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TESIS DOCTORAL

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Desarrollo de bio-nanoencapsulaciones para la mejora de la estabilidad y biodisponibilidad de ingredientes bioactivos de crucíferas

D.ª Paula García Ibáñez

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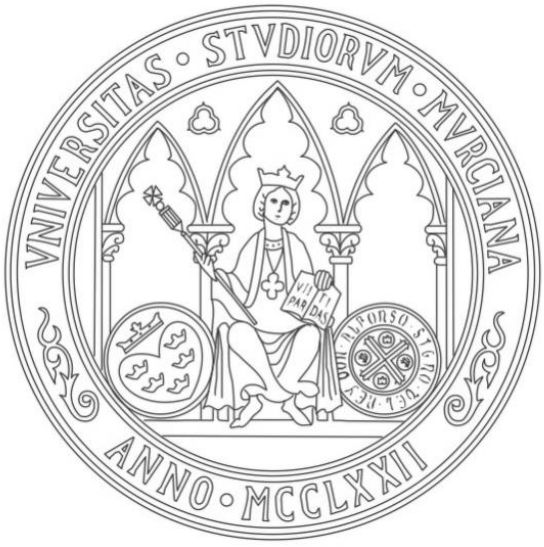

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La presente Tesis Doctoral es un compendio de las siguientes publicaciones:

- **Garcia-Ibañez, P.**, Moreno, D. A., Nuñez-Gomez, V., Agudelo, A., & Carvajal, M. (2020). Use of elicitation in the cultivation of Bimi® for food and ingredients. *Journal of the Science of Food and Agriculture*, *100*(5), 2099-2109. DOI: 10.1002/jsfa.10233.

- **Garcia-Ibañez, P.**, Nicolas-Espinosa, J., & Carvajal, M. (2021). Plasma membrane vesicles from cauliflower meristematic tissue and their role in water passage. *BMC Plant Biology*, *21*(1), 1-12. DOI: 0.1186/S12870-020-02778-6.

- **Garcia-Ibañez, P.**, Moreno, D. A., & Carvajal, M. (2022). Nanoencapsulation of Bimi® extracts increases its bioaccessibility after in vitro digestion and evaluation of its activity in hepatocyte metabolism. *Food Chemistry*, *385*, 132680.
  DOI: 10.1016/j.foodchem.2022.132680.

- **Garcia-Ibañez, P.**, Roses, C., Agudelo, A., Milagro, F.I., Barceló, A. M., Viadel, B., Nieto, J. A., Moreno, D. A. & Carvajal, M. (2021). The influence of red cabbage extract nanoencapsulated with brassica plasma membrane vesicles on the gut microbiome of obese volunteers. *Foods*, *10*, 1038. DOI: 10.3390/foods10051038.

Durante la realización de la presente Tesis Doctoral, la alumna también ha obtenido las siguientes publicaciones y capítulos de libro:

- Martinez-Ballesta, M. C., **Garcia-Ibañez, P.**, Yepes-Molina, L., Rios, J. J., & Carvajal, M. (2018). The expanding role of vesicles containing aquaporins. *Cells*, *7*(10), 179. DOI: 10.3390/cells7100179.

- Rios, J. J., **Garcia-Ibañez, P.**, & Carvajal, M. (2019). The use of biovesicles to improve the efficiency of Zn foliar fertilization. *Colloids and Surfaces B: Biointerfaces*, *173*, 899-905. DOI: 10.1016/j.colsurfb.2018.10.057.

- Hassini, I., Rios, J. J., **Garcia-Ibañez, P.**, Baenas, N., Carvajal, M., & Moreno, D. A. (2019). Comparative effect of elicitors on the physiology and secondary metabolites in broccoli plants. *Journal of plant physiology*, *239*, 1-9. DOI: 10.1016/j.jplph.2019.05.008.

- Quirante-Moya, S., **García-Ibañez, P.**, Quirante-Moya, F., Villaño, D., & Moreno, D. A. (2020). The role of brassica bioactives on human health: are we studying it the right way?. *Molecules*, *25*(7), 1591. DOI: 10.3390/molecules25071591.

- **Garcia-Ibañez, P.**, Sanchez-Garcia, M., Sánchez-Monedero, M. A., Cayuela, M. L., & Moreno, D. A. (2020). Olive tree pruning derived biochar increases glucosinolate concentrations in broccoli. *Scientia Horticulturae*, *267*, 109329. DOI: 10.1016/j.scienta.2020.109329.

- **García-Ibañez, P.**, Yepes-Molina, L., Ruiz-Alcaraz, A. J., Martínez-Esparza, M., Moreno, D. A., Carvajal, M., & García-Peñarrubia, P. (2020). Brassica bioactives could ameliorate the chronic inflammatory condition of endometriosis. *International Journal of Molecular Sciences*, *21*(24), 9397. DOI: 10.3390/ijms21249397.

- Barzana, G., Rios, J. J., Lopez-Zaplana, A., Nicolas-Espinosa, J., Yepes-Molina, L., **Garcia-Ibañez, P.**, & Carvajal, M. (2021). Interrelations of nutrient and water transporters in plants under abiotic stress. *Physiologia Plantarum*, *171*(4), 595-619. DOI: 10.1111/ppl.13206.

- **Garcia-Ibañez, P.**, Ben-Romdhane, O., & Moreno, D. A. (2022). Glucosinolates and their bioactive metabolites as functional compounds modulating inflammation. In *Current Advances for Development of Functional Foods Modulating Inflammation and Oxidative Stress* (pp. 189-204). Academic Press. DOI: 10.1016/B978-0-12-823482.00017-0.

- Ramírez-Pavez, T., García-Peñaranda, A., **Garcia-Ibañez, P.**, Yepes-Molina, L., Carvajal, M., Ruiz-Alcaraz, A. J., Moreno, D.A., García-Peñarrubia, P., & Martínez-Esparza, M. (2022). Potential of Sulforaphane and Broccoli Membrane Vesicles as Regulators of M1/M2 Human Macrophage Activity. *International Journal of Molecular Sciences*, *23*(19), 11141. DOI: 10.3390/ijms231911141.

- Ortiz-Delvasto, N., **Garcia-Ibañez, P.**, Olmos-Ruiz, R., Bárzana, G., & Carvajal, M. (2023). Substrate composition affects growth and physiological parameters of blueberry. *Scientia Horticulturae*, *308*, 111528. DOI: 10.1016/j.scienta.2022.111528.

Además de las siguientes aportaciones a congresos de carácter nacional e internacional:

- IV International Congress of Food Techonology, Quality and Safety (23/10/2018-25/10/2018). **Paula Garcia-Ibañez**; Lucia Yepes; Diego A. Moreno; Micaela Carvajal. Elicitation of Brassicas for increasing bioactive

compounds. Institute of Food Technology in Novi Sad, pp. 29-33. ISBN 978-86-7994-056-8.

- V Jornadas Doctorales del Campus Mare Nostrum (29/05/2019-31/05/2019). **Paula Garcia-Ibañez**, Juan José Ríos, Micaela Carvajal. Aumento de la eficiencia de la fertilización foliar del Zinc mediante biovesículas.

- 18th International Workshop on Plasma Membrane Biology (7/07/2019-12/07/2019). **Paula Garcia-Ibañez**, Diego A.Moreno, Micaela Carvajal. Relevance of lipid-protein composition in water passage through cauliflower meristematic membranes. The Plant Membrane Biology Workshops.

- 1st International Electronic Conference on Food Science and Functional Foods (10/11/2020-20/11/2020). **Paula Garcia-Ibañez**, Diego A. Moreno, Micaela Carvajal. Increased Stability of Bimi® Glucosinolates by Bioencapsulation. Proceedings, 2020. MDPI.

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Agradecimientos

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ÍNDICE

Índice

LISTA DE ABREVIATURAS-LIST OF ABBREVIATIONS

Abreviatura	English	Español
90d ICVs	*90-days inflorescences cauliflower vesicles*	*Vesículas derivadas de inflorescencia de coliflores de 90 días de edad*
AITC	*Allyl-isothiocyanate*	*Alil isotiocianato*
AQPs	*Aquaporins*	*Acuaporinas*
Avr	*Avirulence genes*	*Genes de avirulencia*
BCAT	*Branched chain amino acid transaminase*	*Transaminasa de aminoácidos de cadena ramificada*
BPMVs	*Broccoli plasma membrane vesicles*	*Vesículas de membrana plasmática de brócoli*
CD9, CD81, CD63	*Cluster of differentiation or CD antigens*	*Clúster de diferenciación o antígeno CD*
CYP	*Cytochrome P450 family*	*Familia de citocromos P450*
CYS	*Cystein*	*Cisteina*
DNA	*Desoxyribonucleic acid*	*Ácido desoxirribonucleico*
EPSs	*Epithiospecific proteins*	*Proteínas epitioespecíficas*
ERF	*Ethilene response factors*	*Factores de respuesta a etileno*
EVs	*Extracellular vesicles*	*Vesículas extracelulares*
FMO	*Flavin monooxigenase*	*Flavin monooxigenasa*
GBS	*Glucobrassicin*	*Glucobrasicina*
GIB	*Glucoiberin*	*Glucoiberina*
GNA	*Gluconapin*	*Gluconapina*
GRA	*Glucoraphanin*	*Glucorafanina*
GRAS	*Generally Recognized As Safe*	*Generalmente reconocidos como seguros*
GSH	*Gluthation*	*Glutatión*
GSLs	*Glucosinolates*	*Glucosinolatos*
GS-OX	*Glucosinolate-S-oxidase*	*Glucosinolato-S-oxidasa*
GST	*Gluconasturtin*	*Gluconasturtina*
HGB	*4-hydroxy-glucobrassicin*	*4-hidroxiglucobrasicina*
HSP	*Heat shock proteins*	*Proteínas de choque térmico*

I3C	*Indole-3-carbinol*	*Indol-3-carbinol*
IL-6	*Inteleukin 6*	*Interleucina 6*
ILVs	*Intraluminal vesicles*	*Vesículas intraluminales*
IPM-DH	*Isopropylmalate deshydrogenase*	*Isopropylmalato deshidrogenasa*
IPMI	*Isopropylmalate isomerase*	*Isopropylmalato isomerasa*
ITCs	*Isothiocyanates*	*Isotiocianatos*
JA	*Jasmonic acid*	*Ácido jasmónico*
JAZ	*Jasmonate ZIM domain family*	*Familia de dominio ZIM de jasmonato*
LUVs	*Large unilamellar vesicles*	*Vesículas grandes unilamelares*
MAM	*Methylthioalkylmalato synthase*	*Metiltioalquilmalato sintasa*
MeJA	*Methyl jasmonate*	*Metil jasmonato*
MEVs	*Mammal extracellular vesicles*	*Vesículas extracelulares de mamíferos*
MGB	*4-methoxy-glucobrassicin*	*4-metoxiglucobrasicina*
MLVs	*Multilamellar vesicles*	*Vesículas multilamelares*
MVBs	*Multivesicullar bodies*	*Cuerpos multivesiculares*
MYB	*Family of genes containing the MYB domain*	*Familia de genes que contienen el dominio MYB*
MYC	Myeloma virus oncogene-like gene	*Gen similar del oncogén del virus del mieloma*
NAC	*N-acetyl-L-cystein*	*N-acetil-L-cisteina*
NF-κB	*Nuclear factor kappa-light-chain-enhancer of activated B cells*	*Factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas*
NGB	*Neoglucobrassicin*	*Neoglucobrasicina*
NPR1	*Nonexpressor genes PR1 factor*	*Factor no expresor de genes PR1*
PA	*Phosphatidic acid*	*Ácido fosfatídico*
PC	*Phosphatidylcholine*	*Fosfatidilcolina*
PDEVs	*Plant-derived extracellular vesicles*	*Vesículas extracelulares derivadas de plantas*
PE	*Phosphatidylethanolamine*	*Fosfatidiletanolamina*

PEG	*Poliethylenglycol*	*Polietilénglicol*
PEITC	*Phenetyl isothiocyanate*	*Fenetil isotiocianato*
PR	*Pathogenis-related proteins*	*Proteínas de respuesta a patógenos*
PRO	*Progoitrin*	*Progoitrina*
RNA	*Ribonucleic acid*	*Ácido ribonucleico*
SA	*Salicylic acid*	*Ácido salicílico*
SFN	*Sulforaphane*	*Sulforafano*
SOT/ST	*Sulfotransferases*	*Sulfotransferasa*
SPIONs	*Superparamagnetic iron oxide nanoparticles*	*Partículas supra-paramagnéticas de óxido de hierro*
SUVs	*Small unilamellar vesicles*	*Vesículas pequeñas unilamelares*
TNFα	*Tumor necrosis factor alpha*	*Factor de necrosis tumoral alfa*
TPL	*Transcription factor TOPLESS*	*Factor de transcripción TOPLESS*
TSG101	*Tumor susceptibility gene 101 protein*	*Proteína del gen 101 de subsceptibilidad tumoral*
UGT	*UDP glycosil transferase*	*Glicosil transferasa de UDP*
ZIM	*Zinc-finger inflorescence meristem domain*	*Dominio del meristemo floral de dedos de zinc*

RESUMEN

Resumen

El cultivo de crucíferas, como el brócoli, el repollo, la mostaza o las coles de Bruselas, se ha extendido a lo largo del mundo en las últimas décadas. Gran parte de su protagonismo se debe a su versatilidad, ya que tanto sus distintos órganos (raíces, hojas e inflorescencias) como el aceite obtenido de sus semillas, son aptos para consumo humano y animal. En concreto, los vegetales pertenecientes a la especie *Brassica oleracea* L. destacan por su bajo contenido en grasas, una elevada proporción de agua y una alta concentración de vitaminas (p.ej. vitamina C y tocoferoles) y minerales (p.ej. fósforo, azufre y calcio). También contienen diversos tipos de fitoquímicos beneficiosos para la salud, como los ácidos fenólicos, los antocianos o los glucosinolatos, por lo que se recomienda su consumo habitual en la dieta.

Los glucosinolatos son un grupo de moléculas organoazufradas pertenecientes al metabolismo secundario de las plantas de la familia *Brassicaceae*. Su papel principal es formar parte del sistema de defensa ante patógenos y situaciones de estrés. Asimismo, contribuyen a configurar sus propiedades organolépticas. Se ha descrito una amplia variedad de glucosinolatos, según las alteraciones en su cadena lateral, las cuales dependen, a su vez, del aminoácido del que derivan. No obstante, los glucosinolatos no presentan actividad *per se* y deben ser hidrolizados mediante la acción de la enzima mirosinasa (EC 3.2.3.1) para proporcionar distintos compuestos bioactivos, entre ellos los isotiocianatos. Durante las últimas décadas, se ha podido comprobar extensamente su capacidad como moléculas reguladoras de la respuesta antiinflamatoria, como antioxidantes y anti-proliferativos. En concreto, se ha descrito que isotiocianatos, como el sulforafano, son capaces de interaccionar con los factores de transcripción Nrf2 y NF-κB, que intervienen en la expresión de múltiples genes relacionados con la inflamación y el estrés celular.

Por un lado, la acumulación y biosíntesis de glucosinolatos en la planta también puede modularse de manera exógena gracias al uso de elicitadores. Los

elicitadores son moléculas capaces de provocar una respuesta de defensa en la planta tras su aplicación exógena. Diversas investigaciones realizadas en crucíferas, principalmente en brócoli, han puesto de manifiesto que el uso de fitohormonas, como el ácido salicílico o el metil jasmonato, es capaz de aumentar el contenido en glucosinolatos en la planta. No obstante, la aparición de nuevos híbridos (como el Bimi®) o la mayor relevancia de variedades menos consumidas (como la col lombarda) en el mercado, han puesto de manifiesto la necesidad de diseñar un protocolo adecuado y eficiente para estas especies en campo.

Por otro lado, estas crucíferas son empleadas como material de partida para la obtención de formulaciones ricas en isotiocianatos que puedan ser empleadas en industrias como la cosmética o la alimentaria. Sin embargo, la variedad en sus grupos funcionales, en su polaridad y peso molecular, entre otras características fisicoquímicas de estas moléculas, condicionan su estabilidad y solubilidad. Esto impide un paso eficiente por las barreras biológicas, disminuyendo su bioactividad y requiriendo la administración de concentraciones más altas, lo cual es indeseado. Como alternativa, el uso de nanoportadores permite aumentar esta estabilidad, preservando así su bioactividad y minimizando los efectos negativos de su metabolización. Principalmente, la investigación se ha centrado en los vehículos de origen orgánico, como los liposomas y sus derivados, debido a su alta eficiencia. No obstante, estos pueden provocar reacciones inesperadas en el sistema inmunitario y presenta un alto coste, lo que supone una baja viabilidad para su uso en industrias como la alimentaria. Ante esto, surge el interés de emplear membranas de origen vegetal con el fin de aumentar la biocompatibilidad y obtener una fuente de materia prima que permita una mayor sostenibilidad. De tal modo, se podrían obtener formulaciones alimentarias ricas en isotiocianatos y altamente estables, que contribuyan a la intervención y prevención de patologías asociadas con enfermedades no transmisibles, como la obesidad y el sobrepeso.

Considerando todo lo expuesto anteriormente, la presente Tesis Doctoral plantea como objetivo principal el desarrollo y elaboración de ingredientes bioactivos, obtenidos a partir de material vegetal previamente elicitado en campo

de plantas adultas de Bimi® (*Brassica oleracea* L. var. *itaica* x *Brassica oleracea* var. *alboglabra*) y col roja o lombarda (*Brassica oleracea* L. var. *capitata* f. *rubra*), y estabilizados mediante nanoencapsulaciones provenientes de membrana plasmática de coliflor (*Brassica oleracea* L. var. *botrytis*) para, posteriormente, estudiar su bioaccesibilidad y bioactividad.

Para poder completar este objetivo principal, se establecieron los siguientes objetivos específicos:

- Evaluación del efecto de la elicitación con metil jasmonato, ácido salicílico y su combinación en la acumulación de glucosinolatos en Bimi® y col lombarda, determinando un protocolo adecuado para cada especie (*Capítulo I y Capítulo IV)*.

- Elaboración de formulaciones derivadas de Bimi® y col lombarda enriquecidos en glucosinolatos e isotiocianatos (*Capítulo I, Capítulo III y Capitulo IV*).

- Análisis y estudio de la membrana plasmática derivada de distintos órganos de la coliflor (hojas e inflorescencias), en distintos estados de maduración, para la obtención de un material de partida óptimo como agente nanoencapsulante (*Capítulo II*).

- Evaluación de la estabilidad de compuestos activos presentes en las formulaciones nanoencapsuladas durante la digestión gastrointestinal, así como la determinación de su bioactividad en modelos de obesidad (*Capítulo III y Capítulo IV*).

El creciente interés por parte del consumidor en nuevos sabores menos pungentes y amargos, ha llevado a la revalorización de otras crucíferas, como la col lombarda, y al desarrollo de nuevos híbridos como el Bimi®. Sin embargo, al ser vegetales poco estudiados, el efecto de tratamientos como la elicitación aún es

desconocido. Para ello, se analizó el contenido de glucosinolatos mediante HPLC-DAD-ESI-MS^n de las muestras de Bimi® (hojas, tallo e inflorescencias) y col lombarda, elicitadas en campo con ácido salicílico, metil jasmonato y su combinación. Los estudios realizados en Bimi® revelaron una mayor acumulación de glucosinolatos en hojas mediante la aplicación de 100 µM de metil jasmonato, mientras que la máxima acumulación en las inflorescencias se observó al emplear la combinación de elicitadores. Por otra parte, la concentración de glucosinolatos totales disminuyó con la nebulización de 200 µM de ácido salicílico, afectando a los glucosinolatos indólicos. De este modo, se obtuvieron dos materias primas vegetales (Bimi® y col lombarda) altamente enriquecidas en glucosinolatos, lo que no solo le aporta un valor añadido en el mercado, sino también los convierte en un buen material de origen para la elaboración de formulaciones.

Una vez obtenido este material enriquecido, se realizaron distintos ensayos de extracción mediante hidrodestilación y maceración, tanto en Bimi® como en col lombarda. De este modo, se obtuvieron formulaciones de base acuosa y enriquecidas en isotiocianatos, como el sulforafano, el indol-3-carbinol o la iberina. Sin embargo, los isotiocianatos varían en su peso molecular y características fisicoquímicas, lo que influye en su estabilidad en medios acuosos. Es por ello que se estudió el uso de vesículas de membrana plasmática de inflorescencias de coliflor de 90 días de edad (90d ICVs) como agentes nanoencapsulantes. Se escogió este material, ya que mostró una mayor permeabilidad al paso del agua, gracias a la presencia de acuaporinas de las subfamilias PIP1 y PIP2, lo que le aporta una mayor estabilidad. Además, la reutilización de los subproductos de coliflor como material de origen favorece a la sostenibilidad y contribuye a la economía circular.

Antes de alcanzar los tejidos u órganos de interés, los isotiocinatos presentes en las formulaciones deben ser previamente extraídos durante la digestión para, posteriormente, ser absorbidos a su paso por el intestino delgado. Por ello, se realizaron estudios en dos tipos de modelos de digestión *in vitro*, empleando uno estático para las formulaciones de Bimi® y, para las de col lombarda, un sistema

dinámico acoplado a una fermentación colónica. En ambos casos, se pudo observar una mayor conservación de los isotiocianatos analizados en las formulaciones nanoencapsuladas. Así pues, se pudo determinar que las 90d ICVs ejercen un papel protector de estas vesículas durante los procesos de digestión y fermentación colónica.

Con el fin de analizar el metabolismo de los isotiocianatos presentes en las formulaciones obtenidas de Bimi®, se empleó la línea celular de hepatocitos humanos HepG2. Asimismo, se ensayaron en condiciones control y de inflamación de bajo grado, simulando un estado fisiológico similar al de patologías presentes en enfermedades no transmisibles, como el sobrepeso o la obesidad. Los resultados obtenidos mostraron diferencias tanto en los tratamientos como entre las condiciones, sugiriendo que las formulaciones nanoencapsuladas proporcionaron una mayor fracción bioactiva.

Por otro lado, el creciente interés sobre el papel de la microbiota intestinal en el desarrollo de enfermedades como la obesidad y el sobrepeso, ha derivado en la búsqueda de coadyuvantes alimentarios capaces de modular este este ecosistema microbiano. Es por ello que se evaluó el efecto de las formulaciones de col lombarda sobre el microbioma de voluntarios con obesidad, alimentando tres fermentadores que representan las partes del colon (ascendente, transversal y descendente) durante 14 días. Los resultados revelaron que las formulaciones no alteraban el índice alfa de diversidad, de modo que no se observó extinción microbiana. Tampoco se reflejaron cambios en la relación *Bacteroidetes/Firmicutes.* Sin embargo, la producción de ácido butírico, un ácido graso de cadena corta relacionado con los mecanismos de saciedad, se vio aumentado principalmente con el uso de la formulación nanoencapsulada.

De acuerdo con los resultados obtenidos de los estudios realizados en la presente Tesis Doctoral, se extrajeron las siguientes conclusiones:

Primera. La relación elicitador-especie es clave y juega un papel diferencial según las condiciones de crecimiento y el órgano vegetal. En el caso

del Bimi® cultivado en campo, se encontraron resultados óptimos para la acumulación de glucosinolatos alifáticos e indólicos en las inflorescencias con la combinación de ácido salicilico y metil jasmonato. Para la col roja cultivada, la aplicación en campo de metil jasmonato proporcionó el mayor aumento de glucosinolatos totales, siendo los indólicos los que más se incrementaron.

Segunda. La extracción óptima de glucosinolatos de Bimi® se determinó ente los 15 y 30 minutos de hidrodestilación, variando en función del órgano vegetal seleccionado. Así pues, la matriz de origen y la composición del extractante influyen directamente en el contenido y estabilidad de los glucosinolatos, siendo los principales factores a considerar para la elaboración de un protocolo adecuado.

Tercera. La adición de 90d ICVs como agentes nanoencapsulates no modifica la composición de isotiocianatos presentes en las formulaciones. Esto les proporciona a las vesículas una alta versatilidad para el diseño de formulaciones alimentarias.

Cuarta. El análisis lipídico de las membranas de las 90d ICV reveló un alto grado de insaturación de los ácidos grasos que, junto con la presencia de sitosterol, aportó mayor permeabilidad al agua de la bicapa lipídica de las vesículas.

Quinta. La elevada presencia de acuaporinas, especialmente de las subfamilias PIP1 y PIP2, en las 90d ICVs analizadas mediante análisis proteómico se correlacionó directamente con los altos valores de permeabilidad osmótica del agua obtenidos. Esto le confiere al agente nanoencapsulante la capacidad de adaptarse a los cambios osmóticos producidos en el medio en el que se encuentra, haciéndolo más estable.

Sexta. La nanoencapsulación de isotiocianatos con las 90d ICVs favorece su conservación durante el proceso de digestión gastrointestinal y también tras la fermentación colónica, en el caso de la col lombarda. De este modo, el agente nanoencapsulante mejoró la bioaccesibilidad y fracción disponible de isotiocianatos e indoles bioactivos al finalizar el proceso.

Séptima. Los estudios de bioactividad realizados en la línea celular de hepatocitos HepG2 revelaron patrones distintos de metabolización de isotiocianatos, en función del tratamiento y de las condiciones de estimulación mediante lipopolisacárido, sugiriendo que en un estado de baja inflamación crónica (presente en patologías como la obesidad), se puede obtener una mayor asimilación isotiocianatos empleando formulaciones nanoencapsuladas. Para este fin, los isotiocianatos nanoencapsulados de col lombarda aportaron una mayor fracción de estos bioactivos a la microbiota intestinal, sin alterar la diversidad microbiana tras su tratamiento crónico durante 14 días y modulando la producción de ácidos grasos de cadena corta.

Octava. El aumento de producción de ácido butírico en el microbioma intestinal observado en los ensayos realizados con la formulación nanoencapsulada de col lombarda ha resultado de gran interés para el desarrollo de ingredientes funcionales que contribuyan a la prevención y el tratamiento del sobrepeso y la obesidad.

ABSTRACT

Abstract

The cultivation of cruciferous vegetables, such as broccoli, cabbage, mustard or Brussels sprouts, has spread worldwide in recent decades. Mainly, their growing protagonism is due to their high versatility, since their different organs (roots, leaves and inflorescences) and the oil obtained from their seeds, are suitable for both human and livestock consumption. Specifically, vegetables that belong to *Brassica oleracea* L. species stand out for their low fat content, am elevated water proportion and a high concentration of vitamins (such as vitamin C and tocopherols) and minerals (like phosphorous, sulphur and calcium). Furthermore, they also contain diverse types of beneficial phytochemicals, such as phenolic acids, anthocyanins or glucosinolates, so their incorporation into daily diet is highly recommended.

Glucosinolates are a group of sulphur-based molecules, which take part in the plant secondary metabolism of the *Brassicaceae* family. Their main role is to participate in the plant defence system against pathogens and stress situations. In addition, they contribute to the organoleptic properties of the vegetable. A vast diversity of glucosinolates has been described, based on the modifications of their side chain, which also depend on the type of amino acid they derive. However, glucosinolates on their own are not bioactive, so they have to be hydrolysed by the enzyme myrosinase (EC 3.2.3.1) to provide different bioactive compounds, including isothiocyanates. During the last decades, their capacity as anti-inflammatory response regulating molecules, as antioxidants and anti-proliferatives, has been extensively studied. Furthermore, it has been described that isothiocyanates, such as sulforaphane, are capable of interacting with the transcription factors Nrf2 and NF-κB, which are involved in the expression of multiple genes related to inflammation and cellular stress.

In addition, the accumulation and biosynthesis of glucosinolates in the plant can be modulated exogenously by using elicitors. This compounds are molecules capable of provoking a defence response in the plant when applied. Various

investigations carried out on cruciferous vegetables, mainly broccoli, have shown that the use of phytohormones, such as salicylic acid or methyl jasmonate, increases the glucosinolates content in the plant. However, the appearance of new hybrids (such as Bimi®) or the increasing relevance of under-consumed varieties (such as red cabbage), has highlighted the need to design an adequate and efficient protocol for these vegetables in field.

On the other hand, cruciferous vegetables are often used as a source material for the elaboration of formulations enriched in isothiocyanates, that could be utilized in the cosmetic and food industry. Nevertheless, the diversity in their functional groups, in their polarity and molecular weight, among other physicochemical characteristics of these molecules, conditions their stability and solubility. This affects their efficient passage through biological barriers, decreasing their bioactivity and, thus, requiring the administration of higher concentrations, which is undesirable. Main investigation has focused on the use of carriers from organic origin, such as liposomes and their derivatives. However, despite their high entrapment efficiency, they might provoke unexpected reactions in the immune system and their final cost is expensive, limiting their use in the food industry. As a solution, the interest in using membranes of plant origins arises, in order to increase biocompatibility and obtain a source of raw material that allows greater sustainability. In this way, new food formulations enriched in isothiocyanates with greater stability could be obtained, contributing to the management of pathologies associated with non-communicable diseases, such as obesity and overweight.

Taking all of this into consideration, the following Ph.D. Thesis proposes as the main objective the development and elaboration of bioactive ingredients, obtained from vegetal material previously elicitated in field of adult plants of Bimi® (*Brassica oleracea* L. var. *itaica* x *Brassica oleracea* var. *alboglabra*) and red cabbage (*Brassica oleracea* L. var. *capitata* f. *rubra*) and stabilized by nanoencapsulations from cauliflower plasma membrane (*Brassica oleracea* L. var. *botrytis*) to subsequently study their bioaccessibility and bioactivity.

In order to achieve this main objective, the following specific objectives were stablished:

- Evaluation of the effect of elicitation with methyl jasmonate, salicylic acid and its combination on the accumulation of glucosinolates in Bimi® and red cabbage, determining a suitable protocol for each plant species (*Chapter I and Chapter IV*).

- Preparation of formulations derived from Bimi® and red cabbage enriched in glucosinolates and isothiocyanates (*Chapter I, III and Chapter IV*).

- Analysis and study of the plasmatic membrane derived from different cauliflower organs (leaves and inflorescences), in different stages of maturation, to obtain an optimal stating material as a nanoencapsulating agent (*Chapter II*).

- Evaluation of the stability of the bioactives present in the nanoencapsulated formulations during gastrointestinal digestion, as well as the determination of their bioactivity in obesity models (*Chapter III and Chapter IV*).

The growing interest in the consumer in new less pungent and bitter flavours, has led to the revaluation of diverse cruciferous vegetables, such as red cabbage, and the development of new hybrids, like Bimi®. Nevertheless, since they are understudied vegetables, their reaction to treatments, like elicitation, is still unknown. For that purpose, the content in glucosinolates of samples from Bimi® (leaves, stems and inflorescences) and red cabbage, previous elicited in field with salicylic acid, methyl jasmonate and their combination, was analysed by HPLC-DAD-ESI- MS^n. The studies performed in Bimi® revealed a higher accumulation of glucosinolates in leaves with the application of 100 µM methyl jasmonate, meanwhile the highest concentration in inflorescences was observed

with the combination of both elicitors. Nonetheless, total glucosinolates concentration decreased with the spraying of 200 µM salicylic acid, mainly affecting indolic glucosinolates. In this way, two different vegetal raw material enriched in glucosinolates were obtained. This, not only gives an increased value in the market, but also they can serve as a great source material for the elaboration of diverse formulations.

Once this enriched material was obtained, different extraction assays were performed by hydrodistillation and maceration, both in Bimi® and red cabbage. In this way, aqueous formulations enriched in isothiocyanates, like sulforaphane, indole-3-carbinol and iberin, were obtained. However, isothiocyanates differ in their molecular weight and physicochemical characteristics, which influences their stability in aqueous media. For that, the use of plasma membrane vesicles derived from cauliflower inflorescences of 90 days (90d ICVs) as nanoencapsulating agents was assessed. This material was selected due to their higher water permeability, correlated with the presence of aquaporins form PIP1 and PIP2 subfamilies, which gives them greater stability. Furthermore, the using of by-products from cauliflower as source material favours the sustainability and contributes to the circular economy.

Before reaching the target tissues or organs, the isothiocyanates present in the formulations should be previously extracted through the digestion process, to subsequently be absorbed as they pass through the small intestine. In this way, studies were carried out in two types of *in vitro* digestion models, using a static one for Bimi® formulations and, for red cabbage formulations, a dynamic system coupled to three colonic fermentation reactors.

In order to analyse the metabolism of the isothiocyanates present in the formulations obtained from Bimi®, the HepG2 human hepatocyte cell line was used. The formulations were tested under control and low-grade inflammation conditions, simulating a physiological environment similar to the pathologies present in non-communicable diseases, such as overweight or obesity. The results

obtained showed differences, both in the treatments and between the conditions, suggesting that the nanoencapsulated formulations provided a higher bioactive fraction to the cells. On the other hand, the growing interest in the role of the intestinal microbiota in the development of diseases such as obesity and overweight has led to the search for coadjuvants capable of modulating the gut microbiome. For this reason, the effect of red cabbage formulations on the microbiome of volunteers with obesity was evaluated by feeding three fermenters representing the parts of the colon (ascending, transverse and descending) for 14 days. The results revealed that the formulations did not alter significantly the alpha index of diversity, so no microbial extinction was observed. Neither were changes reflected in the *Bacteroidetes/Firmicutes* ratio. However, the production of butyric acid, a short-chain fatty acid related to satiety mechanisms, was increased mainly with the use of the nanoencapsulated formulation.

From all the results obtained, the following conclusions were extracted:

First. The elicitor-species relationship is crucial and plays a differential role depending on the growth conditions and the plant organ. In the case of Bimi® grown in field, finding optimal results for the accumulation of aliphatic and indolic GSLs in the inflorescences with the combination of salicylic acid and methyl jasmonate. According to red cabbage, field elicitation with methyl jasmonate provided the greatest increase in total glucosinolates, being the indolic the ones that increased the most.

Second. The optimal extraction of GSLs from Bimi® was determined at 15 and 30 minutes of hydrodistillation, varying depending on the selected plant organ. Thus, the source matrix and the selected method directly influence the content and stability of GSLs, being the main factors to consider for developing an adequate protocol.

Third. The addition of 90d ICVs as nanoencapsulated agents does not modify the composition of isothiocyanates present in the formulations. This provides vesicles with high versatility for food formulations design.

Fourth. Lipid analysis of the 90d ICV membranes revealed a high degree of fatty acids unsaturation which, together with the presence of sitosterol, contributed to greater fluidity in the lipid bilayer of the vesicles.

Fifth. The high presence of aquaporins, especially from the PIP1 and PIP2 subfamilies, in the 90d ICVs analysed by proteomic analysis correlates directly with the high values of osmotic permeability obtained. Thus, acquiring a nanoencapsulating agent capable of adapting to the osmotic changes produced in the medium in which it is found, increasing its stability.

Seventh. Bioactivity studies carried out on the HepG2 hepatocyte cell line revealed different metabolization patterns of isothiocyanates, depending on the treatment and the conditions of stimulation by lipopolysaccharide, suggesting that in a state of low chronic inflammation (present in pathologies such as obesity), a greater assimilation using nanoencapsulated formulation could be obtained. For this purpose, the nanoencapsulated red cabbage isothiocyanates provided a higher fraction of this bioactives to the intestinal microbiota without altering the microbial diversity after chronic treatment for 14 days and modulating the production of short chain fatty acids.

Eighth. The increased production of butyric acid in the intestinal microbiome observed in the trials carried out with the nanoencapsulated formulation of red cabbage has been of great interest for the development of functional ingredients that contribute to the prevention and treatment of overweight and obesity.

~ Introducción ~

Introducción

1. Crucíferas como fuentes de compuestos bioactivos

1.1. Familia Brassicaceae y especies principales

La familia de las brásicas (*Brassicaceae*) o crucíferas (*Cruciferae*) se clasifica dentro del grupo de las plantas angiospermas dicotiledóneas y comprende un vasto número de especies (aproximadamente unas 3.709), divididas en 338 géneros (Chen et al., 2011; Al-Shehbaz et al., 2006). Dentro de éstos, podemos destacar a *Arabidopsis, Brassica, Camelina, Raphanus* y *Sinapis*. Se caracterizan por presentar flores con una corola cruciforme y un androceo tetradínamo, además de producir frutos capsulares. Sus cultivos se extienden por todo el mundo y son altamente consumidas, ya que la mayoría presentan inflorescencias, hojas o raíces comestibles y de sus semillas también se obtienen aceites. Todo ello, las convierte en especies vegetales con un alto valor socioeconómico y nutricional.

En concreto, el género *Brassica* tiene una gran relevancia dentro de la familia *Brassicaceae*, al haber sido cultivado y desarrollado en casi todos los continentes. *Brassica spp*. se divide en tres grupos principales; mostazas, colzas y coles. Dentro de estas últimas, la especie vegetal con mayor relevancia es *Brassica oleracea* L, que incluye a la coliflor (*B. oleracea* L. var. *botrytis*), el brócoli (*B. oleracea* L. var. *italica*), la col lombarda (*B. oleracea* L. var. *capitata* f. *rubra*), las coles de Bruselas (*B. oleracea* L. var. *gemmifera*) y la col crespa o kale (*B. oleracea* L var. *sabellica*), entre otros, siendo la especie más consumida para alimentación humana (Al-Shehbaz et al., 2006).

Con respecto a la composición nutricional de las variedades de *B. oleracea* L., éstas presentan unos altos porcentajes de agua en su composición (89-92%), mientras que su contenido en grasas es relativamente bajo (aproximadamente unos 0.37 g en el brócoli). También albergan un elevado contenido en vitaminas, como carotenos, tocoferoles, vitamina C y ácido fólico. Asimismo, los vegetales pertenecientes a *B. oleracea* son ricos en minerales como el fósforo, el azufre y el

calcio (Favela-González et al., 2020). Además, la presencia de distintos tipos de fitoquímicos con efectos beneficiosos en la salud, como ácidos fenólicos, antocianos o glucosinolatos, aporta un valor añadido a su consumo en la dieta.

1.2. Glucosinolatos

Las crucíferas presentan una amplia variedad de metabolitos secundarios responsables del sistema de defensa de la planta ante patógenos y situaciones de estrés, y que les confieren sus propiedades organolépticas. La producción de estos fitoquímicos depende de diversos factores, como el estado de desarrollo de la planta, el órgano de ésta, las condiciones de cultivo y el grupo de crucíferas al que pertenece (Isah, 2019).

1.2.1. Estructura de glucosinolatos y sus productos de hidrólisis; los isotiocianatos

Los glucosinolatos (GSLs), son un grupo de metabolitos secundarios sintetizados principalmente por plantas de la familia *Brassicaceae* (Wittstock & Halkier, 2002). Los GSLs son aniones que presentan una estructura general formada por un grupo tiohidroximato-*O*-sulfonato ligado a un residuo de glucosa, junto con una cadena lateral de alquilo, aralquilo o indolilo, según el aminoácido del que deriven (Barba et al., 2016). No obstante, en la literatura se han citado y descrito más de 200 cadenas laterales (Clarke, 2010). Esta alta diversidad se debe a la elongación de la cadena lateral en el aminoácido precursor antes de la formación de la estructura central del GSL y de una amplia gama de alteraciones secundarias, como la desaturación, oxidación, hidroxilación, metoxilación, sulfatación y glucosilación. También se pueden observar sustituciones por conjugaciones aciladas en los residuos de azúcar (Agerbirk & Olsen, 2012). Sin embargo, su clasificación se simplifica en tres grupos principales, según el aminoácido del que derivan (Prieto et al., 2019):

- Alifáticos: si derivan de la metionina, alanina, leucina, isoleucina o valina.
- Indólicos: si su precursor es el triptófano.

- Aromáticos: cuando proceden de la fenilalanina o la tirosina.

Los GSLs son moléculas con una alta estabilidad dentro de la célula vegetal y, generalmente, se consideran compuestos inocuos. No obstante, cuando la integridad de la estructura vegetal se ve comprometida (por masticación, calor o ataques de plagas), los GSLs entran en contacto con la enzima β-thioglucosidasa, conocida como mirosinasa (EC 3.2.3.1). Los productos de degradación directos de esta reacción son una β-D-glucosa y un grupo tiosulfonato-*O*-sulfonato inestable que, posteriormente, se reorganizará en una variedad de moléculas bioactivas. Con respecto a su almacenamiento, los GSLs se encuentran diseminados en tejidos de todos los órganos de las plantas, mientras que la mirosinasa se localiza en células de mirosina, que no contienen GSLs (Redovniković et al., 2008). De esta manera, la planta se protege contra herbívoros y patógenos con un sistema de defensa altamente efectivo.

En cuanto a la reorganización de la aglicona inestable, denominada como transposición de Lossen, da lugar a la liberación de un ion sulfato y a la formación de distintos metabolitos. Estos varían según la cadena lateral del GSL y las condiciones fisicoquímicas de la reacción, como el pH, la presencia de iones de hierro (Fe^{2+}) y la presencia o ausencia de proteínas epitioespecíficas (EPSs).

Las reacciones descritas, podrían clasificarse de la siguiente manera (Blažević et al., 2020) (Figura 1):

1. A pH neutro, se favorece la reorganización de la aglicona en forma de isotiocianatos (ITCs). Estos compuestos son altamente reactivos y son capaces de inducir enzimas de detoxificación de la fase II *in vivo*. Además, diversos estudios previos demuestran su actividad anti-tumorogénica, y antiinflamatoria. Su función natural es bactericida, fungicida y nematicida (Ramirez et al., 2020).
2. Si la cadena lateral del GSL precursor está hidroxilada en el C3, se puede producir una ciclación espontánea del ITC, dando lugar a una oxazolidina-2-tiona.

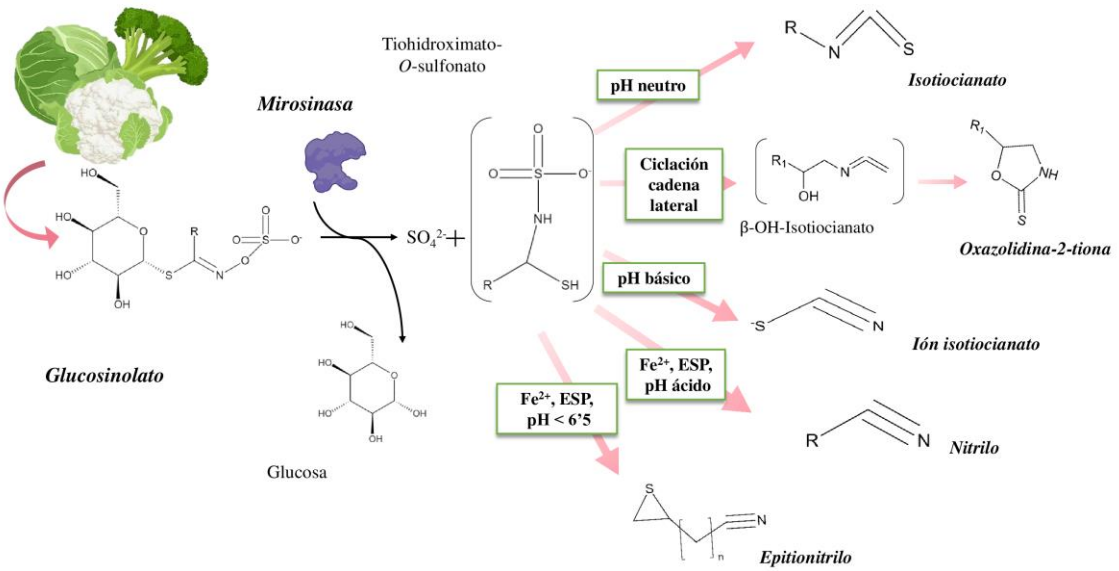

Figura 1. Proceso de hidrólisis de los glucosinolatos y sus principales productos derivados. ESP: proteínas epitioespecíficas.

3. Podemos obtener nitrilos en presencia de EPS, favorecidos también por un pH bajo, inferior a 3.

4. En el caso de que el GSL presente un doble enlace terminal en la cadena lateral, el átomo de azufre liberado por la formación del nitrilo, puede ser captado por éste, resultando en la producción de epitionitrilos.

5. Algunos GSLs pueden ser hidrolizados a tiocianatos bajo condiciones de pH superiores a 8.

Este conjunto de biomoléculas resultantes de la degradación de los GSLs, destacando los ITCs, debe tenerse en cuenta, ya que no solo son los que confieren el sabor característico a las crucíferas, sino que les otorgan una potencial actividad biológica. Por ello, también es crucial conocer sus rutas de biosíntesis, su metabolismo y absorción para conseguir un adecuado procesamiento de estos productos en la industria alimentaria.

1.2.2. Biosíntesis de glucosinolatos y regulación genética

Actualmente, la identificación de genes y enzimas partícipes de la ruta biosintética de los GSLs ha sido descrifrada casi por completo en el modelo *Arabidopsis thaliana*, gracias a herramientas como las colecciones de mutantes, la clonación o la identificación de loci de rasgos cuantitativos (QTLs en inglés) (Borevitz & Chory, 2004; Kroymann et al., 2001). De manera análoga, en *B. oleracea* se han llegado a identificar hasta 105 genes relacionados con el metabolismo de los GSLs, de los cuales 22 están implicados en su biosíntesis (Liu et al., 2014).

Este proceso de biosíntesis puede separarse en tres etapas bien diferenciadas:

A. ELONGACIÓN DE LA CADENA LATERAL DEL AMINOÁCIDO PRECURSOR

Esta etapa solo se produce cuando el aminoácido precursor es metionina o fenilalanina. Resumidamente, comienza con una desaminación llevada a cabo por la enzima aminoácido transferasa BCAT, de la que resulta un 2-oxoácido. Éste, a su vez, entrará en un ciclo de tres reacciones sucesivas de condensación, isomerización y descarboxilación, llevadas a cabo por las enzimas MAM, IPMI e IPM-DH, respectivamente (Sønderby et al., 2010). El resultado final es un 2-oxoácido con una elongación de un grupo metileno. A su vez, éste puede ser de nuevo transaminado por BCAT para proceder a la generación de la estructura central del GSL o someterse a un nuevo ciclo de elongación (Figura 2). Con respecto a la localización subcelular, BCAT actúa en el citosol, mientras que el resto de enzimas de elongación se encuentran en el cloroplasto (Mitreiter & Gigolashvili, 2021; Ida E. Sønderby et al., 2010)

B. FORMACIÓN DE LA ESTRUCTURA CENTRAL

En este paso están implicadas unas 13 enzimas y, a su vez, puede resumirse en 5 pasos bioquímicos.

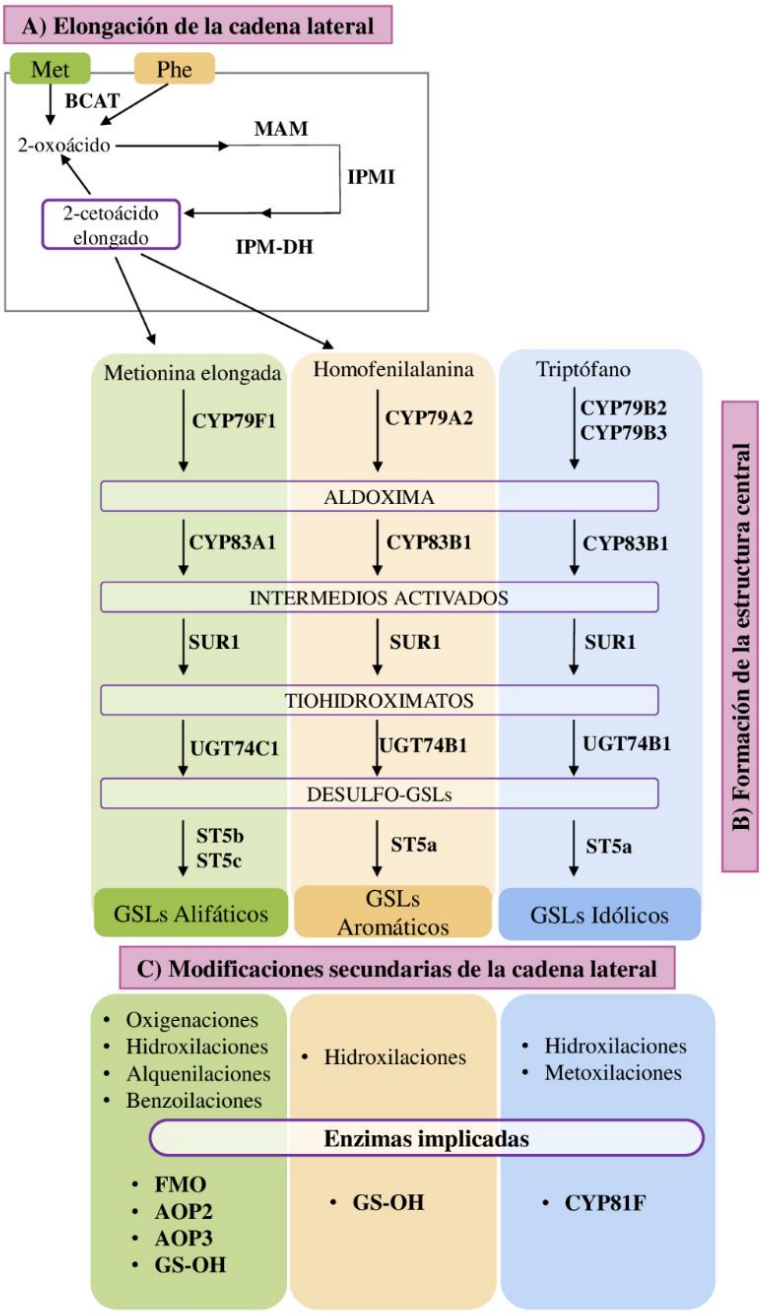

Figura 2. Rutas biosintéticas de los glucosinolatos alifáticos, aromáticos e indólicos. La ruta de los alifáticos incluye a metionina, leucina, isoleucina, valina alanina y glutamato. La ruta aromática comienza con la fenilalanina y tirosina. Dentro de los aminoácidos indólicos se encuentra el triptófano. **A) Elongación de la cadena lateral**: BCAT, aminotransferasa de aminoácidos de cadena ramificada; MAM, metiltioalquilmalato sintasa; IPMI, isopropilmalato isomerasa; IPMDH, isopropilmalato dehidrogenasa. **B) Formación de la estructura central**: CYP79F1, CYP79A2, CYP79B2 y CYP79B3, citocromos P450 de la familia CYP79; CYP83A1 y CYP83B1, citocromos P450 de la familia CYP83; SUR1, super root 1; UGT74C1 y UGT74B1, UDP-glucosiltransferasa; ST5a, ST5b y ST5c, sulfotransferasa 5**. C) Modificaciones secundarias de la cadena lateral:** FMO, flavina monooxigenasa; AOP2 y AOP3, productor de alquenil hidroxialquil; GS-OH, dioxigenasa dependiente de 2-oxoglutarato; CYP81F, citocromo P450 familia CYP81.

Primero, los aminoácidos precursores son convertidos en aldoximas por la acción de los citocromos P450 de la familia CYP79. Se ha observado que CYP79B2 y CYP79B3 metabolizan triptófano, mientras que CYP79A2 metaboliza fenilalanina. Así mismo, se ha demostrado que CYP79F1 pertenece a la ruta de la metionina y sus derivados elongados (Figura 2).

Tras ello, las aldoximas resultantes son oxidadas hasta obtener distintos compuestos activos, mediante los citocromos P450 de la familia CYP83. CYP83B1 es el encargado de las acetaldoximas derivadas del triptófano y la fenilalanina. Por lo contrario, CYP83A1 está implicado en la oxidación de las aldoximas alifáticas.

En tercer lugar, se produce la conjugación de una aldoxima activada con un donante de azufre, mediante reacciones que pueden ocurrir de manera no enzimática. Los *S*-alquil-tiohidroximatos resultantes son convertidos en tiohidroximatos por la acción de la *C*-*S* liasa SUR1.

Los tiohidroximatos son *S*-glicosilados por las glicosiltransferasas de la familia UGT74, siendo UGT74B1 la responsable de los derivados de la fenilalaina, mientras que UGT74C1 actúa en la ruta alifática. Esta reacción proporciona desulfo-GSLs, que finalmente, serán sulfatados mediante las sulfotransferasas. En *A. thaliana,* estas enzimas se conocen como SOT16 (encargada de los derivados de fenilalanina y triptófano), SOT17 y SOT18 (ambos encargados de los derivados alifáticos de cadena larga). No obstante, estudios recientes han denominado a estas enzimas como ST5a, ST5b y ST5c, respectivamente, en *Brassica oleracea* (Yi et al., 2016).

C. MODIFICACIONES SECUNDARIAS DE LA CADENA LATERAL.

Esta tercera etapa de la biosíntesis es un punto clave para incrementar la biodiversidad de GSLs que se encuentran en la naturaleza.

Modificaciones de los GSLs alifáticos

Dentro de las modificaciones secundarias podemos encontrar oxigenaciones, hidroxilaciones, alquilaciones y benzoilaciones. Además, se han identificado distintos *loci* encargados de estas reacciones, como GS-OX (del que destaca la flavina monooxigenasa o FMO), GS-AOP (el cual engloba a dos dioxigenasas, AOP2 y AOP3) y GS-OH (Sønderby et al., 2010).

Modificaciones de los GSLs indólicos

Para este tipo de GSLs principalmente se producen reacciones de hidroxilación y metoxilación. Destaca principalmente la familia de citocromos CYP81F, encargados de la hidroxilación de la glucobrasicina (GBS).

En cuanto a la expresión génica, los factores de transcripción MYB pertenecientes a la subfamilia R2R3 son los reguladores más específicos de la biosíntesis de GSLs. Se caracterizan por presentar dos dominios MYB en su extremo N-terminal, cada uno de los cuales alberga tres α-hélices. La tercera hélice de las dos repeticiones son las que coordinan la unión a un rango de secuencias de DNA específicas (Kelemen et al., 2015). Los factores R2R3 MYBs también pueden dividirse en distintos subgrupos según la similitud de sus secuencias y la conservación de su función. En la planta modelo *Arabidopsis thaliana,* seis miembros del subgrupo 12 son los encargados de regular positivamente genes responsables de la biosíntesis de GSLs. Tres de ellos son clave en la síntesis de los GSLs alifáticos (MYB28, MYB29 y MYB76), y los otros tres en la de los indólicos (MYB34, MYB51 y MYB122).

En el resto de crucíferas se han hallado homólogos para estos genes, pudiendo encontrarse sus secuencias en su totalidad o parcialmente, dado que estos *loci* tienen una función redundante en la síntesis de GSLs. Estos factores de transcripción se inducen mediante estreses mecánicos o heridas, pero los factores pertenecientes a alifáticos e indólicos presentan funciones distintas, expresándose

de manera diferencial según el tejido o el elicitador. Por ejemplo, MYB28 se ha relacionado con la producción de GSLs alifáticos de cadena lateral larga (Sønderby et al., 2010). También se ha observado una mayor expresión del factor de transcripción de indólicos MYB34 en raíces (Frerigmann & Gigolashvili, 2014).

1.2.3. Composición de glucosinolatos en especies vegetales de Brassica

Como se ha mencionado previamente, la composición de GSLs varía entre especies de *Brassica* de manera cualitativa y cuantitativa. Por ejemplo, en las especies de *Brassica rapa* (que incluyen el nabo o la col china), la composición parece ser similar, incluyendo principalmente gluconapina (GNA), progoitrina (PRO), gluconapoleiferina y glucobrasicanapina. Sin embargo, entre las variedades de *B. oleracea* se ha observado una mayor diversidad en la composición de GSLs. No obstante, los más relevantes, dentro de los tres tipos principales, son:

- Alifáticos
  - Glucoiberina (GIB), precursor de la iberina. Se ha estudiado la capacidad de la iberina para inhibir la proliferación celular e inducir la apoptosis en células cancerosas (Gong et al., 2021).
  - Sinigrina (SIN), cuyo producto de degradación es el alil-isotiocianato (AITC), conocido por sus aplicaciones como pesticida natural (Zhang et al., 2021).
  - Glucorafanina (GRA), precursora del sulforafano (SFN), uno de los ITCs más estudiados. Se ha demostrado que es capaz de interaccionar con el factor nuclear eritroide 2 (Nrf2), interviniendo en la regulación de procesos inflamatorios (Ruhee & Suzuki, 2020). Además, presenta actividad anti-tumorogénica, provocando el arresto celular y la apoptosis en células cancerosas (Wang et al., 2021). Recientemente, se ha descrito su papel como modulador de la microbiota intestinal (Jun et al., 2020).
  - Progoitrina (PRO), del que deriva la goitrina. Este ITCs es uno de los más controvertidos, ya que se ha demostrado que tiene actividad como inhibidor de la tiroperoxidasa, una encima clave

en la síntesis de las hormonas tiroideas (Petroski & Minich, 2020). Además, recientemente se ha observado Gque la goitrina puede sufrir una nitrosación en el estómago en presencia de altos niveles de agua, formando N-nitroso-oxazolina, que presenta potencial mutagénico (Matthews et al., 2020).

- Indólicos
  - Glucobrasicina (GBS), de la que se obtiene el indol-3-carbinol (I3C). El producto de la condensación de dos moléculas de I3C es el dímero natural 3,3,-diindolilmetano (DIM). Ambos son productos de hidrólisis bioactivos, reconocidos por su actividad anticancerígena (Du et al., 2019; Megna et al., 2016).
  - 4-hidroxiglucobrasicina (HGB), 4-metoxiglucobrasicina (MGB) y neoglucobrasicina (NGB), son derivados de la GBS. Representan a los GSLs indólicos presentes en la mayoría de las crucíferas comestibles.
- Aromáticos
  - Gluconasturtina (GST), de la que deriva el fenetil-isotiocianato (PEITC), que se comercializa como biocida natural (Poveda et al., 2020).

1.3. Elicitación en crucíferas

1.3.1. Definición y tipos de elicitadores

Se puede definir como "elicitador" a aquellas sustancias, aplicadas de manera exógena, capaces de inducir cambios a nivel fisiológico en la planta. De esta manera, la planta activa distintos mecanismos de defensa, similares a los de respuesta a infecciones, a patógenos o estímulos ambientales. El uso de elicitadores es una herramienta útil para incrementar el contenido en fitoquímicos de la planta durante su desarrollo. Estos pueden emplearse individualmente o en combinación, pero no se deben confundir con los fertilizantes, ni con otros tipos de estreses naturales.

Principalmente, se pueden dividir en abióticos y bióticos. Los elicitadores abióticos incluyen una serie de factores relacionados con el estrés ambiental. Pueden ser de procedencia química, como metales y sales que alteran la integridad de membrana, o factores físicos, como la temperatura, la salinidad o la radiación, entre otros. En cuanto a los bióticos, su origen es biológico y pueden provenir de moléculas exógenas de hongos, bacterias, insectos o herbívoros o moléculas liberadas por la propia planta tras la acción de enzimas del patógeno (endógenas). Dentro de los elicitadores bióticos, también se incluyen las fitohormonas, ya que son moléculas responsables de respuestas celulares en tejidos situados lejos de donde se sintetizan. Los más relevantes son el ácido salicílico (SA), el ácido jasmónico (JA) y su derivado, el metil jasmonato (MeJA), también denominado como jasmonato de metilo.

1.3.2. Elicitación en la respuesta a patógenos de plantas: el papel del ácido salicílico y el ácido jasmónico

A nivel molecular, la respuesta de plantas frente a patógenos está inicialmente regulada por genes de resistencia de plantas (PR) o genes de avirulencia a patógenos (Avr). Además, al ser las plantas organismos sésiles, se ha observado que cada célula presenta la habilidad de producir distintas señales de transducción que pueden inducir resistencia a patógenos en otras partes de la planta. Este estado fisiológico de mayor capacidad defensiva se conoce como resistencia sistémica adquirida (SAR) y está provocado por estímulos ambientales específicos, como hongos, bacterias, virus, nematodos, plantas parasitarias e incluso algunos herbívoros (van Loon, 2016; McDowell & Dangl, 2000). Fitohormonas, como el SA o el MeJA, juegan un papel clave en la transducción de señales en este proceso (Figura 3).

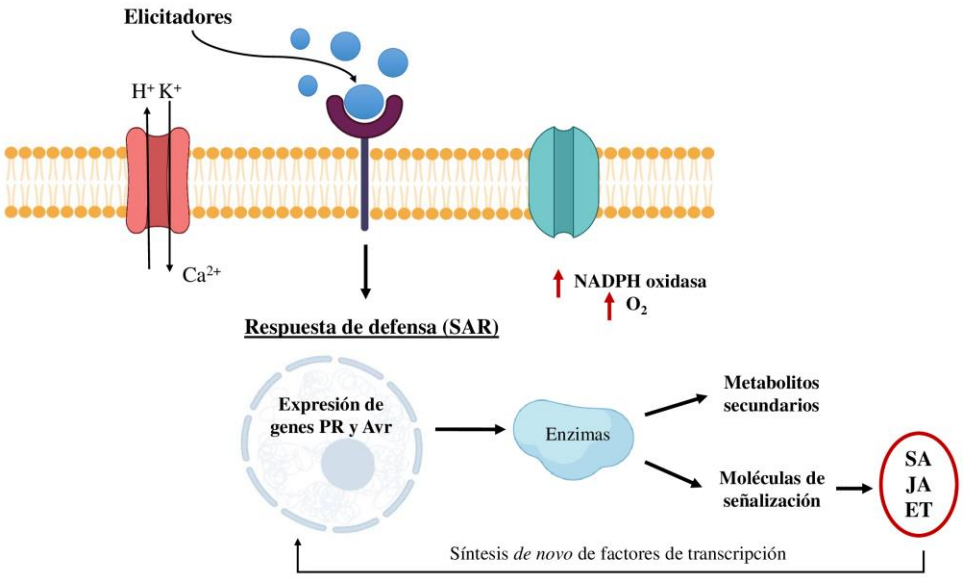

Figura 3. Mecanismo de acción de los elicitadores en la membrana celular. PR: resistencia a patógenos, Avr: avirulencia, SA: ácido salicílico, JA: ácido jasmónico, ET: etileno, SAR: resistencia sistémica adquirida. Imagen extraída y modificada de Baenas et al.,(2014).

Dentro de una amplia variedad de plantas, incluidas las crucíferas, el SA es capaz de inducir la expresión de genes PR mediante la interacción con el factor no expresor de genes PR1 (NPR1) (Wu et al., 2012). El SA es un compuesto fenólico capaz de unirse directamente con NPR1 y sus homólogos, regulando positivamente su estabilidad y actividad. Niveles altos de SA intracelulares desencadenan un cambio redox en el citoplasma que hace que NPR1 pase de dímeros a monómeros (Mou et al., 2003). Estos monómeros activos serán translocados al núcleo (Figura 4). Dado que los NPR1 no presentan dominios de unión al DNA, éste factor de transcripción necesita interaccionar con los factores de transcripción de la familia TGA (que presentan una cremallera de leucina básica) para desempeñar su función (Beckers & Spoel, 2006; Mou et al., 2003).

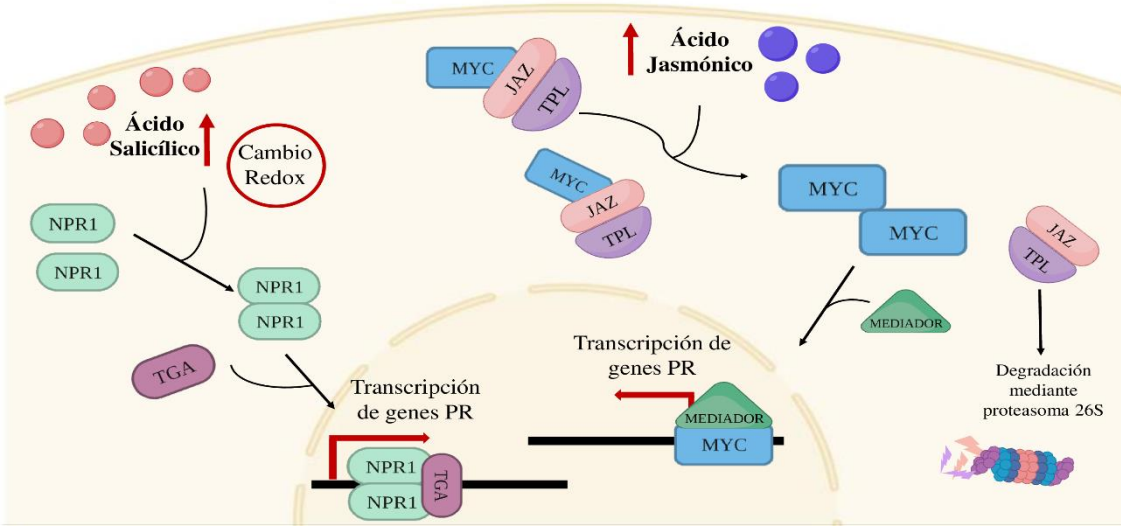

Figura 4. Regulación de genes de resistencia a patógenos (PR) mediante el ácido salicílico y la vía MYC del ácido jasmónico. NPR1: factor no expresor de genes PR1. JAZ: Familia de dominio ZIM de jasmonato, TPL: Factor de transcripción TOPLESS, MYC: gen similar al oncogén del virus del mieloma.

El JA y sus derivados son hormonas del grupo de los lípidos oxigenados (oxilipinas) también juegan un papel relevante en la respuesta sistémica de defensa. De manera muy simplificada, la ruta de señalización regulada por JA puede dividirse en dos ramas diferentes; la ruta MYC, llamada así por el gen similar al oncogén del virus del mieloma, y la ruta condicionada por los factores de respuesta a etileno (ERF) (Zhang et al., 2015). Ante heridas o ataques inducidos por insectos, la rama MYC es la principalmente responsable, y está regulada por los factores MYC2, MYC3 y MYC4, que presentan una cremallera de leucina con un domino hélice-bucle-hélice. En ausencia de JA y derivados, los factores MYC se encuentran reprimidos por el complejo formado por los factores JAZ y TPL (Figura 4). Cuando aumenta la concentración de JA en el citosol, JAZ es degradada por el proteasoma 26S, liberando a MYC para interaccionar con los mediadores transcripcionales (Chini et al., 2007). Estos factores de transcripción coordinan respuestas dependientes del triptófano, aumentando de este modo la producción de GSLs indólicos (Guo et al., 2022). Por otro lado, la vía ERF es inducida ante una

infección necrotrófica en la planta y está co-regulada por el etileno (Pré et al., 2008).

Tanto la ruta del SA como la del JA están interrelacionadas y aunque todavía no se ha elucidado por completo su interacción, varios autores han descrito que genes presentes en la biosíntesis de JA son reprimidos por SA (Leon-Reyes et al., 2010). Además, los factores TGA implicados en la ruta del SA también han demostrado tener un doble papel, tanto positivo como negativo, en la ruta de señalización del JA (Zander et al., 2010).

1.3.3. El uso de metil jasmonato y ácido salicílico para aumentar la producción de glucosinolatos

Con el fin de diseñar una estrategia adecuada para poder producir variedades de crucíferas enriquecidas en GSLs, es necesario comprender los mecanismos que gobiernan su biosíntesis y acumulación. La elicitación, principalmente con MeJA y SA ha sido ampliamente utilizada en campo para poder aumentar el contenido en GSLs (Hassini et al., 2019, 2017). De manera general, se ha podido comprobar que en cultivos como el brócoli, la col rizada o la mostaza, la aplicación exógena de MeJA favorece la acumulación de GSLs indólicos, como la neoglucobrasicina (Augustine & Bisht, 2015; Sun et al., 2012; Pérez-Balibrea et al., 2011). En cuanto al SA, se ha observado que su aplicación estimula la biosíntesis y acumulación de los tres tipos de GSLs (Sun et al., 2012; Smetanska et al., 2007).

En cuanto a su regulación a nivel genético, el estudio de (Guo et al., 2022), en el que analiza perfiles de expresión, muestra que algunos factores de transcripción MYB y genes biosintéticos están altamente inducidos tras aplicar JA (Guo et al., 2013). Además, estudios realizados *A. thaliana* han relacionado su elicitación con MeJA con la inducción de genes CYP, aumentando la acumulación de GSLs indólicos (Mikkelsen et al., 2003). Con respecto a los factores de transcripción, también se ha observado que principalmente MYB51 es un regulador clave en la señalización de la ruta del SA, mientras que MYB34 está implicado en la vía del JA (Frerigmann & Gigolashvili, 2014). Estudios recientes realizados en bulbos de

rábano, también han relacionado positivamente una sobre-expresión en los genes CYP79F1 y $FMO_{GS\text{-}OX}$ tras la elicitación con SA durante seis días (Chen et al., 2019).

No obstante, la acumulación y biosíntesis de los GSLs en la planta depende también de otros factores. Además del tipo de elicitador aplicado, el órgano elicitado o analizado (raíz, parte comestible u hojas), la variedad o la subespecie y el tiempo de aplicación, son otros determinantes (Yi et al., 2016). Por ello, en la presente memoria se pretende diseñar un protocolo de elicitación adecuado para variedades menos estudiadas, en concreto, el Bimi® (híbrido tipo broccolini o Tenderstem ®) y la col roja o lombarda.

2. Metabolismo de las crucíferas y sus beneficios para la salud

2.1. Biodisponibilidad y absorción de los glucosinolatos e isotiocianatos

Se puede definir como biodisponibilidad a la fracción de una biomolécula que es absorbida por el organismo y que llega hasta su diana fisiológica. Los ITCs son la principal molécula bioactiva derivada de los GSLs a pH fisiológico. Es por ello que, comprender las rutas de absorción de estas moléculas y su metabolismo es relevante. Sin embargo, la información disponible, tanto para GSLs como ITCs es más abundante en modelos animales e *in vitro* (Baenas et al., 2016), pero más limitada en estudios intestinales en humanos (Clarke et al., 2011).

Asimismo, existen diversos parámetros, endógenos y exógenos, que afectan tanto a la conversión de GSLs a ITCs, como a su absorción a nivel intestinal. De entre los cuales, destacan:

- La naturaleza del material vegetal, su procesamiento previo y almacenamiento. También influye el grado de disrupción del material vegetal durante la masticación, ya que las amilasas no son capaces de degradar a los GSLs, sino la mirosinasa procedente del material vegetal de origen.

- La concentración de cada tipo de GSLs en el material vegetal de partida. Así como la solubilidad, estabilidad y las propiedades fisicoquímicas particulares de cada GSL y sus productos de degradación.
- La concentración y estabilidad de la enzima mirosinasa en el material vegetal.
- La digestión gástrica y la absorción intestinal, ya que existen grandes diferencias intra e inter individuales. Además, la microbiota juega un papel relevante en la degradación de los GSLs, ya que algunas especies presentan una mirosinasa bacteriana (Bhat & Vyas, 2019). No obstante elucidar cómo influyen de manera directa es altamente complejo, debido a la diversidad del microbioma intestinal, su constante cambio y la interacción entre especies.

En cuanto a la digestión y absorción de los GSLs, si partimos de un material cocinado, este procesado desnaturaliza a la enzima mirosinasa, inactivándola total o parcialmente (Moreda-Piñeiro et al., 2011). En este caso, se conserva una alta proporción de GSLs intactos, que pueden ser parcialmente absorbidos en el estómago. Se ha observado que la fracción restante suele transitar al colon, donde la microbiota intestinal fermentará dichos GSLs. Sin embargo, si partimos de un material crudo o poco cocinado, la mirosinasa comenzará a degradar a los GSLs en la boca con la masticación, generando ITCs (Barba et al., 2016). Al llegar al intestino delgado, este proceso continúa, a la vez que se produce su absorción. De igual manera que el ejemplo anterior, los ITCs restantes también acabarán en el colon (Figura 5).

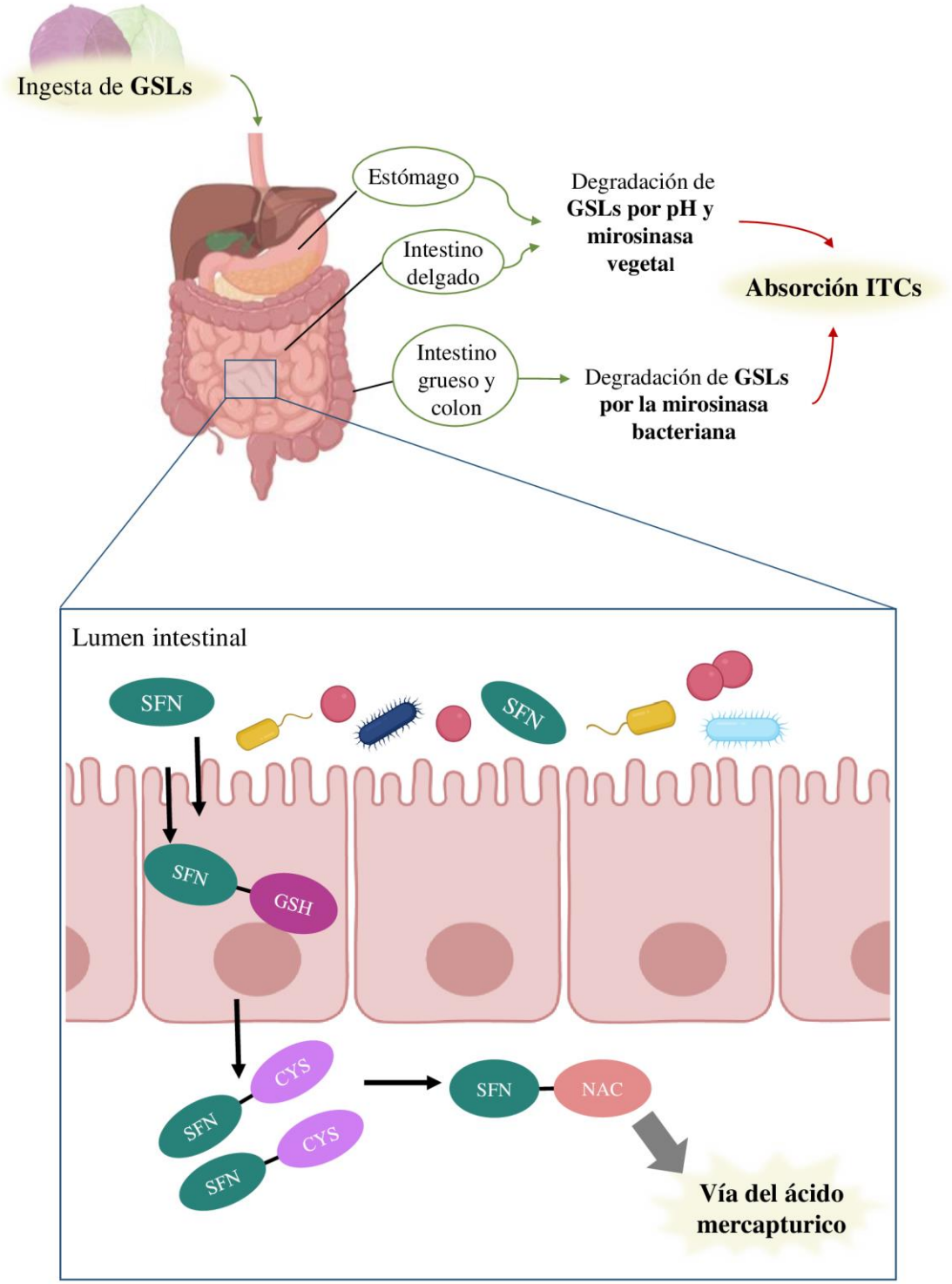

Figura 5. Resumen del destino de los glucosinolatos (GSLs) e isotiocianatos (ITCs) en el tracto gastrointestinal. Descripción de la absorción intestinal y metabolismo del sulforafano (SFN). SNF-GSH: sulforafano-glutatión, SFN-CYS: sulforafano-cisteína, SFN-NAC: sulforafano-*N*-acetilcisteína. Extraído y modificado de Barba et al., (2016)

No obstante, la parte más estudiada hasta la fecha ha sido el metabolismo post-absorción de los ITCs, con especial énfasis en el SFN. Cuando los ITCs pasan del lumen intestinal al interior del enterocito, se conjugan con la glutatión (GSH) y salen a circulación sistémica, siendo su destino final la ruta del ácido mercaptúrico (Traka et al., 2008). En el caso concreto del SFN, este se conjuga rápidamente dando lugar al SFN-GSH gracias a la glutatión-S-transferasa (Figura 5). Este SFN-GSH puede ser liberado al torrente sanguíneo, pero se ha observado un alto porcentaje de escisión, tanto química como enzimática, dando lugar a SFN libre. Tras ello, el SFN-GSH será gradualmente catalizado por una c-glutaniltranspeptidasa y una cysteinilglicinasa, formando SFN-CYS-GLY y SFN-CYS. Este conjugado SFN-CYS es el que se distribuye por los órganos diana, como el hígado. Finalmente, el SFN-CYS es acetilado, dando lugar a una *N*-acetil-S-L-cisteína conjugada al SFN (SFN-NAC). Este metabolito es el que principalmente será excretado por la orina. De manera general, el conjugado ITC-NAC supone entre un 12-80 % de la dosis ingerida de ITCs (Prieto et al., 2019). De este modo, la proporción excretada de estas moléculas se puede correlacionar con la cantidad consumida de ITCs, lo que convierte a estos metabolitos en excelentes biomarcadores.

Con respecto a otros productos de hidrólisis provenientes de los GSLs, existe poca información con respecto a su metabolización por la microbiota y su absorción. Varios estudios respaldan la capacidad de formar nitrilos a partir de GSLs por algunas cepas de géneros como *Bifidobacterium*, *Escherichia coli* VL8 o *Entrerobacter cloacae* (Narbad & Rossiter, 2018). Por otro lado, se ha observado la presencia de enzimas con actividad glucosidasa similar a la mirosinasa en distintos géneros presentes en el intestino, como *Lactobacillus*, *Staphylococcus* y *Bifidobacterium*, entre otros. Sin embargo, elucidar por completo el papel de la microbiota intestinal en la degradación de los GSLs es altamente complejo, debido a las múltiples interacciones microbianas, su alta variabilidad interindividual y su dependencia de la actividad metabólica del hospedador (Sikorska-Zimny & Beneduce, 2021).

2.2. El consumo de crucíferas y su efecto en la salud

Las crucíferas son ampliamente consumidas a nivel mundial, llegando a producirse en España unas 746.510 toneladas de brócoli y coliflor en el año 2020 (FAO, 2020). Asimismo, fue el país responsable de exportar el 38,6% de la producción a nivel mundial ese mismo año. A pesar de esto, no toda la biomasa generada se destina a alimentación y parte de la producción no supera los estándares de comercio, generando grandes mermas en la producción de estos vegetales.

Dada su relevancia, es de gran interés conocer el efecto que ejerce su ingesta habitual en la dieta y cómo influyen sobre el metabolismo y el sistema inmunitario del consumidor. Es por ello que, durante las últimas décadas se han estudiado distintas funciones de los ITCs provenientes de crucíferas beneficiosas para la salud humana, como antioxidantes, antiinflamatorios o anti-proliferativos, entre otros.

Regulación de la respuesta antioxidante y antiinflamatoria

La regulación principal de los ITCs en las rutas de detoxificación y antiinfamatorias a nivel celular es a través de la interacción positiva del factor de transcripción Nrf2 y la regulación negativa del factor NF-κB (Sailaja et al., 2021). Se ha observado que el SFN es capaz de unirse a los residuos de cisteína presentes en el represor del factor Nrf2, Keap1. Bajo condiciones fisiológicas, este represor promueve la degradación de Nrf2 por el proteasoma, pero cuando el SFN interacciona con él, Nrf2 queda libre, translocándose al núcleo y promoviendo la transcripción de genes implicados en la respuesta antiinflamatoria y antioxidante (Greaney et al., 2016). También se ha descrito una interacción de ITCs como el SFN o la erucina con la ruta del factor NF-κB, disminuyendo su capacidad de unirse al DNA, reduciendo la transcripción de genes, incluyendo TNFα, la interleucina 6 (IL-6), y la enzima óxido nítrico sintasa inducible (Cho et al., 2013). Por otro lado, el AITC ha demostrado ser un modulador del receptor de hidrocarburos de arilos, el cual está implicado en la detoxificación de

contaminantes ambientales y es un regulador negativo de la respuesta inmune (Rajakumar et al., 2018).

Efecto quimioprotector de los ITCs en el cáncer

La aplicación de los ITCs como agentes quimioprotectores ha sido ampliamente demostrada, no solo en distintos tipos de cáncer sino también en diversos modelos animales (Prieto et al., 2019). Los ITCs son capaces de inhibir la fase metabólica I mediante la supresión de las enzimas del citocromo P450, que activan muchos agentes cancerígenos, e inducen las enzimas de detoxificación de fase II (Soundararajan & Kim, 2018). Asimismo, la realización de estudios retrospectivos en voluntarios ha vinculado el consumo habitual de crucíferas con una reducción en la incidencia de varios tipos de cáncer, como el colorrectal (Johnson, 2018), el de mama (Bosetti et al., 2012) o el de vejiga (Nguyen et al., 2021), entre otros.

No obstante, a pesar de que se han realizado estudios pre-clínicos de intervención a medio y largo plazo en pacientes, aún es necesario evaluar la seguridad, dosis y momento de intervención apropiado para poder ser empleados como coadyuvantes o fármacos una vez la enfermedad se ha desarrollado (Quirante-Moya et al., 2020).

Influencia de los ITCs en el síndrome metabólico, obesidad y diabetes

La inflamación prolongada de bajo grado suele asociarse con el desarrollo de enfermedades crónicas, como el síndrome metabólico. Bajo esta denominación se han incluido distintos factores de riesgo, como la dislipidemia, la resistencia a la insulina o una alta presión arterial, que incrementan la incidencia de enfermedades cardiovasculares, diabetes mellitus tipo 2 o la obesidad.

Con respecto a la obesidad, estudios realizados en roedores han demostrado que el SFN es capaz de mejorar el control de peso y reducir los niveles de lípidos (Du et al., 2021). Además, el SFN administrado en cultivos *in vitro* de células 3T3-L1 ha demostrado presentar una capacidad para regular la acumulación de grasa

de los adipocitos y de promover la biogénesis de mitocondrias, lo que sugiere que puede modular el paso de la grasa blanca a grasa parda, encargada de mantener el calor corporal (Martins et al., 2018). Estudios realizados en voluntarios humanos con obesidad, se ha observado que el consumo diario de 30 g de brotes de brócoli, favorece la disminución de marcadores inflamatorios como la IL-6 (López-Chillón et al., 2019).

Con respecto a la diabetes mellitus tipo 2, está íntimamente relacionada con la obesidad y el sobrepeso. Esto se debe a que un mayor porcentaje de tejido adiposo implica una mayor resistencia a la insulina, junto con un estado de baja inflamación sistémica, lo que, a su vez, puede desencadenar en un mal funcionamiento hepático (Klein et al., 2022). Trabajos como el de Axelsson et al. (2017), ponen de manifiesto que el extracto concentrado en SFN de brotes de brócoli, reduce la glucosa en sangre en ayunas y la hemoglobina glicosilada en pacientes con obesidad y diabetes tipo 2 desregulada. Incluso se ha observado una sensibilización a la glucosa en modelos murinos tras la ingesta de floretas de brócoli (Zandani et al., 2021).

Crucíferas como prebióticos

Recientemente se ha descubierto el papel de las crucíferas como moduladores del microbioma intestinal. En el trabajo realizado por Kaczmarek et al., (2019) la intervención nutricional con 200 g de brócoli, realizada durante 15 días en voluntarios, reveló un aumento del 10% en la población de *Bacteroidetes* y una reducción del 9% en el phylum *Firmicutes*, en comparación con el control. A pesar de que la degradación de GSLs por la mirosinasa bacteriana es un fenómeno conocido, tal y como se ha descrito previamente en el presente manuscrito, aún es necesario un mayor estudio de cómo los GSLs y los ITCs son capaces de influir en el microbioma.

3. Nanoportadores como sistemas de estabilización de fitoquímicos

Generalmente, los grupos funcionales, polaridades y pesos moleculares de los fitoquímicos presentan una gran variabilidad, lo que conduce a diferencias en su

solubilidad y estabilidad química. Esto dificulta su paso tanto por la barrera hematoencefálica como por los revestimientos endoteliales, las mucosas o por el tracto gastrointestinal. A su vez, esto hace que sea necesario administrar una dosis mayor, lo que podría provocar un indeseado aumento de la toxicidad en órganos periféricos (McClements, 2020; Xiao, 2016).

Ante esta situación, surge como alternativa el uso de nanoportadores para mejorar la bioaccesibilidad y controlar la bioactividad de dichos compuestos, evitando los efectos negativos de su metabolización en condiciones deletéreas. Estas nanopartículas están diseñadas para mejorar la bioeficiencia de los fitoquímicos, aumentando su llegada al sitio diana. Para ello, suelen ser elementos diseñados a nivel atómico o molecular, incluyendo nanoestructuras de pequeño tamaño.

3.1. Tipos de nanoportadores

3.1.1. Origen inorgánico

Este tipo de nanoportadores presenta un núcleo inorgánico (oro, sílice, óxido de hierro o plata, entre otros) y un revestimiento compuesto por polímeros orgánicos o metal. Este es el encargado de proteger a las moléculas "cargadas" de los cambios fisiológicos del exterior y, a su vez, proporcionar un sitio de unión para otras biomoléculas o receptores (Shi et al., 2020).

Su principal ventaja es que la presencia de materiales inorgánicos le otorga a estos nanoportadores una naturaleza plasmónica y magnética, lo que implica una alta detección óptica, pudiendo ser detectados por distintas técnicas de imagen, como la resonancia magnética nuclear (Elzoghby et al., 2016). No obstante, su uso para liberación de fármacos es limitado, ya que muestran una poca capacidad de carga y una alta toxicidad periférica, restringiendo su uso a intervenciones farmacológicas puntuales (Choi et al., 2013). Dentro de esta categoría, las más conocidas son las nanopartículas de plata u oro, las nanorpatículas de sílice mesoporosas y las partículas super-paramagnéticas de óxido de hierro (SPIONs) (Figura 6).

3.1.2. Origen orgánico

Este tipo de nanoportadores se caracterizan por estar basados en el carbono y presentan una mayor capacidad de carga y biocompatibilidad que los de origen inorgánico.

A. POLIMÉRICOS

Son nanopartículas obtenidas mediante una reacción de polimeración de subunidades monoméricas. Dependiendo del método de preparación, las moléculas pueden encapsularse en su interior o quedar retenidas o asociadas a una malla polimérica (Avramović et al., 2020). Su principal ventaja es su alta biocompatibilidad y son altamente biodegradables. Además, al poder variar su composición, es posible modular su cristalinidad y su hidrofobicidad/hidrofilidad dentro del polímero, adaptándolos a la molécula cargada.

Pueden ser de origen sintético, natural o semi-sintético. Dentro de los polímeros sintéticos, los más conocidos son el ácido poliglicólico y el ácido poliláctico (Lombardo et al., 2019). Con respecto a los naturales, estos suelen ser empleados por su baja o nula respuesta inmunitaria. El quitosano, derivado de los caparazones de quitina de los crustáceos, o la goma xantana, proveniente de *Xanthomonas campestris*, suelen emplearse por su naturaleza mucoadhesiva, favoreciendo su absorción en el lecho epitelial (Xing et al., 2019). No obstante, las pectinas (derivadas de las frutas) y los alginatos (obtenidos de las paredes celulares de las algas pardas) presentan una ligera toxicidad farmacológica, por lo que suelen estar destinados a alimentación (Ahmad et al., 2021). Las dextrinas y ciclodextrinas también son ampliamente utilizadas en la industria alimentaria, textil y cosmética, gracias a su capacidad de encapsulación y liberación sostenida en el tiempo (Peng et al., 2017).

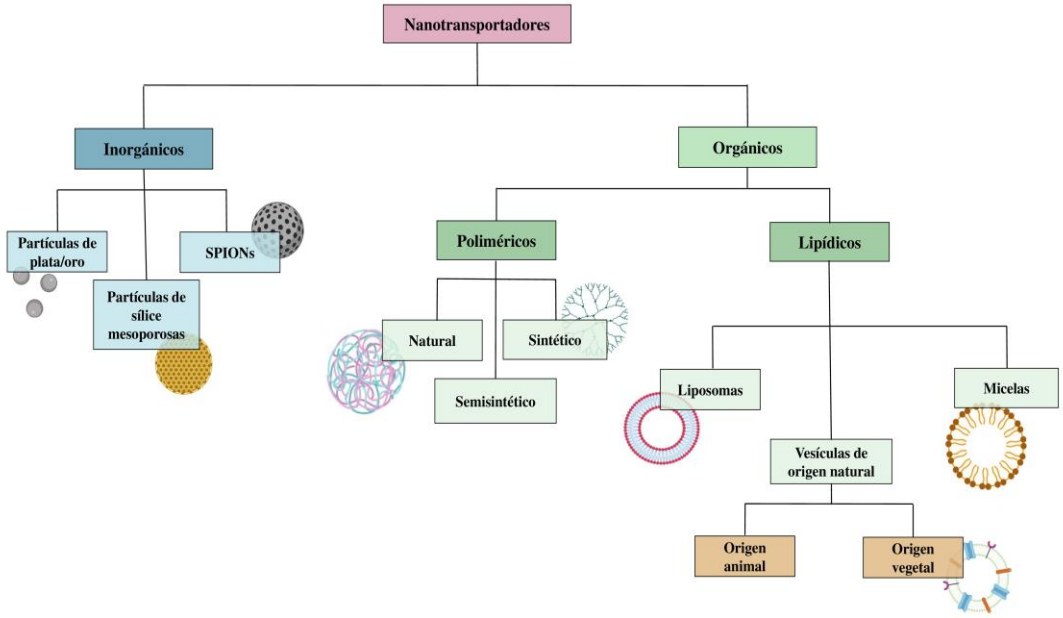

Figura 6. Esquema de los principales tipos de nanoportadores.

B. ORIGEN LIPÍDICO

Este tipo de nanoportadores están basados en moléculas de origen lipídico, que encapsulan el fármaco o compuesto en su interior para prevenir su degradación frente a cambios en el medio. Suelen aumentar el índice terapéutico de su carga, pues promueven su estabilidad en el medio. Sin embargo, algunos pueden ser difíciles de estabilizar en ciertas matrices y presentar problemas de solubilidad. También se caracterizan por tener un tiempo de vida media limitado y suelen ser más costosos que los poliméricos (Ahmad et al., 2021). Dentro de ésta categoría se incluyen principalmente a las micelas, liposomas y vesículas de origen natural (Figura 6).

Micelas

Se obtienen mediante la dispersión de moléculas anfifílicas de bajo peso molecular. Son ser estructuras con un centro hidrofóbico y una corona exterior hidrofílica. Su tamaño es pequeño, oscilando entre los 10-50 nm de diámetro. Este

tipo de micelas se caracterizan por ser sistemas dinámicos, en los que se produce un intercambio de material entre micelas o entre el medio y su interior. Este fenómeno puede disminuir la estabilidad del sistema, reduciendo su bioactividad (Hevus et al., 2012). Para poder controlar la formulación de micelas es clave conocer su concentración micelar crítica, el tamaño de partícula y su morfología, y la estabilidad coloidal de la formulación.

Liposomas

Son vesículas compuestas por una bicapa de fosfolípidos, con las cabezas polares dispuestas hacia el medio y las colas hidrofóbicas interaccionando entre sí. Dentro de sus componentes principales está la fosfatidilcolina, a la que también se le suele añadir colesterol para incrementar la fluidez de la membrana. Su principal diferencia con las micelas es que éstas generalmente se conforman por una monocapa (Akbarzadeh et al., 2013). Al estar compuestos por bicapas, son nanoportadores más biocompatibles, mejorando la estabilidad y solubilidad de los compuestos encapsulados. Suelen dirigirse mejor a los órganos diana, aumentando así la respuesta terapéutica y minimizando su toxicidad.

En función de las propiedades fisicoquímicas de sus componentes, se pueden formular para obtener variaciones en tamaño (generalmente entre 50-450 nm), número de lamelas, estructura y composición. De tal modo, se pueden clasificar en tres tipos principales; vesículas multilamelares (MLVs), vesículas grandes unilamelares (LUVs) con tamaño entre 100-100 nm, y vesículas pequeñas unilamelares (SUVs) con un tamaño menor de 100 nm.

A raíz de su alta versatilidad, durante las últimas décadas han surgido distintos tipos de liposomas, de entre los cuales destacan:

- **Liposomas modificados.** Son modificaciones menores de los liposomas clásicos compuestos por fosfolípidos.
    - *Liposomas PEGilados*. El componente principal de sus bicapas es el polietilénglicol (PEG). Presentan baja

antigenicidad, evitando su detección por el sistema inmunitario.

- *Liposomas dirigidos a ligandos*. Estos contienen carbohidratos, péptidos o anticuerpos unidos a la superficie de la bicapa.
- *Liposomas mixtos*. Son una combinación de un liposoma clásico y las dos modificaciones mencionadas previamente.

- **Niosomas.** Vesículas ultradeformables resultantes de añadir un surfactante no-iónico al colesterol, para posteriormente rehidratar la mezcla en medio acuoso. Presentan un mayor tamaño y capacidad de carga que los liposomas convencionales, aumentando su eficiencia de atrapamiento. Esto se traduce en una mayor estabilidad y una reducción en los costes de producción (Makeshwar & Wasankar, 2013).
- **Etosomas.** Su peculiaridad reside en una alta proporción de etanol en su mezcla (20-50%). Esto los convierte en altamente polares, siendo capaces de permear el estrato córneo, aumentando la penetración transdérmica. Además, el etanol reduce la compactación de la bicapa, siendo vesículas más maleables, pero con misma estabilidad (Zhang et al., 2012).

Vesículas de origen natural

Son vesículas obtenidas a partir de material biológico, ya sea de células eukariotas o procariotas. En hongos, micobacterias y bacterias Gram positivas, se ha observado que juegan un papel relevante en el intercambio de factores de virulencia, implicados en la patogénesis y la interacción con el organismo hospedador (Brown et al., 2015). Debido a su capacidad de provocar fuertes respuestas inmunitarias, no suelen aplicarse en la industria. No obstante, como alternativas surgen las vesículas derivadas de células de mamíferos y plantas.

3.2. Vesículas derivadas de membranas naturales

3.2.1. Vesículas extracelulares de mamíferos

Los exosomas son membranas lipídicas de pequeño tamaño, unos 40-150 nm de diámetro, secretadas por diversos tipos celulares y están presentes en múltiples fluidos biológicos, como la sangre, la saliva o la orina. Además, son capaces de transportar proteínas de señalización, ácidos nucleicos, y lípidos, principalmente (Doyle & Wang, 2019). El creciente interés en estas vesículas extracelulares derivadas de mamíferos (MEVs) reside en su capacidad como nanoportadores, debido a que, por su propia naturaleza, presentan mayor biocompatibilidad, menor toxicidad y muestran una alta capacidad para atravesar las distintas barreras biológicas (Villa et al., 2019).

En cuanto a su biogénesis, se forman a partir de la invaginación de la membrana endosomal, en el citosol de la célula, produciendo cuerpos multivesiculares (MVBs), que engloban vesículas de pequeño tamaño, conocidas como vesículas intraluminales (ILVs). Posteriormente, las MVBs se fusionarán con la membrana plasmática, liberando al medio las ILVs, que pasarán a ser exosomas (Hessvik & Llorente, 2018). Una vez alcanzan la célula diana, pueden o bien interaccionar con los receptores de membrana y desencadenar distintas vías de señalización, o fusionarse con la célula, liberando su carga en el interior (Mathivanan et al., 2010).

Según la célula de la que derive el exosoma, tanto su composición proteíca y lipídica, como su carga, pueden variar. No obstante, se han caracterizado proteínas marcadoras características de MEVs, como CD9, CD81, CD63, flotilina y TSG101 (Yue et al., 2020; Y. Zhang et al., 2019) . La técnica más empleada para su obtención es la ultracentrifugación diferencial, aunque la velocidad y el tiempo dependerán del origen de la muestra de partida, por ejemplo, si son medios de cultivo celulares o fluidos biológicos (Malhotra et al., 2021).

Tal y como se ha mencionado previamente, el rol fisiológico de las MEVs es la comunicación intercelular (Gézsi et al., 2019). Estudios llevados a cabo en la última década, han demostrado que están implicadas en múltiples procesos

fisiológicos. Por ejemplo, se ha observado que los MEVs son capaces de transferir antígenos desde células infectadas o tumorales hasta las células dendríticas, que, a su vez, estimularán el sistema inmunitario (Wolfers et al., 2001). También se ha observado que en situaciones patológicas, las MEVs son capaces de transferir pequeños RNAs y proteínas entre células tumorales, otorgándoles una mayor resistencia a fármacos (Li et al., 2016).

Por otro lado, el estudio principal de las MEVs como nanoportadores se ha enfocado en su uso terapéutico y diagnóstico. Gracias a que presentan una interacción específica ligando-receptor y la posibilidad de incorporar proteínas o péptidos funcionales, se ha demostrado que son una buena opción como vehículo para fármacos o quimiterapéuticos (Dargani & Singla, 2019). Así mismo, al poder albergar pequeños RNA y plásmidos de DNA, también se han estudiado para terapia génica o el desarrollo de vacunas (Duan et al., 2021; Santos & Almeida, 2021). También se ha observado que MEVs derivadas de osteoblastos, osteoclastos y sus precursores son capaces de regular la remodelación ósea, por lo que podrían aplicarse como terapia regenerativa (Xie et al., 2017).

3.2.2. Vesículas extracelulares derivadas de plantas

El descubrimiento de las vesículas extracelulares derivadas de plantas (PDEVs), se remonta a 1967, donde se había descrito que la fusión de MVBs con la membrana plasmática de plantas podría resultar en la liberación de pequeñas vesículas en el medio extracelular (Halperin & Jensen, 1967). No obstante, el interés por su biogénesis y aplicación es reciente, dado que son capaces de superar algunas limitaciones técnicas de las MEVs, como sus largos y laboriosos tiempos de obtención o su bajo rendimiento (P. Li et al., 2017; Lobb et al., 2015).

Con respecto al papel fisiológico de las PDEVs, algunos trabajos han observado un aumento de MVBs en los sitios de infección frente a un ataque fúngico. De este modo, estarían implicados en la respuesta a diversos estreses bióticos y abióticos, incluidos el ataque e infección por patógenos (An et al., 2007, 2006).

En cuanto a su biogénesis, esta no ha sido tan ampliamente estudiada como la de las EVs procedentes de mamíferos, sin embargo, los estudios realizados defienden que se producen de manera similar (Liu et al., 2021; Zhang et al., 2019). La presencia de proteínas de la familia PEN1 también se ha visto relacionada como marcadores de estos PDEVs (Liu et al., 2021).

A. MÉTODOS DE OBTENCIÓN DE LAS PDEVs

La separación mediante gradientes de densidad y ultracentrifugación diferencial es el enfoque estándar para la obtención de PDEVs. Este método parte de la formación de un homogenado del material vegetal de partida, que posteriormente es filtrado para eliminar fibras antes de proseguir con las centrifugaciones (Figura 7). El tiempo y velocidad se suelen ajustar según el material vegetal y se suele emplear un tampón fosfato salino en el proceso de homogenado. Otros métodos combinan la precipitación con la cromatografía de exclusión de tamaño o el uso de fluido apoplástico (Şahin et al., 2019; Rutter & Innes, 2017).

No obstante, existe cierta controversia con respecto a la nomenclatura de las PDEVs, ya que para su obtención se necesita una disrupción previa de los tejidos, a diferencia de las MEVs que están presentes en fluidos o medios de cultivo. Tal y como expone Rome (2019) en su trabajo de revisión, algunas EVs obtenidas de distintos vegetales, como la uva o el jengibre también incluyen marcadores citosólicos o apoplásticos.

Figura 7. Esquema del proceso de obtención de PDEVs.

Actualmente, se está mediando para poder conseguir una nomenclatura adecuada para estas vesículas derivadas de plantas (Pinedo et al., 2021). Es por ello, que, en el presente manuscrito, se emplea el término PDEVs para que coincida con el vocabulario empleado en la literatura. No obstante, debe tenerse en cuenta que probablemente representa una mezcla de todos los tipos de vesículas contenidas en plantas.

B. CARACTERIZACIÓN Y COMPOSICIÓN DE LAS PDEVs

Dependiendo del tejido y el vegetal empleado, las PDEVs pueden presentar diversas características fisicoquímicas. Parámetros como el tamaño (que oscila entre 10 nm y 1 µm), polidispersidad y potencial Z se analizan mediante técnicas de dispersión de luz dinámica, mientras que para el análisis morfológico se emplea microscopía electrónica de transmisión o de barrido (Karamanidou & Tsouknidas, 2022). Por otro lado, la caracterización bioquímica de los PDEVs suele emplear análisis ómicos, que permiten definir su composición lipídica, proteica y metabólica.

Lípidos

Son los principales encargados de mantener la integridad estructural, así como de interaccionar con las membranas de mamíferos. Las principales especies de lípidos que se encuentran en las PDEV son el ácido fosfatídico (PA), la fosfatidiletanolamina (PE) y la fosfatidilcolina (PC) (Urzì et al., 2021). Un estudio reciente realizado por Teng et al. (2018), ha demostrado que la presencia de PA en EVs derivadas de jengibre promueve su internalización por la bacteria *Lactobacillus rhamnosus*, mientras que la presencia de PC promovía una mayor absorción por bacterias de la familia Ruminococcaceae. Asimismo, PA ha demostrado ser un importante mediador lipídico, controlando los procesos de fusión y fisión de membrana (Zhukovsky et al., 2019). También se ha observado una posible regulación del crecimiento y proliferación en células de mamíferos mediante la interacción de este fosfolípido con la ruta de señalización mTOR (Menon et al., 2017).

Proteínas

El perfil proteico de las PDEVs depende de su origen, en términos de vías secretoras y matrices. Sin embargo, se han caracterizado algunas comunes para la mayoría de estas EVs, como las anexinas, que juegan un papel relevante en la secreción de vesículas (Pocsfalvi et al., 2018; Regente et al., 2009). Otras proteínas presentes son las incluidas en la familia de proteínas de choque térmico (HSPs). Por ejemplo, HSP70 se ha encontrado en EVs derivadas de cítricos (Pocsfalvi et al., 2018). Además, también se ha observado la presencia de lipoxigenasas y ATPasas (Bokka et al., 2020). Destaca el papel de las acuaporinas (AQPs), principales responsables del paso del agua y pequeños solutos (como gases o iones), a través de las membranas biológicas (Li et al., 2014). Éstas determinan la permeabilidad osmótica (*Pf*) de la vesícula, regulando su estabilidad en el medio.

Metabolitos

De manera natural, las EVs incluyen moléculas bioactivas presentes en el material de origen. Suelen ser comunes azúcares, como la fructosa, la glucosa y el myo-inositol (Stanly et al., 2020). Vesículas obtenidas de frutas, como cítricos o fresas, también presentan ácido ascórbico, proporcionándoles un alto poder antioxidante (Takakura et al., 2022; Perut et al., 2021). PDEVs obtenidas del jengibre presentaron shogaol, compuesto que le confiere el sabor picante, asociado (Zhuang et al., 2015). Asimismo, el estudio de vesículas obtenidas a partir de brócoli reveló un porcentaje de sulforafano asociado (Martínez-Ballesta et al., 2018).

C. APLICACIÓN DE LAS PDEVs

Agentes anti-tumorogénicos

Durante las últimas décadas, las nanopartículas han sido una herramienta clave para el tratamiento del cáncer, ya sea por su acción como agentes anti-cancerígenos *per se* o como vehículos dirigidos de fármacos quimioterapéuticos (Taléns-Visconti et al., 2022). En su papel como vehículos, sus objetivos principales son

aumentar el tiempo de circulación en sangre del fármaco y lograr una direccionalidad más específica al tumor (Thierry, 2009).

En este contexto, diversos estudios han resaltado las propiedades anticancerígenas de las PDEVs como un potencial candidato como agente terapéitoco en combinación con los tratamientos actuales (Urzì et al., 2021) . Por ejemplo, el uso de PDEVs obtenidas de *Citrus*, mostró una capacidad inhibición de la proliferación celular en líneas de carcinoma pulmonar humano (A549), leucemia mieloide crónica humana (LAMA84) y adenocarcinoma colorrectal humano (SW480) (Raimondo et al., 2015). Recientemente, también se ha descrito la posibilidad de que las PDEVs derivadas de la dieta contengan microRNAs capaces de modular la proliferación de células cancerosas en el tracto digestivo (Dávalos et al., 2021).

Nanoportadores de fármacos

Los agentes nanoencapsulantes convencionales, como los de síntesis artificial y liposomas, presentan diversas limitaciones, como su bajo rendimiento o su alto coste de producción (Reddy et al., 2012). Los PDEVs, sin embargo, ocurren de manera natural, lo que les confiere una alta estabilidad biológica, mientras que su toxicidad es reducida. Asimismo, al poder extraerse de subproductos vegetales, presentan un mayor rendimiento y sostenibilidad, contribuyendo a la economía circular (Buchman et al., 2019).

Se han estudiado múltiples aplicaciones *in vivo* e *in vitro* de estos nanoportadores, como el uso de EVs obtenidas de uva para aumentar la eficacia del metotrexato en modelos murinos de colitis ulcerosa (Lakhani 2017). A pesar de que la investigación actual se centra en tratamientos o fármacos administrados por vía oral o cutánea, en el caso del uso de fármacos que necesitan un alto grado de pureza, se pueden emplear los lípidos purificados de las PDEVs para producir nanoportadores diseñados (Yang et al., 2018). De manera similar, los PDEVs pueden modificarse para incorporar distintos ligandos en la membrana y conseguir una mayor especificidad con la diana (Sterzenbach et al., 2017). Otro método

empleado para modular su composición es la co-extrusión con otras membranas, en concreto se ha observado que el uso de membranas celulares de leucocitos junto con EVs de uva aumentó su direccionalidad hacia ambientes inflamatorios (Wang et al., 2015) (Figura 8).

Agricultura

El uso de nanoportadores en agricultura beneficia el aumento del rendimiento de los cultivos, la inhibición de la proliferación de patógenos, y la eliminación de malas hierbas e insectos no deseados a un menor coste (Singh et al., 2021). Generalmente, se han empleado nanopartículas de origen natural, como el quitosano en la biofortificación con micronutrientes (Deshpande et al., 2017). No obstante, el uso de PDEVs está emergiendo como una alternativa, dado que al ser vesículas derivadas de subproductos vegetales presentan una mayor compatibilidad con los tejidos de la planta, consiguiendo una mayor penetración (Rios et al., 2020).

Industria cosmética

Desde sus primeras investigaciones en la época de 1980s, la incorporación de ingredientes a nanoescala en el sector cosmético ha aumentado exponencialmente debido a su pequeño tamaño y su elevada relación superficie-volumen (Khezri et al., 2018). Su uso proporciona múltiples ventajas, como una mejor protección del bioactivo, mayor penetración de la fórmula y un aumento de la textura y cosmeticidad del producto final (Bilal & Iqbal, 2020). En concreto, el uso de PDEVs ha demostrado en varios estudios *in vitro* e *in vivo* diversas propiedades asociadas al cuidado de la piel, como antioxidantes, regenerativas, anti-melanogénicas y unificadoras del tono de la piel Además, presentan una alta citocompatibilidad en comparación con nanoportadores artificiales (Kee et al., 2022).

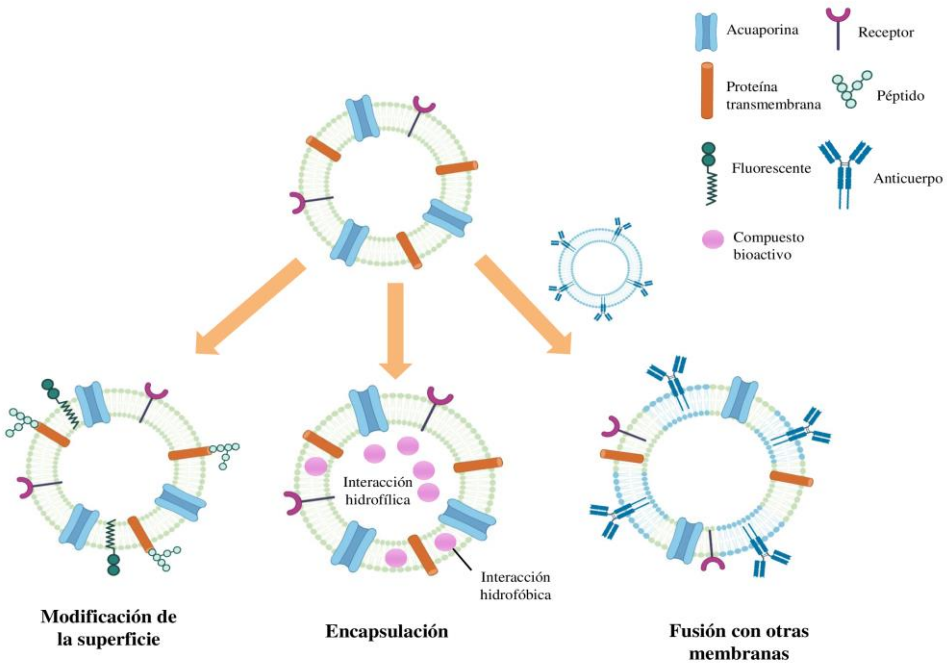

Figura 8. Representación de los distintos tipos de modificaciones de las PDEVs.

Industria alimentaria

Se han explorado múltiples enfoques nanotecnológicos en diversos aspectos del sector alimentario, incluido el procesamiento, el envasado, la seguridad y la nutrición. Por ejemplo, se ha demostrado que el uso de conservantes y aditivos alimentarios nanoencapsulados contribuye a mejorar la dispersabilidad en el producto final, potencia su sabor (reduciendo la cantidad necesaria) y disminuye la necesidad de incluir grasas, sales y azúcares para su conservación (Maurya & Aggarwal, 2019). Sin embargo, el uso de PDEVs en la industria alimentaria todavía no está ampliamente explorado, ya que, hasta la fecha, se suelen emplear liposomas de origen sintético.

El foco actual de las PDEVs en alimentación es como moduladores del microbioma intestinal. Como ejemplo, las vesículas derivadas de limón son capaces de inhibir al patógeno *Clostridioides difficile*, y promover la supervivencia de cepas probioticas como *Streptoccous thermophilus* ST-21 y *Lactobacillus rhamnosus* GG (LGG) (Lei et al., 2020).

3.2.3. Vesículas derivadas de Brassica

Las vesículas derivadas de *Brassica* han sido estudiadas durante las últimas décadas, ejerciendo especial interés en las de membrana plasmática obtenida de brócoli (*Broccoli Plasma Membrane Vesicles* o BPMVs). Las BPMVs son una gran herramienta para caracterizar sus cambios en su composición lipídica ante variaciones en el medio (Chalbi et al., 2015; Casado-Vela et al., 2010). Por ejemplo, el trabajo de López-Pérez et al. (2009), mostró un aumento de la insaturación de ácidos grasos, junto con un incremento en la expresión de acuaporinas en vesículas de membrana plasmática de raíz de brócoli obtenidas en condiciones de salinidad. De este modo, el uso de BPMVs también ha contribuido al estudio de las AQPs. Éstas pertenecen a la familia de proteínas intrínsecas de membrana (MIPs) y, tal y como se ha mencionado previamente, son las encargadas de modular el paso del agua y pequeños solutos a través de la bicapa lipídica. En plantas superiores, destacan 5 subfamilias: proteínas intrínsecas de membrana plasmática (PIPs), proteínas intrínsecas de tonoplasto (TIPs), proteínas intrínsecas similares a nodulina 26 (NIPs), pequeñas proteínas intrínsecas básicas (SIPs) y proteínas intrínsecas sin categorizar (XIPs) (Maurel et al., 2015). Respecto a su conformación, las acuaporinas se disponen en homo u heterotetrámeros formando un poro central, y cada monómero presenta seis dominios transmembrana unidos por cinco *loops* situados en la cara interna o externa de la membrana. En el trabajo publicado por Casado-Vela et al. (2010), se pudieron describir varias isoformas de PIP1 y PIP2 presentes en estas BPMVs. Además, en el trabajo de Martínez-Ballesta et al. (2018) se mostró que las AQPs confieren una alta estabilidad a las BPMVs provenientes tanto de raíces como de hojas de brócoli. También se describe una mayor estabilidad de la GRA conferida por estas vesículas, que, según el estudio mediante *docking* molecular, podría estar debido a su interacción con estas proteínas transmembrana (Martínez-Ballesta et al., 2016).

No obstante, gracias a sus cualidades, se ha considerado el uso de BPMVs para distintas aplicaciones industriales, como el diseño de fertilizantes o su uso cosmético. En el caso del uso de estos nanoportadores, han demostrado ser

altamente eficientes, mejorando la permeabilidad foliar de micronutrientes (zinc, hierro y boro), en diversas especies vegetales, como el pack choi, la batata o el almendro (Nicolas-Espinosa et al., 2022; Rios et al., 2020; Rios et al., 2019). Asimismo, trabajos con BMPVs han resaltado su eficiencia de atrapamiento relativamente alta, capacidad de liberación, y penetrabilidad en los tejidos de la piel (Yepes-Molina et al., 2020).

Otra de las aplicaciones recientes de las BPMVs es su uso para encapsular ITCs o GLSs. En concreto, el SFN es un compuesto con baja estabilidad y solubilidad, ya que se ve afectada fácilmente por la temperatura, el pH, la luz y el oxígeno (Wu et al., 2014). Además, su tiempo de metabolización y excreción es breve, apareciendo hasta un 80 % del SFN ingerido tras 12-24 h desde su consumo (Angelino & Jeffery, 2014). Por ello, han emergido diversas estrategias de nanoencapsulación y formulación para conseguir aumentar su estabilidad y biodisponibilidad (Zambrano et al., 2019). Entre ellas, los trabajos realizados con BPMVs demuestran su alta capacidad para mejorar la estabilidad de este ITC, aumentando su capacidad antiproliferativa en cultivos de melanocitos (SK-MEL) y controlando su liberación en cultivos de macrófagos HL-60, con el fin de incrementar su actividad antiinflamatoria (Yepes-Molina et al., 2022; Yepes-Molina & Carvajal, 2021). No obstante, poco se sabe del funcionamiento de las vesículas derivadas de crucíferas en el tracto digestivo, donde las variaciones de pH y la acción enzimática son factores clave para integridad estructural.

~ Justificación y Objetivos ~

Justificación y objetivos

Justificación

Las biomoléculas presentes en los vegetales, como las crucíferas, presentan diversas funciones beneficiosas para la salud humana. En concreto, los glucosinolatos y sus derivados bioactivos, los isotiocianatos han demostrado presentar un alto potencial en distintas áreas, no solo como agentes antitumorales, sino también como antiinflamatorios y antioxidantes. No obstante, sus características físico-químicas condicionan su aplicación en la industria farmacéutica, alimentaria y cosmética. Es por ello, que los nanoportadores son una de las herramientas más empleadas, ya que confieren protección a los bioactivos frente a la degradación y permiten su paso a través de las barreras biológicas. Sin embargo, su producción suele ser costosa y, según su procedencia, pueden presentar cierta toxicidad e inmunogenicidad. Además, su uso como agentes encapsulates de isotiocianatos es interesante en la búsqueda de ingredientes funcionales que actúen como coadyuvantes frente a factores de riesgo, destacando la disbiosis microbiana o la inflamación crónica de bajo grado, presentes en enfermedades no transmisibles, como la obesidad. Por lo tanto, los nanoportadores empleados deben ser capaces de preservar los compuestos bioactivos a través del tracto gastrointestinal, permitiendo que un mayor número de biomoléculas llegue al lugar de absorción.

Ante ello, nuestro grupo de investigación ha generado un amplio conocimiento en el estudio de las membranas biológicas derivadas de brócoli, su adaptación a cambios medioambientales y su potencial uso como agentes nanoencapsulantes. Además, al derivar de subproductos del brócoli, su producción es sostenible y contribuye a la economía circular. De esta manera, surge la patente "*Method for obtaining plasma membrane vesicles extracted from plants enriched in membrane transport proteins, and uses thereof*" (Patente EP2716280B1). Sin embargo, el uso del brócoli presenta algunos inconvenientes, como el

pardeamiento oxidativo o el poco rendimiento obtenido de sus raíces, por lo que surge la necesidad de explorar otras materias primas.

Por otro lado, para la elaboración de formulaciones de calidad y altamente enriquecidas en bioactivos, es indispensable que la materia prima vegetal presente una alta concentración en estos fitoquímicos. No obstante, el uso de los protocolos de elicitación adecuados para cada especie vegetal es crucial para conseguir un incremento óptimo sin comprometer el rendimiento del cultivo.

Teniendo en consideración todos estos antecedentes, se plantea el desarrollo de la presente Tesis Doctoral, estableciendo el objetivo principal y los objetivos específicos que se exponen a continuación.

Objetivo general

El objetivo general de la presente Tesis Doctoral es el desarrollo y elaboración de ingredientes bioactivos, obtenidos a partir de material vegetal previamente elicitado en campo de plantas adultas de Bimi® (*Brassica oleracea* L. var. *itaica* x *Brassica oleracea* var. *Alboglabra*) y col roja o lombarda (*Brassica oleracea* L. var. *capitata* f. *rubra*), y estabilizados mediante nanoencapsulaciones provenientes de membrana plasmática de coliflor (*Brassica oleracea* L. var. *botrytis*) para, posteriormente, estudiar su bioaccesibilidad y bioactividad.

Objetivos específicos

Los objetivos específicos establecidos incluyen (Figura 9):

- Evaluación del efecto de la elicitación con metil jasmonato, ácido salicílico y su combinación en la acumulación de glucosinolatos en Bimi® y col lombarda, determinando un protocolo adecuado para cada especie (***Capítulo I y Capítulo IV)***.

- Elaboración de formulaciones derivadas de Bimi® y col lombarda enriquecidos en glucosinolatos e isotiocianatos (***Capítulo I, Capítulo III y Capitulo IV***).

- Análisis y estudio de la membrana plasmática derivada de distintos órganos de la coliflor (hojas e inflorescencias), en distintos estados de maduración, para la obtención de un material de partida óptimo como agente nanoencapsulante (***Capítulo II***).

- Evaluación de la estabilidad de compuestos activos presentes en las formulaciones nanoencapsuladas durante la digestión gastrointestinal, así como la determinación de su bioactividad en modelos de obesidad (***Capítulo III y Capítulo IV***).

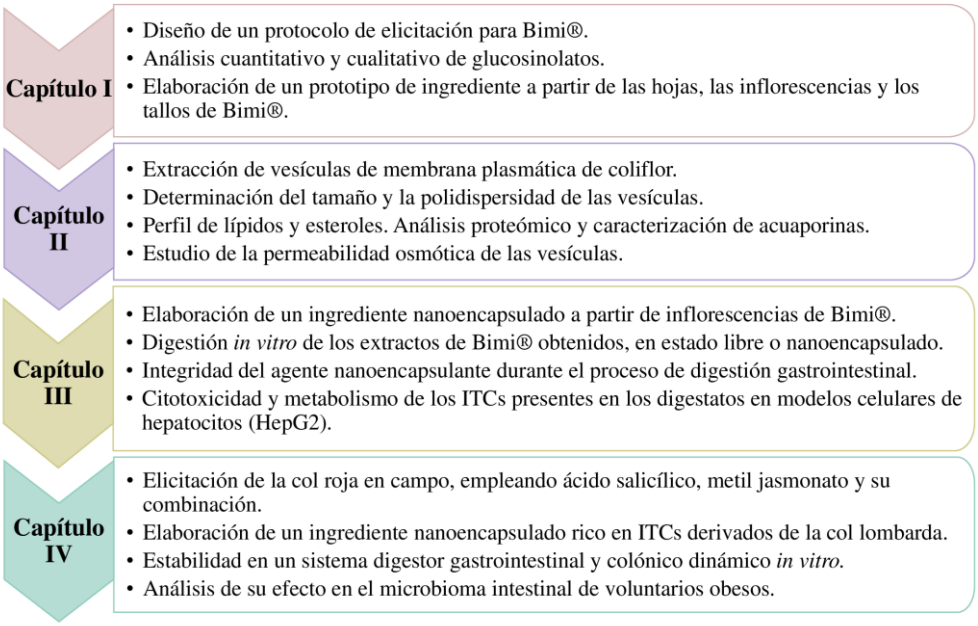

Figura 9. Capítulos de la Tesis Doctoral en representación esquemática.

~ Justification & Objectives ~

Justification and objectives

Justification

Bioactives obtained from plants, such as brassicas, have diverse beneficial functions for human health. Specifically, glucosinolates and their bioactive products, isothiocyanates, have shown great potential in different areas, not only as antitumor agents, but also as anti-inflammatory and antioxidant molecules. However, its physical-chemical characteristics condition its application in the pharmaceutical, food and cosmetic industries. For this reason, nanocarriers are one of the most widely used tools, since they protect bioactives against degradation and allow their passage through biological barriers. Nevertheless, their production is usually expensive and, depending on their origin, they can present certain toxicity and immunogenicity. In addition, their use as encapsulating agenst for isothiocyanates is interesting in order to search for functional ingredients that act as adjuvants against health risk factors, highlighting microbiome dysbiosis or low-grade chronic inflammation, present in non-communicable diseases, such as obesity. Therefore, the nanocarriers used must be able to preserve the bioactive compounds through the gastrointestinal tract, allowing a higher percentage of biomolecules to reach the absorption sites, such as the intestine.

In this way, our research group has generated extensive knowledge in the study of biological membranes derived from broccoli and their adaptation to environmental changes and their potential use as nanoencapsulating agents. Furthermore, as they are obtained from by-products, its production is sustainable and contributes to the circular economy. This has led to the patent "*Method for obtaining plasma membrane vesicles extracted from plants enriched in membrane transport proteins, and uses thereof*" (Patent EP2716280B1). Nonetheless, the use of broccoli presents some problems, such as the browning by oxidation or the low yield provided from its roots, which is why the need to explore new materials arises.

On the other hand, for the production of quality extracts highly enriched in bioactives, it is essential that the raw plant material has a high concentration of these phytocompounds. However, the use of appropriate elicitation protocols for each plant species is crucial to achieve optimal growth without compromising crop performance.

Taking all of this into consideration, we planned to develop the following Ph.D. Thesis, establishing the main objective and the specific objectives that are set out below.

Main objective

The general objective of this Ph.D. Thesis is the development and elaboration of bioactive ingredients, obtained from plant material previously elicited in the field of adult Bimi® plants (*Brassica oleracea* L. var. *italica* x *Brassica oleracea* L. var. *alboglabra*) and red cabbage *(Brassica oleracea* L. var. *capitata* f. *rubra*), and stabilized by nanoencapsulations from cauliflower plasma membrane (*Brassica oleracea* L. var. *botrytis*), to subsequently study their bioaccesibility and bioactivity.

Specific objectives

The following specific objectives include (Figure 10):

- Evaluation of the effect of elicitation with methyl jasmonate, salicylic acid and its combination on the accumulation of glucosinolates in Bimi® and red cabbage, determining a suitable protocol for each plant species (***Chapter I and Chapter IV***).

- Preparation of formulations derived from Bimi® and red cabbage enriched in glucosinolates and isothiocyanates (***Chapter I, III and Chapter IV***).

- Analysis and study of the plasmatic membrane derived from different cauliflower organs (leaves and inflorescences), in different stages of maturation, to obtain an optimal stating material as a nanoencapsulating agent (***Chapter II***).

- Evaluation of the stability of the bioactives present in the nanoencapsulated formulations during gastrointestinal digestion, as well as the determination of their bioactivity in obesity models (***Chapter III and Chapter IV***).

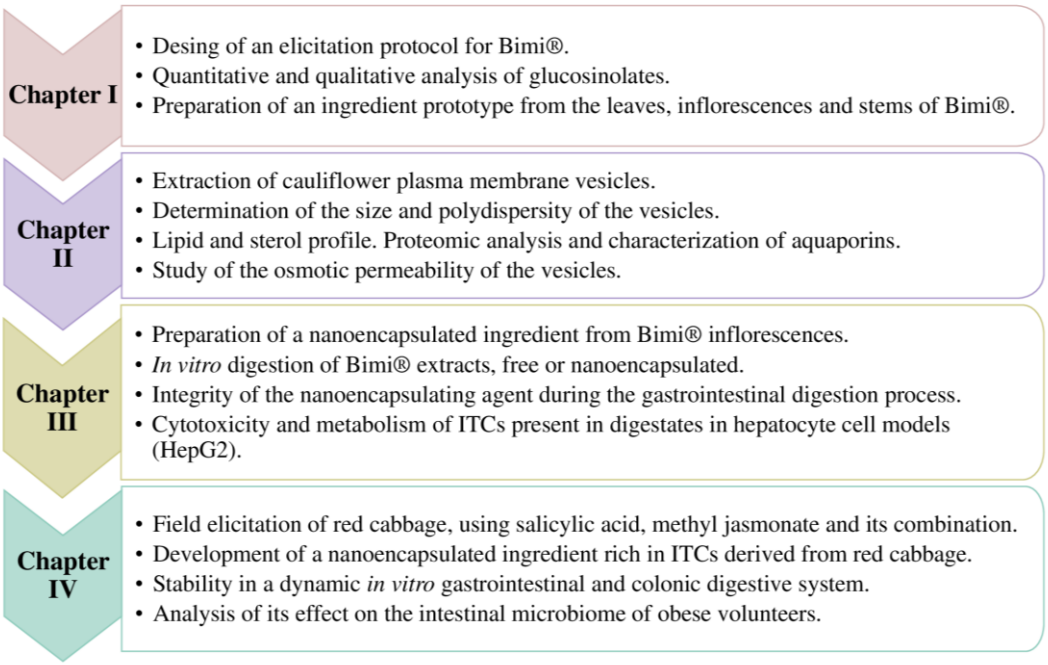

Figura 10. Chapter scheme of the PhD thesis.

· Capítulo 1

Capítulo I

Use of elicitation in the cultivation of Bimi® for food and ingredients

Garcia-Ibañez P., Moreno D.A., Nuñez-Gomez V., Agudelo A., Carvajal M.

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Background: Cruciferous foods rich in health-promoting metabolites are of particular interest to consumers as well as being a good source of bioactives-enriched ingredients. Several elicitors have been used to stimulate the biosynthesis and accumulation of secondary metabolites in foods; however, little is known about the response of new hybrid varieties, such as Bimi®, under field-crop production conditions. Therefore, this study was designed to evaluate the effect of salicylic acid (200 μmol L^{-1} , SA), methyl jasmonate (100 μmol L^{-1} , MeJA), and their combination on Bimi plant organs (inflorescences and aerial vegetative tissues - stems and leaves). For this, the composition of the glucosinolates present in the tissues was evaluated. Also, aqueous extracts of the plant material, obtained with different times of extraction with boiling water, were studied .Results: The results indicate that the combined treatment (SA + MeJA) significantly increased the content of glucosinolates in the inflorescences and that MeJA was the most effective elicitor in leaves. Regarding the aqueous extracts, the greatest amount of glucosinolates was extracted at 30 min - except for the leaves elicited with MeJA, for which 15 min was optimal. Conclusion: The elicitation in the field enriched leaves in glucobrassicin (GB), 4-methoxyglucobrassicin (MGB), and neoglucobrassicin (NGB) and stems and inflorescences in glucoraphanin, 4-hydroxyglucobrassicin, GB, MGB, and NGB. In this way, this enhanced vegetable material favored the presence of bioactives in the extracts, which is of great interest regarding enriched foods and ingredients with added value obtained from them.

Research Article

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Use of elicitation in the cultivation of Bimi® for food and ingredients

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Abstract

BACKGROUND: Cruciferous foods rich in health-promoting metabolites are of particular interest to consumers as well as being a good source of bioactives-enriched ingredients. Several elicitors have been used to stimulate the biosynthesis and accumulation of secondary metabolites in foods; however, little is known about the response of new hybrid varieties, such as Bimi®, under field-crop production conditions. Therefore, this study was designed to evaluate the effect of salicylic acid (200 μmol L^{-1}, SA), methyl jasmonate (100 μmol L^{-1}, MeJA), and their combination on Bimi plant organs (inflorescences and aerial vegetative tissues – stems and leaves). For this, the composition of the glucosinolates present in the tissues was evaluated. Also, aqueous extracts of the plant material, obtained with different times of extraction with boiling water, were studied.

RESULTS: The results indicate that the combined treatment (SA + MeJA) significantly increased the content of glucosinolates in the inflorescences and that MeJA was the most effective elicitor in leaves. Regarding the aqueous extracts, the greatest amount of glucosinolates was extracted at 30 min – except for the leaves elicited with MeJA, for which 15 min was optimal.

CONCLUSION: The elicitation in the field enriched leaves in glucobrassicin (GB), 4-methoxyglucobrassicin (MGB), and neoglucobrassicin (NGB) and stems and inflorescences in glucoraphanin, 4-hydroxyglucobrassicin, GB, MGB, and NGB. In this way, this enhanced vegetable material favored the presence of bioactives in the extracts, which is of great interest regarding enriched foods and ingredients with added value obtained from them.

Keywords: glucosinolates; methyl jasmonate; salicylic acid; ingredients; vegetables

INTRODUCTION

Bioactives-enriched foods, functional ingredients, and other food products are emerging as dietary coadjutants to improve human health.[1] In fact, during the last few years, the prevention and management of non-communicable diseases, including diabetes and obesity, are being approached with nutritional interventions.[2–4] In relation to this, vegetables and fruits are the main sources of phytochemicals in our diets and, around the world, the recommendation for the general population is to eat at least five portions of fruits and vegetables per day in order to reduce the risk of many diseases and to maintain physical and mental wellness.[5,6]

Particularly rich in bioactive compounds are the vegetables of the Brassicaceae family, not only for their nutritional profile but also for the presence of health-promoters such as glucosinolates (GLSs), phenolic acids and flavonoids, vitamin C, and minerals.[7] Broccoli (both as a mature vegetable or as sprouts) has been one of the main subjects of consumer and research interest for the last 20 years, since diverse studies have shown that its regular consumption decreases the risk of colon and lung cancer, among other chronic health conditions.[8] In particular, the main focus has been on the GLSs, which are almost *Brassica*-exclusive secondary metabolites with a defense role against pathogens.[9] They are constituted by a β-thioglucoside *N*-hydrosulfate bound to a sulfur β-glucopyranose, and they vary in the amino acid-derived side chain. Isothiocyanates (ITCs) are produced by the hydrolysis of GLSs, catalyzed by myrosinase (EC 3.2.1.147), after mechanical disruption of the cell tissue – for instance, due to chopping or chewing by herbivores.[10] In broccoli, the ITC that has been studied most is sulforaphane (SFN), an inductor of Nrf2 transcription factor and of phase II detoxification enzymes, with anti-carcinogenic properties.[11–15] More recently, additional roles of the bioactives in *Brassica* species, in relation to diabetes and obesity, have been reported in preclinical studies[16,17] and in nutritional interventions.[18] Furthermore, not only the ingredient by itself, but also *Brassica*-derived extracts are being used in the food industry as nutraceuticals, for food storage, or as prototype ingredients in the cosmetic industry.[19–21] However, these extracts are usually

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based in ITCs content with a lower shelf-life stability than GLSs.[22,23]

The phytochemical composition of plant foods can be enriched by using elicitors, such as jasmonic acid and its methyl ester (methyl jasmonate, MeJA).[24] These signal molecules have been shown to have a key role in plant defense against biotic or abiotic stresses by inducing MYB transcription factors, which regulate a set of genes involved with the GLSs biosynthetic pathway.[25,26] Similarly, salicylic acid (SA) is involved in systemic acquired resistance, to defend the plant against pathogens.[27] Owing to its efficiency, elicitation has been widely used in order to stimulate the accumulation of health-promoting GLSs in *Brassica* species.[28,29] However, only a small set of publications has been obtained from field experiments.[30,31]

Nevertheless, the intense and bitter taste of broccoli affects its acceptability by consumers, hindering the promotion of the daily consumption of vegetables. As a solution, novel varieties of broccoli with milder flavor and less pungency have emerged – such as Bimi®, also known as Tenderstem® and Aspabroc®. It is variety with loose heads that is the result of the crossbreeding of a conventional broccoli variety (*Brassica oleracea* var. *italica*) with a green Chinese kale (*B. oleracea* var. *alboglabra*). Its acceptability from the organoleptic point of view is higher than for other cruciferous varieties, which can be important when attempting to increase the frequency of consumption of these vegetables.

Considering the interest in developing bioactives-enriched foods and ingredients, the aim of this study was to analyze the effects of two elicitors (MeJA and SA), in separate or combined applications, on the biomass and phytochemical composition of the new hybrid brassica variety Bimi. The idea was to obtain phytochemically enriched foods for both fresh consumption and health-promotion and a prototype food ingredient compatible with food-grade extractions and characteristics (not using organic solvents).

MATERIAL AND METHODS

Plant material and treatments

Two hundred Bimi seeds from Sakata Seed Iberica S.L.U. (Valencia, Spain) were induced to germinate by pre-hydration with deionized water and were then aerated continuously for 24 h. After that, the seeds were germinated in vermiculite at 28 °C and 60% relative humidity, in darkness, for 2 days. Then, 5-day-old seedlings were transplanted to agricultural soil in an experimental farm (37° 47′ 52.7″ N, 0° 52′0 0.7″ W, 15 m asl, Murcia, Spain). They were grown under a semiarid Mediterranean climate from March to May 2018. All plants were drip irrigated with a diluted (1:4) Hoagland nutrient solution. The temperature and relative humidity were recorded every 10 min using dataloggers (AFORA S.A., Barloworld Scientific, Murcia, Spain). Twenty-five plants were assigned to each treatment. The different elicitation treatments were as follows: 100 µmol L^{-1} MeJA dissolved in 0.2% ethanol, 200 µmol L^{-1} SA dissolved in 0.2% ethanol, a combined treatment (SA + MeJA), and control plants treated only with distilled water. Concentrations were based on previous experiments performed under controlled conditions.[28] Additionally, this solution was supplemented with a patented concentration of surfactant (PCT/ES2019/070457). The applications were performed spraying 100 mL of elicitor solution individually to each plant. Elicitation started when the appearance of the central flower bud was general among the plants. After 5 days, another elicitation was performed. Thereafter, the plants were allowed to grow for another 4 days and were harvested at commercial stage (around 90 days after transplanting). Then, leaves, stems, and inflorescences from control and treated plants were sampled and weighed fresh. For further analysis, samples from 15 plants from each treatment, randomly chosen, were mixed and distributed in four technical replicates. After that, the samples were transported to the laboratory for processing and analysis (coolers were used, and the duration of sampling and transport was always under 3 h).

Extraction of intact GLSs

Samples (100 mg) of ground, freeze-dried material were extracted with 1 mL of 70% methanol in water, in a bath kept at 70 °C for 30 min, with vortexing every 5 min. After that, the samples were first cooled in an ice bath and then centrifuged at 10 000×*g* for 15 min, at room temperature. The supernatants were transferred to a rotary evaporator unit for complete removal of the methanol. Then, 300 µL of MilliQ water were added to each sample and, after homogenization, the samples were filtered through 0.22 µm diameter Millipore filters (Billerica, MA, USA) into vials for HPLC-DAD analysis.

Elaboration of the GLS-rich ingredient prototype

Bearing in mind the possibility of using by-products and non-commercial parts of the Bimi plants as sources of functional ingredients and reduction of agrowastes, we prepared aqueous extracts using freeze-dried Bimi powder in MilliQ water (1:20 w:v) at 100 °C in a shaker bath with separate samples and extraction times ranging from 15 to 60 min. The vials were continuously agitated at 150 rpm for dynamic extraction. Samples were collected at different time points (15, 30, 45, and 60 min) and cooled in ice slurry for 5 min. After that, the vials were centrifuged at 10 000×*g* for 15 min. The supernatants were collected and filtered through 0.22 µm diameter Millipore filters (Billerica, MA, USA) into vials for high-performance liquid chromatography (HPLC) with diode array detection (DAD) analysis.

Qualitative and quantitative determination

First, the GLSs extracted from the samples were identified by HPLC-DAD–electrospray ionization (ESI) multistage mass spectrometry (MS^n), according to their MS^2 $[M-H]^-$ fragmentation patterns. The conditions employed were the same as in Baenas *et al.* For quantitative analysis, 20 µL of extract was introduced in an Agilent 1100 HPLC-DAD system (Agilent Technologies, Santa Clara, CA, USA). Intact GLSs were identified according to their ultraviolet spectra and elution order. For quantification, sinigrin and glucobrassicin (GB) were used as external standards (Phytochem, Neu-Ulm, Germany).

Statistical analysis

For the field elicitation experiment, one-way analysis of variance (ANOVA) was performed, using Tukey honestly significant difference (HSD) as the *post hoc* test. For the extraction experiments, two-way ANOVA was employed, also followed by a *post hoc* Tukey HSD test. All the analyses were executed in RStudio (version 3.5.1).

RESULTS

Biomass

The fresh weights of the Bimi plant organs were registered during the sampling in the field, at the moment of harvest. Statistical analysis (ANOVA and Tukey) did not find significant differences among the treatments in the biomass total aerial part

(inflorescences and other aerial vegetative tissues – stems and leaves), obtaining data ranged from 389 ± 20 to 407 ± 11 g (average plus/minus standard error).

Glucosinolate analysis of Bimi

The identification and quantification of the intact GLSs in the samples of Bimi (mg g^{-1} dry weight (DW)) showed differing effects of the elicitors applied according to the plant part analyzed and the profile of GLSs (Table 1). The molecular ion $[M - H]^-$ (m/z) of the GLSs, their fragmentation patterns, and the retention time of each compound allowed the identification of 11 GLSs in the Bimi samples studied. In leaves, progoitrin, glucoiberin, and gluconapin were detected by HPLC-DAD–ESI-MS^n in the four treatments, but not in quantifiable amounts. Traces of 4-hydroxyglucobrassicin (HGB) were detected when MeJA was applied. Glucoraphanin (GRA) and GB appeared in control leaves, whereas 4-methoxyglucobrassicin (MGB) and neoglucobrassicin (NGB) were observed after application of the three treatments. For GRA, only plants treated with MeJA showed a slight decrease, relative to control plants, whereas GB increased strongly with MeJA application (fivefold) and with SA + MeJA (threefold). Similarly, the MeJA and SA + MeJA treatments favored the appearance of MGB and NGB, but the latter was also observed in leaves of plants treated with SA.

For Bimi inflorescences (Table 1), traces of progoitrin, gluconapin, glucoalyssin, and gluconasturtiin were found in the MS^2 analysis, while GRA, HGB, GB, MGB, and NGB appeared in the four treatments. For GRA, a slight increase, relative to control plants, was observed when MeJA and SA + MeJA were applied ($P < 0.05$). However, no difference was found for this GLS in the inflorescences when Bimi plants were treated with SA ($P > 0.05$). Regarding HGB, a significantly higher concentration was produced by application of the three treatments (three times higher for SA and MeJA, two times higher for SA + MeJA). For GB, the highest concentration (eight times higher than in control plants) was achieved when the elicitation was performed with SA + MeJA, but a great increase was also found for SA (by 5.5 times) and MeJA (by seven times). For MGB, a slight increase, relative to control plants, was obtained with MeJA, but SA + MeJA gave a higher value (two times higher). The SA treatment gave a significant increase in MGB, compared with the control value, but MeJA did not have a statistically significant effect ($P > 0.05$). Surprisingly, NGB was only present as traces when SA was applied, but its concentration was increased by MeJA (three fold, compared with control plants) and SA + MeJA.

The stems showed traces of progoitrin, gluconapin, glucoalyssin, and gluconasturtiin, according to the HPLC-DAD–ESI-MS^n analysis. For GRA, no statistically significant differences were observed between the control and the treatments ($P > 0.05$). For HGB, a significant increase in concentration (two times) was achieved when SA was applied. Similar results were obtained for GB and NGB, neither differing significantly between the control and the SA treatment ($P > 0.05$). However, MeJA and the combination (SA + MeJA) yielded significantly higher concentrations of GB (three times higher for both). In the same way, the NGB concentration was raised by up to sevenfold by MeJA and SA + MeJA exposure. Nevertheless, no effects were observed for MGB after applying SA or SA + MeJA ($P > 0.05$). Furthermore, application of MeJA lowered the concentration of this GLS by 23% ($P < 0.05$), when compared with the control.

Figure 1(A) shows the total aliphatic GLSs concentrations. For leaves, the MeJA and SA + MeJA treatments provoked a decrease

Table 1. Effect of elicitors on glucosinolates of Bimi® leaves, inflorescences, and stems

	Content (mg g^{-1} DW)															
	Leaves				Inflorescences				Stems							
GLS	Control	SA 200 µmol L^{-1}	MeJA 100 µmol L^{-1}	SA + MeJA	Control	SA 200 µmol L^{-1}	MeJA 100 µmol L^{-1}	SA + MeJA	Control	SA 200 µmol L^{-1}	MeJA 100 µmol L^{-1}	SA + MeJA
PRO	*	*	*	*	*	*	*	*	*	*	*	*
GIB	*	*	*	*								
GRA	1.4 ± 0.04ab	1.67 ± 0.07a	1.10 ± 0.07b	1.52 ± 0.01ab	1.56 ± 0.11b	1.7 ± 0.1 ab	2 ± 0.12a	1.94 ± 0.25a	2.5 ± 0.02a	2.25 ± 0.05a	2.13 ± 0.02a	1.95 ± 0.35a
GNA	*	*	*	*	*	*	*	*	*	*	*	*
GAL					*	*	*	*	*	*	*	*
HGB			*		0.45 ± 0.01b	1.3 ± 0.19a	1.3 ± 0.2a	1.03 ± 0.05a	0.24 ± 0.04b	0.46 ± 0.01a	0.26 ± 0.01b	0.24 ± 0.04b
GST					*	*	*	*	*	*	*	*
GB	0.72 ± 0.03c	0.75 ± 0.12c	3.9 ± 0.04a	2.26 ± 0.09b	0.56 ± 0.04c	3.11 ± 0.11b	3.74 ± 0.21b	4.37 ± 0.08a	0.38 ± 0.04b	0.62 ± 0.24b	1.05 ± 0.04a	1.26 ± 0.26a
MGB			1 ± 0.01a	0.71 ± 0.07b	0.89 ± 0.07c	1.36 ± 0.3ab	1.17 ± 0.01b	1.59 ± 0.1a	0.83 ± 0.01a	0.84 ± 0.05a	0.65 ± 0.03b	0.76 ± 0.18ab
NGB	*	0.19 ± 0.02c	1.63 ± 0.03a	0.5 ± 0.003b	0.64 ± 0.04c	*	1.66 ± 0.3b	2.32 ± 0.07a	0.1 ± 0.01b	0.18 ± 0.01b	0.78 ± 0.04a	0.69 ± 0.16a

The numbers show the average values per treatment ($n = 4$) plus/minus standard deviation. Statistical analysis was performed in order to find the differences between treatments in each part of Bimi and type of glucosinolate. Different letters indicate statistically significant differences ($P < 0.05$).
*The presence of the GLSs was under the limit of quantification for HPLC-DAD–ESI-MS^n (<0.02 mg g^{-1} DW).
SA: salicylic acid; MeJA: methyl jasmonate; GLS: glucosinolate; PRO: progoitrin; GIB: glucoiberin; GRA: glucoraphanin; GNA: gluconapin; GAL: glucoalyssin; HGB: 4-hydroxyglucobrassicin; GST: gluconasturtiin; GB: glucobrassicin; MGB: 4-methoxyglucobrassicin; NGB: neoglucobrassicin.

in total aliphatic GLSs, when compared with the control ($P < 0.05$), and no differences were observed when applying SA ($P > 0.05$). In Bimi inflorescences, the SA treatment performed similarly as in leaves, but the total aliphatic GLSs concentration increased slightly when using MeJA or SA + MeJA ($P < 0.05$). The total aliphatic concentration in stems did not vary with the SA application ($P > 0.05$); furthermore, SA and SA + MeJA gave a decrease when compared with the control ($P < 0.05$). Considering the total indole GLSs (Fig. 1(B)), the greatest increases in leaves were obtained with MeJA (ninefold) and SA + MeJA (fivefold). No significant differences were observed with SA ($P > 0.05$). Regarding inflorescences, the total indole GLSs concentration increased when any elicitor was applied ($P < 0.05$), with SA + MeJA giving the highest value (a fourfold increase). The total indole GLSs concentration in stems did not vary when SA was employed ($P > 0.05$), whereas MeJA and SA + MeJA provoked a twofold rise. Figure 1(C) shows the total GLSs concentration in all Bimi plant parts. In leaves, the total concentration was highly increased by MeJA (fourfold) and SA + MeJA (twofold), but no effect was shown by SA. Regarding inflorescences, all three treatments had an effect on the total GLSs concentration ($P < 0.05$), the combination treatment giving the best result (a three times increase). In addition, when comparing total GLSs content between parts, the highest concentrations were shown in inflorescences (up to 11.25 ± 0.55 mg g^{-1} DW), followed by leaves (up to 7.78 ± 0.18 mg g^{-1} DW). The lowest amount of GLSs was observed in stems, with up to 4.9 ± 0.09 mg g^{-1} DW.

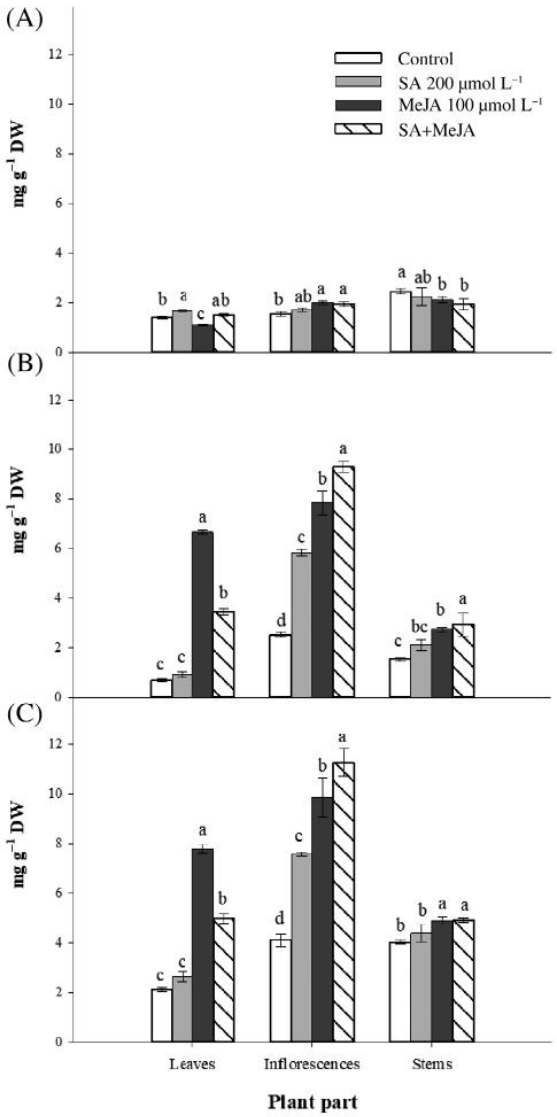

Figure 1. (A) Total aliphatic glucosinolates (GLSs) in Bimi® leaves, inflorescences, and stems. (B) Total indolic GLSs concentration in Bimi leaves, inflorescences, and stems. (C) Total GLSs concentration in Bimi inflorescences, leaves, and stems. Data are means plus/minus standard deviation ($n = 4$). Columns with different letters indicate statistically significant differences between treatments.

Elaboration of a GLSs-rich ingredient prototype

In order to prepare new ingredients enriched in GLSs and compatible with food-grade requirements, boiling-water crude extracts were studied regarding their GLSs concentrations (milligrams per liter). In addition, using non-commercial plant parts of Bimi and harvest remains to produce these ingredients is also contributing to the future challenges of a circular economy and reduction and reuse of agrowastes. The inhibition of myrosinase is relevant to keep the extract rich in GLSs; and for that purpose, temperatures of 70 °C and a minimum of 30 min of extraction are necessary to guarantee the inhibition.[32] Therefore, after preliminary tests (data not shown), we established a set for a 100 °C temperature during extraction in order to use from 15 to 60 min of extraction, to test for both content of GLSs and the possibility of losses originating in the process (e.g. time of extraction, thermal instability, myrosinase activity). Table 2 shows the results obtained for each of the GLSs analyzed in different extracts from Bimi leaves. At 15 min, differences between the treatment and control extracts were found for GRA ($P < 0.05$). The MeJA treatment yielded the highest concentration of this GLS (20-times higher), but SA (two times higher) and SA + MeJA (five times higher) also gave higher concentrations than the control. However, a strong decrease was found when comparing the SA, MeJA, and SA + MeJA samples obtained at 15 min and 30 min (55%, 85%, and 95% respectively). In addition, no differences were found between control extracts and those from treated plants ($P > 0.05$). At 45 min and 60 min of boiling, GRA was not detected by HPLC-DAD. Regarding the GB concentrations, the 15 min samples revealed differences between the control and the treatments. Also, the GB concentration did not differ between the SA and SA + MeJA treatments ($P > 0.05$). With 30 min of boiling, the control (by two times), MeJA (by four times), and SA + MeJA (by four times) extracts showed an increase in GB concentration, compared with 15 min. The GB concentration in the SA samples did not vary with time ($P > 0.05$). At 45 min, only the SA samples exhibited an increased GB concentration ($P < 0.05$), whereas the MeJA and SA + MeJA extracts had a decreased GB concentration at this time of boiling when compared with 30 min ($P < 0.05$). The GB values in the control extract did not vary from 30 to 45 min of boiling ($P > 0.05$). The GB levels were lessened at 60 min for all four treatments ($P < 0.05$). Regarding MGB, it only appeared in the MeJA and SA + MeJA extracts at 15 min. Furthermore, the MGB concentrations in the samples were three times higher for MeJA than for SA + MeJA. At 30 min, MGB was quantified in all treatments,

Table 2. Glucosinolate concentrations of boiling-water extracts of Bimi® leaves at 15, 30, 45, and 60 min of extraction

($mg·L^{-1}$)	15 min				30 min				45 min				60 min			
GLS	Control	SA 200 µM	MeJA 100 µM	SA+MeJA	Control	SA 200 µM	MeJA 100 µM	SA+MeJA	Control	SA 200 µM	MeJA 100 µM	SA+MeJA	Control	SA 200 µM	MeJA 100 µM	SA+MeJA
HGB	350±8.3 de	38.2±1.7c	350±8.3a	89.3±3.5b	17±1.7 de	16.9±0.5de	23.7±0.21d	12.2±0.7e								
GB	26.4±1.64jk	41±2hi	60.1±1de	43.2±3.8ghi	51.6±0.5efg	43±0.21ghi	204.6±7a	167.6±2.4b	48.2±1.8 fgh	55±3.2def	79.1±5.6 c	61.5±1.8d	35.3±1.4ij	40.2±1.8hi	25.8±0.75k	24.8±3.3k
MGB			94.5±6a	32±4.4c	50±3.5b	37.5±2.2 c	37.3±1.1c	35.7±1.4c								

The numbers show the average values per treatment ($n = 3$) ± standard deviation. Different letters indicate statistically significant differences ($P < 0.05$).
SA: salicylic acid, MeJA: methyl jasmonate, GLS: glucosinolate, HGB: 4-hydroxy-glucobrassicin, GB: glucobrassicin, MGB: 4-methoxy-glucobrassicin.

but there was a decrease in its concentration for MeJA and SA + MeJA ($P < 0.05$). No differences were observed among treatments ($P > 0.05$).

Data for the extracts obtained from Bimi inflorescences are shown in Table 3. GRA, HGB, GB, MGB, and NGB appeared in all extracts for all four extraction times. At 15 min, significantly higher values of GRA were observed for SA (five times higher), MeJA (four times), and SA + MeJA (five times), relative to the control. At 30 min of boiling, control samples showed an increase in the GRA concentration, whereas SA extracts suffered a decline ($P < 0.05$ for both). For MeJA and SA + MeJA treatments the 30 min extracts did not show statistically significant differences from their 15 min samples or between themselves ($P > 0.05$). Regarding the 45 min inflorescence extracts, no differences from the 30 min values were found for SA, MeJA, or the combination. Nonetheless, the GRA levels were reduced in control samples after 45 min of boiling ($P < 0.05$). In relation to the treatments, significant differences were not found between the SA and control extracts ($P > 0.05$) but were found for MeJA and SA + MeJA when compared with the control ($P < 0.05$). At 60 min, the GRA concentrations in the control, SA, and MeJA samples did not differ ($P > 0.05$). However, the quantity of this GLS was decreased when compared with the same treatment at 45 min ($P < 0.05$). At 60 min, when compared with 45 min, a statistically significant decrease in GRA was detected only in the SA + MeJA extract ($P < 0.05$). There was no difference between the MeJA and SA + MeJA treatments ($P > 0.05$), these being the samples with the highest concentrations of GRA at 60 min. When analyzing HGB in inflorescences, no differences due to the boiling time were found, except for a decrease in the SA + MeJA samples at 30 min when compared with 15 min ($P < 0.05$). Only at 15 min were differences among the treatments observed for HGB, the combined treatment showing the highest concentration ($P < 0.05$ for all three comparisons). In the inflorescence samples, GB was increased, relative to the control, by the SA (by 1.2 times), MeJA (by 1.3 times), and SA + MeJA (twofold) treatments. At 30 min, its concentration was higher in MeJA samples ($P < 0.05$), did not vary in the control and SA extracts ($P > 0.05$), and was slightly decreased in the SA + MeJA extracts, when compared with 15 min ($P < 0.05$); hence, differences were found among the four treatments ($P < 0.05$). The GB concentrations were lower at 45 min than at 30 min for SA, MeJA, and SA + MeJA ($P < 0.05$) but did not change for the control ($P > 0.05$). The highest GB concentrations at 45 min were found in MeJA (by 1.5 times) and SA + MeJA (by two times). In addition, samples taken at 60 min of boiling did not show a decrease in GB concentration when compared with 45 min ($P > 0.05$ in all four cases). No differences were found between the treatments or time of boiling for MGB ($P > 0.05$).

For NGB, the 15 min extracts obtained from material treated with MeJA or SA + MeJA presented higher concentrations ($P < 0.05$). No statistically significant differences were found between SA and the control ($P > 0.05$). At 30 min, the NGB concentrations were lower than at 15 min of boiling ($P < 0.05$) in the control and SA extracts, but were two times higher in the SA + MeJA extracts. No differences were observed for the MeJA extracts between 15 and 30 min. Among the treatments, the highest concentrations of NGB were found for MeJA (four times higher than the control) and SA + MeJA (five times higher). When boiling for 45 min, a decrease in the NGB concentration was only observed for SA + MeJA ($P < 0.05$). No differences were found between the control and SA extracts ($P > 0.05$), but the NGB

Table 3. Glucosinolate concentrations of boiling-water extracts of Bimi® inflorescences at 15, 30, 45, and 60 min of extraction

	Content (mg L^{-1})																	
	15 min				30 min				45 min				60 min					
GLS	Control	SA 200 μmol L^{-1}	MeJA 100 μmol L^{-1}	SA + MeJA	Control	SA 200 μmol L^{-1}	MeJA 100 μmol L^{-1}	SA + MeJA	Control	SA 200 μmol L^{-1}	MeJA 100 μmol L^{-1}	SA + MeJA	Control	SA 200 μmol L^{-1}	MeJA 100 μmol L^{-1}	SA + MeJA
GRA	42.97 ± 0.26 j	183.5 ± 0.24 abcd	169.45 ± 8.6 cde	194 ± 24a	131.1 ± 6.23 ef	126.5 ± 1.6 gh	155.5 ± 9.4 bcde	165.4 ± 6.3 ab	142 ± 1.6 h	108 ± 2.8hi	170 ± 7.4cde	190 ± 2.8abc	161 ± 4.3fgh	139 ± 2i	186.5 ± 2.68 efg	168 ± 2de
HGB	34.65 ± 1 e	49.4 ± 3.9 bcde	41.05 ± 1.7cde	103 ± 5.5a	48 ± 1bcde	46 ± 1.3bcde	57.2 ± 6 bcde	41.8 ± 2.4b	46.7 ± 1.9bcde	43 ± 0.5bcde	38 ± 0.5de	54.3 ± 5.4bc	46 ± 2e	35.5 ± 2.8bcde	38.6 ± 0.5cde	52 ± 0.5bcd
GB	353.9 ± 15.6gh	431.9 ± 20.7 ef	442.8 ± 5 e	437 ± 7.5bc	352 ± 6.4 g	405 ± 9f	539 ± 7b	582 ± 2.35a	318 ± 2.8ghi	265 ± 5j	488 ± 5.6 cd	508 ± 4.28bcd	307 ± 15hi	295 ± 30ij	490 ± 20 cd	481 ± 3.51d
MGB	35.65 ± 16.4ab	39.9 ± 1.9 bc	30.6 ± 4.5abc	160 ± 9.8ab	32.8 ± 0.85a	25.4 ± 0.6a	29.7 ± 0.17 abc	24 ± 1.6 ab	36 ± 1ab	17.3 ± 0.4bc	23.4 ± 1.6bc	33.8 ± 0.5abc	42.7 ± 0.6ab	24.4 ± 1.2c	26 ± 0.31bc	30 ± 3.15c
NGB	71.65 ± 21e	73.8 ± 1e	163 ± 5.1 bc	232 ± 7.7d	38.5 ± 0.21 f	58.3 ± 2.1 f	170.4 ± 0.45 b	213.3 ± 19 a	36 ± 0.5f	38.2 ± 0.13f	172 ± 3.6b	139.3 ± 1.5b	38 ± 4f	56 ± 7f	172 ± 1.9 cd	130 ± 1.5d

The numbers show the average values per treatment ($n = 3$) ± standard deviation. Different letters indicate statistically significant differences ($P < 0.05$).
SA: salicylic acid; MeJA: methyl jasmonate; GLS: glucosinolate; GRA: glucoraphanin; HGB: 4-hydroxyglucobrassicin; GB: glucobrassicin; MGB: 4-methoxyglucobrassicin; NGB: neoglubobrassicin.

concentrations in the MeJA and SA + MeJA extracts were four times that in the control samples. In the MeJA and SA + MeJA extracts, the NGB concentrations were lower ($P < 0.05$) after 60 min of boiling than at 45 min. As observed at shorter boiling times, the SA and control extracts did not differ regarding NGB ($P > 0.05$).

Table 4 shows the results for boiling-water extracts of Bimi stems. There was a quantifiable amount of GB for all plant treatments and extraction times, but MGB and NGB were only detected at 30 min. For GB, an increase in concentration at 15 min existed for SA (by 1.5 times), MeJA (two -fold), and SA + MeJA (two fold), compared with the control. At 30 min of boiling, a decrease in GB concentration was found for all four treatments ($P < 0.05$). Relative to the control, no difference in GB concentration existed for SA ($P > 0.05$), but for SA + MeJA it was four times higher. Surprisingly, at 45 min, the GB concentrations were higher than at 30 min ($P < 0.05$). There was no significant difference between the control and SA + MeJA extracts ($P < 0.05$) or between the MeJA and SA extracts. Finally, at 60 min, the GB concentration was slightly decreased for the control and MeJA extracts ($P < 0.05$) but did not vary for SA and SA + MeJA ($P > 0.05$). Regarding MGB, only the MeJA extracts exhibited higher concentrations than the control extracts ($P < 0.05$), but no differences were found when comparing the control and SA extracts ($P > 0.05$). With regard to SA + MeJA extracts, its concentration was less than the control ($P < 0.05$). For NGB, an increase in its concentration was found when comparing the treatments with the control ($P < 0.05$), but there were no differences among the treatments ($P > 0.05$).

The total GLSs concentrations in leaves are shown in Fig. 2(A). At 15 min, the extracts obtained from MeJA-elicited leaves contained the highest amount, 11 times more when compared with the control. An increase (threefold) was also observed in the SA + MeJA extracts, but no differences were found for the extracts from SA-elicited leaves ($P > 0.05$). At 30 min of boiling, the MeJA extracts suffered a 54% decrease, relative to 15 min. However, the rest of the samples showed an increase in their total GLSs concentration ($P < 0.05$): MeJA (three times higher than the control) and SA + MeJA (two times higher) remained the extracts with the highest concentrations. When boiling for 45 min, all the samples suffered a decrease in their total concentration ($P < 0.05$), and no differences existed among treatments ($P > 0.05$). Similar results were found at 60 min, but there was a slight decrease in the concentrations ($P < 0.05$).

The total GLSs concentrations in the inflorescences are presented in Fig. 2(B). At 15 min, it was highest in the SA + MeJA samples ($P < 0.05$), compared with the control, but it was also higher for SA and MeJA than in the control ($P < 0.05$). At 30 min, the control and MeJA samples had an increased total GLSs concentration ($P < 0.05$). However, the total GLSs concentration in the SA samples had decreased ($P < 0.05$). The concentration in the SA + MeJA extracts had not altered ($P > 0.05$). By 45 min, all treatments had suffered a decrease in the concentration ($P < 0.05$). At 60 min, the MeJA samples showed a significant decrease in total GLSs ($P < 0.05$), so that no differences were found among the SA, MeJA, and SA + MeJA treatments ($P > 0.05$).

The total GLSs in the boiled-water extracts of the stems (Fig. 2 (C)) at 15 min differed between the control and treatments ($P < 0.05$ in the three comparisons). The concentrations increased from 15 to 30 min ($P < 0.05$ for all samples). The total GLSs concentration of the SA + MeJA samples was double that of the

Table 4. Glucosinolate concentrations of boiling-water extracts of Bimi® stems at 15, 30, 45, and 60 min of extraction

	Content (mg L^{-1})																	
	15 min				30 min				45 min				60 min					
GLS	Control	SA 200 µmol L^{-1}	MeJA 100 µmol L^{-1}	SA + MeJA	Control	SA 200 µmol L^{-1}	MeJA 100 µmol L^{-1}	SA + MeJA	Control	SA 200 µmol L^{-1}	MeJA 100 µmol L^{-1}	SA + MeJA	Control	SA 200 µmol L^{-1}	MeJA 100 µmol L^{-1}	SA + MeJA
GB	41 ± 1.1 g	81.5 ± 3.1 d	61.2 ± 3.4ab	87.8 ± 1.7a	22 ± 3i	21.8 ± 0.5i	30 ± 1.8	79.5 ± 2.4b	70 ± 0.23c	53.2 ± 2.2ef	54 ± 2.3e	75.1 ± 1.8 bc	61 ± 1.4 d	49 ± 2ef	47 ± 4.2 fg	70 ± 1.3c
MGB	*	*	*	*	40 ± 4.6b	43 ± 1.1b	55.7 ± 1.9a	33.5 ± 0.2c								
NGB	*	*	*	*	17 ± 1b	34.7 ± 1.2a	31.4 ± 0.7a	31 ± 2.4a								

The numbers show the average values per treatment ($n = 3$) plus/minus standard deviation. Different letters indicate statistically significant differences ($P < 0.05$).
*The presence of the GLSs was under the limit of quantification for HPLC-DAD–ESI-MS^n (<0.02 mg g^{-1} DW).
SA: salicylic acid; MeJA: methyl jasmonate; GLS: glucosinolate; GRA: glucoraphanin; GB: glucobrassicin; MGB: 4-methoxyglucobrassicin; NGB: neoglubobrassicin.

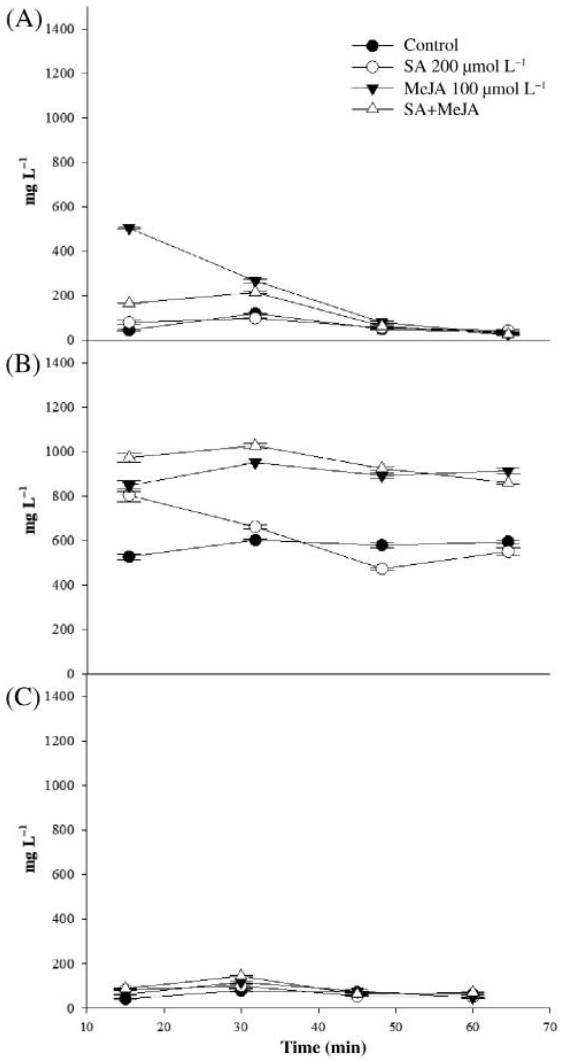

Figure 2. (A) Total glucosinolates (GLSs; mg L^{-1}) found in boiling-water extracts of Bimi® leaves, (B) inflorescences and (C) stems at different extraction times. Each point represents the average of three different extractions plus/minus standard deviation ($n = 3$). Differences between time and treatments were considered when $P < 0.05$.

control extracts. Nevertheless, when comparing the 30 min and 45 min samples, the concentration only remained the same in the control stem extracts ($P > 0.05$) whereas in the other samples it diminished ($P < 0.05$). No differences were found between the control and SA + MeJA extracts ($P > 0.05$). Surprisingly, the concentrations in the SA and MeJA extracts were even lower than in the control ($P < 0.05$). At 60 min, no differences were found for the SA, MeJA, or SA + MeJA samples, compared with 45 min ($P > 0.05$), but the control value decreased ($P < 0.05$).

Comparing between Bimi plant parts, the extracts with the highest concentrations were obtained when using inflorescences as source material (up to 1027 ± 19 mg L^{-1}) when compared with leaves (504 ± 3.3 mg L^{-1}) and stems (144 ± 4.9 mg L^{-1}).

DISCUSSION

Bimi has emerged as a novel variety of *Brassica* with a milder flavor.[33] Apart from the company knowledge about the variety, few reports have addressed the field performance or the phytochemical quality. For this reason, we first elucidated the effects on Bimi biomass and GLSs of different elicitors. The similar fresh weight of the aerial part of the harvested plants in all treatments suggests that the application of elicitors does not affect the yield. In other studies, such as Hassini *et al.*,[24] no differences were observed in broccoli shoots' fresh weight when MeJA or SA was used. Furthermore, treatment with JA or SA did not affect the biomass in turnip plants.[34] Although elicitors induce physiological and morphological changes in plants, these results show that the treatments applied do not affect the agronomic performance of Bimi.

To study the effect of elicitors in our farm experiment, MeJA and SA were chosen since they have been widely investigated in relation to increasing the biosynthesis and accumulation of GLSs.[29–31,34] In our study, the 100 μmol L^{-1} MeJA treatment mainly increased the amount of indole GLSs in leaves and inflorescences (Fig. 1(B)). In previous work performed with other brassicas, such as kale or oilseed rape, field elicitation with MeJA also produced a rise in this type of GLS.[30,35] Nevertheless, treatment of cabbage with 200 μmol L^{-1} MeJA only showed an effect on aliphatic GLSs, suggesting that the effect of this elicitor on individual GLSs also depends on the *Brassica* species.[36] In this respect, in agreement with the finding that aliphatic GLSs usually are affected more by the genotype than by the environment,[10] both GB and its derivatives in inflorescences of Bimi increased after MeJA application (Table 1). However, Liu *et al.*[27] found that NGB showed a seven times increase in broccoli after a 4 day treatment with 250 μmol L^{-1} MeJA, but GB was not affected, suggesting that their interconversion was occurring. In our experiments, the fact that both GB and NGB were increased indicates that not only interconversion between GLSs but also the induction of *de novo* synthesis was happening in Bimi inflorescences. In control leaves, NGB was only detected in trace amounts and MGB was absent (Table 1), but the MeJA treatment elicited their quantifiable accumulation. This also supports the idea of a certain degree of induction of GLSs by MeJA.[37,38]

Treatments with SA have been shown to have an effect on indole, aromatic, and aliphatic GLSs, as well as increasing the total amount of GLSs in broccoli or turnip plants.[29,39] Thiruvengadam *et al.*[29] found that a 100 μmol L^{-1} SA spray applied to turnip plants produced different responses among GLSs of the same type. For example, GB and MGB increased whereas NGB and HGB declined. Similar effects were observed here in Bimi inflorescences, since an increase occurred for HGB ($P < 0.05$), MGB ($P < 0.05$), and GB ($P < 0.05$) but not for NGB ($P > 0.05$), which was unquantifiable, compared with control plants. Nevertheless, neither in leaves nor in stems was a change in total GLSs detected after SA application. Regarding the combined SA + MeJA treatment, the results were surprisingly unexpected. The crosstalk between the JA and SA pathways reveals an antagonism between them. For example, when SA was applied to *B. oleracea* roots, downregulation of JA-related genes was found in the same plant part.[37] However, the highest increase in total GLSs in Bimi inflorescences was obtained by applying the combination of elicitors. Since few are the studies that have combined MeJA and SA in field conditions, maybe Bimi (as a new variety) possesses certain differences from other *Brassica* crops. A relationship between the transcription factors that regulate GLSs biosynthesis and the signaling pathways in which these elicitors are involved has been reported; for example, *MYB34* is a key regulator of JA signaling, and *MYB51* is involved in SA-regulated pathways, and both modulate *de novo* synthesis of indolic GLSs.[25] Yi *et al.*[37,40] found an upregulation of *MYB51*-related genes after SA elicitation in kale leaves. As Bimi is a kalian-hybrid, its genetic regulation in leaves and inflorescences could differ, the genes present in the edible part being sensitive to both elicitors (SA + MeJA), but in leaves the expression might be mainly of JA-related genes with an antagonistic effect when both SA and MeJA were applied together. This interesting aspect should be investigated further to elucidate the factors modulated by SA and MeJA in Bimi.

Another interesting fact was the low amount of progoitrin found in Bimi. This GLS seems to interfere in iodine utilization in the synthesis of thyroid hormones.[41] Furthermore, *Brassica* species low in GLSs have been harvested for broiler diets in order to avoid this detrimental effect.[42] In this work, progoitrin could only be detected in trace amounts by HPLC-DAD–ESI-MS^2, even when the plants were treated with elicitors. This differs from previous studies performed in parental species, in which progoitrin appeared in quantifiable amounts.[38,43] Concerning its reaction to elicitation, similar results (the progoitrin levels did not vary) were observed in broccoli heads and sprouts after treatment with MeJA or SA.[29–31] On the other hand, our elicitation experiment resulted in an edible part enriched in GRA, HGB, GB, MGB, and NGB. Since the ITCs obtained from this precursors (SFN, indole-3-carbinol, and 3,3-diindolylmethane) have reported antitumorigenic and anti-inflammatory activity,[15,44] the elicited material could have a greater health-improving effect. In this way, not only does it seem that field elicitation is a useful tool to enhance the beneficial GLSs content of Bimi, but also that this new generation of brassica-derived foods is safe for human consumption. However, the season for cropping should be taken into account, since different values of glucosinoles would be obtainted.[45]

For the Bimi materials obtained from the elicitation experiments, we studied their possible use as food ingredients by obtaining boiling-water crude extracts rich in GLSs. Aqueous extracts were made, since hydrodistillation is a widely used technique in the food industry and more environmentally friendly than the use of organic solvents.[46] In other work, brassica extracts were obtained by static extraction of plant material at room temperature, in order to obtain ITCs.[21,47] However, it has been reported that some ITCs have poor aqueous stability, such as SFN or erucin, which are partially degraded due to myrosinase activity.[22,23] Hence, the samples were extracted with boiling water in order to inactivate myrosinase, and with long enough extraction time (60 min) to also allow degradation because of thermal instability,[48] so we could see the different range of results from short to long or excessively long extraction procedures that allowed the selection of optimal extraction conditions for further work.

The resistance to temperature is highly interesting in inflorescences, especially in the samples treated with MeJA or SA + MeJA – which had only suffered 5% and 15% ($P < 0.05$) degradation of total GLSs respectively after 60 min, remaining stable at the other times. This result fits quite well with previous studies that showed sulfur-containing aliphatic GLSs have better thermal stability.[49] The indole GLSs appeared to be more thermolabile. In previous work, indole GLSs were degraded in an aqueous medium at 100 °C in the following order: HGB > MGB > GB > NGB.[50,51] In our inflorescences samples treated with MeJA or SA + MeJA, no

significant degradation ($P > 0.05$) was found for NGB. Surprisingly, the NGB degradation percentages were highest for the control and SA treatments, with 50% and 47% degradation respectively after 60 min. This contrasts with the information provided in some previous studies.[50,51] However, another report showed that boiling fresh material produced the highest rate of NGB loss in white cabbage.[52] The GB in the SA and SA + MeJA samples showed 32% and 27% degradation respectively after 60 min of boiling. Nonetheless, MGB and HGB did not show statistically significant degradation, which contrasts with the results obtained by Hennig *et al.*,[53] when HGB was almost completely degraded after 60 min of boiling.

Regarding the leaves and stems samples (Tables 2 and 4), no GRA was found by HPLC-DAD. However, both types of sample yielded GB during the entire boiling process. The extracts obtained from leaves showed interesting kinetics, since at 15 min MGB was only quantifiable in the MeJA and SA + MeJA samples. However, this indole GLS appeared in quantifiable concentrations in the samples of all four treatments at 30 min. Subsequently, at 45 or 60 min, it was not detected. Although HGB was extracted from leaves in the first 15 min of boiling, it then started to degrade to the point where it was not detectable at 45 min. Similar results were obtained by Henning *et al.*,[54] who found the highest HGB degradation rates in double haploids obtained from a broccoli × Chinese kale cross. In extracts obtained from stems, a similar phenomenon was observed: at 30 min, GB showed a significant decrease in its concentration (except for SA + MeJA) whereas MGB and NGB appeared in quantifiable amounts.

The differences found when comparing our results with the previously mentioned studies or even when comparing different plant organs may be due to the matrix effect,[52,54] since the starting plant material is highly complex and there are many effects that can influence the degradation kinetics of GLSs. For example, it has been reported that GLSs degradation rates could be affected by environmental factors.[54] As we have observed in our experiments, elicitation of the starting material influences the GLSs concentration and content of the subsequent extracts. Elicitors, as stimulants, can also affect other pathways not studied in this work (such as flavonoids or vitamin C), determining the differing behavior of the individual GLSs.[55] In our work, in the inflorescences samples at 15 and 30 min, treatment with SA + MeJA gave the highest concentrations of total GLSs (Fig. 2(B)), whereas for the leaf samples, at 15 min, the highest concentration was found for the MeJA treatment (Fig. 2(A)).

In the determination of the best time of extraction of the samples with boiling water, the highest total concentration of GLSs was achieved at 15 and 30 min. In our work, it seems that the plant organ directly affected the optimum time of extraction for aqueous solutions. As an example, for stems (Fig. 2(C)), 30 min of boiling was needed for a better extraction, since this is a tissue with more fiber, but for MeJA-treated leaves this time of boiling was excessive when compared with 15 min (Fig. 2(A)). Furthermore, the total GLSs extraction from leaves and stems showed kinetics typical of a dynamic extraction (Fig. 2(A), (C)).[56] However, the extraction of the total GLSs present in Bimi inflorescences (Fig. 2(B)) was more similar to an exhaustive extraction; that is, the concentration of GLSs remained constant over time, without increasing.[57]

If we compare the different extraction methods performed throughout this work (GLS in tissues extracted with hydro-methanol *versus* GLSs in water extracts), in the case of Bimi inflorescences treated with SA + MeJA, similar total concentrations were obtained with the aqueous extraction (at 15 min) and the hydro-methanolic extraction at 70 °C, as shown in Table 1(calculated from data of mg g D.W. it was obtained 1125.5 ± 39 *versus* 1126 ± 19 mg L^{-1} respectively). However, for MeJA leaves and SA + MeJA stems, the concentration obtained in the hydro-methanolic extract was higher than after 15 min and 30 min of boiling respectively (765 ± 13 *versus* 164.3 ± 4 mg L^{-1}, and 484.6 ± 69 *versus* 143.5 ± 3.7 mg L^{-1}). This indicates that the boiling durations are more critical than the use of a hydro-methanolic solvent and should be determined for each GLS matrix (species, tissue, and agronomic conditions).

CONCLUSIONS

In summary, elicitors such as MeJA led to the enrichment in total GLSs of the inflorescences (edible part) and leaves of Bimi, whereas the combined treatment of SA + MeJA only yielded increases in the inflorescences. Therefore, the response to each elicitor was different according to the plant part analyzed, finding optimal results with SA + MeJA for inflorescences, with MeJA alone for leaves, and with both these treatments in stems. In addition, when Bimi plant material was used as a source of ingredients, the treatment applied and the plant part used influenced the GLSs content and stability when different times of boiling-water extraction were applied. This opens a new pathway for optimizing the process chain from field to food. Furthermore, the development of ingredients using elicitation would maximize the amounts of health-promoting compounds in by-products that could be further used in industry. Therefore, fresh Bimi represents a bountiful source of GLSs after elicitation, and its by-products could be used in the elaboration of new ingredients enriched in phytochemicals, such as nutraceuticals or functional ingredients. Considering the results of this work, elicitation with MeJA or SA + MeJA represents an agricultural practice that would help achieve a circular economy in which wastes are converted into resources.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any conflict of interest in the publication of the results reported in it.

AUTHORS' CONTRIBUTIONS

DAM and MCA contributed to the conception and design of this work. PGI and AA performed the field experiments, and PGI, VNG, and DAM carried out the analytical work. PGI prepared the figures and tables and prepared the first draft of the manuscript. DAM and MCA contributed to manuscript revisions and approved the submitted version. MCA obtained the funding.

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· Capítulo II

Capítulo II

Plasma membrane vesicles from caulifower meristematic tissue and their role in water passage

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Background: Cauliflower (*Brassica oleracea* L. var. *botrytis*) inflorescences are composed mainly of meristematic tissue, which has a high cellular proliferation. This considerable cellular density makes the inflorescence an organ with a large proportion of membranes. However, little is known about the specific role of the lipid and protein composition of the plasma membrane present in this organ. Results: In this work, we analyzed the lipids and proteins present in plasma membrane from two different stages of development of cauliflower inflorescence and compared them with leaf plasma membrane. For this purpose, plasma membrane vesicles were obtained by centrifugation for each sample and the vesicular diameter and osmotic permeability (*Pf*) were analyzed by dynamic light scattering and the *stopped-flow* technique, respectively. In addition, fatty acids and sterols were analyzed by gas chromatography and HPLC. The protein composition of the inflorescences and leaves was characterized by HPLC-ESI-QTOF-MS and the data obtained were compared with *Brassicaceae* proteins present in the UniProt database in relation to the presence of aquaporins determined by western blot analysis. The highest *Pf* value was found in 90 day inflorescences-derived plasma membrane vesicles (61.4 ± 4.14 μms^{-1}). For sterols and fatty acids, the concentrations varied according to the organ of origin. The protein profile revealed the presence of aquaporins from the PIP1 and PIP2 subfamilies in both inflorescences and leaves. Conclusion: This study shows that the composition of the sterols, the degree of unsaturation of the fatty acids, and the proteins present in the membranes analyzed give them high functionality for water passage. This represents an important addition to the limited information available in this field.

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Plasma membrane vesicles from cauliflower meristematic tissue and their role in water passage

Paula Garcia-Ibañez, Juan Nicolas-Espinosa and Micaela Carvajal*

Abstract

Background: Cauliflower (*Brassica oleracea* L. var. *botrytis*) inflorescences are composed mainly of meristematic tissue, which has a high cellular proliferation. This considerable cellular density makes the inflorescence an organ with a large proportion of membranes. However, little is known about the specific role of the lipid and protein composition of the plasma membrane present in this organ.

Results: In this work, we analyzed the lipids and proteins present in plasma membrane from two different stages of development of cauliflower inflorescence and compared them with leaf plasma membrane. For this purpose, plasma membrane vesicles were obtained by centrifugation for each sample and the vesicular diameter and osmotic permeability (*Pf*) were analyzed by dynamic light scattering and the *stopped-flow* technique, respectively. In addition, fatty acids and sterols were analyzed by gas chromatography and HPLC. The protein composition of the inflorescences and leaves was characterized by HPLC-ESI-QTOF-MS and the data obtained were compared with *Brassicaceae* proteins present in the UniProt database in relation to the presence of aquaporins determined by western blot analysis. The highest *Pf* value was found in 90 day inflorescences-derived plasma membrane vesicles ($61.4 \pm 4.14\ \mu ms^{-1}$). For sterols and fatty acids, the concentrations varied according to the organ of origin. The protein profile revealed the presence of aquaporins from the PIP1 and PIP2 subfamilies in both inflorescences and leaves.

Conclusion: This study shows that the composition of the sterols, the degree of unsaturation of the fatty acids, and the proteins present in the membranes analyzed give them high functionality for water passage. This represents an important addition to the limited information available in this field.

Keywords: Plasma membrane, Aquaporin, Brassica, Osmotic permeability

Background

Nowadays, crops from the *Brassicaceae* family are among the ones most cultivated worldwide. Of these, the cauliflower (*Brassica oleracea* L. var. *botrytis*) stands out for its high production and economic relevance. For example, about 26 million tons were produced along with broccoli in 2018 (http://faostat.fao.org/). The main reason for its great demand is the presence of diverse health-promoting bioactives - such as glucosinolates, polyphenols, or vitamin C - that add great nutritional value to its edible part [1].

However, little recent information on the phytochemistry of the cauliflower inflorescence molecular structures is available. Smyth [2] described how this inflorescence begins to develop with the formation of secondary meristems. After that, a continuous proliferation of meristematic tissue takes place, generating a highly branched compact pattern with a whitish color

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and spiral-like shape [2]. Following this, the immature inflorescence initiates its maturation, finally reaching total floral differentiation [3]. Although the intervention of genes related to the identity of the floral meristem, such as *TFL1, LFY, AP1*, or *CAL*, has been studied [4], further comprehension of the biochemical mechanisms underlying this process is needed. In addition, Grevsen et al. [5] observed that environmental factors, mainly temperature, influence the generation of the inflorescences.

As the main focus of the research to date has been the genetic control of the inflorescence development, little is known about the lipid and protein composition of the membranes existing in it. However, a high cellular proliferation is present in the edible part of cauliflower, giving a high membrane concentration [6]. Biological membranes are relevant to cell functionality, not only for their role of compartmentalization, but also by their ability to create an adequate environment for diverse types of proteins, such as transmembrane proteins. The latter mainly determine the specific functionality of each type of membrane present in cells and control diverse constitutive functions, such as endocytosis and ion and water transport [7].

Among the important transmembrane proteins, aquaporins are one of the main protagonists, since they are responsible for the water transport through biological membranes [8]. In cauliflower meristematic tissue cells, it has been observed that there is a high abundance of aquaporins embedded in the vacuolar membrane, which allows swelling of growing cells, while maintaining the cellular turgor [9]. However, the functionality of these proteins can be affected by the lipids of the membrane in which they are embedded [10, 11]. This suggests that the lipid composition of the membrane determines not only its physical characteristics but also the activity and functionality of the proteins present in it [12]. One of the main components of lipid membranes are sterols. These molecules have been linked with different functions, such as lipid packing, since they are able to interact with membrane proteins and fatty acids [13]. Furthermore, membrane sterols have been documented to regulate membrane water permeability [14]. For example, it has been reported that the presence of cholesterol in the bilayer makes it less permeable to water [15]. On the other hand, the composition of fatty acids in cellular membranes also contributes to the thickness, stability, and permeability due to its degree of unsaturation [16].

Therefore, both the lipid and the protein composition are determinants of the membrane transport activity, giving each type of membrane specific functional characteristics. For the above reasons, and with the aim of determining the specific functions of the plasma membrane of the cauliflower meristematic tissue, in this work the presence of fatty acids, sterols, aquaporins, and other proteins was analyzed in two different stages of inflorescence development. To allow a comparison with vegetative tissue, leaf plasma membranes were also analyzed.

Methods

Plant material

Fifty commercial cauliflower (*Brassica oleracea* L. var. *botrytis*) seeds from the Whiton cultivar (CAU02417, provided from Sakata Seed Iberica S.L.U., Valencia, Spain) were induced to germinate by imbibition with water and continuous aeration for 24 h. Then, the seeds were transplanted to vermiculite and were kept in darkness, at 28 °C and 60% relative humidity, for 2 days. The seedlings (5 days old) were transferred to the agricultural soil of an experimental farm (37°47′52.7″N, 0°52′00.7″W, 15 m asl, Murcia, Spain) to be cultured in accordance with local legislation. The experiment was carried out from December to February with average temperatures and relative humidities of 17 °C and 60% (day), and 4 °C and 65% (night), under a semi-arid Mediterranean climate. The daily average temperature and relative humidity were recorded with dataloggers (AFORA S.A., Barloworld Scientific, Murcia, Spain). All plants were drip-irrigated with ¼-strength Hoagland nutrient solution. They were harvested at 70 and 90 days after transplanting. The intermediate leaves and inflorescences (15 of each) were sampled at random and weighed fresh in three technical replicates. After that, samples were kept in storage at 4 °C for 1 day until processing. The whole protocol was performed taking into account the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [17].

Plasma membrane extraction

Plasma membrane isolation was performed as described in Casado-Vela et al. [18]. Samples of fresh material (100 g) were sliced in small pieces and vacuum-infiltrated with a 1:1.6 (w/v) proportion of an extraction buffer (0.5 M sucrose, 1 mM DTT, 50 mM HEPES, 1.30 mM ascorbic acid, pH 7.5) and 0.5 g of PVP (polyvinylpyrrolidone). After 10 min, samples were homogenized and filtered through a nylon mesh with a pore diameter of 100 μm. Then, the filtrate was centrifuged at 10000×*g* for 30 min, at 4 °C; the supernatants were collected and centrifuged for 35 min at 50000×*g*, at 4 °C. The pellet obtained was resuspended in 500 μl of a buffer containing 5 mM PBS and 0.5 M sucrose (pH 6.5) (FAB). Three different extractions per sample type were performed. Two milliliters of this microsomal fraction were introduced in a two-phase system composed of PEG-3350/Dextran-T500–6.3% (w/w), 5 mM KCl, 330 mM sucrose, 2.5 mM NaF, and 5 mM K_3PO_4 (pH 7.8). The system was centrifuged for 5 min at 4000×*g*. Then, the upper phase was

collected and washed with a buffer containing 9 mM KCl, 0.2 M EGTA, 0.5 mM NaF, and 10 mM Tris-borate (pH 8.3). Then, a centrifugation at 55000×*g* for 35 min, at 4 °C, was performed. The pellet obtained was resuspended in FAB. The final protein concentration was determined using an RC DC protein assay kit (BioRad, California, USA), with bovine serum albumin as the standard. The purity of the PM was estimated as described in Casado-Vela et al. [18] (Table S1).

Vesicle size

The mean size of the vesicles obtained from different samples was determined by dynamic light scattering, using a Malvern ZetaSizer Nano XL (Malvern Instruments Ltd., Orsay, France) as described by Barrajón-Catalán et al. [19]. This instrument allows the analysis of particles with diameters from 1 nm to 3 μm.

Stopped flow light scattering

These measurements were performed in a PiStar (Applied Photophysics, Leatherhead, UK) spectrophotometer at 20 °C, as described in Maurel [8]. The kinetics of each vesicle volume adjustment were monitored by dynamic light scattering at 90° and with a λ_{ex} of 515 nm. Purified plasma membrane vesicles from each sample type were subjected to a 100x dilution in a buffer with 30 mM KCl and 20 mM Tris-Mes (pH 8.3, final osmolarity of 360 mOsmol kg^{-1} H_2O). For the measurement, the diluted vesicle preparation was mixed in a 1:1 proportion (v:v) with the same buffer supplemented with 540 mM sucrose (630 mOsmol kg^{-1} H_2O). In this way, an osmotic gradient of 270 mOsmol kg^{-1} H_2O was generated. The osmotic permeability (*Pf*) was calculated using this formula:

$$Pf = \frac{K_{exp}\, V_0}{A_v\, V_w\, C_{out}}$$

Where K_{exp} is the adjusted exponential velocity constant, V_0 is the mean vesicular volume, A_v is the mean vesicular surface area, V_w is the water molar mass, and C_{out} is the external osmolarity.

Lipids and sterols analysis

Five hundred microliters of plasma membrane were mixed with a chloroform-methanol (1:2) mixture [20]. As an internal standard for further sterol analysis, β-colestanol (20 μl, at 0.1 mg ml^{-1}) was added. Then, 0.25 ml of chloroform was added to the mixture before centrifugation at 10000×*g* for 6 min. The resultant interphase, corresponding to the protein content, was collected for further proteomic analysis. The chloroformic phase was removed to another tube and evaporated with N_2. For sterol analysis, 50 μl samples of the chloroformic phase were dried with N_2 and then acetylated using pyridine (50 μl) and Ac_2O (100 μl). After 2 h, the solvents were evaporated with N_2 and 20 μl of ethyl acetate were added. Sterols and fatty acids were determined by gas chromatography, employing an HP5 capillary column (30 m × 0.25 mm × 0.25 μm). This was coupled to a flame ionization detector (FID). Helium was used as the mobile phase (1 ml min^{-1}) and a heat gradient was imposed: from 150 to 195 °C, increasing 3 °C per min, then from 195 to 220 °C at 2 °C per min, and from 220 to 300 °C at 6 °C per min.

Proteomic analysis

Samples were processed following the method described in Stetson et al. [21]. The isolated plasma membrane proteins from 500 μl of plasma membrane vesicles were mixed with 100 μl of 50 mM ammonium bicarbonate (pH 8.3) with 0.01% Protease Max (Promega, Madison, USA). Then, the samples were reduced by adding 100 μl of 20 mM DTT at 56 °C, for 20 min. After that, alkylation was performed by incubation with 100 μl of 100 mM IAA for 30 min, at room temperature and in the dark. Digestion was performed by incubation with 1 μg of trypsin (1:100 w/w) for 3 h, at 37 °C. The samples were dried in a speed vacuum concentrator. The dry samples were resuspended in 20 μl of water/acetonitrile/formic acid (94.9:5:0.1). Then, they were injected onto an Agilent Advance Bio Peptide Mapping HPLC column (2.7 μm × 100 mm × 2.1 mm, Agilent technologies) thermostatted at 55 °C and with a flow rate of 0.4 ml/min. A mixture of water/acetonitrile/formic acid (10:89.9:0.1) was used as the eluent. For detection, an Agilent 6550 Q-TOF coupled with a dual electrospray (AJS-Dual ESI) was used. The experimental parameters were set in MassHunter Workstation Data Acquisition software (Agilent Technologies, Santa Clara, CA, USA), as described in Martínez-Ballesta et al. [22]. The data were processed with Spectrum Mill MS Proteomics Workbench (Agilent Technologies).

The data obtained were compared with the information available in the UniProt database (www.uniprot.org) for the *Brassicaceae* family. Protein function and location were determined from the Gene Ontology database [23].

Gel electrophoresis and immunoblotting

Plasma membrane isolated from cauliflower leaves and inflorescences was employed. Ten micrograms of protein per lane were loaded for 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as shown in Muries et al. [24]. Then, the proteins were transferred to a PVDF membrane and maintained for 20 min at 15 V in an electrophoretic transfer cell (Trans-Blot SD cell, BioRad, CA, USA), using Towing transfer buffer supplemented with 0.05% SDS [24]. Blocking

solution (TBS containing 2% (w/v) skimmed dry milk) was applied to the membrane for 1 h at room temperature. After that, the membrane was again incubated for 1 h at room temperature, with TBS containing 0.05% Tween 20 and one of the selected antibodies. An antibody raised against the first 45 N-terminus residues of *Arabidopsis thaliana* PIP1;1 (dilution 1:3000, kindly provided by Prof. Dr. Anthony Schäffner) and another raised against 17 residues from the C-terminal peptide of PIP2;2 of *A. thaliana* (dilution 1:20000, kindly provided by Dr. Veronique Santoni) were used. Incubation was performed overnight at 4 °C. Goat anti-rabbit IgG coupled to horseradish peroxidase was employed as a secondary antibody (dilution 1:20000). A chemiluminescent signal was developed with West-Pico Super Signal substrate (Pierce, Rockford, IL, USA). The quantification was carried out using ImageJ software and by performing a densitometry analysis.

Data analysis

The statistical analysis comprised a one-way ANOVA followed by a Tukey HSD post hoc test, performed using RStudio (version 3.4.4.).

Results

Mean size of plasma membrane vesicles

The results for the mean vesicle size (nm), represented in Fig. 1, show significant differences between inflorescences and leaves and between the maturation stages ($p < 0.05$). The mean vesicle size was greater for leaves-derived plasma membrane vesicles than for inflorescences-derived ones ($p < 0.05$). The polydispersity data (Fig. 2) show that the variability in the size of inflorescences-derived plasma membrane vesicles was higher than for leaves-derived ones ($p < 0.05$). Hence, vesicles obtained from leaves were more homogeneous in size.

Osmotic water permeability

Figure 3 shows that the osmotic water permeability (*Pf*, $\mu m\ s^{-1}$) of the plasma membrane vesicles differed significantly between the two maturation stages of the inflorescences ($p < 0.05$), those derived from 90-day inflorescences having the highest *Pf* ($64.4 \pm 4.14\ \mu m\ s^{-1}$). Lower values were obtained for leaves, with no significant differences between the two maturation stages ($p > 0.05$).

Lipid analysis

Fatty acids

The results of the fatty acids (% of total fatty acids) analysis are shown in Table 1. The percentage of palmitoleic acid (C16:1) did not vary between 70 days and 90 days for inflorescences-derived plasma membrane vesicles ($p > 0.05$). Furthermore, a similar percentage of palmitoleic acid was found in vesicles derived from 90-day leaves ($p > 0.05$), but a significant decrease was observed for vesicles from 70-day leaves (~ 29–31% vs ~ 19%). For

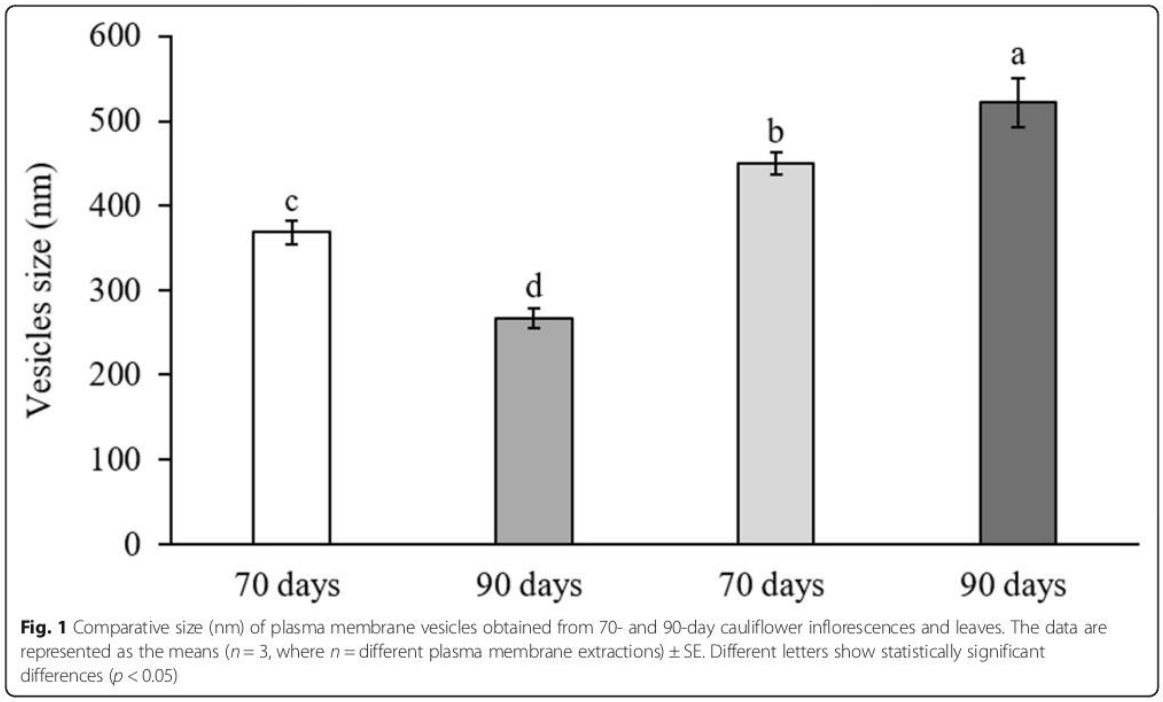

Fig. 1 Comparative size (nm) of plasma membrane vesicles obtained from 70- and 90-day cauliflower inflorescences and leaves. The data are represented as the means ($n = 3$, where n = different plasma membrane extractions) ± SE. Different letters show statistically significant differences ($p < 0.05$)

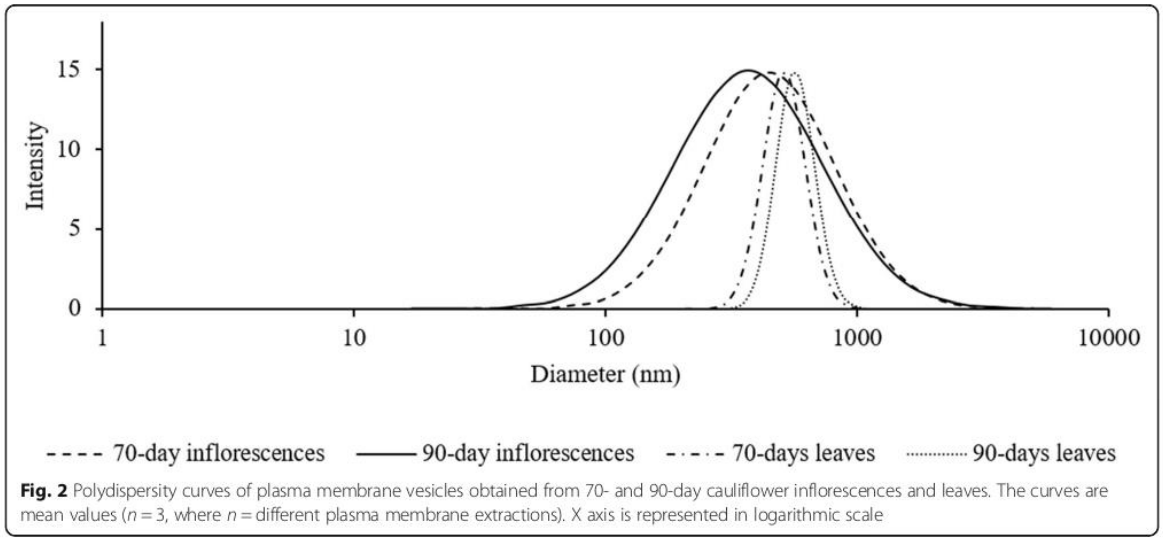

Fig. 2 Polydispersity curves of plasma membrane vesicles obtained from 70- and 90-day cauliflower inflorescences and leaves. The curves are mean values ($n = 3$, where n = different plasma membrane extractions). X axis is represented in logarithmic scale

oleic acid (C18:1), similar percentages were found in vesicles derived from inflorescences at the two maturation stages ($p > 0.05$). Surprisingly, the presence of oleic acid in leaves-derived vesicles greatly differed, the percentage in 70-day leaves being double that in inflorescences and almost 14-times higher when compared to 90-day leaves ($p < 0.05$). For linoleic acid (C18:2), a statistically significant decrease was found between day 70 and day 90 for inflorescences-derived plasma membrane vesicles ($p < 0.05$). However, the opposite was observed in leaves-derived vesicles, the content of linoleic acid being increased at 90-days ($p < 0.05$). The linolenic acid (C18:3) percentage differed significantly among the four types of sample ($p < 0.05$). Higher proportions were found in

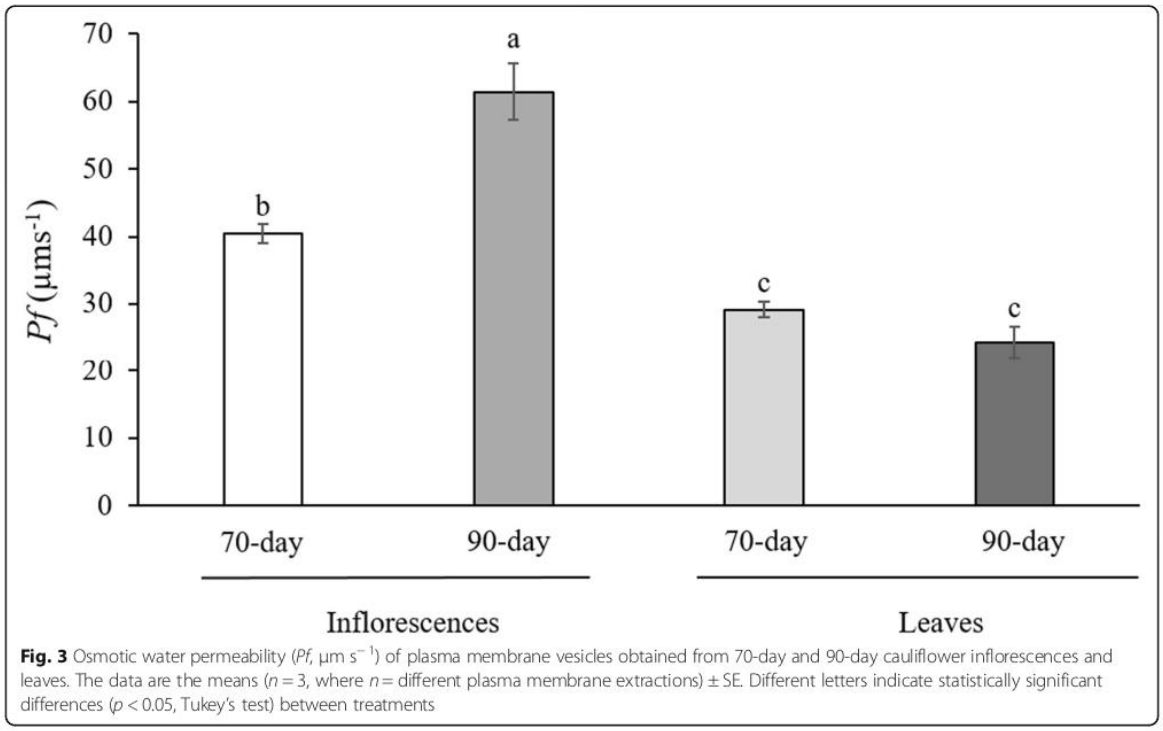

Fig. 3 Osmotic water permeability (Pf, μm s^{-1}) of plasma membrane vesicles obtained from 70-day and 90-day cauliflower inflorescences and leaves. The data are the means ($n = 3$, where n = different plasma membrane extractions) ± SE. Different letters indicate statistically significant differences ($p < 0.05$, Tukey's test) between treatments

Table 1 Fatty acid percentage, percentage of monounsaturated fatty acids (MUFA) double bond index (DBI = ∑ (unsaturated fatty acids x number of double bonds)), ratio of unsaturated fatty acids (RUFA = (C18:2 + C18:3)/(C18:1), for plasma membrane from cauliflower inflorescences and leaves at 70 days and 90 days of development. The data are represented as the means ($n = 3$, where n = different plasma membrane extractions) ± SE. Different letters indicate statistically significant differences ($p < 0.05$, in HSD Tukey's test) between treatments

	Inflorescences		Leaves	
% Fatty acids	70-day	90-day	70-day	90-day
Palmitoleic acid (C16:1)	31.41 ± 0.44a	30.99 ± 1.22a	19.09 ± 0.4b	29.42 ± 0.75a
Oleic acid (C18:1)	6.42 ± 0.70b	5.59 ± 0.78b	11.66 ± 0.51a	0.79 ± 0.07c
Linoleic acid (C18:2)	26.71 ± 1.09a	22.66 ± 0.68b	21.24 ± 0.69b	25.35 ± 1.01a
Linolenic acid (C18:3)	35.46 ± 0.91d	40.91 ± 0.8c	48.08 ± 0.64a	44.44 ± 0.51b
MUFA	37.88 ± 0.26a	36.5 ± 0.45a	30.78 ± 0.27b	30.31 ± 0.47b
RUFA	9.94 ± 1.13d	11.8 ± 1.64b	5.95 ± 0.12c	89.1 ± 5.76a
DBI	166.28 ± 1.27c	173.74 ± 0.3b	198.65 ± 3.8a	184.82 ± 3.62b

leaves, the vesicles from 70-day leaves having the highest percentage (~ 48%, vs ~ 44% for 90-day leaves). For inflorescences-derived vesicles, the proportion of linolenic acid was higher in 90-day samples than in those taken at 70 days ($p < 0.05$, ~ 40% vs ~ 35%).

The total percentages of monounsaturated fatty acids (MUFA) and the ratio of unsaturated fatty acids (RUFA) were also determined (Table 1). The MUFA percentages for inflorescences-derived samples were significantly higher ($p < 0.05$) when compared to leaves. About RUFA, the highest ratio was observed in the 90-day leaves derived plasma membranes ($p < 0.05$). As well, a statistically significant increase ($p < 0.05$) in the double bond index (DBI) was found between the 70-day and 90-day inflorescences cauliflower-derived vesicles. Furthermore, the DBI was significantly higher ($p < 0.05$) in samples from 70-day leaves than in those from 90-day leaves.

Sterol content

The sterol content (μg mg^{-1} of protein) of plasma membrane vesicles was also assessed (Table 2). Campesterol concentration showed a statistically significant increase in vesicles derived from 90-day inflorescences, being 3-fold higher than in those from 70-day ones ($p < 0.05$). No differences in the campesterol concentration were found between vesicles derived from 70- and 90-day leaves ($p > 0.05$). For stigmasterol, no statistically significant differences were found between vesicles from inflorescences at the two maturation stages ($p > 0.05$). However, in leaves-derived vesicles, the concentration of stigmasterol was higher for 90-day leaves (0.16 ± 0.07 vs 0.25 ± 0.09 μg mg^{-1} of protein, $p < 0.05$). A 2-fold, statistically significant increase in β-sitosterol was found in vesicles from 90-day inflorescences when compared to those of 70-day inflorescences ($p < 0.05$). A similar difference was seen when comparing vesicles from 70-day leaves with those of 90-day leaves (0.95 ± 0.11 vs 2.14 ± 0.37 μg mg^{-1} of protein, $p < 0.05$). The stigmasterol/β-sitosterol ratio was also analysed. The highest ratio was found in vesicles derived from 70-day inflorescences, 2-times higher than for 90-day inflorescences and 5-times higher than for both 70-day and 90-day leaves ($p < 0.05$). A statistically significant difference in the stigmasterol/β-sitosterol ratio was not found between 70-day and 90-day leaves (0.17 ± 0.02 vs 0.12 ± 0.03 μg/mg of protein, $p > 0.05$).

Proteomic analysis and immunoblotting

A proteomic analysis was performed with the samples of isolated plasma membrane vesicles form inflorescences and leaves in order to assess qualitatively the proteins present in the vesicles obtained for further analysis. As shown in Fig. S1, the proteins obtained were organized

Table 2 Sterol content (μg mg^{-1} of protein) of plasma membrane from cauliflower inflorescences and leaves at 70 days and 90 days of plant development. The data are represented as the means ($n = 3$, where n = different plasma membrane extractions) ± SE. Different letters indicate statistically significant differences ($p < 0.05$, HSD Tukey's test) between treatments

	Inflorescences		Leaves	
μg mg^{-1} of protein	70-day	90-day	70-day	90-day
Campesterol	1.94 ± 0.39b	5.68 ± 0.64a	1.72 ± 0.46b	2.10 ± 0.35b
Stigmasterol	0.53 ± 0.16a	0.60 ± 0.19a	0.16 ± 0.07c	0.25 ± 0.09b
β-Sitosterol	0.79 ± 0.11b	1.71 ± 0.05a	0.95 ± 0.11b	2.14 ± 0.37a
Stigmasterol/β-Sitosterol ratio	0.67 ± 0.08a	0.35 ± 0.1b	0.17 ± 0.02c	0.12 ± 0.03c

according to their cellular characteristics among soluble, membrane and unclassified. In all samples studied (inflorescences and leaves at both maturation stages) the number of soluble proteins identified was higher (40–48%) than the number of membrane proteins (40–44%). Also, high percentages of unclassified proteins were identified in leaves (13–14% in 90-d and 70-d, respectively) than in inflorescence (8.6–8.9% in 90-d and 70-d, respectively). The number of membrane proteins determined in inflorescence was very similar (42%) for both times (90-d and 70-d) while the number of these proteins determined in leaves was higher at 70-d (44%) than at 90-d (40%).

Within the plasma membrane proteins, they were also classified by their molecular function (Fig. 4). They were grouped in seven functional clusters (catalytic, structural molecules activity, signalling receptor activity, binding, protein folding, transporter activity and antioxidant activity). All the samples showed the similar protein distribution, being the catalytic (35–44%) and the binding proteins (36–39%) standing out as the main groups, followed by transporter (11–18%). The number of transporter proteins were lower identified in inflorescences (11%) than in leaves (18–19%). The structural proteins were higher in inflorescence (6–7%) than in leaves (0.5%). The rest of the proteins identified were very low in all the samples, appearing the signalling receptor proteins only in 90-d leaves.

A search focused on the aquaporins present in plasma membrane derived from cauliflower inflorescences and leaves was also performed. Aquaporin-related peptides were spotted in all sample types (Table 3). In both inflorescences and leaves, peptides corresponding to a wide group of PIP1 aquaporin subfamilies (PIP1;1, PIP1;2, PIP1;3, PIP1;4, and PIP1;5) were identified. Nevertheless, only in the inflorescence samples were peptides related to the PIP2 subfamily detected, PIP2;5 and PIP2;7. Special emphasis can be placed on PIP2;7, for which three different peptide fragments were detected, while only one peptide determined PIP2;5. Members of the Tonoplast Intrinsic Protein (TIP) subfamily were also identified in both types of sample, although this group of aquaporins is generally targeted to the vacuolar membrane. In particular, TIP1;2 and TIP2;1 were found in 70-day inflorescences and 90-day leaves, while only TIP1;2 peptides were detected in 90-day inflorescences and 70-day leaves samples (Table 3).

The results obtained from SDS-PAGE analysis of plasma membrane proteins from leaves and inflorescences are shown in Fig. 5. The presence of two bands was detected; an upper band of 60 kDa, corresponding to dimeric (D) forms of PIPs, and a lower band of ca. 30 kDa,

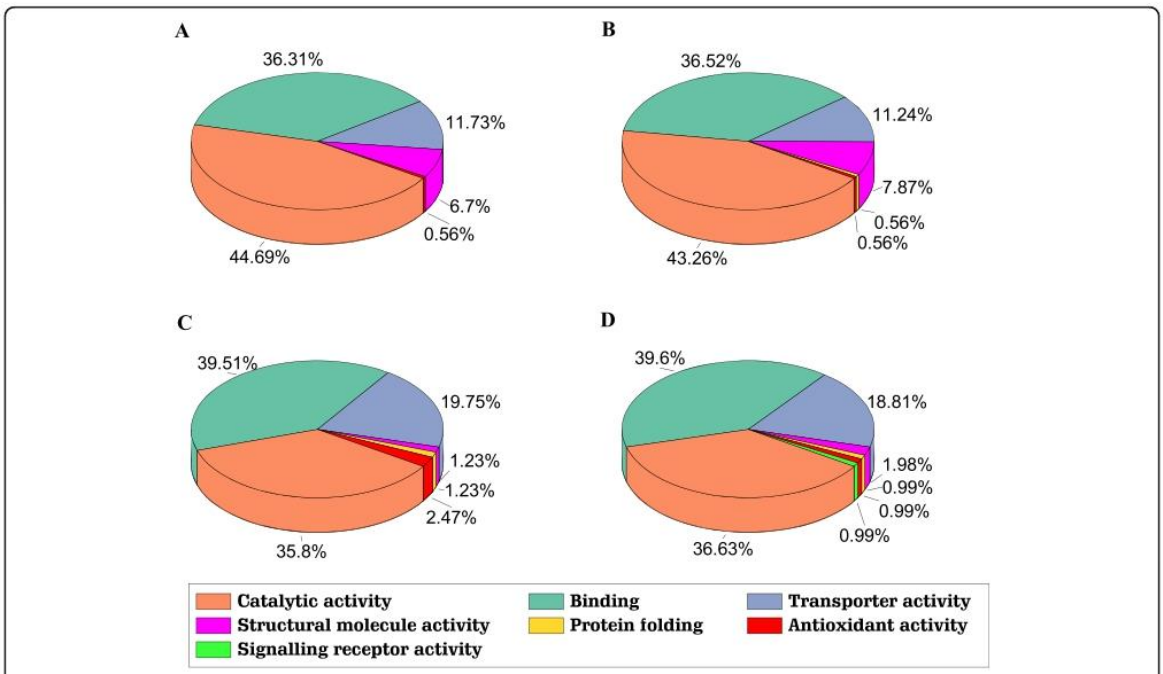

Fig. 4 Functional classification of plasma membrane proteins identified in cauliflower. The proteins identified were classified based on seven categories described in the Gene Ontology database [23]. **a** 70-day inflorescences, **b** 90-day inflorescences, **c** 70-day leaves, and **d** 90-day leaves. The figure design has been done with Origin (Pro), Version 2019. OriginLab Corporation, Northampton, MA, USA

Table 3 Aquaporin proteins identified in plasma membrane samples of cauliflower inflorescences and leaves. All protein sequences were retrieved from *Brassica oleracea* L. var. *oleracea* information in the NCBI and UniProt databases (ID). The symbols **'+'** and **'-'** indicate the presence or absence of the protein in the samples, respectively

Protein	NCBI ID	UniProt ID	Score	Inflorescences		Leaves	
				70-day	90-day	70-day	90-day
PIP1;1	XP_013604594.1	A0A0D3DUU2	31.8	+	+	+	+
PIP1;2	XP_013637020.1	A0A0D3C6I1	34.9	+	+	+	+
PIP1;3	XP_013600561.1	A0A0D3C6T1	22.3	+	+	+	+
PIP1;4	XP_013612790.1	A0A0D3D7M3	22.3	+	+	+	+
PIP1;5	XP_013599049.1	A0A0D3DFM5	22.3	+	+	+	+
PIP2;5	XP_013599897.1	A0A0D3DT38	11.9	+	+	–	–
PIP2;7	XP_013629883.1	A0A0D3BL75	27.4	+	+	–	–
TIP1;2	XP_013613430.1	A0A0D2ZPE6	18.3	+	+	+	+
TIP2;1	XP_013587105.1	A0A0D3CJP0	14.7	+	–	–	+

corresponding to the monomeric form (M). Two PIPs groups were analysed, PIP1 and PIP2. For PIP1, bands from samples of 70-day and 90-day inflorescences (23.4 and 15.7% D + M) were less dense than those of 70-day and 90-day leaves-derived plasma membrane proteins (31 and 28.9% D + M). For PIP2 aquaporins, much denser bands were found for 70-day (27.7% D + M) and 90-day inflorescences (61.4% D + M) when compared with 70-day and 90-day leaves-derived samples (5.2 and 5.7% D + M).

Discussion

The isolation of plasma membrane vesicles using the two-phase aqueous polymer technique [25] has been reported to produce homogeneous material in terms of yield and composition [26]. However, the vesicles isolated from plant tissues can vary depending on the type of plant [20], the organ, and the culture conditions [22]. In our work, the vesicles obtained from adult plants were bigger than those obtained previously from seedlings [22]. Furthermore, the vesicles obtained from inflorescences were smaller but more heterogeneous in size than those obtained from leaves. Also, the vesicles yield from inflorescences was double that from leaves (data not shown). Although these results, that could have been due to differences in cell size and tissue lignification, may be unimportant in a plant physiological study they could be important if an industrial application is considered [27].

One of the main functions of the plasma membrane is the regulation of the passage of diverse molecules and water through it. The *Pf* usually is the parameter chosen to describe water fluxes across the plant membranes isolated vesicles that are driven by the osmolarity gradient [28]. In fact, the osmotic shock applied to our vesicles should not produce any small pore that suppressed convection that have been reported to leaded to a vesicles rupture [29]. The plasma membrane vesicles derived from broccoli leaves by Martínez-Ballesta et al. [30] had *Pf* values similar to the ones obtained in our work. Nevertheless, the *Pf* values obtained for the plasma membrane vesicles derived from 70-day and 90-day inflorescences were 1.6- and 2.5-times higher, respectively, than those of vesicles from 90-day leaves. Since little or no information concerning *Pf* in protoplasts or vesicles derived from *Brassica* inflorescences exists, these results shed light on this matter. Similar values of *Pf* have been reported for plasma membrane vesicles and protoplasts obtained from pepper roots (30 and 40 $\mu m\ s^{-1}$) [31]. Furthermore, *Pf* values as high as 540 $\mu m\ s^{-1}$ have been found in plasma membrane from *Beta vulgaris* roots [32]. This suggests that the water osmotic permeability in inflorescences might be similar to that in roots, due to their requirement for water to maintain turgor. Indeed, a relationship between cell turgor in meristematic tissue and cellular division has been reported [33].

One of the main structural components affecting the physical characteristics of biological membranes are fatty acids. The proportions of different saturated and unsaturated fatty acids may affect the permeability of the bilayer [34]. In our study, the plasma membrane vesicles obtained from cauliflower had a high proportion of unsaturated fatty acids, which provides greater fluidity [35]. In leaf plasma membranes from other species - such as broccoli, *Cakile maritima* L., and *Brassica napus* L. - linolenic acid (C18:3) was a minor component [20]. However, the proportion of this fatty acid was greater in plasma membrane vesicles produced from cauliflower leaves and inflorescences. This difference in fatty acids distribution might have a protective effect against temperature changes, since previous work was carried out in a crop chamber but we grew cauliflowers in the field; an increase in linolenic acid (C18:3) was found in peach fruits under low-temperature stress [36]. Also, a greater degree of unsaturation produces looser packing of the polyunsaturated carbon chains,

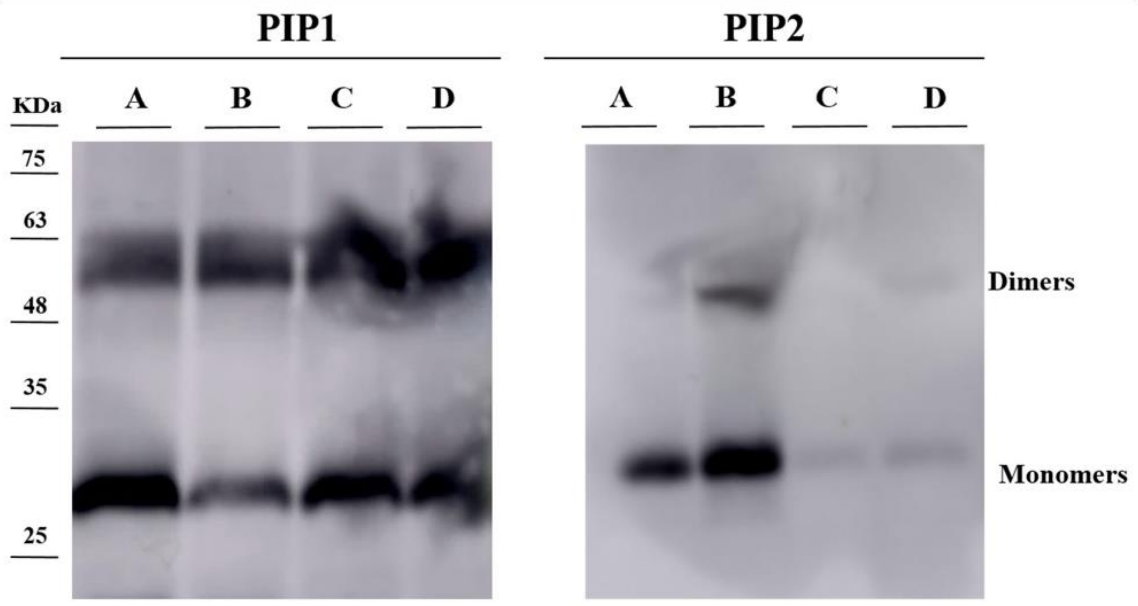

Fig. 5 Immunoblotting analysis for PIP1 and PIP2 aquaporins present in cauliflower plasma membrane vesicles. **a** plasma membrane from 70-day inflorescences, **b** plasma membrane from 90-day inflorescences, **c** plasma membrane from 70-day leaves, **d** plasma membrane from 90-day leaves

decreasing the interaction with other molecules and allowing deeper penetration of water into the bilayer [37]; this could be related to the higher *Pf*. But, the fact that *Pf* was higher in 90-day inflorescences than in 70-days inflorescences, must be related to the aquaporins presence. In our vesicles derived from cauliflower leaves the oleic acid (C18:1) proportion was lower than that reported for *Brassica oleracea* L. var. italica in Chalbi et al. [20]. When comparing the data of *B. oleracea* var. italica leaves [20] with our work, a higher RUFA (ratio of unsaturated fatty acids) was obtained in cauliflower, due to the low percentage of oleic acid (5.95 ± 0.13 and 89.1 ± 5.76 for 70 and 90-day leaves vs 1.19 ± 0.18 in broccoli leaves). In the same way, the DBI was also affected by the different proportions of unsaturated fatty acids. In our analysis, the highest DBI was found in 70-day leaves, since the percentage of linolenic acid (C18:3) was higher in this sample than in *B. oleracea* var. italica (48.08 ± 0.64% vs 5.52 ± 0.8%). In previous studies of soy (*Glycine max* L.) plasma membrane, a rise in oleic acid (C18:1) and a decrease in linoleic acid (C18:2) and linolenic acid (C18:3) were observed [38], which could possibly lead to an increase in membrane rigidity. Since the plants studied in Chalbi et al. [20] were grown in a controlled environment chamber, the lipid proportions might be quite different from those of plants cultivated in the field, where the climatic conditions, such as temperature and humidity, are highly variable.

Sterols also contribute to the bilayer permeability [26]. Campesterol has been studied regarding its contribution to increasing the spatial organization of the lipid bilayer and, thus, its order [39]. Furthermore, it has been linked with a decrease in ionic permeability through lipid membranes [40]. In our work, plasma membrane from 90-day cauliflower inflorescences had the highest content of campesterol per mg of protein. This might be due to the dual function of campesterol, as a structural component and also the precursor of brassinosteroid hormones that are required for normal plant development [41]. As has been reported in *Arabidopsis thaliana,* brassinosteroids help root meristem growth [42]. The fact that the stigmasterol/β-sitosterol ratio was much higher in the plasma membranes of inflorescences, mainly in the young ones (70 d), could have contributed to the increase in *Pf*. In fact, sitosterol has been pointed out as the main regulator of water permeability though membranes along with aquaporins [26, 43]. However, the fact that the highest P_f value was obtained for 90-day inflorescences, which present a lower sitosterol/stigmasterol ratio provide to aquaporins higher contribution to water transport.

By means of the proteomic analysis performed by HPLC-ESI-QTOF-MS, the whole batch of identified proteins in each sample were analysed and categorized according to their cellular location (Fig. S1). However, a few proteins could not be assigned to defined categories in each fraction due to the lack of information. Although

a high percentage of membrane proteins were identified in all samples (42–44%), a notable presence of soluble proteins was also found (40–49%). This could be considered a contamination of the membrane samples, although the number of identified proteins is high, the total amount should be very low.

In addition, we classified proteins based on their functional category within the plasma membrane identified proteins (Fig. 4) [23]. The presence of different transporter activity proteins (11–19%) was detected; these comprised transmembrane ion transporters and ion channels, also located mainly in the plasma membrane. The number of detected transporter proteins revealed a decrease in inflorescence. This could be related with the specialization of the tissue since the meristematic tissue (inflorescence) probably need lower category of plasma membrane transporters.

Table 3 shows the aquaporins identified in plasma membrane extracts of cauliflower inflorescences and leaves. As PIPs aquaporins showed a very conservative structure, the digestion by trypsine enzyme and solubilisation is very similar in all of them. Although the knowledge of the sequences of all *B. oleracea* aquaporins was very useful to investigate the presence of MIPs isoforms, and the analysis of peptides revealed five PIP1 isoforms (PIP1;1, PIP1;2, PIP1;3, PIP1;4, and PIP1;5) (Table 3), due to the high homology within the PIP1 subfamily, we could not identify isoforms unambiguously. Also, two TIPs were identified in our plasma membrane samples. Since TIPs are usually located in the tonoplast [44], their presence could be related with vacuolar contamination. However, the fact that TIPs have been found located in the plasma membrane in pea cotyledons [45] points to the possibility that TIPs were present in our plasma membrane.

Additionally, PIP2 subfamily proteins were found only in inflorescences (Table 3), in particular PIP2;5 and PIP2;7. Of these, only PIP2;7 was unambiguously identified; this aquaporin has been shown to be expressed mainly in *B. oleracea* flowers [46]. Furthermore, when PIP2;7 was overexpressed in *A. thaliana* roots the hydraulic conductivity increased six-fold, showing the important role of PIP2;7 in water transport.

Further information about plasma membrane aquaporins in cauliflower was obtained from immunoblotting (Fig. 5). The results show that PIP1 was present in both leaves and inflorescences, although its density in inflorescences was lower. The greater presence of PIP1 proteins in leaves could be explained by its regulatory role in CO_2 transport [47]. This CO_2 transport would be indispensable for the photosynthetic activity in leaves, whereas in inflorescences the constant cell division and growth would produce CO_2 that would need to be carried to the leaves. In addition, the other PIP1 members have been postulated as O_2 transport facilitators; in particular, PIP1;3 in tobacco plants [48].

In addition, the results for PIP2 show a greater density in samples from inflorescences - which mirrors the information obtained in the proteomic assay- in which PIP2 could only be detected in those samples (Fig. 5). The exclusive identification of PIP2 proteins in inflorescences (PIP2;1, PIP2;2, PIP2;5, and PIP2;7) could be related to the elevated demand for water in cauliflower inflorescences, to maintain nutrient uptake and turgor [49]. The localization of PIP2 in plasma membrane samples from inflorescences (Fig. 5) could also explain the differences observed in *Pf*; the 90-day inflorescences showed the highest *Pf*, in accordance with the higher concentration of PIP2 found in these samples (Fig. 3). The non-detection of PIP2 in leaves could indicate that the function of PIPs is performed here by TIPs or that only limited water transport across the lipid bilayer occurs. However, this aspect needs to be investigated further.

Conclusions

In summary, this study shows how the different elements present in the cauliflower inflorescences plasma membrane play a key role in water passage. This membrane is characterized by a low degree of unsaturation, which could increase the rigidity, decreasing water transport, but the high content in sitosterol (highly correlated with water passage) would compensate this fact. In relation to this, the high presence of aquaporins in inflorescences, especially PIP2;5 and PIP2;7, indicates a potential role of aquaporins in the water transport required for the continuous development of the meristematic tissue. Furthermore, the fact that the aquaporins contribution to water transport in inflorescences must be higher than in leaves, with a potential correlation with the stage of development, provides to sterols and aquaporins a specific role in development. Our work highlights the need for further research on specific aquaporins in relation to adult plant development under natural conditions.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-020-02778-6.

Additional file 1: Figure S1. Classification of proteins identified in cauliflower plasma membrane isolated vesicles. Proteins identified were classified based on three categories corresponding the information available in the Gene Ontology database [23]. (A) 70-day inflorescences, (B) 90-day inflorescences, (C) 70-day leaves, and (D) 90-day leaves. The figure design has been done with Origin(Pro), Version 2019. OriginLab Corporation, Northampton, MA, USA. **Figure S2.** Raw images of immunoblotting analysis for PIP1 and PIP2 aquaporins present in cauliflower plasma membrane vesicles. A: plasma membrane from 70-day inflorescences, B: plasma membrane from 90-day inflorescences, C: plasma membrane from 70-day leaves, D: plasma membrane from 90-day leaves. **Table S1.** Average of the enzymatic activities (nmol min-1 mg-1 Protein) of plasma membrane and microsomal fractions measured in the purification fraction after aqueous polymer two-phase partitioning method.

Abbreviations
DBI: Double bond index; ER: Endoplasmic reticulum; FID: Flame ion detector; HPLC: High Performance Liquid Chromatography; MUFA: Monounsaturated fatty acids; PIP: Plasma membrane intrinsic proteins; PVP: Polyvinylpyrrolidone; RUFA: Ratio of unsaturated fatty acids; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS: Tween 20 blocking solution; TIP: Tonoplast intrinsic protein

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Authors' contributions
MCA contributed to the conception and design of this work. PGI carried out the experiments and JNE performed the analytical work of proteomics. PGI and JNE prepared figures and tables, and prepared the first draft of the manuscript. MCA contributed to manuscript revisions, reads and approved the submitted version. MCA obtained the funding. All authors have read and approved the manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

· Capítulo III

Capítulo III

Nanoencapsulation of Bimi® extracts increases its bioaccesibility after *in vitro* digestion and evaluation of its activity in hepatocyte metabolism

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Isothiocyanates (ITCs) have low stability in aqueous conditions, reducing their bioavailability when used as food ingredients. Therefore, the aim of this work was to increase the stability of the ITCs present in extracts of Bimi® edible parts by nanoencapsulation using cauliflower-derived plasma membrane vesicles. The bioactivity of these nanoencapsulates was evaluated in a HepG2 hepatocyte cell line in a model for low-grade chronic inflammation. The vesicles showed a higher capacity of retention in the *in vitro* gastrointestinal digestion for 3,3-diindolylmethane (DIM), indole-3-carbinol (I3C) and sulforaphane (SFN). Furthermore, Transmission Electron Microscopy (TEM) analysis of the vesicles revealed a decreased size under acidic pH and a release of their cargo after the intestinal digestion. The HepG2 experiments revealed differences in metabolism under the condition of chronic inflammation. The cauliflower-derived plasma membrane vesicles are able to enhance the stability of ITCs through the *in vitro* gastrointestinal digestion, improving their bioaccesibility and potential bioavailability.

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Nanoencapsulation of Bimi® extracts increases its bioaccessibility after *in vitro* digestion and evaluation of its activity in hepatocyte metabolism

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ABSTRACT

Isothiocyanates (ITCs) have low stability in aqueous conditions, reducing their bioavailability when used as food ingredients. Therefore, the aim of this work was to increase the stability of the ITCs present in extracts of Bimi® edible parts by nanoencapsulation using cauliflower-derived plasma membrane vesicles. The bioactivity of these nanoencapsulates was evaluated in a HepG2 hepatocyte cell line in a model for low-grade chronic inflammation. The vesicles showed a higher capacity of retention in the *in vitro* gastrointestinal digestion for 3,3-diindolylmethane (DIM), indole-3-carbinol (I3C) and sulforaphane (SFN). Furthermore, Transmission Electron Microscopy (TEM) analysis of the vesicles revealed a decreased size under acidic pH and a release of their cargo after the intestinal digestion. The HepG2 experiments revealed differences in metabolism under the condition of chronic inflammation. The cauliflower-derived plasma membrane vesicles are able to enhance the stability of ITCs through the *in vitro* gastrointestinal digestion, improving their bioaccesibility and potential bioavailability.

1. Introduction

Since the beginning of 1990s, the health benefits associated with the presence of vegetables from the *Brassicaceae* family in the human diet have been widely studied. Wide epidemiological and empirical evidence has linked their consumption with a chemoprotective effect, ameliorating diseases such as obesity, diabetes and cancer (Cipolla et al., 2015; Ma et al., 2018; Mazarakis, Snibson, Licciardi, & Karagiannis, 2019). These biochemical activities have been mainly attributed to the hydrolysis products of glucosinolates (GSLs) after plant myrosinase (EC 3.2.1.147) hydrolysis; the isothiocyanates (ITCs). GSLs belong to the plant's defence secondary metabolism, composed by a glycosylated thiohydroximate-*O*-sulphate group with a side chain that varies depending on which amino acid they derive from (Blažević et al., 2020). Numerous epidemiological studies have been performed, which have associated the potential beneficial role of consumption of dietary ITCs with human health (Quirante-Moya, García-Ibañez, Quirante-Moya, Villaño, & Moreno, 2020). Enterocytes have shown the ability to take sulforphane (SFN) from the gut lumen and immediately conjugate it with glutathione in the cell cytosol. Then, this conjugate undergoes further enzymatic modifications, producing derivatives from the mercapturic acid pathway (Gu, Mao, & Du, 2021). In addition, the presence of conjugative metabolism in cell cultures has been reported after the application of SFN in the culture media of Caco-2 and Hep2 cells (Baenas et al., 2015). However, further investigation is required on aspects associated to the stability and metabolism of ITCs from complex matrices such as extracts to reach the cells, and on the systemic circulation to provide health-promoting benefits.

Broccoli has been the main brassica specie investigated for the effect of GSLs and ITCs on human health, leaving an open path for other understudied *Brassica* species. However, due to its pungent and bitter flavour, the acceptance of broccoli by costumers is usually low. As a response, new varieties of broccoli with less pungency and a milder taste have emerged in the market. One of them is the crossbreed between *Brassica oleracea* var. *italica* L. (a conventional broccoli) and *B. oleracea* var. *alboglabra* L. (green Chinese kale), named Bimi®. Previous works with Bimi® have characterized its glucosinolates and their response to different elicitors, suggesting that this vegetable, just as its relative, has

Abbreviations: CYS, cysteine; DIM, 3,3-diindolylmethane; DMSO, dimethyl sulfoxide; GRA, glucoraphanin; GSH, glutathione; GSLs, glucosinolates; HGB, 4-hydroxy-glucobrassicin; I3C, indole-3-carbinol; ITCs, isothiocyanates; LPS, lipopolysaccharide; MGB, 4-methoxy-glucobrassicin; MTT, 3-(4,5-dimathylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetyl-cysteine; NGB, 1-methoxy-glucobrassicin, SFN, sulforaphane; TEM, transmission electron microscopy.

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potential benefits for human health (Garcia-Ibañez, Moreno, Nuñez-Gomez, Agudelo, & Carvajal, 2020). Furthermore, broccoli has been reported to present progroitin in various cultivars at a small but quantifiable amount (Li et al., 2021). This glucosinolate can interfere in thyroxine production (Petroski & Minich, 2020). However, progoitrin present in Bimi® was detected at very low content (<0.02 mg g D.W.$^{-1}$). Since the demand for nutraceuticals, functional food and beverages has grown during the last decade, Bimi® seems to be an interesting source material for further food prototypes (Garcia-Ibañez et al., 2020). This, together with a higher acceptance of its flavour, could make Bimi® and its derived products a more customer-friendly choice, which could increase the intake of dietary phytochemicals.

Although diverse *Brassica* species have been used as a source material to date (Garcia-Ibañez et al., 2020), the extracts elaborated are usually enriched in ITCs, which have low water solubility and limited stability (Cirilli et al., 2020; Danafar, Sharafi, Kheiri Manjili, & Andalib, 2017). In this way, encapsulation techniques have emerged as a solution, as they provide a suitable "coating", "carrier" or "matrix", that can help increase the stability and bioavailability of the bioactive compounds. The literature confirms that these techniques are usually successful in increasing the stability of ITCs (between 30% and 70%) under storage conditions (Zambrano, Bustos, & Mahn, 2019). However, little is known about the performance of these vehicles in the digestion process, as there must be a balance between the carrier survival to the low stomach pH and the release of its cargo in the small and large intestine, where the ITCs must be absorbed (Fahey et al., 2019).

Plant plasma membranes have been proposed as a delivery system for bioactive compounds (Chalbi, Martínez-Ballesta, Youssef, & Carvajal, 2015; Yepes-Molina, Martínez-Ballesta, & Carvajal, 2020). For example, cauliflower-derived plasma membrane vesicles have shown high values of osmotic permeability in previous works, a parameter that is directly related with vesicle functionality and stability (Garcia-Ibañez, Nicolas-Espinosa, & Carvajal, 2021). Furthermore, the size of the vesicles described (between 300 and 400 nm) is suitable for multiple biotechnological applications, such as in the agricultural or the cosmetics industry (Rios, Garcia-Ibañez, & Carvajal, 2019; Yepes-molina, Hernández, & Carvajal, 2021). In our previous works, these plasma membrane vesicles reported a high retention of ITCs present in a red cabbage extract, when assessed in an *in vitro* dynamic gastrointestinal system conditioned with the gut microbiome from obese volunteers (Garcia-Ibañez, Roses, et al., 2021).

Obesity is an increasing non-communicable disease with dramatic and global epidemic prevalence, with an estimation that >50% of the global population will have a Body Mass Index (BMI) >30 kg/m^2 by 2030 (Finkelstein et al. 2012). This condition have been reported to involve a chronic systemic inflammatory state (Artemniak-Wojtowicz, Pyrżak, & Kucharska, 2020). Mainly, this state is triggered by an increase in the adipose tissue size (hyperplasia), which was associated to a state of hypoxia and the release of pro-inflammatory cytokines by the adipocytes (Cussotto et al., 2020). In addition, changes in the gut microbiome of obese subjects have been demonstrated to affect intestinal permeability, leading to a high entrance of immunogens, such as lipopolysaccharide (LPS) (Mulders et al., 2018). This alteration could induce an endotoxin-dependent activation of the hepatic Kupffer cells, provoking an inflammatory state in the liver that may cause further damage when sustained in time (Frazier, DiBaise, & McClain, 2011).

Therefore, the aim of this work was to assess the effect of cauliflower-derived plasma membrane nanoencapsulation on the stability of a ITCs-enriched aqueous extract derived from Bimi® edible parts, to evaluate its performance under *in vitro* gastrointestinal digestion and its bioactivity, by deciphering its metabolism in a human liver cell line (HepG2). In this way, analyses of the derived extracts, the samples obtained from the digestions, and its metabolic fate in the cell culture, were performed to elucidate the efficiency of the nanoencapsulation in subsequent food prototypes development.

2. Material and methods

2.1. Plant material

Fifty Bimi® (*Brassica oleracea* L. var. *italica* × *Brassica oleracea* L. var. *alboglabra*) seeds from Sakata Seed Ibérica S.L.U. (Valencia, Spain) were pre-hydrated in deionized water and continuous aeration for 24 h. The seeds were germinated in vermiculite for 2 days in darkness and at 28 °C with a 60% relative humidity. Then, the seedlings were transferred to an experimental farm and grown in soil (37°47′52.7″ N, 0°52′00.7″ W, 15 m asl, Murcia, Spain). Bimi® plants were grown from March to August 2020 under a semi-arid Mediterranean climate. Drip irrigation with ¼ Hoagland solution was provided. Since an increase in secondary metabolites was previously reported by our group when a combination with 200 μM salicylic acid and 100 μM methyl jasmonate (Sigma Aldrich, Darmstad, Germany) in 0.2% ethanol was provided (Garcia-Ibañez et al., 2020), three applications were performed (150 mL per plant, procedure patent PCT/ES2019/070457). The first application was performed at the appearance of the central bud. After 5 days, another application was performed, followed by another 5 days resting period. A third application was carried out 5 days before harvesting. Plants were sampled and the edible part was separated from the leaves. Samples were kept at −80 °C, freeze-dried, and ground for preservation.

2.2. Plasma membrane vesicles extraction

One hundred milligrams of cauliflower inflorescences (*Brassica oleracea* L. var. *botrytis*, kindly provided by Sakata Seed Ibérica (S.L.U., Valencia, Spain) were processed until plasma membrane was obtained as described in Garcia-Ibañez, Nicolas-Espinosa, et al. (2021). Final protein concentration was measured using an RC DC protein assay kit (BioRad, California, USA), with bovine serum albumin as a standard.

2.3. Extract elaboration

Freeze-dried powder from samples was mixed with water (w:v) at a 1:20 ratio. Then, the mixture was vortexed and incubated in a continuously-agitated water bath for 30 min at 100 °C. Then, the samples were placed on ice for 10 min and the extracts were centrifuged at 10,000*g* for 15 min. The supernatant obtained was collected and filtered through and albet filter. Two different sample extracts were made: i) mixing the plasma membrane vesicles with the Bimi® extract at a ratio of 1:2, and ii) free Bimi® extract. For the encapsulation, the mixture of plasma membrane vesicles and extract was vortexed during a minute, in order to induce a mechanical disruption of the vesicles. Then, samples were kept at 4 °C. Both samples were kept at −80 °C for further analysis.

2.4. In vitro digestion of Bimi® extracts

The protocol used for the *in vitro* gastrointestinal digestion was adapted from Minekus et al. (2014). 1 mL of each extract was added to 15 mL of a porcine pepsine (Sigma-Aldrich, MO, USA) solution at pH 2 (2000 Units mL^{-1} in 100 mM HCl). Since the extracts are not sterile and the experimental conditions of the whole gastrointestinal digestion process favour the growth of microorganisms, 75 μL of a 0.5 mM merthiolate (Sigma-Aldrich, MO, USA) solution were added (Garcia-Campayo, Han, Vercauteren, & Franck, 2018). Samples were kept in a thermal bath at 37 °C for 3 h. Samples from this gastric digestion were taken, stored at 4 °C, overnight. After the gastric digestion phase, 7.5 mL of 0.2 M sodium hydroxide solution were added. After neutralization, another 7.5 mL of a pancreatin solution (2000 Units mL^{-1}, Sigma-Aldrich, MO, USA) in a pH 8 phosphate buffer were added. Samples were kept in the bath at 37 °C for 24 h. After that, they were kept overnight at 4 °C. Both, samples from the gastric digestion phase and the complete gastrotintestinal process, were filtered through a 0.22 μm pore diameter PVDF membrane and freeze-dried for further analysis. For the

nanoencapsulated Bimi® extract digestion, samples from the gastric and intestinal digestion were centrifuged at 10,000*g* for 30 min and the pellets were kept at 4 °C.

2.5. Transmission electron microscopy

In order to understand the performance of the cauliflower-derived plasma membrane vesicles, pellets obtained form: i) cauliflower-derived plasma membrane vesicles, ii) vesicles with Bimi® extract, iii) vesicles with Bimi® extract after the gastric digestion and d) vesicles with Bimi® after the complete gastrointestinal digestion, were chemically fixed as described in Rios et al. (2019) for further observation using a JEOL 1011 transmission electron microscope (JEOL USA, Inc., MA, USA) coupled to a GATAN ORIUS SC200 digital camera (GATAN, PA, USA).

2.6. Cells and culture conditions

Human hepatocellular carcinoma cell lines HepG2 (ATCC© HB-8065™, LGC standards S.L.U., Barcelona, Spain) were cultured in EMEM medium supplemented with 2 mmol L^{-1}, 50 IU Ml^{-1} penicillin, 50 mg mL^{-1} streptomycin, 0.1 mM non-essentials amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS), at 37 °C and a humidified atmosphere with 5% CO_2. When the cell growth reached a confluence of 70–80%, they were trypsinized (0.25% trypsin and 0.02% EDTA) and seeded at 1:5 in a 75 cm^2 flask and incubated. All reagents and culture media were provided by Biowest LLC (MO, USA).

2.7. Cell viability assay in cells

HepG2 cell viability to the digested samples was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, MO, USA) colorimetric assay in a 96-well plate, seeding 10,000 cells/well in a final volume of 100 μL. The plates were cultured in a humidified atmosphere with 5% CO_2 and at 37 °C. When cell growth reached 80% confluence, the cells were treated with the following concentrations: 2, 1, 0.5 and 0.25 mg mL^{-1} of digestates from the edible part extracts (free and nanoencapsulated) dissolved in EMEM for 24 h. The same experiment was carried out after incubating the samples for 48 h. For assessing chronic basal inflammation, the cells were treated with the same concentrations of gastrointestinal digestions for 1 h. Then, lipopolysaccharide (LPS) was added to a final concentration of 2 μg mL^{-1} in each well. Since LPS was previously dissolved in dimethylsulfoxide (DMSO), controls with only the solvent were utilized in order to assess its cytotoxicity. Cells were incubated with 200 μL of MTT (1 mg mL^{-1}) in EMEM, for 4 h at 37 °C and 5% CO_2. After that, formazan crystals were solubilized in 100 μL DMSO (Panreac, Barcelona, Spain). Cell viability was calculated setting as 100% of viability the untreated control cells. For the experiments with LPS, stimulated cells with LPS were used as 100% of viability. DMSO was non-toxic to cells at these experimental concentrations. Absorbance at 570 nm was measured with a FLUOStar Omega (BMG Labtech, Ortenberg, Germany). MTT substrate is a yellow tetrazole that, under the action of mitochondrial enzymes produces purple formazan crystals. A higher absorbance at 570 nm is correlated with a major production of these purple-coloured products and, thus, more cellular viability.

2.8. Cell metabolism assays

HepG2 cell metabolism was assessed in 6-well culture cells. Experimental conditions were the same as described for the cytotoxicity assays. When cells reached 80% confluence, a final concentration of 1 mg mL^{-1} of gastrointestinal digestion from each type of sample (free and nanoencapsulated) was incubated for 1 h. Then, a stimuli of 2 μg mL^{-1} of LPS was employed to mimic inflammatory conditions. Samples were incubated for 24 and 48 h. Supernatants were collected and stored at −80 °C. Cells were detached with 500 μL of a solution of 0.25% trypsin and 0.02% EDTA. Then, the samples were centrifuged at 300*g* for 4 min in order to eliminate the trypsin. Resultant pellets were stored at −80 °C. Both, supernatants previously collected and the pellets containing the cells were used for further ITCs quantification in order to analyse the metabolisms of these biomolecules by HepG2 cells.

2.9. Glucosinolates and isothiocyanates quantitative determination

Glucosinolates in raw Bimi® samples were analysed as described in (Garcia-Ibañez et al., 2020) by HPLC-DAD. Data are showed as mean n = 3 ± SD (mg g $D.W.^{-1}$). In order to decipher the presence of isothiocyanates in samples obtained from the reference extracts (free and nanoencapsulated Bimi® extract), their gastrointestinal digestion and from their application in cell metabolism of HepG2 cells (supernatants and pellets), were quantified with a high-throughput UHPLC-QqQ-MS/MS method, employing the protocol described in Baenas et al. (2017). The standards used as a reference for quantification were SFN, I3C and DIM from Santa Cruz Biotech (CA, USA). Data is represented as n = 3 ± SD (μmol mL^{-1}).

2.10. Statistical analysis

One-way ANOVA was used in order to identify differences in ITCs composition in the reference extracts. For the rest of the experiments, a two-way ANOVA was applied. Both were followed by a Tukey's HSD as a *post hoc* test. All the statistical analyses were performed in RStudio (version 3.6.3).

Table 1
Glucosinolates (GSLs) present in raw Bimi® edible part powder (mg g $D.W.^{-1}$) and their bioactive hydrolysis products, isothiocyanates (ITCs), present in the free and nanoencapsulated Bimi® extracts derived from raw material (μmol L^{-1}). For GSLs, data is showed as a mean (n = 3) ± standard deviation. For ITCs, data is shown as a mean (n = 3) ± standard deviation. Different letters show statistically significant differences in the HSD Tukey test ($p < 0.05$) in order to see differences between the extracts.

	Sample type	Biomolecule	Quantity	Total concentration
GSLs (mg g $D.W.^{-1}$)	Bimi® edible part	GRA	2.37 ± 0.06	12.3 ± 0.05
		HGB	1.12 ± 0.06	
		GB	4.62 ± 0.08	
		MGB	1.83 ± 0.01	
		NGB	2.41 ± 0.1	
ITCs (μmol mL^{-1})	Free Bimi® extract	DIM	1.54 ± 0.28a	11.5 ± 0.23a
		I3C	9.95 ± 0.53a	
		SFN	0.005 ± 0.001b	
	Nanoencapsulated Bimi® extract	DIM	0	8.57 ± 0.1b
		I3C	8.24 ± 0.78a	
		SFN	0.33 ± 0.03a	

DIM: 3,3′-diindolylmethane, GSL: glucosinolate; GRA: glucoraphanin; HGB: 4-hydroxy-glucobrassicin; GB: glucobrassicin; I3C: indole-3-carbinol; ITC: isothiocyanate; GB: 4-methoxy-glucobrassicin; NGB: neoglucobrassicin; SFN: sulforaphane.

3. Results

3.1. Characterization of the raw material and the extracts.

Table 1 shows the glucosinolates (GSLs) quantified in the raw Bimi® edible part samples (inflorescences plus edible stems). Data was obtained as mean n = 3 ± SD (mg g D.W.$^{-1}$). The major representative GSL was the indole glucobrassicin (GB, 2.37 ± 0.06mg g D.W.$^{-1}$), and its relatives 4-hydroxy-glucobrassicin (HGB, 1.12 ± 0.06mg g D.W.$^{-1}$), 4-methoxy-glucobrassicin (MGB, 1.83 ± 0.01mg g D.W.$^{-1}$) and 1-methoxy-glucobrassicin (NGB, 2.41 ± 0.1mg g D.W.$^{-1}$). In this way, indole GSLs represent 81% of the total GSL content. On the other hand, the only aliphatic GSL detected in a quantifiable amount was glucoraphanin (GRA, 2.37 ± 0.06mg g D.W.$^{-1}$), accounting for 19% of the total GSLs.

The preparation of GSLs led to the production of GSL-hydrolysis products, and therefore, the reduction of GLSs and the presence of bioactive aliphatic ITCs and indoles (Table 1), including DIM, which was only quantifiable in the free Bimi® extract. The DIM precursor, I3C, was present and quantified in both forms of the extract (free and nanoencapsulated), and in a similar range in concentration (Table 1). For SFN, the GRA derivative ITC, 66% more content was found in the nanoencapsulated extract ($p < 0.05$). Therefore, the total content of ITCs and indoles was significantly higher in the free Bimi® extract ($p < 0.05$), than in the nanoencapsulated form.

3.2. Comparison between the gastric and the intestinal in vitro digestion

The comparison between the gastric and the intestinal digestion on the GSL-hydrolysis products (ITCs) is shown in Fig. 1, since no GSLs were detectable during the gastrointestinal process. DIM concentrations (μM, Fig. 1A) in the free Bimi® extract increased by 2 orders after the intestinal digestion. No statistically significant differences were observed between the gastric and the intestinal digestion for the nanoencapsulated treatment ($p > 0.05$). However, when comparing both types of samples, the DIM concentrations were 4- and 2-fold higher in the nanoencapsulated form, after the gastric and the intestinal digestions, respectively ($p < 0.05$).

According to I3C (Fig. 1B), the free Bimi® extract showed a statistically significant increase in concentration of 1.5 times after the intestinal digestion ($p < 0.05$). However, the nanoencapsulated treatment revealed a statistically significant decrease of 23% when comparing both types of digestions ($p < 0.05$). As for the differences between treatments, concentrations were 3 times higher after the gastric digestion, and 2 times higher after the intestinal digestion, for the nanoencapsulated samples ($p < 0.05$).

Fig. 1C shows the effects of digestion on SFN. The free Bimi® samples showed an increase of almost 10 times when comparing the gastric compartment simulation with the intestinal digestion ($p < 0.05$). Surprisingly, SFN increased 100 times in the nanoencapsulated treatment after the intestinal digestion ($p < 0.05$). When comparing both treatments, no statistically significant differences were observed after gastric digestion ($p > 0.05$). Nevertheless, SFN concentrations were 6 times higher in the nanoencapsulated samples after the lapse of the gastrointestinal digestion ($p < 0.05$).

3.3. Vesicles integrity through in vitro digestion

Fig. 2 shows the evolution of the plasma membrane vesicles integrity through the *in vitro* gastrointestinal digestion with TEM (white bar represents 500 nm). Vesicles with no cargo (Fig. 2A), showed an average diameter of 500 nm and a homogenous size. However, after adding the extracts (Fig. 2B), the vesicles size showed more heterogeneity in their size (red arrows). After gastric *in vitro* digestion (Fig. 2C), the vesicles were smaller (about 250 nm) and heterogeneous (red arrows). Finally, no vesicles were detected after the intestinal digestion (Fig. 2D).

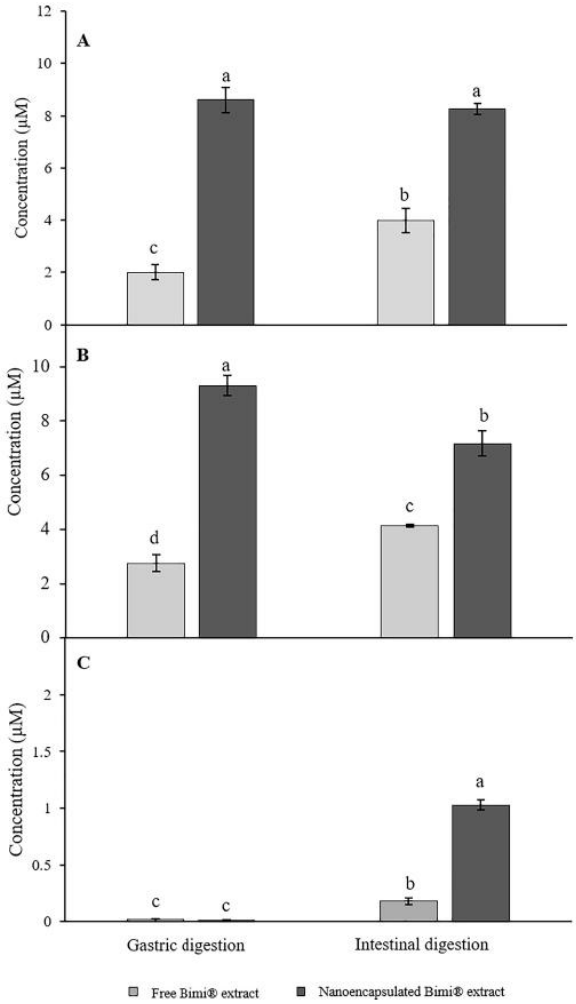

Fig. 1. Comparison between the concentrations of A) DIM, B) I3C and C) SFN found in the gastric and the intestinal digestion, for both free and nanoencapsulated Bimi® extracts. Samples are represented as a mean (n = 3) ± SD. Different letters indicate statistically significant differences in the HSD Tukey Test ($p < 0.05$).

3.4. Cell viability assays

HepG2 cell viability was assessed to determine the toxicity of the digested extracts in a cell culture (Fig. 3). First, Fig. 3A shows the effect of the free and nanoencapsulated Bimi® extract at 24 h. Only the nanoencapsulated treatment at a concentration of 0.5 mg mL^{-1} decreased cell viability at 95% when compared to the control (bar set at 100% corresponds to a b, $p < 0.05$). After 48 h of treatment, none of the treatments showed a statistically significant decrease when compared to the control (bar set at 100% corresponds to a c). However, an increase by approximately 20% of cell viability was observed in the cells treated with 2 mg mL^{-1} of nanoencapsulated Bimi® digested extracts and for both types of digestions at 1 and 0.5 mg mL^{-1}. Furthermore, an increase by 25% was observed when the free Bimi® digested extract was applied at 0.25 mg mL^{-1}.

The cell viability results of samples previously stimulated with LPS (Fig. 3C) revealed that after 24 h of incubation with the treatments, cells with 2 mg mL^{-2} of free Bimi® digestates showed no statistically significant differences with control cells (bar set at 100% corresponds to a

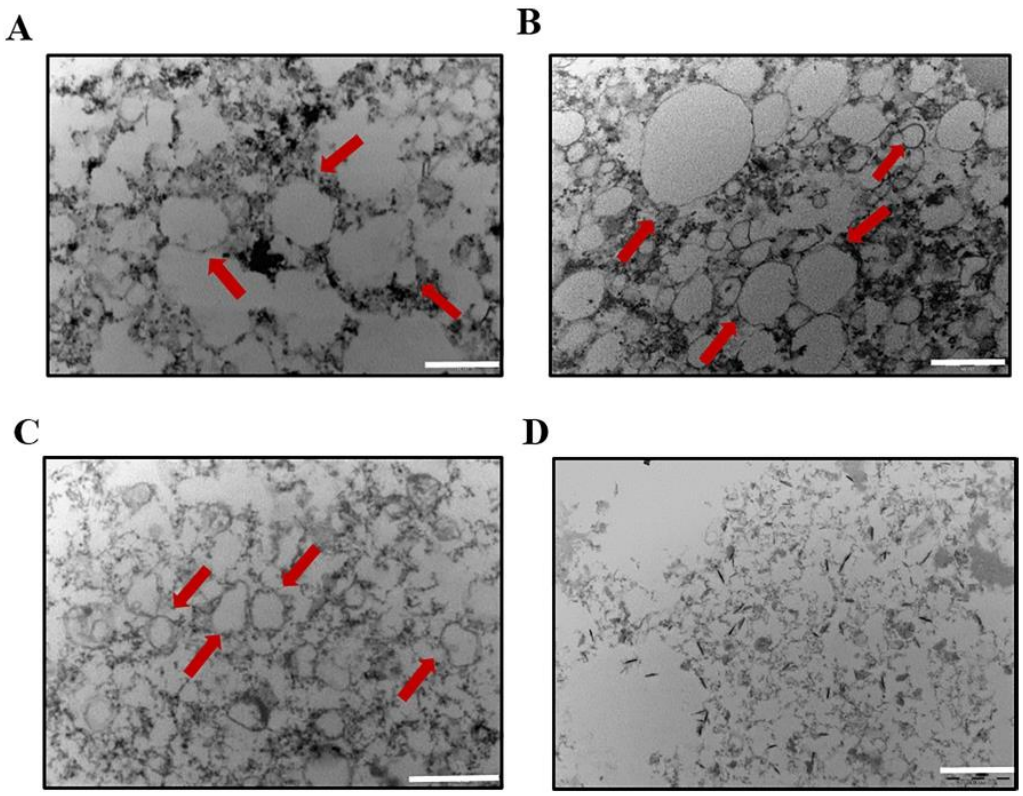

Fig. 2. Transmission Electron Microscopy (TEM) of A) cauliflower-derived plasma membrane vesicles, B) vesicles with Bimi® extract, C) vesicles with Bimi® extract after gastric digestion, and D) vesicles with Bimi® extract after gastrointestinal digestion. Scale bar = 500 nm.

c, $p > 0.05$). However, a 38% increase in cell viability was observed with the nanoencapsulated treatment at the same concentration ($p < 0.05$). For 1 mg mL^{-1}, again no differences were appreciated between the free Bimi® digestates and the control ($p > 0.05$). Surprisingly, cell viability decreased to 75% with the nanoencapsulated treatment ($p < 0.05$). Similar results were observed with a 0.5 mg mL^{-1} free Bimi® digestion ($p > 0.05$), but an increase of 50% was observed with the nanoencapsulated treatment ($p < 0.05$). Regarding the 0.25 mg mL^{-1} treatment, no statistically significant differences were found between treatments ($p > 0.05$). But an approximately 30% increase in cell viability was observed when compared to the control ($p < 0.05$). According to experiments with 48 h of incubation (Fig. 3D), no statistically significant differences were found between the control samples and the treatments (bar at 100% corresponds to $p < 0.05$).

3.5. Cell metabolism assays

Fig. 4 shows the isothiocyanates quantification in cell culture media supernatants after the incubation with free and nanoencapsulated Bimi® digestates. As for DIM, no statistically significant differences were found between treatments at 24 h and 48 h ($p < 0.05$). However, a 1.5 times increase was observed after 48 h of incubation when compared to 24 h ($p > 0.05$). When cells were stimulated with LPS to induce a low grade inflammatory stage, no differences were found between treatments at 24 h ($p > 0.05$), but at 48 h, the nanoencapsulated treatment showed a concentration higher by 1.5 times ($p < 0.05$). According to I3C (Fig. 4B), at 24 h, the nanoencapsulated treatment showed a concentration 3 times higher than the free Bimi® digestates ($p < 0.05$). Nevertheless, no statistically significant differences were found after 48 h for the nanoencapsulated treatment when compared with 24 h ($p > 0.05$). However, I3C levels in the free Bimi® extract were 100% higher than the nanoencapsulated treatment at 48 h ($p < 0.05$). Regarding the cells treated with LPS, no differences were found between treatments at 24 h and between 24 and 48 h for the free Bimi® digestates ($p > 0.05$). At 48 h, the stimulated cells showed a higher concentration of I3C in the nanoencapsulated treatment (2.5 times, $p < 0.05$). SFN quantification (Fig. 4C.), revealed a lower quantity at 24 h and 48 h in the free Bimi® treatment ($p < 0.05$). In addition, no statistically significant differences were found between 24 and 48 h of incubation in both treatments ($p < 0.05$). As for the stimulated cells, no differences were found between treatments at 24 and 48 h ($p > 0.05$). Similarly, no differences were found for the free Bimi® digestates ($p > 0.05$), but a 30% increase was found between 24 and 48 h of incubation for the nanoencapsulated ones ($p < 0.05$). No sulforaphane-glutathione (SFN-GSH) complexes were detected in the cells supernatants. However, sulforaphane coupled to cysteine (SFN-CYS) was analysed (Fig. 4D). At 24 h, no statistically significant differences were found between both treatments ($p > 0.05$), but a decrease of 38% and 62% (for free and nanoencapsulated digestates, respectively) was detected at 48 h ($p < 0.05$). In stimulated cells, at 24 h no differences were found between treatments ($p > 0.05$). Nevertheless, SFN-CYS concentrations in cells treated with the free Bimi® digestates at 48 h, showed a 1.4 times increase when compared to 24 h ($p < 0.05$). Surprisingly, for the nanoencapsulated treatments, a decrease by 57% was found after 48 h of treatment when compared with the 24 h quantification ($p < 0.05$). Finally, sulforaphane-*N*-acetylcysteine complexes (SFN-NAC) were quantified (Fig. 5E), revealing no differences at 24 h when comparing both treatments ($p < 0.05$). At 48 h, a 20% decrease was found for the nanoencapsulated Bimi® digestates when comparing between treatments ($p < 0.05$). According to the stimulated cells, concentrations at 24 h and 48 h were 3 times higher in

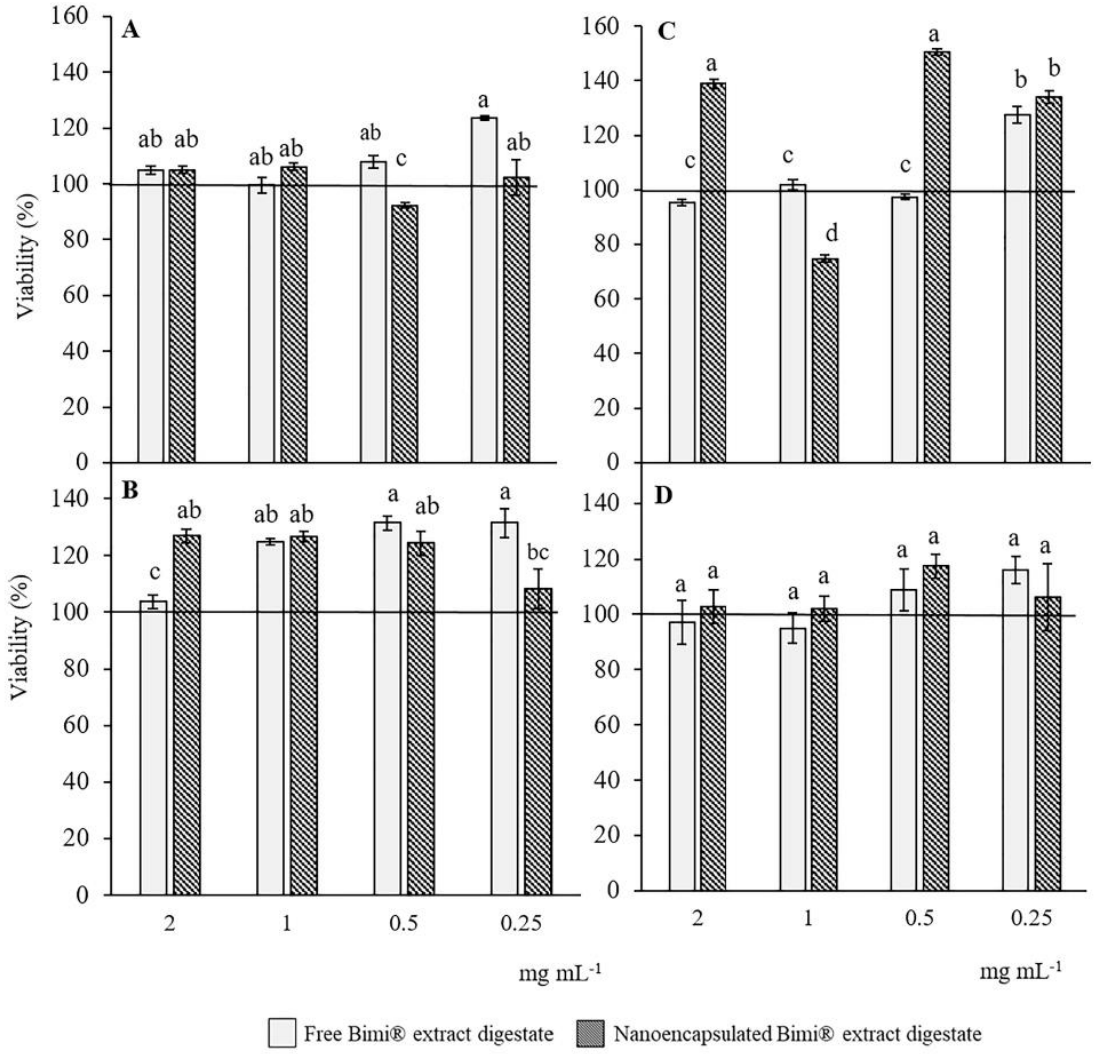

Fig. 3. Cytotoxicity assays in HepG2 of digestates from free and nanoencapsulated Bimi® digestates, at A) 24 h, B) 48 h, and with LPS stimulated cells at C) 24 h and D) 48 h. Samples are represented as a mean (n = 3) ± SD. Different letters indicate statistically significant differences in the HSD Tukey test ($p < 0.05$).

the nanoencapsulated treatment ($p < 0.05$). Furthermore, no significant differences were found between incubation times (24 h vs 48 h) for both treatments ($p > 0.05$).

HepG2 cells (Fig. 5) were also analysed. DIM (Fig. 5A) concentrations were 1.6 times higher in the cells treated with nanoencapsulated Bimi® digestates ($p < 0.05$). However, no differences were found for this treatment after 48 h ($p > 0.05$). After LPS treatment, a similar pattern was found, but intracellular concentrations were lower (about 35% and 300%) when compared to 24 h and 48 h in non-stimulated cells ($p < 0.05$). No I3C was detected in a quantifiable amount in the cells. With regard to SFN concentrations, (Fig. 5B), no statistically significant differences were found between treatments at 24 and 48 h ($p > 0.05$). However, a decrease by 50% (approx.) in both treatments was observed after 48 h of incubation ($p < 0.05$). LPS-stimulated cells at 24 h showed higher concentrations when treated with the nanoencapsulated digestates ($p < 0.05$). But no differences in SFN concentrations were observed at 48 h between treatments ($p > 0.05$). After analysing SFN-GSH concentrations in the cells (Fig. 5C), a higher amount (1.4 times higher) was found when treated with the nanoencapsulated Bimi® extract digestions ($p > 0.05$) at 24 h. Surprisingly, no SFN-GSH was detected after 48 h of incubation with the treatments. When cells were stimulated with LPS, mimicking low grade inflammation, a 15% higher concentration was found in the nanoencapsulated treatment ($p < 0.05$) at 24 h. At 48 h, no statistically significant differences were found between treated cells ($p > 0.05$). When comparing 24 h and 48 h stimulated cells, the concentrations of the free and nanoencapsulated Bimi® digestates were 1.7 and 2.6 higher, respectively, at 24 h ($p < 0.05$). For SFN-CYS (Fig. 5D), a 3 times higher concentration was observed in the free Bimi® digestates when compared to the nanoencapsulated treated cells ($p < 0.05$). Nonetheless, no SFN-CYS concentrations were detected at 48 h in either of the treated cells. Regarding the LPS-stimulated cells, a 20% lower concentration of SFN-CYS was found in the nanoencapsulated Bimi® digestates treated cells ($p < 0.05$) at 24 h. Although a reduction in SFN-CYS concentration was observed in both treatments at 48 h in stimulated cells ($p < 0.05$), no significant differences were found between treatments ($p > 0.05$). Lastly, SFN-NAC was quantified (Fig. 5E), with no statistically significant differences found between treatments in unstimulated cells ($p > 0.05$). However, in LPS-stimulated cells, SFN-NAC quantification was 11 times higher in cells treated with nanoencapsulated Bimi® digestates at 24 h ($p < 0.05$). After 48 h, no

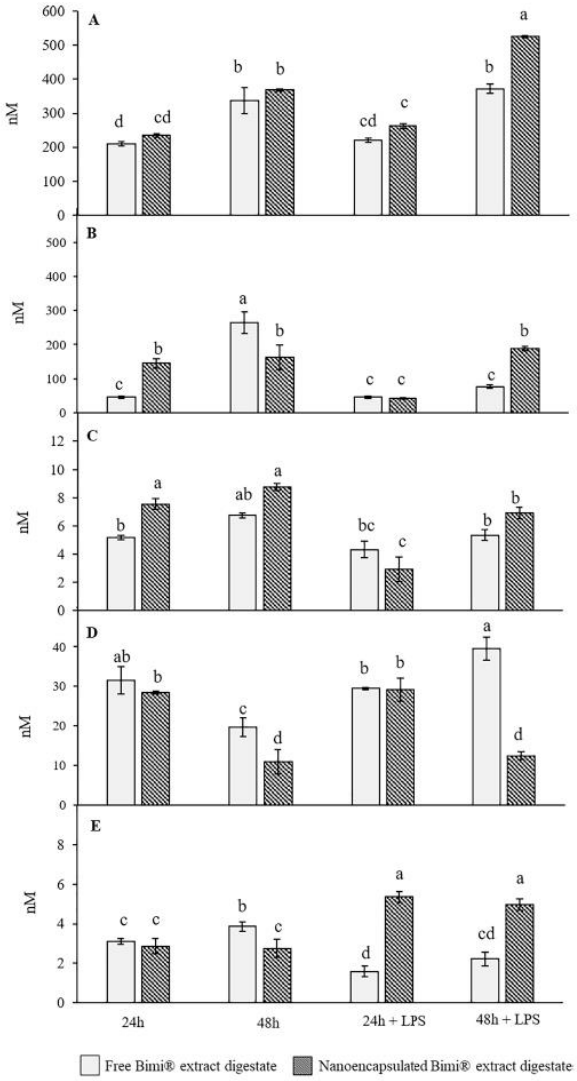

Fig. 4. Supernatant analysis of A) DIM, B) I3C, C) SFN, D) SFN-CYS and E) SFN-NAC by ESI-UHPLC-QqQ, after 24 and 48 h of incubation with free and nanoencapsulated Bimi® digestates in LPS-stimulated and unstimulated HepG2 cells. Samples are represented as a mean (n = 3) ± SD. Different letters indicate statistically significant differences in the HSD Tukey test ($p < 0.05$).

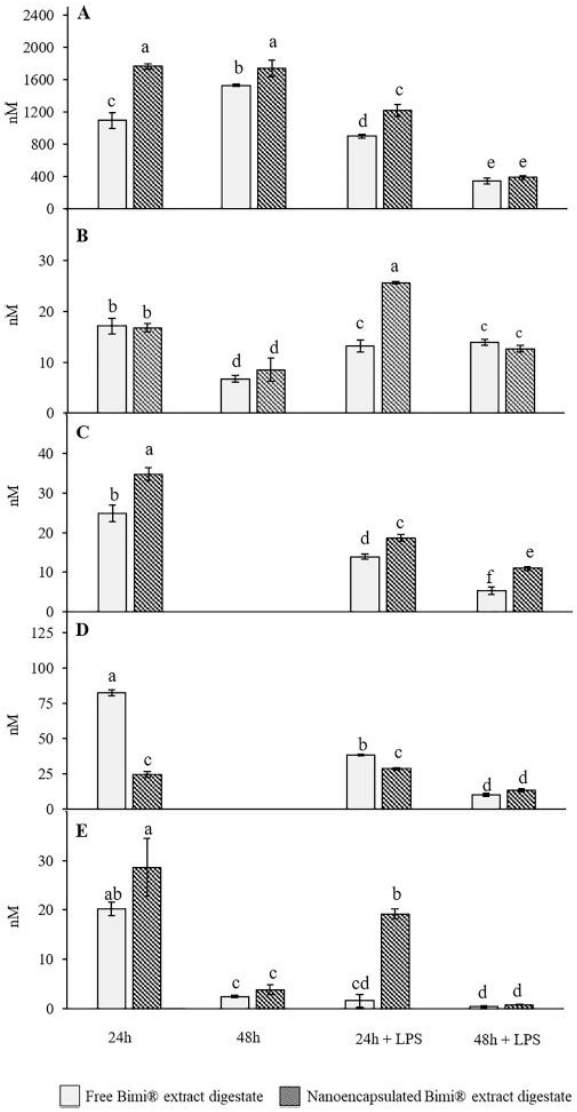

Fig. 5. LPS- stimulated and unstimulated HepG2 cells analysis of A) DIM, B) I3C, C) SFN, D) SFN-CYS and E) SFN-NAC by ESI-UHPLC-QqQ, after 24 and 48 h of incubation with free and nanoencapsulated Bimi® digestates. Samples are represented as a mean (n = 3) ± SD. Different letters indicate statistically significant differences in the HSD Tukey test ($p < 0.05$).

differences were observed between treatments ($p < 0.05$), although a decrease by 95.5% was observed in the nanoencapsulated treated cells after 48 h when compared with 24 h of incubation ($p < 0.05$).

4. Discussion

Recently, Bimi® has emerged as a mild-flavoured option when compared to the habitual pungency of broccoli. As it is often used as a raw gourmet material, its production is highly and carefully selected. This results in that part of the Bimi® edible part production is discarded. In our work, we propose the use of this source material, as it has a high concentration of GSLs, even similar to broccoli leaves (Hassini et al., 2019). However, although we were able to produce an ITC-enriched extract from this material, these biomolecules are often unstable in aqueous medium (Cirilli et al., 2020). In an effort to increase their stability, bioavailability and bioactivity, our group has developed the use of plant-derived plasma membrane vesicles (Garcia-Ibañez, Roses, et al., 2021; Yepes-molina et al., 2021). For example, encapsulated SFN with plasma membrane derived from broccoli, revealed a higher absorption by melanoma cells (Yepes-Molina & Carvajal, 2021). Specifically, cauliflower-derived plasma membrane vesicles have demonstrated to have a high osmotic permeability, thus providing them with a high plasticity for water passage (Garcia-Ibañez, Nicolas-Espinosa, et al., 2021). This information led us to select this type of nanocarrier for encapsulating the proposed aqueous Bimi® extract enriched in ITCs. Also, we decided to focus on aqueous extracts, as they are more compatible with food-grade products, as no organic solvents are needed. The resulting extract provided a higher amount of ITCs derived from indole GSLs, which correlates with their presence in the raw material, composing 81% from the total. Nonetheless, when the nanoencapsulated extracts were analysed and compared with free Bimi®

extracts (Table 1), a lower concentration was found. A possible explanation is that small molecules with higher hydrophobicity, such as DIM, often interact with the plasma membrane, being unable to be detected unless the nanocarrier is degraded (Lee, 2020).

As for the gastrointestinal digestion process, little information is available on the stability of ITCs in their passage through the stomach and intestinal digestion (Oliviero, Verkerk, & Dekker, 2018). In this work, we decided to use an *in vitro* digestion model since it allows to control the experimental conditions ensuring a great first approach for further *in vivo* experiments (Moreda-Piñeiro et al., 2011). Since intact vesicles will never reach liver cells, we previously performed the *in vitro* gastrointestinal digestion. In this way, our main target was to asses if the nanoencapsulation is able to increase the total concentration of ITCs that could arrive to the intestinal lumen, where they will be absorbed. In addition, we decided not to include a phase with saliva, since the α-amylase mainly targets complex sugars, which are not present in our extracts. On the one hand, nanoencapsulated Bimi® extracts reported a higher concentration for DIM and I3C (Fig. 1). Furthermore, an increased amount of both ITCs was found at the endpoint when compared to the initial extracts. This phenomenon correlates with the TEM microscopy observations of a reduction in vesicle size after the gastric digestion (from 500 nm to 250 nm approx.) (Fig. 2C). Moreover, I3C biomolecules are well known for their low stability, as they form oligomers, forming a mixture of diverse condensation products at low pH (Shertzer & Senft, 2000). Regarding SFN (Fig. 1C), only small amount was quantifiable by ESI-UHPLC-QqQ in the gastric digestion. Because the amount of GRA (2.37 ± 0.06 mg g D.W.$^{-1}$) present in Bimi® plants is lower than GB and its derivatives, the final concentration of SFN in the extracts was smaller.

On the other hand, after gastrointestinal digestion (Fig. 1), the nanoencapsulated Bimi® extract showed higher concentrations of DIM, I3C and SFN. Similar results were obtained when cauliflower-derived plasma membrane vesicles were used for red cabbage extracts in an *in vitro* dynamic gastrointestinal digestor (Garcia-Ibañez, Roses, et al., 2021). As reported by Abellán et al. (2021), indole GSLs-derived ITCs from *brassica* sprouts provided a low bioaccessibility after gastrointestinal digestion. However, the cauliflower-derived plasma membrane vesicles were able to increase the stability of DIM and I3C, providing a higher supply of these biomolecules at the end of the entire process, where intestinal absorption takes part. Furthermore, higher amounts were observed for both treatments when the complete gastrointestinal digestion was performed (Fig. 2). Changes in pH from the gastric to the intestinal digestion (from 2 to 8), may have an influence on both the remaining myrosinase and the chemical degradation dynamics (Bones & Rossiter, 2006). Therefore, the presence of cauliflower-derived plasma membrane vesicles ensured a higher fraction of the three ITCs present in the extract after the gastrointestinal digestion.

As for the performance of the cauliflower-derived plasma membrane vesicles, the proteoliposomes changed in size when they were encapsulating the extract (Fig. 3B) and after the stomach digestion (Fig. 2C). Surprisingly, some remaining membrane vesicles were found after the gastrointestinal digestion (Fig. 2D). Our previous work on the characterization of these plasma membrane vesicles revealed a high ability for water passage, what could explain their stability under low pH (Garcia-Ibañez, Nicolas-Espinosa, et al., 2021). In addition, these results suggest that our nanocarrier would increase the bioaccessibility of the cargo not only by avoiding degradation in the stomach, but also releasing the compounds in the intestine, where they are absorbed. Even though changes in pH affect the vesicular integrity, considering that ITCs are small molecules with some hydrophobicity, they could still interact with the lipid bilayer, acting as a reservoir (Rostamabadi, Falsafi, & Jafari, 2019). Furthermore, the reduction observed in vesicle size might be due to the coupling of denser packed domains, such as lipid rafts, being more stable under acidic pH conditions (Yepes-Molina, Carvajal, & Martínez-Ballesta, 2020).

As mentioned before, obesity is a disease that is highly linked to low grade systemic inflammation, affecting the correct development of key organs, such as the liver (Zhou, Urso, & Jadeja, 2020). It has been reported that a dysregulation of the spleen-liver axis can led to alterations in the inflammatory response, ultimately causing a non-alcoholic fatty liver disease pathology (Barrea et al., 2018). Therefore, our interest was to analyse the effect of the digestates obtained from both types of extracts under a liver model cell culture (HepG2). In addition, treatments were assessed during 24 and 48 h in order to know how cell metabolism adapts to a longer exposure to our digestates. When trying to mimic a low-grade inflammatory condition, we used a low concentration of LPS for stimulating this cell line. Firstly, cell cytotoxicity assays were performed in order to determine if the treatments negatively affected cell viability (Fig. 4). Both treatments showed no detrimental effects on cell viability in unstimulated cells after 24 and 48 h of incubation (Fig. 4A and B). In LPS-stimulated cells, an increase in cell viability was observed in all treatments except for 1 mg mL^{-1} concentrations, in which even a reduction of up to 70% was analysed (Fig. 4C). In this way, we decided to assess the digestates at 1 mg mL^{-1} (same concentration as obtained at the endpoint of the gastrointestinal digestion) in HepG2 cells, as the increases in viability may be due to an increased activity in the mitochondrial electron chain after the incubation with the treatments (Rai et al., 2018).

Even though studies on the anti-carcinogenic and cardioprotective effects of DIM and I3C have been performed in HepG2 model cells, little to no information about its metabolism is available (Jiang et al., 2019; Maiyoh, Kuh, Casaschi, & Theriault, 2007). Surprisingly, in our experiments, I3C was detected in quantifiable amounts in cell supernatants (Fig. 4B), but not in the cellular lysates, within which only DIM was detected (Fig. 5A). In addition, high concentrations were found in the cellular lysates (up to 1776 ± 31.2 μM, Fig. 5A). Since it has been reported that I3C spontaneously forms DIM under cell culture conditions, and as this molecule has been characterized as being more bioactive, a feasible explanation could be that HepG2 cells incorporate and accumulate DIM for further metabolisation (Bradlow & Zeligs, 2010). Furthermore, in the work by Staub et al. (2002) an accumulation of DIM in the nucleus of breast tumour cells after I3C treatment was observed. In addition, when comparing stimulated and LPS-stimulated cells, lower concentrations were found in the stimulated cells (Fig. 5A, $p < 0.05$), what could mean that HepG2 are metabolizing DIM faster when they are under inflammatory conditions. On the other hand, unstimulated cells showed almost no statistically significant differences between treatments at 24 h and 48 h for DIM concentrations (Fig. 4A). As higher concentrations of DIM were in the nanoencapsulated digestates, and intracellular concentrations were higher or equal (Fig. 5A), suggests a higher incorporation of this compound due to an increased bioavailability.

SFN is well known to be metabolized in cells via the mercapturic acid pathway. After cell incorporation, SFN reacts with glutathione thanks to glutathione S-transferase, resulting in SFN-GSH. This first reaction may explain why SFN-GSH was only detected in the cellular lysates (Fig. 5C). In addition, SFN-GSH could be released by cells in order to reach systemic circulation, but a high dissociation percentage has been found (Traka & Mithen, 2009). In Baenas et al. (2015), SFN-GSH concentrations were undetectable at 24 h in HepG2, which suggests the higher dissociation of these molecules in culture conditions. This also helps us to understand why the concentration of SFN did not show differences in cellular supernatants between incubation times (Fig. 4C). Perhaps a fraction of this SFN comes from the dissociation of the possible SFN-GSH molecules secreted from the cells. The next key step downstream the mercapturic acid pathway is the formation of SFN-CYS after diverse catalysis reactions. This conjugate comes into systemic circulation, reaching other organs. It has been reported that SFN-CYS is bioactive, inducing apoptosis in tumorigenic cells (Lin et al., 2017). When analysing our results, the fact that no concentrations of SFN-GSH and SFN-CYS were detected at 48 h in unstimulated cell lysates (Fig. 5C and D), suggests that no further accumulation of this metabolite was needed,

secreting SFN-CYS and SFN-NAC, that is, the final product excreted in the urine (Gu et al., 2021). In addition, a 95% increase in concentrations of SFN-NAC at 24 h was observed in LPS-stimulated cells treated with the nanoencapsulated Bimi® digestates (Fig. 5E), which suggests that this treatment is able to provide higher concentrations of metabolites.

5. Conclusions

From the results of this work, we can conclude that cauliflower-derived plasma membrane vesicles provided a higher preservation of ITCs during the gastrointestinal process. Thus, a higher supply of these *Brassica*-derived biomolecules improved the bioaccessibility and bioavailable fraction of bioactive ITCs and indoles under cell culture conditions. Furthermore, different metabolisation patterns were observed according to the studied bioactive molecules and the treatments under LPS stimulation conditions, suggesting that under low-grade and chronic inflammatory status (prevalent under obesity conditions) a higher incorporation is possible using nanoencapsulated ingredients. In this way, the cauliflower-derived plasma membrane vesicles are a biocompatible option in the development of food-grade formulas for increasing the stability and bioaccessibility of ITCs and indoles (GSL-hydrolysis bioactive products) by using agronomical by-products or adding value to side streams.

Authors' contributions

P.G.I., D.A.M. and M.C.A. conceived and designed the experimental plan. P.G.I. and D.A.M., performed experiments, analyzed the data, and wrote the first draft of manuscript. P.G.I., D.A.M., and M.C.A., revised the manuscripts and corrections. M.C.A. managed the funding of project by National Programme. D.A. M. managed funding from Regional Programme. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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· Capítulo IV

Capítulo IV

The influence of red cabbage extract nanoencapsulated with brassica plasma membrane vesicles on the gut microbiome of obese volunteers.

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The aim of the study was to evaluate the influence of the red cabbage extracts on the bioaccessibility of their isothiocyanates, and their effect on the intestinal microbiota using a dynamic model of human digestion treated with the gut microbiome of obese adults. The elicitation of red cabbage plants with methyl jasmonate (MeJA) duplicated the content of glucosinolates (GSLs) in the plant organs used for elaborating the encapsulated formula. The use of plasma membrane vesicles, according to a proper methodology and technology, showed a high retention of sulforaphane (SFN) and indol-3-carbinol (I3C) over the course of the 14-day digestion study. The microbiome was scarcely affected by the treatments in terms of microbiota composition or the *Bacteroidetes/Firmicutes* ratio, but a 3 to 4-fold increase was observed in the production of butyric acid with the encapsulated extract treatment. Based on our pilot red cabbage extract study, the consumption of this extract, mainly encapsulated, may play a potential role in the management of obesity in adults.

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The Influence of Red Cabbage Extract Nanoencapsulated with Brassica Plasma Membrane Vesicles on the Gut Microbiome of Obese Volunteers

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Abstract: The aim of the study was to evaluate the influence of the red cabbage extracts on the bioaccessibility of their isothiocyanates, and their effect on the intestinal microbiota using a dynamic model of human digestion treated with the gut microbiome of obese adults. The elicitation of red cabbage plants with methyl jasmonate (MeJA) duplicated the content of glucosinolates (GSLs) in the plant organs used for elaborating the encapsulated formula. The use of plasma membrane vesicles, according to a proper methodology and technology, showed a high retention of sulforaphane (SFN) and indol-3-carbinol (I3C) over the course of the 14-day digestion study. The microbiome was scarcely affected by the treatments in terms of microbiota composition or the *Bacteroidetes/Firmicutes* ratio, but a 3 to 4-fold increase was observed in the production of butyric acid with the encapsulated extract treatment. Based on our pilot red cabbage extract study, the consumption of this extract, mainly encapsulated, may play a potential role in the management of obesity in adults.

Keywords: red cabbage; isothiocyanates; stability; encapsulation; gut microbiome; obesity

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1. Introduction

Brassica vegetables stand out as dietary coadjutants, functional ingredient sources, or natural bioactive-rich foods, and their consumption may result in a healthier life or prevent some illnesses [1,2], including the modulation of inflammation in overweight and obese adults (a BMI $\geq$ 25 for overweight subjects, and a BMI of 29.9–34.9 for obese subjects) [3]. Particularly, red cabbage (*Brassica oleracea* L. var. *capitata* f. *rubra*) has been studied as a source of health-promoting phytochemicals, including glucosinolates (GSLs) and different classes of phenolic compounds, such as acylated anthocyanins, flavonol glycosides, and hydroxycinnamic acid derivatives [4,5]. GSLs are stable in their natural matrix, but after tissue disruption, they are hydrolyzed by myrosinase (EC 3.2.1.147), resulting in the

production of bioavailable and bioactive compounds, namely, isothiocyanates (ITCs) and indoles [6]. These have been classically studied as the compounds responsible for the effects on Phase II detoxification enzymes, which have anti-tumorigenic effects [7]. More recently, the bioactive compounds of the *Brassica* species have been associated with the modulation of the gastrointestinal microbiome [8,9].

In order to increase the phytochemical content in plant-derived foods and products, the use of elicitation has emerged as a useful tool [10]. The content of glucosinolates in cruciferous plants and sprouts can be manipulated through treatments with elicitors, such as plant hormones (methyl jasmonate (MeJA), jasmonic acid (JA), salicylic acid (SA), ethylene (ET), or abscisic acid (ABA), among others), or mineral or amino acid solutions [11]. These various compounds act as stressors in the plants, and activate an array of mechanisms similar to the defense responses to pathogen infections or environmental stimuli, ultimately affecting the plant's metabolic pathways by enhancing the synthesis of phytochemicals [12]. Jasmonic acid and its methyl ester (MeJA), and methionine, were applied to broccoli and radish sprouts [13] to increase the individual and total contents of GSLs. More recently for Bimi® (a hybrid between broccoli and green Chinese kale) plants in field experiments, the content of GSLs was also increased by the application of 100 μmol L^{-1} of MeJA [14]. SA is a widely used elicitor [15], and it has been reported that it increases the contents of GSLs in broccoli, China rose radish, and red radish sprouts [15,16]. The evidence on the use of elicitors for the enrichment of plant-derived products are widely available in literature [13,14,17].

Presently, a global trend has been observed on the demand for highly nutritious and easy-to-consume-foods, including functional foods or beverages [18]. Different sources of ingredients from *Brassica* species have been used to date [14,19], which are usually prepared as extracts rich in ITCs [20,21]. The problems with using ITCs as ingredients include their low water solubility and very limited stability [22,23], when used in nutritional interventions and compared to the intake of GSLs from a natural matrix (e.g., broccoli sprouts) [24]. With the aim of increasing the stability and bioavailability of ITCs, the use of encapsulation could provide a solution [25]. Recently, the use of plant plasma membrane vesicles as delivery systems for bioactive compounds has been studied [26,27]. Diverse types of plant membrane vesicles could be good candidates for this purpose, such as extracellular vesicles, which are spheroids of cytosolic material surrounded by a lipid bilayer, or extracted plasma membrane from fresh plant tissue [28]. As an example of the latter, we used cauliflower plasma membrane vesicles, which are proteoliposomes with a high proportion of unsaturated fatty acids. Furthermore, the proteomic analysis performed demonstrated the presence of aquaporins, such as PIP1 or PIP2, which grant a high osmotic permeability to this vesicles [29]. However, the information of the performance of these encapsulated formulas in the GI tract is rather limited [30], and one of the objectives of this work was to evaluate this possibility.

The current trends and demands for a healthier diet include consuming more vegetables and fruits on a daily basis [31]. Worldwide national health systems recommend such improvements in diets in order to help counteract the high prevalence of metabolic-related diseases, such as obesity, diabetes, or hyperglycemia [32–34], along with economic and social considerations [35,36]. Nevertheless, these recommendations have not achieved the expected impact, and this situation has led to one of the major causes of death globally [37]. Only recently, the role of the gut microbiome in this context has taken the main stage, and reports on the association between alterations in the gut microbiome (dysbiosis), and modifications in the gut barrier permeability [38], have been shown to be present in obese subjects with a dysbiotic gut microbiome [39]. Furthermore, associations between the gut microbiome and energy imbalances have also been found in obesity [40].

Therefore, the aims of this work were to determine the influence of the elicitation method (MeJA, SA, or its combination) on the content of glucosinolates in red cabbage, in order to use the plant material as a source of bioactive ITCs; to improve the stability of these ITCs by means of nanoencapsulation of red cabbage-derived aqueous extracts;

and, to evaluate their bioaccessibility and their effect on the intestinal microbiota, by using an in vitro dynamic gastrointestinal system, conditioned with gut microbiome from obese adults.

The characterisation of the composition of the red cabbage, the derived extracts, and the samples obtained from the digestion system were carried out to establish the basis for future food product developments for the management of obesity.

2. Materials and Methods

2.1. Plant Material and Treatments

One hundred ninety-two red cabbage (*Brassica oleracea* L. var. capitata f. rubra) seeds from Sakata Seed Iberica (Valencia, Spain) were pre-treated with deionized water and continuous aeration for 24 h. Then, seeds were planted in vermiculite for 2 days in darkness and at 28 °C and 60% relative humidity. Seedlings were transplanted to experimental soil in a farm (37°47′52.7″ N, 0°52′00.7″ W, 15 m asl, Murcia, Spain). The plants were grown from September 2018 to February 2019 under a semiarid Mediterranean climate. Plants were drip irrigated with $\frac{1}{4}$ Hoagland solution. Twenty-four plants were assigned to each treatment, with two replicates. Elicitation treatments (150 mL of solution sprayed per plant, using an elicitation backpack) were as follows: (i) control, 0.2% ethanol; (ii) 100 µM MeJA in 0.2% ethanol; (iii) 200 µM SA in 0.2% ethanol; and iv) combined administration of SA + MeJA. The selection of dosages was based on previous experiments [14,16]. Treatments were also supplemented with a patented concentration of surfactant (Patent # PCT/ES2019/070457). The treatments were applied with the appearance of the flower bud and for 5 days the plants were kept growing, and 5 days after, a second round of 5 additional days of elicitors was applied. The plants were then allowed to grow for another 4 days, and then harvested. For the analyses, 10 plants per treatment were randomly chosen, thoroughly mixed, and distributed into four technical replicates. The samples were quickly transported to the laboratory and kept at −80 °C.

2.2. Extraction of Intact GSLs

One hundred milligrams of freeze dried, grounded material were extracted with 1 mL of 70% methanol using a water bath at 70 °C for 30 min, with vortex agitation every 5 min. The samples were cooled in an ice bath and centrifuged at 10,000× *g* during 15 min, at room temperature. Supernatants were collected and transferred to a rotary evaporator until the complete removal of methanol. After that, 300 µL of MilliQ water were added. After homogenization, samples were filtered through a 0.22-µm-Ø Millipore filter (Billerica, MA, USA) into vials for HPLC-DAD analysis.

2.3. Elaboration of the ITC-Rich Ingredient Prototype

Free red cabbage aqueous extracts were prepared using freeze dried and ground powder extracted by maceration and agitation using MilliQ water (1:20 w:v), at room temperature and in the dark. Then, the samples were centrifuged at 12,000× *g* for 15 min at room temperature. The supernatants were collected and filtered with an Albet filter. For further storage, aqueous extracts were lyophilized to obtain 50 g batches of freeze dried extracts. Resultant batches were stored at −80 °C.

2.4. Microsomal Fraction Extraction

One hundred milligrams of cauliflower inflorescences (*Brassica oleracea* L. var. botrytis), kindly provided by Sakata Seed Ibérica (S.L.U., Valencia, Spain), were cut into small pieces and vacuum-infiltrated with 0.5 g of PVP and 160 mL of extraction buffer (0.5 M sucrose, 1 mM dithiotreitol (DTT), 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1.30 mM ascorbic acid at pH 7.5. After that, the samples were blended and filtered through a nylon mesh (with 100 µm of pore diameter). The collected filtrate was centrifuged at 10,000× *g* for 30 min, at 4 °C. The supernatants were again centrifuged

at 50,000× *g* for 35 min, at 4 °C. The pellet was then resuspended in 500 µL of phosphate buffer (5 mM) with 0.5 M sucrose (pH 6.5).

2.5. Plasma Membrane Isolation

Two milliliters of the cauliflower-derived (previously obtained) microsomal fraction were introduced into a two-phase system mixture, with a final composition of PEG-3350/Dextran-T500-6.3% (*w*/*w*), 5 mM KCl, 330 mM sucrose, 2.5 mM NaF, and 5 mM K_3PO_4 (pH 7.8). After being centrifuged for 5 min at 4000× *g*, the upper phase was collected and washed with a solution containing 9 mM KCl, 0.2 M EGTA, 0.5 mM NaF, and 10 mM Tris-borate (pH 8.3). Next, the samples were centrifuged at 55,000× *g* for 35 min at 4 °C. Then, 1 g of pellet was resuspended in 1 mL of red cabbage aqueous extract for further sample preparation.

2.6. Dynamic Gastrointestinal and Colonic Fermentation Model

Before the colonic fermentation assays, different treatments were prepared: for the non-encapsulated treatment, 1g of red cabbage aqueous lyophilized extract was resuspended and homogenized in 1 mL MilliQ water. For the nanoencapsulated treatment, 1 mg mL^{-1} of red cabbage aqueous extract was nanoencapsulated in 1 mL of cauliflower plasma membrane vesicles. Glycerol was added until obtaining 20 g of total weight, in order to increase the stability of the vesicles during their transport. These samples (1 mL of free red cabbage aqueous lyophilized extract, and 20 mL of nanoencapsulated red cabbage mixture with glycerol) were introduced into the in vitro dynamic gastrointestinal model.

The Dynamic-Colonic Gastrointestinal Digester (D-CGD) was developed by AINIA Technology Center (Valencia, Spain) [41–43], which consists of five interconnected double jacket vessels that simulate the physiological conditions of the stomach, small intestine, and the three colonic sections; that is, the ascending colon (AC), transverse colon (TC), and descending colon (DC). The selection of residence times, pH values, temperature (37 °C), and the volume of each reactor were computer-assisted. All the compartments were communicated by peristaltic pumps, working semi-continuously in the stomach and small intestine and continuously for the colonic vessels. Gastric digestion was simulated by continuously adding a 0.03% (*w*/*v*) pepsin solution (2100 units/mg) for 2 h (for a total volume of 60 mL). A typical gastric digestion pH curve (based on in vivo data) was simulated by adding an HCl (1 M) solution. Digestion in the small intestine was mimicked by the continuous addition of a solution containing pancreatin (0.9 g/L), NaHCO3 (12 g/L), and Oxgall dehydrated fresh bile (6 g/L) in distilled water (total volume of 440 mL), maintaining the intestinal content at pH 6.5. In order to maintain anaerobiosis, gaseous N_2 was flushed for 15 min twice a day. A pool with the feces from four adult volunteers was utilized. The volunteers had dysfunctions or pathologies associated with obesity and/or metabolic syndrome, but had not received any antibiotic treatment during the previous three months, had not consumed enriched foods or supplements with vitamins, probiotics/prebiotics, herbal products, and did not follow weight loss diets or did not have any bowel disease. The inclusion criterion was obesity ($34.9 \geq$ BMI ≥ 30 kg/m^2). The exclusion criteria that were considered were: following a weight loss diet, suffering from gastrointestinal diseases (Crohn's, colitis, IBD, ...), clinical diagnosis of cardiovascular diseases, diabetes or cancer, pregnant or lactating women, and current use of drugs (lipid-lowering, antihypertensive, proton-pump inhibitors, ...). The diet of the fecal donors was not controlled. The only requisite was that they did not use either antibiotics (in the previous three months) or supplements (probiotics, vitamins, herbs). The objective was to include different phenotypes of obesity-related microbiota patterns in the microbiota sample, a wide age range (22–65 years old), sex (two males/two females), BMI (from 26 to 39), and dietary patterns (from vegetarianism to Western diets, including Mediterranean). With this premise, we intended to obtain a high diversity of microorganisms. The volunteers (of Caucasian ethnicity) were recruited from a population that participated in a previous study from the University of Navarra (Cuevas-Sierra et al., 2020) [44].

A 20% (*w*/*v*) fecal solution was prepared with regenerated thioglycolate, inoculated in the colon compartments (50, 80, and 60 mL for AC, TC, and DC, respectively), and completed with a culture medium up to 1000, 1600, and 1200 mL, respectively. The culture medium was elaborated according to Molly et al. [42,43]. The culture medium provided all the necessary nutritional components to simulate the conditions of the human colon and allow the growth of the intestinal microbiota.

A stabilization period of 11 days was required to allow for the growth of the human fecal microbiota in the colon compartments. This time interval corresponded to the stabilization time required (from 10 to 20 days) to overcome the stabilization of the microbiota (latency period), and to reach a bacterial density that was similar to the colonic environment [45,46]. During this period, 200 mL of culture medium were added to the stomach compartment 3 times a day. Samples from the fermentation liquids (FL) from the AC, TC, and DC compartments, corresponding to time 0, were taken at the end of the microbiota stabilization period. After that, a treatment period of 14 days was started by adding the sample to the in vitro digestion system once per day, and the culture medium twice per day. At the end of the treatment period, the samples were taken from the fermentation liquids from the three compartments. These samples were centrifuged (15,000× *g*, 15 min) and filtered through a 0.22-µm-Ø Millipore filter (Billerica, MA, USA) into vials for UHPLC-ESI-QqQ-MS/MS analysis. The maintenance of the microbial population after the stabilization (time 0) and during the treatment period (time 14) was checked by plate counts of total anaerobic bacteria (on Schaedler agar under anaerobic incubation, 37 °C/48 h) and the other bacterial groups. No relevant differences were observed between both periods (>7 log CFU for the AC, TC, and DC compartments).

In addition, the short and medium fatty acids composition of the fermentation medium was determined according to the relative percentage of chromatographic areas of their corresponding methyl esters. The fat was extracted according to the Folch method (cold extraction) and the esterification of the free fatty acids was carried out using a methanolic potassium hydroxide solution. The methyl esters of the fatty acids were analyzed by gas chromatography coupled to a FID detector. The results were expressed as mg of the compound per kg of fecal medium.

2.7. Microbiota Composition and 16S rRNA Analysis

Next Generation Sequencing (NGS) was performed using MiSeq Reagent Kits (Illumina Inc., San Diego, CA, USA). A first PCR was performed on 12.5 ng of genomic DNA obtained from the samples, and 16S-Fw and 16S-Rv primers. After that, a second PCR reaction was performed using 5 µL of DNA and the Nextera® XT DNA Index kit (FC-131-1002, Illumina). Then, the process quality was verified in a Labchip Bioanalyzer (Agilent Technologies Spain S.L., Madrid, Spain). When all the samples were obtained, they were multiplexed by mixing equimolar concentrations from each sample and the internal standard Phix. The mix was diluted until obtaining a concentration of 8 pM. The sequencing was performed in a MiSeq using a MiSeq® Reagent Kit V2 (MS-102-2003).

2.8. Bioinformatic Analysis

The 16S rRNA sequences obtained were curated following the quality criteria from the OTUs processing protocol, using the LotuS pipeline [47] This protocol includes the clustering of de novo sequences by UPARSE and the deleting of chimeric and contaminant sequences for OTUs identification. In addition, this program generates the corresponding abundance matrix. An OTU is defined as organisms that are clustered according to the similarity of their DNA sequence. The taxonomy was assigned by using BLAST and HITdb, reaching a species sensitivity level. The abundance matrix was curated and normalized in R and Bioconductor. A global normalization was performed using the library size as a correction factor. Data was transformed to Log2.

2.9. Glucosinolates and Isothiocyanates Quantitative Determination

Glucosinolates extracted from fresh red cabbage were identified by HPLC-DAD-ESI-MS^n, according to their [M-H] and MS^2 fragmentation patterns. The conditions for analysis were the same as described in Baenas et al. [48]. For the quantitative analysis of intact glucosinolates, 20 µL of extract were introduced into an Agilent 1100 HPLC-DAD system (Santa Clara, CA, USA). The glucosinolates were identified according to their UV spectra and elution order. Sinigrin and glucobrassicin were used as external standards (Phytochem, Neu-Ulm, Germany). Isothiocyanates were measured by a high throughput UHPLC-QqQ-MS/MS method, as described in Baenas et al. [49]. The standards employed for quantification were sulforaphane (SFN), SFN-glutathione (SFN-GSH), SFN-cysteine (SFN-CYS), SFN-N, acetylcysteine (SFN-NAC), iberin, and indole-3-carbinol (I3C) from Santa Cruz Biotechnology (Dallas, TX, USA), via Quimigen S.L. (Madrid, Spain).

2.10. Data Analysis

For the field elicitation experiment, a one-way ANOVA was performed, using Tukey's HSD as a *post hoc* test. For the dynamic digester experiments, a two-way ANOVA was applied, also followed by Tukey's HSD as a *post hoc* test. All of these analyses were carried out in RStudio (version 3.6.3).

3. Results

3.1. Field Elicitation of Red Cabbage

Six different glucosinolates were identified in the red cabbage samples: glucoiberin (GIB), glucoraphanin (GRA), sinigrin (SIN), gluconapin (GNA), 4-hydroxy-glucobrassicin (HGB), and glucobrassicin (GB). The HPLC-DAD analyses allowed the quantification of the three major compounds together with the study of the effect of the different elicitors (Table 1) on the red cabbage GSLs. The concentration of SIN significantly decreased after the application of 200 µM SA ($p < 0.05$). No differences were found between 100 µM MeJA and the combination treatment, when compared with the control ($p > 0.05$). The results for indolic HGB were similar, in that the 200 µM SA treatment decreased its content ($p < 0.05$), but 100 µM MeJA significantly increased it (2-fold) ($p < 0.05$). The combination treatment also reduced the HGB content ($p < 0.05$). Similarly, the GB was not affected by the 200 µM SA ($p < 0.05$) treatment, but increased dramatically with the other treatments: 3-fold with the 100 µM MeJA treatment, and 2-fold in the combination treatment. The total GSLs content followed the response observed in the individual compounds, and the 200 µM SA treatment decreased its value ($p < 0.05$), although the 100 µM MeJA (2-fold) and the combination treatment (1.5-fold), were very positive, when compared to the untreated controls.

Table 1. Effect of elicitors on glucosinolates of red cabbage inflorescences. The numbers show the average values per treatment ($n = 4$) ± standard error. Different letters in a row indicate statistically significant differences in the HSD Tukey's test ($p < 0.05$).

Glucosinolate (mg g D.W.$^{-1}$)	Control	200 µM SA	100 µM MeJA	SA + MeJA
GIB	*	*	*	*
GRA	*	*	*	*
SIN	4.12 ± 0.13 a	3.73 ± 0.08 b	4.95 ± 0.04 a	4.12 ± 0.06 ab
GNA	*	*	*	*
HGB	0.77 ± 0.02 b	0.41 ± 0.03 c	1.79 ± 0.02 a	0.54 ± 0.02 c
GB	2.93 ± 0.05 c	2.24 ± 0.08 c	8.1 ± 0.06 a	5.01 ± 0.1 b
Total GSLs	8.13 ± 0.2 b	6.39 ± 0.03 c	14.82 ± 0.01 a	9.71 ± 0.08 b

SA: salicylic acid, MeJA: methyl jasmonate, GSL: glucosinolate, GIB: glucoiberin, GRA: glucoraphanin, SIN: sinigrin, GNA: gluconapin, HGB: 4-hydroxy-glucobrassicin, GB: glucobrassicin. * The presence of the GSLs was under the limit of quantification for HPLC-DAD-ESI-MS^n (<0.02 mg g D.W.$^{-1}$).

3.2. Characterization of Extracts

The results presented in Table 2 showed that no statistically significant differences were obtained between treatments for SFN, I3C, or iberin ($p > 0.05$). The absence of GSLs was also corroborated by the HPLC-DAD analysis of the samples.

Table 2. Extract composition of the red cabbage aqueous extract for the free and the nanoencapsulated treatment. The numbers show the average values ($n = 3$) ± standard error. Different letters in a row indicate statistically significant differences in the HSD Tukey's test ($p < 0.05$).

	Red Cabbage Aqueous Extract	
ITCs Composition (μg/mL)	**Free Extract**	**Nanoencapsulated**
Sulforaphane (SFN)	6.72 ± 0.68 a	5.64 ± 0.28 a
Indole-3-carbinol (I3C)	1.81 ± 0.15 a	1.05 ± 0.1 a
Iberin	1.82 ± 0.04 a	1.97 ± 0.06 a

ITCs: isothiocyanates.

3.3. Dynamic Gastrointestinal and Colonic Fermentation Model

The presence of ITCs was clear in the two types of samples, free and encapsulated (Figure 1A), and more importantly, regarding the digestion treatment with the aqueous (free) extract, 1.77% of the starting concentration was still available after the gastrointestinal (GI) digestion process. No statistically significant differences were found when comparing the GI digestion with the ascending colon fermentation ($p > 0.05$). However, a decrease in SFN (from 14% to 6% from the initial dosage) was observed when comparing the ascending colon reactor with the transversal and descending ones ($p < 0.05$). With respect to the nanoencapsulated extract, the percentage of bioaccessible SFN did not decrease after GI digestion (retention of 99.4%), and no variations were observed in the three reactors of colonic fermentation ($p < 0.05$). When comparing both treatments, the nanoencapsulated red cabbage aqueous extracts showed the highest percentages of abundance of SFN ($p < 0.05$).

Regarding indole-3-carbinol (I3C, Figure 1B), when analyzing the free red cabbage aqueous extract digestions, 4% remained present after the digestion process ($p < 0.05$). No statistically significant differences were found when compared with the contents after the colonic fermentation in the three reactors ($p > 0.05$). On the other hand, from the nanoencapsulated red cabbage aqueous extract, a higher retention of I3C (by 12%) was observed after the GI digestion when compared with the crude extract ($p < 0.05$), and a statistically significant decrease (1.5-fold) was found after passing through the ascending colon reactor ($p < 0.05$). Nevertheless, no differences were found between the three colonic reactors (ascending, transversal, and descending colon) ($p > 0.05$).

As for the presence of iberin (Figure 1C), no statistically significant decreases were observed after GI digestion ($p > 0.05$). However, when compared with the ascending colon reactor, a 1.5-fold decrease was observed ($p < 0.05$), with an increase observed in the transversal and descending colon (1.5 and 2-fold, respectively) ($p < 0.05$). In addition, the final retention of iberin increased in the descending colon reactor as compared to the initial dosage ($p < 0.05$). Lastly, with respect to the nanoencapsulated form, a significant increase was observed between the GI digestion and the starting extract ($p < 0.05$). However, no differences were observed between the GI digestion and the three reactors of colonic fermentation ($p > 0.05$).

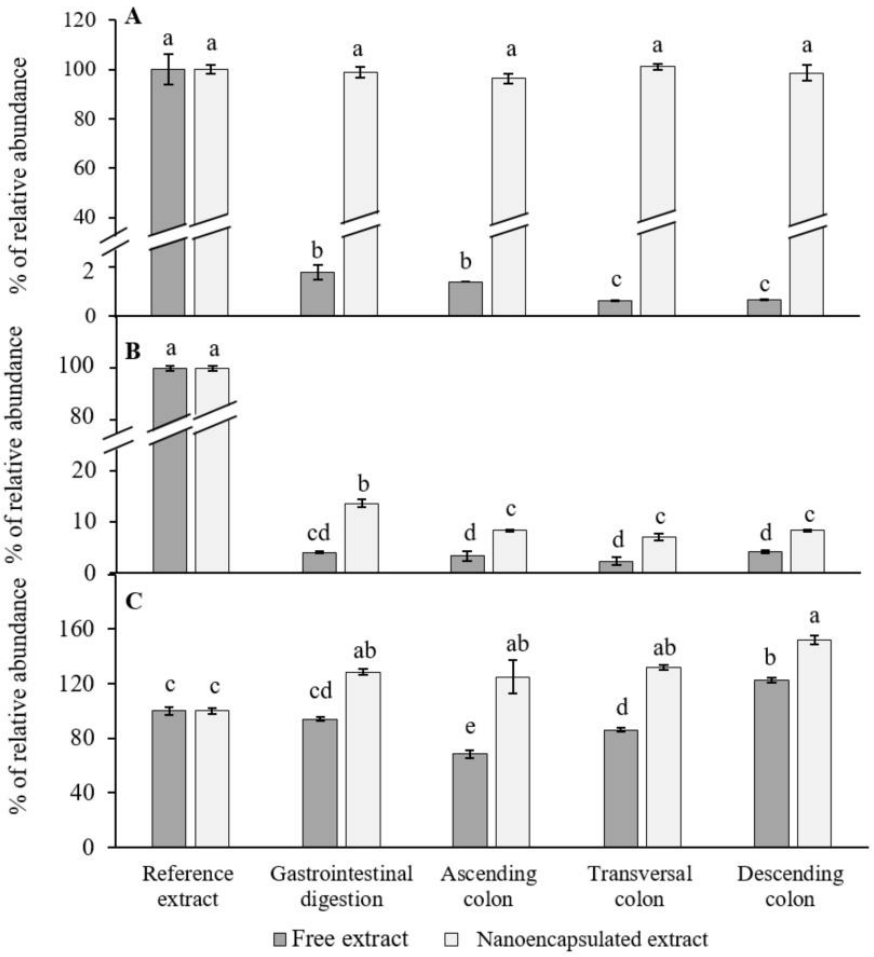

Figure 1. Percentage of the relative abundance of (**A**) sulforaphane (SFN), (**B**) indole-3-carbinol, and (**C**) iberin, when feeding the dynamic colonic-gastrointestinal digester (D-CGID) with the red cabbage aqueous extract, both free and nanoencapsulated ($n = 3 \pm$ SE). The reference extract was taken as 100% and a two-way ANOVA analysis with an HSD Tukey's test as a *post hoc* test was performed. Different letters indicate statistically significant differences ($p < 0.05$).

3.4. Effects of the Red Cabbage Extracts on the Microbiome

As shown in Figure 2A, no differences were found between the alpha index of the two treatments' inoculations ($p > 0.05$). For the ascending colon (Figure 2B) no differences were found between the stabilization and the 14-day treatment, or between treatments. In the transversal colon, a statistically significant decrease ($p < 0.05$) in the alpha index was observed after the 14-day treatment with the nanoencapsulated treatment (Figure 2C). Similar results as observed in the ascending colon were observed in the descending colon reactor (Figure 2D, $p > 0.05$).

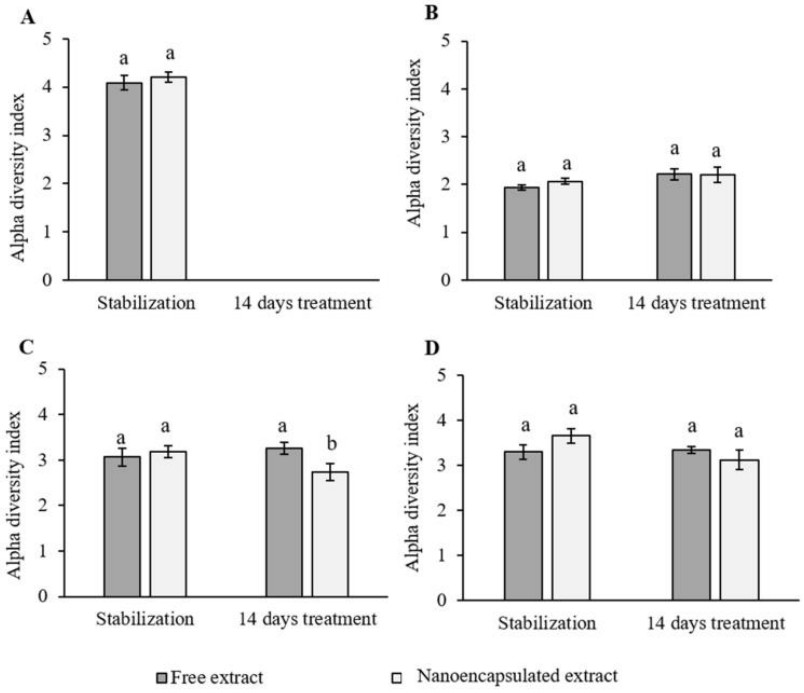

Figure 2. Representation of the alpha diversity index obtained from the inoculation (**A**) and each reactor: (**B**) ascending colon, (**C**) transversal colon, and (**D**) descending colon, before and after the treatment for 14 days with the red cabbage aqueous extract in its free and nanoencapsulated forms ($n = 3 \pm$ SE). Data were analyzed with a two-way ANOVA and the HSD Tukey's test as a *post hoc* test. Different letters mean statistically significant differences ($p < 0.05$).

The effect of the treatments on the percentage of relative sequences, from the six most-relevant phyla present in the human microbiome obtained from obese subjects, was analysed. For both fermentation processes, the percentage of sequences was similar at the inoculation point for the six phyla: 10–12% Actinobacteria, 28–26% Bacteroidetes, 4–7% Cyanobacteria, 28–27% Firmicutes, 9.5–9.6% Lentisphereae, and 19–17% Proteobacteria (Figure 3A,B). As for the assay performed with the free red cabbage aqueous extract, the ascending colon stabilization lacked the representation of the Cyanobacteria and Lentisphareae phyla (Figure 3A). However, 1.7% of the sequences corresponding to Lentisphaerae was detected when the treatment was applied after 14 days. In regard to the assay performed with the nanoencapsulated red cabbage aqueous extract (Figure 3B), sequences from four phyla were identified in the ascending colon: Proteobacteria, Firmicutes/Bacteroidetes/Actinobacteria. Nevertheless, no sequences related with the Lentisphaerae phyla or Cyanobacteria were found either at the end of the stabilization period or after the treatment. The samples from the analysed transverse colon (Figure 3B) showed sequences belonging to the six most relevant phyla after the stabilization treatment, but no sequences corresponding to the Lenstisphaerae phylum were identified after 14 days of treatment with the nanoencapsulated red cabbage aqueous extract. According to the descending colon, sequences corresponding with the six main phyla were identified in both the stabilization process and after the treatment. However, the percentage of sequences corresponding to Firmicutes varied from 19% in the stabilization process to 31% after the treatment with the nanoencapsulated red cabbage aqueous extract (Figure 3B). The relative percentage of sequences identified with Lentisphaerae and Cyanobacteria also differed after the treatment, from 11.7% to 1.5% for the first phylum, and from 16.7% to 9.6% for the second.

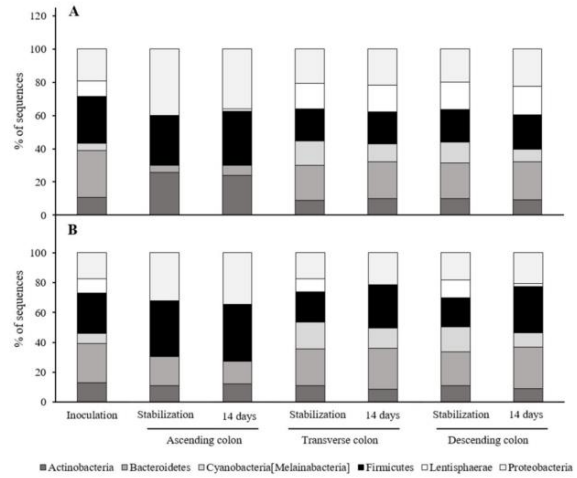

Figure 3. Relative presence (% of sequences identified) of the six most abundant phyla in the human colon in the dynamic in vitro gastrointestinal digestion and colonic fermentation model when fed with (**A**) free red cabbage aqueous extract, and (**B**) nanoencapsulated red cabbage aqueous extract. Data were obtained from the stabilization period (no treatment) and after 14 days of treatment. Represented data are means ± SD. Different letters mean statistically significant differences in the HSD Tukey's test ($p < 0.05$).

The *Bacteroidetes/Firmicutes* ratio (B/F ratio) was also obtained for both fermentation assays (Table 3). No statistically significant differences were observed for the inoculation in both treatments ($p > 0.05$). For the ascending colon, only a decrease between the stabilization and the 14-day treatment was observed when using the nanoencapsulated treatment ($p < 0.05$). Similar results in the B/F ratio were observed for the transversal and the descending colons, showing a decrease after the nanoencapsulated treatment ($p < 0.05$).

Table 3. *Bacteroidetes/Firmicutes* ratio obtained in the inoculation time and in the ascending, transversal, and descending colon reactors after the feeding with red cabbage aqueous extract (free or nanoencapsulated) for 14 days. The results show $n = 3 \pm$ SE. Different letters indicate statistically significant differences in the HSD Tukey's test, performed after a two-way ANOVA ($p < 0.05$).

***Bacteroidetes/ Firmicutes* Ratio**	**Free Red Cabbage Aqueous Extract**		**Red Cabbage Nanoencapsulated Aqueous Extract**	
	Stabilization	14 Days Treatment	Stabilization	14 Days Treatment
Inoculation	1.00 ± 0.02 a	-	0.98 ± 0.07 a	-
Ascending colon	0.15 ± 0.01 c	0.19 ± 0.08 c	0.5 ± 0.03 a	0.39 ± 0.02 b
Transversal colon	1.08 ± 0.1 ab	1.14 ± 0.08 a	1.21 ± 0.16 a	0.95 ± 0.05 b
Descending colon	1.08 ± 0.03 ab	1.09 ± 0.04 ab	1.16 ± 0.08 a	0.90 ± 0.01 b

3.5. Butyric Acid Production by the Microbiota

The butyric acid production by gut microbiota was analysed in the three digester reactors ($mg \cdot Kg^{-1}$ fermentation liquid). For the ascending colon reactor, an increase in its production was observed after the 14-day treatment with the free red cabbage aqueous extract (Figure 4A, $p < 0.05$). No statistically significant differences were observed with the nanoencapsulated treatment ($p > 0.05$). As for the transversal colon reactor (Figure 4B), a 3-fold increase after the 14-day treatment was observed for the free red

cabbage aqueous extract when compared to the stabilization period ($p < 0.05$). Additionally, a 3.5-fold increase was observed when using the nanonencapsulated extract ($p < 0.05$). Regarding the descending colon (Figure 4C), similar results as observed in the transversal colon were obtained. A 2-fold increase was produced after 14 days of treatment with the free red cabbage aqueous extract ($p < 0.05$). In addition, a statistically significant increase (4-fold) was observed in the production of butyric acid after the treatment with the nanoencapsulated extract ($p < 0.05$). Lastly, propionic and acetic acids were studied, but no changes were observed ($p > 0.05$, data not shown).

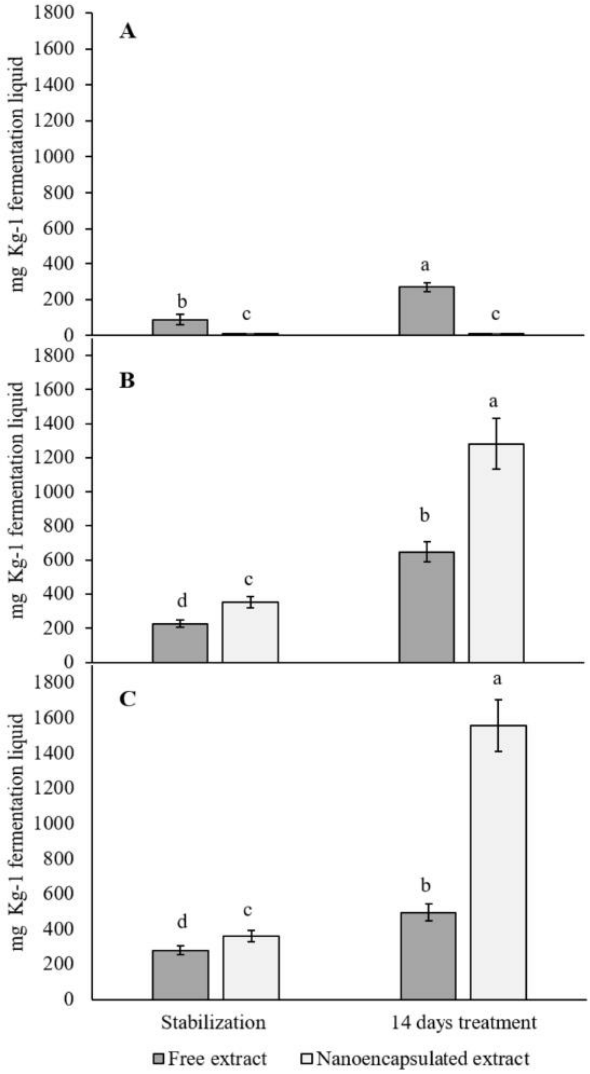

Figure 4. Butyric acid production (mg·Kg^{-1} fermentation fluid) analysed in the stabilization period and after 14 days of treatment with the red cabbage aqueous extract (free and nanoencapsulated) in the (**A**) ascending colon, (**B**) transversal colon, and (**C**) descending colon. Represented data are means ± SD. Different letters mean statistically significant differences in the HSD Tukey's test ($p < 0.05$).

4. Discussion

At present, there is a growing interest in functional foods and beverages obtained from vegetables, as they concentrate bioactive compounds and are considered less time-wasting by health-conscious consumers [18]. However, good quality starting plant material is required for creating a formula enriched in bioactive compounds. For this purpose, the use of elicitors on red cabbage to improve the contents of GSLs was positive under field conditions, as studied here. Our results showed that a foliar application with 100 μM MeJA greatly increased the concentrations of indolic GSLs (HGB and GB; Table 1). Previous works [50] found the elicitation with 200 μM jasmonic acid as positive for red cabbage, but with only an increased content of SIN. However, in our work, SIN remained unaffected. In Hassini et al. [51], MeJA elicitation on red cabbage sprouts did not report increases in SIN, but dramatically increased GB (7-fold increase). Jasmonic acid and its derivatives (such as MeJA) are known to mainly increase the accumulation of indole GSLs [15–17,52], due to their key role as regulator in the jasmonic acid-signaling pathway *MYB34* [53]. Our experimental design under field conditions, the application protocol, as well as the harvesting time, were all factors involved in the response of the plant to the treatments. Similar results were found for GB in kale and cabbage after MeJA elicitation [53], but also with an increased content of GSLs when using SA; as opposed to our findings, which agreed with the work by Thiruvengadam et al. [54], where decreases in HGB were also observed after 100 μM SA spray applications to turnips. Therefore, the results of the elicitation using these compounds as inducers vary depending on the species under study. The performance of the elicitor is not only based on the elicitor–species relationship, but such interaction also plays a differential role under different growing conditions.

Once a GSLs-enriched plant material was obtained, we opted for an aqueous extract enriched in ITCs from red cabbage, since hydrodistillation is more environmentally-friendly, more compatible with a food grade product than the use of organic solvents, and more representative of a dietary approach for the use of the bioactive compounds in a nutritional intervention.

Different types of stabilization methods for ITCs have been studied up to the present, for example, using cyclodextrins in an inclusion complex, or chitosan as coating material [55,56]. However, the coating or wall material is usually very expensive, restricting this type of carrier to high value-added industries, such as the pharmaceutical industry. In this way, ITCs encapsulation for stabilization in other industries still remains a field to be further investigated. In our work, a nanoencapsulation method based on plasma membrane vesicles obtained from cauliflower inflorescences [29] was studied. The use of plant-derived plasma membrane vesicles has been widely studied by our group and provided very positive results in different fields. For example, when used as nanobiofertilizers along with iron and boron for almond trees, a great increase in leaf concentration was observed [57]. Moreover, their potential in the dermatological industry has been assessed in skin keratinocytes, demonstrating their delivery and high penetrability [30]. These previous studies have led us to evaluate cauliflower-derived plasma membrane vesicles as ITCs stabilizers in a food prototype.

On the other hand, before arriving to their target cells or tissues, ITCs should be extracted from a complex matrix during digestion, and then absorbed by the small intestine [58]. For that purpose, a dynamic in vitro gastrointestinal digestion model was employed to estimate bioaccessibility. This in vitro digestion model offers a high reproducibility, ease of control and handling, and can provide a great approach to further in vivo experiments [59]. As for bioaccessibility, it is defined as the quantity of a compound present in food that is released from the digestive bolus into the GI tract, becoming available for absorption [60]. Regarding our results, the SFN analysis from the digestion simulation performed with the free red cabbage aqueous extract, revealed a dramatic decrease in its content after GI digestion ($p < 0.05$, Figure 1A). Xiangang et al. [61] reported on the high stability of SFN obtained from fresh broccoli seeds and sprouts after performing an in vitro GI digestion. This is in contrast with our results, suggesting that the food matrix plays a

key role in the further extraction of bioactives during the digestion process. In this way, when the plasma membrane vesicles were added to the red cabbage aqueous extract, a high percentage of SFN was conserved through the gastrointestinal digestion (Figure 1A). The work by Martínez-Ballesta et al. [62] reported a putative interaction by molecular docking between some proteins (aquaporins) present in the nanocapsules (nanovesicles enriched in aquaporins) obtained from broccoli plants and the glucosinolate glucoraphanin, which increased molecule stability. Therefore, there could also be an interaction between the plant aquaporins found in our vesicles [29] and the ITCs present in the red cabbage aqueous extract, which could have increased stability. In this way, plasma membrane vesicles may act as stabilizing carriers and feeding agents for enzymes and bile salts rather than an encapsulating agent *per se*. However, this aspect should be further studied.

As for I3C (Figure 1B), its stability also increased by the presence of cauliflower-plasma membrane vesicles, with a higher percentage remaining after the gastric digestion ($p < 0.05$). The stability of I3C is known to be a major problem when studying its bioavailability, as these molecules undergo oligomerization and form a mixture of diverse acid condensation products under acidic conditions [63]. Although encapsulation with zein reported an increase in I3C stability against thermal treatments, little is known about its performance under low pH conditions [64]. Regarding iberin (Figure 1C), it has been reported that it is usually more stable under acidic pH conditions. This could explain the high preservation of iberin even in the free red cabbage aqueous extract after the gastrointestinal digestion. Furthermore, for the nanoencapsulated extract, an increase in its relative abundance was observed after the gastrointestinal digestion. An explanation for this phenomena could be that after 14 days of treatment, an accumulation of iberin took place, as it has a low hydrophobicity, and the interaction with the proteolipidic vesicles may have stabilized it through time [65]. Furthermore, the high increase observed in the nanoencapsulated treatment after the colonic fermentation may also be due to the myrosinase activity of some enzymes from the colonic bacteria [66]. In addition, although many studies exist on the bioconversion between glucoraphanin and SFN, and its bioaccessibility [61,67,68], little information is available about the performance of other ITCs under GI digestion and colonic fermentation.

In the last decade, the high importance of the gut microbiome has been brought to light. Many studies which focused on fecal transplants revealed that the disturbance of gut microbiota is involved in the pathogenesis of NCDs [69–71]. Therefore, adjuvants such as specific diets, probiotics and prebiotics, and fecal transplants emerged as new approaches for modulating the gut microbial ecosystem [72]. Usually, the term dysbiosis is employed to define the disturbance of the relative abundance of the microbial groups, often classified by the sequencing of the 16S rRNA [73]. Nevertheless, it is not clear what a "dysbiotic" or "healthy" profile is, as a specific profile could be dysbiotic for an individual, but the same profile could describe a healthy subject [74]. Even though there is extensive discussion on this topic, we wanted to assess the effect of our red cabbage aqueous extracts (free and nanoencapsulated) during 14 days of treatment on the gut microbiome obtained from obese volunteers.

One of the main parameters that provides information about a microbiome is the alpha diversity index, as it describes the species richness based on the OTU count [75]. Our study did not show large variations in the alpha index between the stabilization period and after the 14-day treatments (Figure 2). Similar results were observed by Kazmarek et al. [8], where no significant changes were observed in their alpha index after an 18-day treatment with a broccoli-based diet consumed by human subjects. In addition, our results showed a higher diversity in the transversal and descending colon, whereas the lowest alpha values were obtained in the ascending colon (Figure 2). In brief, it seems that our extracts neither affected the species richness nor caused the extinction of OTUs, but rather decreased them. SFN has been reported to have anti-microbiological effects against gut pathogens in vitro [58]. Since our red cabbage-derived extracts were rich in SFN, the 14-day

treatments might have affected not only the gut pathogens present in the inoculus, but also the beneficial bacteria populations, thus decreasing the OTUs count.

The relative presence of the six most abundant phyla analysed in our experiment was also determined (Figure 3). Among these phyla, we found the four major bacterial groups reported by the bibliography to colonize the adult human gut: Bacteroidetes (Gram-negative anaerobes), Firmicutes (Gram positive), Actinobacteria (Gram positive), and Proteobacteria (Gram negative) [76]. Although they have been described as less abundant [77], Lentisphaerae and Cyanobacteria phyla were also reported as some of the most representative in our study. Our results did not report differences between the stabilization period and the treatment after 14 days when applying the two treatments (Figure 3). Since it has been reported that dietary interventions could cause a shift in the gut microbiome within 24 h, perhaps the time set for our analysis was too long [78]. Nevertheless, some changes were observed after the treatments: in the ascending colon, the percentage of sequences belonging to the phylum Lentisphaerae (Figure 3A) increased from 0% to 1.67% after the treatment with the free red cabbage aqueous extract. In addition, a decrease in the percentage of sequences identified as Cyanobacteria was observed in the transversal (14% down to 10%) and descending colons (13% down to 8%). A similar result was observed for Cyanobacteria when the nanoencapsulated treatment was applied (Figure 3B); a decrease from 17% to 13% in the transversal colon, and a decrease from 17% to 9% in the descending colon. Recently, these phyla have been positively associated with the low-density lipoprotein cholesterol (LDL-C), which decreases the effect of rosuvastatin in patients with hyperlipidemia [77]. Although the conditions were not similar, the decrease in the Cyanobacteria group observed in our work may have a health-related influence on the blood cholesterol levels of obese patients. Proteobacteria are currently the largest phylum in the bacterial domain and are Gram negative, which means that they contain lipopolysaccharides in the outer membrane. Thus, a putative deleterious role has been associated with a Proteobacteria bloom and intestinal inflammation due to the increase in lipopolysaccharide levels [79]. Furthermore, it has been reported that the monocolonization of a germen-free mouse gut with *Enterobacter cloaceae* B29 induced obesity in its host. Therefore, Proteobacteria have been defined as putative gut dysbiosis markers [80]. Nevertheless, in our study, only subtle changes were found in the Proteobacteria phylum when the treatments were applied. For the free red cabbage aqueous extract (Figure 3A), a decrease at the ascending colon was observed after the treatment (40% vs. 35%). Regarding the nanoencapsulated treatment (Figure 3B), a slight increase was found in the transversal (17% vs. 20%) and descending (18% vs. 21%) colons when the nanoencapsulated treatment was applied. Similar results have been reported in rats' gut microbiome after broccoli ingestion, in which a decrease in the Proteobacteria population was observed [81].

According to experiments performed by Ridaura et al. [82], microbial inoculation from an obese model into a lean twin resulted in a progressively greater increase in fat mass and body weight. Hence, many efforts have been made to pinpoint what composes a "healthy" and "obese" microbiome. Derived from these works, an obese phenotype has been linked to a lower *Bacteroidetes/Firmicutes* ratio, whereas this ratio increases in lean phenotypes [83]. In our study, the *Bacteroidetes/Firmicutes* ratio remained close to the one found in the inoculation and in the transversal and descending colon reactors, even after the treatments with the red cabbage aqueous extracts (free or nanoencapsulated). Only in the ascending colon were the ratios obtained below 0.5. Since the studies performed in humans usually did not focus on the three parts of the colon separately, perhaps this colon compartment is a better niche for *Firmicutes*. An increase in the *Bacteroidetes* phyla has been reported in obese mice models after being fed with rice bran [84]. Furthermore, probiotics such as *Lactobacillus salivarius* Ls-33 have been observed to increase the *Bacteroidetes/Firmicutes* ratio in obese adolescents [85]. Nevertheless, this association is not exempt from controversy, as other studies have criticized this ratio for its lack of specificity [86]. For example, recent research performed by Cortés-Martín et al. [87] revealed that no distinctive gut microbiota signature was found related with metabolic syndrome after analysing samples

from 69 obese volunteers and 50 patients with metabolic syndrome. On the other hand, authors such as Aoun et al. [88] manifested that the gut microbiome has an influence in the host nutrient metabolism and energy expenditure, suggesting that further clinical studies are needed to understand how species affect weight gain. In brief, since the influence of the gut microbiome on obesity not only underlies the colon bacteria, but also interconnects genetics, the environment, the immune system, and the brain, it is highly complicated to decipher the exact consequences on metabolic alterations.

Lastly, for butyric acid production, short chain fatty acids (SCFAs) are products that mainly come from the microbes performing anaerobic metabolism of non-digestible carbohydrates. Butyric acid has been reported to regulate the immune function in the intestine [89]. However, recent studies have shown that butyric acid is able to interact with the orexigenic neurons present in the hypothalamus, whose role is to mediate food intake and provide a protective effect against the effects of a high-fat diet [90]. Furthermore, a lower presence of butyrate-producing bacteria has been linked with an increased risk of metabolic disease [91]. A study was conducted with obese volunteers suffering from metabolic syndrome, who were administered with sodium butyrate (4 g/day), showing a positive anti-inflammatory and immunomodulatory effect [92]. As observed in our results, an increase in butyrate production by the gut microbiome was observed for both treatments ($p < 0.05$), although this was significantly higher with the nanoencapsulated extract. In this way, our treatments might exert an effect on the butyrate-producing bacteria taxa, such as *Roseburia intestinalis*, *Faecalibacterium prausnitzii*, or *Eubacterium rectale* [89]. As the microbiome was obtained from obese volunteers, the improved production of this SCFAs may have a direct effect on the food intake by modulating the afferent neurons in the vagal nervous system [93]. Further in vivo research should be conducted to prove the direct effect of the increase in the SCFAs produced by the intake of our extracts on obese subjects and the effect of the treatments on the butyrate-producing bacteria.

5. Conclusions

The present comprehensive study, from plant to food ingredient and health, evaluated the potential for the red cabbage encapsulated prototype enriched in GSL to improve its bioaccessibility for gastrointestinal absorption. This was conducted after evaluating its composition, and with improved stability of its ITCs by encapsulation with plant membrane vesicles. The results showed that the encapsulation helped with reaching the GI microbiota, with the interest placed on modulating the interaction between phyla to improve the metabolic and energy state of human adults suffering from overweightness and obesity. Furthermore, the fact that the encapsulated red cabbage extract provided a higher production of butyric acid, pointed to future developments for the design of a functional ingredient or food product for the management of overweightness and obesity in the adult population. This is of great interest to the public health systems worldwide because of its pandemic proportions. The search for natural alternatives to medications, to be incorporated into diets and nutritional interventions, and to help in the management of overweightness and obesity, has garnered interest worldwide and will have a global impact. However, there is a current need for further research on the bioavailability, metabolism, and bioactivity of natural plant-based products.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of University of Navarra (protocol code 2019.169, date 12 December 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data supporting reported results and generated during the study are available upon requesting them from the corresponding author.

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~ Discusión ~

Discusión

1. Efecto de la elicitación sobre los cultivos de Bimi® y col roja

En los últimos años, la demanda por parte del consumidor de hortalizas crucíferas con un sabor menos pungente y amargo, ha llevado a la búsqueda de nuevos híbridos naturales (Bimi®) y a revalorizar algunas menos protagonistas (col lombarda) en comparación con el brócoli. No obstante, tanto su composición fitoquímica, como su respuesta frente a la bioestumulación con el uso de elicitación, está poco caracterizada. Por ello, uno de los objetivos en la presente Tesis Doctoral ha sido elucidar cómo la elicitación con SA, MeJA y su combinación, puede enriquecer la composición de Bimi® (inflorescencias, tallos y hojas) y en la col lombarda. Estos dos elicitadores se seleccionaron para experimentos en campo y a dosis establecidas en base a la amplia investigación previa en relación a su uso para el aumento y biosíntesis de GSLs (Baenas, et al., 2016; Hassini et al., 2019; Thiruvengadam et al., 2016; Ku et al., 2014). Nuestros experimentos revelaron que, para el Bimi®, la nebulización de 100 µM de MeJA es capaz de aumentar la concentración de GSLs totales tanto en las hojas como en las inflorescencias (Capítulo I, Figura 1C). No obstante, la combinación más efectiva para aumentar la concentración de GSLs totales en las inflorescencias del Bimi® se obtuvo con SA+ MeJA (Capítulo I, Figura 1, Tabla 1). Sin embargo, en la col lombarda el tratamiento con una dosis alta de 200 µM de SA redujo el total de GSLs, mientras que el MeJA y la aplicación combinada de MeJA + SA, fue positiva (Capítulo IV, Tabla 1).

Existe una relación antagónica entre las rutas biosintéticas del SA y del JA en la planta (Li et al., 2019). Por ejemplo, estudios realizados en *Brassica rapa* L. var. *pervirdis* revelaron que cuando se inducían genes relacionados con la ruta del SA, aquellos pertenecientes a la del JA disminuían su expresión (Miyaji et al., 2021). No obstante, la interacción entre ambas aún no se ha elucidado por completo.Por ello, es plausible pensar en la implicación de la regulación genética y bioquímica en el proceso de acumulación de GSLs en el Bimi ®, en el que se obtuvo un mayor

contenido en la parte comestible con la aplicación combinada MeJA+SA (Capítulo I, Figura 1C). En concreto, *MYB34* que actúa como regulador clave en la vía de señalización del JA y *MYB51* que está implicado en la del SA, son capaces de modular la síntesis *de novo* de GSLs indólicos (Frerigmann & Gigolashvili, 2014). Asimismo, se ha descrito una regulación positiva de genes relacionados con *MYB51* después de la elicitación con SA en hojas de col rizada (Yi et al., 2016). En el caso del Bimi®, que es un híbrido de la col rizada y brócoli, la regulación de su expresión génica puede diferir entre órganos. De este modo, la parte comestible sería más sensible a ambos elicitadores (SA+MeJA), pero en las hojas, la expresión de GSLs puede ser más dependiente de genes relacionados con el JA, lo que explicaría el efecto antagónico en este órgano cuando se aplican en combinación.

Por otro lado, en col lombarda se observó una disminución del contenido de SIN e HGB al ser tratadas con 200 µM de SA (Capítulo IV, Tabla 1). Una reducción similar en la HGB se describió en plantas de nabo (Thiruvengadam et al. 2016). Asimismo, la elicitación con 100 µM de MeJA y su combinación (SA+ MeJA) sobre los GSLs alifáticos fue positiva, tanto en el caso de la col lombarda como Bimi® (Capítulo I, Tabla 1 y Capítulo IV, Tabla 1). En trabajos previos realizados en col rizada y colza, la elicitación con MeJA proporcionó resultados similares (Ku et al., 2014; Loivamäki et al., 2004). Sin embargo, en el caso de la col, la elicitación con 200 µM de MeJA ejerció principalmente su efecto sobre los GSLs alifácticos, lo que sugiere que el efecto de este elicitador sobre los distintos GSLs también depende de la especie de *Brassica* (Fritz et al., 2010).

Desde el punto de vista de alimento seguro, cabe destacar el bajo contenido de progoitrina en el Bimi®, estudiado y analizado por HPLC-DAD-ESI-MS^n, incluso en las plantas tratadas con elicitador (Capítulo I, Tabla 1). Esto supone una gran ventaja, ya que este GSL puede interferir con la utilización de iodo para la síntesis de hormonas tiroideas (Felker et al., 2016). En resumen, la aplicación en campo de elicitadores ha dado lugar a alimentos enriquecidos en GSLs, como GRA, HGB, MGB y NGB en el caso del Bimi® y en HGB y GB, en el de la col lombarda.

2. Obtención de formulaciones nanoencapsuladas y su estabilidad en el tracto gastrointestinal

2.1. Obtención de formulaciones enriquecidas en GSLs e ITCs

A partir de Bimi® y col lombarda, se elaboraron formulaciones en base acuosa, ya que la hidrodestilación y la maceración son procesos muy utilizados en la industria alimentaria y son más respetuosos con el medio ambiente que el uso de disolventes orgánicos (Azmir et al., 2013). En primer lugar, en el Capítulo I del presente manuscrito, se desarrolló una formulación mediante hidrodestilación a 100ºC, empleando tanto las inflorescencias, los tallos y las hojas del Bimi® elicitados (Capítulo I, Tablas 2 a 4). De este modo se determinó un tiempo de extracción óptimo de 30 min para la mayoría de los materiales (Capítulo I, Figura 2). A pesar de que este tipo de tratamiento térmico debería inactivar a la enzima mirosinasa, las formulaciones obtenidas y analizados de la parte comestible (inflorescencias + tallos) del Bimi® mostraron una alta presencia de ITCs como el I3C (Capítulo III, Tabla 1). Esto podría deberse a que la inactivación de la mirosinasa no es inmediata, de manera que, en los primeros minutos de la hidrodestilación la enzima sigue activa (Oliviero et al., 2014). Con respecto a la col lombarda, el método empleado fue la maceración, obteniendo así una formulación acuosa enriquecida en ITCs como el SFN, el I3C o la iberina (Capítulo IV, Tabla 2).

Los ITCs se caracterizan por una baja estabilidad en medio acuoso, degradándose rápidamente (Yuanfeng et al., 2021; Cirilli et al., 2020). Ante esto, el uso de nanoportadores surge como una herramienta útil para aumentar la estabilidad, bioaccesibilidad y bioactividad de estas biomoléculas (Danafar et al., 2017). Sin embargo, la mayoría de materiales empleados para el revestimiento o complejación con nanoportadores suelen ser costosos y restringen el producto final a industrias de alto valor añadido, como la farmacéutica (Ahmad et al., 2021). Por ello, en la presente Tesis Doctoral se han seleccionado como agente

nanoencapsulante las vesículas derivadas de membranas plasmáticas de inflorescencias de coliflor (Capítulo II).

Al analizar la composición de las formulaciones nanoencapsuladas, tanto en el caso del Bimi® (Capítulo III, Tabla 1) como en el de la col lombarda (Capítulo IV, Tabla 2), se observó una menor concentración de ITCs al analizarlos mediante UHPLC-ESI-QqQ-MS/MS cuando se añadía el agente nanoencapsulante. Quizás esto se deba a que pequeñas moléculas con mayor hidrofobicidad, como el DIM, a menudo interactúan con la membrana plasmática y no pueden detectarse a menos que el nanoportador esté completamente degradado (Lee, 2020).

2.2. Vesículas de membrana plasmática de coliflor como agentes nanoencapsulantes

El estudio de las distintas aplicaciones de vesículas derivadas de membranas vegetales y, en concreto, las obtenidas de membrana plasmática de brócoli como nanoportadores ha sido investigado recientemente en nuestro grupo de investigación y presenta una gran biocompatibilidad y alta penetración de tejidos, como la piel (Yepes-Molina et al., 2021). No obstante, la presente Tesis Doctoral explora la búsqueda de nuevos materiales que presenten una alta estabilidad y adaptación a los cambios en el medio para poder emplearse en formulaciones alimentarias. En este sentido, las vesículas de membrana plasmática de inflorescencias de coliflor de 90 días de edad o *90-**d**ays **i**nflorescences **c**auliflower **v**esicles* (90d ICVs) presentaron características interesantes para esta finalidad. A pesar de presentar menor tamaño y polidispersidad (Capítulo II, Figura 2), las 90d ICVs presentan una alta proporción de ácidos grasos insaturados, como el linolénico (C18:3), lo que permite una mayor fluidez de membrana (Stubbs & Smith, 1984) (Capítulo II, Tabla 2). Esta alta proporción, tanto en hojas como en las inflorescencias, es superior en coliflor a la encontrada en trabajos previos realizados con otras especies, como brócoli o *Cakile marítima* (Chalbi et al., 2015). Los experimentos realizados en la presente Tesis Doctoral se han realizado en campo, por lo que los cambios ambientales pudieron influir en la composición de los ácidos grasos en la bicapa. En cuanto a los esteroles, las 90d ICVs también

mostraron una alta proporción de campesterol y β-sitosterol (Capítulo II, Tabla 2). Esto puede traducirse en una mayor organización estructural de la membrana y un mejor paso del agua a través de la bicapa, respectivamente (López-Pérez et al., 2007).

En cuanto a la permeabilidad osmótica del agua, este parámetro se emplea para describir los flujos de agua a través de las membranas vegetales aisladas en vesículas, que son impulsados por los gradientes de osmolaridad y se ha relacionado con la estabilidad (Maurel, 1997). Los resultados de las 90d ICVs mostraron un valor muy alto para la P*f* (64,4 ± 4,4 μm s^{-1}) (Capítulo II, Figura 3), en comparación con la obtenida en vesículas derivadas de raíces u hojas de brócoli (Chalbi et al., 2015). Esta alta permeabilidad puede correlacionarse con la presencia de AQPs, ya que el análisis de proteómica inicial, reveló la presencia de cinco AQPs pertenecientes al subtipo PIP1 (PIP1;1, PIP1;2, PIP1;3, PIP1;4, y PIP1;5) y de dos PIP2 (PIP2;5 y PIP2;7) (Capítulo II, Tabla 3). En concreto, se observó una mayor densidad de proteínas PIP2 en las inflorescencias que en las hojas (Capítulo II, Figura 5).

Por lo tanto, las 90d ICVs son un buen candidato como agente nanoencapsulante para formulaciones alimentarias, debido a su alta permeabilidad osmótica, principalmente conferida por la elevada densidad de acuaporinas PIP, presentando una mayor capacidad de adaptación a los cambios en el medio. Además, el uso de inflorescencias maduras que presenten taras o no superen las características establecidas por el mercado es una gran alternativa para poder reaprovechar los subproductos agrícolas.

2.3. Estabilidad de las formulaciones obtenidas frente a la digestión gastrointestinal "in vitro"

Antes de alcanzar los tejidos u órganos diana, los ITCs deben extraerse de una matriz compleja durante la digestión para, posteriormente, poder ser absorbidas en el intestino delgado (Abukhabta et al., 2020). Con tal fin, se emplearon dos tipos de modelos de digestión *in vitro*, tanto estático (Capítulo III) como dinámico acoplado a una fermentación colónica (Capítulo IV), ya que ofrecen una alta

reproducibilidad, son de fácil manejo y proporcionan una primera aproximación para realizar futuros experimentos *in vivo* (Moreda-Piñeiro et al., 2011).

En cuanto a los experimentos realizados con formulaciones libres y nanoencapsuladas de Bimi®, se observó una concentración mayor de DIM e I3C en ambas fases (gástrica e intestinal) en la formulación nanoencapsulada (Capítulo III, Figura 1). Con respecto al SFN, en la fase gástrica solo una pequeña concentración fue detectable mediante UHPLC-ESI-QqQ-MS/MS. Sin embargo, durante la fase de digestión intestinal y al final del proceso de digestión completo, se obtuvo una mayor concentración de SFN en la formulación nanoencapsulada (Capítulo III, Figura 1).

Los experimentos realizados con las formulaciones de col lombarda se realizaron en un modelo dinámico de digestión gastrointestinal y de fermentación colónica *in vitro*, acondicionado con microbiota de voluntarios con obesidad y siendo alimentado durante 14 días (Capítulo IV). El análisis mostró una disminución en la concentración de SFN tras el proceso gastrointestinal completo para la formulación libre, en contraste con el alto porcentaje preservado en el caso de la nanoencapsulada (Capítulo IV, Figura 1A). También se observó una mayor conservación del I3C en el caso de la formulación nanoencapsulada durante todo el proceso de digestión y fermentación (Capítulo IV, Figura 1B). Este ITC suele presentar problemas a la hora de estudiar su biodisponibilidad, ya que estas moléculas oligomerizan en condiciones de pH ácido, formando una mezcla de diversos productos de condensación (Amare et al., 2020). Con respecto a la iberina, se ha descrito que presenta una mayor estabilidad en rangos de pH bajos (Kyriakou et al., 2022), lo cual explicaría su alta conservación durante el proceso gastrointestinal y la fermentación colónica en la formulación libre (Capítulo IV, Figura 1C). También se observó un aumento, en comparación con la referencia, en la concentración de iberina en la formulación nanoencapsulada a su paso por los fermentadores correspondientes a las tres partes del colon (ascendente, transversal y descendente), lo que podría deberse a la acción de enzimas de carácter similar a la mirosinasa presentes en la microbiota (Sikorska-Zimny & Beneduce, 2021)

(Capítulo IV, Figura 1C). Esto, junto con la suplementación continua y la acción del agente nanoencapsulante, explicaría las altas concentraciones tanto de SFN como de iberina en los reactores (Capítulo IV, Figura 1).

Con respecto a la acción del agente nanoencapsulante, el análisis mediante microscopía de transmisión electrónica (TEM) reveló una disminución del tamaño de las vesículas (aproximadamente de 500 nm a 250 nm) durante la digestión estomacal y su desaparición por completo en la fase intestinal (Capítulo III, Figura 2C, 2D). Esto podría relacionarse con la interacción de los ITCs con las vesículas (Capítulos III y IV), ya que estudios previos realizados en BPMVs han descrito la posible interacción de GSLs con las AQPs presentes en estas vesículas (Martínez-Ballesta et al., 2016), por lo que quizás sus derivados también presenten esta cualidad. Incluso al ser moléculas con cierta hidrofobicidad y de pequeño tamaño, podrían interactuar con la bicapa lipídica, actuando como reservorios incluso si la integridad del vehículo está comprometida (Rostamabadi et al., 2019). Además, las 90d ICVs pueden ejercer como cebadores de proteínas, protegiendo a los ITCs no solo de los cambios en el medio, como el pH, sino también de enzimas digestivas y ácidos biliares.

3. Bioactividad de las formulaciones en dos modelos relacionados con la obesidad

3.1. Efecto en un modelo de inflamación hepática (línea celular HepG2)

Actualmente, existen una amplia variedad de trabajos focalizados en la acción antiinflamatoria, cardioprotectora y anticancerígena de los ITCs (Ruiz-Alcaraz et al., 2022; Favela-González et al., 2020; Jiang et al., 2019). No obstante, poca es la información disponible sobre cómo estas biomoléculas son metabolizadas por los distintos modelos celulares. Por ello, en el Capítulo III se realizaron experimentos de metabolismo en la línea de hepatocitos humanos HepG2, tanto en condiciones normales como en un estado de inflamación de bajo grado, simulando condiciones similares a las que se podrían dar en una enfermedad no transmisible, como la obesidad. Con tal fin, se emplearon como tratamientos las formulaciones de Bimi®

tanto libres como nanoencapsuladas, tras ser sometidas a un proceso de digestión gastrointestinal *in vitro*.

En los experimentos realizados, destaca la detección de DIM en los lisados celulares, pero no en los sobrenadantes, encontrándose solo I3C en éstos (Capítulo III, Figura 4B y Figura 5A). Puesto que se ha descrito previamente la formación espontánea de DIM a partir de I3C en condiciones de cultivo celular, y que éste presenta una mayor bioactividad (Bradlow & Zeligs, 2010), una explicación viable podría ser que las células HepG2 incorporan y acumulan DIM para una posterior metabolización. Asimismo, diferencias en su metabolización se pudieron observar entre las células control y las tratadas con LPS, detectando menos concentraciones de DIM en estas últimas (Capítulo III, Figura 5A).

Por otro lado, el análisis de la metabolización del SFN y sus derivados de la vía del ácido mercaptúrico, mostró la ausencia de SFN-GSH y SFN-CYS en los lisados de células no tratadas a 48 horas (Capítulo III, Figura 5C y D). Esto podría sugerir que la célula no necesita una mayor acumulación de estos compuestos, siendo o bien secretados al medio para poder alcanzar otras células (SFN-CYS) o bien metabolizándose a SFN-NAC, que posteriormente será excretado (Gu et al., 2020). Asimismo, la formulación nanoencapsulada mostró una mayor acumulación de metabolitos en la mayoría de las condiciones, tanto en los sobrenadantes como en lisados celulares, lo que sugiere que este tratamiento es capaz de conservar y proporcionar una mayor concentración de metabolitos.

3.2. Evaluación del efecto de las formulaciones sobre el microbioma intestinal

En las últimas décadas, se ha puesto de manifiesto la relevancia de la microbiota intestinal en la prevención y desarrollo de enfermedades no transmisibles. Por ello, coadyuvantes como dietas específicas, probióticos, prebióticos y trasplantes fecales, surgen como nuevos enfoques para modular y estudiar el ecosistema microbiano y su relación con la salud (Zhang et al., 2019). En este campo, se define disbiosis como la alteración en la abundancia relativa de

los grupos microbianos, clasificados mediante secuenciación del ARNr 16S (Carding et al., 2015). No obstante, es complejo clasificar un perfil como "disbiótico" o como "saludable" debido a la alta variabilidad intra e interindividual. A pesar de que existe una amplia discusión por establecer los parámetros de un perfil y otro, el trabajo realizado en el Capítulo IV de la presente Tesis Doctoral evaluó la acción de las formulaciones de col lombarda en la microbiota intestinal de voluntarios con obesidad, mediante fermentadores que simulan los distintos compartimentos del colon (Rinninella et al., 2019).

Uno de los parámetros principales que aportan información sobre el microbioma es el índice de diversidad alfa, ya que describe la riqueza de especies en función del recuento de unidades taxonómicas operativas (OTU) (Luz Calle, 2019). El presente trabajo no obtuvo variaciones en el índice alfa tras comparar el periodo de estabilización del fermentador con las muestras recogidas tras 14 días de tratamiento (Capítulo IV, Figura 2). Resultados similares se han observado en voluntarios tras consumir brócoli durante 18 días (Kaczmarek et al., 2019). En resumen, ambas formulaciones no afectaron a la riqueza de especies ni causaron la extinción de ninguna OTU, solo reduciendo su proporción en el colon ascendente (Capítulo IV, Figura 2). Previamente, se ha demostrado que el SFN presenta una alta actividad antimicrobiana contra patógenos intestinales *in vitro* (Abukhabta et al., 2020). Dado que las formulaciones obtenidas presentan una alta concentración de este ITC, podría suponer que el tratamiento durante 14 días afectase no solo a los patógenos intestinales presentes en el inóculo, sino también a las poblaciones de bacterias beneficiosas, disminuyendo el número de OTUs.

También se determinó la presencia relativa de los seis *phyla* microbianos más abundantes (Capítulo IV, Figura 3). Entre ellos, encontramos cuatro grupos que previamente se han definido por la bibliografía como colonizadores del intestino humano adulto: Bacteroidetes (anaerobios Gram negativos), Firmicutes (Gram positivos), Actinobacteria (Gram positivos) y Proteobacteria (Gram negativos) (Harmsen & de Goffau, 2016). También se encontraron los *phyla* Lentisphaerae y Cyanobacteria, a pesar de que son menos abundantes. Asimismo, se observaron

algunos cambios en los porcentajes de los *phyla* tras el suministro de col roja durante 14 días. Por ejemplo, en la formulación nanoencapsulada (Capítulo IV, Figura 3B) se observó una disminución de abundancia del 17% al 13% en el colon transversal y del 17% al 8% en el descendente en el caso del *phylum* Cyanobacteria. Recientemente, este *phylum* se ha asociado positivamente con la presencia de lipoproteínas de baja densidad, las cuales afectan el efecto de estatinas en pacientes con hiperlipidemia (Liu et al., 2018). De este modo, la disminución del grupo Cyanobacteria observada en el presente estudio, puede modular los niveles circulantes de colesterol de los pacientes con obesidad. Las Proteobacteria son actualmente el *phylum* más grande en el dominio bacteriano y son Gram negativas, lo que significa que contienen lipopolisacáridos en la membrana externa, lo que se ha asociado a un posible efecto negativo por Proteobacteria en inflamación intestinal por el aumento en los niveles de lipopolisacárido (Shin et al., 2015). Sin embargo, en esta investigación solo se encontraron cambios sutiles en el *phylum* Proteobacteria cuando se aplicaron los tratamientos. Para las formulaciones libres de col lombarda (Capítulo IV, Figura 3A), se observó una disminución en el colon ascendente después del tratamiento (40% vs. 35%), mientras que con la formulación nanoencapsulada (Capítulo IV, Figura 3B), se encontró un ligero aumento en el colon transversal (17% vs 20%).

A pesar de los esfuerzos realizados para describir qué compone un microbioma en un individuo saludable y un paciente con obesidad, de los trabajos realizados hasta la fecha se acepta una relación entre *Bacteroidetes/Firmicutes,* más alta en fenotipos delgados y menor en fenotipos con obesidad (Ley et al., 2006). En la presente Tesis Doctoral, la relación *Bacteroidetes/Firmicutes* permaneció cercana a 1, tanto en el inóculo, como en el colon transversal y descendente (Capítulo IV, Tabla 3). Incluso tras la administración de las formulaciones libres y nanoencapsuladas de col lombarda. Únicamente se obtuvieron relaciones inferiores a 0’5 en el colon ascendente. Dado que los estudios realizados en humanos, generalmente, no se centran en las tres partes del colon por separado, tal vez este compartimento sea un nicho biológico más favorable para los Firmicutes. No obstante, la asociación entre la relación de *phyla* y el fenotipo no está exenta

de controversia, ya que algunos estudios no han conseguido encontrar una firma distintiva de microbiota intestinal relacionada con patologías como el síndrome metabólico (Cortés-Martín et al., 2020; Finucane et al., 2014). Puesto que la influencia del microbioma en la obesidad no solo subyace en las bacterias del colon, sino que también interconecta la genética, el ambiente, el sistema inmunológico y el cerebro, es muy complejo descifrar las consecuencias exactas sobre las alteraciones metabólicas.

Por otro lado, los ácidos grasos de cadena corta (SCFAs) son productos que provienen principalmente de los microorganismos que metabolizan de manera anaeróbica los carbohidratos no digeribles. Entre ellos, se ha observado que el ácido butírico es capaz de regular la función inmunitaria en el intestino (Louis & Flint, 2017). Asimismo, estudios recientes han demostrado que el ácido butírico es capaz de interaccionar con las neuronas orexigénicas presentes en el hipotálamo, cuyo papel es mediar la ingesta de alimentos (Li et al., 2018). Tal y como se muestra en la Figura 4 (Capítulo IV), se observó un aumento en la producción de butirato por parte del microbioma intestinal para las formulaciones de col lombarda, siendo significativamente mayor en la formulación nanoencapsulada. Puesto que el microbioma se obtuvo de pacientes con obesidad, la mayor producción de este SCFA podría relacionarse con un efecto directo en la ingesta de alimentos, al modular las neuronas aferentes en el sistema nervioso vagal (Goswami et al., 2018).

4. Consideraciones finales

Durante décadas, el brócoli ha sido el protagonista principal en la investigación sobre crucíferas. No obstante, las nuevas variedades que surgen como alternativas para el consumidor, fuentes de bioactivos para elaboraciones alimentarias y nutracéuticas, se están abriendo paso. En la presente Tesis Doctoral, se ha demostrado que el Bimi® y la col lombarda son capaces de reaccionar de manera positiva a un protocolo adecuado de elicitación, para obtener una matriz alimentaria de alto valor añadido (Capítulo I y Capítulo IV). Asimismo, ambas matrices son muy versátiles para proporcionar formulaciones con altas

concentraciones de GSLs e ITCs sin la necesidad de procesos excesivamente costosos o contaminantes (Capítulo I, Capítulo III, Capítulo IV). Este hecho es altamente beneficioso, puesto que facilitaría un futuro escalado industrial, reduciéndose los costes de producción y favoreciendo el desarrollo sostenible de nuevos productos agroalimentarios.

Por otro lado, el estudio de vesículas derivadas de membranas vegetales es cada vez más amplio, con fuentes como los cítricos, jengibre, tomate y brócoli, entre otros. En el presente manuscrito se muestra cómo el estado de maduración y el órgano vegetal influyen en las características de las vesículas vegetales (Capítulo II). Por ello, se escogieron las inflorescencias obtenidas a 90 días de edad como las óptimas, gracias a su elevada estabilidad. Además, el uso de este tipo de vegetal permite reutilizar una gran proporción de inflorescencias de destrío, lo que no influye en su extracción y son una oportunidad para disminuir la producción de residuos de cosecha.

Con respecto a la estabilidad de los ITCs en las formulaciones, los resultados obtenidos *in vitro* demuestran que las formulaciones nanoencapsuladas son capaces de conservar una mayor fracción de ITCs biodisponibles (Capítulo III y Capítulo IV). Además, esta mayor bioaccesibilidad y bioactividad se ha evaluado en modelos relacionados con la obesidad. Los resultados obtenidos tanto en el estudio de su metabolismo en líneas celulares de hepatocitos (HepG2), como su influencia en un modelo de fermentador acondicionado con el microbioma de pacientes con obesidad, sugieren que las formulaciones nanoencapsuladas podrían ser la base para el desarrollo de coadyuvantes nutricionales (como prebióticos o complementos alimenticios) que contribuyesen a mejorar la patología de pacientes que sufran obesidad o sobrepeso.

~ Conclusiones ~

Conclusiones

De los resultados obtenidos en la presente Tesis Doctoral, se han extraído las siguientes conclusiones en relación a los objetivos establecidos, siendo consideradas las más relevantes del trabajo:

Objetivo 1. Evaluación del efecto de la elicitación con metil jasmonato, ácido salicílico y su combinación en la acumulación de glucosinolatos en Bimi® y col lombarda, determinando un protocolo adecuado para cada especie (Capítulo I y Capítulo IV).

Primera. La relación elicitador-especie es clave y juega un papel diferencial según las condiciones de crecimiento y el órgano vegetal. En el caso del Bimi® cultivado en campo, se encontraron resultados óptimos para la acumulación de GSLs alifáticos e indólicos en las inflorescencias con la combinación de SA y MeJA. Para la col lombarda cultivada, la aplicación en campo de MeJA proporcionó el mayor aumento de GSLs totales, siendo los indólicos los que más se incrementaron.

Objetivo 2. Elaboración de extractos derivados de Bimi® y col lombarda enriquecidos en glucosinolatos e isotiocianatos.

Segunda. La extracción óptima de GSLs de Bimi® se determinó ente los 15 y 30 minutos de hidrodestilación, variando en función del órgano vegetal seleccionado. Así pues, la matriz de origen y la composición del extractante influyen directamente en el contenido y estabilidad de los GSLs, siendo los principales factores a considerar para la elaboración de un protocolo adecuado.

Tercera. La adición de 90d ICVs como agentes nanoencapsulates no modifica la composición de ITCs presentes en las formulaciones. Esto les proporciona a las vesículas una alta versatilidad para el diseño de formulaciones alimentarias.

Objetivo 3. Análisis y estudio de la membrana plasmática derivada de distintos órganos de la coliflor (hoja e inflorescencias), en distintos estados de

maduración, para la obtención de un material de partida óptimo como agente nanoencapsulante (Capítulo II).

Cuarta. El análisis lipídico de las membranas de las 90d ICV reveló un alto grado de insaturación de los ácidos grasos que, junto con la presencia de sitosterol, aportó mayor permeabilidad al agua de la bicapa lipídica de las vesículas.

Quinta. La elevada presencia de acuaporinas, especialmente de las familias PIP1 y PIP2, en las 90d ICVs analizadas mediante análisis proteómico se correlacionó directamente con los altos valores de permeabilidad osmótica del agua obtenidos. Esto le confiere al agente nanoencapsulante la capacidad de adaptarse a los cambios osmóticos producidos en el medio en el que se encuentra, haciéndolo más estable.

Objetivo 4. Evaluación de la estabilidad de compuestos activos presentes en los extractos nanoencapsulados durante la digestión gastrointestinal, así como la determinación de su bioactividad en modelos de obesidad (Capítulo III y Capítulo IV).

Sexta. La nanoencapsulación de ITCs con las 90d ICVs favorece su conservación durante el proceso de digestión gastrointestinal y también tras la fermentación colónica, en el caso de la col lombarda. De este modo, el agente nanoencapsulante mejoró la bioaccesibilidad y fracción disponible de ITCs e indoles bioactivos al finalizar el proceso.

Séptima. Los estudios de bioactividad realizados en la línea celular de hepatocitos HepG2 revelaron patrones distintos de metabolización de ITCs, en función del tratamiento y de las condiciones de estimulación mediante LPS, sugiriendo que en un estado de baja inflamación crónica (presente en patologías como la obesidad), se puede obtener una mayor asimilación ITCs empleando formulaciones nanoencapsuladas. Para este fin, los ITCs nanoencapsulados de col lombarda aportaron una mayor fracción de estos bioactivos a la microbiota intestinal, sin alterar la diversidad microbiana tras su tratamiento crónico durante 14 días y modulando la producción de ácidos grasos de cadena corta.

Octava. El aumento de producción de ácido butírico en el microbioma intestinal observado en los ensayos realizados con la formulación nanoencapsulada de col lombarda ha resultado de gran interés para el desarrollo de ingredientes funcionales que contribuyan a la prevención y el tratamiento del sobrepeso y la obesidad.

~ Conclusions ~

Conclusions

From the results obtained in this PhD thesis, the following conclusions have been drawn in relation to the established objectives, being considered the most relevant of the work.

Objective 1. Evaluation of the effect of elicitation with methyl jasmonate, salicylic acid and its combination on the accumulation of glucosinolates in Bimi® broccolini and red cabbage, determining a suitable protocol for each plant species (Chapter I and Chapter IV).

First. The elicitor-species relationship is crucial and plays a differential role depending on the growth conditions and the plant organ. In the case of Bimi® grown in field, finding optimal results for the accumulation of aliphatic and indolic GSLs in the inflorescences with the combination of SA and MeJA. According to red cabbage, field elicitation with MeJA provided the greatest increase in total GSLs, being the indolic the ones that increased the most.

Objective 2. Preparation of extracts derived from Bimi® and red cabbage enriched in glucosinolates and isothiocyanates (Chapter I, III and Chapter IV).

Second. The optimal extraction of GSLs from Bimi® was determined at 15 and 30 minutes of hydrodistillation, varying depending on the selected plant organ. Thus, the source matrix and the selected method directly influence the content and stability of GSLs, being the main factors to consider for developing an adequate protocol.

Third. The addition of 90d ICVs as nanoencapsulated agents does not modify the composition of ITCs present in the formulations. This provides vesicles with high versatility for food formulations desing.

Objective 3. Analysis and study of the plasmatic membrane derived from different cauliflower organs (leaves and inflorescences), in different stages of

maturation, to obtain an optimal stating material as a nanoencapsulating agent (Chapter II).

Fourth. Lipid analysis of the 90d ICV membranes revealed a high degree of fatty acids unsaturation which, together with the presence of sitosterol, contributed to greater fluidity in the lipid bilayer of the vesicles.

Fifth. The high presence of aquaporins, especially from the PIP1 and PIP2 families, in the 90d ICVs analysed by proteomic analysis correlates directly with the high values of osmotic permeability obtained. Thus, acquiring a nanoencapsulating agent capable of adapting to the osmotic changes produced in the medium in which it is found, increasing its stability.

Objective 4. Evaluation of the stability of the bioactives present in the nanoencapsulated extracts during gastrointestinal digestion, as well as the determination of their bioactivity in obesity models (*Chapter III and Chapter IV*).

Sixth. The nanoencapsulation of ITCs with the 90d ICVs favours their preservation during the gastrointestinal digestion process and also after colonic fermentation, in the case of red cabbage. In this way, the nanoencapsulating agent improved the bioaccessibility and available fraction of ITCs and bioactive indoles at the end of the process.

Seventh. Bioactivity studies carried out on the HepG2 hepatocyte cell line revealed different metabolization patterns of ITCs, depending on the treatment and the conditions of stimulation by LPS, suggesting that in a state of low chronic inflammation (present in pathologies such as obesity), a greater assimilation using nanoencapsulated formulation could be obtained. For this purpose, the nanoencapsulated red cabbage ITCs provided a higher fraction of this bioactives to the intestinal microbiota without altering the microbial diversity after chronic treatment for 14 days and modulating the production of short chain fatty acids.

Eighth. The increased production of butyric acid in the intestinal microbiome observed in the trials carried out with the nanoencapsulated

formulation of red cabbage has been of great interest for the development of functional ingredients that contribute to the prevention and treatment of overweight and obesity.

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