Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Use of Raman spectroscopy and chemometrics for the quantification of metal ions attached to *Lactobacillus kefir*

E. Gerbino[1], P. Mobili[1], E. E. Tymczyszyn[1], C. Frausto-Reyes[2], C. Araujo-Andrade[3] and A. Gómez-Zavaglia[1]

1 Center for Research and Development in Food Cryotechnology, La Plata, Argentina
2 Centro de Investigaciones en Óptica, A.C. Unidad Aguascalientes, México
3 Unidad Académica de Física de la Universidad Autónoma de Zacatecas, Zacatecas, México

Keywords
biosorption, *Lactobacillus kefir*, metal ions, multivariate analysis, prediction models, quantification.

Correspondence
Andrea Gómez-Zavaglia, Center for Research and Development in Food Cryotechnology, La Plata, RA-1900, Argentina.
E-mail: angoza@qui.uc.pt

2011/1436: received 25 August 2011, revised 25 August 2011 and accepted 20 November 2011

doi:10.1111/j.1365-2672.2011.05210.x

Abstract

Aims: To set-up an experimental and analytical methodology to evaluate the feasibility of developing simple, accurate and quantitative models based on Raman spectroscopy and multivariate analysis for the quantification of metal ions adsorbed to the bacterial surface of *Lactobacillus kefir*.

Methods and Results: One millilitre cultures from two strains of *Lact. kefir* in the stationary phase were harvested and washed twice with ultra pure water. The bacterial pellets were resuspended into 1 ml solutions of Pb^{+2}, Cd^{+2} or Ni^{+2} ranging from 0 to 0·9 mmol l^{-1}. The suspensions were further incubated for 1 h at 30°C at pH 5·5. After centrifugation, the pellets were kept to register the Raman spectra and the supernatants were used for the analytical determination of Pb^{+2}, Cd^{+2} and Ni^{+2}. Micro-organisms nontreated with metal ions were used as controls. Principal component analysis (PCA) was performed over the preprocessed Raman spectra to evaluate whether the clusters obtained could be correlated with the concentration of metal ions attached to the bacterial biomass. After that, partial least squares (PLS) models were calibrated with the aim of quantifying the metal ions adsorbed to the bacterial surface. According to the analytical determinations, the maximum binding capacity of all the metals (q_{max}) attained values that are comparable with those observed for other lactic acid bacteria (ca. 0·200 mmol g^{-1}). The spectral analysis revealed that the main functional groups involved in the bacteria/metal interaction are carboxylates, phosphates and polysaccharides. In PCA, the first two principal components explain more than 72% variance of the spectral data set contained in the data structure, allowing a clear discrimination among samples of different concentrations. Based on this information and using as reference the results obtained by analytical methods, PLS prediction models were successfully defined for the quantification of Pb^{+2}, Cd^{+2} and Ni^{+2} attached to the bacterial surface.

Conclusions: The calibration and validation of methods based on multivariate analysis allowed the definition of models for the quantification of Pb^{+2}, Cd^{+2} and Ni^{+2} attached to bacterial surfaces. The high percentages of explained variances in PCA gave a strong support to calibrate the prediction models, depicting very good correlations with the reference method (correlations ~0·90 in all cases).

Significance and Impact of the study: *Lactobacillus kefir* CIDCA 8348 and JCM 5818 bind Pb^{+2}, Cd^{+2} and Ni^{+2} in an efficient way. This fact gives support for their potential use as sequestrants of traces of these metals in products addressed to human and animal consume. The prediction models developed

would be useful for the determination of the investigated metal ions in unknown samples giving at the same time, structural information about this interaction. This is certainly the most important contribution of this work.

Introduction

Toxic metals are not degradable and tend to accumulate in the exposed organisms causing serious health effects. Therefore, their removal represents an important task for the care of both human and animal health and the environment. The use of inactivated microbial biomass as adsorbents (biosorption) has become an efficient tool to remove such metals from different substrates (i.e. water, food, etc.) (Volesky and Holan 1995; Davis *et al.* 2003; Mehta and Gaur 2005). Several species of micro-organisms have been successfully employed for this purpose. The biosorbent capacity of lactic acid bacteria is particularly relevant taking into account their GRAS (generally recognized as safe) status (Halttunen *et al.* 2003, 2007, 2008; Ibrahim *et al.* 2006; Mrvčić *et al.* 2009; Schut *et al.* 2011). This makes them a potential tool to remove traces of toxic metals from food and water intended to human or animal consumption.

From a chemical point of view, it has been reported that removal of heavy metals occurs at the bacterial surface mainly through electrostatic interactions involving phosphate and carboxylate groups (Halttunen *et al.* 2008). Studies carried out on pure S-layers proteins have demonstrated that these superficial structures are able to interact with metal ions through the carboxylate groups of the side chains of aspartate and glutamate residues, and the coordination S-layer/metal is unidentate (Gerbino *et al.* 2011).

Quantification of metal removal is an important issue to evaluate the efficiency of a given micro-organism in bioremediation. The methods generally used to evaluate metal biosorption are based on the quantification of the nonbound metal ion remaining in the supernatant solution after the bacteria/metal interaction. Analytical reactions and atomic absorption spectrometry are the methods currently used to determine the concentrations of these nonbound metal ions (Halttunen *et al.* 2003, 2007, 2008; Velazquez and Dussan 2009).

The use of vibrational spectroscopy is widely extended among microbiologists because the vibrational spectra allow obtaining a global picture of the microbial molecular composition (i.e. nucleic acids, proteins, lipids and cell wall components) (Naumann *et al.* 1991; Mobili *et al.* 2011). For this reason, spectroscopic-based methods have been used for many different purposes including taxonomy, production of metabolites, diagnostics, etc. (Naumann *et al.* 1991; Mobili *et al.* 2011). In spite of that, up to our knowledge these methods have never been used to evaluate the bacterial efficiency in metal removal.

The implementation of spectroscopic methods for the quantification of metal ions requires a proper calibration. In this sense, the methods based on multivariate analysis, such as partial least squares (PLS), have been widely used for the quantification of different analytes, which represents a support to achieve the objectives pursued (Araujo-Andrade *et al.* 2004, 2005, 2009).

For this reason, the aim of this work was to set-up an experimental and analytical methodology to evaluate the feasibility of developing simple, accurate and quantitative models based on Raman spectroscopy and multivariate analysis [principal component analysis (PCA) and PLS] for the quantification of metal ions (Cd^{+2}, Pb^{+2} and Ni^{+2}) adsorbed to the bacterial surface of two strains of *Lact. kefir* (CIDCA 8348 and JCM5818). The Raman spectra also enabled to structurally characterize the bacteria/metal interaction. Analytical methods have been used to validate the multivariate models, thus giving support to the application of Raman spectroscopy for the quantification of metal ions.

Materials and methods

Bacterial strains and growth conditions

Two strains of *Lactobacillus 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. They were cultured in de Man, Rogosa and Sharpe broth (de Man *et al.* 1960) (Difco, Detroit, MI, USA) at 30°C for 48 h (stationary phase).

Metal ions binding assays

A general biosorption assay was designed for testing bacterial retention of Pb^{+2}, Cd^{+2} and Ni^{+2} in nongrowth conditions. One millilitre of micro-organisms in the stationary phase (containing 1 g dry weight per litre of culture) was harvested and washed twice with ultra pure water (Milli-Q plus; Millipore Corp., Billerica, MA, USA). The bacterial pellets were resuspended into 1 ml milli Q water containing concentrations of Pb^{+2} [from

$Pb(NO_3)_2$], Cd^{+2} [from $Cd(NO_3)_2$] or Ni^{+2} [from $Ni(NO_3)_2.6H_2O$] ranging from 0 to 0·9 mmol l^{-1}.

The suspensions were further incubated for 1 h at 30°C at pH 5·5 and after that centrifuged at 6600 ***g*** for 4 min. The pellets were kept to register the Raman spectra, and the supernatants were used for the analytical determinations of Pb^{+2}, Cd^{+2} and Ni^{+2}. The Spectroquant® lead and Nanocolor® cadmium and nickel test Kits (Merck and Macherey-Nagel, Germany) were used for the analytical determinations. Supernatants of micro-organisms nontreated with metal ions were used as controls.

Calculation of biosorption isotherm parameters

The amount of metal ions adsorbed to *Lact. kefir* strains was adjusted using the Langmuir isotherm described in Equation 1:

$$q = q_{\max}[bC_f/(1 + bC_f)] \quad (1)$$

where C_f is the concentration of metal in equilibrium state, q the bound concentration of metal 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. Plotting C_f *vs* q, both obtained from the experimental data, enables the calculation of $q_{\max}$ and b from Eqn (1).

Raman spectra

The Raman spectra of the bacterial samples (both bacteria containing the metal ions adsorbed and nontreated bacteria controls) were measured by placing them onto an aluminium substrate and then under a Leica microscope integrated to the Raman system (Renishaw 1000B). To retain the most important spectral information from each strain, multiple scans were conducted in different points of the sample by moving the substrate on an X-Y stage. 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, 1028, 1157, 1265, 1347 and 1443 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 over the samples was 45 mW. Principal component analysis (PCA) was conducted independently over the six sample sets corresponding to each bacteria/metal interaction. One hundred eighty-three Raman spectra were collected from the six samples containing different metal ions concentrations. On the other side, a total of 149 Raman spectra acquired from the six sample sets were used for the calibration of the model. Each spectrum was registered with an exposure of 30 s, two accumulations, and collected in the 1800–200 cm^{-1} region with 2 cm^{-1} spectral resolution.

The fluorescence contribution was removed by approximating a polynomial function to the spectra and then subtracting it from the spectra.

Data analysis

The recorded Raman spectra were collected using Grams software (ver. 3.04; Thermo Galactic, Salem, NH, USA). Multivariate analysis and data preprocessing as baseline correction, autoscaling (centring and standardization) and multiplicative scatter correction (MSC) were performed on the Raman spectra, using The Unscrambler® software (ver. 9.8; CAMO, Oslo, Norway).

Principal component analysis was performed over the preprocessed Raman spectra to evaluate the spectral differences among strains that have previously interacted with different concentrations of a single metal ion in the PC space. After that, PLS models were calibrated, allowing a quantification of the different metal ions adsorbed to the surface of *Lact. kefir* strains (Martens and Næs 1989; Esbensen 2005).

Statistics

All metal ions removal experiments were performed in triplicate and in three independent assays. Analysis of variance (anova) of the percentage of metal ions removed was carried out for both strains and for all the treatments, using the statistical program InfoStat 2008 (Infostat/Estudiantil, Grupo Infostat/FCA. Universidad Nacional de Córdoba. Ed. Brujas, Córdoba, Argentina). Comparison of means was tested using Tukey methods, and if $P < 0{\cdot}05$, the difference was considered statistically significant.

Results

Biosorption isotherms

Figure 1 depicts the removal of the three metal ions for each of the strains analysed. Removal ranged between 20 and 40% at the highest concentrations tested, the efficiency in the removal following the pattern: $Pb^{+2} > Cd^{+2} > Ni^{+2}$ in both strains (Fig. 1). Although metal removal is strain dependent, the differences between the strains were not statistically significant ($P > 0{\cdot}05$).

The Langmuir isotherm (Eqn 1) allowed the determination of $q_{\max}$ and b from the experimental data. Figure 2a shows that $q_{\max}$ for the interaction bacteria/metal is

approximately 0·2 mmol g^{-1} in all the conditions analysed. However, some differences in the affinity of binding (*b* values) for the three metals were observed. Figure 2b indicates that there exists a correlation between *b* values and the ionic radius of the metal ion assayed. It appears that the larger the ion (larger ionic radius), the greater the affinity (higher *b* values).

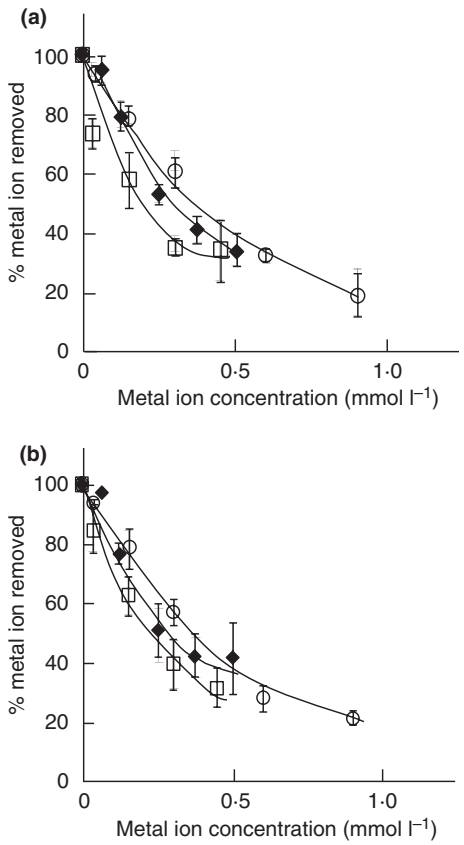

Figure 1 Removal of Pb^{+2}, Cd^{+2} and Ni^{+2} from aqueous solution by *Lactobacillus kefir* CIDCA 8348 (a) and JCM 5818 (b). *Lact. kefir* (a) and (b) were exposed to metal ions concentration in the following ranges: (○) Pb^{+2} 0·03–0·90 mmol l^{-1}, (♦) Cd^{+2} 0·06–0·50 mmol l^{-1}, (□) Ni^{+2} 0·03–0·45 mmol l^{-1}. Results are the average of three independent observations.

Structural analysis

As a first step in the spectral analysis, the Raman spectra of both nontreated *Lact. kefir* strains (controls) were compared with those obtained after the treatment with each of the three metals. Several qualitative differences were observed. Figure 3 depicts an example of the preprocessed average raw Raman spectra of both strains after the treatment with Cd^{+2} together with the corresponding controls. Similar qualitative differences were observed for the other bacteria/metal samples when compared with their respective controls (data not shown).

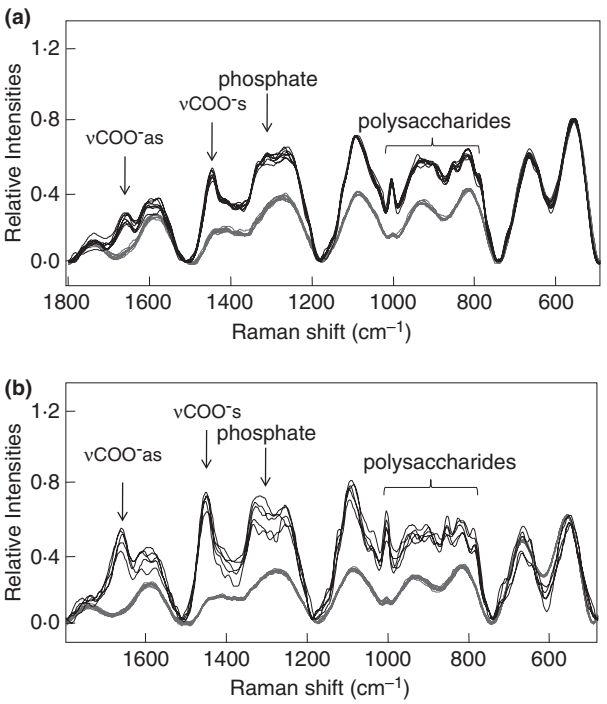

Figure 3 Raman spectra corresponding to (a) *Lactobacillus kefir* CIDCA 8348/Cd^{+2} and (b) *Lact. kefir* JCM 5818/Cd^{+2} (grey spectra). Spectra corresponding to the non-treated strains (controls) are also shown for comparison (black spectra).

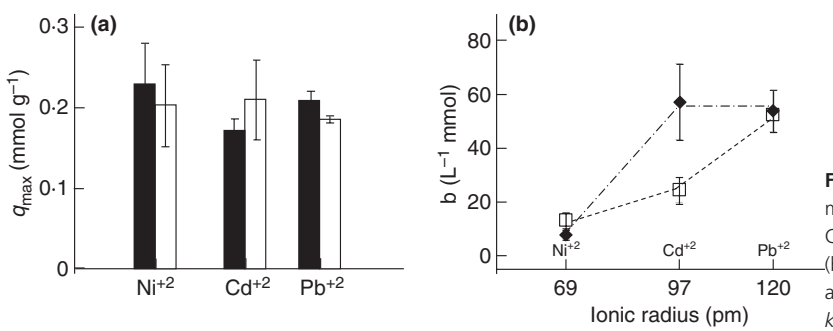

Figure 2 (a) Bar graph showing q_{max} for each metal in both strains (black bars, *Lact. kefir* CIDCA 8348; white bars, *Lact. kefir* JCM 5818). (b) Plot showing the coefficient related with metal affinity *b* for each metal in both strains. (♦, *Lact. kefir* CIDCA 8348; □, *Lact. kefir* JCM 5818).

The main differences observed between controls and bacteria/metal samples clearly denote the sites where metals are attached. The band at 1655 cm^{-1}, corresponding to the asymmetric stretching of carboxylate groups (νCOO^- as), vanishes after the treatment with metals. The band at 1445 cm^{-1} is clearly transformed in a shoulder after the interaction with metals. This band can be ascribed to the symmetric stretching of carboxylate groups (νCOO^- s). Modifications of both νCOO^- s and νCOO^- as indicate that the bacteria/metal interaction occurs through carboxylate groups. The band at 1260 cm^{-1} disappears after the treatment with metals and is related with phosphate groups. Finally, the differences observed in the range 1000–850 cm^{-1} are mainly related with polysaccharides.

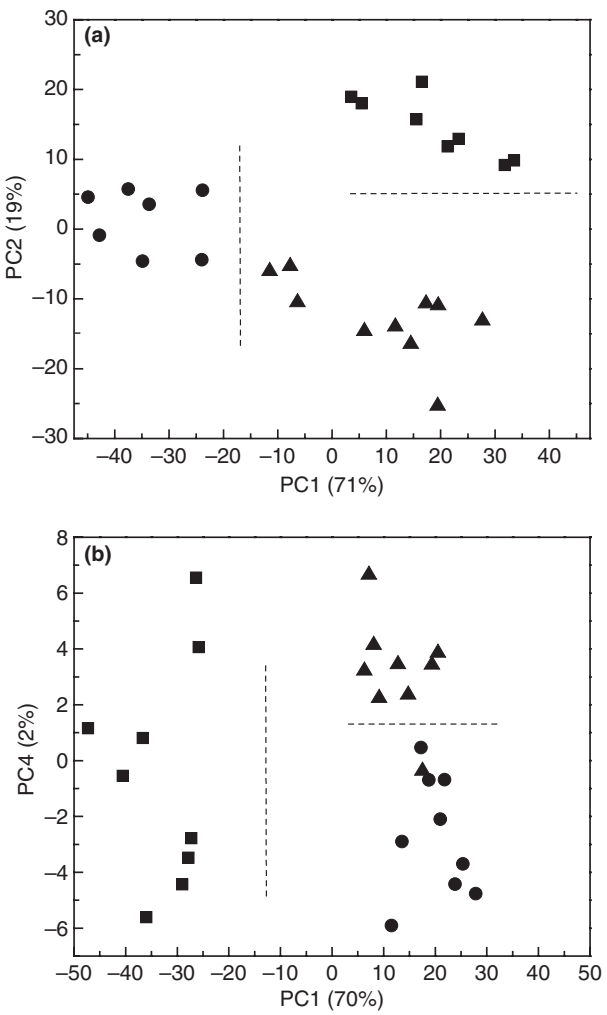

Figure 4 Score plots from the Principal component analysis performed on the raw Raman spectra (1800–200 cm^{-1}) for the interaction of *Lactobacillus kefir* with different concentrations of Pb^{+2}. (a) *Lact. kefir* CIDCA 8348: ■ corresponds to 0·028 mmol l^{-1}, ●, to 0·181 mmol l^{-1} and ▲, to 0·217 mmol l^{-1} Pb^{+2}. (b) *Lact. kefir* JCM 5818: ■ corresponds to 0·028 mmol l^{-1}, ●, to 0·165 mmol l^{-1} and ▲, to 0·184 mmol l^{-1} Pb^{+2}.

Multivariate analysis

The first step for data exploration aiming to calibrate prediction models is the use of unsupervised methods (i.e. PCA). These unsupervised methods do not require a prior knowledge about the spectral data set to be analysed (i.e. properties, classes, metal concentrations, etc.). In particular, PCA allows finding similarities, groupings and associations between objects and variables of the data set, obtained from the analysis of their variance. Furthermore, it provides useful information to evaluate the best parameters and conditions to calibrate prediction models allowing quantification and/or classification. For this reason, to assess the feasibility of calibrating quantification models, PCA was carried out on every data set (contained in the Raman spectra) corresponding to each bacteria/metal interaction as described in Materials and Methods. Afterward, with the information provided by PCA (spectral region/s of greatest influence, correlation between groupings and metal concentrations, outliers identification), the PLS prediction models were calibrated to quantify the metal ion concentration attached to the bacterial biomass.

Principal component analysis

Principal component analysis was carried out for each of the three metals under study and for both strains, to investigate in an unsupervised manner whether the clusters obtained can be correlated with the concentration of metal ions attached to the bacterial biomass. It must be mentioned that taking into account that the Raman spectra corresponding to the nontreated micro-organisms were so different from those corresponding to the bacteria/metal ion samples (for all the concentrations used), they were not considered for the PCA*. Figure 4 depicts the PCA graphs, carried out on the whole preprocessed Raman spectra (1800–200 cm^{-1}), for the interaction of both strains with Pb^{+2}. Figure 4a depicts the PC2 *vs* PC1 scores plot corresponding to *Lact. kefir* CIDCA 8348, and Fig. 4b, the PC4 *vs* PC1 scores plot for *Lact. kefir* JCM

*The inclusion of samples that are clearly different by just looking at the raw Raman spectra (Fig. 3), decreases the sensitivity of PCA since samples corresponding to the non-treated microorganisms cluster together and separated from those corresponding to all the metal ion concentrations used (data not shown). Therefore, the correlation between PCA clusters and metal ion concentrations becomes not possible if controls are included.

Table 1 Percentage of explained variance in each principal component after Principal component analysis

	PC1 (%)	PC2 (%)	PC4 (%)	Total explained variance (%)
Lactobacillus kefir CIDCA 8348				
Pb^{+2}	71	19	–	90
Cd^{+2}	49	47	–	96
Ni^{+2}	58	23	–	81
Lactobacillus kefir JCM 5818				
Pb^{+2}	70	–	2	72
Cd^{+2}	65	24	–	89
Ni^{+}	60	25	–	85

5818. In both cases, three separated groups corresponding to the three concentrations analysed were observed.

The percentages of explained variance in each principal component after PCA carried out on the whole preprocessed Raman spectra for all the bacteria/metal ion interactions are shown in Table 1. The total explained variance for the two components shown in the table is higher than 72% in all cases: 90, 96 and 81% for the interaction of *Lact. kefir* CIDCA 8348 with Pb^{+2}, Cd^{+2} and Ni^{+2}, respectively; and 72, 89 and 85% for the interaction of *Lact. kefir* JCM 5818 with Pb^{+2}, Cd^{+2} and Ni^{+2}, respectively.

These PCA results indicate the existence of a very good correlation between the information contained in the Raman spectra and the metal ion concentrations adsorbed to the bacterial biomass. The high percentages of explained variance represent a great support to calibrate the desired prediction models.

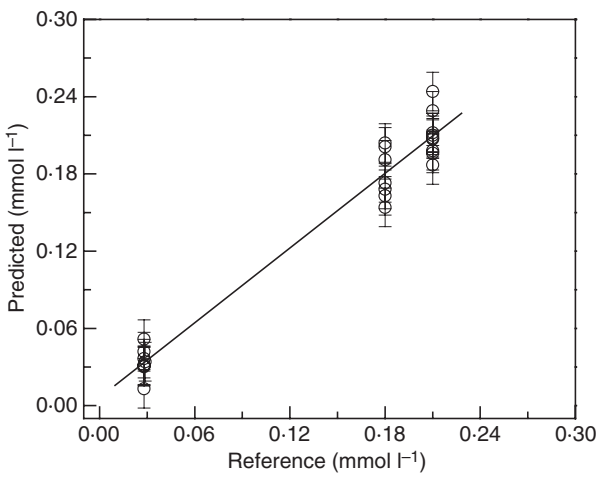

Figure 5 Cross-validation results for the concentration prediction model for the interaction *Lactobacillus kefir* CIDCA 8348/Pb^{+2}. The values corresponding to the cross-validation of the models developed for all the other conditions (strains and metals) are depicted in Table 3 and the statistical values, in Table 4.

Partial least squares

Having demonstrated that an unsupervised method such as PCA allows a clear discrimination between different concentrations of metals, an effort was made to calibrate prediction models allowing the quantification of metal concentrations in unknown samples. For this purpose, models based on PLS were developed. PLS operates in a supervised manner, meaning that a prior knowledge of the metal ion concentration is required (Martens and Næs 1989).

Figure 5 depicts the cross-validation results for the prediction model developed for the quantification of Pb^{+2} adsorbed to *Lact. kefir* CIDCA 8348. The model was calibrated using the preprocessed raw Raman spectra in the 1800–200 cm^{-1} range and validated using the leave-one-out-cross-validation (LOOCV) method, because of the limited number in samples (Martens and Næs 1989). The root mean square error of prediction (RMSEP) is depicted in the Y-error bars. The results obtained by analytical methods (Fig. 1) were used as reference concentrations.

The same process was carried out for all the metal ions and for both strains. The complete data set of the performance calibration/validation process is shown in Table 2. The cross-validation compares the concentrations obtained using analytical methods (Fig. 1), with those obtained using the built prediction model. The PLS plot depicted in Fig. 5 is a representative example of the results obtained from the cross-validation processes carried out. From these results, it can be concluded that the mean of predicted values fits nicely the results obtained from the analytical methods (Table 3).

Table 4 summarizes the statistical values obtained for each PLS prediction model calibrated and validated. These values (correlation, R-square, RMSEP and SEP) indicate the predictive capacity of each PLS prediction model.

Discussion

The efficiency of *Lactobacillus kefir* to remove heavy metals has never been addressed before. The results obtained in this work demonstrated that the sequestrant capacity of *Lact. kefir* CIDCA 8348 and JCM 5818 is comparable with that observed for other lactic acid bacteria. In fact, Halttunen *et al.* reported that q_{max} of Pb^{+2} and Cd^{+2} for different strains of lactobacilli and bifidobacteria range 45–175 mg metal/g dried biomass and 12–54 mg metal/g dried mass, respectively (Halttunen *et al.* 2007). Q_{max}

Table 2 Data set preparation and performance results of partial least squares (PLS)

Strain	*Lactobacillus kefir* CIDCA 8348			*Lactobacillus kefir* JCM 5818		
Sample	Pb^{+2}	Cd^{+2}	Ni^{+2}	Pb^{+2}	Cd^{+2}	Ni^{+2}
No. total of spectra collected	29	31	32	31	30	30
No. spectra used for calibration	25	22	27	27	25	23
No. spectra removed from the calibration (outliers)	4	9	5	4	5	7
Validation method	LOOCV*	LOOCV	LOOCV	LOOCV	LOOCV	LOOCV
No. spectra for validation	25	22	27	27	25	23
Mathematical treatment	raw	raw	raw	raw	raw	raw
Pre-processing	BLR†	BLR	BLR	BLR	BLR	BLR
	MSC‡	MSC	MSC	MSC	MSC	MSC
	1/Std§	1/Std	1/Std	1/Std	1/Std	1/Std
	MC¶	MC	MC	MC	MC	MC
PLS-factors for prediction	4	1	4	4	4	4

*LOOCV, Leave-one-out-cross validation.
†BLR, Base line remove.
‡MSC, Multiplicative scatter correction.
§MC, Mean centering.
¶Standardization.

Table 3 Metal concentrations obtained by partial least squares (PLS) for the set of samples compared with those obtained by analytical methods

	Value determined by analytical methods (mmol l^{-1})	Mean value obtained by PLS (mmol l^{-1})
Lactobacillus kefir CIDCA 8348		
Pb^{+2}	0·028	0·033
	0·181	0·179
	0·217	0·210
Cd^{+2}	0·060	0·065
	0·133	0·131
	0·172	0·164
Ni^{+2}	0·022	0·025
	0·110	0·121
	0·182	0·149
Lactobacillus kefir JCM 5818		
Pb^{+2}	0·028	0·032
	0·165	0·161
	0·184	0·174
Cd^{+2}	0·062	0·066
	0·124	0·122
	0·210	0·195
Ni^{+2}	0·026	0·033
	0·118	0·102
	0·179	0·163

obtained in this work for both metal ions were about 0·200 mmol g^{-1} in the samples assayed (Fig. 2a). These values correspond to *c.* 65 mg lead per gram dried mass and *ca.* 45 mg cadmium/g dried mass. The *b* values indicated that the metal ions of higher ionic radius (Cd^{+2} and Pb^{+2}) are those with the highest affinity (Fig. 2b). This observation suggests that the largest metal ions induce structural changes in the bacterial surface that allow the exposure of sites with higher affinity for metal ions. In this regard, the most outmost structures in the *Lact. kefir* strains under study are the S-layer proteins, and it has been reported that larger ions induce more pronounced alterations on their secondary structure (Gerbino *et al.* 2011). In fact, the treatment of pure S-layers with metal ions let them change their secondary structure from α-helix to β-sheets in a greater extent (Gerbino *et al.* 2011). The largest metals are those contributing the most for these structural changes. The higher *b* values obtained for the largest metal ions should be interpreted considering that in going to higher percentage of β-sheet structures, the metal binding sites of higher affinity become more exposed.

The great differences observed in the Raman spectra of the bacteria/metal samples and those of the controls also support the participation of S-layers in the bacteria/metal interaction (Fig. 3). In agreement with the information reported by other authors (Halttunen *et al.* 2007; Mrvčić *et al.* 2009; Gerbino *et al.* 2011), the Raman spectra of Fig. 3 indicate that the bacteria/metal interaction involves carboxylate and phosphate groups and also superficial polysaccharides. Considering that biosorption is a process occurring at the bacterial surface, the main structures involved in the bacteria/metal interaction are the polysaccharides associated with the cell wall and the S-layer proteins, because they are the most abundant surface proteins in the strains under study (Sara and Sleytr 2000; Mobili *et al.* 2010). Previous results carried out on pure

Table 4 Statistical values calculated in the validation step for the calibration models corresponding to each bacteria/metal interaction assayed in the 1800–200 cm^{-1} region

Strain	*Lactobacillus kefir* CIDCA 8348			*Lactobacillus kefir* JCM 5818		
Sample	Pb^{+2}	Cd^{+2}	Ni^{+2}	Pb^{+2}	Cd^{+2}	Ni^{+2}
Correlation	0·98	0·96	0·88	0·96	0·95	0·93
R-square*	0·96	0·93	0·80	0·93	0·92	0·88
RMSEP†	0·015	0·010	0·028	0·017	0·016	0·020
SEP‡	0·015	0·011	0·029	0·017	0·016	0·020

*R-square, Coefficient of determination.
†RMSEP, Root Mean Square Error of Prediction.
‡SEP, Standard Error of Performance.

S-layers have reported the participation of the side chain carboxylates of aspartate and glutamate residues in the S-layer/metal ion interaction.

On the other hand, the changes observed in the phosphates and polysaccharides bands support the participation of cell wall structures in the bacteria/metal interaction.

Calibration of the models

The results shown in Fig. 4 supported the construction of quantification models based on the Raman spectra (Table 3 and Fig. 4). The statistical values reported in Table 4 support the use of these models for the quantification of metal ions adsorbed to bacterial surfaces.

Up to our knowledge, Raman spectroscopy has never been used for the quantification of heavy metals attached to micro-organisms. Hence, the most important contribution of this work is the development of a useful prediction model for the determination of heavy metals in unknown samples. To obtain this information, the only experimental request is the *in situ* registration of the Raman spectra (meaning that no further treatments of the samples are required).

Moreover, the use of Raman spectroscopy in the study of the adsorption of metal ions to bacterial cells has an added value: besides its successful implementation as a quantification method, it simultaneously provides structural information about the sites involved in the bacteria/metal interaction.

Conclusions

The two main contributions of this work were: (a) the identification of the bacterial structures involved in the bacteria/metal interaction, (b) the development of a quantification model allowing a direct and quick evaluation of this interaction.

The main conclusions drawn from this work can be summarized as follows:

1. *Lact. kefir* CIDCA 8348 and JCM 5818 bind heavy metals in an efficient way. This fact gives support for their potential use as adsorbents of traces of heavy metals in products addressed to human and animal consume.
2. The affinity of both strains to the investigated heavy metals could be correlated with their ionic radius.
3. The bacteria/metal interaction occurs mainly through the carboxylate and polysaccharides exposed at the bacterial surface. This is in agreement with our previous results obtained for the spectral analysis of the S-layer/ion interaction (Gerbino *et al.* 2011).
4. Principal component analysis allowed a clear discrimination of the spectra corresponding to the bacteria/metal interaction at different concentrations.
5. The PLS model developed in this work is potentially useful for the quantification of Pb^{+2}, Cd^{+2} and Ni^{+2} in unknown samples.

Acknowledgments

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica, Argentina (Projects PICT/2008/145 and PICT/2010/2145), PROMEP, Mexico (Project UAZ-PTC-092) and CONACyT, Mexico [Project no. 119491 (2009) and no. 153066 (2010)], CYTED Program (Ciencia y Tecnología para el Desarrollo) Network P108RT0362 and CONACyT-CONICET (México, Argentina) (bilateral project res. no. 962/07-05-2009). A.G.Z., P.M. and E.E.T. are members of the research career CONICET (National Research Council, Argentina). E.G. is doctoral fellow from CONICET.

References

Araujo-Andrade, C., Ruiz, F., Martinez-Mendoza, J.R. and Terrones, H. (2004) Non-invasive *in-vivo* blood glucose levels prediction using near infrared spectroscopy. *AIP Conf Proc* **724**, 234–239.

Araujo-Andrade, C., Campos-Cantón, I., Martínez, J.R., Ortega, G. and Ruiz, F. (2005) Prediction model based on

multivariate analysis to determine concentration of sugar in solution. *Rev Mex Fis* **E 51**, 67–73.

Araujo-Andrade, C., Ruiz, F., Martínez-Mendoza, J.R., Padrón, F. and Hernández-Sierra, J. (2009) Feasibility for non invasive estimation of glucose concentration in newborns using NIR spectroscopy and PLS.*Trends Appl. Spectrosc* **7**, 27–37.

Davis, T.A., Volesky, B. and Mucci, A. (2003) A review of the biochemistry of heavy metal biosorption by brown algae. *Water Res* **37**, 4311–4330.

Esbensen, K.H. (2005) In *Multivariate Data Analysis – In Practice*, 5th edn, Esbjerg, Denmark: CAMO Process AS.

Garrote, G.L., Abraham, A.G. and De Antoni, G.L. (2001) Chemical and microbiological characterisation of kefir grains. *J Dairy Res* **68**, 639–652.

Gerbino, E., Mobili, P., Tymczyszyn, E.E., Fausto, R. and Gómez-Zavaglia, A. (2011) FTIR spectroscopy structural analysis of the interaction between *Lactobacillus kefir* S-layers and metal ions. *J Mol Struct* **987**, 186–192.

Halttunen, T., Kankaanpää, P., Tahvonen, R., Salminen, S. and Ouwehand, A.C. (2003) Cadmium removal by lactic acid bacteria. *Bioscience Microflora* **22**, 93–97.

Halttunen, T., Salminen, S. and Tahvonen, R. (2007) Rapid removal of lead and cadmium from water by specific lactic acid bacteria. *Int J Food Microbiol* **114**, 30–35.

Halttunen, T., Salminen, S., Meriluoto, J., Tahvonen, R. and Lertola, K. (2008) Reversible surface binding of cadmium and lead by lactic acid and bifidobacteria. *Int J Food Microbiol* **125**, 170–175.

Ibrahim, F., Halttunen, T., Tahvonen, R. and Salminen, S. (2006) Probiotic bacteria as potential detoxification tools: assessing their heavy metal binding isotherms. *Can J Microbiol* **52**, 877–885.

de Man, J.C., Rogosa, M. and Sharpe, M.E. (1960) A medium for cultivation of Lactobacilli. *J Appl Bacteriol* **23**, 130–135.

Martens, H. and Næs, T. (1989) Methods for calibration. In *Multivariate Calibration. Chichester, England*, Wiley and Sons Ltd: Chapter 3, pp. 97–112.

Mehta, S.K. and Gaur, J.P. (2005) Use of algae for removing heavy metal ions from wastewater: progress and prospects. *Crit Rev Biotechnol* **25**, 113–152.

Mobili, P., Gerbino, E., Tymczyszyn, E.E. and Gómez-Zavaglia, A. (2010) S-layers in lactobacilli: structural characteristics and putative role in surface and probiotic properties of whole bacteria. In: *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology* ed. Méndez-Vilas, A., Vol. 2, pp 1224–1234. Badajoz, Spain: Formatex Research Center.

Mobili, P., Araujo-Andrade, C., Londero, A., Frausto-Reyes, C., Ivanov-Tzonchev, R., De Antoni, G.L. and Gómez-Zavaglia, A. (2011) Development of a method based on chemometric analysis of Raman spectra for the discrimination of heterofermentative lactobacilli. *J Dairy Res* **78**, 233–241.

Mrvčić, J., Prebeg, T., Barišić, L., Stanzer, D., Bačun-Družina, V. and Stehlik-Tomas, V. (2009) Zinc binding by lactic acid bacteria. *Food Technol Biotechnol* **47**, 381–388.

Naumann, D., Helm, D. and Labischinski, H. (1991) Microbiological characterizations by FT-IR spectroscopy. *Nature* **351**, 81–82.

Sara, M. and Sleytr, U.B. (2000) S-layer proteins. *J Bacteriol* **182**, 859–868.

Schut, S., Zauner, S., Hampel, G., König, H. and Claus, H. (2011) Biosorption of copper by wine-relevant lactobacilli. *Int J Food Microbiol* **145**, 126–131.

Velazquez, L. and Dussan, J. (2009) Biosorption and bioaccumulation of heavy metals on dead and living biomass of *Bacillus sphaericus*. *J Hazard Mater* **167**, 713–716.

Volesky, B. and Holan, Z.R. (1995) Biosorption of heavy metals. *Biotechnol Prog* **11**, 235–250.