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ORIGINAL PAPER

Role of S-layer proteins in the biosorption capacity of lead by *Lactobacillus kefir*

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Abstract The role of S-layer proteins (SLP) on the Pb^{2+} sequestrant capacity by *Lactobacillus kefir* CIDCA 8348 and JCM 5818 was investigated. Cultures in the stationary phase were treated with proteinase K. A dot blot assay was carried out to assess the removal of SLP. Strains with and without SLP were exposed to 0–0.5 mM $Pb(NO_3)_2$. The maximum binding capacity (q_{max}) and the affinity coefficient (b) were calculated using the Langmuir equation. The structural effect of Pb^{2+} on microorganisms with and without SLP was determined using Raman spectroscopy. The bacterial interaction with Pb^{2+} led to a broadening in the phosphate bands (1,300–1,200 cm^{-1} region) and strong alterations on amide and carboxylate-related bands (νCOO^- as and νCOO^- s). Microorganisms without SLP removed higher percentages of Pb^{2+} and had higher q_{max} than those bearing SLP. Isolated SLP had much lower q_{max} and also removed lower percentages of Pb^{2+} than the corresponding whole microorganisms. The hydrofobicity of both strains dramatically dropped when removing SLP. When bearing SLP, strains do not expose a large amount of charged groups on their surfaces, thus making less efficient the Pb^{2+} removal. On the contrary, the extremely low hydrofobicity of microorganisms without SLP (and consequently, their higher capacity to remove Pb^{2+}) can be explained on the basis of a greater exposure of charged chemical groups for the interaction with Pb^{2+}. The viability of bacteria without SLP was not significantly lower than that of bacteria bearing SLP. However, microorganisms without SLP were more prone to the detrimental effect of Pb^{2+}, thus suggesting that SLP acts as a protective rather than as a sequestrant layer.

Keywords S-layer proteins · *Lactobacillus kefir* · Biosorption · Lead

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Introduction

S-layer proteins (SLP) constitute the outermost bacterial structures. They are composed of protein or glycoprotein subunits that self-assemble to form a two-dimensional lattice that completely covers the organism during all stages of growth (Sleytr et al. 2001).

S-layer proteins (SLP) of the genus *Lactobacillus* are among the smallest so far described, with molecular mass ranging from 25 to 71 kDa, and isoelectric point within 9.35 and 10.40 (Garrote et al. 2004; Ventura et al. 2002). They have some similarities with the SLP of other microorganisms in terms of amino acid composition [i.e. high content of hydrofobic amino acids (31.9–38.7 %), hydroxylated amino acids (23–33 %) and lysine residues

(20 %), and very low sulfur amino acid content (<2 %)] (Åvall-Jääskeläinen and Palva 2005; Sara and Sleytr 2000).

The role of SLP has been usually associated with bacteria superficial properties, in particular the bacterial adherence to various substrates (Chen et al. 2007; Jakava-Viljanen and Palva 2007). Different authors reported that the chemical removal of SLP leads to a decrease of bacteria aggregation (Chen et al. 2007; Frece et al. 2005; Garrote et al. 2004; Jakava-Viljanen and Palva 2007), co-aggregation with yeasts (Golowczyc et al. 2009), adherence to eukaryotic cells (Jakava-Viljanen and Palva 2007) or gastric mucin and intestinal mucus (Carasi et al. 2014). In addition, co-aggregation of microorganisms bearing SLP with pathogens like *Salmonella* decreases the capacity of the pathogen to adhere and invade Caco-2/TC-7 cells (Golowczyc et al. 2007) probably because of the murein hydrolase activity of SLP (Prado-Acosta et al. 2008).

SLP have also been reported to be responsible for the protection of different strains of lactobacilli against detrimental environmental conditions. The presence of the SLP decreases the susceptibility of *L. helveticus* ATCC 12046 to mutanolysin (Lortal et al. 1992), the susceptibility of *L. acidophilus* M92 to gastric and pancreatic juice (Frece et al. 2005) and the susceptibility of *L. hilgardii* B706 to wine-related conditions (i.e. presence of copper sulphate or tannic acid) (Dohm et al. 2011).

The capacity of certain microorganisms to adsorb metals has been ascribed to their SLP (Velazquez and Dussan 2009; Dohm et al. 2011; Schut et al. 2011; Gerbino et al. 2012). In particular, species of lactobacilli used in wine-making demonstrated to adsorb higher concentrations of copper, zinc, iron and manganese when they bear SLP than when SLP are removed (Schut et al. 2011).

Isolated SLP showed a high metal-binding capacity (Raff et al. 2003, 2004; Fahmy et al. 2006; Pollmann et al. 2006). On this basis, it has been reported that these proteins may act as protective barriers, preventing the cellular uptake of environmental metals (Fahmy et al. 2006; Velazquez and Dussan 2009; Gerbino et al. 2011, 2012). From a molecular point of view, glutamic and aspartic carboxylate side chains and nitrogen groups were found to be the main molecular fragments involved in bacteria-metal interactions (Fahmy et al. 2006; Gerbino et al. 2011).

In spite of the relevance of these results, the development of novel applications of microorganisms bearing SLP requires a comprehensive study on the interaction SLP-metals and its consequences on the bacterial properties. Considering the role of SLP on the bacterial superficial properties (Garrote et al. 2004; Frece et al. 2005; Chen et al. 2007; Jakava-Viljanen and Palva 2007) and the capacity of *Lactobacillus kefir* strains to remove lead cations (Pb^{2+}) (Gerbino et al. 2012), the goal of this work was to elucidate the contribution of SLP on the removal of Pb^{2+} by *L. kefir*. To fulfill this aim, the capacity of two strains of *L. kefir* (CIDCA 8348 and JCM 5818) to remove Pb^{2+} was investigated before and after the extraction of SLP. The ability of the extracted SLP in removing Pb^{2+} was also assessed. The information obtained from the mentioned assays was then correlated with the bacterial viability and hydrofobicity of the studied strains. This approach allowed gaining an insight on the contribution of SLP to the removal of Pb^{2+} by *L. kefir* strains.

Materials and methods

Bacterial strains and growth conditions

Two strains of *L. kefir*, CIDCA 8348 isolated from kefir grains (Garrote et al. 2001), and JCM 5818, obtained from the Japanese Collection of Microorganisms (Reiken, Japan) were used in this work. The strains, stored frozen at −80 °C, were activated under aerobic conditions in de Man, Rogosa, Sharpe (MRS) broth (de Man et al. 1960; Difco, Detroit, MI, USA) at 30 °C for 48 h. The composition of the culture medium was: proteose peptone, 10.0 g/L; beef extract, 10.0 g/L; yeast extract, 5.0 g/L; dextrose, 20.0 g/L; polysorbate 80, 1.0 g/L; ammonium citrate, 2.0 g/L; sodium acetate, 5.0 g/L; magnesium sulfate, 0.1 g/L; manganese sulfate, 0.05 g/L; dipotassium phosphate, 2.0 g/L; pH = 6.5 ± 0.1.

Preparation of samples

Cultures in the stationary phase (grown 48 h at 30 °C) were harvested by centrifugation (6,600*g* for 4 min), washed twice with phosphate buffered saline (PBS) (Composition: KH_2PO_4, 0.144 g/L; NaCl, 9 g/L; Na_2HPO_4, 0.795 g/L; pH 7.2) and resuspended in PBS to obtain an OD_{550nm} of 10. The dry weight of 1 mL of this suspension was determined by desiccation of the corresponding pellet at 70 °C until constant weight. One mL of bacterial suspensions (containing ca. 1 mg of dried cells) was treated with 1 g/L proteinase K (Sigma Chemical Co., St. Louis, MO., USA) for 30 min at 37 °C (Frece et al. 2005). The microorganisms treated with proteinase K (hereafter, enzymatically treated microorganisms) were then centrifuged and washed twice with PBS. Controls were performed by incubating 1 mL of microorganisms resuspended in PBS (OD_{550nm} = 10) for 30 min at 37 °C without proteinase K (hereafter, not enzymatically treated microorganisms).

A dot blot assay was carried out to check whether SLP were removed (Garrote et al. 2005). Briefly, 6 μL of enzymatically and not enzymatically treated microorganisms were placed onto nitrocellulose membranes (Micron

Separations Inc., USA). After drying for 30 min at room temperature, the membrane was saturated with 3 % w/v non-fat dry milk dissolved in T-TBS buffer [Tris 0.05 M (hydroxymethyl aminomethane, Mallinckrodt, Baker Inc.) NaCl 0.15 M plus Tween 20 0.05 % v/v (Sigma Aldrich, Inc., St. Louis, MO, USA)] at 37 °C for 1 h (pH 7.5). Then, nitrocellulose membranes were washed three times with T-TBS and incubated with polyclonal rabbit antibody antiSLP Ac21 diluted 1:100 (Garrote et al. 2005) for 1 h at 37 °C. After washing, peroxidase-labeled second antibody (horseradish peroxidase conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Inc., USA) diluted 1:2,000 was applied to the membranes and incubated at 37 °C for 1 h. After another cycle of washing, the reaction was visualized by adding a substrate solution consisting of 9 mg of 4-chloro-1-naphthol (SIGMA) and 18 mL of 30 % H_2O_2 (Merck) dissolved in 3 mL of methanol and 15 mL T-TBS.

Bacteria binding assays

After the enzymatic treatment described in "Preparation of samples" section, both enzymatically and not enzymatically treated bacteria were centrifuged and washed twice with decarbonated ultra-pure water (Milli-Q plus; Millipore Cop., USA). Bacterial pellets obtained from enzymatically and not enzymatically treated microorganisms were resuspended in 1 mL decarbonated ultra-pure water (Milli-Q plus; Millipore Cop., USA) containing 0–0.5 mM $Pb(NO_3)_2$. Decarbonation was carried out by boiling milli Q water for 15 min. The concentrations of $Pb(NO_3)_2$ were obtained by diluting a stock solution of 50 mM $Pb(NO_3)_2$ prepared as follows: 165 mg $Pb(NO_3)_2$ were dissolved into 50 mL decarbonated milli Q water and the resulting solution was acidified by adding 1 mL HNO_3 (Sigma Aldrich, Inc., St. Louis, MO, USA). The acidified solution was then diluted with ultrapure milli Q water up to 1,000 mL, to obtain a 50 mM $Pb(NO_3)_2$ solution.

The pH of the bacterial-metal suspensions was adjusted to 5.5 with dilute HNO_3 or NaOH and kept constant for the first 5 min, according to Halttunen et al. (2007, 2008). The suspensions were incubated for 1 h at 30 °C under shaking and were then centrifuged at 6,600*g* for 4 min. The concentration of Pb^{2+} was immediately determined in the supernatants using Spectroquant lead kit (Merck and Macherey–Nagel, Germany).

A calibration curve was performed by diluting the 50 mM $Pb(NO_3)_2$ stock solution in decarbonated milli Q water to attain concentrations within 0–0.025 mM. Appropriate dilutions of samples were carried out to obtain absorbance determinations within the lineal region of calibration curve.

Two kinds of controls were performed: (a) $Pb(NO_3)_2$ solutions whose concentrations were within 0–0.5 mM were incubated for 1 h at 30 °C in the absence of microorganisms. No changes in the concentration of Pb^{2+} were observed in the absence of microorganisms; (b) the bioassay was also carried out on both enzymatically and not enzymatically treated microorganisms resuspended in milli Q water without the addition of $Pb(NO_3)_2$. No Pb^{2+} was detected with the methods used in this work.

To ensure the quality of the analytical determinations, the Pb^{2+} concentrations of one replicate per sample (both in the calibration curve and in the bacterial and SLP samples) were also determined by atomic absorption in an atomic absorption spectrophotometer (SHIMADZU 6800, Shimadzu Corporation, Kyoto, Japan) (reference method).

Calculation of biosorption isotherm parameters

The concentration of Pb^{2+} adsorbed to whole microorganisms enzymatically treated or not, was adjusted using the Langmuir isotherm described in Eq. 1:

$$q = q_{max} \times \left[b\, C_f / \left(1 + b\, C_f\right)\right] \quad (1)$$

where C_f is the concentration of Pb^{2+} in equilibrium state, q the concentration of Pb^{2+} bound in equilibrium state, q_{max} the maximum binding capacity at given conditions, and b a coefficient related to the initial slope of the curve and to the affinity of binding. C_f versus q is plotted using program GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, 2007) and is shown in Fig. 1. These experimental data enable the calculation of q_{max} and b from Eq. 1.

Raman spectra

After the treatment (or not) with proteinase K according to "Preparation of samples" section, cell suspensions (OD_{550nm}: 10 containing 1 mg of dry weight) were washed twice with milli Q water. The pellets were then resuspended in 0.5 mM $Pb(NO_3)_2$ (prepared in decarbonated milli Q water) and pH adjusted to pH 5.5. Samples were then incubated for 1 h at 30 °C.

After the exposure to $Pb(NO_3)_2$, samples were washed twice with ultra-pure water (milli Q plus; Millipore Cop., USA) and the pellets were then dehydrated on desiccators containing silica gel until no changes in water desorption were detected. Microorganisms enzymatically treated or not, that did not interact with $Pb(NO_3)_2$ (washed and resuspended in milli Q water) were used as controls.

Afterward, the samples were placed onto an aluminum substrate located under a Leica microscope (DMLM) integrated to the Raman system (Renishaw 1000B). The Raman spectra were collected in the 1,800–200 cm^{-1}

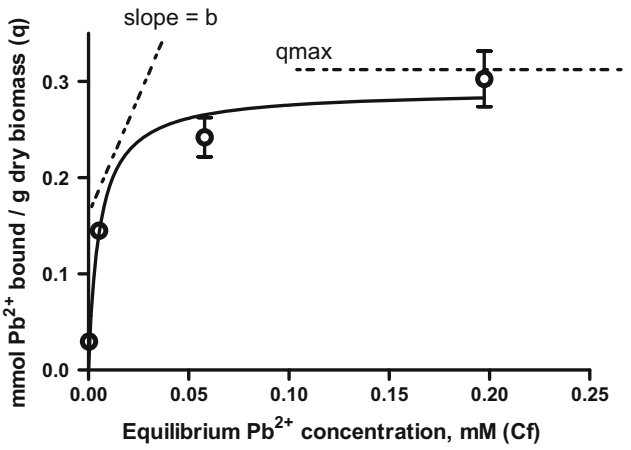

Fig. 1 Effect of metal concentration on Pb^{2+} binding to *L. kefir* JCM 5818 enzymatically treated. The theoretical parameters q_{max} (-maximum binding capacity of the biomass) and b (affinity of binding), calculated from the Langmuir isotherm, are indicated in this representative plot. This regression was carried out for all the samples (whole microorganisms with and without SLP and isolated SLP from both strains) and results are shown in Table 1

region with 2 cm^{-1} spectral resolution and an exposure of 30 s. The fluorescence contribution was removed by approximating a polynomial function to the spectra and then subtracting it from the spectra (Chan et al. 2008; Praveen et al. 2012).

The Raman system was calibrated with a silicon semiconductor using the Raman peak at 520 cm^{-1}, and further improved using samples of chloroform ($CHCl_3$) with bands at 261, 364 and 667 cm^{-1} and cyclohexane (C_6H_{12}) with bands at 383, 426, 801, 1,028, 1,157, 1,265, 1,347 and 1,443 cm^{-1}. The wavelength of excitation was 830 nm and the laser beam was focused (spot size of approximately 2.0 μm) on the surface of the sample with a 50× objective. The laser power irradiation of the samples was 45 mW.

Plate counts

Enzymatically and not enzymatically treated microorganisms exposed to 0.5 mM $Pb(NO_3)_2$ for 1 h at 30 °C ("Bacterial metal ions binding assays" section) were plate counted. The pellets obtained after centrifugation ("Bacterial binding assays" section) were suspended in 1 mL of 0.85 % w/v sodium chloride and bacterial suspensions were serially diluted and plated on MRS agar plates. Bacterial counts were determined after 48 h of incubation at 30 °C.

Extraction of SLP and binding assays

For SLP extraction, cultures of *L. kefir* CIDCA 8348 and JCM 5818 in the stationary phase were collected by centrifugation, washed twice with PBS, and resuspended to an OD_{550nm} of 10 in the same buffer ("Preparation of samples" section). SLP were extracted from bacterial cells using LiCl 5 M (J. T. Baker, Mallinckrodt Baker S. A., Mexico) in a proportion of 4 mL of solution per mL of bacterial suspension. The mixture was incubated in a shaking incubator (Environ Shaker, Lab-line Instruments Inc., Melrose Park, IL, USA) at 200 rpm and 37 °C for 1 h to extract non-covalently bound proteins. Then, the mixture was centrifuged (12,000*g* at 10 °C for 15 min). The supernatant containing the SLP protein was concentrated by ultrafiltration in an Amicon stirred cell 8050 (Millipore Corporation, US) equipped with a regenerated cellulose membrane (Ultracell PLGC04310, MWCO 10000, Millipore Corporation, US), and dialyzed against bidistilled water at 4 °C for 24 h using a cellulose membrane (SpectraPor membrane tube, MWCO 6000–8000, Spectrum Medical Industries, California, US). Protein concentration was determined using the Bradford method (Bradford 1976).

Sorption experiments were carried out onto the dialysis cellulose membranes, containing 1 mg SLP according to Pollmann and Matys (2007). The dialysis cellulose membranes (containing SLP) were shaken at 30 °C for 1 h in 5 mL of $Pb(NO_3)_2$ solutions whose concentrations were within 0–0.15 mM. The pH was adjusted to 5.5 with HNO_3. The concentration of Pb^{2+} bound to the SLP was calculated by determining the residual concentration of Pb^{2+} in the metal solutions with a Spectroquant lead kit (Merck and Macherey–Nagel, Germany). $Pb(NO_3)_2$ solutions within 0–0.15 mM incubated for 1 h at 30 °C in the absence of SLP were used as controls. In addition, the bioassay was also carried out on SLP to whom no $Pb(NO_3)_2$ was added. In this case, no Pb^{2+} was detected with the methods used in this work.

The experimental values obtained were adjusted using the Langmuir isotherm described above in Eq. 1 ("Calculation of biosorption isotherm parameters" section).

Hidrofobicity assays

One mL of enzymatically and not enzymatically bacterial suspensions were put in contact with 0.2 mL of xylene by vortexing for 120 s. Phases were allowed to separate by decantation. The aqueous phase was carefully removed and the optical density was recorded at 600 nm. The decrease of optical density in the aqueous phase was taken as a measure of the bacterial surface hydrofobicity and was calculated as:

$$H\% = [(ODo - OD)/ODo] \times 100 \quad (2)$$

where ODo and OD are the optical densities before and after extraction with xylene, respectively (Gomez-Zavaglia et al. 2002; Pérez et al. 1998).

Statistical analysis

All assays were conducted in triplicate and two independent experiments were carried out. In the case of isolated SLP, the assays were performed in triplicate and repeated four times. Average values were used for data analysis. The statistical data analysis was carried out using program GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, 2007). Means were compared by using one-way ANOVA followed by Tukey's HDS (honesty difference significance) test, and if $P < 0.05$ the difference was considered statistically significant.

To support quality assurance and control (QA/QC) other analytical methods were used. In particular, infrared spectroscopy was used to support the quality of results obtained by Raman spectroscopy, and atomic absorption, to support the quality of the colorimetric determinations of lead [Spectroquant lead kit (Merck and Macherey–Nagel, Germany)].

Results

The efficiency of the proteinase K treatment on SLP removal was evaluated on both strains by performing a dot blot assay using antibodies anti–SLP. Figure 2 shows that microorganisms not enzymatically treated as well as isolated SLP gave a positive reaction. On the contrary, bacteria treated with proteinase K gave a negative reaction. This assay evidenced the removal of SLP up to undetectable values.

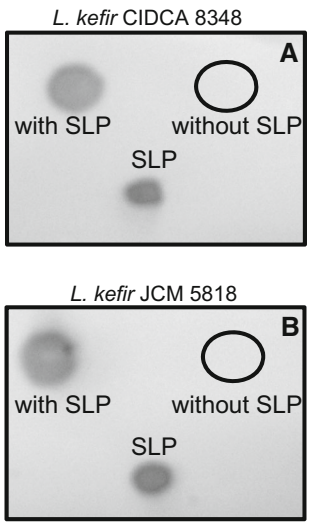

Fig. 2 Dot-blot assay with specific polyclonal rabbit antibody anti–SLP Ac21 diluted 1:100 against isolated SLP and microorganisms with and without SLP. **a** *L. kefir* CIDCA 8348; **b** *L. kefir* JCM 5818

Figure 3 shows the structural differences of microorganisms with and without SLP exposed or not to Pb^{2+}. The interaction of intact microorganisms (bearing SLP) with Pb^{2+} led to the major spectral differences (compare full line spectra with dashed double dot spectra in Fig. 3). The Raman spectra clearly denote that bacteria- Pb^{2+} interactions occur through carboxylate and phosphate groups. Indeed, after the treatment with Pb^{2+}, the band at 1,655 cm^{-1} (asymmetric stretching of carboxylate groups, νCOO^- as) weakened, the band at 1,445 cm^{-1} was transformed in a shoulder (symmetric stretching of carboxylate groups, νCOO^- s) and the band at 1,260 cm^{-1}, related with phosphate groups, broadened. Similar alterations were observed when microorganisms without SLP (dash spectra in Fig. 3) were exposed to Pb^{2+} (dash dot spectra in Fig. 3), indicating that also in this group, bacteria–Pb^{2+} interaction occurs through phosphate and carboxylate groups.

When comparing microorganisms with and without SLP (full line and dash spectra; and dash double dot and dash dot spectra in Fig. 3), the differences are essentially related with the intensity of the bands, especially those ascribed to proteins (i.e. amide I, II and III;[1] Mobili et al. 2009a). This is not surprising considering that SLP represent about 80 % of the total bacterial proteins (Sleytr et al. 2001). The same behavior was observed in both strains analyzed (Fig. 3).

The percentage of Pb^{2+} removed by *L. kefir* CIDCA 8348 and JCM 5818 with and without SLP is shown in Fig. 4a, b. Both strains bearing SLP removed significantly lower percentages of Pb^{2+} than the corresponding strains without SLP ($P < 0.05$) (see squares and diamond plots in Fig. 4a, b). The isolated SLP from both strains also removed Pb^{2+} but significantly lower ($P < 0.05$) than those of the whole microorganisms with and without SLP (Fig. 4c; Table 1). No Pb^{2+} was detected in the supernatants of both bacterial and SLP samples when no external Pb^{2+} was added.

The Langmuir isotherm (Eq. 1) allowed the determination of q_{max} and b from the experimental data. Table 1 shows that q_{max} for the interaction of bacteria with and without SLP as well as their corresponding SLP with Pb^{2+}. The first remarkable observation is that bacteria without SLP showed a significantly higher maximum binding capacity than the corresponding microorganisms bearing SLP

[1] Amide I band ($\sim$1,650 cm^{-1}) arises mainly from the $\nu C = O$ vibrational mode of the protein backbone. The amide II mode ($\sim$1,550 cm^{-1}) is the out-of-phase combination of the NH inplane bend and the CN stretching vibration with smaller contributions from the CO in-plane bend and the CC and NC stretching vibrations. The amide III mode ($\sim$1,400–1,200 cm^{-1}) arises from the in-phase combination of the NH bending and the CN stretching vibration with small contributions from the CO in-plane bending and the CC stretching vibration.

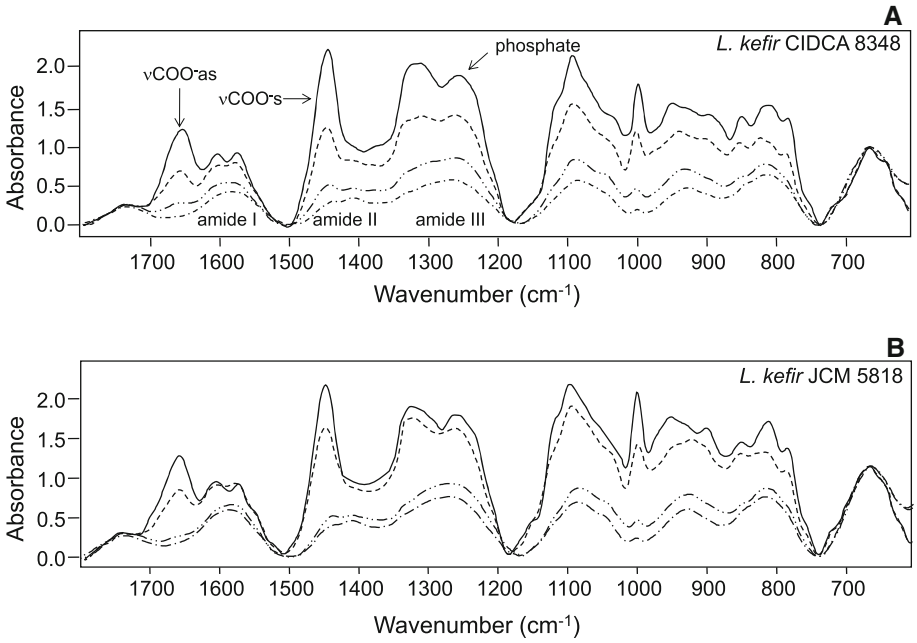

Fig. 3 Raman spectra corresponding to *L. kefir* CIDCA 8348 (**a**) and *L. kefir* JCM 5818 (**b**). *Full line* spectra correspond to microorganisms bearing SLP (not treated with proteinase K) and not exposed to Pb^{2+}. *Dash* spectra correspond to microorganisms without SLP but not exposed to Pb^{2+}. *Dash double dot* spectra correspond to microorganisms bearing SLP exposed to 0.5 mM $Pb(NO_3)_2$, and *dash dot* spectra, to microorganisms without SLP exposed to 0.5 mM $Pb(NO_3)_2$

Table 1 Maximum binding capacity (q_{max}), affinity coefficient (b), and hydrofobicity of isolated SLP and *L. kefir* strains with and without SLP

Conditions	q_{max} (mmol Pb^{2+}/g biomass)	b (L/mmol)	R^{2a}	Hydrofobicity (%)
L. kefir CIDCA 8348				
With SLP	0.205 (c*)[b]	53.83 (c)[b]	0.97	50 ± 5[c]
Without SLP	0.605 (a*)	4.66 (d)	0.92	4 ± 3
SLP	0.030 (f*)	65.61 (b)	0.88	nd
L. kefir JCM 5818				
With SLP	0.181 (d*)	52.47 (c)	0.93	62 ± 4
Without SLP	0.292 (b*)	180.24 (a)	0.81	5 ± 2
SLP	0.060 (e*)	61.65 (b)	0.92	nd

nd not determined

[a] R^2 values indicate adequacy of fitting of the experimental data to the Langmuir equation

[b] Different letters (a*,b*,c* for q_{max} and a,b,c for b) indicate significant differences ($P < 0.05$), according to multiple test comparison HSD Tukey's test

[c] ±, standard deviations

($P < 0.05$). Indeed, *L. kefir* CIDCA 8348 without SLP was able to bind 0.605 mM Pb^{2+}/g biomass, a concentration more than three times higher than that corresponding to the strain bearing SLP (Table 1). In turn, *L. kefir* JCM 5818 without SLP bound 60 % more Pb^{2+} than the corresponding strain without enzymatic treatment. It must also be pointed out that strain CIDCA 8348 with and without SLP was able to bind significantly higher concentrations of lead per gram of biomass (q_{max}) than strain JCM 5818 (Table 1).

In regard to the affinity (b) to lead, both strains showed similar values when bearing SLP ($P > 0.05$). The removal of SLP led to a dramatic drop of b in strain CIDCA 8348 (4.66 L/mmol) and a significant increase in strain JCM 5818 (180 L/mmol) ($P < 0.05$; Table 1).

The q_{max} for the isolated SLP was below 0.060 mM Pb^{2+}/g biomass in both strains (Table 1). Both isolated SLP showed higher affinities of binding than the corresponding whole microorganisms bearing SLP ($P < 0.05$; Table 1).

The hydrofobicity of both strains bearing SLP was above 50 %. After the treatment with proteinase K, it dramatically dropped to values close to 0 (Table 1).

The viability of both strains with and without SLP was not significantly different ($P > 0.05$; Table 2). The exposure to Pb^{2+} of both strains (with and without SLP) led to a significant decrease of viability with regard to microorganisms not exposed to Pb^{2+} (controls) ($P < 0.05$; Table 2). In the case of *L. kefir* JCM 5818, Pb^{2+} was even more detrimental for the strain without SLP ($P < 0.05$).

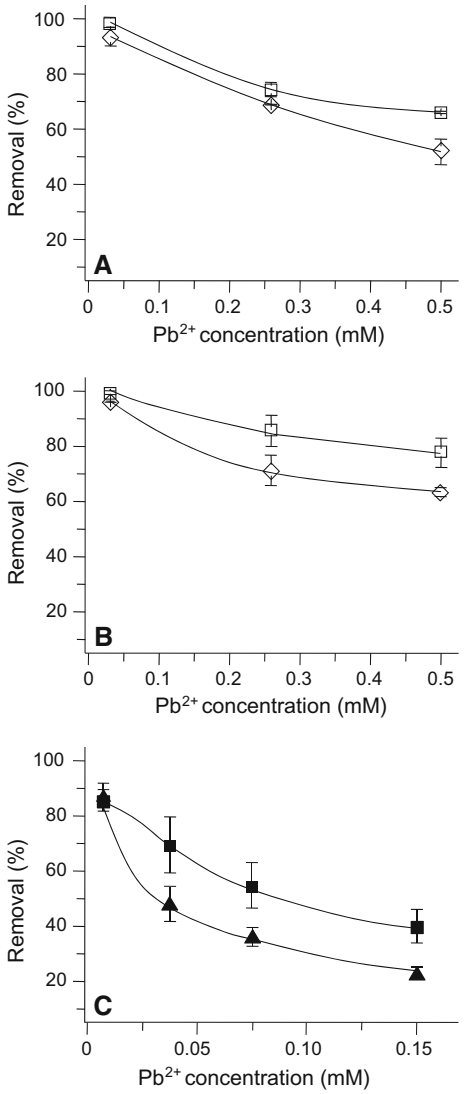

Fig. 4 Removal of Pb^{2+} by microorganisms bearing SLP (*opened diamonds*); microorganisms without SLP (*opened squares*). **a** *L. kefir* CIDCA 8348; **b** *L. kefir* JCM 5818. Results are the average of three independent observations and two independent bioassays. *Error bars* indicate one standard deviation from triplicate analyses (n = 6) of 2 independent bioassays. **c** Removal of Pb^{2+} by SLP extracted from *L. kefir* JCM 5818 (*full squares*) and *L. kefir* CIDCA 8348 (*full triangles*). Results are the average of three independent observations and four independent bioassays. *Error bars* indicate one standard deviation from triplicate analyses (n = 12) of 4 independent bioassays. Note the different x-axis scale in script C

Discussion

SLP are ubiquitous proteins covering the surface of different species of microorganisms from very different environments. Even when there are general structural patterns that are common for the SLP of different genera and species, their specific role is largely related to the activity of the microorganisms bearing them. For example, the role of SLP in pathogens is not the same as their role in probiotics

Table 2 Viability of *L. kefir* strains with and without SLP before and after interacting with 0.5 mM $Pb(NO_3)_2$

	Viability (Log CFU/mL)
L. kefir CIDCA 8348	
With SLP	8.80 ± 0.04 (a)
Without SLP	8.79 ± 0.02 (a)
With SLP + Pb^{2+}	8.3 ± 0.2 (b)
Without SLP + Pb^{2+}	8.4 ± 0.2 (b)
L. kefir JCM 5818	
With SLP	8.8 ± 0.1 (a)
Without SLP	8.8 ± 0.2 (a)
With SLP + Pb^{2+}	8.47 ± 0.03 (b)
Without SLP + Pb^{2+}	7.92 ± 0.03 (c)

Different letters (a,b,c) indicate significant differences ($P < 0.05$), according to multiple test comparison HSD Tukey's test

or in microorganisms isolated from effluents (Åvall-Jääskeläinen and Palva 2005; Chen et al. 2007; Fahmy et al. 2006; Pollmann et al. 2006). For this reason the role of SLP is somehow determined by the environment and applications of microorganisms that bear them.

A thorough analysis of SLP function requires experiments on both microorganisms with and without SLP. When physiological activities are to be determined after removing SLP, the methods selected to extract these superficial proteins must fulfill two requirements: from one side, be efficient in the removal of SLP, and from the other side, not be detrimental for the microorganisms. Chaotropic agents like lithium or guanidinium chloride are generally used for the extraction of SLP (Frece et al. 2005; Mobili et al. 2009a, b; Mulani and Majumder 2013). In spite of their doubtless efficiency, these agents are harmful for microorganisms. Therefore, they are useful only for studies requiring isolated SLP. When the physiological properties (viability, probiotic properties, enzymatic activity, etc.) of microorganisms without SLP are to be investigated, less drastic methods are needed. The treatment with proteinase K fulfills these two requirements and was already used to efficiently remove SLP from different species of lactobacilli (Frece et al. 2005; Schut et al. 2011). This treatment also demonstrated to be useful to successfully remove the SLP of strains *L. kefir* CIDCA 8348 and JCM 5818 (Fig. 2) without deleterious effect on microorganisms (Table 2).

The capacity of lactobacilli bearing SLP to remove metals has been reported some years back (Mrvčić et al. 2009, 2012; Dohm et al. 2011; Schut et al. 2011; Gerbino et al. 2012). In some of them, the role of SLP as responsible structures for metal removal was suggested but not directly proved (Mrvčić et al. 2009; Gerbino et al. 2012). In

this regard Raman spectroscopy is a useful tool for the analysis of bacteria-metal interaction at a molecular level, providing information about the functional groups involved. The interaction of both *L. kefir* CIDCA 8348 and JCM 5818 with Pb^{2+} led to a broadening in the phosphate bands (1,300–1,200 cm^{-1} region) and strong alterations on the carboxylate-related bands (νCOO^- as and νCOO^- s). Phosphate groups in the spectra of whole bacteria arise from teichoic acid (polymer of glycopyranosyl glycerol phosphate), important constituent of grampositive cell walls (Jiang et al. 2004). In turn, the interaction Pb^{2+}-carboxylates arises from superficial structures, including SLP and other cell wall constituents (Jiang et al. 2004).

The Langmuir model is the most widely used when assessing biosorption of heavy metals by lactic acid bacteria (Halttunen et al. 2007, 2008; Mrvčič et al. 2009, 2012). The results obtained in this work using this model showed that isolated SLP of *L. kefir* CIDCA 8348 and JCM 5818 had a very low q_{max} but their affinity to Pb^{2+} was significantly higher than that of the whole microorganisms bearing SLP (Table 1). This indicates that the interaction SLP–Pb^{2+} is more specific when SLP are out from the microorganisms, supporting the use of pure SLP for nano and biotechnological purposes (Pollmann et al. 2006). In spite of that, this behavior of pure SLP is not directly related with their role when bearing whole microorganisms, thus reinforcing the importance of carrying out experiments on microorganisms with and without SLP. Only after removing SLP, their role in the metal sequestrant capacity can be proved and in this regard, the reported information is rather scarce (Schut et al. 2011). The results obtained in this work demonstrated that SLP from both *L. kefir* strains play an important role in the protection of cells against Pb^{2+}. After removing SLP, *L. kefir* CIDCA 8348 increased more than three times the maximum capacity of binding and this capacity was highly unspecific (as b dropped to 4.66 L/mmol) (Table 1). The q_{max} of *L. kefir* JCM 5818 without SLP also increased but Pb^{2+} was removed more specifically ($b = 180.24$ L/mmol) (Table 1). The differential affinity of both strains without SLP may be originated in a differential exposure of negatively charged functional groups available for the interaction with Pb^{2+}. In turn, the increase of q_{max} after removing SLP can be explained considering that the enzymatically treated strains loss their capacity to autoaggregate (Garrote et al. 2004; Golowczyc et al. 2009), thus increasing the available contact surface to interact with Pb^{2+}.

The dramatic drop of hydrofobicity values explains the increased q_{max} of microorganisms without SLP upon exposure to Pb^{2+} (Table 1). Hydrofobicity is a strain dependent superficial property determined by the chemical groups exposed on the surface that may affect aggregation (i.e.: auto and coaggregation) and adhesion of microorganisms to different surfaces (Gomez-Zavaglia et al. 2002; Kos et al. 2003; Jakava-Viljanen and Palva 2007). Autoaggregation of probiotic strains appears to be necessary for adhesion to intestinal epithelial cells (Gomez-Zavaglia et al. 1998, 2002; Kos et al. 2003). The capacity of probiotic strains to coaggregate may contribute to form a barrier that prevents colonization by pathogenic microorganisms (Kos et al. 2003). In this context, SLP have been related with autoaggregation and adherence of *L. acidophilus* to porcine ileal epithelial cells (Frece et al. 2005; Kos et al. 2003) and coaggregation with *Salmonella* (Golowczyc et al. 2007). In addition, removing SLP from *L. kefir* strains leads to the loss of autoaggretation (Golowczyc et al. 2009).

From a chemical point of view, SLP of aggregative strains have similar MALDI-TOF peptide maps, a common pattern recognition by antibodies (Mobili et al. 2009b) and lower contents of β-sheet structures (Mobili et al. 2009a). The effect of removing SLP on superficial properties of *L. kefir* CIDCA 8348 and JCM 5818, together with the molecular structure of SLP supports the relation between the drop of hydrofobicity and the different capacity to bind Pb^{2+} upon removal of SLP (q_{max} and b) (Table 1). Hydrofobic strains, like *L. kefir* CIDCA 8348 and JCM 5818, do not expose a large amount of charged groups on their surfaces, thus making less efficient the Pb^{2+} removal (Table 1). On the contrary, the extremely low hydrofobicity of microorganisms without SLP (and consequently, their higher capacity to remove Pb^{2+}) can be explained on the basis of a greater exposure of charged chemical groups for the interaction with Pb^{2+}. In summary, disaggregation of the enzymatically treated strains increases the bacterial surface contact leading to a greater exposure of charged groups, both factors contributing to the increase of q_{max}.

Plate counts of microorganisms with and without SLP indicate that removal of SLP did not have a deleterious effect on microorganisms (Table 2). However, microorganisms without SLP showed a differential effect when exposed to Pb^{2+}. The viability of *L. kefir* CIDCA 8348 without SLP exposed to Pb^{2+} did not significantly decrease with regard to the same strain bearing SLP exposed to Pb^{2+} ($P > 0.05$). On the contrary, *L. kefir* JCM 5818 without SLP exposed to Pb^{2+} experimented a significant decrease in comparison with the viability of the same strain with SLP exposed to Pb^{2+} ($P < 0.05$; Table 2). This indicates that cells without SLP were more prone to the detrimental effect of Pb^{2+}, in particular the enzymatically treated *L. kefir* JCM 5818, which showed a higher affinity (b) to Pb^{2+} (Table 1). This suggests that SLP acts as a protective rather than as a sequestrant layer. The protective role of SLP against other detrimental factors like digestive juice and enzymes was already reported (Frece et al. 2005).

In this work we showed that these superficial proteins can also act as a barrier against Pb^{2+}. Even when food-grade microorganisms bearing SLP, like *L. kefir*, are able to sequestrate concentrations of Pb^{2+} largely exceeding the tolerated weekly intake (10 μg/L) (World Health Organization 2006), the role of SLP is not determinant in this removal. Other structures carrying carboxylate and phosphate groups (Fig. 3), like oligosaccharides of the cell wall may also be involved in the interaction (Gerbino et al. 2012), and play a role even more important than the SLP.

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Conflict of interest The authors declare no conflicts of interest.

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