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Comparison of Biotransformation of Inorganic Selenium by *Lactobacillus* and *Saccharomyces* in Lactic Fermentation Process of Yogurt and Kefir

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The aim of the present study was to characterize, quantify, and compare the different selenium species that are produced when lactic fermentation with two different types of microorganisms, bacteria (*Lactobacillus*) and yeast (*Saccharomyces*), take place to produce yogurt and kefir, respectively, and to study the transformation process of these species as a function of time. These two dairy products were chosen for the study because they are highly consumed in different cultures. Moreover, the microorganisms present in the fermentation processes are different. While the bacteria *Lactobacillus* is the one responsible for yogurt fermentation, a partnership between bacteria and the yeast *Saccharomyces* causes kefir fermentation. A comparative study has been carried out by fermenting Se(IV) enriched milk in the presence of both types of microorganisms, where the concentration range studied was from 0.5 to 20 μg g^{-1}. Enzymatic extraction enabled selenium speciation profiles, obtained by anionic exchange and ion-pairing reversed phase high performance liquid chromatography (IP-RP-HPLC) with inductively coupled plasma mass spectrometry (ICP-MS) detection. Scanning electron microscopy (SEM) applied to the enriched samples showed segregated Se^0, at added concentrations higher than 5 μg g^{-1}. The main Se species formed depended on the type of microorganism involved in the fermentation process, $SeCys_2$ and MeSeCys being the main species generated in yogurt and SeMet in kefir. The results obtained are different for both kinds of samples. Lactic fermentation for yogurt produced an increment in selenocystine ($SeCys_2$) and Se-methylselenocysteine (MeSeCys), while fermentation to produce kefir also incremented the selenomethionine (SeMet) concentration. The Se species are stable for at least 10 and 15 days for kefir and yogurt, respectively. After this period, selenocystine concentration decreased, and the concentration of Se-methylselenocysteine was found to significantly increase.

KEYWORDS: Selenium speciation; biotransformation; selenium-enriched yogurt; selenium-enriched kefir

INTRODUCTION

Selenium is recognized as a nutritionally essential element and its importance has been recently reviewed (*1, 2*). The human recommended intake is about 55 μg/day, and the highest tolerable intake level has been set to 400 μg/day (*3*). Significant interest arose over the last years regarding the administration of selenium supplements because of their antioxidant and cancer preventive properties (*4*−*6*). It has been shown that those properties strongly depend on its chemical form (*7, 8*), that is, the more bioavailable compounds being more active (*9*).

While the incorporation of selenocysteine into selenoproteins in animals and bacteria has been recognized to be done through a process directed by a UGA codon (*10*), there are still doubts regarding the role of selenium in the plant kingdom. For plants, the most important amino acid is selenomethionine (*11*) and for man and animals the main amino acid is selenocysteine (*7*).

The most widely investigated nutritional supplement containing selenium has been selenium-enriched yeast (*1, 12, 13*). During the growth of *Saccharoyces cerevisiae* yeast, inorganic selenium, which is a potentially toxic and poorly bioavailable species, is converted to safer organic selenium species, namely, selenomethionine (SeMet) (*14, 15*).

Mounicou et al. (*16*) compared the water soluble selenium containing proteins extracted from accumulating and non-accumulating plant species. They found some differences and concluded that SeMet was the primary constituent of the proteins of the non-accumulator plant, while $SeCys_2$ and SeMet were found in the same proportion in the accumulator extract.

Identification of selenoamino acids in food is relevant because despite the similarity in chemical structure, their metabolism significantly depends on the particular species. The two similar amino acids Se-methylselenocysteine and selenomethionine have rather different metabolic pathways. While selenomethionine is metabolized to hydrogen selenide (*17*), Se-methylselenocys-

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Table 1. Experimental Conditions for HPLC

Chromatographic Column	ODP2 HP-4E Shodex
mobile phase	0.05% TFA, 0.1% HFBA solution (pH 2.6); 1% MeOH
flow rate	0.7 mL min^{-1}
injection volume	100 μL

Chromatographic Column	PRP-X-100
mobile phase	10 mM ammonium citrate (pH 5.0); 2% MeOH
flow rate	1.0 mL min^{-1}
injection volume	100 μL

Chromatographic Column	Biosep-SEC-2000; BioBasic60
mobile phase	5 mM phosphate buffer (pH 7.5)
flow rate	1.0 mL min^{-1}
injection volume	100 μL

Table 2. Effect of the Amount of Kefir Fermentation on the Percentage of Biotransformation (Three Replicates with RSD 5−15%)

	Se-biotransformation (%)		
	amount of kefir added		
Se (IV) added ($\mu g.g^{-1}$)	0.25 g	0.50 g	1.0 g
0.50	76 ± 9	103 ± 5	90 ± 7
1.0	55 ± 15	99 ± 5	95 ± 5
2.5	47 ± 16	76 ± 6	75 ± 13
5.0	24 ± 7	35 ± 6	34 ± 5

Table 3. Percentage of Biotransformation in Yogurt and Kefir after 24 h Fermentation after Dialysis (MW >3.5 kDa)

	Se-biotransformation (%)	
Se (IV) added ($\mu g\ g^{-1}$)	kefir	yogurt
0.50	103 ± 5	99 ± 2
1.0	99 ± 5	
2.5	76 ± 6	94 ± 1
5.0	35 ± 6	69 ± 1
20		27 ± 0.5

teine is metabolized in vivo by β-lysase to methylselenol (*18*). This fact is very important because there is evidence that the facile endogenous production of monomethylated selenium is a critical factor in Se chemoprotection (*19*). Selenomethionine is known to be nonspecifically incorporated into proteins in place of methionine during protein synthesis. Thus, selenomethionine is not as readily accessible as Se-methylselenocysteine for further metabolism (*18, 20*). Recently, S. Cuello et al. have reported that MeSeCys protects human hepatoma cells against oxidative stress (*21*).

In a recent publication (*22*), the biotransformation of Se (IV) has been studied, when the process of lactic fermentation was carried out with bacteria *Lactobacillus* in the presence of increasing amounts of Se(IV) to produce Se-enriched yogurt. The main species found were selenocystine ($SeCys_2$) and Se-methylselenocysteine (MeSeCys).

Selenomethionine was found at the same level of concentration in plain and enriched yogurt, indicating that *Lactobacillus* does not metabolize Se(IV) to SeMet.

Kefir is a fermented milk drink, and kefir grains are a combination of bacteria and yeasts in a matrix of proteins, lipids, and sugars, which produce a lactic-alcoholic fermentation, yielding a slighty alcoholic beverage with a consistency similar to thick yogurt. Nowadays, kefir is becoming increasingly popular because of its health benefits.

The aim of this study is to compare the biotransformation of inorganic Se during the lactic fermentation process yielding yogurt and kefir. Nature, concentration and biotransformation of selenium species obtained when yogurt and kefir are produced in a medium with increasing concentrations of Se(IV) were compared. Size exclusion chromatography (SEC) coupled to inductively coupled plasma-mass spectrometry (ICP-MS) was used to evaluate the different molecular weight distribution of water soluble selenium-containing proteins in fermented samples, in the presence of the two different types of microorganisms. Additionally, enzymatic digestion of aqueous extracts was carried out to evaluate the content of selenoamino acids in the high molecular weight proteins. It is also important to quantify the transformation of Se species in dairy products such as yogurt and kefir, at least during the maximum time elapsed from production to consumers. Thus, a stability study of Se species in both, fresh and lyophilised yogurt and kefir samples, for the maximum period in which these kind of samples are generally stored, was performed.

MATERIALS AND METHODS

Instrumentation. A Thermo-X Series ICP-MS, fitted with a Meinhard nebulizer and a Peltier cooled spray chamber (2 °C), was used for ^{82}Se monitoring. The instrumental conditions were forward power 1250 W, plasma gas flow rate 15 L min^{-1}, auxiliary gas flow rate 0.8 L min^{-1}, and carrier gas flow rate 1.1 L·min^{-1}. The ICP-MS instrument was coupled to a liquid chromatographic system, which consisted of a high-pressure pump, Jasco PU-2089 (Italy), and a six port Rheodyne 7725i injection valve. For Size-exclusion chromatography, Biosep-SEC-2000 and BioBasic60 columns were used. Anion-exchange chromatography was performed using a Hamilton PRP-X100 (Reno, NE) column. Reversed-phase chromatography was performed using a ODP2 HP-4E Shodex (Torrance, CA) column.

Selenium species extraction for enzymatic hydrolysis was carried out by using a Sonopuls ultrasonic homogenizer (Bandelin, Germany). It was fitted with an HF generator 2200 at a frecuency of 20 kHz and a titanium microtip of 3 mm diameter. Aqueous extraction was made with an ultrasonic bath, P-Selecta (Varian, Spain). The obtained extracts were centrifuged on an Eppendorf centrifuge 5804 F34-6-38 (Germany).

Samples were digested for total selenium determination in double-walled advanced composite vessels using a microwave oven (CEM, MSP 1000 Matheus, NC). For the determination of total selenium content, an Atomic Fluorescence Spectrometer, AFS, Excalibur, (P.S. Analytical Ltd. Orpington, Kent, UK) was used.

Selenium hydride was generated in a flow injection system, with a peristaltic pump (Gilson HP4Villiers-le-bel, France), a mixing and reaction coil (0.5 mm i.d. PTFE tubing), and a U-tube gas−liquid separator. The separator was connected to a commercial dryer membrane (Perma Pure Products, Farmingdale, NJ) to eliminate the moisture, which was also used as an interface to the AFS system.

Scanning electron microscopy used JEOL, model JSM 6400, accelerated tension 0.2 to 40 kV, resolution 35 Å, and distance of work 8 mm and 35 kV. A spectrometer of dispersion of X-ray energy incorporated a resolution of 133 eV to 5.39 keV; OXFORD, model INCA.

Reagents and Standards. All reagents were of analytical grade and were used without further purification. Selenium standards selenocystine ($SeCys_2$) ($COOH(NH_2)CH\ CH_2$-Se-Se-CH_2-$CH(NH_2)COOH$), selenomethionine (SeMet) (CH_3-Se-CH_2-CH_2-$CH(NH_2)COOH$), Se-methylselenocysteine (MeSeCys), (CH_3-Se-CH_2-$CH(NH_2)COOH$), Na_2SeO_3, and Na_2SeO_4 were purchased from Sigma (St. Louis, MO, USA). Stock solutions of 1000 mg L^{-1} (expressed as selenium) were stored at 4 °C, whereas working solutions were prepared daily by dilution with Milli-Q water.

The molecular weight standards for SEC were Blue dextran (2000 kDa); alcohol dehydrogenase (150 kDa); albumin (66 kDa), carbonic

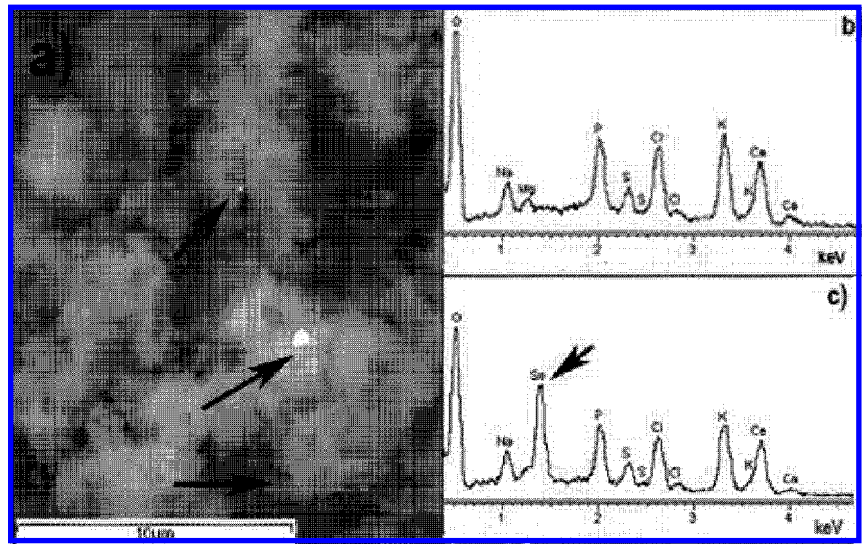

Figure 1. (**a**) Scanning electron microscopy images of yogurt enriched with 10 μg g^{-1}of selenium. Clear points, indicated by arrows, correspond to the segregated selenium. (**b** and **c**) EDX analysis of the matrix and the clear points, respectively.

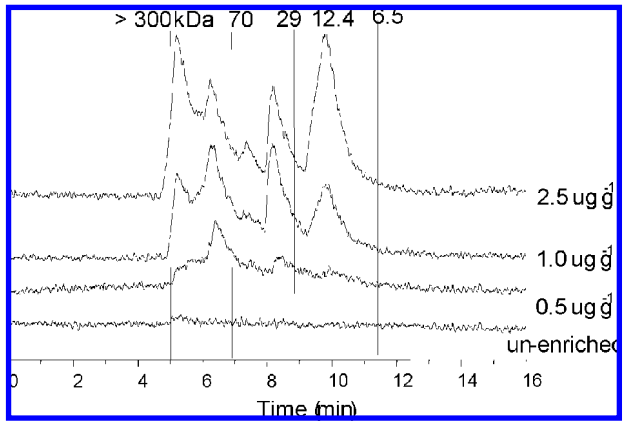

Figure 2. Size exclusion chromatograms (SEC/ICP-MS) using a Biosep-Sec 2000 column, of the aqueous extracts of Se-enriched (0.5, 1.0, 2.5 μg g^{-1}) and undialyzed kefir samples. Elution times for the molecular weight markers at 6.5, 12.4, 29, 70, and >300 kDa are highlighted.

anhydrase (29 kDa), cytochrome C (12.4 kDa), aprotinin (6.5 kDa); coenzyme B12 (1.6 kDa); and selenourea (123 Da) from Sigma Chemical (St. Louis, MO, USA). All of the standards were dissolved in a solution containing 50 mM Tris-HCl, from Fluka (Neu Ulm, Germany) and 100 mM KCl (Merck, Germany) at pH 7.5.

A 1% (w/v) sodium tetrahydroborate solution was prepared by dissolving $NaBH_4$ powder (Merck, Steinheim, Germany) in deionized water, stabilized in 0.1% (w/v) NaOH, and filtered to eliminate turbidity. A 4 M HCl solution was prepared by diluting concentrated HCl (Merck, Suprapur).

H_2O_2 (35%) from Panreac and HNO_3 (65%), distilled in our laboratory in a distilling system for acids, (Model BSB 9391 IR, Berghof, (Germany) were used to digest the samples.

The mobile phase for anion exchange chromatography was 10 mM citric acid (Sigma) in 2% (v/v) MeOH (Sharlab), pH 5, adjusted with ammonium hydroxide (Fluka) and 0.05% trifluoroacetic acid (TFA). 0.1% Hepta-fluorobutiric acid (HFBA), and 1% (v/v) MeOH at pH 2.6 were used for IP-RP-HPLC. The mobile phase for the SEC columns was a 5 mM phosphate buffer (Merck) at pH 7.5. All HPLC solutions were filtered using 0.45 μm Millipore Nylon filters and degassed before use, except for SEC, where 0.22 μm Millipore Millex-HV filters were used.

NIST 1549 Nonfat Milk powder was used as certified reference material for validation of total selenium content.

Procedures. *Kefir Fermentation Process.* Sodium selenite or selenate from 15 μg to 150 μg (as Se) was added to 30 mL of reconstituted skimmed milk (containing 3 g of milk powder). The fermentation process was started by the addition of 0.5 g of kefir. Fermentation was allowed for 24 h at room temperature. The fermentation process for yogurt was carried out as described in a previous work (*22*).

Dialysis. About 15 g of either unenriched or Se-enriched kefir were dialyzed. The dialysis process was performed for 20 h at 4 °C against Milli-Q water, using dialysis membranes with a 3.5 kDa molecular weight cutoff. During this period, the Milli-Q water was changed twice.

Total Selenium Determination. Total selenium content was determined in undialyzed and dialyzed kefir samples, in aqueous and enzymatic extracts. Two grams of sample were mineralized with 2 mL of (3:1) HNO_3/H_2O_2 in a microwave oven. Se (VI) in the digested sample was, by addition of 1.9 mL of 8 M HCl and a microwave oven treatment of 6 min at 650 W (*22, 23*), directly reduced to Se (IV). The samples were then diluted with 4 M HCl to a final volume of 10 mL. Measurements were carried out by HG-AFS and using external calibration (*22*)

Extraction of Water Soluble Proteins. Proteins from 5 g of kefir samples were extracted with Milli-Q water in an ultrasonic bath at room temperature for 30 min (*22*). Extracts were then centrifuged for 30 min at 14,000*g* and 4 °C, and the supernatants were then injected into the SEC column and measured by ICP-MS.

The SEC column was calibrated with molecular mass standards. The logarithm of molecular mass versus the ratio elution volume/void volume followed a linear response and was obtained using either an UV detector at 280 nm or by ICP-MS, monitoring ^{59}Co, ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, and ^{82}Se isotopes, which are the elements bound to protein standards. Stock protein standard solutions were prepared at a concentration of approximately 1000 mg L^{-1} in 50 mM Tris-HCl/100 mM KCl at pH 7.5. Diluted solutions with protein concentrations of 20−100 mg L^{-1} were prepared daily from the stock solutions with deionized water.

Proteolytic Digestion. Enzymatic digestion was carried out by 50 s sonication after the addition of 20 mg of Protease XIV to 2 g of unenriched and Se-enriched kefir and yogurt. The obtained extracts were then filtered through a 10 kDa cutoff centrifuge filter at 7500 rpm for 20 min. Reversed-phase and/or anionic exchange HPLC columns coupled to ICP-MS were used for selenium speciation in the filtrates (*22*).

Determination of Selenium Species by HPLC-ICP-MS. Extracts obtained by enzymatic hydrolysis were injected in anion-exchange HPLC or IP-RP-ICP-MS systems. Operating conditions are summarized in **Table 1**. Identification and quantification of selenium species were performed comparing the retention times of standards and using the standard addition method.

Species Transformation over the Storage Time. For the study of Se species transformation in fresh and freezed-dried yogurt and kefir

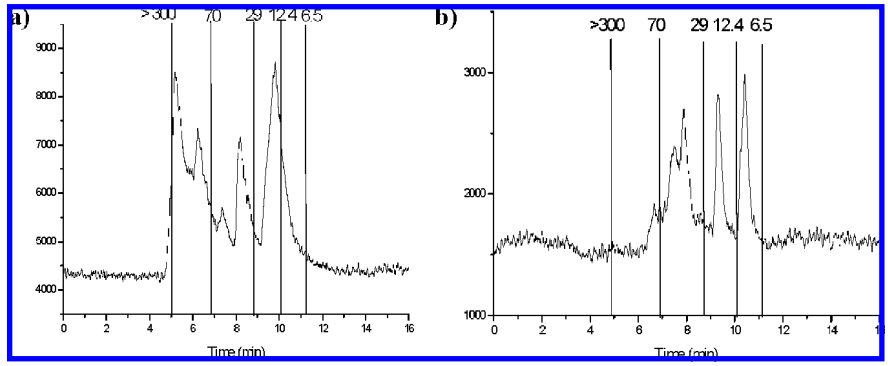

Figure 3. SEC/ICP-MS using a Biosep-Sec 2000 column of the aqueous extracts of Se-enriched (2.5 μg g^{-1}) kefir (**a**) and yogurt (**b**) samples. Elution times for the molecular weight markers at 6.5, 12.4, 29, 70, and >300 kDa are highlighted.

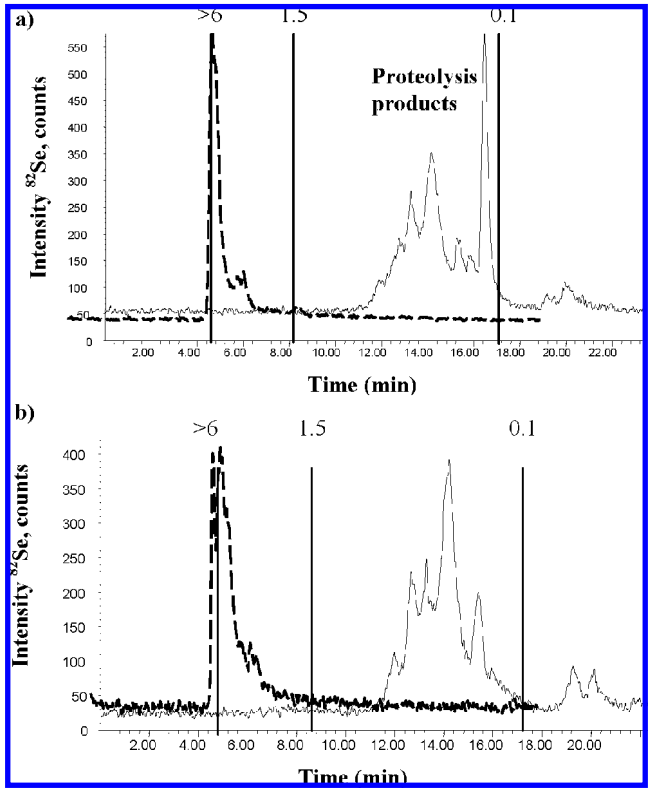

Figure 4. SEC/ICP-MS using a BioBasic60 column of extracts of Se-enriched (2.5 μg g^{-1}) kefir (**a**) and yogurt (**b**) samples before proteolysis (---) and after proteolysis with protease XIV (—). Molecular weight markers at 0.1, 1.5, and >6 kDa are highlighted.

samples, 2.5 g of each spiked sample (previously homogenized by 10 min of automatic stirring) was stored in polyethylene containers and maintained in the dark at 4 °C. Each vial was only used once for further experiments. The storage containers used were previously washed and immersed in a 10% HNO_3 bath for 24 h and rinsed several times with Mill-Q water before use.

Selenium-enriched yogurt and kefir were analyzed at different times (0, 1, 2, 8, 15, 17, 22, and 30 days) in order to evaluate the stability or transformation of the Se species. Three aliquots from each of the prepared vials were taken and treated following the procedure previously described. The reference Se concentration of each Se species (S_{ref}) was considered to be the one obtained at time 0.

RESULTS AND DISCUSSION

Lactic Fermentation of Kefir with Increasing Inorganic Selenium Concentrations: Comparison with Yogurt. Lactic fermentation of kefir and yogurt in the presence of up to 1000 μg g^{-1} of Se(IV) and Se(VI) has been evaluated in order to investigate the effect of Se concentration on fermentation and biotransformation yield.

The maximum Se (IV) concentration that allows kefir to be fermented was found to be 2.5 μg g^{-1}, much lower than the 50 μg g^{-1} found for yogurt.

A reduction of Se (IV) to Se^0 was observed when the Se concentration was higher than 5 μg g^{-1}. This phenomenon was observed by the red color of Se^0 and confirmed by scanning electron microscopy of lyopihilized samples (**Figure 1**). These results are in good agreement with those reported by Andreoni (*24*), who reports that Se^0 appears as result of a detoxification mechanism of microorganisms, allowing survival in the presence of high Se concentration.

The lactic fermentation to produce yogurt is compatible with the presence of Se(VI) in the whole range studied. However, this species is less efficiently biotransformed (below 20%) to organic Se than Se(IV). On the contrary, the addition of Se(VI) in the same concentration range inhibits kefir fermentation. Thus, Se(IV) has been chosen as the most appropriate species for further Se enrichment experiments.

Biotransformation Process. This study was carried out by fermenting the samples in the presence of Se(IV) (added as Na_2SeO_3) in the range from 0.5 μg g^{-1} to 5 μg g^{-1}.

As a first step for a fast evaluation of whether inorganic selenium has been biotransformed during the fermentation process, dialysis through a 3.5 kDa filter membrane was applied to both yogurt and kefir samples. Then, the total selenium content was determined in both samples before and after dialysis. The Se content present after dialysis can be assumed to be bound to molecules of a molecular weight higher than 3.5 kDa and thus to have been biotransformed during fermentation. Total selenium was quantified by hydride generation atomic fluorescence spectroscopy, using the conditions given in a previous work (*22*).

In order to select the optimum amount of fermented kefir to be added in the fermentation process, a preliminary study was carried out. The effect of the amount of kefir fermentation on inorganic selenium biotransformation (after 24 h) was evaluated and the results, shown in **Table 2**, reflect that the degree of biotransformation increases with increasing amounts of fermentation used, until a maximum yield is reached at 0.5 g of fermentation. Thus, this amount has been chosen for further experiments.

A comparative evaluation of the biotransformation in both yogurt and kefir samples is shown in **Table 3**. As can be seen, the degree of biotransformation (calculated from total selenium in samples after dialysis) is lower for kefir than for yogurt samples, being quantitative for Se(IV) concentration of up to 1 μg g^{-1} for kefir and 2.5 μg g^{-1} for yogurt. The different

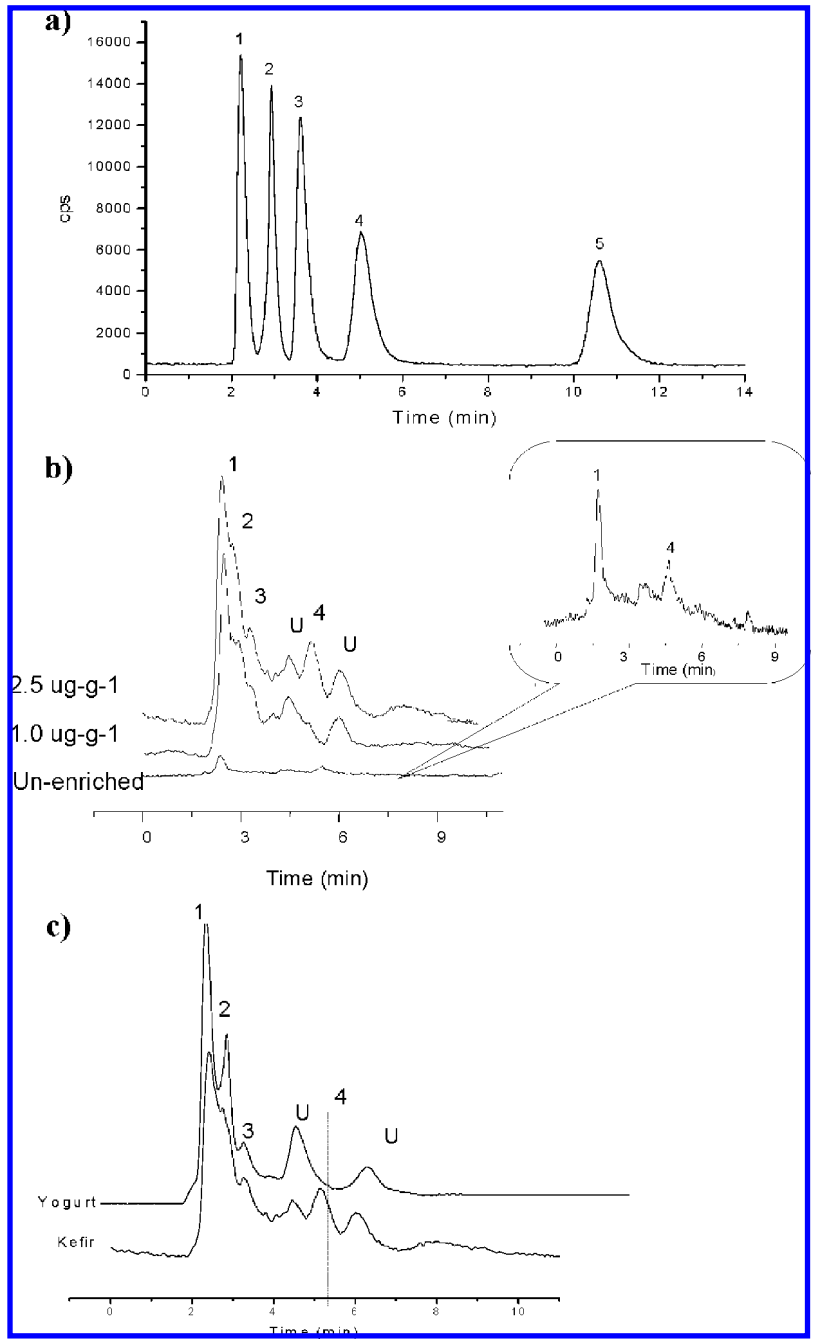

Figure 5. Anionic exchange chromatograms using a Hamilton PRP X100 column. (**a**) Mixture of Se standards containing 20 μg $L^{-1}$of each Se species: (1) $SeCys_2$; (2) MeSeCys; (3) Se(IV); (4) SeMet; (5) Se(VI). (**b**) Enzymatic extracts of unenriched and Se-enriched (1, 2.5 μg g^{-1}) kefir samples at 24 h fermentation time. (1) $SeCys_2$, (2) MeSeCys, (3) Se(IV), (4) SeMet, and (U) unknown. Amplified chromatogram shows the enzymatic extracts of unenriched kefir at 24 h fermentation time; (1) $SeCys_2$ and (4) SeMet. (**c**) Enzymatic extracts of 2.5 μg g^{-1} Se-enriched kefir (**a**) and Se-enriched yogurt (**b**). The dashed line indicates the retention time for SeMet.

behavior between yogurt and kefir samples is due to the different microorganisms involved in the lactic fermentation process. In the case of yogurt, the responsible organisms are bacteria from the *Lactobacillus* genus, while the kefir samples also contain yeast such as *Saccharomyces*.

Chromatographic Profiles of Aqueous Soluble Kefir Extracts Obtained by SEC. Total selenium concentration in aqueous extracts from kefir was determined by the experimental procedure described before. Selenium recoveries in the aqueous extracts were about 20% for the enriched kefir, higher than the recoveries found for yogurt (10%). The aqueous extracts of kefir were injected on a Biosep-SEC-2000 column coupled to ICP-MS, and the ^{82}Se was monitored. Five chromatographic peaks (**Figure 2**) were obtained, two of them in the mass interval from 300 to 70 kDa, two in the range of 70−29 kDa, and a very intense peak with a molecular weight around 10 kDa. These peaks are not present in the original kefir (see **Figure 2**), and it can be clearly observed that in general the intensity of the chromatographic peaks increases with increasing Se (IV) concentration. This fact confirms the incorporation of selenium into high molecular weight molecules when the lactic fermentation of kefir was carried out in the presence of Se(IV).

In order to compare the behavior of the different microorganisms involved in the lactic fermentation process in yogurt and kefir, SEC chromatograms of aqueous extracts of both samples,

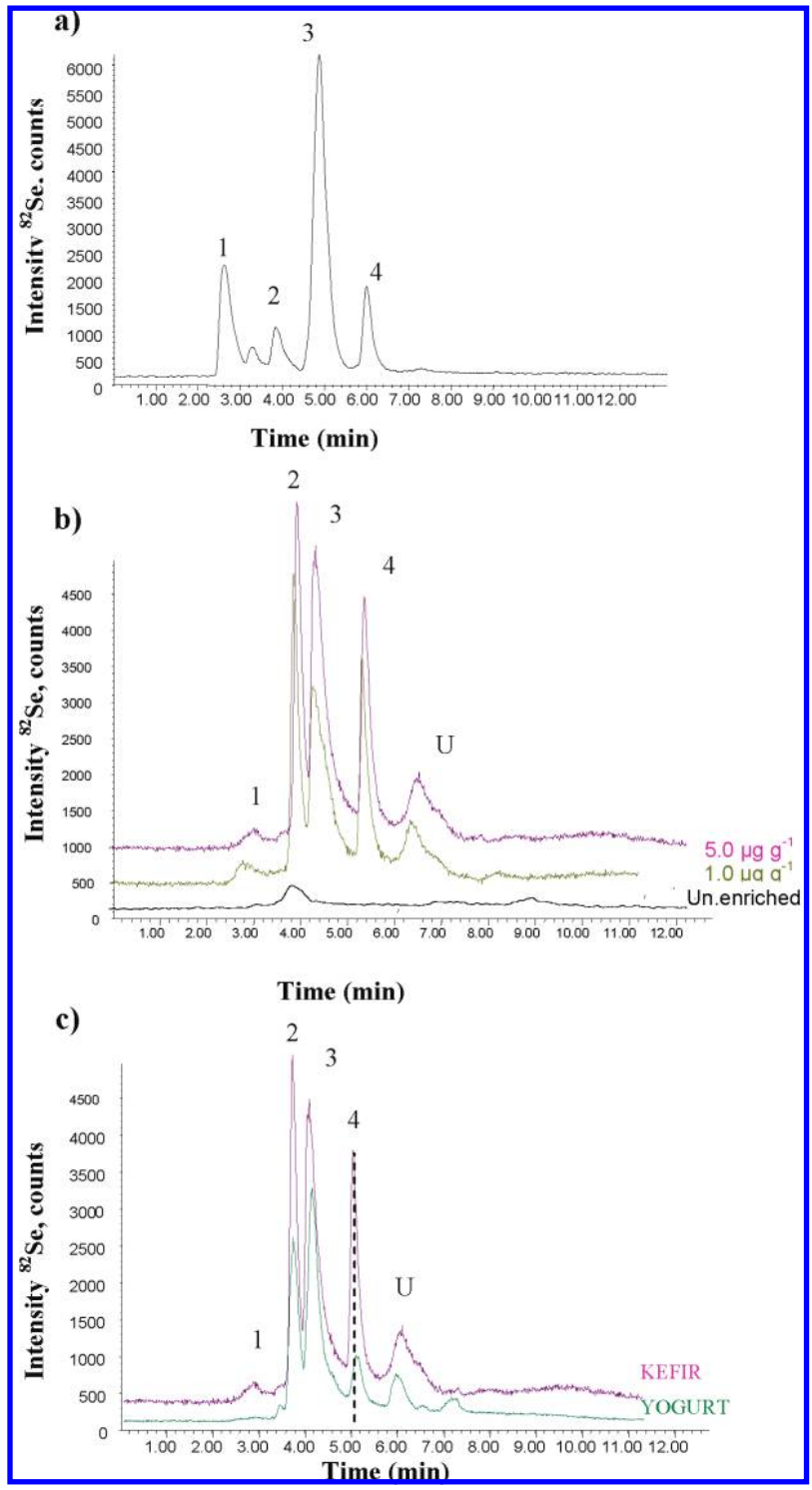

Figure 6. Ion pairing reversed phase chromatograms using a ODP2 HP-4E Shodex column. (**a**) Se standards containing 50 μg L^{-1} of Se each species: (1) Se (IV) + (VI); (2) $SeCys_2$; (3) MeSeCys; (4) SeMet. (**b**) Se species after enzymatic digestion of dialyzed kefir samples unenriched and enriched with 1.0, 5.0 μg g^{-1} of Se(IV). (1) Se (IV)+(VI), (2) $SeCys_2$, (3) MeSeCys, (4) SeMet, and (U) unknown. (**c**) Enzymatic extracts of 5.0 μg g^{-1} Se-enriched kefir (**a**) and Se-enriched yogurt (**b**). The dashed line indicates the retention time for SeMet.

enriched with 2.5 μg g^{-1} of Se(IV), were obtained. The chromatographic profiles are different (see **Figure 3a** and **b**). The most important difference is the high molecular weight of the Se binding compounds present in the aqueous extracts of kefir (ranging from 300 to 70 kDa), which are not found in the case of yogurt. This fact allows one to confirm the different behavior of both microorganisms.

In order to determine the nature of the high molecular weight Se-compounds, enzymatic hydrolysis was carried out. Aliquots of aqueous extracts from yogurt and kefir were subjected to digestion with a proteolytic enzyme, protease XIV, using an ultrasonic probe (USP). The digests were analyzed by size exclusion chromatography coupled to ICP-MS, with a column having a working range between 6.0 to 0.1 kDa. Chromatograms of the aqueous extracts of yogurt and kefir samples, before and after enzymatic digestion, are shown in **Figure 4a** and **b**. As can be observed, selenium peaks shifted to lower molecular weight regions after the

Table 4. Selenium Species Quantification[a] in Hydrolysed Extract of Non-Enriched and Enriched (a) Yogurt and (b) Kefir after 24 h Fermentation Time

(a)

species	non-enriched yogurt	Se-enriched yogurt 0.5 μg.g^{-1}	1.0 μg.g^{-1}	2.0 μg.g^{-1}
$Se(Cys)_2$	3.3 ± 0.2	102 ± 2	107 ± 1	581 ± 3
MeSeCys			145 ± 0.5	312 ± 2
Se (IV)		5.7 ± 0.4	110 ± 1	162 ± 2
SeMet	24 ± 0.5	24 ± 0.5	33 ± 2	10 ± 1

(b)

species	non-enriched kefir	Se-enriched kefir 1.0 μg.g^{-1}	2.5 μg.g^{-1}	5.0 μg.g^{-1}
$Se(Cys)_2$	97.5 ± 0.5	311 ± 5	690 ± 5	1277 ± 10
MeSeCys		70.2 ± 0.3	377 ± 5	525 ± 2
Se (IV)		17.5 ± 0.4	114 ± 2	221 ± 3
SeMet	10.3 ± 0.2	31.7 ± 0.2	155 ± 2	180 ± 1

[a] ng g^{-1} average of three replicates.

enzymatic digestion of the extracts, confirming the high efficiency of the simultaneous USP-enzymatic hydrolysis procedures and suggesting that selenium is bound to proteins.

Speciation Studies Using Anion Exchange and Reversed-Phase HPLC with ICP-MS. Enriched and non-enriched kefir samples were incubated with protease XIV using an ultrasonic probe, following the procedure described above. After enzymatic hydrolysis, samples were centrifuged, and the supernatants were filtered through 10 kDa cutoff membranes. The selenium recovery for enzymatic extracts was higher than 92% after filtering them.

Selenium species released by the enzymatic hydrolysis of the enriched kefir at different levels (1.0−2.5 μg g^{-1}) were analyzed using two chromatographic methods: anionic exchange and ion-pair reversed phase.

Separation of selenium species standards by anionic exchange chromatography was performed in a Hamilton PRP-X100 column, using 10 mM ammonium citrate, pH 5.0, in 2% MeOH as the mobile phase (**Figure 5a**). The dead volume (evaluated with trimethyselenonium) was 1.6 min.

The chromatographic profile of hydrolyzed kefir samples is shown in **Figure 5b**. The species identified in the chromatogram, $SeCys_2$,(t_R 2.4 min), MeSeCys (t_R 2.8 min), Se(IV) (t_R 3,3 min), and SeMet (t_R 5.2 min), have been identified by their retention times and by spiking of the samples. Additionally, two unidentified peaks (U) appear in the chromatogram. The amounts of $SeCys_2$, MeSeCys, and SeMet, the three major species to which Se is transformed, increased proportionally with the concentration of inorganic selenium present in the growing media.

The chromatograms of kefir and yogurt, both enriched samples with 2.5 μg g^{-1} of Se(IV), reflecting an important difference: the SeMet species is not present in the yogurt sample (**Figure 5c**).

A second chromatographic mode based on ion pairing RP-HPLC was performed in order to support the identification of the peaks. A mixture of 0.05% trifluoroacetic acid (TFA) and 0.1% heptafluorobutyric acid (HFBA) at pH 2.6 in 1% MeOH was used for effective ion pairing. Under optimized conditions and at a flow rate of 0.7 mL min^{-1}, the separation of Se compounds was achieved within 7 min (**Figure 6a**).

Chromatograms resulting from ion-pairing reversed phase chromatography of kefir samples shown in **Figure 6b** confirm the identification of $SeCys_2$, SeMet, and MeSeCys. It is important to remark that a good correlation between the amount of the three major species and the amount of selenium added during kefir fermentation was found.

If the results obtained for enriched kefir samples are compared to those obtained for enriched yogurt samples (see **Figure 6c**), the most important difference is the formation of SeMet as a biotransformation product of inorganic selenium during lactic fermentation in kefir samples, with an increase at higher Se (IV) concentrations added. On the contrary, the concentration of SeMet in yogurt remains constant, its content being similar to that of plain yogurt.

Selenium species present in the dialyzed enriched kefir enzymatic hydrolysis extracts were quantified using the standard additions method. Results illustrated in **Table 4** show that an increase of Se(IV) during lactic fermentation does not produce an increase of SeMet in the yogurt samples, while a directly proportional behavior between both compounds (SeMet after lactic fermentation and Se(IV) added during the fermentation process) is found in the case of kefir samples.

This is in fact in accordance with previously published results, clearly demostrating the different behaviors of the microorganisms *Lactobacillus* (*24, 25*) and *Saccharomyces* used for kefir production (*1, 12, 13*) when exposed to inorganic selenium.

Study of the Transformation of the Se Species with Time. The aim of this study was to evaluate the transformation suffered by the three main selenium species present in yogurt and kefir, over the maximum storage time to be expected before consumption of these diary products. The experimental procedure for this evaluation is previously described.

Recently prepared yogurt and kefir samples were stored in polyethylene vials at 4 °C, in which standards of selenium of 0.4 μg mL^{-1} are stable for at least one year (*26*).

The transformation degree (R_x), was evaluated as the ratio of the average of three signal measurements made in samples a different storage times (S_x), to the value of the signal at time = 0 (S_{ref}), the latter being the average of six measurements.

$$R_x = [(S_x)/(S_{ref})]$$

The uncertainty U_x of the ratio R_x was obtained from the variation coefficient of each set of measurements according to the following expression:

$$U_x = (CV_x^2 + (CV_{ref}^2)^{1/2} R_x$$

where CV_x is the variation coefficient of three independent measurements under each storage condition, and CV_{ref} is the variation coefficient obtained for the reference conditions.

The value R_x should be betwen the limits $1 \pm U_{ref}$ to conclude that the species has not been transformed (see **Figures 7b** and **8b**, dashed lines).

The results of the experiment in the case of the fresh yogurt sample are shown in **Figure 7a** and **b**. As can be seen, $SeCys_2$ remains stable for 15 days (**Figure 7a**), after which a drastic reduction in its concentration is observed. This is associated with an increase in the concentration of MeSeCys, which then becomes the major species. The chromatograms for $t = 1$ day, $t = 15$ days, and $t = 30$ days obtained by anionic exchange HPLC are also shown in **Figure 7a** to illustrate the behavior mentioned above.

The results obtained for the kefir sample are shown in **Figure 8a** and **b**. In this figure, the same transformation of $SeCys_2$ to MeSeCys can be observed. These results agree with those found by Dumont et al. (*1*). The main difference was that Se species transformation in kefir starts before the one occurring in yogurt,

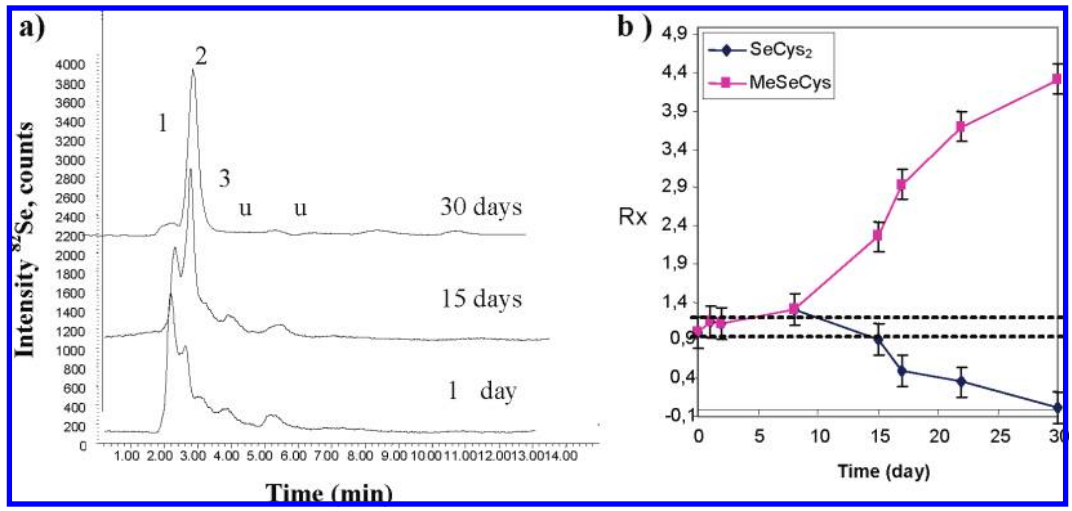

Figure 7. Selenium species transformation as a function of time of storage for Se-enriched yogurt with 2.0 μg g^{-1}. (**a**) Chromatograms obtained by anionic exchange chromatography. (**b**) Rx vs storage time.

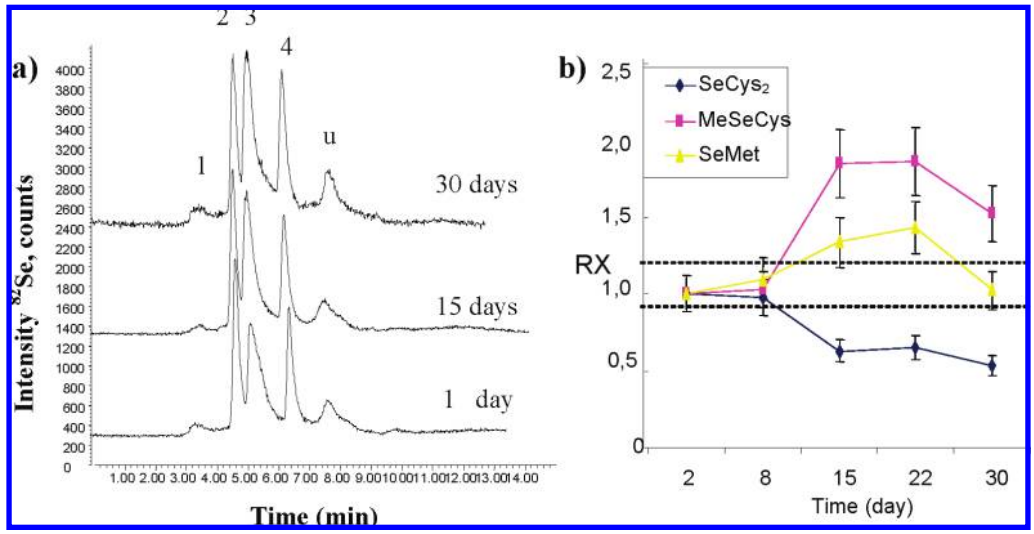

Figure 8. Selenium species transformation as a function of time of storage for Se-enriched kefir with 2.0 μg g^{-1}. (**a**) Chromatograms by ion pair reversed phase chromatography. (**b**) Rx vs storage time.

from the 10th day after fermentation and reaching a plateau from day 15. SeMet in kefir remains constant over the whole period studied.

Similar conditions of storage have been applied to lyophilized samples. In this case, the concentration of the three species ($SeCys_2$, MeSeCys, and SeMet) remained constant over the evaluated period, indicating that the transformation process during storage may be due to microbiological action on the selenium species present.

Conclusions. The different behaviors of the microorganisms present in two different lactic fermentations processes to obtain yogurt and kefir have been studied. A relationship between the amount of Se(IV) added and the concentration of SeMet in the enriched sample has been found only in the case of kefir. Furthermore, the molecular weight of Se-compounds in aqueous extracts of kefir was found to be higher than those of yogurt.

In both cases, an increase in the concentration of Se-methylselenocysteine in the final enriched product is obtained. This is important because this molecule is known to be a precursor for methylselenol, which is considerably more active than selenite or selenomethionine. Future experimentation will be focused on the study of the potential effects against chemical oxidative stress induced by hydroperoxidos of these selenium-enriched products in some biological systems.

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