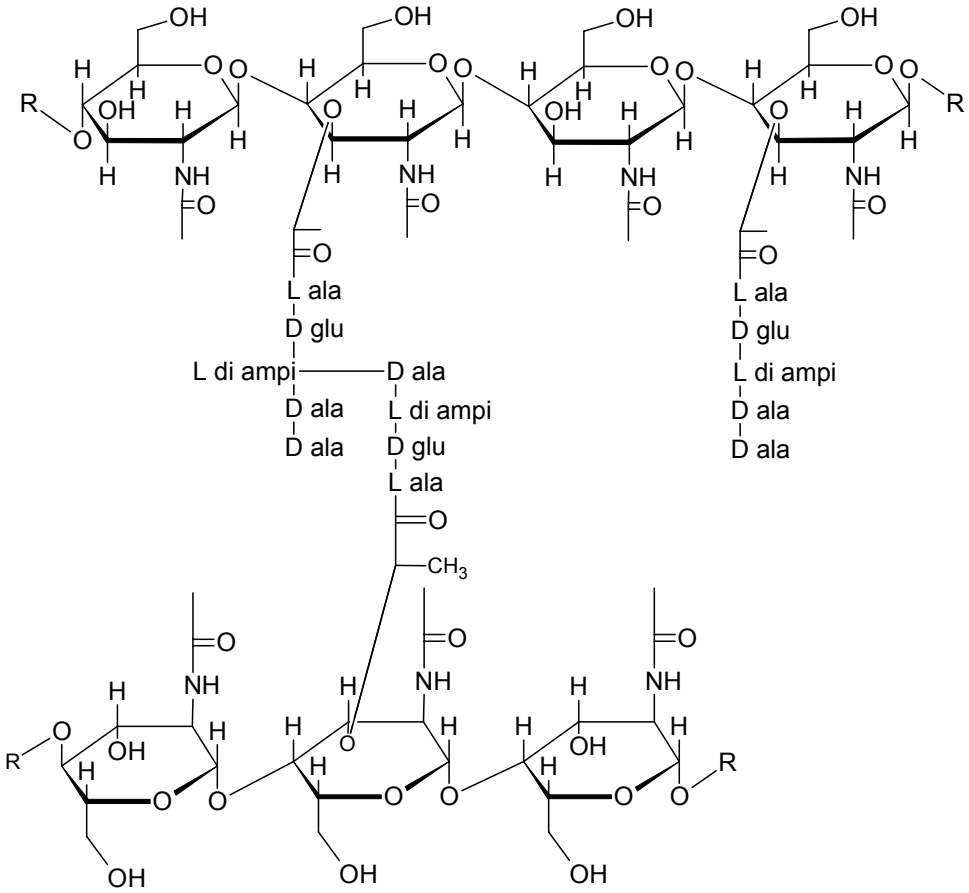

Figure 3. Structure of peptidoglycan of lactic acid bacteria showing repeating disaccharide units and pentapeptide bridge.

Ambrosini et al., 1996) (Figure 4). Additional glucose and D-alanine moieties may be attached to hydroxyl groups of glycerol and ribitol (Archibald et al., 1961).

Lipoteichoic acids (LTA) are compounds structurally similar to TAs but instead of peptidoglycan they are attached to the plasma membrane by a glycolipid anchor. In general, the glycolipid anchor is composed of diacylglycerol attached to a di- or trisaccharide unit (Fischer, 1994). By far the most frequently detected LTA in lactic acid bacteria is the poly(glycerophosphate) LTA (Fischer et al., 1990; Fischer, 1994; Räisänen et al., 2004) . The structure of poly(glycerophosphate) LTA is

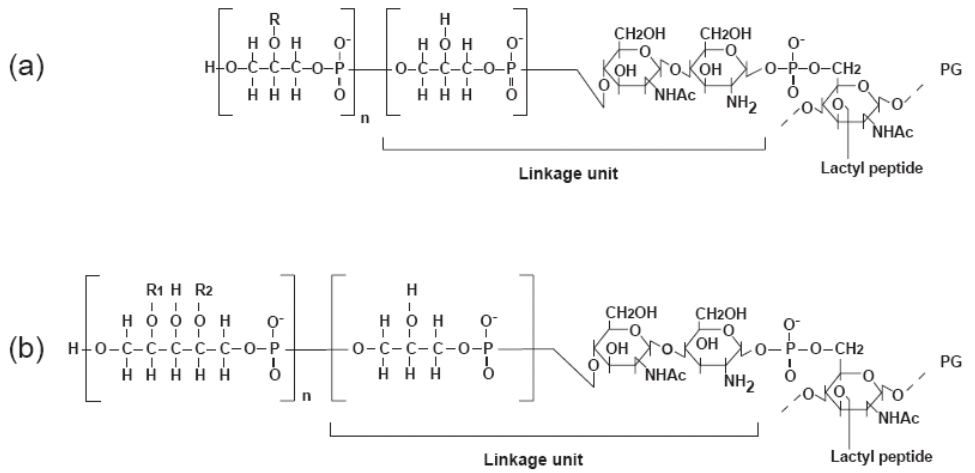

Figure 4. Structures of poly(glycerol-) (a) and poly(ribitolphosphate) teichoic acids (b). Reprinted with kind permission of Springer Science and Business Media from: Springer and Kluwer Publisher/Antonie van Leeuwenhoek, 76, 1999, page 160, The biosynthesis and functionality of the cell-wall of lactic acid bacteria, Jean Delcour, Thierry Ferain, Marie Deghorain, Emmanuelle Palumbo & Pascal Hols, Figure 8.

almost identical to the structure of poly(glycerophosphate) TA with the only difference being the chirality of the glycerol. Similar to TAs, LTAs have glycosyl and D-alanyl substituents attached to hydroxyl groups of glycerol.

Many lactic acid bacteria from the genus *Lactobacillus* have been reported to produce surface layer proteins (S-layer proteins) (Åvall-Jääskelainen and Palva, 2005). These non-covalently bound proteins have a size of 25-50 kDa and they are highly basic, with calculated pI's ranging from 9.35 to 10.88 (Vidgrén et al., 1992; Boot et al., 1993; Jakava-Viljanen et al., 2002; Ventura et al., 2002). Lactic acid bacteria not producing S-layer proteins are reported to have a negative surface charge at neutral pH (Schär-Zammaretti and Ubbink, 2003). Despite the basic nature of S-layer proteins, the surface charge of S-layer producing *Lactobacillus* has also been reported to be negative (Schär-Zammaretti and Ubbink, 2003). An explanation for this may be the involvement of positively charged areas of S-layer proteins in their attachment to peptidoglycan (Smit et al., 2001).

Lactic acid bacteria possess at least two types of cell wall polysaccharides: neutral cell wall polysaccharides and exopolysaccharides. Cell wall polysaccharides are the most common polysaccharides produced by lactic acid bacteria. Compared to peptidoglycans their structures are very complicated and large variations exist between different bacteria. In general, cell wall polysaccharides are branched, chargeless and often contain rhamnose but other sugars such as galactose, glucose, mannose and galactosamine, including negatively charged diaminomannuronic acid have been detected (Wicken et al., 1983; Nagaoka et al., 1985; Habu et al., 1987; Nagaoka et al., 1990; Nagaoka et al., 1995; Nagaoka et al., 1996).

Like cell wall polysaccharides, exopolysaccharides are a structurally diverse group of polysaccharides. Exopolysaccharides are either excreted into the surrounding medium, or bound to the bacterial surface, and form a capsule. Therefore, the latter are also referred to as capsular polysaccharides. Lactic acid bacteria from genera such as *Lactobacillus*, *Enterococcus*, and *Sterptococcus* and some other bacteria commonly used in dairy industry (*Bifidobacterium* and *Propionibacterium*) have been reported to produce exopolysaccharides containing glucose, galactose, rhamnose, mannose, N-acetylglucosamine and N-acetylgalactosamine (Micheli et al., 1999; Landersjö et al., 2002; Nordmark et al., 2005a; Nordmark et al., 2005b; Mozzi et al., 2006; Sanchez et al., 2006)

Based on the above reports, the cell wall of lactic acid bacteria is seen to contain a large number of negatively charged functional groups, mainly carboxyl and phosphoryl. Electrophoretic studies have established that the net surface charge of lactic acid bacteria is negative at neutral pH (Pelletier et al., 1997; Boonaert and Rouxhet, 2000; Schär-Zammaretti and Ubbink, 2003; Schär-Zammaretti et al., 2005).

2.4.2. Removal of toxic compounds and pathogenic bacteria by lactic acid bacteria

The *Aspergillus* and *Fusarium* species are well known producers of aflatoxins and *Fusarium* toxins. Aflatoxins often contaminate corn and corn products, peanuts and milk, whereas *Fusarium* toxins (trichothecenes, zearalenone and fumosins) are contaminants of cereal products (Belitz et al., 2004). The link between oral ingestion of aflatoxins and liver cancer is well established, and the most toxic aflatoxin, aflatoxin B_1, is listed as a class 1 carcinogen by IARC (IARC, 1993).

Fusarium toxins have various adverse health effects that have been observed in farm animals and humans (Bennett and Klich, 2003).

During the last decade, aflatoxin binding (mainly AFB_1) by lactic acid bacteria has been extensively studied. A number of different strains of lactic acid bacteria and bifidobacteria have been tested for AFB_1 removal from buffered aqueous solution *in vitro* (El-Nezami et al., 1998a; Haskard et al., 2001; Peltonen et al., 2001). The removal has been established to be strain specific. Removal by the two most effective strains, *L. rhamnosus* GG and *L. rhamnosus* LC705, was rapid and up to 99.9 % of the initial 5 μg/L could be removed (El-Nezami et al., 1998a). The removal was influenced by both temperature and bacterial concentration, but not pH. *Lactobacillus rhamnosus* GG and *L. rhamnosus* LC705 have also been reported to remove AFM_1, a metabolite of AFB_1 contaminating milk, from skimmed milk and cream (Pierides et al., 2000). In addition to many other lactic acid bacteria (Niderkorn et al., 2006; Niderkorn et al., 2007), the strains most effective at removing AFB_1 have also been reported to bind trichothecenes (El-Nezami et al., 2002a) and zearalenone (El-Nezami et al., 2002b) from liquid media *in vitro.*

Different physical treatments have been established to influence AFB_1 and zearalenone removal by lactic acid bacteria. El-Nezami et al. (1998a, 1998b, 2002a), Haskard et al. (2001) and Niderkorn et al. (2006) demonstrated that heat- and acid- treatments increase AFB_1 and the zearalenone removal capacity of *L. rhamnosus* GG and LC705. The observation that bound AFB_1 and zearalenone were close to fully recovered after washing the bacterial pellet with chloroform (Haskard et al., 2001) and methanol (El-Nezami et al., 2002a), respectively, indicates that the removal of these toxins occurred primarily at the bacterial surface. The effects of heat- and acid-treatments have been suggested to result in denaturation of cell surface proteins and partial breakdown of peptidoglycan which would lead to exposure of new binding sites (Haskard et al., 2001).

To identify the possible binding sites of AFB_1 and zearalenone on the surface of *L. rhamnosus* GG, the effect of various chemical, physical and enzymatic treatments on binding have been studied. Haskard et al. (2000) and El-Nezami et al. (2004) reported the involvement of hydrophobic interactions in AFB_1 and zearalenone binding, respectively. Both AFB_1 (Haskard et al., 2000) and zearalenone (El-Nezami et al., 2004) removal has been observed to decrease after treatment with periodate, which causes oxidation of *cis* OH groups to aldehydes and carboxylic

acid groups, and likewise, when treated with a protease, pronase-E, indicating involvement of cell wall carbohydrates and proteins in binding. Involvement of carbohydrates in AFB_1 binding, mainly peptidoglycan, has also been reported in another study (Lahtinen et al., 2004). The results also ruled out the participation of exopolysaccharides in AFB_1 binding.

Aflatoxin B_1 binding has also been studied in a number of *ex vivo* and *in vivo* experiments. *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Propionibacterium freudenreichii ssp. shermanii* JS (PJS) and a combination of LC705 and PJS have been reported to bind AFB_1 and thereby reduce its uptake to ligated chicken duodenal tissue (El-Nezami et al., 2000a; Gratz et al., 2005). Administration of a single dose of AFB_1 together with *L. rhamnosus* GG has been shown to increase fecal AFB_1 excretion in rats (Gratz et al., 2006). This probably results from increased excretion of an AFB_1-*L. rhamnosus* GG complex, since binding of AFB_1 has been reported to reduce adhesion of *L. rhamnosus* GG to Caco-2 cells (Kankaanpää et al., 2000). Preliminary results by El-Nezami et al. (2000b), suggest that the reduced AFB_1 uptake after administration of lactic acid bacteria observed with chickens and rats may also occur in human subjects. These results were supported in a recent clinical study, showing reduced urinary excretion of aflatoxin B_1-N^7-guanine, a marker for recent aflatoxin B_1 exposure, in subjects receiving *L. rhamnosus* LC705 and *P. freudenreichii* subsp. *shermanii* (1:1, wt:wt) at a dosage of $2\text{-}5x10^{10}$ cfu/day (El-Nezami et al., 2006).

Cyanotoxins are a group of hepato- and neurotoxins produced by cyanobacteria, and their production during cyanobacterial blooms in eutrophic water bodies is a global problem. In addition to mycotoxin removal, lactic acid bacteria have also been reported to remove cyanotoxins from water. Nybom et al. (2007) reported removal of microcystin-LR (a cyanobacterial hepatotoxin) by eleven different lyophilized *Lactobacillus* and *Bifidobacterium* species. Compared to mycotoxins, removal of microcystin-LR was slow and only about 25 % of the toxin was removed after a 24 hour incubation when an initial concentration of 100 µg/L was used (Nybom et al., 2007). Higher pH, temperature and bacterial concentration were observed to enhance the removal. Removal improved significantly, from 25.7 to 58 % when metabolically active *B. lactis* Bb12 was used, but only about 20 % of the bound amount was recovered from the bacterial pellets after methanol extraction. Contrary to observations from mycotoxin removal experiments, treatments with heat, acid and ethanol have been reported to decrease the removal

of MC-LR (Nybom et al., 2007; Surono et al., 2007), although the opposite results have been reported for heat treated *L. rhamnosus* GG and *B. lactis* Bb12 (Meriluoto et al., 2005). Based on the relatively slow removal process, the enhanced removal at the optimal growth temperature of lactic acid bacteria, increased removal by viable bacteria and the low recovery of eliminated microcystin, Nybom et al. (2007) suggested that the removal of microcystin-LR may occur through a metabolism dependent mechanism rather than by passive adsorption.

It has been established that probiotic lactic acid bacteria protect against rotavirus, traveller's, and antibiotic associated diarrhoea (Sazawal et al., 2006; de Vrese and Marteau, 2007). Their protective effects have been related to mechanisms such as competition for adhesion sites and nutritional sources, secretion of antimicrobial substances, toxin inactivation, and immune stimulation. Recently, it has been reported that probiotic lactic acid bacteria may also form aggregates with enteropathogenic bacteria, *Bacteroides vulgatus*, *Clostridium histolyticum*, *Enterobacter sakazakii* and *Staphylococcus aureus* (Collado et al., 2007a; Collado et al., 2007b). Collado et al. (2007a and b) showed that the coaggregation ability of lactic acid bacteria with pathogenic bacteria is dependent on both the probiotic(s) and pathogen studied.

3. AIMS OF THE STUDY

The main purpose of this work was to screen and assess the ability of specific lactic acid bacteria to remove cadmium, lead and arsenic from water. The target was to evaluate the applicability of the best lactic acid bacteria as tools for heavy metal removal from water and characterize their properties for further development of a biofilter.

The specific goals of this study were

1) To select the most efficient lactic acid bacterial strains for the removal of toxic, cationic, heavy metals, cadmium and lead, from water and to evaluate the effect of chemical and physical conditions on the removal and maximum metal elimination capacity of each strain (Studies I, II and V).

2) To assess the ability of specific lactic acid bacteria in native and chemically modified forms to remove anionic arsenic species from water (study III).

3) To compare the effectiveness of single strains and their combination in the removal of heavy metals, microcystin-LR and aflatoxin B1 from aqueous solution (Study IV).

4) To characterize the components responsible for removal of cadmium and lead and study the regeneration of the bacterial biomass for repeated removal attempts (Study V).

4. MATERIALS AND METHODS

4.1 Bacteria and culturing conditions

Both pure single strains and combinations of strains were used in this work. The single strains and combinations used are presented in Table 4. The bacteria were used either as fresh cultures (Study I) or in lyophilized form (Studies II, III, IV and V). Bacteria for fresh cultures and lyophilized cultures were first grown under anaerobic conditions at 37^{o}C for 24, 40 or 48 hours in de Man, Rogosa and Sharp broth (MRS, Merck, Darmstadt, Germany). The biomass was then washed twice (centrifuged and resuspended) with ultra pure water (Milli-Q plus, Millipore S.A., Molsheim, France) or phosphate buffered saline (PBS, pH 7.4). Fresh cultures were used immediately in metal/toxin removal experiments whereas lyophilized bacteria were first frozen to -20^{o}C and then dried in a freeze-dryer. Prior to use, lyophilized cultures were stored at -20^{o}C. *Lactobacillus acidophilus* NCFM, *Bifidobacterium lactis* Bb12, *B. longum* 2C and the two commercial starter cultures were purchased in commercial lyophilized form. These cultures were only washed twice with ultra pure water before use. Commercial lyophilized cultures of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bbi 99/E8, *B. longum* 46 and *Propionibacterium freudenreichii* ssp. s*hermanii* JS were also occasionally used in a similar manner. Part of the washed biomass was inactivated by boiling for one hour.

4.2 Preparation of metal/toxin stock solutions

Commercial stock solutions of Cd ($Cd(NO_3)_2$, Fluka Chemie Gmbh, Schwitzerland), Pb ($Pb(NO_3)_2$, Fluka Chemie Gmbh, Schwitzerland), As(III) (As_2O_3 in 1 M HCl, Reagecon, Shannon, Ireland) and As(V) (H_3AsO_4, Merck KgaA, Darmstadt, Germany) were purchased. Microcystin-LR (MC-LR) was extracted from a culture of *Anabaena* sp. 90 (culture collection of Professor Kaarina Sivonen, University of Helsinki, Finland) and purified by HPLC (Meriluoto and Spoof, 2005). Aflatoxin B_1 (AFB_1) was purchased from Sigma (St. Louis, MO, USA) and dissolved in PBS (Haskard et al., 2001)

4.3 Chemical modifications of bacterial surface

4.3.1 Methylation of carboxyl groups (Studies III and V)

Lactobacillus acidophilus NCFM, *L. casei* DSM20011, *L. crispatus* DSM20584, *L. fermnetum* ME3 and *B. longum* 46 were methylated to neutralize the negative

Table 4. Bacterial biomasses used, their place of purchase, and the conditions and metals/toxins studied in this work.

Biomass	Study numbe	Metals/toxins studied	Studies performed[a]
Lactobacillus acidophilus NCFM Danisco USA Inc., Madison, WI, USA	III	As	t, pH, chemical modification, surface charge
L. casei DSM20011 DSMZ GmbH, Braunschweig, Germany	III	As	t, pH, C_{metal}, chemical modification, surface charge
L. casei Shirota Professor Y-K Lee, Yakult Singapore Pty. Ltd., Singapore	I, II	Cd, Pb	t, C_{metal}
L. crispatus DSM20584 DSMZ GmbH, Braunschweig, Germany	III	As	t, pH, chemical modification, surface charge
L. fermentum ME3 (DSM 14241) Tartu University, Tartu, Estonia	II, V	Cd, Pb	t, pH, C_{metal}, chemical modifications, other cations, de/resorption, TEM
L. johnssonii Lj1 Isolated from Nestlé LC1 product	I	Cd	t, C_{metal}
L. rhamnosus LC705 Valio Ltd., Helsinki, Finland	I, IV	Cd, Pb, AFB_1, MC-LR	t, $C_{metal,}$ hydrophobicity, surface charge, autoaggregation
L. rhamnosus GG (ATCC 53103) Valio Ltd., Helsinki, Finland	I, II, IV	Cd, Pb, AFB_1, MC-LR	t, pH, T, C_{bakt}, C_{metal}, hydrophobicity, surface charge, autoaggregation
Bifidobacterium breve Bbi 99/E8 Valio Ltd., Helsinki, Finland	IV	Cd, Pb, AFB_1, MC-LR	hydrophobicity, surface charge, autoaggregation

Table 4. Continued.

Biomass	Study number	Metals/toxins studied	Studies performed[a]
B. lactis Bb12 Chr. Hansen, Horsholm, Denmark	I, II	Cd, Pb	t, pH, C_{metal}
B. longum 2C Probiotical srl, Novara, Italy	II	Cd, Pb	t, C_{metal}
B. longum 46 Probiotical srl, Novara, Italy	II, V	Cd, Pb	t, pH, C_{metal}, chemical modification, other cations, de/resorption, TEM
Propionibacterium freudenreichii ssp. *shermanii* JS, Valio Ltd., Helsinki, Finland	IV	Cd, Pb, AFB_1, MC-LR	hydrophobicity, surface charge, autoaggregation
FV-DVS XT-303-eXact[b] Chr. Hansen Ltd., Hørsholm, Denmark	II	Cd, Pb	t, C_{metal}
YO-MIX 401[c] Danisco Niebüll GmbH, Niebüll, Germany	II	Cd, Pb	t, C_{metal}
L. rhamnosus GG, *L. rhamnosus* LC705, *B. breve* *Bb*i 99/E8 and *P. freudenreichii* ssp. *shermanii* JS, Valio Ltd., Helsinki, Finland	IV	Cd, Pb, AFB_1, MC-LR	hydrophobicity, coaggregation, surface charge

[a] t = contact time, T = temperature, C_{bakt} = bacterial concentration, C_{metal} = metal concentration, TEM = transmission electron microscopy, [b] contains *Lactococcus lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Leuconostoc pseudomesenteroides* and *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*, [c] contains *Streptococcus thermophilus* and *L. bulgaricus*.

charge of carboxylic groups as described by Fraenkel-Conrat and Olcott (1945). Lyophilized culture was suspended in methanol with 0.1 M HCl to give a final bacterial concentration of 10 g/L and incubated with agitation for 24 h at room temperature. After incubation, methylated biomasses were washed three times (centrifuged and resuspended) with ultra pure water, lyophilized and stored at -20°C.

4.3.2 Amination of carboxyl groups (Study III)

Lactobacillus casei DSM20011 was aminated to change the negative charge of carboxylic groups to positive of amino groups according to the method of Beveridge and Murray (1980). A mixture of 0.5 M ethylenediamine (Fluka Chemie GmbH, Buchs, Switzerland), 0.2 M carbodiimide (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, Sigma-Algrich Inc., Saint Louis, Missouri, USA) and 5 g/L of bacteria was stirred (500 rpm) under nitrogen for 6 h at room temperature. The pH was adjusted to 4.75 with dilute HNO_3 and kept constant. After incubation, aminated biomass was washed three times (centrifuged and resuspended) with ultra pure water, lyophilized and stored at -20°C.

4.3.3 Esterification of phosphoryl groups (Study V)

L. fermentum ME3 and *B. longum* 46 were esterified according to the method of Tobin et al. (1990) to neutralize the negative charge of phosphoryl groups. One gram of biomass was heated under ref1ux conditions with 40 ml of triethyl phosphite (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 30 ml of nitromethane (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for 6 h. Esterified biomasses were then washed three times (centrifuged and resuspended) with ultra pure water, lyophilized and stored at -20°C.

4.4 Metal/toxin binding experiments by study bacteria

The concentration of washed, freshly cultured bacteria in aqueous solution was adjusted to 8 x 10^8 cells/mL after counting by flow cytometry (Virta et al., 1998) (Study I). The suspension was divided into 2 mL aliquots, centrifuged, and the supernatants discarded. Finally, the pellets were resuspended in MQ-water containing 0—1000 µg/L cadmium. When lyophilized bacteria were used (Studies II, III, IV and V), a given amount of dry biomass was suspended to MQ-water or PBS. The bacterial suspension was spiked with the same solvent containing either

one of the metals, MC-LR or AFB_1 to give a final bacterial concentration of 1 g/L, 2 g/L or 10^9 cells/mL, respectively. When necessary, the pH of the suspension was immediately adjusted to a desired value with dilute HNO_3 or NaOH. Samples were incubated at 22°C or 37°C from 5 minutes to 24 hours. After incubation, bacteria were separated from the supernatant by centrifugation and a sample from the supernatant was taken for either metal, MC-LR or AFB_1 analysis. The samples for metal analyses were preserved by addition of a small volume of concentrated ultra-pure 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.

4.5 Effect of contact time, pH, bacterial concentration, metal concentration and temperature (Studies I, II and III)

To assess the effect of different physical and chemical conditions on metal binding, the binding experiments were performed as described above in Section 4.4. In each experiment, one of the conditions was changed while the others were kept constant. The experiments varied the contact time (5—1440 minutes), pH (2—7), bacterial concentration (0.5—1.5 g/L), metal concentration (0.01—100 mg/L) and temperature (4—37°C). The parameters varied with each bacterial preparation are given in Table 4.

Experimental results from metal removal studies with different metal concentrations were fitted to a theoretical model which enabled calculation of specific descriptive parameters. In this work (Studies II and III), the metal removal ability of different LAB strains was evaluated using a Langmuir isotherm defined in Equation (1) (Davis et al., 2003) :

Equation 1. $$q = q_{\max} \frac{bC_f}{1 + bC_f}$$

By plotting C_f (free concentration of metal in equilibrium state) and q (bound concentration of metal in equilibrium state), which were obtained from experimental data, it was possible to calculate q_{max} (maximum binding capacity at given conditions) and b (the coefficient related to the initial slope of the curve and to the affinity of binding) from Equation 1.

4.6 Effect of other cations on cadmium and lead removal (Study V)

The effect of Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Pb^{2+} and Cd^{2+} on cadmium and lead binding was tested using *L. fermentum* ME3 and *B. longum* 46. After spiking the solution with Cd or Pb, an equimolar concentration (0.5 mM) of one of the metals listed above was added and the samples were incubated for 30 minutes at pH 5. Otherwise, the binding method used followed the one described earlier in Section 4.4.

4.7 Localization of bound cadmium and lead by TEM (Study V)

After the metal binding experiment (metal concentration 100 mg/L, pH 5, contact time 30 minutes, 22^{o}C, bacterial concentration 1g/L) the bacterial pellets were prefixed with 5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.16 M s-collidine buffer (pH 7.4) and dehydrated with a series of ethanol washes. Dry pellets were embedded in epoxy resins (Glycidether 100, Merck) and cut into thin sections as described earlier (Sundström et al. 1999). The thin sections were viewed under a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan).

4.8 Desorption of bound metals (Studies III and V)

Desorption of bound metal was studied by washing the bacterial pellets after a binding experiment with ultra pure water (Studies III and V), 0.1 mM and 1.0 mM EDTA (Study V), 1.5 mM and 15 mM HNO_3 (Studies III and V) and 1.5 mM NaOH (Study III). The binding experiment was performed as described above in Section 4.4, the conditions being pH 5, metal concentration 50 mg/L and contact time 10 minutes for cadmium and lead, and for arsenic, pH 7, metal concentration 0.5 mg/L and contact time 5 minutes. After the first binding experiment the bacterial pellet was separated from the supernatant by centrifugation and resuspended to an equal volume of one of the desorbents tested and incubated at room temperature for 10 minutes. The suspension was centrifuged and a sample from the supernatant was taken for metal analysis. Desorption was repeated from three to five times depending on the desorbent and metal studied.

4.9 Resorption of cadmium and lead (Study V)

The reusability of biomass used in cadmium and lead binding was assessed as follows. The binding experiment was first performed as described above in Section 4.4 at pH 5 (metal concentration 100 mg/L and incubation time 10 minutes). The

bacterial pellet was then suspended in 10 mM EDTA or 15 mM HNO_3 for the desorption of the bound metal. After desorption, the pellet was washed twice with ultra pure water to remove residual EDTA/HNO_3 and used again in metal binding. This cycle was repeated in total three times.

4.10 Characterization of bacterial surface

4.10.1 Hydrophobicity (Study IV)

The hydrophobicity of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bbi99/E8 and *P. freudenreichii* ssp. *shermanii* JS was characterized using the microbial adhesion to hydrocarbons (MATH) test, according to the method of Rosenberg et al. (1980). The lyophilized cells were washed twice with 10 mmol/L PBS, pH 7.4 and resuspended in the same buffer to an absorbance (λ = 600 nm) of about 0.25 ± 0.03 in order to standardize the number of bacteria (10^7 to 10^8 CFU/ mL). After addition of an equal volume of p-xylene (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) or n-hexadecane (Fluka Chemie AG, Buchs, Switzerland), the two-phase system was thoroughly mixed by vortexing for 1 min. The aqueous phase was removed after an 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: $[(A_0\text{-}A)/A_0]$ x 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 percentual decrease in the absorbance of the original bacterial suspension due to partitioning of cells into the hydrocarbon layer.

4.10.2 Auto-/coaggregation (Study IV)

Bacterial suspensions of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bbi99/E8, *P. freudenreichii* ssp. *shermanii* JS and their combination were prepared as described for the MATH test. The bacterial suspensions were incubated in aliquots at room temperature without agitation and the absorbance (λ = 600 nm) of bacterial suspensions was measured for determination of auto/coaggregation 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 A_t the A_{600} of the bacterial suspension after incubation of t hours. For comparison of auto- and coaggregation data, a predicted value for

coaggregation was calculated by summing up the autoaggregation indexes of each strain, multiplied by the proportional bacterial concentration of each strain in the combination.

4.10.3 Surface charge (Studies III and IV)

The net surface charge of *L. acidophilus* NCFM, *L. casei* DSM20011 and *L. crispatus* DSM20584 in native and modified forms and *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bbi99/E8 and *P. freudenreichii* ssp. *shermanii* JS in native form was analyzed by measuring the zeta potential (ζ) of the bacteria by microelectrophoresis (Zetasizer 3000, Malvern Instruments Ltd., Worchestershire, UK). Lyophilized bacteria were suspended in 1 mM KNO_3, and after pH adjustment with dilute HNO_3 or NaOH, the suspension was diluted to a final concentration of about 10^8 cells/mL. The pH range studied was from 2 to 12. For each biomass, the isoelectric point (pI) was interpolated from the ζ vs. pH plot. Each analysis was made in triplicate.

4.10.4 Potentiometric titration (Study III)

The efficiency of methylation was assessed by potentiometric titration of native and methylated bacteria. Lyophilized bacteria were suspended in 0.01 mM KNO_3 to give a final concentration of 5 g/L. KNO_3 had been purged with N_2 for several hours in order to remove solubilized CO_2. After pH adjustment to 2.5 with HNO_3, the suspension was titrated with 0.1 M KOH to pH 11 under N_2. The pH of the solution was monitored with a pH meter (PHM 80, Radiometer A/S, Copenhagen, Denmark).

4.11 Analysis of metals, MC-LR and AFB_1

Prior to use, all laboratory ware used in heavy metal experiments were thoroughly rinsed with distilled water, soaked in 10 % (v/v) nitric acid over night, thoroughly rinsed with distilled water and ultra pure water, and finally dried and stored protected from dust. Cadmium and lead samples were analyzed by atomic absorption spectrometry using flame (FAAS) and graphite furnace (GFAAS) methods. Arsenic samples were analyzed either by the hydride generation method (HGAAS) using AAS equipped with VGA-76 Vapor Generation Accessory (Varian Techtron Pty. Limited, Mulgrave, Australia) or FAAS. Prior to HGAAS analysis, arsenic in the samples and standards was reduced to As(III) by adding 1 mL 37 %

HCl (J.T. Baker, Deventer, Holland) and 0.6 mL 20 % (w/v) KI (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) per 10 mL of sample and incubating the samples for three hours at 22°C. Blanks and samples spiked with a given amount of metal to be analyzed were used as quality control samples in all analyses. Instruments used in metal analysis were the Solaar M6 Dual Zeeman AAS Spectrometer (Thermo Electron Spectroscopy Ltd., England) and Varian SpectrAA-300 AAS (Varian Techtron Pty. Limited, Mulgrave, Australia).

AFB_1 and MC-LR concentrations were analyzed by high performance liquid chromatography (HPLC) according to the methods of Lee et al. (2003) and Meriluoto et al. (2005), respectively.

The percentage of toxin/heavy metal removed (bound by bacteria) was expressed as removal % = 100 x $[(C_0-C_1)/C_0]$, where C_0 and C_1 are the initial concentration of toxin and residual concentration of toxin after removal, respectively.

4.12 Statistics

Statistical analyses were performed using the paired t-test or analysis of variance (ANOVA). When ANOVA was used, either Tukeys HSD or the Tamhane test was used to test differences between groups, depending on the homogeneity of variances. Statistical work was performed with SPSS version 11.0 or 13.0.

5. RESULTS

5.1 Characterization of bacterial surface

5.1.1 Hydrophobicity (Study IV)

Adhesion percentages of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bbi99/E8, PJS and their combination (COMB) to n-hexadecane and p-xylene are presented in Figure 5. In general, the adhesion to n-hexadecane was lower than adhesion to p-xylene. The highest adhesion to p-xylene, 82.1, 73.0 and 66.0 %, was observed with *L. rhamnosus* LC705, PJS and COMB, respectively. Adhesion to n-hexadecane of *L. rhamnosus* GG, *L. rhamnosus* LC705, PJS and COMB was observed to be at a similar level ranging between 45.0—50.8 %. Only the adhesion of *B. breve* Bbi 99/E8 (26.3 %) was clearly below this range.

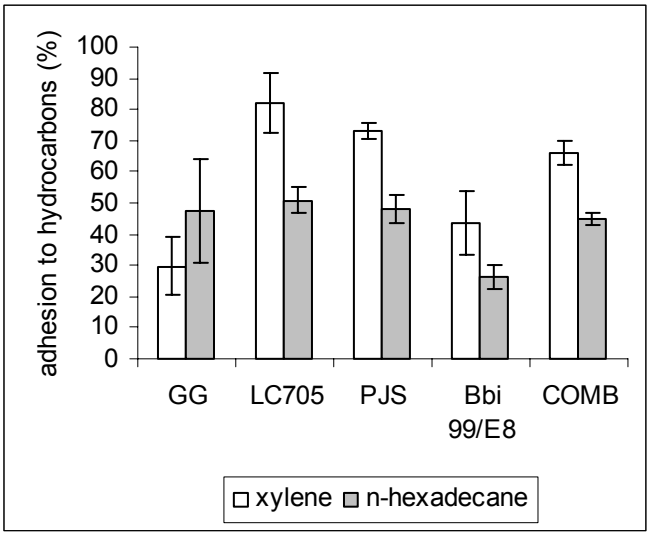

Figure 5. Adhesion percentages of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bbi99/E8, PJS and their combination (COMB) to n-hexadecane and p-xylene. Error bars show standard deviation of three separate determinations.

5.1.2 Auto-/coaggregation (Study IV)

The aggregation ability of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bbi99/E8, PJS and their combination (COMB) increased with longer incubation time and ranged between 13.6±1.2 and 28.0±1.7% after an incubation of 4 h (Figure 6). The aggregation ability followed the order of *L. rhamnosus* LC705 > COMB > *L. rhamnosus* GG ≈ PJS > *B. breve* Bbi 99/E8 at all time points studied.

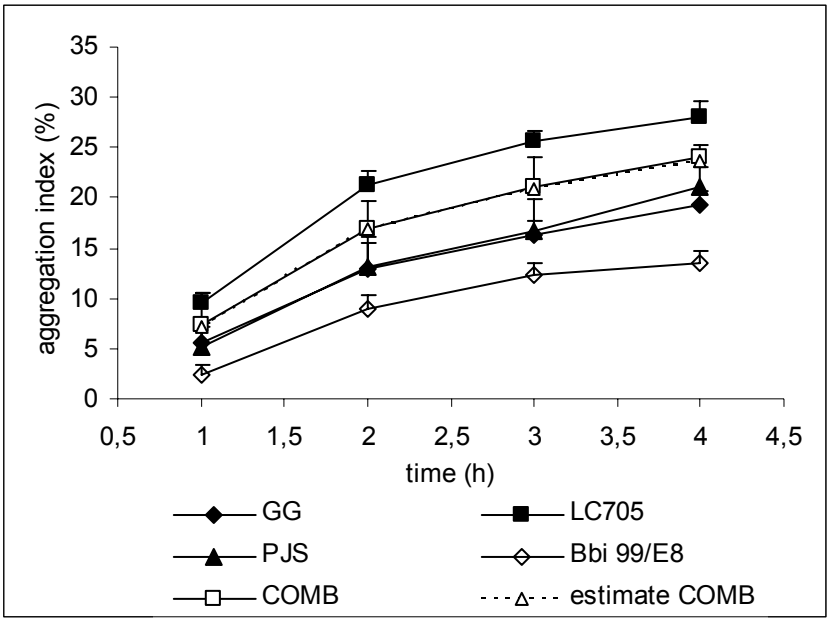

Figure 6. Aggregation index of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bbi99/E8 and PJS, and an estimate for aggregation index of their combination (COMB). Error bars show standard deviation of three separate determinations.

5.1.3 Surface charge (Studies III and IV)

Microelectrophoresis was used to characterize the net surface charge of bacteria used in arsenic removal experiments and when toxin/heavy metal removal by single strains and their combination was compared. Zeta potential graphs of studied strains in native and chemically modified forms are presented in Figures 7 and 8. Graphs of *L. rhamnosus* LC705, *B. breve* Bbi 99/E8 and PJS were quite similar, indicating an almost identical net surface charge. The net surface charge of *L. rhamnosus* GG was clearly higher than with other strains throughout the pH range studied. In general, the net surface charge of these strains was negative.

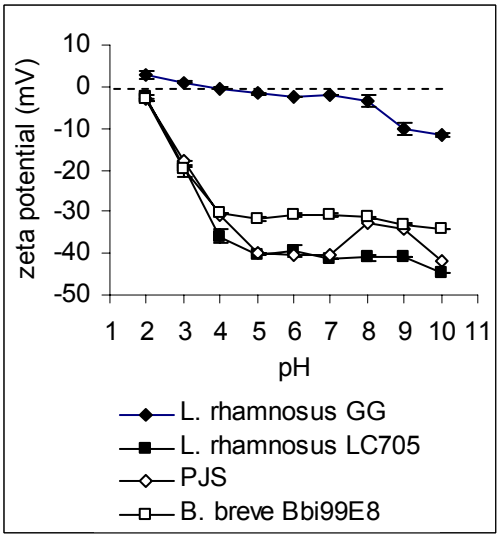

Figure 7. Zeta potentials of bacteria used in study IV.

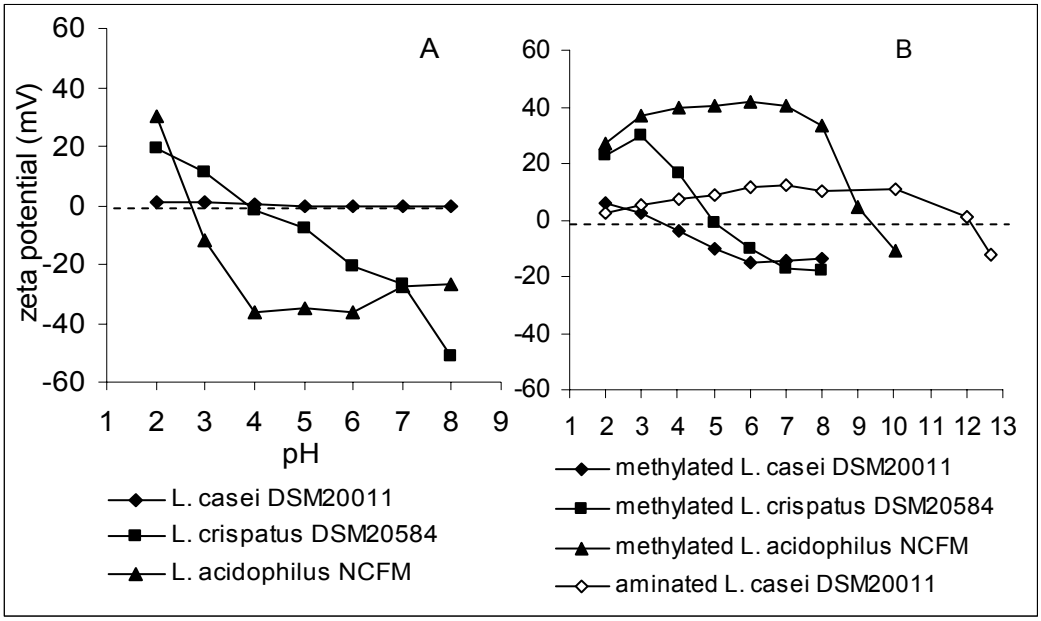

Figure 8. Zeta potentials of studied bacteria in native (A) and chemically modified forms (B) used in study III.

The isoelectric point (pI) (i.e. pH where the zeta potential was zero) was interpolated for the bacteria used in the arsenic removal study (see Table 5). Overall, the zeta potential of methylated and aminated LAB was higher (more positive) when compared to the native bacteria. A similar trend was also seen in pI, as expected. Only methylation of *L. casei* DSM20011 led to a pI lower than that observed with native bacteria. The zeta potential of native *L. casei* DSM20011 was very close to zero which made the interpolation of the exact pI difficult. Therefore, the observed low pI for native *L. casei* DSM20011 as compared to the methylated cells probably resulted from difficulties in interpolation. This was confirmed in potentiometric titration, which showed that less titrant was needed for the titration of methylated *L. casei* DSM20011 as compared to the native cells.

Table 5. Isoelectric points interpolated from zeta potentials as a function of pH. Average and standard deviations of three experiments are presented.

	native	methylated	aminated
L. casei DSM20011	4.5±0.1	3.4±0.2	12.0±0.2
L. crispatus DSM20584	3.9±0.1	4.9±0.1	-[a]
L. acidophilus NCFM	2.7±0.1	9.3±0.1	-

[a] - amination was not done

5.1.4 Potentiometric titration (Study III)

The data from potentiometric titration of the native and methylated LAB showed that less titrant was needed for the methylated LAB as compared to the native LAB to reach the endpoint (pH 11) of the titration (Figure 9). Since the methylation method used was specific for carboxylic groups, this indicated that methylation decreased the number of free carboxylic groups on the bacterial surface. Potentiometric titration could be used for the identification of functional groups on the surface and the determination of methylation degree. However, this could not be performed in this work since equivalence points for different functional groups were not visible.

Potentiometric titration and microelectrophoresis confirmed that the chemical modifications (methylation and amination of carboxyl groups) performed for *L. acidophilus* NCFM, *L. casei* DSM20011 and *L. crispatus* DSM20584 were successful.

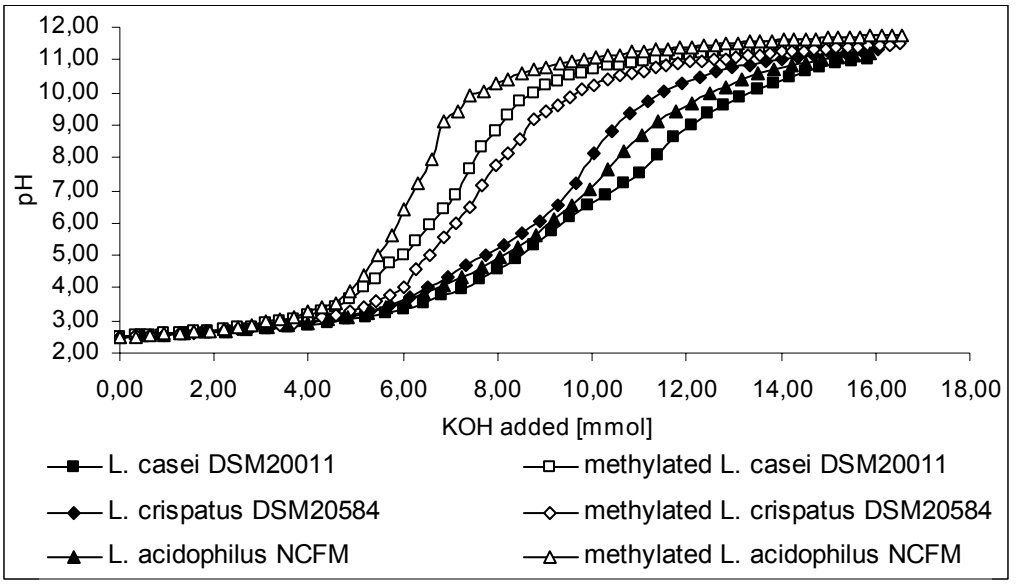

Figure 9. Titration curves of native and methylated *L. casei* DSM20011, *L. crispatus* DSM20584 and *L. acidophilus* NCFM.

5.2 Metal binding by study bacteria (Studies I, II, III and V)

All strains studied removed cadmium and lead, but only aminated *L. casei* DSM20011 removed arsenate (As(V)) (III). None of the strains studied removed arsenite (As(III)). All lyophilized bacterial strains studied were effective in removing low concentrations (0.01—1 mg/L) of cadmium and lead in water. Up to 99% of cadmium and 97% of lead were removed from solutions with initial metal concentrations of 100 and 1000 µg/L, respectively (II). The percentual removal of both cadmium and lead decreased with increasing initial metal concentration and ranged between 11.1—49.0 % for cadmium and 37.5—94.3 % for lead when an initial metal concentration of 100 mg/L was used. Lower cadmium removal ranging from 31.5—90.2, 23.3—63.7 and 28.2—53.2 % was observed for freshly

cultured bacteria when initial cadmium concentrations of 10, 100 and 1000 µg/L, respectively, were used (I).

The specific binding (mg metal bound/g dry biomass) of cadmium and lead increased when the initial metal concentration was increased, until a saturation point was reached (Figure 10). The maximum cadmium binding (q_{max}) calculated from the Langmuir equation ranged from 12.1 to 54.7 mg/g (Table 6). Lead binding was higher, with maximum values ranging from 32.3 to 175.7 mg/g. The affinity of cadmium binding (b) varied from 0.05 to 0.51 L/mg. For lead, the range was from 0.03 to 0.59 L/mg. Only boiled *B. lactis* Bb12 in lead binding did not fit this model. Otherwise, the Langmuir model described the cadmium and lead binding well. Lead binding by boiled *B. lactis* Bb12 was low at low metal concentrations but increased steeply at higher concentrations (data not shown). The most efficient strains in both cadmium and lead binding were *B. longum* 46, *B. lactis* Bb12 and *L. fermentum* ME3.

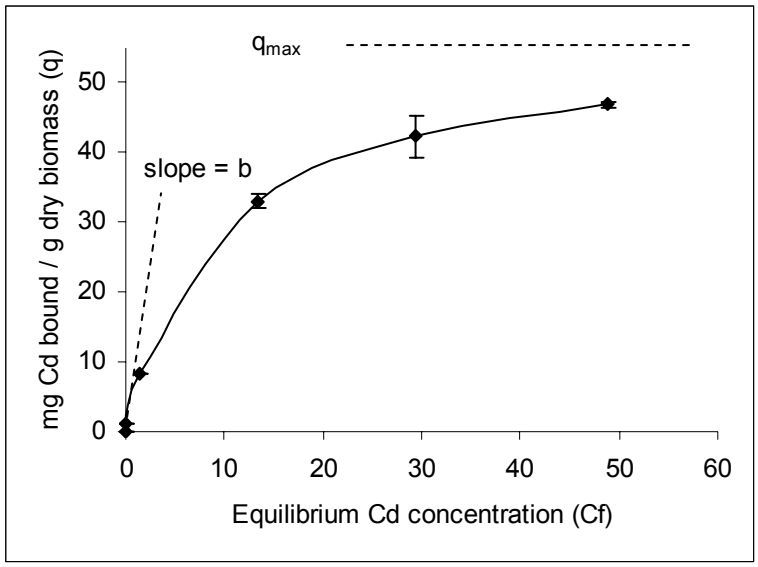

Figure 10. Specific cadmium binding of lyophilized *B. longum* 46 biomass as a function of equilibrium cadmium concentration. Error bars show standard deviation of three separate determinations (II).

Table 6. Parameters (q_{max} and b) obtained from the Langmuir isotherm for Cd (pH 6) and Pb (pH 5) binding of boiled and lyophilized (italicized) lactic acid bacteria at +37 °C (II).

	Cd			Pb		
	q_{max}[a]	b[b]	R^2[c]	q_{max}	b	R^2
B. lactis Bb12	32±1[d]	0,05±0,01	0,99	-[f]	-	-
	34±1[e]	*0,13±0,01*	*0,99*	*111±11*	*0,50±0,22*	*0,87*
B. longum 2C	15±0	0,15±0,06	0,94	47±19	0,12±0,1	0,99
	14±1	*0,33±0,04*	*0,97*	*45±16*	*0,50±0,55*	*0,99*
B. longum 46	32±3	0,09±0,02	0,97	176±19	0,03±0,01	0,93
	55±4	*0,12±0,01*	*1,00*	*94±17*	*0,33±0,27*	*0,95*
L. casei Shirota	19±2	0,51±0,11	0,98	98±3	0,06±0,01	0,99
	12±2	*0,51±0,17*	*0,97*	*70±3*	*0,15±0,01*	*0,92*
L. fermentum ME3	27±0	0,06±0,01	0,97	136±25	0,10±0,04	0,91
	28±4	*0,11±0,03*	*0,99*	*143±33*	*0,15±0,05*	*0,88*
L. rhamnosus GG	13±4	0,32±0,23	0,83	107±18	0,05±0,01	0,97
	13±2	*0,18±0,01*	*0,90*	*47±0*	*0,09±0,01*	*0,99*
starter 1[g]	22±2	0,22±0,03	0,98	32±1	0,20±0,02	0,99
	23±1	*0,51±0,13*	*0,99*	*36±1*	*0,36±0,01*	*0,99*
starter 2[h]	26±1	0,27±0,01	1,00	36±1	0,27±0,01	0,97
	29±1	*0,25±0,04*	*0,99*	*33±2*	*0,59±0,19*	*0,96*

Mean of at least two replicates and standard deviation are shown.
[a] Maximum binding capacity (mg metal bound/g dry biomass).
[b] Coefficient related to affinity of binding (L/mg).
[c] Regression coefficient.
[d] Boiled biomass.
[e] Freeze-dried biomass.
[f] Data did not fit to the Langmuir model.
[g] FV-DVS XT-303-eXact (*L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis*, *L. mesenteroides* subsp. *cremoris*, *L. pseudomesenteroides* and *L. lactis* subsp. *lactis* biovar. *diacetylactis*).
[h] YO-MIX 401 (*S. thermophilus* and *L. bulgaricus*)

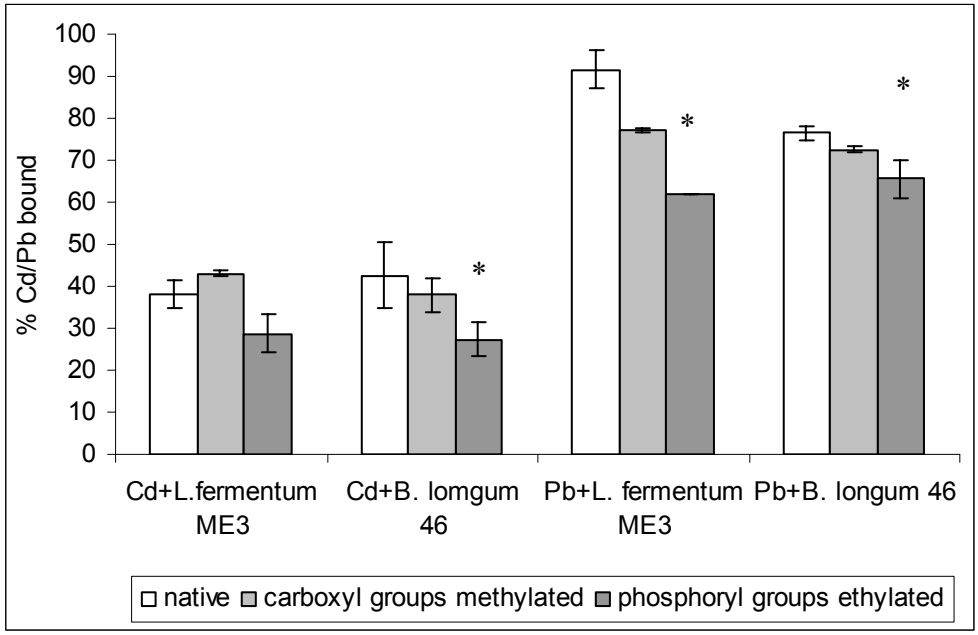

Figure 11. Effect of chemical treatments of carboxyl and phosphoryl groups on cadmium and lead binding by *L. fermentum* ME3 and *B. longum* 46. (t = 30 minutes; pH 5; C_{metal} 50 mg/L; C_{bakt} 1 g/l; +37°C). Significant difference (paired t-test, $p<0.05$) from the removal of cadmium or lead by native biomass is indicated with an asterisk above the bars. Error bars show standard deviation of three separate determinations (V).

In general, boiling only had a minor effect on the maximum cadmium and lead binding capacities (q_{max}) of lyophilized bacteria. In the case of cadmium removal by freshly cultured cells, boiling increased the removal in most cases, and removal decreased only when fresh biomass of *L. rhamnosus* LC705 was used. Both methylation of carboxyl groups (not statistically significant) and ethylation of phosphoryl groups ($p<0.05$) decreased cadmium and lead binding by *L. fermentum* ME3 and *B. longum* 46 when compared to lyophilized biomass (Figure 11) except in the case of cadmium binding by methylated *L. fermentum* ME3, where slightly higher removal was observed compared to native biomass.

Compared to the removal of cadmium and lead, arsenic(V) removal was low and ranged from 7.8 ± 1.7 to 38.1 ± 9.0 %, when the initial concentration ranged between 5 and 0.1 mg/L, respectively (III). The arsenic data fitted well to the

Langmuir isotherm (R^2 = 0.93), and q_{max} and b were 312 ± 68 µg/L and 0.0022 ± 0.0013 L/µg, respectively.

5.3 Removal of cadmium, lead, microcystin-LR and aflatoxin B_1 by single strains and their combination (Study IV)

The removal of cadmium, lead, MC-LR and AFB_1 by *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bbi99/E8, PJS and their combination are presented in Figure 12. In general, the removal of MC-LR and AFB_1 were low ranging from 7.4 to 18.8 % and from 8.9 to 21.4 %, respectively. Removal of cadmium and lead were higher ranging between 22.1—49.1 and 39.7—69.6 %, respectively. The removal of all model toxins was strain dependent.

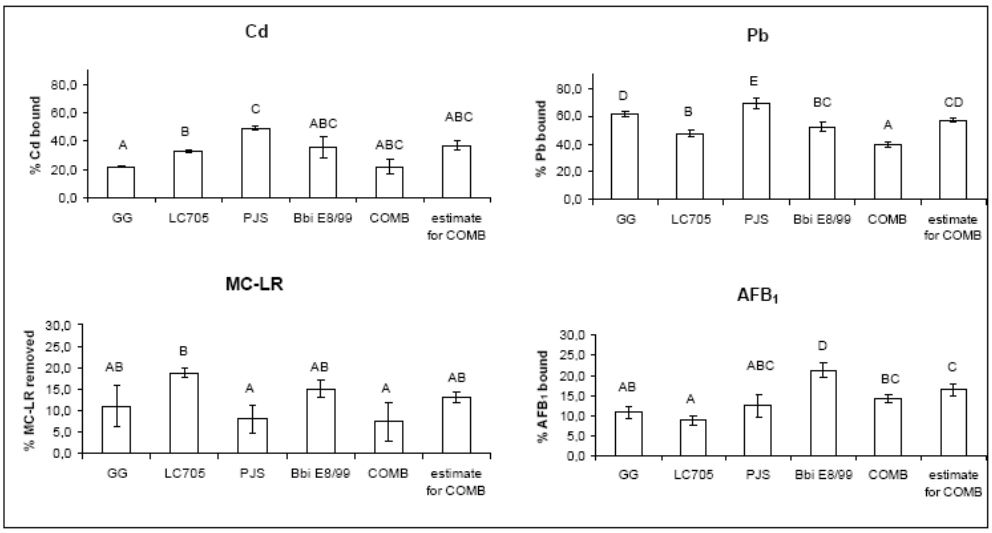

Figure 12. Percentages of cadmium, lead, MC-LR and AFB_1 removed by LAB from aqueous solution. Average of three replicates and standard deviation are presented. Significant differences (ANOVA, $p<0.05$) between the strains is indicated with different letters above bars.

Based on the data obtained with single strains, an estimate was calculated for the removal of model toxins by COMB by summing together the observed removal of each toxin multiplied by the relative cell concentration of that strain in the COMB. When the observed removal by COMB was compared to the calculated estimate, the removal of all the tested model toxins with COMB was observed to be lower (significantly only in case of Pb ($p<0.05$)) than the calculated estimate.

In order to determine the factors causing the reduced removal by combination, and possible associations in removal of different model toxins, the hydrophobicity, aggregation ability and surface charge of the strains and combination used was determined (see Chapter 4.1). No correlation between any of the surface characteristics and the removal of cadmium, lead and MC-LR was observed (data not shown). No correlation between the removal of different toxins was observed (data not shown). A negative linear correlation between AFB_1 removal and both adhesion to n-hexadecane (% AFB_1 removed = -0.48 x adhesion to n-hexadecane + 34, R^2 = 0.95) and aggregation index (% AFB_1 removed = -0.73 x aggregation index + 29, R^2 = 0.67) was observed. A positive linear correlation between aggregation index and adhesion to n-hexadecane (adhesion to n-hexadecane = 1.5 x aggregation index + 11, R^2 = 0.70) was also observed.

5.3 Effect of contact time (Studies I, II and III)

Binding of cadmium, lead and arsenate (As(V)) (Figure 13) occurred rapidly when lyophilized cells were used. After 5 min of incubation, binding of cadmium (10 mg/L) at pH 6 and lead (50 mg/L) at pH 5 ranged between 61.8±3.3–87.8±2.9% and 30.2±7.9–92.6±1.9%, respectively (II). With most of the strains, further incubation had no effect on the binding but a small increase, and decrease, was observed in the cadmium removal by *L. casei* Shirota and *L. fermentum* ME3, respectively (II). The effect of contact time on cadmium removal was slightly different when freshly cultured cells were used. In this case, removal was slower and increased throughout the time range (1-1440 minutes) studied (I). In the case of aminated *L. casei* DSM20011, the highest removal of arsenate (28.7±5.2%) was observed after 5 min at pH 7, and prolonged incubation reduced the removal (III).

5.4 Effect of pH (Studies II and III)

The effect of pH on cadmium, lead and arsenate (As(V)) removal was observed to be significant, with the highest binding occurring at a pH close to neutral (Figure 14). The impact of pH on cadmium removal with all strains studied was quite similar (II). The removal was negligible at pH ≤ 3 with *L. rhamnosus* GG, *L. fermentum* ME3 and *B. longum* 46, and about 20% with *B. lactis* Bb12 at pH 2.

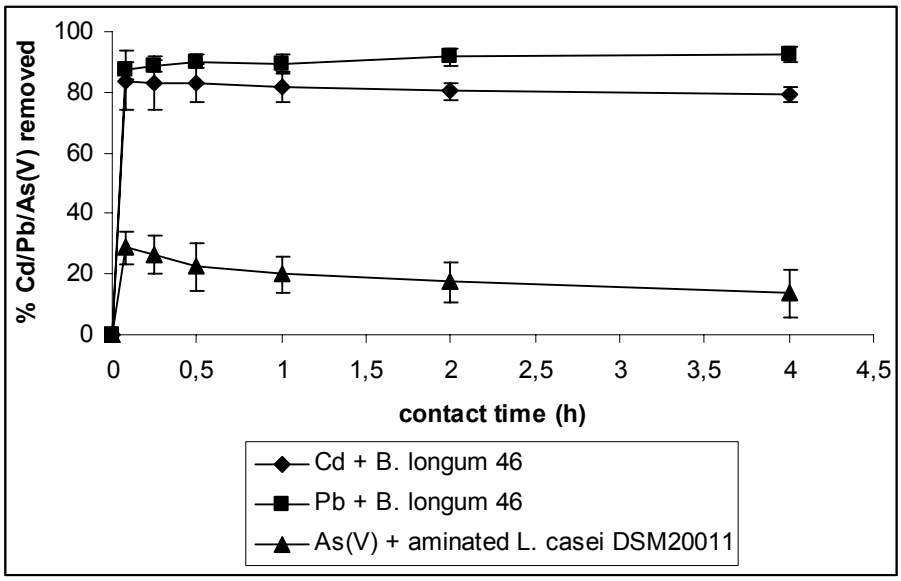

Figure 13. Effect of contact time on Cd (c(Cd) 10 mg/L; C_{bakt} 1 g/L; +37°C; pH 6) and Pb (c(Pb) 50 mg/L; C_{bakt} 1 g/L; +37°C; pH 5) removal by lyophilized *B. longum* 46 and As(V) (c(As(V)) 1 mg/L; C_{bakt} 1 g/L; +22°C; pH 7) removal by lyophilized aminated *L. casei* DSM20011. Average and standard deviation of three replicates are presented (II and III).

Increasing the pH caused an almost linear increase in removal, and the highest binding of cadmium (60–73%) was achieved at pH 6. The removal of lead with *L. rhamnosus* GG, *L. fermentum* ME3 and *B. lactis* Bb12 followed a pattern similar to that of cadmium, although the increase in binding with ME3 started at a lower pH than with the other strains (II). With *B. longum* 46 the removal of lead was high (55%) even at pH 2. The binding increased linearly as a function of pH, and the highest removal (95 %) was achieved at pH 6. To avoid precipitation of metals as metal hydroxides, pH values higher than 6 were not tested. The removal of arsenate by aminated *L. casei* DSM20011 increased from 8.9±4.4 to 28.7±5.2 % when the pH was raised from 3 to 7, respectively (Figure 14) (III). However, when longer contact times were used, the effect of pH decreased, and only small differences in arsenate removal were observed after an incubation of 24 hours at pH's 3, 5 and 7 (see Figure 2 in original article III).

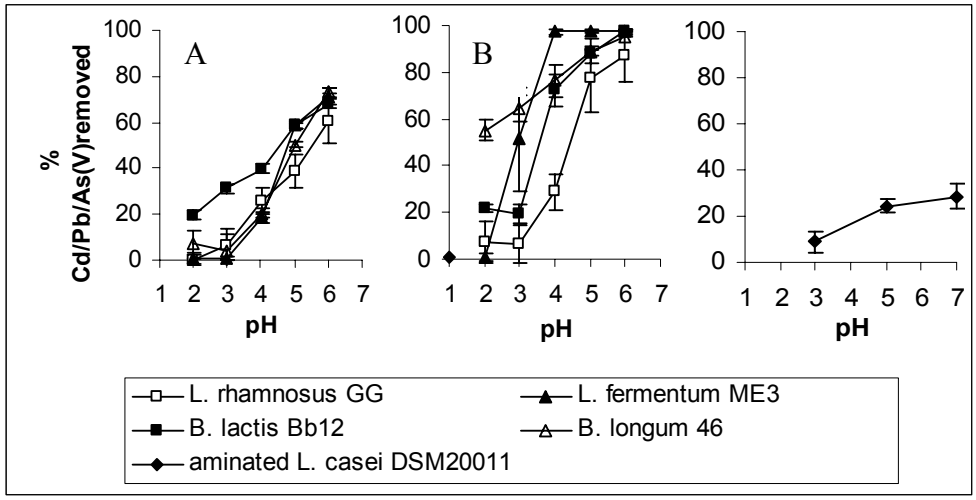

Figure 14. Effect of pH on cadmium (A) and lead (B) removal by lyophilized *L. rhamnosus* GG, *L. fermentum* ME3, *B. lactis* Bb12 and *B. longum* 46 (t = 60 minutes; C_{metal} 10 mg/l (Cd), 50 mg/L (Pb); C_{bakt} 1 g/L; +37°C) and As(V) removal by lyophilized aminated *L. casei* DSM20011 (t = 5 min; $C_{As(V)}$. 1 mg/L; C_{bakt} 1 g/L; +22°C). Average and standard deviation of three replicates are presented.

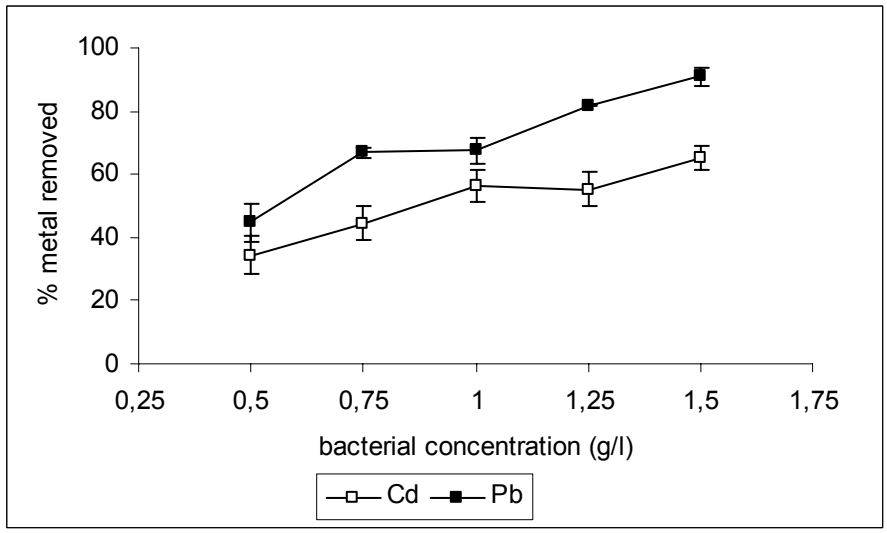

Figure 15. Effect of bacterial concentration on cadmium and lead removal by lyophilized *L. rhamnosus* GG (t = 60 minutes; pH 6 (Cd), 5 (Pb); C_{metal} 10 mg/L (Cd), 50 mg/L (Pb); +37°C). Average and standard deviation of three replicates are presented (II).

5.5 Effect of bacterial concentration and temperature (Studies I and II)

Increasing the bacterial concentration of *L. rhamnosus* GG enhanced the binding of both cadmium and lead (Figure 15) in both lyophilized (II) and freshly cultured forms (I). The effect of temperature on the removal of these two metals by *L. rhamnosus* GG was small (Figure 16) (II).

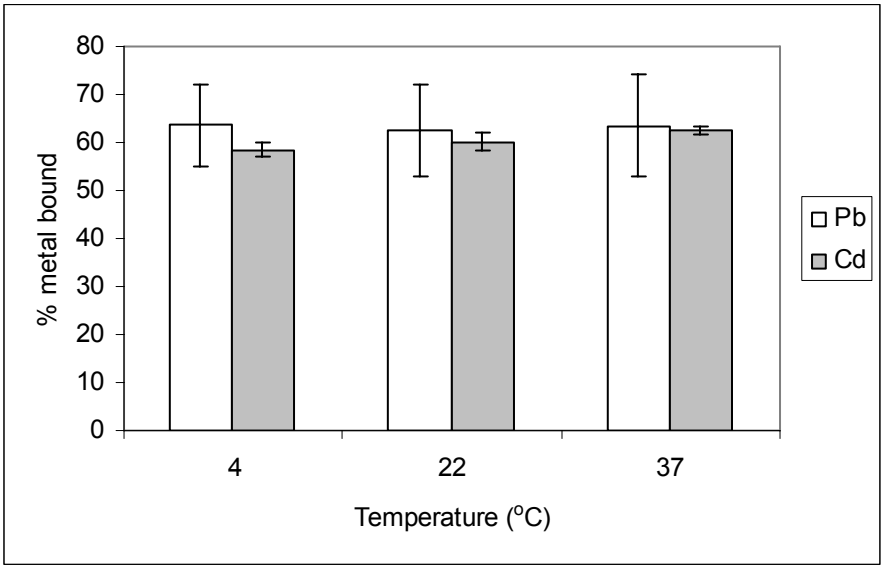

Figure 16. Effect of temperature on cadmium and lead removal by lyophilized *L. rhamnosus* GG (t = 60 minutes; pH 6 (Cd), 5 (Pb); C_{metal} 10 mg/L (Cd), 50 mg/L (Pb); C_{bakt} 1 g/L). Average and standard deviation of three replicates are presented (II).

5.6 Effect of other cations on the removal of cadmium and lead (Study V)

The presence of other cations significantly reduced cadmium binding by both strains studied, whereas the effect of other cations was much smaller in the case of lead (Figure 17). The greatest interference with cadmium binding by *B. longum* 46 and *L. fermentum* ME3 was observed with zinc and lead, respectively. Lead binding by *L. fermentum* ME3 remained unchanged in the presence of all the cations studied when compared to lead binding from a single metal solution. Lead binding by *B. longum* 46 followed a similar pattern but a significant reduction ($p<0.05$) in binding was observed in the presence of iron.

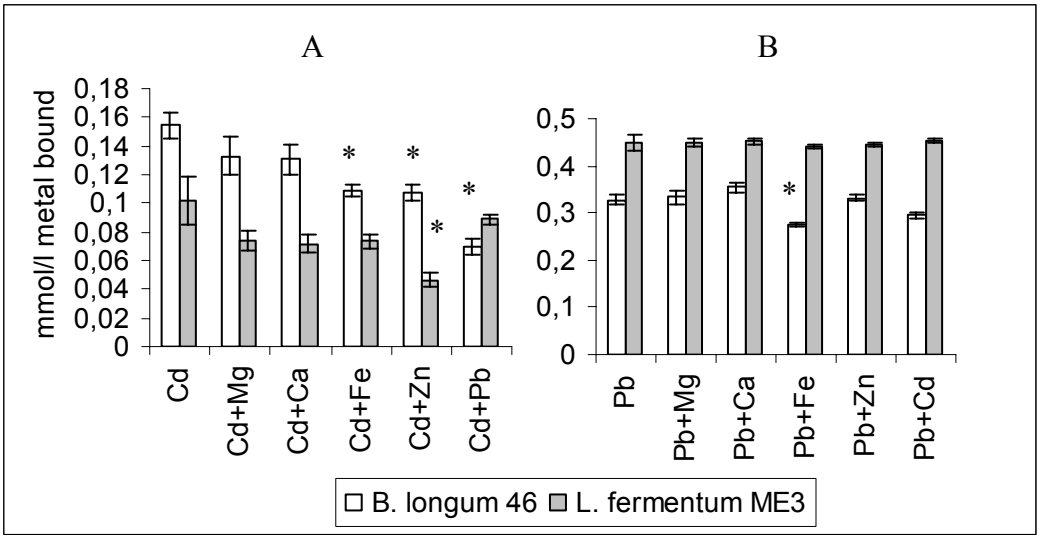

Figure 17. Effect of calcium, magnesium, iron, zinc, cadmium and lead on removal of cadmium and lead by *L. fermentum* ME3 and *B. longum* 46 (t = 30 minutes; pH 5; C_{metal} 0.5 mmol/L; C_{bakt} 1 g/L; +22°C). Significant difference (paired t-test, $p<0.05$) from the removal of cadmium or lead alone is indicated with an asterisk above bars. Average and standard deviation of three replicates are presented.

5.7 Desorption of bound metals (Studies III and V)

The desorption of cadmium and lead from the biomass of *B. longum* 46 and *L. fermentum* ME3 (Figure 18), and arsenate from the biomass of aminated *L. casei* DSM20011 (Table 7) was very low when MQ-water was used as a desorbent. However, effective desorption of cadmium and lead was achieved with 0.1/1.0mM EDTA and 1.5/15 mM HNO_3, and arsenate with 1.5 mM HNO_3 and 1.5 mM NaOH. Practically all bound arsenate was released in the first wash with 1.5 mM HNO_3 and 1.5 mM NaOH. Similar results were obtained for cadmium and lead with all biomasses tested when 1 mM EDTA was used. Desorption with 0.1 mM EDTA was not as effective, and from 33 to 45 % of the metal remained bound after the first wash. However, after the second wash, full desorption of cadmium and lead was achieved also with 0.1 mM EDTA. Since there were no great differences in the desorption capacity of EDTA and HNO_3 they were both used as a desorbent in cadmium and lead resorption experiments.

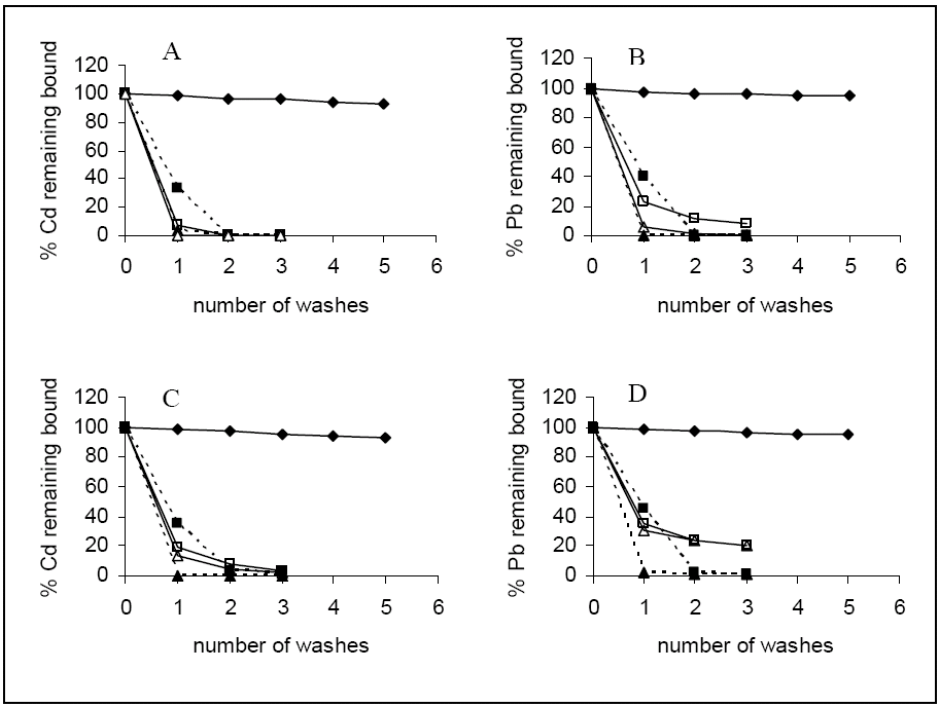

Figure 18. Desorption of cadmium and lead from *B. longum* 46 (A and B) and *L. fermentum* ME3 (C and D) by MQ-water (solid diamonds and solid line), 0.1 mM EDTA (solid square and dashed line), 1.0 mM EDTA (solid triangle and dashed line), 1.5 mM HNO_3 (open square and solid line) and 15 mM HNO_3 (open triangle and solid line). % metal remaining bound = (1 - amount of metal released after desorption/amount of metal bound initially)*100 (V).

Table 7. Percentage of bound As(V) released in three washes with ultra pure water, 1.5 mM NaOH and 1.5 mM HNO_3 (III).

	% released of the bound amount (112 µg As(V)/g)		
	MQ (pH 5.6)	1.5 mM HNO_3 (pH 2.8)	1.5 mM NaOH (pH 11.1)
Wash 1	9.1±5.2	102.3±8.9	103.5±7.2
Wash 2	5.4±2.7	3.4±1.7	0.7±0.9
Wash 3	7.1±3.5	0.2±0.4	0.3±0.5

The pH of the suspension during desorption is given in parentheses. Average and standard deviations of three experiments are presented.

5.8 Resorption of cadmium and lead (Study V)

The data from the resorption of Cd and Pb by *B. longum* 46 and *L. fermentum* ME3 is presented in Figure 19. Cadmium and lead binding by both strains was significantly reduced ($p<0.05$) when 10 mM EDTA was used as a desorbent. Cadmium binding by *B. longum* 46 and *L. fermentum* ME3 was only about 50% and 32% of the initial binding, respectively. Lead binding was reduced even more, being about 34% (*B. longum* 46) and 15% (*L. fermentum* ME3) of the initial binding. When desorption was performed with 15 mM HNO_3 cadmium binding by *B. longum* 46 remained at the initial level. Similar results were observed for *L. fermentum* ME3 for the first resoprtion. However, when *L. fermentum* ME3 was used for the third time, cadmium binding was reduced to 77% of the initial amount but the difference was not statistically significant. The use of 15 mM HNO_3 as a desorbent significantly reduced lead resorption capacity by both *B. longum* 46 and *L. fermentum* ME3 ($p<0.05$). But in the case of *B. longum* 46, the lead binding capacity was much better retained when 15 mM HNO_3 was used compared to 10 mM EDTA.

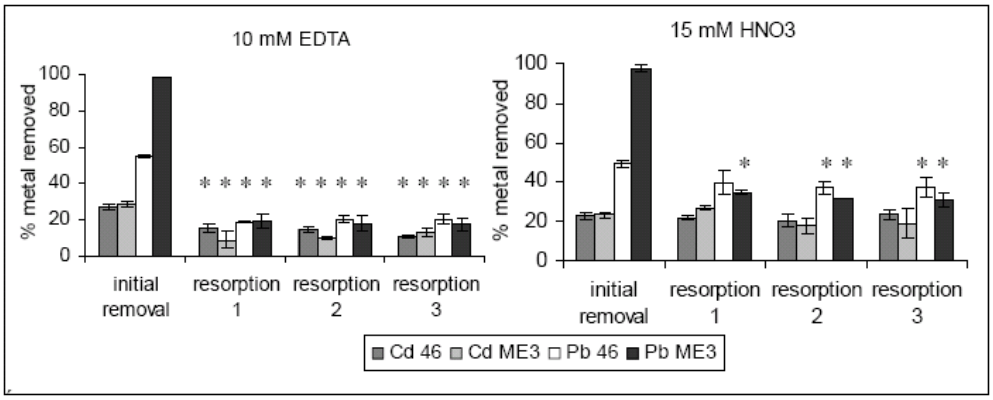

Figure 19. Cadmium and lead resorption capacity of *B. longum* 46 and *L. fermentum* ME3 after desorption with 10 mM EDTA and 15 mM HNO_3. The average resorptions and standard deviations of three replicates are presented. Significant differences (paired t-test, $p<0.05$) from the initial removal of cadmium or lead are indicated with an asterisk above bars (V).

5.9 Transmission electron microscopy (TEM) (Study V)

Transmission electron micrographs of lyophilized *B. longum* 46 and *L. fermentum* ME3 before, and after, lead binding are presented in Figure 20. Lead was clearly

visible on the surface of both strains after binding (B and D) whereas no lead was visible on control micrographs (A and C). Small deposits of lead were also visible inside the bacteria in Figure 21 D. It is believed that these deposits were transferred from the bacterial surface during the sample preparation for electron microscopy. Transmission electron micrographs clearly established that lead binding occurred at the surface of the bacteria. Cadmium was not visible in electron micrographs.

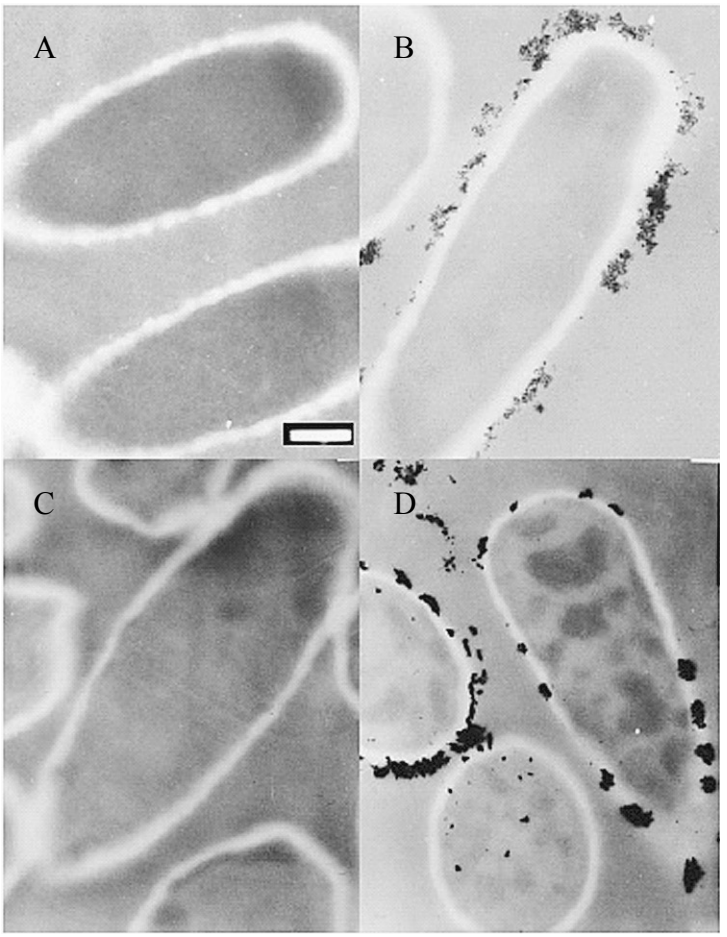

Figure 20. Transmission electron micrographs of *B. longum* 46 (A and B) and *L. fermentum* ME3 (C and D) before and after lead binding. Scale bar 200 nm.

6. DISCUSSION

6.1 Cadmium and lead removal

6.1.1 The capacity of lactic acid bacteria for cadmium and lead removal

Mathematical models are often applied to predict different parameters describing heavy metal binding by microbial biomasses. Metal binding by single strains and combinations of lactic acid bacteria used in this work fitted well to the Langmuir isotherm, except in the case of boiled *B. lactis* Bb12. The Langmuir isotherm was originally developed to describe gas adsorption to solid surfaces. Several assumptions were made when the model was developed: 1. the number of adsorbed molecules does not exceed the number of adsorbing sites, 2. all adsorption sites have the same energy for the adsorbed molecules, 3. one molecule interacts only with one adsorption site. In the case of metal adsorption by microbial biomass at least one of these assumptions is usually not met. However, this isotherm is useful and enables calculation of two important parameters: maximum binding capacity (q_{max}) and adsorption affinity (b) (sometimes also presented as a dissociation constant ($K_d = 1/b$)) (Davis et al., 2003).

In addition to the lactic acid bacteria used in this work, other workers have used the Langmuir model to study metal binding by microbial biomass for many other micro-organisms (reviewed by Volesky and Holan, 1995; Davis et al., 2003; Mehta and Gaur, 2005; Romera et al., 2006). Compared to other micro-organisms studied, the maximum binding capacities of cadmium (12.1 —54.7 mg/g) and lead (32.3—175.7 mg/g) observed in this study were moderate. For example, maximal removal capacities as high as 136 mg/g (Cd) and 347 mg/g (Pb) have been reported for Ca-loaded brown marine algae *Laminaria japonica* (Lee et al., 2004). The adsorption affinity (b) of cadmium binding by the lactic acid bacteria studied varied from 0.05 to 0.51 L/mg. For lead, the range was from 0.03 to 0.59 L/mg. Values ranging from 0.01 to 0.50 and from 0.003 to 13 L/mg have been reported for cadmium and lead binding by algal biomass, respectively (Romero et al., 2006)

When lower concentrations (100 and 1000 µg/L) of cadmium and lead were used, removal by lyophilized lactic acid bacteria was high (78—99%) and the observed differences between biomasses used were quite small. However, differences were

observed when maximum binding capacities were compared. Since the binding of metals has been established to mainly occur at anionic functional groups on the cell surface, differences in the quantity of these groups may be explained by inter-bacteria differences. Ngwenya et al. (2003) reported the characteristics of the surface chemistry of two gram-negative (unknown and *Schewanella putrefaciens*) and two gram-positive bacteria (*Bacillus subtilis* and *Bacillus licheniformis*) and found that surface densities of carboxyl, phosphoryl and hydroxyl/amine groups were all slightly 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). 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, Chang et al. (1997) reported a decrease in cadmium removal when older cells of *Pseudomonas aeruginosa* were used. However, the opposite was observed when lead removal was studied with the same biomass. It has also been reported that stability constants of a given metal vary with functional groups (Fein et al., 1997; Ngwenya et al., 2003). Therefore, the observed inter-biomass differences in maximum metal binding capacities in this work may be attributed to differences in the quantity of functional groups involved in metal binding.

6.1.2 Effect of physical treatments

In general, heat treatment of the lyophilized biomass only had a minor effect on the maximum cadmium and lead binding capacities (q_{max}), whereas heat treatment increased cadmium removal in most cases, when freshly cultured bacteria were used. Results from other studies reporting effects of heat treatment on cadmium and lead biosorption, have been contradictory. For example, heat treatment has been reported to enhance cadmium and lead removal by *Saccharomyces cerevisiae* (Göksungur et al., 2005), but reduce removal by the *Citrobacter* strain MCM B-181 (Puranik and Paknikar, 1999). The enhancement of removal by heat treatment probably results from the increased availability of metal binding sites on the bacterial surface (Göksungur et al., 2005) as a result of the partial breaking up of, for example, the peptidoglycan layer. Alternatively, heat or ethanol treatments are

known to fix soluble cell wall proteins to the cell surface that could otherwise be solubilized and compete with surface binding sites (Huang et al., 1990). The similar cadmium and lead removal observed for lyophilized and boiled lyophilized lactic acid bacteria in this work indicated that lyophilization may have caused partial deterioration of the cell wall, in a manner similar to heat treatment, leading to an increased number of available binding sites. Usually, the aim of lyophilization is to maintain the viability and cell wall integrity of, for example, bacteria, and protecting agents are used to reach this goal. However, cryoprotectants were not used in this work since they could have interfered with the metal removal. Lower cadmium removal by fresh lactic acid bacteria cultures compared to boiled cells may also result from other effects. Urrutia Mera et al. (1992) reported that scandium and uranium removal by metabolically active *B. subtilis* was lower than by bacteria treated with uncouplers of proton pumps or inactivated by radiation. They stated that protons excreted by active proton pumps competed for available binding sites with cationic scandium and uranium species. Another explanation may be heavy metal resistance of viable bacteria. At least *Streptococcus thermophilus* and *Lactococcus lactis* have been reported to express cadmium efflux ATPases (Schirawski et al., 2002).

6.1.3 Effect of contact time

Initial removal of cadmium by freshly cultured *L. rhamnosus* GG was rapid. After the first few minutes of contact, cadmium removal slowly continued throughout the time range studied (5 minutes—24 hours). Similar two phase kinetics were observed when lead sorption by a gram-negative marine bacterium, *Pseudomonas atlantica*, was studied (Harvey and Leckey, 1985): initial rapid binding was followed by slow, nearly constant, removal lasting for hours. Harvey and Leckey (1985) suggested the first phase was sorption of lead to the outermost structures of the lipopolysaccharide (LPS) layer, and the second slow phase to be slow diffusion of lead further into the LPS layer. On the other hand, accumulation of cadmium by an energy-dependent mechanism has been reported to take place in *Lactobacillus plantarum* (Hao et al., 1999). Therefore, the second slow phase of cadmium removal by fresh *L. rhamnosus* GG cells may have resulted from both active transport into cytoplasm and slow diffusion deeper into cell wall structures, e.g peptidoglycan. Cadmium and lead binding by all studied lyophilized lactic acid bacteria and bifidobacteria occurred rapidly and equilibrium was reached in a few

minutes. Similar results have been reported for other bacteria, such as *Bacillus subtilis* (Fein et al., 1997), *Bacillus cereus* (Pan et al., 2006), *Pseudomonas putida* (Pardo et al., 2003), *Pseudomonas aeruginosa* (Chang et al., 1997; Komy et al., 2006), *Citrobacter* (Puranik and Paknikar, 1999) and *Streptomyces rimosus* (Selatnia et al., 2004). In contrast to freshly cultured *L. rhamnosus* GG, a slow second phase was not observed. This indicates that cadmium and lead may have had easier access to binding sites buried deeper in the bacterial cell wall (probably as a result partial degradation of the peptidoglycan caused by lyophilization) leading to rapid, single phase removal.

6.1.4 Effect of pH

Cadmium and lead removal by the lactic acid bacteria studied was enhanced at a higher pH. Similar effects of pH on cadmium and lead binding have also been observed for bacteria such as *Lactobacillus rhamnosus* LC705 (Ibrahim et al., 2006), *Lactobacillus plantarum* (Seki et al., 2006), *Micrococcus luteus* (Seki et al., 2006), *Bacillus subtilis* (Fein et al., 1997), *Pseudomonas putida* (Pardo et al., 2003), *Pseudomonas aeruginosa* (Chang et al., 1997), *Sphaerotilus natans* (Esposito et al., 2002) and a *Citrobacter* strain (Puranik and Paknikar, 1999). Magnesium binding by isolated poly(glycerol phosphate) teichoic acids of *Lactobacillus buchneri* N.C.I.B. 8007 was similarly affected by pH (Lambert et al., 1975a). The effect of pH probably results from competition for negatively charged binding sites between heavy metal cations and protons (H^+) (Huang et al., 1991). In general, an increase in cadmium and lead removal was observed when the pH was raised over 3. This indicated that the increased binding may result from deprotonation of carboxyl groups, as the reported acid dissociation constants (pK_a) of bacterial carboxyl groups, 4.3 (Ngwenya et al., 2003), 4.8 (Fein et al., 1997) and 4.9 (Esposito et al., 2002) are quite close to the pH at which binding increased. The higher lead removal at a pH below 3, observed especially with *B. longum* 46, may result from binding to available phosphate groups of teichoic acids (TA) as these groups are reported to be mainly in their deprotonated form at low pH (Lambert et al., 1975a; Huang et al., 1991). Supporting this, phosphorylation of cyanobacterial biomass, *Lyngbya taylorii*, was reported to enhance its cadmium and lead binding at low pH (Klimmek et al., 2001). Boyanov et al. (2003) reported that the primary cadmium binding sites of *Bacillus subtilis* shift from predominantly phosphoryl to predominantly carboxyl and again to phosphoryl as the pH is raised from below 3

to over 7. Structural differences in TAs may explain the varying effects of pH. According to the studies of Lambert et al. (1975 a and b), the affinity of magnesium ions for TAs varied greatly between structures composed of either poly(glycerol-) or poly(ribitolphosphate). They also reported D-alanylation of teichoic acids to reduce magnesium binding by isolated poly(ribitol phosphate) teichoic acids of *Staphylococcus aureus* (Lambert et al., 1975b).

6.1.5 Effect of bacterial concentration and temperature

The increase in cadmium and lead removal with increasing biomass concentration of both lyophilized and freshly cultured *L. rhamnosus* GG may be explained by a higher biomass/metal ratio (i.e. higher number of binding sites available). A similar effect of biomass concentration has been observed in other studies (Esposito et al., 2002; Ngwenya et al., 2003). In some studies, reduced metal removal has been observed at high biomass concentrations. This may result from sorption of metals to dissolved organic acids that interfere with sorption to bacterial surface structures (Harvey and Leckey, 1985; Puranik and Paknikar, 1999) or formation of cell aggregates that reduce the surface area available for binding. Such a phenomenon was not observed in this work, probably because excessive bacterial concentrations were not used.

Incubation temperatures of +4 to +37°C had no effect on cadmium or lead removal by lyophilized bacteria, indicating that the binding process is energy-independent. Similar observations were made with a *Citrobacter* strain in lead, cadmium and zinc binding (Puranik and Paknikar, 1999) and with yeast biomass in lead binding (Cho and Kim, 2003). Cadmium removal by fresh biomass increased significantly when the incubation temperature was raised from +4 to +37^{o}C. This may result from active transport of cadmium into the bacterial cells. Accumulation of cadmium by an energy-dependent mechanism has been reported to take place in *Lactobacillus plantarum* (Hao et al., 1999).

6.1.6 Effect of other cations

More lead than cadmium was bound by all bacteria tested in this work. A similar preference for lead over cadmium has been observed for microbes such as *Saccharomyces cerevisiae* (Göksungur et al., 2005), *Pseudomonas aeruginosa* (Chang et al., 1997), *Pseudomonas putida* (Pardo et al., 2003), *Citrobacter* (Puranik

and Paknikar, 1999). In this work, the presence of other cations reduced cadmium binding by both *B. longum* 46 and *L. fermentum* ME3, whereas the effect of other cations was much smaller for lead. The greatest interference with cadmium binding by *B. longum* 46 and *L. fermentum* ME3 was observed with zinc and lead, respectively. Puranik and Paknikar (1999) observed a similar high interference by zinc on cadmium binding. Lead binding by *L. fermentum* ME3 remained unchanged in the presence all the cations studied when compared to lead binding from a single pure metal solution. Lead binding by *B. longum* 46 followed a similar pattern, but a slight reduction in binding was observed in the presence of iron and cadmium. Similarly, only a minor decrease in lead removal was observed by *Citrobacter* MCM B-181 (Puranik and Paknikar, 1999) and *Rhodotorula glutinis* (Cho and Kim, 2003) from binary metal solutions with zinc, nickel, cobalt and copper, and potassium, sodium, calcium and magnesium, respectively. Many explanations have been suggested for the selectivity of some metals over others. Doyle et al. (1980) studied the removal of cationic metals (Na, Ca, Mn, Ni, Sr, Zn and Mg) by both native and chemically modified extracted cell walls of *Bacillus subtilis,* and observed that both the affinity and number of binding sites for each metal were different. Fourest and Volesky (1997) found a correlation between metal uptake and the electronegativity of metals in four different brown seaweeds. Pardo et al. (2003) reported a positive linear correlation between metal adsorption constant and stability constant of first metal-hydroxy complex (e.g. $PbOH^+$). Nieboer and Richardson (1980) classed metal ions into three groups (a, b and borderline) based on their preference for N-, O- and S-containing ligands. This classification is also known as Pearson's Hard and Soft Acid Base (HSAB) theory (Atkins et al. 2006). Nieboer et al. (1980) have classed magnesium and calcium to group 'a', lead to group 'b' and zinc, iron and cadmium to the 'borderline' group. According to them, the metal ions belonging to the same group prefer the same types of ligand donor atoms, and therefore should exert the highest interference on each other. When the metal removal data for the lactic acid bacteria is compared to Nieboer and Richardson's classification, it explains the observations made in the present work quite well. Interference of all the cations tested on lead binding was neglible, whereas the interference of the borderline cations, zinc and iron, was the largest on cadmium, which belongs to the same group classification.

6.1.7 Desorption and resorption

Desorption of bound metals from the bacterial surface and its reuse (resorption) are important for the practical applicability of the biomass when water purification is considered. In an optimal situation, the desorbent used should be able to release all bound metal repeatedly without affecting resorption capacity of the biomass. Usually dilute acid solutions (HCl, HNO_3, H_2SO_4) or EDTA have been tested for this purpose. In this work, MQ-water, HNO_3 and EDTA at different concentrations were tested for desorption of bound cadmium and lead. The observed desorption by MQ-water was low (≤ 3 %). Efficient desorption of cadmium and lead was achieved with HNO_3 and EDTA, although often more than one wash was needed to reach full desorption. Efficient and fast desorption of cadmium and lead using dilute acid solutions has been reported in other studies (Chang et al., 1997; Puranik and Paknikar, 1997; Chojnacka et al., 2005). Resorption of cadmium by *B. longum* 46 and *L. fermentum* ME3 biomasses regenerated with 15 mM HNO_3 remained close to the original level but there was reduced lead removal, especially in the case of *L. fermentum* ME3. Reduction in the removal of cadmium and lead was observed after regeneration of *B. longum* 46 and *L. fermentum* ME3 with 10 mM EDTA. Reduced metal removal has also been reported in other studies after regeneration steps with dilute acid (Puranik et al., 1995; Chang et al., 1997; Puranik and Paknikar, 1997) and EDTA (Puranik et al., 1995) solutions. Ethylenediamine tetraacetic acid has been reported to extract lipopolysaccharide-protein complexes concomitantly with magnesium from *E. coli* (Beveridge and Koval, 1981). The observed reduction in metal removal in this work (Study V) may also be related to concomitant removal of some surface components by acids and EDTA.

6.1.8 Removal mechanism

Mechanisms such as complex formation, ion exchange, adsorption, chelation and microprecipitation, have all been proposed to be involved in metal biosorption. The dependence of pH in this work (Studies II and III) indicated that ion exchange is probably at least partly responsible for the observed cadmium and lead binding. The observed drop in pH during the incubation, which is probably a result of proton replacement by heavy metal ions, supports this conclusion. Involvement of anionic surface groups in metal binding has been reported for the gram-positive bacterium, *Bacillus subtilis*. Extraction of the teichoic acid moieties (phosphodiester groups), and reduction of the number of free carboxyl groups, reduced the cation uptake by

isolated *B. subtilis* cell walls (Beveridge and Murray, 1980; Doyle et al., 1980). Some cationic metals, e.g. copper, prefer binding to neutral amino groups, whereas some, such as lead, form negatively charged complexes in water such as $Pb(OH)_3^-$ and $Pb(OH)_4^{2-}$ that can interact electrostatically with positively charged amino groups (Beveridge and 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). In this work (Study V), cadmium and lead 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 of these groups have a significant role in binding of these metals, and they may be the sites where ion exchange occurs. Transmission electron micrographs of *L. fermentum* ME3 and *B. longum* 46 that had been used in lead binding, clearly established the presence of lead deposits on the bacterial surface, suggesting the involvement of another chemical mechanism, such as the reduction of cationic lead to metallic lead, in addition to ion exchange. A similar two step mechanism in lead removal has been reported for other biomasses tested. Lead binding by *B. subtilis* has been reported to start with a stoichiometric reaction between metallic cations and surface binding sites, followed by inorganic deposition of more metal, the first metal cation acting as a nucleation centre (Beveridge and Murray, 1980). Lead removal by biomass of *Sargassum vulgaris* has been reported to occur by a combination of ion-exchange, chelation and reduction reactions leading to formation of lead precipitates on the cell wall (Raize et al., 2004). Lead deposits observed on the surface of lactic acid bacteria may have resulted from redox-reaction between cationic lead and cell wall constituents of lactic acid bacteria (such as reducing sugars) leading to the formation of metallic lead.

6.2 Arsenic removal

Compared to the other biomasses tested in the literature, the maximum arsenic(V) removal capacity, calculated from Langmuir isotherm, of aminated *L. casei* DSM20011 (312±68 µg/L) was very low. Maximum binding capacities of As(V) as high as 24.5 and 45.2 mg/g of dry biomass have been reported for native biomasses of *Penicillium chrysogenum* (Loukidou et al., 2003) and *Lessonia nigrescens* (Hansen et al., 2006), respectively. Chemical modifications are reported to further increase As(V) removal. Modification of *Penicillium chrysogenum* with the

cationic surfactant hexadecyl-trimethylammonium bromide increased the q_{max} to 57.9 mg As(V) /g dry biomass (Loukidou et al., 2003), while methylation of a yeast increased the As(V) removal from close to zero with the unmodified yeast, to about 0.1 mmol/g = 7.5 mg/g (Seki et al. 2005).

The observation that only aminated *L. casei* DSM20011 was able to remove As(V) in water, indicated that amino groups were the most probable binding sites of As(V). There are only a small number of other reports available where As(V) removal from water by different microbes has been studied. The available data indicates the involvement of electrostatic interactions between anionic As(V) species and the cationic surface groups of the biomass. Native, non-viable *Penicillium chrysogenum* biomass was reported to remove As(V) under conditions similar to this work (Loukidou et al., 2003), and the positively charged amino groups were probably responsible for the binding of anionic As(V) species. Seki et al. (2005) observed low As(V) and Cr(VI) removal with a yeast biomass, amino and imidazol groups being the most probable binding sites. Although anionic carboxylic and phosphoryl groups are the most abundant ionic groups, and give lactic acid bacteria their net negative charge, the peptidoglycan layer and surface proteins, such as the S-layer proteins, are known to also contain positively charged groups. *L. acidophilus* strains and *L. crispatus* DSM20584 are known to produce S-layer proteins, and they are reported to be the main surface component of *L. crispatus* DSM20584 (Schär-Zammaretti and Ubbink, 2003). The positive zeta potential of native and methylated lactic acid bacteria at certain pH's also indicates the existence of positively charged surface groups. Therefore, it is expected that native lactic acid bacteria should also have cationic binding sites for the removal of anionic As(V), although no removal was observed in practice.

Seki et al. (2005) reported that methylation of carboxylic groups increased the removal of anionic As(V) and Cr(VI) by a yeast biomass. They speculated that carboxylic groups may have masked the cationic surface groups and inhibited the binding of metals when native biomass was used. However, in this work (Study III) no improvement in As(V) removal was observed when methylated lactic acid bacteria were used, although methylation clearly changed the surface charge of lactic acid bacteria to be more positive. Since methylation only neutralizes the negative charge of carboxylic groups, some other anionic groups like the

phosphoryl groups of (lipo)teichoic acids may still have caused interference. In addition, methylation does not increase the number of positively charged groups and so the ineffectiveness of methylated lactic acid bacteria may be a consequence of the low number of positively charged groups on the bacterial surface. Seki et al. (2005) reported that interactions between As(V) and amino groups are very weak as compared to the imidazol group. Since the amino and guanido groups of lysine and arginine residues, respectively, are the main positively charged groups of S-layer proteins (Åvall-Jääskeläinen and Palva, 2005) and the peptidoglycan layer (Delcour et al. 1999), weak interactions combined with the low number of positively charged functional groups may be the best explanation for the ineffectiveness of native and methylated lactic acid bacteria to remove As(V).

Arsenic(III) removal has been reported to occur by a different mechanism than As(V) removal. Immobilized biomass of a cyanobacterium, *Scytonema,* was observed to effectively remove As(III) from water (Prasad et al., 2006). The tentative removal mechanism was reported to be complex formation between arsenious acid and sulfhydryl groups of *Scytonema* surface proteins (Figure 21). In other studies, biomass from tea fermentation (Murugesan et al., 2006) and the fungus, *Aspergillus niger* (Pokhrel and Viraraghavan, 2006), treated with iron compounds, $FeCl_3$ and iron oxide, respectively, have been observed to effectively remove both As(V) and As(III) probably by complexation with iron (Figure 21) (Thirunavukkarasu et al., 2003). Arsenic(III) removal was also observed from water containing iron, probably by a similar mechanism, when native, inactivated

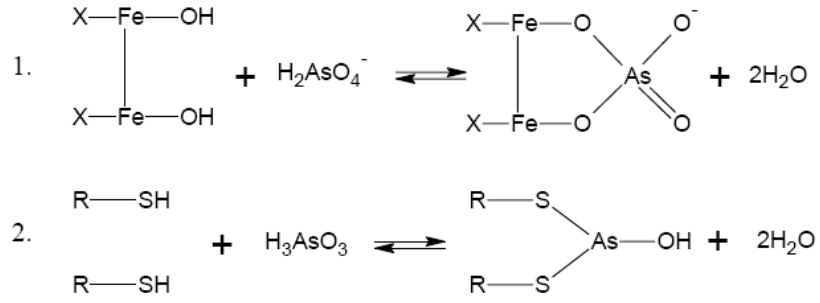

Figure 21. Examples of reactions for aqueous arsenic species with an iron oxide coated biomass (reaction 1) and sulfhydryl groups (reaction 2).

biomass from tea fermentation was used (Murugesan et al., 2006). The observations made in this work (Study V) indicate that the lactic acid bacteria do not have the ligands required for As(III) complexation. Chemical treatments used in this work only modified the surface charge of the biomass and therefore did not enhance the removal of electrically neutral As(III).

Arsenic(V) removal by aminated *L. casei* DSM20011 occurred rapidly. Contrary to the observations in this work with cadmium and lead, arsenic(V) removal decreased when the contact time was prolonged. In other studies, arsenic removal has been reported to be slower, and contrary to observations with lactic acid bacteria, it increases with time (Mcafee et al., 2001; Hansen et al., 2006; Murugesan et al., 2006). The rapid removal of arsenic(V) by aminated *L. casei* DSM20011 indicated that the binding occurred at the bacterial surface. The observed reduction in arsenic(V) removal during prolonged incubation may result from deterioration of the bacterial surface.

A low pH was observed to reduce arsenic(V) removal by aminated *L. casei* DSM20011 and the highest removal was achieved at pH 7. This probably results from the formation of anionic species such as, $H_2AsO_4^-$ and $HAsO_4^{2-}$, at higher pH which increases the probability of electrostatic interactions between arsenic oxyanions and surface quaternary-amino groups. Contrary to observations made in this work, the optimal pH for As(V) removal by *Lessonia nigrescens* (Hansen et al., 2006) and *Rhizopus oryzae* (Mcafee et al., 2001) was acidic.

6.3 Toxin and heavy metal removal by a combination of lactic acid bacteria

A positive linear correlation between hydrophobicity and aggregation index suggests that hydrophobic interactions were at least partly involved in the aggregation of the bacteria studied here. Hydrophobic interactions have also been reported to be involved in the autoaggregation of bifidobacteria (Canzi et al. 2005). A negative linear correlation was observed between AFB_1 removal and both the hydrophobicity and aggregation index. This indicated that aflatoxin B_1 removal probably occurred by hydrophobic interactions and aggregation, where similar interactions were involved between the bacteria, interfered with the removal. This finding is in accordance with earlier observations by Haskard et al. (2000) and

Lahtinen et al. (2004) who reported involvement of hydrophobic interactions in AFB1 removal.

The lack of correlation between removal of different toxins and heavy metals was probably a result of the different binding mechanisms of different toxins and strain specific properties in each case. In fact, it seems that AFB_1 is mainly bound to surface carbohydrates and proteins by hydrophobic interactions (Haskard et al. 2000; Lahtinen et al. 2004), whereas in heavy metal binding, ion exchange and metal complexation to surface constituents seems to be involved. Cultured lactic acid bacteria have been established to be more efficient in microcystin removal as compared to the lyophilized cells (Nybom et al., 2007) which indicates that in addition to physical adsorption, removal may occur through the metabolism.

Physical adsorption to the bacterial surface appears to be the main mechanism for heavy metal and AFB_1 removal (Haskard et al. 2001) but it is only partly responsible for MC-LR (Meriluoto et al., 2005; Nybom et al., 2007; Surono et al., 2007) removal. Therefore, a lower number of available binding sites or smaller surface area may explain the lower than predicted removal with COMB. Auto/coaggregation of bacteria would reduce the total surface area of the biomass. In support of this idea, a negative linear correlation was observed between AFB_1 removal and aggregation index. However, coaggregation by COMB was not higher than what could be predicted from the autoaggregation data of single strains. In fact, the observed coaggregation index was practically identical to the predicted value. Therefore, the coaggregation of bacteria cannot explain the lower than predicted removal of model toxins.

7. CONCLUSIONS

Specific lactic acid bacteria were observed to have a strain-specific capacity to bind the toxic cationic heavy metals, cadmium and lead, from water. Based on the maximum binding capacity, the most efficient strains for cadmium and lead removal were *B. longum* 46 and *L. fermentum* ME3. After chemical modification, *L. casei* DSM20011 also removed anionic arsenic(V). Removal of arsenic(V), cadmium and lead was a rapid, pH-dependent and reversible surface process. Arsenic(V), cadmium and lead were bound to charged functional groups on the bacterial cell wall possibly by ion exchange, complexation and precipitation mechanisms. Bound cadmium and lead were efficiently desorbed from the bacterial surface by dilute HNO_3 and EDTA solutions. Desorption reduced the cadmium and lead resorption capacity of the bacteria, especially when EDTA was used as a desorbent. The bacteria studied are also able to remove aflatoxin B_1 and microcystin-LR from aqueous solution.

In general, the results obtained in this work indicate that the heavy metal removal ability of lactic acid bacteria and the effect of different factors on removal resemble what has also been reported for other biomasses studied: rapid, reversible binding to charged functional groups on the biomass surface. Compared to the other biomasses studied, cadmium and lead removal capacity by lactic acid bacteria was moderate and arsenic(V) removal low. Therefore, the lactic acid bacteria studied are a potential option as biosorbents for cadmium and lead removal from water, but not for arsenic. However, based on the current data, the use of lactic acid bacteria only for heavy metal removal does not seem to offer any additional advantage when lactic acid bacteria are compared to some of the more effective biomasses studied.

In addition to heavy metal binding, the lactic acid bacteria used in this work were also able to remove other toxic compounds such as cyanobacterial hepatotoxins from aqueous solution. Removal of several toxic compounds simultaneously may prove to be a feature that gives lactic acid bacteria the additional advantage over other biomasses. Therefore, future research should be directed from studies with single compounds to the development of immobilized bacterial filters for the removal of several harmful targets simultaneously from water.

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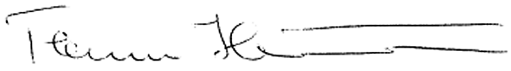

Teemu Halttunen
Turku, December 2007

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