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DOCTORADO EN CIENCIAS DE LOS ALIMENTOS Y SALUD HUMANA

TESIS DOCTORAL

PRODUCCIÓN BIOGÉNICA DE SELENIO ORGÁNICO Y NANOPARTÍCULAS DE SELENIO PARA LA EVALUACIÓN DEL EFECTO SOBRE PARÁMETROS DE CALIDAD SEMINAL: ALTERNATIVA DE USO TECNOLÓGICO

Para obtener el grado de
Doctora en Ciencias de los Alimentos y Salud Humana

PRESENTA

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Por este medio se informa que el comité tutorial asignado a la M. en C. Lourdes González Salitre con número de cuenta 217160, estudiante del Doctorado en Ciencias de los Alimentos y Salud Humana ha terminado el trabajo de tesis titulado "Producción biogénica de selenio orgánico y nanopartículas de selenio para la evaluación del efecto sobre parámetros de calidad seminal: alternativa de uso tecnológico", y por lo tanto se autoriza la impresión del documento en extenso propuesto por la estudiante después de haber sido revisado, analizado y evaluado de acuerdo a lo estipulado en el Artículo 73, VI del Reglamento General de Estudios de Posgrado.
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Debido a lo anterior, la estudiante cumple con los requerimientos de egreso establecidos por el programa de posgrado, al contar con un artículo aceptado en una revista indizada. Por lo que solicitamos a usted tenga a bien permitir al doctorando dar continuidad al proceso necesario que conlleve a la obtención del grado de Doctor en Ciencias de los Alimentos y Salud Humana.

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Dedicatoria

El presente trabajo esta dedicado al esfuerzo de toda la familia, evidentemente no pude haberlo logrado sola, todos son pieza clave en cada paso y este logro también es de ustedes.

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Lourdes González Salitre

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Resumen

El selenio es un oligoelemento esencial para la salud humana y animal. Involucra diferentes mecanismos metabólicos que ofrecen un beneficio a la salud, en lo que las funciones biológicas son realizadas por selenoproteínas. Las especies de selenio con mayor biodisponibilidad y bioaccesibilidad son producidas por microorganismos, como las levaduras, las cuales bioconvierten el selenio inorgánico a selenio orgánico. Por lo tanto, el objetivo de este estudio fue evaluar la producción biogénica de selenoaminoácidos y nanopartículas de selenio por *Saccharomyces boulardii* y su potencial uso tecnológico a través de un estudio *in vitro* para analizar su efecto sobre la motilidad espermática. La bioacumulación de selenio por la levadura superó los 22,000 ppm, además la bioconversión fue a selenometionina, selenocisteína y nanopartículas de selenio. Además, el estudio *in vitro* en semen de verraco, determinó que tanto la levadura selenizada como selenito de sodio conserva la motilidad superior al 65% en un lapso de 48 horas. Finalmente, se logró la producción de una bebida fermentada a base de cebada y *Saccharomyces boulardii* selenizada, que permitió la transferencia de selenio a la bebida, con una concentración de 0.378 ppm. Estos resultados demuestran que la producción biogénica de selenio orgánico por parte de la levadura probiótica *S. boulardii* podría representar una alternativa potencial en el desarrollo de bebidas fermentadas con alto contenido de selenio, que pudieran impactar de manera positiva la salud huma.

Abstract

Selenium is an essential trace element for human and animal health. It involves different metabolic mechanisms that offer a health benefit, in which the biological functions are carried out by selenoproteins. The selenium species with the highest bioavailability and bioaccessibility are produced by microorganisms, such as yeasts, which bioconvert inorganic selenium to organic selenium. Therefore, the objective of this study was to evaluate the biogenic production of selenoamino acids and selenium nanoparticles by Saccharomyces boulardii and their potential technological use through an in vitro study to analyze their effect on sperm motility. The bioaccumulation of selenium by yeast exceeded 22,000 ppm, in addition the bioconversion was to selenomethionine, selenocysteine and selenium nanoparticles. In addition, the in vitro study in boar semen determined that both selenized yeast and sodium selenite retain motility greater than 65% in a period of 48 hours. Finally, the production of a fermented drink based on barley and selenized Saccharomyces boulardii was achieved, which allowed the transfer of selenium to the drink, with a concentration of 0.378 ppm. These results demonstrate that the biogenic production of organic selenium by the probiotic yeast S. boulardii could represent a potential alternative in the development of selenium-rich fermented beverages that could positively impact human health.

Capítulo -I-.

Introducción al trabajo de investigación

1.1 Introducción

El selenio es un oligoelemento natural y esencial para la vida. Se le considera importante ya que forma parte de algunas enzimas con funciones específicas para el organismo. Un ejemplo de ello es la glutatión peroxidasa, donde uno de sus objetivos es salvaguardar y proteger a las células espermáticas del estrés oxidativo. Este efecto se logra al catalizar la reducción del peróxido de hidrógeno. Además, en una etapa temprana de la espermatogénesis, se cree que la glutatión peroxidasa protege a los espermatozoides en desarrollo del daño en el ADN inducido por el estrés oxidativo.

Debido a la importancia de este oligoelemento es necesario consumirlo en una dosis mínima de 55 µg/día. Las principales fuentes de consumo de selenio pueden ser diversas, como el maíz, trigo, cebada, el pepino, apio y brócoli, donde el contenido de selenio va a depender de la cantidad de selenio en el suelo donde crezcan. En este tipo de alimentos, el selenio se encuentra principalmente como selenio inorgánico (selenito, selenato), la desventaja de estas especies de selenio es que no se absorben de manera eficiente en el organismo.

De esta manera, las investigaciones se centran en proporcionar fuentes de selenio orgánico, como selenoaminoácidos, que se absorben de manera más eficiente por el organismo. Una de estas fuentes de selenio orgánico son las levaduras selenizadas, que pueden bioconvertir el selenito a través de su metabolismo a selenoaminoácidos.

En este contexto, se investigó la capacidad de bioconversión de selenito de sodio a selenoaminoácidos por *Saccharomyces boulardii*, con aplicación en fertilidad masculina y aplicación tecnológica en una bebida fermentada. Este trabajo se divide en siete capítulos. En el primer capitulo se muestra una introducción general al trabajo de investigación, justificación, objetivos y diagrama metodológico. En el segundo capítulo se presenta un artículo de revisión de levaduras selenizadas y sus implicaciones en salud humana, así como sus aplicaciones para obtener alimentos enriquecidos con selenio, dicho artículo se encuentra publicado en la revista Food Reviews International.

Más adelante, en el tercer capítulo se presenta una investigación *In silico* en la cual se buscaron los genes implicados en la bioconversión de selenio inorgánico a selenoaminoácidos por *S. boulardii* y *S. cerevisiae.* Se realizó la comparación de genes ortologos y se presento la propuesta de la ruta metabolica de bioconversión de selenato a selenometionina y selenocisteína. Esta parte de la investigación se encuenta publicada en la revista Biointerface Reserch in Applied Chemistry.

La primera parte experimental de la investigación se presenta en el capítulo cuarto. Se presenta el artículo original publicado en el Food Bioscience, donde explica la producción biogénica de selenoaminoácidos y nanopartículas de selenio por *Saccharomyces boulardii*, difundiendo la primera publicación con respecto a estos resultados obtenidos para una levadura probiótica.

Como capítulo cinco, abarca el estudio *in vitro* que se realizó con semen de verracos, para probar el efecto de la levadura selenizada, esta parte experimental se realizó durante una estancia de investigación en el Laboratorio de Imagenología, Zootecnia y Gestión Ambiental de la Universidad Autónoma Metropolitana, Unidad

Xochimilco, dentro del Proyecto Académico Recuperación del cerdo criollo Hidalguense “Ts’udi Xirgo”.

El capítulo seis presenta un artículo de revisión publicado en el Food Chemistry, dicho artículo se presenta como parte introductoria previa a la aplicación tecnológica de la levadura selenizada, la revisión abarca temas de cerveza artesanal y beneficios a la salud humana. Esta revisión da la pauta para el capítulo siete el cual se tradujo en un artícuo enviado a la revista Heliyon en el cual se presenta la parte de elaboración de la bebida fermentada con la levadura selenizada, esta parte experimental se realizó durante la estancia de investigación en el Laboratorio de Higiene, Inspección y Control de Alimentos del Departamento de Química Analítica, Nutrición y Bromatología del Campus de Lugo en la Universidad de Santiago de Compostela.

1.2 Justificación

De acuerdo con datos de la Organización Mundial de la Salud, la infertilidad afecta a 48 millones de parejas, por lo que alrededor de 186 millones de personas en el mundo se ven afectadas por la esterilidad o infertilidad. Entre las parejas que presentan infertilidad, se atribuye que el factor principal o al menos el 50% es debido a la infertilidad masculina. Las principales causas de infertilidad en el aparato reproductor masculino son problemas para eyacular semen, ausencia o baja concentración de espermatozoides y anomalías en la morfología o motilidad espermática.

Reconociendo los efectos de la esterilidad y su importancia en la calidad y bienestar de las personas que buscan concebir y enfocándonos en formas de infertilidad masculina, aunado a que la deficiencia de selenio representa un problema de salud y puede ayudar a contribuir al co-tratamiento de infertilidad idiopática, este estudio se centra proporcionar una alternativa de fuentes orgánicas de selenio, basándose en el metabolismos y capacidad de bioconversión de selenio inorgánico a selenio orgánico por *Saccharomyces boulardii*, una levadura probiótica aprobada por la FDA para consumo humano.

1.3 Objetivos

1.3.1 Objetivo general

Determinar la capacidad de bioacumulación de selenio por *Saccharomyces boulardii* y su potencial uso tecnológico a través de un estudio *in vitro* para analizar su efecto sobre la motilidad espermática.

1.3.2 Objetivos específicos

1) Realizar una revisión bibliográfica a través de una metabúsqueda para publicar un artículo en una revista de alto impacto

2) Proponer la ruta metabólica de selenización de *Saccharomyces boulardii* a través de un estudio *in silico* para determinar su potencial en la aplicación de alimentos funcionales

3) Determinar la concentración mínima inhibitoria de selenito de sodio sobre *Saccharomyces boulardii* a través de una cinética microbiana para desarrollar la levadura selenizada.

4) Identificar la concentración de selenio orgánico metabolizado por *Saccharomyces boulardii* y la presencia de nanopartículas de selenio analizando por medio de HPLC, ICP y TEM para determinar su capacidad de bioconversión de selenio inorgánico

5) Analizar el efecto del selenio orgánico proveniente de la fermentación de *Saccharomyces boulardii* selenizada sobre la calidad espermática, mediante un estudio *in vitro* para determinar su potencial bioactivo

6) Elaborar y caracterizar una cerveza artesanal baja en alcohol mediante la fermentación con *Saccharomyces boulardii* selenizada para su uso como vehículo de la ingesta de selenio orgánico

1.4 Diagrama metodológico

Para el desarrollo de los objetivos de investigación se siguió la metodología ejemplificada en la Figura 1. Dicha figura esta diseñada por objetivos, donde cada objetivo presenta la parte más reelevante.

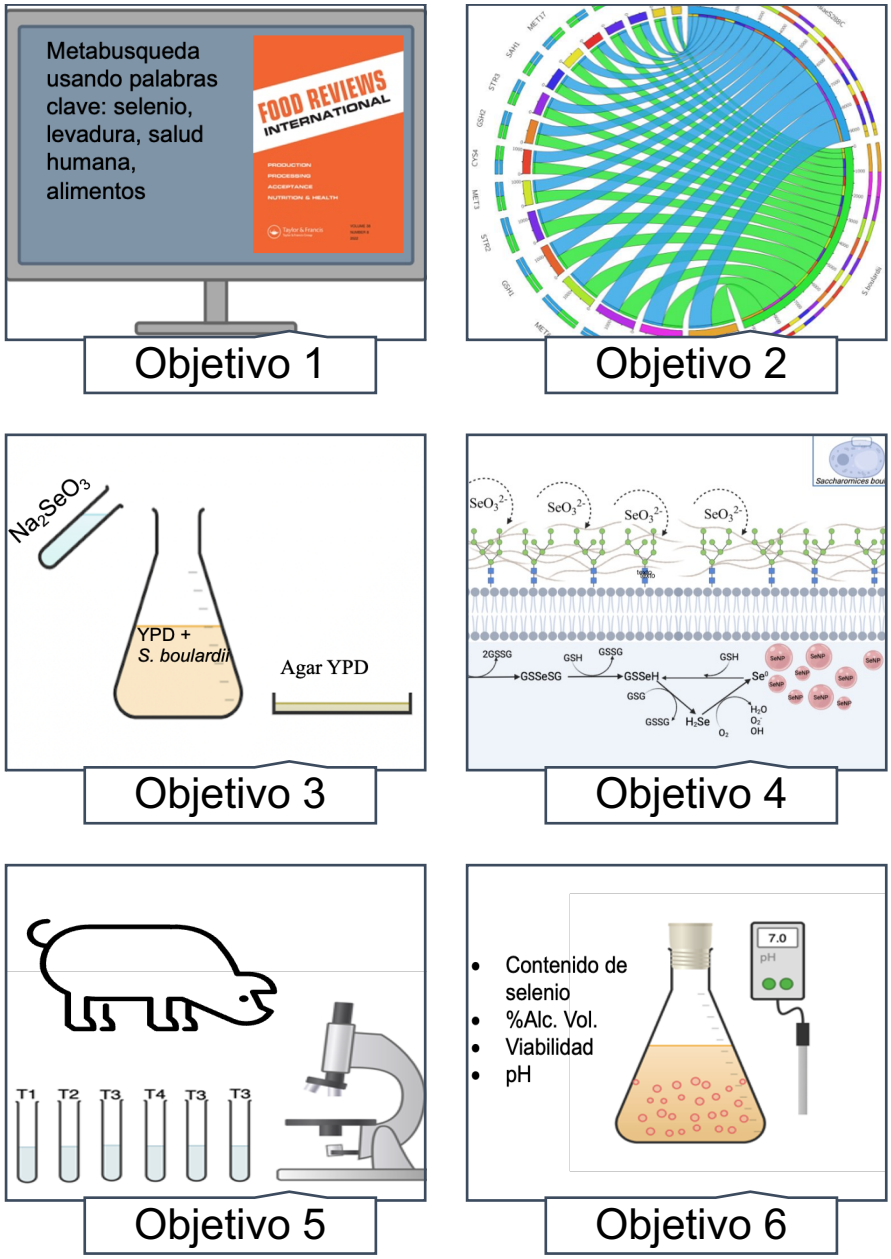

Figura 1.Diagrama metodológico del proyecto doctoral presentado en base a objetivos.

Capítulo -II-
Promising use of selenized yeast to develop new enriched food: Human health implications.

2.1 Introducción

En este capítulo se realizó un extensa revisión bibliográfica publicada en la revista Food Reviews International. En el se habla acerca del selenio y salud humana, la dosis mínima recomendada, así como la dosis que genera toxicidad. Sus características químicas, sus formas inorgánicas y orgánicas. Sus actividades terapéuticas, como en el tratamiento de diferentes tipos de cáncer, como agente antimicrobiano, tratamiento para Alzheimer, de manera específica los beneficios de las nanopartículas de selenio. Así mismo, se presentan los avances en investigación sobre la producción de levaduras selenizadas, las principales especies de levaduras estudiadas para la producción biogénica de selenoaminoácidos, así como la ruta propuesta de bioconversión por *S. cerevisiae*. Se habla del uso de levaduras selenizadas en el procesamiento de alimentos, como leche y productos lácteos, el uso de levadura selenizada como alimento de animales para la producción de huevos de gallina enriquecidos con selenio, en la producción de carne de puerco, de conejo y de pollo, ricos en selenio y finalmente en la producción de bebidas fermentadas. Al final, se dan a conocer las posibles aplicaciones futuras y las potenciales oportunidades y retos en la investigación sobre el mecanismo bioquímico de bioconversión, que nos brindan oportunidad para investigar acerca de levaduras probioticas como *S. boulardii.*

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Promising Use of Selenized Yeast to Develop New Enriched Food: Human Health Implications

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ABSTRACT

Selenium is an essential micronutrient and has been shown to have an antioxidant effect. This can prevent different diseases such as coronary illness or cancer. Its deficiency generates neuromuscular disorders, which is why a recommended daily intake for humans of 55 µg/day has been proposed but it is not always covered. Selenium is integrated as selenoaminoacids to glutathione peroxidases, which are enzymes involved in redox reactions. Some yeast are capable of introducing inorganic selenium into the polypeptide chains of ribosome to later transform it into selenoaminoacids. In some cases, selenized yeasts are used as starter cultures in fermented foods processing. These yeasts are considered bioavailable selenium supplements and currently food science has developed strategies to include them within innovation processes of enriched products. Due to the importance of selenium in human health, the objective of this review is to present a state of the art of selenized foods, going through the importance of selenium in human health and the biochemical mechanism of biotransformation of inorganic selenium to organic species by yeasts.

KEYWORDS

Selenium; selenized yeast; selenomethionine; selenocysteine

Introduction

Microorganisms have different mechanisms to tolerate metals in the environment. These mechanisms are involved in the interaction that exists between the cell and its ability to metabolize them. Cells mainly act through extracellular interactions with proteins and other macromolecules; by interactions on the cell surface and by intracellular interactions involved in metabolism of each microorganism.[1] At the same time, when there are concentrations of metals in growth medium that are higher than metabolic needs, these accumulate many times and are incorporated into proteins. This is the specific case of yeasts, which are capable of accumulating metals such as selenium through conjugation of these with proteins.[2] The bioconversion metabolism of inorganic selenium to an organic species is a route that has been studied for a long time. The importance of this mechanism is related to the large concentrations of selenium that yeast could biotransform, making this metal a bioavailable trace element. It is known that 99% of the selenium that humans consume is inorganic and of this amount only 10% is absorbed. Therefore, it is estimated that the majority of the world's population has deficiencies of this metalloid. Selenium, in turn, has a direct effect on human metabolism since it is

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related to antioxidant activity, as well as its chemopreventive, anti-inflammatory and antiviral properties. Its effect has been studied in cancer, diabetes, cardiovascular diseases, thyroid function and male fertility.[3,4]

From the second half of the 20th century, sufficient evidence was gathered to show that selenium was an essential micronutrient, recognizing different diseases and physiological disorders associated with its deficiency. Thus, for example, Keshan's disease, which is a cardiomyopathy that mainly affects children, and Kashin-Beck's disease, which causes serious disorders in bone development.

For this reason, attempts have been made to supplement the intake of selenium through bioavailable species such as selenomethionine and selenocysteine. These amino acids are generated by the metabolism of yeast biotransforming inorganic species of selenium. Thus, in recent years, studies have been carried out on the use of selenized yeasts in human health improvement. It has been verified through in vivo models that the application of selenium-enriched yeasts to the diet are capable of reducing cholesterol levels. When these are applied in combination with glutathione, there is a direct effect not only in lowering cholesterol, but also in atheroscellosis and lipid oxidation.[5–7] Likewise, selenized yeasts have shown an effect in colorectal cancer treatment.[8] But beyond these studies, there is evidence that the application of this type of yeast has an effect on the reduction of reactive oxygen species, which has implications in the improvement of sperm motility.

With all these evidences, this review aims to show opportunities by the use of selenized yeasts in food production. The first part of this article shows generalities of selenium and its implication in human health, the second part shows an approach to selenium metabolism by yeasts, and finally, the most important studies on selenized-foods production are reviewed, which yeasts are the central axis.

Selenium and human health

Selenium is a natural trace element found in four oxidation states: in its elemental form Se (0), selenide (Se^{2-}), selenite (SeO_3^{2-}) and selenate (SeO_2^{-4}) (Fig. 1). [11] Selenium could be integrated in some antioxidant enzymes.[9,12] According to WHO, the necessary dose for humans is 55 µg/day, however, the range between essential concentrations for humans (55 µg/day) and toxicity (> 400 µg/day) is wide.[13]

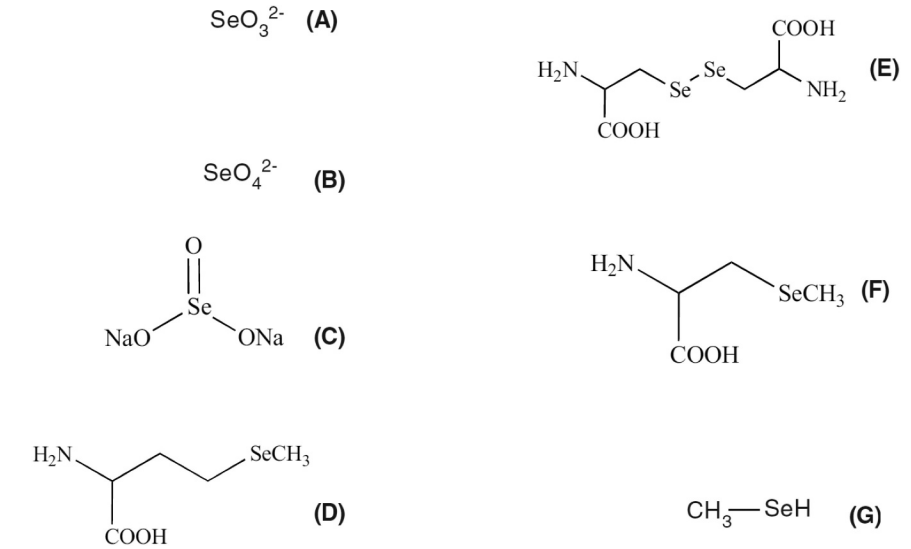

Figure 1. Chemical selenium forms. (A) selenite, (B) selenate, (C) sodium selenite, (D) selenomethionine, (E) selenocysteine, (F) methylselenocysteine y (G) melthylselenol.[9,10].

Due to its chemical characteristics that give its beneficial activity, this trace element has multiple health benefits. But, inorganic selenium such as selenate or selenite, produce genotoxic effects, while organic selenium compounds are safer and better absorbable by humans. These organic forms are generally found as seleno-amino acids: selenocysteine (SeCys), selenomethionine (SeMet) and methylselenocysteine (MeSeCys) (Fig. 1). Additionally, SeMet and MeSeCys or methylseleninic acid can be converted endogenously into methylselenol, which is necessary for anticancer expression.[10]

Selenium has the potential to be used as a therapeutic agent in treatment of inflammatory lung diseases.[14] On the other hand, the use of sodium selenite combined with probiotics (*Lactobacillus acidophilus, Bifidobacterium bifidum* and *Bifidobacterium longum*) has also been reported to improve cognitive function and some metabolic profiles in patients with Alzheimer's (AD).[15]

Selenium could be used in patients with inflammatory bowel disease minimizing cardiovascular risk associated with an increase in biomarkers of inflammation.[16,17] An adequate intake of selenium is essential for thyroid gland function, since the deficiency of this trace element is associated with a greater probability of suffering from thyroid disease.[13,18] Selenium intake has also been related to an inhibitory effect on the human immunodeficiency virus (HIV), due to antioxidant capacity of glutathione peroxidase and other selenoproteins. .[19,20] Recent study has reported that selenium could help to diminish both the probability to infection with SARSCoV-2 and the development of severe symptoms acquire by CoVID-19, activating the immune system .[21] Similarly, selenium and various selenoproteins are related in male reproductive performance, [22] because selenium is essentially necessary for spermatogenesis and male fertility.[23] Although there is evidence that both,

Table 1. Associated mechanism in therapeutic activity of selenium nanoparticles (SeNP).

Therapeutic activity		Mechanism of action	SeNP and/or conjugations	Reference
Cancer	Liver	Induction of mitochondrial diffusion over human hepatocarcinoma (HepG2) and drug-resistant hepatocellular carcinoma (R-HepG2). Cell lines study	PEG-SeNP of 5 nm diameter	[27]
		Apoptosis induction in cell lines of human hepatocarcinoma (HrpG2)	Ch-SeNP of 40 and 60 nm diameter FA-SeNP of 105 nm diameter	[28,29]
		Apoptosis induction caspase-mediated in HepG2 cells	SeNP @ ATP of 40 nm diameter	[30]
		Oxygen reactive species produced through selenium reduction tested in Kunming male mice with murine hepatocarcinoma cells (H22)	SeNp of 40 nm diameter	[31]
	Colorectal	Autophagy and apoptosis activation in cell lines of human colorectal carcinoma (HCT116)	Se-CurNP of 57.5 nm diameter	[32]
	Lung	Apoptosis induction through mitochondria-mediated pathway in cell lines of human lung (L-132) and lung cancer (A549). Inhibition tumor growth in mice inoculated with A549 cells	PEG-SeNP of 40–50 nm diameter	[33]
	Ovary	Decreased metastatic potential in cell lines of ovarian cancer (SKOV-3 y OVCAR-3)	SeNP of 25–50 nm diameter	[34]
	Peritoneal cavity	Oxygen reactive species production in mice inoculated with hepatocarcinoma cells H22	SeNP of different diameters	[35]
Dalton lymphoma		Optimal conditions of immunity system in mice MALB/c with malignant tumors (H2d)	SeNP of 12–30 nm diameter	[36]
Nosocomial infection		Antimicrobial activity against *E. coli S. aureus, K. pneumonia y B. subtilis*	SeNP of 30–100 nm diameter	[37]
Antimicrobial agents		Antibacterial and bactericide activity against *Staphylococcus aureus* resistant to methicillin (MRSA)	Qu – Ach @ SeNPs of 120 nm diameter	[38]
Induced toxicity by As(III) over human lymphocytes		Protective effect against cell death and DNA damage induced by As (III) in human blood	SeNP of 10 to 80 diameter	[39]
Alzheimer		Inhibition of β-amiloide (Aβ) aggregation	B6-SA-SeNP of 95 nm diameter	[40]
Drug administration		Controlled release of drugs induced by pH	P @ ZIF-8 of 85 nm diameter	[41]

a deficiency and an excess of selenium have harmful effects on male fertility, therefore, it is necessary to consume at the recommended doses.[24]

Benefits of seleno-nanoparticles

Advances in selenium research have been made and there are those who claim that nanoparticles of selenium (NPSe) have a greater benefit than only the administration of inorganic or organic selenium.[25] The first study that was conducted, reported antioxidant properties of NPSe with a reduced risk of selenium toxicity.[26] With advances in research, SeNPs have been found to have various benefits to human health (Table 1).

Nanomaterial containing selenium shows anticancer activity by inducing cell apoptosis, by achieving inhibition of tumor growth and by achieving cytotoxicity triggering intracellular generation of reactive oxygen species (ROS).[42] SeNP are used to enhance the immune system and to induce the suppressed function of tumor-associated macrophages and inhibit the proliferation of Dalton's lymphoma.[36] As seen in Table 1, the SeNP and synergistic nanocompounds conjugated with quercetin (Qu) and acetylcholine (Ach) with the surface of selenium nanoparticles (Qu – Ach @ SeNPs) present antimicrobial activity against pathogenic bacteria, this activity could be due to irreversible damage to bacterial membrane causing cytoplasm leakage, allowing the entry of nanocomposites into bacterial cell and causing DNA damage.[38,43] Another positive result of the SeNP is its protective effect against damage induced by As (III).[39]

The use of selenium nanoparticles modified with sialic acid conjugated with an alternative peptide, peptide-B6 (B6-SA-SeNP) has been reported as a promising drug for the treatment of Alzheimer's, by inhibiting the aggregation of β-amyloid (Aβ) and pass the blood-brain barrier (BBB), in addition to protecting PC12 cells against Aβ-induced toxicity.[40] Finally, the P @ ZIF-8, which is a selenium-containing polymer @ metal with organic framework nanocomposites, have been studied with multiple responsiveness under low pH conditions, which makes it a promising system for controlled delivery of drug.[41]

Selenium biotransformation by yeast and its effect on human health

Some yeasts are capable of biotransforming inorganic selenium to organic selenium forms, mainly as SeMet, which has an effect on human health.[2] The selenium biotransformed by yeast is well absorbed and retained in the body.[44] Furthermore, the consumption of yeast-derived selenium is safe and its toxicity is lower than that of inorganic selenium:[45] In addition, consumption of selenized yeasts has been shown to have an effect on atherosclerosis and diabetes.[5,46] In the case of diabetes, the administration of selenized yeast prevents the formation of free radicals, which are inducers of said disease.[47] It has been reported that selenized yeasts may exhibit potential anticancer properties, by increasing oxidative stress and stimulate growth inhibitory effects and induction of apoptosis in breast cancer cell lines.[8,48,49] and colorectal cancer.[8] Regarding prostate cancer, no statistically significant differences have been found in the incidence when patients with prostate and lung cancer are treated with the administration of selenized yeast.[50–53]

Selenized yeast production

Conversion of inorganic selenium to organic selenium by selenized yeasts is characterized by the formation of selenomethionine (SeMet). This selenoamino acid is less toxic and more bioavailable than selenium salts, such as selenite (SeO32-) or selenate (SeO42-).[2,54,55]

Currently, selenized yeasts are used as a dietary supplement to reach the suggested daily dose recommended for human. Different yeast genera have been studied as shown in Table 2, in which it is observed that yeasts such as *Yarrowia, Kluyveromyces* and *Rhodotorula* genera are the least studied compared to the studies carried out in the *Saccharomyces* and *Candida* genera. Kieliszek

Table 2. Selenium bioaccumulation by yeast according to the selenium concentration in media.

Yeast		Na_2SeO_3 concentrations	Selenium accumulated	Selenium species identified	Analytical analysis	Reference
Saccharomyces	*cerevisiae*	10 µg /mL in white wine	6 µg/mL in white wine	SeMet	ICP/MS LC-ICP-MS	[56]
	cerevisiae	200 µg	200 µg	Se-(methyl-L-selenocysteine)	Spectroscopy XANES.	[57]
	cerevisiae NCYC 1026	0–150 ppm	Reduction of yeast biomass	Organic Se	Volumetric valorization a (British Pharmacopeia).	[58]
	cerevisiae 405	15 mg/L	1095 mg/kg	NI	ICP-OES	[59]
	cerevisiae 174	15 mg/L	1193 mg/kg	NI	ICP-OES	[59]
	cerevisiae 193	15 mg/L	833 mg/kg	NI	ICP-OES	[59]
	cerevisiae Lalvin ICV D47	15 mg/L	659 mg/kg	NI	ICP-OES	[59]
	cerevisiae	30 µg/ml	1200 a 1400 µg/g	NI	ICP-AES	[60]
	cerevisiae EC1118	200 µmol/ L	3.1 mg/g	NI	ICP-MS	[61]
	cerevisiae AW063	200 µmol/ L	1 mg/g	NI	ICP-MS	[61]
	cerevisiae AW106	200 µmol/ L	3.5 mg/g	NI	ICP-MS	[61]
	cerevisiae Lalmin® SE2000	200 µmol/ L	6.3 mg/g	NI	ICP-MS	[61]
	cerevisiae	25 mg/L		SeMet	Atomic absorption spectroscopy (AAS)	[2]
	cerevisiae ATCC MYA-2200	60 mg/L	5.64 mg/g		Iodine staining spectroscopy	[62]
	cerevisiae 15–6252	50 mg/L	2.354 mg/g	SeMet	HPLC-ICP-MS	[63]
	cerevisiae BY106	100 mM de SeSug1 SeSug2 TMSe SeMet MeSeCys	438 nmol/g 365 nmol/g 911 nmol/g 2781 nmol/g 1567 nmol/g	SeMet, MeSeCys, SeSug1 y SeSug2, TMSe	ICP/MS HPLC-ICP-MS ESI-TOF-MS y LC-ICP-MS	[64]
	cerevisiae	4000 ppm	4940 µg/g	SeMet	GC-MS	[65]
	Cerevisiae BY4741	1 mM	SeNP	SeNP of 20–30 nm diameter	Transmission electronic microscopy (TEM), SEM	[66]
bayanus	10 µg /mL in white wine	6 µg/mL in white wine	SeMet	ICP/MS LC-ICP-MS	[56]	

(*Continued*)

Table 2. (Continued).

Yeast		Na_2SeO_3 concentrations	Selenium accumulated	Selenium species identified	Analytical analysis	Reference
Candida	*utilis* ATCC 9950	30 mg/L	1841 µg/g.	SeMet	HPLC ICP-MS	[67]
	utilis ATCC 9950	20 mg/L	629 µg/g	SeMet Dehydroselenomethionine-oxide Selenomethionine-NH3. Se-S de selenoglutation-cysteine. Methyltioselenoglutation. 2,3-DHP-selenocysteine	HPLC-ICP-MS y UHPLC-ESI-Orbitrap MS	[68]
	utilis UFMG-RVC -4 36	15 mg/L	1946 mg/kg	NI	ICP-OES	[59]
	utilis CCTCC M 209298	15 mg/L in acid stress conditions	1080 µg/g	NI		[69]
	utilis ATCC 9950	60 mg/L	5.47 mg/g	NI	Iodine staining spectroscopy	[62]
Yarrowia	*lipolytica* ACA DC 50109 lipolytica ALE_70	10 mg/L	Inhibition by selenium presence	NI		[70]
Kluyveromyces	*marxianus* CCT 4086 (=ATCC46537)	15 mg/L	2359 mg/kg	NI	ICP-OES	[59]
Rhodotorula	*glutinis* X-20	30 mg/L	5349.6 µg/g	NI	Hydride generating atomic fluorescence spectrometry (HG-AFS)	[71]

et al.[62,67,68,70,72] have worked on selenization of yeasts such as *Candida utilis* ATCC 9950, *Saccharomyces cerevisiae, Yarrowia lipolytica* ACA DC 50109 and *Yarrowia lipolytica* ALE_70. The best selenium conversion rates were found in both *Candida utilis* and *Saccharomyces cerevisiae*. These authors have shown that at high doses of selenium the lipid peroxidation process is also increased due to oxidative stress due to enzymatic activity of glutathione peroxidase and glutathione reductase.

Candida utilis, in addition to bioconverting sodium selenite to SeMet (Table 2), [67] is capable of bioaccumulating dehydroselenomethionine-oxide, selenomethionine-NH3, Se-S of selenoglutathione-cysteine, methylselenoglutathione and 2, 3- DHP-selenocysteine. Kieliszek and Błażejak, [68] demonstrate using a speciation technique performed by HPLC-ICP-MS and UHPLC-ESI-Orbitrap-MS, that concentration of selenium retained by the yeast depends on both the concentration of the enrichment salt and the yeast species (see Table 2). According to the analyzes performed by ICP-OES, *Candida utilis* UFMG-RVC-4 36 showed to have the best selenium retention.[59]

According to data presented in Table 2, the highest concentration of sodium selenite (60 mg/L), which *Candida utilis* has been enriched, is a concentration of selenium that causes a reduction in cell density of 19%.[73] Similarly, Zhang et al.[69] demonstrated that acid stress has a direct effect on the enrichment of yeasts with selenium. These authors evaluated the growth of *Candida utilis* CCTCC M 209298 in media supplemented with selenium (15 mg/L) at pH: 3.5. The authors observed that there was an increase in the activity of γ-glutamylcysteine synthetase and the proportions of NADH/NAD + and ATP/ADP. Additionally, they demonstrated that the performance of selenized yeast was improved.

Saccharomyces cerevisiae is the most studied yeast in relation to the production of SeMet (Table 2). The absence of the SeCys conversion pathway has been demonstrated because SeMet is the aminoacid

forming by conversion of inorganic selenium. This pathway is the only one for the incorporation of selenium in the active site of selenoenzymes.[74]

The stage of microbial development also has an effect on the bioconversion in SeMet. Ponce de León et al.[63] determined that when the objective is to enrich yeast for the formation of SeMet, the best procedure is to add selenium during the growth phase with small concentrations, reaching this conclusion after inoculating sodium selenite at the beginning of the growth phase. Finally, they observed higher SeMet production when inoculating sodium selenite at the beginning of the growth phase.

On the other hand, Kaur and Bansal, [75] evaluated the addition of sodium selenite on *Saccharomyces cerevisiae* MTCC Code-1766 and its effect on oxidative stress, when evaluating antioxidant defenses, selenium improved the levels of glutathione peroxidase (GSH- Px), total glutathione and glutathione-s-transferase. This demonstrates the importance of selenium in the yeast's enzymatic defense system against oxidative damage. Rajashree and Muthukumar[58] evaluated the addition of sodium selenite from 0 to 150 ppm to *Saccharomyces cerevisiae* NCYC 1026 with the objective of evaluating the toxicity of selenium in yeast cells, and detecting the tolerance to selenium for the production of biomass of selenium tolerant yeast strains that proliferate at high concentrations of selenium. At the highest concentration tested (150 ppm) there was a reduction in biomass by 96.18%. In addition there was cellular damage when biomass concentration decreased.

Selenium tolerance analysis could be performed with different concentrations of selenium (25 ppm, 50 ppm and 75 ppm). In this way, it was observed that the highest absorption of selenium (97.5%) presented at the same way the highest biomass production of *S. cerevisiae*.[58] In another similar study, Zare et al.[2] collected 85 samples of fermentable fruits, 40 yeasts (S1 to S40) were isolated, which were developed in a medium supplemented with selenium at a concentration of 25 mg/L. After 24 hours, the production of SeMet was measured by AAS, finding that this time is enough to increase biomass and selenium accumulation as SeMet.

Martiniano et al.[59] used agroindustrial residues such as sugarcane bagasse and corn bran enriched with sodium selenite for the selenization of *Candida utilis* UFMG-RVC-436, *Kluyveromyces marxianus* CCT 4086, *Saccharomyces cerevisiae* 405, *Saccharomyces cerevisiae* 174, *Saccharomyces cerevisiae* 193 and *Saccharomyces cerevisiae* Lalvin ICV D47. Of all the yeasts tested, those of the genus *Saccharomyces* presented the highest biomass production (7.97 g/L) as a maximum absorption of selenium (1193 ppm). Cell viability was 99%, showing that the best support was corn bran. Pérez-Corona et al.[56] evaluated the addition of sodium selenite to white grape juice for the production of white wine, for the bioconversion of selenium they used *Saccharomyces cerevisiae* and *Saccharomyces bayanus*, the species of selenium found in wine by an analysis by ICP-MS and LC-ICP-MS was SeMet.

Enrichment of *Saccharomyces cerevisiae* has been reported by Suhada et al.[60] These authors evaluated the addition of 30 µg/ml of sodium selenite, resulting in an accumulation of 1.2 to 1.4 mg of selenium per g of yeast. Kieliszek et al.[62] evaluated the addition of 60 mg/L of sodium selenite in *Saccharomyces cerevisiae* ATCC MYA-2200, which managed to accumulate 5.64 mg of selenium per g of yeast. On the other hand, Yoshinaga et al.[61] enriched different yeast strains (see Table 2) at a concentration of 200 µmol/L of selenium in a growth medium called it optimized selenium accumulation medium (OSAM), where the *Saccharomyces cerevisiae* strain Lalmin® SE2000 accumulated the highest amount of selenium, while *Saccharomyces cerevisiae* AW063 was the least efficient yeast in accumulation. Differences in selenium bioaccumulation by yeast are related to genotypes between different yeasts of the same species.

Other species of selenium different to sodium selenite have been tested in yeast enrichment. Ogra et al.[64] tried to enrich *Saccharomyces cerevisiae* BY106 with SeSug1, SeSug2, TMSe (trimethylselenonium ions), SeMet and MeSeCys with the aim of studying how yeast metabolize organic compounds of selenium (see Table 2). These authors found that SeSug1, SeSug2, SeMet and MeSeCys are metabolized in SeMet, whereas TMSe cannot be metabolized in other forms of selenium. On the other hand, Ouerdane and Mester[65] evaluated the enrichment of wild type *Saccharomyces cerevisiae* with selenomethionine or selenium (VI) with the aim of evaluating the substitution of Met by SeMet,

using a synthetic medium to ensure maximum incorporation of SeMet. The medium containing SeMet was able to replace Met by SeMet up to 98%, while the medium enriched with Se (VI) was able to replace 50%. These studies demonstrate that the metabolic pathway of selenium is dependent on the presence of specific enzymes that allow bioconversion of inorganic selenium to organic species.

It is common to find selenium supplements made from selenized yeast. Prange et al.[57] achieved the speciation of selenium from selenized yeast of the Spring Valley ™ Selenium brand using the XANES spectroscopy technique. Each tablet contains 200 µg of selenium, of which 75% corresponded to SeMet and 18% to Se- (methyl-L-selene) cysteine.

Porto et al.[76] achieved the enrichment of *Saccharomyces cerevisiae* UFMG A-905 with sodium selenite at different concentrations (0–100 mg/L). They determined that at 100 mg/L there is a survival of 7%, likewise in a comparative proteomic study it was revealed that sodium selenite at high concentrations (> 50 mg/L) produces oxidative stress in yeast. This effect is due to the fact that SeMet is a precursor of methylselenol. This compound, together with hydrogen selenide, are the initiators in the production of reactive oxygen species (ROS) through a glutathione-dependent reaction inside the cell. However, there are specific mechanisms of *S. cerevisiae* that allow counteracting oxidative stress.

Finally, Mapelli et al.[77] designed a strain of *Sacchromyces cerevisiae* CEN.PK113-7D to express an optimized codon of heterologous selenocysteine methyltransferase and endowed with high intracellular levels of S-adenosyl-methionine. This study was carried out with the objective that the yeast accumulated Se-methylselenocysteine (SeMCys) at higher levels than commercial selenized yeasts. Due to this modification a ~ 24-fold increase in the concentration of SeMCys was achieved. In

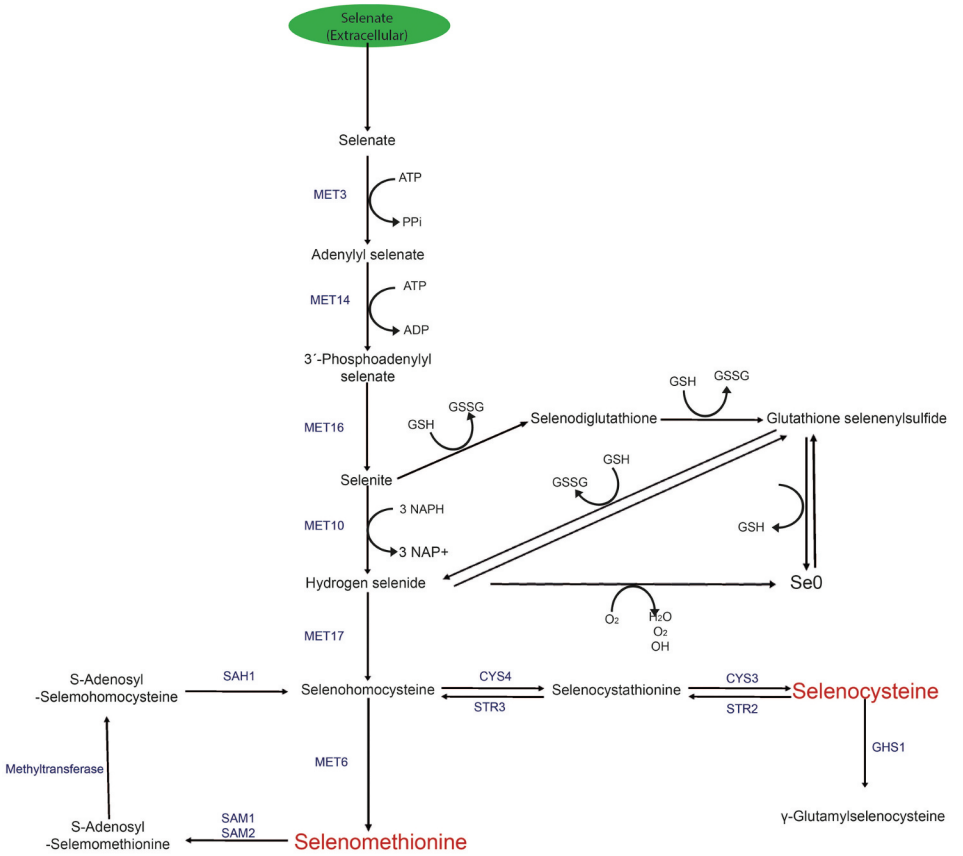

Figure 2. Selenium metabolism in *Saccharomyces cerevisiae*. Modified from:[55,74,78].

addition, the selenization of yeasts, especially *Saccharomyces cerevisiae*, is used for the production not only of inorganic selenium but also of SeNP with an approximate size of 20–30 nm.[66] Thus, there are new proposals for the use of selenized yeasts and their metabolites, which also provides new research opportunities on the benefits to human health.

Inorganic selenium metabolism: Biochemical pathway to biotransformation

Bioaccumulation and metabolism of selenium in yeast is very complex (Fig. 2). Bioaccumulation process of selenium occurs from the extracellular binding by membrane assembly ligands and intracellular accumulation associated with transport of ions through the cytoplasmic membrane into the cell.[78]

The intracellular process of selenium occurs through detoxification that allows yeasts to survive in culture conditions with high concentrations of selenium. Therefore, conversion of selenium begins with reduction of selenate (VI) to adenylyl selenate (APSe) by a reaction catalyzed by ATP sulfurylase. APSe is phosphorylated again by adenylyl sulfate kinase to produce 3′-phosphoadenylyl selenate (PAPSe), later, through the reaction catalyzed by PAPSe reductase, it is converted into selenite (IV), which is subsequently reduced to selenide (Se2-) by 3 '-phosphoadenylyl sulfate reductase using NADPH as a reducing agent.[78]

In a second route (Fig. 1), selenite reacts spontaneously with the reduced form of glutathione (GSH), to produce selenodiglutathione (GS-Se-SG) and the oxidized form of glutathione (GSSG), additionally, selenodiglutathione in presence of a glutathione excess is reduced to glutathione selenyl sulfide (GS-Se-H), which can undergo further transformations, then it spontaneously dissolves into elemental selenium (Se0) and glutathione in presence of superoxide dismutase, or is further reduced by glutathione to produce hydrogen selenide (H2Se/HSe-) with simultaneous formation of the oxidized form of glutathione (glutathione disulfide, GSSG).[74]

Hydrogen selenide is readily oxidized with oxygen with the concomitant generation of ROS. Hydrogen selenide is the main intermediate metabolite involved in the synthesis pathway of all forms of selenium found in microbial cells. Selenide is introduced into O-acetylhomoserine (OAH) by homocysteine synthase (Hcy), which is encoded by MET17 in yeast to produce selenohomocysteine (SeHcy) and acetic acid. Consequently, SeHcy is a branch point in the Se-amino acids biosynthetic pathway (Fig. 2). SeHcy can produce SeMet which is synthesized directly from SeHcy catalyzed by the cobalamin-independent Hcy methyltransferase, which is encoded by MET6.[55,74,78]

Additionally, SeMet is converted to Se-adenosyl-selenomethionine (SeAM) in a reaction catalyzed by S-adenozylomethionine synthase. Subsequently, SeAM undergoes an enzymatic methylation process releasing adenosyl homo-selene cysteine (SeAHCys), this compound is subjected to hydrolysis, resulting in the formation of selenohomocysteine (SeHCys). Then, formation of selenocysteine (SeCys) results from the transformation of selenomethionine with the help of cystathionine γ-lyase (Fig. 2). Subsequently, SeCys reacts with S-adenosylmethionine (SAM) to form seleno-methyl-selenocysteine (SeMeCys) and S-adenosyl-homo-selenocysteine via selenomethyltransferase (SMT). Additionally, SeMeCys is converted to γ-glutamyl-Se-methylcysteine. Finally, formation of selenoproteins through the incorporation of SeCys into proteins, is carried out through a specific Sec-tRNASec complex.[55,78]

Seleno-yeasts into food processing

In addition to its use as a functional supplement, selenized yeasts have been used for food processing. However, companies like Altech. Lallemand, Selko, Orffa and Angel Co. offer selenized yeasts for use in livestock feed. In this way, bioavailable and bioabsorbable organic selenium is given.

Table 3. Use of selenized yeasts in food production for human consumption.

Product		Selenized yeast	Selenium concentration	Reference
Milk and dairy products	Milk	*Saccharomyces cerevisiae*	35–41 µg/kg	[79]
		Saccharomyces cerevisiae NCYC R397	51.8 µg/kg	[80]
		Saccharomyces cerevisiae CNCM I-3060	3.937 L/day in milk production	[81]
		Saccharomyces cerevisiae CNCM I-3060	42.3–60.2 ng/g	[82]
		Saccharomyces cerevisiae	35.81 µg/L	[83]
		Saccharomyces cerevisiae CNCM I-3060	60–270 µg/kg	[84]
		Saccharomyces cerevisiae CNCM I-3060	80–160 µg/kg	[84]
		SeMet	48.34 µg/kg	[85]
	Caciotta cheese	*SeMet*	241 µg/kg	[85]
	Edam cheese	*Saccharomyces cerevisiae* NCYC R397	361 µg/kg	[80]
	Mozzarella cheese	*Saccharomyces cerevisiae*	146 µg/kg	[83]
		Saccharomyces cerevisiae	22.3–31.9 µg/kg	[86]
Chicken meat and eggs	Eggs	*Saccharomyces cerevisiae*	2.6 mg/kg	[87]
		Saccharomyces cerevisiae	0.56 mg/kg	[88]
	Chicken	*Saccharomyces cerevisiae*	1.3 mg/kg	[87]
		Saccharomyces cerevisiae	0.24 mg/kg	[88]
		Saccharomyces cerevisiae CNCM I-3060	0.19 mg/kg	[89]
Enriched Meat	Rabbit	*Saccharomyces cerevisiae* CNCM I-3060	400 µg/kg in loin and 389 µg/kg in hind legs	[90]
		Saccharomyces cerevisiae CNCM I-3060	0.24 mg/kg	[91]
		Saccharomyces cerevisiae CNCM I-3060	0.098 µg/g in hind legs	[92]
		Saccharomyces cerevisiae	169.15–172.85 µg/kg	[93]
	Pork	*Saccharomyces cerevisiae CNCM I-3060*	0.26 mg/ kg	[89]
Fermented beverages	White wine	*Saccharomyces cerevisiae* and *Saccharomyces bayanus*	0.3–6 µg/mL	[56]
	Beer	*Saccharomyces cerevisiae*	17.9–73 µg/kg	[94]
		Saccharomyces cerevisiae W34/70	17.5 µg/g	[95]
		Saccharomyces cerevisiae and *Saccharomyces uvarum*	0.086–6.0 µg /ml	[96]

Obtaining milk

Studies have been carried out on the administration of selenized yeasts to cows with the aim of obtaining milk with a high content of selenium, as described in Table 3.

Faccenda et al.[79] selected Holstein cows to be fed with diets based on malt bagasse enriched with selenized yeast with the objective of investigating selenium absorption. At the end of the treatment there was an increase between 40 to 70% in the content of selenium. Ling et al.[80] chose Estonian Red dairy cows, which were fed Alkosel® selenized yeast for 64 days, along with a total mixed ration (TMR). At the end of the treatment, a 202% increase in selenium was observed in the milk, compared to the control (without selenized yeast).

Liu et al.[83] studied that when administering 0.3 mg/kg of selenized yeast to dairy cows, the concentration of selenium in milk increased from 5.53 µg/L to 35.81 µg/L. Stockdale and Gill[97] and Stockdale et al.[84] administered different concentrations of Sel-Plex selenized yeast and they determined an increase of selenium in milk of 4.5 to 5 µg/kg for each milligram of selenium consumed per day.

Ullah et al.[81] evaluated the administration of different concentrations of Sel-Plex to Achai dairy cows, for milk production. When supplementing with 0.3 mg/kg, production increased by 24%. In addition to this result, selenium supplementation promoted postpartum progesterone production reducing hormonal stress. Calamari et al.[82] studied the administration of Sel-Plex at two different concentrations, to determine the effect of the source (sodium selenite and selenized yeast) and the dose of selenium in Italian Friesian dairy cows. They observed an increase in the concentration of selenium in milk, in addition to the fact that SeMet was detected in milk when the source was selenized yeast. This selenoamino acid was not detected in milk from cows to which sodium selenite was administered. This demonstrates a greater efficiency in the production of selenoproteins when the diet is supplemented with selenized yeast.

Dairy products

Selenized milk can be used to make dairy products. Thus, elaboration of dairy products produced with milk from cows fed with selenized yeast has been studied. Ling et al.[80] elaborated Edam-type cheese with milk that contained 17.1 μg/kg and 51.8 μg/kg of selenium. The final product had a selenium concentration of 146 μg/kg to 361 μg/kg. Liu et al.[83] made mozzarella cheese with milk that contained 35.81 μg of selenium per liter, they obtained cheese with a selenium concentration of 146 μg/kg. They observed that the functional properties were better in cheeses with selenium than those elaborated without selenium. In addition, pH and water activity were lower, in enriched cheeses the total count of microorganisms decreased.

Peng et al.[86] made mozzarella cheese, which was put at different concentrations of selenized yeast overnight. These cheeses were vacuum packed and stored. The selenium content was monitored at 3, 30 and 60 days of storage and samples were taken from the exterior and the center of the cheese. Table 3 shows the selenium content of the center of the cheese after its storage for 60 days. The selenium content in the cheese increased significantly at the end of storage, which improved the functional properties. Additionally, the pH and water activity decreased. Ianni et al.[85] elaborated caciotta cheese, from milk previously enriched with SeMet, which was supplemented to Friesian cows diet. Alzate et al.[98] elaborated Kefir enriched with selenium, using sodium selenite as a source of selenium and *Saccharomyces cerevisiae* for bioconversion to organic selenium. Se (Cys) 2, MeSeCys, Se (IV) and SeMet were detected in the final product.

Chicken meat and eggs

The use of selenized yeast is not restricted to livestock feed. In the same way, it is used to feed chickens and rabbits. In the case of chickens, it is mainly used to obtain eggs or muscle with a high content of selenium. Bakhshalinejad et al.[99] evaluated the administration of Sel-Plex, and different selenium sources to broilers at different concentrations in order to evaluate the quality and performance of the meat. Supplementing with nano particles of selenium and its organic forms, the meat quality was better compared to meat supplemented with only inorganic selenium. Lu et al.[87,88] evaluated the administration of YeaSel® to Hy-Line Brown laying hens, in both studies the increase in selenium in the breast was significantly high compared to the control groups. The highest dose tested was 3 mg/kg, which did not represent toxicological damage to hens.

Lu et al.[87,88] evaluated the products of laying hens. Eggs with a high content of selenium were obtained, 2.6 mg/kg for a supplementation with 3 mg/kg and 0.56 mg/kg for a supplementation with 0.3 mg/kg. Borilova et al.[100] evaluated the effects of supplementation with different selenium sources on the functional properties of eggs. They used sodium selenite, selenized yeast, selenomethionine and a hydroxy selenomethionine analog and observed that when supplementing the feed with selenized yeast, the albumin foaming capacity, the stability and the foaming capacity of the yolk improved, compared to other selenium sources.

Selenium Enriched Meat

Due to the accumulation of selenium in animal tissues, the supplementation of diets with selenized yeasts exerts a benefit in obtaining enriched meat.[89] During supplementation with selenized yeast in rabbit diets, an increase in selenium has been observed in muscle. This benefit has been reported by various authors who have done it in fully supplemented or form diets. .[90–93]

An added benefit of the administration of organic selenium is to improve stress during weaning of mammals, [93] without affecting the growth of the animal.[91,92] Danuta et al.[101] when comparing the quality of rabbit meat, incorporating odorless fish oil, selenized yeast and lycopene into their diet, found that selenium and lycopene expressed antioxidant activity by slowing down the oxidation

process of fats. Same antioxidant capacity reported by Minardi et al.[102] when testing the administration of organic selenium together with Vitamin E.

On the other hand, the use of selenium enriched pork could offer health benefits. Pešut et al.[89] tested administering selenized yeast to fattening pigs, at the end of the treatment they observed an increase in selenium in muscle tissue. Zhang et al., [103] after the administration of selenized yeast and seleniomethionine to the piglet diet, observed improvements in healthy life, growth and antioxidant capacity of piglets. Finally, Zhang et al.[104] showed that by administering organic selenium (SeMet and MeSeCys), the immune function of the serum improved in pigs, in addition to increasing the water retention capacity of the pig muscle.

Fermented beverages

Production of fermented beverages, such as beer and wine, has been innovated with the use of sodium selenite as a source of selenium, which yeasts are incorporated into those processes to carry out the bioconversion of selenite from sodium to organic selenium. Pérez-Corona et al.[56] evaluated the administration of different concentrations of sodium selenite to must from white grapes. Bioconversion was performed during fermentation by aggregated *Saccharomces cerevisiae* and *Saccharomces bayanus*. Selenium content in wine was dependent on the dose of sodium selenite added. And the main form of selenium in wine was SeMet. Furthermore, it was observed that *Saccharomces cerevisiae* had a better transformation efficiency to organic selenium.

In the case of beer, *Saccharomyces cerevisiae* has mainly been used. Rodrigo et al.[94] investigated the production of beer using barley previously enriched with selenite or sodium selenite. The selenium content in the final product was lower than the content initially in the barley grains (see Table 3). SeMet was detected in barley grains, malt, and wort, but not beer. Similar results were found by Rodrigo et al., [95] in this case isotopically enriched ^{77}Se wheat was used for brewing beer, where the selenium content in the final product was 17.5 μg/g. Another alternative to obtain SeMet in beer is the one proposed by Sánchez-Martínez et al.[96] they evaluated the addition of sodium selenite at different concentrations to the malt must and observed changes caused by the presence of *Saccharomyces cerevisiae* and *Saccharomyces uvarum*. Alcohol production was not affected by the incorporation of selenium, and SeMet was the main selenium compound identified from beer, demonstrating the biotransformation of sodium selenite to SeMet by yeast.

Conclusions

Food science and technology are still doing research in order to improve foods by enriching them with bioactive principles. Yeast selenization is one of the most promising research fields, above all because these microorganisms are used in different fermentation processes. Likewise, animal feed for enrichment of secretions such as milk, poultry products such as eggs and meat production, are fields of research that offer opportunities and challenges in the development of functional foods. These opportunities and challenges are complemented by investigations of biochemical mechanisms of trace elements biotransformation such as selenium. Selenium is an essential metalloid for human health and proper metabolic function. Research in this field is of utmost importance since the assurance of food not only requires sustainability as the main axis, but also functional, nutraceutical and safe foods. According to this review, selenium metabolism by yeast aimed not only at new enriched food manufacturing, but also at conducting studies of basic biochemistry to find the complete mechanism of selenium biotransforming. Thus, the combination of basic and applied science suggests a change in our way of seeing food processes and the development of food processing technology.

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Capítulo -III-.
Mechanistic insight into biotransformation of inorganic selenium to selenomethionine and selenocysteine by *Saccharomyces boulardii*: *In-silico* study.

3.1 Introducción

Este capítulo presenta un estudio *in silico* publicado en la revista Biointerface Research in Applied Chemistry. En el se realizó una comparación genetica entre *Saccharomyces cerevisiae* s288c y *Saccharomyces boulardii* para buscar los genes implicados en la biotransformación de selenato de sodio a selenometionina y selenocisteína, usando lo reportado para *S. cerevisiae*. La información genetica de ambas levaduras se recopilo de la base de datos del NCBI. Se realizó la comparación de genes ortologos de ambas levaduras y se construyó la ruta metabólica de bioconversión por *S. boulardii.* Los resultados del presente estudio, mostraron que son 17 genes implicados en la bioconversión de selenato hasta llegar a selenometionina, selenocisteina y selenoglutation. Dichos genes presentes en *S. cerevisiae*, se encuentran de manera putativa en *S. boulardii*, y realizan la misma función que en *S. cerevisiae.* De esta manera, se logró proponer la ruta bioquímica de bioconversión se selenato a selenoaminoácidos por *S. boulardii*, que es la única levadura probiotica aprobada por la FDA para consumo humano. Mediante este estudio bioinformático se observó que *S. boulardii* es capaz de bioconvertir selenio inorgánico a selenio orgánico con potenciales aplicaciones tecnológicas y en el campo de la medicina.

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Mechanistic Insight into Biotransformation of Inorganic Selenium to Selenomethionine and Selenocysteine by *Saccharomyces boulardii*: *In-silico* Study

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Abstract: In recent years, the biosynthesis of seleno amino acids from inorganic selenium has been a subject of investigation. *Saccharomyces cerevisiae* has been reported to bioaccumulate selenium through the metabolism of selenate. Different authors have postulated a metabolic pathway in which selenate is converted into selenomethionine or selenocysteine based on the research carried out so far. However, little has been known how other types of yeast achieve this bioconversion. For that reason, and due to the importance of *Saccharomyces boulardii* as a probiotic yeast, the present study proposes a biosynthetic route used by this yeast to incorporate inorganic selenium into organoselenium compounds. A comparative in-silico study was carried out using *Saccharomyces boulardii* ASM141397V1 and a metabolic model at the genomic scale of *Saccharomyces cerevisiae* S288C. Basic local alignment database BLASTp-NCBI was used to identify orthologous genes in both strains, and the generated data were visualized in a circular layout using CIRCOS software. The metabolic route of selenium assimilation was proposed based on the obtained results.

Keywords: selenium; *Saccharomyces boulardii*; selenocysteine; selenomethionine; probiotic.

1. Introduction

Yeasts can permanently incorporate ions found in their natural environment into their cellular structures [1]. In the recent decade, selenium enrichment in yeasts has been intensively investigated, and the most studied species include *Saccharomyces boulardii*, *Candida utilis*, *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *Rhodotorula glutinis* [2-9]. The focus has been placed on the yeast's metabolism and their biotransformation capability to incorporate inorganic selenium into proteins, particularly enzymes [1]. Bioaccumulated selenium's concentration varies between species and can reach up to 5.64 mg of selenium/g d.w. of yeast in *Saccharomyces cerevisiae* and 1.64 mg of selenium/g DW of yeast in *Candida utilis* [10].

As for *Saccharomyces boulardii* was originally isolated from the lychee fruit and has been the only probiotic yeast approved by the Food and Drug Administration (FDA) for human

consumption [11,12]. *Saccharomyces boulardii* has been commonly used to prevent or treat severe diarrhea associated with bacterial infections and other gastrointestinal disorders [12]. In addition to probiotic properties [13], *Saccharomyces boulardii* has been reported to produce selenium nanoparticles [14]. Therefore, the yeast-assisted production process of organic selenium has been considered a green technology [14,15].

Nevertheless, few works have been focused on applying *Saccharomyces boulardii* to produce selenomethionine (SeMet) and/or selenocysteine (SeCys). The capacity of this yeast to synthesize selenoamino acids offers the possibility to gain access to selenium in a more bio-available and less toxic way [16, 17].

Previous studies have demonstrated similarities between the biosynthetic pathway of sulfur and selenium. The latter was found to replace sulfur and is incorporated in cells as SeMet and/or SeCys [1, 18]. Another proposed mechanism known as trans-sulfuration involves an unspecified enzymatic route that has not been proven yet [19]. Interestingly, not all the yeasts synthesize both selenoamino acids. In the case of *Saccharomyces cerevisiae* BY4741, it is also known that methionine is produced through a route different from the incorporation of inorganic sulfur [19]. In this context, it is possible that certain genes that encode enzymes expressed by *Saccharomyces cerevisiae* to synthesize organoselenium compounds could be orthologous to those found in *Saccharomyces boulardii*. Moreover, the genome of *Saccharomyces cerevisiae* has been the most studied and characterized among eukaryotes [20]. Therefore, the present *in-silico* study focused on identifying probable orthologous implied genes implied in the biosynthesis of organic selenium by *Saccharomyces boulardii* ASM141397v1 to propose a bioconversion route of sodium selenate into SeMet and SeCys.

2. Materials and Methods

2.1. Identification of the study object.

The genetic comparison was carried out using an *in-silico* study. *Saccharomyces boulardii* ASM141397v1 was selected as a study object compared to the *Saccharomyces cerevisiae* s288c genome due to the high genetic similarity between the two yeasts. The iMM904 model [21] and Kegg model [22] for *S. cerevisiae* were used to carry out a genetic comparison. The genetic information of both yeasts was retrieved from the NCBI database [23].

2.2. Bioinformatic search.

Genes involved in the production of SeMet and SeCys were identified using the Entrez database provided by NCBI [23]. Gene sequences reported in selenium metabolism were searched for *Saccharomyces cerevisiae* s288c. A similar procedure was carried out for *Saccharomyces boulardii* ASM141397v1. Sequences for orthologs identification were prepared.

2.3. Orthologs.

With the information collected, orthologs were searched using the BLAST server at the NCBI [23, 24]. BLASTp (protein-protein BLAST) program was selected, and the query sequence was submitted in FASTA format. In addition, the sequence alignment was carried out exclusively with *S. cerevisiae* s288c to compare and find local sequences similarity regions

between the two strains [25]. A total number of 9361 amino acids were analyzed, and the data were visualized on a circular plot using Circos software that permitted exploring the genetic relationship between the two species (http://circos.ca/).

2.4. Construction of Metabolic Route.

Following the analysis of genetic homology, a metabolic route of selenium assimilation and the consequent production of SeMet and SeCys by *Saccharomyces boulardii* were postulated, considering previous proposals made by previous proposals Kieliszek *et al*. [26] and Asghari-Paskiabi *et al*. [27] for *S. cerevisiae*.

3. Results and Discussion

3.1. Bioinformatic search.

Biotransformation of selenate into organic selenium begins with the detoxification process that yeast performs in response to the excess of sodium selenate.

Table 1. Genes involved in the biotransformation of selenate into selenoamino acids in *Saccharomyces cerevisiae* s288c and *Saccharomyces boulardii* ASM141397v1.

Gene	**Chromosome**	**locus_tag *Saccharomyces cerevisiae* s288c**	**locus_tag *sachharomyces boulardii ASM141397v1***	**Enzyme expressed**
SUL1	II	YBR294W	AB282_00450	High-affinity sulfate permease of the SulP anion transporter family
SUL2	XII	YLR092W	AB282_03394	High-affinity sulfate permease
MET3	X	YJR010W	AB282_02749	ATP sulfurylase
MET14	XI	YKL001C	AB282_03058	Adenylylsulfate kinase
MET16	XVI	YPR167C	AB282_05395	3'-phosphoadenylsulfate reductase
MET10	VI	YFR030W	AB282_01793	Subunit alpha of assimilatory sulfite reductase
MET17	XII	YLR303W	AB282_03569	O-acetyl homoserine-O-acetyl serine sulfhydrylase
MET6	Va	YER091C	AB282_01662	Cobalamin-independent methionine synthase
SAM1	XII	YLR180W"	AB282_03468	S-adenosylmethionine synthetase
SAM2	IV	YDR502C	AB282_00999	S-adenosylmethionine synthetase
SAH1	Va	YER043C	AB282_01610	S-adenosyl-L-homocysteine hydrolase
CYS4	VII	YGR155W	AB282_01996	Cystathionine beta-synthase
STR3	VII	YGL184C	AB282_02293	Peroxisomal cystathionine beta-lyase
CYS3	I	YAL012W	AB282_00053	Cystathionine gamma-lyase
STR2	X	YJR130C	AB282_02643	Cystathionine gamma-synthase converts cysteine into cystathionine
GSH1	X	YJL101C	AB282_02849	Gamma glutamylcysteine synthetase
GSH2	XV	YOL049W	AB282_04624	Glutathione synthetase

The metabolic pathway resembles the bioconversion route of sulfur in producing sulfur amino acids. In this pathway, selenium replaces sulfur and is incorporated in the chemical structure of methionine and cysteine [18]. According to Lazard *et al*. [28], genes implied in the biotransformation of selenate into SeMet and SeCys for *Saccharomyces cerevisiae* have been shown in Table 1. The reported genes were included in the search query of genetic sequence in *S. boulardii*. Although the identified genes for *S. boulardii* have received putative names, the encoded enzyme has the same function in both species.

3.2. Orthologs.

Once the relevant genes in *S. boulardii* were identified, homology analysis was carried out to verify whether the genes were orthologous in both strains [24]. The results were visualized on a circular plot, as shown in Figure 1, to determine orthologs in *S. boulardii* and *S. cerevisiae*. Seventeen genes were arranged ascending to the left according to the number of amino acids analyzed. On the right side, the two yeasts species *S. boulardii* and *S. cerevisiae* can be found. Each gene is homologously linked to *S. cerevisiae* (blue) and *S. boulardii* (green). In agreement with the BLASTp search results, they encode the same enzymes that have the same function. For that reason, *S. boulardii* can biosynthesize organoselenium compounds in the same way as *S. cerevisiae* does. The main difference was observed for *SAH*1 and *MET*6 genes which are found in chromosome V of *S. cerevisiae* and chromosome Va in *S. boulardii*.

Despite the high homology between the two genetic sequences, *S. boulardii* has unique physiological and metabolic properties, such as resistance to temperature and acid stress [29, 30]. Another distinctive characteristic is the absence of the following genes in the genome of *Saccharomyces boulardii* ASM141397v1: hexose transporter genes (*HXT*11, *HXT*9), genes implied in asparagine catabolism (*ASP*3-1, *ASP*3-2, *ASP*3-3, *ASP*3-4), transporter gene ARN2, genes involved in the biosynthesis of thiamine or pyridoxine (*SNZ*2, *SNZ*3), and metallothionein gene CUP1 [31]. Nevertheless, none of these genetic differences affects the *S. boulardii* capacity to transform inorganic selenium into selenoproteins, produce selenoparticles and reduce to elemental selenium in detoxification processes.

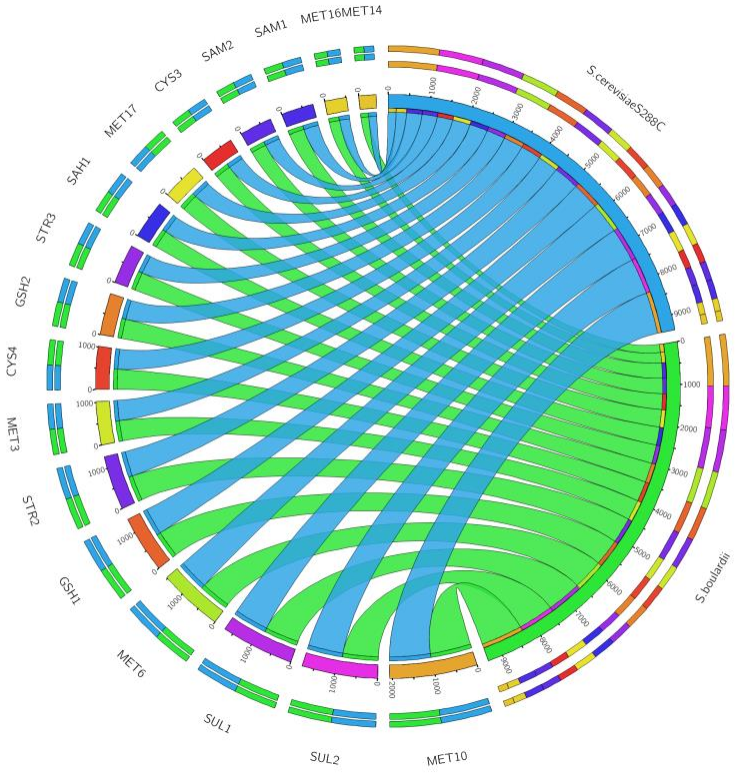

Figure 1. The circular plot of orthologs identified for *Saccharomyces boulardii* ASM141397v1 (green) and *Saccharomyces cerevisiae* s288c (blue).

3.3. Biosynthetic route of SeMet and SeCys in S. boulardii ASM141397v1.

Based on the search results for genes implied in the biotransformation of selenium and information retrieved from the KEGG/PATHWAY database [22] combined with the available

reports on biotransformation of selenium by *S. cerevisiae* [26, 27], a biosynthetic pathway of selenoamino acid production by *S. boulardii* was proposed.

Assuming that biosynthesis starts with detoxifying selenium through a metabolic pathway similar to that of sulfur, the following beginning of absorption is proposed. Selenium could be absorbed in two different ways. The first one involves sulfur ABC membrane transporters encoded by operon cysAWTP. In this mechanism, selenium ions are transported using the energy derived from the hydrolysis of bound ATP. The second system comprises the enzymatic transport of selenium by sulfate permeases [26], encoded by AB282_00450 and AB282_03394 operons. These enzymes are involved in transferring selenate through the plasmatic membrane from the cell's exterior.

Once the selenate is found inside the cell, biotransformation begins with selenate activation. This process is carried out in two subsequent reactions. In the first step, the adenosyl-phosphoryl residue of ATP is transferred to selenate in a reaction catalyzed by ATP sulfurylase that AB282_02749 encodes. As a result, adenylyl selenate is produced, which in turn undergoes phosphorylation to 3'phosphoadenylyl selenate in a reaction catalyzed by an adenylyl-sulfate kinase (AB282_03058). Activated selenate is reduced to selenite prior to the biosynthesis of SeMet and SeCys. First, 3'-phosphoadenylsulfate reductase (AB282_05395) catalyzes the reduction of 3'phosphoadenylyl selenate to 3',5'-bisphosphate, and free selenite, using reduced thioredoxin as substrate. Next, the assimilatory sulfite reductase alpha subunit (AB282_01793) converts selenite into hydrogen selenide. The latter is transferred to selenohomocysteine in the presence of O-acetylserine-O-acetylhomoserine sulfhydrylase (AB282_03569).

Biosynthesis of SeMet from selenohomocysteine is catalyzed by cobalamin-independent methionine synthase (AB282_01662). In this reaction, selenohomocysteine undergoes methylation necessary for SeMet formation. It has been observed that activation of methyltransferases is a cobalamin-dependent reaction similar to that of MetH isolated from *E. coli* [32]. However, homocysteine methyltransferase found in *S. cerevisiae* and *S. boulardii* is a cobalamin-independent enzyme. It has been verified that B12 was not required for these yeast strains to grow.

Moreover, SeMet is converted to S-adenosyl-selenomethionine through the action of S-adenosylmethionine synthetase (AB282_03468/AB282_00999) that catalyzes the transfer of an adenosyl group of ATP to the selenium atom of selenomethionine. In the next step, S-adenosyl-selenomethionine is transformed into S-adenosyl-selenohomocysteine upon the action of methyltransferase. Selenohomocysteine is released again in a reaction catalyzed by S-adenosyl-L-homocysteine hydrolase (encoded by AB282_01610) that catabolizes S-adenosyl-L-homocysteine. The latter is formed following the donation of the activated methyl group of S-adenosyl-L-methionine (AdoMet) to a receptor. Substitution of methionine by SeMet in proteins does not significantly alter the kinetic properties of the enzymes [17].

As for the biosynthesis of SeCys from selenohomocysteine begins with the conversion of selenohomocysteine to selenocystathionine in a reaction catalyzed by cystathionine β-synthase (encoded by AB282_01996). Since the reverse reaction is catalyzed by peroxisomal cystathionine β-lyase (AB282_02293), selenocystathionine is converted back to selenohomocysteine. The next step consists of selenocystathionine transformation to SeCys in the presence of cystathionine γ-lyase (AB282_00053). Furthermore, γ-glutamyl-selenocysteine catalyzes the transformation of SeCys to γ-glutamyl-selenocysteine which is the first step in the biosynthesis of selenoglutathione. Finally, selenoglutathione is produced in the reaction of

γ-glutamyl-selenocysteine and glycine catalyzed by an ATP-dependent glutathione synthetase (AB282_04624), as shown in Figure 2. The sulfur amino acids pathway is correlated with the synthesis of selenocysteine and selenomethionine in yeast such as *Saccharomyces* species [33].

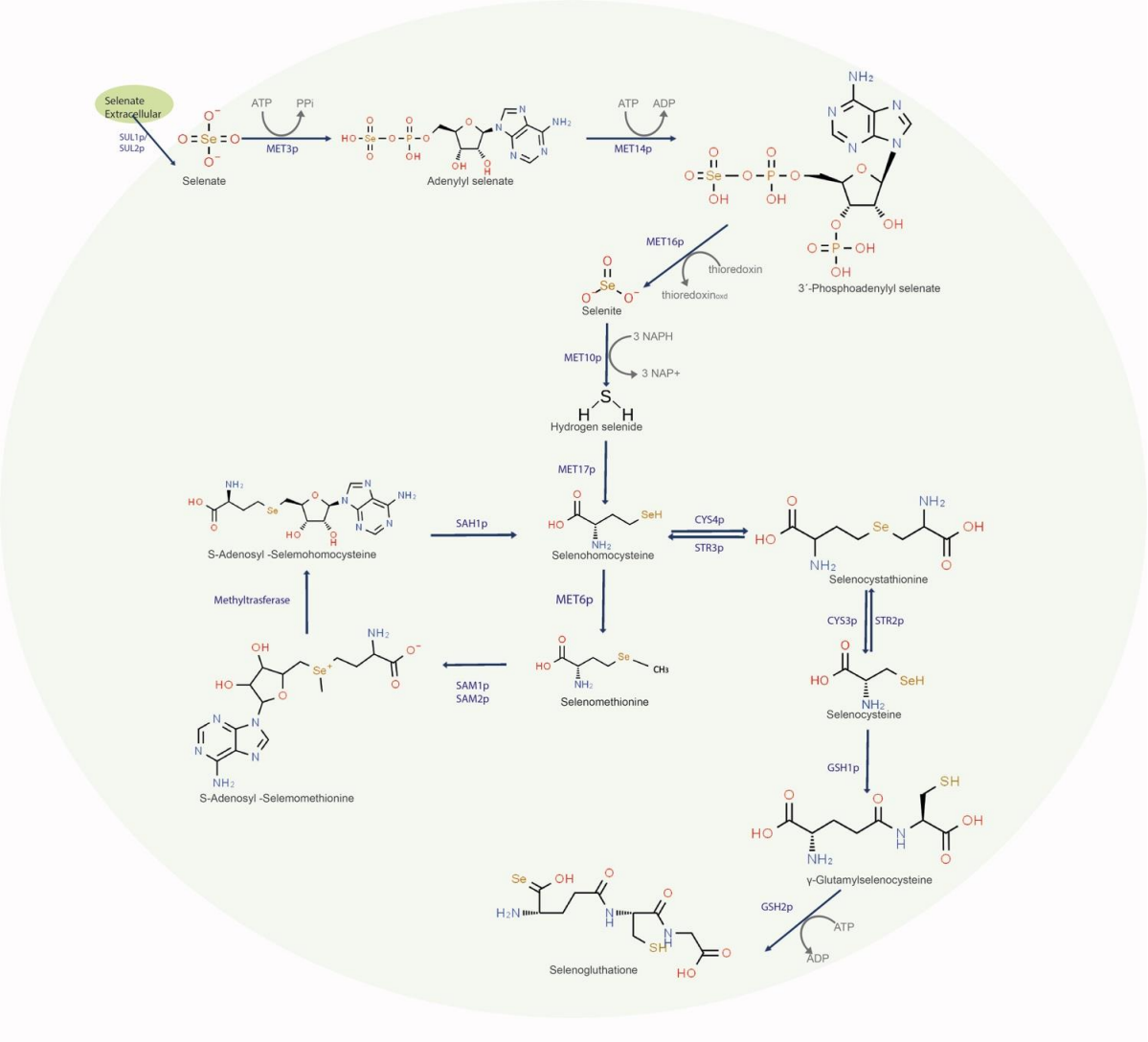

Figure 2. Biosynthetic pathway of organic selenium proposed for *Saccharomyces boulardii* ASM141397v1

4. Conclusions

Identification of orthologs between *S. cerevisiae* and *S. boulardii* permitted determining the biochemical route used by the probiotic yeast to convert inorganic selenium into selenomethionine and selenocysteine. *In-silico* studies have been used to gain theoretical insight into the biochemical mechanisms that govern *S. boulardii*. These biotransformations are of great relevance for technological applications. The addition of *S. boulardii* during the processing of fermented foods offers advantages that go beyond the proven probiotic capacity. Recent studies have shown the importance of organic selenium as a metabolite of high bio-accessibility and bioavailability in the human body compared to the commonly consumed inorganic selenium. Finally, the ability of *S. boulardii* to accumulate selenium could be used in the production of seleno nanoparticles, which have a vast potential of applications including medicine, such as alternative therapies in the fight against cancer, among others.

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Conflicts of Interest

The authors declare no conflict of interest.

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Capítulo -IV-
Biogenic production of seleno-aminoacids and seleno-nanoparticles by *Saccharomyces boulardii.*

4.1 Introducción

En este capítulo se presenta un artículo original publicado en la revista Food Biosciencie. En éste se explica de manera detallada el trabajo realizado para obtener una levadura selenizada. Se calculó la concentración mínima de inhibición de selenito de sodio metabolizado por *Saccharomyces boulardii* y por medio de la técnica ICP-OES se observó que la absorción de selenio total realizado por la levadura fue de 3.4 mg de selenio por gramo de levadura. Además se determinó la capacidad de bioconversión de selenio inorgánico a orgánico, mediante la identificación de dos selenoaminoácidos, a saber, selenometionina y selenocisteína, la identificación de selenoaminoácidos se realizó por RP-HPLC. Adicionalmente se identificó la presencia de nanopartículas de selenio por TEM así como la dispersión de dichas partículas por DLS, donde el tamaño de las nanopartículas oscilaba entre los 100-200 nm. Esta parte de la investigación permitió cumplir con dos objetivos específicos del trabajo doctoral, además de que los resultados demuestran que es posible la producción biogénica de selenoaminoácidos y nanopartículas de selenio por parte de la levadura probiótica *Saccharomyces boulardii.* Finalmente los resultados obtenidos representan un área de oportunidad para seguir estudiando otros campos como la tecnología de alimentos o bien campos de medicina en salud humana y animal.

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Biogenic production of seleno-amino acids and seleno-nanoparticles by *Saccharomyces boulardii*

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ABSTRACT

Selenium is an essential element for humans as it involves different metabolic mechanisms. More bioavailable and bioaccessible selenium species are produced by microorganisms bioconverting inorganic selenium into nanoparticles and seleno-amino acids, which are of technological and health interest. Therefore, the objective of this study was the biogenic production of seleno-amino acids and selenium nanoparticles by *Saccharomyces boulardii*. It was found that the minimum inhibitory concentration of selenite that allowed the viability of the yeast, the biotransformation of selenium, and the production of selenium nanoparticles was 74 ppm of sodium selenite in YPD broth. The yeast accumulated 3.4 mg/g of selenium after 9 h of fermentation in the YPD broth enriched with the calculated minimum inhibitory concentration of Na_2SeO_3. The presence of selenomethionine, selenocysteine, and selenium nanoparticles was verified through RP-HPLC and TEM analysis. The results demonstrated that the biogenic production of seleno-amino acids and seleno-nanoparticles by the probiotic yeast *S. boulardii* is possible. These compounds could have applications in the fields of food technology and medicine.

1. Introduction

Selenium (Se) is an essential trace element in almost all living organisms and is found in nature as inorganic Se: selenite and selenate. Although these compounds are potentially toxic, they could be biotransformed into elemental Se (Se0) and organic forms such as selenocysteine (SeCys), selenomethionine (SeMet), and selenomethylselenocysteine (SeMeCys). It is obtained through the diet in inorganic form (selenate and selenite) or organic form (SeCys and SeMet). SeMet could be incorporated into proteins instead of methionine or transformed into SeCys through a *trans*-sulfidation pathway. SeCys is converted to hydrogen selenide, a crucial metabolite for inserting SeCys into proteins. On the other hand, selenate is reduced to selenite by glutathione, and selenite undergoes further reduction by glutathione to hydrogen selenide. Therefore, all forms of Se present in food can be used for selenoprotein synthesis after conversion to hydrogen selenide (Escobar-Ramírez et al., 2022; Kieliszek & Bano, 2022).

Later, SeCys could be incorporated into the primary structure of selenoproteins (Barchielli et al., 2022). These selenoproteins are involved in the biosynthesis of hormones. They are also related to biochemical mechanisms of prevention or treatment of some conditions such as cancer, cardiovascular diseases, thyroid disease, infertility, HIV, and recently SARS-CoV-2 (Barchielli et al., 2022; González-Salitre et al., 2021). In addition to conversions, the beneficial effects of Se on human health are attributed to the presence of functional selenoproteins involved in physiological processes such as defense antioxidant

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(glutathione peroxidases), thyroid homeostasis (iodothyronine deiodinase), redox regulation of cellular processes (thioredoxin reductase), Se transport and delivery to peripheral tissues (selenoprotein P), protein folding and endoplasmic stress (selenoprotein 15, selenoprotein M, selenoprotein N, and selenoprotein S) (Kieliszek et al., 2022).

Selenium deficiency is associated with Keshan disease, nutritional muscular dystrophy, and muscle and heart defects due to a lack of specific selenoproteins. In the same way, selenium deficiency in pregnancy has recently been reported to cause fetal growth restriction (Chen, 2012; Hofstee et al., 2022). Additionally, it has been reported that selenium can increase humoral immunity and consequently increase the immune response to the COVID-19 vaccine (Dalan & Boehm, 2022; Kieliszek, 2022). Due to the low absorption of inorganic selenium obtained through the diet, it is essential to incorporate 100% bioavailable organic selenium supplements. One way to carry out such supplementation is using selenized yeasts (Jach & Serefko, 2018) because they carry out the bioconversion of inorganic salts of selenium to seleno-amino acids, making selenium a more bioavailable and accessible compound for humans (González-Salitre et al., 2021).

Yeasts have been a study model in the molecular biology of eukaryotes. Due to their ease of cultivation, null toxicity, and pathogenicity, they are good research material as preclinical models. They are a valuable tool for analyzing fundamental cellular processes in eukaryotes, which has greatly facilitated the study of the genomes of various organisms. In addition, the heterologous expression of genes from other eukaryotes has become a subject of study since it is known that many pathogenic and even lethal mutations are found in human genes that have homologous genes in the yeast genome. Scientists estimate that about 23% of the yeast genome is the same as the human genome. This information provides an opportunity to study the effects of selenium in human cell lines over previous studies in yeast (Kieliszek et al., 2022).

Saccharomyces cerevisiae has been the primary yeast studied to carry out the biotransformation of selenite to SeMet. However, other yeasts are interested in carrying out this biotransformation (Kieliszek & Dourou, 2021; Kieliszek et al., 2017; Martiniano et al., 2020; Wang et al., 2019; Yoshinaga et al., 2018). In this sense, the biotransformation pathway of selenocysteine and selenomethionine by *Saccharomyces boulardii* has recently been determined (González-Salitre et al., 2023). This yeast is a probiotic organism approved by the FDA for human consumption due to its resistance to gastric juice and viability at low pH (Fietto et al., 2004). Its usual use is for treating disorders of the gastrointestinal tract, progressing the result of acute diarrhea, antibiotic-associated diarrhea, and traveler's diarrhea (Pais et al., 2020; Shruthi et al., 2022).

In silico studies of using *S. boulardii* to produce seleno-nanoparticles (SeNP) and the biochemical mechanism of the biogenic output of seleno-amino acids were recently reported (Bartosiak et al., 2019; González-Salitre et al., 2023; Patel et al., 2013). Furthermore, studies of organic selenium production by this probiotic yeast are an opportunity to develop fermented foods enriched with organic selenium. Therefore, this study evaluated the biogenic production of seleno-amino acids and nanoparticles from the bioconversion of inorganic selenium during a fermentation process of *S. boulardii.*

2. Materials and methods

2.1. S. boulardii strain and culture conditions

Saccharomyces boulardii ATCC was obtained from the Microbial Ecology Laboratory of Universidad Autónoma Metropolitana (Iztapalapa, CDMX, Mexico), and it was stored (−5 °C) in a solution of glycerol and Yeast Peptone Dextrose (YPD) broth (1:1) (Sigma Aldrich, St. Louis. USA).

2.2. Determination of minimal inhibitory concentration (MIC)

The effect of selenium on yeast growth was evaluated by inoculating 10^7 CFU/mL of *S. boulardii* in 5 mL of YPD broth enriched with known concentrations of sodium selenite (Na_2SeO_3) (0,40, 80, 120, 160, and 200 ppm). The fermentation process was performed in triplicate and carried out in aerobic conditions at 37 °C for 24 h. The viability assay was performed according to the plate count method using YPD agar, incubating in aerobic conditions at 37 °C for 48 h. The average log CFU/mL of *S. boulardii* versus selenium concentration was graphed. The minimal inhibition concentration was calculated using the Talmage and Fitch graphical method, with a modification proposed by González-Olivares et al. (2016). The calculation was performed using GeoGebra software (https://www.geogebra.org).

2.3. Selenization of S. boulardii

As the minimal inhibition was developed, the growth kinetics of *S. boulardii* in YPD broth enriched with Na_2SeO_3 were calculated. A 10^7 CFU/mL yeast concentration was inoculated in testing tubes containing 5 mL of the enriched broth. Fermentation lasted 48 h at 37 °C. Fermentation without selenium was the control, and the growth rate (μ), the generation time (g), and the velocity (k) of both fermentations were measured.

2.4. Absorption of selenium by ICP-OES

Fermentation samples (0, 3, 6, and 9 h) were taken in the logarithmic phase. They were centrifuged (30 000×*g*) for 15 min at 4 °C (SORVALL-fresco, Thermo Fisher Scientific, USA). The supernatant was recovered by analyzing the selenium content, and the biomass was placed in Eppendorf vials. The biomass was dried in a vacuum oven at 60 °C for 4 h. Weight differences calculated mass. For selenium determination, 5 mL of concentrate HNO_3 (Ultrex, JTBaker) was added at 4 g of supernatant. Samples were digested by microwave (ETHOS ID 414) using a temperature gradient (25 min at 20 °C and 15 min at 210 °C). The selenium content of the digested samples was analyzed using the inductively coupled plasma technique (ICP-OES). A concentration curve (0.01–0.2 ppm) of elemental selenium (Se^0) was used. The selenium concentration absorbed was calculated from the difference between the enriched medium's initial and final selenium concentrations. The concentration calculated was expressed as selenium mass per gram of dried yeast.

2.5. Seleno-amino acids determination by RP-HPLC

The determination of SeCys and SeMet by RP-HPLC (PerkinElmer 200 series) was performed. Acetate buffer (0.1 M, pH 7.2) with 0.1 acetonitrile and methanol were mobile phases A and B, respectively. A Zorbax Eclipse C18 column was used for separation (Agilent Technologies Inc., Santa Clara, CA; 250 × 4.6 mm, 5 μm). The elution was performed with 50% A and 50% B for 30 min. A UV–Vis detector was used at 340 nm. The injection volume was 20 μL with a 1 mL/min rate. With some modifications, samples were derivatized before the injection using the technique proposed by Castañeda-Ovando et al. (2019).

2.5.1. Carboximethylation

L-selenomethionine and seleno-L-cystine standards (Santa Cruz Biotechnology, Dallas, Texas) were used. The seleno-L-cystine was subjected to a carboxymethylation reaction to obtain selenocysteine and carboxymethyl-selenocysteine (CSeCys). For getting SeCys, a mol of seleno-L-cystine and 3 mol of KBH_4 (Sigma Aldrich, St. Louis, USA), and 1 mL of deionized water were reacted in a Schlenk flask. The reaction was carried out in a nitrogen atmosphere for 5 min and stopped with HCl (2 M) until it stopped bubbling. The same reaction was performed by adding 3 mol of iodoacetic acid (Sigma Aldrich, St. Louis, USA) to obtain CSeCys. The response was performed for 5 min, and NaOH (20%) was

Table 1
Survival of *Sacharomyces boulardii* in YPD broth enriched with different concentrations of Na_2SeO_3.

Na_2SeO_3 concentration (ppm)	Log CFU/mL
0	9.00 ± 0.00^a
40	4.97 ± 0.02^b
80	3.15 ± 0.15^c
120	3.00 ± 0.00^c
160	3.00 ± 0.00^c
200	3.15 ± 0.15^c

Values with the same superscripts do not differ significantly at $p < 0.05$. Results are expressed as the mean ± standard deviation (n = 3).

added until pH 7.5. After 5 min, a 2 M HCl solution was added until the pH reached 5. The solution was refrigerated (5 °C) overnight and filtered, and the supernatant was lyophilized. The selenized dried yeast (30 g) was lysed sonicating for 1 h, and the carboxymethylation reaction was performed with the same procedure.

2.5.2. Derivatization

Derivatization was performed using the O-phthalaldehyde (OPA) method. The solution was prepared with 50 mg of OPA (Sigma Aldrich, St. Louis. The USA) in 4 mL of methanol HPLC grade (Fermont, N.L. Mexico) and 500 μL of borate buffer (0.4 M) and 50 μL of 2-mercaptoethanol (Sigma Aldrich, St. Louis. USA). Before HPLC analysis, 10 mg of standard or sample (yeast) were dispersed in 1 mL of borate buffer (4 M), adding 30 μL of OPA solution. The reaction was carried out for 1 min before HPLC analysis.

2.6. Selenium nanoparticles (SeNP) characterization by TEM

Nanoparticles of selenium produced by the selenized yeast were characterized by transmission electron microscopy (TEM). The yeast was placed in carbon-coated copper grids measuring 3.05 mm in diameter. A JEOL transmission microscope (JEM 2100), operated at 200 kV of acceleration, was used to obtain the images. Images were taken in brightfield TEM mode. Analysis was compared to yeast without selenium enrichment.

2.7. Particle size distribution analysis by DLS

A study by Dynamic Light Scattering (DLS) was carried out to estimate the selenium nanoparticles' particle size distribution (measured as hydrodynamic diameter). A Malvern model Zetasizer Nano seriesmzs90 equipment was used. 2 mg of sample were weighed, 10 mL of deionized water were added, then dispersed by ultrasonic bath for 30 min. Before analyzing the samples, dispersion by ultrasonication was repeated for 60 s. The reading was performed in triplicate.

2.8. Statistical analysis

Results were analyzed by one-way ANOVA ($p = 0.05$) and through Tukey's contrast with the NCSS statistical software (NCSS 2007, v.0, Kaysville, UT, USA, 2007).

3. Results and discussion

3.1. Determination of MIC of Na_2SeO_3 in the growth of S. boulardii

Results obtained for the survival of *S. boulardii* in YPD broth enriched with different concentrations of Na_2SeO_3 are shown in Table 1.

A decrease of 4 logarithmic cycles in the yeast viability was observed at 40 ppm. The initial concentration in the unenriched medium was 9 log CFU/mL. Despite the decrease observed, the medium with the maximum concentration of Na_2SeO_3 had 3.15 log CFU/mL of viable microorganisms. Total inhibition was not observed. With the data obtained, the MIC was calculated. The MIC of Na_2SeO_3 determined was 74.4 ppm.

Previous studies have described that yeast growth inhibition by Na_2SeO_3 is directly proportional to the salt concentration. For example, in media with concentrations ranging between 40 and 60 ppm of Se^{4+}, it has been reported that the growth of *S. cerevisiae* ATCC MYA-2200 is affected (Kieliszek et al., 2019). The authors demonstrated a significant reduction of cell concentration compared to with results obtained in the controlled growth. In addition, the highest concentration was obtained in the first 22–30 h incubation in all concentrations tested. Experiments showed that during experimentation, yeast took the highest time to adapt to the new environment. Furthermore, the adaptation phase was much longer than that in control, similar to our study. The slowdown is related to oxidative stress due to selenium in the medium.

Similarly, Porto et al. (2015) found growth deacceleration of *S. cerevisiae* UFMG A-905 in media enriched with Na_2SeO_3. The authors described that a concentration of 4 mg of Na_2SeO_3/L, a substantially reduced cell viability (59%) compared with the control. In contrast, they observed a 13 and 7% reduction with 50 and 100 ppm, respectively. The final concentration with 100 ppm was 3.33 E+6 log UFC/mL, two cycles lower than the control. However, it has been shown that whit a higher concentration of Na_2SeO_3 (150 ppm) in media growing *S. cerevisiae* NCYC-1026, total inhibition and damage in the cellular surface is observed (Rajashree & Muthukumar, 2013).

3.2. Selenization of S. boulardii

The growth kinetics of *S. boulardii* in a YPD medium enriched with the MIC of Na_2SeO_3 (74 ppm) were calculated to evaluate the selenization time of the yeast. Results were contrasted with those obtained in an experiment developed in unenriched media. Fig. 1 shows that the

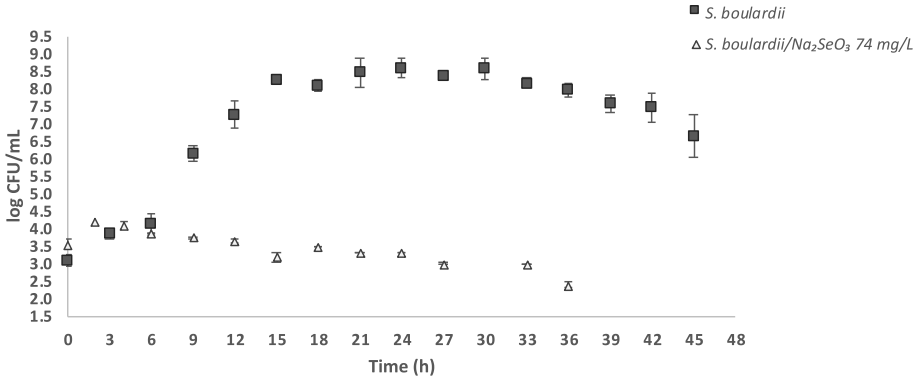

Fig. 1. Growth of *Saccharomyces boulardii* (■) in YPD broth and *Saccharomyces boulardii* in YPD enriched with 74 ppm of Na_2SeO_3 (Δ). Error bars indicate standard deviation (n = 3).

Table 2
Kinetic parameters during growth and selenization of *Saccharomyces boulardii*.

	Velocity rate μ (h^{-1})	Generations (g)	Growth constant *k* (g/h)	Deceleration time (h)
Control	1.039	0.667	1.499	14.51
Selenized yeast	0.71	0.976	1.025	5.97

Table 3
Selenium accumulation by *Sacharomyces boulardii* during fermentation.

Time (h)	Se concentration (mg/g of dry yeast)	Accumulation (%)
0	0.00 ± 0.555^{a}	0.00
3	0.509 ± 0.253^{b}	3.20
6	0.788 ± 0.738^{c}	3.07
9	3.402 ± 0.336^{d}	9.95

Values with the same superscripts do not differ significantly at $p < 0.05$. Results are expressed as the mean ± standard deviation (n = 3).

presence of selenium is implied in the growth rate. Furthermore, the viable count at 24 h in the enriched media was 3.3 log CFU/mL, whereas the control had 8.6 log CFU/mL. The salt's influence was visible from the first time the yeast grew. The logarithmic phase reached its maximum at 14.51 h in the control experiment, and the time for the selenization curve was 5.97 h.

At the same time, the kinetic parameters showed differences, especially for velocity rate (μ) and the growth constant (k). On the other hand, the generation time (g) was higher for the selenized yeast (Table 2).

Studies of *S. cerevisiae* selenization have reported a growth decrease from 84.24% at 50 ppm (Ponce et al., 2002) to 92–93% at 100 ppm of selenite (Porto et al., 2015; Rajashree & Muthukumar, 2013). This study's results are like reports for *S. cerevisiae*, observing a growth decrease of 61.62% at 24 h and 74 ppm of Na_2SeO_3.

A red coloration was also observed in both colonies of yeast and broth in the selenization process. This change of color has also been reported by Lampis et al. (2017), who indicate that the red color in the selenization process demonstrates the reduction process from selenite to elemental selenium (Se^0).

According to Kieliszek et al. (2019), the growth deceleration is due to oxidative stress and the detoxification process initiated by the selenium excess in the medium. Many microorganisms, primarily yeast, could biosynthesize SeNPs through detoxification under anaerobic or aerobic conditions. The bioaccumulation and metabolism of selenium in yeast are very complex. The bioaccumulation process of selenium occurs from the extracellular binding by membrane assembly ligands and intracellular accumulation associated with the transport of ions through the cytoplasmic membrane into the cell (González-Salitre et al., 2021). Selenite and selenate oxyanions are reduced in the form of non-toxic Se^0 or methylated Se species by a cellular detoxification mechanism that maintains redox potential as part of its respiratory chain of electron transfer. Microorganisms biosynthesize SeNPs, while Se oxyanions are reduced and could be accumulated in different forms, such as intracellular, extracellular, or bonded to the membrane (Escobar-Ramírez et al., 2021).

3.3. Absorption of selenium by S. boulardii

Most studies have shown that concentration is directly proportional to growth inhibition, and salt concentration affects the absorption and accumulation of selenium. However, fermentation time directly affects the amount of selenium yeast absorbs (Kieliszek et al., 2015). That is why the selenium absorption by *S. boulardii* at different fermentation times was measured. The initial concentration of Na_2SeO_3 in YPD broth

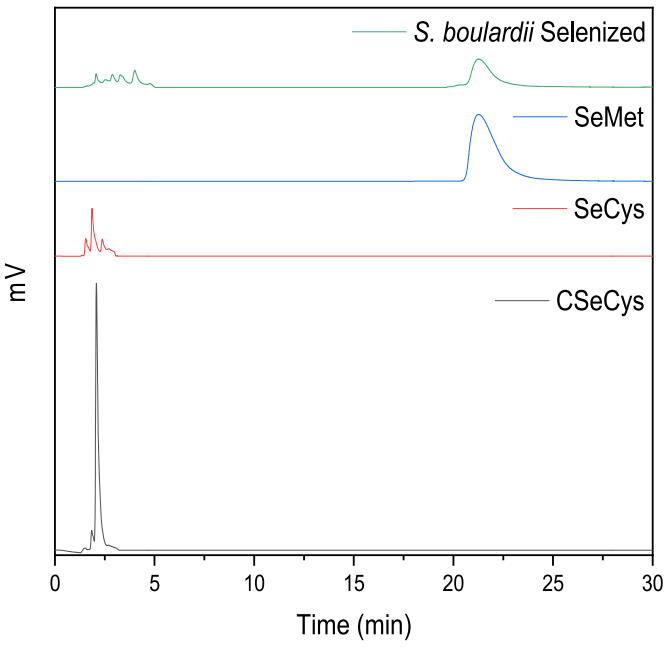

Fig. 2. RP-HPLC chromatogram of yeast lyophilized extracts compared with standards of Carboxymethyl selenocysteine (CseCys), Selenocysteine (SeCys), selenomethionine (SeMet).

was 74 ppm, and the yeast absorbed 0.788 mg/g of dried yeast at the end of the logarithmic phase (6 h). Then, in the stationary phase (9 h), *S. boulardii* reached an absorption level of 3.0402 mg/g of dried yeast. Results are shown in Table 3.

Yeast's capacity for selenium accumulation depends on the culture conditions, the selenium concentration in the experimental medium, and the microorganism used (Kieliszek et al., 2015). For example, Martiniano et al. (2020) found that after adding 15 ppm of Na_2SeO_3 to the media, *S. cerevisiae* Lalvin and *S. cerevisiae* 405 absorbed 0.659 and 1.094 mg/g of dried yeast, respectively. On the other hand, Yoshinaga et al. (2018) demonstrated that after adding 34.58 ppm of Na_2SeO_3, two species of *S. cerevisiae* (EC1118 and AW106) absorbed 3.1 and 3.5 mg/g of dried yeast, respectively.

In contrast, different studies demonstrate that even at high concentrations of salt in the media, yeast absorbs a smaller amount of selenium. Thus, Ponce et al. (2002) found a selenium accumulation of 2.354 mg/g in *S. cerevisiae* 15–6252 enriching the media with 50 ppm of Na_2SeO_3, while at a similar concentration of enrichment (60 ppm), Kieliszek et al. (2016) observed an accumulation of 5.64 mg/g in *S. cerevisiae* ATCC MYA-2200.

Ponce et al. (2002) studied different methods for obtaining selenium enriched-*S. cerevisiae*. They determined that lower doses of sodium selenite (IV) (from 10 to 50 ppm) in yeast's early logarithmic growth phase is the best method. In addition, temperature and pH are the factors that have an impact on the selenization process. Yin et al. (2010) showed that the optimal parameters for the enrichment of *S. cerevisiae* with selenium are 27 °C and acid media (pH of 5.8).

This difference in the selenium accumulation for the same yeast species could be due to its metabolic differences (González-Salitre et al., 2021). Four different species of *S. cerevisiae* were tested, and it was determined that the carbon source determined both the resistance of the selenium presence and the subsequent accumulation. Additionally, phosphate transporters, and their regulation via phosphate concentration, could be another variable contributing to the interplay of selenite resistance and accumulation. Low phosphate conditions have been shown to upregulate low-affinity phosphate transporters, which causes an increase in selenite uptake (Yoshinaga et al., 2018). In this study, *S. boulardii* accumulated 3.404 mg/g of dried yeast in a medium

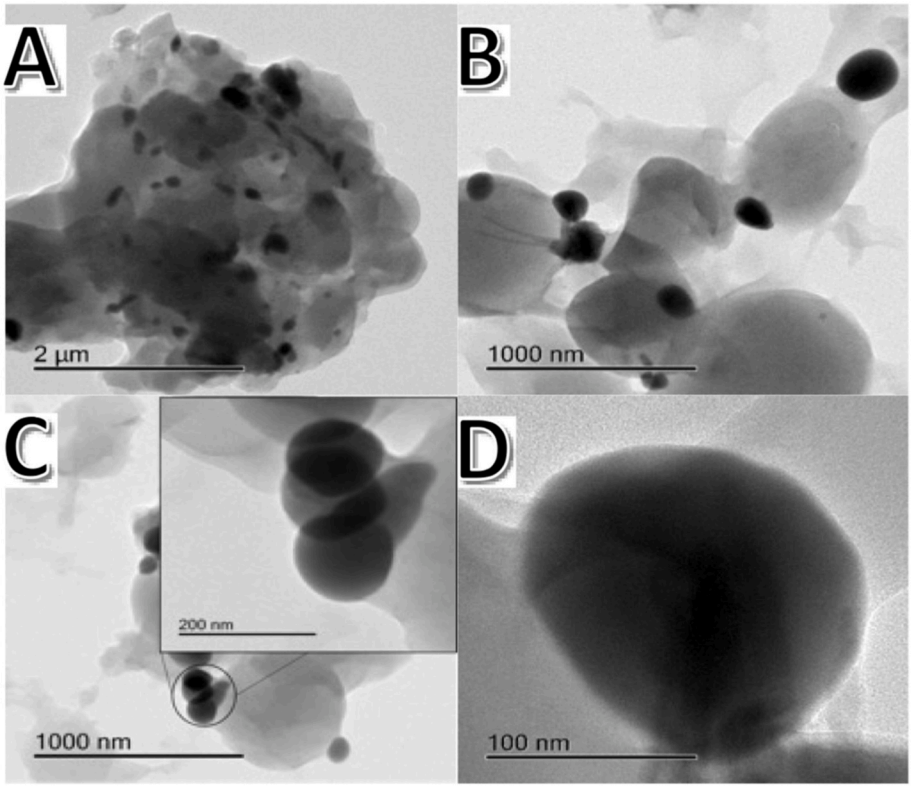

Fig. 3. TEM micrographs of seleno-nanoparticles produced by *Saccharomyces boulardii*, in which can be observed several magnifications with a maximum particle size of: (A) 2 µm, (B) 1000 nm, (C) 1000 nm and sphericity of the particle (<200 nm), (D) 100 nm and the verification of spherical morphology of seleno-particles. All micrographs were taken randomly.

enriched with 74 ppm Na_2SeO_3. Although the genes involved in selenium biotransformation are orthologous between *S. boulardii* and *S. cerevisiae* (González-Salitre et al., 2023), the selenium accumulation capacity in both yeasts differs.

3.4. Determination of seleno-amino acids bioconverted by S. boulardii

The separation of selenium species was analyzed using the RP-HPLC technique to determine the biogenic production of SeMet and SeCys by *S. boulardii*. The chromatographic profile is observed in Fig. 2. The retention time for SeMet was 21.24 min and for SeCys 2.08.

The analysis demonstrated the presence of both seleno-amino acids in the yeast. However, it was observed that the most significant area was found in the peak corresponding to SeMet. This result corresponds to previous reports that account for the production of SeMet in yeasts in more significant quantities than SeCys (Ponce et al., 2002; Prange et al., 2019; Zare et al., 2017), even though both amino acids follow the same metabolic pathway.

The biosynthesis of seleno-amino acids for *S. boulardii* begins with detoxifying the excess of selenite in the growth medium, following a metabolic pathway similar to that of sulfur. First, sulfate permeates the cytosol and is transported from the growth medium across the membrane. Next, selenite is converted to hydrogen selenide by the action of the alpha subunit of sulfite reductase. Subsequently, hydrogen selenide is transferred to selenohomocysteine in the presence of O-acetylserine-*O*-acetylhomoserine sulfhydrylase. Finally, for the biosynthesis of SeMet from selenohomocysteine, the action of cobalamin-independent methionine synthase is necessary (González-Salitre et al., 2023).

SeCys was previously believed that due to the absence of the UGA codon in *S. cerevisiae*, it was not synthesizing (Dernovics & Lobinski, 2008). However, recent studies have shown that, despite the lack of UGA codon, SeCys can be found in selenium-enriched yeast (Bierla et al., 2013). The incorporation route would be that of sulfur substituting selenium for sulfur in the biosynthesis route, similar to the way described above until the formation of selenohomocysteine. Selenohomocysteine is then converted to selenocystathionine by the action of the enzyme cystathionine-synthase, and selenocystathionine is finally converted to SeCys by a reaction catalyzed by the enzyme cystathionine-lyase (González-Salitre et al., 2023).

However, the analysis to verify SeCys in yeast has presented difficulties due to the absence of a SeCys standard and problems in identification and quantification (Dernovics & Lobinski, 2008). Recently, Bierla et al. (2018) tested the presence of SeCys, SeMet, and other selenium species in selenized yeast using a method based on the reduction of Se–Se and S–Se bridges with dithiothreitol derivatized with iodoacetamide (carbamidomethylation) followed by HPLC-ICP MS. In our study; the identification was carried out in similar conditions, using an adaptation proposed by the team. Therefore, this study suggests a new methodology to verify the presence of SeCys in yeasts through a carboxymethylation and derivatization reaction. In addition, results agree with those reported by Bierla et al. (2018), who found high signals for SeMet and lower intensity signals for SeCys in yeast enriched with selenium.

3.5. Nanoparticles of selenium produced by S. boulardii

According to Wadhwani et al. (2017), the natural coating on the biomolecules allows a biogenic production of stable selenium nanoparticles (SeNP). SeNP formation by *S. boulardii* was verified with TEM (Fig. 3), and it can see black and spherical particles of an approximate size between 200 and 100 nm. However, yeast without selenium enrichment showed no a signal corresponding to selenium particles.

Some studies have reported selenium nanoparticle production by yeast. For example, Hariharan, Al-Harbi, Karuppiah, and Rajaram (2012) synthesized SeNP from *S. cerevisiae*, obtaining nanoparticles with a 30–100 nm diameter. However, Faramarzi et al. (2020) reported that

Table 4
Particle size distribution of SeNP produced by *Saccharomyces boulardii* during the selenization process.

Volume percentage	Diameter range (μm)		
	Replicate 1	Replicate 2	Replicate 3
<10%	0.379	0.379	0.365
<25%	1.829	1.353	1.239
<50%	5.084	4.135	3.681
<75%	10.020	9.060	8.187
<90%	24.150	23.360	19.770

this yeast species produced SeNP ranging from 75 to 709 nm. In addition, the size diversification of SeNP has been reported by Garza-García et al. (2022), who found particles from 80 to 250 nm, and Asghari-Paskiabi et al. (2019) synthesized selenium nanoparticles from 6 to 153 nm using *S. cerevisiae* in media enriched with selenite.

There are few studies on the case of nanoparticles produced by *S. boulardii.* Bartosiak et al. (2019) synthesized SeNP from *S. boulardii* as an alternative to green chemistry, in which the SeNP diameter was approximately 235 nm. Patel et al. (2013) synthesized SeNP from *S. boulardii* as an alternative to evaluating them as an anticancer agent. According to Shoeibi and Mashreghi (2017), the size of the nanoparticle influences biological activity, where nanoparticles in the range of 5–200 nm bind free radicals in a size-dependent manner.

According to the results obtained by the DLS analysis (Table 4), in a replicate, a size range was detected that goes from 365 nm (volume percentage <10%) to 25.15 μm (volume percentage <95%). This diameter variability is due to the dispersion made with yeast cells, which does not discriminate between suspended organic matter. However, according to the TEM analysis, it is considered that there is some variability in the size of the selenium nanoparticles, which is due to aggregation mechanisms. Additionally, this difference in size could be due not only to the presence of organic material but also to the state of aggregation in the solution. It has been observed that different concentrations of selenium in solution have different forms of aggregation, which is directly proportional to the concentration (Ashengroph & Tozandehjani, 2022).

Some authors have determined that small nanoparticles are produced in the early stages of fermentation that become longer with the passage of time. This effect, although little studied, has been attributed to aggregation by nucleation in which the coalescence process takes effect (Lampis et al., 2014; Tugarova et al., 2020). The final stage of SeNP formation is due to assembly after the reduction of Se oxyanions. Although this mechanism has not been widely studied and is partly unknown, it has been associated with an Ostwald mechanism in which smaller particles coalesce among themselves to form larger particles without size control due to their size high surface energy (Escobar-Ramírez et al., 2022).

This size diversity is due to the biogenic production based on the enzymatic reduction of yeast from Se^{2-} to Se^0 (Shoeibi and Mashreghi (2017). When yeasts obtain selenite from the medium and transport it through the membrane, it will react with glutathione (GSH) to produce selenodiglutathione (GSSeSG), which GSH will further reduce to glutathione selenenylsulfide (GSSeH), which will then spontaneously dismutate in Se^0 and GSH (González-Salitre et al., 2021; Lazard, 2021) (Fig. 4).

The results were obtained to account for the selenium accumulation capacity of a probiotic yeast, bioconverting inorganic selenium into seleno-amino acids and SeNP. These data are of great importance since, in addition to the health benefits that the consumption of probiotics implies, the selenization of this yeast is a promising process in the search for new sources of organic selenium that counteract the negative health consequences associated with a deficiency of selenium. In addition, there is a field of action in the market since products are made from yeasts such as *Saccharomyces cerevisiae* and *Saccharomyces uvarum* (Kieliszek et al., 2015), which are used for the biogenic production of selenium. This type of product aims to reach the recommended daily intake, which is around 60 μg/g. Furthermore, biogenically produced selenium is fully absorbable by the body. Therefore, it is indicated as an antioxidant product limiting the destructive processes of lipid peroxidation, DNA, and RNA, which has the consequence of cell protection against deformation and genetic damage. Furthermore, as part of glutathione peroxidase, selenium acts as an antioxidant, including the capture of free radicals used in cancer therapies. Thus, diseases such as AIDS, multiple sclerosis, and cancer could be treated with nanoparticles of selenium and seleno-amino acids biogenically produced by this probiotic yeast.

4. Conclusions

The presented study represents an advance in studying the biogenic

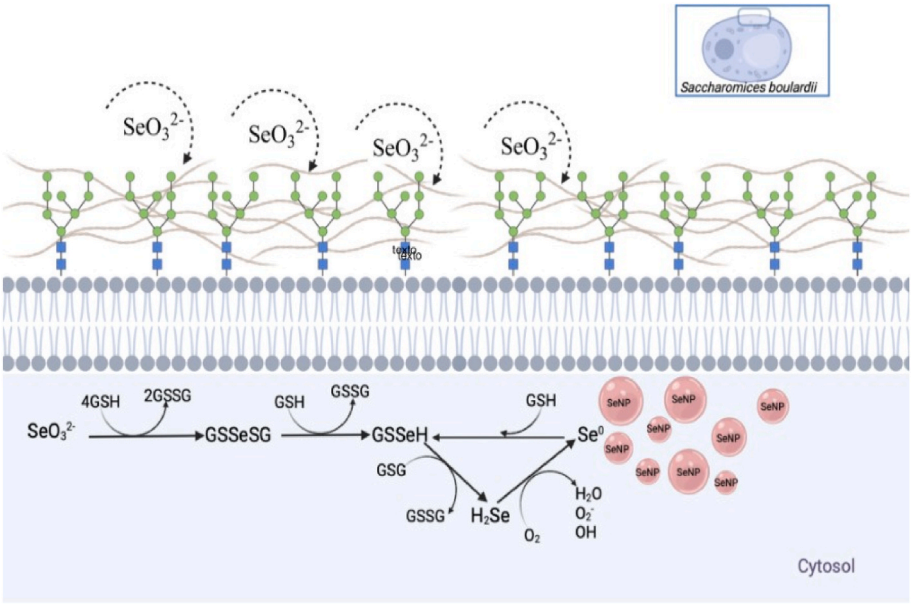

Fig. 4. Proposed mechanism for reduction from selenite to Se^0 by *S. boulardii*.

production of seleno-amino acids and seleno-nano particles by a probiotic yeast. The minimum inhibitory concentration of selenite placed in growth media for *S. boulardii* directly affects the detoxification processes, consistent with the production of seleno-amino acids and selenium nanoparticles. In addition, the output of SeMet in concentrations more significant than SeCys has been verified. However, it is necessary to conduct studies of selenium metabolism by *S. boulardii* since the biochemical mechanism has yet to be fully elucidated. Nevertheless, these results represent an opportunity to respond to challenges that exist in the fields of medicine and food technology.

Authors statement

Conceptualization, L.G.G.O. and A. D.R.G.; writing original draft prepara-tion, L.G.S., L.G.G.O. and A.C.C.; funding acquisition and experimental support, G.M.R.S., A.C.G.C., U.A.B.C.; review and editing, L.G.G.O., A.C.C. and A.C.O.; developing of figures and tables, A.C.O. and L.G.S.; project administration, L.G.G.O, A. D.R.G. and G.M.R.S. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Data availability

No data was used for the research described in the article.

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Capítulo -V-
Efecto del uso de *Saccharomyces boulardii* selenizada sobre parámetros de calidad seminal en semen de verraco: estudio *in vitro.*

5.1 Introducción

El capítulo presenta un estudio *in vitro* realizado con semen de verraco, bajo el Proyecto Académico Recuperación del cerdo criollo Hidalguense “Ts’üdi Xirgo” a cargo de la Dra. Adelfa del Carmen García Contreras. El estudio se llevó a cabo en el rancho “Ts’üdi Xirgo” ubicado en Tepatepec, Hidalgo. El objetivo principal del estudio fue analizar el efecto del selenio orgánico proveniente de la fermentación de la levadura selenizada sobre la calidad espermática de semen de verraco. El diseño experimental se basó en seis tratamientos los cuales incluyeron levadura selenizada y selenito de sodio a diferentes concentraciones, las cuales se incubaron a 17 °C y se mantuvieron hasta 48 horas para llevar a cabo análisis de motilidad espermática. Se trabajó con tres verracos en edad adulta previamente entrenados para saltar al potro y obtener el eyaculado de manera manual. Se utilizó extensor de semen (Acromax), donde se distribuyó el eyaculado para obtener una concentración uniforme de espermatozoides 3,000x 10^9. Los resultados de motilidad espermática no mostraron diferencia significativa respecto al control, pero sí mantuvieron una motilidad superior al 60%, lo que sugiere que es necesario un estudio *in vivo* para evaluar el efecto del consumo de la levadura selenizada, debido a que el selenio orgánico que contiene es más biodisponible a comparación del selenito de sodio.

Efecto del uso de *Saccharomyces boulardii* selenizada sobre parámetros de calidad seminal en semen de verraco: estudio *in vitro.*

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5.2 Introducción

El selenio es un oligoelemento natural y esencial para la vida con funciones esenciales a nivel celular en la salud animal y humana (Qazi et al., 2018). Las funciones biológicas del selenio son realizadas por selenoproteínas con funciones estructurales y enzimáticas (Rayman, 2000). Es así que, en el caso de la espermatogénesis de mamíferos, esta se encuentra mediada por hidroperóxido de fosfolípido glutatión peroxidasa (PHGPx/GPx4) y la selenoproteína P (Boitani and Puglisi, 2008). La selenoproteína P (SEPP1), derivada del hígado, es una proteína plasmática necesaria para el suministro de selenio a los testículos (Boitani and Puglisi, 2008; Michaelis et al., 2014). Mientras que GPx4 es un vínculo entre el

selenio, la calidad del esperma y la fertilidad masculina (Boitani and Puglisi, 2008). GPx4 se expresa en los testículos y se cree que en una etapa temprana de la espermatogénesis protege a los espermatozoides en desarrollo del daño del DNA inducido por el estrés oxidativo (Qazi, et al., 2018). Por otra parte, los espermatozoides maduros dependen de la capacidad de GPx4 de usar hidroperóxidos para la formación de un elemento estructural. Esta estructura brinda integridad a la pieza intermedia del esperma, que es fundamental para la fertilidad masculina (Ursini et al., 1999; Qazi et al., 2019).

En el caso de los verracos, el semen se caracteriza por tener una proporción de ácidos grasos poliinsaturados de cadena larga fácilmente oxidables. Es por ello que requieren de una defensa antioxidante eficaz (Surai and Fiinin, 2015). Se considera que concentraciones óptimas de selenio en la dieta de los animales están asociadas con una mejor protección antioxidante y podría tener efectos positivos sobre la calidad y producción de semen porcino (Surai and Fiinin, 2015). La suplementación con selenio inorgánico (Selenito o selenato) en dieta de verracos se recomienda a dosis de hasta 0.3 ppm (Ahsan et al., 2014). Sin embargo, el selenio orgánico, como selenometionina, tienen una mayor eficacia de asimilación en la dieta y mejora las posibilidades de crear reservas de selenio en el cuerpo (Surai and Fiinin, 2015).

Las levaduras de selenio son una fuente importante de selenometionina, así lo han reportado estudios sobre acumulación y bioconversión de selenio por *Saccharomyces cerevisiae* (Zare et al., 2017; Pérez-Corona et a., 2011; Ponce et al., 2002; Ourdane and Mester, 2008). Recientemente se reportado la producción biogénica de seleniometionina, selenocisteína y nanopartículas de selenio por una

levadura probiótica (*Saccharomyces boulardii)* que tiene un uso prometedor en la industria alimentaria y campos de la medicina (González-Salitre et al., 2023).

Debido a que el selenio orgánico puede proporcionar defensa antioxidante para mantener la integridad, la motilidad y la capacidad de fertilización de la membrana plasmática en mamíferos (Surai and Fiinin, 2015), en este estudio se analizó el efecto del selenio orgánico, proveniente de la fermentación de *Saccharomyces boulardii* selenizada sobre la calidad espermática de verraco mediante un estudio *in vitro* para determinar su potencial bioactivo.

5.3 Materiales y métodos

5.3.1 Material biológico

El eyaculado se obtuvo de tres verracos de la línea genética T´südi Xirgo entrenados para saltar al potro de recogida. Los verracos fueron identificados de acuerdo con la Tabla 1. .

Tabla 1. Identificación de verracos T´südi Xirgo, edad y peso al momento de iniciar el estudio.

ID	Edad	Peso
1308	2 años 3 meses	180 kg
4-1	2 años 10 meses	180 kg
A721	1 año 1 mes	90 kg

5.3.2 Recogida de semen

El eyaculado se obtuvo semanalmente con 6 días de descanso entre recogidas. El semen se recolectó en un recipiente térmico que contenía un vaso desechable cubierto con filtro y mantenido a una temperatura de 37 °C.

5.3.3 Evaluación básica del semen

El semen se mantuvo a 37 °C hasta el análisis de calidad seminal, la cual se evaluó a través de las siguientes variables.

Motilidad. El porcentaje de motilidad espermática se evaluó depositando una gota de semen en un portaobjetos seguido de un cubreobjetos, ambos atemperados a 37 °C y puesto sobre una platina de calentamiento a la misma temperatura (37 °C), la platina estaba situado en un microscopio marca Olympus CX31. Se observó la muestra en el microscopio de campo claro a una resolución de 40X para motilidad en masa y para motilidad individual. El porcentaje de espermatozoides con movimiento se evaluó en un porcentaje de 0-100% para motilidad en masa y de 1-6 para motilidad individual de acuerdo con la técnica reportada por Kubus, (2010).

Concentración espermática. El conteo de células espermáticas se realizó mediante cámara de Neubauer. Primeramente, en una pipeta de Thoma, se mezcló semen con solución formulada (3%) a una dilución 1:200. Se mezcló durante tres minutos y posteriormente de deshecho las primeras 3 gotas, las siguiente se colocó en cada una de las plataformas de la cámara de Neubauer. Se observaron inmediatamente en el microscopio de campo claro a una resolución de 400X. Los cuadros fueron contados de acuerdo con lo descrito por Kubus, (2010)., y se calculó

la concentración de espermatozoides por mililitro de acuerdo con la siguiente ecuación:

$$SpzmL = x*5*10*100*200$$

Donde:

X: representa el promedio obtenido de ambas plataformas de la cámara de Neubauer

5: evaluación de los 25 cuadrantes de la cámara

10: conversión en microlitros

100: conversión a mililitros

200: factor de dilución

Formas anormales. Se tomó una muestra de 20 μL del eyaculado y se colocó sobre un portaobjetos, adicionalmente se añadieron 30 μL de eosina. Se homogeneizaron ambas muestras y se extendieron de forma horizontal sobre todo el portaobjetos con ayuda de uno más. La muestra se dejó secar a temperatura ambiente (22 °C). Se observaron las formas anormales observadas en microscopio de campo claro a 40X de resolución, realizando un conteo de 200 células espermáticas. Se obtuvo la proporción de cada forma anormal y se consideró el total de ellas, entre las formas anormales que se consideraron fueron Gota proximal, Gota distal y Cola de látigo. Para la determinación se usaron las definiciones de anormalidad reportadas por Kubus (2010).

5.3.4 Tratamiento experimental *In vitro*

Los eyaculados recogidos de los tres verracos se diluyeron en diluyente Acromax, a una concentración de espermatozoides (spz) de 3000 $x10^6$ spz/mL. Antes de la

dilución del semen, se añadió levadura selenizada y selenito de sodio a diferentes concentraciones de acuerdo a la Tabla 2. Los tratamientos se colocaron en tubos de ensayo de vidrio y se mantuvieron en oscuridad a una temperatura de 17 °C. La motilidad de los espermatozoides se determinó a tres tiempos distintos, 30 min, 24 y 48 horas posterior a la dilución. Se determinó la motilidad de acuerdo a la técnica descrita..

Tabla 2. Distribución de los tratamientos *in vitro* con levadura selenizada y selenito de sodio

ID	Tratamiento	Concentración de Se (ppm)
T1	Diluyente (Acromax)	
T2	Diluyente, levadura selenizada	0.05
T3	Diluyente, levadura selenizada	0.1
T4	Diluyente, levadura selenizada	0.3
T5	Diluyente, selenito de sodio	0.05
T6	Diluyente, selenito de sodio	0.1

5.4 Resultados

5.4.1 Calidad seminal de verracos

Para garantizar semen de alta calidad se determinaron parámetros seminales visuales del eyaculado del verraco. Los resultados de la calidad seminal se muestran en la Tabla 3. La evaluación visual del semen mostró un color blanco lechoso, casi amarillento para los tres verracos y sin olor perceptible. La

concentración espermática es una herramienta para monitorear la salud y el rendimiento reproductivo del verraco (Rozeboom, 2000). En este caso, hubo diferencias notables entre cada verraco.e observó que el verraco A721 presentó la mayor concentración espermática por mililitro. Contrariamente, el verraco 1308 presentó un mayor volumen de eyaculado. Sin embargo, el verraco 4-1 presentó la mayor concentración total de espermatozoides.

Estos resultados podrían estar relacionados con la edad de los verracos estudiados. El verraco A721 fue el más joven (1 año 1 mes) seguido de 1308 (2 años 3 meses) y finalmente 4-1 (2 años 10 meses). De acuerdo con Smital (2009) el volumen de eyaculado aumenta con la edad, y la concentración de espermatozoides disminuye, lo cual coincide con lo reportado en este estudio. Los verracos con más edad presentaron un mayor volumen de eyaculado y una menor concentración de espermatozoides.

De acuerdo con Falkenberg et al., (1992) la producción óptima de espermatozoides de los verracos es durante la terminación del crecimiento (alrededor de los 2.5 a 3 años). Otro factor que también afecta la calidad dell semen es la frecuencia de colecta. En este estudio se dejó un periodo de descanso de 6 días. Falkenberg et al., (1992) recomienda periodos de descanso entre 2 a 5 días, ya que si la frecuencia de eyaculado es mayor, la concentración de espermatozoides disminuye igual que la libido.

La motilidad en masa e individual para los tres verracos fue muy similar, superior al 90% y en grado 4, lo que indica un movimiento progresivo y sinuoso. En el caso del semen de verraco, no se dispone de umbrales de calidad, sin embargo, Maside et al., (2023) establecieron un valor ≥ 70-80% de espermatozoides móviles totales.

Tabla 3. Calidad espermática del eyaculado de tres verracos adultos.

	1308	4-1	A721
Volumen (mL)	203.69	139.5	73.05
Concentración (Spz/mL)	$317x10^6$	$513x10^6$	$619.5x10^6$
Concentración total	$64351x10^6$	$71307x10^6$	$45254.47x10^6$
Motilidad (%)	91.25	93	92
Motilidad individual (grado)	4	4	4
Anormalidades (%)	2.21	2.56	1.952

Las formas anormales encontradas en los tres verracos estuvieron alrededor del 2%, en las cuales las formas anormales predominantes fueron cola en látigo, gota distal, cola suelta, tracto intermedio flexionado, gota citoplasmática proximal, macro cabeza, estas dos últimas se muestran en la figura 1.

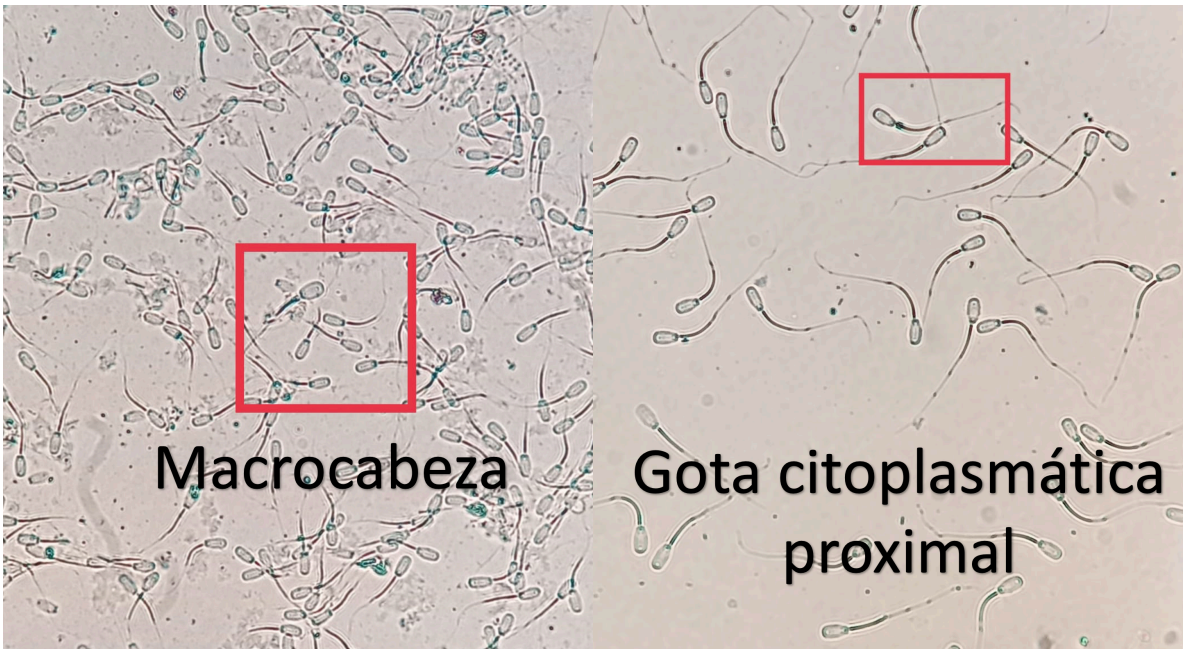

Figura 1. Formas anormales encontradas en semen de verracos

La incidencia de formas anormales encontradas fue baja. En el caso de gotas citoplasmáticas se ha sugerido que su incidencia no debe exceder el 15% cuando el semen se usará para almacenamientos prolongados (Rozeboom, 2000). De acuerdo con Maside et al., (2023) en condiciones normales para verracos, se espera una proporción ≥80% de espermatozoides morfológicamente normales, <20% de espermatozoides morfológicamente anormales y <15% de espermatozoides con gotas citoplasmáticas. La suplementación de concentraciones adecuadas de selenio en la dieta puede mejorar la maduración de los espermatozoides en el epidídimo y reducir el número de espermatozoides con gotas citoplasmáticas (Marín-Guzman et al., 2020)

5.4.2 Influencia de selenio en semen de verraco

El selenio biogénicamente producido por *S. boulardii* estudiado , y el selenito de sodio a diferentes concentraciones, no tuvieron efecto significativo sobre la motilidad total comparada con el tratamiento control solo con extensor (Acromax). Sin embargo, la motilidad espermática total se vio afectada de manera negativa por el tiempo de almacenamiento. Esta condición física disminuyó conforme pasaba el tiempo. El semen de los tres verracos tuvo el mismo comportamiento (Figura 2). Sin embargo, el semen del verraco A721 en presencia con el tratamiento con levadura selenizada y selenito de sodio fue el más afectado al disminuir la motilidad por debajo del 40% a las 48 horas (Figura 2-A721). De acuerdo con Johnson et al., (2000) se considera que el semen almacenado es satisfactorio si los valores de motilidad son superiores al 60% (Johnson et al., 2000). Esta condición se observó

en el verraco 1308 y el 4-1,en los cuales después del almacenamiento a 17 °C y 48 horas se observó una motilidad superior al 65%.

La disminución de la motilidad entonces es debida a la temperatura y el tiempo de almacenamiento, aunque en un estudio in vitro realizado por Marín-Guzman et al., 2020) determinaron que la motilidad de los espermatozoides disminuye cuando se agrega selenio al diluyente. La disminución se vio afectada con concentraciones creciente de selenio. La concentración mínima de selenio utilizada por Marín-Guzman et al., (2020) fue de 0.3 ppm, que corresponde a la máxima utilizada para levadura selenizada en este estudio.

Hace falta un estudio más profundo y a nivel celular para evaluar el efecto del selenio sobre las células espermáticas. Así mismo, es necesario realizar un estudio *in vivo* para suministrar en la dieta valores adecuados de levadura selenizada. La desviación de las cantidades óptimas de Se en la dieta, tanto por encima como por debajo, puede causar múltiples anomalías en los espermatozoides y afectar la motilidad y la fertilidad (Ahsan et al., 2014). Se ha demostrado que dietas bajas en selenio y vitamina E disminuyen la motilidad en verracos y aumentan las formas anormales de los espermatozoides. También afecta su actividad metabólica en el eyaculado, lo que conlleva a una menor motilidad y tasa de fertilización (Marin-Guzman et al., 1997). Por ello, es importante suplementar la alimentación animal con especies de selenio mayormente absorbibles.

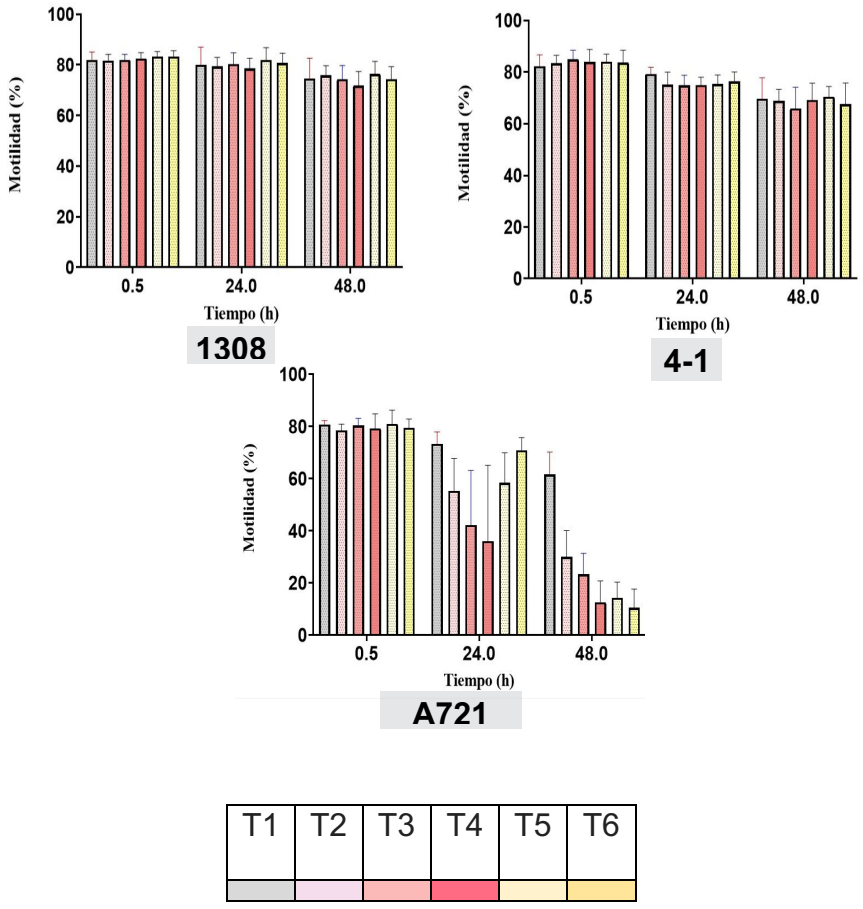

Figura 2. Motilidad espermática de tres verracos (1308, 4-1, A721) y 6 tratamientos 0.5, 24 y 48 horas.

5.5 Conclusiones

En este estudio se observó que el selenio orgánico, proveniente de la fermentación de *Saccharomyces boulardii* podía mantener la motilidad espermática favorables en condiciones de almacenamiento. Este trabajo marca pauta para seguir realizando estudios *in vivo* sobre *Saccharomyces boulardii* selenizada como fuente orgánica de selenio para la suplementación en la alimentación de verracos y evaluar su biodisponibilidad y bioaccesibilidad en el organismo.

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Capítulo -VI-
Humulus lupulus L. a potential precursor to human health: High hops craft beer.

6.1 Introducción

Este capítulo presenta un artículo de revisión enfocado en el lúpulo como precursor potencial para la salud humana y que ha sido publicado en la revista Food Chemistry. Aunque la revisión se enfoca en hablar de los compuestos bioactivos del lúpulo, también tiene como objetivo ser una parte introductoria para hablar acerca de la cerveza, cerveza artesanal y los avances que se tienen sobre la levadura y los beneficios reportados sobre la salud. Hasta el momento *Saccharomyces cerevisiae* es la levadura mayormente utilizada en la industria cervecera para tipo de fermentación Ale (16-25 °C) y *Saccharomyces pastarianus* para fermentación lager (8-15 °C). De igual forma, se reporta los avances que se tienen en la industria de la cerveza artesanal o cerveza independiente, donde existen varias propuestas innovadoras sobre el uso de ingredientes alternativos. Sin duda alguna, abre el panorama sobre las áreas de oportunidad para el uso de *Saccharomyces boulardii* selenizada, con la finalidad de obtener una bebida alcohólica enriquecida con selenio y de igual forma utilizar la levadura residual como subproducto para la industria ganadera. Cabe destacar que, las nuevas tendencias respecto a las cervezas artesanales y cervezas funcionales tienen como objetivo promover un consumo responsable y sustentable.

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Humulus lupulus L. a potential precursor to human health: High hops craft beer

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1. Introduction

Hops (*Humulus lupulus* L.) is a dioecious climbing plant of the *Cannabaceae* family and is one of the main ingredients for brewing. However, their physicochemical composition is related to the cultivation and harvest conditions, especially the climate (arid, semi-arid, and dry) (Hrnčič, Španinger, Košir, Knez, & Bren, 2019; Šrédl, Prášilová, Svoboda, & Severová, 2020). The variety of volatile compounds at different concentrations provides the characteristic aroma and bitterness of hops in beer, which is a complex of sensory impressions. In addition, the mixtures of two or more varieties could generate synergies with the aim of potentiating the aroma or bitterness in each style of beer.

Different bioactive compounds (e. g. volatile oils, alpha/beta acids, phenolic compounds, flavonoids, prenylflavonoids, phytoestrogens, or essential oil, among others) present in multiple varieties of hops have been shown to have positive health benefits. In this sense, once the hops are harvested, all components reach maturation showing different functional properties (e. g. antioxidant, anti-inflammatory, antimicrobial, antimutagenic, anti-allergic, anti-stress and cognitive, among others). So, hops are a beneficial option for using in functional, nutraceutical, therapeutic, and pharmaceutical applications (Almeida et al., 2019; Bortoluzzi, Menten, Silveira, Melo, & Rostagno, 2016; Oliveira Neto, Lopes de Macedo, Lopes de Oliveira, de Queiroz Ferreira, & de Souza Gil, 2017; Sanz, Torres, Vilariño, & Domínguez, 2019).

Therefore, polyphenols, essential oils, and α and β acids, among others, are compounds of hop with the greatest industrial interest. Furthermore, different compounds are associated with the flavor and aroma of beer: α-acids (e. g. cohumulone, humulone, adhumulone), β-acids (e. g. lupulones, colupulone, adlupulone,) and the main volatiles in hops such as methyl octanoate, germacrene B, β-myrcene, geraniol, linalool, *trans*-α-bergamotene, α-cubebene, caryophyllene, *cis*-β-farnesene, α-humulene, β-selinene, β-citronellol among others terpenoids (Cibaka et al., 2017; Rettberg, Biendl, & Garbe, 2018; Su & Yin, 2021). However, both the volatile and non-volatile compounds contained in hop oil might be oxidized or could be totally lost during the brewing process (De Keukeleire, 2000; Moir, 2000; Sanz et al., 2019).

Due to the high boiling temperatures reached during beer cooking, the organoleptic profile is modified and many chemical changes, like isomerizations in the α-acids, are presented. These changes enhance the bitterness of the beer. It is because of that, that bitter beers (e. g. Indian pale ale, bitter, hazy Indian pale ale styles, among others) use one to four different varieties of hops with a high α-acid content. The addition of hops is carried out at the beginning of boiling to obtain high bitterness levels. Other compounds that provide bitterness are polyphenols (e. g. gallic acid, vanillic acid, coumarin, flavonoids and tannin among others). Currently, regardless of the compound that provides the bitterness of the beer, the level is expressed as the International Bitter Unit (IBU). In the case of hops with lower amounts of α-acids, they are added at the end of boiling at temperatures $\leq$ 90 °C without applying further heat, allowing aromatic volatile compounds to remain in the wort (Oladokun et al., 2016). The hop compounds described are mainly consumed in beer production, but they are also used in the manufacture of essential oils, soft drinks and even as food supplements with the aim of obtaining well-being on human health.

Millions of people around the world have experienced high levels of stress as well as chronic degenerative diseases (cardiovascular, obesity, and type II diabetes among others), in relation to that they affect health condition, busy life and bad eating habits. Because this condition has increased in the last decade, people are closer to consume functional foods and beverages (Biendl & Pinzl, 2009; Olsovska et al., 2016; Rietjens, Louisse, & Beekmann, 2017). As hops have been used since ancient times as folk medicine, these functional properties are a topic of interest. Therefore, this review describes the main uses, benefits and future trends that hops when consumed moderately through conventional and unconventional infusions.

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2. Bioactive compounds in hops

Bioactive compounds in foods are those that are not required to support life, but still have a beneficial effect. They have beneficial properties for health to prevent, counteract and/or inhibit multiple diseases. Although, wort bioactive compounds (e. g. polyphenols, anthocyanins, polyunsaturated fatty acids, essential oils and even vitamins) are prone to oxidation, thermolabile, or antagonistic losing their beneficial functional properties. In general, bioactive compounds are obtained from natural ingredients from different morphological parts of plants (e. g. leaves, stems, roots, fruits, shoots and flowers) (Bedini et al., 2016; Bolton et al., 2019).

Currently, various investigations are focused on the preservation, inclusion, potentiation and identification of different natural bioactive compounds such as terpenes, carotenoids, phenolic acids, flavonoids, coumarins, alkaloids, and polyacetylenes. These compounds are studied in the pharmaceutical and food industry application, with the purpose of improving, preserving and/or preventing different diseases or even increasing well-being in human health (Chandrasekara & Shahidi, 2018).

On other hand, the polyphenolic content of hops has long been a widely used component of ancient medicine with beneficial effects in different chronic diseases and positive biological effects for health well-being such as: oxidative stress, cognitive disorders, insomnia, inflammation, insulin sensitivity, type II diabetes, menopause, antioxidants, antibacterial, antifungal, antiviral, anti-inflammatory, antiallergic, antithrombotic, vasodilator, antimutagenicity, anticancer and anti-aging, among others (Fig. 1). The beneficial activity has been evidenced in different publications *in vivo*, *in situ* and *in vitro*. In addition, there are various products available for sale to counteract, regulate or reduce degenerative ailments to maintain successful body health confirming that hop biomolecules are beneficial to human health. For example, xanthohumol (XN), isoxanthohumol (IX), 6-prenylnaringenin (6-PN) and 8-prenylnaringenin (8-PN) are compounds isolated from hops that been extensively studied for its great benefits (Good Night Drink, 2020; Hop Tea, 2022; Míracle Botánicals, 2022; Palo Nutrition, 2022; Herbal terra, 2021; Cam Formulas, 2022; Florida Herbal Pharmacy, 2021 Swanson, 2021; Ayabe et al., 2018; Gulati, Anand, & Ray, 2016; Vázquez-Cervantes et al., 2021; Astray, Gullón, Gullón, Munekata, & Lorenzo, 2020; Inui, Okumura, Matsui, Hosoya, & Kumazawa, 2017; Liu

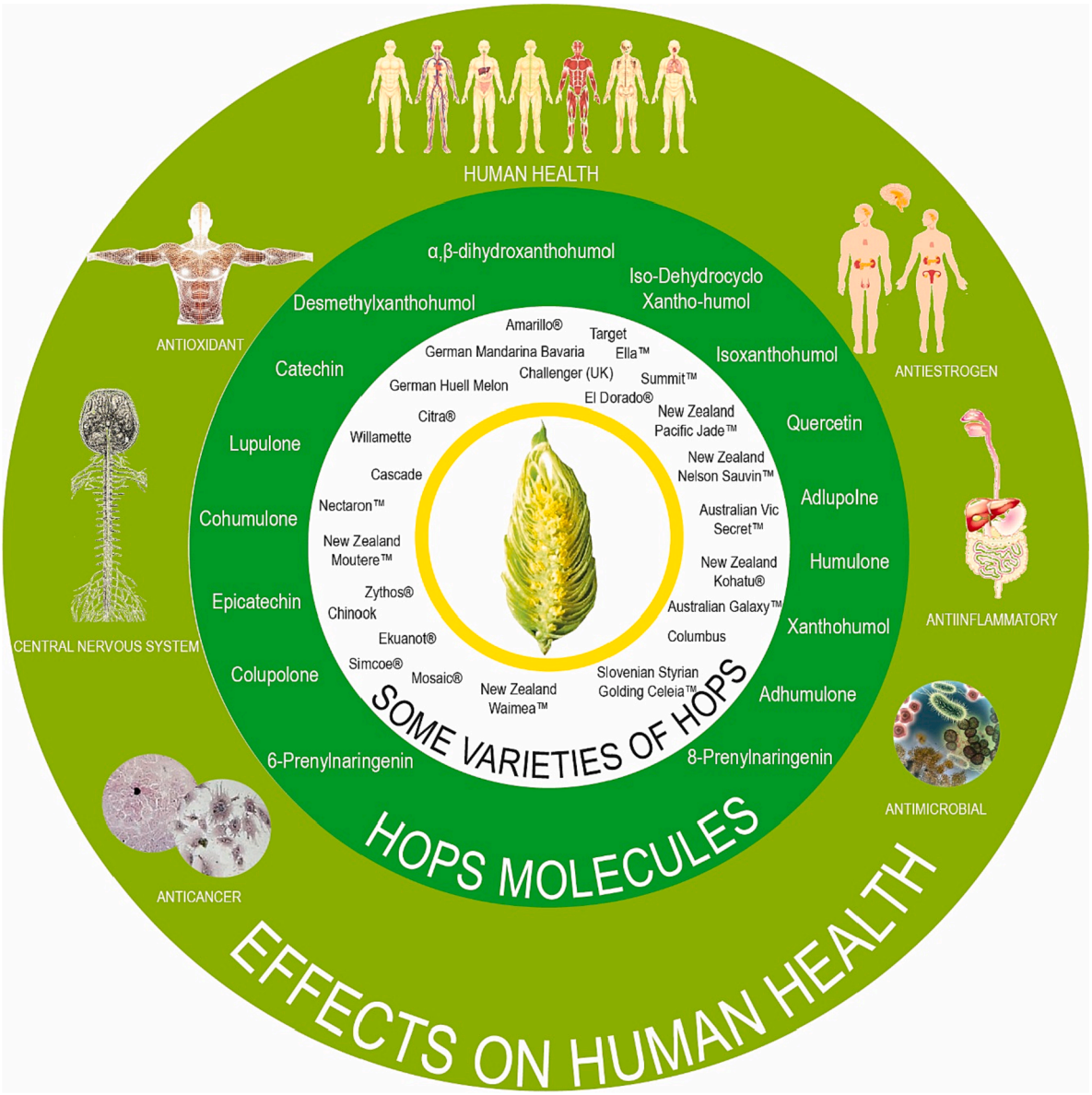

Fig. 1. Effects on human health.

et al., 2015; Fugh-Berman, 2003).

2.1. Potential in human health

The literature has shown that each variety of hops (*Humulus lupulus* L.) contains bitter acids, flavonoids, phytoestrogens and essential oils in different amounts. However, consumers do not currently ingest hops directly due to its strong bitter taste and aroma, but craft beer, is a favorable means to consume hops in a simple and pleasant way in moderation to obtain a preventive benefit to the well-being of human health.

The phytoestrogen 8-prenylnaringenin (8-PN) is a hop compound that causes regulatory effects through cyclization, demethylation and metabolism processes (Di Vito et al., 2012; Dietz, Hajirahimkhan, Dunlap, & Bolton, 2016). Other different bioactive compounds are essential oils of hop, which have an inducing effect to fall asleep. They are also found in dietary supplement products to regulate symptoms of menopause. In this sense, essays *in vivo* to counteract menopause in women using different doses of 100 to 250 μg and even 500 mg of hops extract (8-NP) versus placebo, have shown a statistically significant reduction in menopausal symptoms and a drastic reduction in hot flashes at certain stage of menopause. Nevertheless, experiments are affected by factors such as purity of the extract, standardization of extraction, storage, conditions and prior preparation of the volunteers to carry out the bioassay. Therefore, more clinical trials are needed before the safety and efficacy of hops extracts for relief of menopausal symptoms can be established (Aghamiri, Mirghafourvand, Mohammad-Alizadeh-Charandabi, & Nazemiyeh, 2016; Erkkola et al., 2010; Heyerick, Vervarcke, Depypere, Bracke, & De Keukeleire, 2006; Overk et al., 2008; Salter & Brownie, 2010; Taylor, 2015). Likewise, it was evaluated the possible side effects or results when hops extracts are regularly consumed at different concentrations, controlling days of randomized trial, clinical conditions of the volunteer patients, administration of the dose on an empty stomach or after food consumption, the lack of standardization of the extract, the recruitment of women from multiple sites, and the lack of a placebo effect. Nevertheless, no adverse effects were reported in any of these trials.

Other studies have been conducted to demonstrate the bioactive activity of beer. Rossi et al. (2021) evaluated the effect of the intake of craft and industrial beer against the risk factor for cardiovascular diseases measuring the reduction of serum homocysteine. The study was a randomized crossover, 12 men (28.7 ± 6.0 years) and 12 women (29.4 ± 7.5 years) participated. All participants were healthy, omnivorous, with normal body mass index, non-smokers and not taking oral supplements or contraceptives. The study was conducted whit a free-living diet (normal/ daily) complemented with 330 ml of industrial beer (4.5 % alcohol) or craft beer (9 % alcohol) during 21 days. Anthropometric measurements and blood samples were taken at the beginning and at the end of each period. Industrial beer consumption reduced homocysteine from 7.35 to 6.50 μM/L and increased folic acid from 3.46 to 3.94 ng/ml. In the case of the craft beer, it had no effect on homocysteine reduction, due to the alcohol concentration compared to industrial beer. Results were expressed regardless of the composition of the study samples.

Osteoporosis is another unfavorable condition associated with menopause. The potential of 8-PN to prevent bone loss has been evaluated. The use of 8-PN it has showed favorable results due to its preferential binding to estrogen receptors, which predominate in bone tissue. The potential of 8-PN to prevent osteoporosis has been evaluated only using *in vitro* and *in vivo* models, however, the possibility of clinical trials in human beings has been considered (Hümpel et al., 2005; Keiler et al., 2017; Štulíková, Karabín, Nešpor, & Dostálek, 2018).

Other important bioactive molecules are phytoestrogens, which are known to exhibit anticancer activities. In general, this effect *in vitro* models is suggested that these bioactive compounds act against cancer cells by inducing autophagy or by modulating the cell cycle and the efficacy to suppress the growth of tumor cells. For that reason, the cytotoxic potential of 8-PN and 6-PN in cancer cells has been proved in fibroblast cells (e. g. human prostate cancer, human renal carcinoma, hormone-dependent breast tumors). This property could be used further in the development of new anticancer drugs (Busch et al., 2015; Hemachandra et al., 2012; Štulíková et al., 2018; Venturelli et al., 2016; Wang et al., 2016).

In order to demonstrate the potential biological activity of 8-PN, XN, IX applicable for therapeutic purposes, different studies have been conducted. *In-vivo* rats bioassays, have reported antidiabetic properties, preventing body weight gain and inhibiting insulin resistance and glucose tolerance. In this case as a result, a regular intake of foods rich in phytoestrogens is associated with a lower risk of cardiovascular diseases. Additionally, experiments are still being carried out *in vivo* to corroborate *in vitro* and *in situ* tests. Results of these studies have reflected significant anti-atherosclerotic effect on the thickness of the intima media of the common carotid arteries and inhibition of the growth of atherosclerotic plaques. Based on these results, many authors conclude with that there are a potential use for hop extracts, not only as nutritional, but as functional and nutraceutical supplement to prevent and improve the human health (Costa et al., 2017; Gulati et al., 2016; Myasoedova et al., 2016).

It has been proposed that the positive effect and benefits could be for consuming beverages with hop contents since the moderate consumption of alcoholic beverages such as beer (15 g alcohol/day in women and 30 g alcohol/day in men) may reduce the risk of cardiovascular disease, dementia and cognitive impairment among others, derived from bioactive compounds. (Di Domenico et al., 2020; Salanță et al., 2020a, Salanță et al., 2020b; Ayabe et al., 2018; Elrod, 2018; De Gaetano et al., 2016). In terms of estrogenic beer, 8-prenylnaringenin serves as one of the most potent phytoestrogens from hops (<100 g/L), while isoxanthohumol can be converted to 8-prenylnaringenin by the microbiota of the consumer's intestinal tract (<0.002 g/L). In addition, 8-prenylnaringenin acts as a prospective estrogen and could be applied as therapy and prevention of postmenopausal problems such as hot flashes, insomnia, irregular menstrual periods, mood swings, bone density loss, changes and regulation of levels cholesterol in blood glucose, and discomfort among others (Possemiers et al., 2006, 2009; Stevens & Page, 2004). 8-prenylnaringenin and xanthohumol have been isolated with an ≥ 99 % of purity for further experimentation obtaining effective pharmacological doses (Chen et al., 2012).

3. Potential hops for brewing

Hops range to different varieties due to their bittering properties and high aroma content (Table 1). Each variety provides unique and incomparable fragrance, such as green, citrus, floral, spicy, herbal, woody and fruity notes. These kinds of characteristics are granted and developed from the type of crop to the harvest, the variety (genotype) of the hops, as well as factors such as climate, relative humidity, subsoil composition, altitude, and irrigation (Rutnik, Knez Hrnčič, & Jože Košir, 2021; Katono, Yonezawa, & Inui, 2018; Marceddu, Carrubba, & Sarno, 2020; Ocvirk, Nečemer, & Košir, 2019; Hopslist, 2022; HPA. 2022; BSG CraftBrewing, 2022; Yakima Chief Hops Inc, 2022; Šrédl et al., 2020; Hop head farms, 2022).

On the other hand, hops used to bitter beer are generally added at different stages of the boiling process, while aroma-providing hop varieties are preferably added after. However, to prevent volatilization and loss of aromatic compounds they are added before and/or after the fermentation process. In the case of hop polyphenols, they isomerize at temperature > 90 °C, cyclohexadienone transforms to cyclopentadienone and xanthohumol to isoxanthohumol. These isomers exhibit strong long-lasting bitterness stimulation and low bitterness threshold (Stevens, Taylor, Clawson, & Deinzer, 1999; Zhao et al., 2005). That is why brewers try to preserve some of the original hop composition by adding "aromatic hop" varieties at the end of the boiling period. This procedure is known as "late hopping", which is combined

Table 1
Properties of hops most used in brewing.

Hop	Country	α acids %	β acids %	Total oil mL/ 100 g	Aroma profile	Beer Styles
Ahtanum	United States	3.5 to 6.5	4 to 6	0.5 to 1.7	Grapefruit floral, cedar	Lager IPA Pale Ale
Amarillo	United States	7 to 11	5.5 to 8	1 to 2.3	Grapefruit, orange, lemon, melon, apricot peach	American IPA
Aurora	Slovenia	7 to 13	2.7 to 4.4	0.9 to 1.6	Lime, floral, pine, bergamot, lemon grass, aniseed.	English or Belgian
Azacca	United States	14 to 16	4 to 5.5	1.6 to 2.5	Mango, papaya, orange, grapefruit, lemon, piney, spicy, pineapple, grassy, tropical fruit, citrus	American IPA Saison
Bitter	United States	12 to 14.5	4.5 to 6	1 to 2.3	Pear, watermelon, stone fruit fresh	Belgian Pale Ale IPA
Bravo	United States	15 to 18	3.5 to 5.5	2 to 3.5	Orange, vanilla	Pale Ale IPA Barley wine
Cascade	United States	5.5 to 9	6 to 7.5	0.8 to 2.5	Grapefruit, floral pine	American IPA
Cashmere	United States	7.7 to 9.1	6.4 to 7.1	1.2 to 1.7	Melon, lemon, lime spice	IPA
Centennial	United States	7 to 12	3.5 to 5.5	1 to 3	Lemon, floral, Orange, blossom	American IPA
Challenger	United Kingdom	6.5 to 8.5	4 to 4.5	1 to 1.7	Cedar, green tea, sweet fruit	English Brown Pale Ale
Chelan	United States	12 to 14.5	8.5 to 9.8	1.5 to 1.9	Mild	American
Chinook	United States	11.5 to 15	3 to 4	1 to 2.5	Grapefruit, spicy, pine	IPA American Stout Porter
Citra	United States	11 to 16	3 to 4.5	1.5 to 3	Grapefruit, melon, lime, gooseberry, passion fruit	IPA Weizen American Hazy IPA
Cluster	United States	6 to 9	4 to 6	0.5 to 1.7	Floral, earthy, sweet fruit	Lager Stout Porter
Crystal	United States	3 to 6	5 to 8.5	0.8 to 2.3	Woody, grren	Belgan English Bitter Pilsner
Ekuanot	United States	13 to 15.5	4 to 5	2 to 4	Melon, berry, lime, apple, papaya, green pepper, mango, mandarin, orange	IPA American Belgian American pale ale
Dorado	United States	13 to 17	7 to 8	2.5 to 3.3	Cherry, apricot, pear citrus, watermelon, grass, wood, mint	Blonde Pilsner American pale ale IPA Cream ale Red Wheat
Ella	Australia	13.3 to 16.3	4.8 to 7.8	2.4 to 3.4	Floral, noble, spicy star anise, grapefruit	Pilsner

(*continued on next page*)

Table 1 (*continued*)

Hop	Country	α acids %	β acids %	Total oil mL/ 100 g	Aroma profile	Beer Styles
Enigma	Australia	13.5 to 16.5	4.8 to 6.4	2.4 to 3.4	Pinot, gris, raspberry, redcurrant tropical fruit	Pale ale IPA Amber
Falconer's Flight	United States	9.5 to 12	4 to 5	1.4 to 2	Lemon, grapefruit	American pale ale IPA
Galaxy	Australia	11.6 to 16	5 to 6.9	3 to 5	Passionfruit, peach, clean citrus	Pale ale IPA
Galena	United States	13 to 15.5	7.5 to 8.5	1.3 to 2.1	Pear, pineapple, lime blackcurrant, grapefruit	American ale Porter Stout
Glacier	United States	4 to 7.5	7 to 10	0.5 to 1.5	Plum, blackberry, wood	English-style Pale ale Porter Stout English-style bitter
Huell Melon	Germany	6.9 to 7.5	7.3 to 7.9	0.8	Honeydew, melon, strawberry, tropical fruit, orange, vanilla	Wheat Belgian Pale ale
Idaho	United States	9.5 to 14	3.5 to 5.5	1 to 2	Pineapple, peach, pine, resin, mango, black tea	IPA Pale ale Wheat
Jarrylo	United States	15 to 17	6 to 7.5	3.6 to 4.3	Banana, grass, pear, orange, spicy, fruit	Pale ale Saison Belgian-style
Magnum	United States	12 to 15.5	5.5 to 8	1.5 to 2.5	subtle spice, fruit, clean bittering	IPA Pilsner Stout
Mandarina Bavaria	Germany	7 to 10	5 to 6.5	2.2	Tangerine, grapefruit, lime	IPA Saison
Mosaic	United States	10.5 to 14	3 to 4.5	0.8 to 3	Blueberry, tangerine, papaya, rose, blossom, bubble gum	Pale ale IPA Stout
Motueka	New Zeland	6.5 to 7.5	5 to 5.5	0.8	Lime, lemon, tropical fruit	English-style ale Pilsner
Nugget	United States	13 to 16	4.4 to 5.5	1 to 3	Green, Wood, ginger	Pilsner Pale ale
Saaz	United States	3 to 4.5	3 to 4.5	0.5 to 1	Earthy, spicy	Pilsner Belgian-style ale Wheat
Saaz	Czech Republic	2.5 to 4.5	4 to 6	0.4 to 0.8	Pleasant, mild	Pilsner Belgian-style ale
Sabro	United States	13 to 17	4 to 6.5	1.8 to 3.4	Citrus, stone fruit, coconut tropical fruit, herbal	IPA Saison Porter Stout Fruit beer American pale ale
Saphuir	Germany	2 to 4.5	4 to 7	0.8 to 1.4	Spicy, floral	German-style lager Belgian-style ale Pilsner Wheat
Simcoe	United States	11.5 to 15	3 to 4.5	0.8 to 3.2	passion fruit berry, pine, citrus, bubble gum	IPA American pale ale Wheat Saison Amber
Sládek	Czech Republic	4.5 to 8	4 to 7	1 to 2	Peach, grapefruit, passion fruit	IPA Pilsner Blonde ale
Sticklebract	New Zeland	12	6.6	0.8	Pine, citrus	English-style Bitter IPA English pale ale Pilsner
Summer	Australian	5.6 to 6.4	4.8 to 6.1	1.4 to 2	Apricot, melon	Pilsner
Summmit	United States	15 to 17	5 to 6.5	1.5 to 3	Pepper, incense, anise, orange, pink grapefruit, tangerine	IPA Double IPA Pale ale Wheat
Waimea	New Zeland	16 to 19	7 to 9	2.1	Citrus, pine herbal	IPA Lager
Wakatu	New Zeland	6.5 to 8.5	8.5	1	lime zest, floral	Pilsner Pale ale

(*continued on next page*)

Table 1 (*continued*)

Hop	Country	α acids %	β acids %	Total oil mL/ 100 g	Aroma profile	Beer Styles
						Bock
Whitbread golding	United Kingdom	5.4 to 7.7	2 to 3.3	0.8 to 1.2	Floral, sweet fruit	English-style Bitter pale ale Saison Wheat
Willamette	United States	4.5 to 6.5	3 to 4.5	0.6 to 1.6	Floral, incense, elderberry	Brown ale Pilsner Pale ale Stout Porter

Rutnik et al., 2021; Katono et al., 2018; Marceddu et al., 2020; Ocvirk et al., 2019; Hopslist, 2022; HPA. 2022; BSG CraftBrewing, 2022; Yakima Chief Hops Inc, 2022; Šrédl et al., 2020; Hop head farms 2022; Independent Brewers Association of Australia, 2018.

with "dry hopping". This special technique involves hop addition to beer just before packaging (three to seven days beforehand). In this case, some original hop constituents are transferred directly into the aqueous matrix imparting a discrete high hop character to the beer (Lafontaine et al., 2019; Machado, Faria, Melo, Martins, & Ferreira, 2019).

4. Craft beer

Beer is one of the most consumed beverages worldwide due to its unique organoleptic properties and characteristics (e. g. aromas, flavors, body and color) that are conferred by the use of duly incorporated inputs (e. g. water, malt, hops and yeast) for its elaboration. These factors are related to fermentation process. Currently there are two types of fermentation: Ale and Lager they can be distinguished by the use of different yeast species according to their flocculation behavior. Species such as *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* (hybrid between *S. eubayanus* and *S. cerevisiae*) conferred characteristics that distinguish the final product (Brewers Association, 2021; Gallone et al., 2018; Gonçalves et al., 2016; Granato, Branco, Faria, & Cruz, 2011).

The demand for specific styles of beer of worldwide consumers are moving from traditional "light style" lagers to "craft". In this context, craft refers primarily to the complexity of the style, rather than the scale of the brewer. Additionally, craft beers could also be seen as higher quality beers, which positively reinforces purchasing and consumption habits. Even so, there is a strong consumer demand for light beers (Lager) due to their ease of drinking without a previous CATA task (check-all-that-apply).

Preference studies have demonstrated that consumers who prefer light beers (lagers) associate their preferences with sensory properties such as taste and flavor, along with non-sensory factors such as caloric content and low cost. According to a study made by Hopfer, McDowell, Nielsen, and Hayes (2021) from a CATA task, two kind of consumers (30 preferring 'light' beer and 32 the "craft") found that light beer (Lager) drinkers verified significantly fewer attributes on the CATA task relative to craft beer drinkers. Specifically, craft beers (fermented ales) are perceived as more aromatic and more complex-bodied. This kind of beer is generally produced with higher concentrations and from one or more varieties of hops compared to conventional light-style lagers (Hopfer et al., 2021).

Currently, consumers are looking for alternatives that present welfare benefits by consuming a beer with an excellent pairing (food). However, conventional Lager beers provide a lower concentration of bioactive compounds compared to Ale beers, due to the proportions of malt and hops for its elaboration (Granato et al., 2011). In addition, Lager beers are produced by different adjuncts and processes for a better appearance (e. g. forced carbonation, dilutions in must or at the end of fermentation, filtrations, clarifiers, among others).

4.1. Beer styles

Ale and lager consumers tend to express and reveal a strong preference for a specific style due to the unique aromas and flavors they experience. In particular, the demand for specific styles of beer is changing around the world, as more consumers are switching from conventional "light style" lagers to "craft" ales. In this context, craft beer refers primarily to the complexity of the style, rather than the scale of production. Ale craft beers are also considered of higher quality, which positively reinforces the consumption habits of a functional product, due to the high quality compared to Lager beers. It is evident that the price is higher owed to the quantity and quality of ingredients, lower production and market positioning (Brewers Association, 2021).

Nowadays, strong demand for light-style beers persists and some studies have examined consumer preferences by light beers. These kinds of studies remark the sensory properties such as taste and flavor as the predominant drivers of purchase, but determining preference factors are combined with non-sensory factors such as caloric content. In this sense, reviews should be disseminated so that consumers find out the properties and benefits that they could have with the consumption of different beer styles and how to differentiate the quality of beers. One option is the one called artisanal nature beer, which is venturing into this area with the aim to increase their consumption. The artisanal nature beer has around 91 styles of Ale beer, 35 styles of Lager beer and taking into account new hybrid trends up to 34 styles as new options (Brewers Association, 2021; Seo et al., 2020; Gómez-Corona, Escalona-Buendía, García, Chollet, & Valentin, 2016; Granato et al., 2011; Chrysochou, 2014).

The beer styles with the highest content of hops are called Indian Pale Ale (IPA). These beers are made from more than 2 to 5 different varieties of hops, which have a high content of α-acids and β-acids depending on both the elaboration and maturation process, as well as the conservation of the final product (Elzinga, Tremblay, & Tremblay, 2015). IPAs are precursors to the production of similar high-hopping styles of different nationalities (e. g. English-Style India Pale Ale, American-Style Imperial or Double India Pale Ale, Juicy or Hazy Imperial or Double India Pale Ale, Session India Pale Ale, New Zealand-Style India Pale Ale) that evidently provide specific organoleptic characteristics (Brewers Association, 2021).

Other styles with a high presence of acidity include Sour styles derived from organic acid production. Lactic acid bacteria such as *Pediococcus damnosus* and *Lactobacillus brevis* are responsible of lactic acid production. In the case of acetic acid production, this attributed to *Acetobacter* sp. The use of yeast consortia, including *Brettanomyces clausenii* is important in the production of organic acids such as lactic acid and acetic acid, as well as phenolic compounds (e. g. 4-vinyl guaiacol and 4-ethylphenol), which are important for the well-being of consumer health (Bossaert et al., 2021; Dzialo, Park, Steensels, Lievens, & Verstrepen, 2017; Spitaels et al., 2015; Sterckx, Saison, & Delvaux, 2012).

5. Craft beer as a functional beverage

Beer is a popular product and represents the most consumed alcoholic beverage in the world. Recently, there has been a significant growth of independent craft breweries (small independent, traditional, conservative brewery, production < 10 thousand hectoliters), generating classic styles according to the Brewers Association, 2022, but innovates methods are included in new trends and very promising proposals for demanding palates. In addition, the growing success of small breweries (microbreweries, brewpubs) suggests a greater recognition of the opportunities presented by the use of different varieties of hops and the rest of the ingredients (malt / yeast), highlighting the beneficial attributes that this set of raw materials brings (Brewers Association, 2022 Brewers Association Acermex, 2022; Beer Canada, 2022; Associação Brasileira de Cerveja Artesanal (Abracerva), 2022; Brewers of Europe, 2022; Journalbeer Beer Business, 2022; Japan Brewers Association, 2022; The Brewers Association of New Zealand Brewing, 2022, Independent Brewers Association of Australia, 2018).

5.1. Craft beer / brewing ingredients

The consideration of beer as functional beverage is supported by the bioactive attributes of the ingredients used for its production. In this case, Brewing is an ancient and biotechnological process, which has evolved in such a way that it is currently produced under methodologies that have improved in recent decades. The scientific study within the brewing industry maintains production standards under quality criteria, which leads to the offering of healthy beverages (Bamforth, 2017). So, the brewing process has a great influence on the phenolic composition of beer, as has recently been shown by the different phenolic profiles and biological activities of craft beers (Ale type) compared to conventional industrial beers (Lager type) (Cheiran et al., 2019; Granato et al., 2011; Viana et al., 2021).

The design of processes is pointed to the homogeneous blend of the ingredients to make beer (barley, hops and yeast) which have been shown to have synergistic effects, enhancing the presence of beneficial bioactive compounds for health. Likewise, the different bioactive compounds at different concentrations could be quantified using instrumental technics (high resolution liquid chromatography) developed to measure compounds in the homogeneous mixture of ingredients (water, barley, hops and yeast). Breda, Barros, and Gouvinhas (2022) had determined the concentration of specific polyphenols, orthodiphenols, and flavonoids present in twenty-four Portuguese craft beers (light/dark) produced by different varieties of hops, malt and type of Ale/lager fermentation. These authors implemented the quantification of bioactive compounds by a reversed-phase high-performance liquid chromatography with a diode array detector (RP-HPLC-DAD). They demonstrated that all beer samples had gallic acid and catechin, with concentrations ranging from 4.20 ± 0.43 to 61.57 ± 0.59 μg/mL and 7.50 ± 0.28 to 147.99 ± 3.83 μg/mL, respectively, and found out that gallocatechin, caffeic acid, p-coumaric acid, quercetin, and trans-cinnamic acid were the compounds with the least presence or no presence at all.

Baigts-Allende, Perez-Alva, Ramirez-Rodrigues, Palacios, & Ramirez-Rodrigues, 2021, quantified the content of gallic acid, vanillic acid, caffeic acid, chlorogenic acid, p-coumaric acid, epicatechin, kaempferol, and rutin by liquid chromatography-mass spectrometer method (LC–MS/MS). They identified and quantified polyphenols and β-carotene on twenty-nine samples of fruity commercial beers from different nationalities, observing that all samples showed the presence of epicatechin. Other quantitative techniques such as Folin-Ciocalteu only generally determine the presence of phenolic compounds in the sample, taking the gallic acid as a reference. However, sophisticated HPLC techniques can accurately quantify and recognize the presence of specific compounds using standards with purities of ≥ 99 % with recognition of fragmentation patterns (intensity) (Nardini & Garaguso, 2020).

In this review it was emphasized that the moderate consumption of craft beer (15 g alcohol/day in women and 30 g alcohol/day in men) could be a vehicle for obtaining benefits without altering or having secondary effects (Osorio-Paz, Brunauer, & Alavez, 2020). In the same sense, Padro et al. (2018) described that one beer, in the case of women and two for men, with ≤ 7 % of alcohol or the equivalent to the degree of alcohol is recommended for acquiring all benefits. In a study conducted by Molina-Hidalgo et al. (2020), showed that beer consumption does not mitigate the positive effect of a 10-week high-intensity interval training (HIIT) on physical fitness in healthy young adults aged 24 ± 6 years (35 women and 38 men). These studies reflect that the moderate consume of beer is placed at different level than other beverages with a higher alcohol content such as wine or spirits.

5.1.1. Water

The water used in brewing plays an important role for the integration and colloidal balance of all the inputs to produce an excellent beer due to the mineral content in it. The main ions in beer are cations (calcium, magnesium, sodium, and potassium) and anions (sulfate, nitrate, phosphate, chloride, and silicate) (Montanari, Mayer, Marconi, & Fantozzi, 2009). Minor ions are iron, copper, zinc, and manganese. The mineral content of the brewing water is particularly important to the brewing process and thus to the quality and taste of the final beer. In beer, most of the minerals come from the barley but about 75 % derives from the malt, while the remaining 25 % originates from the water.

Hops contribute a negligible amount of minerals in beer due to the small quantities used (200 g to produce 100 L of beer). In contrast, hops make a notable contribution of nitrate to the beer wort. In addition, some cereals (e. g. corn, rye, wheat, oats, rice, among others) matrices typically contain fewer minerals than malt, so the metal level is lower than that of a malt wort, but the large amount of minerals in the raw materials decreases during the manufacturing process because some minerals are removed through precipitation (Montanari et al., 2009). Among the mineral salts in the water there are: magnesium and sulfate, nutrients that help to highlight the bitterness of the hops the chloride that highlights the sweetness of the malt sodium, which provides texture and calcium that is important during the maceration (Montanari et al., 2009).

5.1.2. Barley

Barley malt is another essential ingredient for brewing, constituting approximately 70–80 %, which contribute to color, flavor, and stability of any style of beer (Piazzon, Forte, & Nardini, 2010). Barley malt provides a great upwelling of polyphenols (e. g. ferulic acid, p-coumaric acid, gallic acid, caffeic acid, vanillic acid, and sinapinic acid) which are beneficial compounds for health (Carvalho & Guido, 2022; Cheiran et al., 2019; Oliveira Neto et al., 2017). In addition to the use of different malts to produce different styles, according to the Brewers Association, barley malt constitutes 0.2 % of polyphenols in the dry matter, as well as providing color, flavor, colloidal stability and shelf life of the beer.

Furthermore, it has been shown that the highest content of beneficial bioactive compounds in beer is provided by hops and hop granule extracts exhibit higher percentages of total polyphenol and flavonoid content, compared to barley malt extracts. Although hops have a higher phenolic concentration compared to malt, most of the phenolic compounds identified in the wort, originate from both ingredients (hops and malt) being a potential beneficial compound content in beer (Carvalho & Guido, 2022). Currently, in order to have a higher content of beneficial compounds, the use of other cereals (e. g. wheat, oats, rice, among others) is being implemented. This addition provides new organoleptic characteristics without losing the origin and type of style in brewing such as a new option for consumers (Ceccaroni et al., 2019; Mastanjević et al., 2018; Zdaniewicz, Pater, Knapik, & Duliński, 2021).

5.1.3. Yeast

Yeast acts on the fermentation process of the must. Various yeasts (e.

g. *Saccharomyces cerevisiae, Saccharomyces pastorianus, Saccharomyces carlsbergensis, Saccharomyces sensu stricto, Saccharomyces bayanus, Saccharomyces cariocanus, Saccharomyces kudriavzevii, Saccharomyces mikatae* and *Saccharomyces paradoxus*) have also been scientifically shown to depend on the concentration and variety of malt and hop components. They metabolize beneficial compounds to generate or potentiate beneficial bioactive compounds for consumer health (Vieira et al., 2016). The most widely used yeasts in the brewing industry have several properties that make them outstanding for industrial brewing use, including rapid growth, good ability to produce ethanol, and tolerance to various types of environmental stresses, such as high ethanol concentration and low oxygen levels (Puligundla, Mok, & Park, 2020).

The fermenting wort widely used yeasts in breweries are conventionally divided into two main classes, bottom fermenting (Lager / 8 to 15 °C / *Saccharomyces pastorianus* or *Saccharomyces carlsbergensis*) and top fermenting (Ale / 16 to 25 °C / S. cerevisiae) (Ferreira, Pinho, Vieira, & Tavarela, 2010). In the brewing process, yeast biomass, also known as residual yeast or leftover yeast, is one of the predominant by-products obtained after the first fermentation (Puligundla et al., 2020). However, there are naturally carbonated beers, through a second fermentation in the bottle (typically in craft beer / Ale fermentation), obtaining a small amount of biomass at the end of the fermentation process. Thus, it is an abundant and economical source of protein (45–60 %), minerals, B-complex vitamins, β-glucans, oligosaccharides and other valuable components. Once the must (blend of water, malt and hops) is obtained, the fermentation process is carried out with the yeast. The first yeast will recognize the environment and identify the essential nutritional components and the secondary metabolites that are present to have an optimal adaptation (genetic coding of the cells to new generations) at ferment generating new efficient secondary metabolites of consumer welfare (Podpora, Świderski, Sadowska, Rakowska, & Wasiak-Zys, 2016; Puligundla et al., 2020).

New trends in beer production highlight the use of various microorganisms and even microbial consortia to obtain new organoleptic properties using barrels made of precious woods (e. g. oak, mahogany, cedar, sandalwood, birch, among others). Currently, consumers are looking for genuine, unique, different and quality products. Craft beer makers have responded to this by introducing a novel combination of raw or malted grains, hop varieties, which have modified the aromas and flavors of the beer with the addition of fruit, spices, colouring, clarifying, flavoring etc. These additions aimed for obtaining greater acceptability and increasing consumption in a responsible manner, as well as promoting the beer culture (Villacreces, Blanco, & Caballero, 2022; Brewers Association, 2021; Cantwell & Bouckaert, 2016).

6. Craft beer storage quality

The storage of lager and ale-type beer can be beneficial or detrimental, the latter being a serious problem to preserve the organoleptic properties (aroma and flavor). Studies have revealed that light and dark beers exhibit greater changes in the chemical composition of the beer during storage and they should be considered the main quality problem faced by brewers. Flavor instability or aging of light beers after 3 to 6 months is a very complex process due to the numerous oxidative and non-oxidative reactions of phenolic compounds conferred by hops. These reactions take place during beer storage at room temperature (20 to 37 °C). The compounds of interest (esters, aldehydes, alcohols, furanic compounds, fatty acids and terpenes) are more stable at refrigeration temperature (4 °C). However, light beers are more sensitive to these changes (Bossaert, Crauwels, De Rouck, & Lievens, 2019; Ferreira et al., 2022; Lehnhardt, Gastl, & Becker, 2019; Sterckx et al., 2012; Tonsmeire, 2014; Wang, Li, Ji, Hu, & You, 2014). In contrast, dark beers which their main component is roasted malts with lower hop content, exhibit characteristic bitterness and acidity. These characteristics are attributed at fewer oxidation reactions resulting in favorable maturation to enhance pleasant aromas and flavors under optimal storage conditions (6 to 18 months).

Long-term storage and/or aging of beer in wooden barrels, cans or dark glass bottles produces an additional complexity of integration of all its components. So, flavors and aromas are obtained with greater accentuation in dark beers, due to the synergies caused by the effect of regulating the pH of dark malts and alcohol, the type of water, types of hops and used yeast. These favorable effects have been presented to typically brew Brown Ale, Porter Ale, Stout Ale, among others (Shayevitz, Harrison, & Curtin, 2020).

In addition of the occurrence of changes in the chemistry and flavor of beer during storage is mainly due to the development of aldehydes, esters, higher alcohols (propanol, isobutanol, isoamyl alcohol, amyl alcohol and aromatic alcohols 2-phenylethanol, tyrosol and tryptophol) and other compounds that arise from various steps of the brewing process. In relation of the composition, aldehydes are a group of compounds with a high contribution to alcohol oxidation creating off-flavors in beer production (Caballero, Blanco, & Porras, 2012; Ferreira & Guido, 2018; Ferreira et al., 2022; Lehnhardt et al., 2019).

If the increase of concentration of short-chain and branched-chain fatty acid esters, present in the wort is possibly the most important volatile compounds in beer. They esters have a positive impact on the overall flavor of the beer, especially aroma, but high or excessive amounts of esters cause off-flavors (odor fruity). Esters, as well as higher alcohols, are produced by yeast metabolism during fermentation, in addition to ethanol. Higher alcohols are the main alcohols that impart a variety of organoleptic attributes such as alcoholic, fruity, and spicy attributes, depending on the concentration and the type of alcohol. On other hand, beer aging comprises many chemical reactions, which occur at different rates depending on the storage conditions applied to beers, with temperature as the precursor of chemical changes (Ferreira et al., 2022).

7. Final remarks and perspectives

The expansion of the brewing industry has allowed many possibilities for improvement in terms of flavor, aroma and functionality of this beverage. Health-related issues and the general desire for healthier lifestyles have led to an increased demand for functional beers. In this way, current technologies provide easier forms to potentiate positive effects that offer additional functional properties for health and wellness benefits with sensory adjustments of classic beer. By comparing different styles of beers, it is also easy to understand that the brewing process and raw materials are the triggers for the beer's phenolic profile. The use of efficient concentrations of hops is a real factor to make and consume a functional beer. In addition, the use of malts and potential adjuncts of bioactive compounds will continue to increase the multiple health benefits that have been scientifically evidenced in this beer. For that reason, new trends in the use of different blends of malts from different cereals, varieties and combinations of traditional and genetically modified hops, additions of fresh and dried fruits with high phenolic content, natural complements to enhance aromas and flavors are increased. Also, the development and growth of the beer industry aims to satisfying the demands of improved functionality of this beverage guaranteeing bioactive properties pointed to human health. That kind of improvement is related to the responsible consumption of these functional beverages avoiding types of consumers, preferences, tastes and medical conditions/illnesses/preventions. The spread of functional beers will establish a new type of health-conscious consumer, who would like to enjoy a beer with another beneficial perspective, as long as they avoid excessive consumption, maintaining a responsible culture and consumption.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Capítulo -VII-
Physicochemical and microbiological parameters during the manufacturing of a beer-type fermented beverage using selenized *Saccharomyces boulardii.*

7.1 Introducción

En este capítulo final del proyecto doctoral se da una aplicación tecnológica de *Saccharomyces boulardii* selenizada. El artículo ha sido enviado a la revista Heliyon. Esta parte experimental se realizó durante una estancia de investigación en el Laboratorio de Higiene, Inspección y Control de Alimentos del Departamento de Química Analítica, Nutrición y Bromatología del Campus de Lugo en la Universidad de Santiago de Compostela, España. El objetivo fue elaborar y caracterizar una cerveza artesanal baja en alcohol mediante la fermentación con *Saccharomyces boulardii* selenizada para dar una propuesta de uso como vehículo de la ingesta de selenio orgánico. La fermentación se realizó con mosto de cebada basándose en un estilo de cerveza tipo Amber Ale. La fermentación se llevó a cabo junto con un control, que consistió en la fermentación con *Saccharomyces boulardii*. De ambas fermentaciones se obtuvo viabilidad, pH, gravedad original, contenido de alcohol y contenido de selenio. La cerveza fermentada con levadura selenizada presentó un contenido final de selenio de 0.378 mg de Se/Kg. Con base a los resultados obtenidos se pudo concluir que la levadura selenizada fermento sin problema el mosto de cebada, obteniendo una bebida baja en alcohol y enriquecida con selenio. Las perspectivas se enfocan en investigar la presencia de selenoaminoácidos y nanopartículas de selenio en la cerveza, pero hasta ahora, estos resultados

muestran una alternativa de ofrecer una bebida funcional con alto contenido de selenio que pudiera brindar un beneficio a la salud humana.

Physicochemical and microbiological parameters during the manufacturing of a beer-type fermented beverage using selenized *Saccharomyces boulardii*

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7.2 Abstract

Selenium is an essential trace element in human health. However, it has been considered a widespread selenium deficiency worldwide, although the recommended daily intake is very low (55 μg per day). That is why strategies have been implemented to comply with this recommendation, trying to induce the intake of bioavailable selenium, mainly in its organic form, as selenoamino acids. Thus, this research aimed to elaborate on a beer-type fermented beverage produced with previously selenized *Saccharomyces boulardii*. For this, the yeast was selenized by adding a minimum inhibitory concentration of Na2SeO3 (74 ppm) to YPD media. Subsequently, barley must fermentations were carried out for 120 hours. Kinetic parameters of the fermentation and physicochemical parameters and selenium content of the beverage were measured. It was observed that the yeast accumulated up to 25.12 mg/g of dry cell, which was higher than that reported for other yeasts. Furthermore, selenization was shown to affect the fermentation rate. This did not influence the physicochemical parameters of the beverage, which were similar to those of the control. Due to the final concentration of selenium in the beverage (0.378 mg/kg), it is considered a process that confers advantages for the safe intake of selenium with bioavailable potential.

Keywords: selenium, selenoamino acid, beer, *Saccharomyces boulardii*

7. 3 Introduction

Selenium is a trace element essential for life. This trace element has been shown to have antioxidant effects, and its intake in adequate concentrations helps to prevent different diseases such as cancer and diabetes, as well as to improve thyroid function and male fertility [1]. The sources of selenium are diverse, such as celery, cucumber, broccoli, and some cereals, such as wheat and barley. Their selenium content will depend on the selenium in the soil where they grow [2]. Despite all sources of selenium, it is generally found in its inorganic form as selenite or selenate, both of which are not fully bioavailable. For this reason, the necessary daily requirements for the human being are not fully satisfied (55 μg/day), especially in regions where this element is still scarce on earth [3].

One way to make organic selenium more bioavailable, and in higher doses, is through the selenization of microorganisms, such as yeasts. This selenization benefits the conversion of inorganic selenium to selenoamino acids such as slenocysteine (SeC) and selenomethionine (SeM). The most studied yeast in selenization techniques is *Saccharomyces cerevisiae* [4, 5, 6, 1]. However, new research supports the use of *Saccharomyces boulardiii*, which is the only yeast approved and used as a probiotic in humans. This yeast has been used to treat gastrointestinal diseases, such as acute diarrhea and traveler's diarrhea [7, 8]. Additionally, it has been shown that *S. boulardii* is capable of metabolically producing selenoamino acids and selenium nanoparticles, enriching growth media with sodium selenite [9].

Currently, and intending to cover selenium deficiencies, research has focused on producing selenium-enriched foods and supplements. Companies such as Altech, Lallemand, Selko, Orffa, and Angel Co., offer selenium-enriched yeasts for use in livestock feed to obtain selenium-enriched products such as eggs, milk, and meat [10, 11, 12]. Regarding beverages, such as beer, research has focused on enriching the soil to obtain barley and wheat with a high selenium content and use them in their production [13, 14]. Another way to obtain selenium-enriched beer is to directly add sodium selenite to the barley wort before adding the yeast [15]. Since beer is one of the most consumed beverages in the world, after tea and water [16], selenium-enriched beer could be considered a vehicle to supplement selenium in the diet and cover its deficiencies. In this context, the objective of the present investigation is to take advantage of the prebiotic characteristics of *Saccharomyces boulardii* and use it in a selenized form to ferment barley must produce beer for its use as a vehicle in the intake of organic selenium.

7.4 Material and methods

7.4.1 Selenization of Saccharomyces boulardii

A study on the selenium accumulation capacity of *Saccharomyces boulardii* was carried out independently [9]. The working group has already reported the minimum inhibitory concentration, so the same protocol was followed. According to this study, it was determined that the minimum concentration of Na_2SeO_3 placed in the yeast growth medium was 74 ppm. Thus, the production of selenized yeast was carried out where YPD broth was enriched with 74 ppm Na_2SeO_3, and 10^7 CFU/mL of *Saccharomyces boulardii* ATCC were inoculated. At the end of 24 hours, it was

centrifuged (7,800 x *g*) for 5 minutes (SORVALL-fresh, Thermo Fisher Scientific, USA), and the biomass was separated from the supernatant.

7.4.2 Inoculum preparation and activation

The supernatant was seeded onto YPD agar previously enriched with 74 ppm Na_2SeO_3, incubating under aerobic conditions at 37 °C for 48 h. Selenized *Saccharomyces boulardii* cells (reddish colonies) were scraped and transferred to YPD broth enriched with the same concentration of Na_2SeO_3, incubated at 37 °C for 24 hours, and after fermentation, centrifuged (7,800 x *g*) for 30 minutes. The resulting biomass was washed with deionized water and finally lyophilized (Labconco) and saved for analysis and fermentation. Some lyophilized selenized *Saccharomyces boulardii* and *Saccharomyces boulardii* (control) were inoculated into test tubes containing YPD broth. They were incubated at 37 °C for 24 hours. This broth was used for the fermentation of the barley must.

7.4.3 Wort preparation

A batch of beer wort was prepared on a laboratory scale in Erlenmeyer flasks of 1 L capacity. It was based on a style of Amber Ale beer. To prepare the wort, previously ground pale ale malt (Simpsons malt) was added to a mash vessel containing water heated to 65 °C. The malt/water ratio was 15% weight/volume. The maceration was carried out for 60 minutes, maintaining the temperature at 65 °C. A filtration process removed the beer bagasse. The resulting wort was brought to a boil with stirring for 60 minutes to achieve a hot break. After 30 minutes of boiling, Yellow hops (Yakima Chief) were added in pellets with 9.8% alpha-acids to achieve a total content of 20

IBUS. The added proportion was 0.08% weight/volume. The wort was cooled to separate hop sediments and proteins at the end of the boiling and the incorporation of hops. The wort was separated into two flasks, and the lost volume of water was recovered. The original gravity (OG) was 1.050 for the control (*S. bulardii*), and 1.048 for the wort inoculated with selenized *S. boulardii*. The inoculation of the yeasts was carried out when the wort reached a temperature of 22 °C. Later, the inoculated wort was manually homogenized, and the fermentation process was carried out at 22 °C for 120 hours. The viability and pH of both yeasts were monitored.

7.4.4 Fermentation kinetic parameters

The speed, the generation time, the growth kinetic constant, and the deceleration zone of the fermentation were calculated to establish the differences in the metabolism of each yeast. The specific growth rate constant (μ) was calculated according to Eq. 1. Equations 2 and 3 were used to determine the generation time (g) and the growth rate constant (K). The initial (N_0) and final (N_x) biomass concentrations correspond to the selected time interval within the logarithmic phase of growth, which were t_0 and t_x, respectively. The determination of the deceleration zone using the GeoGebra software (https://www.geogebra.org) using the Talmage and Fitch graphic method with modifications [17].

Ec 1. $\text{Ln}(N_x) - \text{Ln}(N_0) = \mu (t_x - t_0)$

Ec. 2. $g = 0.693 / \mu$

Ec. 3. $K = 1 / g$

7.4.5 Selenium content

Selenium content analysis was performed by ICP-OES according to the unmodified methodology of [9]. The analysis was carried out on the biomass of selenized *S. boulardii*, on the fermented beverage after the separation of biomass resulting from fermentation, and on spent yeast.

7.4.6 Physicochemical parameters

The pH was measured in triplicate during the 120 hours the fermentation lasted. The specific gravity was measured with the help of a QWORK hydrometer with a specific gravity scale of 0.99 to 1.16. For this, a sample of wort was taken in a 250 mL capacity test tube, the hydrometer was inserted, and the reading was taken. The procedure was carried out in triplicate. To determine the alcohol content, the Balling equation [18] was used by first multiplying the specific gravity by 1000:

$$A_{v/v} = 0.125 * (OG – FG)$$

7.5 Results and discussion

7.5.1 Selenization of Saccharomyces boulardii

The selenium-rich probiotic yeast produced a reddish yeast with a selenium accumulation of 25.12 mg/g of dried biomass. This high accumulation of selenium has not been previously achieved; studies report an accumulation for *S. cerevisiae* of up to 1.19 mg/g of dried biomass when enriching the medium with 15 mg/L of selenite [19], and 3.4 mg/g for *S. boulardii* at 9 hours of growth in a medium enriched

with 74 ppm of selenite. The technique used in this study resulted in adaptation and probably selenium dependence, which, when reseeded, was followed by selenium accumulation.

7.5.2 Fermentation

The fermentation was followed for 120 hours, where viability (Fig. 1) and pH (Fig. 2) were monitored. The first figure shows an adaptation period of more than 12 hours for the selenized yeast, reaching its stationary phase up to 72 hours but reaching the same viability as the control yeast at 120 hours. A consistent behavior concerning pH was observed, where pH decreased up to 24 hours for the selenized yeast; both results indicate that the fermentation of selenized *Saccharomyces boulardii* in barley wort began up to 24 hours later compared to control yeast. This longer period of adaptation by the selenized yeast may be mainly because the selenized yeast is growing on a medium that does not contain selenium.

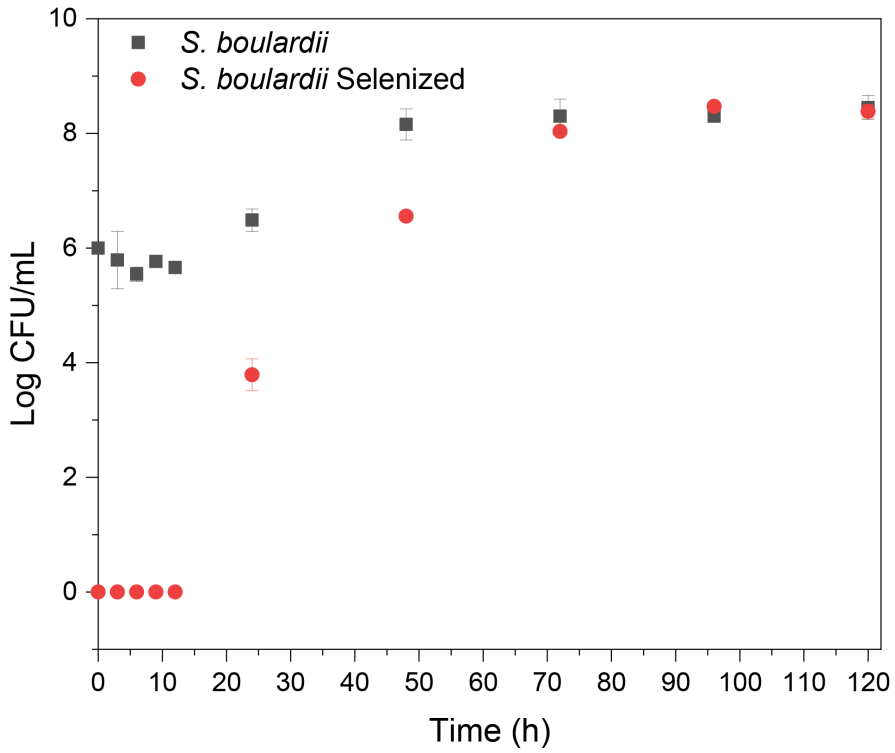

Fig. 1. Growth of *Saccharomyces boulardii* selenized (•) and Saccharomyces boulardii (■) in barley must. Results are expressed as the mean ± standard deviation (n = 3).

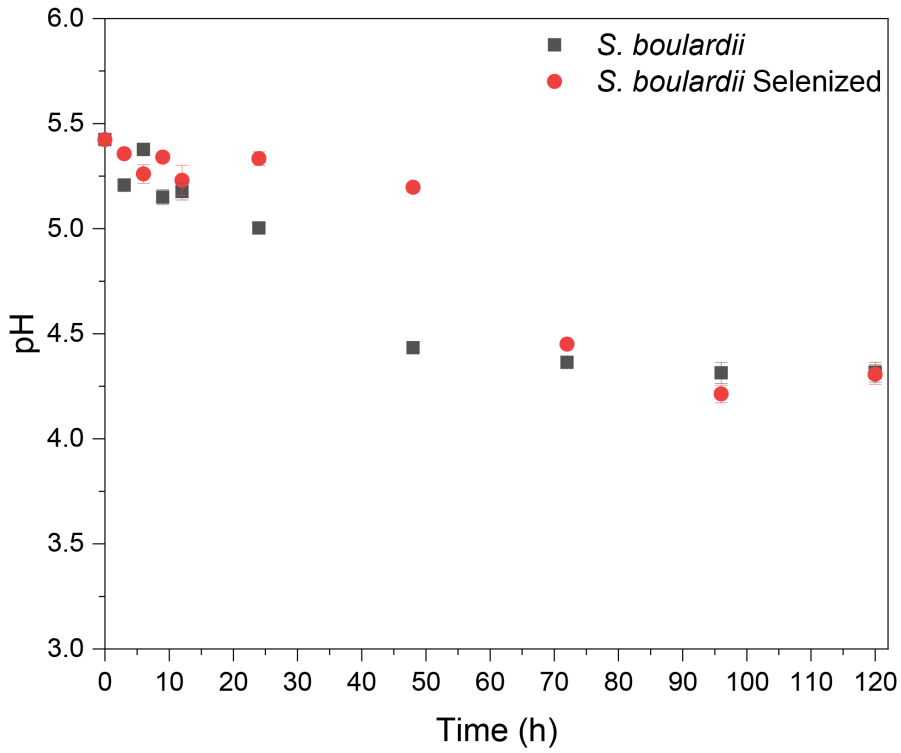

Fig. 2. pH of *Saccharomyces boulardii* selenized (•) and *Saccharomyces boulardii* (■) in barley must. Results are expressed as the mean ± standard deviation (n = 3).

As shown in Table 1, the selenized yeast has a lower speed (μ) concerning the control. This behavior coincided with the generations (g) per hour, which were lower than those observed in the control. Therefore, the constant (K) was lower in control than in the selenized yeast. Likewise, the deceleration phase (end of the logarithmic phase and beginning of the stationary) was reached faster in control yeast than in selenized yeast, and this is due to the correlation that exists with the growth rate and the acceleration of metabolism.

Table 1

Kinetics parameters of *Saccharomyces boulardii* selenized and *Saccharomyces boulardii* (control)

	***S. boulardii* selenized**	**Control yeast**
Velocity rate μ (h^{-1})	**0.289**	**0.145**
Generations (g)	**2.39**	**4.77**
Growth constant *k* (g/h)	**0.41**	**0.2**
Deceleration time (h)	**113.84**	**98.25**

It has been observed that in selenized microorganisms, there are differences in the kinetic parameters of each fermentation, identifying that both the generations and the doubling rate were lower in fermentations of selenized microorganisms than in those where the same microorganisms were used without selenized. Likewise, due to these decreases, the stationary phase was reached more slowly in fermentations with selenized microorganisms [20]. This same behavior has been observed by Ye et al. [21], who determined that in a YPD medium prepared for selenized *S. cerevisiae*, a logarithmic phase was determined up to 32 hours, while for the control, it was reached at 21 hours. Very few studies have realized the importance of fermentation kinetics in systems in which a selenized yeast is used or in the selenization of food products and beverages.

7.5.3 Physicochemical parameters of the fermented beverage

The physicochemical parameters between the fermented beverage using selenized yeast and the control fermentation varied remarkably. One of the most important

parameters of a beer is the final gravity. Regarding the final gravity, a better attenuation was observed for the fermented with the control yeast. However, it is not ideal for a beer to obtain a better attenuation. The final gravity should be around between 1.008 and 1.012, depending on the style of beer [22]. A study by de Paula et al. [23] mentions that fermenting wheat beer wort with *Saccharomyces cerevisiae* var. *boulardii* also did not match the performance of *Saccharomyces cerevisiae* since it did not reach the same final gravity values. This is due to the preference of *S. boulardii* for glucose over other sugars, such as maltose, which is only consumed when glucose is scarce [24]. Therefore, probably the fermentation must take a longer time.

Table 2

Physicochemical parameters, yeast viability and selenium content of beers produced by *S.boulardii* selenizase and *S. boulardii*.

Parameter	***Saccharomyces boulardii* selenized**	**Control yeast**
OG	1.048±0.00	1.050±0.00
FG	1.029±0.00	1.020±0.00
%Alc. Vol.	2.5±0.007	3.75±0.006
pH	4.307±0.047	4.317±0.047
Log10CFU/mL	8.385±0.120	8.450±0.212
Se (mg/kg)	0.378 mg/Kg	----------

Results are expressed as the mean ± standard deviation (n = 3).

Alcohol production was 1.25% lower for the beer that used selenized yeast. Previous investigations on the production of beer with *S. boulardii* have reported that the alcohol production (3.83 (%v/v)) is like the control yeast using *Saccharomyces cerevisiae* (3.75 (%v/v)) [25]. In the same way, the authors mention that the attenuation is low, for which they have opted for mixed fermentations with *S. cerevisiae* for better attenuation and alcohol production. Other studies using *S. boulardii* for elaborating wheat beer report an alcohol production of 4.01-4.40 (% v/v). Therefore, the production of alcohol depends on the amount of glucose in the medium and the attenuation mentioned above. However, in this study, the alcohol production by selenized yeast was affected. Although Sánchez-Martínez et al. [15] mention that, during beer production in the presence of sodium selenite the alcohol production was not affected. More studies would be needed to elucidate why alcohol production is decreased when the wort is fermented with selenized yeast.

In the case of pH, the decrease as a function of time was similar in both fermentations, reaching a value of 4.3. Similar results were reported by Mulero-Cerezo, et al. [26] for an India Pale Ale style beer. After fermentation with *S. boulardii*, the pH reached a value of 4.7. On the other hand, de Paula et al. [23] reports a pH of 4.17 and 4.52 for wheat beer, and they conclude that this effect is due to the production of organic acids by the probiotic yeast, which has been reported by Chan and Liu [27].

7.5.4 Selenium content

Finally, this is the first report of brewing with a selenized probiotic yeast. A craft beer style was chosen since these beverages do not undergo filtration, clarification, or pasteurization, allowing the yeast's viability and a greater amount of selenium in the beverage. The initial selenium content in selenized *S. boulardii* was 25.12 mg/g. For the starter culture, 230±0.2 mg of yeast was weighed at the end of fermentation, and after the ICP-OES analysis, it was found that 0.378 mg/kg were present in the beer and 5.302 mg/kg in the spent yeast.

Similar studies carried out with *S. cerevisiae* report that when enriching the wort with increasing concentrations of Na_2SeO_3 (0.2 to 20 μg/mL), the selenium concentration in the beer also increased with minimum values of 0.086 μg/mL and maximums of 6.00 μg/mL [15]. Another study was based on enriching barley with Na_2SeO3 (10 and 20 g/ha), of which 20.6 and 17.9 μg/kg respectively were present in the beer [14]. This demonstrates a new way to obtain selenium-enriched beer, which allows greater retention of selenium in the beverage, in addition to the fact that selenium from selenized *S. boulardii* is present as selenoamino acids and selenium nanoparticles, as reported by our research group previously [9]. In addition, it would be necessary to elucidate the amount of organic selenium present in the beer and in the spent yeast, which could be given a second use either for subsequent fermentations or feeding livestock.

7.6 Conclusion

This study observed that selenized yeast could ferment barley must under the same fermentation conditions used for control yeast. However, it is necessary to conduct some studies to obtain the best fermentation kinetic controls and the physicochemical parameters for a beer made with a selenized yeast comparable to those of a non-selenized yeast. Using a selenized probiotic yeast for elaborating a beer-type beverage is a great advantage and represents an opportunity among the challenges of manufacturing functional foods. This has to do with the accumulation of selenium, probably more bioaccessible and bioavailable since it could represent an organic source of this element. The results obtained in this research represent a potential alternative to developing fermented beverages with a high selenium content, which could positively impact human health.

7.7 References

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