VNIVERSITAT DE VALÈNCIA

Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal

Programa de Doctorado con Mención hacia la Excelencia en Ciencias de la Alimentación

Obtención de nutrientes y compuestos bioactivos a partir de biomasa marina y evaluación de la toxicidad con bioensayos

Nutrients and bioactive compounds recovery from marine biomass and toxicity evaluation using bioassays

Tesis Doctoral Internacional

Valencia, junio 2023

Presentada por: **Francisco Juan Martí Quijal**

Dirigida por: **Dr. Francisco J. Barba Orellana**

Dra. María José Ruiz Leal

Los Drs. Francisco José Barba Orellana y María José Ruiz Leal, Profesor Titular del Área de Nutrición y Bromatología y Catedrática del Área de Toxicología, respectivamente, del Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal, de la Universitat de València, en calidad de directores de la tesis doctoral de D. Francisco Juan Martí Quijal

INFORMAN QUE:

La presente Tesis Doctoral se ha realizado en los grupos de investigación Alisost (GIUV2021-494) y RiskTox (GIUV2021-513) y se enmarca en los proyectos AQUABIOPRO-FIT (Identificador subvención: 790956) (financiado por EU Commission y BBI-JU Horizon H2020), IDIFEDER/2018/046 (financiado por la Generalitat Valenciana y el Fondo Europeo de Desarrollo Regional), PID2020-115871RB-100 (financiado por el Ministerio de Ciencia e Innovación de España) y AGROALNEXT/2022/060 (financiado por el MCIN con fondos de la Unión Europea NextGenerationEU (PRTR-C17.I1) y la Generalitat Valenciana).

D. Francisco Juan Martí Quijal ha disfrutado de una ayuda para la formación de personal investigador de carácter predoctoral dentro del Programa "Atracció de Talent" (UV-INV-PREDOC19F1-1010001) de la Universitat de València desde enero del 2020 (Resolución de 20 de diciembre 2019).

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El Dr. Francisco J. Barba Orellana, profesor titular de Universidad del Área de Nutrición y Bromatología, y la Dra. María José Ruiz Leal, Profesora Catedrática del Área de Toxicología, del Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal, de la Universitat de València

CERTIFICAN QUE:

El graduado en Farmacia D. Francisco Juan Martí Quijal ha realizado bajo su dirección el trabajo que lleva por título **"Obtención de nutrientes y compuestos bioactivos a partir de biomasa marina y evaluación de la toxicidad con bioensayos" / "***Nutrients and bioactive compounds recovery from marine biomass and toxicity evaluation using bioassays*" y autorizan su presentación para optar al título de Doctor con Mención Internacional por la Universitat de València.

Y para que así conste, expiden y firman el presente certificado

Burjassot (Valencia), junio 2023

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El doctorando agradece a la Universitat de València:

La beca de investigación de carácter predoctoral del Programa “Atracció de Talent” (UV-INV-PREDOC19F1-1010001) de la Universitat de València (Resolución de 20 de diciembre 2019).

La presente Tesis Doctoral Internacional se engloba dentro de los siguientes proyectos

• BBI-JU through the H2020 Project AQUABIOPRO-FIT “Aquaculture and agriculture biomass side stream proteins and bioactives for feed, fitness and health promoting nutritional supplements” (Grant number 790956).

• Generalitat Valenciana: IDIFEDER/2018/046 - "Procesos innovadores de extracción y conservación: pulsos eléctricos y fluidos supercríticos" through European Union ERDF funds (European Regional Development Fund).

• Exposición combinada a micotoxinas y plaguicidas. Evaluación del riesgo, seguridad y estrategias de mitigación (PID2020-115871RB-I00), financiado por el Ministerio de Ciencia e Innovación

• Programa AGROALNEXT (AGROALNEXT/2022/060) “Desarrollo y optimización de procesos innovadores y sostenibles de extracción de aceite y proteínas a partir de microalgas, insectos, residuos y subproductos agroalimentarios: Evaluación de propiedades biológicas (EXTRAOLIOPRO))”, financiado por el Ministerio de Ciencia e Innovación con fondos de la Unión Europea NextGenerationEU (PRTR-C17.I1) y la Generalitat Valenciana

Además, ha contado con la participación y el apoyo de:

• Red Iberoamericana de Investigación en Valorización de Residuos y Subproductos de Origen Alimentario (VALORAL), auspiciada por la Asociación Universitaria Iberoamericana de Postgrado (AUIP).

• Red CYTED “Aprovechamiento sostenible de recursos biomasicos vegetales iberoamericanos en cosmetica (BIOLATES)”

• Servei Central de Suport a la Investigació Experimental (SCSIE), Universitat de València, València.

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La presente tesis doctoral ha dado lugar a 11 artículos publicados o que se publicarán en las siguientes revistas:

1. *A chemometric approach to evaluate the impact of pulses, Chlorella and Spirulina on proximate composition, amino acid, and physicochemical properties of turkey burgers. Journal of the Science of Food and Agriculture (2019), 99, 3672-3680.*
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2. *Influence of different sources of vegetable, whey and microalgae proteins on the physicochemical properties and amino acid profile of fresh pork sausages. LWT-Food Science & Technology (2019), 110, 316-323.*
   Índice de impacto JCR (2019): 4,006 (28/139) Q1. Food Science & Technology.

3. *Isolation, identification and investigation of fermentative bacteria from sea bass (Dicentrarchus labrax): Evaluation of antifungal activity of fermented fish meat and by-products broths. Foods (2020), 9, 576.*
   Índice de impacto JCR (2020): 4,350 (37/143) Q2. Food Science & Technology.

4. *Fermentation in fish and by-products processing: an overview of current research and future prospects. Current Opinion in Food Science (2020), 31, 9-16.*
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5. *Impact of fermentation on the recovery of antioxidant bioactive compounds from sea bass byproducts. Antioxidants (2020), 9, 239.*
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6. *Obtaining antioxidants and natural preservatives from food by-products through fermentation: A review. Fermentation (2021), 7, 106.*
   Índice de impacto JCR (2021): 5,123 (55/168) Q2. Biotechnology & Applied Microbiology.

7. *Extraction of antioxidant compounds and pigments from spirulina (Arthrospira platensis) assisted by pulsed electric fields and the binary mixture of organic solvents and water. Applied Sciences (2021), 11, 7629.*
   Índice de impacto JCR (2021): 2,838 (100/179) Q3. Chemistry, Multidisciplinary.

8. *Enhancing nutrient recovery and bioactive compound extraction from spirulina through supercritical fluid extraction: implications for SH-SY5Y cell viability. Foods, en revisión*
   Índice de impacto JCR (2021): 5,561 (35/144) Q1. Food Science & Technology.

9. *Sea bass side stream extracts obtained by pulsed electric fields: nutritional characterization and effect in SH-SY5Y cells Foods, en revisión*
   Índice de impacto JCR (2021): 5,561 (35/144) Q1. Food Science & Technology.

10. *Effect of natural extracts on critrinin-induce cytotoxicity in SH-SY5Y cells. Toxins, en revisión*

    Índice de impacto JCR (2021): 5,075 (20/94) Q1. Toxicology*, en revisión*.

11. *Citrinin's toxic effects on SH-SY5Y cells: unraveling mechanisms of cell damage. Archives of Toxicology, en revisión*

    Índice de impacto JCR (2021): 6,168 (12/94) Q1. Toxicology

Autorizan la presentación de la Tesis Doctoral para optar al Grado de Doctor en Ciencias de la Alimentación.

Y para que conste a los efectos oportunos,

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" Todo hombre puede ser,
si se lo propone,
escultor de su propio cerebro."

Santiago Ramón y Cajal

ÍNDICE GENERAL/ *GENERAL INDEX*

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Resultados/*Results*

Impact of fermentation on the recovery of antioxidant bioactive compounds from sea bass byproducts

Isolation, identification and investigation of fermentative bacteria from sea bass (*Dicentrarchus labrax*): evaluation of antifungal activity of fermented fish meat and by-products broths

Sea bass side stream extracts obtained by pulsed electric fields: nutritional characterization and effect on SH-SY5Y cells

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ABREVIATURAS

AFB1: aflatoxina B1

AFs: aflatoxinas

BPE: beta-ficoeritrina

CAT: catalasa

CFM: concentración fungicida mínima

CIM: concentración inhibitoria mínima

CIT: citrinina

DHA: ácido docosahexaenoico

DMSO: dimetilsulfóxido

DON: deoxinivalenol

DPP-IV: dipeptidil peptidasa IV

DPPH: 2,2-difenil-1-picrilhidracilo

ECA: enzima convertidora de angiotensina

EFS: extracción con fluidos supercríticos

EPA: ácido eicosapentaenoico

FAO: Organización de las Naciones Unidas para la Alimentación y la Agricultura

FSol: fermentación en estado sólido

FSum: fermentación sumergida

GPx: glutation peroxidasa

GSH: glutatión

HHP: altas presiones hidrostáticas

hpf: horas post-fertilización

HPO:

IARC: Centro Internacional de Investigaciones sobre el Cáncer

IC_{50}: concentración inhibitoria media

LC_{50}: concentración letal media

LD_{50}: dosis letal media

MDA: malondialdehido

MMP: potencial de membrana mitocondrial

MS: materia seca

NIV: nivalenol

ODS: Objetivos de Desarrollo Sostenible

OPLS-DA: análisis discriminante de proyecciones ortogonales a estructuras latentes

OTA: ocratoxina A

PCR: reacción en cadena de la polimerasa

PE: pulsos eléctricos

PUFAs: ácidos grasos poliinsaturados de cadena larga

RASFF: Sistema de Alerta Rápida para Alimentos y Piensos

ROS: especies reactivas de oxígeno

SOD: superóxido dismutasa

STE: esterigmatocistina

TE: equivalente de Trolox

ZEA: zearalenona

Resumen de la tesis

Esta Tesis Doctoral se centra en la utilización de la fermentación con bacterias ácido-lácticas aisladas de pescado para la obtención de compuestos antioxidantes y antifúngicos a partir de subproductos de pescado. Se identificaron ácidos fenólicos como el ácido DL-3-fenil-láctico y el ácido benzoico tras fermentar caldos elaborados con carne de pescado y subproductos durante diferentes tratamientos. Los extractos obtenidos de la fermentación de subproductos de pescado por bacterias aisladas del colon y del estómago mostraron la mejor actividad antioxidante. Además, demostraron actividad antifúngica frente a diferentes cepas de hongos de los géneros Penicillium, Aspergillus y Fusarium.

Para optimizar la extracción de compuestos bioactivos de subproductos de pescado, se emplearon pulsos eléctricos (PE). Este pretratamiento mejoró la extracción de proteínas y compuestos antioxidantes de la cabeza y la piel del pescado, aunque no mejoró la extracción en el caso de las vísceras. Los PE alteraron la distribución del peso molecular de las proteínas extraídas y de la recuperación de minerales, la cual varió según el subproducto y mineral estudiado. Por otra parte, la exposición de los péptidos bioactivos antioxidantes y de los extractos obtenidos de la cabeza de pescado en cultivos de células SH-SY5Y mostraron un aumento en la viabilidad celular.

Además, se estudió el efecto de los PE en la recuperación de compuestos bioactivos de la microalga espirulina (*Arthrospira platensis*). Se probaron diferentes tiempos de extracción y disolventes, obteniéndose mejores resultados con tiempos de extracción cortos en la extracción de clorofilas, carotenoides y compuestos fenólicos totales en los primeros minutos de extracción.

La eficiencia de la extracción con fluidos supercríticos (EFS) en la recuperación de compuestos de la espirulina también fue evaluada. La EFS mejoró la extracción de ficocianina, clorofila, carotenoides y minerales en comparación con la extracción convencional. Además, se observó una mayor recuperación de ácidos grasos específicos. El extracto obtenido con EFS también mostró una mejora en la viabilidad celular cuando se expuso a las células SH-SY5Y.

Siguiendo con el estudio de las microalgas, se evaluó cómo la adición de espirulina y chlorella (*Chlorella vulgaris*), además de diversas legumbres, a productos cárnicos procesados afecta a los parámetros fisicoquímicos y nutricionales. Se observó un cambio significativo en los parámetros de color tras la incorporación de proteínas de microalgas y un aumento del contenido total de aminoácidos con la inclusión de proteínas de espirulina.

Finalmente, se estudió la actividad citoprotectora de los extractos obtenidos en cultivos de células SH-SY5Y expuestos a la micotoxina citrinina (CIT). Se evaluaron los efectos citotóxicos de la CIT expuesta de forma individual y combinada con los extractos. Se compararon los resultados en dos modelos *in vitro*, cultivos de células SH-SY5Y en monocapa (2D) y esferoides (3D) y un modelo *in vivo*, embriones de pez cebra (Danio rerio). Los resultados demostraron que la CIT afectó el potencial de membrana mitocondrial, indujo apoptosis, detuvo el ciclo celular y causó alteraciones en el retículo endoplásmico en células SH-SY5Y. En el modelo *in vivo* de embriones de pez cebra, la exposición a altas concentraciones de CIT provocó malformaciones. La exposición combinada de CIT con los extractos optimizados de cabeza de lubina obtenidos mediante PE y extracto etanólico de espirulina, mostró un aumento de la viabilidad celular y una reducción en la apoptosis en comparación con la exposición a CIT sola en células SH-SY5Y.

PhD thesis abstract

This doctoral thesis focuses on the use of fermentation with lactic acid bacteria isolated from fish to obtain antioxidants and antifungals from fish side streams. Phenolic acids such as DL-3-phenyl-lactic acid and benzoic acid were identified after fermenting broths made with fish meat and side streams during different treatments. The extracts obtained from the fermentation of fish side streams by bacteria isolated from the colon and stomach showed the best antioxidant activity. In addition, they demonstrated antifungal activity against different strains of fungi from the Penicillium, Aspergillus, and Fusarium genera.

To optimise the extraction of bioactive compounds from fish side streams, pulsed electric fields (PEF) were used. This pre-treatment improved the extraction of proteins and antioxidant compounds from the head and skin of the fish, although it did not improve extraction in the case of the viscera. The PEF treatment altered the molecular weight distribution of the extracted proteins and the recovery of minerals, which varied according to the by-product and mineral studied. On the other hand, the exposure of antioxidant bioactive peptides and extracts obtained from the fish head in SH-SY5Y cell cultures showed an increase in cell viability.

In addition, the effect of PEF on the recovery of bioactive compounds from the microalgae spirulina (*Arthrospira platensis*) was studied. Different extraction times and solvents were tested, with better results obtained with short extraction times in the extraction of chlorophylls, carotenoids, and total phenolic compounds in the first minutes of extraction.

The efficiency of extraction with supercritical fluids (SFE) in the recovery of compounds from spirulina was also evaluated. The SFE improved the

extraction of phycocyanin, chlorophyll, carotenoids, and minerals compared to conventional extraction. In addition, a higher recovery of specific fatty acids was observed. The extract obtained with SFE also showed an improvement in cell viability when exposed to SH-SY5Y cells.

Continuing with the study of microalgae, it was evaluated how the addition of spirulina and chlorella (*Chlorella vulgaris*), in addition to various legumes, to processed meat products affects the physicochemical and nutritional parameters. A significant change in colour parameters was observed after the incorporation of microalgae proteins and an increase in the total amino acid content with the inclusion of spirulina proteins.

Finally, the cytoprotective activity of the extracts obtained in SH-SY5Y cell cultures exposed to the mycotoxin citrinin (CIT) was studied. The cytotoxic effects of CIT exposed individually and combined with the extracts were evaluated. The results were compared in two *in vitro* models, SH-SY5Y cell cultures in monolayer (2D) and spheroids (3D), and an *in vivo* model, zebrafish embryos (*Danio rerio*). The results showed that CIT affected the mitochondrial membrane potential, induced apoptosis, stopped the cell cycle, and caused alterations in the endoplasmic reticulum in SH-SY5Y cells. In the *in vivo* model of zebrafish embryos, exposure to high concentrations of CIT caused malformations. The combined exposure of CIT with the optimised extracts of sea bass head obtained by PEF and ethanolic extract of spirulina, showed an increase in cell viability and a reduction in apoptosis compared to exposure to CIT alone in SH-SY5Y cells.

ANTECEDENTES BIBLIOGRÁFICOS

LITERATURE REVIEW

CAPÍTULO 1. PROCESOS DE EXTRACCIÓN Y OBTENCIÓN DE NUTRIENTES Y COMPUESTOS BIOACTIVOS A PARTIR DE SUBPRODUCTOS DE PESCADO Y MICROALGAS

1.1 BIOMASA MARINA

1.1.1 Subproductos de pescado

Según la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO, por sus siglas en inglés), la producción mundial de animales acuáticos se estimó en 178 millones de toneladas en el año 2020. De estos 178 millones, la pesca de captura contribuyó con el 51 % (90 millones de toneladas) y la acuicultura con el 49 % (88 millones de toneladas). Por otro lado, centrándonos en la acuicultura, la producción acuícola total se estimó en 87,5 millones de toneladas de animales acuáticos, destinados al consumo humano principalmente para su uso como alimentos, 35,1 millones de toneladas de algas (macro y microalgas) para usos tanto alimentarios como no alimentarios y 700 toneladas de conchas y perlas para uso ornamental, alcanzando un total de 122,6 millones de toneladas en peso vivo en 2020 (FAO, 2022).

Teniendo en cuenta la importancia del pescado como fuente de proteínas, cabe destacar su aporte a nivel mundial, representando aproximadamente el 20% de la proteína animal consumida por la población (FAO, 2022). Además, la FAO estimó 158 millones de toneladas el consumo mundial de alimentos acuáticos en 2019, lo que implica un aumento anual medio del 3% desde 1961, donde el consumo mundial fue de 28 millones de toneladas (FAO, 2022). El consumo aumentó a un ritmo anual medio del 3,0% desde 1961, en comparación con una tasa de crecimiento demográfico del 1,6%. Asimismo, los productos provenientes del mar son una valiosa fuente de compuestos bioactivos como péptidos, aminoácidos, ácidos grasos

poliinsaturados de cadena larga omega-3 (PUFAs), vitaminas (por ejemplo, vitaminas A y D) y minerales como calcio, potasio y zinc (Kundam et al., 2019). La composición nutricional del pescado consiste en un 15-30% de proteínas, un 0-25% de grasa y un 50-80% de humedad en función de la especie, edad, género, salud y temporada de captura (Caldeira et al., 2018).

Por ejemplo, los pescados blancos, como el bacalao y la merluza, contienen alrededor del 20% de proteínas, un 80% de agua, un 0,5-3% de grasa, minerales, vitaminas, carbohidratos y otros compuestos. Por otro lado, los pescados grasos, como la caballa y el salmón, contienen un 20% de proteínas, un 10-18% de aceite y un 62-70% de agua (Kundam et al., 2019). Entre un 20% y un 80% del peso total del pescado se considera como desperdicio o subproducto por la industria pesquera, dependiendo de varios parámetros como el tipo de pescado y las especificaciones de procesamiento (Caldeira et al., 2018). Este residuo generalmente incluye cabeza, vísceras, piel, espinas y escamas, que representan aproximadamente el 9-12%, 12-18%, 1-3%, 9-15% y 5% del peso total del pescado, respectivamente (Villamil et al., 2017). Cabe destacar que estudios recientes han demostrado que estos residuos pueden considerarse una valiosa fuente de compuestos de alto valor añadido. En consecuencia, se ha prestado una considerable atención a los nutrientes y compuestos bioactivos presentes en estos subproductos de pescado. De hecho, estos materiales se consideran fuentes sostenibles para las industrias alimentaria, farmacéutica, nutracéutica y cosmética, entre otras (Kundam et al., 2019).

1.1.1.1 Nutrientes y compuestos bioactivos de los subproductos del pescado

En los subproductos de pescado se pueden encontrar una gran diversidad de compuestos, los cuales pueden tener una acción beneficiosa sobre la salud humana. Estos beneficios para la salud se logran a través de múltiples actividades biológicas, que incluyen la actividad antioxidante, la

mediación hormonal, el fortalecimiento del sistema inmunológico, y la mejora de la salud digestiva, entre otras (Kundam et al., 2019). Por lo tanto, los subproductos del pescado son una fuente efectiva de compuestos bioactivos que se pueden utilizar como suplementos nutricionales y proporcionar beneficios para la salud. Se han llevado a cabo numerosos estudios para extraer nutrientes y compuestos bioactivos de diferentes subproductos, algunos de los cuales se detallan a continuación:

a) Proteínas

Los subproductos de pescado pueden contener hasta un 58% de proteínas (Nirmal et al., 2022). Las proteínas del pescado son fuentes ricas en aminoácidos tanto esenciales como no esenciales (Rana et al., 2023). Los subproductos ricos en proteínas incluyen espinazo, piel, cabeza, vísceras y sangre, que se pueden utilizar para producir colágeno/gelatina, proteoglicanos, péptidos bioactivos e hidrolizados de proteínas, entre otros. Tanto estos aminoácidos esenciales como los péptidos bioactivos obtenidos a partir de los subproductos del pescado tienen un gran potencial como compuestos beneficiosos para mejorar la salud (Nirmal et al., 2022).

- Colágeno y gelatina

El colágeno es una proteína fibrosa y estructural presente en el espacio extracelular del pescado y contribuye a la función fisiológica de los tejidos en piel, escamas, huesos, cráneo, vejiga natatoria y resto de vísceras (Rajabimashhadi et al., 2023). Es la proteína más abundante en el pescado, representando el 25% de la proteína total (Caldeira et al., 2018). El colágeno tiene una amplia gama de aplicaciones en los sectores relacionados con la salud, específicamente en cosmética, industria farmacéutica y cuidado médico (que incluye cirugía plástica, ortopedia, oftalmología y odontología) (Silva et al., 2014). De hecho, existen muchos tipos de colágeno, aunque la

forma más común en los subproductos del pescado es el colágeno tipo I, y se encuentra en los tejidos conectivos, piel, músculos, huesos y córnea (Caldeira et al., 2018; Raman & Gopakumar, 2018). Se ha obtenido colágeno a partir de la piel de diferentes tipos de peces (Chi et al., 2014). Además, el colágeno del pescado, después de su extracción, puede ser hidrolizado enzimáticamente para liberar péptidos bioactivos. Algunos péptidos derivados del colágeno podrían exhibir actividad antioxidante (Chi et al., 2014), actividad antimicrobiana frente a diferentes cepas de bacterias (Ennaas et al., 2015), y una potente actividad antihipertensiva a través de propiedades inhibidoras de la enzima convertidora de angiotensina (ECA) (Alemán et al., 2013).

La gelatina es una macromolécula proteica obtenida por la desnaturalización térmica del colágeno mediante un proceso irreversible cinético. Comparte algunas de las propiedades del colágeno debido a su composición similar. Por lo tanto, se puede utilizar para mejorar la consistencia, elasticidad y estabilidad de los alimentos, así como para producir películas comestibles y biodegradables que aumentan la vida útil de los productos alimentarios (Caldeira et al., 2018). Además, algunos autores también han observado como la gelatina de pescado tiene una mayor actividad antioxidante que las sintéticas (Ishak & Sarbon, 2018). Varios estudios han extraído gelatina de los subproductos del pescado, siendo la piel de pescado la principal fuente de gelatina (Irwandi et al., 2009). Por ejemplo, diferentes autores han extraído gelatina de la piel del pez barramundi (*Lates calcarifer*) (Sae-leaw et al., 2016) y el bacalao del Pacífico (*Gadus macrocephalus*) (Ngo et al., 2016). Otros también han recuperado gelatina a partir de los huesos de la tilapia del Nilo (*Oreochromis niloticus*) (Zakaria et al., 2015), las escamas de la carpa cabezona (*Hypophthalmichthys nobilis*) (T.

Huang et al., 2017) y la cabeza de la caballa del Atlántico (*Scomber scombrus*) (Zied Khiari, Daniel Rico, Ana Belen Martin-Diana, 2011).

- Péptidos bioactivos

Los péptidos bioactivos consisten principalmente en secuencias de entre 2 y 20 aminoácidos. Sin embargo, estos péptidos son inactivos dentro de las proteínas nativas y se activan después de ser liberados por la digestión *in vivo* (proteólisis) o por hidrólisis enzimática *in vitro*, que es el mejor método para obtener hidrolizados proteicos con propiedades bioactivas (Zamora-Sillero et al., 2018). Además, los péptidos activos extraídos de los subproductos del pescado muestran múltiples actividades biológicas en función de su composición de aminoácidos y secuencia de estos. También desempeñan un gran papel en aplicaciones farmacéuticas y médicas, lo que resulta en la promoción de la salud humana y puede ser útil en la prevención y tratamiento de varias enfermedades crónicas. Por lo tanto, los péptidos obtenidos pueden actuar como antioxidantes, antidiabéticos, inmunomoduladores, agentes antitumorales y antimicrobianos, entre otros (Kim & Wijesekara, 2010).

Diferentes subproductos de pescado son ricos en péptidos bioactivos. En este sentido, varios estudios han demostrado que la cabeza, las vísceras, la piel y la espina dorsal son buenas fuentes para el desarrollo de hidrolizados de proteínas con propiedades bioactivas. Entre ellos, es posible destacar el trabajo realizado por Bougatef et al. (2010) en el que se purificaron siete péptidos antioxidantes a partir de la cabeza y las vísceras de la sardina (*Sardinella aurita*). Estos autores observaron que los péptidos obtenidos presentaron una alta actividad antioxidante, medida con el ensayo de eliminación de radicales del 2,2-difenil-1-picrilhidracilo (DPPH) (Bougatef et al., 2010). También es interesante resaltar el estudio realizado por Robert et al. (2015) en el cual obtuvieron un alto contenido y diversidad de péptidos,

concretamente 1374 en el hidrolizado desarrollado a partir de subproductos de tilapia (cabeza, esqueleto y vísceras).

Por otro lado, los péptidos bioactivos aislados de la cabeza y huesos del atún han mostrado buenas actividades antioxidantes (Bougatef et al., 2012; Je et al., 2007). Además de los péptidos antioxidantes que pueden estar naturalmente presentes, se ha observado por diferentes autores que los péptidos obtenidos tras el proceso de hidrolizado de las proteínas pueden tener también actividad biológica. En este contexto, los péptidos bioactivos aislados de la piel, los huesos y la extracción de músculo de gelatina hidrolizada de salmón del Atlántico (*Salmo salar*) presentan actividad antioxidante, inhibición de la ECA y actividad antidiabética mediante la inhibición de la enzima dipeptidil peptidasa IV (DPP-IV) (Neves et al., 2017).

Otros autores han obtenido hidrolizados de la piel de pez barramundi (*Lates calcarifer*) y pez lija (*Aluterus monoceros*) con péptidos con actividades inmunomoduladoras y antiproliferativas (Karnjanapratum et al., 2016; Sae-leaw et al., 2016). Además, Gu et al. (2011) aislaron 11 péptidos del colágeno de la piel de salmón (*Salmo salar*) después de una hidrólisis enzimática, los cuales mostraron una importante actividad inhibidora de la ECA y podrían ser útiles como alimentos funcionales y agentes antihipertensivos. En otro estudio, Guo et al. (2013) utilizaron colágeno de la piel de abadejo de Alaska (*Theragra chalcogramma*) para generar péptidos quelantes de hierro después de ser tratados con enzimas comerciales, identificando que el tripéptido Ser-Cys-His presentaba una alta actividad quelante de hierro.

b) Lípidos

Respecto a los lípidos, el uso más habitual de los subproductos de pescado es la obtención de aceites que contienen ácidos grasos omega-3 (Soldo et al., 2019). Estos aceites pueden contribuir a disminuir el riesgo de

padecer enfermedades cardiovasculares, cáncer, diabetes y depresión (Ivanovs & Blumberga, 2017), afectando también al sistema inmunológico y asegurando un desarrollo neurológico adecuado (Ivanovs & Blumberga, 2017).

Una de las principales fuentes de ácidos grasos omega-3 como el ácido eicosapentaenoico (EPA) y el ácido docosahexaenoico (DHA) son los pescados grasos como el arenque, la sardina, el salmón y la caballa (Hamed et al., 2015; Kundam et al., 2019). La cantidad y composición de estos aceites dependen en gran medida de la especie, la temporada y el lugar de captura (Hamed et al., 2015). En el caso de los subproductos del pescado, los ácidos grasos se pueden obtener principalmente de la piel, el intestino, la cabeza y los huesos de diferentes especies de peces. Por ejemplo, diferentes autores han obtenido PUFAs a partir de las espinas de bacalao, abadejo, salmón, trucha, arenque, caballa y jurel y de las vísceras de tilapia (*Oreochromis niloticus*) y carpa común (*Cyprinus carpio* L.) (Lisichkov et al., 2014; Shirahigue et al., 2016; Toppe et al., 2007). Además, en otro estudio se extrajo PUFA (omega-3) de la piel de caballa (Sahena et al., 2010). Asimismo, en un trabajo llevado a cabo por T. B. Ahmad et al. (2019), se extrajeron lípidos de las vísceras de sardina australiana (*Sardinops sagax*) y de la cabeza de salmón (*Salmo salar*), encontrándose grandes cantidades de ácidos grasos omega-3, EPA y DHA.

Los aceites de pescado son una fuente rica en vitaminas (A y D). La vitamina A se concentra principalmente en los aceites de hígado de pescado. El halibut, la sardina y el bacalao contienen vitamina A y D en su hígado (Kundam et al., 2019), mientras que el arenque, la caballa, la trucha y el salmón tienen vitamina D en sus tejidos, y el atún amarillo contiene vitamina D en sus huesos (Talib & Zailani, 2017). Estas vitaminas se incluyen comúnmente en suplementos dietéticos para diversas aplicaciones, como la salud ósea o formulaciones antioxidantes (Harris et al., 2017).

c) Minerales

Las espinas de pescado presentan una gran cantidad de minerales, constituyendo los minerales inorgánicos aproximadamente el 60% de los huesos de pescado. Por lo tanto, son una fuente importante de hidroxiapatita, calcio, fosfato, zinc, selenio y hierro (Bruno et al., 2019).

Diferentes autores han aislado minerales de varias especies de pescados. Respecto a esto, las espinas de pez barramundi (*Lates calcarifer*) representan una fuente de calcio y fósforo (Pal et al., 2017). Además, se aislaron calcio, fósforo, magnesio y estroncio de las escamas del pez *Catla catla* (Paul et al., 2017). Estos minerales son compuestos importantes en formulaciones nutracéuticas destinadas a mejorar la salud, principalmente la salud ósea, pero también enfermedades cardiovasculares o inmunológicas (Webb, 2015).

1.1.2 Microalgas

1.1.2.1 Valor nutricional

a) Proteínas

Desde el punto de vista nutricional, las algas son una interesante fuente alternativa de proteínas. El contenido de proteínas en las algas varía según la especie, el hábitat y el período estacional. En este sentido, las microalgas presentan una concentración de proteínas más alta que las macroalgas. En general, la fracción proteica (peso seco) de las algas verdes y rojas oscila entre el 10% y el 47%, mientras que el porcentaje de proteínas de las algas pardas es inferior al 15% (excepto para el wakame (*Undaria pinnatifida*), cuyo nivel de proteínas se encuentra entre el 11% y el 24%) (Fleurence, 1999; Herrero et al., 2012; Min Wang et al., 2022). Las microalgas pueden contener más del 60% de proteínas, presentando la cianobacteria *Spirulina platensis* una

composición proteica del 43% al 63%, por lo que se considera un suplemento alimenticio (Villarruel-López et al., 2017).

Además, el perfil de aminoácidos esenciales de las algas cumple con los requisitos de la FAO (FAO, 2018). En este sentido, el contenido de aminoácidos esenciales de las microalgas y cianobacterias más comunes ha sido revisado en literatura (Eilam et al., 2023; Maddiboyina et al., 2023; Mittal & Ranade, 2023). Sin embargo, los estudios de bioaccesibilidad *in vitro* sugieren que las proteínas de las algas sin procesar tienen una digestibilidad reducida en comparación con otras fuentes de proteínas (Villarruel-López et al., 2017). Por otro lado, se ha considerado que otros compuestos proteicos no nutricionales, como enzimas producidas por las algas y péptidos derivados de ellas, también actúan como compuestos bioactivos. Por ejemplo, se ha revelado que varios tipos de enzimas, como la manuronan C5 epimerasa, pueden ser producidos por las algas (Parte et al., 2017). Muchas de las enzimas producidas por las algas son conocidas por ser importantes para las industrias alimentaria y farmacéutica (Inoue et al., 2016; Levy-Ontman et al., 2015). Por ejemplo, se ha visto que las enzimas de *Closterium*, *Cylindrotheca* y *Chaetoceros muelleri* son efectivas en la degradación del dietil ftalato (Gao & Chi, 2015). Además, las algas pueden producir glutatión peroxidasa, ascorbato peroxidasa y catalasa (Babu et al., 2014; Moenne et al., 2016). Asimismo, el alginato puede ser producido a partir de las algas pardas, donde una asociación simbiótica entre bacterias y algas marinas da como resultado la producción de alginato liasas (Ertesvåg, 2015). Por otro lado, se han producido una variedad de péptidos bioactivos mediante la hidrólisis enzimática de las proteínas de las algas (Beaulieu, 2019). En este contexto, una investigación mostró que la elaboración de pan con hidrolizado de proteínas de alga dulce (*Palmaria palmata*) presentaba un potencial efecto cardioprotector (Fitzgerald et al., 2014). Estos estudios indican que los péptidos bioactivos

obtenidos de las algas pueden considerarse ingredientes valiosos que se pueden utilizar para la producción de alimentos.

b) Polisacáridos

Las algas marinas contienen mucopolisacáridos y polisacáridos estructurados en forma de almacenamiento y pared celular. Algunas especies de algas contienen polisacáridos en un rango del 4% al 76% (peso seco), siendo los niveles más altos determinados en especies como *Ascophyllum*, *Palmaria*, *Porphyra* y *Ulva* (Usman et al., 2017). Tanto la estructura de la pared celular como los polisacáridos de almacenamiento son específicos de cada especie.

Las algas verdes contienen polisacáridos de ácido sulfúrico, galactanos sulfatados y xilanos. En cambio, las algas pardas presentan ácido algínico, fucoidan, laminarina y sargassano. Por su parte, las algas rojas contienen agar, carragenano, xilanos, almidón florideano, galactano sulfatado soluble en agua, así como el mucopolisacárido porfirina (Chandini et al., 2008; Kraan, 2012).

Los polisacáridos no participan en el valor nutricional de las algas, pero se consideran una fuente de fibra dietética resistente a la hidrólisis enzimática de la microflora intestinal presente en el tracto digestivo humano. Las fibras dietéticas incluidas en las algas marinas se dividen en fibras insolubles (celulosa, mananos y xilenos) y fibras solubles (agares, ácido algínico, furonano, laminarina y porfirina) (Kraan, 2012). La diversa composición química de los polisacáridos de fibra dietética se ha considerado responsable de sus posibles actividades biológicas. En este sentido, se ha demostrado que los polisacáridos sulfatados tienen diversos beneficios para la salud, como agentes anticoagulantes, antioxidantes, antiproliferativos, antitumorales, antiinflamatorios, antivirales y reductores de colesterol (Mišurcová et al., 2015).

En particular, se ha demostrado que el fucoidano exhibe propiedades antivirales y antiinflamatorias, así como efectos antimetastásicos en células cancerosas invasivas de pulmón humano metastásico (Khalid et al., 2018). Además, una revisión reciente concluyó que el fucoidano, el sulfato de laminarina y el carragenano ralentizan la progresión de la lesión aterosclerótica, mientras que el alginato, el ulvano (lechuga de mar) y el agar reducen los factores de riesgo asociados (Patil et al., 2018).

c) Lípidos

El contenido lipídico en las algas marinas difiere entre las macroalgas y las microalgas. Las algas marinas suelen presentar un bajo porcentaje de lípidos (1-3% del peso seco), mientras que muchas microalgas contienen un 20-50% de lípidos (biomasa seca) e incluso se han documentado valores que oscilan entre el 1% y el 70% (Barkia et al., 2019). Sin embargo, el perfil lipídico de ambos tipos de algas ha despertado un considerable interés en los últimos años debido al alto contenido de PUFAs. Los PUFAs típicamente observado en las algas marinas son los ácidos α-linolénico (18:3n-3), octadecatetraenoico (18:4n-3), araquidónico (20:4n-6) y EPA (20:5n-3) (Kendel et al., 2015).

Por otro lado, los perfiles lipídicos completos (saturados, monoinsaturados y PUFAs) de las microalgas y cianobacterias más comúnmente utilizadas han sido detallados en la literatura (Parveen & Patidar, 2022; Tzima et al., 2023; Udayan et al., 2022). Además de desempeñar un papel importante en la prevención de enfermedades cardiovasculares, osteoartritis y diabetes, estos PUFAs poseen propiedades antimicrobianas, antivirales, antiinflamatorias y antitumorales (Kendel et al., 2015). Se ha demostrado que los PUFAs y los glicolípidos obtenidos de *U. armoricana* y *S. chordalis* presentan actividades antitumorales prometedoras (Kendel et al., 2015). Dado que las dietas desequilibradas actuales no proporcionan cantidades suficientes de PUFAs omega-3 para satisfacer los requerimientos fisiológicos

humanos, las algas marinas son una de las nuevas fuentes alternativas que pueden ayudar a mantener dietas saludables (Tocher et al., 2019).

d) Vitaminas

En general, las algas marinas contienen vitaminas hidrosolubles y liposolubles. Además de desempeñar funciones vitales en el cuerpo, se ha considerado que la vitamina E (α-tocoferol), la vitamina C (ácido ascórbico) y parcialmente la vitamina B1 y la vitamina B3 (niacina) son responsables de la actividad antioxidante de las algas (Škrovánková, 2011).

1.1.2.2 Compuestos bioactivos y antioxidantes

Los pigmentos de las algas marinas son fundamentalmente clorofilas y carotenoides como carotenos (β-caroteno) y xantofilas (fucoxantina, violaxantina, antheraxantina, zeaxantina, luteína, neoxantina, entre otros) (Aryee et al., 2018). Las algas más estudiadas por su contenido en carotenoides incluyen las algas pardas (*Laminaria spp.* y *Undaria pinnatifida*), las algas rojas (*Corallina elungata* y *Jania rubens*) y las microalgas verdes (*Dunaliella salina*, *Chlorella spp.*, *Haematococcus pluvialis* y *Spirulina spp.*) (Christaki et al., 2013). Los principales carotenoides de las algas son astaxantina, fucoxantina, β-caroteno, luteína y zeaxantina. Hay que destacar que la capacidad antioxidante de la astaxantina, el carotenoide principal encontrado en la microalga verde unicelular *Haematococcus pluvialis*, es aproximadamente 10 veces mayor que la del β-caroteno, la luteína, la zeaxantina, la cantaxantina y más de 500 veces mayor que la del α-tocoferol. Además, estudios *in vitro* e *in vivo* han demostrado la efectividad de la astaxantina frente a enfermedades coronarias, inflamatorias crónicas, diabetes, gastrointestinales, hepáticas y neurodegenerativas, así como frente a la aterosclerosis, el desarrollo cerebral isquémico y el síndrome metabólico (Christaki et al., 2013). Además, el β-caroteno producido por la microalga

halófila *Dunaliella salina* inhibió las células neoplásicas y redujo el fibrosarcoma en ratas Wistar (Villarruel-López et al., 2017). La fucoxantina, el carotenoide bioactivo más importante en los cloroplastos de algas pardas como *Ascophyllum nodosum* y *Laminaria spp.*, ha demostrado efectos antiproliferativos en células de cáncer de próstata y colon humano, eficacia en el tratamiento de la obesidad y la diabetes tipo 2, así como propiedades antiinflamatorias y antioxidantes (Christaki et al., 2013; Herrero et al., 2012). Además, se han encontrado compuestos complejos constituidos por pigmentos unidos a proteínas que exhiben bioactividad. Por ejemplo, las ficobiliproteínas, presentes solo en las algas rojas y las cianobacterias, se han relacionado con propiedades hepatoprotectoras, antiinflamatorias y antioxidantes (Herrero et al., 2012).

Los polifenoles son metabolitos secundarios cuyas estructuras varían desde moléculas simples hasta compuestos altamente polimerizados. Como plantas acuáticas, las macro- y microalgas son las principales fuentes marinas de compuestos polifenólicos. Las algas verdes y rojas contienen bromofenoles, ácidos fenólicos y flavonoides, mientras que solo se han encontrado florotaninos en las algas pardas (Gómez-Guzmán et al., 2018). En general, estos fitoquímicos se consideran compuestos bioactivos con posibles beneficios para la salud en numerosas enfermedades humanas debido a su actividad antioxidante, así como a su efecto inhibidor de enzimas y actividades antimicrobianas, antivirales, antitumorales, antidiabéticas, antialérgicas y antiinflamatorias (Gómez-Guzmán et al., 2018).

En particular, se ha asociado a los florotaninos con actividades biológicas beneficiosas para la salud, como actividades anticancerígenas, bactericidas, radioprotectoras y antialérgicas, demostradas por *Ecklonia cava*, *Ecklonia stolonifera*, *Ecklonia kurome*, *Eisenia bicyclis*, *Ishige okamurae*, *Sargassum thunbergii*, *Hizikia fusiformis*, *Undaria pinnatifida* y *Laminaria japónica* (Freile-

Pelegrín & Robledo, 2013; Khalid et al., 2018). En cuanto a las microalgas, existe información limitada sobre compuestos fenólicos específicos en estos organismos y la actividad que proporcionan. Jerez-Martel et al. (2017) identificaron y cuantificaron los seis fenoles ampliamente distribuidos en la naturaleza (ácido gálico, (+)-catequina, (-)-epicatequina, ácido siríngico, ácido protocatecuico y ácido clorogénico) en extractos crudos de varias cianobacterias y microalgas. Determinaron su actividad antioxidante y observaron una relación directa entre ésta y los compuestos fenólicos. La mayor concentración de compuestos fenólicos se encontró en la microalga *Euglena cantabrica*), en la cual se identificó la presencia de ácido clorogénico, catequina, epicatequina, ácido protocatecuico y ácido gálico.

Los esteroles son otro grupo interesante de compuestos extraídos de las algas marinas. No solo los esteroles, sino también algunos de sus derivados, han mostrado propiedades hipocolesterolemiantes, antiinflamatorias y anticancerígenas (Ibañez et al., 2012; Michalak & Chojnacka, 2015). Por otro lado, al igual que en bacterias y plantas, los glicolípidos también están presentes en las algas marinas y podrían desempeñar un papel importante en enfermedades inflamatorias (Talero et al., 2015).

Por último, varios metabolitos secundarios (productos naturales basados en quinonas, hierridina B y pequeñas amidas) producidos por diferentes cianobacterias han mostrado citotoxicidad en las células cancerosas de colon HT-29 (Olsen et al., 2014; Talero et al., 2015).

1.2 FERMENTACIÓN PARA LA RECUPERACIÓN DE COMPUESTOS BIOACTIVOS A PARTIR DE SUBPRODUCTOS DE LA INDUSTRIA AGROALIMENTARIA

La fermentación se ha utilizado ampliamente como método de conservación de alimentos o para la producción de compuestos de interés,

por ejemplo, etanol, en el caso de las bebidas alcohólicas. En los últimos años se ha investigado el uso de la fermentación para la obtención de compuestos con un impacto directo sobre la salud o de interés a nivel industrial, tales como los compuestos antioxidantes (Diez-Ozaeta & Astiazaran, 2022; Garcia & Remize, 2022). Por otra parte, el uso de la fermentación en subproductos derivados de determinadas actividades de la industria agroalimentaria permite la reducción del impacto ambiental causado por estos residuos, contribuyendo de esta manera a proteger el medioambiente (Almaraz-Sánchez et al., 2022; Erskine et al., 2023).

Por último, existe una demanda creciente por parte de la sociedad en la reducción del uso de aditivos alimentarios de origen químico y su sustitución por otros de origen natural. Se debe destacar que gran parte de los aditivos añadidos a los alimentos actúan como conservantes, ya que permiten extender la vida media del alimento reduciendo las pérdidas económicas. Por lo tanto, la fermentación puede ser una fuente alternativa de obtención de compuestos de origen natural que permitan el reemplazo de los compuestos químicos utilizados actualmente como conservantes (Rivero-Pino et al., 2023; Mingxuan Wang & Rong, 2022).

1.2.1 Procesos de fermentación

El proceso de fermentación se ha optimizado a lo largo del tiempo con el objetivo de mejorar la eficiencia y aumentar la productividad del proceso. Actualmente destacan dos estrategias de fermentación en función del sustrato que se quiere fermentar: fermentación sumergida (FSum) o fermentación en estado sólido (FSol) (Koubaa et al., 2021).

La FSum se lleva a cabo en un medio líquido, ya sea directamente en sustratos líquidos o bien mediante la adición de agua a sustratos sólidos. Es el método de preferencia cuando los microorganismos involucrados en la

fermentación requieren una humedad elevada (Doriya et al., 2016). Los compuestos bioactivos producidos por los microorganismos durante la fermentación se secretan en el medio líquido. Por lo tanto, la FSum se usa principalmente para la obtención de metabolitos secundarios de los microorganismos en estado líquido. Entre las ventajas de esta técnica destaca la posibilidad de producir compuestos a gran escala, la buena transferencia de masa y calor durante el proceso y la mejor difusión de los microorganismos. Sin embargo, los rendimientos obtenidos son bajos, consume una gran cantidad de energía, y no es un método respetuoso con el medio ambiente debido al gran volumen de agua residual generado (Doriya et al., 2016).

A pesar de que la FSum no es la técnica más utilizada para el aprovechamiento de residuos alimentarios, diversos autores han investigado su aplicación en la obtención de compuestos bioactivos. En este sentido, Zhang et al., (2017) utilizaron una FSum sucesiva en tres etapas con *Serratia marcescens*, *Lactobacillus plantarum* y *Rhizopus japonicus* sobre subproductos del procesado de gambas para la obtención de quitina y quitosano. Por otro lado, Klempová et al., (2020) obtuvieron ácido γ-linolenico y β-caroteno al fermentar subproductos de cereales (salvado, granos de malta usados, granos de destilería…) con el microorganismo *Mucor wosnessenskii* (Klempová et al., 2020). Bartkiene et al. (2019) utilizaron subproductos procedentes de la cebada para obtener compuestos antioxidantes y antimicrobianos mediante la fermentación con la bacteria *Pediococcus acidilactici*.

En la FSol, se utiliza un sustrato sólido en ausencia de agua. En este caso, el crecimiento de los microorganismos se produce directamente sobre el sustrato, aprovechándose al máximo de esta manera los nutrientes presentes en el sustrato (Thomas et al., 2013). Para ello se utilizan microorganismos con

un bajo requerimiento de humedad, como por ejemplo los hongos (Subramaniyam & Vimala, 2012). Las principales ventajas de este proceso es su alto rendimiento, ya que permite la obtención de un producto final con una elevada actividad, así como la posibilidad de usar directamente subproductos alimentarios para la recuperación compuestos de interés, reduciendo el impacto medioambiental mediante la utilización de estos subproductos. Además, cabe destacar que este tipo de fermentación es más respetuosa con el medio ambiente, debido a su bajo o nulo consumo de agua. Por lo tanto, este proceso permite la obtención de compuestos de alto valor económico mediante la revalorización de residuos. Sin embargo, entre sus principales desventajas se encuentran la dificultad en el escalado industrial y en el control de los parámetros de la fermentación (Doriya et al., 2016).

Los residuos agroalimentarios generalmente contienen todos los nutrientes necesarios para el crecimiento de los microorganismos, por lo que su utilización como sustrato genera buenos rendimientos en el proceso de fermentación. Los subproductos de la industria agroalimentaria utilizados como sustratos en FSol son diversos: tallos, hojas, raíces, semillas y cáscaras, cortezas, melaza, orujo y bagazo (Sadh et al., 2018).

Diversos autores han investigado el uso de la FSol en la obtención de diferentes compuestos bioactivos a partir de subproductos agroindustriales. En concreto, mediante la fermentación tiene lugar la acción de enzimas bacterianas que permiten la hidrólisis de las paredes celulares del sustrato, liberando de esta manera compuestos como polifenoles, vitaminas, ácidos orgánicos o péptidos bioactivos (Arte et al., 2015). Por ejemplo, se ha investigado la obtención de ácido poli-**γ**-glutámico a partir de tallos de maíz y harina de soja tras la fermentación con *Bacillus amyloliquefaciens* y una comunidad microbiana compleja natural (Fang et al., 2020).

Los subproductos alimentarios se pueden utilizar también en la obtención de enzimas, que pueden ser utilizadas en una segunda etapa para la obtención de compuestos bioactivos. En este sentido, Teles et al. (2019) emplearon residuos de uva y salvado de trigo como sustrato para la producción de enzimas hidrolíticas mediante fermentación con *Aspergillus niger*, las cuales se aplicaron posteriormente a los residuos de uva para la recuperación de polifenoles.

Como se ha descrito anteriormente, el uso de la fermentación para la recuperación de compuestos con finalidad conservante a partir de residuos de la industria agroalimentaria, tales como antioxidantes o antifúngicos, está en auge y cada vez recibe más atención por parte de la comunidad científica. Esto se debe, entre otros motivos, a que es un proceso simple que permite valorizar los subproductos alimentarios, reduciendo el impacto que estos provocan en el medio ambiente. A continuación, se detallan algunos estudios relacionados con la obtención de compuestos antioxidantes y antifúngicos mediante la fermentación de subproductos agrícolas.

1.2.2 Obtención de compuestos bioactivos mediante la fermentación de pescado y sus subproductos

Los principales productos obtenidos a partir de pescado y sus subproductos son péptidos bioactivos y aceite. Como ya se ha comentado anteriormente, los péptidos bioactivos son secuencias de 2 a 20 aminoácidos, con una función específica y beneficios para la salud, como actividad antihipertensiva, antitumoral o antioxidante. Los péptidos bioactivos están inactivos dentro de la proteína original, pero al liberarse por hidrólisis pueden presentar varias funciones fisiológicas (Fu et al., 2019). Esta actividad específica está condicionada por la secuencia y tipo de aminoácidos, así como por la longitud de la cadena. Además, debe mencionarse que un péptido puede mostrar diferentes efectos bioactivos (Bhandari et al., 2019).

Hasta ahora, el método de elección en la industria para la obtención de péptidos bioactivos se centra en el uso de ácidos o bases para promover la hidrólisis de las proteínas. Este tipo de método es un procedimiento simple y económico (Suresh et al., 2018). Sin embargo, el uso de la fermentación puede proporcionar diferentes beneficios en comparación con la hidrólisis asistida por cambios de pH. En este sentido, se ha observado que la fermentación de vísceras de salmón Atlántico produce un aumento en la efectividad de los péptidos antioxidantes que actúan en conjunto con el glutatión (GSH) protegiendo frente a los daños causados por el estrés oxidativo (C. K. Rajendran et al., 2018). Además, la fermentación produce proteínas más digeribles debido a su hidrólisis y degradación en péptidos más cortos y aminoácidos (Ramírez et al., 2013). Por lo tanto, podría ser una herramienta útil en la mejora de la calidad nutricional de los productos pesqueros que se utilizan como alimentos fuente de proteínas (Özyurt et al., 2019). Por último, mediante la fermentación, la calidad del aceite obtenido de residuos de lubina aumenta en comparación con el tratamiento con ácido fórmico (Özyurt et al., 2018).

Entre los compuestos obtenidos mediante la fermentación de subproductos de origen marino, se puede destacar la generación de péptidos con diferentes actividades biológicas, ya que es posible recuperar péptidos con diferentes efectos fisiológicos a partir de pescado o sus subproductos. Por ejemplo, Robinson Wenno et al. (2016) obtuvieron péptidos inhibidores de la ECA mediante la fermentación de vísceras de atún. En otro estudio, Rinto et al. (2017) obtuvieron péptidos a partir de una salsa de pescado fermentada con capacidad para reducir los niveles elevados de colesterol. Sin embargo, las principales actividades biológicas de los péptidos bioactivos obtenidos a partir de la fermentación de subproductos de pescado se basan tanto en su función antioxidante como biocida, aunque también se han obtenido otros

conservantes y compuestos como ácido láctico o bioplásticos, además de compuestos aromáticos.

En lo que respecta a los subproductos de pescado, lo más común es su utilización para la obtención de compuestos antioxidantes. Por ejemplo, Choksawangkarn et al. (2018) obtuvieron péptidos bioactivos a partir de salsa de subproductos de pescado fermentados. En otro estudio, Fang et al., (2017) utilizaron la fermentación con *Aspergillus oryzae* de piel de rodaballo descartada para obtener péptidos bioactivos con actividad antioxidante. Por último, y como se ha descrito en el apartado anterior, Ruthu et al. (2014) obtuvieron péptidos antioxidantes tras la fermentación de cabezas de pescado (de carpas Rohu y Catla) con bacterias lácticas.

Hay que destacar que la fermentación con bacterias lácticas se ha utilizado durante siglos en pescado y otros mariscos para mejorar su conservación. Una forma de mejorar la conservación de los alimentos es reducir la proliferación microbiana, y en este sentido las bacterias lácticas tienen un papel relevante, ya que pueden producir compuestos antimicrobianos que evitan la proliferación de patógenos transmitidos por los alimentos o microorganismos causantes de deterioro, prolongando así la vida útil de los alimentos (Manivasagan et al., 2014). Por ejemplo, a partir del fermento de una cepa de *Lactobacillus plantarum* se recuperó una sustancia antimicrobiana que se utilizó como aditivo alimentario para mejorar la conservación de los alimentos (Lin & Pan, 2017).

En consonancia con esto, Angiolillo et al. (2018) utilizaron la fermentación de glicerol en la superficie de lomos de lubina para obtener reuterina, que actúa como un agente antibacteriano previniendo la contaminación microbiana. Otro ejemplo es el uso de bacterias lácticas aisladas de subproductos de pescado (específicamente *Lactobacillus curvatus* y *Enterococcus faecium*) para evitar la contaminación microbiana, mejorando así

la conservación del pescado fresco (merluza, gallo y salmón) (Gómez-Sala et al., 2016; Leroi et al., 2015).

Al mismo tiempo, uno de los mayores problemas actuales en los alimentos es la contaminación de productos con microorganismos patógenos o compuestos producidos por ellos. Entre las principales causas de intoxicación, la contaminación por bacterias como *Salmonella*, *Campylobacter*, *Escherichia coli* enterohemorrágica (0157:H7) y *Listeria*, así como las micotoxinas, que son compuestos químicos producidos por hongos, son algunos de los más importantes (World Health Organization (WHO), 2019). La fermentación con bacterias lácticas ayuda a combatir la contaminación de los alimentos por microorganismos y hongos patógenos a través de diferentes mecanismos. Por ejemplo, pueden hidrolizar compuestos tóxicos resultantes de la contaminación por hongos patógenos (Luz et al., 2018). Otro posible mecanismo de acción es la generación de sustancias antibacterianas que afectan directamente al crecimiento de patógenos transmitidos por los alimentos (Hashemi. et al., 2017).

En este sentido, las bacteriocinas desempeñan un papel relevante como péptidos con actividad antimicrobiana frente a ciertas bacterias, entre las cuales se encuentran las responsables de enfermedades transmitidas por los alimentos (Kumariya et al., 2019). Se ha observado que las bacterias aisladas de subproductos de pescado (principalmente vísceras) pueden producir bacteriocinas contra los principales microorganismos patógenos, como *Staphylococcus aureus*, *Salmonella sp.*, *Escherichia coli*, *Listeria monocytogenes*, entre otros. Gómez-Sala et al. (2015) aislaron 197 bacterias con actividad antibacteriana directa contra 20 patógenos transmitidos por los alimentos, de las cuales 57 provienen de subproductos de pescado fresco, como la piel y las vísceras principalmente, y en menor medida del músculo (Gómez-Sala et al., 2015). Por otro lado, Sahnouni et al. (2016) identificaron 30

bacterias de los géneros *Carnobacterium* y *Lactobacillus* del tracto gastrointestinal de jurel Atlántico, sardina europea y bonito del Atlántico con actividad antibacteriana contra *Staphylococcus* aureus, *Escherichia* coli, *Pseudomonas aeruginosa*, *Listeria innocua*, *Salmonella sp.* y *Vibrio sp.*.

La fermentación puede contribuir también a cambiar el sabor de los alimentos (Sun et al., 2016). En general, los alimentos fermentados a base de pescado tienen un sabor umami, debido al alto contenido de ácido glutámico y ácido aspártico responsables de este aroma. Tras la fermentación de salsa de pescado a partir de subproductos de pescado de agua dulce, Zhao et al. (2017) determinaron un aumento en el ácido glutámico, además de otros compuestos volátiles aromatizantes como aldehídos de 2-metilbutanal y 3-metilbutanal. Estos compuestos se consideran responsables del sabor y olor característicos de las salsas de pescado fermentadas.

Por otro lado, Sun et al. (2016) observaron un aumento en los aminoácidos libres responsables del sabor umami y dulce tras la fermentación de salsa de pescado de anchoa (*Engraulis japonicus*) por *Aspergillus oryzae*. También determinaron que el sabor de la salsa fermentada era más intenso que el de la muestra no fermentada. Además, se obtuvo una gran cantidad de compuestos aromáticos volátiles, como alcoholes, aldehídos, cetonas, ésteres y fenoles. De estos, los alcoholes representaron el grupo principal, siendo casi el 10% del total de los compuestos volátiles, y entre los aldehídos obtenidos, se destaca el 2-metilbutanal. Finalmente, Kasankala et al. (2012) obtuvieron mediante fermentación de carpas plateadas con *Aspergillus oryzae* una salsa de pescado con una gran cantidad de compuestos volátiles, como alcoholes (siendo el etanol el principal), cetonas como la 1-butanona, ácidos (principalmente ácido acético y ácido butanoico), aldehídos y fenoles. Todos estos resultados demuestran que la fermentación a partir de pescado

o sus subproductos puede ser una herramienta muy útil en la obtención y potenciación del aroma.

Además de lo descrito anteriormente, la fermentación de residuos y subproductos de pescado puede utilizarse en la obtención de compuestos de interés para la formulación de nutracéuticos. En este sentido, Özyurt et al. (2018) obtuvieron un aceite de alta calidad para la producción de complementos alimenticios a partir de la fermentación de residuos de lubina. Tang et al. (2018) produjeron mediante fermentación de espinas de carpa herbívora (*Ctenopharyngodon idellus*) una solución rica en calcio que también se puede utilizar como nutracéutico.

De manera similar, se está investigando el uso de los subproductos de pescado para la producción de plásticos respetuosos con el medio ambiente. En este sentido, Mohapatra et al. (2017) lograron obtener bioplástico a partir de la fermentación de desechos de pescado con *Bacillus subtilis*. Por último, también se investigó el uso de los subproductos de pescado (tilapia y siluro) para la obtención de ácido láctico mediante fermentación, con posibles usos en la industria alimentaria, cosmética y farmacéutica (Shi et al., 2018).

1.3 PULSOS ELÉCTRICOS

La aplicación de pulsos eléctricos (PE) con el fin de revalorizar los subproductos del pescado ha sido utilizada previamente por el grupo de investigación, centrando su atención en la capacidad antioxidante de los extractos, considerando también su efecto antiinflamatorio y actividad probiótica (Wang, Zhou, Collado, et al., 2021; Wang, Zhou, Pallarés, et al., 2021). Sin embargo, no se ha profundizado en la composición de los extractos más allá del contenido total de proteínas, así como tampoco se han realizado estudios sobre la cuantificación de minerales y la presencia de metales pesados, ambos compuestos de gran relevancia para una buena

caracterización de la biomasa marina. Además, tampoco se ha explorado su actividad sobre la viabilidad celular en líneas celulares humanas. Por lo tanto, en la presente tesis se ha profundizado en estos aspectos.

Por otro lado, entre las aplicaciones más reconocidas de la tecnología de PE destaca su uso para facilitar la extracción de compuestos bioactivos a partir de microalgas. En este sentido, nuestro grupo de investigación también ha realizado estudios previos en los que se ha evaluado la mejora de la extracción de estos compuestos tras la aplicación de PE. Concretamente, se han llevado a cabo estudios en microalgas como *Phaeodactylum tricornutum*, *Tetraselmis chuii*, *Chlorella vulgaris* y *Arthrospira platensis* (Kokkali et al., 2020; Ramon-Mascarell et al., 2022; Ricós-Muñoz et al., 2023; Wang et al., 2023; Zhou et al., 2022). En este caso, es de gran relevancia conocer los parámetros óptimos para una extracción eficiente, además de explorar el uso de diferentes disolventes que permitan recuperar una gran variedad de compuestos bioactivos. Por todo ello, se ha profundizado en estos aspectos utilizando la microalga espirulina para ello, por ser la microalga más consumida a nivel mundial, y con una seguridad alimentaria demostrada.

1.3.1 Fundamento de la tecnología de pulsos eléctricos

El tratamiento con PE consiste en la aplicación de pulsos de alto voltaje a alimentos u otros productos situados entre dos electrodos durante cortos períodos de tiempo. De esta manera, se provocan modificaciones estructurales en la membrana de las células biológicas, interrumpiendo la consistencia de la membrana celular, y en consecuencia aumentando su permeabilidad (Barba et al., 2015; Chauhan & Unni, 2015). El tratamiento se realiza a temperatura ambiente, minimizando la pérdida de energía por calentamiento y permitiendo ajustar la intensidad del proceso según las condiciones de operación (Pourzaki & Mirzaee, 2008; Ricci et al., 2018).

Los PE son un método no térmico de permeabilización de la membrana celular que se caracteriza por su bajo consumo de energía, tiempos de procesamiento cortos y por ser un proceso libre de residuos. Esto lo convierte en una opción innovadora, rentable y sostenible para la industria alimentaria, biotecnológica y farmacéutica (Barba et al., 2015; Puértolas et al., 2016; Puértolas & Barba, 2016). Además, también permite la inactivación de microorganismos sin afectar a la calidad ni el sabor de los alimentos (Ohshima et al., 2007).

El equipo de PE consiste en un generador de pulsos, una cámara de tratamiento, electrodos y un sistema de control y adquisición de datos (Puértolas et al., 2016) (Figura 1). Al diseñar un proceso de PE, se deben definir varios parámetros, como el número, la duración (en μs), la amplitud, la forma (exponencial, unipolar, bipolar…), la intensidad de campo (kV/cm) y la frecuencia (Hz) del pulso (Puértolas et al., 2012; Puértolas & Barba, 2016). La energía específica de entrada del proceso, que se relaciona con el costo económico y la huella ambiental, se utiliza para comparar la eficiencia e impacto económico del tratamiento de PE con métodos de extracción convencionales (Puértolas & Barba, 2016). Las formas de pulso más utilizadas son decaimientos exponenciales y onda cuadrada, siendo esta última más adecuada para optimizar el efecto de una aplicación de PE, ya que mantiene la intensidad sin cambios durante toda la duración del ancho del pulso (Ricci et al., 2018).

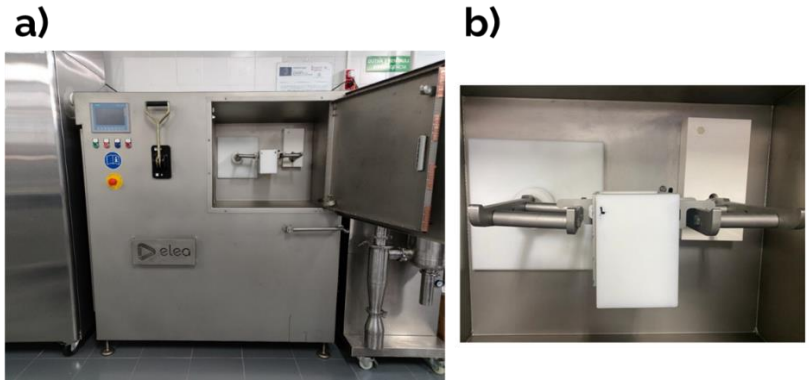

Figura 1. a) Equipo de pulsos eléctricos utilizado para la realización de la presente Tesis Doctoral y situado en la Facultat de Farmàcia de la Universitat de València. b) Cámara de tratamiento donde se pueden ver los electrodos y la cubeta donde se deposita la muestra.

A pesar de la preocupación por la inversión de capital inicial en la industrialización de la aplicación de PE en el procesamiento de alimentos, existen numerosas ventajas, como la continuidad del proceso, alta retención de nutrientes y vitaminas, y alta calidad organoléptica del producto. La tecnología de PE puede ser más efectiva cuando se combina con otros métodos, como las altas presiones hidrostáticas (HPP) y los ultrasonidos (Huang et al., 2006; Jaeger et al., 2010; Riener et al., 2008). Sin embargo, la combinación y optimización de parámetros depende del material y del objetivo del tratamiento, por lo que es esencial continuar investigando. La tecnología de PE ofrece el potencial de mejorar de manera eficiente y económica el consumo de energía, controlar la presencia de microorganismos en los alimentos de manera rápida y homogénea y preservar las propiedades nutricionales.

Esta tecnología presenta un gran potencial en diversas aplicaciones dentro de la industria alimentaria, destacando la extracción de nutrientes y compuestos bioactivos, la mejora de la calidad y la vida útil de los alimentos,

y la reducción de los tiempos de procesamiento en comparación con los métodos convencionales.

En la extracción de nutrientes y compuestos bioactivos, como antioxidantes, pigmentos y aceites esenciales, el tratamiento con PE puede aumentar la eficiencia de extracción al facilitar la liberación de estos compuestos de las células vegetales o animales. Esto se debe a la permeabilización de las membranas celulares y la formación de poros, lo que permite una mayor penetración de los solventes y una extracción más efectiva (Barba et al., 2015).

En cuanto a la mejora de la calidad y la vida útil de los alimentos, el tratamiento con PE puede inactivar microorganismos y enzimas responsables del deterioro de los alimentos, sin afectar negativamente sus propiedades organolépticas y nutricionales. Esto resulta en productos con una mayor vida útil y una mejor calidad en comparación con los tratamientos térmicos convencionales (Ohshima et al., 2007).

Además, la tecnología de PE puede reducir los tiempos de procesamiento en comparación con los métodos convencionales. Por ejemplo, en la deshidratación osmótica de frutas y verduras, el tratamiento con PE puede acelerar la transferencia de masa y reducir el tiempo necesario para alcanzar un contenido de humedad deseado (Ricci et al., 2018).

1.3.2 Aplicación de pulsos eléctricos a la extracción de compuestos bioactivos a partir de subproductos de pescado

La técnica de PE puede de gran interés en la valorización de subproductos de las industrias de procesamiento de pescado. En esta línea, Zhou et al., (2012) diseñaron un método mejorado para extraer calcio a partir de espinas de pescado utilizando PE de alta intensidad. Al comparar los resultados con la técnica de ultrasonidos, se puede concluir que el

tratamiento con PE permitió alcanzar una mayor eficiencia de extracción en un corto período de tiempo. Este hallazgo concuerda con los resultados negativos sobre la aplicabilidad de los ultrasonidos para acelerar un proceso de extracción (Gavahian et al., 2017). Asimismo, otra investigación utilizó el mismo material de partida, las espinas de pescado, para extraer sulfato de condroitina mediante PE de alta intensidad (He et al., 2014). Este estudio resaltó los beneficios de esta técnica, incluyendo un tiempo de procesado reducido, una mayor eficiencia y aspectos relativos al medio ambiente. Además, He, Yin, Yan y Wang (2017) combinaron los PE (formas de onda de pulso triangular bipolar en decaimiento exponencial con una duración de pulso de 2 μs) con extracción semibiónica para optimizar no solo la extracción del calcio y sulfato de condroitina, sino también del colágeno a partir de las espinas de pescado. Los contenidos máximos de los ingredientes activos en el extracto se obtuvieron con un campo eléctrico efectivo de 22,79 kV/cm y 9 pulsos, obteniendo unos resultados de 19,8; 39,268 y 3,875 mg/mL para calcio, sulfato de condroitina y colágeno, respectivamente.

Li et al., (2016) también investigaron la valorización de residuos de pescado utilizando PE. Desarrollaron una extracción asistida por PE y enzimas para el aislamiento de proteínas de las vísceras de abulón. Estudiaron los efectos de varias condiciones de PE, incluyendo el tiempo de tratamiento, la intensidad y la relación material-solvente, observando el rendimiento de extracción más alto cuando la relación solvente-material fue de 1 a 4 y se aplicó PE durante 600 μs a una intensidad de 20 kV/cm. Según los autores, la técnica de extracción propuesta resultó en un alto rendimiento de proteínas de vísceras de abulón, que poseía propiedades emulsionantes prometedoras en comparación con los métodos convencionales de extracción enzimática. En cambio, la viscosidad y las propiedades espumantes del producto extraído disminuyeron cuando se aplicó PE (Li et al., 2016). Este estudio resaltó la

posibilidad de combinar otras técnicas con PE para mejorar la eficiencia del proceso.

1.3.3 Aplicación de pulsos eléctrico a la extracción de compuestos bioactivos a partir de microalgas

La extracción mediante PE ha sido ampliamente estudiada en el caso de las microalgas, ya que se considera como un método eficiente y selectivo para la extracción de nutrientes y compuestos bioactivos, principalmente carbohidratos, proteínas, aceite y pigmentos, entre otros. Las microalgas espirulina (*Arthrospira platensis* y *Arthrospira maxima*) y chlorella (*Chlorella vulgaris*) son las más consumidas por la población, debido a su popularidad entre los ciudadanos, y debido también al gran número de estudios científicos realizados en los que se describe su alta densidad de nutrientes y compuestos bioactivos, así como su potencial para apoyar la salud en general.

En este sentido, Han et al. (2019) aplicaron PE como pre-tratamiento para aumentar el rendimiento en la extracción de aceite a partir de *Chlorella pyrenoidosa*. El tratamiento se aplicó en continuo, y los parámetros utilizados fueron un voltaje máximo de 20 kV, una frecuencia máxima de 150 Hz y una amplitud de pulso de 0-10 µs. Tras el tratamiento y la posterior extracción de los lípidos utilizando cloroformo/metanol (2:1, v/v) como disolvente, se observó que el pre-tratamiento con PE aumentó un 32% el rendimiento de la extracción de ésteres metílicos de ácidos grasos respecto al proceso sin PE. Zhang et al. (2021) también aplicaron PE con el fin de mejorar la extracción de aceite a partir de chlorella. En este caso, los autores obtuvieron un aumento en el rendimiento de extracción de lípidos de hasta un 166,67 % al comparar la muestra tratada con PE frente a una muestra control.

Por otro lado, también se ha estudiado la aplicación de PE a la microalga *Auxenochlorella protothecoides* para la extracción de lípidos. Concretamente, Silve et al. (2018) aplicaron un tratamiento de PE en continuo con un aporte energético de 1,5 MJ/kg materia seca (MS) y realizaron una extracción posterior utilizando una mezcla de agua/etanol/hexano (1:18:7,3 v/v/v), obteniendo un rendimiento del 97% en la extracción de lípidos.

Buchmann et al. (2019) utilizaron los PE con el fin de extraer proteínas a partir de *Chlorella vulgaris*. Estos autores concluyeron que la eficiencia del proceso dependía de la fase de crecimiento en la que se encontrase la célula. También confirmaron que el aumento en la extracción de proteínas es directamente proporcional al campo eléctrico utilizado. En este caso, el mejor rendimiento en la extracción tras la aplicación de PE fue del 96,6 % del total de proteínas libres. Carullo et al. (2018) estudiaron la influencia de los diferentes parámetros implicados en un tratamiento por PE sobre la extracción de compuestos intracelulares, tales como proteínas, carbohidratos y compuestos iónicos, de la microalga anteriormente citada. Los intervalos de cada parámetro evaluado fueron 10-30 kV/cm para el campo eléctrico y 20-100 kJ/kg para la energía aportada, con una duración del pulso de 5 µs, y el tratamiento se realizó en continuo. Los resultados obtenidos mostraron que la electroporación producida por el tratamiento con PE independientemente de los valores seleccionados para cada parámetro promovió una rápida liberación de compuestos iónicos intracelulares, aumentando rápidamente en todos los casos la conductividad desde los 1,78 mS/cm obtenidos en la muestra control hasta 2,08–2,21 mS/cm obtenidos en los diferentes tratamientos con PE. Además, la aplicación de PE también permitió aumentar 20 y 8 veces la extracción de proteínas y carbohidratos, respecto a una muestra control no tratada. Se observó también que el parámetro más

influyente en la mejora de la eficiencia fue la energía total aplicada, especialmente en la extracción de proteínas.

Diversos estudios han evaluado la obtención de ficocianina, un pigmento azul con capacidad antioxidante, a partir de espirulina. En esta línea, Jaeschke et al. (2019) realizaron diferentes tratamientos en continúo aplicando un campo eléctrico de 40 kV/cm y 28,56 y 112 J/mL. Los pulsos tuvieron una duración de 1 µs y una frecuencia entre 2 y 6 Hz. Los mejores resultados se alcanzaron con los tratamientos de 122 y 56 J/mL, alcanzando un valor máximo de 85,2 mg de ficocianina/g y 48,4 g de proteínas/100 g. Käferböck et al. (2020) aplicaron un tratamiendo de PE con una energía especifica de 100 kJ/kg y un campo eléctrico de 15,20 y 25 kV/cm. Se obtuvo un notable aumento en la extracción de ficocianina, pasando de 6,38 ± 0,22 mg/g MS en la extracción control (sin tratamiento de PE) hasta los 15,95 ± 1,71 mg/g MS obtenidos en la extracción tras el tratamiento con PE a 25 kV/cm y 100 kJ/kg.

Por último, también se ha estudiado la extracción de pigmentos a partir de otras microalgas. En este aspecto, Martínez et al. (2019) estudiaron la extracción de β-ficoeritrina (BPE) de la microalga *Porphyridium cruentum* utilizando un medio acuoso. En este caso, se investigó la influencia del campo eléctrico y de la duración del pulso, utilizando un intervalo de 2-10 kV/cm y 30-150 µs, respectivamente. Los resultados aportados por estos autores destacan que tras el tratamiento a 8 o 10 kV/cm y con una duración de pulso de 150 µs, aplicando una extracción posterior de 24 h, se consiguió recuperar el 100% de BPE.

Como se ha demostrado, los PE son una herramienta muy útil en la mejora de la extracción de nutrientes y compuestos bioactivos a partir de microalgas. Sin embargo, todavía es necesario explorar la influencia de los diferentes disolventes utilizados en la extracción, así como la eficacia de

tratamientos de mediana intensidad (1-3 kV/cm) en la recuperación de compuestos de interés.

1.4 FLUIDOS SUPERCRÍTICOS

1.4.1 Fundamento de la extracción con fluidos supercríticos

La extracción con fluidos supercríticos (EFS) consiste en la utilización de disolventes en su punto crítico de presión y temperatura, ya que en estas condiciones los disolventes tienen propiedades intermedias entre los gases y los líquidos. Esto conlleva que se obtenga una viscosidad más baja y un coeficiente de difusión más alto, mejorando la penetración en la matriz (Khawli et al., 2019; Zhou et al., 2021). Esto, sumado a la compactación de la matriz por efecto de la presión, facilita la extracción de compuestos bioactivos (pigmentos, polifenoles, carotenoides).

A pesar de que se pueden utilizar diferentes compuestos como disolventes supercríticos, el más utilizado es el CO2 ya que tiene diversas ventajas, entre las que se pueden destacar su gran disponibilidad y que es barato y respetuoso con el medio ambiente, con lo que contribuye a hacer más sostenible esta técnica (da Silva et al., 2016; Pimentel-Moral et al., 2019; Wrona et al., 2017). Sin embargo, su baja polaridad hace que sea necesario utilizarlo con un cosolvente polar, para una eficiente extracción de compuestos más polares. En este caso, el cosolvente más utilizado es el etanol, ya que es un disolvente respetuoso con el medio ambiente y además es seguro (Pinelo et al., 2007). Por lo tanto, todo esto hace que la EFS, utilizando CO_2 y etanol como disolventes, encaje perfectamente en los principios de la Química Verde, ya que se utilizan disolventes seguros tanto para los seres humanos como para el medio ambiente.

Los parámetros que condicionan el proceso de EFS son la temperatura del horno, la presión del sistema, el flujo de CO_2, el ratio CO_2:cosolvente y el

tiempo de extracción. La temperatura del horno condiciona la temperatura a la que se encuentra la muestra durante el proceso de extracción. La presión del sistema es aquella que se aplica a la muestra para realizar la extracción. Por otro lado, el ratio CO_2:cosolvente, generalmente CO_2:etanol, es de importancia ya que determina la polaridad del disolvente, y por tanto influye de forma determinante en las características de los compuestos extraídos. Por último, el flujo de CO_2 y el tiempo de extracción también condicionan el rendimiento final de la extracción. La combinación de todos estos parámetros y la selección de los valores óptimos para cada uno de ellos permite la extracción selectiva y eficiente de los compuestos de interés (Khawli et al., 2019).

Los principales componentes de un equipo de fluidos supercríticos son las bombas (una para el fluido supercrítico y otra para el cosolvente), el recipiente de extracción donde se ubica la muestra, el horno que aloja el recipiente de extracción a la temperatura deseada, y las válvulas que controlan el flujo del fluido (Ahmad et al., 2019). Además, también se debe tener en cuenta el recipiente donde se almacena el gas, el refrigerador, y otros accesorios como puede ser un colector de fracciones o un detector (Figura 2).

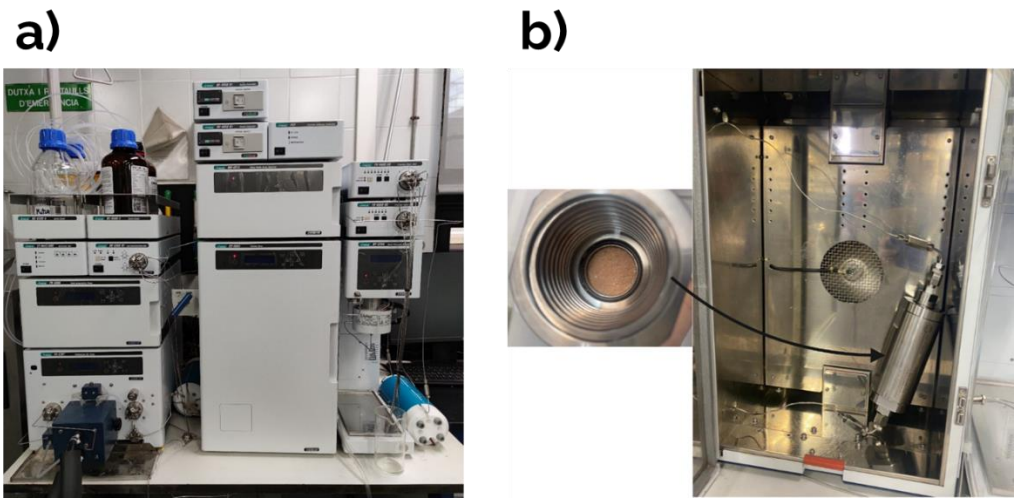

Figura 2. a) Equipo de extracción con fluidos supercríticos utilizado para la realización de la presente Tesis Doctoral y situado en la Facultat de Farmàcia de la Universitat de València. b) Interior del horno del equipo donde se localiza la vasija en la que se introduce la muestra.

Entre las ventajas de esta tecnología es posible destacar, como ya se ha comentado anteriormente, que es una extracción respetuosa con el medio ambiente ya que no se utilizan disolventes orgánicos persistentes, y que mantiene la calidad del producto final, ya que se utilizan temperaturas relativamente bajas (entre 40 y 80 °C). Además, el proceso tiene una elevada eficiencia debido a la baja viscosidad y elevada difusividad del fluido supercrítico. Sin embargo, entre los inconvenientes que presenta cabe destacar que es una tecnología cara y con un equipamiento complejo y que la extracción de moléculas polares está limitada, debido al carácter apolar del CO_2, disolvente generalmente utilizado como fluido supercrítico durante la extracción (Khawli et al., 2019).

1.4.2 Uso de la extracción con fluidos supercríticos para la recuperación de compuestos bioactivos a partir de microalgas

En el ámbito de las microalgas, la principal aplicación de la EFS es la recuperación de lípidos, debido al carácter apolar del CO_2. De acuerdo con esto, Leone et al (2019) utilizaron la EFS para extraer ácidos grasos omega-3 a partir de la microalga *Nannochloropsis sp*. Las condiciones aplicadas en este trabajo fueron una presión en el rango de 100 a 550 bar, una temperatura desde 50 hasta 75 °C, y un flujo de etanol de 7,24 o 14,48 g/min. En este caso, estudiaron la influencia de los diferentes parámetros en el proceso de extracción, observando que al aumentar tanto la presión como la temperatura del proceso, se mejoró la selectividad hacia la extracción de EPA, mientras que la presión intermedia y la presión más baja promovieron la recuperación del DHA. El rendimiento más alto en la extracción de EPA se obtuvo al aplicar las condiciones de 75 °C y 550 bar con un caudal de CO_2 de 14,48 g/min, llegando a extraer 5,69 mg/g, lo que supone un 15,59%. En cambio, en el caso del DHA, las condiciones a las que se obtuvo el mayor rendimiento fueron 50 °C y 400 bar con un caudal de CO_2 de 14,48 g/min, recuperando un 79,63%, lo que equivale aproximadamente a 0,12 mg/g. Como se puede observar, la modulación de los parámetros de presión y temperatura permitió hacer más selectiva la extracción.

Por otro lado, Esquível-Hernández et al. (2016) recuperaron 0,283 mg de carotenoides/g MS de espirulina utilizando la EFS con un flujo de 15 g/min de CO_2 durante 50 min y 26,70% (v/v) etanol 96%/agua (v/v) como cosolventes, a una presión de 15 MPa y una temperatura de 60 °C. Yen et al. (2012) también se centraron en la obtención de carotenoides, concretamente luteína, a partir de la microalga *Scenedesmus sp*. Para ello, llevaron a cabo un proceso de EFS, y observaron que el máximo rendimiento se obtuvo al aplicar

400 bar, 70 °C y etanol como cosolvente en una proporción del 30%, recuperando el 76,7% del total de luteína.

Otro carotenoide que acapara un gran interés es la astaxantina. Como se ha comentado previamente, la microalga *Haematococcus pluvialis* produce una gran cantidad de este carotenoide. Wang et al., (2012) aplicaron una presión de 43,5 MPa, una temperatura de 65 °C y 2,3 mL etanol/g de muestra como cosolvente, recuperando un 87,42% de astaxantina.

Por último, en el caso de la espirulina, el compuesto más importante es el ácido γ-linolénico, un PUFA que tiene efectos positivos en una gran variedad de enfermedades, tales como cáncer, enfermedades neurodegenerativas, enfermedades cardiacas, o artritis, entre otras (Choopani et al., 2016). Respecto a esto, Esquível-Hernández et al. (2017) utilizaron EFS para recuperar compuestos bioactivos de esta microalga. Tras analizar la influencia de cada factor implicado en el proceso, obtuvieron los contenidos máximos de nutrientes y compuestos bioactivos con las condiciones de presión de 450 bar, temperatura de 60 °C y un flujo de cosolvente de 11 g/min. Los contenidos máximos de metabolitos bioactivos en los extractos fueron 0,69 ± 0,09 µg/g de riboflavina, 5,49 ± 0,10 µg/g de α-tocoferol, 524,46 ± 0,10 µg/g de β-caroteno, 1,44 ± 0,10 µg/g de luteína y 32,11 ± 0,12 mg/g de ácidos grasos con 39,38% de ácido palmítico, 20,63% de ácido linoleico y 30,27% de ácido γ-linolénico.

También se han estudiado otros compuestos antioxidantes. Wang et al. (2007) estudiaron el efecto de la temperatura (de 32 a 48 °C), la presión (de 20 a 40 MPa) y el tiempo de extracción (de 2 a 4 h) en la recuperación de antioxidantes a partir de *Arthrospira platensis*. Las condiciones óptimas obtenidas por estos autores fueron 48 °C, 20 MPa y un periodo de 4 h de extracción, obteniendo que los extractos contenían 85,1 g/kg de flavonoides, 77,8 g/kg de β-caroteno, 113,2 g/kg de vitamina A y 3,4 g/kg de α-tocoferol.

Como se puede observar, la EFS ofrece muchas posibilidades como metodología de extracción sostenible y segura de diferentes compuestos bioactivos a partir de microalgas. Sin embargo, es necesario seguir estudiando este proceso para optimizarlo en función de la microalga y las diferentes condiciones en cada parámetro.

CAPÍTULO 2. BÚSQUEDA DE FUENTES ALTERNATIVAS DE NUTRIENTES Y COMPUESTOS BIOACTIVOS CON EL FIN DE REEMPLAZAR Y MEJORAR LA COMPOSICIÓN DE PRODUCTOS CÁRNICOS PROCESADOS PARA HACERLOS MÁS SALUDABLES.

La carne es un alimento que contiene una gran cantidad de nutrientes, como vitaminas (B12 y B9), minerales (hierro y zinc), y otros compuestos lipídicos o proteicos de interés. Es por ello por lo que se debe tener en cuenta el consumo de carne para el mantenimiento de una dieta equilibrada y saludable. Se ha estimado que en 2022 la producción mundial de carne y derivados alcanzó alrededor de los 360 millones de toneladas (FAO, 2022), con un consumo medio por parte de la población en constante aumento desde mediados del siglo pasado (Chung et al., 2021). Sin embargo, el consumo excesivo de carne, sobre todo carne procesada (salchichas, hamburguesas…) se ha relacionado con el aumento del riesgo de padecer diversas enfermedades crónicas y neurodegenerativas (Chung et al., 2021; Giromini & Givens, 2022). En línea con lo descrito anteriormente, también se ha observado que el consumo elevado de carne aumenta el riesgo de sufrir enfermedades cardiovasculares, como la isquemia cardiaca y la hipertensión (Allen et al., 2022; Papier et al., 2023).

Con el fin de prevenir estos problemas de salud, es necesario implementar estrategias que promuevan un menor consumo de carne y, al mismo tiempo, fomenten la ingesta de alimentos de origen vegetal. De hecho, el consumo actual de legumbres, frutas y verduras se encuentra por debajo de las recomendaciones diarias para la población, lo cual tiene un impacto negativo en una dieta equilibrada y saludable. Esto también puede contribuir al desarrollo de diversas enfermedades crónicas como cáncer (Miller et al., 2017; Yip et al., 2019; Čižmárová et al., 2023).

Los principales compuestos de interés nutricional que es posible encontrar en estos alimentos de origen vegetal son proteínas, carbohidratos, grasas saludables y fibra dietética, y otros compuestos bioactivos tales como vitaminas, flavonoides, polifenoles y carotenoides, entre otros (Langyan et al., 2022). En concreto, las legumbres son fuente de proteínas y aminoácidos (por ejemplo, las judías y los guisantes contienen de 16,7 a 32,9 g de proteínas/100 g y 22,4-31,7 g de proteínas/100 g, respectivamente) (Carbas et al., 2021).

Las legumbres además contienen numerosos compuestos bioactivos, con efectos beneficiosos para el organismo. Una de las funciones bioactivas más destacadas es su capacidad antioxidante. Debido a la presencia de compuestos como polifenoles, flavonoides y otros compuestos antioxidantes, las legumbres ayudan a neutralizar los radicales libres y proteger las células del daño oxidativo (Singh et al., 2017). También se ha visto que las legumbres presentan propiedades antiinflamatorias (Juárez-Chairez et al., 2022). Esto es particularmente importante debido a la relación constatada entre la inflamación crónica y diversas enfermedades como la diabetes, la obesidad y las enfermedades cardiovasculares. Otra bioactividad relevante de las legumbres es su efecto antitumoral. Las lectinas presentes en legumbres han demostrado tener propiedades antitumorales al interferir con el crecimiento de células cancerosas (Cavada et al., 2023). Además de por sus propiedades antioxidantes y antiinflamatorias, el contenido de fibra soluble aportado por las legumbres puede ayudar a reducir los niveles de colesterol en la sangre, lo que contribuye a reducir uno de los factores de riesgo asociado al desarrollo de enfermedades cardiovasculares. Además, el consumo regular de legumbres puede ser beneficioso para el mantenimiento de unos niveles estables de glucosa en sangre. La presencia de carbohidratos complejos en este alimento provoca que se digieran lentamente, lo que ayuda a evitar picos

de azúcar en la sangre y ayudar a reducir el riesgo de padecer diabetes tipo 2 (Conti et al., 2021).

La utilización de legumbres en la elaboración de productos cárnicos procesados es un procedimiento ampliamente establecido, concretamente el empleo de la soja. Su adición a este tipo de productos permite mejorar las propiedades tecnológicas y funcionales y, a su vez, abaratar el coste final del mismo (Canan et al., 2020). Sin embargo, la soja presenta una gran alergenicidad, causada por algunas de las proteínas contenidas en esta legumbre (Taylor et al., 2021). Por lo tanto, es necesario la búsqueda de alternativas que puedan solucionar este problema a la vez que mantengan las funciones y el aporte nutritivo de la soja. Por ello es de interés investigar la función de otras legumbres como aditivos en productos cárnicos que podrían en un futuro sustituir a las proteínas de soja como ingrediente cuando fuese necesario.

Por otro lado, el suero lácteo supone el principal subproducto derivado de la industria quesera (Buchanan et al., 2023). Sin embargo, las proteínas del suero de la leche son reconocidas como una fuente de nutrientes de alta calidad ya que contienen diversos componentes bioactivos. Son ricas en aminoácidos esenciales como la cisteína, aminoácidos de cadena ramificada como la leucina, valina e isoleucina, y péptidos bioactivos. Además, se ha demostrado que estas proteínas tienen efectos beneficiosos para la salud humana, como propiedades antioxidantes, antiinflamatorias, de reducción de la obesidad y reducción de la presión arterial (Yiğit et al., 2023). El suero de la leche se ha investigado como componente para la obtención de una gran diversidad de productos (películas y recubrimientos comestibles, ácido láctico y otros compuestos bioquímicos, bioplásticos, biocombustibles…) siendo de gran relevancia nutricional su uso en la elaboración de una gran

variedad de alimentos procesados, tales como productos cárnicos, galletas y bollería, salsas… (Królczyk et al., 2016; Zandona et al., 2021).

Por último, cabe destacar la gran relevancia nutricional de las microalgas, consideradas como una importante fuente de nutrientes y compuestos bioactivos. Las microalgas más relevantes y con un consumo más extendido son la espirulina (*Arthrospira sp.*) y la chlorella (*Chlorella vulgaris*), las cuales reciben cada vez más atención desde el punto de vista nutricional debido a que pueden ser un complemento valioso de una dieta sana.

Tanto espirulina como chlorella son ricas en ácidos grasos esenciales, entre los que podemos destacar el DHA y EPA, los cuales se ha demostrado que son beneficiosos para el sistema cardiovascular y tienen actividad neuroprotectora. Otros ácidos grasos también presentes en estas microalgas son: ácido palmítico, linoleico, oleico, α-linolénico, γ-linolénico, hexadecatetraenoico, palmitoleico, hexadecadienoico, hexadecatrienoico, láurico, estearidónico y araquidónico (Daki et al., 2022; Jahromi et al., 2022).

Estas microalgas también son una gran fuente de aminoácidos esenciales (leucina, triptófano, metionina, fenilalanina, lisina, tionina, isoleucina y valina), fundamentales para la síntesis de proteínas y el desarrollo del tejido muscular (Liestianty et al., 2019). Esto hace que sean una excelente fuente de proteína vegetal completa para dietas vegetarianas y veganas, además de para diversificar la ingesta de proteínas en una dieta normal.

Aparte de los valiosos nutrientes comentados anteriormente, las microalgas, concretamente espirulina y chlorella, también aportan minerales y compuestos antioxidantes. De hecho, unos de los antioxidantes más importantes que podemos encontrar en estos organismos son los polifenoles y los pigmentos. Los pigmentos sirven a las microalgas para realizar el metabolismo fotosintético, y tienen actividades biológicas muy importantes

como por ejemplo actividad antitumoral, antiinflamatoria, neuroprotectora y antioxidante. Entre estos pigmentos, los más destacables son los carotenoides y las ficobiliproteínas, como la ficocianina (Vaz et al., 2016). Además, también podemos encontrar minerales como sodio, magnesio, fósforo, potasio, calcio y hierro, entre otros (Darwish et al., 2020). El consumo regular de estas dos microalgas se ha relacionado con efectos beneficiosos para la salud a nivel de salud cardiovascular, mejora del sistema inmunitario, control de la glucemia y equilibrio del sistema digestivo (Barghchi et al., 2023; Daki et al., 2022; Sherafati et al., 2022).

En conclusión, espirulina y chlorella son microalgas con un alto valor nutricional debido a su contenido relevante en ácidos grasos esenciales, aminoácidos, vitaminas, minerales y compuestos antioxidantes, lo que las convierte en una fuente completa de nutrientes. Por lo tanto, la incorporación de microalgas en alimentos procesados puede aportar beneficios para la salud humana a la vez que se contribuye a un proceso más sostenible, teniendo en cuenta tanto el aumento progresivo de la población como la dieta y los hábitos de salud actuales (Caporgno & Mathys, 2018).

CAPÍTULO 3. APLICACIÓN DE LOS COMPUESTOS OBTENIDOS A PARTIR DE SUBPRODUCTOS DE PESCADO Y ESPIRULINA EN LA REDUCCIÓN DE LA TOXICIDAD DE MICOTOXINAS

3.1 MICOTOXINAS

Las micotoxinas son metabolitos secundarios producidos por diferentes hongos toxigénicos que de forma natural contaminan los alimentos. Los principales hongos productores de estas toxinas pertenecen a los géneros *Alternaria*, *Aspergillus*, *Claviceps*, *Fusarium*, *Penicillium*, y *Stachybotrys* (Malir et al., 2023).

Las primeras micotoxinas descubiertas fueron las aflatoxinas (AFs), en 1960. En la actualidad se han identificado más de 500 micotoxinas y entre ellas se incluyen la ocratoxina A (OTA), deoxinivalenol (DON), nivalenol (NIV), toxinas T2 y HT2, fumonisinas y citrinina (CIT) (Malir et al., 2023).

Las micotoxinas se encuentran principalmente en los cereales (trigo, maíz, cebada…), y de forma minoritaria en especias, semillas y otros alimentos de origen vegetal. En este sentido, es importante señalar que la alimentación de animales a partir de piensos elaborados con cereales contaminados con micotoxinas puede dar lugar a la acumulación en sus tejidos y la consecuente contaminación de los alimentos de origen animal como los huevos, la leche o la carne (Janik et al., 2020). Esta contaminación no suele producirse por una sola micotoxina, sino que en un mismo alimento pueden detectarse diversas micotoxinas. Esto puede ser debido al hecho de que un mismo hongo produzca diferentes micotoxinas o bien que los alimentos estén contaminados con diferentes hongos. En el caso de productos de origen animal, la elaboración de piensos a partir de diferentes cereales favorece la presencia de varias micotoxinas de manera simultánea (Smith et al., 2016).

La exposición a micotoxinas a través de los alimentos es un problema a nivel mundial tanto para la población humana como para los animales. Se ha demostrado que la toxiinfección por la ingesta de micotoxinas puede generar principalmente trastornos gastrointestinales (vómitos, diarrea…), enfermedades hematológicas, lesiones hepáticas y renales, así como presentar efectos estrogénicos, mutagénicos y carcinógenos (Patriarca & Fernández Pinto, 2017). En este sentido, las micotoxinas que conforman el grupo de las AFs, están clasificadas como carcinógenas por la Agencia Internacional de Investigaciones sobre el Cáncer (IARC, por sus siglas en inglés) (Ostry et al., 2017). Esto es de especial interés teniendo en cuenta que el proceso de cambio climático actual conllevará un aumento de la temperatura y humedad a nivel global que favorecerá el crecimiento de hongos micotoxigénicos en zonas del mundo en las que actualmente no están presentes, incrementándose de esta manera la contaminación por micotoxinas (Zingales et al., 2022).

A pesar de que multitud de estudios destacan que la contaminación por micotoxinas tiene una prevalencia del 25% en alimentos, basándose en un informe del año 2007 de la FAO, cada vez hay más autores que ponen este dato en entredicho (Eskola et al., 2019). De hecho, la incidencia de micotoxinas en grano, el principal alimento contaminado por estos compuestos se estima en un 60-80% (Eskola et al., 2019).

Respecto a la exposición dietética a estas toxinas, se ha observado que la OTA es la micotoxina con mayor prevalencia, seguida de los tricotecenos (DON, NIV, T2…), las AFs, la zearalenona (ZEA), las fumonisinas, la patulina, las enniatinas y la beauvericina. Sin embargo, los contenidos determinados en alimentos son inferiores a los límites establecidos por la legislación europea vigente, a excepción de muestras puntuales de vino y leche (Carballo et al., 2019).

Por otro lado, el Sistema de Alerta Rápida para Alimentos y Piensos (RASFF, por sus siglas en inglés) ha recibido en la última década (enero 2013 - diciembre 2022), 1360 notificaciones validadas sobre micotoxinas, siendo el principal motivo de las mismas la determinación de niveles elevados de AFs (*RASFF Window - Search*, 2023). De estas 1360 notificaciones, 494 se realizaron en el año 2022.

Todo esto pone de manifiesto la importancia de conocer los mecanismos de toxicidad de este tipo de contaminantes alimentarios, con el fin de poder prevenir los efectos tóxicos sobre la salud debido a la presencia de las micotoxinas en los alimentos. También es de interés la investigación de estrategias de reducción y prevención de su toxicidad, con el fin de minimizar sus implicaciones tóxicas para la salud, contribuyendo a disponer de unos alimentos más seguros.

En la presente Tesis Doctoral se investigará la micotoxina CIT, la cual ha atraído la atención tanto de investigadores como de autoridades debido a que la falta de datos dificulta su regulación a nivel europeo (Narváez et al., 2021; Sáncheza et al., 2017).

3.1.1 Citrinina

La CIT es una micotoxina producida por hongos *Aspergillus, Penicillium y Monascus*, y puede estar presente principalmente en cereales, aunque también se puede encontrar en frutas, hierbas y especias, con una amplia distribución a lo largo de diferentes zonas geográficas de todo el mundo (Narváez et al., 2021). Es habitual encontrarla junto a otras micotoxinas, principalmente OTA (Geisen et al., 2018).

Respecto a su estructura química, es una quinona con dos puentes de hidrógeno intramoleculares. Además, la CIT es una micotoxina policétida, con un peso molecular de 250,25 g/mol y cuya fórmula química es $C_{13}H_{14}O_5$. Se

encuentra en forma sólida y tiene un color amarillo limón (de Oliveira Filho et al., 2017).

Esta micotoxina afecta principalmente a los riñones, habiéndose observado diversos efectos como necrosis renal y degeneración del túbulo renal con presencia de cilindros proteínicos (Ahamad, 2018). Además, se ha demostrado que puede actuar sobre el hígado, induciendo la apoptosis en los hepatocitos mediada por el estrés oxidativo en el retículo endoplásmico a través de una alteración del calcio (D. Wu et al., 2022; J. Wu et al., 2022). Por último, también puede afectar a otros órganos como el corazón, la médula ósea o el sistema digestivo (de Oliveira Filho et al., 2017; Jeswal, 1996; Wu et al., 2013).

A nivel molecular, su mecanismo de acción se ha estudiado mediante ensayos celulares *in vitro* y ensayos *in vivo*. Se ha observado que en función del tipo de línea celular ensayada y de la concentración utilizada, la CIT provoca el bloqueo del ciclo celular en varias fases, incluidas las fases G1 y G2/M (Chang et al., 2011; Kumar et al., 2011). Por otro lado, también se ha demostrado que esta micotoxina puede producir daño en el ADN, estimular la autofagia y producir disfunción mitocondrial en embriones de ratón (Huang et al., 2021).

También se ha descubierto que los mecanismos de muerte celular se ven afectados significativamente por la CIT. Según las investigaciones, la exposición a la CIT puede causar apoptosis mediante la activación de varias vías de señalización celular, como la vía mitocondrial, el estrés del retículo endoplásmico y la activación de caspasas (Kumar et al., 2011; D. Wu et al., 2022; Yu et al., 2006). Como se ha comentado previamente, la CIT puede modificar el potencial de la membrana mitocondrial (MMP), lo que provoca alteraciones en el funcionamiento mitocondrial y puede desembocar en la muerte celular por apoptosis (Chan, 2007; Chen & Chan, 2009). Por último, se ha demostrado

que los orgánulos y las estructuras celulares pueden sufrir daños como resultado de la exposición a la CIT (Sun et al., 2020).

Sin embargo, aún no existe suficiente evidencia científica sobre los mecanismos moleculares y las vías implicadas en la toxicidad de la CIT. Prueba de ello es el hecho de que la Comisión Europea no ha establecido un límite para el contenido de esta micotoxina en los alimentos debido a los limitados datos disponibles. A nivel legislativo, tan solo se ha regulado el contenido máximo de CIT para un complemento alimenticio a base de arroz fermentado con levadura roja (*Monascus purpureus*) (The Commission of the European Communities, 2006).

Por todo ello, el estudio en profundidad de los mecanismos bioquímicos y las rutas involucradas en la toxicidad de la CIT es necesario para garantizar la seguridad de los alimentos y la protección de la salud humana y animal.

3.1.2 Uso de compuestos de origen natural para reducir la toxicidad producida por micotoxinas

Como se ha comentado anteriormente, la contaminación de los alimentos por la presencia de micotoxinas tiene un gran impacto sobre la salud, por lo que es necesario buscar estrategias para reducir su toxicidad. Diversos estudios han demostrado que compuestos de origen natural podrían tener la capacidad de reducir los efectos tóxicos producidos por las micotoxinas. En este sentido, se ha observado que el flavonoide quercetina aumenta la viabilidad en células SH-SY5Y expuestas a la esterigmatocistina (STE). Además, es capaz de contrarrestar los cambios producidos por esta micotoxina respecto a la generación de estrés oxidativo y al aumento de HO-1 y NOS-2, dos enzimas inducidas en presencia de estrés y cuya expresión está aumentada tras la exposición a STE. Estos autores han demostrado un efecto antiinflamatorio al reducir la expresión génica de las citoquinas

proinflamatorias TNF-α e IL-6, aumentadas por la exposición a la micotoxina (Zingales, Sirerol-Piquer, et al., 2021). Martínez-Alonso et al. (2022) evaluaron el efecto protector de un extracto de judía roja frente al efecto tóxico de la toxina T-2 en células hepáticas HepG2. Los principales polifenoles detectados en el extracto fueron la epicatequina y la delfinidina, con una concentración de 3,297 y 3,108 mg/Kg, respectivamente. En este caso, el tratamiento simultáneo y el pretratamiento de las células HepG2 con extracto de judía roja no dio lugar a ninguna modificación del efecto citotóxico de la micotoxina. En cambio, la combinación de T-2 a 7,5 nM con el extracto diluido (1:8) de judía roja mostró una disminución de la producción de especies reactivas de oxígeno (ROS) tras 120 min de exposición, en comparación al control.

Por otro lado, las algas pueden tener un efecto detoxicante frente a micotoxinas probablemente debido a su contenido en compuestos bioactivos con capacidad antioxidante (como polifenoles) o a otros compuestos como los polisacáridos (Yadavalli et al., 2023). En concreto, las macroalgas *Laurencia obtusa* (alga roja) y *Caulerpa prolifera* (alga verde), han demostrado un efecto protector frente a la hepatotoxicidad producida por la aflatoxina B1 (AFB1) en ratas (Abdel-Wahhab et al., 2006). Los extractos obtenidos a partir de estas algas, ricos en esteroles y diterpenos, produjeron una reducción en los niveles de marcadores tumorales (α-fetoproteína y antígeno carcinoembrionario) y pro-inflamatorios (TNF-α, NO e IL-1α) en suero, siendo el resultado más significativo el obtenido con el extracto de *Caulerpa prolifera*. Los autores también observaron una potenciación de la actividad de las enzimas antioxidantes catalasa (CAT), glutatión peroxidasa (GPx) y superóxido dismutasa (SOD), lo que conllevó la reducción de la peroxidación lipídica hepática.

De manera similar, Guo et al. (2021) observaron un efecto protector de un extracto de *Enteromorpha prolifera* (alga verde) rico en polisacáridos frente a

los efectos tóxicos de la AFB1 en pollos, reduciendo el estrés oxidativo y la apoptosis producidos por la micotoxina. Banu et al. (2008) elaboraron una suspensión a partir de *Sargassum wightii* (alga parda) en concentración de 1,8% (p/v) que demostró una actividad detoxicante y fungicida frente a la micotoxina AFB1 producida por *Aspergillus flavus*. Los autores sugieren que la eliminación tanto de la micotoxina como del hongo, probablemente se deba a la acción de las clorofilas presentes en el extracto de alga.

Por otro lado, el efecto de las microalgas sobre la acción tóxica de las micotoxinas también ha sido evaluado. En el estudio desarrollado por Elbasuni et al. (2022) se evaluó el efecto protector de complementar la dieta con *Chlorella vulgaris* (1 g/kg dieta) en codornices expuestas a AFB1 (50 ppb). La suplementación con la microalga mejoró la actividad de enzimas hepáticas antioxidantes y disminuyó la expresión de citoquinas pro-inflamatorias inducidas por la micotoxina, como TNF-α, IL-1β y IL-6. Además, la administración conjunta de *Chlorella vulgaris* y AFB1 mejoró el valor nutricional de la carne y redujo significativamente los residuos de micotoxina en comparación a la administración de la micotoxina únicamente. Por último, la microalga mitigó el daño hepático, la reducción del crecimiento y la disminución de la carne inducidos por la AFB1, probablemente debido a sus componentes antioxidantes y nutricionales, como aminoácidos esenciales, vitaminas o minerales, entre otros.

Esto coincide con lo descrito en otro estudio en el que se evaluó el efecto protector de *Chlorella pyrenoidosa* frente a la hepatotoxicidad causada por AFB1 en pollos (Subhani, 2018). En este caso, la complementación con un extracto de *Chlorella pyrenoidosa* (500 mg) en una dieta contaminada con AFB1 (350 ppb) contrarrestó los efectos tóxicos producidos por la micotoxina. Entre las alteraciones producidas por la ingesta de AFB1 se puede destacar una reducción en la ingesta de pienso por parte de los animales con la

consecuente disminución en su peso. Respecto a los parámetros bioquímicos, un nivel bajo de AFB1 en la dieta no sólo aumentó la peroxidación lipídica, sino que también redujo significativamente la capacidad antioxidante total, así como la actividad de las enzimas hepáticas CAT y GPx. En cambio, al complementar la dieta con el extracto de microalga, estas funciones se mantuvieron en niveles similares o cercanos al control, contrarrestando los efectos perjudiciales inducidos por la micotoxina. Esto sugiere que la complementación de *Chlorella pyrenoidosa* en la dieta de pollos de engorde podría ser un método seguro y barato para reducir los efectos tóxicos de AFB1.

Por último, el efecto protector de compuestos de origen animal ha sido evaluado por diversos autores. En concreto, Taroncher et al. (Taroncher et al., 2021) analizaron el impacto de diferentes hidrolizados de pescado obtenidos a partir de subproductos de salmón, caballa y arenque, frente a la micotoxina T-2 tras 24 h de exposición en células Caco-2/TC7. En este caso, el mayor efecto protector se obtuvo con los hidrolizados de vísceras de salmón, que alcanzó un 76,18% de aumento de la viabilidad celular en comparación con las células tratadas únicamente con la micotoxina T-2.

Esto demuestra que, a partir de recursos naturales como microalgas y residuos de pescado entre otros, se pueden obtener compuestos que permitan reducir la toxicidad de las micotoxinas. Por lo tanto, es necesario la investigación en este aspecto con el fin de descubrir y caracterizar esta función protectora en diferentes tipos de extractos y matrices disponibles.

3.2 MÉTODOS ALTERNATIVOS PARA LA EVALUACIÓN DE LA TOXICIDAD DE MICOTOXINAS

3.2.1 Cultivos celulares en 3D

Tradicionalmente los cultivos celulares en 2D, con las células formando una monocapa, se han utilizado para la evaluación de la toxicidad *in vitro*, pasando después a los ensayos con animales para la etapa *in vivo*. Actualmente, existe una tendencia hacia la búsqueda de ensayos alternativos a la experimentación animal con el fin de cumplir los requerimientos propuestos por el principio de las 3 R: Reducción, Refinamiento y Reutilización (Diederich et al., 2022; Verderio et al., 2023).

En este contexto, están apareciendo nuevas técnicas *in vitro* que aportan una información más completa y próxima a las condiciones *in vivo* con respecto a las técnicas habituales en 2D. Una de estas técnicas alternativas es el uso de cultivos en 3D, en los que las células conforman una estructura tridimensional como esferoides u organoides. De esta manera, se obtiene una mejor simulación del microambiente celular que en los modelos celulares en monocapa, obteniéndose información más fiable y realista, y contribuyendo además a reducir el uso de animales en investigación (Augustyniak et al., 2019; Zingales, Torriero, et al., 2021).

Diversos autores han utilizado este tipo de cultivos celulares con el objetivo de estudiar los efectos tóxicos producidos por diversas micotoxinas. En este aspecto, Štampar et al. (2019) generaron esferoides de células hepáticas HepG2 con el fin de realizar ensayos de genotoxicidad y obtener unos resultados más sensibles respecto a los cultivos en monocapa. Los autores testaron diversos compuestos, entre ellos la AFB1, a concentraciones de 10, 20 y 40 µM y 24 h de exposición. La AFB1 indujo alteraciones en el ADN a concentraciones iguales o superiores a 20 µg/mL, tanto en el modelo de

esferoides como en el modelo de monocapa. Se determinó que los resultados obtenidos en 3D eran comparables a los obtenidos en los ensayos 2D.

La toxicidad de AFB1 a nivel hepático también ha sido estudiada en modelos 3D por Ma et al. (2022), con el fin de explorar diferentes mecanismos moleculares inducidos por esta micotoxina y de esta forma evaluar los efectos en la viabilidad celular, en las mitocondrias, estrés oxidativo y membrana celular. Para ello se emplearon tres modelos diferentes de esferoides: un monocultivo de células HepG2, un co-cultivo con células HepG2 y EA.hy926 (células de endotelio vascular) y un co-cultivo triple formado por células HepG2, EA.hy926 y LX-2 (células estrelladas hepáticas). En este caso, los autores demostraron que la sensibilidad de los modelos 2D y 3D difiere a medida que se incrementa la concentración de micotoxina. En concreto, basándose en la detección del marcador de apoptosis Caspasa-3, la sensibilidad del modelo 3D superó la del modelo 2D al aumentar la concentración de AFB1. También se evidenció que, aunque la AFB1 a una concentración igual o inferior a 30 µg/mL puede inducir la apoptosis en células HepG2 en el cultivo 2D, no afecta significativamente a las células 3D, especialmente a las de triple co-cultivo.

El efecto de otras micotoxinas también se ha evaluado mediente el uso de esferoides como modelo celular 3D. En el estudio de Zingales, Torriero, et al. (2021), se utilizaron esferoides de células SH-SY5Y y SK-N-DZ para evaluar los efectos tóxicos producidos por la micotoxina STE. En este trabajo se observó que los esferoides derivados de SH-SY5Y demostraron una resistencia significativamente mayor al efecto tóxico de la exposición a STE en comparación con los cultivos celulares 2D. Esto probablemente se deba a que la ausencia de una organización tridimensional en los modelos monocapa puede dar lugar a una sobreestimación de la toxicidad celular. En este

sentido, se observó que el aumento de ROS producido por la toxina en los esferoides se produjo tras 2 días de exposición a la concentración más alta ensayada (5 µM), y se necesitaron 3 días de exposición para que la concentración más baja (2,5 µM) tuviese un efecto significativo sobre este parámetro. Esto contrasta con lo descrito en células cultivadas en monocapa, donde se observaron cambios tras 1 día de exposición a la micotoxina (Zingales et al., 2020). Los resultados también mostraron que la STE inducía una mayor mortalidad en los esferoides de las células SH-SY5Y que en los de las células SK-N-DZ, lo que revela diferencias significativas en la sensibilidad a la micotoxina entre las dos líneas celulares.

3.2.2 Pez cebra

El pez cebra es un modelo *in vitro*/*in vivo* ampliamente utilizado en la investigación toxicológica. Este modelo presenta la particularidad de considerarse como método *in vitro* en los ensayos llevados a cabo desde las 0 h hasta las 120 h post-fertilización (hpf) tras la fecundación del huevo y como método *in vivo* en ensayos a partir de las 120 hpf (Strähle et al., 2012). Este modelo ofrece muchas alternativas respecto a la experimentación tradicionalmente realizada con roedores. Una de estas ventajas es el hecho de que los embriones y larvas de pez cebra se desarrollan rápidamente y son transparentes, lo que permite visualizar fácilmente los órganos y estructuras internas. Esto facilita el estudio de los efectos de los compuestos tóxicos sobre el desarrollo y la función de los órganos. Por otro lado, el mantenimiento del pez cebra es barato y además requiere menos espacio y recursos en comparación a los roedores u otros animales utilizados en investigación. Finalmente, el rápido crecimiento de este organismo junto con el gran número de huevos que libera en cada puesta hace que pueda utilizarse para el cribado de un gran número de compuestos de forma relativamente rápida y con una menor implicación ética en comparación al

uso de mamíferos, lo que es muy útil en la realización de ensayos de toxicidad (Bauer et al., 2021).

Este modelo ha sido utilizado por diversos autores para evaluar los efectos tóxicos producidos por diferentes micotoxinas. En este sentido, He et al. (2023) utilizaron este modelo para evaluar el efecto tóxico de AFB1 durante el desarrollo y más concretamente el impacto sobre la generación de músculo. Los resultados mostraron que la AFB1 causaba disfunción motora en embriones de pez cebra, además de provocar un desarrollo muscular anormal en las larvas, mediante la inducción de anomalías en la arquitectura del tejido muscular. Estos autores concluyeron que la AFB1 puede inducir toxicidad en el desarrollo e inhibir el desarrollo muscular a través del daño oxidativo, la apoptosis y la alteración de los complejos de unión estrecha en larvas de pez cebra.

El pez cebra se ha empleado como modelo en el estudio de la hepatotoxicidad de ZEA (Zhang et al., 2022). En este caso, los autores describieron que esta micotoxina producía daño hepático al observar una reducción en el tamaño del hígado y un aumento en los niveles de aspartato aminotransferasa. También observaron alteraciones en la expresión génica de diversas enzimas antioxidantes y un aumento en la oxidación lipídica, causada por el aumento del estrés oxidativo.

Por otro lado, Rong et al. (2023) utilizaron embriones de pez cebra para evaluar el daño producido por la co-exposición a ZEA y DON y sus consecuencias sobre el estrés oxidativo, la hepatotoxicidad, la apoptosis y la inflamación. Los embriones fueron expuestos desde las 4 hpf hasta las 96 hpf. Los resultados mostraron que el tratamiento combinado de ZEA (200, 400, 800 µg/L) y DON (4000 µg/L) no causó la muerte de los embriones de pez cebra en ninguno de los grupos. Sin embargo, disminuyeron los tiempos de movimiento y los latidos del corazón del pez cebra, lo que implica que estas

micotoxinas inducen toxicidad en el desarrollo. A las 96 hpf, la co-exposición de ZEA y DON, concretamente las combinaciones ZEA 400 µg/L + DON 4000 µg/L y ZEA 800 µg/L + DON 4000 µg/L, condujeron a un estrés oxidativo significativo, que se demuestra por el aumento del nivel de ROS y el contenido de malondialdehido (MDA), así como cambios de las enzimas antioxidantes (SOD, CAT y GPx) y sus genes. Además, el tratamiento combinado de ZEA y DON desencadenó la apoptosis a través de la sobreexpresión de genes relacionados (p53, Caspasa-9, Caspasa-3) y la regulación a la baja del gen Bcl-2 (anti-apoptótico). Estas combinaciones también provocaron un aumento en la expresión génica de IL-1β, IL-6, TNF-α, TLR4, MyD88 y NF-κBp65, lo que implica la inducción de un proceso inflamatorio. Por tanto, estos resultados indican que la co-exposición de ZEA y DON causó estrés oxidativo, dando lugar a mayores efectos tóxicos potenciales para los embriones de pez cebra que sus respectivos tratamientos individuales.

Por otra parte, Y. Wang et al. (2021) evaluaron la combinación de AFB1 y DON en embriones de pez cebra. Tras evaluar la toxicidad a los 7 días de exposición, se constató que el DON tenía menor toxicidad para los embriones de pez cebra que la AFB1 con una concentración letal media (LC_{50}) de 218,3 mg/L para el DON y de 0,031 mg/L para la AFB1. También se observaron diferencias significativas en los resultados obtenidos para los genes relacionados con estrés oxidativo, inflamación, apoptosis y función endocrina en comparación a la exposición individual a cada micotoxina.

En definitiva, se ha demostrado que el uso de pez cebra en los diferentes estadios de desarrollo es una buena herramienta en la investigación de los efectos tóxicos producidos por las micotoxinas y sus combinaciones. Además, la aplicación de este modelo contribuye a realizar una investigación más ética y reducir el número de mamíferos utilizados en investigación.

CAPÍTULO 4. APORTACIÓN DE LA PRESENTE TESIS DOCTORAL A LA IMPLEMENTACIÓN Y DESARROLLO DE LOS OBJETIVOS DE DESARROLLO SOSTENIBLE

La implementación de los Objetivos de Desarrollo Sostenible (ODS) ha adquirido una importancia creciente en el ámbito de la investigación y la industria alimentaria con el fin de satisfacer las necesidades de la población sin comprometer la salud del planeta y los recursos naturales. Entre los principales desafíos planteados se encuentra la búsqueda de métodos sostenibles para la extracción de compuestos bioactivos de interés, así como la reducción y la reutilización de residuos mediante la búsqueda de alternativas para estos desechos. Es dentro de este marco donde se plantea y adquiere una gran importancia a valorización mediante el aprovechamiento para la obtención de compuestos de interés.

Por lo tanto, el uso de métodos de extracción sostenibles para la obtención de compuestos de alto valor añadido a partir de subproductos de la industria agroalimentaria, la inclusión de proteínas procedentes de fuentes alternativas en productos cárnicos y la investigación de los mecanismos de toxicidad de contaminantes alimentarios y su prevención supone un paso más hacia la completa implementación de los ODS.

La investigación abordada en la presente Tesis Doctoral se relaciona con diversos ODS, contribuyendo en el desarrollo e implementación de estos. En primer lugar, se puede destacar que la utilización más eficiente de los recursos se alinea con el ODS 12, "Producción y Consumo Responsables". Como se ha explicado anteriormente, la aplicación de estas tecnologías no convencionales de extracción permite reducir el uso de disolventes orgánicos y el tiempo de procesado, disminuyendo de esta manera el impacto ambiental y dando lugar a una gestión más sostenible de los recursos naturales. Además, la inclusión de proteínas de origen vegetal o procedentes

de microalgas en alimentos cárnicos procesados puede contribuir a disminuir la dependencia actual a los alimentos de origen animal, y por tanto reducir el impacto ambiental generado por la cría intensiva de animales. Este trabajo también se alinea con el ODS 9, "Industria, Innovación e Infraestructura", ya que el uso de subproductos agroalimentarios y microalgas como materia prima fomenta la economía circular, reduce los desperdicios y favorece de nuevo la gestión eficiente de los recursos y una menor huella ambiental. Cabe destacar que el uso de fuentes alternativas de proteínas en alimentos cárnicos requiere del desarrollo de nuevas técnicas de procesado y formulación en la industria, un aspecto que también se contempla en este ODS.

Otro ODS en el que se puede incluir la presente investigación es el ODS 2, "Hambre Cero", ya que las tecnologías abordadas permiten la valorización de subproductos y el mejor aprovechamiento de los recursos mediante el aumento de la disponibilidad de nutrientes esenciales, contribuyendo así a la mejora de la calidad nutricional. Además, la inclusión de microalgas y otras fuentes alternativas de proteínas en productos cárnicos procesados contribuye a mejorar la calidad nutricional de los alimentos, y de esta forma a mejorar la disponibilidad de nutrientes esenciales. Por otra parte, la línea de investigación planteada puede contribuir a que los alimentos ricos en proteínas sean más accesibles y asequibles, ayudando a abordar la prevención y la lucha contra la desnutrición y el hambre. Por último, el estudio de las micotoxinas y sus mecanismos tóxicos puede ayudar a mejorar las prácticas agrícolas y el almacenamiento de los productos, reduciendo el desperdicio alimentario y mejorando la calidad y la seguridad de los alimentos, impulsando el aumento de la disponibilidad de alimentos y por lo tanto contribuyendo a reducir el hambre.

La investigación relacionada con la mejora del perfil nutricional de productos cárnicos procesados también se encuentra en línea con en el ODS 3, "Salud y Bienestar", debido a que se disminuye la presencia de grasas saturadas y se aumentan los nutrientes beneficiosos para la salud humana, promoviendo dietas más equilibradas y contribuyendo a la reducción de los riesgos para la salud asociados al elevado consumo de carne roja. De la misma manera, la investigación realizada sobre los efectos tóxicos de la CIT se puede relacionar con este ODS Los efectos perjudiciales de las micotoxinas sobre la salud han sido ampliamente estudiados. Por lo tanto, la investigación de sus mecanismos de toxicidad y la manera de prevenirlos puede ayudar a identificar riesgos y desarrollar estrategias de prevención de enfermedades relacionadas con la exposición a estas toxinas, contribuyendo así a promover la salud y el bienestar.

Por último, las micotoxinas no son solo un problema relacionado con la salud humana, sino que también afectan a los animales, lo que puede causar una reducción de la biodiversidad y por tanto provocar un impacto negativo en el ecosistema. Es por ello por lo que el estudio de estrategias de mitigación de la toxicidad causada por las micotoxinas puede abordarse dentro del marco del ODS 15, "Vida de Ecosistemas Terrestres", contribuyendo a conservar el medioambiente y minimizando su impacto en el entorno natural.

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OBJETIVOS

OBJECTIVES

El objetivo general de la presente *Tesis Doctoral* es la utilización de biomasa marina como fuente de ingredientes para su uso como aditivos alimentarios, nutracéuticos, y/o sustitución parcial de alimentos, con el fin de obtener productos con un perfil nutricional y bioactivo más saludable. Además, también se evaluará la aplicación de la biomasa marina en la mitigación de efectos tóxicos de contaminantes alimentarios mediante modelos alternativos.

Para lograr el objetivo general, se han desarrollado diferentes objetivos específicos, los cuales se detallan a continuación:

Objetivo 1. Obtención de compuestos antioxidantes y antifúngicos a partir de subproductos de pescado mediante fermentación con bacterias ácido-lácticas.

Objetivo 2. Optimización del proceso de extracción de proteínas y compuestos bioactivos a partir de subproductos de lubina utilizando pulsos eléctricos, y recuperación de minerales y péptidos bioactivos.

Objetivo 3. Recuperación de nutrientes y compuestos bioactivos a partir de la microalga espirulina mediante la extracción con pulsos eléctricos y fluidos supercríticos.

Objetivo 4. Evaluar los cambios en las propiedades fisicoquímicas y el valor nutricional de productos cárnicos (hamburguesas de pavo y salchichas de cerdo) obtenidos tras la adición de diferentes proteínas de origen vegetal (guisantes, lentejas y judías), algas (chlorella y spirulina) y/o suero de leche.

Objetivo 5. Determinación de los efectos tóxicos y mecanismos de acción de la citrinina mediante modelos in vitro (modelos celulares 2D y 3D de células SH-SY5Y) e in vivo (embriones de pez cebra).

Objetivo 6. Evaluación del efecto protector de extractos de biomasa marina obtenidos en la presente tesis frente a los efectos tóxicos producidos por la citrinina mediante modelos alternativos.

RESULTADOS

RESULTS

Impact of Fermentation on the Recovery of Antioxidant Bioactive Compounds from Sea Bass Byproducts

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Abstract

The aim of the present research was to obtain antioxidant compounds through the fermentation of fish byproducts by bacteria isolated from sea bass viscera. To that purpose, bacteria from sea bass stomach, intestine, and colon were isolated. With the selected bacteria, growing research was undertaken, fermenting different broths prepared with sea bass meat or byproducts. After the fermentation, the antioxidant activity, phenolic acids, and some proteins were evaluated. The main phenolic acids obtained were DL-3-phenyl-lactic acid and benzoic acid at a maximum concentration of 466 and 314 ppb, respectively. The best antioxidant activity was found in the extracts obtained after the fermentation of fish byproducts broth by bacteria isolated from the colon (6502 μM TE) and stomach (4797 μM TE). Moreover, a positive correlation was found between phenolic acids obtained after the fermentation process and the antioxidant activity of the samples. It was also concluded that the lactic acid bacteria isolated from sea bass had an important proteolytic capacity and were able to synthesize phenolic acids with antioxidant capacity. This work has shown the relevance of fermentation as a useful tool to valorize fish byproducts, giving them an added economic value and reducing their environmental impact.

Keywords: fermentation; fishing industry byproducts; bioactive peptides; antioxidant activity; phenolic acids

1. Introduction

The latest FAO (Food and Agricultural Organization) report, published in 2018, showed an important growth in fish caught over the last few years due to the increased fish population's consumption (from 17.6 kg per capita in 2007 to 20.3 kg per capita in 2016) [1].

During the fish production process, several byproducts are produced, representing more than 30% of fish weight, and in some cases, up to 70% [2]. Among the different parts of fish that can be considered byproducts, muscle pieces, viscera, thorns, heads, skin, fins, and scales are the most representative [3]. These must be properly managed in order to avoid environmental problems and to maintain the sustainability of the resources. Moreover, it should be noted that since each byproduct has a different composition, different alternatives have been evaluated for each of them [4].

For instance, different innovative approaches have been studied in order to valorize fish byproducts due to the environmental problem they represent; for instance, the application of green extraction technologies such as ultrasound-assisted extraction, pulsed electric fields, and supercritical fluid extraction to obtain valuable compounds from fish byproducts. These technologies allow the recovery of interesting compounds with the minimum environmental impact, reducing the use of organic solvents, which can be toxic and/or improving the extraction efficiency [5,6].

Fermentation is conventionally used for food preservation, but not so frequently for valorization purposes, as a tool to obtain high added-value compounds. In the food industry, fermentation is considered as any process in which the activity of microorganisms promotes the development of a profitable change in a food or drink [7]. Through fish byproducts, fermentation is possible to obtain quite different high added-value compounds such as

bioactive peptides, high-quality oils, or protein hydrolysates, as well as many others like bioplastics, lactic acid, or preservative compounds, which are very useful in food, pharmaceutical or cosmetic industries [8]. For fermentation processes, the main microorganisms used are lactic acid bacteria (LAB), mainly due to their safety, since they are present in a multitude of fermentative processes of food aimed for human consumption, such as dairy products or alcoholic beverages.

The lactic acid bacteria (LAB) is a group of microorganisms characterized by the production of lactic acid as the main product of carbohydrate fermentation. They are Gram-positive, not sporulated, and have a coralline or bacillary shape [9,10,11]. These bacteria, through the phosphorylation of carbohydrates, obtain the metabolic energy forming the main metabolite, lactic acid. As the acid is accumulated, the structure of the proteins is modified, as happens with the texture of the product. Such bacteria have been used throughout history for the fermentation of a multitude of foods, producing changes in taste and texture, acting as a preservative, and increasing their shelf life [9,12].

Among the different high added-value compounds obtained from fish byproducts, it is possible to highlight nutrients (e.g., proteins and lipids) as well as bioactive compounds (e.g., polyphenols and bioactive peptides). For instance, during fermentation, LAB play a very important role due to their proteolytic capacity, which allows them to fractionate the proteins into peptides and free amino acids. Generally, small peptides of 3 to 20 amino acids are obtained [13,14]. Some of these peptides have biological activity; therefore, they are considered bioactive peptides [15]. Furthermore, not only are bioactive peptides produced during fermentation, but different phenolic acids have also been found, such as benzoic acid or phenyl-lactic acid, identified in silages inoculated with LAB [16]. These compounds produced by

the LAB are also related to the antimicrobial activity and the antioxidant activity [17].

Sea bass is one of the most produced fish worldwide, but especially in Europe. The main production system is aquaculture, with a production of 165,915 tons in 2016, representing 96% of the total production [18]. Sea bass culture is mainly focused on the geographical area of the Mediterranean Sea, with Turkey, Egypt, Tunisia, Italy, and Spain being the main producers [19]. Due to the new trends in both consumption and commercialization (for example, the sale of frozen fillets), a large number of byproducts are generated during the sea bass production process, which needs to be managed properly, assuming an additional cost for the processing industries [20]. In fact, studies have already been carried out focusing on obtaining high added-value compounds from seabass byproducts [20,21].

Therefore, the present study aims to explore the potential application of fermentation, using LAB isolated from sea bass viscera, in order to valorize sea bass byproducts by obtaining high added-value antioxidant compounds.

2. Materials and Methods

2.1. Samples

Sea bass samples were purchased from a local supermarket, and they were obtained from the Mediterranean Sea. They were quickly preserved using an ice bath after their death. The same day the samples were obtained, they were eviscerated, and the meat was separated from the byproducts (skin, head, tail, thorns, and backbone). Subsequently, all the parts, except the viscera, were frozen until needed for use.

2.2. Chemicals

The radical ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Folin-Ciocalteau reagent 1N, gallic acid, and potassium persulfate ($K_2S_2O_8$) were purchased from Sigma–Aldrich (Steinheim, Germany). The Gram color kit containing crystal violet, Lugol PVP, safranin, and decolorizing solution was purchased from Liofilchem Bacteriology Products (Roseto, Italy). Anaerocult® anaerobic environment system was purchased from Merck (Darmstadt, Germany). The enzyme ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) catalyst used for the polymerization of polyacrylamide gels were obtained from Sigma-Aldrich (St. Louis, USA). Acetonitrile and dithiothreitol (DTT) were purchased from VWR (Leuven, Belgium). The molecular weight pattern 10–250 kDa was obtained from BioRad (Hercules, California). Ethanol 96° was purchased from GUINAMA S.L.U. (Valencia, Spain).

2.3. Isolation of Bacteria

Once the fish samples were eviscerated, those bacteria existing in the digestive tract were isolated. For that purpose, the digestive system of the sea bass samples was separated into three parts, the stomach, small intestine, and colon. Each of these parts was immersed in 200 mL of Man, Rogosa, and Sharpe broth (MRS Broth, Liofilchem Bacteriology Products, Roseto, Italy) and incubated at 37 °C for 48 h under anaerobic conditions, using an Anaerocult® device. Then, serial dilutions were made and growth in Petri dishes with Man, Rogosa, and Sharpe agar (MRS Agar, Liofilchem Bacteriology Products, Roseto, Italy) and incubated at 37 °C for 48 h. Finally, 8 strains were isolated from each part of the digestive tract, and they were identified using a code about the part of the intestinal tract from where they were isolated ("S" for stomach, "I" for small intestine, and "C" for colon) followed each one by a

number. All these operations were carried out under sterile conditions in a Telstar MH 100 laminar flow hood (Telstar, Terrassa, Spain).

2.4. Identification of Bacteria

To identify the type of bacteria isolated, the colonies were characterized by their characteristic morphologies and their type of fermentation [22,23]. For the analysis of morphological characteristics, shape, surface, edge, and color of the colonies were considered. Moreover, a Gram stain was performed to study the characteristics of the cell membrane and the morphology of the bacteria. The production of acid during fermentation was measured by the decrease of pH.

In order to identify bacteria genetically, a 16S rRNA gene sequencing was performed. The identification of isolates was performed using the method described by Chenoll et al. [24] with some modifications. Briefly, the main modification was the primers; in the cited reference, the primers used were 616V and 630R, however, in our case, we used 616V and 699R. The DNA culture was extracted using the high pure PCR template preparation kit (Roche). The 16S rRNA sequence was amplified and sequenced using an Applied Biosystems ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Foster City, CA, USA). The DNA templates were amplified by PCR using the universal primers amplifying a 1000 bp region of the 16S rRNA gene; 616V: 5'-AGAGTTTGATYMTGGCTCAG-3' for the forward primer and 699R: 5'-RGGGTTGCGCTCGTT-3'for the reverse primer. The 616V and 699R primers, Taq DNA polymerase, and dNTP mix were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The DNA templates were amplified by initial denaturation at 94 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. The integrity of the PCR products was assayed by the development of single bands following electrophoresis for 1 h at 100 V

in 2% (w/v) agarose gels in Tris–borate EDTA buffer. Amplicons were purified using the commercial Metabion GmbH mi-PCR purification kit (Planegg, Germany), followed by sequencing reactions using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems), premixed format. The resulting sequences were automatically aligned and inspected visually and then compared with the on-line tool BLAST [25]. The strain was identified based on the highest scores. The isolated and identified bacteria are kept at the Department of Preventive Medicine and Public Health (Faculty of Pharmacy, Universitat de València) and are available to any research group which is interested in working with them.

2.5. Elaboration of Fish Meat and Fish Byproducts Broths

Four different growth broths were elaborated: waste broth (WB; fish byproducts), meat broth (MB; fish fillets), waste broth 2% glucose (WBG; fish byproducts + 2% glucose), and meat broth 2% glucose (MBG; fish fillets + 2% glucose).

The procedure carried out to prepare the broths was the same in all of them. Firstly, an Oster 4655 crusher (London, United Kingdom) was used for crushing fish byproducts or meat, following a ratio of 1:3 (w/v) with water. Subsequently, the broths were centrifuged (4000 rpm, 15 min) using an Eppendorf 5810R centrifuge (Hamburg, Germany). The supernatants were poured into 1 L bottles, and 2% glucose was added to the corresponding broths. Then, they were pasteurized using an SBS30 bath (Staffordshire, United Kingdom) at 85 °C for 20 min [26]. Once pasteurized, they were again centrifuged at 4000 rpm for 15 min. The different broths were frozen until use.

2.6. Fermentation

To carry out the fermentation process, 2 mL of exponentially growing bacteria (37 °C for 12 h) were inoculated in 100 mL MRS Broth at a final

concentration of 10^7 CFU/mL and incubated in different tubes at 37 °C for 24, 48, and 72 h. To verify that the fermentation was performed appropriately, the pH was measured, and the bacterial load count was carried out every 24 h.

After the incubation period, the tubes were centrifuged in an Eppendorf 5810R centrifuge (Hamburg, Germany) at 4000 rpm for 10 min. The supernatants, once cleaned of cells, were frozen at −20 °C.

2.7. SDS-PAGE Electrophoresis

In order to evaluate the different sizes of fish protein residues after 72 h fermentation, SDS-PAGE electrophoresis was performed according to the method previously described [27].

2.8. Extraction and Identification of Phenolic Acids

To determine the phenolic acids produced by the LAB, the method described by Denardi-Souza et al. [28] was carried out. For that purpose, LC-ESI-qTOF-MS was used.

The equipment used for the analysis consisted of LC Agilent 1200 (California, USA) chromatography. The column used was a Gemini C18 50 × 2 mm, with 100 Å and a particle size of 3 µm (Phenomenex). The mobile phases used were water as solvent A and acetonitrile as solvent B, both acidified with 0.1% formic acid. The chosen elution gradient was 0 min, 5% B; 30 min 95% B; 35 min, 5% B, at a flow rate of 0.3 mL/min. Finally, the volume injected was 20 µL, and the column equilibrated 3 min before the next analysis.

Mass spectrometry was performed with an Agilent ultra high definition accurate mass Q-TOF-MS 6540, equipped with Agilent dual jet stream (Dual AJS ESI) electrospray ionization source. It was programmed in the negative ionization mode with the following conditions: drying gas flow at 8 L/min, drying gas temperature at 350 °C, nebulizer pressure at 30 psi, capillary

voltage at 3.5 kV, voltage fragmentor at 175 V, and scan range from 20 to 380 m/z. Collision energies of 10, 20, and 40 eV were used for the targeted MS/MS experiments. Finally, integration and data elaboration were managed using the Masshunter qualitative analysis B.08.00 software.

2.9. Antioxidant Activity

To determine the total antioxidant capacity, the TEAC (Trolox equivalent antioxidant capacity) assay was used as previously described [29].

Briefly, 25 mL of ABTS (7 mM) was mixed with 440 µL of $K_2S_2O_8$ (140 mM) and kept at room temperature for 12–16 h in darkness to obtain the radical $ABTS^{\bullet+}$. This solution was diluted 1/100 in ethanol in order to obtain a working solution with an absorbance of 0.700 ± 0.020 measured at 734 nm (A_0, initial absorbance). Then, 100 µL of the appropriately diluted extracts were added, and the absorbance was measured at 3 min (A_f) in a Perkin–Elmer UV/Vis Lambda 2 spectrophotometer (Perkin–Elmer, Jügesheim, Germany). The measurements were made in triplicate. The percentage of inhibition (% Inhibition) was calculated using the following equation (1);

$$\%\, Inhibition = \left(1 - \frac{A_f}{A_0}\right) \times 100 \quad (1)$$

In order to obtain the antioxidant capacity, a standard curve was first established with Trolox, and the percentage of inhibition of the samples was interpolated. The results were expressed as micromolar Trolox equivalent (µM TE).

2.10. Statistical Analysis

The statistical analysis was performed using the InfoStat® software version 2018. All experiments were performed in triplicate, and the differences between the groups were analyzed using a one-way analysis of the variance

(ANOVA) followed by the Tukey HSD post-test for multiple comparisons. The level of significance was considered as $p \leq 0.05$.

The correlations were established using the StatAdvisor® software version 2018 and Pearson's test. The range of correlation coefficients ranged from −1 to +1, and they measured the strength of the linear relationship between the variables. A p values ≤ 0.05 indicate correlations significantly different from zero, with a confidence level of 95.0%.

3. Results and Discussion

3.1. Isolation and Identification of Bacteria

Although 64 different strains were initially isolated (8 from each part of the digestive tract of different sea bass fishes), only 30 were finally obtained (7 from the stomach, 7 from the intestine and 16 from the colon), due to the rest having either stopped growing or not showing adequate growth. After the strains were correctly isolated and identified, different aspects of the colonies were analyzed. All had a white, circular shape and were opaque. In addition, they did not present any type of pigment, which fits perfectly with the description of LAB provided by Astuti [22].

Subsequently, a Gram stain of each of the isolated colonies was performed, and, as a result, Gram-positive cocci and bacilli were obtained. After the first identification of the morphology, different bacteria from different parts were selected and fermented with two types of broths to see if the pH was modified. The pH decreased independently of the colonies studied as fermentation time increased, whereas this effect was not observed in the controls (Table 1).

Table 1. pH results during fermentation.

Sample	0 h	24 h	48 h	72 h
MB CONTROL	6.97	6.98	6.89	6.84
WB CONTROL	6.87	6.80	6.76	6.63
S3 MB	6.97	6.36	6.01	5.71
S3 WB	6.87	6.15	5.92	5.70
S4 MB	6.97	5.70	3.99	3.83
S4 WB	6.87	5.55	3.80	3.73
S6 MB	6.97	6.34	5.93	5.69
S6 WB	6.87	6.27	5.81	5.53
S7 MB	6.97	6.75	4.45	4.73
S7 WB	6.87	6.67	5.10	3.74
I1 MB	6.97	6.60	5.99	5.68
I1 WB	6.87	6.43	5.80	5.55
C7 MB	6.97	5.54	4.45	4.30
C7 WB	6.87	5.45	5.10	5.23
C8 MB	6.97	6.45	6.12	4.54
C8 WB	6.87	6.30	4.38	4.35

MB, meat broth; WB, waste broth; S, bacteria from stomach; I, bacteria from small intestine; C, bacteria from colon.

With the results obtained after carrying out the above-mentioned tests, it could be concluded that the isolated bacteria could belong to the LAB family since their morphology was identical to the LAB, the compounds produced during the fermentation were acidic and promoted a decrease in pH, and also because they were isolated in MRS medium, which is suitable for LAB growth.

However, in order to obtain a clear bacteria identification, a 16S rRNA gene sequencing was performed for four of these bacteria. The results obtained showed that isolated bacteria were *Lactobacillus plantarum*, concluding that the isolated bacteria were LAB. For instance, several authors have isolated lactic bacteria (mainly *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Carnobacterium*, and *Lactococcus* genera) from the intestinal

tract of different types of fish [11,30,31], and the stomach, intestine, and gills using the same method [32].

3.2. Fermentation

Regarding the results of the bacterial load test performed to choose the best culture broth, as can be seen in Table 2, there were no statistically significant differences ($p > 0.05$) between the broths containing 2% glucose (MBG and WBG) and those that do not (MB and WB).

Table 2. Bacterial load (logarithms) from the stomach, intestine, and colon.

Heading	MB	MBG	WB	WBG
Stomach bacteria	9.96 ± 1.63 ^{A}	10.23 ± 1.62 ^{A}	9.97 ± 2.13 ^{B}	9.93 ± 1.62 ^{B}
Intestine bacteria	8.92 ± 3.84 ^{A}	7.85 ± 1.69 ^{A}	9.21 ± 1.80 ^{B}	9.36 ± 2.01 ^{B}
Colon bacteria	10.35 ± 1.30 ^{A}	10.47 ± 2.53 ^{A}	10.19 ± 2.69 ^{B}	10.84 ± 1.32 ^{B}

Different letters in the same column mean statistically significant differences ($p \leq 0.05$). MB, meat broth; MBG, meat broth with 2% glucose; WB, waste broth; WBG, waste broth with 2% glucose.

According to Table 2, there were no significant differences between any of the broths tested. However, it was finally decided to use those samples containing 2% glucose in order to provide more carbohydrates to the broth, and thus ensuring bacterial growth.

3.3. Electrophoresis

Regarding the results obtained after using the electrophoresis, a great difference was observed in the protein profile between the different fermented broths. The fermented MBs had higher protein concentration and molecular weight than the WB, which showed a reduced protein concentration and lower molecular weight (10–15 kDa). Figure 1 shows the proteolytic activity of different bacteria strains on the WB. As can be seen in

Figure 1, the strains S4, S7, and C8 showed hydrolysis of the proteins present in the WB. However, the rest of the fermented WBs did not show great differences compared to the control.

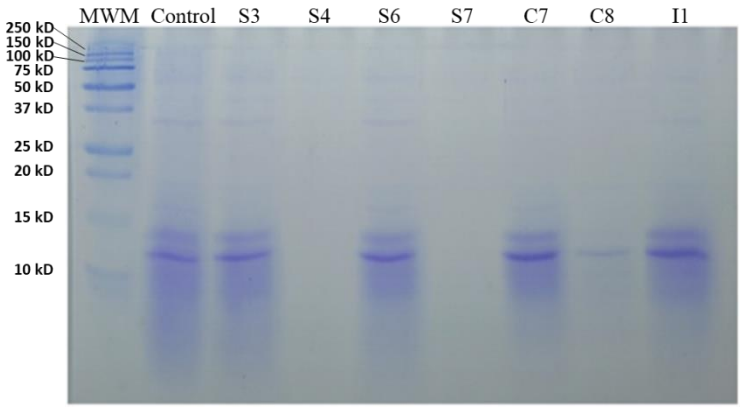

Figure 1. SDS-PAGE analysis of fermented waste broth with different isolated bacteria; MWM, molecular weight marker.

Regarding the MB (Figure 2), there was greater proteolysis when strains S4, C7, and C8 were used; thus, promoting the hydrolysis of proteins of high molecular weight (20–250 kDa). The rest of the strains had a similar amount of protein, like the control, despite the fermentation process.

The results obtained in our study were similar to previous works carried out by other authors. For instance, Altinelataman et al. [26] analyzed the protein profile of sea bass and sea bream muscle hydrolysates by SDS-PAGE. These authors concluded that the peptides often appeared to be included within larger fragments because incomplete hydrolysis occurred. In the same study, the authors were able to identify different proteins such as parvalbumin beta-2 (15 kD) or triosephosphate isomerase B (24–26 kD), which is in accordance with our results.

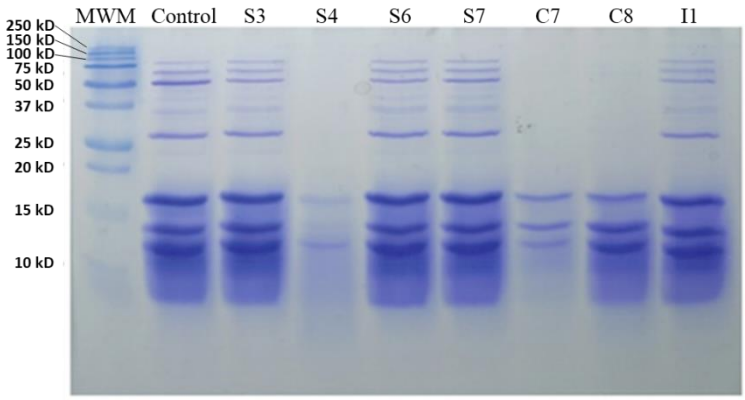

Figure 2. SDS-PAGE analysis of fermented meat broth with different isolated bacteria; MWM, molecular weight marker.

3.4. Phenolic Acids

Two phenolic acids, DL-3-phenyllactic acid, and benzoic acid, were isolated from the broths used in this study resulting from the fermentation process. The results showed an increase in the content of these compounds was obtained during the fermentation time, thus, it was demonstrated that these acids were products of fermentation since these compounds were not found in any of the control samples.

As shown in Table 3, the bacteria isolated from the stomach produced the greatest amount of phenolic acids in MB and WB, being the predominant compound DL-3-phenyllactic acid, reaching up to 467 ppb when the S7 strain was used. Benzoic acid was also found, with lower values, up to 314 ppb when the S4 strain was used. Both phenolic acids increase when fermentation time increases.

Other authors have studied the production of phenolic acids after using the fermentation process in different matrices. For example, Lavermicocca et al. [33] observed an important amount of DL-3-phenyllactic acid after fermentation with 9 LAB in MRS. On the other hand, the study by Lau and

Liong [34] concluded that the concentrations of the acids produced by the LAB species differed according to the strain studied. Urbiene and Leskauskaite [35] investigated the formation of benzoic, sorbic, and nucleic acids during fermentation with LAB. They found concentrations up to 14–23 mg/kg of benzoic acid in the milk fermented by LAB.

In the study conducted by Quattrini et al. [36], the potential of 25 strains of *Lactobacillus plantarum* was explored. They were able to produce 1,2-dihydroxybenzene, benzoic acid, p-hydroxyphenyllactic acid, and DL-3-phenyllactic, which agree with our results. Ramos et al. [37] studied strains of *Lactobacillus plantarum*, finding that these strains produced mevalonolactone, 5-methyl-hydantoin, and benzoic acid. Moreover, Yu et al. [38] examined the use of *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, and *Streptococcus thermophilus* at different incubation temperatures and optimized the production of benzoic acid.

Table 3. Phenolic acids in fermented and control samples. Results expressed in ppb, parts per billion.

Samples		DL-3-phenyllactic acid			Benzoic acid		
		24 h	48 h	72 h	24 h	48 h	72 h
Meat broth (MB)	CONTROL	nd	nd	nd	nd	nd	nd
	S3	11.4 ± 2.5 ^{b}	30.9 ± 1.2 ^{d}	33.0 ± 1.1 ^{b}	52.8 ± 4.3 ^{c}	101.0 ± 2.5 ^{f}	166.4 ± 2.2 ^{e}
	S4	nd	12.7 ± 0.4 ^{b}	29.4 ± 0.7 ^{b}	76.4 ± 3.6 ^{d}	179.8 ± 3.4 ^{g}	314.2 ± 5.7 ^{f}
	S6	64.1 ± 1.9 ^{d}	109.5 ± 3.5 ^{g}	289.9 ± 1.6 ^{f}	29.0 ± 4.2 ^{b}	19.4 ± 0.6 ^{b}	12.9 ± 0.9 ^{b}
	S7	21.0 ± 1.5 ^{c}	192.9 ± 2.3 ^{h}	466.7 ± 3.5 ^{g}	35.0 ± 0.7 ^{b}	51.7 ± 2.3 ^{d}	51.0 ± 2.4 ^{c}
	I1	10.5 ± 1.2 ^{b}	20.7 ± 2.2 ^{c}	163.0 ± 4.1 ^{e}	nd	66.5 ± 2.3 ^{e}	13.2 ± 0.8 ^{b}
	C7	9.6 ± 2.6 ^{b}	38.4 ± 2.3 ^{e}	76.7 ± 1.4 ^{d}	0.7 ± 0.3 ^{a}	4.5 ± 0.8 ^{a}	4.6 ± 0.2 ^{a}
	C8	20.8 ± 1.2 ^{c}	49.7 ± 2.1 ^{f}	51.7 ± 1.1 ^{c}	4.7 ± 0.9 ^{a}	28.0 ± 2.2 ^{c}	92.1 ± 2.3 ^{d}
Waste broth (WB)	CONTROL	nd	nd	nd	nd	nd	nd
	S3	21.9 ± 1.5 ^{b}	145.4 ± 4.2 ^{d}	138.4 ± 4.2 ^{e}	172.7 ± 2.6 ^{f}	59.5 ± 4.3 ^{c}	74.8 ± 6.7 ^{e}
	S4	23.1 ± 1.1 ^{b,c}	186.4 ± 2.4 ^{e}	71.8 ± 2.2 ^{c}	117.9 ± 4.3 ^{e}	101.8 ± 3.3 ^{c}	48.7 ± 1.1 ^{d}
	S6	26.5 ± 2.7 ^{c,d}	22.6 ± 3.6 ^{b}	20.1 ± 1.2 ^{b}	89.5 ± 4.7 ^{c}	82.3 ± 5.2 ^{c}	92.2 ± 6.6 ^{f}
	S7	21.0 ± 1.1 ^{b}	19.7 ± 5.4 ^{b}	146.7 ± 3.2 ^{f}	101.9 ± 2.5 ^{d}	7.6 ± 1.6 ^{a,b}	17.5 ± 4.4 ^{b}
	I1	30.7 ± 2.1 ^{d}	160.1 ± 1.5 ^{d}	175.0 ± 3.3 ^{g}	88.9 ± 5.2 ^{c}	64.7 ± 3.3 ^{c}	21.3 ± 3.1 ^{b,c}
	C7	53.8 ± 1.3 ^{e}	65.8 ± 2.3 ^{e,c}	81.5 ± 0.6 ^{d}	44.8 ± 2.2 ^{b}	22.8 ± 2.1 ^{b}	25.4 ± 3.2 ^{b,c}
	C8	28.0 ± 1.2 ^{d}	80.5 ± 3.3 ^{c}	198.2 ± 4.5 ^{h}	460.5 ± 3.3 ^{g}	120.0 ± 5.2 ^{d}	32.5 ± 4.1 ^{c}

Different letters in the same column mean statistically significant differences ($p \leq 0.05$); nd, not detected.

3.5. Antioxidant Activity

Table 4 shows the results of the antioxidant activity of the fermented samples. As can be seen, the results are quite different, reaching the highest antioxidant activity after using a fermentation process of 24 h. The only sample that achieved significant differences in antioxidant activity respect to control was C8. The C7 sample, and all those from the stomach (S), reached values with a large difference compared to control after 48 h of fermentation. A similar response was observed for stomach samples (S) after 72 h. Considering the results obtained, it can be affirmed that, in the case of MBG, the samples with the highest antioxidant activity are those that have been fermented by bacteria from the stomach (Table 4).

Table 4. Antioxidant activity (μM Trolox equivalent) of the meat broth with 2% glucose fermented by the selected bacteria at 37 °C for 24, 48, and 72 h.

Sample	24 h	48 h	72 h
Control	793.12 ± 63.54 ^{a}	463.70 ± 22.12 ^{a}	452.84 ± 21.68 ^{a}
S3	865.39 ± 44.62 ^{a,b}	1106.30 ± 21.36 ^{e}	1073.35 ± 37.11 ^{d}
S4	987.57 ± 14,66 ^{a,b,c}	1030.92 ± 10.28 ^{e}	882.42 ± 20.72 ^{c}
S6	831.32 ± 78.12 ^{a,b}	815.63 ± 47.03 ^{d}	683.97 ± 112.19 ^{b}
S7	845.94 ± 66.62 ^{a,b}	664.60 ± 20.43 ^{c}	601.92 ± 7.36 ^{b}
I1	1079.94 ± 45.08 ^{c,d}	442.33 ± 23.39 ^{a}	645.77 ± 26.70 ^{b}
C7	1021.60 ± 93.76 ^{b,c}	570.56 ± 18 ^{b}	436.43 ± 28.88 ^{a}
C8	1231.60 ± 118.03 ^{d}	624.76 ± 44.78 ^{b,c}	651.49 ± 46.72 ^{b}

Different letters in the same column mean statistically significant differences ($p \leq 0.05$) from control samples.

As for the samples obtained from the WB (Table 5), the strains S3 and I1 stand out and showed a greater antioxidant activity compared to the control (without bacteria) samples. With this broth, the highest antioxidant activity was reached at 48 h. The C8 strain showed the highest activity of all samples after 72 h of fermentation. Once again, bacteria from the stomach are, in general, the ones with the greatest antioxidant activity.

The results obtained in this study were in close agreement with others previously obtained by different authors such as Sampath et al. [39], who obtained similar results in extracts obtained after the fermentation with LAB of viscera of horse mackerel. In another study, Altinelataman et al. [26] found antioxidants at maximum concentrations, between 0.25 and 0.5 mg/mL, in hydrolyzed sea bass. Sae-Leaw et al. [40] and Vázquez et al. [41] also found similar results with broths prepared with hydrolyzed gelatine and fermented byproducts with LAB of hake, grenadier fish, and horse mackerel among others.

Table 5. Antioxidant activity (μM Trolox equivalent) of waste broth with 2% glucose fermented by the selected bacteria at 37 °C for 24, 48, and 72 h.

Sample	24 h	48 h	72 h
Control	1160.11 ± 95.75 ^{a}	1968.92 ± 104.04 ^{b}	1629.99 ± 63.39^{bc}
S3	2559.33 ± 271.05 ^{c}	4797.41 ± 407.22 ^{e}	2370.39 ± 248.17 ^{d}
S4	1474.13 ± 5.29 ^{a,b}	3726.24 ± 64.46 ^{d}	1355.54 ± 44.41 ^{b}
S6	1218.92 ± 47.74 ^{a}	1189.09 ± 13.98 ^{a}	1085.19 ± 22.04 ^{a,b}
S7	1243.28 ± 74.25 ^{a}	3058.00 ± 71.93 ^{c}	1223.49 ± 75.49 ^{a,b}
I1	2228.97 ± 129.09 ^{c}	2256.52 ± 54.28 ^{b}	2130.25 ± 59.39 ^{c,d}
C7	1362.59 ± 18.57 ^{a}	1140.01 ± 39.86 ^{a}	603.14 ± 39.86 ^{a}
C8	1714.81 ± 60.56 ^{b}	2016.59 ± 147.17 ^{b}	6502.60 ± 638.78 ^{e}

Different letters in the same column mean statistically significant differences ($p \leq 0.05$).

Moreover, Raghavan et al. [42] showed an important antioxidant activity in tilapia hydrolysates. In addition, there are more studies that found antioxidant

peptides in the skin of blacktip shark, blue mussel protein, cod protein, *Trichiurus japonicus* protein, skate skin, and oysters [43,44,45].

3.6. Correlations between Phenolic Acids and Total Antioxidant Capacity

In order to know the correlations between the different antioxidant compounds studied and the total antioxidant capacity (TAC), Pearson's test was performed. Significant positive correlations ($p \leq 0.05$) were found between benzoic acid and TAC ($r = 0.2967$) in fermented MB. On the other hand, the correlation between DL-3-phenyllactic acid and TAC was also significant ($p \leq 0.05$), but it showed a negative correlation ($r = -0.2744$).

However, when the existing correlations in phenolic acids and TAC of the fermented WB were evaluated, the different behavior was evidenced. There was no significant correlation between benzoic acid and TAC ($r = 0.1099$; $p > 0.05$). Nevertheless, there was significant ($p \leq 0.0001$) and positive correlation between DL-3-phenyllactic acid and TAC ($r = 0.5793$). The difference between the results obtained in both samples may be due to the difference in the composition of the broths.

Although no studies have been found that relate the presence of phenolic acids with antioxidant activity in sea bass byproducts, correlations were observed between phenolic compounds and antioxidant capacity in other marine and vegetable food products (e.g., *Laminaria* and *Porphyra* algae) [17,46–48].

5. Conclusions

In conclusion, it has been shown that the fermentation of fish byproducts with lactic acid bacteria (LAB) is a useful tool for obtaining antioxidant compounds. As has been seen, there is a strong positive correlation between DL-3-phenyllactic acid and the total antioxidant capacity. In addition, DL-3-

phenyllactic acid is obtained as a result of the fermentation of fish byproducts, which is an economical, clean, and environmentally friendly process. For all these reasons, the fermentation of fish byproducts can be a good strategy for the reduction of fish byproducts through the valorization of them, contributing to achieving a sustainable development.

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Conflicts of Interest: The authors declare no conflict of interest.

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Isolation, Identification and Investigation of Fermentative Bacteria from Sea Bass (Dicentrarchus labrax): Evaluation of Antifungal Activity of Fermented Fish Meat and By-Products Broths

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Abstract

During fish production processes, great amounts of by-products are generated, representing ≈30–70% of the initial weight. Thus, this research study is investigating 30 lactic acid bacteria (LAB) derived from the sea bass gastrointestinal tract, for anti-fungal activity. It has been previously suggested that LAB showing high proteolitic activity are the most suitable candidates for such an investigation. The isolation was made using a MRS (Man Rogosa Sharpe) broth cultivation medium at 37 ºC under anaerobiosis conditions, while the evaluation of the enzymatic activity was made using the API® ZYM kit. Taking into account the selected bacteria, a growing research was made fermenting two kinds of broths: (i) by-products (WB), and (ii) meat (MB). Both were fermented at three different times (24, 48 and 72 h). Then, the antifungal activities of both fermented by-products and meat broths were determined qualitatively and quantitatively in solid and liquid medium against two different strains of the genera *Penicillium*, *Aspergillus* and *Fusarium*. After the experiments, a total of 30 colonies were isolated, observing a proteolytic activity in 7 of the isolated strains, which belong to *Lactobacillus* genus, and the two more active strains were identified by polymerase chain reaction (PCR) as *L. plantarum*. Several strains evidenced antifungal activity showing an inhibition halo and Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values between 1–32 g/L and 8–32 g/L, respectively. In conclusion, the isolated bacteria of sea bass had the ability to promote the antifungal activity after the fermentation process, thus being a useful tool to give an added value to fish industry by-products.

Keywords: sea bass; by-products; fermentative process; antifungal activity; lactic acid bacteria

1. Introduction

Nowadays, the increased fish production, up to 171 million tons in 2016 [1], is also generating an increase in food waste and by-products, which can represent from 30% to 70% of the total weight of the fish [2].

Among the main by-products obtained from fish, eyes (0.8–1.5%), skin (1–3%), head (9–12%), viscera (12–18%), spines (9–15%) and muscle discards (40–55%) are the most representative [3], all of them having an important content of nutrients and bioactive compounds. For the fish industry, meat constitutes the main useful product, accounting for more than 50% of the fish. However, there are several potential uses for the inedible parts, including skin, viscera, heads and cartilage. Therefore, the industry has been forced to propose several options for the valorization of these by-products in order to develop an integral and sustainable use of fish [4,5].

Thus, taking into account the current trend regarding the increased production of high-added-value compounds from natural sources, which can control biological contamination, fish by-products can constitute a useful tool [6]. In this sense, the use of fermentation can be an interesting strategy to recover the valuable compounds from fish by-products [7]. Fermentation is a conventional process used since ancient times for food preservation, however, it has not been traditionally used to recover high-added-value compounds from fish by-products, although it presents a clear potential for this purpose [8]. For instance, some previous research evaluated lactic acid fermentation produced by lactic acid bacteria (LAB) to recover biomolecules from different by-products, observing its great potential to extract oil [9,10,11,12], chitin [13,14], proteins and antimicrobial and antioxidant compounds from fish by-products [7,9,15]. However, at this stage of development, there are no studies evaluating the effect of fermentation assisted by LAB to recover compounds from sea bass by-products with an

antifungal activity. One of the factors to take into account to maximize the recovery of compounds is the isolation and use of bacteria belonging to the targeted fish, as using microorganisms other than the ones belonging to the natural microbial flora of the fish would cause non-desired alterations at the end products.

For instance, microorganisms isolated from sea bass viscera or metabolic products from these microorganisms could be used in order to inhibit growth or eliminate unwanted biological contamination, thus increasing food safety and product shelf life [16]. In this sense, other previous studies have confirmed the use of LAB, especially *Lactobacillus plantarum*, as the most relevant antifungal species in food, being an alternative to conventional preservatives, and showing an important inhibition of different fungi of genera *Aspergillus*, *Penicillium* and *Rhizopus*, among others [17].

In this sense, as it is shown in Figure 1, the general objective of the present study was to screen for the enzymatic activity of lactic acid bacteria strains isolated from sea bass gastrointestinal tract in order to select the most proteolytic ones. These were then used to ferment by-products from sea bass to obtain compounds endowed with antifungal activity.

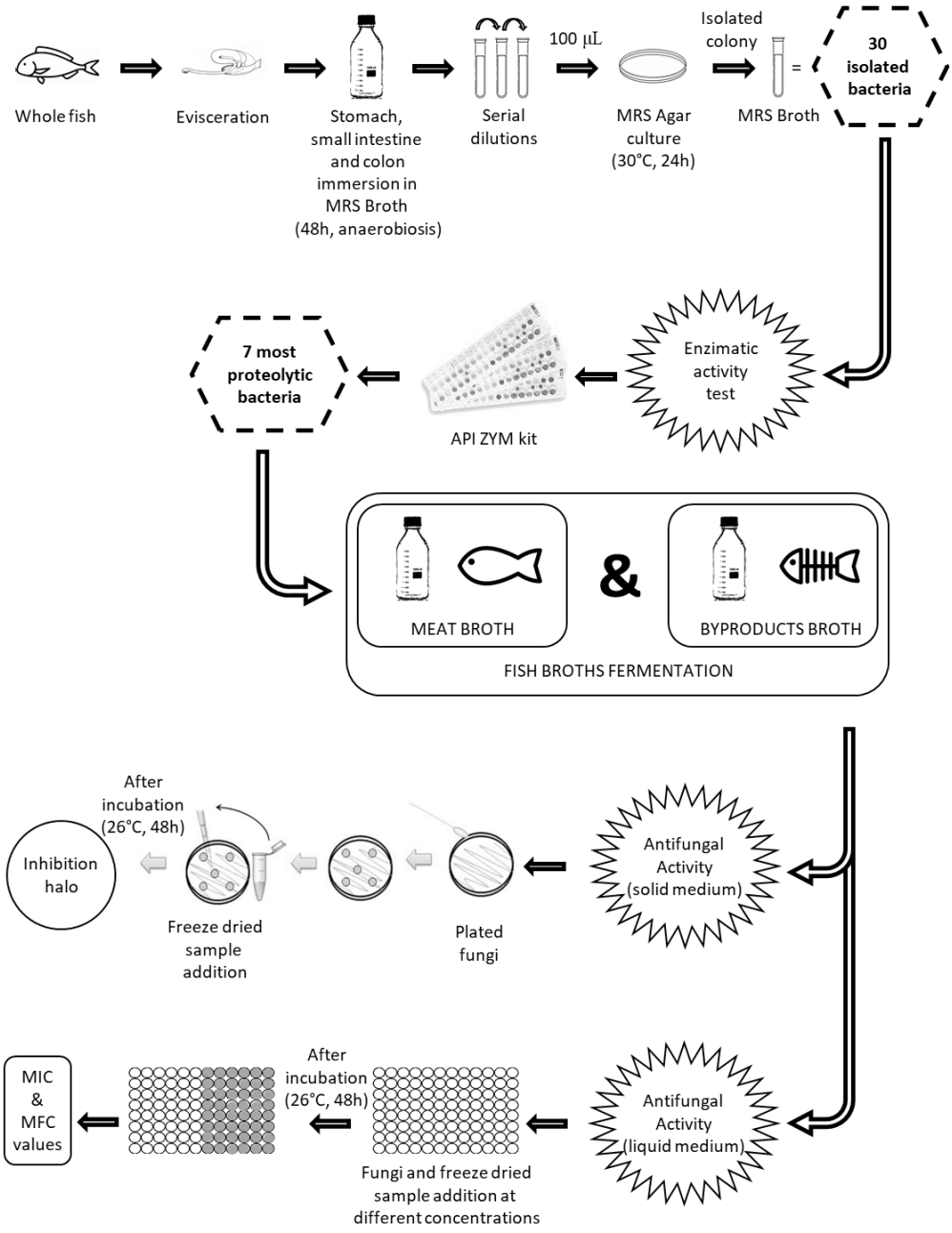


Figure 1. Schematic representation of the experimental set-up. MRS: Man, Rogosa & Sharp; MIC: Minimum Inhibitory Concentration; MFC: Minimum Fungicidal Concentration.

2. Materials and Methods

2.1. Materials

2.1.1. Samples

Fresh sea bass (*Dicentrarchus labrax*) samples (whole fish) fished from the Mediterranean Sea were bought at a local market in Tarragona, Spain. They were kept fresh at a temperature of 4 °C until dissection.

2.1.2. Microorganisms

A total of 30 bacteria were isolated from sea bass (sixteen from the colon, seven from the intestine and seven from the stomach). To carry out the different tests, we selected the seven bacteria with the highest proteolytic activity [18] (two from the colon, one from the intestine and four from the stomach).

In order to evaluate the antifungal activity, strains of *Aspergillus parasiticus* CECT 2681, *Penicillium expansum* CECT 2278 and *Penicillium flavus* CECT 2949 were obtained from the Spanish Type Culture Collection (CECT Valencia, Spain). *Fusarium graminearum* ITEM 126 and *Fusarium verticillioides* ITEM 1205 strains were obtained from the Agro-Food Microbial Culture Collection of the Institute of Sciences of Food Production (Bari, Italy). The fungus *Penicillium verrucosum* VTT D 01847 was obtained at the VTT Technical Research Center of Finland (Turku, Finland).

2.1.3. Consumables

The culture media used consisted of MRS (Man Rogosa Sharpe) Broth, MRS Agar, PDA (Potato Dextrose Agar) and PDB (Potato Dextrose Broth). They were purchased from Liofilchem Bacteriology Products (Roseto, Italy). The anaerobic microbiology incubation system (Anaerobic®) was obtained from Merck (Darmstdt, Germany). For the Gram test, the Gram Color Kit containing

Crystal Violet, Lugol PVP, Safranina and Decolorizing solution was used, and it was purchased from Liofilchem Bacteriology Products (Roseto, Italy). To study the enzymatic activity of the bacteria, the API® ZYM kit, containing the ZYM A + ZYM B reagents (BioMérieux, Marcy-l'Etolie, France), was used. Deionized water was obtained from a Mili-Q water purification system (Millipore, Bedfore, MA, USA). Likewise, methanol was purchased from Fisher Scientific (Madrid, Spain) and acetonitrile from VWR (Leuven, Belgium).

For the polymerase chain reaction (PCR), the "High Pure PCR Template Preparation Kit" from Roche Molecular Systems (Pleasanton, CA, USA) was used. To extract the DNA from the bacteria, ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and the BigDye Terminator v3.1 cycle drying kit were used, which were acquired from Applied Biosystem Inc. (Foster City, CA, USA), primers 616V and 699R, Taq Dna Polymerase and the five nucleobases were obtained from Thermo Fisher Scientific (Waltham, MA, USA), the "my PCR purification kit" was purchased from Metabion (Gmbh, Germany).

2.1.4. Fish By-Products Broths

Two types of broth were prepared from sea bass: (i) meat (MB) and (ii) by-products (WB), which consisted of a mixture of by-products (37 g of head/100 g of by-products, 29.7 g of guts/100 g of by-products, 13.3g of skin/100 g of by-products and 20g spines/100 g of by-products).

To obtain the broth, the meat was crushed with distilled water, 1:3 ratio (w/v), with an OSTER crusher (Albacete, Spain), using the following conditions: 220–240 V, 50/60 Hz and 600 W for 10 min. The mixture was then centrifuged using an Eppendorf 5810R centrifuge (Hamburg, Germany) for 15 min at 4000 rpm. The supernatant was taken in order to obtain a broth without impurities. Then, it was pasteurized in a heat bath at 85 °C for 20 min [19]. Finally, it was

centrifuged again in order to obtain a cleaner broth and to facilitate the absence of interferences in the performance of the different tests.

2.2. Methods

2.2.1. Isolation and Identification of Bacteria by Morphology, Catalase Test and PCR

To get the bacteria isolated from sea bass, the sample was firstly dissected. The stomach (S), small intestine (I) and colon (C) were separated and grown in a 900 mL MRS Broth liquid medium (LAB selective medium). They were incubated in a culture oven at 37 °C during 48 h under anaerobic conditions using Anaerocult® A.

In order to isolate the different colonies after the incubation, serial dilutions from 10^{-2} to 10^{-8} were made. Then, 100 µL of each dilution were plated in MRS Agar. The plates were incubated at 37 °C for 24 h.

After the incubation, different colonies were selected. The isolated colonies were placed in a 15 mL tube with MRS Broth medium. A total of 30 bacteria, 7 from the stomach, 7 from the small intestine and 16 from the colon, were isolated.

In order to determine the morphology of the bacteria, a Gram stain test was performed, and the morphologies were observed with an XJS500 binocular microscope at 40X. The catalase test was performed by mixing an isolated colony in MRS agar with hydrogen peroxide 3%. Moreover, the positive reaction of the catalase test was evaluated by observing bubble formation.

For the identification of the isolated bacteria, they were grown in MRS medium supplemented with 0.05% (w/v) cysteine and incubated anaerobically at 37 °C from 36 to 72 h. The DNA of the samples was extracted

using the "High Pure PCR Template Preparation kit" (Roche), quantified spectrophotometrically and adjusted to a final concentration of 40 ng/µL with ultrapure water, obtained from Sigma-Aldrich (St Louis, MI, USA, USES). To confirm the DNA microarray results, the specific amplification of 16S rRNA gene fragments was performed with "ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit" from Applied Biosystems Inc. (Foster City, CA, USA) by using the previously described primers (616V: 5'-AGAGTTTGATYMTGGCTCAG-3' and 699R: 5'-RGGGTTGCGCTCGTT-3'). DNA purity was checked by standard methods.

For 16S rRNA gene amplification, reaction mixtures contained 2 µL (50 pmol/µL) of primers 616V and 699R, 0.5 µL (2U/L) of Taq Polymerase DNA, 10 µL of 10× buffer and 10 µL of dNTP containing 1 mM of dATP, dGTP, dCTP and dTTP (70 µL of miliQ water and 5.5 µL of DNA, with a total of 100 µL). All reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

The DNA template was amplified, with an initial cycle of denaturation at 94 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 1 min, a second cycle at 55 °C for 1 min for hybridization of the primers, a third 1 min cycle at 72 °C for extension and a final cycle of 72 °C for 10 min. The final sequence was analyzed by individual bands in 2% agarose gels (w/v) in tris-borate EDTA buffer for one hour at 100 V, then read by electrophoresis. The amplicons were purified using the commercial "mi-PCR purification kit" purchased from Metabion Gmbh (Steinkirchen, Germany) and subsequent sequencing reactions were carried out with the "Big Dye Terminator v3.1 cycle sequencing kit" purchased from Applied Biosystems. The resulting sequences were aligned and compared with the BLAST online tool. The strain was identified on the highest score.

2.2.2. Enzymatic Activity

The enzymatic activity of the 30 isolated bacteria was studied, using the API® ZYM kit, a semiquantitative method. The results were obtained according to the intensity of the colored reaction, comparing with the table provided by the manufacturer. The results were expressed as nanomoles of hydrolyzed substrate.

To select the strains with the highest proteolytic activity, the trypsin and α-chymotrypsin were selected, taking into account that both are essential for the digestion process, as they are responsible for the degradation of proteins and polypeptides, although others such as cystine arylamidase and valine arylamidase are also proteolytic indicators.

2.2.3. Fermentation of Isolated Bacteria in Meat and By-Products Broths

Once the 7 most proteolytic bacteria were selected, they were inoculated in the different broths (MB and WB) as follows: 1 mL of MRS Broth with bacteria in the exponential growth phase (12 h at 37 °C) was inoculated in 40 mL of the broth. Then, the broths were fermented at 24, 48 and 72 h at 37 °C. A control, using identical conditions, was made for each type of broth.

In order to assess the fermentation process, a bacterial growth study was carried out at 24, 48 and 72 h. Serial dilutions of the different broths were carried out as follows: one hundred microliters of sample were inoculated in a MRS Agar plate to assess bacterial count.

On the other hand, a pH measurement was carried out with the Ph8 + DHS equipment (LabProcess, Badalona, Spain) at different fermentation times, in order to assess the production of organic acids during the fermentation period. The entire procedure was done under sterile conditions in a Telstar MH 100 laminar flow hood (Terrassa, Spain).

2.2.4. Antifungal Activity

In order to evaluate the antifungal activity of the selected bacteria in the different broths after a 72 h fermentation, an antifungal activity test was performed on solid medium, according to the method described by Varsha et al. [20], with some modifications. The test was carried out against fungi of the genera *Penicillium*, *Fusarium* and *Aspergillus*.

Firstly, the samples were lyophilized in order to concentrate and stabilize them. Then, samples (250 g/L) were resuspended in PDB and filtered using a filter (0.22 µm). The PDA plates, specific for fungal growth, were prepared following the manufacturer's specifications. To facilitate the growth of the fungi *Penicillium*, *Fusarium* and *Aspergillus*, a cotton swab dipped in water was used.

Once the fungus was inoculated, a total of eight wells were made with the tip of a sterile pipette, in which 100 µL of the resuspended samples were added. The plates were incubated for 48 h at 26 °C. After the incubation, the results demonstrated that the higher halo of inhibition was related to the higher antifungal capacity of the bacteria present in the fermented broth.

In order to determine the Minimum Inhibitory Concentration (MIC), the method previously described by Fothergill [21] was used. The test was performed in a 96-well plate, as follows: a negative control where only PDB medium was added, and secondly, in order to verify that the medium was not contaminated, a positive control consisting of 100 µL of the suspension fungi at 10^5 spores/mL in PDB, and a volume of 100 µL of sample at final concentration from 0.5 to 250 g/L was used. Immediately, 100 µL of the fungi suspension at 10^5 spores/mL in PDB was added to all wells, except the negative control.

All trials were performed in quadruplicate. After 72 h of incubation, the MIC was estimated. The MIC is defined as the lowest concentration of antifungal agent in which there was a visual absence of growth compared to that produced by the growth in the control well. Moreover, in order to confirm the MIC, 10 µL of the concentrations with no growth observed were inoculated in plates with PDA medium, which were incubated in an oven at 26 °C in order to proceed to their visible growth after 72 h. The Minimum Fungicidal Concentration (MFC) was defined as the minimum concentration of an agent promoting a reduction in the number of viable colonies ≥ 99%, with respect to inoculum. All these operations were carried out under sterile conditions in a Telstar MH 100 laminar flow hood (Terrassa, Spain).

2.3. Statistical Analysis

The statistical analysis was performed using the InfoStat software® version 2018. All experiments were carried out in triplicate and the differences between the groups were analyzed using a one-way analysis of variance (ANOVA) followed by the Tukey HSD (Honestly Significant Difference) post-test, for multiple comparisons. The level of significance considered was $p \leq 0.05$.

The correlations were established using the StatAdvisor software® version 2018 and Pearson´s test. The range of correlation coefficients ranges from –1 to +1, and they measure the strength of the linear relationship between the variables. A p-values ≤ 0.05 indicate correlations significantly different from zero, with a confidence level of 95%.

3. Results and Discussion

All isolated bacteria were Gram-positive since the cell wall remained purple after Gram staining, thus indicating that these bacteria were LAB. Moreover, the main form of the cells was coco-bacilli and catalase-negative, these being the most characteristic of LAB [22].

Subsequently, the enzymatic activity of these 30 bacteria was evaluated by using a semi-quantitative method (API® ZYM kit). The activities of 19 enzymes, including alkaline phosphatase, glucosidase and trypsin, were studied, observing great differences according to the fish matrix (stomach, small intestine or colon) studied (Table 1). Bacteria from the stomach had more esterase and alkaline phosphatase activities, while those isolated from the colon had more α-β-glycosidase and n-acetyl-β-glucosaminidase activities. On the other hand, few bacteria had α-mannosidase, α-frucosidase and β-glucuronidase activities. Likewise, it was observed that stomach bacteria had a similar enzymatic activity, since most of them had the same nanomoles value for the same enzymes.

The main aim of this test was the selection of the most proteolytic bacteria to carry out the fermentation tests, in order to maximize protein hydrolysis and the release of small peptides or amino acids. These bacteria were selected taking into account the presence of proteolytic enzymes, trypsin and α-chymotrypsin; therefore, a total of 7 bacteria were selected, four from the stomach, two from the colon and one from the intestine.

Once the seven most proteolytic bacteria were selected, they were fermented in the different culture broths and a bacterial growth test was carried out on a MRS agar plate at different times (24, 48 and 72 h). Figure 2 shows the bacterial growth in meat and by-product broths.

Table 1. Results of the enzymatic activity of the isolated bacteria (in nmol).

Enzymes	Stomach Bacteria							Small Intestine Bacteria							Colon Bacteria														
	S1	S2	S3	S4	S5	S6	S7	I1	I2	I3	I4	I5	I6	I7	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16
Alkaline Phosphatase	10	≥40	≥40	≥40	≥40	5	5	5	5	5	0	30	5	5	20	10	10	20	10	10	10	0	0	0	0	0	10	5	5	0
Esterase (C4)	5	20	20	20	5	≥40	≥40	20	10	5	0	20	10	5	0	20	30	20	20	20	20	0	0	0	0	0	0	5	5	5
Esterase Lipase (C8)	5	20	10	10	10	20	30	20	30	0	0	10	20	20	0	20	10	10	10	10	10	5	0	0	5	0	5	5	5	5
Lipase (C14)	5	0	0	0	0	0	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leucine Arylamidase	≥40	30	30	30	20	30	20	0	5	5	0	30	≥40	10	20	30	30	≥40	30	30	20	0	5	0	0	0	≥40	0	10	5
Valine Arylamidase	30	5	0	0	0	5	0	5	5	5	0	0	≥40	10	0	5	0	20	5	0	5	0	5	0	0	0	≥40	10	10	5
Cystine Arylamidase	20	0	0	10	0	0	10	10	5	0	0	5	10	0	5	10	0	5	0	0	0	10	0	5	0	5	10	10	10	0
Trypsin	0	5	0	10	0	10	0	0	5	0	0	0	0	0	0	5	0	0	0	0	0	0	5	0	0	0	5	10	10	5
α-chymotrypsin	0	0	10	5	0	0	10	10	0	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	5	0	0	0	0
Acid Phosphatase	30	10	10	10	10	5	5	10	5	0	5	5	≥40	10	5	5	0	10	5	10	10	20	5	10	5	10	≥40	30	30	5
Naphthol-as-bi-phosphohydrolase	30	10	5	10	20	5	10	10	5	5	5	10	≥40	10	5	10	20	10	5	10	10	10	5	20	10	5	30	30	20	10
α-Galactosidase	10	0	0	0	0	0	0	0	5	0	0	0	≥40	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	5
β-Galactosidase	30	0	0	0	0	0	0	0	0	0	0	0	≥40	0	0	5	0	0	0	0	0	0	0	0	5	0	≥40	0	0	5
β-Glucuronidase	0	0	0	0	0	0	0	0	0	0	0	0	≥40	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-Glucosidase	20	10	10	10	10	5	0	0	0	5	0	0	≥40	0	0	≥40	0	5	10	≥40	30	≥40	≥40	5	0	≥40	10	≥40	≥40	5
β-Glucosidase	≥40	10	5	0	10	0	0	5	0	0	0	0	≥40	0	0	30	0	0	20	≥40	30	0	0	30	≥40	0	≥40	0	0	≥40
N-Acethyl-**β**-glucosaminidase	0	0	5	0	0	0	5	0	5	0	0	5	0	10	5	10	20	0	0	0	0	10	5	5	0	0	0	5	0	5
α-Mannosidase	0	0	0	0	0	0	0	0	5	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-Fucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	5	0	0

S: Bacteria isolated from stomach; I: Bacteria isolated from small intestine; C: Bacteria isolated from colon.

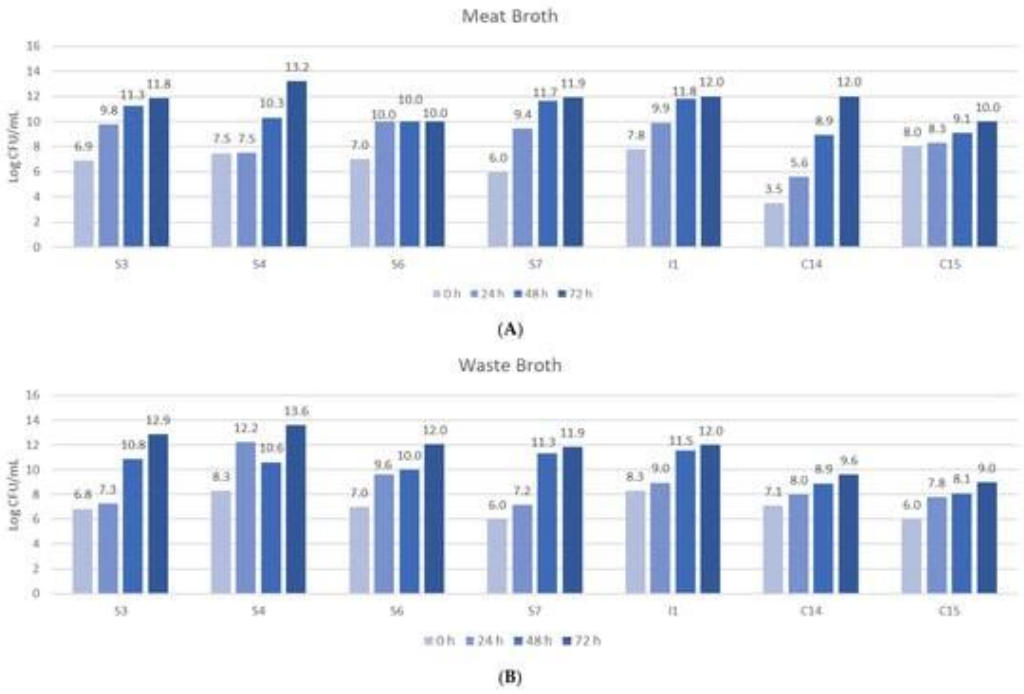


Figure 2. Bacterial growth in (A) meat broth (MB) and (B) by-products broth (WB) at three fermentation times (24, 48 and 72 h). S: Bacteria from stomach; I: Bacteria from small intestine; C: Bacteria from colon.

Regarding the bacterial growth in the by-products broth (WB) after 72 h of incubation, a growth from 6–11, 6–13.6 and 9–12 log CFU/mL occurred for bacteria from the colon, stomach and intestine, respectively. The results demonstrated that stomach bacteria had the highest growth. For meat broth (MB), after 72 h of fermentation, the isolated bacteria from the colon showed a growth from 5 to 12 log CFU/mL, those from the stomach, 6–13.2 log CFU/mL, and those from the intestine, 10–12 log CFU/mL. Thus, in MB, the highest growth was also observed in stomach bacteria.

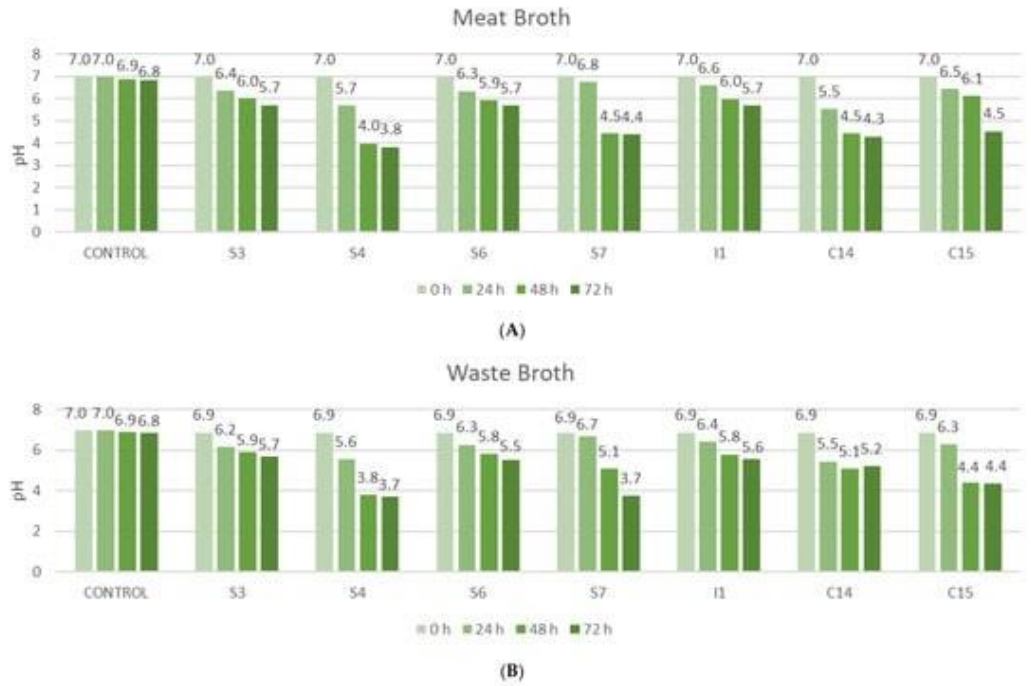


Figure 3. pH values of (A) meat broth (MB) and (B) by-products broth (WB) at three fermentation times (24, 48 and 72 h). S: Bacteria from stomach; I: Bacteria from small intestine; C: Bacteria from colon.

If the broths are compared, there is no difference regarding bacterial growth, but colon bacteria had a faster growth in the WB, while the gut bacteria grew faster in the MB. No differences in growth may be attributed to a similar nutrient content in the different broths. On the other hand, the bacterial growth was checked and the pH of the broths was evaluated for the same purpose, checking that fermentation was taking place.

Figure 3 shows the pH values for the seven bacteria studied. In all strains, it was observed that the pH decreases with the elapse of fermentation period, which means that fermentation occurred, since during the lactic acid

fermentation the bacteria convert simple carbohydrates into lactic acid, ethanol and CO_2, and consequently, pH decreases. Stomach bacteria S4 showed the highest decrease in pH (from 6.9 to 3.7) in the two broths studied. Moreover, the highest decrease of pH in WB (from 6.9 to 3.7) was also shown after using stomach bacteria S7.

Regarding the results obtained about the antifungal activity, strain S3, isolated from the stomach, presented the highest antifungal activity against all fungi, in the different fermented broths, except for *P. verrucosum*, which showed an important resistance to MB (Table 2).

If the results of the broths are observed separately, it is possible to find that the WB produced a greater number of antifungal compounds since strains S3 and S4 showed an important antifungal activity against all the fungi studied. The S7 strain presented an 8 mm inhibition halo against P. expansum and the C14 strain for F. graminearum. In the MB, strain C14, isolated from the colon, presented an important antifungal activity against the fungi *P. verrucosum* and *F. gramineraum*, while strain S3 had antifungal activity against all the fungi except for *P. verrucosum*. Strains S6, I1 and C15 did not show any antifungal activity for the tested broths (Table 2).

If the results are compared by fungi, *P. expansum* was the most sensitive fungus, especially with those compounds obtained from WB. On the other hand, *P. verrucosum* was the fungus presenting the highest resistance against all strains (Table 2).

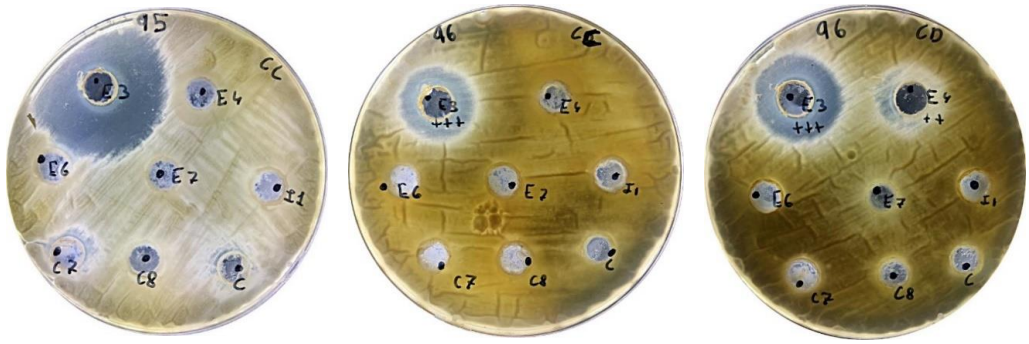
As in our study, other authors also observed an important antifungal activity of the compounds generated by LABs (*Lactobacillus* strains) against *A. parasiticus*, *A. flavus*, *F. verticilloides* and *P. expansum* [23]. Moreover, in another study with bacteria isolated from gills and stomach, Bajpai et al. [24] also observed that some LAB strains showed an important antimicrobial

activity against pathogenic bacteria. In the same line, another study also showed the antifungal activity of 11 LABs strains against *Fusarium oxysporum* [20]. Furthermore, the halo of inhibition presented by strain S3 against fungi *P. expansum* and *A. parasiticus* is also shown in Figure 4.

Table 2. Results of antifungal activity of fermented MB and WB in solid medium.

Fungi	Control	S3	S4	S6	S7	I1	C14	C15
	Meat Broth							
Aspergillus parasiticus	-	+++	-	-	-	-	-	-
Aspergillus flavus	-	+	-	-	-	-	-	-
Penicillium expansum	-	+++	-	-	-	-	-	-
Penicillium verrucosum	-	-	-	-	-	-	+	-
Fusarium graminearum	-	+++	-	-	-	-	+	-
Fusarium verticillioides	-	+++	-	-	-	-	-	-
	By-Products Broth							
Aspergillus parasiticus	-	+++	++	-	-	-	-	-
Aspergillus flavus	-	+	+	-	-	-	-	-
Penicillium expansum	-	+++	+++	-	+	-	-	-
Penicillium verrucosum	-	+	+	-	-	-	-	-
Fusarium graminearum	-	+	+	-	-	-	+	-
Fusarium verticillioides	-	+++	+++	-	-	-	-	-

S: Bacteria from stomach; I: Bacteria from small intestine; C: Bacteria from colon. +: <8 mm, ++: 8–10 mm, +++: >10 mm of inhibition halo. "–": it did not show antifungal activity.



(a) **(b)** **(c)**

Figure 4. Antifungal activity represented by the halo of inhibition in Petri dishes of: (a) fermented MB against *P. expansum*, (b) fermented MB against *A. flavus*, and (c) fermented WB against *A. parasiticus*.

The tests performed to determine the antifungal activity in liquid medium are shown in Table 3 (bacteria not shown did not have any antifungal activity). The compounds produced by the MB lacked antifungal activity with all strains except for the C14 bacteria, isolated from the colon, which presented an important resistance against *P. verrucosum*, with a MIC value of 16 g/L and a MFC value of 32 g/L. Moreover, C14 bacteria also showed a great ability to delay the growth of *F. graminearum*, with a MIC value of 16 g/L.

Regarding the WB, strain C14 had a MIC of 16 g/L against the fungus *F. graminearum*, although it was not possible to establish a MFC value. As in the test conducted on solid medium, strain S4 gave positive results regarding the growth inhibition for all fungi studied, ranging from 1 g/L for *F. gramineraum* to 32 g/L for *A. flavus*. However, MFC values were only established for *Fusarium* genus. Strain S3 had an antifungal activity against all fungi (1–16 g/L). However, as for strain S4, it was not possible to establish a MFC value for the

Aspergillus family. On the other hand, strains I1, C15, S6 and S7 did not show either MIC or MFC values.

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Fungicide Concentration (MFC).

Fungi	C14		S3		S4		C14	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
	Meat Broth		Waste Broth					
Aspergillus parasiticus	nd	nd	8	nd	16	nd	nd	nd
Aspergillus flavus	nd	nd	16	nd	32	nd	nd	nd
Penicillum expansum	nd	nd	1	16	8	16	nd	nd
Penicillium verrucosum	16	32	4	8	4	16	nd	nd
Fusarium graminearum	16	nd	2	16	1	31	16	nd
Fusarium verticillioides	nd	nd	1	8	4	16	nd	nd

The results obtained in our study are similar to those obtained by other authors in freshwater fish. So, Ruthu et al. [12] found an inhibitory activity in fermented carp heads. They obtained a MFC value ranging from 60 to 96 mg/ml against the different strains of *Penicillium*. In the same study, the authors also obtained negative results against *A. flavus*.

The MIC and MFC values are related to the pH values obtained. For instance, the S4 and C14 strains were those presenting more acidic values (3.7 and 4.3, respectively) after 72 h of fermentation. This fact can be attributed to the mechanism of action of LABs against fungi and bacteria, since the accumulation of lactic acid and other organic acids produced by them reduce the pH and consequently, have an inhibitory effect on fungi [25].

In a previous study, it was demonstrated that *L. plantarum* presented an antifungal activity against *Penicillium* in deteriorated citrus and yogurt [26]. Moreover, in the study by Cray et al. [27], *L. plantarum* delayed the growth of *Fusarium culmorum* in oat drink. In contrast, in the study conducted by Ryu et al. [28], a strain of *L. plantarum* isolated from kimchi presented antifungal activity against *Aspergilllus* and *Penicillium* genera fungi.

Bacteria S3 and S4 were identified by PCR, due to the highest antifungal activity in solid and liquid medium. They were identified as *Lactobacillus plantarum*. According to the studies of Ringo et al. [29], this microorganism was also isolated in Atlantic salmon and Arctic trout [30], pollock [31] and cod [32].

In contrast, there are no previous studies identifying *L. plantarum* in sea bass. It is necessary to emphasize that the intestinal microflora of fish is very variable, and depends on many factors, including environmental conditions and temperature [33], but usually, LABs are the main bacteria in the digestive tract of a healthy fish. According to the study of Nair et al. [34], genera *Carnobacterium* and *Lactobacillus* are the most common bacteria found in the digestive tract of fish. Moreover, Sahnouni et al. [35] also stated that the most common genus found in fish digestive tract is *Lactobacillus*.

One of the main advantages of using *L. plantarum* deals with its use in the industry as a probiotic. For instance, different studies assessed the potential use of fish intestinal tract isolates as probiotics [36]. Moreover, *L. plantarum* is

considered as GRAS (Generally Recognized as Safe) and its nutritional applications have been previously evaluated [37]. Since the isolated bacteria are adapted to very low pH, the potential use in humans is considered, but further in vivo research is still needed to verify their application in fish by-products' valorization to obtain antifungal compounds.

4. Conclusions

From the results obtained in this work, it can be concluded that 30 lactic acid bacteria strains were identified and isolated from sea bass gastrointestinal tract. When the most proteolytic strains were used to ferment by-products from the same matrix (sea bass), the fermented extracts obtained presented an antifungal activity against several toxigenic fungi, especially those obtained from sea bass by-products. These results can be useful for the valorization of fish by-products, and also for the development of new natural compounds, which can be used as preservatives in the food industry. However, more studies should be carried out in order to optimize the fermentation process as well as the best way to isolate the targeted antifungal compounds.

Author Contributions: F.J.B. and G.M. conceived and designed the study; F.J.M.-Q., A.P., A.T. and C.L. performed the experiments; F.J.M.-Q., A.P. and F.J.B. wrote the manuscript; M.-J.R., P.T., F.J.B and J.M. supervised the study and reviewed the manuscript. All authors have read and approved the final manuscript.

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Sea Bass Side Stream Extracts Obtained by Pulsed Electric Fields: Nutritional Characterization and Effect on SH-SY5Y Cells

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Foods (Under review)

Abstract

Fish side streams are an environmental and economic problem. In this work, pulsed electric fields (PEF) extraction was optimized and used as a new way for their valorization. Sea bass head, skin, viscera and backbone were used for the study. PEF technology improved the extraction of proteins and antioxidant compounds from head and skin, while it was not successful for viscera. SDS-PAGE showed that the protein molecular weight distribution was affected by the extraction process, revealing differences between the control and PEF extraction conditions. In addition, the extraction of macro-minerals and micro-minerals were also evaluated. The effect of PEF on this parameter differed according to the matrix and the mineral studied. Heavy metals were also taken into account, studying the presence of As, Cd, Hg and Pb in the extracts. PEF pre-treatment reduced the presence of As in skin, viscera and backbone, ranging from 18.25 to 28.48%, according to the matrix evaluated. The potential antioxidant bioactive peptides were also analysed, revealing that the treatment of the sample directly affects to the variety of them. Finally, the effect of the extracts on cell viability was assessed using SH-SY5Y cells, observing an increase in cell viability for the extracts obtained from head.

Keywords: pulsed electric fields; fish side streams; antioxidants; minerals; heavy metals; bioactive peptides, cell viability, SH-SY5Y

1. Introduction

Recently, the European Commission adopted a circular economy action plan, as a part of the European Green Deal. Among others, it is designed to promote circular economy processes and also to ensure that waste is prevented and the resources used are kept in the EU economy for as long as possible [1].

The fishing industry would be a good starting point to tackle this strategy, considering several factors such as: i) the increase in consumption and the expansion of fish pro-duction over the last decades; ii) the large amount of waste generated and iii) the potential commercial interest of this waste. The latest estimates indicate that global fish production reached about 178 million tonnes in 2020 and consumption increased at an average an-nual rate of 3.0% between 1961 and 2019 [2].

Fish processing side streams can range between 30% to 70% of the fresh weight (dif-fering according to the fish species). In this sense, muscle cuts represent 15-20%, while other products are also discarded as a low-value material such as viscera, bones, head, skin and fins representing 12-18%, 9-15%, 9-12% and 1-3%, respectively [3]. These sides streams have been traditionally used primarily to produce fishmeal and fish oil for ani-mal feeding. However, this is a small portion (currently estimated at 27-48%) and most of the side streams are discarded [2].

With these data on mind, it is necessary to drive our attention to the development of additional valorization approaches and the research about their beneficial properties that could enhance the minimization of this fish side stream while, at the same time, higher economic benefits are achieved, and environmental impact reduction is promoted.

However, it should be considered that the choice of the extraction method for these side streams is just as important as the valorization of the side streams themselves. In the context of the European Green Deal, much effort has been devoted to developing more sustainable and environmentally friendly extraction processes. These innovative ap-proaches, so-called "green technologies", reduce some of the limitations associated with conventional extraction methods by requiring less time, energy, and solvent, thus miti-gating the impact on the environment [4–6].

Several studies have been focused on the utilization of fish side streams through the application of green technologies [6–8]. Specifically, pulsed electric fields (PEF) is a tech-nology based on the application of short electrical pulses at high voltage for brief periods and have been demonstrated to be a sustainable alternative to conventional extraction methods [4,5]. Some applications of PEF in fish side streams can be found in literature, confirming their potential to be utilized as an ecologically acceptable and cost-effective tool to valorize them [6,8]. Specifically, in our research group, PEF has been applied to trout and sole side streams to obtain extracts with antioxidant [9], antibacterial and anti-inflammatory activities [10].

In the framework of the European community, sea bass (*Dicentrarchus labrax*) is one of the most widely consumed products, as well as one of the six most farmed species [11]. Accordingly, both the characterization and utilization of sea bass side streams have at-tracted recent research interest. The chemical composition analysis of skin, guts, gills, liv-er, head and fish bones from sea bass has demonstrated that they are an interesting source of proteins, minerals (calcium and phosphorus) and lipids. The lipid fraction contains high amounts of unsaturated fatty acids (particularly monounsaturated and omega-3 fatty acids) [12]. In addition, our research

group has obtained antioxidant compounds through the fermentation of sea bass by-products by bacteria isolated from their fish viscera [13].

On the other hand, other techniques have been used for the valorization of sea bass side streams, obtaining extracts with antioxidant capacity through the application of ul-trasound-assisted extraction (UAE) [14] and pressurized liquid extraction (PLE) [15]. Specifically, studies on nutritional value have been carried out. For instance, it has been shown that protein recovery percentages of 7-28% (muscle), 11-40% (heads), 26-99% (vis-cera), 7-34% (skin), 24-75% (bones), and 10-30% (tails) were obtained after the application of green technologies such as UAE [14] and PLE [15].

Regarding pulsed electric fields technology (PEF), several researchers have used PEF to extract various compounds from fish by-products. For instance, Burnett et al. [16] used PEF to extract lipids from Hoki male gonad. In their study, an electric field strength rang-ing from 0.625 to 1.875 and a frequency between 25 and 100 Hz was applied. They found that the highest lipid extraction yield was obtained at 1.25 kV/cm and 50 Hz. Similarly, Wang et al. [9] used PEF to extract proteins and antioxidant compounds from rainbow trout and sole by-products. The authors used a specific energy ranging from 123 to 300 kJ/kg and an electric field between 1 and 3 kJ/kg to obtain the compounds. These same parameters were also used by the authors to obtain extracts with anti-inflammatory activ-ity and the ability to modulate the intestinal microbiota from the same by-products [10]. Lastly, Franco et al. [17] used PEF pre-treatment to extract antioxidant compounds from sea bream and sea bass by-products. They used 100 pulses, a frequency of 10 Hz, a pulse width of 20 µs, and a specific energy ranging from 17.4 to 29.4 kJ/kg. These studies demonstrate the potential of PEF technology for the recovery of valuable compounds from fish by-products.

As mentioned before, only one study has applied PEF for the valorization of sea bass side streams [17]. However, in this study, the authors only focused on the analysis of total antioxidant activity of the methanolic and aqueous extracts obtained from sea bass gills, bones and heads assisted by PEF and conventional extraction. However, at this stage of development there is a need to characterize the full profile of compounds instead of total antioxidant capacity. Therefore, given the lack of knowledge in this regard, it is important to expand the information and data on the recovery of individual high-added-value compounds from sea bass side streams assisted by PEF. Moreover, it should be also consid-ered the presence of heavy metals, as they are the main contaminants in fish and fish-derived products. Finally, it is necessary to complete the information with other nutri-tionally interesting compounds like bioactive peptides or minerals.

2. Materials and Methods

2.1 Chemicals and reagents

Sodium fluorescein was acquired from Fluka Chemie AG (Bunds, Switzerland). Coomassie brilliant blue R250, Precision Plus Protein™ 5–250 kDa (molecular weight marker), and 8–16% Mini-PROTEAN® TGX™ Precast gels were bought from BioRad company (Hercules, CA, USA). Dithiothreitol (DTT) was purchased from VWR (Leuven, Belgium). In order to obtain highly pure water (resistivity >18 MΩ/cm), a Milli-Q SP Rea-gent Water System (Millipore Corporation, Bedford, MA, USA) was used. The $K_2S_2O_8$, Trolox, ABTS and AAPH reagents were procured by Sigma–Aldrich (Steinheim, Baden-Württemberg, Germany). Hydrochloric acid (HCl), and Sulfuric acid 96% (H_2SO_4) were purchased from Merck (Whitehouse Station, NJ, USA). Potassium phosphate mono-basic (KH_2PO_4), boric acid (H_3BO_3), sodium phosphate dibasic (Na_2HPO_4)

and potassium phosphate dibasic (K_2HPO_4), were purchased from Merck (Darmstadt, Germany). Lastly, ethanol 96% was bought from Panreac (Castellar del Vallés, Barcelona, Spain).

2.2 Samples

Sea bass (*Dicentrarchus labrax*) fresh deceased fish samples were obtained from a local market and were kept at 4 ˚C until the analysis. The different side streams (head, skin, viscera and backbone) were separated manually and stored at -20 ˚C.

2.3 Optimization process

For the optimization study, a response-surface methodology was used by means of a Box-Behnken design with two central points. The studied parameters were specific energy (50-300 kJ/Kg), field strength (1-3 kV/cm) and time of extraction (0-24 h). The responses analysed were: i) total protein and ii) antioxidant capacity measured with two methods, TEAC and ORAC. As it is shown in Table S1, 15 different experiments were set with the combination of the minimum, central and maximum values of each parameter. Moreover, a replicate of the central point was done to check the variability and reproducibility. Stat-graphics Centurion XVI® (Statgraphics Technologies, Inc., The Plains, VA, USA) was used for the optimization process.

2.4 Pulsed Electric Fields (PEF) treatment

For the PEF treatment of the different side streams, the PEF-Cellcrack III equipment (ELEA, Quakenbrück, Germany) available at the Faculty of Pharmacy of the University of Valencia was used. A 900 mL treatment chamber and a sample:water ratio of 1:15 were chosen. The specific energy (kJ/kg) and field strength (kV/cm) parameters were set according to the experimental design (Table S1). The pulse duration and frequency remained

constant at 100 ms and 2 Hz, respectively. The pulse form was unipolar square wave pulse. The conductivity and temperature of the sample were measured before and after the treatment using a portable conductivity meter ProfiLine Cond 3310 (WTW, Xylem Analyt-ics, Weilheim in Oberbayern, Germany). Despite a significant increase in temperature when using a high specific energy (e.g.: 300 kJ/kg for skin optimal extract), its influence was minimized when comparing the control extraction and the sample pre-treated with PEF, since both extractions were carried out at the same temperature. Therefore, the only factor that was different was the pre-treatment PEF, and only changes due to this treatment were observed, which was the aim of the study.

2.5 Supplementary aqueous extraction

After the PEF treatment, samples were stirred at 200 rpm from 0.5 to 24 h, according to the experimental design (Table S1). Then, samples were centrifuged at 3050 x g for 10 min in a 5810R centrifuge (Eppendorf AG, Hamburg, Germany). The supernatant was collected and kept frozen at −20 °C for further analysis.

2.6 Minerals and heavy metals determination and quantification

The mineral composition (Ca, Mg, P, Fe, Se and Zn) and heavy metals (As, Pb, Hg and Cd) of each extract were analyzed according to the methods described by de la Fuente et al. [18]. Briefly, samples were digested in a microwave oven with H_2O_2 and HNO_3 and then filtered. Finally, the liquid fraction was injected in an inductively coupled plasma spectrometer mass detector (ICP-MS model 7900, Agilent Technologies, CA, USA) for the detection and quantification of the minerals and heavy metals.

2.7 Proteins

2.7.1 Total protein content

The total protein content of the obtained extracts was determined as described by Al Khawli et al. [14].

2.7.2 Molecular weight distribution

For the analysis of molecular weight distribution, an SDS-PAGE electrophoresis was performed based on the method previously described by de la Fuente et al. [15]. First, proteins were precipitated with cold acetone (ratio sample:acetone 1:4 (v/v)). Then, samples were centrifuged at 9000 x g, the supernatant was removed, and the pellet was resuspended in deionized water. Sample buffer (62.5 mmol/L Tris-HCl (pH 6.8), 2g/100g SDS, 20g/100g glycerol, 0.01g/100g bromophenol blue, and 50 mmol/L dithiothreitol) was added to that samples and they were denaturalized at 95 °C for 5 min. Subsequently, 10 µL of the mixture were loaded on an 8–16% Mini-PROTEAN® TGX™ Precast gel and the electrophoresis was run 30 min at 120 V and then at 80 V until the end. Glycine (192 mmol/L), Trizma® base (25 mmol/L) and SDS (0.1g/100g) were mixed to prepare the running buffer. Precision Plus Protein™ 5–250 kDa was used in order to estimate the molecular weight of the bands. Once electrophoresis was finished, 0.125% Coomassie brilliant blue R–250 was used to stain the gel. Subsequently, a mixture of acetic acid (10g/100g) and methanol (20g/100g) (in water) was utilized to distain the gel.

2.7.3 Bioactive peptides identification

Bioactive peptide identification was carried out according to the method proposed by de la Fuente et al. [19]. Once the soluble peptides were isolated, they were analyzed using a nanoESI qTOF mass spectrometer (6600plus

TripleTOF, ABSCIEX, Framingham, MA, USA), equipped with a trap column (ChromXP C18, 3 µm 120 Å, 350 µm, 0.5 mm; Eksi-gent). After the LC-MS/MS was performed, the identification of the different peptides was done using the software ProteinPilot v5.0 search engine (AB SCIEX). Finally, the bioactivity and potential bioactivity of the different peptides was checked using the BIOPEP-UWM database [20].

2.8 Total Antioxidant Capacity determination

In order to determine Total Antioxidant Capacity (TAC), both Trolox Equivalent An-tioxidant Capacity (TEAC) and Oxygen Radical Absorbance Capacity (ORAC) assays were used. Regarding TEAC, 440 µL of $K_2S_2O_8$ 140 mmol/L were added to 25 mL of ABTS 7 mmol/L and kept in darkness at room temperature for 12-16 h to obtain $ABTS^{\bullet+}$ radical. The solution was diluted with ethanol until an absorbance of 0.700 ± 0.020 was reached at 734 nm, which was considered the initial absorbance. Next, appropriately diluted extracts (100 µL) were mixed with 2 mL of $ABTS^{\bullet+}$ radical, and the absorbance was measured after 3 minutes using a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer (Perkin-Elmer, Jügesheim, Germany) with triplicate measurements. To determine the antioxidant activity, a standard curve was prepared using Trolox, and the percentage of inhibition (% Inhibition) was calculated for each sample. The percentage of inhibition was then interpolated to determine the antioxidant activity, expressed as µmol trolox equivalent/L extract (µmol TE/L).

The ORAC assay was employed to assess antioxidant capacity based on the ability to eliminate peroxyl radicals, using the method outlined by de la Fuente et al. [18].

2.9 Cell cultures and assessment of cell viability

2.9.1 Cell culture

The human neuroblastoma SH-SY5Y cells were cultured in DMEM Ham's-F12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were incubated under specific conditions: pH 7.4, 5% CO_2 at 37 °C, and 95% air atmosphere with constant humidity. The culture medium was changed every 2-3 days. Different concentrations of fish by-products extracts (head, skin, viscera and backbone) were tested. Extract concentrations tested were 1:2 dilutions (25% to 0.78%). Control groups were included in each experiment.

2.9.2 Assessment of cell viability

In order to determine cell viability of sea bass by-product extracts (head, skin, viscera and backbone) obtained by PEF and by agitation (control), the MTT assay in SH-SY5Y cells was performed following the method described by Zingales et al. [21]. Briefly, 30,000 cells/well were seeded in 96-well plates for 48 hours until they reached 80% of confluence. Then, the culture medium was removed and cells were exposed individually to sea bass by-products extracts at increasing concentrations, from 0.78 up to 25%, obtained by both methods carried out, for 24 hours. After the incubation period, the culture medium containing the extract was replaced with fresh medium containing 50 µL of MTT salt (5 mg/mL PBS). After 3 hours of incubation at 37 °C in the dark, the resulting formazan crystals were dissolved in DMSO. An automatic plate reader (MultiSkanEX, Labsystem, Helsinki, Finland) was utilized to measure absorbance at 540 nm. Cell viability was expressed as a percentage relative to the control.

2.10 Statistical analysis

Significant differences between the results were determined by performing a t-test or an analysis of variance (ANOVA). In addition, the least significant differences (LSD) test was used. A $p<0.05$ was considered significant. The software GraphPad Prism 8 (GraphPad Software, Inc., California, USA) was used for the statistical analysis.

3. Results

3.1. Optimization process and comparison with the control sample

The obtained results for each response (total proteins, TEAC and ORAC) and each side stream are shown in the supplementary material (Tables S2-S5 and Figures S1-S4). As were expected the increment of temperature and conductivity due to the PEF treatment was greater as the electric field strength and the specific energy increased.

The optimization was carried out in order to maximize the value of each studied response. The optimal conditions for each side stream are presented in Table 1.

Table 1. Optimal conditions regarding protein recovery, TEAC and ORAC based on the studied side streams.

By-product	Head	Skin	Viscera	Backbone
Specific energy (kJ/Kg)	220	300	123.72	300
Field strength (kV/cm)	1	3	3	1
Time of extraction (h)	21.35	24	15.17	21

Then, the extractions were performed following the abovementioned optimal conditions. The obtained results were compared to those obtained after control process (without PEF pre-treatment) (Figure 1). As can be observed in the figure 1, the PEF pre-treatment in-creased protein recovery for head (from 21.01 ± 1.14 (control) up to 28.92 ± 3.22 % (PEF Optimum) of total proteins) and skin (from 37.25 ± 1.63 up to 51.34 ± 1.98 % of total pro-teins) extracts, while it had a negative effect on viscera extracts, decreasing the recovery compared to control. Regarding the antioxidant activity, the behavior was similar for both TEAC and ORAC assays, observing an increase in the antioxidant capacity values of head and skin extracts obtained by PEF, and decreased antioxidant values for viscera extracts compared to control samples. For the TEAC assay, the values for head and skin improved by 21.74 and 29.11%, respectively, while for viscera it decreased by 17.65 %. In addition, ORAC results increased by 22.11% (head) and 40.93% (skin), while for viscera extracts the value was reduced by 19.88%. Finally, the backbone extracts did not show any significant differences in total protein or TEAC values after PEF compared to control, while ORAC values were higher in control samples than in PEF extract (526.38 vs. 379.29 μM TE, respectively). The improvement in protein and antioxidant compounds extraction from the head and skin can be explained by the electroporation process promoted by PEF processing. In general, PEF affects biological cells and leads to specific structural changes and destruction of the cell membrane. Then, it helps releasing intracellular components, thus increasing the extraction yield of different compounds, like protein or antioxidant compounds. However, in the case of viscera extracts, no improvement was observed compared to the control samples. This can be explained because the electric field strength used was 3 kV/cm, and it has been observed by other authors up to 10 kV/cm the effect is not significant. At least electric fields of 10 kV/cm are required to electroporate fish viscera, being 20 kV/cm the optimal one [22]. Therefore, the intensity used in our study

is lower than the required to electroporate and promoting an improvement in the extraction yield.

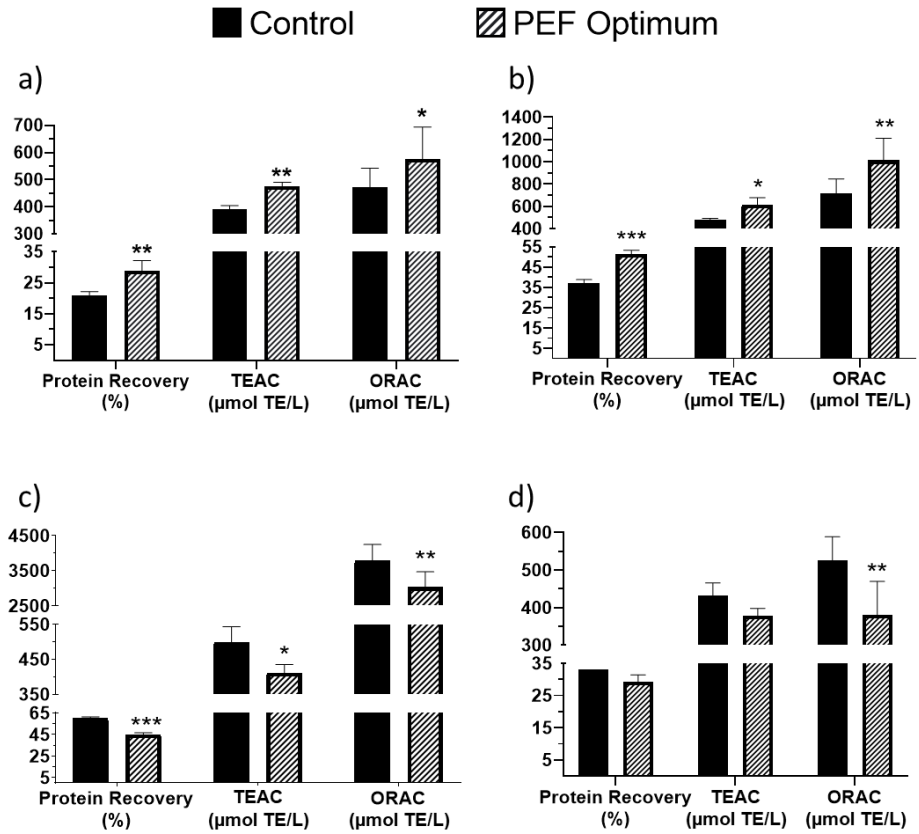

Figure 1. Comparison between PEF-pretreatment at optimal conditions and control sample at the same time of extraction for the studied side streams: a) Head, b) Skin, c) Viscera and d) Backbone. Results are expressed as mean ± standard deviation (SD). *-p<0.05; **-p<0.01; ***-p<0.001.

These results do not agree with the data showed by Franco et al. [17], who did not detect any significant difference in antioxidant capacity measured by the TEAC method for sea bass head and bone extracts after PEF treatment. However, this can be explained by the way of preparation of the sample. In our study, the sample was treated directly, putting the side stream directly in

the treatment chamber, while in the study of Franco et al., the sample was intensively crushed and vortexed. This step can hide the effect of PEF, as the cells are not intact and then the intracellular components are already released after the crushing process. On the other hand, our results fully agree with the data reported by Wang et al. [9], who obtained a higher protein recovery in sole head and skin extracts after PEF pre-treatment, while no significant differences were observed for viscera. Moreover, the highest protein recovery was also obtained with skin extract, as in our study.

3.2. Distribution of protein molecular weight

The molecular weight of the control and PEF extracts' protein profile was analysed. Figure 2 depicts the SDS-PAGE of the various samples. Notably, this method also offers insight into the overall protein content. As can be seen, skin extracts had the highest amount and variety of proteins. Moreover, it is also remarkable the ability of PEF to preserve high molecular weight proteins. In this sense, it can be observed that for the PEF pre-treated backbone extract, there is a band at 50 kDa which is lost in the backbone control extract. This effect can be also observed for skin samples, in which the PEF sample has a more intense band at 50 kDa, while in the control sample, this band seems that has been degraded, showing a more intense color in the range from 25 to 37 kDa.

These results also show that skin extracts had the highest protein content, while viscera extracts had the lowest values. However, this gel does not allow peptides to be retained and studied, so they have been analyzed qualitatively using a nanoESI qTOF mass spectrometer and it will be discussed in the next section.

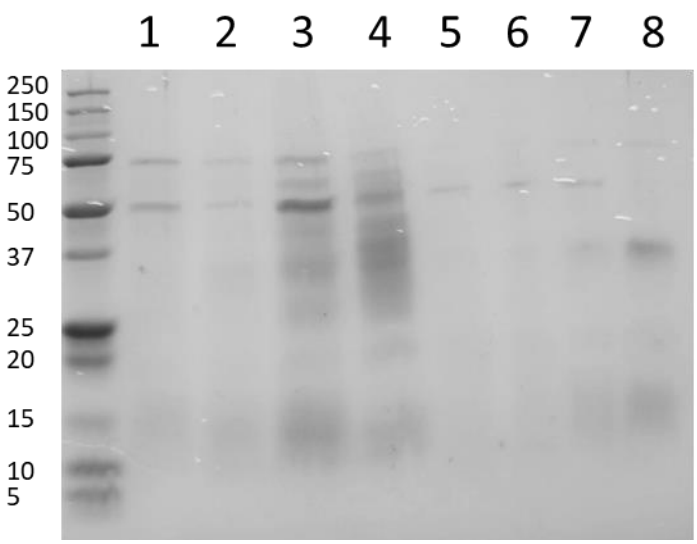

Figure 2. SDS-PAGE gel of the by-product extracts. 1: Head with pulsed electric fields (PEF) pre-treatment; 2: Head control; 3: Skin with PEF pre-treatment; 4: Skin control; 5: Viscera with PEF pre-treatment; 6: Viscera control; 7: Backbone with PEF pre-treatment; 8: Backbone control.

3.3 Bioactive peptides identification

The presence of peptides with antioxidant or potential antioxidant activity in each extract was studied. In the backbone extract obtained after PEF treatment was found the peptide LEQQVDDLEGSLEQEKK, which has antioxidant activity, previously demonstrated by Je et al. [23]. Moreover, other potential antioxidant peptides were found, which are presented in Table 2 (only peptides with more than 90% of confidence were selected). The BIOPEP-UMP database did not reveal any association between these peptides and antioxidant activity. Nevertheless, a bioinformatic analysis revealed the presence of several short amino acid sequences with antioxidant properties within these peptides, as shown in Table 2.

The potential antioxidant activity of the peptides evaluated was based on the presence of specific amino acids in the peptide sequence. It is known that amino acids with aromatic rings in their molecule have good antioxidant capacity. These amino acids are tryptophan (W), tyrosine (Y) and phenylalanine (F), which are aromatic amino acids, associated with a higher antioxidant capacity due to the presence of an aromatic ring and the ability to transfer protons and/or electrons. Moreover, histidine (H) is also associated with strong radical-scavenging activity due to its imidazole ring. In addition, the pyrrolidine and indole rings of proline (P) and W are able to act as hydrogen donors due to the presence of hydroxyl groups, being hydroxyl radical scavengers [24]. As can be seen in Table 2, PEF pre-treatment can affect the peptides variability. For head extracts, PEF increases the diversity of peptides, resulting in a wider range of peptides found in the pre-treated sample compared to the control sample. However, for skin extracts the effect is the opposite: PEF reduces the variety of peptides compared to the control sample. It should be noted that the highest amount of potential antioxidant peptides was found in backbone samples, probably due to the presence of remnants of fish flesh adhering to the backbone. Finally, it should be mentioned that the analysis of potential bioactive peptides was just qualitative, not quantitative. Then, it cannot be correlated the different amounts of obtained peptides with antioxidant capacity, as it can be found a different proportion of each peptide in each extract.

Table 2. Peptides found in the different extracts with potential antioxidant capacity and the group responsible for this bioactivity.

	Sequence	Involved amino acids
HEAD CONTROL	ATDGGAHGVINVSVSEAAIEASTR	AH; GAH
	IIDQDKSGFIEEDELKL	EL; LK
	IIDQDKSGFIEEDELKLFLQN	EL; LK
	MEHDPQARDRKAQEL	EL
	SANLMAGHWVAISGAAGGLGSLAVQYAK	GAA
HEAD PEF OPT	AISEELDHALNDMTSI	EL
	AVRNDEELNKLLGGVTI	EL
	AVRNDEELNKLLGGVTIAQGGVLPNIQA	EL
	AVRNDEELNKLLGGVTIAQGGVLPNIQAVLLPK	EL
	ENKNLQQEISDLTEQLGETGKSIHELEK	EL
	GTEDELDKYSEALKDAQEKLELAEKKATDAEGD-VAS	DAQEKLE; EL; KD; LK
	GTEDELDKYSEALKDAQEKLELAEKKATDAEGD-VASLNR	DAQEKLE; EL; KD; LK
	ISEELDHALNDMTSI	EL
	LLIVYPWTQR	PWT; PW; VY; YPW; YPWT
	MSADAMLKALLGSK	LK
	NDEELNKLLGGVT	EL
	NDEELNKLLGGVTIAQGGVLPNIQAVLLPK	EL
	NLQQEISDLTEQLGETGKSIHELEK	EL
	VAKLEKTIDDLEDELYAQK	LY; EL
	VFLENVIRDAVTYT	IR; TY; VFL
	VFLENVIRDAVTYTEHAK	IR; TY; VFL
	VFLENVIRDAVTYTEHAKR	IR; TY; VFL
	VGAGAPVYLAAVLEYLTAEILELAGNAAR	EL; VY
	VLEYLTAEILELAGNAARDNKKT	EL
	YKAISEELDHALNDMTSI	EL; KAI
SKIN CONTROL	AFGLFDRVGDNKVAYNQ	AY
	AGLLTSRSPTGSLWVVTA	LWV; LW
	AVGKVIPELNGKITGMA	EL; KVI; PEL
	AVGKVIPELDGKLTGMA	EL; KVI; PEL

Table 2. (Cont.)

	Sequence	Involved amino acids
SKIN CONTROL	AVGKVIPELNGKITG	EL; KVI; PEL
	AVGKVIPELNGKLTG	EL; KVI; PEL
	AVGKVIPELNGKLTGMA	EL; KVI; PEL
	FAGDDAPRAVFPS	AGDDAPR
	FGLFDRVGDNKVAY	AY
	IIPASTGAAKAVGKVIPELNGK	EL; KVI; PEL; GAA
	IIPASTGAAKAVGKVIPELNGKITGMA	EL; KVI; PEL; GAA
	IIPASTGAAKAVGKVIPELNGKLTGMA	EL; KVI; PEL; GAA
	LRVFDKEGNGTVMGAELR	EL
	SSSLEKSYELPDGQVITIGNER	EL
	SVAELGEQIDNLQR	EL
	SVAELGEQIDNLQRVKQKLEKEKSE	EL
	SYELPDGQVITIGNER	EL
	TQQLEDLKRQLEEEVKAKN	LK
	TQQLEDLKRQLEEEVKAKNALAH	AH; LK
	VAELGEQIDNLQRVKQKLEKEKSE	EL
	VGKVIPELNGKITGMA	EL; KVI; PEL
	VGKVIPELNGKLTGMA	EL; KVI; PEL
	VLSGGTTMYPGIADRM	MY
	VLSGGTTMYPGIADRMQKE	MY
	VLSGGTTMYPGIADRMQKEITA	MY
SKIN PEF OPT	AFGLFDRVGDNKVAYNQIADIMR	AY
	DDEETTALVCDNGSGLVKAGFAGDDAPRA	AGDDAPR
	GAAAGAGAGAAGAGAAAGAEGPAGGPTGGP	GAA
	GKKMFGKQAGEDESDDFAIGGSTPTNKLK	LK
	GPAGAGAGDEAVDGATLYVKNLSF	LY
	IITNWDDMEKIWH	WDDMEK
	IITNWDDMEKIWHHT	HH; WHH; WDDMEK
	PIEHGIITNWDDMEKIWHHT	HH; WHH; WDDMEK
	QAGAAGGQPGAKAGGDDDVVDA	GAA
	VLSGGTTMYPGIADRMQKEITAL	MY
	YPIEHGIITNWDDMEKIWHHT	HH; WHH; WDDMK

Table 2. (Cont.)

	Sequence	Involved amino acids
VISCERA CONTROL	AIGLPDELIQK	EL
	AWGPGLEGGVVGK	AW; WG
	EVHLGWAAKGLGRKIQAMM	HL; MM
	FGGEHIPNSPF	GGE
	NIPTSGAEIGGAFGGEK	GGE
VISCERA PEF OPT	AIGLPDELIQK	EL
	AIIDQDKSGFIEEDEL	EL
	AIIDQDKSGFIEEDELK	EL; LK
	AWGPGLEGGVVGK	AW; WG
	GIELPYQDPAIK	EL
	GKDLFVSDLK	KD; LK; LFV
	IIDQDKSGFIEEDELK	EL; LK
	ILDQDKSGFIEEDELQ	EL
	IVNGEEAVPHSWPW	PHS; PW
	KEADAMAVDGGQVY	VY
	KVMGFVGIQTGFR	GFVG
BACKBONE CONTROL	AAVPSGASTGVHEALELR	EL
	AAVPSGASTGVHEALELRDGDKSRY	EL; RY
	AAVPSGASTGVHEALELRDGDKSRYLG	EL; RY; RYL; RYLG; YLG
	AAVPSGASTGVHEALELRDGDKSRYLGKGT	EL; RY; RYL; RYLG; YLG
	AAVPSGASTGVHEALELRDNDKANY	EL
	AGTNGETTTQGLDGLYER	LY
	AHQQTLDDLQAEEDKVNT	AH
	AKAVGKVIPELNGKLTGMA	EL; KVI; PEL
	AKYGKDATNVGDEGGF	KD
	AVGKVIPELNGKLT	EL; KVI; PEL
	AVGKVIPELNGKLTG	EL; KVI; PEL
	AVGKVIPELNGKLTGMA	EL; KVI; PEL
	AVPSGASTGVHEALELRDGDKSRY	EL; RY
	AVPSGASTGVHEALELRDGDKSRYLGKGT	EL; RY; RYL; RYLG; YLG
	AVRNDEELNKLLGGVTIAQGGVLPN	EL

Table 2. (Cont.)

	Sequence	Involved amino acids
BACKBONE CONTROL	EEALDHLETLKRENKNLQQEISDLTEQLGETGKSI-HELEKA	HL; EL; LK
	EHGIVNNWDDMEKIWHHT	HH; WHH; WDDMEK
	EKTIDDLEDELYAQK	LY; EL
	ELPDGQVITIGNER	EL
	FDMFDTDGGGDISTKELGT	EL
	FMLELDGTENKSK	EL
	GIITNWDDMEK	WDDMEK
	GPMKGILGYTEHQ	LGY
	HIADLAGHKDVILP	KD
	IADLAGNEDVILPVPAFNVINGGSHAGNK	GSH
	IITNWDDMEKIWH	WDDMEK
	IITNWDDMEKIWHHT	HH; WHH; WDDMEK
	ISDLTEQLGETGKSIHELE	EL
	ISDLTEQLGETGKSIHELEK	EL
	ISDLTEQLGETGKSIHELEKA	EL
	ITATQKTVDGPSGKLWR	LWR; LW
	IVNNWDDMEKIWH	WDDMEK
	KLEKTIDDLEDELY	LY; EL
	KYGKDATNVGDEGGF	KD
	LAGTNGETTTQGLDGLYER	LY
	LAVRNDEELNKLLGGVTIAQGGVLPN	EL
	LEKTIDDLEDELY	LY; EL
	LPDGKVITIGNER	KVI
	LQLAVRNDEELNKLLGGVTIAQGGVLPN	EL; LQL
	LTEQLGETGKSIHELEK	EL
	LTGCTDIQIVGDDLTVTNPKR	TGC
	LTKLEEAEKAADESERGMKVIENR	KVI
	MLELDGTENKSKFGANA	EL
	MLELDGTENKSKFGANAILGVS	EL
	QGGGGWGGGPGGGQQGGGAP	WG
	SEALKDAQEKLELAE	DAQEKLE; EL; KD; LK

Table 2. (Cont.)

	Sequence	Involved amino acids
BACKBONE CONTROL	SEALKDAQEKLELAEK	DAQEKLE; EL; KD; LK
	SEALKDAQEKLELAEKKATDAEGDVASLNR	DAQEKLE; EL; KD; LK
	SEALKDAQEKLELAEKKATDAEGDVASLNRR	DAQEKLE; EL; KD; LK
	SELKKDIDDLELTL	EL; KD; LK
	SELKKDIDDLELTLAK	EL; KD; LK
	SELKKDIDDLELTLAKVEKE	EL; KD; LK
	SELKKDIDDLELTLAKVEKEKHATEN	EL; KD; LK
	SELKKDIDDLELTLAKVEKEKHATENK	EL; KD; LK
	SKQLEDDLVALQKKLKGTEDELDKYSE	EL; LK
	SVAELGEQIDNLQR	EL
	SYELPDGQVITIGNER	EL
	TEQLGETGKSIHELEKA	EL
	VAKLEKTIDDLEDELY	LY; EL
	VEEELDRAQERLATALTKLEEAEKAADESERG	EL
	VEEELDRAQERLATALTKLEEAEKAADESERGMK	EL
	VEEELDRAQERLATALTKLEEAEKAADESERGMKVIEN R	EL; KVI
	VGKVIPELNGKLTGMA	EL; KVI; PEL
	VINGGSHAGNKLAMQEFM	GSH
	VLSGGTTMYPGIADR	MY
	VVESTGVFTTIEKASAH	AH
	VVESTGVFTTIEKASAHIKGGAKR	AH
	YELPDGQVITIGNER	EL
BACKBONE PEF OPT	AALTGGAAAGVAGAAAAGPAGDIA	GAA
	AHQQTLDDLQAEEDKVNT	AH
	AISEELDHALNDMTS	EL
	AVRNDEELNKLLGGVTIAQGGVLPN	EL
	DAQEKLELAEKKATDAEGDVAS	DAQEKLE; EL
	EKTIDDLEDELYAQK	LY; EL
	FMIELDGTENK	EL
	GIITNWDDMEK	WDDMEK
	GPAGAGAGDEAVDGATLYVKNLSF	LY

Table 2. (Cont.)

	Sequence	Involved amino acids
BACKBONE PEF OPT	GTEDELDKYSE	EL
	GTEDELDKYSEALKDAQEKLE	DAQEKLE; EL; KD; LK
	HLQLAVRNDEELNKLLGGVTIAQGGVLPN	HL; EL; LQL
	IIDQDKSGFIEEDELKL	EL; LK
	IITNWDDMEK	WDDMEK
	IITNWDDMEKIWHHT	HH; WHH; WDDMEK
	ILDQDKSGFIEEDELQLFLQN	EL; LQL
	ISDLTEQLGETGKSIHELEK	EL
	ISEELDHALNDMTS	EL
	ISEELDHALNDMTSI	EL
	KKQADSVAELGEQIDNLQR	EL
	KLKGTEDELDKYSEALKDAQEKLELAEKKATDAEGDVASLNR	DAQEKLE; EL; KD; LK
	LAVRNDEELNKLLGGVTIAQGGVLPN	EL
	LEKTIDDLEDELYAQK	LY; EL
	LTKLEEAEKAADESERGMKVIENR	KVI
	MSADAMLKALLGSK	LK
	NLQQEISDLTEQLGETGKSIHELEK	EL
	PGPNKGDSRGPPNHHMGP	HH; NHH; GPP
	PGSPAGAATSAPGAPAPG	GAA
	PIEHGIITNWDDMEK	WDDMEK
	RIQLVEEELDRAQERLATA	EL
	SADTLWGIQKDLKDL	LWG; KD; LK; LW; WG
	SEALKDAQEKLELAEKKATDAEGDVAS	DAQEKLE; EL; KD; LK
	SEALKDAQEKLELAEKKATDAEGDVASLNR	DAQEKLE; EL; KD; LK
	SEALKDAQEKLELAEKKATDAEGDVASLNRR	DAQEKLE; EL; KD; LK
	SKQLEDDLVALQKKLKGTEDELDKYSEALKDAQEKLELAEKKATDAEGDVASLNR	DAQEKLE; EL; KD; LK
	SQKEDKYEEEIKVLTDKLK	LK
	SQKEDKYEEEIKVLTDKLKEAETR	LK
	SQKEDKYEEEIKVLTDKLKEAETRAE	LK
	TIDDLEDELYAQK	LY; EL

Table 2. (Cont.)

	Sequence	Involved amino acids
BACKBONE PEF OPT	VAKLEKTIDDLEDELY	LY; EL
	VEEELDRAQERLATALTKLEEAEKAADESERGMK	EL
	VEEELDRAQERLATALTKLEEAEKAADESERG	EL
	VRNDEELNKLLGGVTIAQGGVLPN	EL
	VTIMPKDIQLAR	KD

A: alanine; C: cysteine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histi-dine; I: isoleucine; K: lysine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: serine; T: threonine; V: valine; W: tryptophan; Y: tyrosine.

Regarding the effect of the treatment on peptides, it has been seen that PEF treatment can modify the secondary structure of peptides, mainly the alpha-helix content. However, the relationship between antioxidant capacity and these changes is not clear and needs further study. On the other hand, it has also been seen that PEF treatment can alter the spatial conformation of the molecule, exposing aromatic amino acids and thus modifying the antioxidant capacity of the peptide [25]. This can contribute to the higher antioxidant capacity observed in head and skin PEF extracts compared to the control. However, the lower antioxidant capacity of PEF viscera extract compared to the control sample should be further studied.

With respect to the peptides obtained, the peptide IITNWDDMEKIWHHT, which contains 3 sequences with antioxidant capacity (HH; WHH; WDDMEK), is noteworthy. The SEALKDAQEKLELAE sequence also has a large presence of amino acid combina-tions with antioxidant capacity, with 4 different groups (DAQEKLE; EL; KD; LK). It can also be observed that LLIVYPWTQR presents 5 different groups with potential antioxidant activity, which are PWT; PW; VY; YPW and YPWT. Finally, the sequence SADTLW-GIQKDLKDLKDL has up to 5 groups with antioxidant potential (LWG; KD; LK; LW; WG) too.

Other authors also obtained a significant influence when using non-conventional technologies to obtain bioactive peptides from fish side streams. In this sense, de la Fuente et al. [19] obtained 137 potential antioxidant peptides after the application of pressurized liquid extraction (PLE) technology on salmon viscera. However, these authors only ob-tained 67 potential antioxidant peptides when using shaking extraction (control). These results confirm that the technology used for the extraction procedure affects the obtained products, allowing the recovery of different peptides and other bioactive compounds. In addition, after the different procedures, the sequences GAA, GPP and EL were found in the antioxidant extracts.

In addition, the same antioxidant peptides sequences after the treatment of different animal foods with different methods were described in other studies. In this sense, Saiga et al. [26] reported the presence of the peptides DAQEKLE, IEAEGE, DSGVT, VPSIDDQEELM and EELDNALN in the extracts obtained after the enzymatic treatment of pork muscles, being the sequence DAQEKLE the one with the highest antioxidant capacity. Zielińska et al. [27] found the sequence AGDDAPR after the *in vitro* digestion of *Tenebrio molitor*. Finally, Oliveira Lima et al. [28] described the presence of the peptide sequence IITNWDDMEK, with the antioxidant group WDDMEK, in hydrolyzed samples from stripped weakfish (*Cynoscion guatucupa*) by-products by enzymatic hydrolysis using Protamex.

3.4 Mineral content

Regarding mineral content, Mg, P, Ca, Fe, Zn and Se were analyzed by ICP-MS (Figure 3). For head side stream, PEF increased the extraction of Mg, P, Ca, Fe, and Zn, while for skin, PEF pretreatment had only a significant effect for P, Fe and Zn. On the other hand, for viscera, the highest concentration of Fe and Zn was observed for the PEF-treated ex-tracts, while the control extracts presented a higher recovery of Mg, P and Ca. Finally, PEF treatment

had a significant effect to recover Mg, P and Fe in backbone extracts. It is worth mentioning that Se was only found in the PEF-pretreated backbone extracts. Although the application of PEF had different effects according to the targeted each mineral and side stream, in general, it can be concluded that it is a promising technology to enhance mineral recovery.

Se intake is extremely variable across the world [29] and the health benefits are still to be defined [30]. However, it is known that is involved in the antioxidant mechanism of the cells through the enzyme GSH, which catalize the reduction of hydrogen and lipid perox-ides [31]. Se is also involved in the regulation of several antioxidant genes, like superoxide dismutase (SOD) and catalase (CAT) [32]. Moreover, is also known its synergic effect with vitamin E as antioxidants, protecting the cell membrane [31]. In addition, Zn is also a relevant mineral regarding antioxidant function in human body. As Se, it also contributes to the correct function of antioxidant enzimes (GSH and SOD) [33]. Moreover, Zn is also able to inhibit NADPH-oxidase, reducing the production of reactive oxygen and nitrogen species [34].

The PEF technology allowed its recovery and the extracts obtained could be used for the development of nutraceuticals. In most cases, PEF enhanced the extraction of Fe and Zn, and certain population intakes of these two minerals are provided by eating small fish whole [35]. However, in order to use the bones of large fish in food products, the bone structure must be softened. This can be achieved through various methods, such as the application of hot water, hot acetic acid, or steam. [36]. In this case, PEF technology is a green alternative that with further processing could provide similar results.

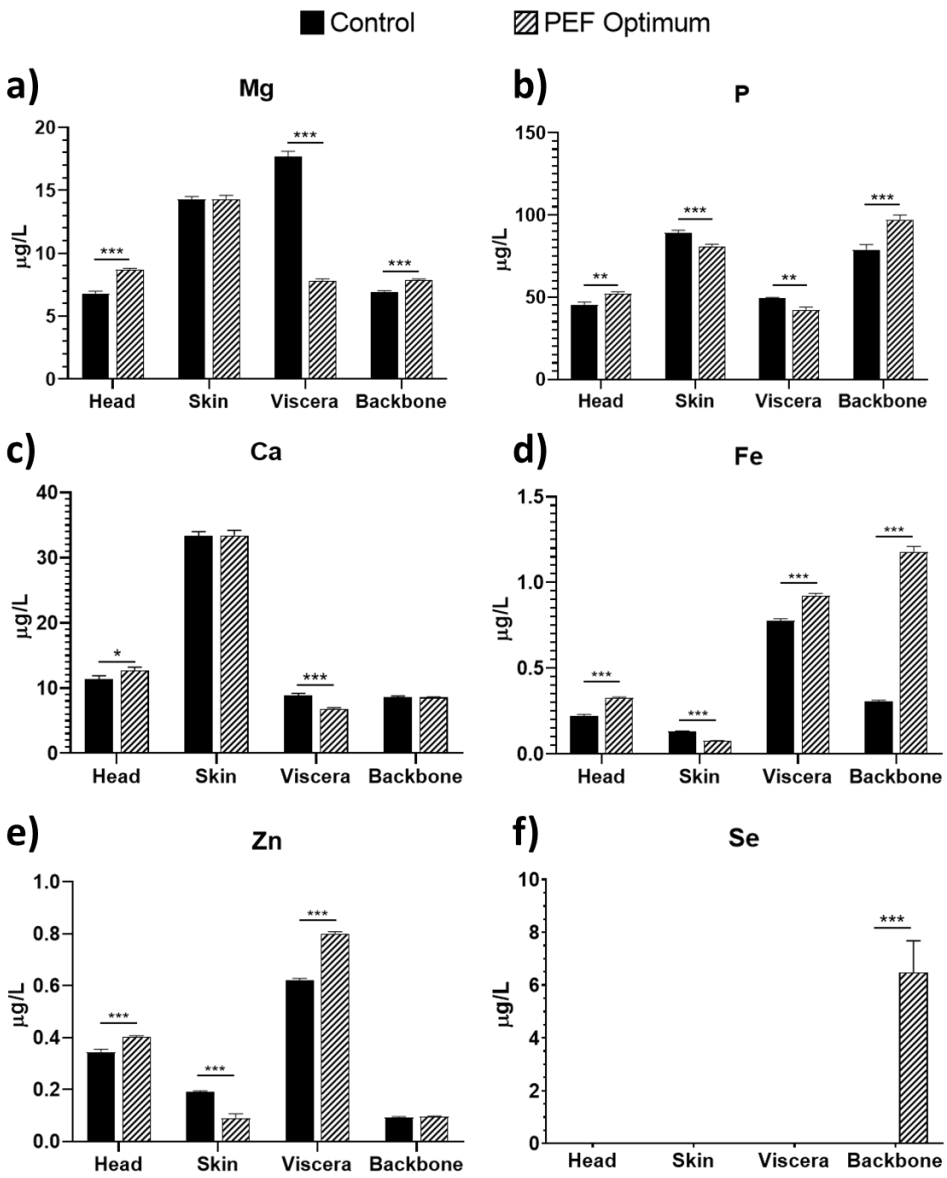

Figure 3. Concentration of: a) Mg, b) P, c) Ca, d) Fe, e) Zn and f) Se in sea bass side streams aqueous extracts, comparing control extraction by soaking vs. PEF pre-treatment. * - p<0.05; ** - p<0.01; *** - p<0.001.

3.5 Heavy metals quantification

As, Cd, Hg and Pb were analyzed by ICP-MS, as they are the main contaminants found in marine fish [37].

As can be observed in figure 4, PEF pre-treatment had a significant effect in reducing the concentration of As of skin, viscera and backbone extracts. Moreover, the PEF treatment also reduced the concentration of Hg in the head extract. On the other hand, the ap-plication of PEF increased the release of Pb to the extract in the head and viscera side streams. Finally, PEF did not have a significant effect on Cd concentrations.

The heavy metals concentration ranges varied from 6.9-16.5, 0-1.3, 0.8-1.48 and 0.197-0.67 µg/L of extract for As, Cd, Hg, and Pb, respectively. As was the most predominant heavy metal found in head, skin, and backbone samples, followed by Hg, Pb, and Cd. Conversely, for viscera samples, the order of toxic metal concentration was As > Hg > Cd > Pb, with a decreasing trend, being the side stream with the highest Cd concentration (1.2-1.3 µg/L). All the values obtained are below the limits set by the EFSA for Cd, Hg and Pb of 0.050, 0.5 and 0.30 mg/Kg wet-weight muscle meat, respectively [38]. In the case of As, there is not a maximum limit set but recommendations about the daily intake. Nevertheless, the type of As (organic or inorganic) needs to be elucidated because the organics forms present in some mollusks and crustaceans have not been shown to produce adverse effects in humans consuming this seafood [39].

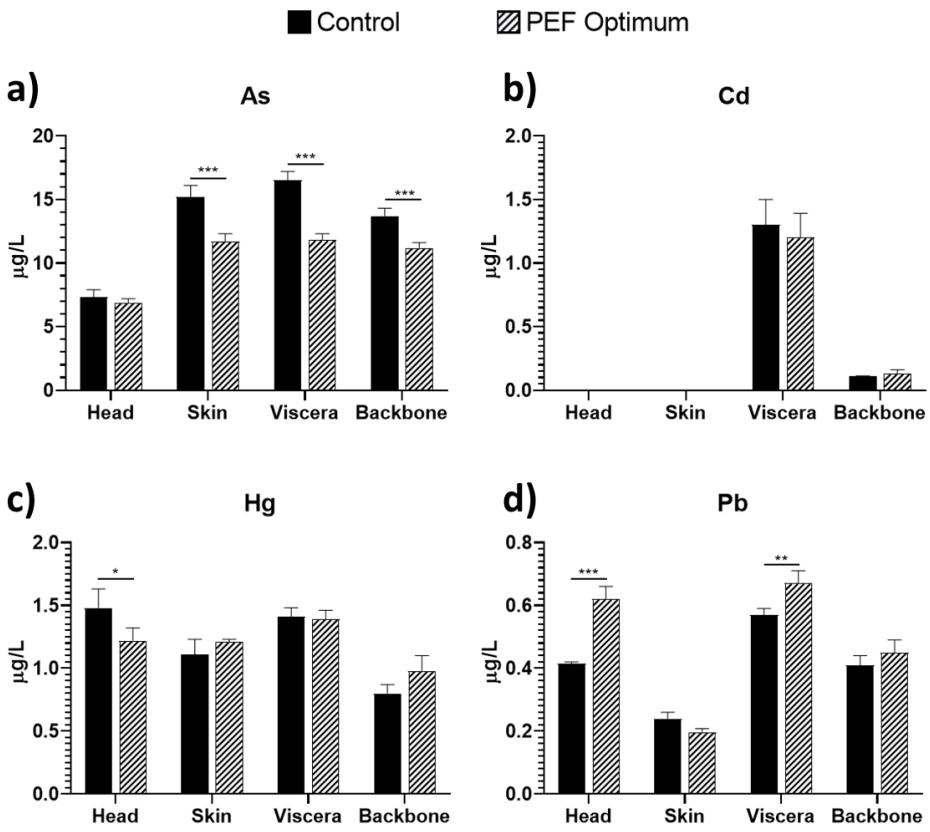

Figure 4. Concentration of a) As, b) Cd, c) Hg and d) Pb in sea bass side streams aqueous extracts, comparing control extraction by soaking vs. PEF pre-treatment. * - p<0.05; ** - p<0.01; *** - p<0.001.

3.6 Effect of fish by-products extracts on cell viability

In order to determine cell viability of each sea bass by-product extracts obtained by PEF and agitation (control) methods, a MTT assay in SH-SY5Y cells for 24 hours was per-formed (Figure 5).

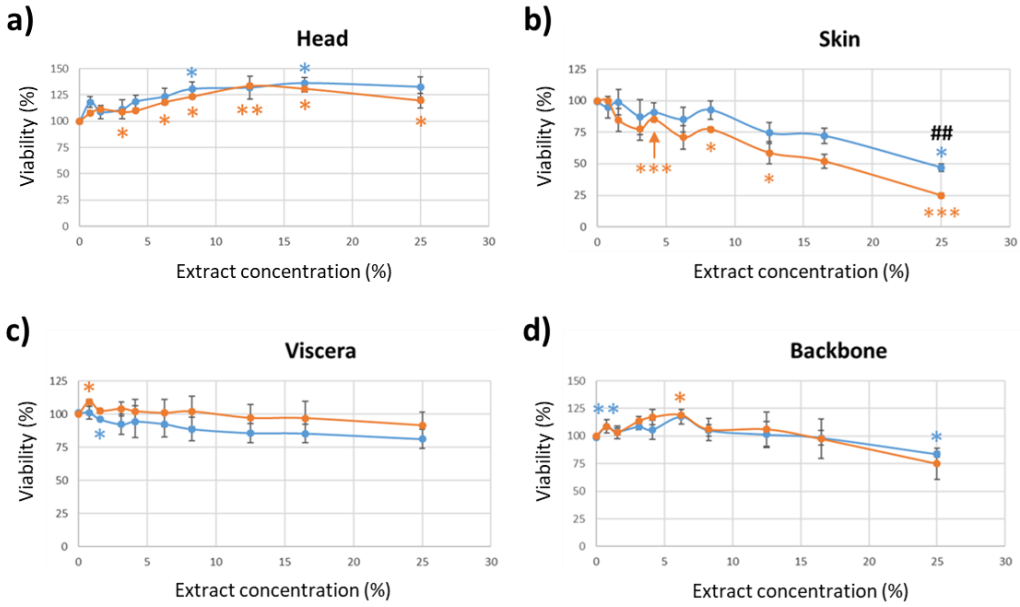

Figure 5. Effect of fish by-products extracts (a) head; b) skin; c) viscera and d) backbone) obtained by pulsed electric fields pre-treatment ("PEF Opt.") and its control on cell viability after 24 h of exposure. Cell viability was measured by MTT assay on SH-SY5Y cells. The results are expressed as mean ± SEM of three independent experiments. * - $p<0.05$ vs. its respective cell control (0% ex-tract); ** - $p<0.01$ vs. its respective cell control (0% extract): *** - $p<0.001$ vs. its respective cell control (0% extract); ## - $p<0.01$ between PEF and control extracts.

The results of the extract concentration-cell viability assay demonstrated that the highest cell viability (33% and 25%) was achieved with 12.5% and 8.5% head extract obtained by PEF and agitation (control), respectively (Fig. 5a). However, both the skin extracts, the one obtained by PEF and the one obtained by agitation (control in Fig. 5b) showed a significant decrease in cell viability, compared to cells not exposed to any extract (0% extract). Moreover,

SH-SY5Y cell exposed to PEF skin extracts resulted in a major cell viability decrease compared to agitation method (control in Fig. 5b). In contrast, the PEF and agitation (control) extracts of backbone and viscera did not exhibit any significant changes when compared to each other. Nevertheless, the 6.25% backbone PEF extract ex-hibited a significant increase in cell viability at 6.25% concentration respect to cell (0% extract).

Our results are similar to the data reported by other authors. The hydrolysates derived from fish by-products are the primary focus of study in literature due to their potential as valuable products with diverse bioactive properties. In this sense, in a recent work, Taroncher et al. [40] studied the effects of fish hydrolysates in Caco-2/TC7 cells viability. They examined different by-products of salmon (S), mackerel (M) and herring (H): heads (HSH), backbones (HSB, HMB) and, viscera (HSV, HMV, HHV). Within the tested concentrations (0.03125 - 1 mg/mL), hydrolysates had minimal impact on cell viability by MTT assay, except for HSB (0.125 mg/mL) and HSV (0.0625 mg/mL and 0.25 mg/mL), which showed significant cell viability increases (27% for HSB and 51.2% for HSV). No cytotoxic effects were observed by these authors. Respect to the total protein content (PC) assay, Taroncher et al., found that all hydrolysates, except HSB and HMV, increased cell viability. Notable increases in PC were observed for HMB (18%), HSV (19%), HHV (139%), HSH (140%) and, HMH (214%). These results indicate that hydrolysate exposure enhanced cell viability in Caco-2/TC7 cells. Compared to our results, similar findings were obtained for heads and backbones, while Taroncher et al. obtained higher cell viability after exposure to viscera hydrolysates.

The cytoprotective effect of other marine by-products has been previously reported. The research conducted by Zhong et al. [41] explores the protective effect of hydrolysates derived from silver carp by-products against oxidative stress. These authors demonstrated a substantial radical-

neutralizing capacity of these hydrolysates on Caco-2 cells exposed to low concentrations of H_2O_2. These findings are consistent with the observations made by Hu et al. [42], who evaluated the oxidative stress in HepG2 cells exposed to hydrolysates obtained from monkfish (*Lophius litulon*) muscle. The study revealed a cytoprotective effect, leading to increased cell viability. Furthermore, these authors reported no cytotoxic effects associated to the studied products. Similarly, Gómez et al. [43] observed a dose-dependent increase in cytoprotective effect in HepG2 cells exposed to hydrolysates derived from side streams of red tilapia for 24 hours.

Moreover, it has been observed that other hydrolysates derived from marine biomass are also non-cytotoxic. Wiriyaphan et al. [44] reported no cytotoxic effects of the hydrolysate derived from *Nemipterus spp*. side streams in Caco-2 cells. Moreover, Zheng et al. [45] demonstrated that human umbilical vein endothelial (HUVECs) cell viability did not decrease when they are exposed to hydrolysate obtained from swim bladders of *Nibea japonica* for a 24-hour.

5. Conclusions

By treating with PEF, the protein extraction rate from sea bass side increased and changed the molecular size distribution compared to control. Moreover, the assessment of the extracts' antioxidant capacity demonstrated that the application of PEF enhanced the antioxidant activity of the sea bass skin and head extracts. It has been also demonstrated that PEF pre-treatment can improve the generation of potential bioactive peptides, in-creasing its variability and the presence of antioxidant peptides. In addition, PEF showed a higher recovery of several minerals like Fe, Zn or Mg, which have a high relevance from a nutritional point of view. Moreover, PEF technology has been

demonstrated to be useful for heavy metal reduction in fish extracts. Finally, head extracts improve cell viability in SH-SY5Y cells at 24 h of treatment, being higher the result obtained for PEF extract com-pared to control. Unfortunately, the application of PEF for the treatment of marine side streams is limited, mainly because of the high financial investment required to acquire the equipment. However, advantages such as the short processing time or the low energy de-mand can make this technology another option for marine waste treatment to valorize the by-products and recover interesting compounds.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Response surface plots for protein recovery (a), TEAC (b) and ORAC (c) values obtained for head extracts and desirability degree (d). Desirability is based on the common response of the various responses analyzed. The less significant parameter was fixed at its optimal condition.; Figure S2: Response surface plots for protein recovery (a), TEAC (b) and ORAC (c) values obtained for skin extracts and desirability degree (d). Desirability is based on the common response of the various responses analyzed. The less significant parameter was fixed at its optimal condition.; Figure S3: Response surface plots for protein recovery (a), TEAC (b) and ORAC (c) values obtained for viscera extracts and desirability degree (d). Desirability is based on the common response of the various responses analyzed. The less significant parameter was fixed at its optimal condition.; Figure S4: Response surface plots for protein recovery (a), TEAC (b) and ORAC (c) values obtained for backbone extracts and desirability degree (d). Desirability is based on the common response of the various responses analyzed. The less significant parameter was fixed at its optimal condition.; Table S1: Specific energy (kJ/Kg), field strength (kV/cm) and time of

extraction (h) conditions for each experiment of the response-surface optimization design.; Table S2: Protein recovery, TEAC and ORAC results for sea bass head extract obtained for each experiment of response-surface optimization.; Table S3: Protein recovery, TEAC and ORAC results for sea bass skin extract obtained for each experiment of response-surface optimization.; Table S4: Protein recovery, TEAC and ORAC results for sea bass viscera extract obtained for each experiment of response-surface optimization.; Table S5: Protein recovery, TEAC and ORAC results for sea bass backbone extract obtained for each experiment of response-surface optimization.

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Conflicts of Interest: The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Specific energy (kJ/kg), field strength (kV/cm) and time of extraction (h) conditions for each experiment of the response-surface optimization design.

Experiment	Specific energy (kJ/ Kg)	Field strength (kV/ cm)	Time of extraction (h)
1	300	2	12
2	300	1	24
3	50	3	24
4	50	2	12
5	50	1	0
6	175	3	12
7	175	2	0
8	300	1	0
9	300	3	0
10	50	3	0
11	300	3	24
12	50	1	24
13	175	1	12
14	175	2	24
15	175	2	12
16	175	2	12

Table S2. Protein recovery, TEAC and ORAC results for sea bass head extract obtained for each experiment of response-surface optimization.

Experiment	Specific energy (kJ/Kg)	Field strenght (kV/cm)	Time of extraction (h)	% Protein Recovery (g/100 g proteins in sample)	Antioxidant Capacity	
					TEAC (µmol TE/L)	ORAC (µmol TE/L)
1	300	2	12	45.73	339.40	341.21
2	300	1	24	78.28	432.16	514.85
3	50	3	24	55.51	300.37	415.51
4	50	2	12	46.98	339.40	265.58
5	50	1	0	27.00	91.64	32.00
6	175	3	12	64.23	347.69	418.80
7	175	2	0	42.43	73.09	36.51
8	300	1	0	35.63	162.53	98.09
9	300	3	0	35.67	124.63	147.47
10	50	3	0	32.92	52.50	18.63
11	300	3	24	89.63	343.65	432.61
12	50	1	24	46.22	405.26	302.77
13	175	1	12	67.80	593.59	625.19
14	175	2	24	61.56	439.79	576.80
15	175	2	12	58.04	421.09	320.57
16	175	2	12	51.90	401.85	438.31

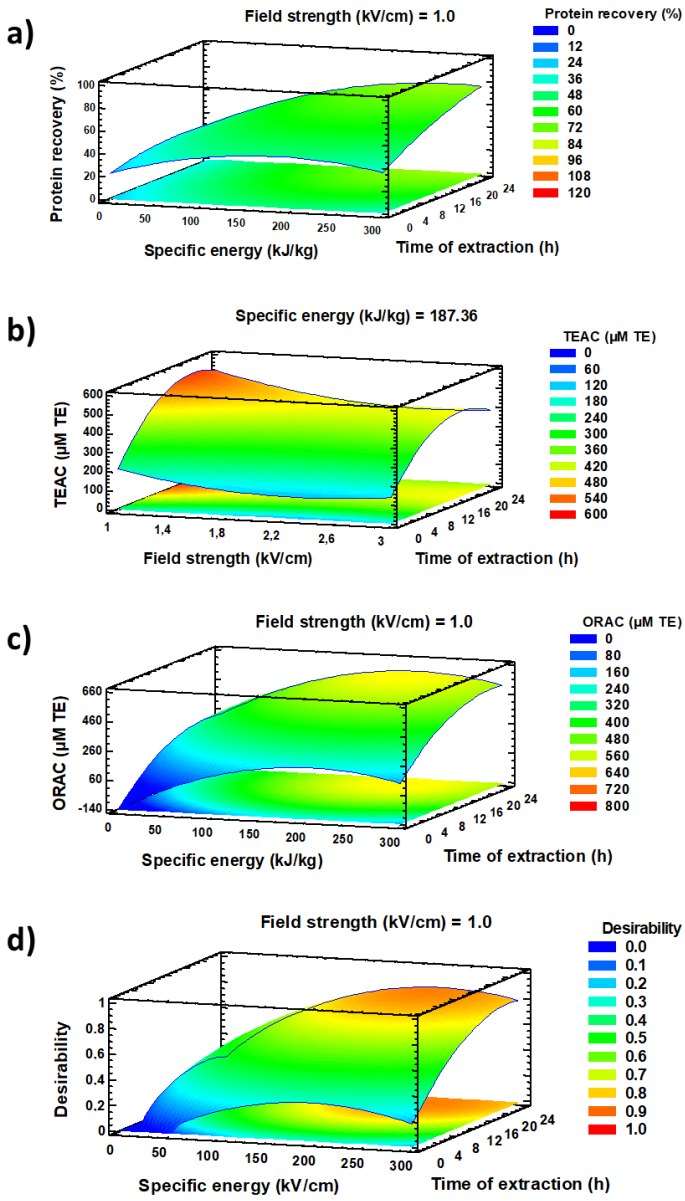

Figure S1. Response surface plots for protein recovery (a), TEAC (b) and ORAC (c) values obtained for head extracts and desirability degree (d). Desirability is based on the common response of the various responses analyzed. The less significant parameter was fixed at its optimal condition.

Table S3. Protein recovery, TEAC and ORAC results for sea bass skin extract obtained for each experiment of response-surface optimization.

Experiment	Specific energy (kJ/Kg)	Field strenght (kV/cm)	Time of extraction (h)	% Protein Recovery (g/100 g proteins in sample)	Antioxidant Capacity	
					TEAC (µmol TE/L)	ORAC (µmol TE/L)
1	300	2	12	12.88	505.92	721.93
2	300	1	24	29.93	645.70	613.07
3	50	3	24	13.87	543.90	442.68
4	50	2	12	13.55	490.88	881.63
5	50	1	0	6.72	119.85	158.65
6	175	3	12	20.58	551.59	604.00
7	175	2	0	5.54	120.02	146.01
8	300	1	0	10.56	166.17	441.91
9	300	3	0	8.88	181.52	435.32
10	50	3	0	5.09	52.10	52.60
11	300	3	24	39.58	749.28	1662.31
12	50	1	24	12.61	265.16	285.03
13	175	1	12	16.72	435.50	917.60
14	175	2	24	13.36	486.52	429.35
15	175	2	12	10.39	445.07	566.29
16	175	2	12	11.46	440.67	330.07

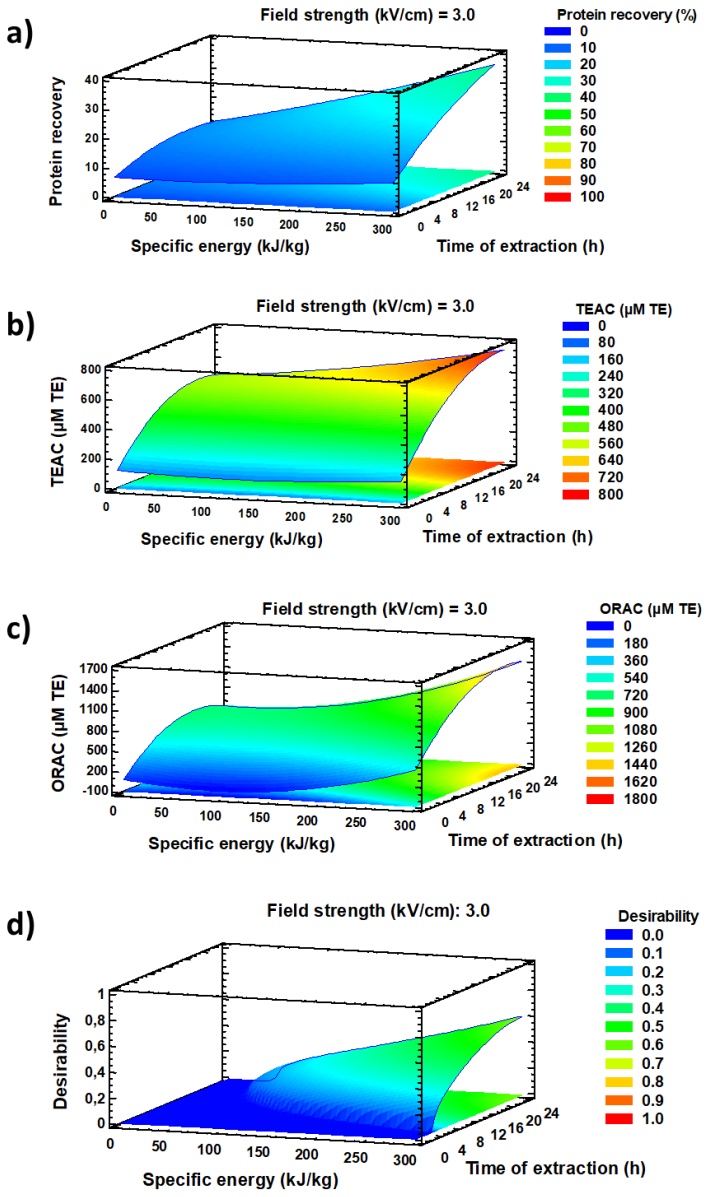

Figure S2. Response surface plots for protein recovery (a), TEAC (b) and ORAC (c) values obtained for skin extracts and desirability degree (d). Desirability is based on the common response of the various responses analyzed. The less significant parameter was fixed at its optimal condition

Table S4. Protein recovery, TEAC and ORAC results for sea bass viscera extract obtained for each experiment of response-surface optimization.

Experiment	Specific energy (kJ/Kg)	Field strenght (kV/cm)	Time of extraction (h)	% Protein Recovery (g/100 g proteins in sample)	Antioxidant Capacity	
					TEAC (µmol TE/L)	ORAC (µmol TE/L)
1	300	2	12	51.85	894.44	2262.10
2	300	1	24	46.23	648.91	1358.19
3	50	3	24	53.93	952.99	2316.29
4	50	2	12	97.31	1041.30	2927.15
5	50	1	0	20.14	173.44	161.82
6	175	3	12	107.25	1129.73	5680.53
7	175	2	0	23.83	341.66	199.51
8	300	1	0	32.81	568.90	707.21
9	300	3	0	23.74	452.11	424.72
10	50	3	0	18.40	139.58	78.45
11	300	3	24	33.27	1046.67	794.38
12	50	1	24	52.85	701.03	2218.30
13	175	1	12	49.77	983.51	2482.46
14	175	2	24	40.20	1020.99	1926.99
15	175	2	12	72.17	921.18	3064.80
16	175	2	12	83.44	922.91	2427.66

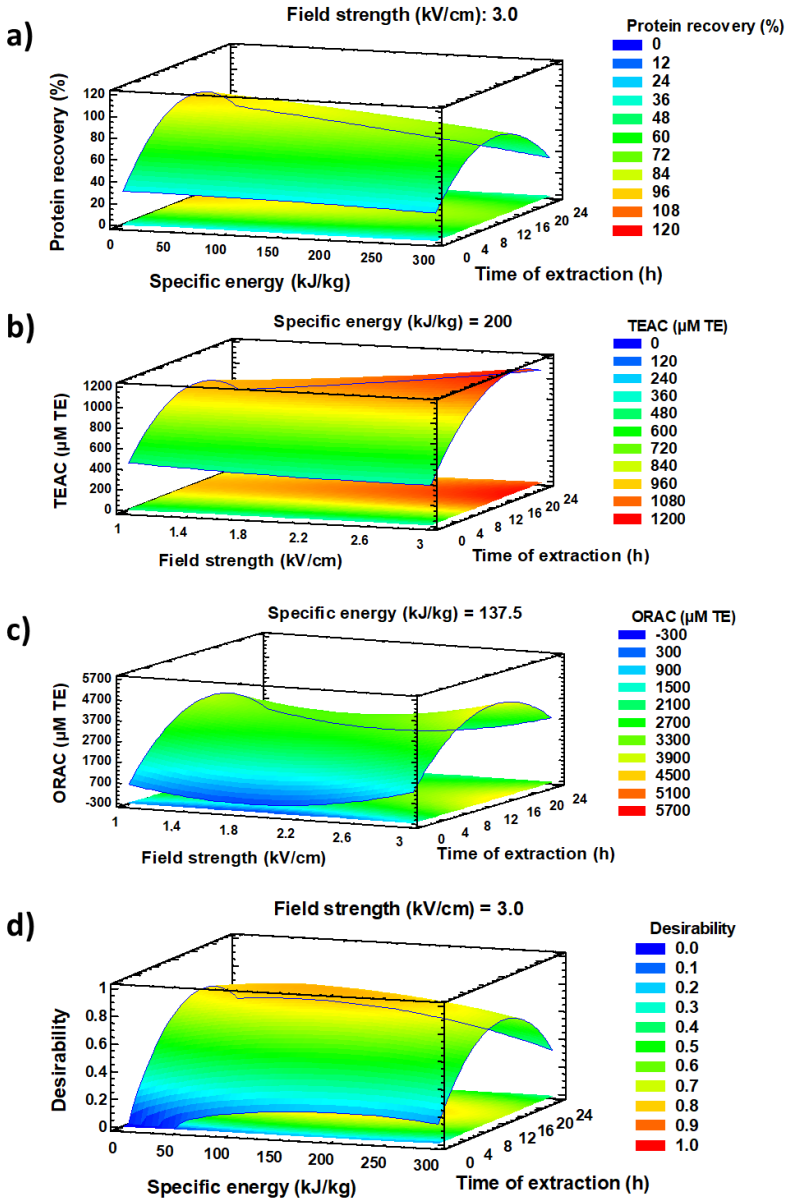

Figure S3. Response surface plots for protein recovery (a), TEAC (b) and ORAC (c) values obtained for viscera extracts and desirability degree (d). Desirability is based on the common response of the various responses analyzed. The less significant parameter was fixed at its optimal condition.

Table S5. Protein recovery, TEAC and ORAC results for sea bass backbone extract obtained for each experiment of response-surface optimization.

Experiment	Specific energy (kJ/Kg)	Field strenght (kV/cm)	Time of extraction (h)	% Protein Recovery (g/100 g proteins in sample)	Antioxidant Capacity	
					TEAC (µmol TE/L)	ORAC (µmol TE/L)
1	300	2	12	25.43	1.14	749.68
2	300	1	24	28.03	23.00	544.48
3	50	3	24	25.41	9.76	469.19
4	50	2	12	29.72	8.65	669.95
5	50	1	0	12.90	13.28	111.31
6	175	3	12	28.75	0.07	443.35
7	175	2	0	10.89	10.09	46.59
8	300	1	0	15.76	11.39	167.48
9	300	3	0	13.61	8.49	154.37
10	50	3	0	12.73	9.92	92.66
11	300	3	24	24.91	0	259.42
12	50	1	24	23.37	18.60	382.67
13	175	1	12	27.92	1.22	746.61
14	175	2	24	25.55	14.61	494.11
15	175	2	12	29.17	21.35	633.47
16	175	2	12	21.77	11.93	391.30

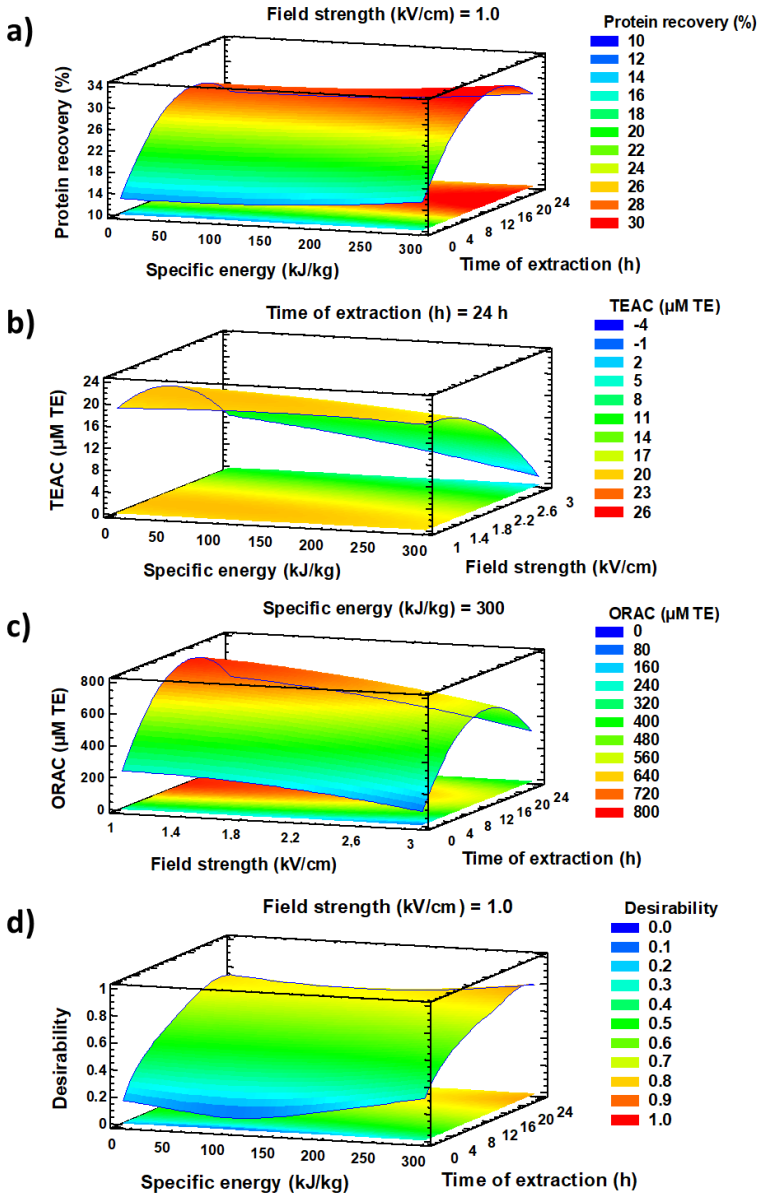

Figure S4. Response surface plots for protein recovery (a), TEAC (b) and ORAC (c) values obtained for backbone extracts and desirability degree (d). Desirability is based on the common response of the various responses analyzed. The less significant parameter was fixed at its opti-mal condition.

Extraction of Antioxidant Compounds and Pigments from Spirulina (*Arthrospira platensis*) Assisted by Pulsed Electric Fields and the Binary Mixture of Organic Solvents and Water

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Abstract

The application of pulsed electric fields (PEF) is an innovative extraction technology promoting cell membrane electroporation, thus allowing for an efficient recovery, from an energy point of view, of antioxidant compounds (chlorophylls, carotenoids, total phenolic compounds, etc.) from microalgae. Due to its selectivity and high extraction yield, the effects of PEF pre-treatment (3 kV/cm, 100 kJ/kg) combined with supplementary extraction at different times (5–180 min) and with different solvents (ethanol (EtOH)/H_2O, 50:50, v/v; dimethyl sulfoxide (DMSO)/H_2O, 50:50, v/v) were evaluated in order to obtain the optimal conditions for the extraction of different antioxidant compounds and pigments. In addition, the results obtained were compared with those of a conventional treatment (without PEF pre-treatment but with constant shaking). After carrying out the different experiments, the best extraction conditions to recover the different compounds were obtained after applying PEF pre-treatment combined with the binary mixture EtOH/H_2O, 50:50, v/v, for 60–120 min. PEF extraction was more efficient throughout the study, especially at short extraction times (5–15 min). In this sense, recovery of 55–60%, 85–90%, and 60–70% was obtained for chlorophylls, carotenoids, and total phenolic compounds, respectively, compared to the maximum total extracted amount. These results show that PEF improves the extraction yield of antioxidant bioactive compounds from microalgae and is a promising technology due to its profitability and environmental sustainability.

Keywords: pulsed electric fields; green extraction; microalgae; antioxidants; pigments

1. Introduction

Over the last decade, several research studies have evaluated the use of microalgae as a source of nutrients and bioactive compounds. This is due to a growing interest in the development of new foods that provide health benefits and that meet basic energy and nutritional requirements [1,2]. It is preferred that functional foods have a natural origin, such as from plants, algae and/or microalgae. In this sense, proteins obtained from microalgae have been used to replace proteins of animal origin in meat-like preparations such as turkey burgers [3] or in the fortification of vegan foods such as kefir produced from soy and almond based beverages [4].

Microalgae are becoming increasingly relevant, especially for their composition, since they are a source of high-added-value compounds [5], such as carotenoids, chlorophylls, and other pigments (antioxidants) [6], and polyunsaturated fatty acids [7,8].

Conventional extraction of antioxidant bioactive compounds from microalgae is often carried out using solvents and using dry biomass [9]. However, this conventional extraction method is very slow, involves the extraction of unwanted compounds, and can promote the degradation of some thermolabile compounds. Therefore, there is a need for innovative approaches such as pulsed electric fields (PEF) that affect the quality of the extracted compounds to a lesser extent and can be applied in a continuous flow to obtain higher extraction efficiency rates, minimize the use of solvents, and thus be a more efficient and sustainable extraction alternative [10,11].

In recent years, several studies have applied PEF technology on microalgae for extraction purposes [12,13,14,15]. This non-thermal technique consists of applying high-voltage electrical pulses between two electrodes in the treatment chamber [16]. Short electric pulses (1–100 μs) at field intensities

of 0.1–1 kV/cm are employed for reversible permeabilization in plant cells for stress induction, at 0.5–3 kV/cm for irreversible permeabilization of plant and animal tissues, and at 15–40 kV/cm for irreversible permeabilization of microbial cells.

The external electric field increases the transmembrane potential and promotes membrane pore formation of the biological cell. High-intensity electric pulses can be generated by the switched discharge of a suitable capacitor bank. The properties of the discharge circuit determine the shape of the time-dependent potential at the treatment chamber. Parallel plate electrodes or colinear type treatment chambers constitute the most commonly used circuits [17]. The PEF technique can be effectively applied in many food processing applications such as microorganism/enzyme inactivation, recovery of bioactive compounds, drying and freezing processes, and to promote the enhancement of some selected properties of food macromolecules and some chemical reactions [16]. It has several advantages compared to conventional techniques as it favours the extraction of bioactive compounds by generating reversible or irreversible micropores in the plasma membrane of the cells, promoting the migration of interesting compounds into the cytoplasm through the membrane with high selectivity/purity, no thermal effect, and short extraction times [18]. The efficiency of PEF to permeabilize cell membranes differs according to process parameters such as electric field strength, treatment time, specific energy, pulse shape, pulse width, frequency, temperature, the properties of the treated food sample such as its pH and conductivity, and the characteristics of the target cells [19].

An example of its usefulness is observed in the extraction of lipids from microalgae. In this regard, a first PEF-assisted extraction of water-soluble bioactive compounds that cross the membrane through the formed micropores can be performed and then a second extraction with ethanol

(EtOH) can be applied to obtain lipids or lipid-soluble metabolites that have remained inside the cell, which provides advantages in subsequent purification processing [20]. The use of PEF is possible in aqueous solutions with a low dry matter content, facilitating the more energy-efficient isolation of high-added-value compounds extracted from microalgae directly from the culture without dehydration or drying [20,21].

Most of the studies evaluating the application of PEF focus on the use of aqueous suspensions of microalgae, which mainly allows for the extraction of water-soluble compounds [22,23], while the extraction of non-polar pigments (e.g., chlorophylls and carotenoids) is very low under these conditions due to their low solubility in water [24]. Some previous studies have evaluated the use of complementary extractions with EtOH (96%), obtaining higher yields in the extraction of pigments from microalgae pre-treated with PEF [25]. Studies combining the use of PEF in suspensions of microalgae (*Nannochloropsis*) in water and the subsequent addition of an organic solvent to improve pigment extraction efficiency have been also carried out [12,13].

PEF-assisted extraction yields of nutritionally valuable compounds (lipids, pigments, and proteins) can vary depending on the microalgae used [26]. Therefore, specific studies are needed to evaluate the extraction in different microalgae species at different times and with different solvents to obtain the necessary information and thus be able to scale up the process to an industrial level in an efficient, sustainable way and with a high extraction yield.

Several studies are currently evaluating PEF-assisted extraction from microalgae biomass such as *Chlorella* and *Nannochloropsis* [12,22,25,27]. However, there is a lack of data on the impact of PEF on the extraction of high-added-value compounds from other microalgae species such as spirulina (*Arthrospira platensis*). Spirulina is an undifferentiated filamentous cyanobacterium [28,29] whose cells are 3–12 μm wide and can reach 16 μm

[30]; it has been used as food for centuries by various cultures as it has a biochemical profile rich in bioactive molecules and there are studies that support its benefits for human health [31].

However, considering the gap that exists regarding different extraction levels of antioxidant compounds according to the different microalgae species, the main aim of this study was to evaluate how PEF pre-treatment combined with the binary mixtures of ethanol (EtOH)/H2O, 50:50, v/v and dimethyl sulfoxide (DMSO)/H2O, 50:50, v/v at different extraction times can affect to the recovery of antioxidant bioactive compounds from spirulina.

2. Materials and Methods

2.1. Sample

The spirulina, in noodle form, was produced by the company Ecospirulina (Serra, Comunitat Valenciana, Spain). The cultured biomass comes from the species *Artrospira platensis* (more recently *Limnospira platensis*), strain paracas 15016. The Paracas reference refers to the lake from which it originated, Lake Paracas, south of Lima, Peru.

In Ecospirulina, cultivation is carried out in ponds in a greenhouse under natural sunlight and without the use of artificial light. Shading is applied to partially cover the culture ponds, which allows pigment production to be controlled. During the production of the sample used in this trial, the average temperature during the day was 32 °C, while the average temperature during the night was 24 °C. The pH of the culture varied between 9.8 and 10.4, being regulated by the addition of CO_2 at the time of each harvest.

The biomass was filtered through a drum filter with 30-micron mesh. The culture substrates were returned to the culture pond, while the biomass was vacuum pressed and then converted into noodle form. In this format, it was

air-dried at low temperature (40 ± 2 °C) to reduce the degradation of poorly resistant bioactive compounds at higher temperatures.

2.2. Extraction Procedure

Four samples of 2% (w/v) aqueous suspensions were made from the spirulina dry matter (DM). For this, 198 mL of deionised water were added to 4 g of dry biomass. In two of four samples, the same volume (198 mL) of EtOH or DMSO was subsequently added to finish with a 1% suspension (Figure 1). These two mixtures only received a conventional shaking treatment under stirring. The other 2% suspensions in water were treated with PEF before mixing with the organic solvents. As can be observed in Figure 1, the extracts were obtained from the 1% suspensions at different times and centrifuged to obtain the supernatant to be analysed. A total of 24 extracts were obtained, 12 of which were obtained by conventional extraction and the other 12 by PEF-assisted extraction.

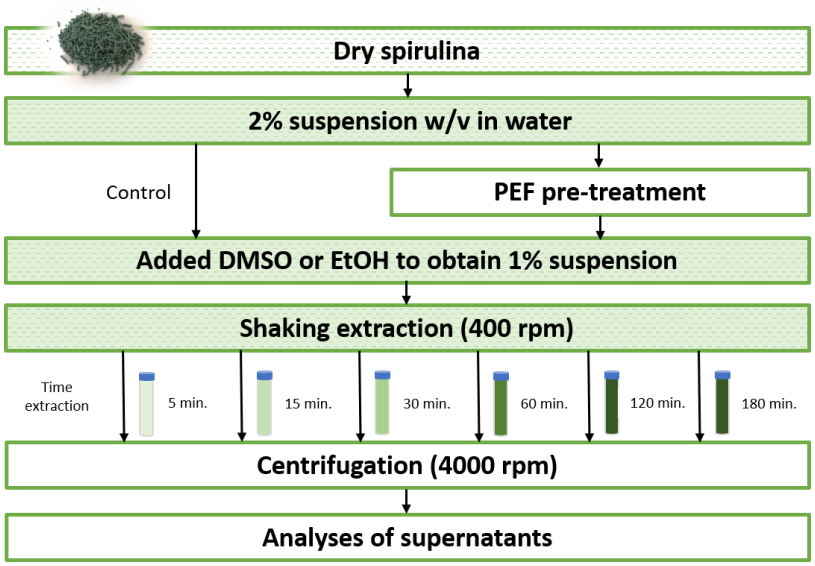

Figure 1. A schematic representation of the extraction process.

2.2.1. Pulsed Electric Fields (PEF) Extraction

For the PEF pre-treatment of the spirulina 2% (w/v) solution was used with the PEF-Cellcrack III equipment (German Institute for Food Technology (DIL)) (ELEA, Ger-many). A treatment chamber of 900 mL capacity was used, the distance between the elec-trodes was fixed at 10 cm, and the total mass added to the treatment chamber was 202 g (198 g of water + 4 g of microalgae). A 100 kJ/kg treatment was applied with an electric field of 3 kV/cm according to a previous work [24]. Before and after the treatment, the temperature and conductivity of each sample was measured with a portable conductivity meter, ProfiLine Cond 3310 (WTW, Xylem Analytics, Weilheim in Oberbayern, Germany). The minimum electric field strength needed to produce changes in the cells is 1 kV/cm, and it has been shown that with a pulse duration of milliseconds, an electric field of 3–4 kV/cm is sufficient to create electroporation [14,32].

2.2.2. Solvent Extractions

First, conventional extraction (Control) was performed on one set of samples. The solvents dimethyl sulfoxide (DMSO) or ethanol (EtOH) (1:1, v/v) in deionised water were added up to a final volume of 400 mL to the dry spirulina samples for nutrient extraction. Once the solvents were added, the samples were shaken at 400 rpm for 5, 15, 30, 60, 90, 120, or 180 min at room temperature to test the effect of shaking time on the extraction of compounds from the processed biomasses. Subsequently, the samples were centrifuged for 10 min at 4000 rpm using a 5810R centrifuge (Eppendorf Ibérica, Madrid, Spain). The extract obtained was kept at −20 °C for further analysis.

Next, the remaining samples were pre-treated with PEF under the conditions described above, and then an extraction process was carried out following the same methodology used for the conventional method.

Figure 2 shows the extracts obtained after an extraction using (a) DMSO/H_2O or (b) EtOH/H_2O at different extraction times (5–180 min) and the extracts obtained after pre-treatment with PEF using (c) DMSO/H_2O and (d) EtOH/H_2O.

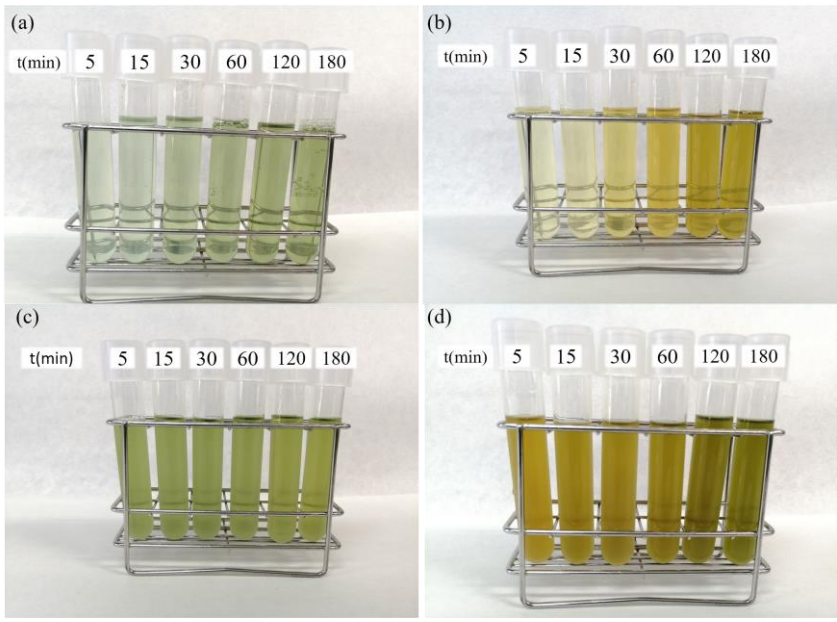

Figure 2. Conventional extraction with (a) DMSO/H2O or (b) EtOH/H2O and their respective extracts using PEF pre-treatment with (c) DMSO/H2O and (d) EtOH/H2O.

2.3. Chemical Analysis

2.3.1. Total Phenolic Compounds (TPC)

For the determination of total phenolic compounds (TPC) (mg gallic acid equivalents (GAE)/g DM), the Folin–Ciocalteu method was used, using the procedure described by Parniakov et al. [12]. This technique is based on the

property of phenols to react against oxidising agents. The Folin–Ciocalteu reagent contains molybdate and sodium tungstate, which react with the phenolic compounds present in the medium to form phosphomolybdic and phosphotungstic complexes. In a basic medium, electron transfer reduces these complexes to tungsten oxide (W_8O_{23}) and molybdenum oxide (Mo_8O_{23}), which are chromogens with an intense blue colour, proportional to the concentration of phenolic groups present in the sample. Gallic acid (Sigma-Aldrich, Steinheim, Germany) was used as a standard. First, Folin–Ciocalteu reagent at 50% v/v, 2% Na_2CO_3, and diluted gallic acid standards were prepared. To carry out the analysis, 3 mL of Na_2CO_3 was added to a test tube, then 100 µL of standard or sample extract was added, and finally 100 µL of Folin–Ciocalteu reagent was added to this mixture. The samples were incubated for 60 min at room temperature under darkness. Finally, the samples were measured at 750 nm wavelength using a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer (Perkin-Elmer, Jügesheim, Germany). All analyses were performed in triplicate.

2.3.2. Trolox Equivalent Antioxidant Capacity (TEAC)

To determine the total antioxidant capacity (TAC), the Trolox equivalent antioxidant capacity (TEAC) assay was used. The TEAC value (micromolar Trolox equivalents, µM TE) measures the antioxidant capacity of a substance, compared to the standard, Trolox (Sigma-Aldrich, Steinheim, Germany). The TEAC value was measured using the method described by Safafar et al. [33] based on the discolouration of the ABTS radical.

The radical cation $ABTS^{\bullet+}$ (chromophore) was produced by reacting a 7 mM ABTS stock solution with 440 µL of an oxidant such as 140 mM potassium persulphate ($K_2S_2O_8$). The mixture was kept in the dark at room temperature for 16 h before use. The solution was then diluted with 96% EtOH until its absorbance at 734 nm was 0.70 ± 0.02 at 30 °C.

Once the initial absorbance was reached, 2 mL of ABTS$^{\bullet+}$ was mixed with 100 µL of extract and the sample was measured after 3 min. The reaction produced a discolouration due to neutralisation of the radical cation of ABTS$^{\bullet+}$, which depended on the concentration of antioxidants in the sample. The absorbance was measured at a wavelength 734 nm in a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer (Perkin-Elmer, Jügesheim, Germany). All analyses were performed in triplicate.

2.3.3. Oxygen Radical Absorbance Capacity (ORAC)

The method used was previously described by Khawli et al. [34]. This assay measures the oxidative degradation of a fluorescent molecule such as fluorescein (Sigma-Aldrich, St. Louis, MO, USA) after the addition of a free radical generator such as 2,2'-azobis(2-aminodinopropane) dihydrochloride (AAPH). The determination measures the degree of antioxidant protection of the sample with respect to the Trolox standard and is expressed in micromolar Trolox equivalents (µM TE).

The automated ORAC assay was performed on a Wallac 1420 VICTOR 2 plate reader (Perkin-Elmer, Jügesheim, Germany). The measurements were carried out in 96-well plates in which only the inner 60 wells were used. For the determination, a phosphate buffer solution (7.5 mM and pH 7–7.4) was prepared. For this purpose, 22.72 g of Na_2HPO_4 and 22.16 g of KH_2PO_4 were weighed and then each dissolved in 200 mL of deionised water. A volume of 61.6 mL of the first solution and 38.9 mL of the second solution were mixed and made up to 1000 mL with deionised water.

The standard (Trolox 100 µM) was prepared each day by adding 12.5 mg of Trolox to 50 mL of the previously prepared phosphate buffer. From this solution, 1 mL was taken and made up to 10 mL with the phosphate buffer to obtain the desired concentration. For fluorescein, 44 mg were weighed and

made up to 100 mL with phosphate buffer. The working solution of 78 nM fluorescein was prepared daily. For this, 0.167 mL of the first solution was taken and made up to 25 mL with phosphate buffer.

Finally, the working dilution of the 221 mM AAPH radical was prepared daily by taking 600 mg of AAPH and bringing it up to 10 mL with phosphate buffer. As the ORAC assay is extremely sensitive, the microalgae extracts were adequately diluted prior to analysis to avoid interferences. In this case, the microalgae samples were diluted between 1:100 and 1:200, v/v.

For plate preparation, 50 µL of fluorescein (78 nM) and 50 µL of sample, blank (phosphate buffer), or standard (Trolox, 100 µM) were placed in each well and finally 25 µL of AAPH (221 mM) was added. As measurement variations may occur from well to well due to the low conductivity of polypropylene plates, the plates were pre-warmed at 37 °C for 10 min after the addition of fluorescein and before the addition of AAPH to avoid this problem. The plates were analysed immediately after the addition of AAPH and measurements were taken every 5 min until the relative fluorescence intensity of the standard (Trolox) was less than 5% of the initial reading value. All analyses were performed in triplicate.

2.3.4. Chlorophyll a, Chlorophyll b, and Carotenoids

Chlorophyll a, chlorophyll b, and carotenoids contents were estimated spectrophotometrically according to the study by Parniakov et al. [35]. They were calculated using the equations of Lichtenthaler and Wellburn [36] for EtOH and Wellburn [37] for DMSO. This method is based on the determination of carotenoid and chlorophyll content based on the maximum absorbances of chlorophyll a (C_a), chlorophyll b (C_b), and total carotenoids (C_{x+c}). Using EtOH as a solvent, the maximum absorbances were found at the wavelengths of 664 nm, 648 nm, and 470 nm for chlorophyll a (C_a), chlorophyll b (C_b) and total

carotenoids (C_{x+c}), respectively. For DMSO, the wavelengths were 665 nm, 649 nm, and 480 nm, respectively. Aliquots of the extracts obtained were diluted and the absorbances (A) were measured at the wavelengths listed above according to the solvent used. All analyses were performed in triplicate.

EtOH equations:

$$C_a\ (\mu g/mL) = 13.36\ A_{664} - 5.19\ A_{648} \quad (1)$$

$$C_b\ (\mu g/mL) = 27.43\ A_{648} - 8.12\ A_{664} \quad (2)$$

$$C_{x+c}\ (\mu g/mL) = (1000\ A_{470} - 2.13\ C_a - 97.64\ C_b)/209 \quad (3)$$

DMSO equations:

$$C_a\ (\mu g/mL) = 12.47\ A_{665} - 3.62\ A_{649} \quad (4)$$

$$C_b\ (\mu g/mL) = 25.06\ A_{649} - 6.5\ A_{665} \quad (5)$$

$$C_{x+c}\ (\mu g/mL) = (1000\ A_{480} - 1.29\ C_a - 53.78\ C_b)/220 \quad (6)$$

2.4. Statistical Analysis

Data were analysed using analysis of variance (ANOVA), where PEF pre-treatment, solvents, and extraction time were the factors and chlorophyll, carotenoids, TPC, TEAC, and ORAC concentrations were the variables. Data were expressed as mean ± standard deviation in all cases. A value of $p < 0.05$ was considered significant. In addition, the LSD (Least Significant Differences) test was performed to determine the differences between the means of the values obtained. All analyses were performed with STATGRAPHICS Centurion XVI 16.1.03 (Statgraphics Technologies Inc., Princeton, NJ, USA).

3. Results and Discussion

In the present study, the effect of extraction time, the use of polar solvents such as DMSO and EtOH (aprotic and protic, respectively) combined with H_2O (50:50, v/v), and the application of a PEF pre-treatment to improve the extraction yield and efficiency of pigments and antioxidant compounds were evaluated.

3.1. Conventional Extraction

3.1.1. Chlorophyll a, Chlorophyll b, and Carotenoids

Figure 3 shows the chlorophyll a, chlorophyll b, and carotenoid content of the extracts obtained after conventional extraction with respect to the extraction time and solvent used. After performing a 2-factor analysis of variance (ANOVA) (time and solvent), it was observed that both extraction time and solvent had a significant effect ($p < 0.05$) on the extraction of chlorophyll a, chlorophyll b, and carotenoids, observing a higher extraction of all these compounds with the longer extraction times, and in general, observing higher values of chlorophyll a, chlorophyll b, and carotenoids when an EtOH/H_2O mixture was used compared to that seen with the DMSO/H_2O mixture. Thus, at 180 min, the highest values of chlorophyll a (0.57 ± 0.01 mg/g DM), chlorophyll b (0.55 ± 0.01 mg/g DM), and carotenoids (0.50 ± 0.01 mg/g DM) were obtained after using the EtOH/H_2O mixture, representing an increase of 34%, 54%, and 84.2%, respectively, compared to those obtained with the DMSO/H_2O solvent at equivalent extraction times.

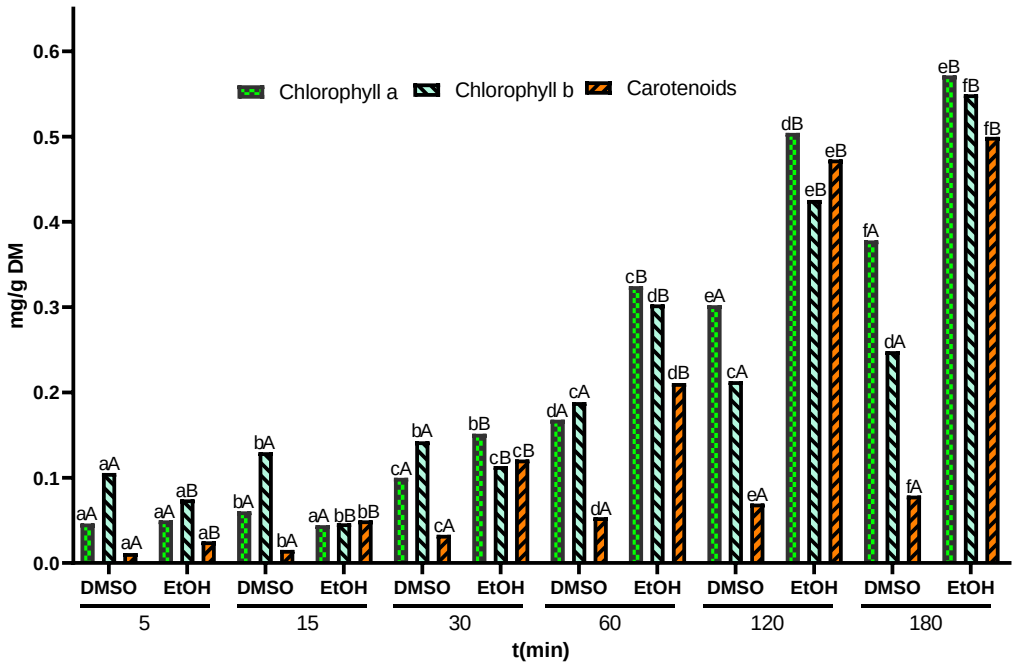

Figure 3. Chlorophyll a, chlorophyll b, and total carotenoids. Conventional extraction (5–180 min) using two binary mixtures (EtOH or DMSO in 50% deionised water). Different lower-case letters in the same parameter and solvent indicate statistical differences as a function of extraction time. Different capital letters in the same parameter and time indi-cate statistical differences as a function of solvent.

However, it should be noted that this was not verified for all the compounds evaluated. For example, when using the solvent EtOH/H_2O, no significant differences were found in the chlorophyll a content after 5 and 15 min of extraction. Moreover, no statistically significant differences in chlorophyll b content were observed after 15 and 30 min of extraction with the solvent DMSO/H_2O. When analysing the statistical results between

solvents, no differences were found in chlorophyll a extraction after 5 and 15 min of extraction.

These results are in agreement with those obtained by other authors, who observed a higher extraction of chlorophyll a, chlorophyll b, and carotenoids when the EtOH/H_2O mixture was used [13]. They attributed this effect to a change in the polarity of the medium, which made it easier for the compounds to cross the lipid membrane of the cell, favouring the extraction of the compounds studied.

3.1.2. Total Phenolic Compounds (TPC) and Total Antioxidant Capacity (TAC)

Figure 4 shows the results obtained for total phenolic compounds (TPC) and total antioxidant capacity (TAC) determined by TEAC and ORAC. After performing a 2-factor analysis of variance (ANOVA) (time and solvent), it was observed that both time and solvent had a significant effect ($p < 0.05$) on the extraction of phenolic compounds.

ORAC values ranged from 295.87 ± 19.01 to 393.24 ± 15.28 µmol TE/g DM after using EtOH/H_2O and from 72.45 ± 5.95 to 155.95 ± 10.78 µmol TE/g DM when the mixture DMSO/H_2O was used. In addition, a greater effect on the extraction of antioxidant compounds was observed at 30 and 180 min, respectively. On the other hand, the TEAC values ranged from 4.52 ± 0.02 to 19.05 ± 1.25 µmol TE/g DM after using the EtOH/H_2O mixture and from 9.19 ± 0.62 to 16.19 ± 1.37 µmol TE/g DM with the DMSO/H_2O mixture. The maximum value for the TEAC assay (19.05 µmol TE/g DW) was obtained after 180 min extraction and using EtOH 50% as the solvent. The highest ORAC value was also obtained after using EtOH, but after 30 min of extraction (393.24 µmol TE/g DW). Regarding DMSO, the best values for TEAC and ORAC were obtained at 60 min (16.19 µmol TE/g DW) and 15 min (155.95 µmol TE/g DW), respectively. The higher ORAC values are due to other antioxidant

compounds not measured in this study, especially fat-soluble compounds such as vitamin E that may have an impact on the antioxidant capacity [38,39,40]. Moreover, ORAC sensitivity for other antioxidant compounds should also be taken into account [34].

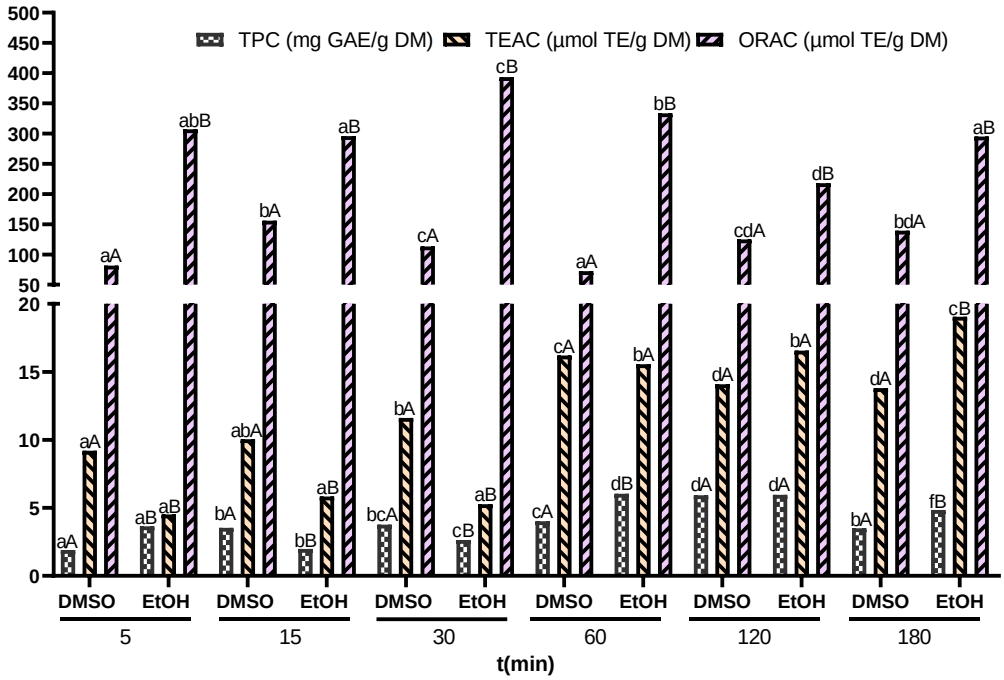

Figure 4. Total phenolic compounds, TEAC and ORAC. Conventional extraction (5–180 min) using two binary mixtures (EtOH or DMSO in 50% deionised water). Different lower-case letters in the same parameter and solvent indicate statistical differences as a function of extraction time. Different capital letters in the same parameter and time indicate statistical differences as a function of solvent.

In the determination of total phenolic compounds (TPC), concentrations between 3.64 ± 0.07 and 6.04 ± 0.28 mg GAE/g DM were obtained for the EtOH/H_2O extraction and between 1.91 ± 0.07 and 5.921 ± 0.175 mg GAE/g DM for the DMSO/H_2O extraction. For both solvents, the highest values were obtained at 120 min, the phenolic content at that time was similar to that obtained by Shanti et al. [41] for spirulina.

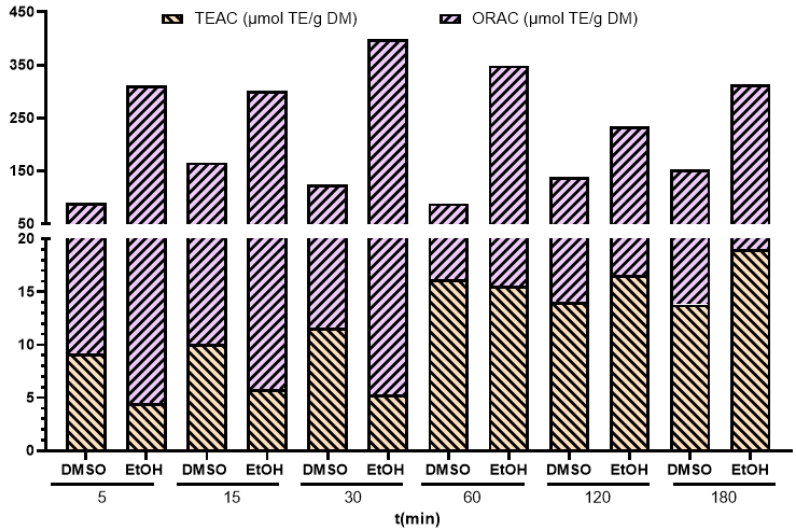

Figure 5. Total antioxidant capacity (TAC) values obtained by adding the mean values of TEAC and ORAC for each time and solvent.

Regarding the mean values of TAC (TEAC + ORAC), it is worth noting that the values obtained with DMSO/H_2O are relatively lower compared to those of the EtOH/H_2O mixture, independent of the extraction time (Figure 5). This may be due to a lower extraction of chlorophylls, carotenoids, and phenolic compounds, as seen above, suggesting a clear contribution of these compounds to TAC. This same correlation has been previously observed by

other authors after evaluating the use of these solvents for the extraction of these compounds from the microalgae *Nannochloropsis* [13].

Table 1. Correlation between antioxidant compounds and total antioxidant capacity (TEAC and ORAC).

	TPC	TEAC	ORAC	Chlorophyll a	Chlorophyll b	Carotenoids
TPC		0.7114 (36) 0.0000	0.0301 (36) 0.8618	0.7016 (36) 0.0000	0.6827 (36) 0.0000	0.5727 (36) 0.0003
TEAC	0.7114 (36) 0.0000		−0.3054 (36) 0.0701	0.7986 (36) 0.0000	0.8419 (36) 0.0000	0.6306 (36) 0.0000
ORAC	0.0301 (36) 0.8618	−0.3054 (36) 0.0701		0.1415 (36) 0.4103	0.1164 (36) 0.4991	0.3586 (36) 0.0317
Chlorophyll a	0.7016 (36) 0.0000	0.7986 (36) 0.0000	0.1415 (36) 0.4103		0.9511 (36) 0.0000	0.8735 (36) 0.0000
Chlorophyll b	0.6827 (36) 0.0000	0.8419 (36) 0.0000	0.1164 (36) 0.4991	0.9511 (36) 0.0000		0.9169 (36) 0.0000
Carotenoids	0.5727 (36) 0.0003	0.6306 (36) 0.0000	0.3586 (36) 0.0317	0.8735 (36) 0.0000	0.9169 (36) 0.0000	

* TPC: Total phenolic compounds; TEAC: Trolox equivalent antioxidant capacity; ORAC: Oxygen radical antioxidant capacity.

To evaluate the possible correlations between the different antioxidant compounds (chlorophyll a, chlorophyll b, carotenoids, and total phenolic compounds) and the determination methods used for total antioxidant capacity (TEAC and ORAC), a Pearson's test was performed for 36 samples (Table 1). The main correlations were observed between TEAC and carotenoids ($R = 0.9094$, $p < 0.05$), chlorophyll a ($R = 0.7986$, $p < 0.05$), chlorophyll b ($R = 0.8419$, $p < 0.05$), and total phenolic compounds ($R = 0.7114$, $p < 0.05$), obtaining moderate to high positive correlation coefficients. These results are in agreement with those of other authors [42,43] who evaluated the

existing correlations between the different antioxidant compounds and the methods used for the joint evaluation of the total antioxidant capacity.

It should be noted that there was no correlation between antioxidant compounds (chlorophyll a and chlorophyll b) and ORAC. This is because the ORAC method is a more sensitive technique and some authors consider it better than TEAC [44] when measuring total antioxidant compounds, as ORAC measures the antioxidant capacity of compounds other than phenolics. A significant correlation was observed, but with a low correlation coefficient between ORAC and carotenoids ($R = 0.3586$, $p < 0.05$).

3.2. Pulsed Electric Fields (PEF)-Assisted Extraction

3.2.1. Ethanol/Water

Figure 6 shows the results obtained after applying both treatments (conventional and PEF) using the mixture EtOH/H_2O. After performing a two-way ANOVA analysis (time and treatment), it was observed that both the extraction time and the use of the PEF pre-treatment had a significant effect ($p < 0.05$) on the extraction of chlorophylls, carotenoids, (Figure 6a), and total phenolic compounds (Figure 6b). This effect is due to the electroporation produced by the PEF, which facilitates the extraction of these compounds more efficiently and with less agitation time.

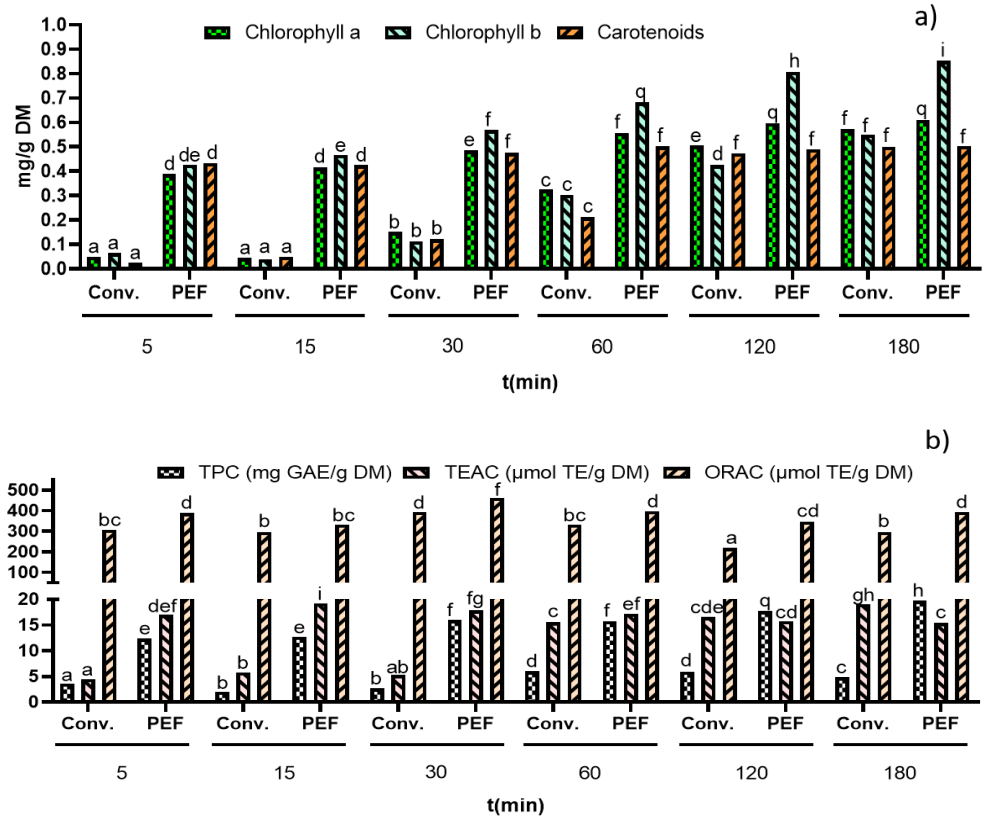

Figure 6. Chlorophyll a, chlorophyll b, and carotenoid content (a); TPC, TEAC, and ORAC (b) after conventional extraction and PEF-assisted extraction with EtOH/H_2O. Different lower-case letters in the same parameter indicate statistical differences depending on the extraction time or treatment used.

It should be noted that no significant differences were observed regarding the maximum carotenoid content obtained after applying PEF pre-treatment for 60 min (0.50 ± 0.01 mg/g DM) or after conventional treatment without PEF for 180 min (0.50 ± 0.01 mg/g DM), which showed that PEF is an effective tool to reduce carotenoid extraction time, being 3 times faster than conventional extraction. A similar effect was observed for chlorophyll a: We obtained similar values after applying PEF and supplementary extraction for 120 min (0.60 ± 0.01 mg/g DM) as those obtained with conventional treatment

for 180 min (0.57 ± 0.04 mg/g DM), observing a reduction of 60 min in the time to obtain the maximum chlorophyll a content. Moreover, PEF pre-treatment increased the extraction of chlorophyll b throughout all the extraction times compared to that of the control sample.

After analysing the statistical data of the TPC values, it was found that the pre-treatment with PEF also had a very positive effect on the extraction, obtaining significant differences ($p < 0.05$) compared to those of the conventional treatment, independently of the extraction time. Moreover, it was also observed that after PEF extraction, the TPC values were 2–3-fold higher. For example, after 180 min of conventional extraction, TPC values of 4.84 ± 0.48 mg GAE/g DM were obtained, while the value obtained at the same time after PEF pre-treatment was 19.75 ± 0.50 mg GAE/g DM, representing a 75% increase. The increase in chlorophylls, carotenoids, total phenolic compounds, and TAC content obtained after PEF application compared to those of conventional extraction is in agreement with the results obtained by other authors after similar experiments with the microalgae *Nannochloropsis spp.* [13].

3.2.2. Dimethyl Sulfoxide/Water

Figure 7 shows the results obtained for chlorophyll, carotenoids, TPC, TEAC, and ORAC content after conventional and PEF-assisted extraction using DMSO/H_2O as a solvent. After performing a two-way ANOVA (time and treatment), it was observed that both extraction time and treatment had a significant effect ($p < 0.05$) on the extraction of chlorophylls, carotenoids (Figure 7a), and TPC (Figure 7b). This effect was less than that observed after using the EtOH/H_2O mixture, mainly due to a lower solvent extraction capacity, as could be observed after using the conventional extraction method (Figure 3 and Figure 4).

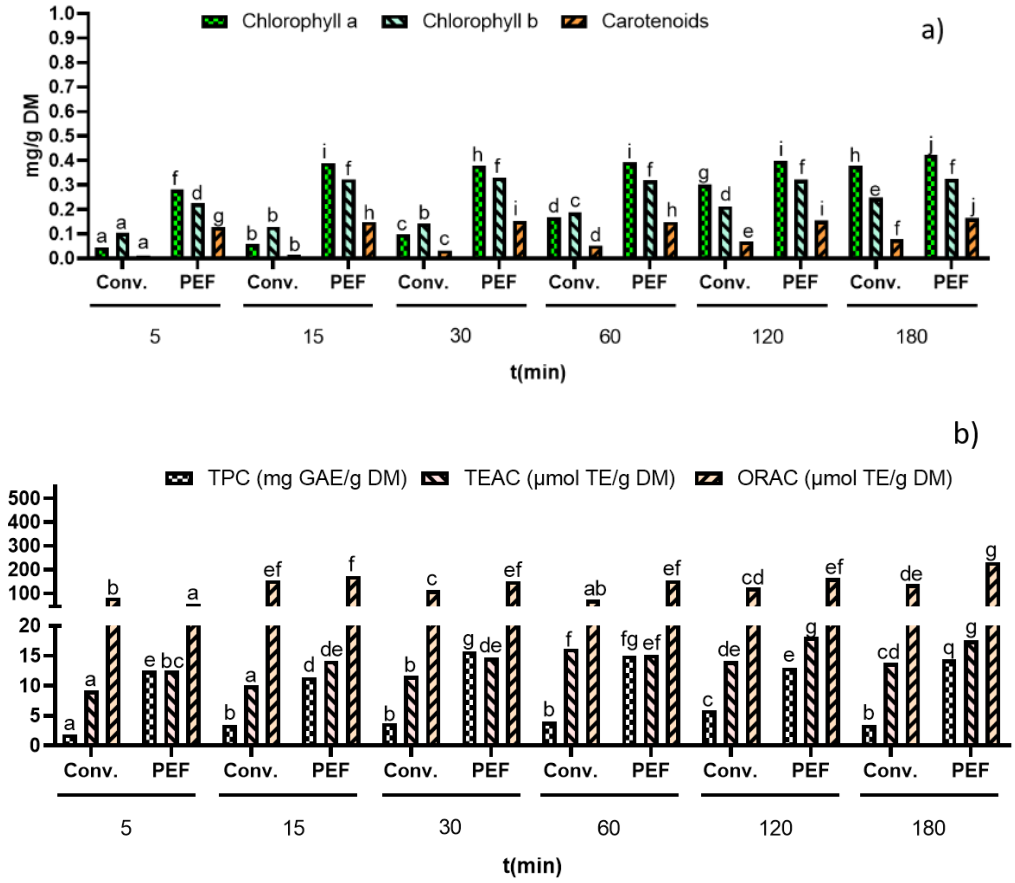

Figure 7. Chlorophyll a, chlorophyll b, and carotenoid content (a); TPC, TEAC, and ORAC (b) after conventional extraction and PEF-assisted extraction with DMSO/H_2O. Different lower-case letters in the same parameter indicate statistical differences depending on the extraction time or treatment used.

Lower TEAC values were also observed due to a decreased extraction of antioxidant compounds (chlorophylls, carotenoids, and phenolic compounds) (Table 1). However, the ORAC values were not significantly affected, although a non-significant increase was found when longer extraction times were used.

It should be noted that the maximum extraction of chlorophyll a and chlorophyll b occurred 15 min after applying the PEF pre-treatment, with values of 0.39 ± 0.01 mg/g DM and 0.32 ± 0.01 mg/g DM, respectively. However, after applying the conventional treatment, these values were not obtained until 180 min. PEF pre-treatment reduced the extraction time by 165 min. Regarding carotenoids, a similar effect was found, with the maximum carotenoid extraction (0.15 ± 0.01 mg/g DM) observed after applying the PEF pre-treatment and subsequent extraction for 15 min, obtaining lower values (0.08 ± 0.00 mg/g DM) than after using conventional extraction for 180 min.

The TPC content was also increased by PEF pre-treatment, for example, the maximum content extracted at 5 min after PEF pre-treatment (12.53 ± 0.31 mg AGE/g DM) was much higher than that obtained at 180 min after conventional treatment (4.84 ± 0.48 mg AGE/g DM).

3.3. Pulsed Electric Fields (PEF) Efficiency

In order to better evaluate the effect of PEF on the extraction of compounds and to compare it with conventional extraction, the Y_{PEF} efficiency coefficient was introduced. This coefficient is defined as the ratio between the values obtained for chlorophylls, carotenoids (Figure 8a), TPC, TEAC, and ORAC (Figure 8b) with PEF-assisted extraction and those same values obtained with conventional extraction.

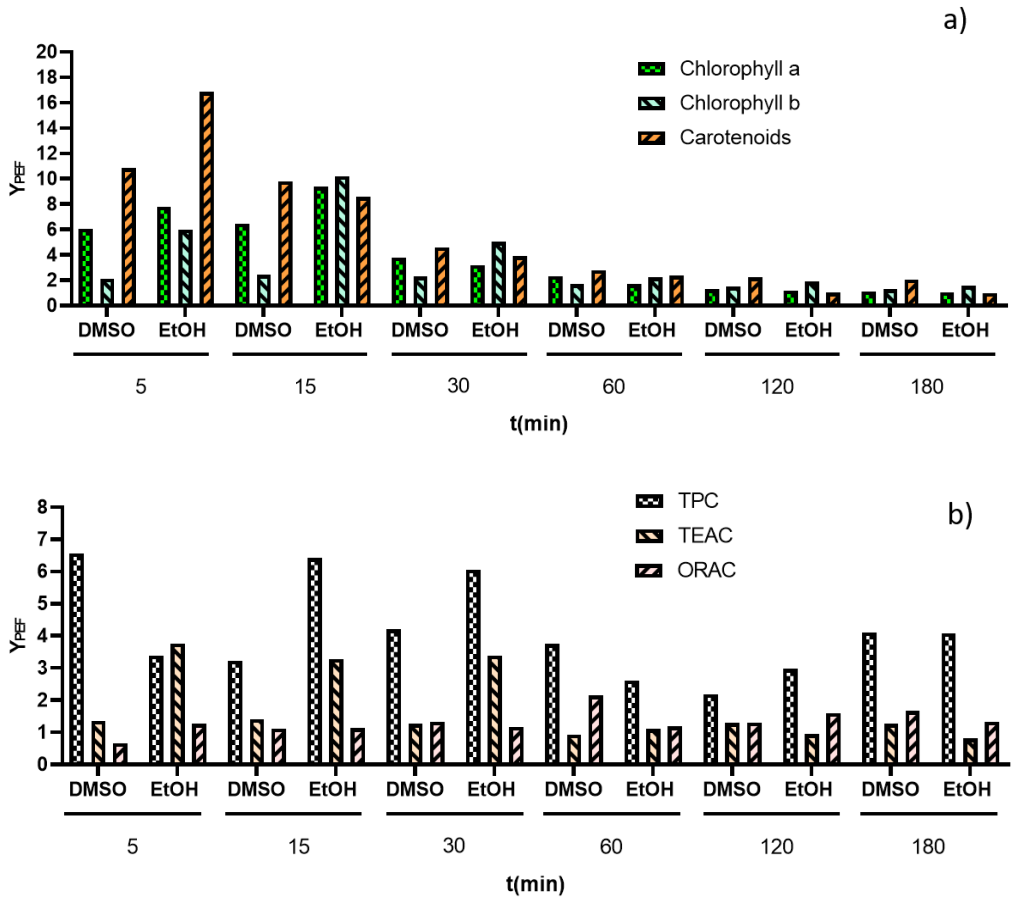

Figure 8. Efficiency ratio (Y_{PEF}) for chlorophyll a, chlorophyll b and carotenoids (a), and TPC, TEAC, and ORAC (b) vs. time.

The value of Y_{PEF} corresponds to $\frac{Ca(\frac{mg}{g}MS)PEF}{Ca(\frac{mg}{g}MS)conv.}$ (for chlorophyll a), $\frac{Cb(\frac{mg}{g}MS)PEF}{Cb(\frac{mg}{g}MS)conv.}$ (for chlorophyll b), etc.

It is important to note that the maximum Y_{PEF} values were observed at the optimum extraction times of 5 and 15 min, independently of the solvent

mixture, obtaining a greater effect in the extraction of carotenoids and TPC. From 30 min onwards, Y_{PEF} values decreased rapidly, which gives an idea of the effectiveness of the PEF in extracting these compounds, suggesting that in a reduced time the extraction can be increased, and the cost reduced. In this regard, a recovery of 55–60% for chlorophylls, 85–90% for carotenoids, and 60–70% for total phenolic compounds was obtained with respect to the maximum total content extracted.

The Y_{PEF} values for TEAC were also increased, as these are directly related to the extraction of the antioxidant compounds. However, this effect was not observed for the ORAC method, as no direct relation between higher extraction and ORAC values was obtained.

4. Conclusions

From the results obtained in this study it can be concluded that both time and solvent have a significant impact on the recovery of antioxidant compounds when conventional extraction is used, obtaining the highest values of phenolic compounds, chlorophyll a, chlorophyll b, and carotenoids when the mixture EtOH/H_2O was used for 180 min. Moreover, a strong relationship of TEAC values with total phenolic compounds, carotenoids, and chlorophylls was found while ORAC values were positively correlated with carotenoids. Both PEF treatment and extraction time had a statistically significant effect on the recovery of antioxidant compounds when the EtOH/H_2O mixture was used, showing a considerable reduction in the extraction time required to recover polyphenols, carotenoids, and chlorophylls compared to those of conventional treatment. When the impact of PEF and the DMSO/H_2O mixture was evaluated, it was found that both the treatment and the extraction time had a statistically significant effect, reducing the extraction times compared to the conventional treatment; however, in this case the maximum content of antioxidant compounds was

lower than that observed for EtOH/H_2O. TAC values were also increased after PEF treatment, mainly due to an increase in the extraction of antioxidant compounds. In addition, the maximum efficiency values were observed at 5 and 15 min for the two solvents, with a greater effect on the extraction of carotenoids and total phenolic compounds. In the future, the implementation of PEF in the extraction of antioxidant bioactive compounds from microalgae would be interesting, as it could be a promising and environmentally sustainable technology.

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Enhancing Nutrient Recovery and Bioactive Compound Extraction from Spirulina through Supercritical Fluid Extraction: Implications for SH-SY5Y Cell Viability

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Foods (Under review)

Abstract

This work explores the efficiency of supercritical fluid extraction (SFE) to recover min-erals, pigments, and antioxidant compounds from the spirulina microalgae. Moreover, the fatty acids and phenolic profile of the extracts obtained were also investigated, and the effect of the extracts on SH-SY5Y cell viability was tested. The extraction of phycocyanin was improved by SFE compared to conventional extraction, from 2.838 ± 0.081 mg/g dry matter (DM) (control) to 6.438 ± 0.411 mg/g DM (SFE). SFE treatment also improved chlorophyll a and carotenoid recoveries increasing from 5.612 ± 0.547 to 8.645 ± 0.857 mg/g DM and from 0.447 ± 0.096 to 0.651 ± 0.120 mg/g DM, respectively. Regarding minerals, the SFE improved Mg recovery a 77% more than the control extraction. Moreover, palmitoleic, stearic, γ-linolenic, eicosadienoic and eicosatrienoc acids recovery was improved by SFE. Phenolic profile was identified by Triple-TOF-LC-MS-MS. Considering heavy metals, a higher Pb extraction was observed for the SFE extract, while no significant differences were observed for Hg between both extractions. Finally, SFE extract improved cell viability compared to control extract. Thus, SFE constitutes an interesting tool to sustainably extract of high-added-value compounds, however, potential contaminants such as Pb need to be controlled in the resulting extracts.

Keywords: supercritical fluid extraction, microalgae, minerals, heavy metals, polyphenols, fatty acids, cell viability

1. Introduction

Over the last years an increased population interest in the consumption of fresh products rich in nutrients and bioactive compounds has been shown, due to their potential use and benefits in medical and pharmaceutical industries [1]. In that regard, microalgae contain polysaccharides and phycobiliproteins showing interesting properties to improve the rheological and nutritional properties of food matrices, while polyphenols and xanthophylls are related with interesting bioactivities, including antioxidant or cytotoxic ef-fects [2]. Owing to the interest in algae as a source of nutraceuticals, novel strategies should be carried on adding value to their derived functional components to be applied in food, animal feed, cosmetics industries as well as in pigments and additives´ production. Moreover, producing bioactive compounds from microalgae is interesting because it con-stitutes a means of sustainable processing [3].

Spirulina is a cyanobacterium considered as a blue microalga, widely found in South America. Because of its composition and the health benefits associated with its consump-tion, this microalga shows interesting potential to become an important food in the future and to be used as an ingredient in the development of functional foods [4]. It includes a high concentration of nutrients, such as proteins, vitamins, minerals, and fatty acids spe-cially, omega-3 and omega-6 fatty acids, which contribute to basic human nutrition [5]. Moreover, it is rich in chlorophylls (Chls), carotenoids (Car), phycocyanins (PC), and phenolic compounds which can be used as colorants and natural antioxidants [6,7].

Conventional extraction techniques such as percolation, maceration, counter-current extraction and Soxhlet involve the use of toxic organic solvents, such as chloroform, methanol, acetone, and diethyl ether in large amounts, for a long time. Moreover, they could affect the biological activity of

the extracted molecules [8,9]. The use of environmentally friendly technologies such as supercritical fluid extraction (SFE), pulsed electric fields, high-voltage electric discharges, high-pressure homogenization, ultrasound, and microwave-assisted extraction have emerged to overcome many limitations of conventional extraction methods. These technologies can extract valuable compounds from microalgae without using toxic solvents minimizing the environmental impact [10,11].

Supercritical fluid extraction constitutes a cost-effective extraction aligned with Green Chemistry principles. It allows to obtain extracts with a great extraction efficiency and selectivity tailoring temperature, pressure, and flow rate parameters. SFE shows promising applications in food processing and in the extraction of metals as complexes and functional ingredients from natural sources, as well as it can constitute an interesting tool in the decontamination of hazardous substances [12]. The major limitation of this technology constitutes the CO_2 chemical behavior in supercritical conditions. In this sense, the addition of appropriate polar cosolvent modifies SFE-CO_2 polarity increasing the solubility of nutrients and bioactive compounds [13]. This technique has been used by several authors to separate and purify nutrients and bioactive compounds from Spirulina achieving good results [14–16].

Lastly, extracts derived from microalgae are often rich in a diverse array of nutrients, vitamins, minerals, antioxidants, and other biologically active substances that can pro-mote cell growth, survival, and overall viability. By creating a nourishing environment, microalgae extracts have the potential to enhance cell viability and stimulate proliferation [17]. Additionally, these extracts typically contain potent antioxidants, such as carote-noids, phycobiliproteins, and tocopherols, which effectively shield cells from oxidative stress [18]. Moreover, certain microalgae extracts possess anti-

inflammatory properties attributed to bioactive compounds like omega-3 fatty acids, PC, and polysaccharides [19]. Considering these factors, it is intriguing to investigate the effects of these extracts on cell viability, aiming to determine whether they exert beneficial effects on cellular function.

The present work explores the recovery of total phenolic compounds (TPC), pigments, PC and antioxidant active compounds from spirulina algae employing SFE. The results obtained by SFE have been compared with a conventional stirred extraction. Fatty acids profile of SFE and conventional extracts was also analyzed. Moreover, the potential of SFE in the recovery of mineral compounds has been studied, as well as has been controlled the presence of heavy metals in the resulting extracts. This is intended to give an overview of the different nutritionally interesting compounds extracted by each method. Finally, we assessed the impact of these extracts on cell viability during a 24-hour exposure period to determine any potential positive effects on cells. It is important to note that there are currently no studies in the literature that evaluate the impact of spirulina extracts obtained through SFE on cell viability.

2. Materials and Methods

2.1 Chemicals

Sodium carbonate (Na_2CO_3), sodium hydrogen phosphate (Na_2HPO_4), and potassium dihydrogen phosphate (KH_2PO_4) were acquired from VWR (Saint-Prix, France). Nitric acid (HNO_3) was obtained from Panreac (Barcelona, Spain). Methanol, absolute ethanol (EtOH) and hexane were purchased from VWR Chemicals (Rosny-sous-Bois, France). CO_2 was obtained from Carburos Metálicos (Massalfassar, Valencia, Spain). EtOH 96° was obtained from Guinama (La Pobla de Vallbona, Valencia, España). Folin–Ciocalteu reagent, potassium persulfate ($K_2S_2O_8$), gallic acid, ABTS (2,2-Azino-Bis-3-

eThylbenzothiazoline-6-Sulphonic Acid), fluorescein, 2,2-Azobis(2-amidinopropane) dihydrochloride (AAPH) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma–Aldrich (Steinheim, Baden-Württemberg, Germany).

2.2 Samples

The spirulina samples were obtained from Hainan Island (China). The microalga was grown in open flow-through ponds. No artificial light was used, and shade nets partially covered the culture ponds, allowing control of pigment production. At the time of harvest, all samples were dried with dry air at 60 °C.

2.3. Extraction process

2.3.1 Shaking extraction

A shanking extraction process was performed following the protocol described by Sansone et al. [20]. Briefly, 5 g of microalgae were suspended in 50 mL of pure EtOH in the dark shaking at 500 rpm for 30 min. Then, the mixture was separated by centrifugation at 4000 rpm for 10 min, and the liquid phase was transferred to a clean tube. The pellet was resuspended in 50 mL of EtOH and extracted a second time. After repeating the separation process by centrifugation, both extracts were pooled.

2.3.2 Supercritical fluid extraction (SFE)

The supercritical fluid extraction (SFE) was conducted using a JASCO system (Tokyo, Japan). The system consisted of various components: an isocratic CO_2 pump (PU-4387) capable of a flow rate between 5 and 40 mL/min, adjustable in increments of 0.01 mL/min, with a maximum pressure of 50 MPa and a pulse extraction system; a cooling circuit (JULABO FL 1201) to cool the pump, offering a temperature range of -20 to 40 °C, a flow rate of

up to 23 L/min, and a cooling power of 1.2 kW at 20°C; an isocratic pump for organic modifiers (PU-4086, HPLC) with a flow rate range of 0.001-10 mL/min, adjustable in 0.01 mL/min increments, and a peak pressure of 70 MPa; a thermostatic oven for glass reactions (CO-4065) with a temperature range of 4 to 90 °C; a thermostatic system employing Peltier and temperature transfer via air flow; a pressure regulator (BP-4340); and a 5 mL glass vessel for SFE (803559-5mL). The extraction process lasted for 1 hour, following a modified version of the method described by Mendes et al. [21]. Specifically, the extraction vessel was filled with 5 g of dried spirulina, and the procedure utilized a temperature of 50 °C, a pressure of 25 MPa, and a CO_2:EtOH ratio of 90:10, with a flow rate of 16 mL/min.

2.4. Measure of antioxidant capacity, pigments and polyphenols

2.4.1 Total Antioxidant Capacity

The measure of total antioxidant capacity was carried out by two different methods: Trolox Equivalent Antioxidant Capacity (TEAC) and Oxygen Radical Antioxidant Capacity (ORAC) tests. These assays were carried out following the protocol described by al Khawli et al. [22]. The results were expressed as µmol Trolox Equivalents (TEs)/g dry matter (DM). The experiments were performed in triplicate.

2.4.2 Pigments and Total Phenolic Content

The content of chlorophyll a (Chla) and total carotenoids (Car) was calculated based on the procedure described by Lichtenthaler and Wellburn [23] with the modifications of Lima et al. [24] for spirulina microalgae. The different equations used are:

$$Chla = 15.65 \times A_{666} - 0.766 \times A_{644} \quad \text{(Eq. 1)}$$

$$Car = (1000 \times A_{470} - 2.86 \times Chla)/245 \quad \text{(Eq. 2)}$$

The total phenolic content (TPC) analysis was performed following the method described by Martí-Quijal et al. [25]. Briefly, 3 mL of Na_2CO_3 were added to a test tube, then 100 µL of standard or sample extract (appropriately diluted) and finally 100 µL of Folin-Ciocalteu reagent were also added. Then, the samples were incubated for 60 minutes in darkness at room temperature, and finally they were measured at a wavelength of 765 nm.

The concentration of PC was determined following the protocol described by Patil et al. [26], measuring the absorbance at 620 nm. The results were expressed as mg/g DM.

2.5. Evaluation of minerals and heavy metals content

The minerals analyzed were Mg, P, Ca, Fe, Zn and Se, as they are the more interesting minerals from a nutritional point of view, being essential for several biological processes [27]. On the other hand, the heavy metals studied were As, Cd, Hg and Pb, as they are the main ones in marine resources and also they are among the ones with the greatest adverse effects [28]. All of them were identified and quantified by ICP-MS, following the method previously used by de la Fuente et al. [29] for heavy metals. To sum up, 1 mL of the obtained extracts diluted at 1 mg/mL was digested with 250 µL of H_2O_2 (35% v/v) and 1 mL of HNO_3 (69% v/v) in a microwave system (MARS, CEM, Vertex, Spain) (800 W and 180 °C for 15 min). Then, the samples were filtered and diluted to an appropriate volume using ultrapure water. Finally, the content of minerals (Mg, P, Ca, Fe, Zn and Se) and heavy metals (As, Hg, Cd, and Pb) was evaluated using an inductively coupled plasma spectrometer mass detector (ICP-MS, Agilent model 7900). For the quantification, a standard calibration curve was used. Results are expressed as µg/g DM of three independent experiments.

2.6. Fatty acids profile analysis

The determination of lipid profile was carried out by gas chromatography coupled to flame ionization detector (GC-FID). Briefly, 1 mL of the extract resuspended at 20 mg/mL was mixed with 1 mL of KOH 2N in methanol and 1 mL of hexane. After mixing them for 2 min, the hexane phase was taken and 2 µL were injected in a PerkinElmer Gas Chromatograph Clarus® 590. A standard FAME Mix (Supelco 37 Component FAME Mix, Sigma-Aldrich, Laramie, Wyoming, USA) was used to identify the fatty acids.

The GC column SP-2560®, CPWAX 52CB silica capillary WCOT column with a di-mension of 30 m of length, 0.25 mm internal diameter with a 0.25 µm thick cover (SUPELCO) SP-2560 Capillary GC Column was operated in split mode. The oven temperature program was established as 180 °C for 5 min, increased to 210 °C at a rate of 4 °C/min, and finally held at 250 °C for 20 min, increasing at a rate of 20 °C/min. Nitrogen was used as a carrier gas at a flow rate of 20 psi. The results were expressed as mg/g DM.

2.7 Phenolic profile

The phenolic profile was analyzed using Triple-TOF-LC-MS-MS characterization, following the method described by Zhou et al. [30]. In this study, the TripleTOF™ 5600 (ABSCIEX) LC/MS/MS system, coupled with the Agilent 1260 Infinity (Agilent, Wald-bronn, Germany), was employed for phenolic compound identification. Chromatographic separation was achieved using a Waters UPLC C18 column (1.7 µm, 2.1 × 50 mm) Acquity UPLC BEH.C18 obtained from Waters (Cerdanyola del Vallès, Spain). The mobile phase consisted of water (0.1% formic acid, A) and methanol (0.1% formic acid, B). The elution gradient of the mobile phase was as follows: 90% (A) and 10% (B) from 0 to 13 min, 100% (B) from 13 to 15 min, and 90% (A) and 10% (B) from 15.1 to 22 min. The flow rate was set at 0.4 mL/min, and an injection volume of 5 µL was

used. Mass spectrometric data were acquired in the mass range of 80-1200 m/z. Prior to sample analysis, calibration was performed using an external calibration system. The MS operated using an information-dependent acquisition (IDA) method, employing the survey scan type (TOF-MS) and the dependent scan type (product ion) with a collision energy of -50 V. The MS parameters were as follows: ion spray voltage of -4500 V, dust removal potential of 90 V, collision energy of -50 V, temperature of 400 °C with a curtain gas of 25 psi, ion source gas 1 at 50 psi, and ion source gas 2 at 50 psi. The IDA MS/MS was performed based on the following criteria: ions exceeding 100 CPS, ion tolerance of 50 MDa, collision energy set at 25 V, and dynamic background subtraction enabled.

2.8 Cell cultures and cell viability assay

2.8.1 Cell culture

For cell viability analysis, SH-SY5Y cells, a type of human neuroblastoma cells, were cultured in DMEM Ham's-F12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were incubated under specific conditions: pH 7.4, 5% CO_2 at 37 °C, and 95% air atmosphere with constant humidity. The culture medium was refreshed every 2-3 days. To test the effects of various concentrations of spirulina extracts, the dry matter obtained after the extraction was resuspended in DMSO, and the solutions were added to the culture medium, ensuring that the final DMSO concentration did not exceed 1% (v/v). Control groups containing the same amount of solvent were included in each experiment.

2.8.2 Cell viability assay

To assess cell viability, the MTT assay was conducted as described by Zingales et al. [31]. In brief, 30,000 cells/well were seeded in 96-well plates

and allowed to grow for 48 hours until reaching 80% confluence. Subsequently, the cells were exposed to different concentrations of spirulina extracts (ranging from 125 to 2000 µg/mL) for 24 hours. After the incubation period, the culture medium containing the spirulina extract was replaced with fresh medium containing 50 µL of MTT salt (5 mg/mL PBS). Following 3 hours of incubation at 37 °C in darkness, the resulting formazan crystals were dissolved in DMSO. An automatic plate reader (MultiSkanEX, Labsystem, Helsinki, Finland) was used to measure absorbance at 540 nm. Cell viability was expressed as a percentage relative to the solvent control (1% DMSO).

2.9 Statistical analysis

The statistical analysis was performed using GraphPad Prism 8. A t-student test was used to compare SFE vs. control samples. A p value <0.05 was considered significant. All the experiments were carried out in triplicate. The results are presented as mean ± SD.

3. Results and Discussion

3.1 Pigments and Total Phenolic Content

Chlorophyll, Car, and polyphenols are bioactive compounds found in vegetables and algae that have been extensively studied for their antioxidant, anti-inflammatory, and cancer-preventive properties [32,33]. PC, a protein extracted from spirulina, has also been shown to have various biological functions, including antioxidant and anti-inflammatory effects, inhibition of bacterial growth, and protection against liver and kidney damage, among others, making it a promising natural molecule for various applications [32,34]. PC and other phycobiliproteins, alongside Chls and Car, are considered essential algal pigments [2].

The Chla, total Car, TPC and, PC in both, control and SFE extracts are shown in Figure 1.

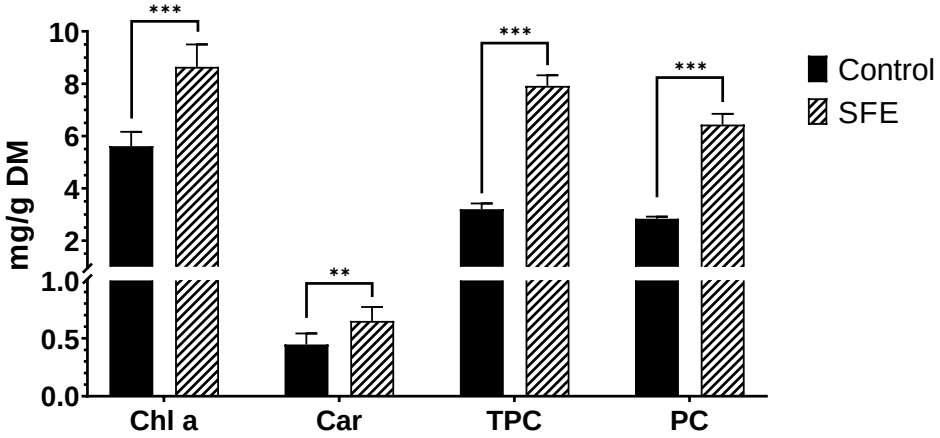

Figure 1. Chlorophyll a (Chl a), total carotenoids (Car), total phenolic content (TPC) and phycocyanin (PC) values comparing SFE vs. control extraction. Results are expressed as mean ± SD. ** - $p < 0.01$; *** - $p < 0.001$

As it can be observed in the Figure 1, SFE treatment significantly improved pigment extraction compared to control, increasing from 5.612 ± 0.547 to 8.645 ± 0.857 mg/g DM for Chl a, and from 0.447 ± 0.096 to 0.651 ± 0.120 mg/g DM for Car. These results are in close agreement with the published literature. In this sense, Tong et al. [35] described an increase in Chl a from 2.73 ± 0.05 up to 6.84 ± 0.18 mg/g when used SFE compared to control for 120 min. These results showed an increase of 2.5 times in Chl a extraction, which is higher than the 1.53 times obtained in our study. However, this fact can be explained taking in account the time of extraction, as it is of 2 h for Tong et al. while in our study we selected a time of extraction of 1h, based on the optimal conditions found in the available literature. In addition, other parameters like pressure also can

modify the result, as Tong et al. performed the extraction at 48.7 MPa, but in our study only 25 MPa were used.

The low yield in Car extraction may be attributed to pressures lower than 30 MPa, as suggested by Marzorati et al. [36]. They found that pressures below 30 MPa were inade-quate to obtain Car-enriched extracts. In addition, a higher yield of Car can be achieved when a modifier phase is not used during extraction. However, in our study, we utilized 10% EtOH as a co-solvent. The same authors reported total Car value of 3.5 ± 0.2 mg/g DM after SFE under 30 MPa and 45 °C at a flow rate of 15 mL/min and without co-solvent. However, other authors also obtained lower values for carotenoid from spirulina by SFE. For instance, Esquivel-Hernández et al. [37] obtained 0.283 mg of carotenoids/g DM using SFE (CO_2) with a flow rate of 15 g/min of CO_2 for 50 min and 26.70% (v/v) EtOH 96%/water (v/v) as a co-solvent under 15 MPa and 60 °C. Despite the similarity in flow rate and extraction time, the pressure used by these authors was lower, and the percentage of EtOH was higher. These differences may account for our higher value of Car.

SFE extraction also increased the recovery of phenolic compounds, resulting in an increase from 3.202 ± 0.547 mg/g DM using conventional extraction to 7.917 ± 0.406 mg/g DM with SFE. These findings align with those reported by Mallikarjun et al. [15], who obtained a value of 340 mg/100 g DW (= 3.4 mg/g DM) using SFE at 40°C, a pressure of 12 MPa, and CO_2 flow rate of 1.2 Kg/h. Additionally, the results obtained through SFE correspond well with the values reported by Dejsungkranont et al. [38], who recorded a TPC value ranging from 6.44 to 11.40 mg Gallic Acid Equivalents (GAE)/g DM. The highest values were achieved using 40 °C, 31.03 MPa, and 90 minutes of extraction (11.10 mg GAE/g DM) and 60 °C, 24.13 MPa, and 90 minutes (11.40 mg GAE/g DM). Notably, the extraction time in both studies was 90 minutes, which is 30 minutes longer than what we used in our study. This, coupled with the use of

a higher pressure or temperature, likely accounts for the higher values obtained. It is evident that the duration and conditions of the extraction process significantly impact the outcome of the experiment.

Regarding the value obtained for PC, a significant increase in the extraction yield of this protein was observed after using SFE, compared to the control extraction under stirring, improving from 2.838 ± 0.081 mg/g DM (control) to 6.438 ± 0.411 mg/g DM (SFE), which means an increase of 126.8%.

Deniz el al. [39] used SFE to optimize the PC extraction from spirulina, studying the influence of temperature, pressure, time and percentage of co-solvent (EtOH) and com-paring to a control. These authors reported that the maximum PC value with the highest purity was obtained at 60 ˚C, 25 MPa, 45 min and 10 % of EtOH as co-solvent. These values are quite similar to the ones used in the present study (50 ˚C, 25 MPa, 60 min and 10 % of EtOH as co-solvent). In addition, Deniz et al. also found a higher extraction of PC when used SFE compared to a control, being 13% higher.

Pinto et al. [40] studied the extraction of PC and other lipids from spirulina comparing SFE (only CO_2), SFE (with 10% EtOH as co-solvent) and pure EtOH at high pressure. The results are in agreement with the obtained in the present study, as they obtained a higher PC extraction when used SFE with 10% EtOH as co-solvent compared with pure EtOH. The PC extraction improved from 0.6 ± 0.2 up to 0.9 ± 0.3 (wt%), which means an increase of 150%.

3.2 Antioxidant capacity

The results obtained regarding the antioxidant capacity (Figure 2) demonstrate an increase in the measured antioxidant capacity by the TEAC assay. The value rose from 20.6 ± 1.35 µmol TE/g DM to 26.4 ± 0.99 µmol TE/g DM after the SFE extraction. This increase in TEAC value could be related to the improved extraction of antioxidant compounds, particularly polyphenols,

previously described in the study. The TEAC assay has a very good correlation with the concentration of polyphenols in the sample, which effectively represents their antioxidant activity [41].

On the other hand, the ORAC assay did not show significant differences between control (189 ± 10.2 µmol TE/g DM) and SFE (175 ± 8.60 µmol TE/g DM) samples.

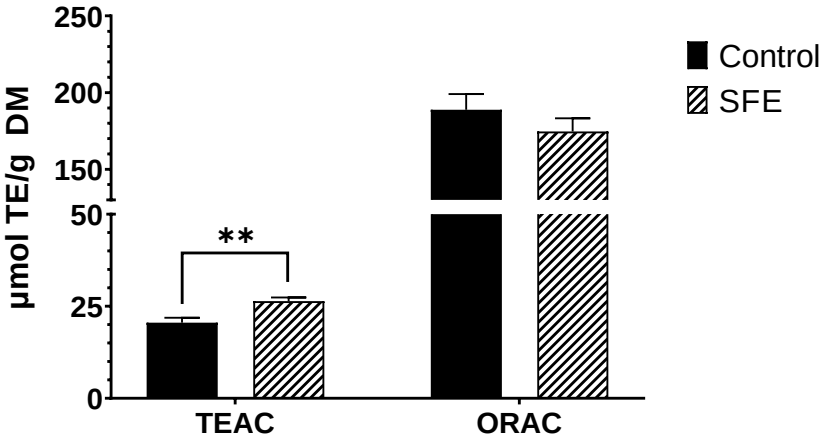

Figure 2. TEAC and ORAC antioxidant assays, comparing SFE vs. control. Results are expressed as mean ± SD. **-p<0.01.

The results obtained in the present study for the TEAC assay were slightly lower compared to those obtained by Dejsungkranont et al. [38]. They achieved antioxidant ac-tivity values, measured by the DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay, in the range of 44.18 to 118 µmol TE/g DM after using SFE in samples of *Spirulina maxima*. However, this can be explained by the higher polyphenol values obtained by those authors, as dis-cussed earlier, which correspond to a higher antioxidant capacity than what was found in our study.

3.3 Mineral content

Macrominerals are essential for a good health. Magnesium is a vital cofactor in over 300 enzyme systems and is essential for energy production, cell membrane transport, nerve impulse conduction, muscle contraction, and heart rhythm. Low Mg levels are associated with various chronic health conditions such as migraines, osteoporosis, Alzheimer's disease, asthma, hypertension and insulin resistance [42]. Calcium and phosphorus are essential macrominerals for neuromuscular function and skeletal mineralization. Cal-cium is abundant in bones and teeth and plays a critical role in blood vessel contraction, muscle tone, and nerve transmission. Phosphorus is primarily found in mineralized bone and is necessary for DNA, RNA, and ATP synthesis. Parathyroid hormone (PTH), vitamin D and, Fibroblast Growth Factor 23 (FGF23) control the levels of both minerals [42].

On the other hand, microminerals (e.g.: Fe, Zn, Se…) are also relevant for several functions in human body. In this sense, selenium's nutritional benefits are achieved through 25 selenoproteins that contain selenocysteine. During low selenium supply, some selenoproteins are prioritized, including glutathione peroxidase. Selenoproteins play a vital role in human health. For example, Se supplementation can stimulate the immune system, including enhancing T cell proliferation and natural killer cell activity [43]. Zinc is a vital trace element in the human body that acts as a signaling factor and regulates chronic inflammation. It also aids in the synthesis of antioxidant enzymes and catalyzes enzymes involved in metabolism. Furthermore, Zn plays a critical role in insulin synthe-sis and storage, making it important for metabolic disorders such as type-2 diabetes mellitus and atherosclerosis [44]. Finally, iron is crucial for various metabolic processes such as oxygen transport, DNA synthesis, and electron transport in living organisms. Anemia or functional impairments can result from Fe deficiency. Even mild or moderate forms of

Fe deficiency anemia can lead to cognitive, immune, and work capacity deficits [45].

Then, concerning the minerals (Mg, P, Ca, Fe, Zn and Se), the SFE recovered 77% more Mg than the conventional extraction, increasing from 49.33 ± 1.00 up to 91.50 ± 2.25 µg/g DM. In addition, Fe extraction was also improved through SFE, reaching 2.00 ± 0.08 µg/g DM for SFE extract while only 0.87 ± 0.04 µg/g DM for the conventional one. However, the conventional extraction obtained a higher yield for the recovery of P (52.98 ± 2.86 vs. 20.93 ± 2.25 µg/g DM) and Ca (52.27 ± 1.43 vs. 35.25 ± 2.25 µg/g DM) (Figure 3). This can be explained due to the lower solubility of minerals in non-polar solvents, like supercritical CO_2 [46]. Nevertheless, the ability of SFE to recover more Mg from spirulina compared to EtOH extraction could be attributed to the fact that Mg is more soluble in nonpolar solvents than Ca and P. Lastly, no significant differences were observed for Zn, while Se was not detected in any of the studied samples.

In this sense, Michalak et al. [46] also obtained a better yield recovering Mg (406 ± 61 mg/L from 4070 ± 810 mg/Kg DM) than Ca (1060 ± 210 mg/L from 14400 ± 2900 mg/Kg DM) and P (43 ± 6 mg/L from 1520 ± 300 mg/Kg DM) after the extraction of supercritical fluids from Baltic seaweeds.

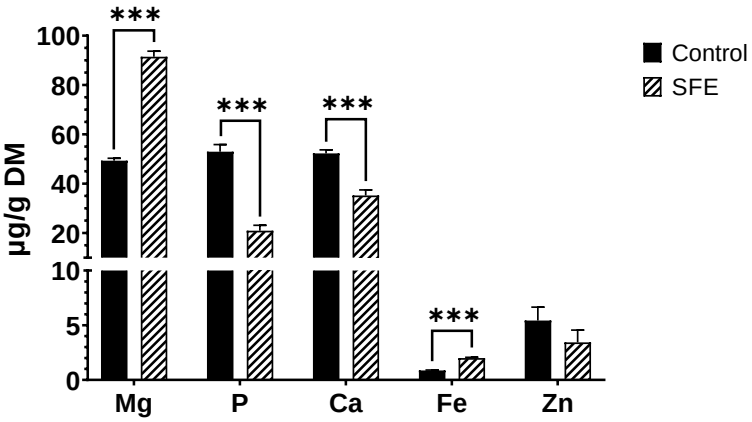

Figure 3. Content of Mg, P, Ca, Fe, and Zn in both SFE and control extracts. Se was not detected. Results are expressed as mean ± SD. ***-p<0.001.

3.4 Heavy metals content

For heavy metals (As, Cd, Hg and Pb), a higher Pb extraction was observed for the SFE extract (0.0953 ± 0.0030 µg/g DM) compared to control (0.0222 ± 0.0014 µg/g DM), while for Hg there are no significant differences between both extractions (Figure 4). Nei-ther As nor Cd were found in any of the samples.

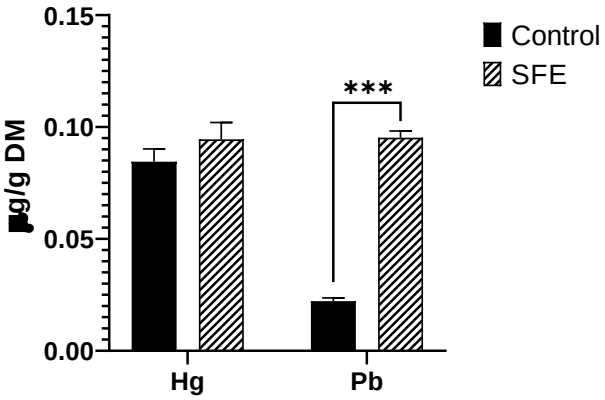

Figure 4. Content of heavy metals Hg and Pb in both SFE and control extracts. As and Cd were not detected. Results are expressed as mean ± SD. ***-p<0.001.

Commission Regulation (EC) No 1881/2006 [47] established maximum levels for some heavy metals (such as As, Cd, Pb, and Hg) in various food products, but did not establish any maximum levels for algae and halophilic plants, except for food supplements composed exclusively or mainly of seaweed. Recently, in the case of Hg, Regulation (EC) No 2018/464 [48] set a maximum residue limit (MRL) of 0.01 mg/Kg for algae and pro-karyotic organisms. However, these levels are regulated based on wet weight, while our samples are measured based on dry weight, resulting in a more concentrated result than the value on a wet weight basis.

To the best of our knowledge, no studies are available in the literature about heavy metals contents in spirulina SFE extracts, so the contents obtained in this study have been compared with the information available in raw spirulina. In this sense, Al-Harbi [49] studied the occurrence of Pb, As and Cd in 25 spirulina products commercialized for hu-man consumption. The concentrations reported by these authors were Cd (0.003 - 0.069), As (0.006 - 0.578), Pb (0.100 - 1.206) expressed in mg/Kg dry weight, being Pb levels similar to those observed in the present study after SFE. Contrary to the present study, Hsu et al., [50] reported As levels up to 2 µg/g in spirulina food samples, while Pb was detected in one sample at 15 µg/g. Pb levels were higher than those determine in the present study. Moreover, these authors detected Hg at lower contents (<0.03 µg/g).

3.5 Fatty acids profile

Fatty acids are vital for human health, serving as building blocks for cell membranes and precursors for signaling molecules. Palmitoleic acid, a monounsaturated fatty acid, has been linked to lower inflammation levels and improved insulin sensitivity [51]. Linoleic acid is also associated with lower type 2 diabetes risk and potential insulin sensitivity improvement [52]. Stearic acid, a saturated fatty acid, has a neutral effect on cholesterol levels and may even

lower cholesterol when replacing other saturated fats in the diet [53]. Additionally, polyunsaturated fatty acids like γ-linolenic acid, eicosadienoic acid, and eicosatrienoic acid have anti-inflammatory properties and may reduce the risk of chronic diseases such as arthritis, diabetes, and heart disease [54].

Figure 5 illustrates the fatty acid profile of both the control and SFE extractions. The SFE method enhanced the recovery of several fatty acids, including palmitoleic, stearic, γ-linolenic, eicosadienoic, and eicosatrienoic acids. The most significant increase was ob-served in γ-linolenic acid, which rose by 110% from 3.056 ± 0.100 mg/g DM in the control to 6.436 ± 0.120 mg/g DM in the SFE sample. Palmitoleic and stearic acids also experi-enced a substantial increase of 55.84% and 45.05%, respectively, from their control values of 0.941 ± 0.038 and 0.200 ± 0.007 mg/g DM. Similarly, eicosadienoic and eicosatrienoic acid increased by around 50% following SFE. Specifically, eicosadienoic acid increased from 0.037 ± 0.001 to 0.056 ± 0.002 mg/g DM, while eicosatrienoic acid increased from 0.024 ± 0.001 to 0.036 ± 0.001 mg/g DM.

However, there was a reduction in linoleic acid content, with the control extraction having a higher concentration (2.200 ± 0.060 mg/g DM) than the SFE extraction (1.180 ± 0.042 mg/g DM).

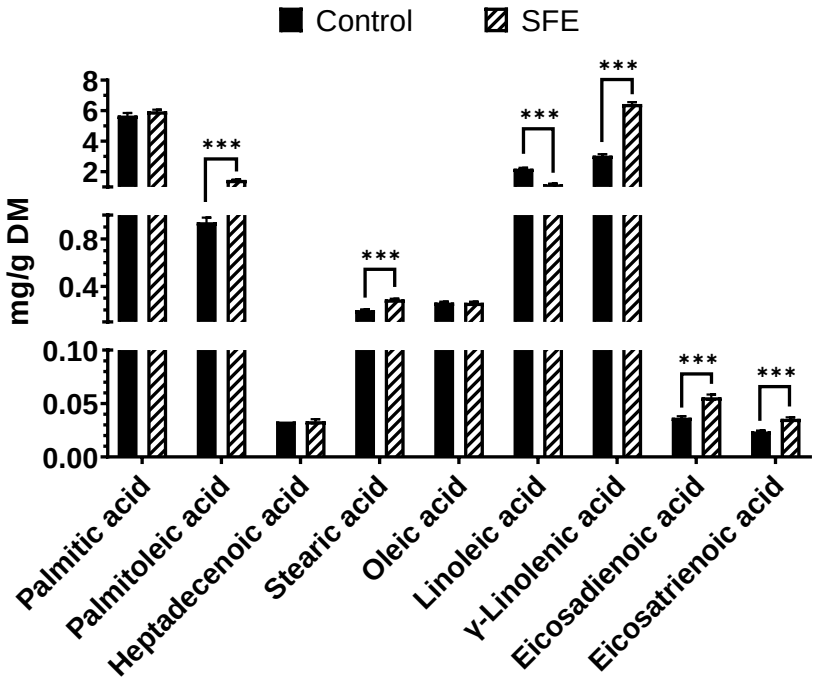

Figure 5. Fatty acids profile in both SFE and control extracts. Results are expressed as mean ± SD. *** - p< 0.001.

On the other hand, Table 1 displays the relative proportion of each fatty acid that was determined in both the control and SFE extracts. As shown in the Table 1, the SFE process promoted a slight modification in the proportion of each acid when compared to the con-trol extraction. The most noticeable changes are observed in palmitic acid and linoleic ac-id, which experience a reduction of around 6-7%, decreasing from 53.24% to 46.90% for palmitic acid and from 15.60 to 7.02% for linoleic acid. The reduction in the case of linoleic acid is particularly striking, as its relative presence in the extract is reduced by 50%, compared to the initial proportion, after the application of SFE.

Table 1. Relative fatty acids concentration both in control and SFE extracts. Results are expressed as mean (%) ± SD.

Fatty acid	Formula	Relative concentration (mean (%) ± SD)		Significance
		Control	SFE	
Palmitic acid	C16:0	53.24 ± 0.07	46.90 ± 2.44	*
Palmitoleic acid	C16:1n-7	6.59 ± 0.08	8.28 ± 0.12	***
Heptadecenoic acid	C17:1n-7	0.23 ± 0.00	0.21 ± 0.01	n.s.
Stearic acid	C18:0	1.47 ± 0.01	1.79 ± 0.09	**
Oleic acid	C18:1n-9	1.95 ± 0.01	1.63 ± 0.01	***
Linoleic acid	C18:2n-6	15.60 ± 0.14	7.02 ± 0.28	***
γ-Linolenic acid	C18:3n-6	20.66 ± 0.01	36.49 ± 1.88	***
Eicosadienoic acid	C20:2n-6	0.25 ± 0.01	0.33 ± 0.01	***
Eicosatrienoic acid	C20:3n-6	0.17 ± 0.01	0.21 ± 0.01	**

n.s.: not significant; * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$.

Conversely, the most significant increase occurs in γ-linolenic acid, which rises from 20.66% in the control extract to 36.49% in the SFE extract. It is also worth noting that long-chain polyunsaturated acids, such as eicosadienoic acid and eicosatrienoic acid, ob-serving an increase in their proportion in the SFE extract compared to the control, with an increment from 0.25% and 0.17% to 0.33% and 0.21%, respectively. This implies that SFE can enhance the lipid profile in a way that promotes the extraction of unsaturated fatty acids.

Finally, the only fatty acid that does not experience a significant modification regarding its proportion in the obtained extract is heptadecenoic acid, obtaining very similar values in both extracts.

The results obtained in this study are similar to those described in the literature. In this regard, the values are within the same range as those obtained by Esquivel-Hernández et al. [55], with a very similar proportion of fatty acids: palmitic acid (39.38%), γ-linolenic acid (30.27%), linoleic acid (20.63%), palmitoleic acid (7.88%), oleic acid (1.06%) and stearic acid (0.75%). The proportion of these fatty acids also fits with that found by Crampon et al. [56] regarding the composition present in the microalgae. It is described that the major component is palmitic acid, with 63.48%, followed by linoleic acid (13.73%), linolenic acid (9.42%), and palmitoleic acid (8.02%), among others. There-fore, in our case, the extraction of γ-linolenic acid has been notably increased compared to the relative content in the microalgae, obtaining values much higher than 9.42% (20.66% for control and 36.49% for SFE).

Mendiola et al. [14] have also reported the ratio of different fatty acid areas after the application of SFE to 75 g of spirulina with 10% EtOH, 220 bar, and 27°C. However, these authors detected the presence of lauric acid (3.53-19.2%) and myristic acid (1.38-2.89%), which were not detected in our study. In contrast, the value reported by these authors for unsaturated fatty acids, specifically linoleic acid (0.66-0.92%) and palmitoleic acid (5.80-5.92%), is lower than that obtained in our experiments.

3.6 Phenolic profile

In Table 2, the phenolic compounds identified using Triple-TOF-LC-MS-MS with a score equal to or greater than 80% are shown. As can be observed, the use of one treatment or another can influence the type of the recovered compounds. It can be ob-served that after performing the extraction with SFE, polyphenols such as apigenin 7-O-glucuronide, 3,4-dihydroxyphenylglycol, or acetyl eugenol, among others, were identified in the extracts, which are not present in the control extract.

The identification of hydroxybenzoic acid, 4-hydroxybenzaldehyde, and protocatechuic acid coincides with what has been described in the literature by Klejdus et al. [57], who identified these compounds after performing an extraction using the combination of solid-phase and supercritical fluid extraction from *Arthrospira platensis*. On the other hand, Zhou et al. [30] detected the presence of phenol in spirulina extracts obtained using pressurized liquid extraction with DMSO as solvent. The presence of hesperidin in spirulina has also been reported in the literature [58] after extraction with ethanol and methanol. Finally, McCarthy et al. [59] also described the presence of apigenin in extracts obtained from *Arthrospira platensis*.

Table 2. Phenolic profile obtained by Triple-TOF-LC-MS-MS for the extracts obtained by super-critical fluid extraction (SFE) and its control from spirulina biomass. The extracts were resus-pended at 10 mg/mL in methanol. Results are expressed as mean ± SD.

	Compound	Formula	Score	Retention time (min)
Control	Phenol	C_6H_6O	91 %	5.52
	Benzoic acid	$C_7H_6O_2$	90 %	6.27
	4-Hydroxybenzaldehyde	$C_7H_6O_2$	90 %	6.27
	2-Hydroxybenzoic acid	$C_7H_6O_3$	83 %	5.51
	3-Hydroxybenzoic acid	$C_7H_6O_3$	83 %	5.51
	4-Hydroxybenzoic acid	$C_7H_6O_3$	83 %	5.51
	Sesamol	$C_7H_6O_3$	83 %	5.51
	Protocatechuic aldehyde	$C_7H_6O_3$	83 %	5.51
	4-Ethylcatechol	$C_8H_{10}O_2$	94 %	7.80
	4-Vinylsyringol	$C_{10}H_{12}O_3$	87 %	8.67
	Hesperidin	$C_{28}H_{34}O_{15}$	95 %	8.46
	Neohesperidin	$C_{28}H_{34}O_{15}$	95 %	8.46
SFE	Phenol	C_6H_6O	88 %	5.76
	Benzoic acid	$C_7H_6O_2$	89 %	6.53
	4-Hydroxybenzaldehyde	$C_7H_6O_2$	89 %	6.53
	Tyrosol	$C_8H_{10}O_2$	87 %	8.01
	4-Ethylcatechol	$C_8H_{10}O_2$	87 %	8.01
	Hydroxytyrosol	$C_8H_{10}O_3$	87 %	7.69
	3,4-Dihydroxyphenylglycol	$C_8H_{10}O_4$	91 %	7.62
	Acetyl eugenol	$C_{12}H_{14}O_3$	81 %	10.47
	Apigenin 7-O-glucuronide	$C_{21}H_{18}O_{11}$	86 %	6.73

3.7 Impact on SH-SY5Y cell viability

The impact of the SFE spirulina extract on SH-SY5Y cell viability was evaluated and compared to the control extract. To the best of our knowledge, this is the first study in the literature evaluating the cytotoxicity of a SFE spirulina extract, which is crucial for future applications related to human health. The extract concentrations were tested across a wide range: 125, 187.5, 250, 375, 500, 750, 1000, 1500, and 2000 µg/mL. It is worth noting that there is a slight increase in cell viability at the concentration of 375 µg/mL, reaching 114% of cell viability. Moreover, it can be observed that at high extract doses, the cell viability decreases in the control group, while it remains around 100% for the SFE extract. Specifically, at a concentration of 1500 µg/mL, cell viability in the control extract was 83.2%, whereas in the SFE extract, it was 100% compared to the cell viability without the spirulina extract (1% DMSO, used as control for cell viability). The same effect is more pronounced and exhibits significant differences ($p<0.05$) in the case of the 2000 µg/mL concentration, where the control extraction reduced cell viability to 64.3%, while the SFE extract was similar to control. This is likely due to the higher content of bioactive com-pounds, as previously described, which help sustain cell viability at high concentrations compared to the control.

Other researchers have examined the cytotoxicity of extracts from this microalga using different methods and solvents. Conversely to our results, Akbarizare et al. [60] found slightly higher results in HepG2 cells. They evaluated the 24-hour exposure of aqueous and methanolic spirulina extracts on HepG2 liver cells and human fibroblasts using the MTT assay. In HepG2 cells, they observed an IC_{50} at concentrations of 1700 ± 140 µg/mL (aqueous extract) and 1280 ± 220 µg/mL (methanolic extract). In human fibroblasts, the IC_{50} was reached at concentrations of 2340 ± 60 µg/mL and 2430 ± 40 µg/mL for the aqueous and methanolic extracts, respectively. These concentrations

were considerably higher, indicating that our study aligns more closely with the behavior observed in fibroblasts. However, at the range of concentration tested we did not obtain IC_{50} neither for SFE nor control extracts.

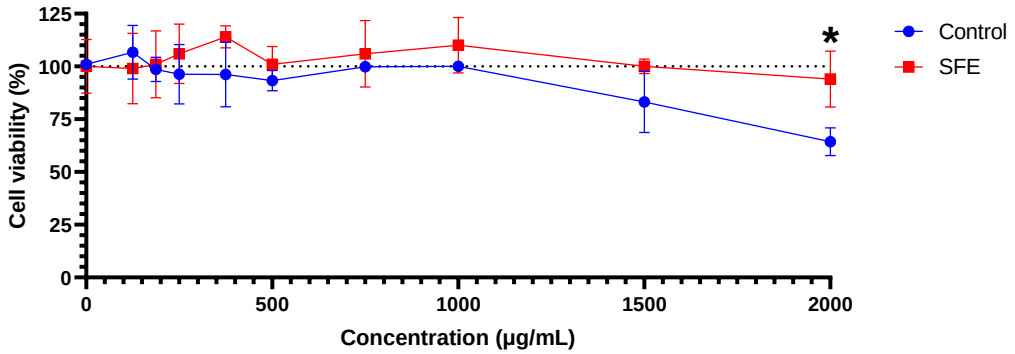

Figure 6. Effect of spirulina extracts (control and SFE) on cell viability in SH-SY5Y cell line. Cell viability was evaluated using MTT assay. Results are expressed as mean ± SD. * - $p< 0.05$ vs. control extract.

In contrast, the results differ significantly from those obtained by Nurani et al. [61] for HeLa cells, a type of human cervical cancer cells. They found that the IC_{50} of the ethanolic spirulina extract after 24-hour exposure to HeLa cells was 260,4 µg/mL, much lower than our values. However, this difference could be attributed to the intrinsic characteristics of HeLa cells, as in the same study, the authors also tested the extract on human dermal fi-broblasts (HDFa cells), obtaining an IC_{50} value of 2065.7 µg/mL, which closely resembles the results of Akbarizare et al. and our results. This confirms that the low IC_{50} value obtained for HeLa cells may be specific to this particular cell line.

Therefore, it is essential to perform cytotoxicity studies considering the cell line used, the duration of exposure, and conducting a comprehensive characterization of the tested extract.

5. Conclusions

The results presented in this study demonstrate the effectiveness of SFE technology in sustainably extracting bioactive and health-relevant compounds from microalgae like spirulina. The extracted compounds include pigments, polyphenols, polyunsaturated fatty acids, and minerals, with demonstrated positive effects on the viability of the SH-SY5Y cell line.

However, it is important to note that SFE can also lead to an increase in potential contaminants like Pb. Therefore, it is crucial to have proper quality control measures in place to ensure the safety and purity of the extracted compounds.

In conclusion, this study highlights the potential of SFE technology as a valuable tool for the extraction of various compounds from microalgae, which can be used in various applications. Moreover, the use of an alternative method to determine cytotoxicity may be useful for food industry. Nonetheless, it is important to monitor and control the levels of potential contaminants that may arise from the extraction process. The findings presented in this study contribute to the understanding of the nutritional and bioactive properties of spirulina and open up new opportunities for its commercial exploitation.

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F.J.M-Q. and N.P.; writing—review and editing, F.J.M-Q., N.P., F.J.B. and M-J.R.; supervision, F.J.B. and M-J.R.; project administration, F.J.B.; funding acquisition, F.J.B. All authors have read and agreed to the published version of the manuscript.

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A chemometric approach to evaluate the impact of pulses, *Chlorella* and *Spirulina* on proximate composition, amino acid, and physicochemical properties of turkey burgers

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Abstract

BACKGROUND: Changes in physicochemical parameters, proximate composition, amino acid and taste profiles of turkey burgers enriched by 1% with soy (control), pulses, *Chlorella* and *Spirulina* proteins were studied.

RESULTS: Color parameters, pH, ash content, total, essential and non-essential amino acids were significantly different among the different types of turkey burgers prepared. In this regard, turkey burgers made with pea protein presented the highest values for pH and lightness, whereas the samples prepared with broad bean showed the highest redness. The inclusion of bean and seaweed produced a marked increase of glutamic acid, lysine and aspartic acid. However, the taste profile was similar in the different six turkey burgers studied (soy, pea, lentil, broad bean, *Chlorella* and *Spirulina* protein). Orthogonal projections to latent structures discriminant analysis (OPLS-DA) allowed to classify turkey burgers according to protein sources, as compared to soy (control). Textural parameters, moisture and color were found to be the most discriminant parameters, able to describe the differences among burgers. Nonetheless, according to the supervised OPLS model, broad beans were found to possess a similar profile to soy (control).

CONCLUSION: Considering all studied parameters, the enrichment of turkey burgers with bean proteins could be used as a promising alternative to soy proteins from a technological point of view.

Keywords: textural properties; color parameters; seaweeds; taste profile; turkey burger

1. Introduction

Among foods of animal origin, it is known that meat and its derivatives are a good source of nutrients, among which we can highlight vitamins such as B_{12}, minerals such as iron and zinc, and lipids and proteins.[1–3] This makes meat products an important group of foods which is also consumed globally by a large number of people. But, despite the nutritional value of its components, an excessive consumption of meat, specially processed meat, is related to the risk of cancer development, colon cancer being the most notable.[4,5] Several processed meat products incorporate soy proteins, due to their beneficial properties for health, and their good nutritional profile, with a large number of essential amino acids.[6,7] However, soy is a recognized allergen, so it is necessary to look for other vegetable sources of good nutritional quality to replace it. Legumes, which include lentils, peas and beans, are rich in protein, comprising 20% of dry weight in the case of beans and peas.[8] Furthermore, products derived from algae are now becoming important.[9] Microalgae are a good source of polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), antioxidants (phenolic acids and flavonoids), polysaccharides and proteins.[10–13] For all these com-ponents of great nutritional relevance and bioactive nature, algae have been used both in the food industry (to make food supplements and nutraceuticals) and in the cosmetic industry.[14–16] The use of these sources has allowed for the improvement of food through nutritional value or oxidation of lipids and so on.[17–19] Another factor to take into account is the environmental impact. As it is known, meat production has a high ecological cost, and is contributing to climate change.[20] In contrast, plants with a high protein value such as soybean, pea or lentil, and also algae have a lower land-use footprint, lower water-use footprint and lower carbon footprint.[21] In addition, legumes can fix atmospheric nitrogen, which would reduce the use of nitrogen

fertilizers.[21] Focusing on the food industry, algae have been used for several purposes such as improving the quality of meat and eggs by using them in feeding animals or using them to reduce cholesterol levels.[15,22] In addition, microalgae contain important amounts of essential amino acids and proteins, thus constituting a good alternative source of protein.[15,22–25] It has been seen that these proteins are of high quality, comparable to others of plant origin such as rice, wheat or beans.[10,26,27] Many algae have been studied in relation to their protein content. For example, Milovanovićet al.[28] noticed that several strains of cyanobacteria have a high protein content (42.8% to 76.5% on a dry weight basis). One of these algae, *Spirulina*, has been found to have protein levels comparable to soy and even meat, so its use in food would be profitable for health, due to its composition of amino acids, polyphenols, essential fatty acids, vitamins and minerals.[29] Incorporating new ingredients into processed foods can alter characteristics such as taste. In this sense, it is known that some amino acids provide the sweet taste (glycine, threonine, alanine, serine and proline), others, a bitter taste (phenylalanine, histidine, leucine, isoleucine, alo-isoleucine, methionine, valine and tryptophan), and finally some of them are responsible for the umami taste (glutamic acid and aspartic acid).[30] All these considerations have to be taken into account when adding *Chlorella*, *Spirulina* or other vegetable proteins to processed food. Furthermore, the term 'chemometrics' describes the statistical and mathematical approaches used to optimize the design of experiments and extract useful information from large and complex datasets.[31] Chemical data commonly include values and properties of various compounds determined by laboratory experiments and having numerous sources of variance.[32] Usu-ally, chemometrics is applied when there is a large and complex dataset, in terms of sample numbers, types, and responses. The results are used for authentication of geographical origin, farming systems, or even to trace adulteration of high value-added commodities.[32] The aim of this work was to determine the changes in the

physic-ochemical properties and nutritional quality of turkey burgers on adding different protein sources of vegetable origin (peas, lentils and beans) and algae (*Chlorella* and *Spirulina*) to turkey meat, and to compare them with soy protein, which was used as a control due to its wide use in the industry.

2. Material and methods

2.1 Experimental design and manufacture of turkey burgers

Novafrigsa S.A.–Grupo Coren (Lugo, Spain) provided the turkey meat. All the additives and spices used were of food grade. Vitessence™ (Manchester, UK) Pulse Proteins supplied soy, pea, lentil and bean proteins. Algaenergy (Madrid, Spain) supplied *Chlorella* and *Spirulina* proteins. All the chemicals used for the analysis were of analytical grade. A total of 30 turkey hamburgers, in six separate batches, were made in the Meat Technology Center (Galicia, Spain), five with each protein source. Initially, the turkey lean meat was ground with a refrigerated mincer machine (La Minerva, Bologna, Italy), and then vacuum-minced with all the additives, except the protein extract, in a vacuum mincer machine (Fuerpla, Valencia, Spain). Next, it was divided into six batches, one for each protein source, the designated protein was added at 1% and was cold stored (4 °C) for 4 h. Finally, the burgers were formed using a burger-maker (Gaser, A-2000, Girona, Spain).

2.2 Physicochemical parameters

Physicochemical parameters were analyzed according to the method previously reported by Lorenzo et al.[33] The pH of the burgers was measured using a digital portable pH-meter (HI99163, Hanna Instruments, Eibar, Spain) equipped with a penetration probe. Color was measured using a portable colorimeter (CM-600d-Konica Minolta, Tokyo, Japan) with pulsed xenon arc lamp, 0° viewing angle geometry and 8 mm aperture size, to esti-mate burger color in the CIELAB space: lightness (L*); redness (a*); yellowness (b*). The color

was measured in three different points of each sample in homogeneous and representative areas, free of fat. Water-holding capacity (WHC) was measured as cooking loss (%), whereas textural profile analysis (TPA) test was conducted using a texture analyzer (TA-XT2, Stable Micro Systems, Godalming, UK) according to the method proposed by Lorenzo and Carballo[34] using a load cell of 5 kg. Hardness (in newtons), adhesiveness (in g×s), elasticity (in millimetres), cohesiveness, gumminess (in newtons) and chewiness (N×mm) were obtained using Texture Exponent 32 software (version 1.0.0.68, Stable MicroSystems).

2.3 Proximate composition

To determinate moisture content, a sample was dried at 105 °C until a constant weight was acheived and then weight loss was measured, according to ISO 1442:1997.[35] For protein determination, Kjeldahl total nitrogen method was used with a nitrogen conversion factor of 6.25 for proteins.[36] Lipid extraction was performed submitting samples to a liquid–solid extraction employing petroleum ether in an extractor apparatus (Ankom HCl Hydrolysis System, Macedon, New York, USA) at 90 °C during 60 min, following the AOCS official method Am 5–04. Finally, ash content was calculated by maintaining the sample at 600 °C in a muffle furnace (Carbolite RWF 1200, Hope Valley, UK) until constant weight, according to ISO 936:1998.[37]

2.4 Amino acid content

Hydrolyzed amino acid composition (g/100 g of meat) of manufactured turkey burgers was estimated using the procedure previously described by Lorenzo et al.[38] 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Waters AccQ-Fluor reagent kit) was used for amino acids derivatization, which were determined by reversed-phase high-performance liquid chromatography

(RP-HPLC) (Waters 2695 Separations Module + Waters 2475 Multi Fluorescence Detector + WatersAccQ-Tag amino acids analysis column).

2.5 Statistical analysis

One-way analysis of variance (ANOVA) was used to examine the results obtained. Furthermore, Duncan's test was completed to compare the means. To determine the coefficients which maximize the differences among samples, a linear discriminate func-tion containing an optimal subset of variables was used. Data were presented as mean ± standard deviation (SD) of five replicates, and was considered significant when $p<0.05$. All statistical analyses were performed using the software Statgraphics CenturionXVI.I® (Statgraphics Technologies, The Plains, VA, USA). Afterwards, the whole dataset was imported into SIMCA 13 (Umetrics, Malmö, Sweden), ultraviolet (UV) scaled, and elabo-rated by means of orthogonal projections to latent structures discriminant analysis (OPLS-DA) supervised modeling. In this regard, the variation between the groups was separated into predictive and orthogonal components (i.e. ascribable to technical and biological variation). The OPLS-DA score plot allowed similarity/unrelatedness between treatments to be described and to identify the parameters better depicting differences. To support and validate the plot observed, the presence of outliers was inspected by means of Hotelling's T2, using 95% and 99% confidence limits for suspect and strong outliers, respectively. Furthermore, the goodness-of-fit (R2Y) and the goodness-of-prediction (Q2Y) were also considered, using a threshold value of > 0.5 for the latter. Cross-validation (CV-ANOVA; $p<0.01$) and permutation testing (N=100) were then carried out to validate and exclude overfitting. Finally, the VIP (i.e. variable importance in projection) selection method was used to select those parameters possessing the highest discrimination potential, setting a VIP score > 1.

3. Results and discussion

3.1 Physicochemical parameters

The changes in pH (Fig. 1(A)) and color parameters (Fig. 1(B)) when adding proteins from different sources are shown in Fig. 1. Slight variations in pH were found, however the statistical analysis revealed that there were significant differences ($p < 0.05$) among samples, with the highest pH value corresponding to the sample made with soy protein (6.38). These results contrast to the results of Parniakov et al.,[39] since in their study, the highest pH values were found in chicken rotti prepared with *Chlorella* and *Spirulina* proteins. However, Cofrades et al.[40] also obtained lower pH values when *Himanthalia elongata* was incorporated into meat products.

Furthermore, in the color parameters, it can be seen that the highest L^* value belongs to the legume proteins, while the algae (*Chlorella* and *Spirulina*) presented the lowest ones. This is also the case with a^*, where the negative value of the algae may be due to their green and blue pigments. Finally, b^* values ranged between 8.11 and 17.72, in the turkey burgers prepared with *Spirulina* protein, which showed the lowest value, while the samples made with pea protein presented the highest one. These results match completely with the results obtained by Parniakov et al.,[39] who obtained similar results in chicken rotti manufactured with different protein sources. However, WHC was not altered by the incorporation of different vegetable proteins to the turkey burgers.

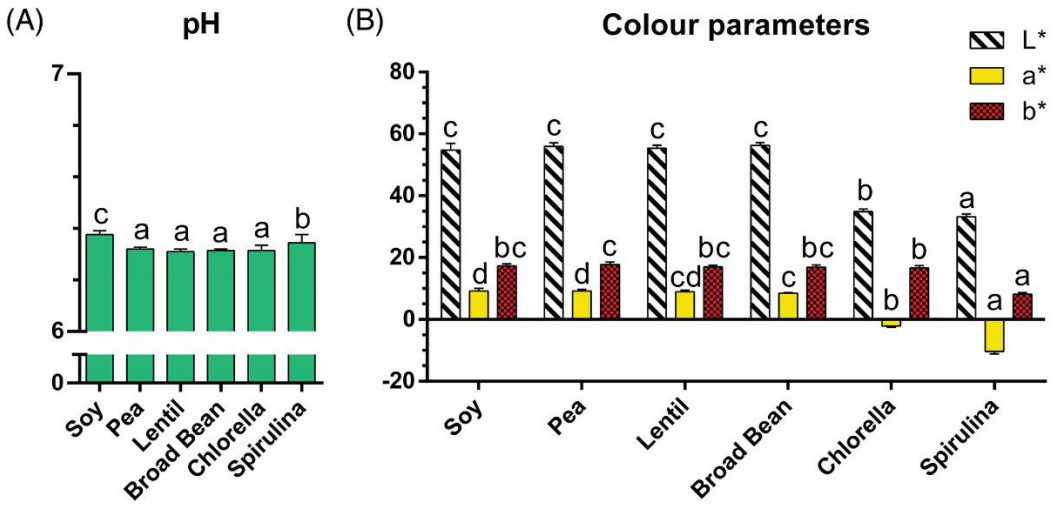

Figure 1. The pH (A) and color parameters (B) of turkey burgers prepared with different proteins (n = 5). Data are presented as mean ± standard deviation (SD). Bars with the different letters differ significantly ($p < 0.005$).

3.2 Proximate composition

Since the formulation of each one of the samples is the same, with exception of the protein used, large changes in chemical composition would not be expected. This is observed in Fig. 2, where neither the moisture nor the lipid content showed significant differences among batches. However, the protein content is different, with the maximum value corresponding to the samples prepared with soy protein (15.44%) and the minimum value for turkey burgers made with *Chlorella* protein (14.81%). The percentage of ash also changed among the different batches. However, the interval of the values is much more narrow, since the minimum value was obtained in the batch prepared with lentil protein (2.00%) and the maximum one corresponds to the batch prepared with the *Spirulina* protein (2.05%). As already mentioned earlier, these ranges of such small values are due to the fact that the formulation is exactly the same in all batches, with the exception of the protein added, which only represents 1% of the final composition.

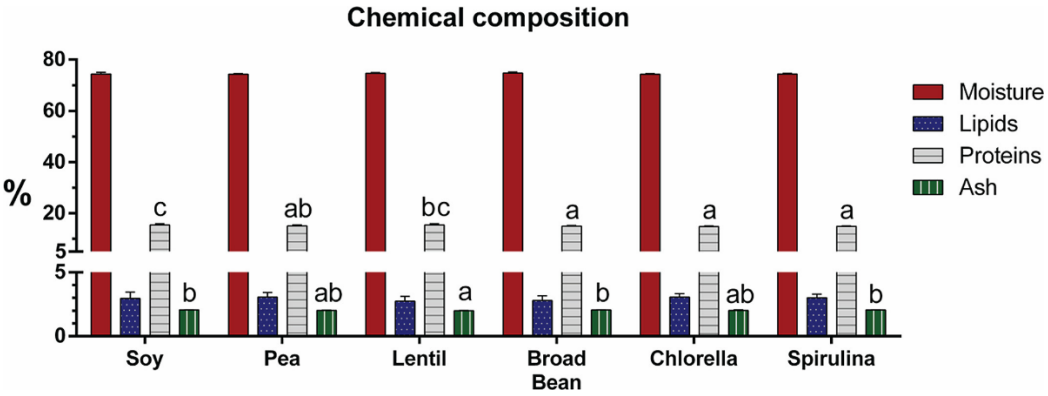

Figure 2. Chemical composition of turkey burgers prepared with different proteins (n = 5). Data are presented as mean ± standard deviation (SD). Bars with the different letters differ significantly ($p < 0.05$).

Neither did Parniakov et al.[39] obtain significant changes in the percentage of moisture. However, they found changes in the lipid content, obtaining a maximum value of 5.82% in the chicken rotti batch made with *Chlorella* protein. In contrast to our study, both protein and ash contents were higher in the batch of lentil protein: 22.06% and 3.22% respectively.

Furthermore, López-López et al.[41] obtained a reduction in moisture and lipid content by adding *Himanthalia elongata* to frankfurters, and compared results with a control sample, containing soy. They also observed an increase in the amount of protein and ash in the products enriched with algae compared to the control. Finally, Cofrades et al.[40] noticed a decrease in moisture and ash content by adding *Porphyra umbilicalis* or *Himanthalia elongata* to meat products.

3.3 Textural parameters

Regarding textural properties, only elasticity and adhesiveness showed significant differences among groups (Fig. 3). Elasticity was higher in samples prepared with soy protein (0.61 mm) than in the other samples. Furthermore, adhesiveness showed its highest value in burgers made with lentil protein (−17.04 g×s), whereas the lowest results were obtained in burgers made with *Chlorella* and *Spirulina* proteins, very close to zero (−0.66 and −1.08 g×s, respectively).

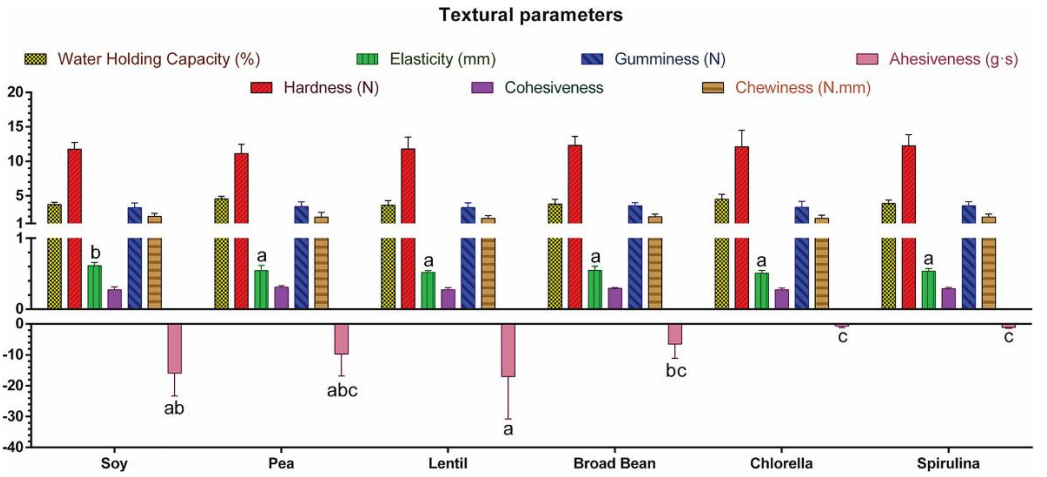

Figure 3. Textural parameters of turkey burgers prepared with different proteins (n = 5). Data are presented as mean ± standard deviation (SD). Bars with the different letters differ significantly ($p < 0.05$ for elasticity; $p < 0.005$ for adhesiveness).

Other studies such as Choi et al.[42] presented a reduction in parameters such as hardness, chewiness or gumminess when adding *Laminaria japonica* to pork patties. Moreover, Parniakov et al.[39] observed a decrease in textural parameters with the exception of adhesiveness in chicken rotti containing *Chlorella* or *Spirulina* proteins. However, authors such as Cofrades et al.[40] or López-López et al.[41] noticed an increase in the parameters of hardness and

chewiness by adding *Himanthalia elongata* to meat gel/emulsion or Frankfurt sausages.

3.4 Amino acid content

Table 1 shows the influence of addition of different proteins of vegetable and microalga origin in turkey burgers. The hydrolyzed amino acid profile of the turkey burgers included 17 out of 20 amino acids constituting food proteins. Arginine was included in the essential amino acid group.[43] Cysteine and methionine were not detected. It can be seen that amino acids such as serine, glycine, arginine, and tyrosine did not show significant changes. In all these cases, the minimum value corresponded to samples prepared with pea protein, while the maximum values correspond to turkey burgers formed with *Spirulina* and broad bean proteins. As for the rest of the amino acids, glutamic acid was the predominant, obtaining a maximum value of 2.21 g/100 g in the case of broad bean protein. This is interesting from a sensory point of view, since it is known that glutamic acid provides umami flavor.[44] It is also observed that histidine was the minority amino acid, with a minimum value of 0.25 g/100 g for the pea protein. The amino acids with the greater difference among the different batches were glutamic acid, valine, lysine, isoleucine, leucine and phenylalanine. These results agree with the data obtained by Cofrades et al.[45] who observed an increase in these amino acids when adding *Porphyra umbilicalis* to meat emulsions.

Table 1. Amino acid content (expressed as g/100 g) of turkey burgers with different protein replacements by pulses and algae proteins (n = 5)

Amino acid	*Protein source*						Sig.
	Soy	Pea	Lentils	Broad beans	*Chlorella*	*Spirulina*	
Asp	0.99±0.09^{ab}	0.79±0.14^{a}	0.85±0.14^{a}	1.20±0.23^{b}	1.00±0.08^{ab}	1.14±0.15^{b}	**
Ser	0.43±0.26	0.41±0.05	0.45±0.09	0.58±0.09	0.48±0.04	0.57±0.05	n.s.
Glu	1.77±0.12^{a}	1.46±0.27^{a}	1.56±0.24^{a}	2.21±0.40^{b}	1.85±0.14^{ab}	2.13±0.25^{b}	***
Gli	0.63±0.17	0.48±0.05	0.45±0.08	0.65±0.10	0.51±0.07	0.60±0.06	n.s.
Hys	0.30±0.03^{a}	0.25±0.04^{a}	0.28±0.06^{a}	0.37±0.05^{b}	0.31±0.03^{ab}	0.35±0.03^{b}	**
Arg	0.86±0.09	0.74±0.11	0.80±0.16	0.88±0.45	0.95±0.05	1.17±0.11	n.s.
Thr	0.51±0.06^{ab}	0.42±0.07^{a}	0.47±0.10^{a}	0.60±0.09^{b}	0.51±0.03^{ab}	0.60±0.05^{b}	*
Ala	0.72±0.12^{bc}	0.56±0.06^{ab}	0.57±0.10^{a}	0.80±0.14^{c}	0.69±0.04^{abc}	0.80±0.10^{c}	*
Pro	0.59±0.11^{bc}	0.47±0.06^{ab}	0.45±0.10^{a}	0.57±0.08^{abc}	0.51±0.02^{abc}	0.61±0.04^{c}	*
Cis	-	-	-	-	-	-	-
Tyr	0.47±0.08	0.36±0.09	0.42±0.15	0.52±0.10	0.39±0.01	0.49±0.06	n.s.
Val	0.52±0.06^{ab}	0.42±0.07^{a}	0.44±0.08^{a}	0.61±0.10^{bc}	0.54±0.02^{abc}	0.62±0.06^{c}	***
Met	-	-	-	--	-	-	-
Lys	0.99±0.06^{a}	0.83±0.14^{a}	0.90±0.14^{a}	1.30±0.28^{b}	1.07±0.07^{ab}	1.22±0.16^{b}	***
Isoleu	0.53±0.03^{a}	0.43±0.08^{a}	0.45±0.08^{a}	0.64±0.10^{b}	0.55±0.02^{ab}	0.65±0.07^{b}	***
Leu	0.89±0.05^{a}	0.73±0.13^{a}	0.76±0.13^{a}	1.08±0.17^{b}	0.94±0.05^{ab}	1.09±0.11^{b}	***
Phe	0.45±0.04	0.38±0.06	0.41±0.09	0.55±0.07	0.48±0.02	0.55±0.05	***
Total	10.66±0.81^{a}	8.74±1.37^{a}	9.26±1.66^{a}	12.53±1.56^{b}	10.73±0.69^{ab}	12.60±1.25^{b}	***
E	5.07±0.38^{a}	4.21±0.67^{a}	4.51±0.82^{a}	6.02±0.46^{b}	5.31±0.30^{ab}	6.26±0.61^{b}	***
NE	5.59±0.48^{ab}	4.53±0.69^{a}	4.76±0.85^{a}	6.51±1.13^{b}	5.42±0.39^{ab}	6.34±0.64^{b}	**
E/ NE	0.91±0.05	0.93±0.01	0.95±0.02	0.94±0.09	0.98±0.01	0.99±0.01	n.s.

E: essentials amino acids; NE: non essential amino acids. All the data are expressed as mean ± standard deviations. Means with the different superscript letters in a row are differ significantly. Sig: Significance; *** ($p < 0.005$). ** ($p < 0.01$). * ($p < 0.05$). n.s.: not significant.

However, the batch of turkey burgers with a higher total amino acid content was the one with *Spirulina* added, as well as a ratio between essential and non-essential amino acids close to one. However, the lower total amount of amino acids belongs to burgers prepared with pea protein, while the worst ratio of essential/non-essential amino acids was found in samples elaborated with soy protein. In this sense, Parniakov et al.[39] also found the highest total amino acid content in chicken rotti made with *Chlorella* and *Spirulina* proteins and the lowest in samples prepared with pea proteins. In addition, these authors obtained a ratio of more than one essential/non-essential amino acids for *Chlorella* and *Spirulina* proteins, which showed that both algae are a good source of essential amino acids. López-López et al.[41] and Dawczynski et al.[46] also found an increase in the total amount of amino acids by incorporating algal proteins into meat products. Therefore, this study suggests that soy protein in meat products can be replaced by broad bean protein or algae such as *Chlorella* or *Spirulina*, thus improving the protein profile of the food.

3.5 Chemometric evaluation

The OPLS-DA was used as a supervised statistical tool to plot similarities/differences among turkey burgers on the basis of all the parameters studied. This multivariate data analysis allows extracting information from complex dataset characterized by multiple variables and using all the variables simultaneously. In particular, OPLS models are able to rotate the projection so that the model focuses on the effect of interest, thus separating data into predictive and uncorrelated information (i.e. orthogonal signal correction). The dataset based on physicochemical parameters (pH, color and texture), proximate composition (moisture, protein, lipid and ash content), and amino acids profile was used for this statistical analysis.

According to our experimental conditions, two OPLS models were considered. In the first model, the protein source (soy, broad beans, lentils,

beans, *Chlorella* and *Spirulina*) was used as class membership criteria, in order to identify those turkey burgers more similar according to the parameters aforementioned. The OPLS score plot obtained by means of the latter considerations is reported in Fig. 4.

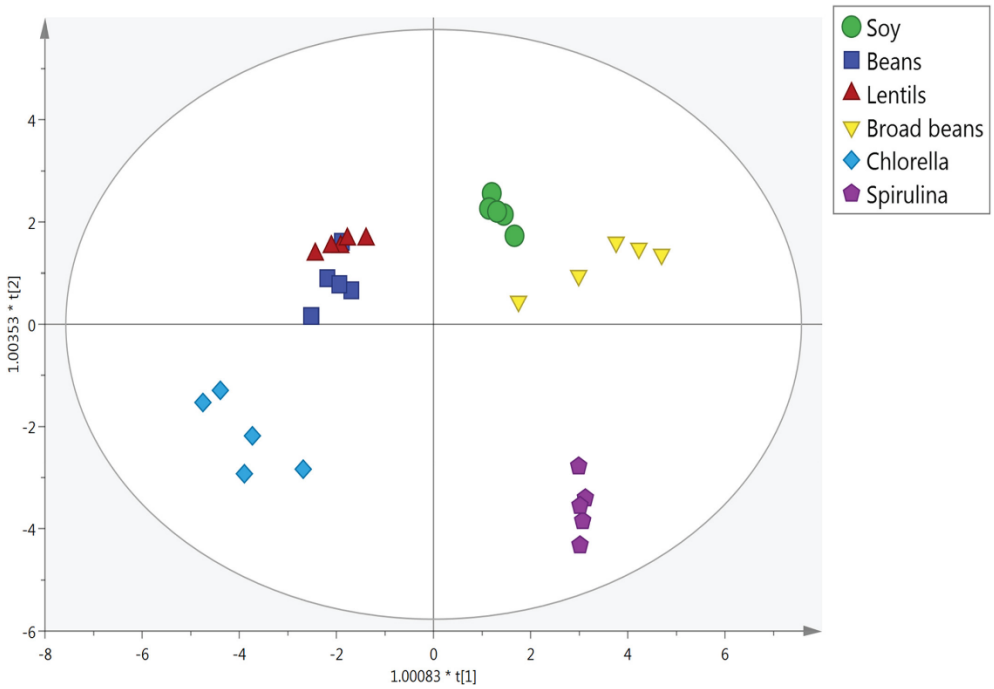

Figure 4. Orthogonal projections to latent structures (OPLS) score plot obtained using the protein source (i.e. soy, broad beans, lentils, beans, *Chlorella* and *Spirulina*) as class membership criteria, in order to identify those turkey burgers more similar.

Interestingly, the separation between groups was very good with bean and lentil proteins possessing a very similar profile and clustering together. Similar information was obtained looking at soy and broad bean proteins, with

them both being included in the same region of the OPLS score plot. Notably, *Chlorella* and *Spirulina* proteins, i.e. those burgers enriched with microalgae, were completely different in terms of the parameters studied, when compared to the other turkey burger samples. The OPLS model obtained suggested that, among pulses, broad bean was the protein source that allowed a burger similar to the control (soy) to be obtained. Afterwards, the variable selection method VIP (variable importance in projection) was used to identify those parameters allowing the score plot hyperspace distribution previously described. In this regard, the most discriminating parameters possessing a VIP score > 1 are reported in Table 2.

As a general consideration, 14 discriminating parameters were identified, with textural parameters such as cohesiveness, elasticity and adhesiveness possessing a very high discrimination potential, followed by those related to color (L^*, a^*, and b^* values). Interestingly, other parameters such as chewiness and hardness were found to be very important, thus confirming that the final texture was affected by the alternative protein replacement. The OPLS model was cross-validated by means of permutation testing (N = 100) and inspected for outliers by means of Hotelling's T2 range, then checking model parameters that were found to be more than acceptable, being R2Y = 0.85 and Q2Y = 0.63.

A second OPLS model was carried out to discriminate the six turkey burgers prepared according to the 'type of protein' (i.e. control versus pulses and microalgae). The OPLS score plot obtained with this second interpretation is reported in Fig. 5.

Table 2. VIP (variable importance in projection) selection method to identify those parameters discriminating burgers according to the protein source (i.e. soy, broad beans, lentils, beans, *Chlorella* and *Spirulina*)

Var ID (Primary)	M1.VIP[5+1+0]	2.44693 * M1.VIP[5]cvSE
Cohesiveness	1.25667	0.677421
Elasticity (mm)	1.20848	0.223379
% Moisture	1.20547	0.407983
Adhesiveness (g.sec)	1.16958	0.139647
pH	1.16599	0.351083
b*	1.12919	0.397432
L*	1.11402	0.179352
a*	1.11217	0.168263
% Fat	1.09554	0.556257
Chewiness (N.mm)	1.08377	0.251971
Hardness (N)	1.04924	0.560976
% Protein	1.03569	0.299671
% WHC	1.01428	1.19757
Gumminess (N)	0.964638	0.467577
% Ash	0.951902	0.440015
Gli	0.910074	0.275342
Pro	0.906318	0.265215
Lys	0.881883	0.330559
Ser	0.881457	0.265732
Thr	0.880821	0.268914
Glu	0.878199	0.292486
Ala	0.877588	0.294838
Asp	0.877331	0.307914
Arg	0.875491	0.35891
Val	0.874921	0.264664
Hys	0.87435	0.279875
Isoleu	0.874288	0.251043
Leu	0.873122	0.257744
Phe	0.872906	0.249869
Tyr	0.858216	0.245838

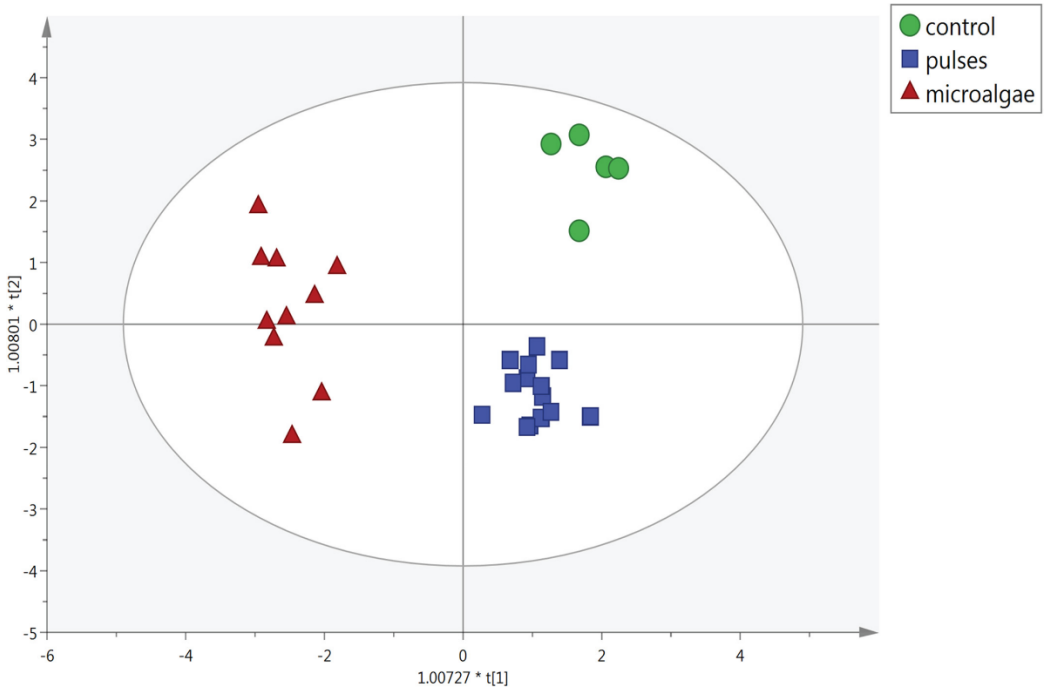

Figure 5. Orthogonal projections to latent structures (OPLS) model to discriminate the six turkey burgers prepared according to the 'type of protein' (i.e. control versus pulses and microalgae).

This analysis confirmed that pulse proteins were the protein source most similar to the control (soy) in comparison to microalgae. Indeed, the second latent vector of the OPLS space clearly discriminated *Chlorella* and *Spirulina* burgers from the other ones. This second OPLS model was cross-validated and checked again for outliers (Supporting Information), and provided very robust fitting parameters (R^2Y = 0.85 and Q^2Y = 0.70). Afterwards, the VIP selection was used again to identity those parameters differentiating the classes of protein sources (Table 3).

Table 3. VIP (variable importance in projection) selection method to identify those parameters discriminating burgers according to the 'type of protein' (i.e. control versus pulses and microalgae)

Var ID (Primary)	M1.VIP[2+3+0]	2.44693 * M1.VIP[2]cvSE
L*	1.74725	0.507909
a*	1.72648	0.589273
pH	1.57491	1.04779
b*	1.39601	0.642895
Elasticity (mm)	1.26783	0.492545
Adhesiveness (g.sec)	1.2546	0.550322
% Protein	1.13053	0.474078
Gli	0.956316	0.423798
Pro	0.950281	0.353726
Ala	0.891026	0.284986
Val	0.866575	0.225304
Tyr	0.866432	0.299584
Isoleu	0.856226	0.19837
Leu	0.853151	0.192762
Asp	0.852202	0.200481
Thr	0.849875	0.223605
Phe	0.84676	0.200456
Hys	0.843147	0.210289
Glu	0.841142	0.18419
Cohesiveness	0.83058	0.760239
Lys	0.825743	0.181145
Arg	0.815714	0.220525
% Ash	0.777262	0.486084
Gumminess (N)	0.772585	0.749919
Chewiness (N.mm)	0.761671	1.39904
Ser	0.732158	0.129513
% Moisture	0.651411	0.668345
Hardness (N)	0.649502	0.543268
% WHC	0.625979	0.572184
% Fat	0.572034	0.797117

Interestingly, a lower number of variables possessing a VIP score > 1 was obtained (i.e. seven), with color (L*, a*, and b* values) and pH being the most discriminant contributors. This indicates that the enrichment of turkey burgers with alternative proteins could affect the visual acceptance, in turn driving the choices by the final consumer. In this regard, as stated by Shan et al.,[47] health-oriented reformulations of processed meat are very promising in terms of addressing increasing health concerns regarding this food category; however, consumer acceptance cannot be taken for granted.

4. Conclusions

The use of different vegetable and microalgae proteins as soy protein replacers in the preparation of turkey burgers with improved nutritional profile, produces changes, both in the physicochemical properties and protein profile. Burgers prepared with *Spirulina* and broad bean protein presented the highest amount of total amino acids. In addition, the ratio between essential and non-essential amino acids increased, indicating that these proteins are a good source of essential amino acids. The color parameters were also greatly altered by *Spirulina* and *Chlorella* proteins, since they were significantly decreased, owing to them acquiring a green-blue color, probably due to the pigments they contain. Finally, there was a reduction in the elasticity and adhesiveness, as well as the pH of the samples with these proteins. The OPLS-DA multivariate modeling carried out from physicochemical, textural and composition parameters suggested that addition of bean and lentil proteins provided a very similar profile, whereas addition of broad bean proteins resulted in a profile very close to soy (control). However, microalgae as proteins source showed the most distinctive and characteristic profiles. Among others, textural parameters (cohesiveness, elasticity and adhesiveness) followed by color (L*, a*, and b* values) possessed the highest

discrimination potential. Overall, the choice of one protein source over another distinctively affected both textural properties and color.

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Influence of different sources of vegetable, whey and microalgae proteins on the physicochemical properties and amino acid profile of fresh pork sausages

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Abstract

The purpose of this study was to evaluate changes in the physicochemical properties and amino acid profiles of pork sausages prepared by including vegetable protein sources (beans, peas, and lentils), microalgae (*Chlorella* and *Spirulina*) or whey, as compared with a control (soy protein). Significant differences were found for all the studied parameters. The protein content was significantly lower in sausages made with pea protein compared with the control. Colour parameters changed significantly after the incorporation of microalgae proteins. Moreover, significant differences among treatments were observed in the amino acid profile. The inclusion of *Spirulina* proteins resulted in an increase in the total amino acid content and the ratio of essential/non-essential amino acids. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) allowed pork sausages to be classified according to the protein source, in comparison with soy (control). Textural parameters (chewiness, gumminess and hardness) followed by colour and pH were the most discriminant parameters. Considering texture traits, physicochemical parameters and amino acid profiles across treatments, proteins from legumes and whey provided profiles closer to that of soy. However, although microalgae-derived proteins altered the colour and texture, they provided nutritionally favourable profiles, thus suggesting that seaweeds could also be used to enrich pork sausages, as an alternative to soy protein.

Keywords

Seaweed, Colour parameters, *Chlorella* and *Spirulina*, textural traits, Beans, Lentils

1. Introduction

Meat and meat products have been part of the human diet since ancient times (Mann, 2018). The average consumption per person per year of this food group worldwide has increased constantly increasing since the mid-twentieth century (FAO, 2018; Godfray et al., 2018).

Despite the large number of nutrients provided by meat products, such as vitamin B_{12} and iron (Banjari & Hjartåker, 2018), it is recommended that red and processed meats are moderately consumed (Lorenzo et al., 2018). There are numerous studies that link excessive meat consumption with a higher probability of suffering from cancer (Banjari & Hjartåker, 2018; Farvid et al., 2018; Godfray et al., 2018; Song & Chan, 2018; You & Henneberg, 2018). In addition, the cholesterol, fat content, and fat composition of meat are important health issues for consumers, because they have been associated with obesity and hypercholesterolaemia (Domínguez et al., 2018a). Moreover, within different types of meat, many studies have established a relationship between the consumption of processed meats, such as sausages, and the risk of cancer (WHO, 2018). Similarly, several studies have related the intake of processed meat with the appearance of chronic diseases such as obesity, type 2 diabetes, cardiovascular diseases and some types of cancer (Bellou, Belbasis, Tzoulaki, & Evangelou, 2018; Boada, Henríquez-Hernández, & Luzardo, 2016; Rouhani, Salehi-Abargouei, Surkan, & Azadbakht, 2014; Zhang, Cogswell, Wang, & Bowman, 2017). This shows that although meat is a main source of proteins and iron, whereas most of the members of the B-complex can be obtained from plant sources, the intake of this group of foods should be moderate. Moreover, apart from being health conscious, consumers also take into account the environmental issues related to increased meat production and food sustainability (Domínguez et al., 2018b).

On the other hand, there are studies showing that a diet rich in plant foods decreases the risk of suffering from chronic diseases, such as cardiovascular and neurodegenerative diseases and cancer (Neuhouser, 2018; Null, Pennesi, & Feldman, 2017). For this reason, alternatives have been sought, such as the incorporation of products of vegetable origin in pork sausages to improve their quality (Eisinaite, Vinauskiene, Viskelis, & Leskauskaite, 2016).

Regarding the protein component of the diet, the human being needs a total of 20 amino acids, of which 9 are classified as essential because the human body cannot produce them itself and must, therefore, obtain them from food (Watford & Wu, 2018). Therefore, a diet providing sufficient amounts of these essential amino acids is very important for growth and health maintenance.

Proteins of vegetable origin are frequently used as meat proteins analogues, usually in meat products such as sausages. This is due to their health benefits, low cost, and lack of cholesterol (Carvalho et al., 2019; Marti-Quijal et al., 2019; 2018; Zamuz et al., 2019). Of all the vegetable proteins, the demand for soy protein is the highest in the food industry (Asgar, Fazilah, Huda, Bhat, & Karim, 2010). As the highest quality vegetable protein, soy is characterized by its favourable amino acid profile, which is comparable to that of meat protein (Kumar et al., 2017; Thirumdas et al., 2018). In addition, it has been shown to have positive effects on cardiovascular health and lipid metabolism (Ruscica et al., 2018; Tang et al., 2017). Apart from soybeans, legumes are another one of the most important sources of vegetable proteins, a key aspect of human nutrition (Havemeier, Erickson, & Slavin, 2017). It has also been shown that the consumption of legumes reduces the risk of suffering from cardiovascular diseases and diabetes (Becerra-Tomás et al., 2018; Blekkenhorst et al., 2018).

Another high-protein food source is microalgae (Barba, 2017). Proteins from microalgae have attracted special interest in recent years, largely as an alternative to proteins of plant origin, mainly due to allergenicity issues. Moreover, proteins obtained from microalgae are inexpensive and sustainable. In addition, they have attracted great interest as functional foods (Matos, Cardoso, Bandarra, & Afonso, 2017; Parniakov et al., 2015). Thus, these proteins have an increasing expectation among consumers due to their relationship between diet and health (Roohinejad et al., 2017). Fundamentally, algae are characterized by their high amino acids content (Lorenzo et al., 2017). *Spirulina* and *Chlorella* are among the algae that are used most often in the food sector; both have a high protein content, excellent nutritional value, and are also very easy to grow (Priyadarshani & Rath, 2012; Spolaore, Joannis-Cassan, Duran, & Isambert, 2006).

Another healthy alternative source of protein is whey (Musina et al., 2018). Whey is a serious environmental problem, because it is a residue derived from the dairy industry, which is produced in large quantities and its disposal is complex (Yadav et al., 2015). Whey proteins are interesting from a nutritional point of view, because they comprise approximately 20% of total bovine milk proteins and they play an important nutritional role as a rich and balanced source of essential amino acids (Almeida, Alvares, Costa, & Conte-Junior, 2016).

The present study investigates the addition of different proteins, i.e., legumes (peas, lentils and beans), algae (*Spirulina* and *Chlorella*), and whey on the nutritional composition (moisture, fat, ash, carbohydrates, proteins and amino acid profile) and physicochemical properties (colour, hardness, adhesiveness, elasticity, gumminess and chewiness) of pork sausages. Pork sausages supplemented with soybeans will be used as a control, since soy protein is commonly added on an industrial level to this type of meat product.

2. Material and methods

2.1. Samples

Fresh pork meat was provided by Frigolouro - Grupo Coren (Pontevedra, Spain). All species and additives used in the preparation of the different types of sausage were purchased at a local market. The different sources of proteins [*Glycine max* (soybean), *Lens culinaris* (lentil), *Vicia faba* (beans), algae proteins (*Spirulina* and *Chlorella*) and whey] were supplied by Ingredion, SA, Algaenergy SA (Madrid, Spain) and Queizuar, SL (Santiago de Compostela, Spain), respectively.

2.2. Preparation of fresh sausages

A total of seven different types of fresh sausages were prepared with each of the added proteins (soy, peas, lentils, beans, *Chlorella*, *Spirulina* and whey), with 15 of repetitions each. To each batch, 1% of the corresponding protein were added.

In the preparation of fresh sausages, the meat was minced with a 6 mm plate, which was placed in a refrigerated chopper (La Minerva, Bologna, Italy). Then, the meat was mixed in a vacuum chopper machine (Fuerpla, Valencia, Spain) for 3 min with water and all additives, except the proteins, until a fine and homogeneous paste was obtained. Once a homogeneous mass was obtained, the corresponding batches were made, and the necessary amount of protein was added to each of them. The composition of the different ingredients used in the preparation of fresh pork sausages is given in Table 1. The processed sausages are shown in Fig. 1.

Table 1. Formulation of fresh sausages.

	Kg	%
Pork shoulder	10.1155	50.58
Fat	6.0693	30.35
Water	2.5289	12.64
Florida NCO	0.6069	3.03
Protein[a]	0.2023	1.01
Potato starch	0.2023	1.01
Vegetable fiber	0.2023	1.01
INBAC ADL	0.0405	0.20
Garlic	0.0192	0.10
LSC1 Coloring	0.0121	0.06
Oregano	0.0007	0.00
Total	20	

[a] Respective protein content in each batch.

Figure 1. Elaborated fresh sausages. Batches: Batch 1 - sausages prepared with soy protein (control); Batch 2 - sausages prepared with pea protein; Batch 3 - sausages prepared with lentil protein; Batch 4 - sausages prepared with broad bean protein; Batch 5 - sausages prepared with Chlorella protein; Batch 6 - sausages prepared with Spirulina protein and Batch 7 - sausages prepared with whey protein.

2.3. Proximate composition

The determination of moisture was done using a gravimetric method. A paste was generated as a result of mixing the sausage and ethanol, which was subjected to stove drying until constant mass, following International Standard ISO R-1442 (ISO, 1978). To determine the protein content, Kjeldahl method was used according to the International Standard ISO R-937 (ISO, 1978). The amount of protein in the meat was determined from its nitrogen content. The fat was extracted with petroleum ether following the AOCS official method Am 5-04 (AOCS official method Am 5-04). Ash content was determined by calcination in muffle following International Standard ISO R-936 (ISO, 1998).

2.4. Physicochemical properties

The determination of the physical parameters was done following the method previously described by Lorenzo, Pateiro, and Franco (2013). The pH was measured using a pH meter (HI 99163-Hanna Instruments, Spain). Colour measurement was performed on the muscle surface after 1 h of exposure to air using a Konica Minolta CR-400 colorimeter (Osaka, Japan). The determination of water holding capacity (WHC), expressed as cooking losses, was carried out following the technique described by Honikel (1997). The texture of the sausages was determined with a texture analyser (TA-XT2, Stable Micro Systems, Godalming, United Kingdom) as described by Lorenzo and Carballo (2015).

2.5. Amino acid profile

Hydrolysed amino acid composition (g/100 g of meat) of sausages was determined using the procedure previously described by Lorenzo, Cittadini, Bermúdez, Munekata, and Domínguez (2015). 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Waters AccQ-Fluor reagent kit) was used for

the derivatization of amino acids and they were subsequently analyzed by RP-HPLC using a Waters 2695 Separations Module with a Waters 2475 Multi Fluorescence Detector equipped with a Waters AccQ-Tag amino acid analysis column (van Wandelen & Cohen, 1997).

2.6. Statistical analyses

The results were analyzed statistically using the IBM SPSS Statistics® 23.0 program (IBM Corp). In order to detect differences in the physicochemical parameters and the amino acid profile among batches, an analysis of variance (ANOVA) was applied. The separation of quadratic means was performed using a Duncan test, performed at a significance level $\alpha < 0.05$. The levels of significance have been indicated with the following notation: *** - $p < 0.001$; ** - $p < 0.01$; * - $p < 0.05$; n. s - non-significant.

Afterwards, the whole dataset containing physicochemical parameters together with amino acid profile was exported into SIMCA 13 (Umetrics, Malmö, Sweden), UV scaled, and elaborated by means of orthogonal projections to latent structures discriminant analysis (OPLS-DA) supervised modelling. Therein, the OPLS model divided the variation between the groups into predictive and orthogonal components (this latter ascribable to technical and biological variation). The presence of outliers was investigated by means of Hotelling's T2, using 95% and 99% confidence limits for suspect and strong outliers, respectively. Thereafter, the goodness-of-fit (R2Y) and the goodness-of-prediction (Q2Y) were calculated. Cross-validation was then made (CV-ANOVA; $p < 0.01$) and permutation testing (N - 100) was used to exclude model overfitting. Finally, the VIP variable selection method (variable importance in projection) was used to select those parameters possessing the highest discrimination potential, considering a VIP score > 1.

3. Results and discussion

3.1. Effect of different protein sources on the chemical composition of fresh pork sausages

Table 2 shows the proximal composition of the different fresh pork sausages following addition of the different protein sources. Since the formulation used to prepare each batch (control, pea, lentil, broad bean, *Chlorella*, *Spirulina* and whey) was the same, the chemical composition of the samples would have to be similar. That said, the moisture content of the samples prepared with lentils (66.57 g/100 g) was slightly higher than that of the control group elaborated with soybeans (64.71 g/100 g), however, the difference was not significant. A similar trend was observed by Parniakov et al. (2018), who did not obtain significant differences in the moisture content of chicken roti samples prepared with different protein sources, reporting values between 66.34 g/100 g and 69.14 g/100 g for samples made with lentil and pea protein, respectively. However, these authors found significant differences in fat, protein and ash content among the different batches studied.

With regard to lipid content, the highest values were observed in the samples prepared with pea protein (14.75 g/100 g) while the lowest were obtained for the samples made with lentil protein (11.63 g/100 g). In this sense, Parniakov et al. (2018) observed a higher fat content in chicken roti samples manufactured with *Spirulina* (5.82 g/100 g) compared with the control group (5.04 g/100 g). In addition, López-López, Cofrades, Ruiz-Capillas, and Jiménez-Colmenero (2009) also found a higher lipid content in Frankfurt sausages made with *Himanthalia elongata* compared with the control batch. On the other hand, the protein content was significantly lower in sausages made with pea protein (14.68 g/100 g) than in sausages made with soy protein (15.40 g/100 g). These results are consistent with those found by Parniakov et

al. (2018) who observed significant differences in the protein content of chicken roti made with different protein sources, the highest values being observed in samples made with lentil protein. Finally, sausage samples made with *Spirulina* had a higher ash content (2.98 g/100 g) than samples made with pea protein (2.83 g/100 g). A similar result was obtained by Parniakov et al. (2018) who found that chicken roti samples made with lentil protein showed higher ash contents than samples containing pea protein.

Table 2. Effect of the inclusion of different protein sources in the chemical composition of fresh pork sausages. All the data are expressed as mean ± standard deviations (n = 15). Means with different superscript letters in a columndiffer significantly. Significance: n.s.: not significant; * = $p < 0.05$.

		% Moisture	% Lipid	% Protein	% Ash
Protein source	Soy	64.71±0.56	12.11±0.34	15.40±0.18^{c}	2.97±0.02^{c}
	Pea	64.24±2.24	14.75±2.58	14.68±0.26^{a}	2.83±0.10^{a}
	Lentil	66.57±0.71	11.63±0.67	14.90±0.23^{abc}	2.94±0.06^{bc}
	Broad bean	64.85±0.46	13.90±0.73	14.80±0.37^{a}	2.86±0.07^{ab}
	Chlorella	64.56±1.27	14.01±1.68	15.32±0.30^{bc}	2.93±0.08^{bc}
	Spirulina	64.12±0.74	13.80±0.79	15.36±0.41^{c}	2.98±0.04^{c}
	Whey	64.62±2.51	14.51±3.21	14.84±0.74^{ab}	2.91±0.05^{bc}
Significance		n.s.	n.s.	*	*

3.2. Effect of different protein sources on pH and colour parameters of fresh pork sausages

The effects of different protein sources on pH and colour parameters of fresh pork sausages are presented in Table 3. The pH showed significant differences among the seven batches of fresh pork sausages studied, since

the samples elaborated with *Spirulina* protein presented the highest values (6.15). These results agree with those previously found by Parniakov et al. (2018), who observed that chicken roti samples made with *Spirulina* and *Chlorella* protein presented a significantly higher pH than the other batches. However, other authors such as Cofrades, Benedí, Garcimartin, Sánchez-Muniz, and Jimenez-Colmenero (2017), Cofrades, López-López, Solas, Bravo, and Jiménez-Colmenero (2008) and López-López et al. (2009) found a pH reduction after incorporating algae (*Himanthalia elongata*).

With regard to colour parameters, luminosity (L*) and redness (a*) were significantly affected by the inclusion of different protein sources in the formulation of fresh pork sausages (Table 3). L* was higher in samples of fresh pork sausage made with bean protein, while lower a* was obtained in samples made with protein from algae (*Spirulina* and *Chlorella*). However, the yellow levels (b*) were not affected by the incorporation of different protein sources into fresh pork sausages, presenting values that were fairly close to each other, and differed fundamentally from those of samples prepared with *Spirulina*. In a study published by Parniakov et al. (2018), these authors observed that colour parameters were not affected by the incorporation of pea, lentil and bean proteins in the formulation of chicken roti. However, the colour parameters were modified by the incorporation of algae proteins (*Spirulina* and *Chlorella*) into chicken roti due to the presence of green and blue-green pigments.

Table 3. Effect of the inclusion of different protein sources on the pH values and colour parameters (L*, a* and b*) of fresh pork sausages.

		pH	L*	a*	b*
Protein source	Soy	6.06±0.04^{c}	50.12±0.97^{b}	22.84±1.05^{d}	13.48±0.79^{bc}
	Pea	6.04±0.05^{bc}	52.67±2.44^{bc}	22.16±0.87^{cd}	13.70±1.19^{c}
	Lentil	5.98±0.05^{a}	52.09±4.13^{bc}	22.27±1.29^{cd}	13.65±1.57^{c}
	Broad Bean	6.09±0.03^{cd}	54.58±2.23^{c}	20.81±1.05^{c}	12.78±0.93^{bc}
	Chlorella	5.98±0.03^{ab}	33.96±2.15^{a}	0.59±0.33^{b}	11.80±2.01^{b}
	Spirulina	6.15±0.07^{d}	32.39±1.67^{a}	-5.05±0.26^{a}	4.60±0.82^{a}
	Whey	5.93±0.04^{a}	52.50±2.68^{bc}	22.35±3.06^{cd}	13.63±1.93^{c}
Significance		***	***	***	***

All the data are expressed as mean ± standard deviations (n = 15). Means with different superscript letters in a column differ significantly. Significance: *** = $p < 0.005$.

With regard to colour parameters, luminosity (L*) and redness (a*) were significantly affected by the inclusion of different protein sources in the formulation of fresh pork sausages (Table 3). L* was higher in samples of fresh pork sausage made with bean protein, while lower a* was obtained in samples made with protein from algae (*Spirulina* and *Chlorella*). However, the yellow levels (b*) were not affected by the incorporation of different protein sources into fresh pork sausages, presenting values that were fairly close to each other, and differed fundamentally from those of samples prepared with *Spirulina*. In a study published by Parniakov et al. (2018), these authors observed that colour parameters were not affected by the incorporation of

pea, lentil and bean proteins in the formulation of chicken roti. However, the colour parameters were modified by the incorporation of algae proteins (*Spirulina* and *Chlorella*) into chicken roti due to the presence of green and blue-green pigments.

3.3. Effect of different protein sources on texture parameters and water holding capacity (WHC) of fresh pork sausages

Table 4 shows the results obtained after the inclusion of different protein sources in the parameters of texture and WHC of fresh pork sausages. Changes in WHC was observed after the incorporation of different protein sources in the formulation of fresh pork sausages, showing the highest values in samples made with lentil, whey, *Spirulina*, and *Chlorella* protein (above 4.30 g/100 g), the samples made with soy, pea, and bean protein presented a WHC lower than 4 g/100 g.

On the other hand, all the texture parameters (hardness, elasticity, cohesiveness, gumminess and chewiness) were affected by the incorporation of different protein sources in the preparation of fresh pork sausage. Hardness, elasticity, cohesiveness, gumminess and chewiness was significantly higher (3.20 kg, 0.83 mm, 0.35, 1.13 kg and 0.94 kg×mm, respectively) in sausage samples made with soy protein (control) compared with the other batches studied. A similar result was obtained by Parniakov et al. (2018) who found that chicken roti samples made with soy protein had higher hardness, elasticity, cohesiveness, gumminess, and chewiness.

Table 4. Effect of the inclusion of different protein sources on texture parameters and water holding capacity (WHC) of fresh pork sausages. All the data are expressed as mean ± standard deviations (n = 15). Means with different superscript letters in a column differ significantly. Significance: n.s.: not significant; *** - $p < 0.005$ * - $p < 0.05$.

		WHC (%)	Hardness (N)	Adhesiveness (g·s)	Elasticity (mm)	Cohesiveness	Gumminess (N)	Chewiness (N·mm)
Protein source	Soy	3.98 ± 0.59^{abc}	31.40 ± 1.95^{c}	-26.05 ± 26.75	0.83 ± 0.02^{e}	0.35 ± 0.02	11.07 ± 1.16^{c}	9.18 ± 1.04^{c}
	Pea	3.64 ± 0.38^{a}	23.61 ± 1.00^{ab}	-7.62 ± 4.92	0.81 ± 0.02^{de}	0.33 ± 0.02	7.90 ± 0.38^{b}	6.37 ± 0.35^{b}
	Lentil	4.33 ± 0.68^{bc}	24.97 ± 3.36^{b}	-10.83 ± 5.26	0.79 ± 0.05^{cde}	0.32 ± 0.05	7.88 ± 1.38^{b}	6.29 ± 1.35^{b}
	Broad Bean	3.70 ± 0.37^{ab}	25.42 ± 2.18^{b}	-14.66 ± 9.66	0.77 ± 0.02^{bc}	0.33 ± 0.08	8.19 ± 1.18^{b}	6.29 ± 1.04^{b}
	Chlorella	4.34 ± 0.28^{c}	21.09 ± 2.26^{a}	-12.63 ± 6.75	0.71 ± 0.04^{a}	0.28 ± 0.02	5.94 ± 0.94^{a}	4.19 ± 0.54^{a}
	Spirulina	4.34 ± 0.42^{c}	24.49 ± 2.04^{b}	-11.76 ± 9.51	0.77 ± 0.03^{bcd}	0.28 ± 0.01	6.98 ± 0.74^{ab}	5.38 ± 0.55^{b}
	Whey	4.57 ± 0.55^{c}	23.93 ± 2.43^{ab}	-16.77 ± 11.52	0.75 ± 0.03^{b}	0.31 ± 0.03	7.43 ± 1.52^{b}	5.55 ± 1.06^{b}
Significance		*	***	n.s.	***	n.s.	***	***

3.4. Effect of different protein sources on the amino acid profile of fresh pork sausages

The effect of the inclusion of different protein sources on the amino acid profile of fresh pork sausages is summarized in Table 5. All the amino acids studied showed significant differences except glycine and proline, which presented average values of 0.90 and 0.64 g/100 g, respectively. The main amino acids were glutamic acid, aspartic acid, lysine, and leucine, which showed the highest values in sausage samples made with algae protein (*Spirulina* and *Chlorella*). These results are in line with those previously observed by other authors (Parniakov et al., 2018; Žugčić et al., 2018) who also found that the addition of algae increased the content of most amino acids.

Fig. 2 shows the average values of total, essential, non-essential amino acids (g/100 g), and the ratio of essential to non-essential amino acids in processed sausage samples. Significant differences were observed in total and non-essential amino acids ($p < 0.01$) and essential amino acids ($p < 0.05$) among the different batches studied. The highest values were found in sausages made with *Spirulina* (17.70 g/100 g of total, 8.77 g/100 g of essentials and 8.82 g/100 g of non-essential amino acids) whereas the lowest were found in sausages made with pea protein (Fig. 2).

With regard to the ratio between essential and non-essential amino acids, the highest values were obtained for sausages made with lentil protein, although no significant differences were observed among the samples studied. However, Parniakov et al. (2018) found values above 1 in roti samples made with algae protein, indicating that *Spirulina* and *Chlorella* are good sources of essential amino acids.

Table 5. Effect of the inclusion of different protein sources in the amino acid content (g/100 g) of fresh pork sausages. All the data are expressed as mean ± standard deviations (n=15). Means with different superscript letters in a row differ significantly. Significance: n.s.: not significant; *** - $p < 0.005$; ** - $p < 0.01$; * - $p < 0.05$.

	Protein source							Significance
	Soy	Pea	Lentil	Broad Bean	Chlorella	Spirulina	Whey	
Asp	1.38 ± 0.28^{abc}	1.18 ± 0.12^{a}	1.25 ± 0.21^{ab}	1.47 ± 0.28^{bcd}	1.58 ± 0.24^{cd}	1.67 ± 0.12^{d}	1.48 ± 0.14^{bcd}	*
Ser	0.77 ± 0.22^{abc}	0.54 ± 0.08^{a}	0.63 ± 0.06^{ab}	0.74 ± 0.08^{abc}	0.86 ± 0.22^{c}	0.80 ± 0.06^{bc}	0.71 ± 0.12^{abc}	*
Glu	2.34 ± 0.42^{abc}	2.05 ± 0.22^{a}	2.15 ± 0.33^{ab}	2.51 ± 0.48^{abcd}	2.64 ± 0.47^{cd}	2.88 ± 0.21^{d}	2.51 ± 0.20^{bcd}	*
Gli	0.78 ± 0.14	0.73 ± 0.12	0.69 ± 0.11	1.09 ± 0.44	0.90 ± 0.05	0.99 ± 0.08	1.09 ± 0.72	n.s.
Hys	0.55 ± 0.06^{bc}	0.47 ± 0.05^{a}	0.52 ± 0.07^{ab}	0.58 ± 0.14^{bc}	0.65 ± 0.06^{cd}	0.68 ± 0.06^{d}	0.56 ± 0.04^{bc}	**
Arg	1.16 ± 0.15^{abc}	1.02 ± 0.09^{a}	1.09 ± 0.16^{ab}	1.36 ± 0.19^{cd}	1.35 ± 0.17^{cd}	1.48 ± 0.12^{d}	1.30 ± 0.24^{bcd}	**
Thr	0.69 ± 0.07^{abc}	0.58 ± 0.07^{a}	0.64 ± 0.09^{ab}	0.74 ± 0.19^{abc}	0.81 ± 0.07^{bc}	0.89 ± 0.08^{c}	0.61 ± 0.27^{ab}	*
Ala	0.95 ± 0.19^{ab}	0.82 ± 0.07^{a}	0.86 ± 0.12^{a}	1.08 ± 0.09^{bc}	1.11 ± 0.13^{bc}	1.19 ± 0.08^{c}	1.08 ± 0.25^{bc}	*
Pro	0.65 ± 0.13	0.60 ± 0.07	0.55 ± 0.09	0.50 ± 0.34	0.68 ± 0.11	0.74 ± 0.09	0.60 ± 0.09	n.s.
Tyr	0.32 ± 0.23^{a}	0.29 ± 0.17^{a}	0.39 ± 0.09^{abc}	0.28 ± 0.19^{a}	0.52 ± 0.07^{bc}	0.54 ± 0.19^{c}	0.35 ± 0.02^{ab}	*
Val	0.74 ± 0.12^{bc}	0.62 ± 0.06^{a}	0.67 ± 0.10^{ab}	0.85 ± 0.10^{cd}	0.85 ± 0.11^{cd}	0.90 ± 0.08^{d}	0.77 ± 0.07^{bc}	***
Lys	1.36 ± 0.28^{ab}	1.19 ± 0.13^{a}	1.28 ± 0.21^{ab}	1.43 ± 0.35^{abc}	1.54 ± 0.32^{bc}	1.70 ± 0.15^{c}	1.47 ± 0.12^{bc}	*
Isoleu	0.73 ± 0.11^{ab}	0.60 ± 0.08^{a}	0.66 ± 0.11^{a}	0.85 ± 0.10^{bc}	0.83 ± 0.11^{bc}	0.88 ± 0.08^{c}	0.74 ± 0.06^{ab}	**
Leu	1.22 ± 0.17^{abc}	1.04 ± 0.12^{a}	1.13 ± 0.17^{ab}	1.45 ± 0.16^{cd}	1.42 ± 0.21^{cd}	1.50 ± 0.13^{d}	1.28 ± 0.09^{bc}	**
Phe	0.62 ± 0.04^{abc}	0.52 ± 0.05^{a}	0.57 ± 0.09^{ab}	0.66 ± 0.14^{bcd}	0.70 ± 0.06^{cd}	0.74 ± 0.07^{d}	0.63 ± 0.04^{abcd}	*

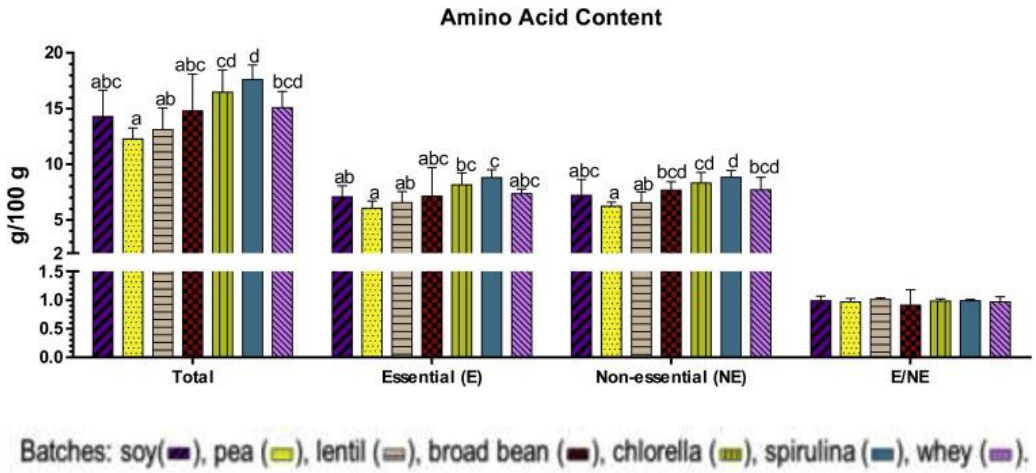

Figure 2. Effect of the inclusion of different protein sources in the total, essential, non-essential amino acids (g/100 g) and essential/non-essential ratio amino acid content (g/100 g) of fresh pork sausages.

3.5. Multivariate classification of the different protein sources

In this work, a chemometric and supervised approach based on orthogonal projection to latent structure discriminant analysis (OPLS-DA) was carried out for data reduction purposes. This model allowed similarities and differences to be described across the different sausages on the basis of all the parameters studied. In more detail, the dataset based on physicochemical parameters (pH, colour and texture), proximate composition (moisture, protein, lipid and ash content) and the amino acidic profiles was used for the supervised modelling. In this regard, OPLS-DA was suitable for highlighting differences between the parameters analyzed as well as for identifying those variables that possessed the highest discriminatory power.

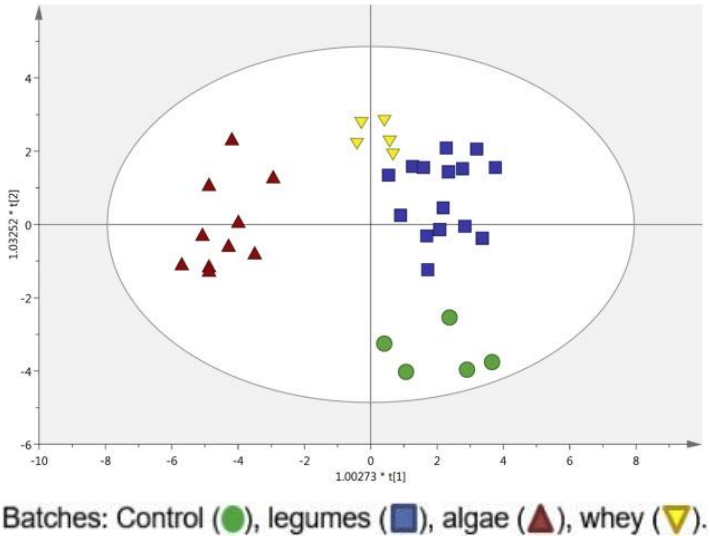

Figure 3. Orthogonal Projection to Latent Structure discriminant analysis (OPLS-DA) carried out considering the different protein source used for replacement purposes algae, legumes, whey and soy). Individual replicates are given in the OPLS-DA score plot.

The OPLS-DA model was built considering the protein source used (algae, legumes and whey when compared to control) as a "class membership" criterion. The OPLS-DA score plot obtained using this interpretation is reported in Fig. 3. The supervised statistical approach allowed us to highlight a clear separation among groups, with an evident discrimination between "algae" and the other protein sources (whey, legumes and soy). In this regard, we can state that the addition of bean, pea and lentil proteins produced a profile comparable to that of sausages prepared with whey proteins, when considering the parameters examined. Furthermore, the OPLS-DA model suggested that legumes (such as lentils and broad beans) were the protein source that produced a final product most similar to the control (soy). The variable selection method VIP (variable importance in

projection) was used to identify the parameters most closely related to the score plot patterns previously described. The parameters accounting for the main differences across treatments, namely those possessing a VIP score >1, are presented in the supplementary material. The OPLS model was cross-validated by means of permutation testing (N = 100) and inspected for outliers by means of Hotelling's T2 range (supplementary material), and finally by checking model fitness parameters (R2Y = 0.69 and Q2Y = 0.46). As an overall consideration, 11 VIP markers could be identified, with textural parameters (chewiness, gumminess and hardness) possessing a very high discrimination potential, followed by those parameters related to colour (L*, a*, b*) and pH. Interestingly, four amino acids were also found to discriminate between the different protein sources, with alanine characterized by a VIP score of 1.06, followed by valine (1.03), aspartic acid (1.01) and histidine (1.00). Therefore, this modelling approach confirmed that the final texture and colour were markedly affected by the partial addition of alternative protein sources to pork sausages.

According to our findings, it was evident that the addition of vegetable and whey proteins produced distinctive texture, colour and amino acid profiles. In this regard, the substitution of animal proteins with alternative sources represents a challenging task in product development, mainly because the partial replacement can lead to final products with different sensory and rheological properties from the original formulations. To date, soy proteins in combination with animal proteins (such as whey, casein or gelatine) have literally dominated the market, with few studies focusing on the use of alternative vegetable sources (Ainis, Ersch, & Ipsen, 2018). However, before using proteins from legume grains and seeds, one should evaluate their structural stability carefully. Nonetheless, even though the structural traits of legume proteins are of primary importance, their potential adverse effects

(such as allergenicity and toxicity) should be considered carefully before the exploitation of these sources. Besides, plant and vegetable proteins are often perceived as incomplete protein sources as they commonly lack essential amino acids, including histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Bleakley & Hayes, 2017). In this regard, microalgae (such as *Chlorella* and *Spirulina*) are generally considered as a protein source that meets the FAO requirement for adults concerning the essential amino acid composition, with few studies available on both their use as an alternative to animal proteins in meat products and their digestibility (Bleakley & Hayes, 2017). Therefore, considering these premises and examining our findings, we can conclude that these proteins can be used as an alternative to soy protein in the preparation of pork sausages. In addition, the final products could be distinguished by their colour and final texture rather than their amino acidic profile. Starting from previous considerations, our data suggest that each protein source has particular advantages together with some drawbacks. Proteins from microalgae likely provide a more favourable amino acid profile, and thus have a better nutritional profile. However, legume-derived proteins can provide a better texture and colour, which might help with consumer acceptance. Overall, the enrichment of pork sausages with proteins from alternative sources can affect the visual acceptance, which in turn drives consumer choice.

4. Conclusions

Depending on the origin of the proteins used in sausages preparation (vegetable, dairy, and seaweed), some changes in physicochemical and textural properties were observed. The protein content decreased significantly with the incorporation of extracts rich in pea protein, while the incorporation of this type of samples led to an increase in lipid content in

sausages. Regarding the pH, significant differences were found in all batches studied, since the sausages prepared with *Spirulina* presented a higher pH. Significant differences were also observed regarding colour parameters (L*, a*, b*) among the different pork sausages, depending on the protein source used. In this sense, the lowest a* was obtained in the samples prepared with algae protein (*Spirulina* and *Chlorella*). On the other hand, the WHC increased after the addition of lentil protein, whey, *Spirulina*, and *Chlorella*, and were reduced after the incorporation of soy, pea, and bean protein. The hardness, elasticity, cohesiveness, gumminess, and chewiness were higher in the control samples (soybean). In addition, significant differences were found in the amino acid profile. The addition of *Spirulina* algae increased the total, essential and non-essential amino acid content of pork sausages. OPLS-DA multivariate statistics carried out on physicochemical, textural and composition parameters suggested that the addition of legume and whey proteins provided a very similar profile, whereas the addition of microalgae as a protein source produced the most distinctive and characteristic profiles. Among others, textural parameters (chewiness, gumminess and hardness) followed by colour (L∗, a∗, and b∗) and pH possessed the highest discrimination potential. Therefore, the choice of one protein source over another may affect both textural and colour properties, thus strongly impacting consumer choice.

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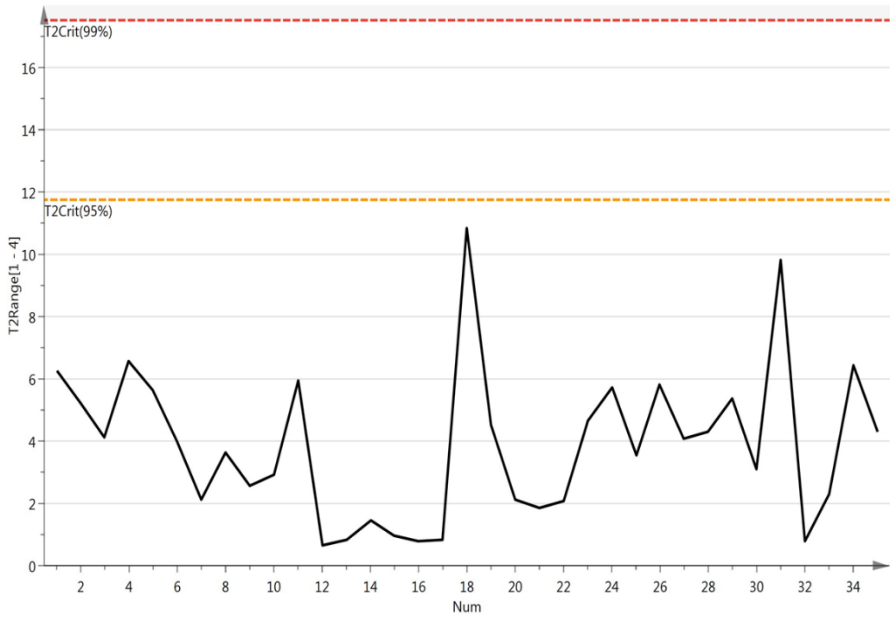
SUPPLEMENTARY DATA

Table. Parameters accounting for the main differences across treatments, namely those possessing a VIP score >1.

Var ID (Primary)	M1.VIP[3+1+0]	2.44693 * M1.VIP[3]cvSE
Masticability (N.mm)	1,30631	0,304691
Gumminess (N)	1,28943	0,323414
pH	1,26763	1,15311
a*	1,22968	0,293093
L*	1,19829	0,281701
Hardness (N)	1,17991	0,338118
b*	1,11069	0,553135
Ala	1,05842	0,353078
% Protein	1,05087	0,274766
Val	1,0311	0,256435
Asp	1,01386	0,244101
Hys	1,00896	0,246995
Leu	0,98934	0,326075
Glu	0,978069	0,279421
Phe	0,977477	0,210912
Adhesivity (g.sec)	0,977285	1,0761
Arg	0,975409	0,196528
Ser	0,961211	0,532716
Elasticity (mm)	0,958171	0,412649
Thr	0,937384	0,337996
Cohesivity	0,934587	0,368987
Isoleu	0,931622	0,401454
Lys	0,924525	0,303942
% Ash	0,887189	0,822217
Tyr	0,869781	0,511202
% water retention capacity cooking	0,866743	0,924796
Pro	0,844058	0,326511
Gli	0,777948	1,18495
% Fat	0,524619	0,975626
% Humidity	0,338536	0,94591



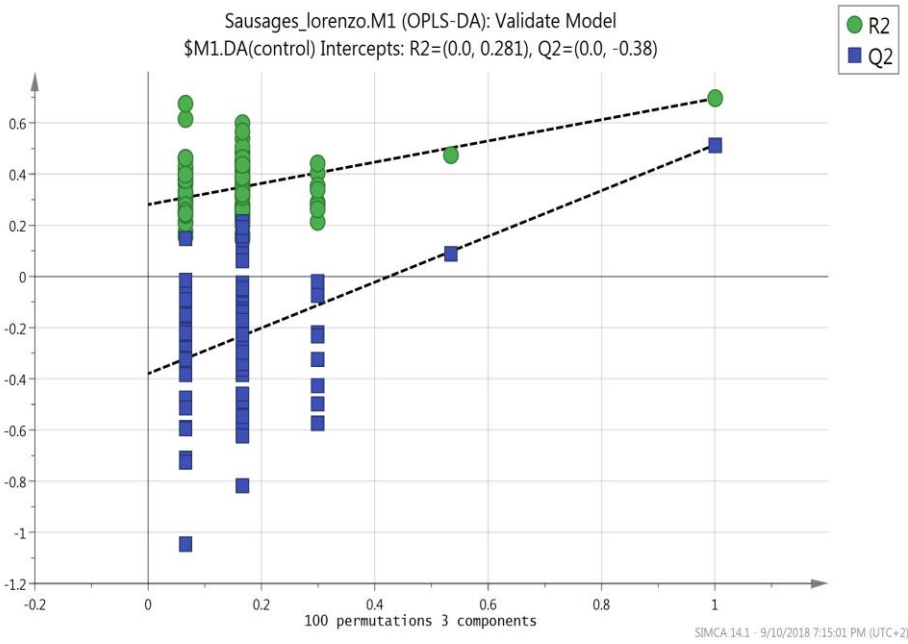


Figure. OPLS model cross-validation by means of permutation testing (N = 100) and inspected for outliers by means of Hotelling's T2 range and evaluation of model fitness parameters (R2Y = 0.69 and Q2Y = 0.46).

Mechanistic Insights into Citrinin-Induced Neurotoxicity In SH-SY5Y Cells: From ROS Production to Apoptosis and Cell Cycle Alterations

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Archives of Toxicology (Under review)

Abstract

Citrinin (CIT) is a mycotoxin commonly found in cereals. It is produced by fungi belonging to the *Aspergillus*, *Penicillium*, and *Monascus* genera. While extensive research has been conducted on its nephrotoxic properties, its impact on neurons remains poorly understood. This study aims to investigate the mechanisms of CIT-induced toxicity in a human neuroblastoma cell line (SH-SY5Y). Our findings revealed the following IC_{50} values for cells treated with CIT: 77.1 ± 10.1 µM at 24 hours and 74.7 ± 9.6 µM at 48 hours using the MTT assay, and 101.0 ± 20.3 µM at 24 hours and 54.7 ± 7.4 µM at 48 hours using the Neutral Red assay. Concentrations ranging from 19.375 µM to 50 µM CIT were selected for further experiments. Interestingly, CIT did not induce an increase in reactive oxygen species (ROS) production at any of the tested concentrations. However, it did lead to a G2/M phase arrest. Specifically, the percentage of cells in the G2/M phase increased from 11.83% (control) to 18.47% at 38.75 µM CIT and 33.10% at 50 µM CIT. Additionally, a significant increase (from 19.83% to 28.80%) in cells in the S phase was observed at 50 µM CIT compared to the control, while no significant differences were observed at any stage of the cell cycle between the control and the lowest concentration tested (25 µM CIT). Moreover, an increase in late apoptosis process was noted in cells exposed to CIT, particularly at higher concentrations (38.75 and 50 µM). We observed a decrease of this anti-apoptotic protein as the concentration of CIT increased in our experimental conditions. In conclusion, CIT induces apoptosis and G2/M arrest in SH-SY5Y cells. However, further investigations are necessary to gain a comprehensive understanding of the specific mechanisms underlying CIT's toxicity in SH-SY5Y cells.

Keywords: citrinin, cell cycle, apoptosis, SH-SY5Y, ROS, mitochondrial membrane potential, western blot

1. Introduction

Mycotoxins, including citrinin (CIT), are secondary metabolites synthesized by various fungi, primarily attributed to genera such as *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Claviceps* (Pallarés et al., 2022). CIT, specifically produced by species of *Aspergillus*, *Penicillium*, and *Monascus*, is commonly found in stored grains, fruits, herbs, and spices, with a global distribution across different regions (Narváez et al., 2021).

According to the EFSA, there is not sufficient scientific evidence on this mycotoxin, which hinders the proper risk assessment. Therefore, more toxicological information is needed regarding this mycotoxin to enhance the accuracy of the assessments and, its toxic effects (Alexander et al., 2012). In addition, the European Commission Regulation (EC) no. 1881/2006, which sets maximum levels for contaminants in food to safeguard animal and public health, as well as agricultural productivity, does not specify a limit for CIT content in food due to the limited occurrence data available (The Commission of the European Communities, 2006). Currently, the regulation only addresses CIT levels in food supplements. Nevertheless, there is ongoing focus and effort to improve the risk assessment of this mycotoxin.

Regarding the toxicological aspects, CIT exhibits antibacterial, antifungal, and antiprotozoal activities. However, it is also recognized as a hepatorenal toxin in several species. Studies conducted *in vitro* have demonstrated that CIT negatively impacts renal mitochondrial function and the synthesis of macromolecules, leading to cell death (Xu et al., 2006).

Studies have demonstrated that exposure to CIT can lead to alterations in cell cycle progression, disrupting the tightly regulated processes that govern cellular division. Specifically, CIT has been found to induce cell cycle arrest at different phases, including the G1 and G2/M phases, depending on the cell

type and concentration of CIT used (Chang et al., 2011; Kumar et al., 2011). This disruption of the cell cycle can have relevant implications for cellular proliferation and function.

The mycotoxin CIT has been found to exert also significant influence on cellular apoptosis and necrosis processes. CIT has been shown to trigger apoptosis by activating different cellular signalling pathways, including the mitochondrial pathway, endoplasmic reticulum stress, and caspase activation (Kumar et al., 2011; Wu et al., 2022; Yu et al., 2006). Additionally, CIT has been associated with the disruption of mitochondrial membrane potential, leading to mitochondrial dysfunction and subsequent apoptotic cell death (Chan, 2007; Chen & Chan, 2009). Moreover, CIT exposure has been linked to induced damage to cellular structures and organelles (Sun et al., 2020).

There is scarce information about cytotoxicity of CIT. So, further investigation on the molecular mechanisms and pathways involved in CIT-induced toxicity are needed.

Therefore, the objective of this study is to investigate the mechanisms of CIT-induced toxicity in a human neuroblastoma cell line (SH-SY5Y). These cells of human origin can contribute to understand the molecular mechanisms of toxicity in cellular levels similarly as in experimental animal models.

2. Materials and methods

2.1 Reagents

The reagent grade chemicals and cell culture compounds used, namely culture medium DMEM Ham's-F12, penicillin, streptomycin, trypsin/EDTA solutions, phosphate buffer saline (PBS), fetal bovin serum (FBS), tetrazolium bromide (MTT), neutral red dye (NR), propidium iodide (PI), HEPES, Rhodamine

123 (Rh123), 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI), 2',7'-dichlorodihydrofluorescein diacetate (H2-DCFDA), were from Sigma Chemical Co (St. Loius, MO, USA). The NaCl and ethanol were from Merck KGaA (Germany). The CaCl2 was from Scharlau Chemie S.A. (Barcelona, Spain). Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific (Geel, Belgium). Precast 7.5% Mini-PROTEAN TGX gels, Immuno-Blot PVDF low fluorescence membrane, Tris/glycine buffer, TBS buffer, Precision Plus Protein Western MW standard and all other routine chemicals required for SDS-PAGE and Western blotting were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Human recombinant annexin V-FITC conjugate was from Invitrogen (USA). Standard of the selected mycotoxin CIT (MW: 250.25 g/mol) was purchased from Sigma-Aldrich (St. Louis Mo. USA). Stock solution of the mycotoxin were prepared in DMSO and maintained at -20°C

2.2 Cell culture and treatment

The human neuroblastoma (SH-SY5Y) cells were maintained in DMEM Ham's-F12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. The cells were cultured under standard conditions with a pH of 7.4, 5% CO2, and a temperature of 37°C in a 95 % air atmosphere with constant humidity. The culture medium was refreshed every 2-3 days. To achieve the desired concentrations of CIT, appropriate solutions were added to the culture medium, ensuring a final DMSO concentration of ≤1% (v/v). Control groups containing equivalent amounts of solvent were included in each experimental setup.

2.3 Cytotoxicity assays

The MTT and NR assays were performed as described by Ruiz et al. (2006) with some modifications. SH-SY5Y cells were cultured at a density of 30,000 cells per well in 96-well plates. After 48 hours, when the cells reach the 80%

confluence, the culture medium was replaced with fresh medium containing the serial dilutions of CIT, ranging from 19.38 to 310 µM by dilution 1/2. Cells were exposed to the mycotoxin for 24 and 48 hours without medium or mycotoxin replenishment.

The MTT assay relies on the conversion of a yellow soluble tetrazolium salt to a blue insoluble formazan product by the mitochondrial succinic dehydrogenase enzyme, indicative of viable cell metabolism.

For the MTT assay, 24 and 48 h of exposure with serial concentrations of CIT, the culture medium containing the mycotoxin was replaced each well received 200 µL of fresh medium containing 50 µL of MTT salt (5 mg/mL in PBS). After 3-hour of incubation at 37 °C in the dark, the resulting formazan crystals were dissolved in DMSO. The absorbance of the solution was measured at 570 nm using a VICTOR2 1420 multilabel plate counter reader (PerkinElmer, Turku, Finland). Cell viability was expressed as a percentage relative to the solvent control (≤1% DMSO). Three independent experiments were performed for each exposure time, with 8 replicates per experiment. The mean inhibition concentration (IC50) values were calculated using SigmaPlot version 11 (Systat Software Inc., GmbH, Germany).

The neutral red (NR) assay assess the uptake and retention of NR dye in lysosomes.

For NR assay, the NR assay, the NR solution was pre-incubated overnight at 37°C and filtered through a 0.22 µm membrane filter to remove any dye crystals. After 24 and 48 h of incubation with CIT concentrations (serial dilution-2), medium containing CIT was removed and 200 µL of freshly prepared NR solution (50 µg/mL) pre-warmed at 37°C was added to each well and all plates returned to the incubator at 37°C for 3 h. The cells were washed once with PBS and fixed with formaldehyde– $CaCl_2$ solution (0.5%

formaldehyde and 1% CaCl2 solution in PBS), and then extracted by adding acetic acid–ethanol solution (1% acetic acid and 50% ethanol in PBS). Plates were gentle shacked for 5 min so that complete dissolution was achieved before measured absorbance at 540 nm with an automatic ELISA plate reader Multiscan EX (Thermo Scientific, MA, USA). Cell viability was expressed in percent relative to control cells (≤1% DMSO). Three independent experiments were conducted and mean inhibition concentration (IC50) values were determined using SigmaPlot version 11 (Systat Software Inc., GmbH, Germany).

2.4 Reactive oxygen species assay

Early intracellular ROS production was assessed using H2-DCFDA, a fluorescent probe. H2-DCFDA is taken up by cells and undergoes deacetylation by intracellular esterases. This conversion results in the formation of non-fluorescent 2',7'-dichlorodihydrofluorescein (H2-DCF), which is further oxidized by ROS to produce highly fluorescent dichlorofluorescein (DCF). The ROS generation assay was performed according to Zingales et al. (2020). Specifically, 30.000 cells/well were seeded in a 96-well black culture microplate. After 48h, the culture medium was replaced, and the cells were incubated with fresh medium containing 20 µM H2-DCFDA for 20 minutes. Then, the H2-DCFDA solution was removed, and 200 µL/well of fresh medium, ≤1% DMSO (control) or medium containing CIT at concentrations ranging from 19.38 tod 38.75 µM was added. These concentrations were selected based on previous cytotoxicity assays. All tested concentrations were found to be non-toxic to SH-SY5Y cells and were below the IC_{50} values obtained. Fluorescence measurements were taken at excitation/emission wavelengths of 485/535 nm using a Wallace Victor2, model 1420 multilabel counter (PerkinElmer, Turku, Finland) at intervals up to 120 min. The results are presented as an increase in fluorescence relative to

the solvent control. The experiments were conducted independently in duplicate, with 24 replicates each.

Additionally, ROS generation after 24 h of CIT exposure was also evaluated. In this assay, after confluence, the SH-SY5Y cells were exposed to CIT by replacing the culture medium with fresh medium containing CIT at final concentrations of 25, 38.75, and 50 µM. After 24 hour of exposure, the supernatant with CIT was removed, and each well received 200 µL of fresh medium with 20 µM H2-DCFDA. The plates were incubated at 37 °C for 30 minutes, in darkness. Fluorescence measurements was determined using a Wallace Victor2, model 1420 multilabel counter (PerkinElmer, Turku, Finland) at excitation/emission wavelengths of 485/535 nm. The results were expressed relative to the solvent control. The experiments were conducted independently in duplicate, with 24 replicates each.

2.5 Mitochondrial membrane potential assay

To evaluate the mitochondrial membrane potential (MMP) in living cells, Rhodamine123 (Rh123) was utilized following the method described by Liu et al. (Liu et al., 2018). Rh123 is a non-toxic, green-fluorescent dye that is quickly taken up by cells with active mitochondria. Briefly, SH-Y5Y cells were seeded in 96-well black culture microplates at a density of 30,000 cells per well. After confluence, they were exposed to CIT at concentrations of 25, 38.75 and 50 µM for 24 hours. Then, the medium was removed, and the cells were incubated with 5 µM Rh123 in fresh medium for 15 minutes at 37 °C in darkness. The fluorescence intensity was measured using a Wallace Victor2, model 1420 multilabel counter (PerkinElmer, Turku, Finland) with excitation/emission wavelengths of 485/535 nm. The results were expressed as a decrease in fluorescence relative to the control (≤1% DMSO). Two independent experiments were conducted to ensure reliability, with 24 replicates each.

2.6 Flow cytometric analysis of apoptosis

Measurement of cell death, which occurs through necrosis and apoptosis, was performed using annexin V-FITC/PI double staining method. Necrosis refers to unregulated cell death, while apoptosis is a genetically controlled process. During apoptosis, phosphatidylserine (PS) is exposed on the outer membrane of cells. To identify different cell populations (early or late apoptotic, necrotic, and dead), annexin V-FITC/PI double staining was utilized following the method described by Vermes et al. (Vermes et al., 1995). Annexin V is a calcium-dependent protein that binds to PS with high affinity, while PI binds to the DNA of necrotic or dead cells. Viable cells with intact membranes exclude PI, whereas dead and damaged cells allow PI to enter. Specifically, viable cells were Annexin V-FITC-/PI-, early apoptotic cells were Annexin V-FITC+/PI-, late apoptotic cells, which have completed apoptosis and entered the necrotic phase, were Annexin V-FITC+/PI+, and necrotic cells were Annexin V-FITC-/PI+.

For the Annexin V-FITC/PI double staining assay, 700,000 cells were seeded per well in six-well plates and cultured for 24 hours. Subsequently, they were exposed to CIT at concentrations of 25, 38.75, and 50 µM for 24 hours. After trypsinization, the cells were suspended in 360 µL of Hepes-Ca^{2+} buffer containing 10 mM Hepes-NaOH (pH 7.4), 135 mM NaCl, and 2.5 mM $CaCl_2$. Following a 30-minute incubation at 4 °C in the dark, 10,000 cells were acquired and analysed using a BD LSRFortessa flow cytometer (BD Biosciences). Quadrant statistics were applied to determine the percentages of viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells within the total cell population. Three independent experiments were performed.

2.7 Flow cytometric analysis of cell cycle

Cell-cycle analysis was conducted using Vindelov's PI staining solution, following the method outlined by Juan-Garcia et al. (2013). PI is a fluorescent dye that binds to double-stranded DNA, enabling the accurate assessment of cellular DNA content through flow cytometric analysis. In brief, 700,000 cells were seeded per well in six-well plates and exposed to CIT at concentrations of 25, 38.75 and 50 µM for 24 hours. Subsequently, the cells were trypsinized and placed on ice for 30 minutes with 860 µL of fresh medium containing Vindelov's PI staining solution. The staining solution was prepared using 40 µg/mL RNase, 0.1% Triton X-100, 10 mM Tris, 10 mM NaCl, and 50 µg/mL of PI in PBS. Three independent experiments were conducted, and 20,000 cells from each sample were analyzed using a BD LSRFortessa flow cytometer (BD Biosciences, Franklin Lakes, NJ).

2.8 Assessment of activation of Bcl2

For the Western blot analysis, proteins were extracted from SH-SY5Y cells were lysed and proteins were extracted in 200 µL of RIPA buffer with protease and phosphatase inhibitor cocktail (Santa Cruz Biotechnology). Samples were centrifuged at 12,000 g and 4 °C for 15 min to collect cellular proteins in the supernatants and they were quantified with Bradford assay (BioRad) with Cydex modification (Rabilloud, 2018). Equal amounts of proteins in samples were separated by 7,5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF low fluorescence membrane (BioRad). Membranes were blocked in 5% non-fat milk in buffer TBS-Tween for 1 hour at room temperature and after blocking the membrane was incubated with anti-β actin 1:1000 (251-006, SynapticSystem), anti-Nrf2 1:1000 (12721, Cell Signaling), anti-Bcl2 1:1000 (12789-1-AP, Proteintech) and anti-Bax 1:1000 (60267-1lg, Proteintech) in blocking buffer at 4 °C overnight. Then, the membrane was incubated with Alexa Fluor 647 (703-605-155,

Jackson ImmunoResearch), IRDye 660 RD (925-68071, LI-COR Biosciences) and IRDye 800 WC (925-32210, LI-COR Biosciences) diluted in blocking buffer for 1 hour at room temperature. Protein bands were visualized via fluorescence in an Amersham ImageQuant 800 (Cityva LifeSciences) machine, and densitometry analysis was performed using QuantityOne (BioRad).

2.9 Statistical analysis

The statistical analysis was conducted using GraphPad Prism version 8 (GraphPad Software, San Diego, CA), a statistical software package. The data were presented as the mean ± SEM from independent experiments. Student's t-test for paired samples was employed to analyze the statistical significances. Additionally, one-way ANOVA followed by the Tukey HDS post-hoc test was utilized for multiple comparisons. Statistical significance was determined at a p-value of ≤ 0.05.

3. Results and discussion

3.1 Cytotoxicity

The cytotoxic effects of CIT on SH-SY5Y cells were evaluated using the MTT and NR assays. The IC_{50} values obtained by both methods are shown in Table 1.

Results obtained by the MTT assay revealed that the viability of cells exposed to CIT decreased significantly in a concentration-dependent manner, but there were no differences between the two time of exposure time tested (dat not shown).

Table 1. IC_{50} values obtained for CIT in SH-SY5Y cells by MTT and NR assays at 24 and 48 h of exposure. Results are expressed as mean ± SEM of three independent experiments.

IC_{50} value (µM)	24 h	48 h
MTT	77.1 ± 10.1 µM	74.7 ± 9.6 µM
NR	101.0 ± 20.3 µM	54.7 ± 7.4 µM

Similarly, in the NR assay, exposure to CIT in SH-SY5Y resulted in a concentration-dependent decrease in cell viability after 24 and 28 h of exposure (data not shown). However, according to the IC_{50} values obtained by the NR assay, higher cytotoxic effect was observed after 48 h of exposure respect to MTT assay (Table 1).

These results are highly consistent with those observed in the literature. The MTT assay results obtained in our study are very similar to the findings of Klarić et al. (2012). These authors determined cell viability of CIT in PK15 cells after 24 hours of exposure, an observed an IC_{50} value of 73.5 µM by the MTT assay and 75.4 µM CIT in the trypan blue assay. Additionally, the result obtained in our study for the NR assay are very similar to the value reported by Föllmann et al. (2014). These authors found that the IC_{50} value of CIT after 24 hours of exposure in V79 cells was 70 µM, and the IC_{50} after 48 hours of exposure was 53 µM. The value obtained at 48 hours is highly similar to the one described in our study (54.7 µM), although the result obtained at 24 hours is lower than obtained by us (101.0 µM).

These results could be explained because the SH-SY5Y cells are more sensitive to CIT compared to other cell types, as these authors evidenced higher IC_{50} values than us in different cell lines. In this sense, Aydin et al. (2019) obtained an IC_{50} value of 116.5 µM in a mouse Sertoli cell line after 24 hours of

exposure. And, Gayathri et al. (2015) obtained an IC_{50} of 155 µM on HepG2 cells exposed to CIT for 24 hours by the MTT assay.

The results obtained by the cytotoxic assays guided the selection of concentrations for subsequent assays. The CIT concentrations selected for further experiments were 19.375, 25, 38.75, 50 and 77.5 µM CIT, depending on the experiment.

3.2 ROS production

3.2.1 Early ROS production

The results obtained measuring for 120 min early ROS production after the addition of CIT to SH-SY5Y cells are shown in Figure 1. ROS production was determined using the DCFH-DA assay. As shown in Figure 1, CIT did not induce ROS generation at the tested concentrations.

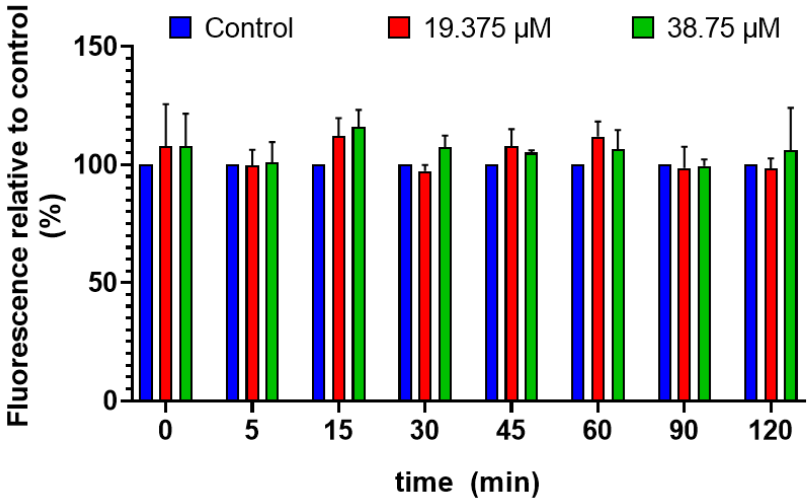

Figure 1. Time dependence of ROS-induced fluorescence in SH-SY5Y cells exposed to CIT at 19.375 and 38.75 µM. Results are expressed as mean ± SEM of two independent experiments.

The levels of ROS production remained similar to the control group during the first 120 minutes of CIT exposure. According to Vanacloig-Pedros et al., CIT rapidly activates genes encoding antioxidant enzymes (Vanacloig-Pedros et al., 2016), which could explain the lack of elevated ROS levels due to the swift antioxidant action of these enzymes.

Similarly, this finding is in line with previous studies conducted with other mycotoxins, such as sterigmatocystin, where no increase in ROS production was observed within the first 2 hours of exposure (Zingales et al., 2020). Similar results were obtained by Agahi et al., who described that concentrations below 2.5 μM of α-zearalenol did not generated ROS production during the initial 120 minutes in SH-SY5Y cells (Agahi et al., 2020). Finally, Taroncher et al. (2020) also reported that exposure to patulin (2.5-10 nM), deoxynivalenol (DON) (25-100 nM), and T-2 toxin (8.5-34 nM) did not increase early ROS production within the first 120 minutes in HepG2 cells.

3.2.2 ROS production at 24 h

After analyzing the early production of ROS, a study was conducted to determine the ROS generation after 24 hours of CIT exposure in SH-SY5Y cells. For this purpose, CIT was added at concentrations of 25, 38.75, and 50 μM, and ROS production was measured using the reactive DCFH-DA assay after 24 hours. The results are shown in Figure 2. As can be observed, there were no significant ROS generation between any of the tested CIT concentrations and the control.

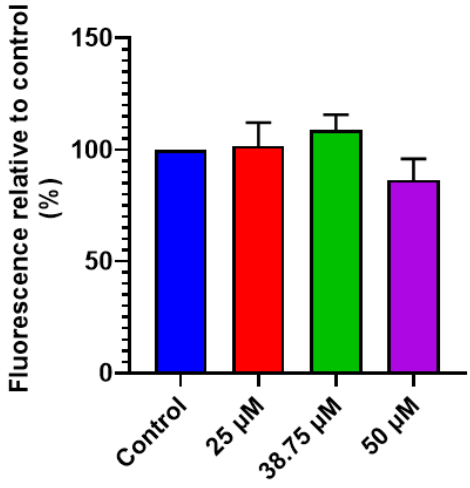

Figure 2. ROS-induced fluorescence in SH-SY5Y cells exposed to CIT (25, 38.75 and 50 µM) for 24 h. Data are expressed as mean ± SEM of two independent experiments.

Despite the fact that many mycotoxins induce cellular oxidative stress by increasing ROS, there are also some studies on different mycotoxins that have shown no increase in ROS generation at 24 h of exposure. In this regard, Cano-Sancho et al. (2015) observed that the production of ROS did not increase in Caco-2 cells following exposure to DON for 6, 24, and up to 48 hours, concluding that the cytotoxicity of this mycotoxin was not related to ROS production. According to the findings of Galvano, Campisi, et al. (2002) and Galvano, Russo, et al. (2002), it was observed that fumonisin B1 (FB1) does not induce an increase in ROS production in rat astrocytes at 48, 72 and 144 h and in human fibroblasts at 48 and 72 h. Finally, Janik-Karpinska et al. (2023) did not observe significant changes in ROS levels after exposing Hs68 cells to the mycotoxin T-2 (0.001-100 µM) for 24 and 48 h.

3.3 Mitochondrial membrane potential

Mitochondrial function was assessed in SH-SY5Y cells exposed to different concentrations of CIT (25, 38.75, and 50 µM) for 24 hours. The changes in mitochondrial membrane potential (MMP) were analysed using Rh123. The results showed no significant MMP alteration when SH-SY5Y cells were incubated with increasing concentrations of CIT (Figure 3). These findings suggest that the cytotoxic effects induced by STE may not be directly associated with MMP alteration. However, further investigation is needed to better understand the impact of CIT exposure on mitochondrial functional activities in cells.

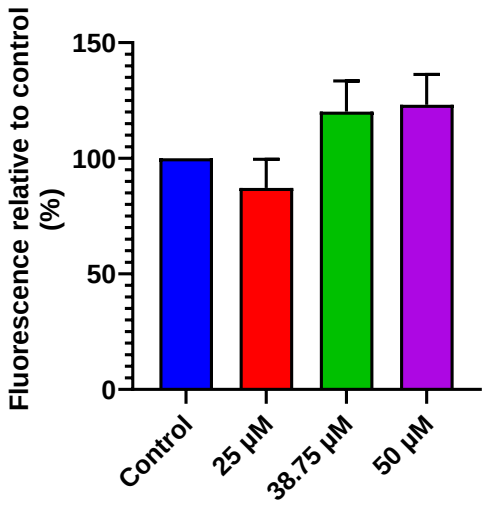

Figure 3. Effect of CIT (25, 38.75 and 50 µM) at 24 h of exposure on mitochondrial membrane potential (MMP) determined by Rhodamine 123 (Rh123) assay. Data are expressed as mean ± SEM of two independent experiments

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Our findings are similar with those obtained in literature in this field. For instance, Pérez-Fuentes et al. (2021) reported no significant changes in MMP when SH-SY5Y cells were exposed from 0.1 to 30 µM FB1 for 6 and 24 hours and DON for 6 hours.

However, conversely our results, other mycotoxins induce mitochondrial dysfunction. Pang et al. (2022) observed that 5 and 10 ng/mL of T-2 toxin reduced the MMP to 60.7% and 41.5% respectively, compared to control cells. Furthermore, FB1 at 20 and 40 µg/mL has been found to cause mitochondrial damage and a loss of MMP in IPEC-J2 cells (Wang et al., 2022). Similarly, Zhao et al. (2021) detected a decrease in MMP in Het-1A cells exposed to OTA for 24 hours.

As can be observed, when comparing the results obtained by Pérez-Fuentes et al. and Pang et al. regarding FB1, it becomes evident that there are notable differences in the MMP effects produced depending on the cell line used in each assay.

3.4 Apoptosis analysis

Due to, there was a decrease in cell viability depending on CIT concentration tested (data not shown), it was essential to study the mechanisms triggering cell death. For this purpose, apoptotic effect was measured in SH-SY5Y cells. Figure 4 presents the distribution of viable, early apoptotic, late apoptotic, and necrotic cells after CIT exposure (25, 38.75, and 50 µM) in SH-SY5Y cells during 24 hours.

Results in Figure 4 showed that for the cells exposed to CIT, the late apoptotic and necrotic cell population increased compared to the control cells. Regarding late apoptotic cells, this increase was similar for 38.75 µM CIT (2.026 ± 0.052 folds) and for 50 µM CIT (2.093 ± 0.117 folds), respect to the control. Concerning necrosis, there was an increase in a concentration-

dependent manner of necrotic cells exposed to CIT, being 1.653 ± 0.074 and 2.229 ± 0.348 times higher for 38.75 and 50 µM CIT, compared to the control.

Early apoptotic cells exposed to CIT for 24 h slightly decreased at 38.75 and 50 µM CIT, probably due to the long exposure time (24 h) and the increase in late apoptotic cell population. Finally, alive cells proportion decreased as expected for 38.75 and 50 µM, while there was a not significant increase in this population at 25 µM, probably explained by the hormetic effect.

No significant change was observed in the lowest concentration tested, 25 µM, for any of the studied processes.

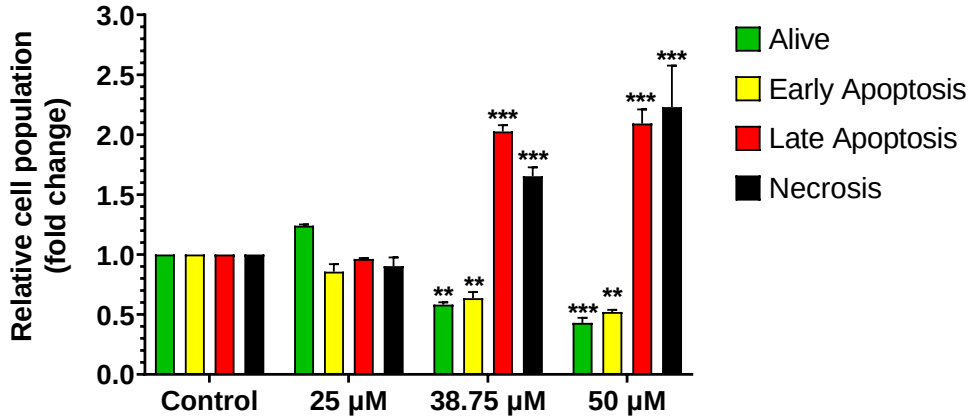

Figure 4. Analysis of apoptosis/necrosis induction in SH-SY5Y cells treated with CIT at 25, 38.75 and 50 µM for 24 h. Data are expressed as mean ± SEM of three independent experiments. ** - $p < 0.01$ vs. control; *** - $p < 0.001$ vs. control for each phase of the cell cycle.

The results obtained in our study are similar to those obtained in the literature. Salah et al. (2017) observed that 150 µM CIT induced apoptosis in HCT116 cells by triggering endoplasmic reticulum stress through the activation of the mitochondrial pathway, after 24 h of exposure. This finding

was also confirmed by Yu et al. (2006) in their study on HL-60 cells exposed to CIT (25, 50, and 100 μM) for 24 hours. Similarly, Sharath Babu et al. (2017) investigated the effects of CIT on C2C12 cells and concluded that this mycotoxin was able to induce apoptosis.

3.5 Cell cycle analysis

To study the impact of CIT (25, 38.75, and 50 μM) on cell cycle, flow cytometry analysis using PI staining was conducted on SH-SY5Y cells after 24 hours of exposure (Figure 5). The highest concentration of CIT (50 μM) demonstrated statistically significant differences compared to the control group across all phases. There was a notable decrease in the percentage of cells in the G0/G1 phase accompanied by an increase in the percentage of cells in the S and G2/M phases. Specifically, the percentage of SH-SY5Y cells in the G0/G1 phase decreased from 67.10 ± 0.58% (in control cells) to 34.77 ± 2.08 %. The S phase showed an increase, reaching 28.80 ± 1.80 % compared to the control (19.83 ± 0.64 %). Similarly, the G2/M phase exhibited an increase, with the number of cells rising to 33.10 ± 1.94 %, compared to the control (11.83 ± 0.84 %). Moreover, the subG0 phase also experimented a significant increase at the highest concentration, reaching 3.07 ± 0.70 %, respect to the control value (0.15 ± 0.04 %). Additionally, at 38.75 μM CIT, there was a significant increase in the G2/M phase (18.47 ± 1.44 %) compared to the control. The lowest concentration tested, 25 μM CIT, did not exhibit any significant differences in any phase when compared to the control cells.

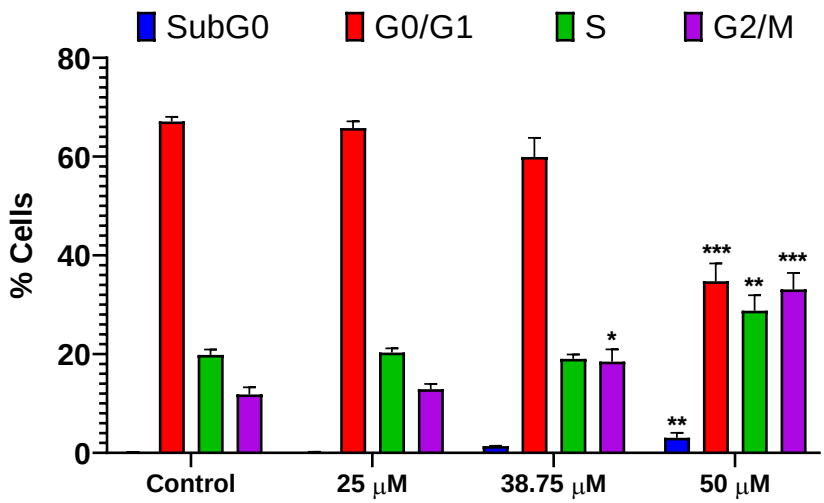

Figure 5. Analysis of cell-cycle distribution of SH-SY5Y cells treated with CIT at 25, 38.75 and 50 μM for 24 h. Data are expressed as mean ± SEM of three independent experiments. * - $p < 0.05$ vs. control; ** - $p < 0.01$ vs. control; *** - $p < 0.001$ vs. control for each phase of the cell cycle.

Our results are similar to those obtained by Föllmann et al. (2014). They observed that ≥ 50 μM CIT induced a G2/M phase arrest in V79 cells. In addition, their study also reported an increase in the SubG0 population (apoptotic cells), which corresponds with the results observed in our study. Similarly, Chang et al. (2011) identified a G2/M phase arrest in HEK293 cells following exposure to 50, 75, and 100 μM CIT for 24 hours. Specifically, they noted a G2/M phase population of 29.0 ± 3.2% at a concentration of 50 μM, which closely resembles our findings of 33.10 ± 1.94%.

Furthermore, Kumar et al. (2011) conducted an *in vivo* study to determine the effects of CIT topically applied in mice. When mice were exposed to CIT (from 24 to 72 hours), there was a notable increase in the cells population in the G0/G1 phase (from 45% to 71%) and there was a decrease in the S phase (from 44% to 59%), compared to the control group. However, there were no

significant changes observed in the G2/M phase following CIT exposure for 12 to 24 hours, but an increase in G2/M phase was evidenced after 48 and 72 hours of exposure (an increase of 65% and 56% more, respectively, compared to control).

3.6 Protein expression analysis by western blot

To confirm the presence of apoptosis generated by exposure to CIT in SH-SY5Y cells, the protein expression of Bcl2 (apoptosis marker) was evaluated by western blot and the signal was detected by two-color infrared fluorescence, greatly improving the sensitivity, quality and precision of the data obtained for this technique (Pillai-Kastoori et al., 2020). Since our cell cycle and flow cytometry to apoptosis assays showed significant data at concentrations of 38.75 µM and 50 µM we decided to use the same concentrations with the addition of 77.5 µM and to evaluate whether the observed apoptosis tends to be dose-dependent in our model. The results are shown in Figure 6.

Bcl-2 is considered an apoptotic marker because its expression plays a critical role in the regulation of this process. It is a member of the Bcl-2 family of proteins, which correspond to anti-apoptotic and pro-apoptotic proteins (Antonsson, 2001). Different authors have demonstrated the anti-apoptotic role of Bcl-2, where high levels of expression protect cells from apoptotic stimuli and promote cell survival, while low levels are associated with increased susceptibility to apoptosis (Pećina-Šlaus, 2010). Wu et al. demonstrated apoptosis and decreased Bcl-2 expression in mouse liver after exposure to CIT at much higher concentrations of mycotoxin than those used in our model (Wu et al., 2022).

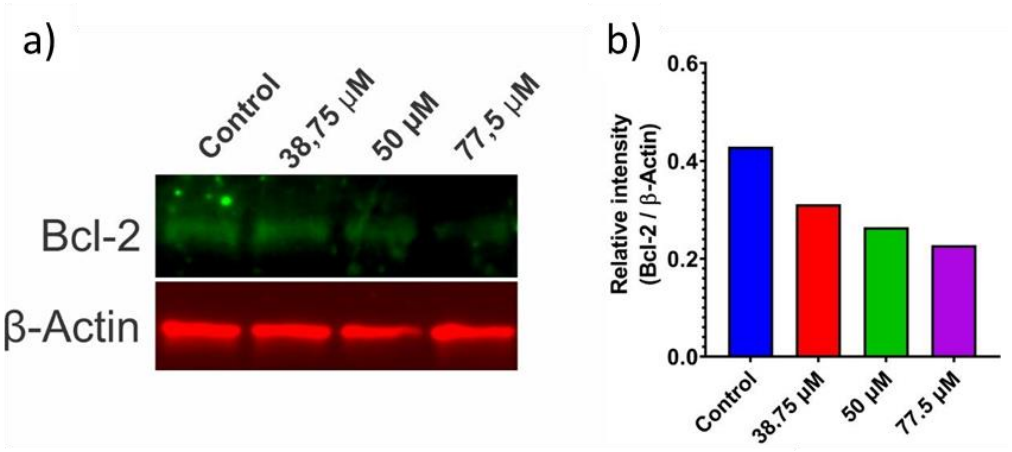

Figure 6. Expression of Bcl-2 in SH-SY5Y cells after exposure to CIT. a) Fluorescent western blot against the apoptosis marker Bcl-2 and the loading control protein β-actin in SH-SY5Y cells after exposure to 38.75, 50 and 77.5 µM CIT for 24h. b) Densitometric analysis plot for Bcl-2 against the loading control β-actin. Results are presented as the value for one experiment.

Although statistical studies cannot be performed as this is a single independent experiment, the decrease in Bcl-2 expression in our model is observed as the concentration of citrinin increases with respect to the control, supporting what was observed by cell cycle and flow cytometry. It is important to highlight the high sensitivity of this technique when determining expression by fluorescence using infrared secondary antibodies (Taylor et al., 2022).

4. Conclusion

In summary, our findings provide additional information on the toxicity of CIT in SH-SY5Y cells, and establish a basis for future research into the full mechanism of this mycotoxin and its health implications. The results of our study reveal that CIT causes S- and G2/M-phase arrest in SH-SY5Y cells. We

also gained new insights into the cell death process induced by CIT in SH-SY5Y cells, showing an increase in late apoptosis in cells exposed to CIT, corroborated by a reduction in Bcl2 protein expression. Ultimately, these findings will help us to develop effective strategies to mitigate the detrimental impact of CIT and safeguard public health.

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Citrinin's Effects on SH-SY5Y Spheroids: A Comprehensive Study

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Abstract

Citrinin (CIT) is a mycotoxin produced by fungi of the Aspergillus, Penicillium, and Monascus genera, primarily contaminating cereals, but it can also be found in fruits, herbs, and certain spices. Despite our understanding of its toxic effects on the kidneys and liver, the mechanisms underlying CIT toxicity have not been fully investigated. Therefore, the objective of this study is to examine the effects of CIT on 3D cultures (spheroids) of a human neuroblastoma cell line (SH-SY5Y) and elucidate the specific toxicity mechanisms induced by CIT. The IC50 values obtained from MTT, ATP, and Presto Blue assays after 24, 48, and 72 hours of exposure ranged from 34.41 ± 4.01 µM to 83.14 ± 5.70 µM. CIT decreases mitochondrial membrane potential (MMP, 25 µM), induce apoptosis and caused cell cycle arrest in the S and G2/M phases (50 µM) and cause alterations in the endoplasmic reticulum. However, CIT did not increase reactive oxygen species (ROS),. Moreover, no chromosomal alterations were observed as a result of CIT exposure. Finally, the use of a 3D cell model is expected to provide more accurate simulations of in vivo conditions compared to 2D assays. However, further studies are required to delve deeper into the toxicity mechanisms described in this work in order to improve the prevention and treatment of CIT-induced food poisoning, thereby enhancing food safety.

Keywords: citrinin, spheroids, SH-SY5Y, apoptosis, cromosomic alterations, oxidative stress, endoplasmic reticulum

1. Introduction

Food safety is an issue of growing concern worldwide. The consumption of food contaminated with mycotoxins, that is, toxic metabolites produced by fungi, represents a risk to human health (). Among these mycotoxins, citrinin (CIT) has received attention due to its presence in a variety of foods, including cereals, fruits, herbs and spices (Narváez et al., 2021). Fungi of the genera *Aspergillus*, *Penicillium* and *Monascus* are the main producers of CIT (Zhang et al., 2021). Although it has been established that CIT can have toxic effects on the kidneys and liver; however, the underlying mechanisms of their toxicity have not yet been fully investigated.

In addition, CIT has been shown to affect the function of other intracellular organelles (Sun et al., 2020). Studies have shown that CIT can influence the function of the endoplasmic reticulum, a key organelle in protein synthesis and control of intracellular calcium balance, affecting also apoptosis process and cell cycle (Kumar et al., 2011; D. Wu et al., 2022; J. Wu et al., 2022). Previous studies have shown that CIT can trigger apoptosis in different cell lines (de Oliveira Filho et al., 2017). However, the exact molecular mechanisms involved in CIT-induced apoptosis in neuronal cells remain to be elucidated. It has also been suggested that CIT may increase the production of reactive oxygen species (ROS) and affect mitochondrial membrane potential (MMP), indicating its possible involvement in oxidative stress and mitochondrial dysfunction (Chan, 2007; Chen & Chan, 2009).

The role of CIT in human health has been investigated by in vivo and in vitro methods. In last decades, in vitro methods have been performed in two-dimensional cell cultures; however recently the tree-dimensional models have been developed for determining potential mechanisms of action that better recreates the cellular microenvironment and provides more relevant results compared to two-dimensional cell culture assays (Zingales et al., 2021).

The use of SH-SY5Y cell spheroids resemble the structure and function of tissues *in vivo*, as this 3D configuration allows for more accurate cell communication, formation of cell junctions and preservation of important cellular features. As a result, SH-SY5Y cell spheroids provide a suitable platform to investigate the mechanisms of CIT toxicity in a more relevant context.

The main objective of this study is to analyse the effects of CIT on SH-SY5Y cell spheroids and to understand the molecular mechanisms underlying their toxicity. To achieve this, different cellular aspects, such as cell viability, ROS production, mitochondrial membrane potential alterations, changes in chromosomal integrity, effect of CIT on endoplasmic reticulum and lysosomes function, and impact of this mycotoxin on apoptosis process and cell cycle will be evaluated.

2. Material and Methods

2.1 Reagents

The chemicals used in the experiment were of reagent grade quality and the cell culture compounds included a culture medium called DMEM high glucose with L-glutamine and fetal bovine serum (FBS), both obtained from ATCC. Additional components such as non-essential amino acids (MEM NEAA) and trypsin/EDTA solutions were sourced from Gibco (USA). The following reagents were also used: penicillin, streptomycin, phosphate buffer saline (PBS), tetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (H2-DCFDA), bovine serum albumin (BSA), sodium hydroxide (NaOH), sodium chloride (NaCl), trizma base (Tris), t-octylphenoxypolyethoxyethanol (Triton-X 100) and propidium iodide (PI). These reagents were obtained from Sigma Chemical Co, (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was acquired from Fisher Scientific (Geel, Belgium). I-Block was obtained from Applied

Biosystems (Foster City, CA, USA). Monoclonal antibody for GAPDH was purchased from GeneTex (Irvine, CA, USA). Primary antibody for cleaved PARP (cPARP) was obtained from Cell Signaling Technology (Beverly, MA, USA). Finally, a standard of citrinin (CIT) was acquired from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of the mycotoxin were prepared in DMSO and stored at a temperature of -20 ˚C.

2.2 Cell culture and spheroids formation

SH-SY5Y cells, derived from human neuroblastoma, were cultured at pH 7.4, 5% CO_2 at 37 ºC and constant humidity. They were cultured in monolayer in DMEM high glucose with L-glutamine medium supplemented with 10% FBS, 1% penicillin-streptomycin and 1% MEM NEAA (100X). The medium was replaced every 48-72 h.

In order to obtain the spheroids, the protocol described by Zingales et al. (Zingales et al., 2021) was used. To that purpose, Corning® ultra-low attachment (ULA) 96-well round bottom plates were used. Briefly, after the trypsinization of the SH-SY5Y monolayer, 2000 cells/well were seeded, with 200 µL of complete medium in each well. Then, plates were centrifuged at 1200 rpm for 10 min to facilitate the cells to accumulate at the bottom of the well. The initial optimal seeding densities were established such that each spheroid for the cell lines sized approximately 500 µm in diameter. This size is considered appropriate for the spontaneous formation of gradients of oxygen and other nutrients, and of differential proliferation rates, all essential for bio-relevant 3D experimental studies (Vinci et al., 2012). Finally, the spheroids were incubated for 7 days in the same conditions than the monolayer cells. The formation of the spheroid during the 7 days is shown in Figure 1.

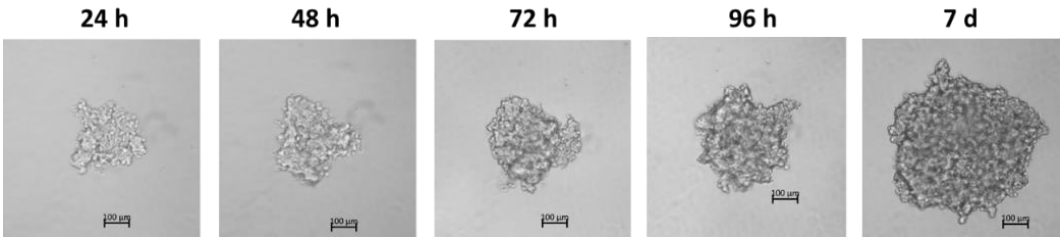

Figure 1. Bright-field images of the formation of SH-SY5Y spheroid from day 1 to day 7. Scale bar: 100 μm.

2.3 Citotoxicity of citrinin on spheroids

The effect of citrinin (CIT) on spheroids viability was determined at 24, 48 and 72h after exposure. For that purpose, both qualitative (Live and Dead) and quantitative (MTT, ATP and Presto Blue) assays were used. Moreover, the spheroids were also observed at the microscope to evaluate the morphology.

2.3.1 Citrinin exposure

SH-SY5Y cultures were exposed to CIT at day 7 after spheroids formation, by replacing 100 µL of culture medium with medium containing CIT to obtain final concentrations ranging from50 to 310 µM (50, 75, 100, 150, and 310 µM). Once the mycotoxin was added, the medium was not replaced during the different tested exposure times (24, 48, and 72 hours).

For the quantitative assays (MTT, ATP, and Presto Blue), cell viability was expressed as a percentage relative to the solvent control (1% DMSO). The mean 50% inhibitory concentration (IC_{50}) values were calculated using SigmaPlot version 11 (Systat Software Inc., GmbH, Germany).

2.3.2 Live and Dead assay

Live and Dead assay was used as qualitative method. These procedure uses calcein to stain in green the alive cells and ethidium homodimer-1 (EthD-1) to mark in red the dead ones. To sum up, a mix containing 5 µL of calcein AM and 20 µL of EthD-1 in 10 mL of PBS was prepared in darkness. Then, the medium was removed from the well and 100 µL of the mix was added. The plate was incubated for 30 min at room temperature and finally the spheroids were observed at the fluorescence microscope.

2.3.3 MTT assay

The MTT assay is based on the ability of the mitochondria to transform yellow soluble tetrazolium salt (substrate) to blue insoluble formazan salt (product). The experiment was performed as described by Zingales et al. (Zingales et al., 2021). Once the exposure time was finished, spheroids were transferred individually to a flat bottom 96 well plate with 100 µL of supernatant. Then, 50 µL of MTT solution (5 mg/mL in PBS) were added to each well. The plate was incubated for 4h at 37 °C protected from light. Finally, the supernatant was removed and the blue formazan was solubilized in 50 µL of DMSO/well. The absorbance was measured at 560 nm using a SPARK® multimode microplate reader (Tecan, Switzerland).

2.3.4 ATP assay

The ATP assay is based on the measurement of intracellular ATP using luciferase, which generates a luminescent signal directly related with the amount of ATP (and also, directly related to cell viability).

To carry out the experiment, each spheroid was transferred individually to an opaque white 96 well plate with flat bottom with 50 µL of supernatant. Then, 50 µL of CellTiter-Glo reagent were added and the plate was incubated

in darkness for 30 min at room temperature. Lastly, the luminescence was measured at 550-570 nm.

2.3.5 Presto Blue assay

The Presto Blue assay is based on the resazurin reaction, which involves the mitochondria in viable cells. This reaction transforms the non-fluorescent blue resazurin into the highly fluorescent pink resorufin. To perform the assay, each individual spheroid was transferred to another plate with 90 µL of medium and, 10 µL of the Presto Blue reagent (Invitrogen, ThermoFisher, Life Technologies Corporation, 29851 Willow Creek, Eugene, OR 97402, USA) was added. The plate was then incubated at 37 °C, protected from light, for 2 hours. Finally, the fluorescence was measured at an excitation and an emission wavelength of 560 and 590 nm, respectively.

2.4 Reactive Oxygen Species production assay

The H2-DCFDA assay was used to detect intracellular ROS production in SH-SY5Y spheroids. Cells metabolise the H2-DCFDA reagent by a deacetylation reaction to 2',7'-dichlorodihy-drofluorescein (H2-DCF), which is a non-fluorescent compound. H2-DCF is then oxidised by intracellular ROS, transforming it into dichloro-fluorescein (DCF) and emitting fluorescence. So, for that purpose, SH-SY5Y spheroids were exposed to 25 and 50 µM of CIT for 48h. Then, the supernatant was removed and 100 µL of H2-DCFDA were added. The plate was incubated for 30 min at 37 °C in darkness. Finally, the fluorescence was measured in a SPARK® multimode microplate reader (Tecan, Switzerland) using an excitation and an emission wavelength of 485 and 535 nm, respectively.

2.5 Mitochondrial Membrane Potential

In order to measure the alterations in Mitochondrial Membrane Potential (MMP), rhodamine 123 (Rh123) was used. The SH-SY5Y spheroids were exposed to 25 and 50 µM of CIT. At 48h of exposure, the medium was removed and Rh123 was added at a final concentration of 10 µM in each well. The spheroids were incubated at 37 °C for 90 min and then, the medium was removed and 150 µL of PBS were added. The fluorescence intensity was measured at a excitation and emission wavelength of 485 and 535 nm, respectively.

2.6 Cell cycle analysis

The cell cycle was analyzed using flow cytometry. Spheroids were exposed to CIT at concentrations of 25 and 50 µM for 48 hours of exposure. Subsequently, the spheroids were collected and resuspended in 500 µL of PBS with an addition of 4.5 mL of 70% EtOH. The samples were then incubated at -20 °C for a period of 2 hours. After the incubation, they were centrifuged at 1200 rpm for 7 minutes, washed with PBS, and centrifuged once more at the same speed and duration. Then, the supernatant was discarded, and the spheroids were resuspended in a mixture consisting of 0.1% (v/v) Triton, 0.4 mg/mL RNAseA, and 1 mg/mL Propidium Iodide (PI) in PBS. The suspension was incubated for 30 minutes. Finally, the samples were analyzed using a Cytoflex flow cytometer (Beckman Coulter), ensuring a minimum of 10,000 recorded events for each analysis. A total of 48 spheroids were utilized for each experimental condition.

2.7 Apoptosis and Necrosis analysis by flow cytometry

The influence of CIT on apoptosis and necrosis processes in spheroids was studied using flow cytometry, using the Annexin V-FITC/PI double staining assay. For this purpose, 48 spheroids were used for each condition.

The spheroids were mechanically degraded and treated with trypsin for 5 minutes. Subsequently, spheroids were washed in binding buffer (0.1 M Hepes/NaOH, pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$) and, centrifuged at 1200 rpm for 5 minutes. After removing the supernatant, the pellet was resuspended in 100 µL of binding buffer, and 1 µL of FITC-Annexin V and 5 µL of PI were added (BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I, BD Biosciences, USA). The mixture was then incubated for 15 minutes in darkness at room temperature. Finally, 400 µL of binding buffer was added, and the samples were measured using a Cytoflex flow cytometer (Beckman Coulter), ensuring a minimum of 10,000 recorded events.

2.8 RNA extraction y RT-qPCR for the evaluation of genic expression

In order to assess the impact of CIT on the endoplasmic reticulum and lysosomes, a study was conducted to examine the expression of genes associated with alterations in these organelles. The genes examined included GRP78, ATF4, and CHOP for the endoplasmic reticulum and, LAMP2 for the lysosomes. The protocol described by Fusco et al. (Fusco et al., 2023) for RNA extraction and RT-qPCR procedure was carried out.

A total of 48 spheroids were utilized for each concentration. Total RNA from the treated spheroids was extracted using Trizol reagent (15596026, Invitrogen) and quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific). The isolated RNA was then used to synthesize cDNA using the TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, A25576) following the manufacturer's instructions. Real-time PCR was prepared using the TaqMan Fast Advanced Master Mix (Applied Biosystems, 4444963) and Platinum SYBR Green (7900 Applied Biosystems). The relative mRNA expression levels for each gene were analyzed using the ddCt method, with GAPDH serving as the reference gene. The gene-specific primers used are shown in Table 1:

Table 1. Gene-specific primers used for real time PCR experiments.

Gene	Forward primers (5' → 3')	Reverse primers (5' → 3')
GRP78	CTGGTGTGCTCTCTGGTGAT	TGACACCTCCCACAGTTTCA
ATF4	AGTCCCTCCAACAACAGCAA	GGCATCCAAGTCGAACTCCT
CHOP	AGCCCTCACTCTCCAGATTC	ACCACTCTGTTTCCGTTTCC
LAMP2	TGGAAGCATTTGTGGGGATG	GCTGCCTTGGTAAAATTCGC

2.9 Protein expression evaluation

The protein expression analysis in SH-SY5Y spheroids was conducted following the method described by (Zingales et al., 2021). Proteins were isolated from a total of 48 spheroids for each condition and quantified using the BCA™ Protein Assay Kit (Thermo Scientific, USA). Five µg of total proteins were loaded onto a 4-12% SDS-PAGE polyacrylamide gel. After electrophoresis, the resolved protein gel was transferred to a nitrocellulose membrane. The membrane was incubated in a blocking solution (0.2 g of I-Block and 0.1% Tween in PBS) and subsequently probed with the primary rabbit monoclonal antibody against cleaved PARP (cPARP) (dilution 1:1000 in the blocking solution) and the primary mouse monoclonal antibody against GAPDH (dilution 1:5000 in the blocking solution), which served as the loading control. After washing the membranes to remove unbound antibodies, they were incubated with appropriate secondary antibodies against rabbit (dilution 1:25000 in the blocking solution) and against mouse (dilution 1:20000 in the blocking solution). Chemiluminescence was developed using the Western Hypernova chemiluminescent reagent (Cyanagen, Bologna, Italy), following the manufacturer's protocol, and detected using the iBright™ CL1500 Imaging System (Invitrogen, USA). The intensities of the cPARP bands were quantified using the iBright Analysis Software (Thermo Fisher Scientific, USA) and normalized to the internal control (GAPDH).

2.10 Array comparative genomic hybridisation profile

The array comparative genomic hybridisation (aCGH) analysis was performed according to Agilent oligonucleotide array-based CGH for Genomic DNA Analysis Protocol (Version 7.3). Samples were compared with control DNA derived from commercial SH-SY5Y cell line. Briefly: Genomic DNA was purified using Quick-DNA™ Microprep Kit (Zymo Research). 500 ng of DNA were digested at high temperature and labelled by random priming with CY5-dCTP for the samples and CY3-dCTP for the controls DNA. Samples were purified using Micron filters YM-30 (Sigma-Aldrich, Missouri, USA); DNA was hybridized on a SurePrint G3 Human CGH Array Kit 8x60K (Agilent Technologies, Santa Clara, USA) with a minimum resolution of 100 kb, at 65 °C for about 24 h. The aCGH was analyzed using DNA microarray Agilent Scanner (G5761A) and digital analysis was carried out with Agilent Genomic Workbench 7.0 using the Aberration Algorithm ADM-1 with an Aberration Threshold of 5.0. Human Genome Browser, February 2009, assembly hg19 was used for DNA sequence information.

2.11 Statistical analysis

The statistical analysis was conducted using GraphPad Prism 8 software (GraphPad Software, San Diego, California, USA). Differences between groups were analyzed using ANOVA with a Tukey *post*-test for multiple comparisons. Statistical significance was determined at a p-value of ≤ 0.05. The results were expressed as the mean ± SEM of several independent experiments.

3. Results and discussion

3.1 Effect of CIT exposure on SH-SY5Y spheroids viability

The effect of CIT on the morphology and integrity of SH-SY5Y cell spheroids is depicted in Figure 2. As can be observed, CIT induces a

disaggregation of the spheroids at all tested concentrations and exposure times.

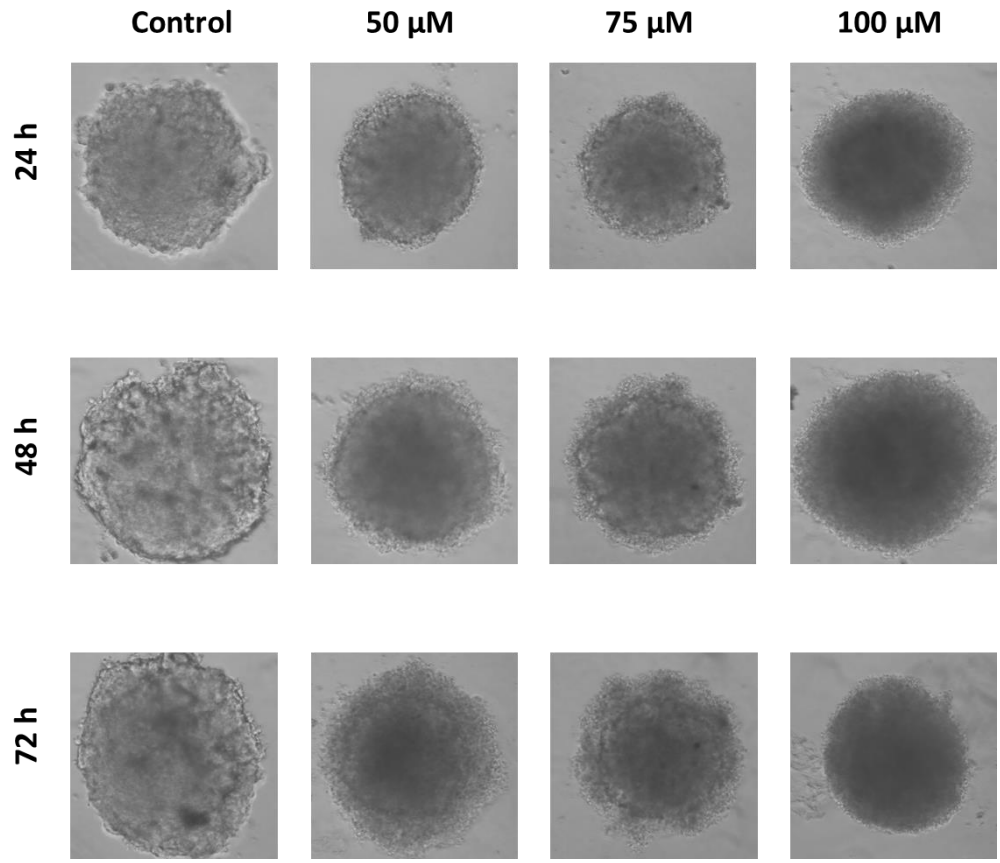

Figure 2. Bright-field images of SH-SY5Y spheroids after 24, 48 and 72 h exposure to 50, 75 and 100 µM CIT. Images were obtained using the Light Microscope Zeiss Axio Observer (Zeiss Microscopy, Germany).

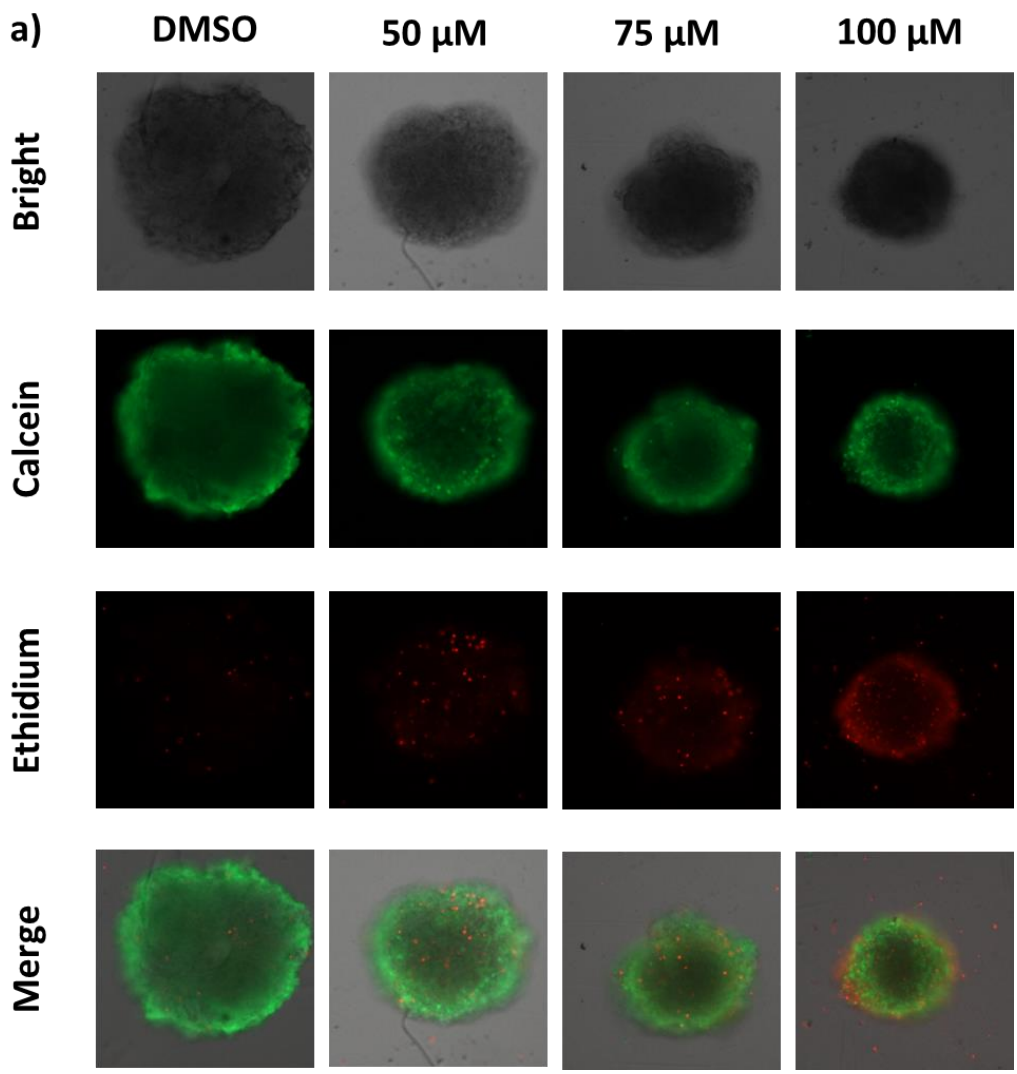

Figure 3a. Live & Dead inmunofluorescence images obtained after the SH-SY5Y spheroids exposure at DMSO (control) and 50, 75 and 100 µM CIT for 24 h. Alive cells are coloured in green while dead cells are coloured in red.

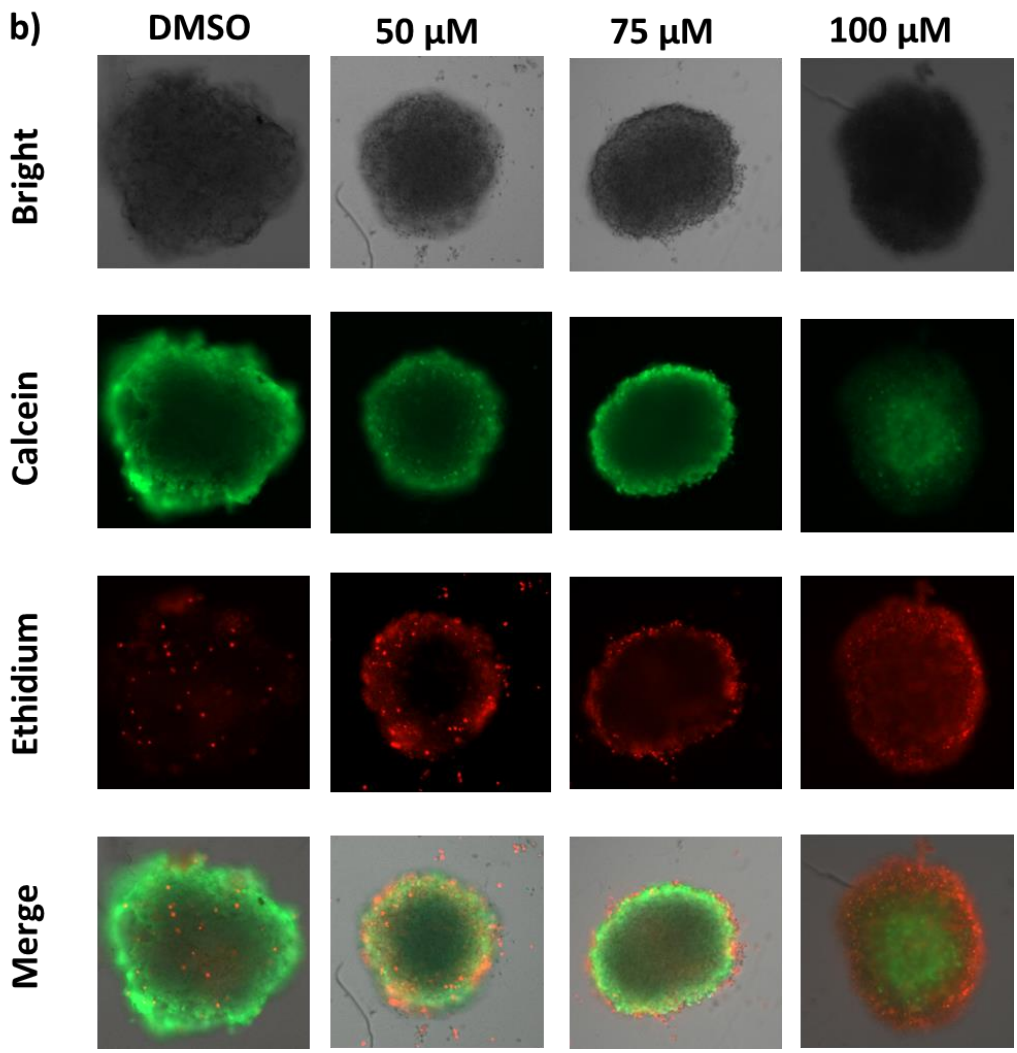

Figure 3b. Live & Dead inmunofluorescence images obtained after the SH-SY5Y spheroids exposure at DMSO (control) and 50, 75 and 100 µM CIT for 48 72 h. Alive cells are coloured in green while dead cells are coloured in red.

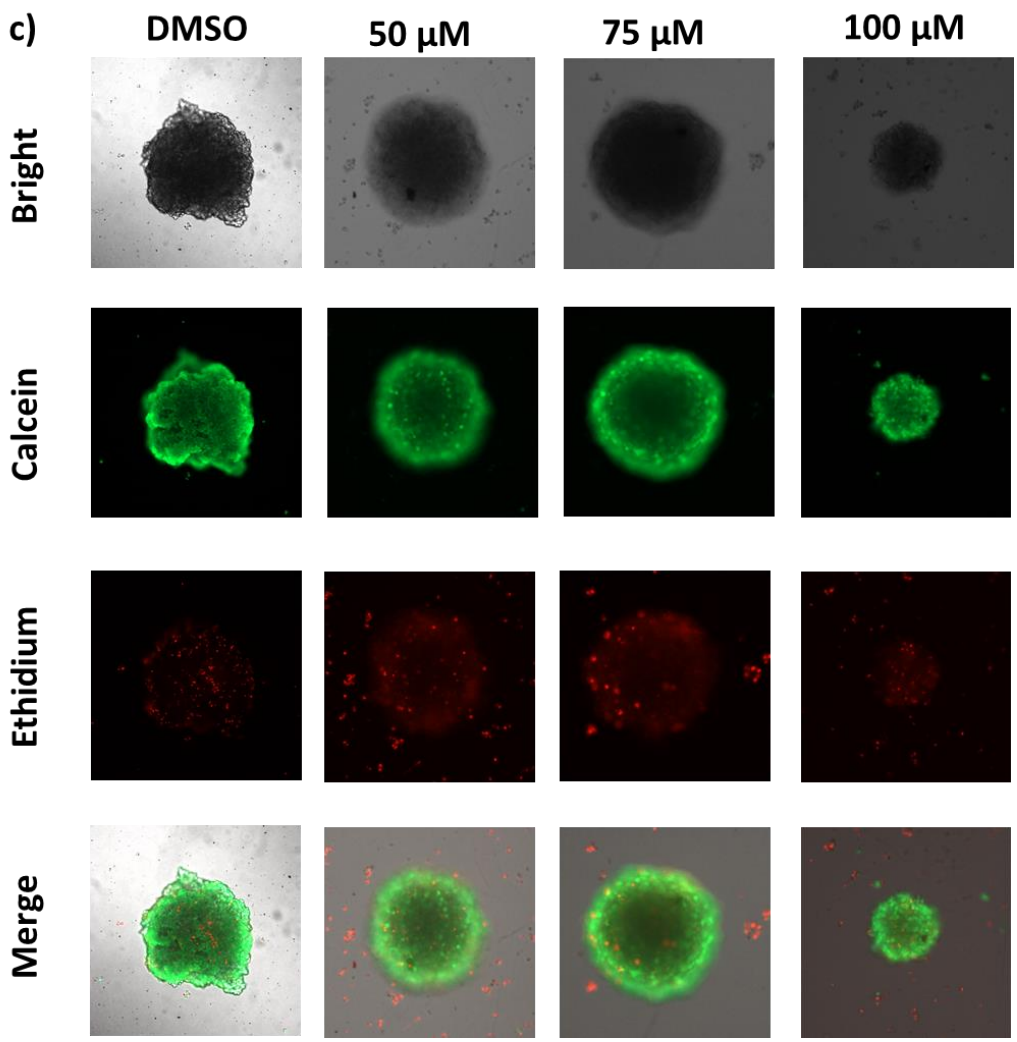

Figure 3c. Live & Dead inmunofluorescence images obtained after the SH-SY5Y spheroids exposure at DMSO (control) and 50, 75 and 100 µM CIT for 72 h. Alive cells are coloured in green while dead cells are coloured in red.

After performing immunofluorescence using the Live&Dead kit, it is observed that CIT mainly affects the outer cells of the spheroid, with a more pronounced effect at longer exposure times. Additionally, at the lowest CIT

concentration (50 µM) and 24 hours of exposure, an increase in dead cells compared to the control was observed (Fig. 3a). And, this cytotoxic effect increase as CIT concentration increases. So, the cytotoxicity of CIT is concentration-dependent. The same effect was observed for all time of exposure (24, 48 and 72 h; Figures 3a-c). Furthermore, cytotoxicity of CIT is time-dependent, because of higher cytotoxic effect was evidenced at the higher time of exposure tested (72 hours; Fig. 3c).

On the other hand, the cytotoxic effect of CIT on SH-SY5Y spheroids was evaluated using the MTT, ATP, and Presto Blue assays after 24, 48 and 72 h of exposure. Table 2 shows the IC_{50} values of CIT, on SH-SY5Y spheroids using the three end points selected.

Table 2. The IC_{50} values of CIT in SH-SY5Y spheroids performed by MTT, ATP and Presto Blue method at 24, 48 and 72 h of exposure. Data are expressed as mean ± SEM of three independent experiments.

IC_{50} values	Time of exposure	MTT	ATP	Presto Blue
	24h	83.14 ± 5.70 µM	82.27 ± 10.65 µM	75.70 ± 6.70 µM
	48h	74.21 ± 12.46 µM	56.78 ± 3.70 µM	64.16 ± 9.58 µM
	72h	47.01 ± 30.05 µM	34.41 ± 4.01 µM	46.73 ± 8.61 µM

As can be observed, the IC_{50} of CIT in SH-SY5Y spheroids ranges from 34.41 ± 4.01 µM (ATP assay after 72 h of exposure) to 83.14 ± 5.70 µM (MTT assay after 24 h of exposure), depending on the exposure time and the assay used. The CIT decreased the number of viable SH-SY5Y cells after 24, 48 and 72 hours of exposure in a significant dose-dependent manner (graphs not shown). However, the cytotoxicity was similar at the same time of exposure by the three methods tested (graphs not shown). And, similar IC_{50} values were obtained by the three assays at the same exposure time tested (Table 1).

Therefore, according to the results obtained, it was concluded to selected 25 and 50 µM of CIT concentrations (both below the IC_{50} at that exposure time) for the subsequent assays developed in this study and related to the mechanism of CIT toxicity. Moreover, considering the results obtained by the live and dead assay, 48 hour of exposure was selected.

3.2 Evaluation of ROS generation

Due to the fact that other authors have described an increase in oxidative stress caused by this mycotoxin, and in order to clarify the mechanism by which its toxic effects occur, a study on the production of intracellular ROS after exposure to CIT has been conducted. The results of the study are presented in Figure 4.

As can be observed in Figure 4, there is a significant decrease in ROS at the highest concentration studied, 50 µM, compared to the control. This could be due to cell viability decreasing between 24.4% and 40.7% after 50 µM CIT exposure in SH-SY5Y spheroids for 48 hours (graphs not shown). Therefore, this decrease in viability could be responsible for the reduction in ROS production. On the other hand, no significant changes were observed after 25 µM CIT exposure in SH-SY5Y respect to control spheroids.

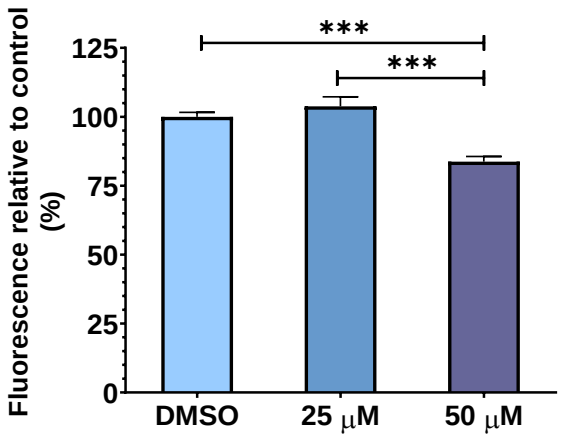

Figure 4. Reactive oxygen species-induced fluorescence in SH-SY5Y spheroids exposed to CIT. SH-SY5Y spheroids were loaded with H2-DCFDA for 20 min in 96-well plates (1 spheroid per well) and then exposed to test agent or vehicle (control). Fluorescence of oxidised DCF was followed by emission at 535 nm (excitation 485 nm). The results are expressed as mean ± SEM of 3 independent experiments. ***$p \leq 0.001$ indicates a significant difference compared to the control.

3.3 Alterations in mitochondrial membrane potential

To determine alterations in MMP, the fluorescent dye Rh123 was used. The results obtained are shown in Figure 5.

As can be observed in Figure 5, there is a significant decrease in MMP at the lowest CIT concentration tested (25 μM), which demostrated that CIT alters de MMP.

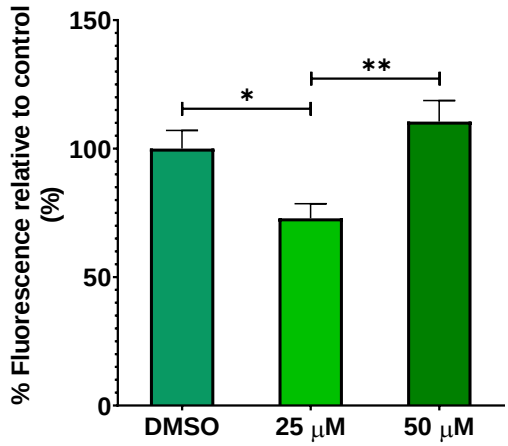

Figure 5. Effect of CIT (25 and 50 μM) on mitochondrial membrane potential in SH-SY5Y spheroids determined by Rh123 assay. The SH-SY5Y spheroids were exposed to CIT or control (1% DMSO) for 48 h in 96-well black plate and then loaded with Rh123 for 15 min. Data are expressed as mean ± SEM of three independent experiments. *p ≤0.05 indicates a significant difference compared to the control. **p ≤ 0.01 indicates a significant difference compared to the control.

3.4 Cell cycle analysis

Cell cytotoxicity could produce DNA damage and alter cell-cycle progression. Determination of CIT alteration on SH-SY5Y spherodis cell cycle was determined by flow cytometry analysis by PI-staining after 48 h of exposure. The results obtained are shown in Figure 6.

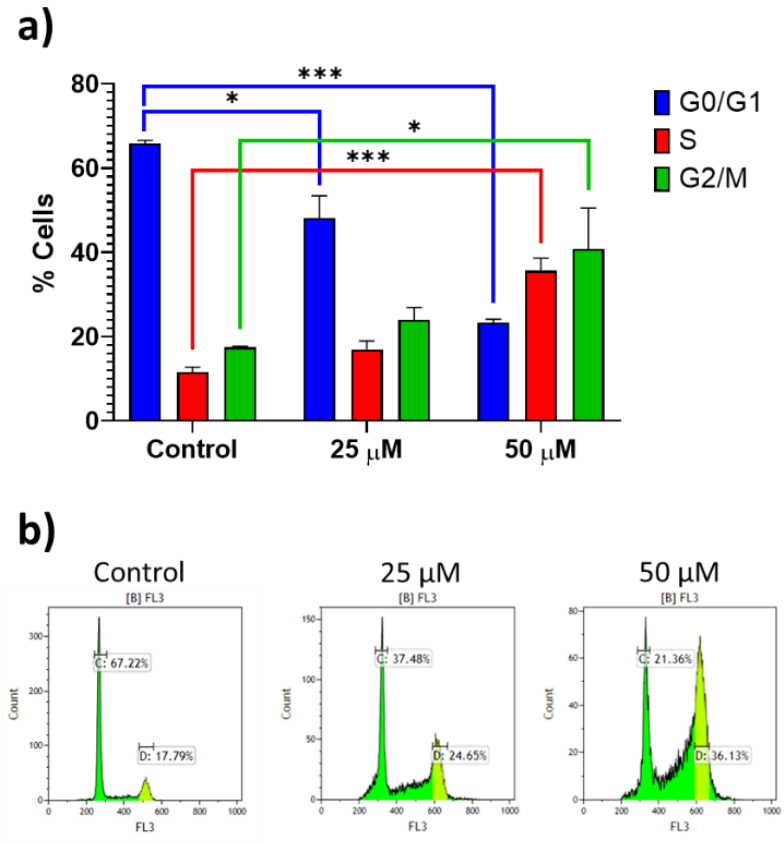

Figure 6. a) Cell cycle analysis of SH-SY5Y spheroids exposed to 25 and 50 µM CIT for 48 h. b) Histograms representative of SH-SY5Y spheroids treated with control, 25 and 50 µM of CIT for 48 h. Results are expressed as Mean ± SEM of three independent experiments, and 10,000 events were recorded each time. *$p \leq 0.05$ indicates a significant difference compared to the control. ***$p \leq 0.001$ indicates a significant difference compared to the control.

As shown in Fig. 6, after 48 h of exposure to 50 µM CIT, a significant percentage of cell reduction in G0/G1 phase accompanied by an increase in the percentage (%) of number of SH-SY5Y spheroids of S and G2/M phases

was observed, as compared to the control. In this study, it has been observed that CIT is capable to disturb the normal progression of proliferating cell. When DNA damage is irreparable, checkpoints eliminate such potentially hazardous cells by permanent cell-cycle arrest or cell death. The induction of cell cycle arrest in G2/M and/or in S-phase is a common response of most eukaryotic cells to a genotoxic challenge (Shackelford et al., 1999 10.1289/ehp.99107s15). Furthermore, the reduction in G0/G1 is still maintained through CIT exposure. G0/G1 phase is a period when cells make critical decisions about their destiny, including the optional requirement to replicate DNA and complete the cell division cycle. Importantly, the checkpoints alarmed by genotoxic stress can delay cell-cycle progression even when cells have already passed this restriction point. Thus, depending on the nature of DNA damage, the cell cycle can maintain G0/G1 block.

3.5 Apoptosis/Necrosis measurement

In order to understand whether the observed decrease in cell proliferation is related to CIT exposure in SH-SY5Y spheroids, apoptosis and necrosis induced by the studied concentrations (25 and 50 µM CIT) was determined by flow cytometry. The results obtained are shown in Figure 7.

Results in Figure 7 demonstrated an increase in cells in the necrosis. In addition, there is an increasing trend in late apoptosis, although it is not statistically significant (p = 0.07). There are not variations in the early apoptosis phase. Due to the 48-hour exposure time used in the assay, it is likely that the cells have already passed through the early apoptosis phase at the beginning of the toxin exposure and at the time of measurement are already in the late apoptosis phase, which would explain the low percentage of population in early apoptosis. Lastly, and as expected, there is a significant decrease in the percentage of viable cells directly related to the dose of CIT used, which correlates with the previous assays conducted in this study.

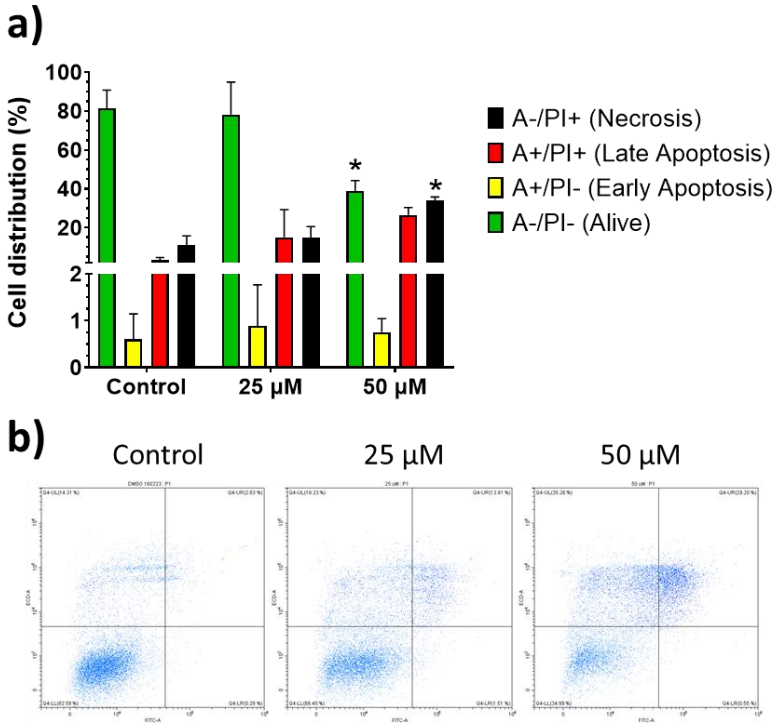

Figure 7. Analysis of apoptosis and necrosis induction in SH-SY5Y spheroids exposed to 25 and 50 µM CIT for 48 h. Cells were stained with Annexin V-FITC and PI to distinguish early apoptotic, apoptotic/necrotic and necrotic cells. a) Percentage of early apoptotic, late apoptotic/necrotic and necrotic cells. b) Two-dimensional dot plots of FITC Annexin V *vs.* PI through flow cytometry. Cells stained negative for Annexin V-FITC and PI in the lower left quadrant shows alive cells. Cells stained positive for Annexin V-FITC and negative for PI in the lower right quadrant are representing early apoptosis. Cells stained negative for Annexin V-FITC and positive for PI in the upper left quadrant representing necrotic cells. Cells stained positive for both Annexin V-FITC and PI in the upper right quadrant are the late apoptotic/necrotic cells. Results are expressed as Mean ± SEM of three independent experiments, and 10,000 events were recorded each time. *$p \leq 0.05$ indicates a significant difference compared to the control.

3.6 Study of the effect of CIT on the endoplasmic reticulum and lysosomes

Due to previous assays demonstrated alterations in various intracellular organelles (Sun et al., 2020), the analysis of gene expression related to the function of the endoplasmic reticulum (GRP78, ATF4, and CHOP) and lysosomes (LAMP2) was carried out in SH-SY5Y spheroids exposed to CIT by RT-qPCR analysis. The results obtained from the RT-qPCR analysis are shown in Figure 8.

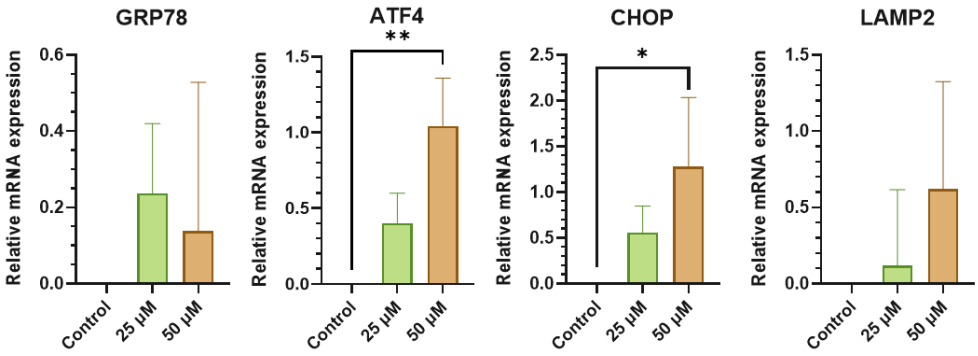

Figure 8. Relative mRNA expression of the genes related to endoplasmic reticulum (GRP78, ATF4 and CHOP) and lysosomes (LAMP2) determined by RT-qPCR in SH-SY5Y spheroids exposed to CIT at 25 and 50 µM for 48 h. Results are expressed as Mean ± SEM of three independent experiments. *$p \leq 0.05$ indicates a significant difference compared to the control. **$p \leq 0.01$ indicates a significant difference compared to the control.

As can be observed in Figure 8, there is a significant increase in the ATF4 gene expression at the highest CIT concentration (50 µM) tested compared to the control. Additionally, a 1.28-fold increase in the expression of CHOP was observed. Regarding lysosomal function, no differences in the expression of

LAMP2 was observed. All these results suggest that both, the endoplasmic reticulum and lysosomes are negatively affected after CIT exposure in SH-SY5Y spheroids.

3.7 Protein expression analysis of cPARP

In order to confirm the previously obtained results regarding the induction of apoptosis by CIT, the analysis of cleaved PARP (cPARP) protein expression, which is directly involved in the process of apoptosis, was performed. The results are shown in Figure 9.

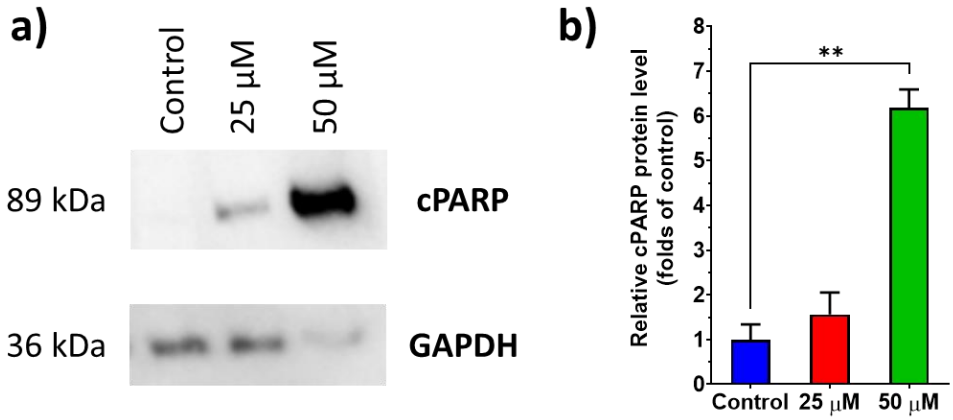

Figure 9. a) Western blot showing cPARP protein expression. b) Densitometric analysis performed using iBright Analysis Software. Results are expressed as Mean ± SEM of two independent experiments. $^{**}p \leq 0.01$ indicates a significant difference compared to the control.

Figure 9 shows that there is a significant increase in cPARP expression in SH-SY5Y spheroids exposed to 50 µM CIT. The increase was 6-fold higher than the control. This result confirms the previous results obtained. Therefore, it can be concluded that CIT induces apoptosis in SH-SY5Y cell spheroids exposed to 50 µM.

3.8 Study of possible chromosomal abnormalities

The study of chromosomal abnormalities was conducted using the Array Comparative Genomic Hybridization (aCGH) assay. The results are presented in figure 10

Array comparative genomic hybridization (array CGH) is a technique employed to identify changes in DNA copy numbers within segments. In recent times, significant progress has been made in this field, allowing for a detailed analysis of genetic alterations and variations in copy numbers across the entire genome. This comprehensive overview delves into the existing genomic array platforms and CGH methodologies, emphasizing their relevance in investigating cancer genetics, constitutional diseases, and human diversity. Additionally, it explores various software programs designed for visualizing and analyzing array CGH data, facilitating computational interpretation (Lockwood et al., 2005).

The results observed in Figure 10 indicate that CIT does not induce chromosomal alterations at CIT tested concentrations (25 and 50 µM) in SH-SY5Y speroids during 48 hours of exposure. No changes in the copy number of genome segments were detected in the analyzed sample, with the control sample exhibiting a similar profile to the samples exposed to CIT.

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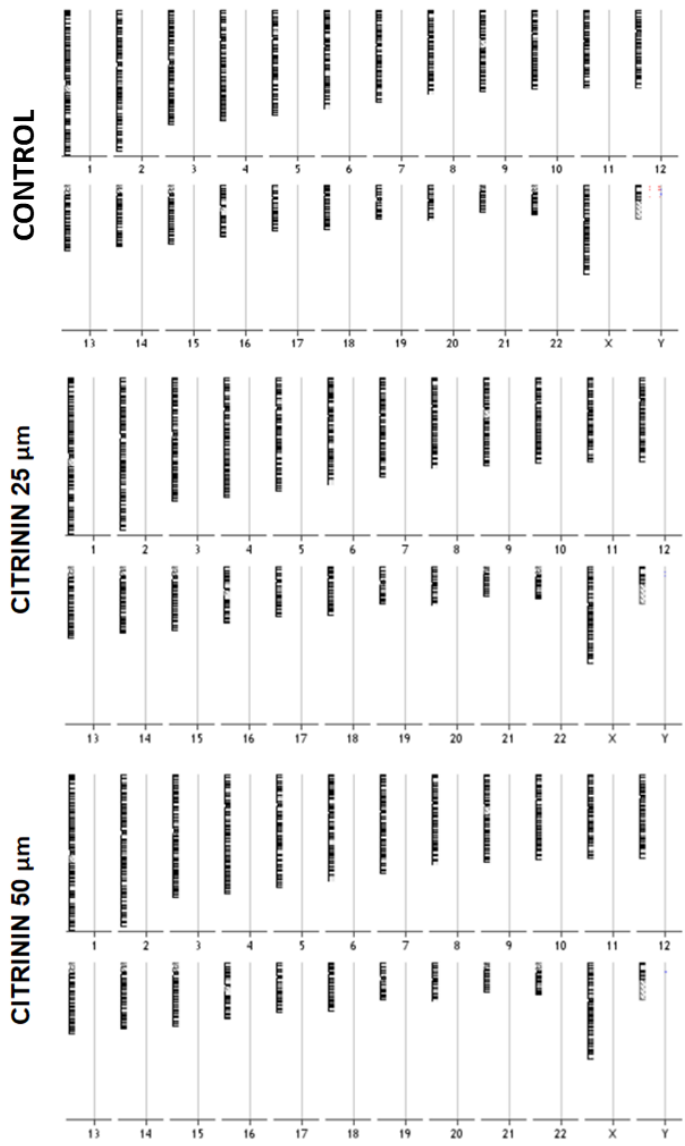

Figure 10. Results obtained from the Array Comparative Genomic Hybridization (aCGH) assay after exposing SH-SY5Y cell spheroids to 25 and 50 µM CIT for 48 hours. Whole-genome idiogram of a SH-SY5Y cell spheroids exposed to CIT hybridized against compared with control DNA derived from commercial SH-SY5Y cell line.

4. Conclusion

After analyzing different mechanisms of action involved in cellular function, it has been observed that CIT induces apoptosis and disrupts mitochondrial membrane potential; causes cell cycle arrest in the S and G2/M phases and, affects the functioning of the endoplasmic reticulum. Moreover, it has also been observed that its toxic effect on cells is time- and concentration-dependent. However, no evidence has been found to suggest that this mycotoxin induces chromosomal alterations.

Due to the use of a three-dimensional cell culture model, these results are more representative of reality as they better recreate the cellular microenvironment. However, further investigation into the toxicity of CIT is necessary to identify other pathways that may be involved. This will provide a comprehensive understanding of its toxicity in order to prevent the effects caused by intoxication with this mycotoxin.

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Impact of Citrinin on Zebrafish Development: Insights into Acute Toxicity

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Article in preparation

Abstract

Citrinin (CIT) is a mycotoxin commonly found in cereals, known for its nephrotoxic effects. *Danio rerio* (zebrafish) is a suitable model organism for studding functional disorders. The aim of the present study is to investigate the toxic effects of CIT using a zebrafish model. Zebrafish embryos up to 96 hours post-fertilization were utilized for the experiments. To gain further insights into the underlying mechanisms of CIT toxicity, the expression levels of apaf1 and bax genes (associated with apoptosis), gpx (antioxidant activity), ripk3 (necrosis), and IL-8 and CXCL-CIC (inflammation) were analysed. The LD_{50} values obtained ranged from 255 ± 10.10 µM (72 hours) to 140.5 ± 21.7 µM (96 hours). Exposure to the highest CIT concentration (300 µM) resulted in malformations in 100% of the zebrafish embryos, whereas no malformations were observed at the lowest concentration tested (50 µM). It was observed an upregulation in the expression of all the genes tested, indicating their potential involvement in CIT-induced toxicity. In addition, it was determined an alteration in reactive oxygen species (ROS) production in zebrafish embryos exposed to CIT. This finding suggests that CIT may modulate oxidative stress pathways, which could contribute to its toxic effects. In conclusion, our study provides valuable insights into the toxic behaviour of CIT using a zebrafish model.

Keywords: zebrafish, citrinin, acute toxicity, ROS, malformations

1. Introduction

Citrinin (CIT) is a mycotoxin produced by fungi belonging to the *Aspergillus, Penicillium*, and *Monascus* genera, primarily found in cereals. Previous studies have identified CIT as a nephrotoxic compound, causing renal damage in both animals and humans. However, the underlying molecular mechanisms and broader toxicological effects of CIT, particularly in developing organisms, remain poorly understood (de Oliveira Filho et al., 2017; Narváez et al., 2021).

On the other hand, zebrafish (*Danio rerio*) has emerged as a valuable research model system for clarification of the roles of specific genes and signaling pathways in development. in various fields, including toxicology, as it offers different advantages. Firstly, the zebrafish genome has been fully sequenced and extensively studied, making it easier to identify genes and signalling pathways affected (Howe et al., 2013). Additionally, their small size, rapid reproduction and, high fecundity allow for cost-effective large-scale experiments. The transparent nature of zebrafish during early developmental stages enables *in vivo* observation of toxic effects on organs and tissues, facilitating the assessment of potential damage and elucidation of underlying mechanisms (Bauer et al., 2021). Lastly, zebrafish share physiological and genetic similarities with humans, enabling the extrapolation of findings from this model to situations more relevant to human health (Bambino & Chu, 2017; Roper & Tanguay, 2018). So, it is an adequate model system to study developmental biology, toxicology, and gene transfer and, for clarifying mechanisms in toxicity.

Currently, several researchers have utilized the zebrafish model to evaluate the toxicity of various mycotoxins regarding their teratogenic activity and developmental effects. This is primarily due to the ease of detecting endpoints, such as malformations (Juan-García et al., 2020). Furthermore, it

contributes to conducting more ethical research with reduced usage of mammals in experimentation. Therefore, the objective of this study was to investigate the toxic effects of CIT during development behaviour using a zebrafish model. For this purpose, the acute toxicity test developed by the OECD (*Test No. 236: Fish Embryo Acute Toxicity (FET) Test*, 2013) on zebrafish embryos was carried out. Then, the expression of genes involved in different pathological processes, including inflammation, apoptosis, necroptosis and, oxidative stress by the production of reactive oxygen species (ROS) were determined.

2. Materials and methods

2.1 Breeding and maintenance of zebrafish

The AB strain of zebrafish (*Danio rerio*) used in this study were bred and cared in compliance with the guidelines outlined in the European Animal Welfare Legislation (Directive 2010/63/EU). The zebrafish were housed in the ZebraLab facility located at the Istituto di Ricerca Pediatrica Città della Speranza in Padova, Italy. Fertilized eggs were collected through natural spawning processes. Subsequently, the embryos were maintained in water supplemented with methylene blue at a constant temperature of 28 °C until the completion of the 96-hour post-fertilization assay.

2.2 Acute toxicity test

The acute toxicity assay was conducted following the Fish Embryo Acute Toxicity (FET) Test, test number 236, as described by the OECD (*Test No. 236: Fish Embryo Acute Toxicity (FET) Test*, 2013). At 1 hour post-fertilization (hpf), the embryos were divided into groups of 20 individuals in 6-well plates, and 3 mL of methylene blue with the corresponding concentration of CIT, ranging from 50 to 300 µM, and 1% DMSO as a control, were added. The water with the respective concentration of mycotoxin was renewed daily.

The fish were observed daily using a stereomicroscope (SMZ1500 stereomicroscope, Nikon) from the addition of CIT until 96 hpf. The mortality of the individuals was assessed by observing the presence or absence of coagulation in the egg and, once dechorionated, the presence or absence of a heartbeat. Furthermore, the formation of different malformations was also evaluated, including hatching rate, pericardial edema, yolk edema, pigmentation, body curvature and, tremors. Additionally, the heartbeat was also measured visually.

2.3 RNA Extraction and RT-qPCR analysis

For the analysis of gene expression related to pathological processes, such as inflammation (IL-8 and CXCL-CIC), oxidative stress (gpx), apoptosis (apaf1 and bax), and necrosis (ripk3), concentrations of 50, 150, and 300 µM CIT were exposed (plus 1% DMSO as control) in 96 hpf zebrafish for a 72-hour exposure period.

The RNA extraction from the zebrafish embrios was performed using Trizol reagent (15596026, Invitrogen) following the manufacturer's protocol. The concentration of the extracted RNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Subsequently, the isolated RNA was utilized for cDNA synthesis using the TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, A25576) according to the provided instructions. For real-time qPCR, the TaqMan Fast Advanced Master Mix (Applied Biosystems, 4444963) and Platinum SYBR Green (7900 Applied Biosystems) were employed. The relative mRNA expression levels of the target genes were analyzed using the ddCt method, with β-actin serving as the reference gene. The gene-specific primers utilized are listed in Table 1.

Table 1. Gene-specific primers used for RT-qPCR experiments

Gene	Forward primers (5' → 3')	Reverse primers (5' → 3')
apaf1	GTGCCAGTGACAAGAAGGTG	TCTGCTCCTCGTGTTCAACT
bax	ATGAGCTGGATGGAAATGC	CCTGTTCCCTGATCCAGTTAA
ripk3	GTGTGTATGAGGGCCGTTTG	AGATCCTCCAGAGAACCCCT
gpx	TCCTGCAGTCTCTGAAATACG	TGTACCTCTTGAATGGTTCCC
IL-8	GTCGCTGCATTGAAACAGAA	CTTAACCCATGGAGCAGAGG
CXCL-C1C	CTGCTGCTTGCGGTAGTTTA	TCAACTTTGTCGCAGTTTGG

2.4 Assessment of the impact of CIT on ROS generation.

In order to evaluate the presence of intracellular ROS in zebrafish, the dichorofluorescein diacetate (H2-DCFDA) probe was utilized. This reagent undergoes a conversion process to form 2',7'-dichlorodihydrofluorescein (H2-DCF), which initially lacks fluorescence. When intracellular ROS is present, H2-DCF undergoes oxidation, resulting in the generation of dichlorofluorescein (DCF) and subsequent fluorescence emission. Therefore, an increase in fluorescence intensity means a higher ROS production within the cells.

For the H2-DCFDA experiment, zebrafish was exposed to 50 and 150 µM CIT over a 48-hour exposure. To conduct the assay, H2-DCFDA was added to the water in each condition to achieve a final concentration of 50 µM. The zebrafish were then incubated for 30 minutes at a temperature of 28°C and darkness. Subsequently, the fish were observed using a confocal microscope (Zeiss LSM800 Airyscan), with an excitation and an emission wavelength of 485 and 535 nm, respectively to detect the increases of fluorescence.

3. Results and Discussion

3.1 Evaluation of the acute toxicity of CIT in zebrafish embryos

Figure 1 illustrates the toxic effects of CIT on the morphology and integrity of zebrafish embryos after 50, 150, and 300 µM CIT exposure for 48, 72, and 96 hours.

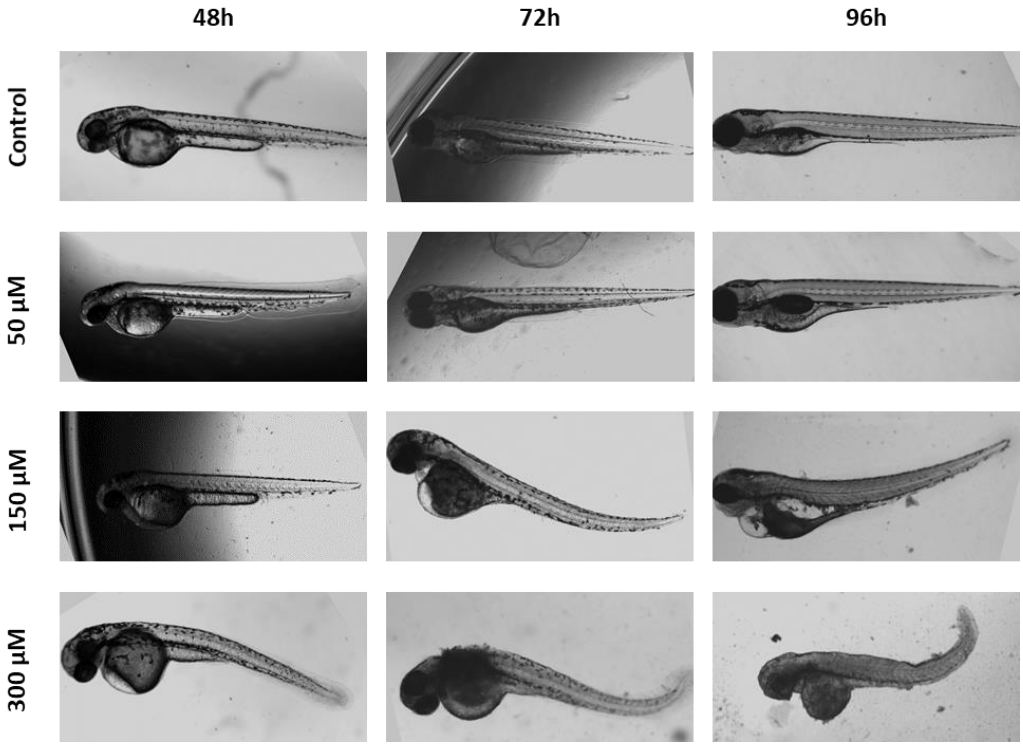

Figure 1. Toxic and sublethal effects produced in zebrafish embryos by exposure to CIT at 50, 150, and 300 µM for 48, 72, and 96 hours.

As can be observed, there are no differences between 50 µM CIT and the control at any time of exposure tested. However, at 150 µM CIT clear signs of toxicity were observed in zebrafish embryos, such as body curvature and oedema. Moreover, these effects increase as the time of exposure increases.

At the 300 µM CIT toxic effects exhibited body curvature and oedema in the heart and yolk (48 h). And, all individuals experience mortality by 96 hours. Furthermore, upon analyzing mortality in the different groups, the lethal dose 50 (LD_{50}) was determined at 72 and 96 hours (Figure 2 and Table 2).

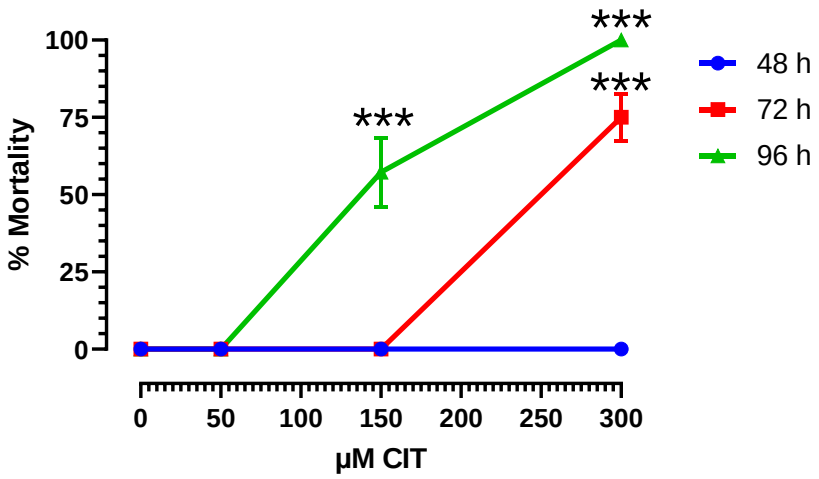

Figure 2. Relationship curves between Zebrafish mortality and CIT concentration (50, 150 and 300 µM) at 48, 72 and 96 h of exposure. The mortality of the individuals was assessed by observing the presence or absence of coagulation in the egg and, once dechorionated, the presence or absence of a heartbeat. Results are expressed as Mean ± SEM of three independent experiments. *** - $p < 0.001$ vs. control at each time of exposure.

Table 2. LD_{50} values for zebrafish exposed to CIT in a concentration range of 50 to 300 µM and during an exposure time of 48 to 96 hours. Mortality of the individuals was assessed by observing the presence or absence of coagulation in the egg and, once dechorionated, the presence or absence of a heartbeat. The results were obtained from three independent experiments and are expressed as Mean ± SEM.

	48 H	72 H	96 H
DL_{50} (µM CIT)	> 300	255 ± 10.10	140.5 ± 21.70

Finally, according to the OECD guidelines for acute toxicity assessment, it was also examined the potential malformations induced by CIT in zebrafish embryos at 48 and 72 h. For this objective, the parameters evaluated include hatching rate, pericardial oedema, yolk oedema, pigmentation, body curvature, and tremors. Table 3 presents these parameters, alongside the corresponding mortality rates, for a better understanding of the data. Furthermore, we analysed variations in heart rate, and the outcomes are illustrated in Figure 3.

Table 3. Main malformations observed in zebrafish exposed to 50, 150 and 300 μM CIT for 48 and 72 h by the OCDE Test No. 236 protocol. Data have been obtained from 3 different experiments, and are presented as the sum of all of them.

		48h treatment			72h treatment		
		Total	Ratio	%	Total	Ratio	%
Control	Dead	0	0/60	0%	0	0/60	0%
	Hatching rate	34	34/60	57%	60	60/60	100%
	Pericardial edema	0	0/60	0%	0	0/60	0%
	Yolk edema	0	0/60	0%	0	0/60	0%
	Pigmentation	60	60/60	100%	60	60/60	100%
	Body curvature [1]	0	0/60	0%	0	0/60	0%
	Tremors	0	0/60	0%	0	0/60	0%
CIT 50 μM	Dead	0	0/60	0%	0	0/20	0%
	Hatching rate	29	29/60	48%	60	60/60	100%
	Pericardial edema	0	0/60	0%	0	0/60	0%
	Yolk edema	1	1/60	2%	0	0/60	0%
	Pigmentation	60	60/60	100%	60	60/60	100%
	Body curvature [1]	0	0/60	0%	0	0/60	0%
	Tremors	0	0/60	0%	0	0/60	0%
CIT 150 μM	Dead	0	0/60	0%	0	0/60	0%
	Hatching rate	37	37/60	62%	60	60/60	100%
	Pericardial edema	2	2/60	3%	33	33/60	55%
	Yolk edema	11	11/60	18%	34	34/60	57%
	Pigmentation	60	60/60	100%	60	60/60	100%
	Body curvature [1]	4	4/37	11%	29	29/60	48%
	Tremors	0	0/60	0%	11	11/40	28%
CIT 300 μM	Dead	0	0/60	0%	48	48/60	80%
	Hatching rate	30	30/60	50%	52	52/60	87%
	Pericardial edema	60	60/60	100%	57	57/57	100%
	Yolk edema	60	60/60	100%	57	57/57	100%
	Pigmentation	60	60/60	100%	60	60/60	100%
	Body curvature [1]	27	27/30	90%	41	41/42	96%
	Tremors	23	23/60	38%	0	0/5	0%

[1] Only for hatched fish

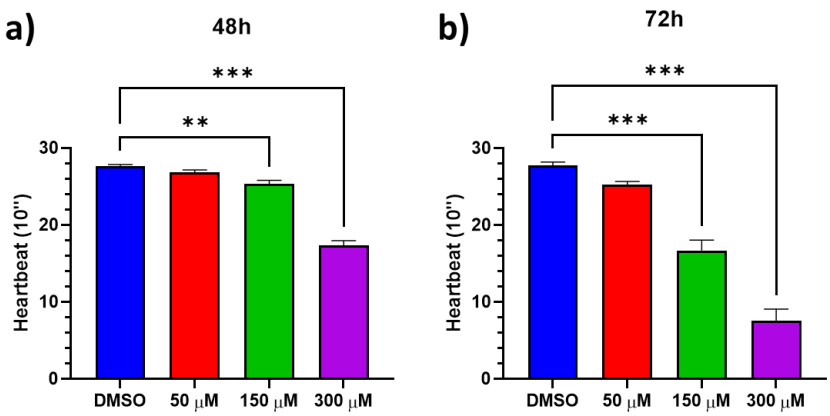

Figure 3. Changes in heartbeat measured for 10 seconds in zebrafish exposed to 50, 150 and 300 µM CIT for a) 48 h and b) 72 h. Results are expressed as Mean ± SEM of three independent experiments. ** - $p < 0.01$ compared to control; *** - $p < 0.001$ compared to control.

It can be observed that at 50 µM CIT, no differences in malformation development were observed at either 48 or 72 hours compared to the control. This confirm the results obtained by Csenki et al. (Csenki et al., 2021), who did not observe any sublethal effects in zebrafish embryos exposed to 25 µM CIT at 120 hpf. However, at the concentration of 150 µM CIT and72 hours of exposure, approx. 50% of individuals have developed malformations, primarily oedema or body curvature. Notably, tremors were also observed at this concentration after 72 hours. This observation suggests a potential neurotoxic effect of CIT (Brotzmann et al., 2021). In fact, other authors have reported that lower CIT concentrations (10 µM) interferes with neural activity and ganglia formation in embryonic zebrafish (Tsai et al., 2023). Lastly, at the highest concentration studied, 300 µM CIT, malformation development has been observed at 48 hours of exposure, with all individuals exhibiting oedema and nearly all individuals (27 out of 30 hatched fish) displaying body curvature.

Therefore, it can be concluded that CIT promotes malformations during zebrafish embryo development in a concentration-dependent manner. On the other hand, exposure to CIT did not affect the embryos' pigmentation.

Regarding alterations in heart function, as it is evidenced in Figure 3, at both times of exposure, 48 and 72 hours, 150 and 300 µM CIT cause a reduction in heartbeat frequency. This reduction could be attributed to the development of malformations and cardiac abnormalities, according to Wu et al, who demonstrated that CIT exposure during embryonic stages can impair heart development, resulting in reduced cardiac cavities, blood accumulation, and improper formation of the cardiac circuit (T. S. Wu et al., 2013).

3.2 Study of the influence of CIT on ROS production in zebrafish embryos

Figure 4 shows the production of ROS in zebrafish embryos after 50 and 150 µM CIT exposure for 72 h.

After 150 µM CIT exposure to zebrafish for 72 h, the production of ROS was distributed more extensively throughout the body, particularly in the outer regions. Their presence was noticeable in areas with deformities, such as the yolk, where oedema formation becomes apparent. However, ROS production was not observed at the visceral level, at 50 µM CIT.

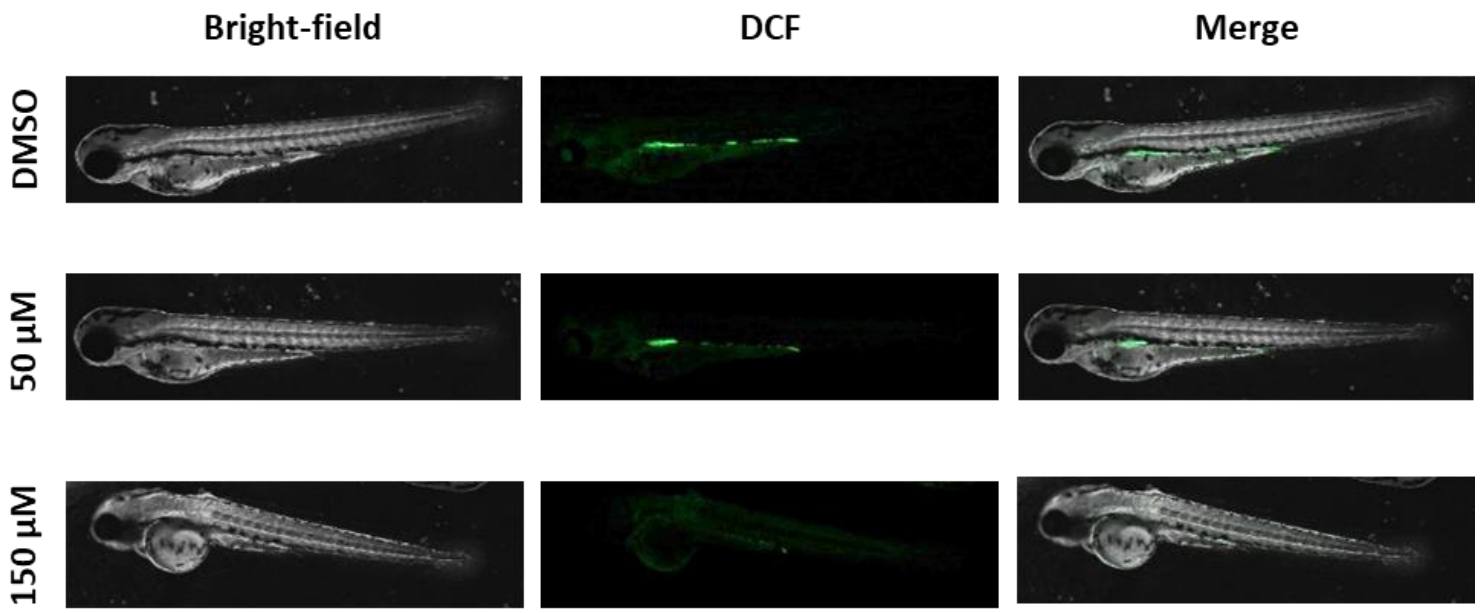

Figure 4. Production of ROS in zebrafish embryos exposed to 50 and 150 µM CIT for 72 hours. The intensity of fluorescence produced by ROS generation was obtained using the dichlorofluorescein di acetate (H2-DCFDA) probe added 30 minutes prior to taking the photos. The bright-field image, the fluorescence image produced by the DCF-ROS reaction, and the overlay of both images are presented.

3.3 Impact of Citrinin on apoptosis, necrosis, inflammation processes and oxidative stress response

In order to a better understanding of mechanisms of toxicity produced by CIT, the expression of genes associated with various pathological processes was determined in zebrafish embryos exposed to 50, 150, and 300 µM CIT for 72 hours. In this context, the genes apaf1 and bax were analyzed to study the apoptosis process, as they are known to play a crucial role in initiating this pathway of cellular death (Anand et al., 2021; Orzáez et al., 2014). To understand the cellular death pathways, the process of necrosis has also been investigated by analyzing the real-time expression of the ripk3 gene. This gene participates in cellular necroptosis, which refers to regulated necrosis (Tonnus et al., 2021; Zhang et al., 2022).

Additionally, the inflammatory process and the oxidative stress produced, which are highly relevant pathological factors, have been also determined. In this regard, the expression of the genes IL-8 and CXCL-CIC, which are induced during an inflammatory process, has been analyzed (Lv et al., 2022; Oehlers et al., 2010). Finally, the expression of gpx has been studied as a marker of the oxidative stress response (Yang et al., 2021). The results obtained from s the expression of these genes are presented in Figure 5. The genes associated with apoptosis, apaf1 and bax, showed a significant increase (1.21 and 1.55, respectively) in their expression ($p < 0.05$) after 72 hours of exposure to 300 µM CIT, compared to the control (Figs. 5a and b). The process of necrosis was also affected by 300 µM CIT, inducing a significant ($p < 0.05$) 1.94-fold increase in the expression of ripk3, compared to the control (Fig. 5c). Additionally, gpx also exhibited a significant ($p < 0.001$) increase in expression, reaching a 1.59-fold increase, compared to the control (Fig. 5d). The CIT induced the expression of CXCL-CIC ($p < 0.05$), a gene associated with the inflammatory

response (Fig. 5e). Although not statistically significantin the expression of IL-8 was observed (Fig. 5f).

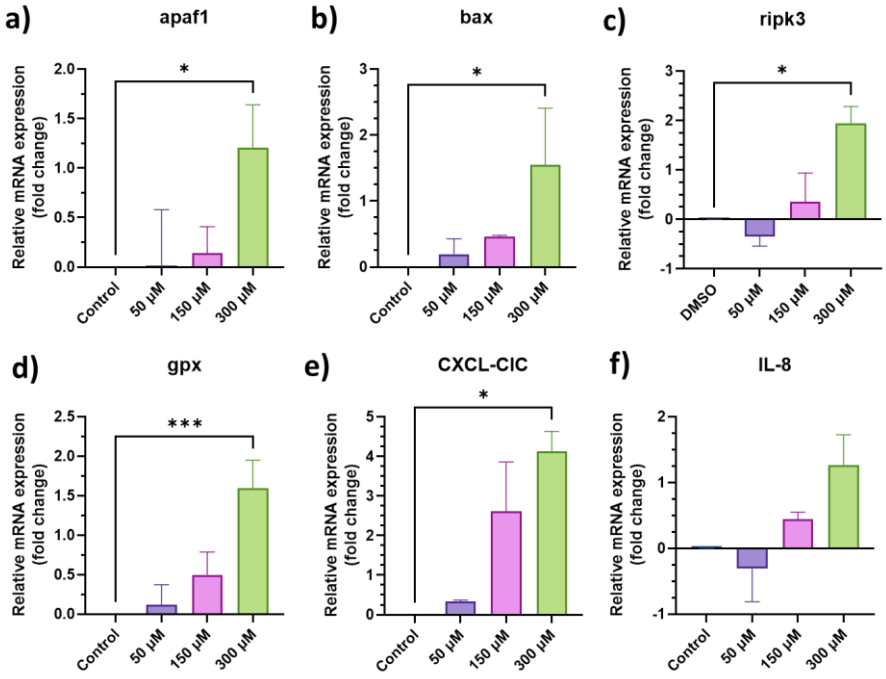

Figure 5. Effect of CIT on a) apaf1, b) bax, c) ripk3, d) gpx, e) CXCL-CIC and f) IL-8 expression in zebrafish embryos exposed to 50, 150 and 300 µM CIT for 72 h. The relative mRNA expression levels were measured by RT-qPCR. The average of the target gene values was normalized to the corresponding β-actin value and expressed as fold change compared to the control. Data are expressed as mean ± SEM of three independent experiments (n = 3) with 3 replicates each. (*) $p \leq 0.05$ indicates a significant difference compared to the corresponding control.

These findings demonstrate that 300 µM CIT causes alterations through the development of inflammation and oxidative stress, and it induces apoptosis and regulated necrosis in zebrafish embryos. These observations are consistent with the results reported by Wu et al. (T. S. Wu et al., 2012), who observed the induction of proinflammatory genes, specifically COX2a, TNF-α, and IL-1β, in zebrafish embryos exposed to CIT at 24 hpf to 72 hpf. Wu et al. (J. Wu et al., 2023) also observed an increase in protein expression of bax and bax/Bcl2 ratio in male Kunming mice after intragastric administration of different doses of CTN (0, 1.25, 5, or 20 mg/kg BW) one of CIT metabolites, confirming the induction of the apoptosis mechanism by CIT. Similarly, Wang et al. (Wang et al., 2023) noted an increase in gene expression of bax in zebrafish exposed to 0.635 mg/L CIT for 7 days.

4. Conclusion

In conclusion, the present study investigated the toxic effects of CIT using the zebrafish model. Exposure to the highest concentration of CIT (300 µM) resulted in malformations in 100% of zebrafish embryos, while no malformations were observed at the lowest concentration tested (50 µM). The influence of CIT on the production of ROS has also been observed, with differences in the distribution of these oxidant molecules in zebrafish compared to the control. Lastly, the induction of genes related to apoptosis, necroptosis, inflammation, and oxidative stress suggests that the toxic effects of CIT are associated with these pathological processes.

In summary, this study provides valuable information on the acute toxic effects of CIT during embryo development using the zebrafish model. These findings contribute to a better understanding of the mechanisms of CIT toxicity and could have important implications for risk assessment and prevention of exposure to this mycotoxin.

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Effect of natural extracts on citrinin-induced cytotoxicity in SH-SY5Y cells

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Toxins (Under review)

Abstract

Citrinin (CIT) is a mycotoxin commonly found in grains, as well as in fruits, herbs, and spices. Its toxicity primarily affects the kidneys and liver. Meanwhile, the by-products of the agri-food industry, particularly from fishing and aquaculture, contribute significantly to environmental concerns but can also serve as valuable sources of bioactive compounds. Additionally, microalgae like spirulina offer compounds of interest, rich in nutrients and antioxidants. This study aims to reduce CIT's toxicity on SH-SY5Y cells using natural extracts from spirulina and fish by-products. The combination of these extracts with CIT has shown increased cell viability up to 15% for fish by-products extract and about 10% for spirulina extract, compared to CIT alone. Furthermore, a notable reduction of up to 63.2% in apoptosis has been observed when combining the fish by-product extract with CIT, counteracting the effects of CIT alone. However, the extracts' effectiveness in preventing CIT toxicity in the cell cycle remains unclear. Overall, considering these nutrient and bioactive compound sources is crucial for enhancing food safety and mitigating the harmful effects of contaminants such as mycotoxins. Nevertheless, further studies are needed to investigate their mechanisms of action and better understand their protective effects more comprehensively.

Keywords: citrinin, spirulina, fish by-products, mycotoxin, SH-SY5Y

1. Introduction

Mycotoxins, toxic metabolites produced by certain fungi, have become increasingly relevant in scientific, health, and agro-food contexts. These compounds can contaminate various agricultural products such as cereals, fruits, nuts, and processed foods, becoming a significant threat to food safety and human health [1].

In particular, citrinin (CIT), a mycotoxin produced by species of the *Aspergillus*, *Penicillium*, and *Monascus* genera, has raised growing concerns due to its toxicity and occurrence in food and feed [2]. CIT has been associated with adverse effects on the renal and hepatic systems, and its presence in grain-derived and fermented products presents considerable challenges for the food industry and regulatory agencies in terms of contamination control and prevention [3].

In addition to mycotoxins, the generation of by-products and waste in the agro-food industry is a critical issue of concern. The production and processing of food result in the generation of by-products such as fruit peels, animal bones, and processing residues. These by-products can present both environmental and economic challenges., but they also hold significant potential for obtaining valuable bioactive compounds, including antioxidants, bioactive peptides, and enzymes.

Among the relevant by-products, those originated from the fishery industry deserve special attention. Fishery by-products, such as heads, backbones, skin and viscera, represent a rich source of bioactive compounds, including peptides, oils, collagen, and minerals, which can find applications in functional foods and pharmaceutical products [4]. To harness these by-products in a sustainable and efficient manner can contribute to the

valorization of the fishery industry and the reduction of environmental impact [5,6].

In the quest for new sources of bioactive compounds, microalgae have emerged as a promising alternative. In particular, spirulina, a green-blue microalga with high nutritional value, has stood out for its exceptional content of proteins, vitamins, minerals and, antioxidant compounds [7]. Spirulina has demonstrated a wide range of health benefits, including antioxidant, anti-inflammatory and, anticancer properties and, has become a key ingredient in the formulation of functional foods and nutritional supplements [8].

The objective of this study is to examine the impact of combining the mycotoxin CIT with two different natural extracts, one derived from fish by-products and the other from the microalgae spirulina, in order to mitigate the toxic effects induced by this mycotoxin by an *in vitro* method. The research will assess cell viability using the MTT assay, alongside other cellular processes including cell cycle and apoptosis by flow cytometry. Finally, in order to determine the cytoprotective effects of the extracts obtained, a comparative analysis will be conducted, between the effects of CIT in a cell culture model tested alone and CIT in combination with each of the extracts proposed.

2. Results and Discussion

2.1. Effect of spirulina and fish by-products extracts on cell viability

2.1.1 Fish by-products extracts

Extracts obtained from fish by-products (head, skin, viscera and backbones) by agitation (control) and pre-treatment with pulsed electric fields (PEF), were characterized in a previous study and evaluated on SH-SY5Y cells

at concentrations ranging from 0.78% to 25% (v/v) by MTT assay, at 24 h of exposure (data not shown).

After assessing each by-product individually at 24 hours of exposure, it was found that the head extract obtained by PEF (HPO) increased cell viability by 25% at a concentration of 8.25% (v/v in medium) and by 33% at a concentration of 12.5% (v/v in medium). However, both the skin extract obtained by PEF and the skin extract obtained by agitation (control) showed a significant decrease in cell viability. In contrast, the extracts of backbone and viscera did not exhibit any significant changes when compared to the control (data not shown).

Based on these results, the HPO extract was selected for future experiments as it had the highest content of antioxidant compounds and showed the best response in terms of cell viability. Concentrations of 8.25% (v/v) and 12.5% (v/v) were chosen to be combined in further experiments with the mycotoxin as they produced the most promising results on cell viability.

2.1.2 Spirulina ethanolic extract

The spirulina (*Arthrospira platensis*) ethanolic extract (Sp) was obtained by agitation with ethanol. It was characterized in a previous study (data not shown). The concentrations chosen for combining the Spirulina extract with citrinin were 31.25 µg/mL and 62.5 µg/mL based on previous tests performed in our laboratory (data not shown). It should be noted that at the concentrations selected, the Sp extract did not exhibit any reduction in cell viability (data not shown).

2.2. Cytotoxic effects respect to the combination of natural extracts with citrinin

2.2.1 HPO extract in combination with citrinin

Figure 1 presents the results obtained for cell viability following the combination of HPO extract at two different concentrations: 8.25% and 12.5% (v/v). At the lowest tested concentration of CIT, 25 µM, no significant differences were observed among CIT tested individually and the HPO extracts. However, a clear protective effect is evident when the HPO extracts (both concentrations) were combined with 38.75 µM and 50 µM CIT. When applied at a concentration of 38.75 µM CIT, cell viability increased from 79.2% to 86.3% (8.25% HPO) and 87.6% (12.5% HPO). It is noteworthy that at 50 µM CIT, the simultaneous use of the HPO extract increases viability by 15%, reaching 83.5% (8.25% HPO) and 85.6% (12.5% HPO), compared to the 70.9% viability observed with CIT alone. It is also important to highlight that no significant differences were observed between the two HPO tested concentrations. Thus, the lowest concentration (8.25% HPO) was selected for the subsequent toxicological studies.

This cytoprotective effect may be attributed to the action of different compounds present in fish extracts, such as antioxidants or bioactive peptides, among other nutrients. Some of these compounds also exhibit potential antioxidant activity, and can help to improve cell viability.

This finding are in agreement with other studies reported in the literature. Taroncher et al. [9] observed that hydrolysates from fish by-products, specifically salmon backbones and salmon viscera, increased 27.0% and 51.2% cell viability, respectively, by MTT assay. In addition, the same authors reported that higher increases in cell viability measured by total protein content assay. Cell viability increased 214% in mackerel head hydrolysate, compared to control, followed by salmon head and herring viscera

hydrolysates, which achieved cell viability values of 140% and 139% compared to control (100%), respectively. These results indicate that hydrolysate exposure enhanced cell viability in Caco-2/TC7 cells. Moreover, it has been demonstrated that these extracts possess antioxidant capacity, because of they contributed to the reduction of oxidative stress caused by reactive oxygen species in Caco-2/TC7 cells [9].

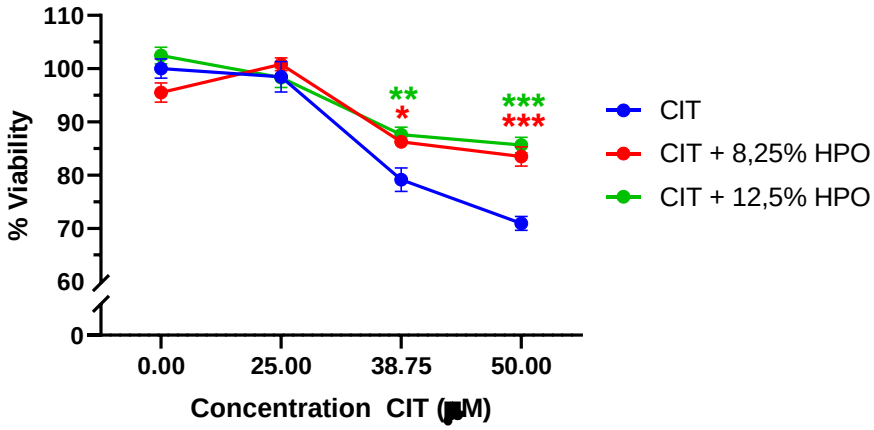

Figure 1. Figure 2. Effect of CIT (25, 38.75 and 50 µM), and CIT with Head PEF Opt. extract (HPO) (8.25 and 12.5% (v/v)) on SH-SY5Y cell viability. The results are presented as Mean ± SEM of three independent experiments. * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$ *vs* CIT.

Additionally, it has been also demonstrated that fish extracts can enhance the antioxidant activity of natural antioxidant compounds. Concerning this, Taroncher et al. [10] demonstrated that cell proliferation was enhanced by the combination of vitamin E or resveratrol with hydrolyzed protein from salmon fish. Similarly, another mixture containing hydrolyzed protein from both

salmon and mackerel fish blend with vitamin C and quercetin, also demonstrated an increased cell proliferation.

On the other hand, Shashikumar et al. [11] observed that primary hepatocytes, which had been damaged by D-galactosamine, exposed to fish oil facilitated the restoration of their functions. That is, the production of albumin and the regulation of various liver enzymes (LDH, ALP, GOT, and GPT), altered by the hepatotoxic injury. The recovery functions were dependent on the fish oil concentration, and the protective effect was observed at 10 and 20 µg/mL of oil.

Finally, Omerovic et al. [12] reported that the administration of an extract obtained from cod muscle decreased mortality of mice treated with the anticancer drug doxorubicin, likely due to cardioprotection through antioxidant activity.

2.2.2 Spirulina ethanolic extract (Sp.) in combination with CIT

Figure 2 shows the results of cell viability obtained from the SH-SY5Y cells exposed to the combination of Sp. extract and CIT. Concentrations of 31.25 µg/mL and 62.50 µg/mL of Sp. extract were selected, and 25 µM, 38.75 µM, and 50 µM were tested for CIT. The results demonstrate the remarkable effectiveness of the Sp. extract in protecting cells against CIT cytotoxicity. Even at the lowest concentration tested (25 µM CIT), an increase in cell viability was observed with both Sp extracts, resulting in viability values of 107.1% (31.25 µg/mL Sp.) and 106.5% (62.5 µg/mL Sp.), compared to control (98.22%). The highest increase in cell viability was observed at the concentration of 38.75 µM CIT, and Sp. extract (31.25 µg/mL), reaching 89.5%; whereas the extract at 62.5 µg/mL only achieves 86.1% viability, which is not significantly different from the viability observed with the mycotoxin alone (80.4%). Furthermore, at

50 µM CIT, both extracts exhibit similar results, significantly increasing cell viability from 77.1% (CIT) to 87.0% (31.25 µg/mL) and 86.0% (62.5 µg/mL).

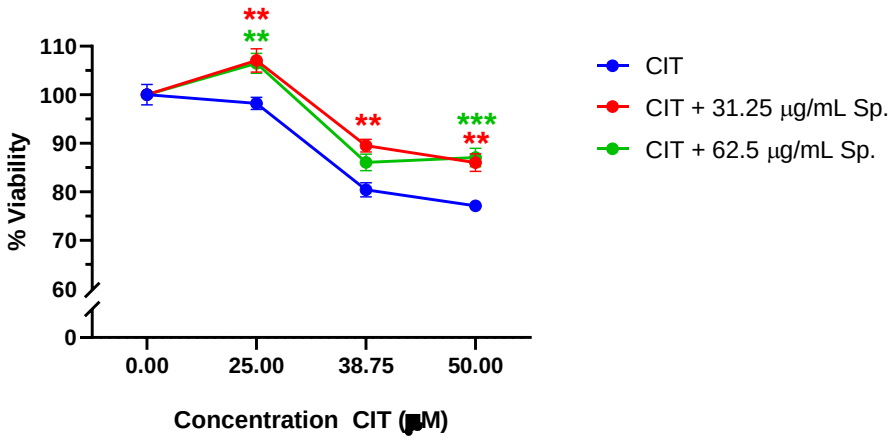

Figure 2. Effect of CIT (25, 38.75 and 50 µM), and CIT with ethanolic spirulina extract (Sp.) (31.25 and 62.50 µg/mL) on SH-SY5Y cell viability. The results are presented as Mean ± SEM of three independent experiments. * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$ *vs* CIT tested individually.

Other researchers have also documented the protective effect of microalgae extracts against toxins exposure in cell cultures. In this context, Kang et al. [14] reported a protective effect of a hydrolysate from the microalga *Navicula incerta* against damage caused by a 1-hour exposure to 1 M ethanol in HepG2/CYP2E1 cells (HepG2 cells transfected with human CYP2E1 cDNA). These authors described that after a 48-hour exposure to the hydrolysate, a protective effect was observed against a subsequent 48-hour exposure to 1 M ethanol. It was due to enhanced mitochondrial antioxidant activity and improved functionality, owing to the components present in this protein hydrolysate.

On the other hand, Ben Saad et al. [15] described a protective effect of an ethanolic extract from the red algae *Alsidium corallinum* against potassium bromate-induced toxicity in mice. These authors observed that the algae extract helped the mice regain body weight compared to those who were only administered with toxicant, resulting in weight loss. It was hypothesized that the weight gain was due to the minerals contained in the extract (elevated levels of Ca, Mg, Fe, Cu, and Zn). Additionally, the administration of the *Alsidium corallinum* extract led to the restoration of normal hematological parameters, such as hematocrit, hemoglobin and, platelet and red blood cell count, which were altered by exposure to $KBrO_3$. The protective effect could be attributed to the presence of flavonoids in the extract, which prevent membrane fragility [15].

As evidenced in a previous study carried out in our laboratory, the selected spirulina extract of this research is rich in antioxidant compounds such as polyphenols and pigments like phycocyanin. Additionally, it contains fatty acids (including γ-linolenic acid, eicosadienoic acid, and eicosatrienoic acid), as well as essential minerals such as Mg, P, Ca, Fe, and Zn. Thus, according to their results, these constituents may account for the observed cytoprotective effect against CIT exposure in SH-SY5Y cells.

2.3 Effect of natural extracts combination with CIT regarding cell cycle

Cell-cycle analysis was conducted following the protocol described by Zingales et al. [15], employing Vindelov's PI staining solution. This fluorescent dye has the ability to intercalate with double-stranded nucleic acids, allowing for accurate and efficient assessment of cellular DNA content through flow cytometric analysis.

Figure 4 describes the results obtained for cell cycle analysis in SH-SY5Y cells after 24 h of exposure to CIT, CIT+Sp. and CIT+HPO. As can be seen, CIT

alone produces a concentration-dependent increase of cells in S-phase and especially in G2/M compared to the control condition, with 50 µM being the condition with the greatest increase in both cases (an increase of 0.58 and 1.74 times for S and G2/M phase, respectively, compared to CIT control). With regard to the combination of CIT with the extracts, as can be observed, during the G2/M phase, there is a higher proportion of cells in this phase in the presence of the CIT+HPO combination compared to CIT alone (in a range from 0.40 to 1.42 times more, compared to each concentration of CIT alone), across all tested conditions (Control, 25, 38, 75, and 50 µM) (Figure 4c). The fact that this increase is already observed in the HPO Control condition raises suspicions that it may be a result of enhanced cell proliferation facilitated by the HPO extract.

Regarding the S phase, no significant differences were observed among the tested conditions. In fact, the behavior remains consistent across all cases, whether it is CIT alone or in combination with the extracts.

Lastly, as shown in Figure 4a, a significant reduction in the proportion of cells in the G0/G1 phase at the highest concentration of 50 µM was observed. There is a reduction in this phase, both in the spirulina extract ($p<0.01$) and in the HPO extract ($p<0.001$) compared to 50 µM CIT alone.

Therefore, it can be concluded that none of the tested extracts induces relevant changes in the effect of the CIT mycotoxin on the cell cycle in SH-SY5Y cells.

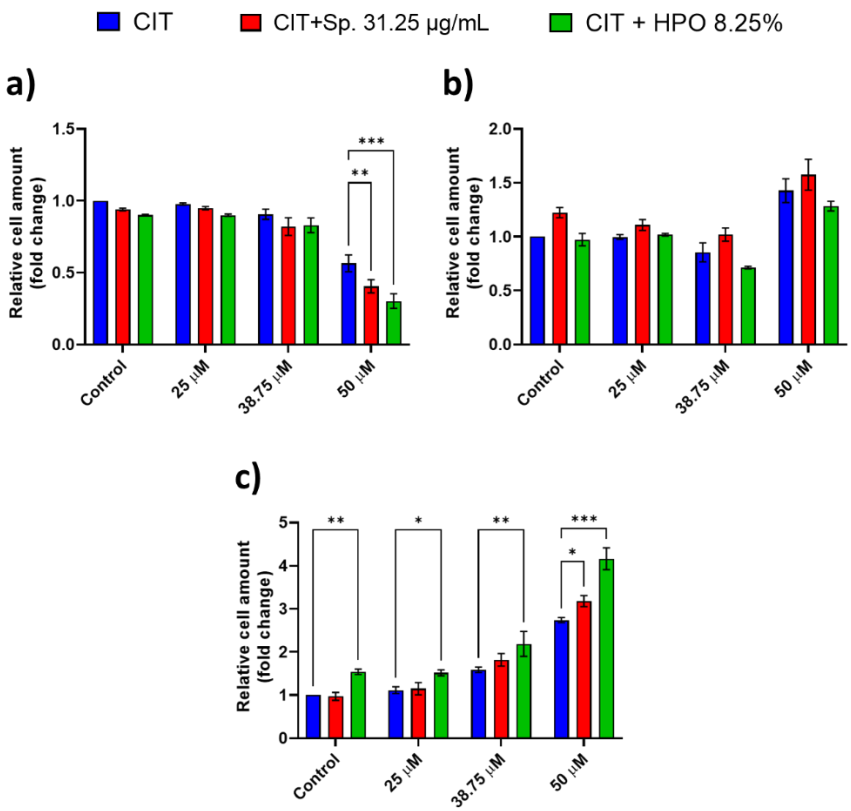

Figure 3. Flow cytometry results for cell cycle being a) G0/G1 phase; b) S phase and c) G2/M phase of SH-SY5Y cells after 24h of exposure to CIT, CIT+EtOH-Sp and CIT+HPO. Results are expressed as Mean ± SEM of three independent experiments. * - p<0.05; ** - p<0.01; *** - p<0.001 *vs* CIT.

The extracts studied in this research did not demonstrate a protective effect on the toxic action of CIT at the cellular level. There are different works in the literature that use extracts obtained from marine biomass to mitigate the harmful effects of different toxins on the cell cycle. For instance, Yang et al. [16] observed that the addition of an aqueous extract of the seaweed *Gracilaria tenuistipitata* (AEGT) protected H1299 cells from damage caused by

24 h of exposure to H_2O_2. Specifically, the authors noted that the extract reversed the G2/M phase arrest induced by H_2O_2 in H1299 cells when it was combined with 4 mg/mL of AEGT, achieving similar results to the control cells.

Furthermore, Lee et al. [17] reported a cytoprotective effect when applying an ethanolic extract of spirulina (*A. platensis)* to nHDF cells after UVB irradiation. They observed an increase in S and G2/M phases (1.7 fold and 1.3 fold, respectively, compared to control) These authors found that the highest concentration of the extract (20 µg/mL) during the UVB radiation exposure, reduced cell population values for the G1/G0 phase(74.23%), for the S phase (8.99%), and for the G2/M phase (11.81%) of the control (79.11%, 6.89%, and 10.50% for the G0/G1, S, and G2/M phases, respectively).

The differences between the results obtained in our study and those reported by Lee et al. may be attributed to the different sources of cellular damage, and the type of cells employed. Lee et al. used human dermal fibroblasts (nHDFs), and Yang et al. used human lung adenocarcinoma cells (H1299), whereas we utilized SH-SY5Y cells, which are a neuroblastoma tumor cell line.

2.4 Effect of natural extracts combination with CIT regarding apoptosis process

Cell death can occur through necrosis or apoptosis. During apoptosis, phosphatidylserine (PS) is externalized to the outer membrane. Annexin V-FITC/PI double staining is used to distinguish between apoptotic and necrotic cell populations. Annexin V, a calcium-dependent phospholipid-binding protein, binds to cells displaying externalized PS, while PI binds to the DNA of necrotic or dead cells. Viable cells exclude PI, early apoptotic cells are Annexin V-FITC positive, and late apoptotic cells, transitioning to necrosis, are both Annexin V-FITC and PI positive [18]. This staining technique helps to

identify and differentiate these cell states based on their membrane integrity and apoptotic progression.

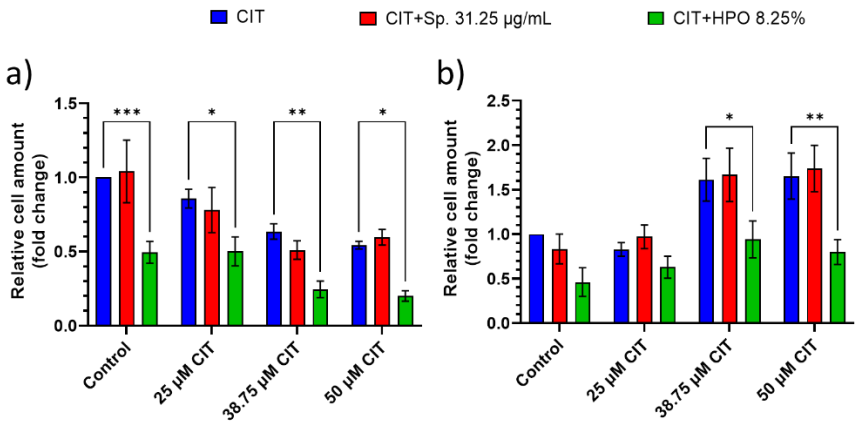

Figure 4. a) Early and b) Late apoptosis induction in SH-SY5Y cells after exposure of CIT, CIT+Sp. and CIT+HPO for 24 h. Results are expressed as Mean ± SEM of three independent experiments. * - p<0.05; ** - p<0.01; *** - p<0.001 *vs* CIT.

The effect of the mycotoxin CIT and its combination with the Sp. and HPO extracts on apoptosis is depicted in Figure 3. As can be observed, there is a significant reduction in apoptotic cells in a concentration-dependent manner for all treatments, CIT and its combination with the extracts. The highest decrease in early apoptotic cells was observed when CIT is combined with the HPO extract compared to CIT tested alone. In particular, early apoptotic cell population decrease to 50.6%, 41.5%, 61.5%, and 63.2% for Control (0 µM CIT), 25, 38.75, and 50 µM CIT, respectively. It is noteworthy that even in the control sample, the HPO extract alone demonstrates the ability to decrease the apoptotic cell population, potentially linked to the higher cell viability

associated with this extract observed. Additionally, a significant decrease in apoptosis is observed even at the highest concentration tested (50 µM), from 0.54 (50 µM CIT) to 0.20 (50 µM CIT + HPO) folds, indicating a protective capacity of the extract even under high toxin concentrations.

On the other hand, regarding late apoptosis, Figure 3B shows late apoptotic of CIT and its extract combination after 24 h of exposure in SH-SY5y cells. Differences between the addition of HPO extract and the CIT tested alone are only observed at the two highest concentrations tested, 38.75 and 50 µM. The value of late apoptosis is approximately half of that obtained with CIT alone, decreasing from 1.61 to 0.94 folds compared to the CIT tested alone for 38.75 µM, and from 1.65 to 0.79 for 50 µM CIT tested alone. The anti-apoptotic activity demonstrated by HPO extract may be attributed to the presence of peptides that could interfere with the apoptosis process induced by the CIT mycotoxin.

On the contrary, there were no changes observed in either the early apoptotic or the late apoptotic cells when comparing SH-SY5Y cells exposed to CIT+Sp. with the ones exposed to CIT alone, at any of the CIT concentrations tested. This result suggests that the mechanism through which cell viability increase in CIT+Sp. SH-SY5Y exposed cells compared SH-SY5Y cells exposed to CIT alone is unrelated to the apoptotic process.

The results obtained in this study are consistent with findings reported in other studies in the literature. Remarkably, Gómez et al. [19] evaluated various cytoprotective properties of a hydrolysate of red tilapia (*Oreochromis spp.*) viscera in Caco-2 cells. The authors found that pre-treatment with the smallest peptide fraction with a molecular weight below 1 kDa (isolated by ultrafiltration from the whole hydrolysate) prior to applying 5 mM of H_2O_2 resulted in a 28.8% reduction in late apoptotic cells, compared to cells exposed only to H_2O_2, achieving values similar to control condition.

The research conducted by Gao et al. [20] investigated the protective effect of a hydrolysate derived from tilapia by-products, specifically the skin. The study revealed that the addition of tilapia skin peptides at a concentration of 50 ng/mL provided protection to CT-26 cells against apoptosis induced by the addition of LPS (1 µg/mL). This effect was also observed in HT-29 cells.

Lastly, Hoon Song et al. [21] described similar results. The authors studied the impact of fish collagen peptides (FCP) on mouse thymic cortical epithelial reticular cells exposed to cisplatin, a commonly used chemotherapy drug. Cisplatin treatment changed proteins associated with the apoptotic process, causing a decrease in the expression of anti-apoptotic proteins and an increase in the expression of pro-apoptotic proteins. However, pre-treatment with 0.08% FCP for 24 hours mitigated these changes, restoring the expression of apoptosis-related proteins closer to their normal levels. FCP demonstrated the ability to enhance the expression of certain protective proteins while reducing the levels of harmful ones when compared to cells treated with cisplatin alone.

4. Conclusions

In conclusion, the results of this study demonstrate the promising cytoprotective effects of fish by-products extract, specifically sea bass head extract, in reducing the toxicity of CIT by improving cell viability and decreasing apoptosis. Additionally, the Sp. extract showed potential in enhancing cell viability when combined with CIT. However, further research is needed to elucidate the underlying mechanism of the cytoprotective effect of Sp. and fish extract. Overall, this study highlights the importance of further exploration and in-depth investigations to fully harness the potential of fish by-products and spirulina extracts in cytoprotection.

5. Materials and Methods

5.1 Reagents

Sigma Chemical Co (St. Loius, MO, USA) provided the following reagents: culture medium DMEM Ham's-F12, penicillin, streptomycin, trypsin/EDTA solutions, phosphate buffer saline (PBS), fetal bovine serum (FBS), Ribonuclease A (RNase), tetrazolium bromide (MTT), trizma base (Tris), propidium iodide (PI), t-octylphenoxypolyethoxyethanol (Triton-X 100), and HEPES. Human recombinant annexin V-FITC conjugate was obtained from Invitrogen (USA). Merck KGaA (Germany) supplied NaCl. The CIT standard was also purchased from Sigma-Aldrich (St. Louis Mo. USA), and dimethyl sulfoxide (DMSO) was acquired from Fisher Scientific (Geel, Belgium). CIT standard was resuspended in pure DMSO.

5.2 Samples

The sea bass head extract obtained by pulsed electric fields (HPO) was obtained following the method described by Wang et al [22]. Briefly, the sea bass head was treated by pulsed electric fields, using the following parameters: a specific energy of 220 kJ/kg, a field strength of 1 kV/cm, a pulse duration of 100 ms and 2 Hz as frequency, with a unipolar square wave pulse. Then, it was stirred at 200 rpm for 21.35 h in water. Finally, the sample was centrifuged at 3050 x g for 10 min in a 5810R centrifuge (Eppendorf AG, Hamburg, Germany) and the supernatant was collected

In addition, spirulina (*Arthrospira platensis*) ethanolic extract (Sp.) was obtained as reported by Sansone et al. in a previous article [23]. To sum up, 5 g of microalgae were suspended in 50 mL of pure EtOH in the dark shaking at 500 rpm for 30 min. Then, the mixture was separated by centrifugation at 4000 rpm for 10 min, and the liquid phase was transferred to a clean tube. The pellet was resuspended in 50 mL of EtOH and extracted a second time. After

repeating the separation process by centrifugation, both extracts were pooled.

5.2 Cell culture

Human neuroblastoma (SH-SY5Y) cells were cultured in DMEM Ham's-F12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. The cells were maintained under standard conditions at 37°C, 5% CO_2, and a pH of 7.4 in a 95% air atmosphere with constant humidity. The culture medium was changed every 2-3 days. To attain the desired concentrations of CIT, CIT+ Sp. and CIT+HPO, appropriate solutions were prepared and added to the culture medium, ensuring a final DMSO concentration of ≤1% (v/v). Control groups, containing equivalent amounts of solvent (DMSO), were included in all experimental.

5.3 Cell viability assay

The cell viability was determined using the MTT assay. For that purpose, SH-SY5Y cells were seeded in 96 well plates at density of 30.000 cells/well. Once the cells reached the 80% of confluence, CIT at different concentrations with or without the different extracts tested (Sp. or HPO) was added. After 24h of exposure, the supernatant was removed and 200 µL of fresh medium with 50 µL of MTT (5 mg/mL) were added. Then, the cells were incubated at 37 °C for 3h in darkness. Finally, the supernatant was removed and DMSO was added to solubilize the formazan crystals. Absorbance was read at 630 nm using a VICTOR3 1420 multilabel plate counter reader (PerkinElmer, Turku, Finland).

5.4 Cell cycle analysis

The effects on SH-SY5Y cell cycle were determined by flow cytometry as previously described by Zingales et al. [15], using Vindelov's PI staining

reagent. For that purpose, 70.000 cells/well were seeded in 6 well plates. Then, 24h later the cells were exposed to the different concentrations of CIT (25, 38.75 and 50 µM), with Sp. or HPO extracts and without them. After 24h of exposure, cells were trypsinized and incubated at 4 ˚C for 30 min in darkness with 500 µL of Vindelov's PI staining solution. Finally, 20,000 events were analysed for each sample in a BD LSRFortessa (BDBiosciences, Franklin Lakes, NJ) flow cytometer. The Vindelov's PI staining reagent was prepared mixing 10 mM Tris, 0.1% Triton X-100, 40 µg/mL RNase, 50 µg/mL of PI and 10 mM NaCl in PBS. Three independent experiments were performed.

5.5 Apoptosis measurement

The apoptosis process in SH-SY5Y cells was performed following the protocol described by Zingales et al. [24]. The double stain Annexin V-FITC/PI was used for this purpose. To carry out the experiment, 70.000 cells/well were seeded in 6 well plates and, 24h later they were exposed to the different concentrations of citrinin (25, 38.75 and 50 µM), with spirulina or fish extracts and without them. After 24h of exposure, cells were trypsinized and resuspended in 500 µL of Hepes-Ca^{2+} buffer containing Annexin V-FITC/PI. Lastly, 10,000 events were acquired and analysed by a BD LSRFortessa (BD Biosciensces) flow cytometer. Viable cells (Annexin V-FITC– / PI–), early apoptotic (Annexin V-FITC+ / PI–), late apoptotic (Annexin V-FITC+ / PI+) and necrotic (Annexin V-FITC+ / PI–) cells were evaluated from the total population of cells. Three independent experiments were performed.

5.6 Statistical analysis

The statistical analysis was performed using the software GraphPad Prism 9 (GraphPad Software, California, USA). Results were expressed as Mean ± SEM of three independent experiments. The statistical analysis of the results was carried out using Student's t-test, comparing each extract combination

vs. CIT (alone) samples. Differences were considered statistically significant when $p \leq 0.05$.

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Conflicts of Interest: The authors declare no conflict of interest.

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DISCUSIÓN

DISCUSSION

La acuicultura se considera una fuente importante de alimentos de alta calidad frente a la reciente problemática del incremento de la densidad de la población. En este sentido, los subproductos marinos y las microalgas presentan diversas propiedades beneficiosas que los convierten en productos valiosos tanto para la industria alimentaria y como para la farmacéutica. El valor nutricional de estos alimentos y la bioactividad de estos subproductos marinos y sus derivados tienen una amplia variedad de aplicaciones. Entre los subproductos marinos se incluyen nutrientes como aceites de alta calidad, proteínas y polisacáridos y compuestos bioactivos como péptidos bioactivos y polifenoles, los cuales pueden ser utilizados por la industria alimentaria. Sin embargo, para ello se requiere el desarrollo de técnicas comerciales que procesen los residuos de subproductos marinos y le aporten un valor añadido.

La investigación sobre la reutilización de residuos de pescado y el aprovechamiento de fuentes alternativas de nutrientes como las microalgas puede contribuir a fomentar el crecimiento industrial a través del desarrollo sostenible. La fermentación, así como el uso de tecnologías innovadoras (PE y EFS) puede dar una segunda vida a los subproductos del pescado provenientes de la pesca o la acuicultura, así como potenciar el uso de microalgas. Por lo tanto, la biomasa marina, y en concreto los subproductos de pescado y las microalgas, como la espirulina o la chlorella, podrían ser una fuente importante de compuestos de interés para la industria alimentaria, mejorando su valor económico y minimizando el impacto ambiental de los residuos, contribuyendo de esta forma a la sostenibilidad de la producción pesquera y la acuicultura.

De los resultados obtenidos en esta tesis, se ha observado que, tras la fermentación de la carne y subproductos de lubina con bacterias lácticas aisladas del propio pescado, los principales ácidos fenólicos obtenidos fueron

el ácido DL-3-fenil-láctico y el ácido benzoico, con una concentración máxima de 466 y 314 ppb, respectivamente. La mejor actividad antioxidante se encontró en los extractos obtenidos tras la fermentación del caldo de subproductos de pescado por bacterias aisladas del colon (6502 µM equivalente de trolox (TE)) y del estómago (4797 µM TE). Además, se encontró una correlación positiva entre los ácidos fenólicos obtenidos tras el proceso de fermentación y la actividad antioxidante de las muestras. También se concluyó que las bacterias lácticas aisladas de lubina tenían una importante capacidad proteolítica y eran capaces de sintetizar ácidos fenólicos con capacidad antioxidante. Asimismo, se investigó la posibilidad de obtener compuestos antifúngicos mediante la fermentación de estos residuos. En este sentido, de un total de 30 bacterias lácticas aisladas de la lubina, se encontraron 7 con actividad proteolítica. Estas bacterias pertenecen al género *Lactobacillus*, y las dos cepas más activas fueron identificadas mediante reacción en cadena de la polimerasa (PCR) como *L. plantarum*. Además, varias cepas mostraron con una Concentración Inhibitoria Mínima (CIM) y Concentración Fungicida Mínima (CFM) de 1-32 g/L y 8-32 g/L, respectivamente.

La obtención de nutrientes y compuestos bioactivos a partir de subproductos de pescado mediante el uso de tecnologías innovadoras, concretamente PE ha sido evaluada. Tras un proceso de optimización mediante la metodología respuesta-superficie, la tecnología de PE mejoró la extracción de proteínas y compuestos antioxidantes de la cabeza y la piel de pescado, mientras que no supuso una mejora de la extracción en las muestras de vísceras. El proceso de extracción utilizado determinó el tipo de proteínas extraídas, observándose diferencias significativas entre las muestras extraídas sólo por agitación (muestras control) y las muestras a las que se les aplicó un pretratamiento con PE. Además, también se evaluó la extracción de macro- y

microminerales. El efecto de los PE influyó en la extracción dependiendo de la matriz y el mineral estudiado. Además, los metales pesados As, Cd, Hg y Pb presentes en los extractos fueron analizado. El pretratamiento con PE redujo la presencia de As en piel, vísceras y espina dorsal, entre el 18,25 y el 28,48%. También se analizaron los péptidos bioactivos potencialmente antioxidantes, revelando que el tratamiento de la muestra afecta directamente a la variedad de los mismos. Por último, se evaluó el efecto de los extractos sobre la viabilidad celular utilizando células SH-SY5Y, observándose un aumento de la viabilidad celular cuando éstas se exponían a extractos de cabeza de pescado.

Por otro lado, la tecnología de los PE también se ha investigado en la recuperación de nutrientes y compuestos bioactivos a partir de microalgas. En concreto, se ha estudiado la espirulina, por ser una de las microalgas más consumidas a nivel mundial. Para este propósito, se evaluaron los efectos del pretratamiento con PE (3 kV/cm, 100 kJ/kg) combinado con extracción a diferentes tiempos (5-180 min) y con diferentes disolventes (etanol/H_2O, 50:50, v/v; dimetilsulfóxido (DMSO)/H_2O, 50:50, v/v) con el fin de obtener las condiciones óptimas para la extracción de compuestos antioxidantes y pigmentos. Además, los resultados obtenidos se compararon con los de un tratamiento convencional (sin pretratamiento con PE pero con agitación constante). Tras realizar los diferentes experimentos, las mejores condiciones de extracción para recuperar los diferentes compuestos se obtuvieron tras aplicar PE combinado con la mezcla binaria etanol/H_2O, 50:50, v/v, durante 60-120 min. Aunque la extracción con PE fue eficiente durante todo el estudio, se obtuvieron mejores resultados a tiempos de extracción cortos (5-15 min). En este sentido, durante los primeros minutos de extracción se obtuvo una recuperación del 55-60%, 85-90% y 60-70% para clorofilas, carotenoides y compuestos fenólicos totales, respectivamente, en comparación con la

cantidad máxima total extraída. Estos resultados demuestran que los PE mejoran el rendimiento de extracción de compuestos bioactivos antioxidantes en microalgas y que ésta es una tecnología prometedora debido a su rentabilidad y sostenibilidad medioambiental.

Siguiendo con los estudios sobre la extracción de nutrientes y compuestos bioactivos a partir de espirulina, y con el fin de explorar otros métodos de extracción además de los PE, se llevó a cabo un análisis de la extracción de minerales, pigmentos y compuestos antioxidantes a partir de esta microalga utilizando la EFS. Para determinar el efecto citoprotector de los extractos obtenidos por EFS, éstos se expusieron a cultivos de células SH-SY5Y y se evaluó su efecto sobre la viabilidad celular. Se observó que la extracción de ficocianina mejoró con EFS en comparación con la extracción convencional, oscilando de 2,838 ± 0,081 mg/g de MS (control) a 6,438 ± 0,411 mg/g de MS (EFS). La EFS también mejoró las recuperaciones de clorofila A y carotenoides, aumentando de 5,612 ± 0,547 a 8,645 ± 0,857 mg/g MS y de 0,447 ± 0,096 a 0,651 ± 0,120 mg/g MS, respectivamente. En cuanto a los minerales, la EFS mejoró la recuperación de Mg, obteniendose un 77% más que con la extracción convencional. Además, la EFS mejoró la extracción de los ácidos palmitoleico, esteárico, g-linolénico, eicosadienoico y eicosatrienoco. El perfil fenólico, identificado mediante espectrometría de masas con Triple-TOF-LC-MS-MS, mostró diferencias entre el perfil de compuestos extraídos en la muestra control frente a los extraídos mediante EFS. En cuanto a los metales pesados, se observó una mayor extracción de Pb con la EFS, mientras que no se observaron diferencias significativas para el Hg entre ambas técnicas de extracción. Por último, los extractos obtenidos con la EFS mejoraron la viabilidad celular de las células SH-SY5Y respecto a los extractos obtenidos por métodos convencionales (control). Así pues, la EFS constituye una herramienta interesante para la extracción sostenible de

compuestos de alto valor añadido procedentes de espirulina, aunque es necesario controlar los contaminantes potencialmente tóxicos, como el Pb, en dichos extractos.

Por otro lado, debido al gran valor nutricional de la espirulina y de otras algas como la chlorella, además de legumbres e incluso subproductos de la industria quesera como el suero, se evaluó el efecto de la incorporación de estas microalgas y determinadas legumbres (soja, guisantes, lentejas y habas) a alimentos cárnicos procesados, particularmente a salchichas de cerdo y hamburguesas de pavo. Se analizaron diferentes parámetros de calidad y físico-químicos para determinar si pueden ser utilizados como sustitutivos de la proteína de soja, empleada actualmente en su elaboración. Los parámetros de color, pH, contenido en cenizas, aminoácidos totales, esenciales y no esenciales fueron significativamente diferentes entre los distintos tipos de hamburguesas de pavo preparadas. En este sentido, las hamburguesas de pavo elaboradas con proteína de guisante presentaron los valores más altos de pH y luminosidad, mientras que las muestras preparadas con haba mostraron el mayor enrojecimiento. La adición de haba y alga produjo un marcado aumento de ácido glutámico, lisina y ácido aspártico. Sin embargo, el perfil de sabor fue similar en las seis hamburguesas de pavo estudiadas (proteína de soja, guisante, lenteja, haba, chlorella y espirulina). El análisis discriminante de proyecciones ortogonales a estructuras latentes (OPLS-DA, por sus siglas en inglés) permitió clasificar las hamburguesas de pavo según las fuentes de proteína, en comparación con la soja (control). Los parámetros de textura, humedad y color resultaron ser los más importantes en la diferenciación entre las hamburguesas. No obstante, según el modelo OPLS supervisado, se observó que las habas poseían un perfil similar al de la soja (control). Por otro lado, respecto al análisis llevado a cabo en las salchichas de cerdo, se encontraron diferencias significativas para todos los parámetros

estudiados. El contenido en proteínas fue significativamente inferior en las salchichas elaboradas con proteína de guisante respecto al control (soja). Los parámetros de color (L*, a* y b*) cambiaron significativamente tras la incorporación de proteínas de microalgas. Además, se observaron diferencias significativas en el perfil de aminoácidos entre las hamburguesas elaboradas con las diferentes matrices. La inclusión de proteínas de espirulina produjo un aumento del contenido total de aminoácidos y de la relación entre aminoácidos esenciales y no esenciales. En este caso, también se llevó a cabo un OPLS-DA, en el que se clasificaron las salchichas de cerdo según la fuente proteica, en comparación con la soja (control). Los parámetros de textura (masticabilidad, gomosidad y dureza), seguidos del color y el pH, fueron los más discriminantes. Teniendo en cuenta los rasgos de textura, los parámetros fisicoquímicos y los perfiles de aminoácidos de los distintos tratamientos, las proteínas de las leguminosas y del suero de leche proporcionaron perfiles más parecidos a los de la soja. Sin embargo, aunque las proteínas derivadas de microalgas alteraron el color y la textura, éstas proporcionaron perfiles nutricionalmente favorables tanto en el caso de las hamburguesas de pavo como en el de las salchichas de cerdo, lo que sugiere que las algas marinas también podrían utilizarse para enriquecer las salchichas de cerdo, como alternativa a la proteína de soja.

Por otra parte, un aspecto muy importante a controlar en la cadena alimentaria es la contaminación de los alimentos, siendo de gran relevancia según los informes de RASFF de las últimas décadas la contaminación por micotoxinas. En este sentido, la micotoxina CIT tiene una gran relevancia debido a que la falta de evidencia científica sobre su mecanismo de toxicidad conlleva que no se haya establecido hasta la fecha un valor máximo para su presencia en diferentes alimentos. Por lo tanto, se llevó a cabo el estudio de la toxicidad de esta micotoxina mediante modelos alternativos a la

experimentación animal, utilizando métodos *in vitro* en diferentes modelos celulares (2D y 3D) utilizando células de neuroblastoma humano (SH-SY5Y), así como un estudio *in vivo* mediante la utilización de embriones de pez cebra. Para determinar el efecto citotóxico de la CIT se utilizó el modelo celular 2D (monocapa) con el que se obtuvieron los siguientes valores IC_{50} para las células tratadas con CIT: 77,1 ± 10,1 µM a las 24 h y 74,7 ± 9,6 µM a las 48 h mediante el ensayo MTT, y 101,0 ± 20,3 µM a las 24 h y 54,7 ± 7,4 µM a las 48 h mediante el ensayo del rojo neutro. Confirmada su toxicidad, se realizaron diferentes ensayos para determinar los mecanismos de acción tóxica de la CIT, entre los que se incluyen: estrés oxidativo mediante generación de ROS, alteración de la proliferación celular por el ciclo celular y muerte celular por apoptosis y necrosis. Para estos experimentos se seleccionaron concentraciones de CIT entre 19,375 µM y 50 µM. La CIT no indujo un aumento de la producción de ROS en ninguna de las concentraciones ensayadas. Sin embargo, sí provocó una detención de la fase G2/M. En concreto, el porcentaje de células en la fase G2/M aumentó del 11,83% (control) al 18,47% a 38,75 µM de CIT y al 33,10% a 50 µM de CIT. Además, se observó un aumento significativo (del 19,83% al 28,80%) de las células en la fase S a 50 µM de CIT en comparación con el control, mientras que no se observaron diferencias significativas en ninguna fase del ciclo celular entre el control y la concentración más baja probada (25 µM de CIT). Además, se observó un aumento del proceso de apoptosis tardía en las células expuestas a CIT, especialmente a concentraciones más altas (38,75 y 50 µM). Se confirmó la apoptosis por expresión de la proteína de apoptosis Bcl2 mediante análisis western blot, y disminución de la proteína anti-apoptótica a medida que aumentaba la concentración de CIT. Estos resultados fueron confirmados posteriormente en un modelo 3D (esferoides) de las células SH-SY5Y. En este caso, Los valores IC_{50} obtenidos en los ensayos MTT, ATP y Presto Blue tras 24, 48 y 72 h de exposición oscilaron entre 34,41 ± 4,01 µM y 83,14 ± 5,70 µM.

La CIT disminuyó el MMP (25 µM), indujo la apoptosis, provocó la detención del ciclo celular en las fases S y G2/M (50 µM) y causó alteraciones en el retículo endoplásmico. Sin embargo, de la misma manera que lo observado en el modelo 2D, la CIT no incrementó la generación de ROS. Además, no se observaron alteraciones cromosómicas como resultado de la exposición a la CIT. El uso de un modelo celular en 3D proporciona información más precisa en relación a las condiciones *in vivo* en comparación con los modelos celulares en 2D. Sin embargo, se requieren más estudios para profundizar en los mecanismos de toxicidad descritos en este trabajo en relación a la CIT. En este sentido, y para complementar los estudios llevados a cabo y la información obtenida, se decidió utilizar un modelo *in vivo* con embriones de pez cebra de hasta 96 hpf. Para profundizar en los mecanismos subyacentes de la toxicidad de la CIT, se analizaron los niveles de expresión de los genes Apaf1 y Bax (asociados a la apoptosis), GPx (actividad antioxidante), RIPK3 (necrosis), e IL-8 y CXCL-CIC (inflamación). Los valores de LD_{50} obtenidos en este modelo *in vivo* oscilaron entre 255 ± 10,10 µM (72 h) y 140,5 ± 21,7 µM (96 h). La exposición a la concentración más alta de CIT (300 µM) produjo malformaciones en el 100% de los embriones de pez cebra, mientras que no se observaron malformaciones en la concentración más baja ensayada (50 µM). Se observó una regulación al alza en la expresión de todos los genes ensayados, lo que indica su posible implicación en la toxicidad inducida por la CIT. Además, se observó una disminución en la producción de ROS en los embriones de pez cebra expuestos a CIT. Este hallazgo, sugiere que la CIT puede modular las vías del estrés oxidativo, lo que podría contribuir a una disminución del efecto tóxico. En conclusión, nuestro estudio proporciona información valiosa sobre el comportamiento tóxico de la CIT utilizando un modelo de pez cebra. Sin embargo, es necesario seguir investigando la toxicidad del CIT para identificar otras vías que puedan estar implicadas. De

este modo, se comprenderá mejor su toxicidad para prevenir los efectos causados por esta micotoxina.

Una vez analizados los efectos tóxicos de la CIT, se estudió la reducción de su toxicidad en células SH-SY5Y mediante el uso de extractos naturales. En este sentido, se utilizaron extractos seleccionados de los estudios llevados a cabo anteriormente, en concreto los extractos optimizados de cabeza de lubina obtenido mediante PE (HPO) y el extracto etanólico de espirulina. La combinación de estos extractos con CIT mostró un aumento de la viabilidad celular de hasta el 15% para el extracto de subproductos de pescado y de alrededor del 10% para el extracto de espirulina, en comparación a una exposición a CIT sola en las células SH-SY5Y. Además, se observó una reducción de hasta el 63,2% en la apoptosis al combinar el extracto HPO con CIT, contrarrestando los efectos tóxicos de la exposición a CIT de forma individual en las células SH-SY5Y. Sin embargo, la eficacia de los extractos para prevenir la toxicidad de CIT en el ciclo celular sigue sin estar clara. En general, la consideración de estas fuentes de nutrientes y compuestos bioactivos es crucial para mejorar la seguridad alimentaria y mitigar los efectos nocivos de contaminantes como las micotoxinas. No obstante, se necesitan más estudios para investigar sus mecanismos de acción y comprender mejor sus efectos protectores de forma más exhaustiva.

CONCLUSIONES

De la presente Tesis Doctoral se extraen las siguientes conclusiones:

Conclusión 1. Se ha demostrado que la fermentación de subproductos de pescado con bacterias lácticas (BAL) es una herramienta útil para la obtención de compuestos antioxidantes y antifúngicos. El ácido DL-3-feniláctico (antioxidante) se obtiene como resultado de la fermentación de subproductos de pescado, que es un proceso económico, limpio y respetuoso con el medio ambiente. Por todo ello, la fermentación de subproductos pesqueros puede ser una buena estrategia para la reducción de estos residuos mediante la valorización de los mismos, contribuyendo a alcanzar un desarrollo sostenible.

Conclusión 2. El tratamiento con pulsos eléctricos (PE) aumentó la extracción de proteínas a partir de subproductos de lubina. También alteró la distribución del tamaño molecular de las proteínas extraídas. Además, el tratamiento con PE mejoró la actividad antioxidante de los extractos de piel y cabeza de lubina, promoviendo la producción de diversos péptidos bioactivos y antioxidantes; así como la recuperación de minerales cruciales para la nutrición como el Fe, Zn y Mg. Los PE demostraron su eficacia en la reducción de metales pesados en los extractos de pescado. Los extractos de cabeza de pescado (control y obtenido por PE) mostraron un aumento de la viabilidad celular en células SH-SY5Y después de 24 horas de exposición.

Conclusión 3. La extracción asistida por PE de compuestos bioactivos a partir de espirulina fue más eficiente durante todo el estudio, especialmente a tiempos de extracción cortos (5-15 min). En este sentido, se obtuvo una

recuperación del 55 al 60% para clorofilas, del 85 al 90% para carotenoides y del 60 al 70% para compuestos fenólicos totales, respecto a la cantidad máxima total extraída.

Por otro lado, los resultados obtenidos demuestran la eficacia de la extracción de compuestos bioactivos como pigmentos, polifenoles, ácidos grasos poliinsaturados y minerales de la espirulina con fluidos supercríticos (SFE). Los compuestos bioactivos obtenidos por SFE mostraron una mayor viabilidad celular en células SH-SY5Y después de 24 horas de exposición. Sin embargo, la SFE también aumenta la extracción de contaminantes como el Pb. Por lo tanto, es crucial disponer de medidas adecuadas de control de calidad para garantizar la seguridad y pureza de los compuestos extraídos.

Conclusión 4. La adición de judías y microalgas a hamburguesas de pavo produjo un aumento del contenido de ácido glutámico, lisina y ácido aspártico. La adición del alga espirulina a salchichas de cerdo aumentó el contenido de aminoácidos totales, esenciales y no esenciales. El enriquecimiento de las hamburguesas de pavo con proteínas de judías podría utilizarse como una alternativa prometedora a las proteínas de soja desde un punto de vista tecnológico. Respecto a las salchichas de cerdo, las proteínas de leguminosas y suero eran similares a la soja en cuanto a textura, parámetros fisicoquímicos y perfiles de aminoácidos. A pesar de alterar el color y la textura, las proteínas de microalgas presentaron perfiles nutricionales favorables. Esto sugiere que las algas podrían ser una alternativa a la proteína de soja para enriquecer las salchichas de cerdo. La elección de la fuente de proteínas, puede afectar a las propiedades del alimento (textura, color, etc.) e influir en la elección del consumidor.

Conclusión 5. La citrinina (CIT) causa toxicidad en las células SH-SY5Y en monocapa. La toxicidad aumenta al aumentar la concentración y el tiempo de exposición. La CIT en las células SH-SY5Y produjo un bloqueo de las fases S y G2/M del ciclo celular y el aumento de la apoptosis tardía con una expresión reducida de la proteína Bcl2. Este mismo efecto se ha observado en esferoides de SH-SY5Y donde la CIT induce apoptosis, altera el potencial de la membrana mitocondrial, provoca la detención del ciclo celular y afecta la función del retículo endoplásmico. Sin embargo, no se encontraron pruebas de alteraciones cromosómicas. El uso de un modelo de cultivo celular tridimensional, frente a un modelo en monocapa, refleja con mayor precisión los mecanismos de acción a nivel celular que se observaría en un organismo vivo.

Conclusión 6. En el estudio in vivo llevado a cabo en embriones de pez cebra se observó que concentraciones elevadas de CIT (300 µM) inducen malformaciones en el 100% de los embriones de pez cebra a las 96 h de exposición. En este modelo, la CIT induce la expresión de genes asociados a la apoptosis, la necroptosis e inflamación.

Conclusión 7. Los resultados de este estudio demuestran efectos citoprotectores del extracto de subproductos de pescado y microalgas. El extracto de cabeza de lubina aumenta la viabilidad celular y disminuye la apoptosis en células SH-SY5Y expuestas a citrinina (CIT). El extracto de espirulina aumenta la viabilidad celular cuando se combinó con CIT. Sin embargo, se necesitan más estudios para dilucidar los mecanismos que causan efecto citoprotector, tanto en los extractos de espirulina como de pescado.

Conclusión 8. En general, este estudio pone de relieve la importancia de las técnicas innovadoras de PE y EFS por su eficiencia y sostenibilidad, permitiendo la obtención de compuestos bioactivos de alta calidad de los alimentos, lo que puede mejorar la nutrición y la seguridad alimentaria, y tiene potencial para innovar en la producción y procesamiento en la industria alimentaria. Así como la capacidad potencial de los subproductos del pescado y los extractos de microalgas en la protección frente al daño celular causado por contaminantes de alimentos, como las micotoxinas.

CONCLUSIONS

The following conclusions are drawn from this doctoral thesis:

Conclusion 1. It has been demonstrated that the fermentation of fish side streams with lactic acid bacteria (LAB) is a useful tool for obtaining antioxidant and antifungal compounds. DL-3-phenyllactic acid (an antioxidant) is obtained as a result of the fermentation of fish side streams, which is an economical, clean, and environmentally friendly process. Therefore, the fermentation of fish side streams can be a good strategy for reducing these waste materials through their valorization, contributing to achieving sustainable development.

Conclusion 2. The treatment with pulsed electric fields (PEF) increased the extraction of proteins from sea bass side streams. It also altered the distribution of the molecular size of the extracted proteins. Additionally, the PEF treatment improved the antioxidant activity of the skin and head extracts of sea bass, promoting the production of various bioactive and antioxidant peptides, as well as the recovery of crucial minerals for nutrition such as Fe, Zn, and Mg. The PEF treatment demonstrated its effectiveness in reducing heavy metals in fish extracts. The head extracts (control and PEF) showed an increase in cell viability in SH-SY5Y cells after 24 hours of exposure.

Conclusion 3. The PEF-assisted extraction of bioactive compounds from spirulina was more efficient throughout the study, especially at short extraction times (5-15 min). In this regard, a recovery of 55 to 60% for chlorophylls, 85 to 90% for carotenoids, and 60 to 70% for total phenolic compounds was obtained, relative to the maximum total amount extracted.

On the other hand, the results obtained demonstrate the effectiveness of extracting bioactive compounds such as pigments, polyphenols, polyunsaturated fatty acids, and minerals from spirulina using supercritical fluids (SFE). The bioactive compounds obtained through SFE showed higher cell viability in SH-SY5Y cells after 24 hours of exposure. However, SFE also increases the extraction of contaminants such as Pb. Therefore, it is crucial to have appropriate quality control measures in place to ensure the safety and purity of the extracted compounds.

Conclusion 5. Citrinin (CIT) causes toxicity in SH-SY5Y monolayer cells. The toxicity increases with increasing concentration and exposure time. CIT in SH-SY5Y cells produced a blockage of the S and G2/M phases of the cell cycle and an increase in late apoptosis with a reduced expression of the Bcl2 protein. This same effect has been observed in SH-SY5Y spheroids where CIT induces apoptosis, alters the mitochondrial membrane potential, causes cell cycle arrest, and affects the function of the endoplasmic reticulum. However, no evidence of chromosomal alterations was found. The use of a three-dimensional cell culture model, as opposed to a monolayer model, more accurately reflects the mechanisms of action at the cellular level that would be observed in a living organism.

Conclusion 6. In the *in vivo* study carried out on zebrafish embryos, it was observed that high concentrations of CIT (300 µM) induce malformations in 100% of the zebrafish embryos at 96 hours of exposure. In this model, CIT induces the expression of genes associated with apoptosis, necroptosis, and inflammation.

Conclusion 7. The results of this study demonstrate cytoprotective effects of fish side streams and microalgae extracts. The sea bass head extract increases cell viability and decreases apoptosis in SH-SY5Y cells exposed to citrinin (CIT). The spirulina extract increases cell viability when combined with CIT. However, further studies are needed to elucidate the mechanisms causing cytoprotective effect, in both spirulina and fish extracts.

Conclusion 8. In general, this study highlights the relevance of innovative PEF and SFE techniques for their efficiency and sustainability, enabling the extraction of high-quality bioactive compounds from food, which can enhance nutrition and food security and have the potential to innovate production and processing in the food industry. Additionally, it highlights the potential capacity of fish side streams and microalgae extracts in protecting against cellular damage caused by food contaminants such as mycotoxins.

ANEXO I: ARTÍCULOS PUBLICADOS

Research Article

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A chemometric approach to evaluate the impact of pulses, *Chlorella* and *Spirulina* on proximate composition, amino acid, and physicochemical properties of turkey burgers

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Abstract

BACKGROUND: Changes in physicochemical parameters, proximate composition, amino acid and taste profiles of turkey burgers enriched by 1% with soy (control), pulses, *Chlorella* and *Spirulina* proteins were studied.

RESULTS: Color parameters, pH, ash content, total, essential and non-essential amino acids were significantly different among the different types of turkey burgers prepared. In this regard, turkey burgers made with pea protein presented the highest values for pH and lightness, whereas the samples prepared with broad bean showed the highest redness. The inclusion of bean and seaweed produced a marked increase of glutamic acid, lysine and aspartic acid. However, the taste profile was similar in the different six turkey burgers studied (soy, pea, lentil, broad bean, *Chlorella* and *Spirulina* protein). Orthogonal projections to latent structures discriminant analysis (OPLS-DA) allowed to classify turkey burgers according to protein sources, as compared to soy (control). Textural parameters, moisture and color were found to be the most discriminant parameters, able to describe the differences among burgers. Nonetheless, according to the supervised OPLS model, broad beans were found to possess a similar profile to soy (control).

CONCLUSION: Considering all studied parameters, the enrichment of turkey burgers with bean proteins could be used as a promising alternative to soy proteins from a technological point of view.

Keywords: textural properties; color parameters; seaweeds; taste profile; turkey burger

INTRODUCTION

Among foods of animal origin, it is known that meat and its derivatives are a good source of nutrients, among which we can highlight vitamins such as B_{12}, minerals such as iron and zinc, and lipids and proteins.[1–3] This makes meat products an important group of foods which is also consumed globally by a large number of people. But, despite the nutritional value of its components, an excessive consumption of meat, specially processed meat, is related to the risk of cancer development, colon cancer being the most notable.[4,5] Several processed meat products incorporate soy proteins, due to their beneficial properties for health, and their good nutritional profile, with a large number of essential amino acids.[6,7] However, soy is a recognized allergen, so it is necessary to look for other vegetable sources of good nutritional quality to replace it.

Legumes, which include lentils, peas and beans, are rich in protein, comprising 20% of dry weight in the case of beans and peas.[8] Furthermore, products derived from algae are now becoming important.[9] Microalgae are a good source of polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), antioxidants (phenolic acids and

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Influence of different sources of vegetable, whey and microalgae proteins on the physicochemical properties and amino acid profile of fresh pork sausages

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ABSTRACT

The purpose of this study was to evaluate changes in the physicochemical properties and amino acid profiles of pork sausages prepared by including vegetable protein sources (beans, peas, and lentils), microalgae (*Chlorella* and *Spirulina*) or whey, as compared with a control (soy protein). Significant differences were found for all the studied parameters. The protein content was significantly lower in sausages made with pea protein compared with the control. Colour parameters changed significantly after the incorporation of microalgae proteins. Moreover, significant differences among treatments were observed in the amino acid profile. The inclusion of *Spirulina* proteins resulted in an increase in the total amino acid content and the ratio of essential/non-essential amino acids. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) allowed pork sausages to be classified according to the protein source, in comparison with soy (control). Textural parameters (chewiness, gumminess and hardness) followed by colour and pH were the most discriminant parameters. Considering texture traits, physicochemical parameters and amino acid profiles across treatments, proteins from legumes and whey provided profiles closer to that of soy. However, although microalgae-derived proteins altered the colour and texture, they provided nutritionally favourable profiles, thus suggesting that seaweeds could also be used to enrich pork sausages, as an alternative to soy protein.

1. Introduction

Meat and meat products have been part of the human diet since ancient times (Mann, 2018). The average consumption per person per year of this food group worldwide has increased constantly increasing since the mid-twentieth century (FAO, 2018; Godfray et al., 2018).

Despite the large number of nutrients provided by meat products, such as vitamin B_{12} and iron (Banjari & Hjartåker, 2018), it is recommended that red and processed meats are moderately consumed (Lorenzo et al., 2018). There are numerous studies that link excessive meat consumption with a higher probability of suffering from cancer (Banjari & Hjartåker, 2018; Farvid et al., 2018; Godfray et al., 2018; Song & Chan, 2018; You & Henneberg, 2018). In addition, the cholesterol, fat content, and fat composition of meat are important health issues for consumers, because they have been associated with obesity and hypercholesterolaemia (Domínguez et al., 2018a). Moreover, within different types of meat, many studies have established a relationship between the consumption of processed meats, such as sausages, and the risk of cancer (WHO, 2018). Similarly, several studies have related the intake of processed meat with the appearance of chronic diseases such as obesity, type 2 diabetes, cardiovascular diseases and some types of cancer (Bellou, Belbasis, Tzoulaki, & Evangelou, 2018; Boada, Henríquez-Hernández, & Luzardo, 2016; Rouhani, Salehi-Abargouei, Surkan, & Azadbakht, 2014; Zhang,

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Article

Isolation, Identification and Investigation of Fermentative Bacteria from Sea Bass (*Dicentrarchus labrax*): Evaluation of Antifungal Activity of Fermented Fish Meat and By-Products Broths

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Abstract: During fish production processes, great amounts of by-products are generated, representing ≈30–70% of the initial weight. Thus, this research study is investigating 30 lactic acid bacteria (LAB) derived from the sea bass gastrointestinal tract, for anti-fungal activity. It has been previously suggested that LAB showing high proteolitic activity are the most suitable candidates for such an investigation. The isolation was made using a MRS (Man Rogosa Sharpe) broth cultivation medium at 37 ºC under anaerobiosis conditions, while the evaluation of the enzymatic activity was made using the API® ZYM kit. Taking into account the selected bacteria, a growing research was made fermenting two kinds of broths: (i) by-products (WB), and (ii) meat (MB). Both were fermented at three different times (24, 48 and 72 h). Then, the antifungal activities of both fermented by-products and meat broths were determined qualitatively and quantitatively in solid and liquid medium against two different strains of the genera *Penicillium*, *Aspergillus* and *Fusarium*. After the experiments, a total of 30 colonies were isolated, observing a proteolytic activity in 7 of the isolated strains, which belong to *Lactobacillus* genus, and the two more active strains were identified by polymerase chain reaction (PCR) as *L. plantarum*. Several strains evidenced antifungal activity showing an inhibition halo and Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values between 1–32 g/L and 8–32 g/L, respectively. In conclusion, the isolated bacteria of sea bass had the ability to promote the antifungal activity after the fermentation process, thus being a useful tool to give an added value to fish industry by-products.

Keywords: sea bass; by-products; fermentative process; antifungal activity; lactic acid bacteria

1. Introduction

Nowadays, the increased fish production, up to 171 million tons in 2016 [1], is also generating an increase in food waste and by-products, which can represent from 30% to 70% of the total weight of the fish [2].

Among the main by-products obtained from fish, eyes (0.8–1.5%), skin (1–3%), head (9–12%), viscera (12–18%), spines (9–15%) and muscle discards (40–55%) are the most representative [3], all of

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Current Opinion in Food Science

Fermentation in fish and by-products processing: an overview of current research and future prospects

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Fish industry has been growing continuously over the last decades and generates huge amounts of by-products. These by-products come from fish head, skin, bones, thorns, and viscera. Part of them are processed for feed, collagen and oil production, and to a lesser extent to produce biofuels and fertilizers, but many other high-value bioactive compounds can be recovered. Fish fermentation, which is traditionally used to increase fish shelf-life, results into the formation of bacteria metabolites of interest. Applied to by-products, fermentation increases the quality of protein hydrolysates, oil and produces antioxidant compounds. This technology, which is safe, environmental-friendly and poor energy consuming, presents advantages for future applications.

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Introduction

Fermentation is a metabolic process that consists of obtaining energy from organic compounds without using any external oxidizing agent. To this purpose, lactic acid bacteria, which are a group of aero-tolerant Gram-positive bacilli or cocci, strictly fermentative, organotrophic and producing lactic acid as the main final product of fermentation, are used [1]. Fermentation has been used since ancient times as a preservative method for any kind of foods. However, it is now of greater interest for nutrition and health, as the fermentation of food produces an increase in the levels of bioactive compounds, in addition to promote a decrease in anti-nutrients through hydrolysis [2•]. The beneficial effects of fermented foods consumption can be due to living microorganisms ingested together with the fermented food, some of them exhibiting a probiotic effect, or can result from compounds synthesized during fermentation as a result of bacterial metabolism [3].

Fermented fish products

Fermented fish is a traditional food in many countries around the world, especially in Asia, but also in Europe and Africa. This is because throughout history humans have sought to improve the preservation of fresh fish, due to its short shelf-life. One of the most common fermented fish products is fish sauce. This sauce is mainly prepared in Asia from the fermentation of fish such as sardines, anchovies or mackerels, being an important source of nutrients in the diet of this region. In addition to fish sauce, in Asia there is also fermented fish paste, widely consumed in the Philippines. On the other hand, in Europe it is also possible to find meals based on fermented fish, such as *rakeorret* in Norway, *surströmming* in Sweden or *maatjes* in Holland. Finally, in Africa many traditional meals contain fermented fish, such as *salanga* in Chad, *guedj* in Gambia or in Senegal, *koobi* in Ghana or *dagaa* in Uganda. Currently, there is a lot of scientific literature around the food made with fermented fish. For that reason, in this review we will not go into more depth and we will focus more on fish by-products.

Fish by-products

The global production of fish has been increasing over recent years. For instance, global fish production was about 20 million tons in 1950, and increased up to 171 million tons in 2016, (≈50% aquaculture). Fish consumption has also increased globally, from 9.0 kg in 1961 to 20.2 kg per person in 2015 [4]. Such a fish production increase has also entailed an increase in fish waste. These wastes, which are in many cases discarded directly without attempting to take advantage of them, represent a major environmental and economic problem that may affect the viability of fishing and aquaculture industry [5]. Therefore, it is necessary to give a second life to this waste, so that it is possible to reduce pollution and make the fish industry more efficient.

Definition of fish by-products

The by-products are obtained as a result of fish processing at an industrial level, either from fishing or from aquaculture. These by-products represent around 60%

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antioxidants

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Article

Impact of Fermentation on the Recovery of Antioxidant Bioactive Compounds from Sea Bass Byproducts

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Abstract: The aim of the present research was to obtain antioxidant compounds through the fermentation of fish byproducts by bacteria isolated from sea bass viscera. To that purpose, bacteria from sea bass stomach, intestine, and colon were isolated. With the selected bacteria, growing research was undertaken, fermenting different broths prepared with sea bass meat or byproducts. After the fermentation, the antioxidant activity, phenolic acids, and some proteins were evaluated. The main phenolic acids obtained were DL-3-phenyl-lactic acid and benzoic acid at a maximum concentration of 466 and 314 ppb, respectively. The best antioxidant activity was found in the extracts obtained after the fermentation of fish byproducts broth by bacteria isolated from the colon (6502 μM TE) and stomach (4797 μM TE). Moreover, a positive correlation was found between phenolic acids obtained after the fermentation process and the antioxidant activity of the samples. It was also concluded that the lactic acid bacteria isolated from sea bass had an important proteolytic capacity and were able to synthesize phenolic acids with antioxidant capacity. This work has shown the relevance of fermentation as a useful tool to valorize fish byproducts, giving them an added economic value and reducing their environmental impact.

Keywords: fermentation; fishing industry byproducts; bioactive peptides; antioxidant activity; phenolic acids

1. Introduction

The latest FAO (Food and Agricultural Organization) report, published in 2018, showed an important growth in fish caught over the last few years due to the increased fish population's consumption (from 17.6 kg per capita in 2007 to 20.3 kg per capita in 2016) [1].

During the fish production process, several byproducts are produced, representing more than 30% of fish weight, and in some cases, up to 70% [2]. Among the different parts of fish that can be considered byproducts, muscle pieces, viscera, thorns, heads, skin, fins, and scales are the most representative [3]. These must be properly managed in order to avoid environmental problems and to maintain the sustainability of the resources. Moreover, it should be noted that since each by-product has a different composition, different alternatives have been evaluated for each of them [4].

For instance, different innovative approaches have been studied in order to valorize fish byproducts due to the environmental problem they represent; for instance, the application of green extraction

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fermentation

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Review

Obtaining Antioxidants and Natural Preservatives from Food By-Products through Fermentation: A Review

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Abstract: Industrial food waste has potential for generating income from high-added-value compounds through fermentation. Solid-state fermentation is promising to obtain a high yield of bioactive compounds while requiring less water for the microorganism's growth. A number of scientific studies evinced an increase in flavonoids or phenolics from fruit or vegetable waste and bioactive peptides from cereal processing residues and whey, a major waste of the dairy industry. Livestock, fish, or shellfish processing by-products (skin, viscera, fish scales, seabass colon, shrimp waste) also has the possibility of generating antioxidant peptides, hydrolysates, or compounds through fermentation. These bioactive compounds (phenolics, flavonoids, or antioxidant peptides) resulting from bacterial or fungal fermentation are also capable of inhibiting the growth of commonly occurring food spoilage fungi and can be used as natural preservatives. Despite the significant release or enhancement of antioxidant compounds through by-products fermentation, the surface areas of large-scale bioreactors and flow patterns act as constraints in designing a scale-up process for improved efficiency. An in-process purification method can also be the most significant contributing factor for raising the overall cost. Therefore, future research in modelling scale-up design can contribute towards mitigating the discard of high-added-value generating residues. Therefore, in this review, the current knowledge on the use of fermentation to obtain bioactive compounds from food by-products, emphasizing their use as natural preservatives, was evaluated.

Keywords: fermentation; food by-products; antioxidant; antifungal

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1. Introduction

The processing of food by the industry generates a large amount of by-products that are generally discarded. In some cases, these by-products are reused for animal feed or as fuel for energy, but they still have a low economic value. It has been estimated that 88 million tons of by-products are generated from the agri-food industry, at a cost of 143,000 million euros [1]. These by-products can be converted into a wide variety of compounds with high added value, such as biofuels or bioactive compounds [2].

Fermentation has been used for thousands of years to preserve food or produce compounds of interest such as ethanol. Over time, this process has been gaining importance in research related to nutrition and health, because through fermentation, bioactive compounds of interest are obtained, such as antioxidant compounds [3]. It also raises interest at an industrial level since it allows the reuse of waste to obtain the desired compounds from them. In this way, it contributes to protecting the environment by reducing the environmental impact caused by these wastes [4].

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applied sciences

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Article

Extraction of Antioxidant Compounds and Pigments from Spirulina (*Arthrospira platensis*) Assisted by Pulsed Electric Fields and the Binary Mixture of Organic Solvents and Water

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Abstract: The application of pulsed electric fields (PEF) is an innovative extraction technology promoting cell membrane electroporation, thus allowing for an efficient recovery, from an energy point of view, of antioxidant compounds (chlorophylls, carotenoids, total phenolic compounds, etc.) from microalgae. Due to its selectivity and high extraction yield, the effects of PEF pre-treatment (3 kV/cm, 100 kJ/kg) combined with supplementary extraction at different times (5–180 min) and with different solvents (ethanol (EtOH)/H_2O, 50:50, *v*/*v*; dimethyl sulfoxide (DMSO)/H_2O, 50:50, *v*/*v*) were evaluated in order to obtain the optimal conditions for the extraction of different antioxidant compounds and pigments. In addition, the results obtained were compared with those of a conventional treatment (without PEF pre-treatment but with constant shaking). After carrying out the different experiments, the best extraction conditions to recover the different compounds were obtained after applying PEF pre-treatment combined with the binary mixture EtOH/H_2O, 50:50, *v*/*v*, for 60–120 min. PEF extraction was more efficient throughout the study, especially at short extraction times (5–15 min). In this sense, recovery of 55–60%, 85–90%, and 60–70% was obtained for chlorophylls, carotenoids, and total phenolic compounds, respectively, compared to the maximum total extracted amount. These results show that PEF improves the extraction yield of antioxidant bioactive compounds from microalgae and is a promising technology due to its profitability and environmental sustainability.

Keywords: pulsed electric fields; green extraction; microalgae; antioxidants; pigments

1. Introduction

Over the last decade, several research studies have evaluated the use of microalgae as a source of nutrients and bioactive compounds. This is due to a growing interest in the development of new foods that provide health benefits and that meet basic energy and nutritional requirements [1,2]. It is preferred that functional foods have a natural origin, such as from plants, algae and/or microalgae. In this sense, proteins obtained from microalgae have been used to replace proteins of animal origin in meat-like preparations such as turkey burgers [3] or in the fortification of vegan foods such as kefir produced from soy and almond based beverages [4].

Microalgae are becoming increasingly relevant, especially for their composition, since they are a source of high-added-value compounds [5], such as carotenoids, chlorophylls, and other pigments (antioxidants) [6], and polyunsaturated fatty acids [7,8].

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ANEXO II:

COMUNICACIONES A CONGRESOS

Autores: Marti-Quijal, FJ., Tornos, A., Princep, A., Ruiz, MJ., Barba, FJ.
Título: Extraction of bioactive compounds from fish by-products assisted by pulsed electric fields
Congreso: VI International Student Congress of Food Science and Technology
Tipo de participación: Póster
Lugar de celebración: Valencia (España)
Año: 2019

Autores: Marti-Quijal, FJ., Manyes, L., Barba, FJ., Ruiz, MJ.
Título: Effect of fish by-products extracts in Caco-2 cells viability
Tipo de participación: Póster
Congreso: XXIII Congreso Español y VII Iberoamericano de Toxicología
Lugar de celebración: Sevilla (España)
Año: 2019

Autores: Marti-Quijal, FJ., Ruiz, MJ., Barba, FJ.
Título: Green technology extraction for the recovery of biological interesting compounds from fish by-products
Congreso: VII International Student Congress of Food Science and Technology
Tipo de participación: Comunicación oral
Lugar de celebración: Valencia (España)
Año: 2020

Autores: Marti-Quijal, FJ., Tolosa J, Barba FJ, Ruiz MJ
Título: Evaluación de la toxicidad de citrinina *in vitro*: Revisión
Tipo de participación: Póster
Congreso: VIII Jornadas de Formación en Toxicología
Lugar de celebración: Valencia (España)
Año: 2021

Autores: Marti-Quijal, FJ., Ramon-Mascarell F, Barba FJ
Título: Effect of pulsed electric fields on the recovery of antioxidant protein extracts from fish side streams
Tipo de participación: Póster
Congreso: The 2nd International Electronic Conference on Foods - "Future Foods and Food Technologies for a Sustainable World"
Lugar de celebración: On-line
Año: 2021

Autores: Marti-Quijal, FJ., Ramon-Mascarell F, Barba FJ
Título: Impact of pulsed electric fields technology on pigments extraction yield from *Arthrospira platensis*
Tipo de participación: Póster
Congreso: The 2nd International Electronic Conference on Foods - "Future Foods and Food Technologies for a Sustainable World"
Lugar de celebración: On-line
Año: 2021

Autores: Marti-Quijal, FJ., Ramon-Mascarell F, Barba FJ
Título: Recovery of polyphenols and compounds with antioxidant activity from spirulina (*Arthrospira platensis*) through the use of different organic solvents assisted by pulsed electric fields
Tipo de participación: Póster
Congreso: The 2nd International Electronic Conference on Foods - "Future Foods and Food Technologies for a Sustainable World"
Lugar de celebración: On-line
Año: 2021

Autores: Marti-Quijal, FJ., Barba FJ, Ruiz MJ
Título: Efectos de la combinación de un extracto etanólico de espirulina y la micotoxina citrinina sobre células SH-SY5Y
Tipo de participación: Póster
Congreso: IX Jornadas de Formación en Toxicología
Lugar de celebración: Valencia (España)
Año: 2022

Autores: Marti-Quijal, FJ., Castagnini, JM, Ruiz MJ, Barba FJ
Título: Antioxidant activity and phenolic composition of extracts obtained from microalgae after the application of innovative processes
Tipo de participación: Comunicación oral
Congreso: 15th World Congress on Polyphenols Applications
Lugar de celebración: Valencia (España)
Año: 2022

Autores: Marti-Quijal, FJ., Torriero N, Fietta A, Zingales V, Barba FJ, Ruiz MJ, Fusco P, Esposito MR, Cimetta E
Título: Study of citrinin toxic effects and its mechanism both *in vitro* and *in vivo* using zebrafish embryos
Tipo de participación: Comunicación oral
Congreso: XXIV Congreso español de toxicología y VIII iberoamericano
Lugar de celebración: Córdoba (España)
Año: 2022

Autores: Marti-Quijal, FJ., Torriero N, Fietta A, Zingales V, Barba FJ, Ruiz MJ, Fusco P, Esposito MR, Cimetta E
Título: Study of citrinin toxic effects on a 3D neuroblastoma model: Cytotoxicity, oxidative stress and cell death
Tipo de participación: Póster
Congreso: 21st International Congress of the European Society of Toxicology *In Vitro* (ESTIV2022)
Lugar de celebración: Sitges (España)
Año: 2022

Autores: Pallarés, N; Castagnini, JM; Martí-Quijal, FJ.; Ruiz, MJ; Barba, FJ
Título: Recovery of polyphenols, antioxidant compounds and minerals form spirulina: Influence of supercritical fluid extraction
Tipo de participación: Póster
Congreso: 4th International Congress on "Green Extraction of Natural Products" (GENP2022)
Lugar de celebración: Poreč (Croacia)
Año: 2022

Autores: Martí-Quijal, FJ; Barba, FJ; Ruiz, MJ

Título: Efecto de la micotoxina citrinina sobre la producción de ROS y la alteración del potencial de membrana mitocondrial en células neuronales SH-SY5Y

Tipo de participación: Póster

Congreso: X Jornada de Formación en Toxicología

Lugar de celebración: Valencia (España)

Año: 2023