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Selenium accumulation by *Lactobacillus* isolated from commercial fermented milk: Minimum inhibitory concentration and kinetic growth changes

Acumulación de selenio por *Lactobacillus* aislados de leches fermentadas comerciales: Concentración mínima inhibitoria y cambios cinéticos de crecimiento

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Abstract

Selenium is essential for human health; however, recommended daily intake is not always met. Thus, studies have been carried out on the biogenic production of more bioavailable selenium. It has been demonstrated that certain lactobacilli can metabolize inorganic selenium to transform it into selenoamino acids. This study aimed to add selenium on *Lactobacillus casei* Shirota and *Lactobacillus johnsonii* La1, isolated from commercial dairy products through an MRS media fermentation enriched with Na_2SeO_3 to determine the minimum inhibitory concentration, the changes in the kinetics growth, and the selenium bioaccumulation. The minimum inhibitory concentration (MIC) was determined using the Talmadge and Fitch method. Kinetic changes were calculated by modeling the growth curve, and an inductively coupled plasma (ICP) assay was used to determine selenium accumulation by the cell. The MIC of Na_2SeO_3 was higher than 190 mg/L in both bacteria, and kinetic changes showed faster growth when media was not enriched. Selenium absorption of 64.50 % was found for *Lb. casei* Shirota and 75.78 % for *Lb. johnsonii* La1. Obtained results demonstrated that these lactic acid bacteria bacteria are a potential ingredient in functional food processing to their ability to accumulate selenium.

Keywords: Selenium, lactic acid bacteria, *Lactobacillus*, selenoamino acid, selenocysteine.

Resumen

El selenio es un elemento esencial para la salud humana; sin embargo, no siempre se cumple la ingesta diaria recomendada. Por lo tanto, se han llevado a cabo estudios para la producción biogénica de selenio más biodisponible. Se ha demostrado que determinados lactobacilos son capaces de metabolizar el selenio inorgánico para transformarlo en selenoaminoácidos. Es por ello que el objetivo de este estudio fue insertar selenio en *Lactobacillus casei* Shirota y *Lactobacillus johnsonii* La1, aislados de productos lácteos comerciales a través de un medio de fermentación MRS enriquecido con Na_2SeO_3 para determinar la concentración inhibitoria mínima, los cambios en la cinética de crecimiento y la bioacumulación de selenio. La concentración mínima inhibitoria (CMI) se determinó utilizando el método de Talmadge y Fitch. Los cambios cinéticos se calcularon modelando la curva de crecimiento, y se usó el ensayo de plasma acoplado inductivamente (ICP) para determinar la acumulación de selenio por parte de la célula. La CMI de Na_2SeO_3 fue superior a 190 mg/L en ambas bacterias y los cambios cinéticos mostraron un crecimiento más rápido cuando los medios no estaban enriquecidos. Se encontró una absorción de selenio de 64.50% para *Lb. casei* Shirota y 75,78% para *Lb. johnsonii* La1. Los resultados obtenidos demostraron que estas bacterias probióticas son un ingrediente potencial en el procesamiento de alimentos funcionales debido a su capacidad de acumulación de selenio.

Palabras clave: selenio, probiotico, *Lactobacillus*, seleno-aminoácidos, selenocisteína.

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1 Introduction

Selenium (Se) is part of 25 selenoproteins, which are enzymes mainly containing selenocysteine (SeC) in their active center (Zoedis *et al*. 2018). Among them are selenoenzymes that include glutathione peroxidase, iodothyronine deiodinase, and thioredoxin reductase (Amit *et al*., 2019). This element is essential for humans (50 to 60 μg/day) because Se exhibits an important antioxidant activity, protecting cells from oxidative stress (Zhang *et al*., 2020a). Different diseases are related to Se deficiency; for example, some implied thyroid disorders (Gheorghiu and Badiu, 2020). In addition, it has been proved that organic selenium of biogenic origin, especially that metabolized by LAB, correlates with the regulation of thyroid hormone (Ibrahim *et al*., 2019). Thus, Se supplementation through food is necessary, especially in regions where Se concentrations in soil are lower, for example, in southern and eastern European countries. However, supplementation with inorganic species of Se, such as selenate or selenite (Se IV and Se VI), is usually most toxic due to their high nephrotoxicity and accumulation in kidneys, liver, and other organs (Krausova *et al*., 2020).

Notably, some strains of lactic acid bacteria (LAB), which are part of the gastrointestinal microbiota of both healthy individuals and those with some degree of dysbiosis, have demonstrated their ability to bind, capture, absorb, and bio-transform metallic ions from the growth media (Mrvčić *et al*., 2012). Similarly, these microorganisms could be part of the food industry, especially in those processes that involve milk transformation. These kinds of processes take advantage of the properties and synergism of LAB, which affect both the rheological and sensory properties of foods (Kousha *et al*., 2017). Taking advantage of the LAB capacity for metallic ions absorption, developing these selenized microorganisms has gained attention in research aimed at searching for safety sources of more bioavailable selenium (Ringuet *et al*., 2021; Gangadoo *et al*., 2020; Pham *et al*., 2019).

Ferreira *et al*. (2021) found in an in vivo study with mice that the Se level supplemented through the diet affects the selenoproteins biosynthesis because there is a competition for selenium between microbiota and the host. Then, other authors have reported the same conclusion according to these results related to obesity increment and selenoproteins production (Watanabe *et al*., 2020). Recently, Zhang *et al*. (2020b) described the importance of the Se transformation into selenoamino acids that subsequently being inserted in selenoproteins structure, which have anti-COVID-19 activity. In addition, it is known that the inserted Se to LAB is converted into Se-methylselenocysteine and γ-glutamyl-Se-methylselenocysteine, which have demonstrated effects on cancer prevention in animal models (Pophaly *et al*., 2014; Alzate *et al*., 2010).

Lactic acid bacteria could be used in symbiotic products when they have probiotic capacity (Ceja-Medina, *et al*., 2021, Martínez-Preciado *et al*., 2021)). Although LAB represents a promising group for obtaining organic selenium, the legislation does not consider its use as a selenized microorganism in food processing. Therefore, it is necessary to increase the information to remark the safety, functionality, and properties of selenized LAB. Thus in recent years, it has been advanced studies on selenization of probiotic and not probiotic LAB, emphasizing absorption and their functional properties related to human health (Chen *et al*., 2019; Cruz *et al*., 2018). However, it is necessary to know selenization conditions and levels of absorption, which are implied during the fermentation process, to observe and analyze the feasibility of selenized LAB.

That is why this work aimed to study the minimum inhibitory selenium concentration for two lactic acid bacteria isolated from commercial dairy products, determining their changes in kinetic parameters during selenization and the concentration of absorbed selenium to verify their ability to survive in the presence of an inorganic salt of selenium and its level of absorption.

2 Materials and methods

2.1 Samples

Lactobacillus casei Shirota and *Lactobacillus johnsonii* La1 were isolated from two dairy products that declared their presence (Yakult® and Chamyto Danone®, respectively). They were propagated in MRS (Man Rogosa and Sharpe) broth incubating at 37 ºC for 24 h. Purity of culture was proved using a Gram-stained. Isolated bacteria were stored at -4 ºC in MRS broth with the same volume of glycerol. A viable cell determination was carried out in each vial using the plate count method.

2.2 Determination of minimum inhibitory concentration of Na_2SeO_3

Enriched media were prepared, adding different dilutions of Na_2SeO_3 from a solution of 500 ppm. The solution was prepared with sterile deionized water (120 ºC, 15 min, 1.5 psi of pressure). The desired concentration of selenium was placed in assay tubes, and MRS broth was added to complete 10 mL. The concentrations of Na_2SeO_3 tested were: 20, 40, 60, 80, 100, 150, 200, 250 and 300 mg/L of Na_2SeO_3. All solutions were sterilized before their use. A concentration of 10^6 CFU of lactic acid bacteria was inoculated in each tube and incubated for 36 h at 37 ºC. The viability of each tested tube was measured through plate count methodology ending the incubated time. Minimum inhibitory concentration (MIC) was determined using the graphical method of Talmadge and Fitch (Peña and Circo 2007), with González-Olivares *et al*. (2016) modifications.

2.3 Fermentation

2.3.1 Cell concentration

A curve of cell growth was developed of each lactobacilli growth in MRS broth enriched with the Na_2SeO_3 calculated as the minimum inhibitory. Each of the curve was compared with one carried out without selenium. Fermentations were carried out at 37 ºC for 36 h in anaerobic conditions. The cell concentration was determined by the plate count method in MRS-agar, sampling each two hours.

2.3.2 Kinetic parameters

Kinetic parameters were calculate aimed at the metabolic differences that lactic acid bacteria presented in the presence of Na_2SeO_3. The specific growth rate constant (μ) was calculated according to equation 1 (Eq. 1). Equations 2 and 3 were used to determine the generation time (g) and the constant growth rate (K). The initial concentration (N_0) and the final (N_x) of biomass corresponded to the time interval selected in the logarithmic phase, which were t_0 and t_x, respectively.

$$\ln(N_x) - \ln(N_0) = \mu(t_x - t_0) \quad (1)$$

$$g = \ln(2)/\mu \quad (2)$$

$$K = 1/g \quad (3)$$

2.4 Concentration of absorbed selenium

2.4.1 Biomass separation

Biomass separation was performed according to González-Olivares *et al*. (2016). It was placed 1 mL of fermented MRS enriched in Eppendorf tubes. The sample was centrifuged at 10,000 × g for 15 min at 4 ºC to separate the biomass of the culture medium. The supernatant was recovery. Cells were washed twice with a 0.3 % (p/v) of dithiothreitol (DTT) (Sigma Aldrich) to eliminate the excess of selenium that could be adhered to the cell membrane, centrifuging, and decanting between each rinse. The supernatant was mixed with its respectively DTT rinse to calculate the Se concentration by ICP. The biomass obtained was stored at -4 ºC for subsequent analysis.

2.4.2 Inductively coupled plasma-optical emission spectrometry (ICP-OES) analysis

Using the methodology of Castañeda-Ovando *et al*. (2019), the absorbed selenium by lactic acid bacteria tested was analyzed. At 1 mL of sample were added 5 mL of concentrate HNO_3 and 4 mL of deionized water. The mixture was digested in an accelerated microwave reaction system (MARS 5 microwave, CEM Corporation, Matthews, NC), using a temperature gradient from room temperature to 175 ºC for 5.5 min and then from 175 ºC to 180 ºC for 4.5 min. The pressure was 110 psi. The solution was made up to 25 mL at the end of the reaction. A calibration curve was made at different selenium concentrations (0.25, 0.50, 0.75, 1, 1.25, and 1.5 ppm), diluting from a Se stock solution of 50 ppm in 5% HNO_3. Standards and samples were analyzed by ICP-OES, using an Optima 8300 spectrometer from PerkinElmer (Waltham, MA) at an emission wavelength of 196 nm.

2.5 Statistical analysis

All experiments were performed in triplicate. ANOVA statistically analyzed experimental data, and means were compared by Tukey's method ($p < 0.05$). The NCSS-2007 (v.07.1.15; NCSS LLC, Kaysville, UT) software was used for the statistical analysis.

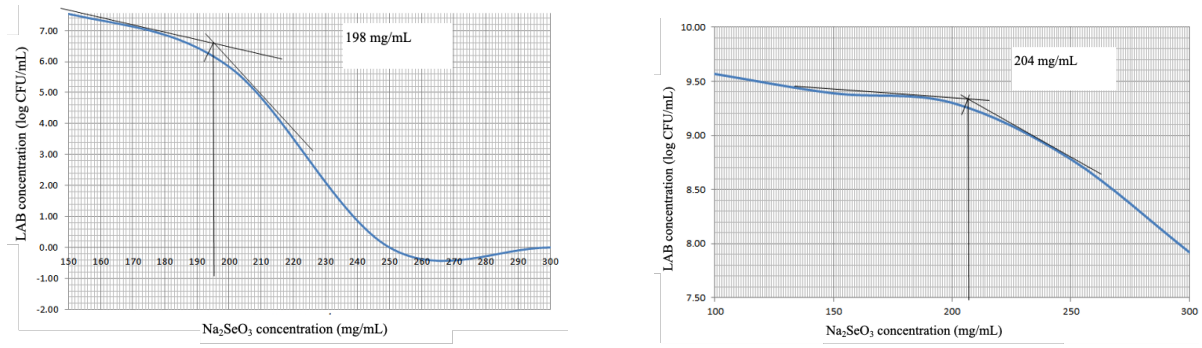

Figure 1. Minimum inhibition concentration (MIC) of lactobacillus exposed to different concentration of Na_2SeO_3 a) *Lb. casei* Shirota and b) *Lb. johnsonii* La1. Use of the Taldmadge and Fitch graphical method.

Table 1. Viability of *Lb. shirota* and *Lb. johnsonii* La1 growth at different concentrations of Na_2SeO_3 in MRS-broth.

Concentration of Na_2SeO_3 (mg/L)	log CFU/mL *Lb. casei*	log CFU/mL *Lb. johnsonii*
0	9.37 ± 0.04^{a}	9.50 ± 0.05^{a}
20	8.82 ± 0.07^{b}	9.99 ± 0.00^{b}
40	8.92 ± 0.10^{b}	9.81 ± 0.03^{c}
60	8.22 ± 0.13^{c}	$9.50 \pm 0.03^{c,d}$
80	8.30 ± 0.11^{c}	$8.58 \pm 0.07^{c,d}$
100	8.20 ± 0.13^{c}	$9.57 \pm 0.01^{d,e}$
150	7.54 ± 0.13^{d}	9.39 ± 0.01^{e}
200	5.83 ± 0.13^{e}	9.30 ± 0.00^{f}
250	0^{f}	8.78 ± 0.09^{g}
300	0^{f}	7.92 ± 0.07^{h}

Values are mean ± SD (n = 3); values in the same column with different superscript letters are significantly different ($p < 0.05$).

3 Results and discussion

3.1 Minimum inhibition concentration

Minimum inhibition concentration was calculated for each microorganism that was tested in the Se concentration using the viability results that are shown in Table 1. It was observed in the viability of *Lb. casei* Shirota, the initial count in not selenium media was 9.37 log CFU/mL. Selenium concentrations in media from 20 to 100 mg/L obtained approximate viability of 8 logarithmic cycles of CFU.

In the case of *Lb. johnsonii* La1, the initial concentration in the MRS medium without selenium was 9.5 log CFU/mL. This microorganism concentration remained constant up to 80 mg of Na_2SeO_3/mL observing decrees of 1 logarithmic cycle but the bacterial concentration was recovered to initial value. Then, a slight decrease in microorganism concentration was observed at 250 mg/mL. At Na_2SeO_3 concentrations of 300 mg/mL, a reduction of one logarithmic cycle showed respect to the previous concentration and two concerning the initial. Comparing these results with those observed for *Lb. casei* Shirota, there was a higher resistance to the presence of selenium by *Lb. johnsonii* La1. Data obtained calculated the MIC, which was 198 mg/L for *Lb. casei* Shirota and *Lb. johnsonii* La1 204 mg/L. Results of the Taldmadge and Fitch graphical method are showed in the figure 1a and 1b.

Authors such as Pusztahelyi *et al*. (2015) and Xu *et al*. (2018) have determined higher minimum inhibition concentrations of Na_2SeO_3 for species of *Lb. casei* (1 g/L and 206 mg/L respectively). Nevertheless, in both studies, LAB was conditioned in enriched media with Na_2SeO_3 to develop the bacteria resistance to high concentrations of inorganic selenium. Both studies aimed to prepare LAB for the biogenic transformation of seleno nanoparticles. In contrast, different studies have aimed to study the selenium accumulation capacity of LAB of inorganic sources (Krausova, *et al*., 2020; Castañeda-Ovando, *et al*., 2019; Xu *et al*., 2018; González-Olivares, *et al*., 2016). Others have been carried out to use selenized LAB as a potential starter in functional dairy products (Pophaly *et al*., 2014; Martínez *et al*., 2019; Crespo *et al*., 2021).

It is known that LAB used as starters are very labile and have little ability to adapt. However, these microorganisms survive at Se concentration higher than 200 mg/L when they are developed in enriched media with an inorganic source of selenium such as Na_2SeO_3. But it has been verified that selenite reduces to elemental selenium (Se^0) or hydrogen

selenide (H_2Se) due to a detoxification mechanism. This mechanism is believed to protect bacteria against damage selenium could cause within the cell (Krausova *et al*., 2020; Martínez *et al*., 2019). In this sense, the detoxification mechanism may not be inactivated at tolerance concentrations, which could cause the inhibition.

In this study, evidence was found that an interaction between cell and selenium at MIC of each lactic acid bacteria exists, activating a probably detoxification mechanism through a bioconversion from selenite to selenide. This activation is started by proteases produced to avoid oxidative damage to cells. According to Wu *et al*. (2015), with higher MIC concentrations, bacteria cannot reduce selenium, causing a growth inhibition because the cell cannot adapt to media enriched with selenium. Consequently, there is a complete growth inhibition as observed in the viability analysis of *Lb*. *casei* Shirota, which showed total inhibition at 250 mg/L.

3.2 Fermentation kinetics

A comparison of lactic acid bacteria growth data developed in enriched and no enriched MRS-broth was made. Results of sampling every two hours are shown in figure 2.

The growth of *Lb*. *casei* Shirota in enriched medium presented a displacement in the deceleration stage for the deceleration time in medium without Se (15 h and 6 h, respectively). In contrast, the deceleration stage during the growth of *Lb*. *johnsonii* La1 in the supplemented medium was presented in the non-enriched medium (10 h and 12 h, respectively). It is known that some LAB activates metabolic processes in the presence of salts such as Na_2SeO_3 due to the detoxification mechanism (Escobar-Ramírez *et al*., 2021). However, the cell concentration was higher in media without supplementation, which coincides with the results reported by Martínez *et al*. (2020). They determined that the cell concentration of some LAB such as species of *Lactobacillus rhamnossus* and species of *Lactococcus* was higher in non-enriched media. A calculation of kinetic parameters was carried out to determine differences caused by the displacement of the deceleration stage. Results were observed in table 2.

It was observed that the presence of Se affects the growth rate of *Lb*. *casei* Shirota. The growth rate was lower in presence of the salt compared to the growth observed in media without enrichment. In addition, the generation time was 3.7 times higher in the presence

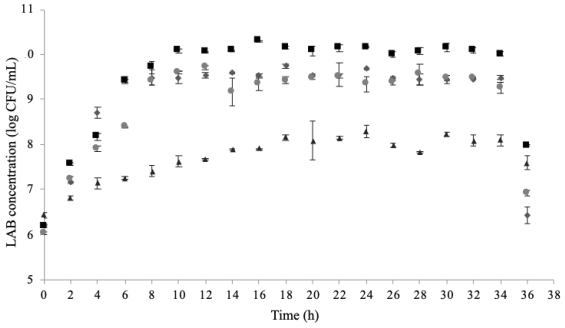

Figure 2. Growth curves of *Lb. casei* Shirota in media (▲) with Na_2SeO_3 and (⧫) without Na_2SeO_3; *Lb. johnsonii* La1 in media (●) with Na_2SeO_3 and (■) without Na_2SeO_3.

of Se. However, in the case of *Lb*. *johnsonii* La1, the presence of Se did not affect kinetic parameters on the microorganism growth, according to statistics analysis.

Some authors such as Martínez *et al*. (2020), Kousha *et al*. (2017), and Andreoni *et al*. (2000) have reported the activation of LAB growth exerted by Na_2SeO_3 at low concentrations (1-5 ppm) in MRS-broth. However, the activation is due to LAB taking up the inorganic Se of the medium, transforming it into organoselenium compounds, and assimilating it into different cell fractions, such as proteins, polysaccharides, and nucleic acids (Pieniz *et al*., 2017; Zhang *et al*., 2009). Additionally, Se accumulation for LAB growth at lower concentrations of the MIC referred to an increase of selenoamino acids concentration. In contrast, the accumulation of selenium in enriched media at a higher concentration of the MIC is related to the transformation of selenite to elemental selenium deposited near the cell membrane (Kousha *et al*., 2017). Escobar-Ramírez *et al*. (2021) reported that selenium accumulated in the cell periphery is accumulated simultaneously in the periplasm, especially in those bacteria producing seleno nanoparticles. However, in different cases, the Se bioaccumulation has not affected the cellular growth, specifically in the stationary phase. Martínez *et al*. (2020) reported that only specie of *Lactobacillus rhamnossus* among 10 *Lactobacillus* tested presented significant differences in growth rate (μ).

3.3 Absorbed Se concentration

Table 3 shows the inorganic Se bioaccumulation of each lactic acid bacterium tested. The higher absorption of Se (75.78 %) was observed in *Lb*.

Table 2. Kinetic parameters during growth of *Lb. casei* Shirota and *Lb. johnsonii* La1 in media added with Na_2SeO_3 and non-enriched media.

Parameter	*Lb. casei* Shirota with Na_2SeO_3	*Lb. casei* Shirota without Na_2SeO_3	*Lb. johnsonii* with Na_2SeO_3	*Lb. johnsonii* without Na_2SeO_3
Growth rate (μ)	$0.01\pm2.9\times10^{-5a}$	$2.9\times10^{-3}\pm4.0\times10^{-4b}$	$5.1\times10^{-3}\pm1.9\times10^{-3b}$	$2.0\times10^{-3}\pm2.0\times10^{-6b}$
Generation time (g)	63.51±0.17	239.11±32.97	146.4±55.8	349.0±0.35
Growth rate constant (K)	$1.6\times10^{-3}\pm4.3\times10^{-5a}$	$4.2\times10^{-3}\pm5.8\times10^{-4b}$	$7.4\times10^{-3}\pm2.8\times10^{-3b}$	$2.9\times10^{-3}\pm2.8\times10^{-6b}$

Values are mean ± SD (n = 3); values in the same line with different superscript letters are significantly different ($p < 0.05$).

Table 3. Concentration of inorganic selenium uptake by *Lb. casei* Shirota and *Lb. johnsonii* La1 during fermentation of MRS enriched with a critical inhibitory concentration of Na_2SeO_3 at deceleration time.

Lactic acid bacteria	$[Se]_1$ (μg/mL)	log CFU/mL	Selenium uptake (%)	Selenium concentration (μg_{Se}/CFU)
Lb. casei Shirota	90.41	7.4	55.61	6.79
Lb johnsonii La1	93.15	7.9	75.78	8.93

$[Se]_1$ Initial concentration of selenium stoichiometrically calculated from Na_2SeO_3 added to MRS broth

johnsonii La1. The concentration absorbed by *Lb. casei* Shirota was 55.61 %.

Se absorption found in this research was higher than the concentration reported by Andreoni *et al.* (2000), which determined values lower than 13 % for some *Lactobacillus* species. Other authors, such as Zhi-Qiang *et al.* (2009) and González-Olivares *et al.* (2016), found a lower concentration of Se accumulated by *Lb. rhamnossus* GG and *Lb. delbruecki*. Nonetheless, these last authors have reported that *Lb. helveticus* IUAMI-70129 can bioaccumulate up to 76 % of Se presented in media MRS enriched at MIC of Na_2SeO_3. Besides, it has been reported Se absorption of *Streptococcus thermophilus* respect CFU obtained, which is lower than some reports of *Lactobacillus* (1.66 μg_{Se}/CFU) (Castañeda-Ovando *et al.*, 2019).

Differences in Se absorption between LAB species are due to each microorganism's tolerance to the presence of Se in media and is according to its detoxification mechanism (Escobar-Ramírez *et al.*, 2021). Furthermore, this tolerance depends on the bioconversion of seleno-amino acids, mainly selenocysteine, that each bacteria carry out. For example, it is known that in the fermentation of milk, which is a medium adequate for LAB growth, the bioaccumulation and subsequent selenocysteine transformation are more efficient (Alzate *et al.* 2008).

Due to the few reports of absorption and accumulation of selenium by species of *Lb. casei* and *Lb. johnsonii*, and being these microorganisms of technological importance in the field of dairy science and technology, they are a potential ingredient in manufacturing products enriched with completely bioavailable and bioaccessible organic selenium.

Conclusions

Lactic acid bacteria isolated from commercial dairy products, *Lactobacillus casei* Shirota and *Lactobacillus johnsonii* La1, accumulate inorganic selenium in concentrations similar to some reported for other lactobacilli. In addition, the minimum inhibitory concentration of inorganic selenium in MRS broth only slows growth in one of the microorganisms studied. In contrast, no effect is observed in the other. This indicates that the effect of an inorganic salt of selenium on the metabolism of lactic bacteria could be dependent on the detoxification system of each of them. Finally, the accumulated concentration of selenium for each microorganism is dependent on both the metabolism and the concentration of selenium in the medium, so the higher the concentration of selenium in the medium, the greater the accumulation. Additionally, the inhibition exerted by selenium on the growth and development of each microorganism could be related to the bioaccumulation capacity, which is directly proportional. Thus, with the results obtained, the potential of these microorganisms in the application of fermented dairy products is observed, but with added value with the high probability of finding organic species of selenium

that are more bioavailable and bioaccessible. These results could be the beginning of the exploration of the selenium accumulation capacity in starters of industrial fermented foods.

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