Rocz Panstw Zakl Hig 2016;67(3):253-262

http://wydawnictwa.pzh.gov.pl/roczniki_pzh/

ORIGINAL ARTICLE

BIO-TRANSFORMATION OF SELENIUM IN SE-ENRICHED BACTERIAL STRAINS OF *LACTOBACILLUS CASEI*

Eliza Kurek[1], Anna Ruszczyńska[1,2], Marcin Wojciechowski[1,2], Anna Łuciuk[1], Magdalena Michalska-Kacymirow[1], Ilona Motyl[3], Ewa Bulska[1,2]*

[1]University of Warsaw, Biological and Chemical Research Centre, Żwirki i Wigury 101 street, 02-089 Warsaw, Poland
[2]University of Warsaw, Faculty of Chemistry, Pasteura 1 street, 02-093 Warsaw, Poland
[3]Lodz University of Technology, Faculty of Biotechnology and Food Sciences, Wolczanska 171/173 street, 90-924 Łodz, Poland

ABSTRACT

Background. Selenium is an element of very great importance for the proper functioning of the human body, mainly due to its antioxidant properties. Selenium exhibits a preventive effect in the case of cardiovascular disease, the immune system, male infertility and inhibits the toxic action of other agents. Selenium is important for Hashimoto's disease. Intake of selenium in the diet slows the aging process. The biological and toxicological effects of selenium strongly depend on its chemical form. Some organisms for example: plant, yeast, are capable of metabolizing low bioavailable selenium compounds (inorganic selenium) into its high bioavailable forms (organic selenium).

Objective. The aim of this study was to investigate the bio-transformation of selenium by *Lactobacillus* bacteria towards the characterisation of selenium metabolites.

Material and Methods. The speciation of selenium was evaluated by high performance liquid chromatography with inductively coupled plasma mass spectrometry detector. The extraction of selenium species from lyophilized bacteria was executed with water, the mixture of lipase and protease, as well as lisozyme and sodium dodecyl sulphate.

Results. All investigated bacteria strains cultivated in the presence of Na_2SeO_3 effectively uptake selenium. Surprisingly, none of the applied extraction media exhibited a strong power to release the majority of the uptaken selenium compounds. Thus a maximum of 10% of the selenium was extracted from bacteria exposed to the enzymes. However, it was found that *Lactobacillus* bacteria are able to metabolize inorganic ions of selenium (IV) into Se-methionine, Se-methyloselenocysteine and other unidentified forms.

Conclusions. The study confirmed the ability of probiotic bacteria to biotransform inorganic selenium into its organic derivatives. Therefore, Se-enriched bacteria can be considered as an addition to the functional food.

Key words: *selenium speciation, extraction procedure, Lactobacillus casei bacteria, Lactic acid bacteria (LAB), HPLC ICP-MS, functional food*

STRESZCZENIE

Wprowadzenie. Selen jest pierwiastkiem o bardzo dużym znaczeniu dla prawidłowego funkcjonowania organizmu człowieka, głównie ze względu na swoje właściwości antyoksydacyjne. Selen odgrywa ważną rolę w profilaktyce chorób układu sercowo-naczyniowego, układu immunologicznego, niepłodności, a także chroni przed szkodliwym działaniem substancji toksycznych. Selen jest istotny dla przebiegu choroby Hashimoto. Jego obecność w diecie wpływa na spowalnianie procesów starzenia się. Biologiczne i toksykologiczne efekty działania selenu w znacznym stopniu zależą od jego postaci chemicznej. Niektóre organizmy, na przykład rośliny i drożdże, mają zdolność metabolizowania słabo przyswajalnych form selenu (nieorganiczny selen) do jego łatwo przyswajalnych związków (selen organiczny).

Cel. Celem niniejszych badań było poznanie procesów biotransformacji selenu przez bakterie *Lactobacillus* pod kątem identyfikacji form chemicznych selenu.

Materiały i metody. Badania specjacji selenu przeprowadzono z wykorzystaniem metody wysokosprawnej chromatografii cieczowej połączonej ze spektrometrią mas z plazmą indukcyjnie sprzężoną. Do ekstrakcji form chemicznych selenu z liofilizowanych bakterii użyto wody, mieszaniny lipazy i proteazy, a także lizozymu oraz SDS-u.

Wyniki. Wszystkie badane szczepy bakterii, hodowane w obecności Na_2SeO_3, efektywnie pobierały selen. Żaden z zastosowanych ekstrahentów nie uwolnił jednak całego związanego w bakteriach selenu. Maksymalnie udało się wyekstrahować 10% pobranego przez bakterie selenu, przy zastosowaniu enzymów.

[*]**Corresponding author:** Anna Ruszczyńska, University of Warsaw, Biological and Chemical Research Centre, Żwirki i Wigury 101, 02-089 Warsaw, Poland, phone: +48 22 82 20 211, fax: +48 22 822 59, 96, e-mail: aruszcz@chem.uw.edu.pl

Niemniej jednak, okazało się, że bakterie z rodzaju *Lactobacillus* są zdolne do metabolizowania nieorganicznych jonów selenu (IV) do Se-metioniny, Se-metyloselenocysteiny oraz niezidentyfikowanych form selenu.
Wnioski. Badania potwierdziły zdolność bakterii probiotycznych do biotransformacji nieorganicznych form selenu do jego organicznych metabolitów. W związku z tym, wzbogacone w selen bakterie mogą być używane, jako dodatek do żywności funkcjonalnej.

Słowa kluczowe: *specjacja selenu, procedura ekstrakcji, bakterie Lactobacillus casei, bakterie kwasu mlekowego, HPLC ICP-MS, żywność funkcjonalna*

INTRODUCTION

Selenium plays an important role as an essential micronutrient for animals and humans. Excess of selenium intake could lead to toxic reactions in living organisms, but its desired content plays an important role in prevention against heart diseases and cancer. Although a small difference between essential and toxic performance of selenium provoked public health awareness, selenium, especially its organic derivatives, have been recognized as an important dietary factor [13]. Plants are the primary distributors of selenium, originally present in soils. The amount of selenium in soil differs geographically and Europe is known for its low content. Therefore, a variety of Se-rich biological products have been developed up to present times including yeast, wheat, fruits and vegetables cultured in selenite enriched soil or medium.

The biological and toxicological effects of selenium strongly depend on its chemical form. Thus the interest in speciation of selenium in various matrices, towards the evaluation of its metabolisms has grown during the recent years. Sample preparation is very important for successful assessment of the concentration and range of selenium species. Solid-liquid extraction is most commonly used for this purpose and different media (e.g. water, surface active compounds, HCl, NaOH, enzymes) can be applied to release the selenium species from biological matrices. The identification of individual selenium species supports understanding of metabolic processes occurring in plants, animals and bacteria. Liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICP-MS) has been commonly used for this purposes [7, 12, 14, 17, 20, 24, 27]. Speciation of selenium has been widely investigated in animal tissues [4, 15, 29] and in plants [20, 21, 23, 28, 30]. Less attention so far was paid to selenium speciation in bacteria, but in all papers a very low extraction efficiency was stressed [9, 10, 11, 22]. Several selenium species have been identified in *Bifidobacterium animalis* cultivated with the addition of sodium selenite [32].

The interest in speciation of selenium in bacteria comes from the growing popularity and importance of the functional food. Selenium is known for its health promoting properties and can be considered as a component of the functional food. Therefore, it is very important to search for different carriers, which will deliver this essential micronutrient to the human body. It appears that *Lactobacillus* bacteria are likely to be this kind of carrier as well as being able to be a functional food component e.g. in the form of dairy products like yoghurt or kefir enriched with *Lactobacillus* bacteria.

Last research show, that selenium-enriched probiotics have several health benefits on the host for their antioxidative, antipathogenic, antimutagenic, anticarcinogenic and anti-inflammatory activities [27].

Lactic acid bacteria (LAB) are generally recognized as safe microorganisms, and have long been used in food industry. Many reports show their usefulness as probiotics for human and animals with extensive physiological effects (antimicrobial, immunomodulatory, anticarcinogenic, antidiarrheal, antiallergenic and antioxidant activities) [6]. Probiotic bacteria should be safe for the host organism, resistant to gastric acids and bile salts in order to survive the transit into the gut.

Several research groups have already demonstrated the ability of bacteria to accumulate selenium. It was found that *Lactobacillus bulgaricus* grown on MRS or *Lactobacillus casei rhamnosus* obtained from the commercial product of fermented milk, could uptake selenium, however no data on the specific selenium compounds were given [5, 31]. Selenium speciation was investigated in details in milk exposed to the fermentation process or in yoghurt [1-3]. A probiotic strain of *Enterococcus durans* was recently reported to accumulate Se from the medium at considerably high levels [25, 26]. The research demonstrated that microorganisms including *Lactobacillus* had the capacity to perform the biotransformation of Se(IV) to selenocysteine or Se-methylselenocysteine, however no all compounds were identified.

The aim of this work was to investigate the performance of pure bacteria strains to execute the bio-trasformation of selenium. We assumed that if bacteria could accumulate and metabolize inorganic selenium into its organic derivatives, this naturally induced bio-transformation by *Lactobacillus* could be explored for the production of functional food enriched with bioactive selenium compounds. In order to investigate the performance of *Lactobacillus* towards biotransformation of selenium, the species extracted

by various media were investigated by HPLC ICP MS. The second part of this work was optimization of extraction conditions, because of problems with efficiency of this process.

EXPERIMENTAL

Instrumentation

Inductively coupled plasma mass spectrometry An Elan 6100 DRC ICP MS (Perkin Elmer SCIEX, USA) was used. A conventional *Mainhardt* nebulizer and a quartz cyclonic spray chamber were used for sample introduction. The ICP-MS conditions were as follows: plasma power 1100 W, plasma argon flow 15 L/min, auxiliary flow 1.21 L/min, nebulizer flow 0.86 L/min. The interference-free isotope ^{82}Se was monitored.

HPLC Agilent 1200 (Agilent, USA) equipped with Hamilton PRP-X100 anion exchange column, 250 mm × 4.6 mm i.d., 10 µm (Hamilton, USA) was used in connection with ICP MS. HPLC column was connected to the nebulizer of the ICP-MS by PEEK tubing.

Chemicals and standards

All chemicals were of analytical grade and were used without further purification. Nitric acid (V) 65%, hydrochloric acid 37%, internal standard (yttrium) and multielemental ICP-MS standard solutions were obtained from Merck, Germany. Water for analysis was obtained from Mili-Q system (Milipore, USA). Lipase and protease were obtained from Aldrich Chemical Company Inc., USA.

Sodium selenite (Na_2SeO_3) and sodium selenate (Na_2SeO_4) were obtained from Sigma, Germany. Selenomethionine (Se-methionine), Se-methylselenocysteine (Se-MET-Selenocysteine), Se-cystine ($Se(Cys)_2$), were purchased from Sigma, USA. Selenomethionine-Se-oxide (SeMetO) was achieved by adding excess of oxidizing agent, 2.5 ml of 30% (v/v) of H_2O_2 to 25 ml aliquot of a 0.1 mol/L HCI solution of selenomethionine (50 mg/L Se) and left overnight in the dark. Sodium dodecyl sulfate (SDS), tris(hydroxymethyl) aminomethane (TRIS) and sodium hydroxide were obtained from Sigma, USA. Peroxide of hydrogen 30% and ammonium acetate were purchased from POCh, Poland. TES solution was composed of TRIS (Sigma, USA), EDTA (POCH, Poland) and saccharose (Sigma, USA).

A reference material BCR-CRM 184, bovine muscle with total selenium content of 0.183 mg/kg (±7%), was obtained from European Commission – Joint Research Centre, Institute for Reference Materials and Measurements, Belgium.

Sample preparation

Acid and bile salt resistant strains with antagonistic activity against several pathogenic bacteria were selected for this work. Those strains present different levels of antagonistic activities against tested pathogens and were chosen due to their highest antagonistic activity in the mixture. *Lactobacillus casei* (strains named as *Lb. casei* ŁOCK 0900 and *Lb. casei* ŁOCK 0908) and *paracasei* (strain named as *Lb. paracasei* ŁOCK 0919) were fed with bacterial growth medium enriched in selenium nutrient (2.5 mg of Na_2SeO_3 per 1 L of medium) for 24 hours. Once the process was completed, bacterial strain was lyophilized. One part of lyophilized material was digested (towards the determination of total content of selenium) and another part was exposed to a different extraction medium (towards investigation of selenium speciation).

The cultivation of bacterial strains was conducted at the Technical University of Łódź by an experienced microbiologist. The final selection of the conditions was preceded by a series of tests in order to bring the appropriate dose of selenium to the agar medium. The influence of different selenium compounds (SeO_2, Na_2SeO_3, Na_2SeO_4) as well as increasing concentrations of selenium in medium (1, 2.5, 5, 10 mg Se/L of medium) on growth and acidifying activity of *Lactobacillus casei* and *Lactobacillus paracasei* and their ability to accumulate selenium was evaluated.

In order to determine the dynamics of growth and acidification activity, bacterial cultures were prepared/made by the method of periodic MRS medium supplemented with different sources and concentrations of selenium. The bacteria were cultured for 48h at the temperature of 37°C (with limited access of oxygen – 5% of CO_2) in medium at pH 6.2 – 6.3. All measurements were performed immediately after inoculation of the medium, then after 3h, 6h, 12h, 18h, 24h, 36h and 48h of the culture time.

It was found that selenium present in medium up to 5 µg/mL act as a stimulator of growth of bacteria, however higher concentrations of selenium in culture inhibit their growth. When Se(IV) is above 5 µg/mL, the reddish colour of biomass was observed, which might be due to the reduction of selenium to its red elementary form.

The most effective grown of bacterial was obtained in the presence of selenium (IV) at the concentration between 1.0 µg to 2.5 µg of Se per mL of medium. Finally, the solution containing 2.5 mg of Na_2SeO_3 per 1 L of medium was used.

Analytical procedures

Determination of selenium

In order to determine the total concentration of selenium, an approximately 0.1 g of lyophilized bacteria was digested in 4 mL of HNO_3 (65 %) under the following conditions: (i) 500 W/10 min.; (ii) 1000 W/15 min.; (iii) cooling/5 min. A microwave-assisted unit (Anton Paar Mutliwave Sample Preparation System, Austria) with Teflon vessels was used. Digested samples were filtered through sterile syringe-driven 0.45 μm nylon membrane filters (Millex, France). Transparent solutions were transferred to plastic tubes, filled with de-ionized water to 20 mL and stored in a refrigerator below 4 °C before measurements.

Selenium content was determined by ICP MS by monitoring ^{82}Se isotope. Calibration was performed with the standard solutions containing 1 μg/L, 10 μg/L and 100 μg/L of selenium.

The validation of the analytical procedure was performed with certified reference material BCR-CRM 184, bovine muscle with the certified value for selenium (0.183 mg/kg ± 7 %), which was digested in the same conditions as used for bacterial strains. The concentration of selenium obtained within this work was 0.169 mg/kg ± 8.5 %, which we consider as being within the uncertainty of certified value.

Extraction procedure

Water or the solutions of surface-active compound SDS, NaOH, HCl and enzymes (lysozyme as well as the mixture of protease and lipase) were used for the extraction with support of ultrasonic shaking. About 0.1 g of dry mass of lyophilized bacteria was weighted in the plastic centrifuge tubes and 5 mL of extractant solution was added. The extraction was performed under the following conditions: (i) water at (37 ± 1)°C for 30 min.; (ii) 4% SDS in 30 mmol/L Tris HCl at (37± 1)°C for 30 min.; (iii) 0.4% protease in 30 mmol/L Tris HCl at (37 ± 1)°C for 30 min.; (iv) 0.4% lipase in 30 mmol/L Tris HCl at (37 ± 1)°C for 30 min.; (v) mixture of protease and lipase aqueous solution at (37 ± 1)°C for 30 min.; (vi) 0.4% lysozyme in 30 mmol/L Tris HCl or 10 g/L lysozyme in TES at (37 ± 1)°C for 30 min; (vii) 0.1 mol/L NaOH solution at (37 ± 1)°C for 30 min.; and (viii) 1 mol/L HCI solution at (37 ± 1)°C for 30 min. The supernatant was separated from the residue by centrifugation for 10 min. at 10000 rpm and filtered through a 0.45 μm membrane filters. The centrifuge MPW-53 (MPW Med. Instruments, Poland) was used for this purpose.

Determination of selenium content in extracts was performed directly after digestion of supernatants. Aliquots used for HPLC separation were kept at -10 °C before measurements.

Chromatographic separation procedure

HPLC: Eluent A: 5 mM acetate buffer (pH 4.7); eluent B: 100 mM acetate buffer (pH 4.7), were used. The mobile phase was delivered at 1.0 mL/min. in gradient mode: (0 – 4) min. - 100% A, (4 – 7) min. – from 100% A to 0% A, (7 – 30) min. - 100 % B.

Filtered solutions were degassed before the injection, then 100 μL or 25 μL of the sample was injected for ICP MS.

RESULTS

Efficiency of accumulation of selenium by bacteria

In order to evaluate the accumulation performance of the *Lactobacillus* bacteria, the total content of selenium was determined in strains before and after being exposed to the agar medium with added Na_2SeO_3. Sodium selenite (Na_2SeO_3) was chosen for the enrichment as it is known to be more likely to be converted into its organic derivatives [32].

Carefully selected strains of bacteria (chapter 2.3) - *Lactobacillus casei* ŁOCK 0900*,* ŁOCK 0908 and *paracasei*, ŁOCK 0919*,* were cultivated on bacterial growth medium with the addition of selenium nutrient. It was found that the content of selenium in original bacteria culture (ŁOCK0900, ŁOCK0908 and ŁOCK0919) was below the limit of detection (LOD) of the entire analytical procedure, including sample digestion and ICP MS measurements. When bacteria were cultivated on the selenium enriched agar, regardless of the type of bacteria strain, the efficiency for selenium accumulation was found to be significant (Table 1). All data given in the Table 1 relates to the total selenium contents in respective bacteria cells.

Table 1. Mean and standard deviation values of content of selenium in bacterial strains (calculated from three replicates of the analytical procedure for each strain). "+ Se" means the addition of selenium to the agar medium. The LOD of the analytical procedure is $0.9*10^{-3}$ mg/kg. The LOQ of the analytical procedure is $1.4*10^{-3}$ mg/kg.

Bacterial strains	Concentration ± SD [mg/kg]
ŁOCK 0900	< LOD
ŁOCK0900 + Se	1357 ± 61
ŁOCK 0908	< LOD
ŁOCK 0908 + Se	1313 ± 33
ŁOCK 0919	< LOD
ŁOCK 0919+ Se	1913 ± 63

Efficiency of extraction of selenium compounds

Extractions were carried in ultrasound as well as using a magnetic stirrer, also various time and temperatures of extraction were tested. Water, SDS, NaOH, HCl, lysosyme, protease, lipase as well as the mixture of lipase and protease were tested. The selected conditions for each extraction solutions are listed in the experimental part (chapter '*Extraction Procedure*').

The amount of selenium extracted from bacteria with the use of various media is presented in Table 2. The comparison of the data from Table 1 and 2 indicates low extraction efficiency, regardless the applied media, which was presumably due to the resistant cell walls of bacteria.

Despite the use of different variants of extraction, very low extraction efficiency was obtained. The next stage of research will be the further optimization of the extraction conditions.

Speciation of selenium by HPLC ICP MS

Although the extraction power of the applied solutions was not very high, the obtained extracts were characterized in respect to their chemical speciation. In order to evaluate a possible bio-transformation of selenium in bacteria, further investigation towards identification of chemical forms of selenium was performed using anion-exchange HPLC followed by ICP-MS detection of ^{82}Se isotope.

Table 2. Content of selenium [µg/kg] in bacteria extractable by various media given with standard deviation (±) calculated from two repetitions of the analytical procedure calculated for each sample. The LOD of the analytical procedure is 0.9 µg/kg. The LOQ of the analytical procedure is 1.4 µg/kg.

Extractant	*Lb. casei* ŁOCK 0900+Se	*Lb. casei* ŁOCK 0908+ Se	*Lb. paracasei* ŁOCK 0919+Se
Water	48.5±0.3	29.1±0.2	19.9±0.2
4 % SDS in Tris HCl	14.4±0.2	17.2±0.4	22.2±0.3
0.4 % protease in Tris HCl	88.5±6.3	87.9±1.9	118.8±2.5
0.4 % lipase in Tris HCl	19.8±1.0	18.6±0.3	24.3±0.5
0.4 % lipase and 0.4 % protease in Tris HCI	109.5±4.3	98.4±1.3	133.7±8.0
Lisozyme in TES	136.2±3.1	116.5±4.1	186.0±9.7
0.1 mol/L NaOH	87.7±2.8	92.4±2,0	127.1±5.3
1 mol/L HCI	15.1±0.6	21.2±0.5	17.4±0.2

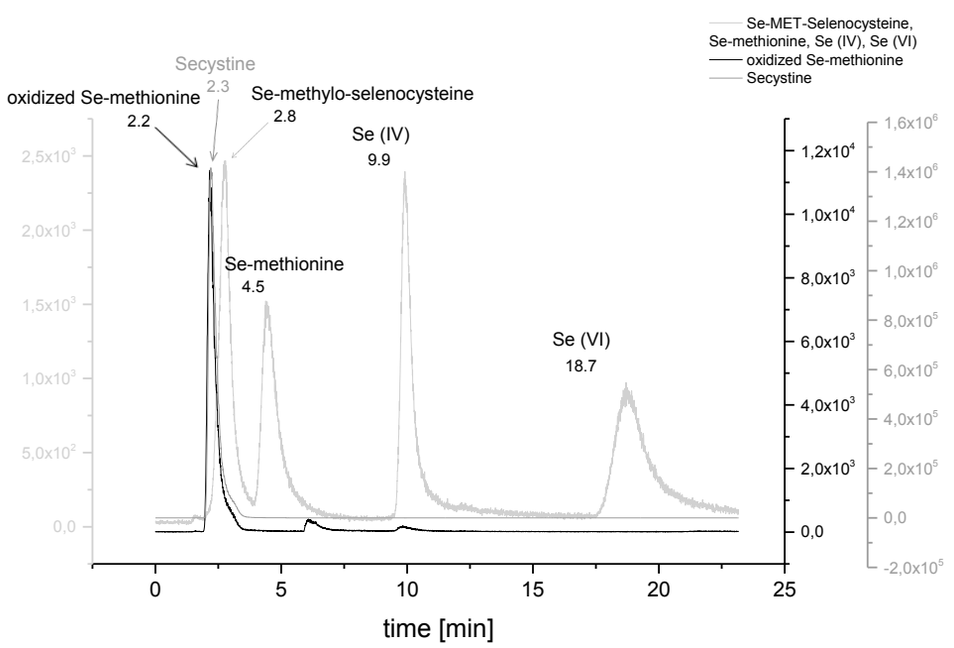

Figure 1. An anion exchange HPLC-ICP-MS chromatograms of selenium standards. Sequence of standards, concentrations and retention times: Se-methionine-Se-oxide (CSe = 10 mg/L, tR = 2.2 min), Secystine (CSe = 10 mg/L, tR = 2,3 min), Se-METSelenocysteine (CSe = 100 µg/L, tR = 2.8 min) and Se-methionine (CSe = 100 µg/L, tR = 4.4 min), Se(IV) (CSe = 100 µg/L, tR = 9.9 min) and Se(VI) (CSe = 100 µg/L, tR = 18.7 min)

a)

b)

c)

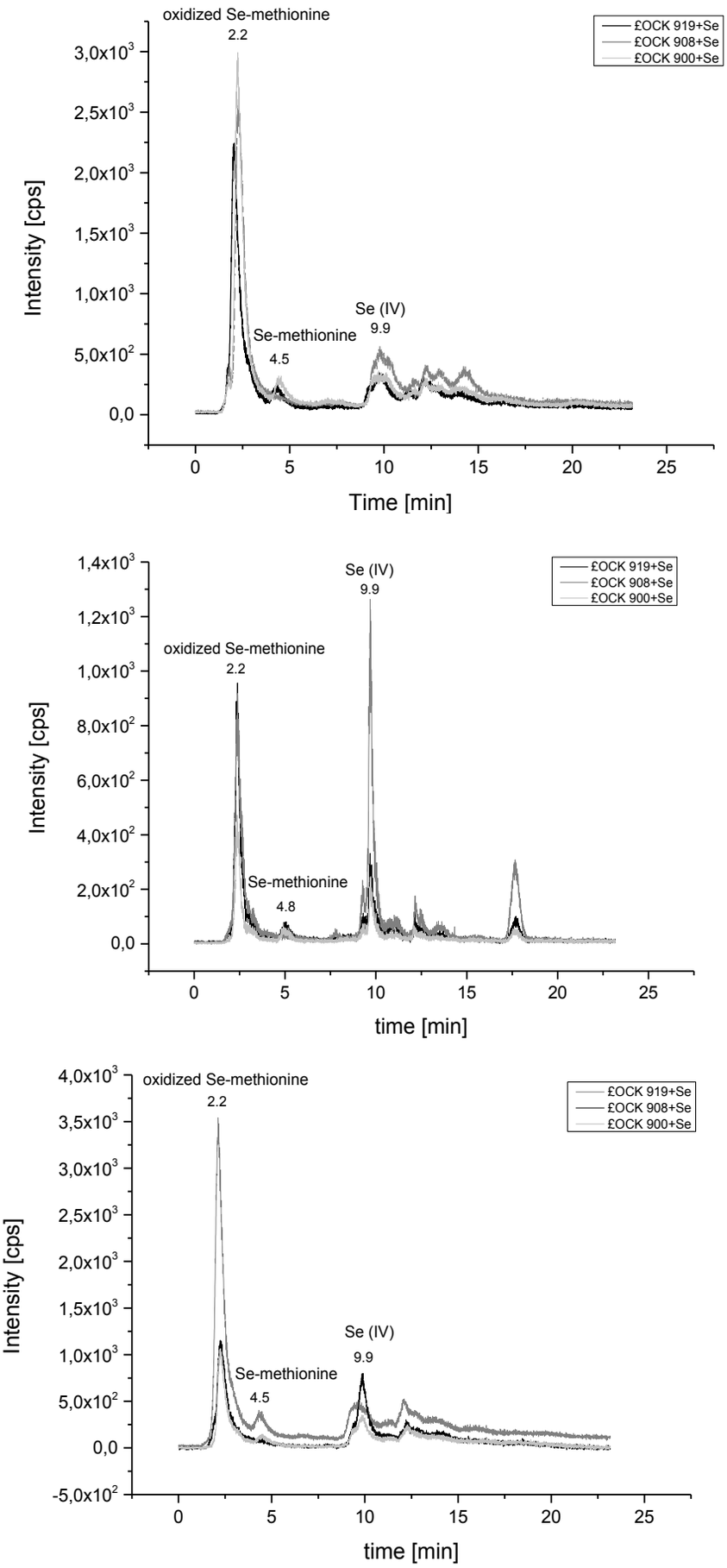

Figure 2. An anion exchange HPLC-ICP-MS chromatograms of extract for: a) water (30 min at (37 ± 1) ºC in ultrasound); gradient elution, pH= 4.7; b) a mixture of protease and lipase (30 min at (37 ± 1) ºC in ultrasound), gradient elution, pH= 4.7; c) lysozyme (30 min at (37 ±1) ºC in ultrasound) gradient elution, pH = 4.7

Calibration of HPLC-ICP-MS system

The commercially available selenium compounds were used to calibrate the retention times of HPLC under described conditions (Figure 1). The standard solutions of the reference selenium compounds were prepared to contain 100 µg/L of selenium. For the mixture of all compounds (Figure 1), selenium signals appeared sequentially: (i) Se-MET-Selenocysteine [2.8 min]; (ii) Se-methionine [4.4 min]; (iii) Se (IV) [9.9 min]; (iv) Se (VI) [18.7min]. Individual chromatograms were prepared for Se-methionine-Se-oxide [2.2 min.] and for Se-cystine [2.3 min.], as to avoid the overlap with the retention time of Se-MET-Selenocysteine (Figure 1).

Selenium speciation in extracts from bacteria Lactobacillus

Using the anion exchange chromatographic separation, the selenium profiles for each of the extracts were registered for all the investigated strains. At first instance it was visible that the common feature of all chromatograms was the presence of intensive selenium signal occurring at 2.3 minutes, indicating the presence of Se-methionine-Se-oxide or Se-cystine (Figure 1). The less intensive signal was also noted at 9.9 minute, indicating the presence of Se(IV). Interestingly, the ratio of both signals, evaluated in terms of peak intensity, varies depending on the extraction media. Water and HCl solution mainly extracts species eluted after 2.3 minutes, while under enzymatic extraction a significant amount of Se(IV) is co-extracted. Apparently none of the extraction medium releases Se(VI), which could possibly indicate that no oxidization of selenium occurs in bacterial cell.

Besides the identified signal for Se(IV) and non-identified signals for species eluted at 2.3 minute, the presence of Se-methionine, eluted at 4.4 minute were confirmed in majority of cases. However, in contrast to the intensive signal occurring at 2.3 minute. The Se-methionine is present in a much smaller amount. As it was already mentioned, selenium signal appearing at 2.3 minute can be assigned to Se-methionine-Se-oxide or Se-cystine, both eluted at the same time. In case of described chromatography separation of the mixture of selenium compounds, a signal for Se-MET-Selenocysteine was obtained at 2.8 minute. The authors assumed that in the bacteria's samples, Se-MET-Selenocysteine might be also present, but its presence is masked by the high, extending signal at the elution time of 2.3 minute.

An example of chromatograms, received for the extracts obtained with water, mixture of protease and lipase or lysozyme are given in Figure 2a, 2b and 2c, respectively. One can see that while the overall patterns of all chromatograms are very close, they are far from being identical. One of the differences comes from the lack of signal present in 4.4 minute (Se-methionine) for strain *Lb. casei* ŁOCK 0908, regardless of the extraction medium being used. Interestingly, also the ratio of the signals at 2.3 min. to the one at 9.9 min. varies between all examined bacterial strains. The most pronounced difference was found for the strain *Lb. casei* ŁOCK 0908, where the highest amount of extracted Se(IV) was detected.

DISCUSSION

Efficiency of accumulation of selenium by bacteria

Lactobacillus bacteria strains meet requirements of probiotics, according the World Health Organization. It is known that some *Lactobacillus* bacteria may possess potentially therapeutic properties including maintenance of healthy digestive juices as well as improving immune function, thus are frequently added to some food products, e.g. yoghurt or cheese.

Various microorganisms, including bacteria, can accumulate selenium inside their cells as organic and inorganic compounds. In general, the process of accumulation of selenium by microorganisms can be due to two mechanisms: extracellular binding by the active groups of biopolimers present in the structure of cell-membrane conjunction and intracellular binding coupled with the ion transportation thorough the biological membrane into the cell [8].

Efficiency of extraction of selenium compounds

The efficiency of extraction was expressed as the ratio of selenium amount extracted from the bacteria to its total content. Hence the extraction efficiency provides the information about the selenium compounds which are soluble in the respective medium under defined extraction conditions (time and temperature).

Although the extraction conditions for animal's and plant's tissues are well described in the literature [4, 16, 21, 22, 29, 30, 31], less information is available in respect to bacteria [9, 10, 11, 22]. Therefore, the detailed studies on the selection of the extraction conditions were required. For this purpose water, SDS, NaOH, HCl, lysosyme, protease, lipase as well as the mixture of lipase and protease were selected. Various conditions for performing the extraction of selenium from bacterial strains *Lactobacillus casei* and *paracasei* (ŁOCK0900 + Se, ŁOCK0908 + Se and ŁOCK0919 + Se) were tested.

The comparison of the data from the above with the total selenium contents indicates low extraction efficiency, regardless of the applied media, which was presumably due to the resistant cell walls of the bacteria.

Lactobacillus bacteria adhere to the Gram (+) type characterized by very good cross-linked cell walls expected to prevent the release of inter alia selenium

compounds. Moreover, *Lactobacillus* bacteria have a surface layer (S-layer) consisting of hard and tightly packed monomolecular layer composed of identical proteins. This structure encloses the whole cell surface. The S-layer protects the bacteria from the chemical and physical agents and may be a barrier against high-molecular-weight substances (e.g. lytic enzymes) or against low pH and mechanical impulses [18].

Interestingly, bacteria exhibit a high efficiency uptake of selenium from the media enriched with inorganic selenium ions, which are then being incorporated into a form which is not easily extracted by the media applied. Regardless of the medium used the efficiency of the extraction did not exceed 10% of the total content of selenium, which means that close to 90% of selenium species were trapped in the bacterial cells and were not accessible for the chemical investigation of speciation. Water medium extracted from 20 µg/kg to 50 µg/kg of selenium, which corresponds to an average of 2% of its total content. The most effective medium, lysozyme, which was expected to breakdown the cell walls of Gram (+) bacteria, released close to 10 % of the total accumulated selenium.

Protease, which is responsible for the hydrolysis of peptide bonds, and lipase, applied for the hydrolyze of ester bonds in fat cells, were expected to release selenium incorporated in selenoamino acids. Surprisingly, in the presence of lipase only 1.5% of selenium was released. More effective protease eluted about 7% of selenium. Whereas, the mixture of both enzymes resulted in 8% of the extraction efficiency, thus did not exhibit any synergistic effects; seems that both enzymes present in the mixture act in the same way when applied separately.

Sodium hydroxide, which was expected to release selenium compounds of high molecular weight, offered the efficiency of extraction close to 7% of the total selenium content. Although hydrochloric acid is often used in speciation studies [19], in the case of bacteria it did not extract more than 1% of selenium compounds.

The overall results indicate that the best efficiency, close to 10%, can be achieved only when lisozyme or the mixture of lipase and protease were used. Slightly less effective is sodium hydroxide alone, whereas the other media exhibit much lower performance to release selenium species from bacteria.

The examination of the extraction efficiency of selenium compounds from the bacterial strains were performed by *Michalke* [19], who pointed out low extraction efficiency, mostly below 10%. Our results were in agreement with the previously described and support the hypothesis that specifically cross linked bacterial cell walls are very resistant to the destruction by chemical agents. The sequential extraction procedure was also used, but this did not bring any significant increase of the extraction efficiency.

The next stage of research will be searching for the new paths of extraction of selenium compounds from microbial biomass. The conditions similar to the human digestive tract were used in order to achieve the most effective conditions of selenium compounds extraction. Finding the right path of selenium release from the bacterial biomass is very important for the possibility of using bacteria as the source of selenium in the diet.

Selenium speciation in extracts from bacteria Lactobacillus

Using the anion exchange chromatographic separation, the selenium profiles for each of the extracts were registered for all the investigated strains. The investigation of the released selenium species was performed by HPLC ICP MS.

It was found that *Lactobacillus* bacteria are able to metabolize inorganic ions of selenium (IV) into Se-methyloselenocysteine, Se-methionine and other unidentified forms. Only a small fraction of selenium stays in the original Se(IV) form. Apart from the identified signals, a very intensive signal for the compound eluted at 2.3 min appears on all chromatograms, regardless of the extraction medium.

In case of described chromatography separation of the mixture of selenium compounds, a signal for Se-MET-Selenocysteine was obtained at 2.8 minute. The authors assumed that in the bacteria's samples, Se-MET-Selenocysteine might be also present, but its presence is masked by the high, extending signal at the elution time of 2.3 minute.

Further investigation in the identification of unknown forms of selenium will be performed with the use of MS/MS and will be describe in the next work.

It is worth emphasising that the amount of selenium compound observed by HPLC ICP MS cannot be directly related to the entire efficiency of extraction, as summarized in chapter '*Efficiency of extraction of selenium compounds'*.

Although the originally up-taken compound of selenium (selenite) could be extracted with a very low efficiency by water, that does not indicate that it is almost completely transformed to the respective organic derivatives (e.g. Se-methionine and compounds eluted at 2.3 min.). The use of enzymatic extraction leads to the release of higher amount of extractable selenium inorganic ions, where the most pronounced effect was found for lysozyme. Although the highest signal was detected in the case of *Lb. casei* ŁOCK 0908, the extractable amount of Se(IV) can be estimated not to be higher than 1% of the total selenium content in bacteria.

The chromatographic profiles, especially the presence of the signals at 2.3 and 4.4 minutes, proved the performance of *Lactobacillus* bacteria for the bio-transformation of the inorganic selenium into its organic derivatives: Se-methionine (4.4 min) and non-identified selenocompounds (2.3 min). The ability to biotransform inorganic selenium compounds to its organic derivatives was also shown in study on different lactic acid bacteria [1, 16, 27, 32]

It should be noted, that due to low efficiency of extraction, still close to 90% of the total up-taken selenium remains incorporated inside the bacteria cell.

CONCLUSIONS

The aim of this study was to evaluate whether the probiotic *Lactobacillus* bacteria could effectively up-take selenium and possibly perform its bio-transformation into seleno-organic derivatives. For this purpose various *Lactobacillus* strains were cultured on bacterial growth medium enriched with Se(IV). By determination of the total content of selenium in pure bacteria, before and after the enrichment, their high ability to up-take selenium was thus confirmed. Surprisingly, none of the applied extraction media exhibited a strong power to release the majority of the uptaken selenium compounds. Thus a maximum of 10% of the selenium was extracted from bacteria exposed to the enzymes. The investigation of the released selenium species was performed by HPLC ICP MS. It was found that *Lactobacillus* bacteria are able to metabolize inorganic ions of selenium (IV) into Se-methionine, Se-methyloselenocysteine and other unidentified forms. Only a small fraction of selenium stays in the original Se(IV) form.

The study confirmed the ability of probiotic bacteria to transform partially inorganic selenium into its organic forms as a result of direct culture on agar medium supplemented with inorganic selenium. It was noted that *Lactobacillus,* which might exists in various strains, exhibits some differences in selenium bio-transformation, which should be taken into account during the control process in the production process of selenium enriched foodstuff. However, because of its ability to biotransform inorganic selenium to its organic derivatives, Se-enriched bacteria can be considered as an addition to the functional food.

Acknowledgments

The study was carried out at the Biological and Chemical Research Centre, University of Warsaw, established within the project co-financed by European Union from the European Regional Development Fund under the Operational Programme Innovative Economy, 2007 – 2013. 2.

This project is financed in the framework of grant entitled: „Investigation of chemical and biological processes of selenium biotransformation in selenophilic plants and probiotic bacteria towards their application as functional food" attributed by the National Center for Research and Development (2012/05/B/ST4/01219).

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

1. *Alzate A., Cañas B., Pérez-Munguía S., Hernández-Mendoza H., Pérez-Conde C., Gutiérrez M., Cámara C.*: Evaluation of the Inorganic Selenium Biotransformation in Selenium-Enriched Yogurt by HPLC-ICP-MS. J. Agric. Food Chem. 2007**;**55:9776–9783.
2. *Alzate A., Fernández-Fernández A., Pérez-Conde M.C., Gutiérrez A.M., Cámara C.:* Comparison of Biotransformation of Inorganic Selenium by *Lactobacillus* and *Saccharomyces* in Lactic Fermentation Process of Yogurt and Kefir. J. Agric. Food Chem. 2008;56:8728–8736.
3. *Alzate A., Pérez-Conde M.C., Gutiérrez A.M., Cámara C.*: Selenium-enriched fermented milk: A suitable dairy product to improve selenium intake in humans. Int. Dairy J. 2010;20:761-769.
4. *Bierla K., Dernovics M., Vacchina V., Szpunar J., Bertin G., Lobinski R.*: Determination of selenocysteine and selenomethionine in edible animal tissues by 2D size-exclusion reversed-phase HPLC-ICP MS following carbamidomethylation and proteolytic extraction. Anal. Bioanal. Chem. 2008;390:1789−1798.
5. *Caballero Araúz I.L., Afton S., Wrobel K., Caruso J.A., Gutiérrez Corona J.F., Wrobel K.*: Study on the protective role of selenium against cadmium toxicity in lactic acid bacteria: An advanced application of ICP-MS. J. Hazard. Mater. 2008;153:1157–1164.
6. *Calomme M., Hu J., Van den Brandend K., Vanden Berghe D.A.*: Seleno -lactobacillus – an organic selenium source. Biological Trace Element Research 1995;47:379−383.
7. *Chan Q., Afton S.E., Caruso J.A.:* Selenium speciation profiles in selenite-enriched soybean (Glycine Max) by HPLC-ICPMS and ESI-ITMS. Metallomics 2010;2:147−153.
8. *Chmielewski J., Tyflewska A.*: Selenium in microorganisms. In: *Wierzbicka M., Bulska E., Pyrzyńska K., Wysocka I., Zachara B.A.* (eds.) Selenium. The element essential to health and fascinating for researchers. Warsaw, Malamut, 2007.
9. *Combs G.J., Garbisu C., Yee B.C., Yee A., Carlson D.E., Smith N.R., Magyarosy A.C., Leighton T., Buchanan B.B.:* Bioavailability of selenium accumulated by selenite-reducing bacteria. Biol. Trace Elem. Res. 1996;52:209−225.
10. *Dernovics M., Stefánka Z., Fodor P.*: Improving selenium extraction by sequential enzymatic processes

for Se-speciation of selenium-enriched *Agaricus bisporus*. Anal. Bioanal. Chem. 2002;372:473−480.
11. *Ghosh A., Mohod A.M., Paknikar K.M., Jai R.K.*: Isolation and characterization of selenite- and selenate-tolerant microorganisms from selenium contaminated sites. World J. Microb. Biot. 2008;24:1607−1611.
12. *Hart D.J., Fairweather-Tait S.J., Broadley M.R., Dickinson S.J., Foot I., Knot P., McGrath S.P., Mowat H., Norman K., Scott P.R., Stroud J.L., Tucker M., White P.J., Zhao F.J., Hurst R:* Selenium concentration and speciation in biofortified flour and bread: Retention of selenium during grain biofortification, processing and production of Se-enriched food. Food Chem. 2011;126:1771−1778.
13. *Hatfield D.L., Berry M.J., Gladyshev V.N.*: Selenium. Its Molecular Biology and Role in Human Health (3rd ed.). New York, Springer. 2012.
14. *Kannamkumarath S.S., Wrobel K., Wrobel K., Vonderheide A., Caruso J.A.*: HPLC-ICP-MS determination of selenium distribution and speciation in different types of nuts. Anal. Bioanal. Chem. 2002;373:454−460.
15. *Kurek E., Ruszczynska A., Wojciechowski M., Czauderna M., Bulska E.*: Study on speciation of selenium in animal tissues using high performance liquid chromatography with on-line detection by inductively coupled plasma mass spectrometry. Chemical Analysis (Warsaw) 2009;54:43−57.
16. *Lamberti C., Mangiapane E., Pessione A., Mazzoli R., Giunta C., Pessione E.*: Proteomic characterization of a selenium metabolizing probiotic *Lactobacillus reuteri* Lb2 BM for nutraceutical applications. Proteomics 2011;11:2212-2221.
17. *Lipiec E., Siara G., Bierla K., Ouerdane L., Szpunar J.:* Determination of selenomethionine, selenocysteine, and inorganic selenium in eggs by HPLC-inductively coupled plasma mass spectrometry. Anal. Bioanal. Chem. 2010;397:731−741.
18. *Messner P., Sleytr U.*: Crystalline bacterial cell-surface layers. Adv. Microb. Physiol. 1992;33:213–75.
19. *Michalke B., Witte H., Schramel P.*: Effect of different extraction procedures on the yield and pattern of Se-species in bacterial samples. Anal. Bioanal. Chem. 2002;372:444−447.
20. *Michalska-Kacymirow M., Kurek E., Smolis A., Wierzbicka M., Bulska E.*: Biological and chemical investigation of *Allium cepa* L. response to selenium inorganic compounds. Anal. Bioanal. Chem. 2014; 15: 3717-3722.
21. *Montes-Bayón M., LeDuc D.L., Terry N., Caruso J.A.*: Selenium speciation in wild-type and genetically modified Se accumulating plants with HPLC separation and ICP-MS/ES-MS detection. J. Anal. At. Spectrom. 2002;8:872−879.
22. *Nelsona D.C., Caseyb W.H., Sisona J.D., Macka E.E., Ahmada A., Pollacka J.S.*: Selenium uptake by sulfur-accumulating bacteria. Geochim. Cosmochim. Acta 1996;60:3531−3539.
23. *Peñas E., Martinez-Villaluenga C., Frias J., Sánchez-Martínez M.J., Pérez-Corona M.T., Madrid Y., Cámara C., Vidal-Valverde C.*: Se improves indole glucosinolate hydrolysis products content, Se-methylselenocysteine content, antioxidant capacity and potential anti-inflammatory properties of sauerkraut. Food Chem. 2012;132:907−914.
24. *Pérez-Corona M.T., Sánchez-Martínez M., Valderrama M.J., Rodríguez M.E., Cámara C., Y. Madrid Y.*: Selenium biotransformation by Saccharomyces cerevisiae and *Saccharomyces bayanus* during white wine manufacture: Laboratory-scale experiments. Food Chem. 2011;124:1050− 1055.
25. *Pieniz S., Andreazza R., Anghinoni T., Camargo F., Brandelli A.:* Probiotic potential, antimicrobial and antioxidant activities of *Enteroccus durans* strain LAB18s. Food Control 2014;37:251-256.
26. *Pieniz S., Andreazza R., Pereira J. Q., Camargo F.A., Brandelli, A.*: Production of Selenium-enriched biomass by *Enteroccus durans.* Biol. Trace Elem. Res. 2013;155:447-454.
27. *Pophaly S.D., Poonam, Singh P., Kumar H., Tomar S.K., Singh R.*: Selenium enrichment of lactic acid bacteria and bifidobacteria: A functional food perspective. Trends Food Sci. Technol. 2014;39:135-145
28. *Pyrzynska K.:* Selenium speciation in enriched vegetables. Food Chem. 2009;114:1183−1191.
29. *Quijano M.A., Moreno P., Gutiérrez A.M., Pérez-Conde M.C., Cámara C.*: Selenium speciation in animal tissues after enzymatic digestion by high-performance liquid chromatography coupled to inductively coupled plasma mass spectrometry. J. Mass Spectrom. 2000;35:878−84.
30. *Wróbel K., Wróbel K., Kannamkumarath S.S., Caruso J.A., Wysocka I.A., Bulska E., Swiatek J., Wierzbicka M.:* HPLC-ICP-MS speciation of selenium in enriched onion leaves - a potential dietary sources of Se-methyloselenocysteine. Food Chem. 2004;86:617−623.
31. *Xia S.K., Chen L., Liang J.Q.*: Enriched Selenium and Its Effects on Growth and Biochemical Composition in *Lactobacillus bulgaricus*. J. Agric. Food Chem. 2007;55:2413−2417.
32. *Zhang, B., Zhou, K., Zhang, J., Chen, Q., Liu, G., Shang, N., Qin, W., Li, P., Lin, F*:. Accumulation and species distribution of selenium in Se-enriched bacterial cells of *Bifidobacterium animalis* 01. Food Chem. 2009; 115, 727−734.

Received: 09.03.2016
Accepted: 20.06.2016