VNIVERSITAT DE VALÈNCIA

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

Biological activity of sterols from foods and food supplements in cardiovascular and intestinal therapeutic targets

Actividad biológica de los esteroles de alimentos y complementos alimenticios en dianas terapéuticas cardiovasculares e intestinales

DOCTORADO EN CIENCIAS DE LA ALIMENTACIÓN

TESIS DOCTORAL INTERNACIONAL

Presentada por:

Diego Miedes Sanz

Dirigida por:

Dr. Antonio Cilla Tatay

Dra. Amparo Asunción Alegría Torán

Valencia, mayo 2025

El **Dr. Antonio Cilla Tatay**, Profesor Titular de Universidad de la Universitat de València y la **Dra. Amparo Asunción Alegría Torán**, Catedrática de Universidad de la Universitat de València,

INFORMAN QUE:

D. Diego Miedes Sanz ha realizado bajo su dirección la Tesis Doctoral que lleva por título **Actividad biológica de los esteroles de alimentos y complementos alimenticios en dianas terapéuticas cardiovasculares e intestinales**, y autorizan su presentación para optar al título de Doctor por la Universitat de València.

Y para que así conste, expiden y firman el siguiente certificado.

Burjassot, 22 de mayo de 2025

Dr. Antonio Cilla Tatay **Dra. Amparo Asunción Alegría Torán**

VNIVERSITAT DE VALÈNCIA Facultat de Farmàcia i Ciències de l'Alimentació

El **Dr. Antonio Cilla Tatay**, Profesor Titular de Universidad de la Universitat de València y la **Dra. Amparo Asunción Alegría Torán**, Catedrática de Universidad de la Universitat de València,

INFORMAN QUE:

La presente Tesis Doctoral Internacional se ha realizado en el grupo Bionutest (GIUV2013-067) y se enmarca en el proyecto PID2019-104167RB-I00, financiado por el Ministerio de Ciencia e Innovación (MCIN/AEI/10.13039/501100011033) y, parcialmente, por la Generalitat Valenciana (CIAICO/2021/076).

El doctorando Diego Miedes Sanz ha disfrutado de una ayuda para la contratación de jóvenes demandantes de empleo como técnico superior de apoyo a la investigación dentro del Programa INVESTIGO (CPI-22-450) de la Generalitat Valenciana, en el periodo entre noviembre 2022-2024.

El doctorando Diego Miedes Sanz ha disfrutado de una beca para estancias internacionales predoctorales en instituciones de países del Programa Erasmus+, cofinanciadas por la Comisión Europea y la Universitat de València, realizada en *Alma Mater Studiorum-Università di Bologna*, Bolonia (Italia), entre el 1 de mayo y el 1 de agosto de 2024.

ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

En Burjassot, a 22 de mayo de 2025

Dr. Antonio Cilla Tatay **Dra. Amparo Asunción Alegría Torán**

AGRADECIMIENTOS

Pese a que habitualmente se lee que escribir los agradecimientos es una de las partes más complicadas de una tesis doctoral, creo que tengo tanto que agradecer y a tanta gente, que esto no podría ser así en mi caso.

En primer lugar, por supuesto, tengo que dar una y mil veces las gracias a las dos personas que me han acompañado en este recorrido desde el día que comencé con mi trabajo final de máster, mis directores. Toni, fue contigo con quien en primer lugar contacté para introducirme en este mundo, todo por el buen recuerdo que guardaba de aquellas clases en 2015 (10 años, como pasa el tiempo) y de la cercanía para comunicar ciencia y nutrición. Aprecio mucho toda la dedicación que tienes por la docencia, incluyendo a un doctorando como yo. Sobre todo, esas veces en las que al volver de Cultivos y verte en la cafetería, no podías aguantar tus ganas de saber "cómo ha ido". Eso es interés y pasión, y quiero que sepas que es algo muy valioso. Gracias por todo. Amparo, lo creas o no, has causado en mí un cambio en muchos aspectos a nivel laboral (y personal). Pese a que aún todavía guardo algunos vicios y aspectos a mejorar, soy hoy mejor profesional de lo que era, sin duda. He tenido una suerte enorme de tener como directora a una apasionada de la docencia como lo eres tú. Tu insistencia en convencerme en ser metódico (pese a que siempre existieron y existirán los tira y afloja) ha sido algo vital para mí. Has estado siempre dispuesta a invertir tu tiempo conmigo para enseñar, predicando con el ejemplo, que es la forma más efectiva. A ambos, gracias por tratarme a nivel personal siempre de una manera profesional, respetuosa y, sobre todo, humana.

Por supuesto, no puedo olvidarme de otras personas como Lupe. Siempre me has aportado esa dosis de positividad y energía que desprendes habitualmente. También tu apoyo y ayuda en momentos críticos con equipos y el liderazgo que has ejercido cuando ha sido necesario. También agradecer a Reyes por sus consejos, su buen humor y por interesarse siempre por mí. Echaré de menos los sustos que a veces me llevo cuando entras en el laboratorio llamando a alguien.

Por razones de espacio, no puedo dedicar unas palabras a cada persona que me gustaría, pero me gustaría dar las gracias a los otros miembros del grupo, Mónica y Amparo; a nuestros "vecinos" de Nutraliment, los profesores Jesús, Mª José, Ana y compañía; a mis casi vecinas de escritorio, las profesoras Ana y Cristina Juan; a todos los miembros de la secretaría del departamento, los que estuvieron o están, especialmente a

Carmen, que me ha sacado de algún que otro laberinto y que siempre ha transmitido la mejor de las actitudes; al personal de la sección de cultivos celulares por su gran trabajo y cercanía; al personal del Hospital Clínico (Pilar, Sergio), de la facultad de Medicina (Raquel) y, por supuesto, a todos y cada uno de los voluntarios que contribuyen a la ciencia con su tiempo y su voluntad, solo puedo decir GRACIAS.

Una tesis en soledad sería difícilmente conseguible para mí y para cualquiera. Los que están en tu misma situación son los que mejor pueden entenderte, escucharte y ayudarte a pasar los malos tragos (y los buenos). Por tanto, tengo que agradecer especialmente a todos mis amigos y compañeros del laboratorio. Mussa, por los cientos (miles) de charlas. Nerea, por tus admirables tesón y buen hacer. Sonia, por tu tranquilidad y tu inestimable ayuda. Virginia, por escucharme tan bien. Marta, por nuestras interminables comidas. Leo, por tu gran humor. Matteo, por tus ideas científicas y no científicas. Clara, por ofrecer siempre tu hombro. Adriana, por esa calma y saber estar. A los que han venido durante un tiempo o forman parte de otro laboratorio y han aportado su experiencia, compañía, amistad y, en muchos casos, idioma (Fran, Ignazio, Romy, Ana María, Sebastián, Sofía, Ilenia, Anna, Antonia, Valentina, Juanfran…). A Raquel, por estar siempre ahí y confiar en mí desde "el otro lado". A todos y muchos más que han pasado por aquí, gracias por haber contribuido a todo esto.

Gracias a la profesora Maria y a Dario por acogerme en la preciosa Bologna en una etapa que, de diversas maneras, produjo un cambio en mi persona. Gracias por dejarme trabajar con vosotros y compartir vuestros conocimientos. No puedo hablar de esta ciudad sin pararme a agradecer a mis compañeras, que, pese al corto periodo, se convirtieron en fundamentales para mí. Claudia "chupeta", por tu incansable ánimo y energía con todo lo que haces. Giulia, por tu cariño y cercanía desde el primer día conmigo. Y por supuesto, Sofía, porque fuiste el mayor y mejor respaldo para mí en distintos momentos allí. Os recuerdo mucho y no puedo más que agradeceros todo.

A todos mis amigos de fuera de este "mundo", por aportar a la vida en general vuestro granito de arena y ayudarme a transitar por este camino.

A mi compañera, colega y, por encima de todo, amiga, por ser siempre el bastón cuando es necesario, el refugio cuando no se puede más, la calma cuando no se encuentra y la emoción siempre que vale la pena. Podría dedicarte un capítulo entero solo con todo lo

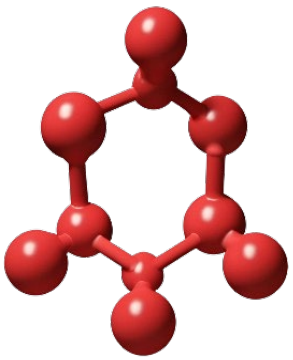
que tengo que agradecerte y espero estar cada día agradeciendo la suerte que tengo de que estés junto a mí. Esta tesis es tuya también, Maite.

Pero todo esto que he escrito no sería posible ni tendría ningún sentido sin las dos personas que no solo me trajeron a este mundo, sino que me han acompañado cada día de mi vida en todos los caminos que he seguido. Gracias papá y gracias mamá. Vuestros valores y educación han tenido mucho que ver, no quizá en lo que aquí se vea escrito, pero en la relación, actitud y comportamiento que he tenido con todos los que forman parte de este viaje. Gracias por apoyarme de tantas maneras y transmitir vuestra sabiduría de vida. Dadles las gracias a los abuelos por haberos criado así y transmitírmelo a mí. Una parte de mí y, por tanto, una parte de todos ellos está también aquí en estas páginas.

Muchísimas gracias a cada persona que ha estado ahí en algún momento, por pequeño que sea.

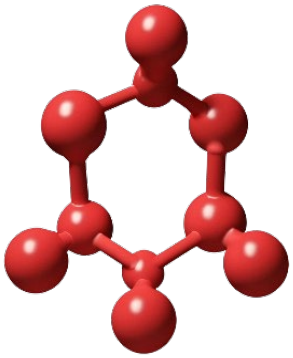
“Es el trabajo que nunca ha comenzado el que tarda más en terminar”

John Ronald Reuen Tolkien



Índice

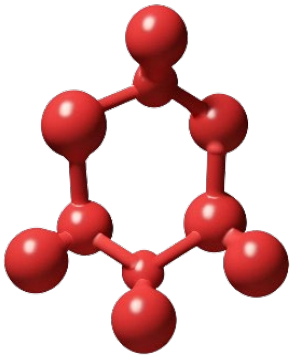
Index



ÍNDICE GENERAL / GENERAL INDEX

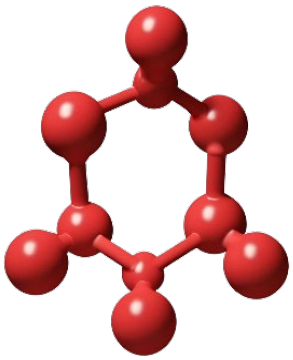
ÍNDICE DE FIGURAS / *FIGURE INDEX*

ÍNDICE DE TABLAS / *TABLE INDEX*



Resumen

Abstract



Las enfermedades cardiovasculares y el cáncer son responsables de la mayor parte de las muertes en el mundo desarrollado. El cáncer colorrectal se sitúa como uno de los más prevalentes y causantes de más defunciones a nivel mundial. Los esteroles vegetales (EV) son compuestos análogos al colesterol con reconocido efecto hipocolesterolemiante debido a la disminución de la absorción intestinal del colesterol. Los mecanismos incluyen la competición por la micelarización y por ser también sustrato de enzimas implicados en la absorción del colesterol a través del enterocito y su distribución sanguínea.

La dieta occidental no proporciona la cantidad recomendada de EV para obtener su efecto hipocolesterolemiante. Únicamente los patrones dietéticos basados en alimentos de origen vegetal, como son las dietas veganas, proporcionan cantidades significativas de estos compuestos. Por esta razón, son necesarias estrategias como el enriquecimiento de alimentos y el uso de complementos alimenticios con EV. Estos últimos tienen como principal ventaja proporcionar la ingesta recomendada de EV en una única toma y con facilidad para el consumidor, que no debe alterar su patrón dietético. Por otra parte, para el enriquecimiento con EV, originalmente las margarinas se han utilizado en mayor medida. Posteriormente, han surgido otras matrices con mejor perfil nutricional tales como las bebidas de fruta a base de leche o el pan integral de centeno, que la normativa europea permite enriquecer con EV.

Las personas de avanzada edad presentan diversas condiciones fisiopatológicas que afectan al riesgo de enfermedad cardiovascular. En este grupo de población, los EV añadidos a alimentos podrían ver modificada su bioaccesibilidad por las condiciones gastrointestinales del anciano. Entre ellas destacan la peor salud oral (incluyendo pérdida de piezas dentales), disminución general de secreción enzimática y ralentización de todas las etapas de la digestión, desde el estómago hasta el colon.

Es bien conocido el efecto hipocolesterolemiante de los EV presentes en algunos alimentos enriquecidos (pueden disminuir la colesterolemia hasta en un 12,5%). Sin embargo, se ha demostrado que poseen efecto antiproliferativo a nivel intestinal en modelos celulares y murinos, reduciendo la viabilidad de células tumorales, aumentando la apoptosis, regulando el ciclo celular y deteniendo su progresión, y modificando la transcripción de genes responsables tanto de la señalización o el desarrollo de la apoptosis, como del control del ciclo celular. Asimismo, han mostrado efecto antieriptótico en modelo *ex vivo*, permitiendo reducir la externalización de fosfatidilserina

en eritrocitos humanos. Este parámetro es un factor de riesgo para enfermedades cardiovasculares debido a su participación en los eventos ateroscleróticos, ya que los sujetos con hipercolesterolemia presentan niveles superiores de eriptosis respecto a los sujetos normocolesterolémicos.

El metabolismo del colesterol puede generar distintos compuestos potencialmente perjudiciales para la salud, entre los que destacan los óxidos del colesterol. Estos se generan durante la elaboración de los alimentos y también están presentes en plasma ya que se forman en el organismo mediante enzimas o por el estrés oxidativo. Entre otros efectos perjudiciales, provocan un mayor estado inflamatorio en el endotelio vascular, aumentando el riesgo de enfermedades cardiovasculares. La mayor concentración de colesterol en plasma de los pacientes con hipercolesterolemia les predispone a presentar niveles superiores de óxidos de colesterol. Por ello, los EV podrían suponer una buena opción para controlar este parámetro.

El objetivo general de la presente Tesis Doctoral es evaluar la bioaccesibilidad y efectos biológicos de los EV, asociados a la ingesta de alimentos o complementos alimenticios enriquecidos con estos, mediante una doble aproximación complementaria *in vitro* e *in vivo*.

Para ello, se desarrolla el siguiente plan de trabajo: (i) caracterización de los EV y su estabilidad en la elaboración de un pan integral de centeno, (ii) comparación de la bioaccesibilidad de EV, bajo condiciones fisiológicas digestivas del anciano, de un pan integral de centeno y una bebida de fruta a base de leche enriquecidos con EV, (iii) evaluación *in vitro* del efecto antiproliferativo de los líquidos de fermentación del pan integral de centeno con y sin EV a nivel colorrectal, y (iv) estudio del efecto antieriptótico, hipocolesterolemiante, sobre la adherencia al endotelio y la oxidación del colesterol de un complemento alimenticio con EV en sujetos hipercolesterolémicos tratados con estatinas.

A continuación, se resume la metodología empleada para cada punto del plan de trabajo:

(i) En primer lugar, se elabora en planta piloto un pan integral de centeno enriquecido con EV (porción individual de 80g, que aporta 2,18g de EV al día). Para ello se adiciona un ingrediente microencapsulado en polvo (Lypophytol® ME dispersable palm free) a la masa constituida por harina integral de centeno, agua, levadura, sal y ácido ascórbico.

Para evaluar la estabilidad del enriquecimiento, se realizan 3 horneados en 3 días diferentes con condiciones idénticas (misma harina, tiempos y fermentación). Paralelamente, se elabora a nivel industrial una bebida de fruta a base de leche (250 mL / ración, que aporta 2,3g de EV al día). Se utilizan como ingredientes leche desnatada, grasa láctea y concentrado de proteína de suero enriquecida con membrana de glóbulos de grasa láctea, zumo de mandarina a partir de concentrado y puré de plátano, a la que también se adicionan EV con un ingrediente (Lypophytol® 146 ME Dispersible).

(ii) El pan integral de centeno y la bebida de fruta a base de leche se someten a una digestión gastrointestinal simulada mediante el método estático INFOGEST 2.0, que comprende las fases oral, gástrica e intestinal. Paralelamente, se preparan blancos de digestión con agua. Se adapta el método a las condiciones fisiológicas del anciano en base a datos publicados en la bibliografía: se aumenta el tiempo de digestión de las fases gástricas e intestinal de 2 a 3 horas; se reduce la actividad de lipasa gástrica (85 %), pepsina (50%) y pancreatina (50%), así como de las sales biliares (50%); se aumenta el pH gástrico de 3 a 6; se disminuye la frecuencia de agitación (de 100 a 50 rpm). Se obtiene la fracción bioaccesible de cada matriz por centrifugación y se determinan los esteroles de las fracciones bioaccesibles y de las matrices sin digerir mediante cromatografía de gases acoplada a detector de ionización de llama. Se cuantifican con curvas de calibrado con la adición de patrón interno (epicoprostanol) y, tras ello, se calcula la bioaccesibilidad de los esteroles.

(iii). Para evaluar la actividad antiproliferativa del pan integral de centeno con o sin EV se aplica un sistema de digestión gastrointestinal semidinámico (simgi®) que incluye los tramos del colon humano (ascendente, transversal y descendente). Previamente se realiza una digestión gastrointestinal en discontinuo debido a la dificultad de introducir un alimento sólido directamente en los reactores. El sistema se inocula con la microbiota de un donante sano y se estabiliza durante 14 días. Tras la digestión de los dos panes, con un periodo de lavado entre ellos de 9 días, se toman muestras de los líquidos de fermentación (LF) en el colon descendente de ambos panes y de los blancos de estabilización y lavado. Se filtran y diluyen con medio de cultivo para preservar la viabilidad celular. Como modelo tumoral se utilizan células de adenocarcinoma de colon (Caco-2) y como modelo no tumoral, células normales del colon (CCD-18co). Se evalúa por citometría de flujo la apoptosis/necrosis, la progresión del ciclo celular y los contenidos intracelulares de calcio, ceramida, glutatión reducido y especies reactivas de

oxígeno. Se determina la transcripción de diversos genes responsables de la apoptosis (*BAX*, *BCL2*, *CASP3*, *CASP8* y *CASP9*), así como de genes relacionados con la progresión del ciclo celular (*CDKN1A*, *TP53*, *CCND1* y *CCNE1*) mediante PCR cuantitativa. Para comprender y explicar las posibles diferencias del efecto antiproliferativo entre ambos panes, se determina adicionalmente el contenido en ácidos grasos de cadena corta mediante cromatografía de gases (acetato, butirato, propionato y valerato) y la actividad antioxidante mediante espectrofotometría y fluorimetría (polifenoles totales y capacidad de absorción de radicales de oxígeno, ORAC).

(iv) Se realiza un ensayo clínico aleatorizado doble ciego controlado con placebo (aceptado por el comité de ética del Hospital Clínico Universitario de Valencia, número de aprobación 2023/069) en pacientes con hipercolesterolemia tratados con estatinas. Para ello se elabora un complemento alimenticio con EV en polvo, utilizando el mismo ingrediente que para el pan integral de centeno, que los participantes ingieren diariamente durante 6 semanas. Se toman muestras de sangre al inicio y al final del tratamiento. Inmediatamente, se aíslan los eritrocitos (por centrifugación) y se separan del plasma (que se almacena a -80ºC hasta su análisis). En los eritrocitos se determina la externalización de fosfatidilserina, el tamaño celular y el glutatión reducido, todas mediante citometría de flujo, para estudiar el estado eriptótico de cada sujeto. Además, se elabora un modelo de endotelio humano a partir de células primarias de vena umbilical humana (HUVECs), por donde se hace pasar los eritrocitos. Mediante grabación de vídeo, se calcula su adhesión al endotelio vascular. En plasma, se evalúan glucosa, colesterol total, triglicéridos, colesterol HDL y LDL, Apo A1, Apo B, proteína C reactiva de alta sensibilidad e insulina, y se calcula el índice de resistencia a la insulina. Asimismo, se determinan los óxidos de colesterol plasmáticos por cromatografía de gases acoplada a espectrometría de masas, previa saponificación y extracción del insaponificable y purificación por extracción en fase sólida. Se cuantifican con curvas de calibrado con patrón interno (19-hidroxi colesterol). El colesterol se cuantifica con la misma técnica sin purificación previa, utilizando como patrón interno el betulinol. Al inicio del tratamiento, se realizan encuestas de estilos de vida a cada sujeto: adherencia a la dieta Mediterránea (cuestionario MEDAS) y actividad física (cuestionario IPAQ-*short*). Los resultados se estratifican en función de la edad, el sexo, el índice de masa corporal y la adherencia al tratamiento (medida por el número de sobres de suplemento no consumidos). Además, se

estudian correlaciones entre los parámetros sanguíneos anteriormente incluidos y los estilos de vida de los participantes.

Los principales resultados que se obtuvieron en los distintos objetivos son los siguientes:

(i) Se confirma la estabilidad de los EV del pan integral de centeno enriquecido ya que no se observan diferencias en los contenidos de EV totales entre los 3 horneados (2,14 – 2,23g / 100g pan integral de centeno). Se detectan 9 EV tanto en el ingrediente como en los panes, en orden decreciente: β-sitosterol, sitostanol, campesterol, campestanol, Δ5-avenasterol, Δ7-estigmastenol, estigmasterol, Δ7-avenasterol y Δ5,24-estigmastadienol. El perfil de EV es similar entre el pan y el ingrediente fuente de estos, mostrando que, pese a que los cereales son alimentos ricos en EV, la porción mayoritaria de EV en el pan integral de centeno enriquecido es proporcionada por el ingrediente.

(ii) Los EV muestran un mayor contenido en la fracción bioaccesible de la bebida de fruta a base de leche en condiciones gastrointestinales del anciano en comparación con el adulto (136 vs. 73 mg EV / 100 g de bebida). El orden en contenido decreciente de esteroles individuales es igual en ambas condiciones (β-sitosterol, sitostanol, campesterol, colesterol, campestanol y estigmasterol). La bioaccesibilidad de EV es más elevada en condiciones del anciano que de adulto (14,9 vs. 8,0 %). El rango de bioaccesibilidad de los EV individuales es de 14,4 a 17,2 % en condiciones de anciano, mientras que en condiciones de adulto es de 5,5 a 16,6 %. De ellos, el estigmasterol es el esterol que más aumenta su bioaccesibilidad (un 162%) y el campesterol el que menos (un 58%).

El contenido de EV en la fracción bioaccesible del pan integral de centeno disminuye en condiciones gastrointestinales del anciano en comparación con el adulto (256 vs. 459 mg EV / 100 g de pan). El orden en contenido decreciente de esteroles individuales es igual en ambas condiciones (β-sitosterol, sitostanol, campesterol, campestanol, Δ5-avenasterol, Δ7-estigmastenol, Δ7-avenasterol, estigmasterol y Δ5,24-estigmastadienol). La bioaccesibilidad también disminuye en condiciones de anciano respecto al adulto (11,2 vs. 20,3 %). El rango de bioaccesibilidad de los EV individuales es de 10,9 a 20,5 % en condiciones de anciano, mientras que en condiciones de adulto es de 19,1 a 36,3 %. La reducción es homogénea entre los distintos EV, con un intervalo de un 39 a un 49% de disminución (Δ5-avenasterol y estigmasterol, respectivamente).

(iii) Los LF del pan enriquecido con EV y sin enriquecer diluidos con medio de cultivo (1/5, v/v) muestran un efecto citotóxico selectivo para células de adenocarcinoma de colon (modelo Caco-2), sin afectar a la viabilidad de las células normales del colon (modelo CCD-18co). Dicho efecto se observa tanto con pan enriquecido como sin enriquecer, aunque se aprecian diferencias entre ellos en función del ensayo. La viabilidad celular (medida mediante el ensayo de actividad mitocondrial MTT) se reduce en mayor medida con los LF del pan enriquecido en comparación a sin enriquecer (34 vs. 15 % de reducción). La apoptosis (mediante el ensayo de Anexina / yoduro de propidio) es mayor con los LF del pan enriquecido debido al aumento en la apoptosis tardía que en los del pan sin enriquecer (47 vs. 9 % de aumento). Esto provoca que el porcentaje de células viables disminuya en mayor medida (35 vs. 21 % de reducción).

De modo opuesto, en el ensayo que evalúa la progresión del ciclo celular, la proporción de células en fase Sub G1 (células que presentan un contenido de ADN inferior al normal y, por tanto, son apoptóticas) es superior en los LF del pan sin enriquecer que en los del pan enriquecido (132 vs. 116 % de aumento). Esto provoca una reducción en ambos casos del porcentaje de células en el resto de las fases del ciclo celular (G0/G1, S y G2/M). La disminución del glutatión reducido es mayor tras el tratamiento con los LF del pan sin enriquecer que en los del pan enriquecido (indicando mayor estrés oxidativo para la célula) (36 vs. 18 % de reducción). El incremento del calcio intracelular es más alto tras el tratamiento con los LF del pan sin enriquecer que en los del pan enriquecido (indicando apoptosis) (134 vs. 27 % de aumento).

Ambos líquidos de fermentación, tras 3 horas de tratamiento, activan el gen de la caspasa 8 (40 y 8 % de aumento con LF de pan enriquecido y sin enriquecer, respectivamente). Tras 6 horas, el LF del pan sin enriquecer activa el gen de la caspasa 3, mientras que el del pan enriquecido produce un aumento no significativo. Ambos activan el gen responsable de la proteína supresora de tumores p53 (166 vs. 37 % de aumento con LF de pan enriquecido y sin enriquecer, respectivamente, tras 6 horas). Además, disminuyen la transcripción del gen que codifica la ciclina D1 (un 55% en ambos casos tras 3 horas), reguladora del ciclo celular. Esto sugiere que la ingesta de ambos panes podría proporcionar un efecto protector a nivel gastrointestinal equivalente, debido al contenido en fibra, polifenoles y compuestos antioxidantes, aunque el enriquecimiento con EV podría suponer un beneficio extra en algunos casos.

(iv) La ingesta de un complemento alimenticio con 2 g de EV durante 6 semanas en pacientes hipercolesterolémicos tratados con estatinas no produce cambios (antes vs. después del tratamiento) estadísticamente significativos en el colesterol total (172 vs. 168 mg/dL) y LDL (99 vs. 95 mg/dL) plasmáticos. Los marcadores de eriptosis no mejoran: la externalización de fosfatidilserina (32,9 vs. 31,7 %), los niveles de glutatión intracelular (300 vs. 266 media geométrica) ni el tamaño celular (519 vs. 518 media geométrica).

Se detectan 6 óxidos del colesterol en plasma, en orden decreciente (μg/mL): β-epoxicolesterol (0,29-0,48), 7-cetocolesterol (0,26-0,43), α-epoxicolesterol (0,21-0,26), 7β-hidroxicolesterol (0,14-0,22), colestanotriol (0,11-0,14) y 7α-hidroxicolesterol (0,11-0,13). El contenido de óxidos de colesterol totales no disminuye tras las 6 semanas de tratamiento con el complemento con EV (1,42 vs. 1,47 μg/mL antes y después del tratamiento, respectivamente).

Solo se produce un aumento de los valores de ApoA1 (6,4 %), así como una disminución de la adhesión de los eritrocitos al endotelio vascular (55%), tras la ingesta del complemento con EV. Además se obtiene una correlación inversa entre ambos parámetros en los sujetos que consumen el complemento con EV al final del tratamiento. Estos resultados sugieren que ambos factores están relacionados y que podrían tener efecto beneficioso en la salud cardiovascular de esta población debido principalmente a un menor riesgo de aterosclerosis (desencadenada por adhesión al endotelio de plaquetas o eritrocitos).

Los participantes del estudio, en general, siguen el patrón de la dieta Mediterránea, ya que un 27% de los participantes se clasifican en el grupo de alta adherencia y los restantes en el grupo de adherencia moderada. El 54 % de los sujetos presenta baja actividad física, mientras que solo un 11,5% presentan alta actividad física. Al evaluar posibles correlaciones del estilo de vida (dieta y ejercicio) con los parámetros evaluados anteriormente, no se obtiene ninguna correlación ni asociación estadísticamente significativa entre ellos.

Las principales conclusiones que se extraen de la presente Tesis Doctoral son:

La bebida de fruta a base de leche enriquecida con EV presenta una bioaccesibilidad superior tras ser digerida bajo las condiciones gastrointestinales del anciano respecto al adulto (14,9 *vs*. 8,0 %), mientras que en el pan integral de centeno enriquecido con EV es

inferior al adulto (11,2 *vs*. 20,3 %), debido a factores como la influencia de la matriz (sólida/líquida) y la actividad de las enzimas, entre otras.

Los LF del pan enriquecido muestran un efecto antiproliferativo más marcado en cuanto a la viabilidad celular y la apoptosis, mientras que los LF del pan sin enriquecer inducen un efecto antiproliferativo más notable en la regulación del ciclo celular, así como en los niveles de glutatión reducido y calcio intracelular. Ambos elevan la expresión de genes pro-apoptóticos y de regulación del ciclo celular.

El consumo del complemento con EV no mejora el perfil lipídico, la oxidación del colesterol, ni los marcadores de eriptosis en sujetos con hipercolesterolemia tratados con estatinas. Sin embargo, aumenta la concentración de ApoA1 y disminuye la adhesión de los eritrocitos al endotelio vascular, lo que supone un factor protector frente a la aterosclerosis.

De todo ello se puede concluir que el enriquecimiento con EV de alimentos con un buen perfil nutricional, como son el pan integral de centeno o las bebidas de fruta a base de leche es una opción adecuada para diversos grupos poblacionales. Esto incluye a las personas mayores, quienes frecuentemente presentan alteraciones en los niveles de colesterol, así como a individuos que buscan prevenir enfermedades crónicas mediante una alimentación saludable. Además, el consumo de complementos alimenticios con EV, pueden suponer una estrategia eficaz y segura para contribuir a la disminución de factores de riesgo cardiovascular, especialmente en personas con hipercolesterolemia tratadas con estatinas.

Cardiovascular diseases and cancer are responsible for the majority of deaths in the developed world. Colorectal cancer is one of the most prevalent cancers and causes the most deaths worldwide. Plant sterols (PS) are cholesterol analogs with a recognized cholesterol-lowering effect due to decreased intestinal absorption of cholesterol. The mechanisms include competition for micellarization and also being a substrate for enzymes involved in the absorption of cholesterol through the enterocyte and its distribution in the blood.

The Western diet does not provide the recommended amount of PSs to obtain their hypocholesterolemic effect. Only dietary patterns based on foods of plant origin, such as vegan diets, provide significant amounts of these compounds. For this reason, strategies such as food fortification and the use of dietary supplements with PS are necessary. The main advantage of the latter is that they provide the recommended intake of PSs in a single intake and with ease for the consumer, who does not have to alter his or her dietary pattern. On the other hand, for fortification with PS, originally margarines have been used to a greater extent. Subsequently, other matrices with a better nutritional profile have emerged, such as milk-based fruit beverages or wholemeal rye bread, which European regulations allow PS fortification.

Elderly people present various pathophysiological conditions that affect the risk of cardiovascular disease. In this population group, the bioaccessibility of PSs added to foods could be modified by the gastrointestinal conditions of the elderly. These include poorer oral health (including loss of teeth), a general decrease in enzyme secretion, and slowing of all stages of digestion from the stomach to the colon.

The hypocholesterolemic effect of PSs present in some enriched foods is well known (they can reduce cholesterolemia by up to 12.5%). However, it has been demonstrated that they have an antiproliferative effect at intestinal level in cellular and murine models, reducing the viability of tumor cells, increasing apoptosis, regulating the cell cycle and stopping its progression, and modifying the transcription of genes responsible for signaling or the development of apoptosis, as well as the control of the cell cycle. They have also shown an anti-eryptotic effect in an ex vivo model, reducing phosphatidylserine externalization in human erythrocytes. This parameter is a risk factor for cardiovascular diseases due to its participation in atherosclerotic events, since subjects with hypercholesterolemia present higher levels of erythrocytosis than normocholesterolemic subjects.

Cholesterol metabolism can generate various compounds that are potentially harmful to health, including cholesterol oxides. These are generated during food processing and are also present in plasma as they are formed in the body by enzymes or oxidative stress. Among other detrimental effects, they cause an increased inflammatory state in the vascular endothelium, increasing the risk of cardiovascular disease. The higher plasma cholesterol concentration in patients with hypercholesterolemia predisposes them to have higher levels of cholesterol oxides. Therefore, PSs could be a good option for controlling this parameter.

The general objective of this Ph.D. Thesis is to evaluate the bioaccessibility and biological effects of PSs, associated with the intake of food or food supplements enriched with PSs, by means of a double complementary approach *in vitro* and *in vivo*.

For this purpose, the following work plan is developed: (i) characterization of PSs and their stability in the preparation of a whole-grain rye bread, (ii) comparison of PS bioaccessibility, under physiological digestive conditions of the elderly, of a whole-grain rye bread and a milk-based fruit beverage enriched with PSs, (iii) *in vitro* evaluation of the antiproliferative effect of rye bread fermentation liquids with and without PS at colorectal level, and (iv) study of the antieryptotic, hypocholesterolemic, endothelial adhesion and cholesterol oxidation effect of a food supplement with PS in hypercholesterolemic subjects treated with statins.

The methodology used for each point of the work plan is summarized below:

(i) First, a whole-grain rye bread enriched with PS (80g individual portion, providing 2.18g of PS per day) is produced in a pilot plant. For this purpose, a microencapsulated powdered ingredient (Lypophytol® ME dispersible palm free) is added to the dough consisting of wholemeal rye flour, water, yeast, salt and ascorbic acid. To evaluate the stability of the enrichment, 3 baking cycles are carried out on 3 different days with identical conditions (same flour, times and fermentation). In parallel, a milk-based fruit beverage (250 mL/serving, providing 2.3 g of PS per day) is produced on an industrial scale. Skimmed milk, milk fat and whey protein concentrate enriched with milk fat globule membrane, mandarin juice from concentrate and banana puree are used as ingredients, to which PS is also added with an ingredient (Lypophytol® 146 ME Dispersible).

(ii) Wholemeal rye bread and milk-based fruit beverage are subjected to simulated gastrointestinal digestion using the INFOGEST 2.0 static method, comprising oral, gastric and intestinal phases. In parallel, digestion blanks are prepared with water. The method is adapted to the physiological conditions of the elderly based on data published in the literature: the digestion time of the gastric and intestinal phases is increased from 2 to 3 hours; the activity of gastric lipase (85%), pepsin (50%) and pancreatin (50%) as well as bile salts (50%) is reduced; the gastric pH is increased from 3 to 6; the frequency of agitation is decreased (from 100 to 50 rpm). The bioaccessible fraction of each matrix is obtained by centrifugation and the sterols of the bioaccessible fractions and the undigested matrices are determined by gas chromatography coupled to a flame ionization detector. They are quantified with calibration curves with the addition of internal standard (epicoprostanol) and, after that, the bioaccessibility of the sterols is calculated.

(iii). To evaluate the antiproliferative activity of wholemeal rye bread with or without PS, a semi-dynamic gastrointestinal digestion system (simgi®) including the sections of the human colon (ascending, transverse and descending) is applied. Previously, discontinuous gastrointestinal digestion is performed due to the difficulty of introducing a solid food directly into the reactors. The system is inoculated with the microbiota of a healthy donor and stabilized for 14 days. After digestion of the two breads, with a 9-day washout period between them, the fermentation liquids (FL) are sampled in the descending colon of both breads and the stabilization and washout blanks. They are filtered and diluted with culture medium to preserve cell viability. Colon adenocarcinoma cells (Caco-2) are used as a tumor model and normal colon cells (CCD-18co) are used as a non-tumor model. Apoptosis/necrosis, cell cycle progression and intracellular contents of calcium, ceramide, reduced glutathione and reactive oxygen species are evaluated by flow cytometry. Transcription of several genes responsible for apoptosis (BAX, BCL2, CASP3, CASP8 and CASP9), as well as genes related to cell cycle progression (CDKN1A, TP53, CCND1 and CCNE1) are determined by quantitative PCR. To understand and explain the possible differences in the antiproliferative effect between the two breads, the short-chain fatty acid content is additionally determined by gas chromatography (acetate, butyrate, propionate and valerate) and the antioxidant activity by spectrophotometry and fluorimetry (total polyphenols and oxygen radical absorbance capacity, ORAC).

(iv) A randomized, double-blind, placebo-controlled clinical trial (accepted by the ethics committee of the Hospital Clínico Universitario de Valencia, approval number 2023/069) was conducted in patients with hypercholesterolemia treated with statins. For this purpose, a food supplement with powdered PS is prepared, using the same ingredient as for wholemeal rye bread, which the participants ingest daily for 6 weeks. Blood samples are taken at the beginning and end of treatment. Immediately, erythrocytes are isolated (by centrifugation) and separated from plasma (which is stored at -80°C until analysis). In erythrocytes, phosphatidylserine externalization, cell size and reduced glutathione are determined, all by flow cytometry, to study the erythrocyte status of each subject. In addition, a model of human endothelium is made from primary human umbilical vein cells (HUVECs), through which erythrocytes are passed. By video recording, their adhesion to the vascular endothelium is calculated. In plasma, glucose, total cholesterol, triglycerides, HDL and LDL cholesterol, Apo A1, Apo B, high-sensitivity C-reactive protein and insulin are evaluated, and the insulin resistance index is calculated. Plasma cholesterol oxides are also determined by gas chromatography coupled to mass spectrometry, after saponification and extraction of the unsaponifiables and purification by solid-phase extraction. They are quantified with calibration curves with internal standard (19-hydroxy cholesterol). Cholesterol is quantified with the same technique without prior purification, using betulinol as internal standard. At the start of treatment, lifestyle surveys were carried out on each subject: adherence to the Mediterranean diet (MEDAS questionnaire) and physical activity (IPAQ-short questionnaire). The results are stratified according to age, sex, body mass index and adherence to treatment (measured by the number of supplement sachets not consumed). In addition, correlations between the previously included blood parameters and the participants' lifestyles are studied.

The main results obtained for the different objectives are as follows:

(i) The stability of the PSs of the enriched wholemeal rye bread is confirmed as no differences are observed in the total PS contents between the 3 baking (2.14 - 2.23g / 100g wholemeal rye bread). Nine PSs are detected in both the ingredient and the breads, in decreasing order: β-sitosterol, sitostanol, campesterol, campestanol, Δ5-avenasterol, Δ7-stigmastenol, stigmasterol, Δ7-avenasterol and Δ5,24-stigmastadienol. The profile of PSs is similar between bread and their source ingredient, showing that, despite cereals being

PS-rich foods, the majority portion of PSs in enriched whole-grain rye bread is provided by the ingredient.

(ii) PSs show a higher content in the bioaccessible fraction of the milk-based fruit beverage under gastrointestinal conditions in the elderly compared to the adult (136 vs. 73 mg PS / 100 g of beverage). The order in decreasing content of individual sterols is equal in both conditions (β-sitosterol, sitostanol, campesterol, cholesterol, campestanol and stigmasterol). The bioaccessibility of PS is higher in elderly than in adult conditions (14.9 vs. 8.0 %). The range of bioaccessibility of individual PSs is 14.4 to 17.2 % in elderly conditions, while in adult conditions it is 5.5 to 16.6 %. Of these, stigmasterol is the sterol that increases its bioaccessibility the most (by 162%) and campesterol the least (by 58%).

The PS content in the bioaccessible fraction of wholemeal rye bread decreases in gastrointestinal conditions of the elderly compared to the adult (256 vs. 459 mg PS / 100 g of bread). The order in decreasing content of individual sterols is equal in both conditions (β-sitosterol, sitostanol, campesterol, campestanol, Δ5-avenasterol, Δ7-stigmastenol, Δ7-avenasterol, stigmasterol and Δ5,24-stigmastadienol). Bioaccessibility also decreases in elderly conditions with respect to adult (11.2 vs. 20.3 %). The range of bioaccessibility of individual PSs is 10.9 to 20.5 % in elderly conditions, while in adult conditions it is 19.1 to 36.3 %. The reduction is homogeneous among the individual PSs, with a range of 39 to 49 % decrease (Δ5-avenasterol and stigmasterol, respectively).

(iii) FLs from PS-enriched and non-enriched bread diluted with culture medium (1/5, v/v) show a selective cytotoxic effect on colon adenocarcinoma cells (Caco-2 model), without affecting the viability of normal colon cells (CCD-18co model). This effect was observed with both enriched and non-enriched bread, although differences between them were observed depending on the assay. Cell viability (measured by MTT mitochondrial activity assay) is reduced to a greater extent with FL from enriched compared to non-enriched bread (34 vs. 15% reduction). Apoptosis (by Annexin/propidium iodide assay) is higher with FLs from enriched bread due to increased late apoptosis than those from non-enriched bread (47 vs. 9 % increase). This causes the percentage of viable cells to decrease to a greater extent (35 vs. 21 % decrease).

Conversely, in the assay assessing cell cycle progression, the proportion of cells in the Sub G1 phase (cells with lower-than-normal DNA content and therefore apoptotic) is

higher in the FL of the non-enriched bread than in those of the enriched bread (132 vs. 116% increase). This causes a reduction in both cases of the percentage of cells in the remaining cell cycle phases (G0/G1, S and G2/M). The decrease in reduced glutathione is greater after treatment with the FLs of the non-enriched bread than in those of the enriched bread (indicating greater oxidative stress for the cell) (36 vs. 18 % reduction). The increase in intracellular calcium is higher after treatment with FL from the non-enriched bread than from the enriched bread (indicating apoptosis) (134 vs. 27% increase).

Both fermentation liquids, after 3 hours of treatment, activate the caspase 8 gene (40 and 8 % increase with FL from enriched and non-enriched bread, respectively). After 6 hours, FL from non-enriched bread activates the caspase 3 gene, while that from enriched bread produces a non-significant increase. Both activate the gene responsible for the tumor suppressor protein p53 (166 vs. 37% increase with FL from enriched and non-enriched bread, respectively, after 6 hours). In addition, they decrease the transcription of the gene encoding cyclin D1 (55% in both cases after 3 hours), a cell cycle regulator. This suggests that the intake of both breads could provide an equivalent protective effect at the gastrointestinal level, due to the content of fiber, polyphenols and antioxidant compounds, although enrichment with PS could provide an extra benefit in some cases.

(iv) The intake of a food supplement with 2 g of PS for 6 weeks in hypercholesterolemic patients treated with statins does not produce statistically significant changes (before vs. after treatment) in plasma total cholesterol (172 vs. 168 mg/dL) and LDL (99 vs. 95 mg/dL). Eryptosis markers do not improve: phosphatidylserine externalization (32.9 vs. 31.7%), intracellular glutathione levels (300 vs. 266 geometric mean) or cell size (519 vs. 518 geometric mean).

Six plasma cholesterol oxides are detected, in decreasing order (μg/mL): β-epoxycholesterol (0.29-0.48), 7-ketocholesterol (0.26-0.43), α-epoxycholesterol (0.21-0.26), 7β-hydroxycholesterol (0.14-0.22), cholestanotriol (0.11-0.14) and 7α-hydroxycholesterol (0.11-0.13). Total cholesterol oxides content did not decrease after 6 weeks of treatment with PS supplementation (1.42 vs. 1.47 μg/mL before and after treatment, respectively).

There is only an increase in ApoA1 values (6.4%), as well as a decrease in erythrocyte adhesion to vascular endothelium (55%), after the intake of PS supplementation. In addition, an inverse correlation between both parameters was obtained in subjects who consumed PS supplementation at the end of treatment. These results suggest that both factors are related and could have a beneficial effect on cardiovascular health in this population mainly due to a lower risk of atherosclerosis (triggered by adhesion of platelets or erythrocytes to the endothelium).

The study participants, in general, follow the Mediterranean diet pattern, as 27% of the participants are classified in the high adherence group and the remaining in the moderate adherence group. Fifty-four percent of the subjects present low physical activity, while only 11.5% present high physical activity. When evaluating possible correlations of lifestyle (diet and exercise) with the previously evaluated parameters, no correlation or statistically significant association was obtained between them.

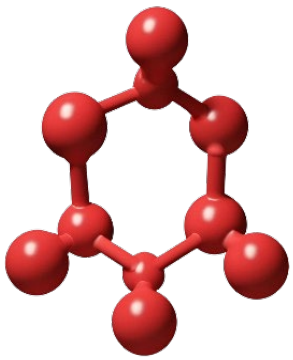
The main conclusions drawn from this Ph.D. Thesis are:

The PS-enriched milk-based fruit beverage presents a superior bioaccessibility after digestion under the gastrointestinal conditions of the elderly with respect to the adult (14.9 vs. 8.0 %), while in the PS-enriched wholemeal rye bread it is lower than in the adult (11.2 vs. 20.3 %), due to factors such as the influence of the matrix (solid/liquid) and enzyme activity, among others.

FLs from enriched bread show a more marked antiproliferative effect on cell viability and apoptosis, while FLs from non-enriched bread induce a more remarkable antiproliferative effect on cell cycle regulation, as well as on reduced glutathione and intracellular calcium levels. Both elevate the expression of pro-apoptotic and cell cycle regulation genes. Supplementation with PSs does not improve the lipid profile, cholesterol oxidation, or erythrocyte markers in subjects with hypercholesterolemia treated with statins. However, it increases ApoA1 concentrations and decreases red blood cell adhesion to vascular endothelium, which is a protective factor against atherosclerosis.

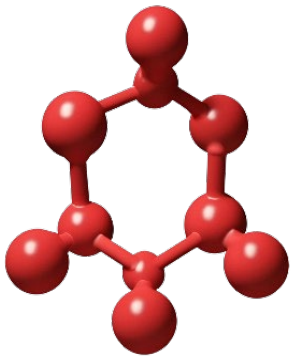
From all this, it can be concluded that PS enrichment of foods with a good nutritional profile, such as whole-grain rye bread or milk-based fruit beverages, is a suitable option for various population groups. This includes older adults, who frequently have cholesterol levels, as well as individuals seeking to prevent chronic diseases through a healthy diet. Furthermore, the use of PS supplements can be an effective and safe strategy to help

reduce cardiovascular risk factors, especially in people with hypercholesterolemia treated with statins.



Abreviaturas

Abbreviations



5-FU: 5-fluorouracile

AA: ácido araquidónico

AAPH: 2,2'-Azobis(2-methylpropionamidine) dihydrochloride

ABCB1a/b: transportadores casete de unión a trifosfato de adenosina B1 a y b

ABCG5/G8: transportadores casete de unión a trifosfato de adenosina G5/G8

AC: *ascending colon*

ACAT2: acil-coenzima-A colesterol aciltransferasa 2

ADP: adenosín difosfato

AGCC: ácidos grasos de cadena corta

AGL: ácidos grasos libres

ANOVA: *analysis of variance*

APO: apolipoproteína / *apolipoprotein*

ATP: adenosín trifosfato

BAX: proteína X asociada a BCL2

BCL2: célula B/linfoma 2

BF: bioaccessible fraction

BMI: body mass index

CB: compuestos bioactivos

CCR: cáncer colorrectal

CDK2: cinasa dependiente de ciclina 2

cDNA: *complementary DNA*

CE: colesterol esterasa / *cholesterol esterase*

CMFDA: 5-chloromethylfluorescein diacetate

COPs: productos de oxidación del colesterol / *cholesterol oxidation products*

COX: ciclooxigenasa

CRC: colorectal cancer

CT: colesterol total

CVD: cardiovascular disease

CXCL16/SR-PSOX: receptor de depuración que se une a la fosfatidilserina y a los lípidos oxidados

DAG: diacilgliceroles / *diacylglycerols*

DC: *descending colon*

DCFDA: 2',7'-dichlorofluorescein diacetate

DMSO: dimethylsulfoxide

ECV: enfermedades cardiovasculares

EGM-2: endothelial cell growth medium

ESIN: *Engineered Stomach and small INtestinal*

EV: esteroles vegetales / *PS: plant sterols*

EPHS: externalization of phosphatidylserine

FADD: *Fas-associated death domain*

FBS: fetal bovine serum

FFA: free fatty acids

FL: fermentation liquids

FLO: fermentation liquid without plant sterols

FLPS: fermentation liquid with plant sterols

FS: fosfatidilserina

FSC: forward scatter

G_1: fase de intervalo 1

G_2: fase de intervalo 2

GAE: gallic acid equivalent

GC-FID: gas chromatography–flame ionization detection

GC-MS: gas chromatography-mass spectrometry

GL: gastric lipase

GSH: glutatión reducido / *reduced glutathione*

HDL: lipoproteína de alta densidad / *high density lipoprotein*

HMDS: hexamethyldisilazane

HMG-CoA: hidroximetilglutaril-CoA

HOMA: homeostasis model assessment

hs-CRP: high sensitive C reactive protein

HUVEC: human umbilical vein endothelial cell

IARC: Agencia Internacional para la Investigación del Cáncer

IPAQ: International Physical Activity Questionnaire

IS: internal standard

LDL: lipoproteínas de baja densidad / *low density lipoprotein*

LG: lipasa gástrica

LF: líquidos de fermentación

M: fase de mitosis

MAG: monoacilgliceroles

MEDAS: Mediterranean Diet Adherence Screener

MEM-NEAA: MEM non-essential amino acids

MET: metabolic equivalent

mRNA: messenger RNA

MTP: proteína microsomal transportadora de triglicéridos

MTT: *3, (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide*

NPC1L1: proteína Niemann-Pick similar a C1

NWRB: non-enriched wholemeal rye bread

OR: oxidation ratio

ORAC: oxygen radical absorbance capacity

PAF: factor de activación plaquetar

PDB: partially dried bread

PGE2: prostaglandina E2

PI: propidium iodide

POPs: productos de oxidación de los esteroles vegetales

qPCR: quantitative polymerase chain reaction

RBC: red blood cell

RCT: randomized controlled trial

RGE: rabbit gastric extract

RH: relative humidity

RNase A: ribonuclease A

ROS: especies reactivas de oxígeno / *reactive oxygen species*

RT: retrotranscription

S: fase de síntesis

SB: *stabilization blank*

SCFA: short chain fatty acids

SGF: simulated gastric fluid

SHIME*: Simulator of the Human Intestinal Microbial Ecosystem*

SIF: simulated intestinal fluid

SIM: single ion monitoring

Simgi*: SIMulator of the Gastro-Intestinal tract*

SPE: solid phase extraction

SSF: simulated salivary fluid

tBOOH: *tert*-butilo hidroperóxido

TAC: total antioxidant capacity

TC: total cholesterol

TG: triglycerides

TIC: total ion current

TICE: vía de excreción transintestinal

TIM: *TNO´s gastrointestinal model*

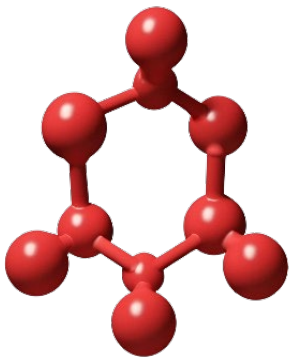
TMCS: trimethylchlorosilane

TPC: total phenolic content

TrC: *transverse colon*

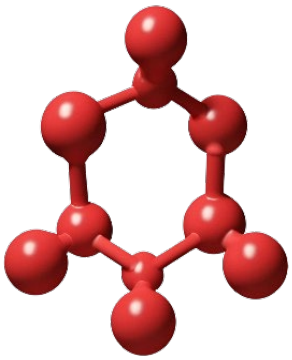
WB: *wash blank*

WRB: plant sterol-enriched wholemeal rye bread



Introducción

Introduction



1. Introducción

Las enfermedades crónicas no transmisibles son la principal causa de mortalidad a nivel mundial, destacando entre ellas la enfermedad cardiovascular (ECV) (WHO, 2021) y el cáncer (WHO, 2022). Las personas de edad avanzada son un grupo de población con mayor prevalencia de estas enfermedades que la población general (Kumar & Alfonso, 2019).

Los principales factores de riesgo de estas enfermedades son la obesidad, el tabaquismo, la hipertensión arterial, la alimentación inadecuada y la hipercolesterolemia, entre otros. En ese sentido, las actuaciones en salud pública deben centrarse en modificar los estilos de vida de la población y una alimentación saludable incorporando alimentos con compuestos bioactivos (CB), con el fin de prevenir estas enfermedades. Unos de los CB con mayor evidencia científica con efectos beneficiosos para la salud son los esteroles vegetales (EV). Estos compuestos son ampliamente conocidos por su efecto hipocolesterolemiante, el cual mejora un factor de riesgo de ECV. Por ello, pueden suponer no solo una alternativa coadyuvante al tratamiento farmacológico en pacientes con hipercolesterolemia leve, sino que también pueden generar una sinergia con fármacos en casos de mayor riesgo cardiovascular. Los EV también presentan un potencial efecto sobre otras enfermedades, sobre todo en el tracto digestivo, donde ejercen efectos beneficiosos a nivel local, debido a su baja absorción, como antiinflamatorio y antiproliferativo. En ese sentido, poseen un potencial efecto preventivo sobre el cáncer colorrectal (CCR), aunque la evidencia es todavía insuficiente para confirmarlo (Salehi et al., 2021).

Debido a la mayor prevalencia de enfermedades crónicas no transmisibles en los ancianos y al envejecimiento paulatino de la población occidental, es importante conocer qué diferencias digestivas presenta este grupo poblacional y cómo estas pueden afectar a la efectividad de los alimentos funcionales. Conocer las diferencias entre matrices alimentarias con buen perfil nutricional de naturaleza líquida (bebidas de fruta a base de leche) o sólida (pan integral de centeno) puede resultar fundamental para, en última instancia, adaptar y mejorar las formulaciones de los alimentos funcionales con EV destinados a dicha población. Para ello, por cuestiones éticas y de ahorro económico y de tiempo, pueden resultar útiles los modelos *in vitro*, no solo para determinar la digestibilidad o bioaccesibilidad de nutrientes y CB, sino para evaluar los efectos

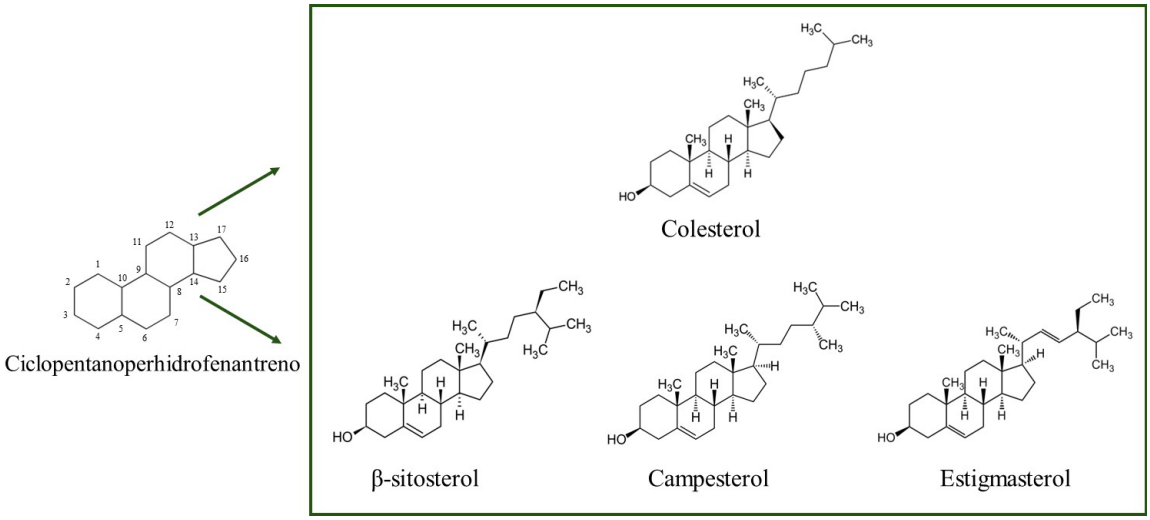
biológicos de estos y generar un conocimiento suficiente para acometer estudios *in vivo* que justifiquen su uso y utilidad en humanos.

2. Esteroles

2.1. Concepto y estructura

Los EV son compuestos lipofílicos de origen vegetal derivados del ciclopentano[α]-perhidrofenantreno, siendo por tanto análogos del colesterol. Sin embargo, se diferencian de este por la cadena hidrocarbonada lateral, ya que los EV presentan una cadena de 9 o 10 C, mientras que en el colesterol es de 8 C (**Figura 1**). Estos se subdividen a su vez en fitoesteroles (presentan un doble enlace en el carbono C-5) y fitoestanoles (no contienen ese doble enlace). Los EV se presentan en los vegetales en forma libre o esterificados (principalmente con ácidos grasos, aunque también con ácidos hidroxicinámicos o con una hexosa mediante una glicosilación) y forman parte de la fracción insaponificable de los lípidos (Feng et al., 2020; Garcia-Llatas et al. 2021a).

Figura 1. Estructura química del colesterol y de los principales esteroles vegetales



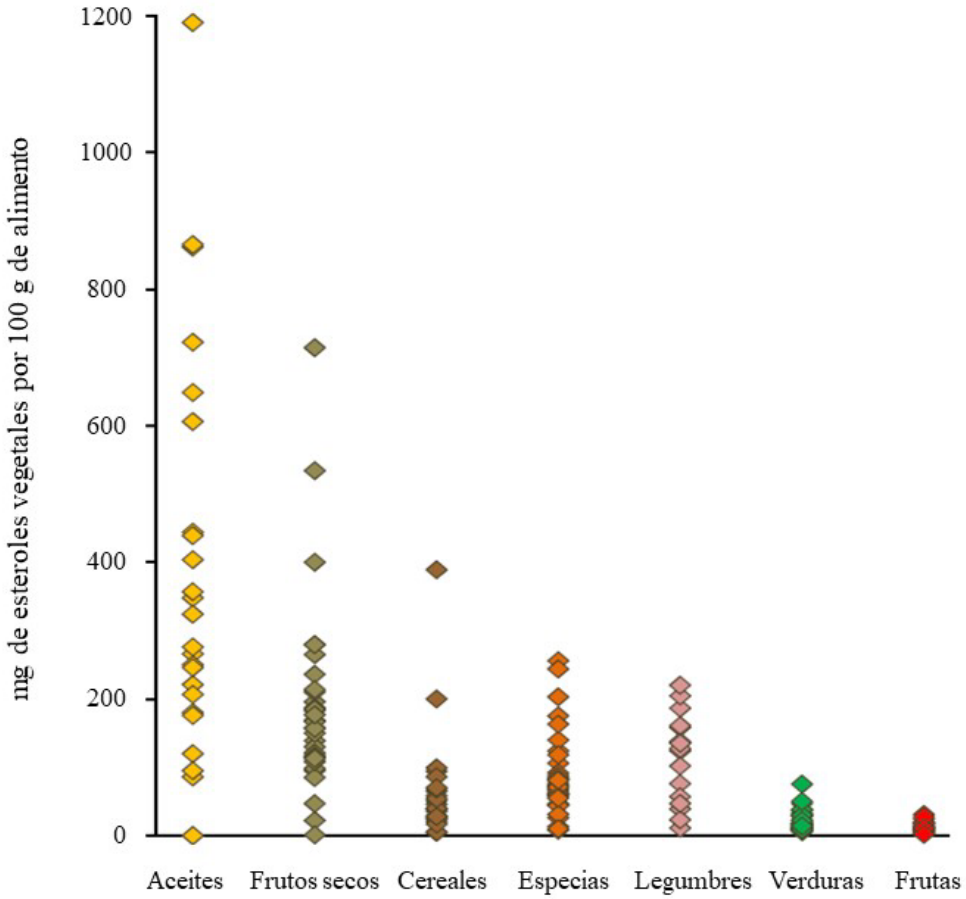
2.2. Ingesta de esteroles vegetales

2.2.1. Dieta

Las principales fuentes de EV son, por orden descendente en contenido, aceites vegetales (como el aceite de maíz), frutos secos y semillas, cereales, verduras y frutas (**Figura 2**). Las dietas con una mayor frecuencia de consumo de estos alimentos son las más ricas en EV, por tanto, las dietas veganas aportan mayor contenido de EV (896-1047

mg/día). Sin embargo, las dietas occidentales aportan hasta cinco veces menos, con un rango de 193-373 mg/día (Racette et al., 2015). En España, la ingesta media de EV se sitúa en 338 mg/día en hombres y 250 mg/día en mujeres (Escurriol et al., 2009), o 276 mg/día en general (Jiménez-Escrig et al., 2006). En la Comunidad Valenciana, la media se mantiene, situándose entre 274-282 mg/día (Garcia-Llatas et al., 2015).

Figura 2. Contenido en esteroles vegetales de distintos alimentos, divididos por categorías



Adaptado de Racette et al. (2015)

La ingesta mínima de 800 mg/día de EV contribuye a mantener los niveles de colesterol plasmático (Reglamento 432/2012/CE). Por tanto, solo las dietas basadas en alimentos vegetales, especialmente las veganas, proporcionan estos aportes. Por otro lado, la ingesta requerida para tener un efecto hipocolesterolemiante oscila entre 1,5 y 3 g/día de esteroles o estanoles vegetales (Reglamentos 983/2009/UE, 376/2010/UE 384/2010/UE y 686/2014/UE). Por ello, es necesario el enriquecimiento de alimentos o el uso de complementos alimenticios, para obtener los mayores beneficios posibles.

2.2.2. Alimentos enriquecidos

Los primeros alimentos autorizados para enriquecimiento con EV fueron las margarinas, debido a la fácil solubilización de los EV en la matriz grasa. Más adelante se comenzó a enriquecer alimentos con menor contenido graso y mejor perfil nutricional, ya que los avances tecnológicos permitieron incorporar los EV no solo a matrices grasas, sino a algunas mixtas e incluso bajas en grasa. En la Unión Europea, existen diversas matrices donde está autorizado el enriquecimiento con EV, entre ellas, las grasas amarillas de untar (Decisión 2000/500/CE), aliños para ensaladas, productos de tipo leche, productos tipo leche fermentada, bebidas de soja y productos tipo queso (Decisión 2004/333/CE), las leches fermentadas (Decisiones 2004/334/CE y 2004/335/CE), bebidas de fruta a base de leche (Decisión 2004/336/CE), otras bebidas con base láctea semi o desnatada (Decisión 2004/845/CE), pan de centeno (Decisiones 2006/58/CE y 2006/59/CE), aceite como nuevo ingrediente (Decisión 2007/343/CE) y bebidas a base de arroz (Decisión 2008/36/CE). Estas normativas regulan no solo los alimentos donde está permitido añadir EV, sino también la abundancia relativa de los distintos EV mayoritarios, siendo obligatorio que los alimentos enriquecidos no superen los siguientes porcentajes: β-sitosterol (80%), campesterol (40%), estigmasterol (30%), sitostanol (15%), campestanol (5%), brasicasterol (3%), y otros esteroles y estanoles (3%).

Las bebidas de fruta a base de leche y el pan de centeno representan ejemplos de alimentos con un perfil nutricional saludable. En el caso de las bebidas, por su contenido en vitaminas, minerales y antioxidantes (carotenoides y polifenoles, entre otros) procedentes de las frutas y por el bajo aporte graso y calórico. El pan integral de centeno, como posible sustituto del pan blanco de trigo, es fuente de fibra alimentaria (Åman et al., 2010) y aporta un contenido lipídico bajo, lo que permite que sea una matriz idónea para el enriquecimiento con EV para personas con hipercolesterolemia moderada.

2.2.3. Complementos alimenticios

Una alternativa para incrementar la ingesta diaria de EV son los complementos alimenticios, que se definen como *los productos alimenticios cuyo fin sea complementar la dieta normal y consistentes en fuentes concentradas de nutrientes o de otras sustancias que tengan un efecto nutricional o fisiológico, en forma simple o combinada, comercializados en forma dosificada* (Directiva 2002/46/CE traspuesta al Real Decreto 1487/2009 modificado por el Real Decreto 130/2018). Pese a que los EV no forman parte

de los ingredientes autorizados por esta normativa para su uso en complementos, la comercialización de estos se ampara en la cláusula de reconocimiento mutuo del Real Decreto, en la que se especifica que los complementos fabricados legalmente en la UE o en países con acuerdo de comercio sí que pueden ser comercializados y no se aplicará la restricción de ingredientes del propio Real Decreto.

Se permite su comercialización con un contenido máximo de 3 g de EV por día. Pueden tener distintas presentaciones y están formulados para que su consumo sea rápido y sencillo, permitiendo alcanzar las dosis recomendadas con una sola toma, facilitando el proceso y, teóricamente, mejorando la adherencia a la ingesta, en contraposición al consumo de alimentos enriquecidos y, sobre todo, a cambios dietéticos que aumenten la ingesta de EV (como podría ser el aumento de consumo de vegetales en detrimento de alimentos de origen animal). Sin embargo, los complementos alimenticios con EV poseen algunos inconvenientes, como la baja solubilidad de los EV. Esto afecta también a sus características organolépticas, haciéndolos menos deseables para la población, lo que supone un importante reto para la industria. Para paliar estos inconvenientes, se hace necesaria una correcta encapsulación de los EV, aspecto que se está consiguiendo en los últimos años, mejorando la formulación a nivel de estabilidad, solubilidad y preservación de características organolépticas (Blanco-Morales et al., 2024). Para ello, se comercializan en formatos como cápsulas (desde 800 mg/cápsula), o *sticks* bebibles (1500 mg/*stick*), a los que se le añade un saborizante que pueda potencialmente elevar la adherencia al tratamiento (datos extraídos

2.3. Absorción y biotransformación de esteroles

2.3.1. Liberación, absorción y distribución

Los EV se absorben en el intestino delgado, previa hidrólisis de las formas esterificadas por la enzima colesterol esterasa (CE). La absorción se produce mediante la incorporación de los EV y el colesterol (biliar y dietético) a las micelas mixtas, las cuales se aproximan al borde en cepillo de los enterocitos (**Figura 3**). Para dicho proceso es clave la presencia de mono y diacilgliceroles, los cuales proceden de la digestión lipídica mediada por la lipasa gástrica (LG) y la lipasa pancreática. Aunque los EV pueden absorberse por difusión pasiva a través del enterocito, fundamentalmente se produce por transporte activo mediante la proteína Niemann-Pick similar a C1 (NPC1L1) (Zhang et al., 2022).

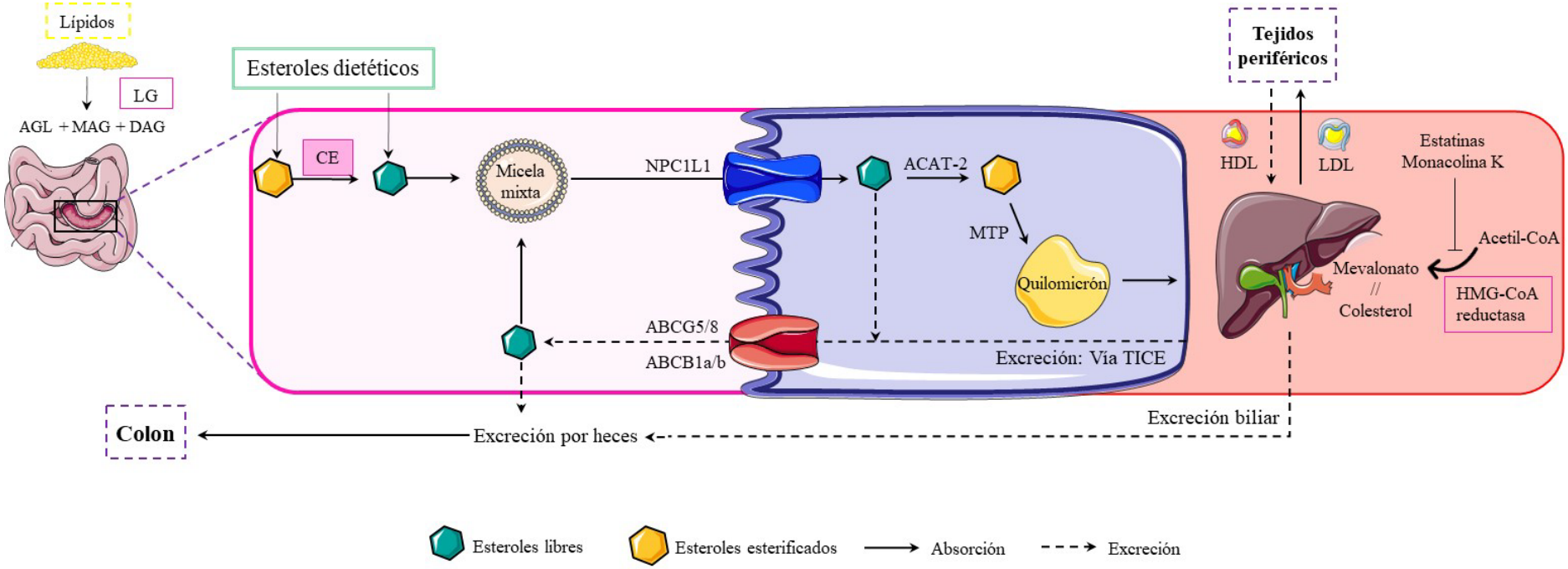
Una vez en el enterocito, los esteroles se reesterifican, mediante la enzima acil-coenzima-A colesterol aciltransferasa 2 (ACAT2), para entrar en los quilomicrones, siendo más eficiente la reesterificación para el colesterol que para los EV. Posteriormente son incorporados en los quilomicrones por la proteína microsomal transportadora de triglicéridos (MTP), y los quilomicrones son transportados al hígado a través del torrente sanguíneo. Seguidamente son integrados en lipoproteínas de baja densidad (LDL) y transportados por la circulación general a los tejidos periféricos, donde ejercen su actividad biológica antes de ser metabolizados y eliminados del torrente sanguíneo.

La absorción del colesterol varía entre individuos por razones de sexo, edad o etnia, aunque también debido a polimorfismos en genes clave en el proceso de absorción o excreción como *ABCG5*, *ABCG8*, *APOE* o *NPC1L1*, entre otros (Mokhtar et al., 2022). El rango de absorción del colesterol es relativamente amplio entre individuos, aunque en promedio es más elevado (40-50%) que la absorción media de los EV (<5%) (Blanco-Morales et al., 2021a). La absorción del campesterol es superior a este valor medio (≈15%), mientas que el β-sitosterol y estigmasterol presentan una absorción inferior (≈2,5 y <1%, respectivamente) (Ikeda, 2015). La baja afinidad de los EV por la ACAT2 en comparación al colesterol es responsable de los valores medios de absorción tan reducidos (Blanco-Morales et al., 2021a).

2.3.2. Metabolismo y excreción

Tras ser distribuidos por el organismo, los esteroles son transportados mediante las lipoproteínas de alta densidad (HDL) en mayor proporción que en las LDL, favoreciendo su excreción por vía biliar. Además, los EV presentes en el enterocito que no han sido esterificados, son devueltos al lumen intestinal mediante los transportadores casete de unión a trifosfato de adenosina G5/G8 (ABCG5/G8). También existe un mecanismo adicional de excreción denominado vía de excreción transintestinal (TICE), por la cual el enterocito capta los esteroles contenidos en las lipoproteínas del torrente sanguíneo en contacto con la membrana basolateral, desplazándolos hasta el borde apical, donde puede seguir la vía de excreción común (de Boer et al., 2018). Sin embargo, parece que la vía TICE no es dependiente de los transportadores ABCG5/8, ya que no resulta afectada si se inhiben específicamente estas proteínas. Por tanto, deben ser otros transportadores diferentes los responsables de esta ruta de excreción, para lo que se

Figura 3. Esquema del proceso de absorción, metabolismo y excreción de los esteroles



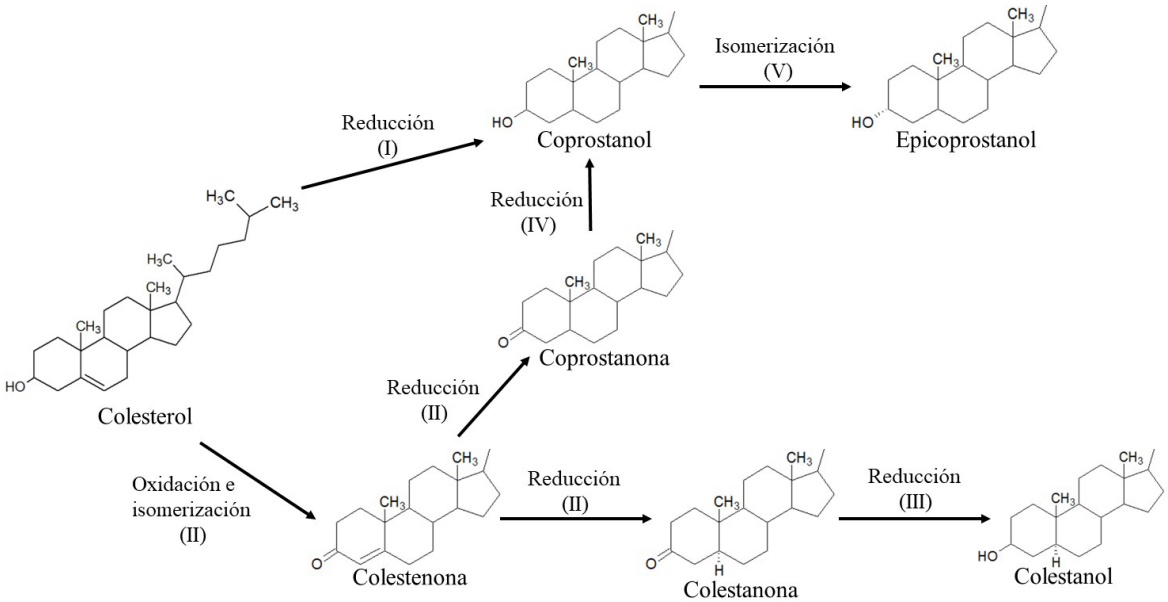
ABCB1a/b: transportadores casete de unión a trifosfato de adenosina B1 a y b; ABCG5/8: transportadores casete de unión a trifosfato de adenosina 5 y 8; ACAT-2: acil-coenzima-A colesterol acetiltransferasa 2; AGL: ácidos grasos libres; CE: colesterol esterasa; DAG: diacilgliceroles; HDL: lipoproteína de alta densidad; LDL: lipoproteína de baja densidad; LG: lipasa gástrica; MAG: monoacil gliceroles; MTP: proteína microsomal transportadora de triglicéridos; NPC1L1: proteína Niemann-Pick similar a C1; TICE: vía de excreción transintestinal

(ABCB1a/b). Esta vía, pese a su reciente descubrimiento, contribuye hasta en un 35% a la excreción total de esteroles (Nakano et al., 2019).

2.3.3. Fermentación colónica

Los esteroles no absorbidos llegan al colon, donde son susceptibles de ser metabolizados por la microbiota. El colon humano presenta una gran variedad de bacterias, destacando entre ellas dos géneros con capacidad de metabolizar los esteroles, *Bacteroides* y *Eubacterium* (Cuevas-Tena et al., 2018). El colesterol puede ser metabolizado directamente a coprostanol mediante una reducción (I). Otra ruta de metabolización del colesterol resulta en la formación de colestenona mediante oxidación e isomerización, la cual puede ser reducida bien a coprostanona o a colestanona (II). Este último metabolito, a su vez, puede sufrir reducción hasta colestanol (III), mientras que la coprostanona es susceptible de ser de nuevo reducida a coprostanol (IV) (Wong, 2014). De este modo, la conversión a coprostanol puede ser realizada tanto directa (I) como indirectamente (II-IV). Adicionalmente, el coprostanol puede formar el isómero epicoprostanol (V) (**Figura 4**).

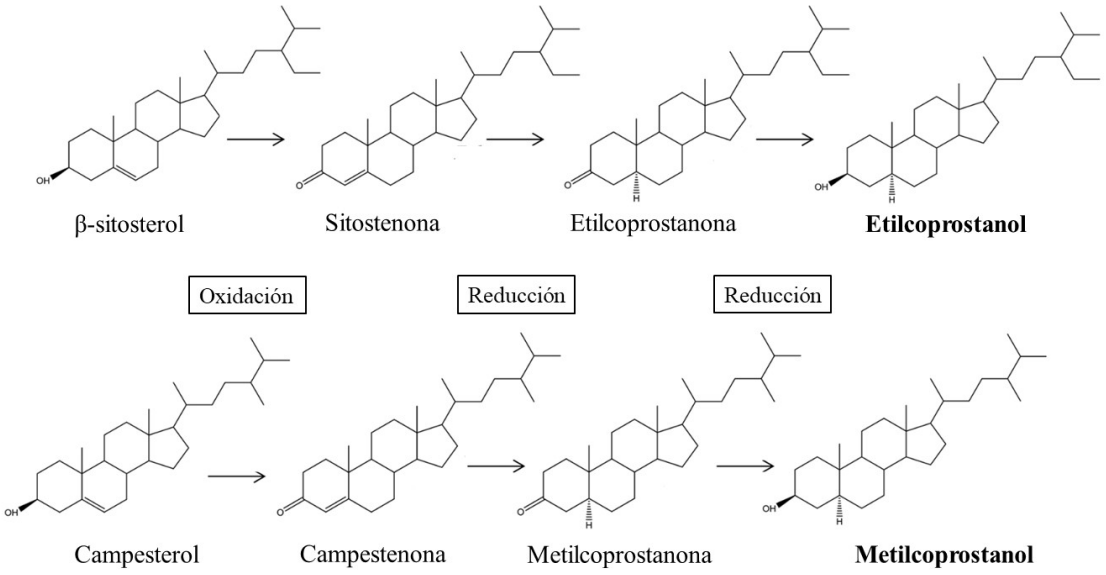
Figura 4. Metabolización del colesterol por la microbiota colónica



Por otra parte, aunque el estudio del metabolismo de EV es escaso en comparación al del colesterol, tienen un metabolismo análogo al de este. En su caso, las transformaciones están principalmente mediadas por bacterias del género *Eubacterium* (**Figura 5)**. Se

forman derivados -3-*ona* (sitostenona o campestenona), los cuales pueden metabolizarse a etil- coprostanona/coprostanol (desde β-sitosterol) o metil- coprostanona/coprostanol (desde campesterol) (Cuevas-Tena et al., 2018).

Figura 5. Metabolización de los principales esteroles vegetales por la microbiota colónica



Adaptado de Cuevas-Tena et al. (2018)

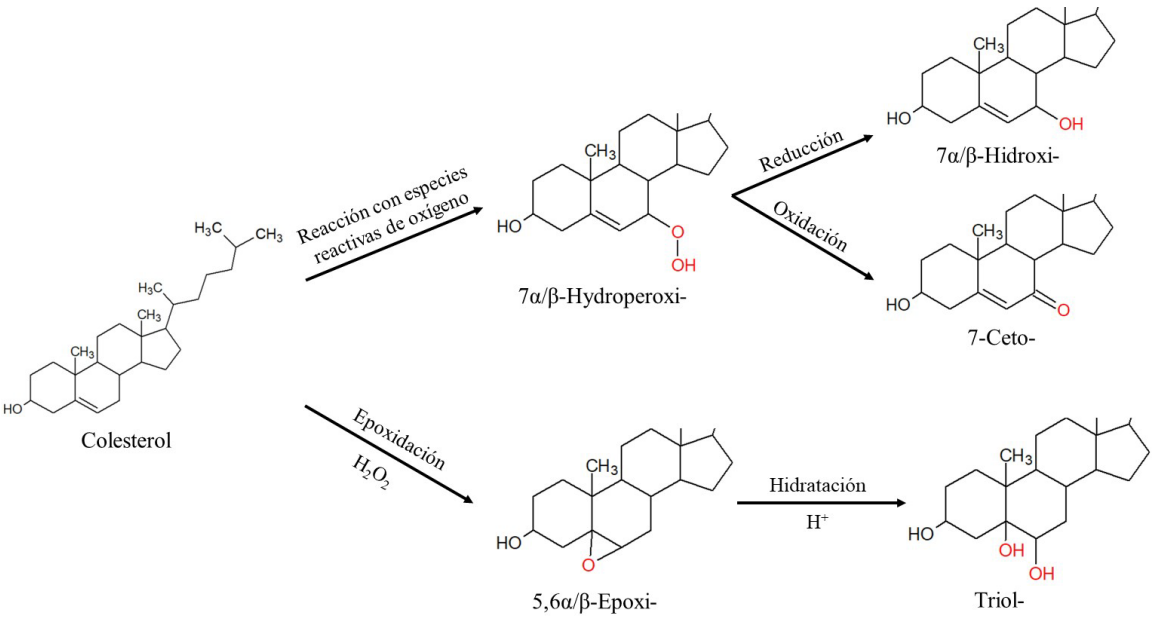
2.3.4. Formación de óxidos de esteroles

Los esteroles pueden ser metabolizados *in vivo*, formándose productos de oxidación del colesterol (COPs) y de los EV (POPs). Todos ellos tienen grupos polares adicionales en la cadena hidrocarbonada, lo que modifica sustancialmente su bioactividad. Este proceso es de suma importancia porque los COPs poseen distintos efectos negativos sobre el organismo, principalmente como proinflamatorios (Garcia-Llatas et al., 2021b).

Estas sustancias están presentes de forma natural en los alimentos debido al almacenamiento, presencia de oxígeno en contacto con el alimento y, sobre todo, a procesos de calentamiento (Lin et al., 2016). La abundancia de los distintos COPs varía entre los alimentos, siendo los más importantes el 7-cetocolesterol, 7α/β-hidroxicolesterol, 7α/β-hidroperoxicolesterol, 5,6α/β-epoxicolesterol y el colestanotriol (**Figura 6**) (Garcia-Llatas et al., 2021b). Trabajos previos de nuestro grupo de investigación han constatado que la diferencia de temperatura en el almacenamiento (4-

37 °C) no afecta a la oxidación en bebidas enriquecidas con EV, sino el tiempo de almacenamiento (mayor en 6 que en 2 meses) (González-Larena et al., 2015).

Figura 6. Productos de oxidación del colesterol



De manera similar a los esteroles no oxidados, POPs y COPs procedentes de la dieta o de la excreción hepática biliar, son esterificados dentro del enterocito para ser introducidos en los quilomicrones y, mediante las distintas lipoproteínas, ser distribuidos por el organismo a través de la circulación general (Kulig et al., 2016). Su excreción sigue las mismas vías que los esteroles no oxidados, mientras que a nivel hepático son metabolizados y, posteriormente, transportados por la bilis al colon para ser excretados. La absorción intestinal de POPs parece ser incluso superior a la de los EV (<16 *vs.* <5 %). Contrariamente, la absorción de los COPs es inferior a la del propio colesterol, entre un 5 a un 25%, dependiendo del COP (Garcia-Llatas et al., 2021b). Esto se debe a la menor solubilidad micelar y menor esterificación dentro del enterocito, lo que puede incrementar el daño potencial que estos ocasionan en el intestino (Cilla et al., 2017).

La presencia de COPs y POPs en plasma proceden de la dieta, así como de su formación endógena, por vía enzimática y no enzimática. La vía enzimática se debe a la actividad de diversas enzimas mitocondriales, mientras que la no enzimática se debe a la acción prooxidante de especies reactivas de oxígeno (ROS), entre otros. Algunos de los

oxisteroles más habituales formados de manera endógena son los 7α/β-OH-colesterol, 7-cetocolesterol y otros derivados hidroxi- en posiciones 24, 25 y 27 (Cilla et al., 2017).

En población sana, la concentración de COPs en plasma es relativamente reducida en comparación a la de colesterol, con un rango de 0,01-0,1 μM, mientras que la de colesterol se sitúa alrededor de 5000 μM (Cilla et al., 2017). Como se puede observar en la **Tabla 1**, la concentración mínima de COPs en pacientes hipercolesterolémicos es de hasta el doble para algunos de ellos en comparación a la de individuos sanos. Esto puede indicar una mayor concentración media de COPs asociada a la hipercolesterolemia (entre un 20 y un 50% superior a individuos sanos), como indican diversos autores (Menéndez-Carreño et al., 2011; Chen et al., 2019), si bien la variabilidad interindividual es elevada.

Tabla 1. Contenido de productos de oxidación del colesterol en plasma de individuos sanos e individuos con hipercolesterolemia

Oxisterol	**Niveles plasmáticos (ng/mL)**	
	Individuos sanos (Menéndez-Carreño et al., 2011; Chen et al., 2019; Lin et al., 2019)	Individuos hipercolesterolémicos (Menéndez-Carreño et al., 2011; Chen et al., 2019)
24-hidroxi-colesterol	53,5–283,5	10,9–238,9
7α-hidroxi-colesterol	148,6–570,2	236,7–583,0
7-cetocolesterol	41,9–589,5	80,0–538,6
4β-hidroxi-colesterol	9,1–103,1	22,6–128,9
5,6β-epoxicolesterol	22,9–255,9	23,0–246,1
27-hidroxi-colesterol	128,3–506,4	92,8–405,6
5,6α-epoxicolesterol	3,8–101,8	1,6–93,5
7β-hidroxi-colesterol	26,3-220	300*
25-hidroxi-colesterol	14,2–153,1	30,6–419,1
20-hidroxi-colesterol	0,03–0,5	0,05–0,6
Colestanotriol	1,4–111,3	2,9–40,0

* Solo disponibles datos medios (Menéndez-Carreño et al., 2011)

Entre los efectos negativos de los COPs en el organismo, destaca su efecto citotóxico a nivel endotelial producido por un aumento de la apoptosis en las células endoteliales, ya sea mediante una inducción de la producción de ROS, entrada de calcio a la célula o la modificación del potencial de membrana mitocondrial (Kulig et al., 2016). Además, son capaces de intercalarse en la membrana celular, aumentando la rigidez del endotelio, lo cual se potencia debido a la ya mencionada estimulación de la producción de ROS. En especial, el radical superóxido reacciona con el óxido nítrico disminuyendo el efecto

vasodilatador de este (Gargiulo et al., 2017). Además, los COPs producen un daño inflamatorio, con la subsecuente liberación de citoquinas proinflamatorias, que, junto a la elevada apoptosis endotelial y a todos los factores previamente comentados, crean un ambiente propicio a la adhesión endotelial (Vejux et al., 2020). Se sabe que este proceso es clave en la lesión aterosclerótica y, por tanto, en el desarrollo de ECV (Messner & Bernhard, 2014). Dada la mayor concentración de COPs en plasma respecto a POPs, así como su mayor potencial citotóxico (Cilla et al., 2017), refuerza la importancia de su cuantificación en plasma.

3. Evaluación de la bioaccesibilidad de nutrientes y compuestos bioactivos en el anciano

3.1. Métodos de digestión in vitro

Los métodos de digestión *in vitro* son ampliamente utilizados dadas las múltiples ventajas que poseen, entre ellas la sencillez, rapidez y costo económico, además de la no necesidad de comité ético para llevarse a cabo comparados con los ensayos *in vivo*. Permiten un cribado extenso de alimentos y de variedad de condiciones digestivas, lo que los convierte en el primer paso de evaluación de la bioaccesibilidad de los alimentos. Además, dichos métodos, si se estandarizan, tienen la ventaja de eliminar la variabilidad interindividual que se presenta inevitablemente en estudios en humanos (Mackie et al., 2020). Mediante estos métodos es posible estimar la bioaccesibilidad de macronutrientes "accesibilidad del nutriente a las enzimas intestinales, lo que lo hace disponible para la absorción" y micronutrientes "liberación del nutriente de la matriz alimentaria en el intestino, haciéndolo disponible para la absorción", así como de los CB. Además, permiten evaluar la digestibilidad de los macronutrientes "hidrólisis de estos en el tracto gastrointestinal" (Grundy et al., 2024).

Los métodos *in vitro* se dividen en dos grupos, los estáticos y los dinámicos, con características muy diferentes. Los **métodos estáticos** comprenden una fase oral, gástrica e intestinal, aunque algunos pueden incluir una fase colónica para simular la fermentación. Estos son muy eficientes, ya que permiten el análisis de una gran variedad de matrices o parámetros con un costo de tiempo y económico reducido. Sin embargo, no reproducen los gradientes de pH que ocurren fisiológicamente en el sistema digestivo, además del vaciado gástrico progresivo. Pese a ello, en general, parecen correlacionarse generalmente bien con los resultados *in vivo* para la digestibilidad de los macronutrientes

(sobre todo el almidón y, en menor medida, proteínas), y la bioaccesibilidad de algunos micronutrientes como el hierro. Respecto a los CB, salvo los carotenoides, los cuales han sido ampliamente estudiados y muestran correlaciones altas, se aprecian correlaciones menos robustas, ampliándose esa debilidad a mayor complejidad de la matriz. Como ejemplo de ello se encuentran los polifenoles, en los que la dificultad de valorar la fermentación, así como la gran variedad de estructuras químicas pueden debilitar la correlación (Bohn et al., 2018).

La metodología de estos sistemas ha sido muy heterogénea a lo largo del tiempo, pero en los últimos años ha surgido un consenso para estandarizar dichos métodos y lograr una uniformidad en su aplicación, la cual permita extraer resultados comparables entre sí. Dicha estandarización se desarrolla en la acción COST INFOGEST, con una primera armonización en 2014 (Minekus et al., 2014), posteriormente actualizada y denominada INFOGEST 2.0 (Brodkorb et al., 2019). En la **Tabla 2** se recopilan las condiciones generales del método INFOGEST y los cambios propuestos en su versión 2.0.

Por otra parte, los **sistemas dinámicos** tienen un coste elevado en tiempo y dinero respecto a los estáticos, así como un mayor requerimiento de personal especializado, por lo que su disponibilidad es menor. Sin embargo, la adición de enzimas, el peristaltismo y el paso de un compartimento a otro están controlados automáticamente, al contrario que ocurre con los estáticos y permiten generar un gradiente de pH en las distintas fases digestivas. Por todo ello, reproducen de forma más precisa la fisiología digestiva humana. Estos sistemas permiten, por tanto, evaluar las transformaciones de los alimentos y el metabolismo de sus compuestos durante el tránsito gastrointestinal, además de conocer los cambios que dichos alimentos producen en la composición de la microbiota colónica. Los sistemas dinámicos pueden dividirse en mono y multi-compartimentales. De los primeros existen distintos modelos, como *The Dynamic gastric model* o *The human gastric simulator*, y únicamente son usados para simular una fase digestiva (principalmente la fase gástrica). Los segundos, más complejos, permiten simular más de una fase digestiva o la digestión completa. Los modelos *TNO´s gastrointestinal model (TIM-1)*, *Engineered Stomach and small INtestinal* (*ESIN*) y *DIDGI®* únicamente comprenden la digestión hasta el intestino delgado, donde puede haber compartimentos para cada sección del órgano o un único compartimento. Sin embargo, existen modelos que, además de las fases gástrica e intestinal, incluyen el colon, como el *Simulator of the*

Tabla 2. Condiciones digestivas del método INFOGEST y modificaciones propuestas en la versión 2.0

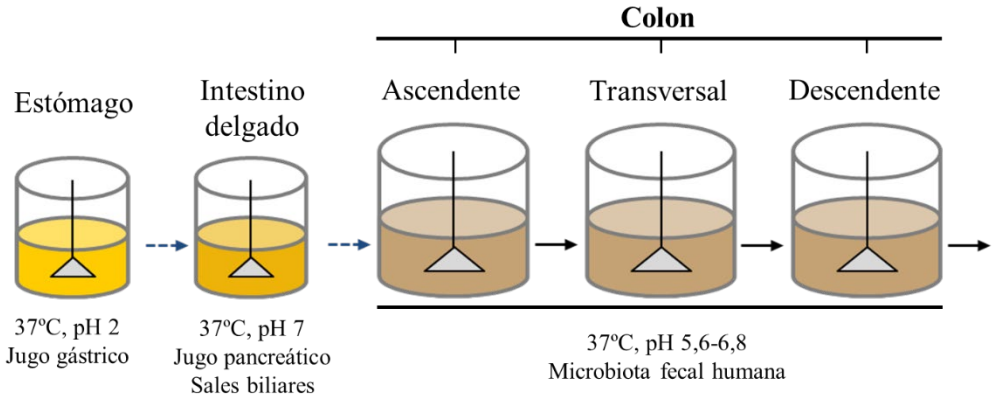
Fase digestiva	Minekus et al., 2014	Brodkorb et al., 2019
Oral	α-amilasa salivar (75 U/mL) pH 7 / 2 min / 95 rpm Ratio alimento:fluido sintético (1:1, p/p) para simular la textura pastosa susceptible de tragarse (similar a mostaza o pasta de tomate)	Aplicación obligada de esta fase
Gástrica	Pepsina (2000 U/mL) pH 3 / 2 h / 95 rpm Ratio alimento:fluido sintético (1:1, p/p)	Adición de lipasa gástrica (60 U/mL)
Intestinal	Tripsina Quimotripsina Lipasa pancreática Colipasa Amilasa pancreática	Sin modificaciones
	Pancreatina (100 U/mL en base a tripsina)	
	Sales biliares (10 mM) pH 7 / 2 h / 95 rpm Ratio alimento:fluido sintético (1:1, p/p)	

Human Intestinal Microbial Ecosystem (*SHIME*), con tres compartimentos para el colon, el *TIM-2* (basado en el *TIM-1* pero con la posibilidad de añadir la microbiota humana), o el *SIMulator of the Gastro-Intestinal tract* (simgi®) (Guerra et al., 2012; Dupont et al., 2019; Mackie et al., 2020). Este último ha sido utilizado en la presente tesis doctoral y comprende compartimentos para el estómago, el intestino delgado y los tres tramos del colon (ascendente, transversal y descendente) (**Figura 7**) (Tamargo et al., 2017). Además, estos sistemas dinámicos, permiten obtener al final del proceso los líquidos de fermentación (LF), que suponen una mejor aproximación a la digestión completa que se desarrolla *in vivo*, pudiendo ser usados no solo para la evaluación de la bioaccesibilidad sino también en la evaluación de distintos efectos biológicos a nivel local en el tracto gastrointestinal.

3.2. Adaptación de la digestión in vitro a las condiciones fisiológicas del anciano

Los sistemas de digestión gastrointestinal simulada deben ser adaptados a las condiciones fisiológicas de distintos grupos poblacionales. La población anciana posee unas características fisiológicas y patológicas diferentes a la del adulto. Algunas de ellas afectan directamente al proceso digestivo en sus diferentes etapas (**Figura 8**). En la boca,

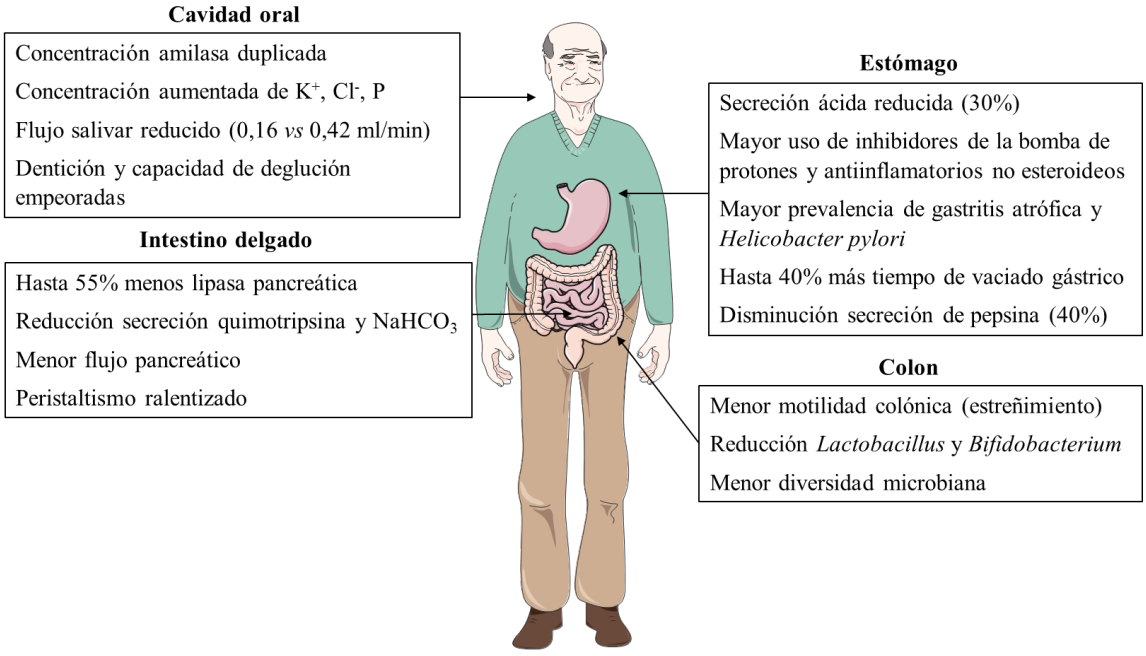
Figura 7. Esquema del modelo de digestión y fermentación colónica *in vitro* simgi®



los ancianos presentan en general peor dentición, aumento relativo de la concentración de la amilasa y de algunos iones, así como una reducción en la producción de saliva (Nagler & Hershkovich, 2005). Además, la prevalencia de disfagia orofaríngea es superior a la del adulto joven (Khan et al., 2014), disminuyendo potencialmente la variedad de la ingesta. A nivel gástrico, la población anciana presenta hipoclorhidria (y aclorhidria en algunos casos) y reducción de la secreción enzimática y de iones, así como un vaciado gástrico más lento (Feldman et al., 1996; Rémond et al., 2015). En el intestino delgado, también se observa una reducción del peristaltismo y de la secreción enzimática y biliar (Laugier et al., 1991; Soenen et al., 2016). Entre los cambios a nivel colónico destacan el aumento del estreñimiento, que incrementa el riesgo de desarrollo de CCR en esta población, así como modificaciones en el contenido y concentración de distintos géneros bacterianos, con una menor diversidad microbiana en general. Así, géneros considerados como potencialmente beneficiosos para la salud colónica como son *Lactobacillus* o *Bifidobacterium* y bacterias productoras de butirato como algunas del género *Eubacterium*, tienden a verse reducidos en beneficio de otras bacterias, principalmente del filo Bacteroidetes (Yatsunenko et al., 2012; Mangiola et al., 2018).

Estos cambios fisiológicos en el anciano van acompañados de malabsorción de nutrientes como proteínas, vitaminas y minerales, y patologías entre las que destaca principalmente la sarcopenia, condición que produce un empeoramiento funcional general, llevando en muchos casos al encamamiento (Muscaritoli et al., 2010). Esta condición se caracteriza por un desequilibrio entre los procesos anabólicos y catabólicos que lleva a una pérdida paulatina de la adecuada función y tamaño muscular. Una de las razones que puede conducir a esta situación es una ingesta inadecuada, principalmente de proteínas, pero también de otros nutrientes y energía (Jang et al., 2023).

Figura 8. Cambios fisiopatológicos relacionados con el envejecimiento que afectan a la digestibilidad de los alimentos (Feldman et al., 1996; Nagler & Hershkovich, 2005; Yatsunenko et al., 2012; Rémond et al., 2015; Soenen et al., 2016)



Los cambios fisiológicos descritos pueden afectar a la digestibilidad de los macronutrientes y bioaccesibilidad de micronutrientes y CB, así como al potencial efecto de los alimentos funcionales. Además, puede servir para mejorar la formulación de estos últimos para adecuarlos a las necesidades y fisiología de los ancianos, destacando en este sentido la mejora de las texturas (Gallego et al., 2022).

Se han publicado numerosos estudios que adaptan una digestión simulada a las condiciones gastrointestinales del anciano. En la presente tesis doctoral, se realiza una revisión de los estudios que han adaptado las condiciones de digestión a la fisiología del anciano (ver Anexo I: Makran, M., **Miedes, D**., Cilla, A., Barberá, R., Garcia-Llatas, G., & Alegría, A. (2022). Understanding the influence of simulated elderly gastrointestinal conditions on nutrient digestibility and functional properties. *Trends in Food Science & Technology*, *129*, 283-295). Posteriormente a este trabajo se han publicado varios estudios en este sentido que evalúan digestibilidad de macronutrientes (Gallego et al., 2023; Hernández-Olivas et al., 2023; Melchior et al., 2023; Moretton et al., 2023; Zhang et al., 2023a,b; Cao et al., 2024; Moretton et al., 2024; Wang et al., 2024; Qiu et al., 2025), y

solo uno de ellos ha evaluado la bioaccesibilidad de micronutrientes (calcio) y CB (polifenoles) (Hernández-Olivas et al., 2023).

Se ha evaluado una amplia variedad de matrices, desde algunas más sencillas (fórmulas enterales, aislados de suero lácteo, péptidos, emulsiones y soluciones acuosas vitamínicas) hasta alimentos completos (aceites de semillas, frutas, carnes y derivados cárnicos, pescados, huevos, legumbres, cereales, semillas y lácteos). En dichos estudios se ha determinado principalmente la digestibilidad proteica utilizando diferentes parámetros (contenido de aminoácidos en el digerido, proporción de péptidos grandes vs. pequeños y concentración de amonio, entre otros). En menor medida también se ha estudiado la digestibilidad de carbohidratos (degradación del almidón y liberación de lactosa) y lípidos (metabolización de triacilgliceroles a ácidos grasos), además de la bioaccesibilidad de algunos micronutrientes. La actividad biológica (capacidad antioxidante, antimicrobiana e inmunomoduladora) de algunos productos y la bioaccesibilidad de CB (entre los que se encuentran los EV) también han sido evaluadas. La digestibilidad proteica (32-49%) disminuye, en general, en las condiciones digestivas del anciano. De igual manera, la bioaccesibilidad de vitaminas liposolubles (9,8-40%) se ve reducida, con excepciones como los pescados magros (vitamina D) y lácteos líquidos (vitamina A). Se observa una reducción en la bioaccesibilidad del calcio (9-60%), así como de los polifenoles (50%). Contrariamente, la digestibilidad lipídica se ve aumentada (2,2-8,0%) en dichas condiciones, aunque sin diferencias en pescados ni en la cinética de la digestión de los lípidos. La digestibilidad de los carbohidratos, por su parte, no muestra una única tendencia, aumentando (36%) al utilizar un sistema dinámico únicamente hasta la fase gástrica y una matriz basada en cereales (*tsampa*), o disminuyendo (15-75%) al utilizar un sistema dinámico completo con distintas matrices. Los estudios recopilados presentan diversas limitaciones como: carecer de homogeneidad metodológica, pues algunos aplican fase oral con voluntario anciano y otros no, no utilizan enzimas clave como la LG (Hernández-Olivas et al., 2020a,b;2021a,b), algunos trabajos no adaptan al anciano la actividad enzimática (Assad-Bustillos et al., 2020; Planche et al., 2022), no aplican fase intestinal (Romano et al., 2019; Aalaei et al., 2021; Peyron et al., 2021; Li et al., 2022a), y existe discrepancia respecto a la duración de las fases digestivas y al pH gástrico.

Recientemente, se ha propuesto un método de digestión *in vitro* estático adaptado a las

condiciones de los ancianos basado en el método estandarizado para el adulto joven INFOGEST (Menard et al., 2023). En la **Tabla 3** se muestran las diferencias en las condiciones gastrointestinales entre el método armonizado y el aplicado en la presente tesis para la población anciana.

Tabla 3. Diferencias en las condiciones digestivas adaptadas al anciano entre el método armonizado y las utilizadas en la presente tesis doctoral

Fase	**Armonizado (Menard et al., 2023)**	**Tesis (adaptado de Shani-Levi et al. 2017)**
Oral	α-Amilasa (75 U/mL) 2 min, 37ºC, 95 rpm, pH 7	
Gástrica	Pepsina (1200 U/mL) Lipasa gástrica (36 U/mL) pH 3,7	Pepsina (1000 U/mL) Lipasa gástrica (9 U/mL) pH 6
Intestinal	Pancreatina (80 U/mL de tripsina) Sales biliares (6,7 mM) Duración 2 horas	Pancreatina (50 U/mL de tripsina) Sales biliares (5 mM) Duración 3 horas

En general, las disminuciones de actividades enzimáticas en el anciano respecto al adulto propuestas por la armonización del método son levemente inferiores a las de la presente tesis, salvo en el caso de la LG, donde la diferencia es notable (40 vs. 85 %, respectivamente). Además, el incremento en el pH gástrico es muy leve (de 3 a 3,7) en comparación al propuesto en esta tesis doctoral (de 3 a 6). La reducción de la actividad enzimática de la pepsina es comparable (40 vs. 50 %, respectivamente), al igual que la concentración de sales biliares (33 vs. 50 %, respectivamente), aunque la diferencia en la reducción aplicada en la actividad de la pancreatina es superior (20 vs. 50 %, respectivamente).

4. Efectos biológicos

El principal efecto biológico de los EV es su acción hipocolesterolemiante relacionada con la reducción de la absorción de colesterol dietético debido a su competición por la entrada en las micelas mixtas. Además, los EV han demostrado otros efectos biológicos tales como antiproliferativo, antiinflamatorio, inmunomodulador o antioxidante.

4.1. Modelos biológicos

Modelos *in vitro*: células Caco-2 y CCD-18co

Los modelos celulares son los más utilizados para evaluar diferentes efectos biológicos de sustancias o alimentos. Representan un paso importante como modelo preclínico, ya que son relativamente simples, eficientes respecto a tiempo y recursos, carecen de dilemas éticos y son fácilmente reproducibles. Permiten observar efectos potencialmente beneficiosos o tóxicos de ciertas sustancias. Sin embargo, no suponen una evidencia definitiva, ya sea por reproducir la estructura y funcionalidad de los distintos órganos humanos de forma limitada, con su complejidad tisular, o las interacciones entre distintos tejidos, órganos o sistemas. En general, son útiles para realizar estudios de cribado con diferentes sustancias y pueden suponer una base previa de evidencia para los estudios con modelos de experimentación animal más complejos, como paso previo a ensayos clínicos (Lea, 2015).

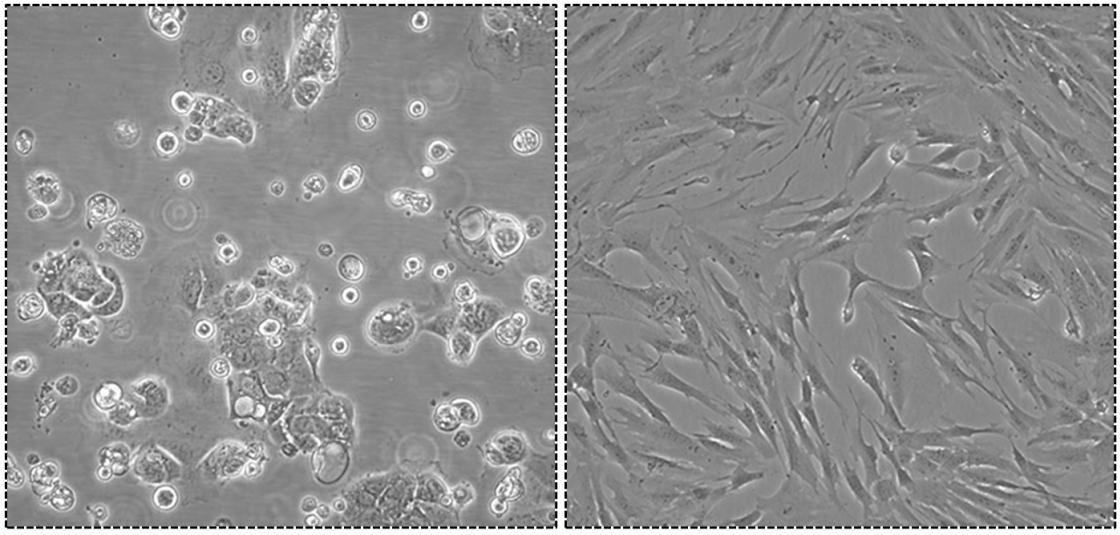
La línea celular Caco-2, consistente en células de adenocarcinoma de colon, ha sido ampliamente utilizada durante muchos años como modelo tumoral de cáncer de colon. Dichas células fueron aisladas en 1977 de un paciente que padecía un adenocarcinoma de colon (Fogh & Trempe, 1975). Son usadas habitualmente tras su diferenciación, con el objetivo de emular a las células normales del epitelio del intestino delgado (Pinto et al., 1983). Cuando se cultivan hasta la confluencia celular, desarrollan uniones estrechas, adquieren polaridad representando el borde en cepillo del epitelio y expresan enzimas características de este tejido (lactasa, aminopeptidasa, dipeptidil peptidasa IV y sucrasa-isomaltasa) (Lea, 2015). Una revisión reciente ha establecido recomendaciones relacionadas con la compatibilidad de las células Caco-2 con los digeridos de alimentos, con el fin de conocer las condiciones de absorción de estos y evaluar sus nutrientes mediante cultivo celular (Kondrashina et al., 2023).

En el contexto de esta tesis, cuando dichas células se encuentran sin diferenciar, esto es, en fase de crecimiento exponencial, suponen un modelo de cáncer de colon que puede ser utilizado para evaluar potenciales efectos antiproliferativos o quimiopreventivos de sustancias aisladas o mezclas complejas (López-García et al., 2017), así como del producto de la digestión de un alimento (Cilla et al., 2022).

La línea celular CCD-18co, consistente en fibroblastos de colon humano, se utiliza habitualmente como modelo no tumoral para comparar con las células Caco-2 no

diferenciadas (modelo tumoral) (**Figura 9**). Para que un compuesto, mezcla o alimento presente un efecto antiproliferativo beneficioso para la salud, este debe ser capaz de dañar de manera selectiva a las células tumorales sin afectar o afectando en menor medida a las células normales, para lo que se utilizan estas células como control. Trasladando el modelo celular *in vitro* a la situación tumoral real en el organismo, hace referencia a una sustancia que sea capaz de estimular la apoptosis o reducir la progresión del ciclo celular en el tumor localizado pero que no sea o sea levemente tóxica para los tejidos no tumorales (Makran et al., 2023a).

Figura 9. Imágenes de microscopio óptico de las líneas celulares utilizadas en la presente tesis



Caco-2 **CCD-18co**

Fuente: *American Type Culture Collection*.

Modelo *ex vivo*: eritrocitos humanos

El cultivo *ex vivo* con eritrocitos se utiliza ampliamente por su facilidad de obtención y relativa manejabilidad. Solo se requiere de una extracción de sangre del voluntario, para posteriormente, mediante centrifugado, aislar los eritrocitos del resto de componentes de la sangre. Para el análisis de la eriptosis (apartado 4.3) se mantienen en una solución Ringer, que contiene sales en diferentes proporciones para conseguir equilibrio osmótico y glucosa para proporcionar el adenosin trifosfato (ATP) necesario para las células. Se pueden utilizar para evaluar el efecto tóxico de compuestos aislados o el efecto protector frente a estrés oxidativo (inducido por *tert*-butilo hidroperóxido (tBOOH), H_2O_2, COPs o

humo de tabaco, entre otros) o frente a estrés energético (no adición de glucosa al Ringer) (Qian et al., 2012; Tesoriere et al., 2014; Álvarez-Sala et al., 2018a; Restivo et al., 2023).

4.2. Antiproliferativo

La prevención y tratamiento del cáncer es uno de los retos sanitarios más importantes a nivel mundial, sobre todo en los países desarrollados, toda vez que el aumento de la esperanza de vida se traduce en una mayor prevalencia de esta enfermedad. Entre todas las neoplasias, el CCR es el tercer tipo de cáncer más común, después del cáncer de mama y de pulmón. En 2022 se diagnosticaron casi 2 millones de casos en todo el mundo. Pese a la existencia de protocolos de detección eficaces, el CCR sigue siendo la segunda causa más común de muerte relacionada con el cáncer. La Agencia Internacional para la Investigación del Cáncer (IARC) estima que la incidencia de CCR aumentará un 56% entre 2020 y 2040, por lo que se pueden esperar más de 3 millones de nuevos casos al año (IARC, 2023).

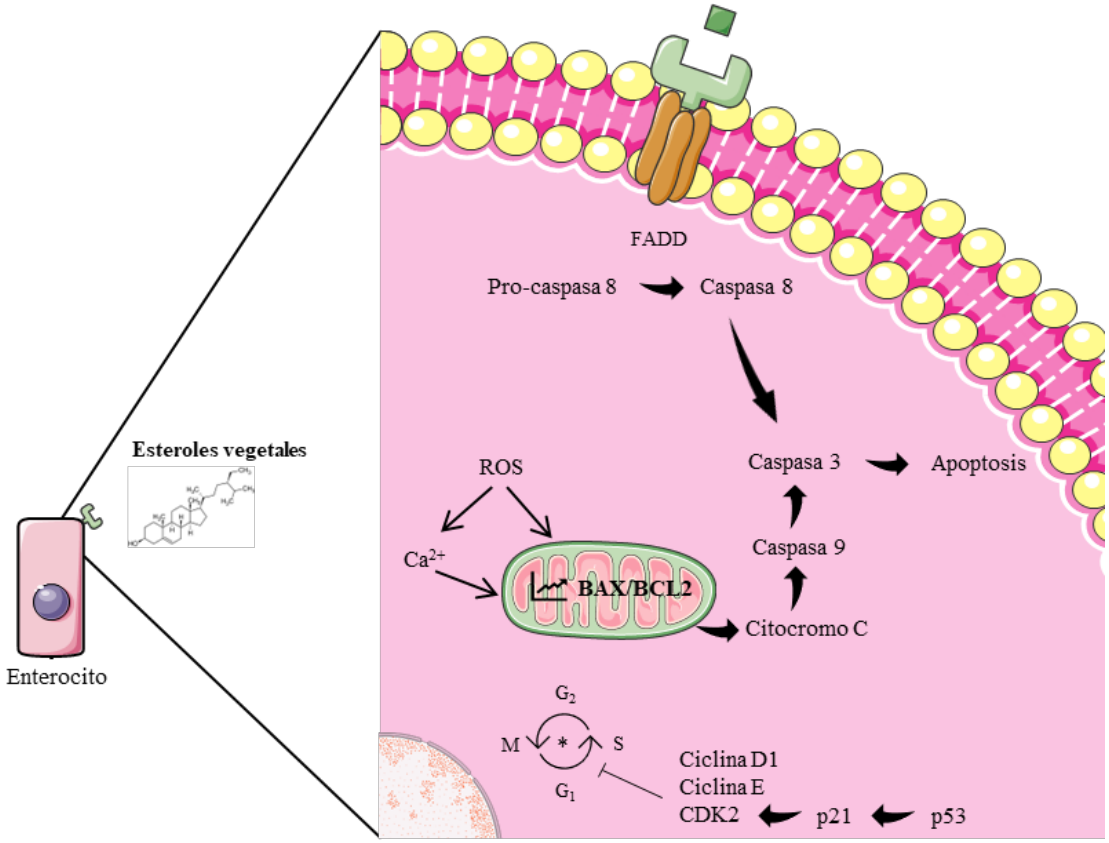
La dieta representa uno de los factores de riesgo más relevantes en su desarrollo, destacando el consumo de carne roja y procesada, y la baja ingesta de fibra dietética. Sin embargo, la dieta también aporta componentes potencialmente protectores respecto al desarrollo de la neoplasia, principalmente la ingesta de vegetales como frutas y verduras, por su contenido, no solo en fibra, sino también en compuestos sulfurados (isotiocianatos y glucosinolatos), polifenoles, carotenoides, EV, entre otros (Kumar et al., 2023). De entre todos, la fibra dietética destaca por su conocido efecto protector, ya observado desde hace más de 20 años en grandes estudios epidemiológicos (Bingham et al., 2003; Peters et al., 2003). Este efecto se debe a su metabolización por la microbiota intestinal, produciendo ácidos grasos de cadena corta (AGCC), principalmente butirato, el cual supone una importante fuente energética para los colonocitos, reduce la proliferación celular y es capaz de inhibir el daño producido por sustancias genotóxicas (Ebert et al., 2001; Xiong et al., 2022). Otros AGCC importantes son el propionato y el acetato, los cuales inhiben la proliferación celular y son capaces de activar la respuesta inmunológica de células T (Xiong et al., 2022). De forma conjunta, aumentan la apoptosis de las células tumorales colónicas, ya sea desestabilizando el estado redox celular, como produciendo arresto en fase G2/M en el ciclo celular (Matthews et al., 2012). Adicionalmente, la fibra dietética previene el estreñimiento, factor de riesgo de desarrollo de la enfermedad (Makharia et al., 2022).

El consumo actual de fibra en España es sensiblemente inferior al recomendado (12,6 *vs.* 25 g/día) (EFSA 2010; González-Rodríguez et al., 2017), por lo que la introducción en la dieta de alimentos con una mayor densidad de este componente es de gran interés. Los cereales integrales destacan por su elevado contenido de fibra alimentaria (entre 9,2 y 37,7 g / 100 g de cereal, exceptuando el arroz, con menor contenido), siendo mayor el contenido de insoluble (entre 5,4 y 33,9 g / 100 g de cereal) que de soluble (entre 1,4 y 20,0 g / 100 g de cereal) (Prasadi & Joye, 2020), por lo que puede ser interesante la sustitución del consumo de pan común de harinas refinadas de trigo (pan blanco), arroces y pastas, por panes 100% integrales de avena o centeno, nutricionalmente más completos (Åman et al., 2010). Además, el pan de centeno podría estar relacionado con un menor riesgo de desarrollo de CCR en comparación con el pan blanco de trigo, como se observa en un ensayo clínico aleatorizado cruzado donde los participantes consumían el equivalente al 20% de la energía total bien con el pan blanco de trigo, bien con pan integral de centeno. El efecto protector observado es atribuible a la reducción del tiempo de tránsito intestinal y el aumento de la masa fecal que produce el consumo de pan de centeno en comparación al de trigo, pero también a la menor presencia tras la digestión de ácidos biliares secundarios, tóxicos para los colonocitos, y a un aumento en la concentración de butirato (Gråsten et al., 2000).

Los EV han demostrado capacidad antiproliferativa tanto en modelos celulares (Caco-2, COLO 320 DM, HCT116 y HT29) como en modelo animal murino de cáncer de colon, actuando directamente en el desarrollo de la enfermedad. La mayor parte de los estudios han utilizado el β-sitosterol (Salehi et al., 2021). Los mecanismos biológicos por los que los EV ejercen su actividad antiproliferativa en cáncer de colon se resumen en la **Figura 10.**

Diversos estudios han utilizado las células Caco-2 sin diferenciar para evaluar los efectos de los EV en la proliferación celular a nivel colónico, ya sea a concentraciones colónicas (6-140 μM) (Maiyo et al., 2016; López-García et al., 2017; Álvarez-Sala et al., 2018b), plasmáticas (0,25-13,25 μM) (Cilla et al., 2015) y no fisiológicas (5-400 μM) (Daly et al., 2009); también de POPs (7β-hidroxi-sitosterol, 30 μM) (Roussi et al., 2005); así como del principal producto metabólico generado por la microbiota de los EV, el etilcoprostanol a concentraciones colónicas (9-300 μM) (Makran et al., 2023a) (**Tabla 4**).

Figura 10. Actividad antiproliferativa de los esteroles vegetales en cáncer colorrectal



BAX: proteína X asociada a BCL2; BCL2: célula B/linfoma 2; CDK2: cinasa dependiente de ciclina 2; FADD: *Fas-associated death domain*; G_1: fase de intervalo 1; G_2: fase de intervalo 2; M: fase de mitosis; ROS: especies reactivas de oxígeno; S: fase de síntesis. *: Ciclo celular

La disminución de la viabilidad observada *vs.* control varía en un rango amplio (22-60 %) entre los estudios, si bien todos muestran una disminución significativa de esta. Esta reducción se justifica por un aumento en la apoptosis (2-6 veces de aumento de la externalización de la fosfatidilserina (FS) *vs.* control) (Cilla et al., 2015; Álvarez-Sala et al., 2018b), ya sea por el aumento en los niveles intracelulares de ROS (Cilla et al., 2015; López-García et al., 2017; Álvarez-Sala et al., 2018b) o por el aumento de calcio intracelular (Cilla et al., 2015). El etilcoprostanol, por su parte, produce una reducción de la viabilidad en Caco-2 (22-43% *vs.* control) debida a un aumento en la apoptosis y en la población celular en fase subG$_1$ del ciclo celular. Dicha apoptosis se ve corroborada por el incremento, de hasta 8 veces *vs.* control, en los niveles de ceramida (mediador proapoptótico) y por una sobreproducción de ROS. A nivel de expresión génica, este metabolito produce un aumento tanto de la ratio *BAX/BCL2* (proapoptótico) como de la

Tabla 4. Efecto antiproliferativo de esteroles vegetales aislados o sus mezclas sobre células Caco-2

Tipo de esteroles y tratamiento	Resultados (cambios vs. control)	Referencia
7β-hidroxi-sitosterol (60 μM), 32 h	↑ actividad de caspasa 3 y 9 Fragmentación del ADN tras 56 h de tratamiento	Roussi et al., 2005
β-sitosterol, campesterol o sitostanol de forma individual (5-400 μM), 48 h	↓ viabilidad (0-47%, MTT)	Daly et al., 2009
Mezcla de β-sitosterol (12 μM), campesterol (1 μM) y estigmasterol (0,25 μM), 24 h	↓ viabilidad (25-45%, MTT) ↑ apoptosis (2-4 veces), calcio intracelular (hasta 3,4 veces), ROS (hasta 10 veces)	Cilla et al., 2015
β-Sitosterol-3-O-glucósido (35-140 μM), 48 h	↓ viabilidad (45-60%, MTT) ↑ apoptosis (13%)	Maiyo et al., 2016
Mezcla de β-sitosterol (115 μM), campesterol (11 μM) y estigmasterol (6 μM), 24-72 h	↓ viabilidad (59%, MTT) ↑ necrosis (3 veces) sin aumento de apoptosis, ROS (3 veces)	López-García et al., 2017
	↓ viabilidad (22-30%, MTT) ↑ apoptosis (3 veces), ROS (2,7 veces)	Álvarez-Sala et al., 2018b
Etilcoprostanol (9-300 μM), 24 h	↓ viabilidad (22-43%, MTT) ↑ apoptosis (85%), ROS (22%), ceramida (8 veces)	Makran et al., 2023a

ROS: especies reactivas de oxígeno

expresión del gen *CASP9* (y el de la caspasa efectora *CASP3*), lo que confirma que la apoptosis se produce por vía mitocondrial (o intrínseca). Además, la expresión de los genes *CCND1* y *CCNE1*, que codifican la ciclina D1 y E1, respectivamente, se ve reducida. De igual manera, se produce una reducción de la transcripción del gen que codifica la proteína p21 (*CDKN1A*) (Makran et al., 2023a). Tanto estas ciclinas como indirectamente la proteína p21, a través de la inhibición de la cinasa dependiente de ciclina 2 (CDK2), producen una regulación del ciclo celular a nivel de la fase G_1 y la progresión a la fase de síntesis (S) por lo que su inhibición se asocia a un mecanismo antiproliferativo (ver **Figura 10**).

Sin embargo, los compuestos no llegan de manera aislada al colon y, junto a otros factores como la digestión, enzimas y resto de componentes de los alimentos y la dieta, ejercen un efecto, sinérgico o no, sobre el colon. En ese sentido, el uso de LF procedentes de una digestión dinámica con fermentación colónica puede simular mejor las condiciones en el colon tras ingerir los alimentos y permiten estudiar el potencial quimiopreventivo de los alimentos en células tumorales. En concreto, se ha evaluado el efecto antiproliferativo sobre células de adenoma de colon (LT97) de LF obtenidos de la digestión gastrointestinal *in* vitro estática y fermentación discontinua (por lotes) de alimentos ricos en fibra como frutos secos (Glei et al., 2017; Schlörmann et al., 2017; Glei et al., 2018), cebada (Schlörmann et al., 2020; 2021), avena (Schlörmann et al., 2021; Glei et al., 2021) y panes de trigo y de centeno (Schlörmann et al., 2012; 2022) (Ver **Tabla 5**). Para obtener los LF, los autores detienen la fermentación a 4ºC y las muestras se centrifugan 2 veces a 4200 *g* durante 15 minutos. Después, se realiza una centrifugación extra a 10300 *g* durante 15 minutos y los sobrenadantes se filtran para esterilizar (tamaño de poro: 0,22µm). En general, tras el aumento de AGCC producido por la fermentación de la fibra dietética (principalmente butirato), se produce un efecto antiproliferativo (aumento de la actividad de caspasa 3 y, consecuentemente, de la apoptosis) en células de adenoma de colon (LT97).

4.3. Antieriptótico

La eriptosis es la apoptosis, o muerte programada, de los eritrocitos. La ausencia de mitocondrias en estas células afecta notablemente a su formación, funciones o suministro de energía, pero también a su capacidad de apoptosis, pues en el resto de las células, estos orgánulos son claves en el proceso apoptótico. Los principales mecanismos usados por los eritrocitos para su muerte programada dependen de la entrada de calcio a la célula, la

Tabla 5. Actividad antiproliferativa de líquidos de fermentación procedentes de alimentos ricos en fibra

Matriz	Resultado y mecanismo	Referencia
Avena	↑ actividad de caspasa 3 Atribuido a producción de butirato	Glei et al., 2021
	↑ actividad de caspasa 3 Atribuido a producción de butirato, contenido de β-glucano y reducción de producción de amonio	Schlörmann et al., 2021
Cebada	↑ actividad de caspasa 3 Atribuido a producción de butirato, contenido de β-glucano y reducción de producción de amonio	Schlörmann et al., 2020
		Schlörmann et al., 2021
Frutos secos	↑ apoptosis y actividad caspasa 3 Atribuido a producción de butirato y otros compuestos como selenio	Glei et al., 2017
	↑ apoptosis y actividad caspasa 3 Atribuido a producción de butirato	Schlörmann et al., 2017
	↑ apoptosis y actividad caspasa 3 Atribuido a producción de butirato y compuestos fenólicos	Glei et al., 2018
Pan de trigo y centeno	↑ apoptosis y expresión de genes relacionados (*CYP2C8, GSTT1, CHST1, SULT2B1, c-JUN, SOD2, GPX1, ERCC4, TNFSF10* y *WNT2B*), equivalente en ambos panes Atribuido a producción de butirato (similar entre panes)	Schlörmann et al., 2012
	↑ actividad caspasas 2 y 3 equivalente en ambos panes Atribuido a producción de butirato (similar entre panes)	Schlörmann et al., 2022

formación de ceramida, la disminución del volumen celular y la exposición de la FS en la superficie celular (Alghareeb et al., 2023) (**Figura 11**). Estos procesos pueden ser estimulados por diferentes vías, desde la exposición a toxinas o antibióticos, hasta procesos fisiológicos como el estrés oxidativo (incremento de ROS y/o disminución de los niveles de glutatión reducido (GSH)), el choque osmótico o el estrés energético (agotamiento de ATP celular). El mecanismo clave en este proceso es la externalización

de la FS, ya que los eritrocitos con la membrana disfuncional son los más propensos a adherirse al endotelio y causar trastornos en la microcirculación (Lang et al., 2012).

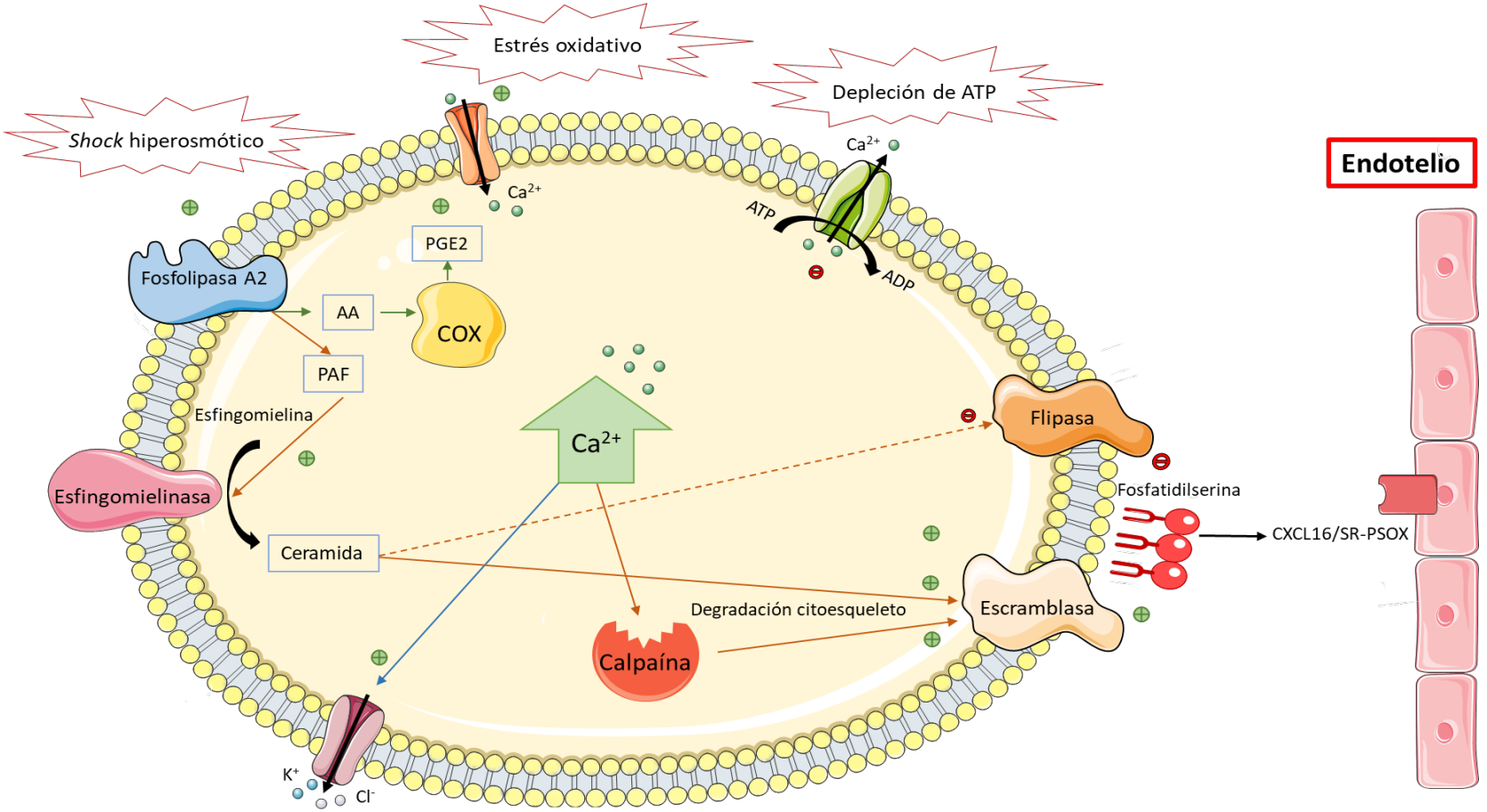
La enzima encargada de externalizar la FS es la escramblasa, estimulada, directa o indirectamente, por el aumento del calcio intracelular. Dicho aumento puede ser provocado por: i) el estrés oxidativo, capaz de activar canales de calcio en la membrana celular, ii) el *shock* hiperosmótico a través de la formación de prostaglandina E2, activadora de los canales de calcio, iii) el estrés energético, ya que, al agotarse las reservas de ATP, se inhibe la salida de calcio a través de los canales ATP-dependientes. El calcio también estimula la activación de la calpaína, una endopeptidasa capaz de degradar las proteínas citoesqueléticas y, por tanto, degradar la estructura de la célula, exponiendo la FS en la cara externa de la membrana celular en última instancia (Restivo et al., 2021).

La afectación en la ósmosis celular también aumenta la producción del factor de activación plaquetar (PAF), que estimula directamente la actividad de la esfingomielinasa. Esta enzima de membrana produce ceramida a partir de la degradación de la esfingomielina, que es capaz de activar la escramblasa al mismo tiempo que inhibe a la flipasa, una enzima encargada de mantener los fosfolípidos en la posición adecuada en el interior de la membrana celular. Por tanto, la producción de ceramida desestabiliza el equilibrio en la membrana celular, exponiendo la FS a la superficie (Alghareeb et al., 2023).

La FS externalizada en los eritrocitos eriptóticos se une a receptores de plaquetas o células endoteliales como el receptor de depuración que se une a la fosfatidilserina y a los lípidos oxidados (CXCL16/SR-PSOX) produciendo su adhesión al endotelio, lo cual conlleva el empeoramiento de la microcirculación y el subsecuente aumento de riesgo cardiaco (Fang et al., 2022).

Pese a que la eriptosis es fenómeno natural mediante el cual se eliminan las células senescentes o con lesiones, el aumento de esta es una condición habitual en patologías como la enfermedad renal crónica, diabetes, leucemia, o algunas anemias, entre otras. La anemia se produce en el caso de que la eritropoyesis no sea suficiente para regenerar el gran número de eritrocitos eliminados, resultando en una disminución paulatina del hematocrito (Alghareeb et al., 2023). Asimismo, se ha observado que la excesiva eriptosis

Figura 11. Esquema del proceso de las distintas rutas de eriptosis y su implicación en la adhesión de los eritrocitos eriptóticos al endotelio



AA: ácido araquidónico; ADP: adenosín difosfato; ATP: adenosín trifosfato; CXCL16/SR-PSOX: receptor de depuración que se une a la fosfatidilserina y a los lípidos oxidados; COX: ciclooxigenasa; PAF: factor de activación plaquetar; PGE2: prostaglandina E2. ⊕: activación; ⊖: inhibición; las flechas naranjas indican el proceso de externalización de fosfatidilserina; las flechas verdes indican el proceso de activación de canales de entrada de calcio; la flecha azul indica proceso de salida de iones y consecuente disminución de tamaño celular.

tiene un efecto protrombótico, ya que los eritrocitos eriptóticos son más propensos a adherirse al endotelio vascular, aumentando de ese modo el riesgo de agregación plaquetaria y de formación de lesiones ateroscleróticas (Lang et al., 2012; Lang et al., 2014; Pretorius et al., 2016) con un aumento del riesgo cardiovascular (Fang et al., 2022).

Una reciente revisión ha evaluado la capacidad preventiva de distintos compuestos de los alimentos sobre la eriptosis. La investigación es amplia para compuestos fenólicos como el resveratrol, el hidroxitirosol o la naringina, entre otros. En general, los compuestos fenólicos son capaces de prevenir la eriptosis en modelos *ex vivo* o murinos, ya sea por disminución de la externalización de la FS o por la reducción del estado oxidativo general (Restivo et al., 2022a). El número de estudios que sugieren un efecto preventivo con otros compuestos es relativamente reducido, destacando algunos alcaloides como la cafeína, que es capaz de reducir la exposición de la FS en modelo *ex vivo* (Floride et al., 2008), o la indicaxantina, un pigmento procedente de los higos chumbos con efecto antioxidante (Tesoriere et al., 2015). La vitamina C, con su potente efecto antioxidante, también es capaz de neutralizar el estrés oxidativo, reduciendo consecuentemente la eriptosis (Mahmud et al., 2010).

Se ha observado, en un estudio clínico observacional, que las personas con hipercolesterolemia tienen niveles de eriptosis más elevados que los sujetos normocolesterolémicos, incluso tras toma de estatinas y subsecuente reducción de la colesterolemia. Además, presentan mayor adhesión *ex vivo* de los eritrocitos al endotelio (12,5 *vs.* 5,5 células adheridas / mm^2 de endotelio en pacientes con y sin hipercolesterolemia, respectivamente), remarcando el ya conocido efecto negativo del exceso de colesterol plasmático en la salud cardiovascular. Además, se aprecia que los niveles de GSH se correlacionan inversamente no solo con la adhesión, sino también con el colesterol plasmático y la ApoB (proteína característica del LDL-c) (Cilla et al., 2021). Estudios previos también han observado mayores niveles de eriptosis en pacientes con dislipemia tratados con estatinas respecto a sujetos sanos (Pinzón-Díaz et al., 2018). No solo la atorvastatina (Rana et al., 2019), sino otras estatinas como la simvastatina, consiguen incluso estimular el desarrollo de la eriptosis, como se aprecia en estudios *ex vivo* (Al Mamun Bhuyan et al., 2017).

En un estudio realizado por nuestro grupo de investigación, los EV previnieron la eriptosis en un modelo *ex vivo* con 8 voluntarios. Tras inducir estrés oxidativo en los eritrocitos mediante tBOOH (75 μM), una mezcla dietética de β-sitosterol, campesterol y

estigmasterol a concentraciones plasmáticas (22 μM) reduce la producción de calcio (≈ 15%) y de ROS (≈ 20%), aumentando las reservas de GSH intracelular (≈ 15%) y, adicionalmente, disminuye la hemólisis de la muestra. Además, los EV también impidieron la inducción de la externalización de la FS tras exponer los eritrocitos a β-criptoxantina (Álvarez-Sala et al., 2018a). Por lo tanto, se plantea como hipótesis que el consumo de EV podría suponer un enfoque dietético preventivo para los factores de riesgo de ECV (hipercolesterolemia y eriptosis), representando un efecto sinérgico unido a la terapia con estatinas.

4.4. Hipocolesterolemiante

Las ECV son la principal causa de muerte a nivel mundial. Se estima que en 2019 fallecieron casi 18 millones de personas a causa de estas patologías, lo que supone un tercio del total de muertes. Entre ellas, destacan el infarto agudo de miocardio y el accidente cerebrovascular, responsables de la mayoría de dichas muertes. Son diversos los factores que pueden provocar estas enfermedades, destacando entre ellos el consumo de tabaco y de alcohol, la hipertensión o la hipercolesterolemia (WHO, 2021). El aumento excesivo de colesterol plasmático, junto a un entorno proinflamatorio, puede provocar la formación de placas de ateroma en las arterias y, en última instancia, el desarrollo de un accidente vascular (Frostegård, 2013).

La regulación de la colesterolemia puede controlarse a nivel dietético mediante la ingesta de EV y fármacos como la ezetimiba, disminuyendo su absorción; a nivel de síntesis endógena, mediante la inhibición de la enzima hidroximetilglutaril-CoA (HMG-CoA) reductasa por fármacos como las estatinas o CB como la monacolina K (ver **Figura 3**).

4.4.1. Efecto del tratamiento dietético

Los EV reducen la colesterolemia al disminuir la absorción del colesterol dietético por diversos mecanismos (ver **Figura 3**). En primer lugar, inhiben la incorporación del colesterol a las micelas mixtas debido a su mayor hidrofobicidad, lo cual hace que estas tengan mayor afinidad por los EV. Todavía en el lumen intestinal, también producen la cristalización de ambos y su subsecuente precipitación. Además, una vez en las micelas, los EV pueden competir con el colesterol por el transportador encargado de introducirlos en el enterocito (NPC1L1), por la posterior esterificación (mediante la ACAT2) en el interior del mismo, y por la incorporación a los quilomicrones (mediante la MTP).

También se ha propuesto que pueden ejercer una regulación sobre la ACAT2 y la MTP, aunque el mecanismo molecular todavía no ha sido esclarecido. Por otra parte, los EV son capaces de activar la vía TICE y, por tanto, incrementar el eflujo de colesterol en el borde en cepillo y su posterior eliminación por vía fecal (ver **Figura 3**) (Li et al., 2022b).

El efecto hipocolesterolemiante presenta heterogeneidad interindividual debido a la presencia de polimorfismos genéticos en genes responsables de la absorción y el metabolismo del colesterol, principalmente los genes *ABCG5/G8, APOE, APOA4/A5, CETP, CYP7A1, HMGCR, LIPC, LPA, MTHFR, NPC1L1 y SCARBI* (Makran et al., 2021). Las personas que, debido a algunos de estos polimorfismos, presenten una absorción de colesterol elevada junto a una síntesis reducida, generalmente se benefician de un mayor efecto hipocolesterolemiante con la ingesta de EV en comparación a las personas con el caso inverso (Fumeron et al., 2017; Makran et al., 2021). Además, la magnitud de dicho efecto depende de factores como la dosis: mayor efecto con dosis por encima de 2 y 2,5 g diarios (Ras et al., 2014; Fontané et al., 2023); la matriz: mayor efecto con margarinas, lácteos o bebidas que en panes o cereales (Fontané et al., 2023); el momento de la toma: mejor en comidas principales (Li et al., 2022b), aunque otras revisiones indican que este factor no es relevante (Gao et al., 2023). Sin embargo, otras variables como la presencia de esteroles o estanoles (He et al., 2018) no parecen influir de una manera relevante a dicha magnitud. Por otro lado, respecto al estado libre o esterificado de los EV no existe un consenso, ya que algunas revisiones muestran que no tiene relevancia (Musa-Veloso et al., 2011), mientras otras sí que aprecian mayor efecto con EV esterificados (Gao et al., 2023).

Un metaanálisis reciente ha evaluado los efectos de la toma de EV en distintos formatos (alimentos enriquecidos y complementos alimenticios) en 58 ensayos clínicos aleatorizados con pacientes hipercolesterolémicos (Gao et al., 2023). En la **Tabla 6** se han recopilado los estudios que evalúan el efecto hipocolesterolemiante de leche y derivados, zumos, bebidas a base de zumo de frutas y leche, pan o complementos alimenticios enriquecidos con EV. La dosis de EV varía entre ensayos desde 1,2 hasta 3 g/día. Los períodos de intervención con EV oscilan ampliamente desde las 2 semanas hasta los 12 meses. La reducción del colesterol total (CT) tras el tratamiento, respecto al placebo, presenta un rango de 1,6 hasta 12,3%, mientras que el LDL-c se reduce entre un 4,3 y 15,9%. Sin embargo, ni la concentración de triglicéridos ni de HDL-c se ven modificadas en el global de los ensayos. La reducción del colesterol no está influenciada por el tipo de

Tabla 6. Efecto hipocolesterolemiante de alimentos enriquecidos con esteroles vegetales (pan y bebidas) y complementos alimenticios

Matriz	**Esterol/estanol (g/día)**	**Duración (semanas)**	**Diseño**	**Resultados**	**Referencia**
Leche / pan	1,6 esteroles	3	Cruzado	CT: -8,7% en leche sin reducción con pan LDL-c: -15,9% en leche y -6,5% en pan	Clifton et al., 2004
Zumo de naranja	2 esteroles	8	Paralelo	CT: -7,2% LDL-c: -12,4%	Devaraj et al., 2004
Yogur líquido	3 esteroles	4	Paralelo	LDL-c: -9,5% junto a comida y -5,1% sin comida	Doornbos et al., 2006
Cápsulas	1,6 estanoles	4	Paralelo	CT: -8% LDL-c: -9%	Woodgate et al., 2006
Cápsulas	1,3 esteroles	4	Cruzado	CT: Reducción no significativa LDL-c: -7%	Acuff et al., 2007
Cápsulas	2,6 esteroles	12	Paralelo	CT: -3,5% LDL-c: -5%	Earnest et al., 2007
Leche fermentada	1,6 esteroles	6	Paralelo	CT: -5,7% LDL-c: -8,6%	Hansel et al., 2007
Yogur líquido	2 esteroles	4	Paralelo	CT: Reducción no significativa LDL-c: Reducción no significativa	Niittynen et al., 2008
Yogur líquido	1,6 esteroles	6	Paralelo	CT: -8% LDL-c: -10,5%	Plana et al., 2008
Leche	1,2 esteroles	6	Paralelo	CT: -9,2 % LDL-c: -11,5%	Sukmaniah et al., 2008
Yogur líquido	2 esteroles	4	Paralelo	CT: -3,2% LDL-c: -4,5 %	Khandelwal et al., 2009
Leche fermentada	1,6 esteroles	6	Paralelo	CT: -12,3% LDL-c: -11,3%	Mannarino et al., 2009
Leche	2 esteroles	12	Paralelo	CT: -6,8% LDL-c: -10%	Bañuls et al., 2010

Tabla 6. Continuación

Matriz	Esterol/estanol (g/día)	Duración (semanas)	Diseño	Resultados	Referencia
Leche	2 esteroles	12	Paralelo	CT: -7,3% LDL-c: -10,2%	Hernández-Mijares et al., 2010
Pan de centeno	2 esteroles	2	Paralelo	CT: -1,6% LDL-c: -4,4%	Söderholm et al., 2012
Cápsulas	2 esteroles	4	Cruzado	CT: Sin reducción LDL-c: Sin reducción	Ottestad et al., 2013
Cápsulas	1,8 esteroles / estanoles	6	Paralelo	CT: -3,5% LDL-c: -4,3%	McKenney et al., 2014
Bebida a base de fruta y leche	1,5 esteroles / estanoles	4	Cruzado	CT: Sin reducción LDL-c: Sin reducción	Granado-Lorencio et al., 2014
Yogur líquido	2 estanoles	52	Paralelo	CT: -7,7% LDL-c: -9%	Párraga-Martínez et al., 2015
Leche	1,5 esteroles	3	Paralelo	CT: -5% LDL-c: -8,2%	Cheung et al., 2017
Bebida a base de fruta y leche	2 esteroles / estanoles	6	Cruzado	CT: -3,2% LDL-c: -6%	Álvarez-Sala et al., 2018c
Smoothie	2 estanoles	4	Paralelo	CT: -5,6% LDL-c: -9%	Lestiani et al., 2018
Pan blanco	2,3 esteroles	4	Paralelo	CT: -6,1% LDL-c: -8,8%	Ferguson et al., 2019

CT: colesterol total; LDLc: colesterol asociado a lipoproteínas de baja densidad

EV (esteroles o estanoles) o el momento de la ingesta de estos. No obstante, la dosis (mayor reducción a dosis superiores a 2 g/día) y la matriz (mayor reducción con matrices ricas en grasa) sí que influyen en los resultados.

Recientemente, la UE ha autorizado el uso de levadura de arroz rojo por su efecto hipocolesterolemiante por inhibición de la HMG-CoA reductasa, análogamente a los tratamientos farmacológicos con estatinas. El reglamento permite la declaración de propiedades saludables en "una combinación de extracto seco de hoja de alcachofa normalizado en ácidos cafeoilquínicos, monacolina K en arroz de levadura roja, policosanoles derivados de la caña de azúcar, oligómeros procianidólicos de corteza de pino marítimo francés, extracto seco de ajo normalizado en alicina, succinato ácido de d-α-tocoferilo, riboflavina y hexanicotinato de inositol" (Reglamento 648/2023/UE). Como se aprecia en un reciente metaanálisis que comprende 17 estudios que utilizan este nutracéutico, esto se traduce en una reducción sustancialmente elevada de la colesterolemia tras su ingesta crónica (entre 4 y 168 semanas), de media 0,94 mmol/L (Osadnik et al., 2022). La ingesta de un complemento con la combinación de monacolina K (10 mg), EV (1,5 g) e hidroxitirosol (5 mg) durante un mes en sujetos con hipercolesterolemia moderada no tratada produce una reducción del CT y de LDL-c de 11,4 y 19,8 %, respectivamente (Domenech et al., 2019). Sin embargo, tanto las estatinas como la monacolina K pueden presentar efectos adversos, como problemas musculares (rabdomiólisis en casos más graves) o hepáticos, aunque son infrecuentes (Norata & Banach, 2024). Por estos motivos y ya que "sigue habiendo una posibilidad de efectos nocivos para la salud asociados al uso de monacolinas procedentes del arroz fermentado con levadura roja", la UE ha decidido retirar la declaración de propiedades saludables para este nutracéutico (Reglamento 2041/2024/UE).

4.4.2. Tratamiento dietético y farmacológico

Para el tratamiento de la hipercolesterolemia moderada se utilizan principalmente las estatinas, como la atorvastatina (10-20 mg), simvastatina (20-40 mg), rosuvastatina (5-10 mg) o pitavastatina (1-4 mg), entre otras. Estos fármacos inhiben la actividad de la HMG-CoA reductasa, por lo que se sintetiza una cantidad de colesterol notablemente menor, reduciendo así la colesterolemia.

En pacientes tratados con estatinas, se puede instaurar un tratamiento con EV actuando de forma sinérgica con el tratamiento terapéutico de la hipercolesterolemia. La reducción

adicional de la colesterolemia por parte de los esteroles (entre el 7-12 %) podría evitar un aumento en la dosis de fármaco suministrado (Anagnostis et al., 2023, Fontané et al., 2023).

En la **Tabla 7** se recopilan los ensayos clínicos aleatorizados que evalúan el efecto hipocolesterolemiante con alimentos enriquecidos en EV (mayoritariamente margarinas) o complementos alimenticios, junto a un tratamiento con estatinas (preferentemente atorvastatina). La ingesta de EV varía entre 1,8 y 3 g/día entre los ensayos. Pese a que la diversidad metodológica dificulta la comparación directa, se observa un efecto sinérgico de los EV en el tratamiento de la hipercolesterolemia con estatinas. Un reciente metaanálisis muestra que los pacientes tratados (estatinas más EV) respecto a los del grupo placebo (solo estatinas) presentan una disminución del CT (2,0-9,0 %) y LDL-c (3,0-15,0 %), sin cambios en la concentración ni de HDL-c ni de triglicéridos (Han et al., 2016). En base a estos resultados, los EV no solo mejoran el perfil lipídico (disminución de LDL-c sin afectar a HDL-c) en pacientes sin tratamiento hipocolesterolemiante, sino que pueden suponer una estrategia como tratamiento coadyuvante con inhibidores de la HMG-CoA reductasa. No se ha evaluado en inhibidores de la absorción, posiblemente por suponer vías de actuación similares, disminuyendo la sinergia entre tratamiento farmacológico y dietético.

Dado que la mayoría de los estudios contemplados utilizan las margarinas, alimento con un perfil nutricional poco saludable, sería de interés conocer el efecto de las estatinas junto a EV contenidos en matrices alimentarias más saludables o complementos alimenticios.

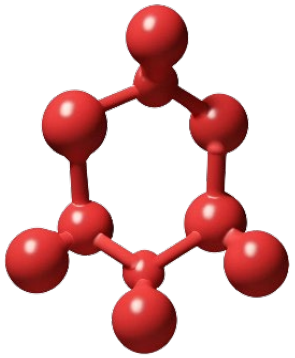
Tabla 7. Características y resultados de los ensayos clínicos con combinación de tratamiento con estatinas y esteroles vegetales

Estudio y duración (semanas)	Número de participantes	Esterol y dosis	Estatinas (mg/día)	Cambio respecto a placebo (%)	Referencia
			Margarinas		
Cruzado (7)	8	β-sitosterol, 3g/día)	Pravastatina (40)	CT: -5,3 LDL-c: -9,7	Gylling & Miettinen, 1996
Paralelo (8)	83 (T) / 84 (P)	Estanoles, 3g/día)	Atorvastatina, pravastatina, simvastatina, o lovastatina *	CT: -6,9 LDL-c: -9,6	Blair et al., 2000
Paralelo (4)	37 (T) / 38 (P)	Esteroles, 2g/día)	Cerivastatina (0,4)	CT: -2,0 LDL-c: -3,0	Simons, 2002
Cruzado (8)	10	Estanoles, 2g/día)	Atorvastatina o simvastatina *	CT: -9,0 LDL-c: -15,0	Cater et al., 2005
Paralelo (6)	11 (T) / 9 (P)	Estanoles, 3g/día)	Atorvastatina o simvastatina (80)	CT: -6,1 LDL-c: -6,7	Castro-Cabezas et al., 2006
Cruzado (4)	30	β-sitosterol, 2,5g/día)	Atorvastatina o simvastatina (40)	CT: -5,2 LDL-c: -7,7	Fuentes et al., 2008
Paralelo (16)	15 + 15 (T) / 11 (P)	Estanoles o esteroles, 2,5g/día)	Atorvastatina, simvastatina o pravastatina *	CT: -5,2 / -7,1 (esterol / estanol) LDL-c: -7,6 / -12,2 (esterol /estanol)	De Jong et al., 2008
Paralelo (85)	11 + 8 (T) / 11 (P)	Estanoles o esteroles, 2,5g/día)	No especificado	CT: -3,9 / -5,4 (esterol / estanol) LDL-c: -9,2 / -9,6 (esterol / estanol)	Kelly et al., 2011

Tabla 7. Continuación

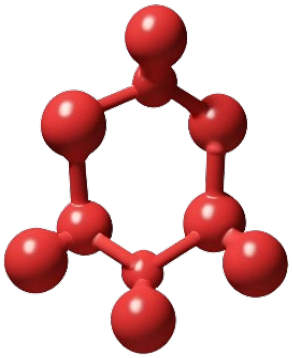
Estudio y duración (semanas)	Número de participantes	Matriz y dosis	Estatinas (mg/día)	Cambio respecto a placebo (%)	Referencia
			Margarinas		
Paralelo (4)	12 (T) / 12 (P)	Estanoles, 3g/día)	Atorvastatina, simvastatina o rosuvastatina (dosis estable)	CT: -7,8 LDL-c: -14,8	Hallikainen et al., 2011
			Lácteos		
Paralelo (9)	8 (T) / 10 (P)	Yogur líquido (estanoles, 2g/día)	Simvastatina (10)	CT: -6,5 Colesterol no HDL-c: -8,9	Plat et al., 2009
Cruzado (6)	35 ancianos	Leche fermentada (esteroles, 2g/día)	No especificado	CT: -5,1 LDL-c: -11,4	Andrade et al., 2015
			Complementos alimenticios		
Paralelo (6)	13 (T) / 13 (P)	Comprimidos (esteroles, 1,8g/día)	Atorvastatina, simvastatina, pravastatina o lovastatina (10-40)	CT: -5,7 LDL-c: -9,1	Goldberg et al., 2006
Cruzado (4)	86	Cápsulas (esteroles, 2g/día)	Atorvastatina (10)	CT: -7,7 LDL-c: -6,5	Malina et al., 2015

CT: colesterol total; LDL-c: colesterol asociado a lipoproteínas de baja densidad; HDLc: colesterol asociado a lipoproteínas de alta densidad; T: tratados (esteroles vegetales y estatinas); P: placebo (solo estatinas). * No se especifica dosis de estatinas



Objetivos

Objectives



El objetivo general de la presente Tesis Doctoral es evaluar la bioaccesibilidad y efectos biológicos de los esteroles vegetales, asociados a la ingesta de alimentos o complementos alimenticios enriquecidos con estos, mediante una doble aproximación complementaria *in vitro* e *in vivo*.

Para ello, se plantean los siguientes objetivos específicos:

Objetivo 1. Caracterizar la composición de esteroles vegetales de un pan integral de centeno enriquecido y su estabilidad durante la panificación.

Objetivo 2. Adaptar el método armonizado de digestión gastrointestinal (INFOGEST 2.0) a las condiciones fisiológicas del anciano para evaluar la bioaccesibilidad de esteroles vegetales presentes en alimentos enriquecidos de distinta naturaleza (sólido y líquido).

Objetivo 3. Evaluar la actividad antiproliferativa y mecanismo de acción de un pan integral de centeno enriquecido con esteroles vegetales, tras su digestión y fermentación colónica, en células de cáncer colorrectal humano.

Objetivo 4. Evaluar el efecto antieriptótico de un complemento alimenticio conteniendo esteroles vegetales tras su ingesta regular en sujetos hipercolesterolémicos tratados con estatinas y su relación con el estilo de vida.

Objetivo 5: Analizar la influencia de la ingesta de un complemento alimenticio con esteroles vegetales sobre los niveles de productos de oxidación del colesterol en sueros de pacientes hipercolesterolémicos tratados con estatinas y la correlación con el estilo de vida.

The general objective of this Ph.D. thesis is to evaluate the bioaccessibility and biological effects of plant sterols, associated with food intake or enriched-food supplements, through a complementary *in vitro* and *in vivo* approach.

For this purpose, the following specific objectives are considered:

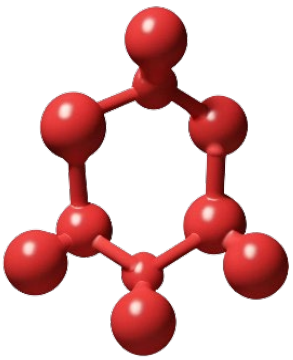
Objective 1. To characterize the composition of plant sterols of an enriched-wholemeal rye bread and its stability during baking.

Objective 2. To adapt the harmonized gastrointestinal digestion method (INFOGEST 2.0) to the physiological conditions of the elderly to assess the bioaccessibility of plant sterols present in enriched foods of different nature (solid and liquid).

Objective 3. To evaluate the antiproliferative activity and mechanisms of action of an plant sterol-enriched wholemeal rye bread, after its digestion and colonic fermentation, in human colorectal cancer cells.

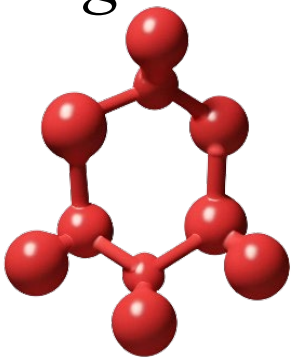
Objective 4. To evaluate the antieryptotic effect of a food supplement containing plant sterols after regular intake in hypercholesterolemic statin-treated subjects and its relationship with lifestyle.

Objective 5: To analyze the influence of the intake of a plant sterol-food supplement on the levels of plasmatic cholesterol oxidation products of hypercholesterolemic statin-treated subjects and its relationship with lifestyle.



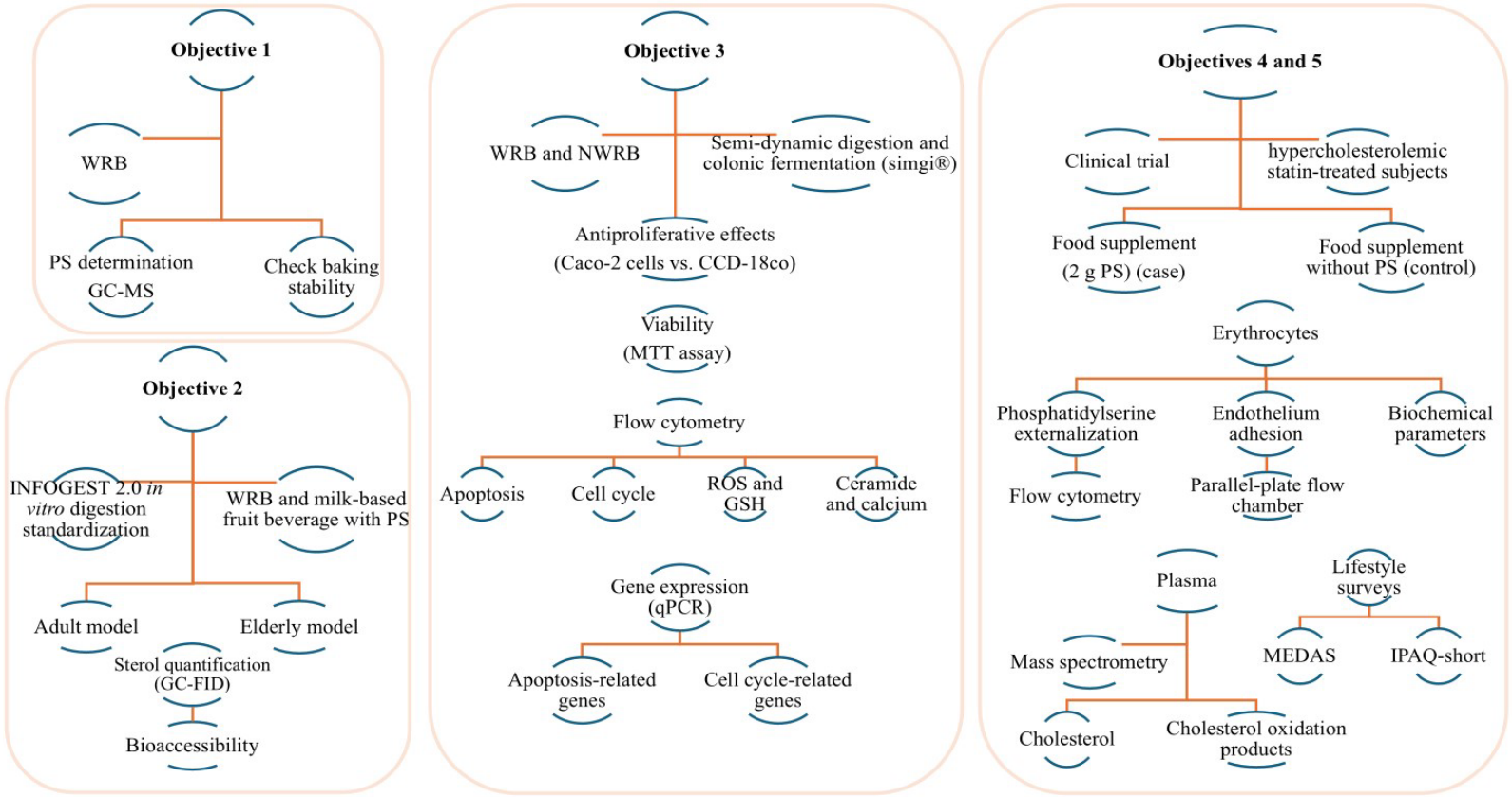
Diseño experimental y difusión de resultados

Experimental design and results dissemination



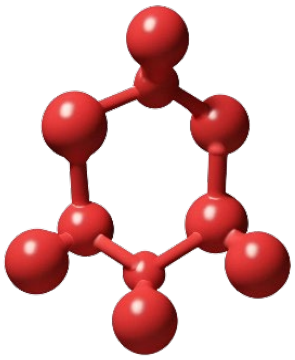
El diseño experimental elaborado en la presente Tesis Doctoral para abordar los objetivos planteados se muestra en la **Figura 12**, mientras que la difusión de resultados se representa en la **Figura 13**.

Figure 12. Description of the working plan followed to address the objectives of the Ph.D. thesis

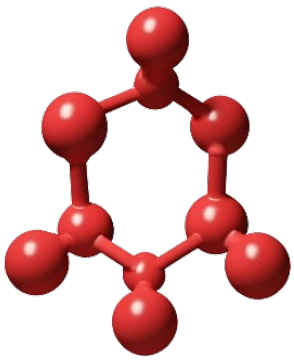


GSH: reduced glutathione; IPAQ-short: International Physical Activity Questionnaire- short form; MEDAS: Mediterranean diet adherence screener; NWRB: non-enriched wholemeal rye bread; PS: plant sterols; ROS: reactive oxygen species; WRB: plant sterol enriched wholemeal rye bread

Figure 13. Dissemination of results



Chapter I:
Assessment of plant sterol bioaccessibility under elderly gastrointestinal conditions: Effect of liquid/solid matrix



1. Materials and methods

1.1. Reagents

5-Cholesten-3β-ol (cholesterol) (purity 99%), 5,22-cholestadien-24-ethyl-3β-ol (stigmasterol) (purity 97%), 24α-ethyl-5α-cholestan-3β-ol (sitostanol) (purity 97%) and the internal standard (IS) 5β-cholestan-3α-ol (epicoprostanol) (purity 99%) were purchased from Merck LifeScience S.L.U. (Madrid, Spain). 24α-Methyl-5-cholesten-3β-ol (campesterol) (purity 98%) and 5-cholesten-24β-ethyl-3β-ol (β-sitosterol) (purity 98%) were supplied from Chengdu Biopurify Phytochemicals Ltd (Sichuan, China).

Trimethylchlorosilane (TMCS) (≥98%, v/v) and anhydrous pyridine (≥99.5%, v/v) obtained from Acros Organics (Geel, Belgium) and hexamethyldisilazane (HMDS) (≥99.9%, v/v) obtained from Merck LifeScience S.L.U. (Madrid, Spain) were used as derivatization reagents. α-Amylase from human saliva (E.C 3.2.1.1), ammonium carbonate, ammonium chloride, anhydrous sodium sulfate, bovine bile salts, butylhydroxytoluene, calcium chloride dihydrate, cholesterol esterase (CE) from porcine pancreas (E.C 3.1.1.13), hydrochloric acid (purity 37% w/w), magnesium chloride hexahydrate, pancreatin from porcine pancreas, pepsin from porcine gastric mucosa (E.C 3.4.23.1), potassium chloride, potassium dihydrogen phosphate, potassium hydroxide, sodium chloride and sodium hydroxide were purchased from Merck LifeScience S.L.U. (Madrid, Spain). Rabbit gastric extract (RGE15, 15 U lipase per mg powder) was acquired from Lipolytech (Marseille, France). Absolute ethanol (≥99.8%, v/v), sodium bicarbonate and sodium hydroxide were purchased from Panreac (Barcelona, Spain). Cyclohexane (≥99.9%, v/v), diethyl ether (≥99.5%, v/v) and n-hexane (≥96%, v/v) were obtained from Scharlau (Barcelona, Spain). Water was purified using a Milli-Q system (Millford, MA, United States).

1.2. Samples

Two matrices of different nature were used, one liquid and the other solid, whose ingredient and nutritional composition are described below:

a) The milk-based fruit beverage containing (%, w/v) skimmed milk, milk fat and whey protein concentrate enriched with milk fat globule membrane (49%), mandarin juice from concentrate (45%), banana puree (4%) and microencapsulated free microcrystalline plant sterols (PS) (1%) from tall oil in a powder form (Lypophytol® 146 ME Dispersible,

Lipofoods, Barcelona, Spain) was used. The manufacturing was performed in the Hero Global Technology Center (Alcantarilla, Spain), as described by Blanco-Morales et al. (2021b). The skimmed milk powder was dissolved in water alongside the other ingredients. Then, the mixture was subjected to pasteurization (90 °C / 30 s) using an indirect heat exchanger, and afterwards it was cooled to 20 °C. Finally, the beverage was aseptically bottled in 250 mL tetra bricks. In a previous work, gas chromatography–flame ionization detection (GC-FID) was used to establish the sterol profile in the beverage (Makran et al., 2022).

b) The plant sterol-enriched wholemeal rye bread (WRB) was made with the following proportions (%, w/w) of ingredients in the bread dough: whole rye flour (57%), compressed yeast (1.4%), common salt (0.9%), ultrapure water (38.2%), ascorbic acid (0.006%) and microencapsulated free PS (2.5 %) from tall oil in a powder form (Lypophytol ME dispersible palm-free). The WRB manufacturing process began by mixing all the ingredients in a mixer with rotating blades. Immediately afterwards, the bread dough was left to rest for 10 minutes and divided into individual portions of 102.5 g. These were manually balled and left to rest for another 15 minutes. Subsequently, the dough was fermented at a temperature of 28 °C with a relative humidity of 85% for 45 minutes. Once fermented, the bread doughs were baked for 25 minutes at a temperature of 180 °C. Four individual loaves with an average weight of 81.32 ± 1.09 g were obtained from each baking process. With the aim of evaluating the influence of the manufacturing process in the PS content, experiments with WRB from different manufacturers (baking 1, baking 2 and baking 3) were carried out with the same batch of flours under identical baking conditions and at three different days. To obtain a more homogeneous and stable sample, fresh WRB was partially dehydrated at 24 °C overnight until the humidity was below 14% (w/w) and further homogenized with a sample homogenizer until a breadcrumb texture was obtained (this is the partially dried bread (PDB)). The nutritional information of both samples (the beverage and the WRB) is summarized in **Table 8**.

1.3. Determination of enzymatic activities and bile salt content

As reported in a previous work by our research group (Makran et al., 2022), all enzymatic activities and bile salt content were analyzed following the guidelines provided by the INFOGEST protocol (Minekus et al., 2014; Brodkorb et a., 2019), except for CE, as its analysis is not contemplated in the INFOGEST method and no standardized methodology for determining its activity has been described (Petry & Mercadante, 2019).

In the INFOGEST protocol, the definition of the activity units of each enzyme can also be found. The only enzyme activity of the oral phase determined was that of α-amylase from human saliva. In the gastric phase, pepsin from porcine gastric mucosa and RGE were determined. In this case, due to its content, the pepsin activity was also determined in RGE. Regarding the intestinal phase, both pancreatin from porcine pancreas and bovine bile were determined. Regarding the CE, the activity of the batch provided by the manufacturer (34.8 U mg−1 powder) was used.

Table 8. Nutritional composition of the plant sterol-enriched milk-based fruit beverage (%, w/v) and the plant sterol-enriched wholemeal rye bread (%, w/w)

	Milk-based fruit beverage[a]	**Wholemeal rye bread[b]**
Humidity	84.6	32.7 ± 1.0
Lipids	2.4	2.8 ± 0.1
Carbohydrates	9.0	44.3 ± 0.4
Fiber	< 0.5	13.7 ± 1.5
Soluble	-	3.4 ± 0.1
Insoluble	-	10.3 ± 1.4
Proteins	3.0	5.1 ± 0.0
Ash	0.5	1.4 ± 0.0

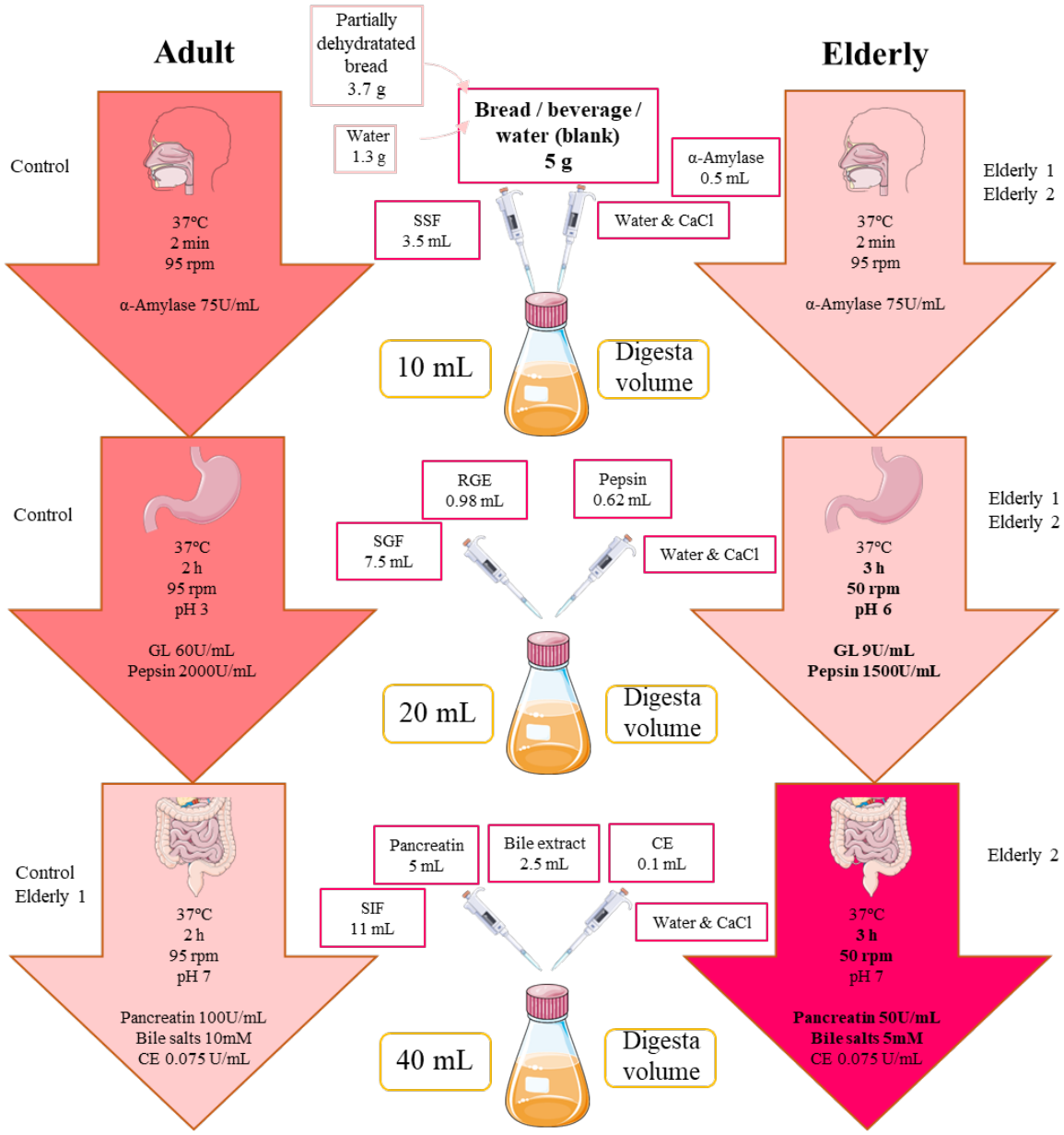
[a]Data from manufacturer. [b]Data analyzed in triplicate.

1.4. Simulated gastrointestinal digestion

Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to the INFOGEST protocol (Minekus et al., 2014). The different conditions tested, and their specific parameters are summarized in **Figure 14**. The healthy adult control conditions were performed following the INFOGEST 2.0 standardized methodology proposed by Brodkorb et al. (2019) with the inclusion of CE to make it more physiological (Makran et al., 2022).

Briefly, 3.7 g of PDB were mixed with 1.3 g of water for one min in a sample homogenizer (Masticator Basic 400, IUL, Spain) until reaching the humidity from fresh bread (26.2%). Then, 5 g of beverage, the rehydrated bread or ultrapure water (as a blank for digestion) and 3.5 mL of SSF were mixed and shaken for one minute. Afterwards, 0.5 mL of α-amylase solution, 25 µL of 0.3 M calcium chloride and 975 µL of ultrapure water

Figure 14. Flow diagram of the simulated digestion process with the adaptations to the elderly population



CE: Cholesterol esterase; GL: Gastric lipase RGE: Rabbit gastric extract; SIF: Simulated intestinal fluid; SGF: Simulated gastric fluid; SSF: Simulated salivary fluid.

were added to obtain a final volume of 10 mL. The mixture was placed in a shaker bath for 2 min at 37 °C and 95 rpm. Once the oral phase was completed, to simulate the gastric phase, 7.5 mL SGF and 5 µL of 0.3 M calcium chloride were added and mixed manually for 1 min. Subsequently, 0.98 mL of RGE solution was added. Since RGE provides the pepsin activity, it was completed with 0.62 mL of pepsin solution. The pH of the mixture was adjusted to the appropriate one for each condition and ultrapure water was added up

to a volume of 20 mL. The mixture was placed in the shaker bath at 37 °C with agitation for 2 or 3 hours (adult and elderly conditions, respectively). To simulate the intestinal phase, 11 mL of SIF, 5 mL of pancreatin solution, 40 µL of 0.3 M calcium chloride, 2.5 mL of bile extract solution of bovine origin and 0.1 mL of CE solution were added. The final mixture was mixed manually for one minute and adjusted to pH 7, then ultrapure water was added to a final volume of 40 mL. The mixture was placed again in the shaker bath at 37 °C with agitation for 2 or 3 hours (adult and elderly conditions, respectively).

In elderly 1 condition (E1), for both samples, the gastric phase was adapted by reducing the activity of pepsin to 75% and the lipase activity to 15%, increasing the pH (3 vs 6) and duration (2h vs 3h), and reducing the frequency of agitation (95 vs 50 rpm). In elderly 2 condition (E2), the changes made in E1 were maintained and additionally some changes in the intestinal phase were made: a reduction of the pancreatin activity and bile salt concentration, 50% in both cases, an increase in digestion time (2h vs 3h), and a reduction of the frequency of agitation (95 vs 50 rpm).

For the beverage and PDB, no changes were carried out in the oral phase of E1 and E2 compared with the control since the assays were performed with a liquid food and the lack of teeth that sometimes occurs in the elderly will not be relevant. In addition, salivary amylase activity was not increased since the beverage does not contain a significant substrate for the enzyme, and due to its high economic cost (PDB and beverage). Therefore, the oral phase was not modified in agreement with previous studies (Aalaei et al., 2021; Lee et al., 2021).

Notwithstanding, for the bread digestion, preliminary tests were carried out by applying an *in vivo* oral phase to fresh WRB with human mastication. A healthy adult volunteer (41 years, amylase activity: 245.3 ± 16.9 U mL^{-1} saliva) was used and mastication cycles were reduced with respect to the adult model (20 vs 40). Changes in the digestion conditions E1 and E2 were selected considering the recommendations provided in the review by Shani-Levi et al. (2017) and in previous elderly adapted static *in vitro* digestion trials that also analyzed lipolysis and lipophilic compounds (i.e., vitamin D), since there is no standardization for applying the conditions of the elderly (Hernández-Olivas et al., 2020a, b; 2021a, b).

The digesta obtained (from blanks, control (adult), E1 or E2) was centrifuged for 90 min at 4 °C and 3100 *g* (Centrifuge 5810 R, Eppendorf AG, Germany) and the supernatant

corresponding to the bioaccessible fraction (BF) was collected. The formula [(sterol content in BF/sterol content in WRB or beverage) × 100] was used to calculate the bioaccessibility of total and individual sterols. In addition, the contents of the digestion blank were subtracted from the samples to obtain the actual PS content provided by the WRB or the beverage.

1.5. Sterol determination

1.5.1. Beverage saponification and extraction

The methodology described by Blanco-Morales et al. (2018) was used for sterol determination in beverage and its BF. The lipid fraction of 5 g of beverage was extracted using chloroform : methanol (1:1, v/v) with 0.05% butylhydroxytoluene at 60 °C. After that, chloroform was added to the tube before filtering the sample (Whatman no. 1.90 mm). Then, a 1M KCl solution was added to the filtered sample, and it was stored overnight at 4 °C. The next day, the sample was completely dried using a rotary evaporator (BÜCHI Labortechnik, Switzerland) and a nitrogen stream. 10 mL of a mixture of hexane : isopropanol (4:1, v/v) were used to dissolve the lipid fraction. Then, 0.5 mL of that solution or 5 g of BF from the beverage digestion were mixed with 200 µg of IS (epicoprostanol) and subjected to hot saponification (2 mL of 1 M KOH in ethanol : water (9:1, v/v)) in a water bath (1 h, 65 °C) and the unsaponifiable fraction was extracted with diethyl ether.

1.5.2. Bread acid hydrolysis, saponification and extraction

Acid hydrolysis and saponification were carried out for the bread following the method proposed by Piironen et al. (2002). The IS (epicoprostanol) (1 mg), and 1 mL of ethanol were added to PDB (0.35 g), or PS-ingredient sample (20 mg), both weighed in a 30 mL test tube at the beginning of the determination. For acid hydrolysis, 5 mL of 6M HCl were added, and the tube contents were mixed for 30 s with a test tube mixer (Vortex MS2, Janke and Kunkel, Ika Labortechnik, Germany), before the tube was placed into a shaking water bath (SBS40, Cole-Parmer, United Kingdom) (80 °C) for 60 min. After the tube was cooled, lipids were extracted with 20 mL of an n-hexane and diethyl ether (1:1) solvent mixture by shaking for 10 min at 400 rpm using a sample tube rocker (KS 260 Basic, Ika, Germany). Then, it was centrifuged for 10 minutes at 500 rpm and the organic layer was transferred to a round-bottom flask and evaporated to dryness using a rotary

evaporator (B490, BÜCHI Labortechnik, Switzerland) at 50 °C, and then resuspended in 8 mL of absolute ethanol.

The determination of BF, since acid hydrolysis was not carried out, was started directly by adding 2 g of BF, (weighed into a round-bottom flask), and then 0.2 mg of IS and 6 mL of absolute ethanol were added and transferred to a 50 mL test tube. Both BF and the previously hydrolyzed samples (PS ingredient and PDB) were saponified with 0.5 mL of saturated aqueous KOH solution. The tube contents were mixed (10 s) using the test tube mixer and they were placed in a shaking water bath (80 °C) for 30 min (100 rpm) and then cooled. For extraction of the unsaponifiable fraction, 12 mL of water and 20 mL of cyclohexane were added, followed by shaking at 400 rpm for 10 min using the sample tube rocker. The organic layer was transferred to a round-bottom flask and evaporated to dryness in a rotary evaporator. The residue was dissolved in 1 mL of hexane. A silica cartridge (Finisterre SPE tube Si 500 mg/6 mL, Teknokroma, Spain) was activated by 5 mL of hexane. After filtering and applying the sample as a hexane extract, the cartridge was washed with 5 mL of hexane and 5 mL of a hexane and diethyl ether (90: 10) solvent. The sterol fraction was eluted with 5 mL of a hexane and diethyl ether (50:50) solvent and evaporated to dryness. In PDB and sterol ingredient, the sample was transferred to a 5 mL volumetric flask, from which 1 mL is taken for derivatization. In the BF, the sample was not diluted, and the derivatization proceeded directly.

1.5.3. Derivatization, identification and quantification in beverage and bread

The derivatization was made following Alvarez-Sala et al. (2016). The trimethylsilylether derivatives, prepared from 600 µL of pyridine : HMDS : TMCS (5 : 2 : 1, v/v/v) (25 min, 40 °C), were dissolved in hexane after the evaporation of the reagent, filtered through a 0.45 µm filter (syringe driven Millex FH with filter 1 mL Millipore, Milford, MA, United States) and dissolved in 100 µL of n-hexane. 1 µL of the above solution was injected into a GC-FID (YL Instrument 6500 GC System, Gyeonggi-do, Korea). A capillary column CP-Sil 8 low bleed/MS (50 m × 0.25 mm × 0.25 µm film thickness) (Chrompack-Varian, Middelburg, The Netherlands) and hydrogen as the carrier gas (2 mL min^{-1}) were used. The oven temperature was increased from the initial 280 °C (held 20 min) to 290 °C (held 5 min) at a rate of 0.7 °C min^{-1} and then increased to 320 °C (5 min) at a rate of 30 °C min^{-1}. The temperature of both the injector and the detector was 325 °C, and a split ratio of 1 : 20 was used.

Sterols were identified by comparing their relative retention times with those of the pure standards. Sterols without commercialized standard (campestanol, Δ5-avenasterol, Δ5,24-stigmastadienol, Δ7-stigmastenol and Δ7-avenasterol) have been identified by mass spectrometry, comparing the main ions with the ones provided by the bibliography (Piironen et al., 2002; Yorulmaz et al., 2009; Drira et al., 2018) and mass spectra library (NIST, 2015), and a single sterol was assigned for each relative retention time. Calibration curves containing 200 μg of IS and increasing amounts of sterol standards were used for quantification. Therefore, due to the mentioned the lack of standard for them, the β-sitosterol calibration curve was used for their determination.

1.6. Statistical analysis

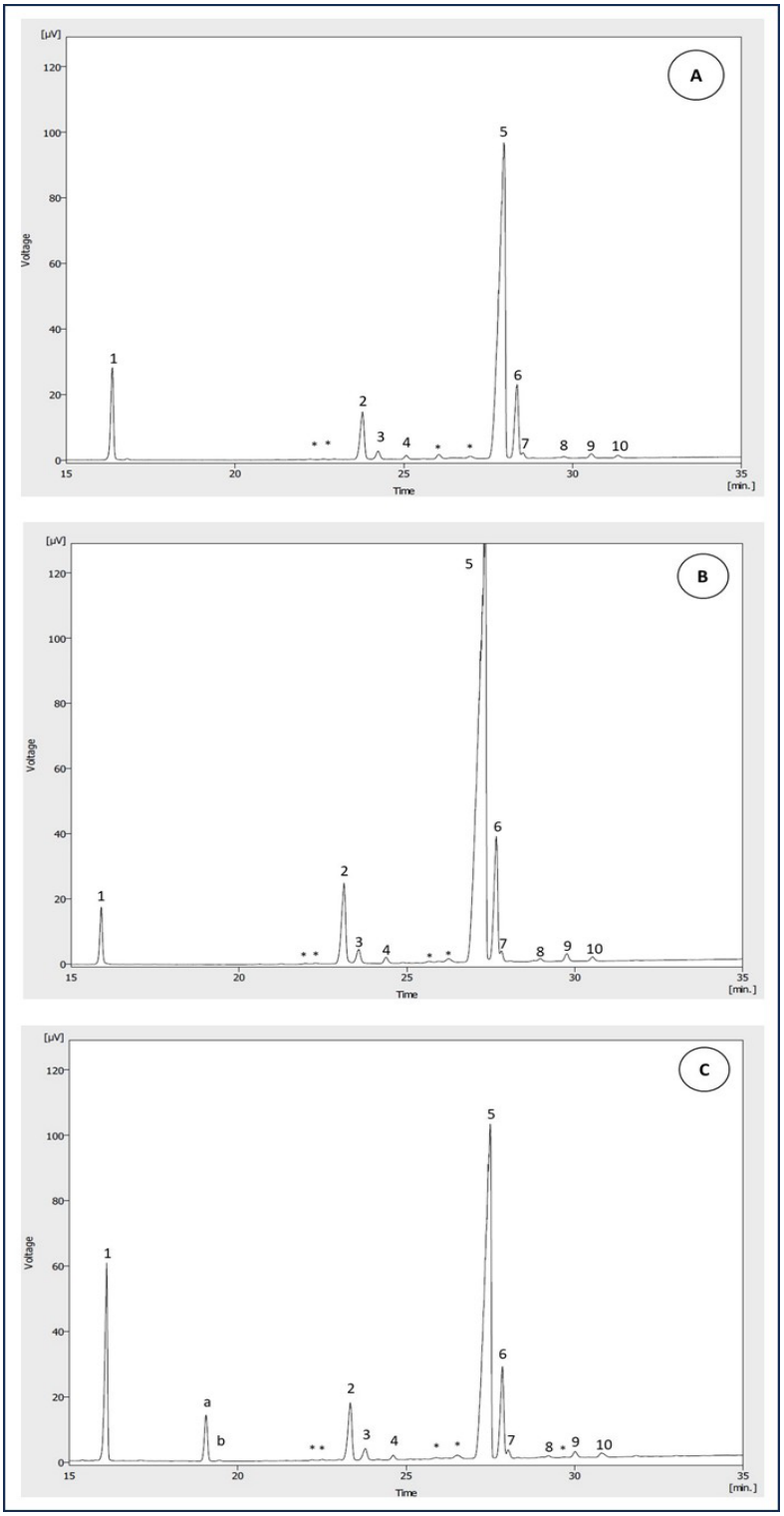
Sterol characterization in the beverage, WRB and PS-ingredient was carried out in triplicate. All assays of sterol bioaccessibility were determined in two independent experiments with at least three replicates for each experiment (n = 6 replicates). Shapiro–Wilk and Levene tests were used to evaluate normality and homoscedasticity, respectively. Then, to determine statistically significant differences ($p < 0.05$) between different baking of the bread or gastrointestinal conditions for the total and individual PS contents in the beverage, WRB, PS-ingredient, BF or bioaccessibility, one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, was used. The program used for statistical analysis was GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, CA, USA).

2. Results

2.1. PS in PS-enriched wholemeal rye bread

PS identified in the ingredient, WRB and BF are shown in **Table 9**, which includes the retention time, relative retention time and characteristic ions of each sterol identified by gas chromatography-mass spectrometry (GC-MS). In a total analysis time of 30 min, seven phytosterols (campesterol, stigmasterol, β-sitosterol, Δ5-avenasterol, Δ5,24-stigmastadienol, Δ7-stigmastenol and Δ7-avenasterol) and two phytostanols (campestanol and sitostanol) were identified (**Figure 15A–C**). After IS (epicoprostanol), cholesterol and cholestanol were detected only in BF. Besides, other peaks eluted corresponding to compounds with steroidal structures, which have not been identified as PS.

Figure 15. Chromatograms of sterols identified in plant sterols (PS)-enriched wholemeal rye bread and its bioaccessible fractions. A. PS ingredient; B. PS-enriched wholemeal rye bread; C. Bioaccessible fraction from elderly gastrointestinal adapted *in vitro* digestion



1. epicoprostanol (internal standard); 2. campesterol; 3. campestanol; 4. stigmasterol; 5. β-sitosterol; 6. sitostanol; 7. Δ5-avenasterol; 8. Δ5,24-stigmastadienol; 9. Δ7-stigmastenol; 10. Δ7-avenasterol; a. cholesterol; b. cholestanol; *. steroid structures.

Table 9. Sterols identified in plant sterol ingredient, plant sterol-enriched wholemeal rye bread or their bioaccessible fractions: absolute retention time (Rt), relative retention time (RRt) and characteristic ions

	Sterols	Rt (min)	RRt (min)	Characteristic ions	References
1	Epicoprostanol (IS)	15.454 ± 0.487		445.4, 370.4, 355.3, 316.3, 257.2, 215.2,161.1	
2	Campesterol	22.442 ± 0.700	1.452 ± 0.001	472.4, 430.2, 382.4, 343.3, 289.2, 255.2	
3	Campestanol	22.882 ± 0.707	1.481 ± 0.002	474.9, 459.4, 417.3, 373.6, 369.3, 343.3, 306.2, 215.2	Mass spectra of sterol standard and Mass spectra library (Drira et al., 2018)
4	Stigmasterol	23.689 ± 0.712	1.533 ± 0.003	484.5, 469.7, 394.3, 379.6, 355, 295.5, 255.2, 213.4	
5	β-sitosterol	26.484 ± 0.834	1.714 ± 0.004	486.4, 471.4, 396.4, 357.4, 275.3, 255.2, 213.2	
6	Sitostanol	26.849 ± 0.806	1.738 ± 0.004	488.3, 473.4, 431.4, 383.4, 305.2, 215.2	
7	Δ5-Avenasterol	27.023 ± 0.785	1.749 ± 0.005	484.4, 473.4, 431.4, 386, 296.0, 357.3, 55.0	Faubel et al., 2022; Alvarez-Sala et al., 2016; Yorulmaz et al., 2009; Ogawa et al., 2016
8	Δ5,24-stigmastadienol	28.206 ± 0.785	1.825 ± 0.007	484.4, 469.3, 386.2, 345, 296.3, 257.0, 55.0	Alvarez-Sala et al., 2016; Yorulmaz et al., 2009; Ogawa et al., 2016
9	Δ7-stigmastenol	28.987 ± 0.799	1.876 ± 0.008	486.4, 471.4, 396, 381.3, 303.2, 255.2, 213.2	Alvarez-Sala et al., 2016; Yorulmaz et al., 2009; Ogawa et al., 2016
10	Δ7-Avenasterol	29.758 ± 0.793	1.92 ± 0.010	The literature indicates that it elutes after Δ7-Stigmastenol	Faubel et al., 2022; Alvarez-Sala et al., 2016; Yorulmaz et al., 2009

Rt and RRt values were expressed as media ± standard deviation of 4 independent assays by triplicate (n = 12) and 2 independent assays by triplicate (n = 6) in wholemeal rye bread and their bioaccessible fractions, respectively. IS: internal standard.

PS contents in PS-ingredient and WRB prepared under different baking days are tabulated in **Table 10**. The total PS content ranged between 2.17 and 2.23 g/100 g of fresh WRB under various baking conditions (mean 2.18 ± 0.09), including the sum of free and covalently bound sterols (steryl esters with fatty acids, or phenolic acids, steryl glucosides, and acylated steryl glycosides) due to acid hydrolysis applied in the analysis that breaks all bonds of conjugated sterols (Garcia-Llatas et al., 2021a).

The order of individual PS relative percentage from the PS-ingredient, determined from the greatest to least abundance, is as follows: β-sitosterol (78.8%), sitostanol (10.2%), campesterol (7.4%), campestanol (1.4%), Δ5-avenasterol (0.7%), Δ7-stigmastenol (0.6%), stigmasterol (0.5%), Δ7-avenasterol (0.4%) and Δ5,24-stigmastadienol (0.2%). This order observed for the ingredient was maintained in the WRB.

No statistically significant differences ($p > 0.05$) were observed in the individual and total PS contents under different baking days (see **Table 10**). It is confirmed that the desired PS-enrichment is maintained under different baking days if they are carried out with the same batch of flour and if baking conditions remain identical between them.

2.2. Adaptation of the digestion conditions to the elderly population

2.2.1. Plant sterol-enriched milk-based fruit beverage

The data for each individual sterol and total PS content in the beverage and its BF are summarized in **Table 11**. Modifications in the gastric phase (E1) produced a significant increase ($p < 0.05$) in the solubility of total PS (1.7-fold) as well as cholesterol (2.5-fold) compared with the control (adult). The significant increase ($p < 0.05$) in individual sterols with respect to the control, except for campestanol, which was not modified, followed the order: stigmasterol (2.2-fold) > sitosterol (1.8-fold) > campesterol (1.4-fold).

When comparing the results obtained in the elderly trial 1 (E1) with the control, the increase in bioaccessibility of the total PSs was 73% ($p < 0.05$), although with substantial variations between the different individual PSs (**Figure 16**). Those most affected by the modification were stigmasterol (101% increase) and β-sitosterol (82% increase) ($p < 0.05$), both above the mean for total PS increase. However, sitostanol (57%) and campesterol (40%) showed a smaller increase ($p < 0.05$). Campestanol was the only one that differed in its bioaccessibility, not showing a statistically significant increase.

Table 10. Individual and total plant sterols (PS) content (mg PS/100 g bread) in different baking of wholemeal rye bread and PS content in the PS-ingredient (g PS/100 g powder). The relative percentage of plant sterol contents were indicated within parenthesis

PS	Baking 1	Baking 2	Baking 3	Mean	PS-ingredient
Campesterol	156.08 ± 7.53 (7.2)	160.95 ± 6.12 (7.2)	151.58 ± 6.86 (7.1)	156.20 ± 7.20 (7.2)	5.55 ± 0.39 (7.4)
Campestanol	21.23 ± 2.03 (1.0)	24.32 ± 0.16 (1.1)	24.45 ± 1.78 (1.1)	23.34 ± 2.08 (1.1)	1.02 ± 0.06 (1.4)
Stigmasterol	8.79 ± 0.51 (0.4)	9.70 ± 0.21 (0.4)	9.38 ± 0.66 (0.4)	9.29 ± 0.59 (0.4)	0.39 ± 0.02 (0.5)
β-Sitosterol	1720.54 ± 9.88 (79.2)	1762.84 ± 56.18 (79.0)	1685.23 ± 79.68 (78.9)	1722.87 ± 72.91 (79.0)	58.82 ± 3.84 (78.8)
Sitostanol	225.65 ± 9.88 (10.4)	232.44 ± 8.82 (10.4)	223.75 ± 11.02 (10.5)	227.28 ± 9.48 (10.4)	7.62 ± 0.41 (10.2)
Δ 5-Avenasterol	15.00 ± 0.86 (0.7)	16.23 ± 0.99 (0.7)	14.57 ± 0.94 (0.7)	15.26 ± 1.10 (0.7)	0.52 ± 0.04 (0.7)
Δ 5,24-Stigmastadienol	4.25 ± 0.04 (0.2)	5.13 ± 0.01 (0.2)	5.67 ± 0.66 (0.3)	5.11 ± 0.74 (0.2)	0.12 ± 0.04 (0.2)
Δ 7-Stigmastenol	13.56 ± 1.85 (0.6)	13.34 ± 0.96 (0.6)	14.12 ± 0.70 (0.7)	13.68 ± 1.15 (0.6)	0.42 ± 0.04 (0.6)
Δ 7-Avenasterol	8.22 ± 1.01 (0.3)	7.03 ± 1.18 (0.3)	8.12 ± 0.37 (0.4)	7.64 ± 1.00 (0.4)	0.31 ± 0.04 (0.4)
Total PS	2173.01 ± 101.26	2231.25 ± 73.12	2136.86 ± 101.51	2180.37 ± 90.43	74.65 ± 5.15

Contents were expressed as media ± standard deviation (n = 3). No statistically significant differences were observed in the individual nor in the total PS content in different baking.

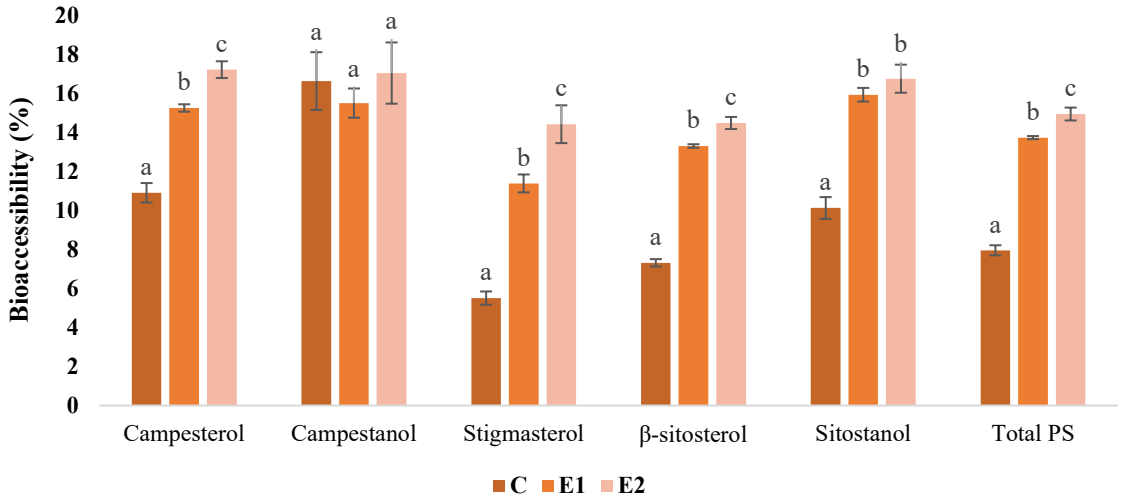
A possible hypothesis for this result is that being a minor sterol in the beverage (1.3% of the total PSs), it is interpolated in the lower part of the sitostanol calibration curve. Regarding cholesterol, the increase in bioaccessibility was 2.5-fold compared with the control ($p < 0.05$), much higher than the increase in PS (**Figure 17**).

Table 11. Individual and total plant sterol (PS) content (mg/100g beverage) in the bioaccessible fraction (BF) after gastrointestinal digestion in control (C) (adult conditions) and both elderly conditions (E1: gastric phase adapted & E2: gastric and intestinal phases adapted) compared to those determined in the beverage

Sterol	**Beverage***	**Bioaccessible fraction**		
		Control	Elderly 1 (E1)	Elderly 2 (E2)
Cholesterol	12.72 ± 1.32	2.65 ± 0.35^{a}	6.49 ± 0.40^{b}	7.79 ± 0.37^{c}
Campesterol	61.95 ± 3.95	6.76 ± 0.31^{a}	9.46 ± 0.12^{b}	10.67 ± 0.26^{c}
Campestanol	11.98 ± 1.02	1.99 ± 0.18^{a}	1.86 ± 0.09^{a}	2.04 ± 0.19^{a}
Stigmasterol	5.03 ± 0.31	0.26 ± 0.04^{a}	0.57 ± 0.02^{b}	0.73 ± 0.05^{c}
β-sitosterol	742.17 ± 55.81	54.23 ± 1.53^{a}	98.77 ± 0.68^{b}	107.54 ± 2.28^{c}
Sitostanol	90.99 ± 1.68	9.22 ± 0.51^{a}	14.51 ± 0.31^{b}	15.25 ± 0.65^{b}
Total PS	911.11 ± 59.83	72.56 ± 2.35^{a}	125.16 ± 0.75^{b}	136.23 ± 3.02^{c}

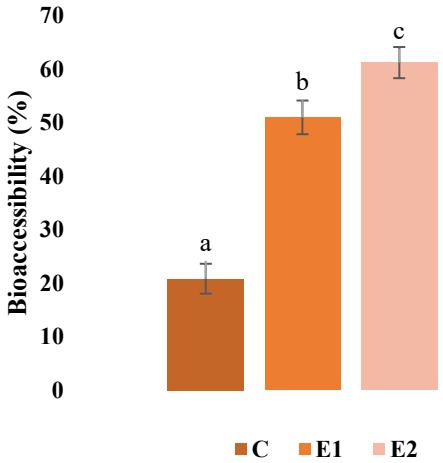
Different lowercase letters (a-c) indicate statistically significant differences ($p < 0.05$) in the BF of each sterol determined. Results are expressed as mean ± standard deviation (n=4). * Data taken from Makran et al. (2022).

Figure 16. Bioaccessibility (%) of plant sterols (PS) of the enriched milk-based fruit beverage assessed under adult (control, C), elderly 1 (E1) and elderly 2 (E2) conditions



Results are expressed as mean ± standard deviation (n = 4). Different lowercase letters (a-c) in each sterol indicate statistically significant differences ($p < 0.05$) in PS bioaccessibility between conditions.

Figure 17. Bioaccessibility (%) of cholesterol of the enriched milk-based fruit beverage assessed under adult (control, C), elderly 1 (E1) and elderly 2 (E2) conditions



Results are expressed as mean ± standard deviation (n = 4). Different lowercase letters (a-c) indicate statistically significant differences ($p < 0.05$) in cholesterol bioaccessibility between conditions.

The increases in the BF of total and individual PSs and cholesterol in the modified gastric and intestinal (E2) phases were higher than those observed in the previous trial (E1). A significant increase ($p < 0.05$) in the solubility of total PSs (2-fold) and cholesterol (3-fold) compared with the control was observed (see **Table 11**). The significant increase ($p < 0.05$) in individual sterols followed the same order: stigmasterol (2.8-fold) > sitosterol (2-fold) > campesterol (1.6-fold), and no changes were observed in the case of campestanol.

In fact, this was the case when comparing bioaccessibility, which was 88% higher ($p < 0.05$) in E2 compared with the control for total PSs and showed a 9% increase ($p < 0.05$) from E1 to E2. Regarding individual PSs, stigmasterol and β-sitosterol showed the highest increases in bioaccessibility with respect to the control (162% and 98%, respectively) whereas sitostanol and campesterol showed the lowest increases (by 66% and 58%, respectively). When comparing differences in bioaccessibility between E2 and E1, it was observed that stigmasterol was the one that most notably increased ($p < 0.05$) (27% more in E2), followed by campesterol and β-sitosterol (13% and 9% increase in E2, respectively). However, sitostanol and campestanol did not significantly increase their bioaccessibility when both elderly conditions were compared. The bioaccessibility of cholesterol also increased significantly (by 3-fold) in relation to the control ($p < 0.05$), which implies that the increase between E1 and E2 was 20%.

2.2.2. Plant sterol-enriched wholemeal rye bread

Previous experiments of gastrointestinal digestion adapted to elderly with *in vivo* mastication were first carried out on fresh WRB with the conditions of gastric and intestinal phases indicated in **Figure 14**. The results showed a great variability (27–64%) in individual and total PS content of BF and bioaccessibility between the different digestion replicates.

Mastication with only 20 cycles does not allow an adequate disintegration of the bread, which could cause variability in the action of the enzymes due to mechanical reasons and hinder the release of the matrix, and the micellarization of PS. That hypothesis could be explained with the results of a trial comparing matrix disintegration in rye bread and wheat bread after mastication and *in vitro* gastric digestion. It was observed that in sourdough wholemeal rye bread a greater number of large-sized particles (>2 mm) and a greater aggregation of these were maintained compared to wheat bread (Nordlund et al., 2016).

Since the data obtained with human mastication adapted to older people could not be used in this work to accurately assess the bioaccessibility of PS, tests with PDB were reproduced to achieve more homogeneous digestion. In addition, due to economic costs (it will be needed to increase the concentration of α-amylase), together with the fact that the oral phase is not a determinant in the bioaccessibility of lipophilic compounds such as plant sterols, lead us to maintain adult oral phase conditions. The PS contents determined in the PDB and the BF of both three conditions (adult, E1 and E2) assessed are shown in **Table 12**.

No statistically significant differences ($p > 0.05$) were observed in the individual and the total PS contents of the BF or their respective bioaccessibility under gastric elderly conditions (E1 modifications) (individual PS range of 17.8–35.1%, total PS 19.3%) with those obtained under healthy adult conditions (individual PS range of 19.1–36.3%, total PS 20.3%) (**Figure 18**).

However, when these same two conditions were compared with the gastrointestinal elderly conditions (E2 modifications), significant reductions ($p < 0.05$) were observed (individual PS range of 10.9–20.5%, total PS 11.2%). The relative magnitude of the reduction was similar in all nine PS determined: Δ5-avenasterol (39%), Δ7-avenasterol

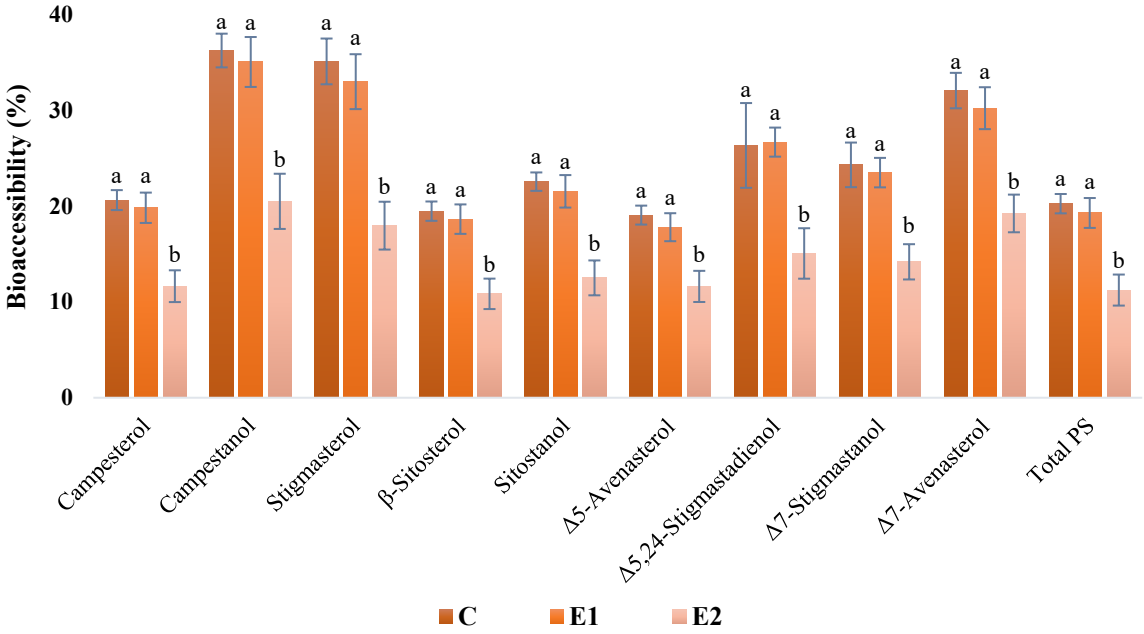
(40%), Δ7-stigmastenol (42%), Δ5,24-stigmastadienol (43%), campestanol (43%), campesterol (44%), β-sitosterol (44%), sitostanol (45%) and stigmasterol (49%).

Table 12. Individual and total plant sterols (PS) content (mg/100g of fresh bread) in partially dehydrated wholemeal rye bread and its bioaccessible fraction (BF) in control (C) (adult conditions) and both adapted elderly conditions studied (Elderly 1: gastric conditions adapted; Elderly 2: gastric and intestinal conditions adapted)

Plant sterol	Rye bread	Bioaccessible fraction		
		Control	Elderly 1	Elderly 2
Campesterol	159.47 ± 6.68	32.90 ± 1.66^{a}	31.63 ± 2.53^{a}	18.57 ± 2.65^{b}
Campestanol	17.61 ± 2.51	6.38 ± 0.31^{a}	6.17 ± 0.46^{a}	3.61 ± 0.51^{b}
Stigmasterol	8.76 ± 0.80	3.08 ± 0.21^{a}	2.89 ± 0.25^{a}	1.57 ± 0.22^{b}
β-Sitosterol	1815.75 ± 116.78	353.68 ± 18.33^{a}	338.56 ± 27.92^{a}	196.93 ± 28.86^{b}
Sitostanol	225.09 ± 30.95	50.78 ± 2.17^{a}	48.51 ± 3.82^{a}	28.17 ± 4.09^{b}
Δ5-Avenasterol	20.56 ± 2.64	3.92 ± 0.20^{a}	3.66 ± 0.30^{a}	2.39 ± 0.33^{b}
Δ5,24-Stigmastadienol	4.65 ± 1.09	1.23 ± 0.21^{a}	1.24 ± 0.07^{a}	0.70 ± 0.12^{b}
Δ7-Stigmastenol	15.58 ± 0.91	3.79 ± 0.36^{a}	3.66 ± 0.24^{a}	2.21 ± 0.29^{b}
Δ7-Avenasterol	10.31 ± 1.58	3.30 ± 0.19^{a}	3.12 ± 0.23^{a}	1.98 ± 0.20^{b}
Total PS	2277.79 ± 158.62	459.06 ± 22.98^{a}	439.44 ± 35.62^{a}	256.14 ± 36.93^{b}

Different lowercase letters (a-b) indicate statistically significant differences ($p < 0.05$) in the BF of each sterol determined. Results are expressed as mean ± standard deviation (n=6).

Figure 18. Bioaccessibility (%) of plant sterols (PS) of the enriched wholemeal rye bread assessed under adult (control, C), elderly 1 (E1) and elderly 2 (E2) conditions



Results are expressed as mean ± standard deviation (n = 6). Different lowercase letters (a-b) in each sterol indicate statistically significant differences ($p < 0.05$) in PS bioaccessibility.

3. Discussion

3.1. Plant sterol content in the ingredient and wholemeal rye bread

The order of individual PS relative percentage from the PS-ingredient (**Table 10**) complies with the European regulations that regulate the enrichment with PS of rye bread and state that the relative percentages of different PS must be: <80% β-sitosterol < 15% sitostanol < 40% campesterol < 5% campestanol < 30% stigmasterol < 3% brassicasterol < 3% other sterols/stanols (Decision 2006/59/EC). The contents of the PS ingredient used in WRB baking (**Table 10**) are consistent with previous determinations with different ingredients but of the same origin, showing stability in this type of PS-source ingredient, not only in their total content (70.9 vs 74.7 g PS/100 g), but also in the relative percentages of different individual PS: β-sitosterol (78.86), sitostanol (11.95), campesterol (7.13), campestanol (1.20), stigmasterol (0.82), and brassicasterol (0.04) (González-Larena et al., 2011). These authors identified brassicasterol (0.04%), not detected in our ingredient. The phytosterols and phytostanols identified in WRB represent an average relative percentage of 88.5% and 11.5%, respectively. In this sense, phytostanols, mainly campestanol, predominated in the steryl ferulates of grain milling fractions (including rye), whereas their proportion of the total sterols was 14–30% (González-Larena et al., 2011), representing about 19.3% in rye bread (Piironen et al., 2002).

The PS content of the WRB is significantly higher than that indicated by other authors for rye bread containing wheat flour (80.3 mg/100 g) and rye bread (90.2 mg/100 g) (Piironen et al., 2002). This was expected, given that the bread has been enriched with an ingredient source of PS with an experimentally determined purity of 74.65%.

The order and magnitude of individual PS in the WRB can be explained because cereal products are one of the most important natural sources of PS, 4-desmethylsterols (sitosterol, campesterol, stigmasterol, Δ5-avenasterol, Δ7-avenasterol) being predominant, specially β-sitosterol (representing in our rye bread 79% of the total PS). The order is similar to that indicated in rye bread by other authors (Piironen et al., 2002), except that campesterol is higher than sitostanol and stigmasterol of the same order as Δ5-avenasterol. These changes are explained by the contribution of the PS-ingredient, whose huge PS content causes the order of these sterols to vary. The content of sitostanol added with the PS-ingredient was higher than that of campesterol (10.21 vs 7.43% of total

ingredient PS) and the content of Δ5-avenasterol added with the PS-ingredient was also higher than that of stigmasterol (0.7 vs 0.52% of total ingredient PS).

Regarding the inter-day stability, it is known that the content of PS in grains of different rye cultivars from the same geographical area and in the same harvest does not differ significantly. The most important determining factor of the PS content is the composition of ingredients from different milling products (wholemeal flour, bran and germ) or flours with different degrees of extraction (% ash) (Hakala et al., 2002; Piironen et al., 2002). The results obtained allows comparing PS bioaccessibility assays in rye bread, carried out under different digestion conditions without influencing individual and total PS contents.

The presence of cholesterol and cholestanol only in BF samples is explained by the contributions of these sterols by porcine pancreatin, bovine bile and GL used during digestion. This was estimated in a previous work, without significant contribution of PS (Makran et al., 2022).

3.2. Influence of the elderly-adapted gastrointestinal conditions in the bioaccessibility of sterols

Different factors may condition the increase in PS bioaccessibility (except campestanol) of the beverage following elderly gastrointestinal conditions such as lower activity of GL, longer duration of the gastric stage, slower stirring and increased pH. The observed increase in the sterol bioaccessibility (vs control) in the E1 condition may be due to the lower activity of GL (only 15% in relation to the control) and the pH used, which was not the optimal one for the enzyme activity (5–5.4) (Amara et al., 2019). These results agree with recent evidence in which it was observed that GL negatively affects the sterol bioaccessibility present in a PS-enriched beverage (Makran et al., 2022). This effect could be attributed to the diacylglycerols (DAG) and free fatty acids (FFA) released by GL (McClements & Li, 2010) that help to solubilize the cholesterol from reagents of digestion, displacing cholesterol and PS from the beverage. In addition, by increasing the duration of the gastric phase by 50%, cholesterol and PS are more available for their micellarization, which could in part explain the increase in the bioaccessibility of sterols. This hypothesis agrees with the results of a dynamic digestion assay evaluating the kinetics of lipids, proteins and peptides released during digestion in a meat matrix, in which a higher release of lipids was detected in aliquots taken after a longer duration of digestion (Peyron et al., 2021). Similar conclusions were drawn from the study by Boyd

et al. (2021) that evaluated the influence of CE addition in an *in vitro* digestion model (INFOGEST method) to assess PS bioaccessibility in soybean oil. The authors reported that PS bioaccessibility is positively correlated with digestion time. In the same study, the effect of the type of agitation on bioaccessibility was evaluated. It was found that the use of a head-over-heels rotation increased the bioaccessibility of sterols, compared with the commonly used orbital agitation at higher speed, due to a greater homogenization of the digestate. Furthermore, as the pepsin added in our trial was lower and its optimal activity is at pH 2 (much closer to the pH of the adult than that of the elderly) (Johnston et al., 2007), at the end of digestion there will still be more protein to digest. This greater amount of protein could increase the bioaccessibility of PSs, as occurred with other lipophilic compounds. In that sense, in a static INFOGEST trial it was observed that the co-digestion under complete digestive conditions of carotenoid-rich foods (tomato, carrot juice and spinach) with different protein preparations increased the bioaccessibility of carotenoids in the case of well-soluble proteins (Iddir et al., 2021).

On the other hand, the same gastric phase adaptations (modification E1) did not produce a change in the bioaccessibility (vs adult) of the WRB (see **Figure 18**). The differences between both food matrices (solid vs liquid) could partially explain this observation. In fact, it has been observed that the GL contributes to a greater extent to the digestion of triglycerides (TG) in liquid foods (25%) than in solid foods (10%) (Amara et al., 2019). In this context, GL has difficult access to its substrate (TG), and its action could be greatly reduced since WRB is a very complex matrix rich in fiber with a solid structure. Besides, this could explain why, even though it has been observed that a decrease in the activity of the GL noted in the adaptation to the elderly translates into a higher bioaccessibility of PS in a liquid matrix by not being able to perform its action correctly (Makran et al., 2022), in a solid matrix, neither in the control nor in the gastric elderly model of the present study did these expected differences occur.

The lack of the effect of GL could be attributed to the influence of the soluble fiber present in the WRB, that contains up to twice as much β-glucan and fructan than wheat bran, while it contains up to half the cellulose (Kamal-Eldin et al., 2009). In this sense, the addition of different soluble fibers (guar gum, pectins or Arabic gum) at increasing concentrations (0.3 and 2% w/v) to a lipid mixture (triolein/phosphatidylcholine/cholesterol) produced larger lipid droplets compared to those without the presence of fiber. This made it difficult for GL to access TG. The authors

attributed this to the higher viscosity in the case of the presence of soluble fiber. In addition, this effect seems to be dose dependent. The presence of Arabic gum and high viscosity guar gum decreases the degree of lipolysis of TG through a decrease in the effectiveness of GL (Pasquier et al., 1996). This may explain why a food as rich in fiber as WRB (and in soluble fiber in particular), has its lipolysis at the gastric level greatly diminished.

Different outcomes were obtained in the beverage and the WRB when all digestive phases were adapted to the elderly. In the beverage, the adaptation increased even more the bioaccessibility of PS (except sitostanol and campestanol). This can be partly explained by the different hydrophobicities of sterols. In this sense, cholesterol has a lower hydrophobicity than PS. The different hydrophobicities of 4 major PS and cholesterol have been studied, obtaining an order of aqueous solubility as follows: cholesterol > campesterol > β-sitostanol > β-sitosterol (Matsuoka et al., 2008).

Bile salts help the solubilization of sterols and other lipophilic compounds (Singh et al., 2019). In fact, the increase in cholesterol bioaccessibility in E2 compared with E1 may be due to the reduction of bile salts and the reduction of pancreatin. Both pancreatin and bovine bile contain cholesterol, as Makran et al. (2022) noticed. Considering the amount of the reagents added, the main cholesterol contributor is porcine pancreatin, followed by bovine bile and RGE (1.7, 1.0 and 0.05 mg per total digesta, respectively), implying a total cholesterol (TC) content of 2.75 mg in the digestion blanks. In this sense, the reduction in bile and pancreatin in the elderly conditions increased the sterol bioaccessibility by reducing the contribution of cholesterol in the reagents used for the digestion, which favors the beverage's sterol solubilization. This observation was also confirmed by Blanco-Morales et al. (2018) and Makran et al. (2022) (in which the concentration of bile salts was reduced from 10 to 1.4 and from 17.5 to 10 mM, respectively). Furthermore, the pancreatic lipase present in the pancreatin produces FFA and DAG from TG that have not yet been digested in the gastric phase, thus promoting the solubilization of cholesterol from reagents. Therefore, the decrease in pancreatin could improve sterol bioaccessibility, as previously mentioned. Previous *in vitro* studies evaluating lipolysis under elderly conditions (applying a reduction in pancreatin activity and bile salt concentration as in E2) found no changes in lipolysis in elderly conditions, and it was even increased in the case of some fatty matrices, such as aged cheese (28.8 g fat per 100 g cheese) (Hernández-Olivas et al., 2020b) and fatty fish such as salmon (14.0

g fat per 100 g fish) (Hernández-Olivas et al., 2020a). The authors conclude that the longer digestion duration could compensate for the enzymatic decrease, although they pointed out that the results depend primarily on the matrix and its fat content.

Furthermore, it seems that the intestinal absorption of cholesterol is positively correlated with age, among other factors (Wang 2007), which is consistent with a greater bioaccessibility. In fact, in a clinical trial with 33 subjects in which some of them were administered drugs to increase the intestinal transit speed for comparison with others without treatment, it was observed that a longer gastrointestinal transit time improved the bioavailability of cholesterol. The authors attributed this observation to a higher incorporation of cholesterol into the mixed micelles (Ponz de León et al., 1982).

On the contrary, the same adaptations reduced PS bioaccessibility in WRB. This opposite effect could be justified by the fiber content. Fiber might cause lower lipid and cholesterol absorption. Lower bioaccessibility (up to 24% reduction in fat bioaccessibility and up to 22% reduction in cholesterol bioaccessibility) was observed after digestion in a multi-compartmental gastrointestinal model (Minekus et al., 2005), when a partially hydrolyzed guar gum at 3 and 6% concentration (cholesterol : fiber ratio 0.04 and 0.02 respectively) was added to yogurt enriched with egg yolk (4%) and sunflower oil (3%). Similar findings were obtained from an INFOGEST simulated digestion trial with pork patties with and without the addition of different fiber extracts (from lemon, pomegranate, lemon albedo, grapefruit and tiger nut). Lower cholesterol solubility (ranging from 10 to 31% reduction depending on the fiber extract used) from fiber-added patties, when compared with fiber-less patties, was observed (López-Marcos et al., 2015). However, in another INFOGEST simulated digestion study in a PS-enriched milk-based fruit beverage, the addition of 2.5 and 5 g of galactooligosaccharides per 250 mL of beverage did not cause any change in the bioaccessibility of sterols compared to the beverage without added galactooligosaccharides. The authors attributed this fact to the low content of fiber in relation with sterols (ratio of sterol : fiber 0.98–0.6), which could have prevented the fiber from having an effect on reducing the bioaccessibility of sterols (Blanco-Morales et al., 2018). This is consistent with the results obtained in our work, since the PS : fiber ratio in this case is 0.17, more similar to that obtained by Minekus et al. (2005). It could explain that, in order to observe the effect of reducing the bioaccessibility of sterols due to fiber, not only the total fiber content must be considered, but also its ratio with respect to sterols.

In addition, the reduction of enzymes and bile salts and agitation during digestion may have contributed to the outcome. The reduction of pancreatin (50%), containing pancreatic lipase, in this case responsible for the complete lipolysis (since in the previous phase the GL could not exert its effect correctly), will produce partial lipolysis. This hypothesis is supported by the results obtained in olive oil by adding different concentrations of high molecular weight soluble fiber extracted from *Lentinula edodes*. An inhibition of the activity of pancreatic lipase in the sample was observed, and this inhibition was correlated with the fiber concentration (up to 81.9% inhibition with 10 mg mL^{-1} of fiber) (Xue et al., 2020).

In addition to the activity of the enzymes themselves, for the correct micellarization of sterols, greater emulsification is necessary. Bile salts increase the bioaccessibility of lipophilic compounds such as sterols since they support correct emulsification of lipids. Fiber is one of the compounds that binds to bile acids, as it occurs when joining methylcellulose and bile salt sodium deoxycholate (Behera et al., 2021), preventing bile acids from emulsifying sterols. When they are bound to the fiber, bile acids are eliminated in the feces, and they are not available to emulsify sterols (Singh et al., 2019). As far as we know, there are no studies that have evaluated the impact of fibers on the action of bile salts and sterol micellarization, all combined. However, it has been observed that compounds such as polyphenols bind with bile salts due to their physicochemical properties, preventing the correct micellarization of sterols. This aspect was observed in a trial combining epigallocatechin gallate and taurocholic acid, where such a combination caused lower solubility of cholesterol and phosphatidylcholine in the micelles (Ogawa et al., 2016). This effect is consistent with the need for a greater amount of bile salts (as is the case under adult gastrointestinal conditions) to emulsify the same amount of sterols in the presence of fiber, which could explain the decrease of bioaccessibility in the elderly due to the halving of bile salts. This explanation could also be supported by the decrease in agitation during intestinal digestion. To achieve the emulsion, it is necessary for the fat drops to be mixed correctly, and the least frequency of agitation could possibly prevent this process, supported by the high viscosity of the mixture due to the presence of soluble fiber.

The dietary fiber content also produces a slower release of carbohydrates, which can also affect fat breakdown and micellarization (Shin et al., 2010). This would support the

hypothesis of reduced intestinal absorption of sterols and hence the change in bioaccessibility by reducing bile salts and pancreatin.

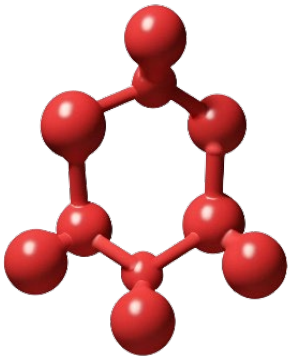
A review that considered different meta-analyses on the impact of various factors on the plasma cholesterol-lowering effect of PS showed that the relative reduction of plasma cholesterol is maintained with age, which means that, starting from a higher basal cholesterol, the decrease in absolute terms increases and, therefore, this age group could benefit to a greater extent from the PS intake (Trautwein et al., 2018).

It is worth noting that after performing those assays on the beverage and bread, a proposal for a standardization of the digestive conditions of the elderly to adapt the INFOGEST method was published (Menard et al., 2023) (see Introduction, section 3.2). We could hypothesize that some results would change using those conditions. For instance, the lower gastric pH could be relevant for enhancing GL activity. This could result in lower PS bioaccessibility in the beverage mainly, due to the aforementioned importance of this enzyme on the micellarization. Nevertheless, the lower duration of the intestinal phase (3 vs 2 h in the present work), could worsen the effect of bile salts on micellarization, resulting in a potential lower bioaccessibility, mostly in the WRB. To sum up, the changes proposed can affect PS bioaccessibility in both directions, so it is difficult to hypothesize whether the expected change would be large or, on the contrary, relatively small.

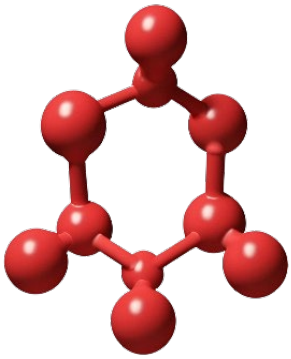
These results have been shared in two publications:

Miedes, D., Makran, M., Barberá, R., Cilla, A., Alegría, A., & Garcia-Llatas, G. (2022). Elderly gastrointestinal conditions increase sterol bioaccessibility in a plant sterol-enriched beverage: Adaptation of the INFOGEST method. *Food & Function*, *13*, 4478-4485.

Miedes, D., Makran, M., Cilla, A., Barberá, R., Garcia-Llatas, G., & Alegría, A. (2023). Aging-related gastrointestinal conditions decrease the bioaccessibility of plant sterols in enriched wholemeal rye bread: *In vitro* static digestion. *Food & Function*, *14*, 6012-6022.



Chapter II:
Chemopreventive effect of an *in vitro* digested and fermented plant sterol-enriched wholemeal rye bread in colon cancer cells



1. Materials and methods

1.1. Reagents

2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), bovine serum albumin, 2′,7′-dichlorofluorescein diacetate (DCFDA), dimethylsulfoxide (DMSO), 3, (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Fluo 3-AM, fluorescein sodium salt, 5-fluorouracile (5-FU), Folin–Ciocalteu reagent, gallic acid, monoclonal anti-ceramide antibody produced in a mouse (primary antibody), goat anti-mouse IgM + IgG + IgA anti-body, F(ab′)2, FITC conjugate (secondary antibody), propidium iodide (PI), ribonuclease A (RNase A), sodium carbonate (Na_2CO_3), and Trolox were purchased from Merck LifeScience S.L.U. (Madrid, Spain). 5-Chloromethylfluorescein diacetate (Green CMFDA) was acquired from Abcam (Cambridge, UK). FITC Annexin V Apoptosis Detection Kit I was purchased from BD Biosciencies (San Jose, CA, USA). TRIzol™ reagent was purchased from Invitrogen™ (Carlsbad, CA, USA). A ReliaPrep™ RNA miniprep system kit was acquired from Promega (Madison, WI, USA). A TaqMan™ reverse transcription kit and PowerUp™ SYBR™ green master mix were purchased from Applied Biosystems™ (Foster city, CA, USA). D-MEM + GlutaMAX™ (4.5 g/L glucose), MEM non-essential amino acids (MEM NEAA) solution (100×), HEPES buffer solution (1 M), antibiotic solution (10,000 U/mL penicillin and 10,000 µg/mL streptomycin), antimycotic solution (250 µg/mL amphotericin B), fetal bovine serum (FBS), PBS pH 7.4 (1×) and trypsin-EDTA solution (2.5 g/L trypsin and 0.2 g/L EDTA) were acquired from Gibco™ (Scotland, UK). Absolute ethanol was obtained from Panreac (Barcelona, Spain). 2-Propanol and chloroform were supplied by Scharlau (Barcelona, Spain). Deionized water was obtained using a Milli-Q water purification system (Millipore™, Bedford, MA, USA).

1.2. Samples

A WRB (containing 1.3 g of PS/portion of 80 g) and a non-enriched wholemeal rye bread (NWRB) were manufactured as previously indicated in Chapter 1, section 1.2., both with the same conditions, as described by Makran et al. (2023b). **Table 13** summarizes the nutritional information of both breads as well as their PS contents.

Table 13. Nutritional information and plant sterol content (g/100 g bread) of enriched and non-enriched wholemeal rye bread

	WRB	**NWRB**
Water	32.7 ± 1.0	26.14 ± 0.02
Lipids	2.76 ± 0.02	1.16 ± 0.02
Carbohydrates	44.3 ± 0.4	51.5 ± 1.3
Soluble fiber	3.4 ± 0.1	4.0 ± 1.3
Insoluble fiber	10.3 ± 1.4	10.7 ± 0.16
Proteins	5.16 ± 0.03	5.85 ± 0.11
Ash	1.37 ± 0.04	1.47 ± 0.02
Plant sterols	1.6 ± 0.04	0.05 ± 0.001

Data are shown as mean ± standard deviation (n = 3). NWRB: non-enriched wholemeal rye bread; WRB: plant sterol-enriched wholemeal rye bread. Source: (Makran et al., 2023b).

1.3. Semi-dynamic in vitro digestion and colonic fermentation

The dynamic gastrointestinal digestion and colonic fermentation system used was simgi®, developed by CIAL (CSIC-UAM) (Madrid, Spain), described by Tamargo et al. (2022) with some modifications. This system consists of five compartments (stomach, small intestine, ascending colon (AC), transverse colon (TrC), and descending colon (DC)) with a maintained temperature (37 °C). Furthermore, it has a constant flow of enzyme solutions, as well as NaOH and HCl to maintain the pH at each stage of digestion (stomach: 1.8; small intestine: 7; AC: 5.6; TrC: 6.3; DC: 6.8).

Gastrointestinal digestion was carried out in a reactor without peristaltic movements and without emptying into the small intestinal compartment due to the high quantity of fiber of the rye bread in the oral bolus. However, the progressive addition of enzymes and solutions to maintain pH was performed in the same way. In the remaining compartments (colonic vessels), pipes connect them, and the peristaltic valve pumps were automatically controlled.

The fecal sample for the colonic fermentation was obtained from a healthy donor with the following exclusion criteria: the donor had not received any anabolic, hormonal, antibiotic or hypocholesterolemic treatment; consumed herbal products, enriched foods, vitamin, carotenoid, pro/prebiotics, PS, or phytoestrogen supplements; suffered any acute inflammation or gastrointestinal diseases; taken any chronic medication; and was not vegan or vegetarian.

Figure 19 shows a schematic diagram of the gastrointestinal digestion and colonic fermentation process and sample collection. Samples of the fermentation liquid (FL) from the DC vessel corresponding to time 0 (stabilization blank, SB) were taken at the end of the microbiota stabilization period of 13 days, before digesting any bread.

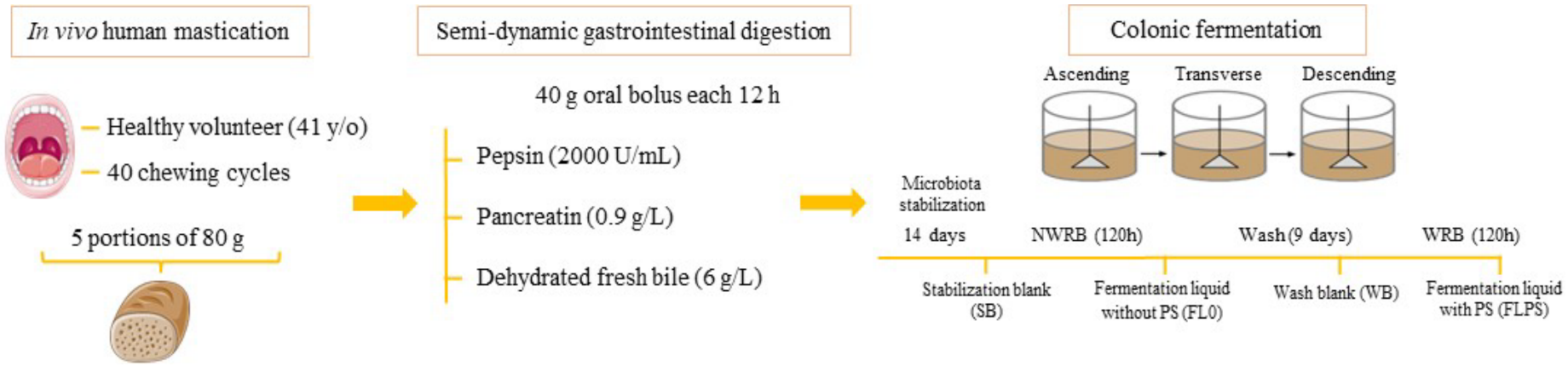
Prior to the dynamic digestion with simgi®, a human oral phase was performed. Five individual breads, representing portions of WRB and NWRB (81.45 ± 1.14 g) were chewed as described previously by Faubel et al. (2022) and five independent oral boluses were obtained for each type of bread. Due to the difficulties presented by the gastrointestinal digestion of the oral boluses in the simgi®, a semi-dynamic gastrointestinal digestion of these oral boluses corresponding to one intake (40 g WRB or NWRB) was performed. To feed the simgi® during 120 h (5 days), gastrointestinal digesta were added twice each day, representing a consumption of one portion of bread per day (80 g of NWRB or WRB). After the microbiota stabilization period, the AC was fed with the gastrointestinal digesta corresponding to the NWRB (145 mL digesta from 40 g bread) (representing the first consumption of bread for one day) and 8 h later, another amount of intestinal digesta (145 mL) (corresponding to the next 40 g of daily consumption of bread) was added. Aliquots of the FL without plant sterols (FL0) were taken at 120 h at the end of the DC and stored at −20 °C.

Next, a washout period was performed during 9 days in order to stabilize the microbiota and remove the sterol residue from the NWRB, and aliquots of the wash blank (WB) were taken at the end of this period. Then, the AC was fed with the WRB in the same way as for the NWRB. Aliquots of the FL with plant sterols (FLPS) were collected from the DC at 120 h and stored at −20 °C.

1.4. Cell culture

CCD-18Co (normal colon fibroblasts) and Caco-2 (colorectal adenocarcinoma) cells were acquired from the American Type Culture Collection (CRL-1459 and HTB-37, respectively) (Rockville, MD, USA). Both cell lines were cultured in 75 cm^2 flasks (Corning™ Falcon™) with D-MEM supplemented with 10% (*v*/*v*) FBS, 1% (*v*/*v*) HEPES, 1% (*v*/*v*) MEM NEAA, 1% (*v*/*v*) antibiotic solution, and 1% (*v*/*v*) antimycotic solution. Cells were maintained at 37 °C in a humidified atmosphere (95% relative humidity, RH) with 5% (*v*/*v*) of CO_2.

Figure 19. Schematic diagram of the semi-dynamic gastrointestinal digestion and colonic fermentation



NWRB: non-enriched wholemeal rye bread; WRB: plant sterol-enriched wholemeal rye bread.

The four samples (SB, FL0, WB and FLPS) were centrifuged (3100 x *g*/15 min/4 °C) and the supernatants were stored. To avoid any cytotoxic artifact, the supernatants were firstly diluted and filtered. A 0.22 µm (PTFE) filter was tested without dilution and with 1/2 and 1/5, v/v dilutions (in D-MEM). But the complexity of the samples, rich in fiber and with a colloidal structure, prevented it. For this reason, the samples were diluted with D-MEM (1/5, *v*/*v*), then filtered through 0.45 µm (PTFE) and stored at −20 °C.

For experimental studies, Caco-2 (passages 19–38) and CCD-18Co (passages 8–15) cells were dissociated from the flask with trypsin-EDTA. Subsequently, cells were plated at 5×10^4 cells/cm^2 onto 96-well (MTT assay), 24-well (cytofluorometric analysis) or 6-well plates (quantitative polymerase chain reaction (qPCR) assays) (Costar Corp., Cambridge, MA, USA) and incubated (37 °C, 95% RH, 5% (*v*/*v*) CO_2) during 24 h with a culture medium. After that, the cells were treated with the samples for 24 h because this reflects a realistic estimate of human intestinal exposure to dietary compounds. However, gene expression assays (qPCR) were performed at 3 and 6 h since transcriptional modifications are initiated during the early exposure to treatment. Untreated cells were used as a control, and 5-FU (25 µM) was used as a positive control, owing to its cytotoxic effect on colon cancer cells.

1.5. MTT cytotoxicity assay

The MTT is based on the formation of purple formazan crystal (in active cells) from yellow tetrazolium salt, allowing to determine the cell metabolic activity (Gerlier & Thomasset, 1986). Cells were incubated with a MTT solution (4 h, 37 °C, 95% RH, 5% (*v*/*v*) CO_2) and the formazan crystals were solubilized with DMSO. Absorbance was measured with a multi-well spectrophotometer (Synergy H1 microplate reader, BioTek, Agilent, Santa Clara, US) (570 nm, background subtraction 690 nm), and was correlated with cell viability. Results were expressed as % of viability compared to cell control.

1.6. Detection of apoptosis: Annexin V/PI assay

The cellular state was evaluated with the two-dimensional gating method of an FITC-Annexin V kit, which detects the externalization of phosphatidylserine; this represents an apoptotic event (Darzynkiewicz et al., 2001). It allows to differentiate between viable (annexin and PI negative), early apoptotic (annexin positive and PI negative), late apoptotic (annexin and PI positive) and necrotic (annexin negative and PI positive) cells and was measured by flow cytometric analysis (FACS Verse, BD Biosciences) (λexc =

488 nm and λem = 527/32 nm for FITC-Annexin V and λexc = 488 nm and λem = 586/42 nm for PI). At least 1×10^4 events were analyzed for each sample.

1.7. Cell cycle progression

The proportion of the different stages of the cell cycle was evaluated using PI, which binds to the DNA and allows to differentiate between cells with a normal DNA amount (G0/G1 phase) and cells with double the DNA amount (G2/M phase) (Darzynkiewicz et al., 2001). The S phase was placed in the intermediate of these and the apoptotic cells, whose DNA content is lower, were placed as the subG1 phase. Cells were incubated with ethanol:PBS 70:30 (*v*/*v*) and after that with a mix of RNase A and PI (40 and 100 µg/mL, respectively in PBS) (30 min, 37 °C, 95% RH, 5% (*v*/*v*) CO_2). The fluorescence was evaluated with flow cytometry (FACS Verse, BD Biosciences) (λexc = 488 nm and λem = 527/32 nm). At least 1×10^4 events were analyzed for each sample.

1.8. Determination of ceramide levels

The levels of ceramide were evaluated using a goat anti-mouse, polyclonal, FITC, secondary antibody after incubation (60 min/37 °C/5% CO_2/95% RH) with a mouse monoclonal anti-ceramide antibody (Restivo et al., 2022b). After centrifugation (450 x *g*/5 min/4 °C), and a PBS wash, cells were incubated with 100 µL of secondary antibody (30 min/37 °C/5% CO_2/95% RH), which binds to intracellular ceramide and can be analyzed by flow cytometry (FACS Verse, BD Biosciences) (λexc = 488 nm and λem = 527/32 nm). At least 1×10^4 events were analyzed for each sample.

1.9. Levels of reactive oxygen species (ROS)

The measurement of ROS was performed with DCFDA, which is oxidized by ROS, producing fluorescence. Briefly, cells were suspended with DCFDA (1 µM, final concentration) and incubated in darkness (30 min/37 °C/5% CO_2/95% RH). After centrifugation (450 x *g*/5 min/25 °C), the pellet was suspended in 300µL of PBS and analyzed by flow cytometry (FACS Verse, BD Biosciences) (λexc = 488 nm and λem = 527/32 nm) (López-García et al., 2017). At least 1×10^4 events were analyzed for each sample.

1.10. Intracellular reduced glutathione (GSH)

The content of GSH was determined with CMFDA, a specific dye able to bind to nonprotein thiol (as GSH). Briefly, the CMFDA was mixed with 500 µL of cell suspension in a cytometer tube (1 µM, final concentration). Cells were incubated with the dye (40 min/37 °C/5% CO_2/95% RH), centrifuged (450 x *g*/5 min/25 °C), suspended in 300 µL of PBS, and analyzed by flow cytometry (FACS Verse, BD Biosciences) (λexc = 488 nm and λem = 527/32 nm). At least 1×10^4 events were analyzed for each sample.

1.11. Intracellular calcium levels

Levels of intracellular calcium were determined using Fluo-3AM probe (Cilla et al., 2015). Fluo-3AM can bind to calcium and emit fluorescence proportionally to the calcium content. Briefly, cells were harvested with trypsin (2.5 g/L), centrifuged (450 x *g*/5 min/25 °C), suspended in 500 µL a Fluo-3AM in PBS solution (2 µM) and incubated for 40 min (37 °C/5% CO_2/95% RH). The supernatant was removed after centrifugation (450 x *g*/5 min/25 °C), 400 µL of PBS were added to each cytometer tube, and analyzed by flow cytometry (FACS Verse, BD Biosciencies) (λexc = 488 nm and λem = 527/32 nm). At least 1×10^4 events were analyzed for each sample.

1.12. Evaluation of gene transcription by qPCR

To evaluate the expression of the regulatory genes of the apoptosis and the cell cycle, a relative quantification of its messenger RNA (mRNA) levels was performed using qPCR prior retrotranscription (RT) to complementary DNA (cDNA).

RNA extraction:

The extraction was performed by isolating and purifying the RNA with the TRIzol™ reagent. After the culture medium was removed, 500 µL of TRIzol™ reagent was added and cells were harvested with cells scrapers (Sarstedt®, Nümbrecht, Germany), and after 5 min, they were harvested again. The cell lysate was transferred to a 1.5 mL Eppendorf® tube, and the whole process was repeated. A total of 200 µL of chloroform were added and the tubes were centrifuged for 15 min at 4 °C and 12,000 × *g* (Sigma® 3K15). Then, the supernatants were transferred to a new tube and 170 µL of isopropanol were added. The RNA was purified using a ReliaPrep™ RNA Miniprep System kit according to the technical manual provided by the manufacturers (but 2 min centrifugation). The concentration and purity of the RNA was determined with a micro-volume

spectrophotometer (NanoDrop Lite, Thermo Fisher Scientific™, Waltham, US). The extract was maintained at −80 °C until analysis.

Primer design:

Primers for each gene were designed using Primer-BLAST software of the National Center for Biotechnology Information. The default criteria were maintained for a PCR melting temperature (min 57, opt 58, max 60, and max melting temperature difference 0.5 °C) and product size (150 to 200 base pairs). The sequence of the target and reference gene primers as well as the amplification efficiency are shown in **Table 14**.

Table 14. Sequence of primers of the evaluated genes and their efficiency

Gene	Primer Sequence (5′→3′)		Efficiency (%)
	Forward	Reverse	
BAX	GGGCCCACCAGCTCTGA	CCTGCTCGATCCTGGATGA	108
BCL2	GGATTGTGGCCTTCTTTGAG	GCCGGTTCAGGTACTCAGTC	121
CASP3	CCTGGTTATTATTCTTGGCGAAA	GCACAAAGCGACTGGATGAA	125
CASP8	CAGGCAGGGCTCAAATTTCT	TCTGCTCACTTCTTCTGAAATCTGA	121
CASP9	TGCTGAGCAGCGAGCTGTT	AGCCTGCCCGCTGGAT	130
CDKN1A	GATGAGTTGGGAGGAGGCAG	GAGCGAGGCACAAGGGTACA	140
TP53	CCACCATCCACTACAACTACAT	CACAAACACGCACCTCAAA	135
CCND1	CCCTCGGTGTCCTACTTCAA	AGGAAGCGGTCCAGGTAGTT	107
CCNE1	CTCCAGGAAGAGGAAGGCAA	TCGATTTTGGCCATTTCTTCA	127
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	112

BAX, *BCL2*, *CASP3*, *CASP8*, *AND CASP9*—Cell apoptosis; *CDKN1A*—p21, involved in phase S of cell cycle; *TP53*—p53, cell cycle regulation; *CCND1*—cyclin D1, involved in cell cycle G1/S phase; *CCNE1*—cyclin E1, involved in cell cycle G1/S phase; *ACTB*—actin beta, housekeeping.

Retrotranscription:

The RT was carried out using a TaqMan™ Reverse Transcription kit, according to the protocol provided by the manufacturers (25 °C for 10 min, 48 °C for 30 min, 95 °C for 5 min and stabilization at 16 °C), obtaining a final concentration of 30 ng/µL of cDNA.

Quantitative real-time PCR:

Amplification reactions were performed with a StepOne Plus Real-time PCR instrument. SYBR™ Green detection chemistry was used for the reaction, carried out in a MicroAmp™ Fast Optical 96-Well Reaction Plate 0.1 mL (Applied Biosystem™). Each

reaction (10 µL, three technical replicates) was composed of 45 ng cDNA, 5 µL of PowerUp™ SYBR™ Green Master Mix, and 2 µL of solution of primer pair (900 nM of both primers). There was an initial holding stage at 95 °C for 5 min, followed by 50 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and an elongation at 72 °C for 15 s. After amplification, melting curve analysis was performed over a gradient (+0.3 °C/min) extending from annealing to the denaturation temperature.

Gene expression analysis:

Changes in gene expression in apoptosis-related (*BAX*, *BCL2*, *CASP3*, *CASP8*, and *CASP9*) and cell cycle-related genes (*CDKN1A*, *TP53*, *CCND1*, and *CCNE1*) were assessed using the $2^{-\Delta\Delta Cq}$ method (Livak et al., 2001). The *ACTB* gene was used as a reference gene since its expression remains constant across the different conditions.

1.13. Total antioxidant capacity (TAC)

Since no standardized TAC method exists, at least two different methods with different mechanisms of action, hydrogen atom transfer reaction mechanism (Oxygen radical absorbance capacity, ORAC) and electron transfer based assay (Folin–Ciocalteu method) were performed (Prior et al., 2005).

ORAC method

The ORAC method was performed following Ou et al. (2001), with modifications. A mixture of 80 µL of fluorescein (1 µM), 80 µL of the samples (1/5 DMEM diluted supernatants of SB, FL0, WB, or FLPS, or Trolox standard) and 40 µL of an AAPH solution in PBS (250 mM) was added. The fluorescence intensity was measured every min during 90 min (λexc = 485 nm and λem = 520 nm) using a multi-well spectrophotometer (Victor^3 multi-label counter, Perkin-Elmer, Waltham, US) and the area under the curve was calculated. The results were compared with a Trolox standard (20 µM) and were expressed as µM Trolox.

Total phenolic content (TPC)

The TPC was determined following the Folin–Ciocalteu method (Singleton & Rossi, 1965). A mixture of 3 mL of a Na_2CO_3 solution (2%, *v*/*v*), 100 µL of sample (1/5 DMEM diluted supernatants of SB, FL0, WB, or FLPS) and 100 µL of Folin–Ciocalteu reagent was incubated for 1 h. Then, the absorbance of samples and a gallic acid calibration curve

was measured with a spectrophotometer at 765 nm (Lambda 365 uv/vis spectrophotometer, Perkin-Elmer, Waltham, US). Results were expressed as mg gallic acid equivalents (GAE)/L.

1.14. Short chain fatty acids (SCFA) determination

Acetic, propionic, butyric and valeric acid from all samples were analyzed (n = 2) by GC-FID (Agilent 6890A, Agilent), using helium as the carrier gas (1.5 mL min^{-1}) and a column DB-WAXtr (60 m, 0.325 mm × 0.25µm). The temperature started at 50 °C for 2 min, at 15 °C min^{-1} was raised until 150 °C, at 5 °C min^{-1} was raised until 200 °C, at 15 °C min^{-1} was raised until 240 °C, and kept for 20 min (Faubel et al., 2025). Results were expressed as a concentration (mM) of SCFA.

1.15. Statistical analysis

All assays were carried out in two independent experiments (at least n = 3 per assay). Shapiro−Wilk was used to check normality and a Levene test to check homoscedasticity. One way ANOVA, followed by a Tukey´s post hoc test, was applied to determine statistically significant differences between treatments ($p < 0.05$). The program used for statistical analysis was Graphpad Prism 8.0.2 (GraphPad Software Inc., San Diego, CA, USA).

2. Results

2.1. Cytotoxicity

The selectivity of the cytotoxic effect of the samples on tumoral cells was assessed (see **Table 15**). No cytotoxic effect was observed on the non-tumoral cell line after all treatments with the three dilutions assayed for 24 h. It is possible that the content of the SCFAs may be the main contributor. SCFAs (butyrate mainly) are a relevant source of energy for colonocytes, and a higher content of SCFAs could improve normal cell development. On the other hand, in the tumoral cell line only 1/5 dilution did significantly increase the cytotoxicity ($p < 0.05$). The viability reduction after treatment with FL compared to their blanks was higher with FLPS (34%) compared to FL0 (15%).

Considering these results, it was decided to use the 1/5 dilution of the treatments for the subsequent assays only with tumoral cells. In addition, the non-tumoral cell line was no longer used, as its only purpose was to ensure that the effect of treatments on tumoral cells does not impact on the normal colonic epithelium.

Table 15. Evaluation of cytotoxicity (MTT) of the different treatments for 24 h and with different dilutions in CCD-18Co (normal colon fibroblasts) and Caco-2 (colorectal adenocarcinoma) cells vs control cells (both lines untreated)

	Viability (% of Control)					
	CCD-18Co			**Caco-2**		
Treatment	**1/5**	**1/10**	**1/20**	**1/5**	**1/10**	**1/20**
SB	97.8 ± 8.1 ^{aA}	98.9 ± 7.4 ^{aA}	88.6 ± 4.0 ^{aA}	73.4 ± 2.5 ^{aB}	82.0 ± 12.4 ^{aB}	119.8 ± 17.7 ^{aA}
FL0	102.7 ± 7.0 ^{aA}	181.4 ± 17.2 ^{bA}	176.0 ± 20.9 ^{bA}	62.3 ± 2.5 ^{bB}	100.3 ± 1.0 ^{bA}	109.5 ± 7.9 ^{aA}
WB	104.3 ± 6.4 ^{aA}	104.2 ± 6.7 ^{aA}	101.6 ± 5.8 ^{aA}	98.2 ± 11.1 ^{cA}	96.9 ± 7.4 ^{abA}	106.4 ± 14.4 ^{aA}
FLPS	105.2 ± 8.8 ^{aA}	103.1 ± 11.1 ^{aA}	102.3 ± 18.0 ^{aA}	65.0 ± 1.7 ^{bB}	97.9 ± 0.6 ^{bA}	104.8 ± 20.1 ^{aA}
5-FU (25µM)		96.4 ± 19.0			85.0 ± 4.9	

Data are shown as mean ± standard deviation (n = 3). Different lowercase letters (a–c) in the same column (dilution) indicate statistically significant differences between treatments for the same cell line ($p < 0.05$). Different uppercase letters (A, B) indicate statistically significant differences between cell lines with the same treatment ($p < 0.05$). SB: stabilization blank; FL0: fermentation liquid without plant sterols; WB: wash blank; FLPS: fermentation liquid with plant sterols; 5-FU: 5-fluorouracil.

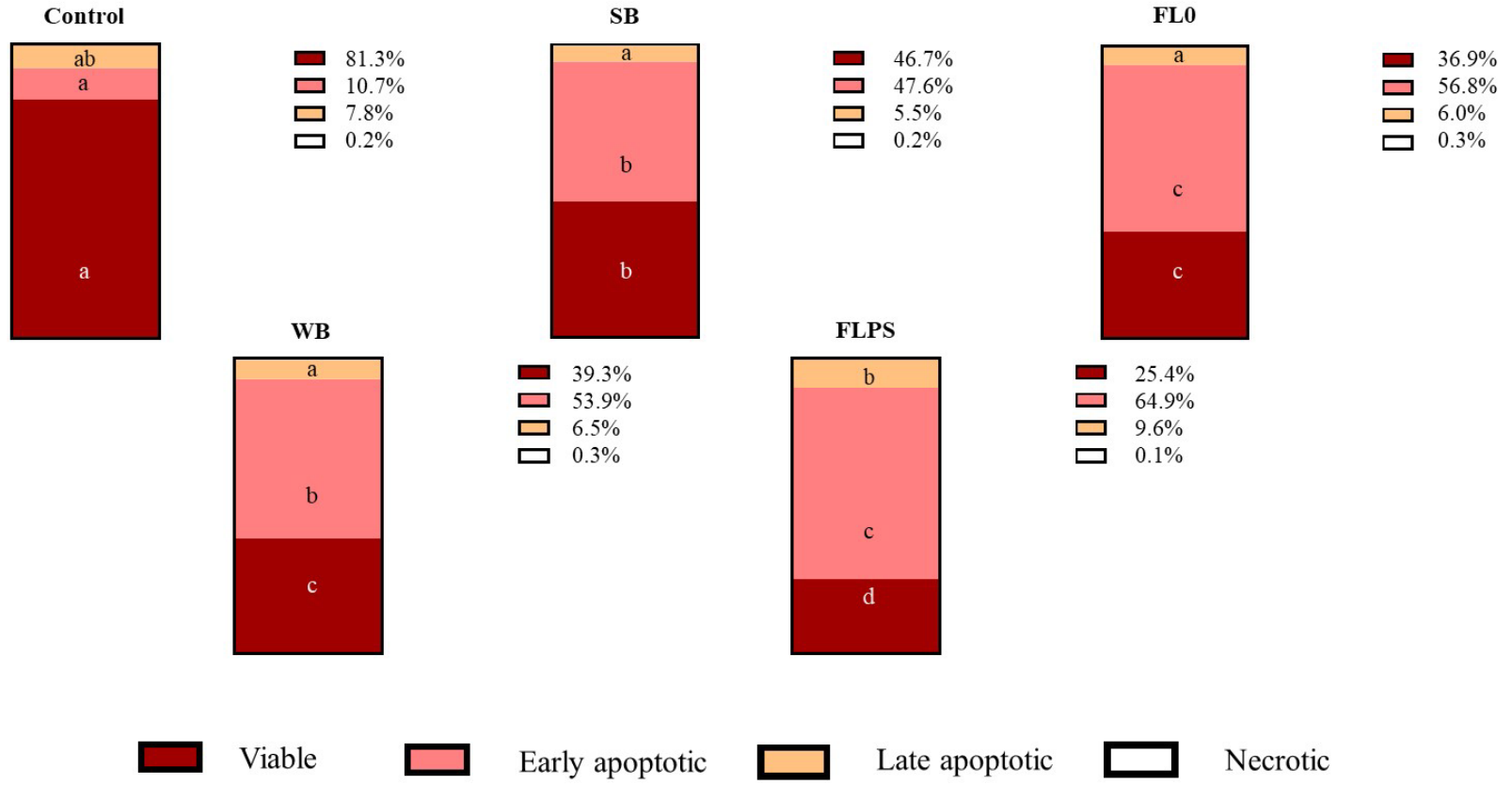
2.2. Apoptosis

The cell status distribution after treatment with the samples is represented in **Figure 20**. The % of apoptotic cells (the sum of early and late apoptotic cells) was higher in both blanks (53 and 60% in SB and WB, respectively) compared to the control (19%). This is increased after treatment with FL samples (63 and 74% in FL0 and FLPS, respectively). Comparing FLs with their respective blanks, viable cells were reduced to a greater extent with FLPS (by 35%) compared to FL0 (by 21%). Early apoptotic cells were increased similarly (by 19%). Otherwise, late apoptotic cells were further increased with FLPS (by 47%) compared to FL0 (by 9%).

2.3. Cell cycle

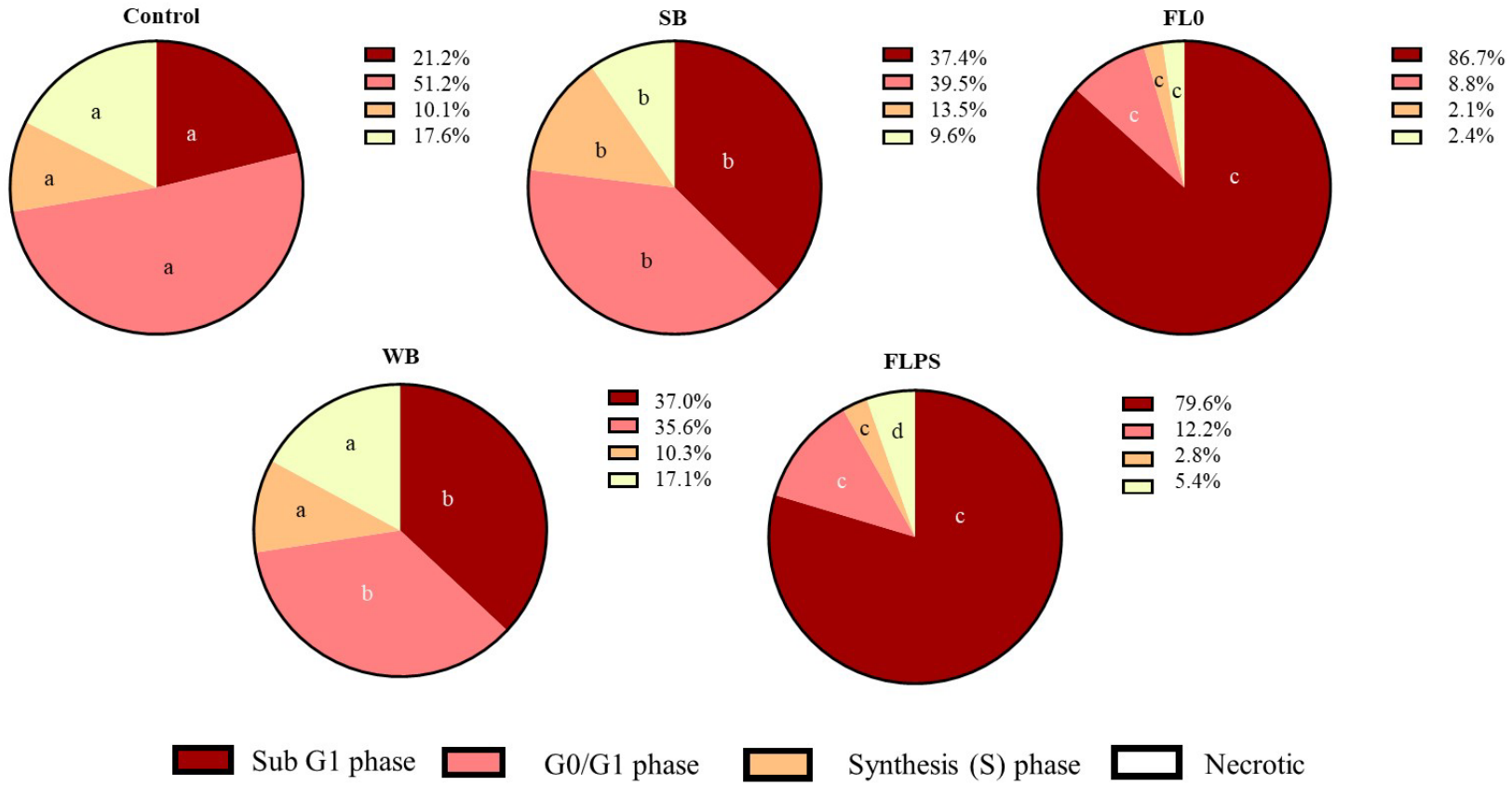
The cell distribution in the different phases of the cell cycle is represented in **Figure 21**. All treatments produced a statistically significant increase ($p < 0.05$) (vs control) of cells in Sub G1 phase (marker of apoptosis). Furthermore, an increase in the Sub G1 population after treatment with FL (vs respective blanks) (by 132 and 116% with FL0 and

Figure 20. Distribution of Caco-2 cells in the different cell states after 24 h of treatment with samples (1/5 dilution)



Data are shown as mean (n = 3). Different letters (a–d) in the same cell state (viable, early or late apoptotic, and necrotic) indicate statistically significant differences between treatments (columns) ($p < 0.05$). FL0: fermentation liquid without plant sterols; FLPS: fermentation liquid with plant sterols; SB: stabilization blank; WB: wash blank

Figure 21. Distribution of Caco-2 cells in the different phases of the cell cycle after 24 h of treatment with samples (1/5 dilution)



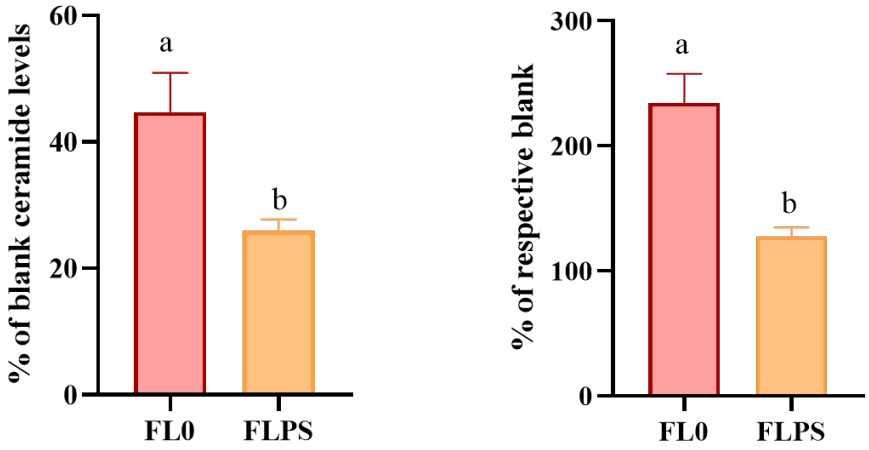
Data are shown as mean (n = 3). Different letters (a–c) in the same phase (Sub G1, G0/G1, S, and G2/M) indicate statistically significant differences between treatments (circles) ($p < 0.05$). FL0: fermentation liquid without plant sterols; FLPS: fermentation liquid with plant sterols; SB: stabilization blank; WB: wash blank

FLPS, respectively) was observed. Consequently, the proportion of all subsequent phases was significantly reduced, and none showed an arrest. These data confirm the results obtained in the Annexin V/PI assay. This means that FL could act mainly through an induction of apoptosis and a reduction in cell proliferation.

2.4. Cell death mechanisms

The next step was to delve into the mechanisms by which cell death occurs. The cellular levels of ceramide (the second messenger in activating the apoptosis cascade) were not increased after treatment with FL (vs respective blanks) (**Figure 22A**). Both blanks did produce an increase in the levels, compared to the control. This fact could indicate a possible cytotoxic effect derived from gastrointestinal digestion and colonic fermentation reagents, mediated by ceramide. Nevertheless, intracellular calcium levels did show an increase ($p < 0.05$) after FL treatment compared to their respective blanks (**Figure 22B**). The increase observed was higher with the FL0 treatment (134%), while with the FLPS it was moderate (27%).

Figure 22. Levels of (A) ceramide and (B) intracellular calcium in Caco-2 after 24 h of treatment with samples (1/5 dilution)

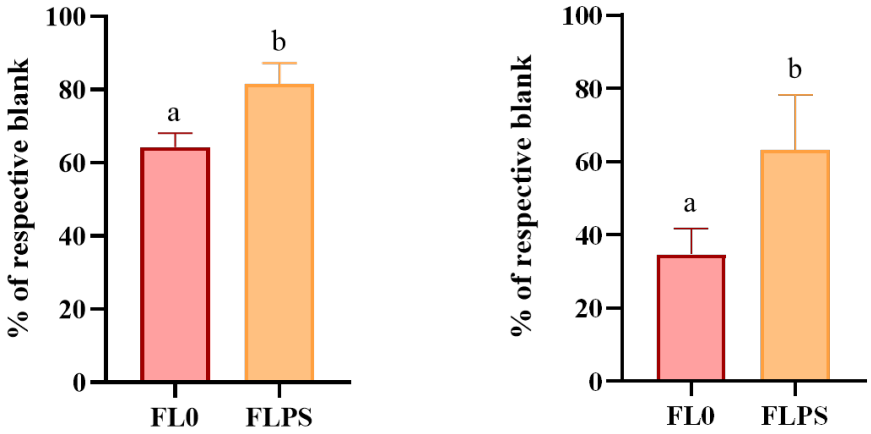


Data are shown as mean ± standard deviation (n = 3). Different letters (a, b) show statistically significant differences between FL0 and FLPS ($p < 0.05$). FL0: fermentation liquid without plant sterols; FLPS: fermentation liquid with plant sterols. Respective blanks are stabilization blank for FL0 and wash blank for FLPS.

2.5. Cell redox status

The alteration in the cell redox status can promote apoptosis in tumoral cells. In this sense, intracellular GSH levels showed a statistically significant reduction ($p < 0.05$) after treatment with both FLs (vs blanks). The reduction was higher with the FL0 treatment (by 36%) compared with the FLPS treatment (by 18%) (**Figure 23A**). However, the ROS assay did not show the same trend, and no increase in ROS levels was observed after the FL treatment (**Figure 23B)**.

Figure 23. Levels of (A) intracellular glutathione and (B) reactive oxygen species in Caco-2 after 24 h of treatment with samples (1/5 dilution)



Data are shown as mean ± standard deviation (n = 3). Different letters (a, b) show statistically significant differences between FL0 and FLPS ($p < 0.05$). FL0: fermentation liquid without plant sterols; FLPS: fermentation liquid with plant sterols. Respective blanks are stabilization blank for FL0 and wash blank for FLPS.

2.6. Transcriptional changes in apoptosis and cell cycle regulation-related genes

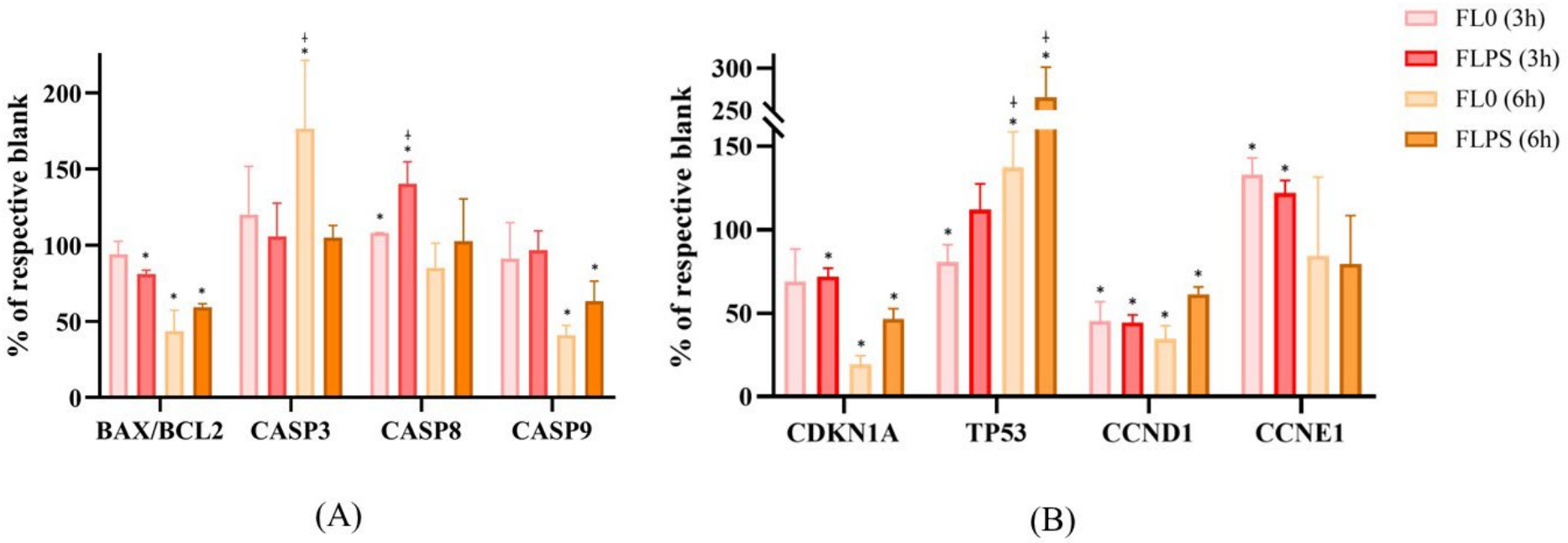
Figure 24 shows changes in gene expression at 3 and 6 h (vs the respective blank). No increase was observed in the *BAX*/*BCL2* ratio, or in *CASP9* expression with the FL treatment at both durations. This fact eliminates the possibility of cell death occurring through the intrinsic (mitochondrial) pathway. However, *CASP8* expression did show a statistically significant increase ($p < 0.05$) after both FL treatments at 3 h. The increase was higher with FLPS (by 40%) compared to with FL0 (by 8%). After 6 h of treatment, there were no changes in the expression of this gene. This indicates that at 3 h, apoptosis

signaling is still occurring, whereas after 6 h, cell death is already well advanced. This is confirmed with the results of the *CASP3* gene expression, which is the executor caspase being at the end of the signaling cascade responsible for carrying out apoptosis. While after 3 h of treatment, neither FL0 nor FLPS produced any increase in expression (the time when cell death signaling is still occurring), after 6 h, FL0 did increase *CASP3* expression (by 76%). In addition, despite the FLPS not producing a statistically significant increase, an upward trend can be observed, although without statistical significance. Furthermore, an increase in *TP53* expression after both FL treatments for 6 h was observed. In this case, the FLPS produced a much larger increase (by 166%) compared to the FL0 (by 37%). Moreover, *CCND1* showed a statistically significant decrease ($p < 0.05$) with both FL and treatment durations. The reduction was higher with FL0 compared with FLPS (65 vs 39% and 55% vs 55% after 6 and 3 h, respectively). The inhibition of cyclin D1 means a higher regulation of the cell cycle, as this cyclin works in the early stages of the cell cycle and may imply a decrease in cell proliferation. The results are consistent with what was observed in the cell cycle assay. Conversely, *CCNE1* expression was slightly increased (at 3 h) or unchanged (at 6 h). This is consistent with the lack of cell cycle arrest previously observed. Proliferation in the present study is downregulated mainly through cell death, so cyclins that act in later phases of the cycle could not show a decrease (as is the case). Finally, *CDKN1A* showed a reduced gene expression with FL compared to blanks, although the decrease is time-dependent, showing a higher decrease after 6 h of treatment with samples compared with 3 h of treatment.

2.7. Total antioxidant capacity

After subtracting the absorbance given by DMEM in both assays (used to dilute the samples), the results were expressed as an increase in antioxidant capacity of FL vs the respective blank. The TPC increase was statistically significant higher ($p < 0.05$) in FLPS (55.6 ± 1.7 mg GAE/L) compared to FL0 (47.8 ± 1.9 mg GAE/L). A slightly higher antioxidant capacity ($p < 0.05$) was also observed by the ORAC method in the FLPS samples. An increase (vs blank) of 2267 ± 585 μM Trolox was observed in FLPS, whereas the increase observed in FL0 was of 2032 ± 506 μM Trolox.

Figure 24. Gene expression of Caco-2 cells after 3 and 6 h of treatment with samples. (A) Apoptosis-related genes; (B) Cell cycle-related genes



Data are expressed as mean ± standard deviation (n = 9). For the same gene and same time, the asterisk (*) indicates statistically significant differences ($p < 0.05$) between the treatment and its blank. The sign ǂ indicates statistically significant differences ($p < 0.05$) between both treatments at the same time (FL0 and FLPS). FL0: fermentation liquid without plant sterols; FLPS: fermentation liquid with plant sterols.

2.8. Short chain fatty acids

The concentrations of the main SCFA in the DC at 120 h after gastrointestinal digestion and colonic fermentation from all samples is summarized in **Table 16**. FLPS contained significantly less total SCFAs compared to FL0. Likewise, the concentration of butyrate was also lower in FLPS.

Table 16. Concentration (mM) of short-chain fatty acids in samples in the descending colon at 120 h

	Acetate	**Propionate**	**Butyrate**	**Valerate**	**Total**
SB	30.99 ± 0.22 ^{a}	12.58 ± 0.30 ^{a}	4.03 ± 0.00 ^{a}	3.35 ± 0.00 ^{a}	55.66 ± 0.54 ^{a}
FL0	31.84 ± 1.84 ^{a}	2.25 ± 0.01 ^{b}	29.09 ± 0.60 ^{b}	0.67 ± 0.02 ^{b}	65.30 ± 1.19 ^{b}
WB	40.29 ± 0.59 ^{b}	15.42 ± 0.22 ^{c}	3.79 ± 0.11 ^{c}	3.54 ± 0.03 ^{c}	67.34 ± 0.99 ^{b}
FLPS	12.65 ± 12.65 ^{c}	1.38 ± 0.01 ^{d}	3.75 ± 0.05 ^{c}	0.37 ± 0.00 ^{d}	18.49 ± 0.06 ^{c}

Data are shown as mean ± standard deviation (n = 3). Different letters in the same column (fatty acid) indicate statistically significant differences between treatments ($p < 0.05$). SB: stabilization blank; FL0: fermentation liquid without plant sterols; WB: wash blank; FLPS: fermentation liquid with plant sterols.

3. Discussion

Our results show that FLs from wholemeal rye bread (enriched or not with PS) can exert a chemopreventive effect via apoptosis, cell cycle regulation and modification of related genes. They have a cytotoxic effect on tumoral cells (Caco-2) without affecting normal cells (CCD-18co).

Diet can be a useful tool to help prevent colorectal cancer (CRC). The use of bioactive compounds such as PSs, in the context of a healthy diet may exert a chemopreventive effect (Huang et al., 2017). The treatment of this disease is generally conducted through chemotherapeutic agents. These substances often cause various side effects such as nausea, mucositis, diarrhea, or vomiting, which can cause a worsening of the diet (Chau & Cunningham, 2002). PSs remain highly unabsorbed at the colon (Le Goff et al., 2019) and perform their antiproliferative effect, targeting cell death in tumoral cells (such as the Caco-2 cell line) without damaging normal epithelial cells (such as the CCD18-Co cell line). Furthermore, the presence of fiber in the digested/fermented samples, with the subsequent formation of SCFA, together with the antioxidants, could have a synergistic action in the antiproliferative effects observed (Kumar et al., 2023).

The chemopreventive effects of PSs extend to numerous types of cancer, but cancers of the digestive tract are the most relevant (Zhu et al., 2023). The low absorption of PSs, which is below 5% in healthy adults (Le Goff et al., 2019), produces a longer time of contact with the tissue, facilitating their effects. In this sense, stigmasterol has shown a chemopreventive effect in gastric cancer cells (SGC-7901 and MGC-803), via apoptosis induction. Stigmasterol inhibits the phosphorylation of mTOR and downregulates this pathway, which produces cell cycle arrest, activates cell apoptosis, and inhibits cell migration and invasion (Zhao et al., 2021). Furthermore, other studies have shown that ethylcoprostanol (β-sitosterol metabolite) also has a chemopreventive effect at colonic concentrations, damaging Caco-2 cells (producing 75% of early apoptosis) without negatively affecting normal cells (only 24% of early apoptosis in CCD-18Co) (Makran et al., 2023a).

Whole grain consumption is associated with less relative risk of mortality by cancer (0.85) and other non-communicable diseases (e.g., cardiovascular diseases, CVD) (Aune et al., 2016). The main reason for this reduced risk seems to be the whole grains' high fiber content. Fiber intake is directly responsible for a lower CRC risk, as observed by the EPIC study (Murphy et al., 2012). Rye bread consumption could also be linked to a lower CRC risk compared with white wheat bread consumption, as observed in a randomized crossover trial. The protective effect can be attributed to a reduced intestinal transit, an increased fecal weight, and less total bile acid concentration in feces, highlighting a lower presence of secondary bile acids, which are potentially negative for the colonic epithelium (Gråsten et al., 2000). In our study, the gastrointestinally digested and colonically fermented wholemeal rye bread (14.7 and 13.7 g fiber/100 g in NWRB and WRB, respectively) produced an antiproliferative effect at colonic level, in line with the observational studies. The first finding was the antiproliferative effect of the products of colonic digestion and fermentation on tumoral cells. In addition, no cytotoxic effect was observed with the samples from both breads on normal colon cells. This observation confirms a selective cytotoxic effect for colonic tumoral cells by FLs. Therefore, the consumption of any of the wholemeal rye breads would be of interest in the primary prevention of the disease.

Two opposite trends were observed in all antiproliferative assays for certain chemopreventive parameters with an increased effect in FL0 vs FLPS, or the contrary. These differences could be attributed to three different factors (PS, SCFA, and TAC).

Firstly, the difference in PS content between the NWRB and the WRB. The enrichment of the bread is directly related to the higher PS content after digestion and colonic fermentation in FLPS. In this sense, in a study with Caco-2 cells, the PS at colonic concentrations (132 μM combined) exerted an antiproliferative effect after 24 h, both individually (β-sitosterol, campesterol and stigmasterol) and in combination. Furthermore, the authors observed that their combination (as occur in our PS ingredient and, consequently in the WRB) had a more powerful effect, producing a decrease in cell viability of 59% compared to the control (López-García et al. 2017). Additionally, it has been observed that the combination of PS and 5-FU enhanced the therapeutic effect that 5-FU performed on Caco-2 and HT-29 colon cancer cells. An increased apoptosis and cell cycle arrest in the S phase were responsible for this effect (Álvarez-Sala et al., 2018b). These findings could support the superior cytotoxic effect elicited by FLPS in MTT and Annexin V/PI assays.

Secondly, SCFA production was higher after feeding the dynamic digester with the NWRB (65 vs 18 mM in WRB), mainly through butyrate production (29 vs 4 mM in WRB). A study using the simgi® system showed a higher total SCFA content in the control sample (gastrointestinal digestion and colonic fermentation blank) compared with food-origin samples (Tamargo et al., 2023). This is in line with what we observed in our study with the WB, which contains a higher concentration of total SCFA (except butyrate) compared with FLPS. The presence of PS would modulate the microbiota (through the fermentation) in such a way that decreases the butyrate-producing bacteria. Also, acetate-producing bacteria may additionally decrease during fermentation due to the presence of PS, and butyrate can be generated from acetate.

The SCFA and, specifically butyrate, has demonstrated a cytoprotective effect at the colonic level (Matthews et al., 2012). Butyrate can prevent the tumor initiation by promoting the metabolization of genotoxic substances such as 4-hydroxy-2-nonenal (Ebert et al., 2001). This is possible because enzymes that participate in the biotransformation of cytotoxic compounds are induced. In addition, butyrate can promote apoptosis and slow the cell cycle progression in tumoral colonic cells (HT-29 and LT-97). Butyrate is also capable of inducing the expression of the *CASP3* gene and consequently producing apoptosis (Putaala et al., 2011). This aligns with the results obtained in our study, where the *CASP3* gene expression of Caco-2 cells was increased with the FL0 treatment, and only a trend was observed in FLPS. The histone hyperacetylation plays a

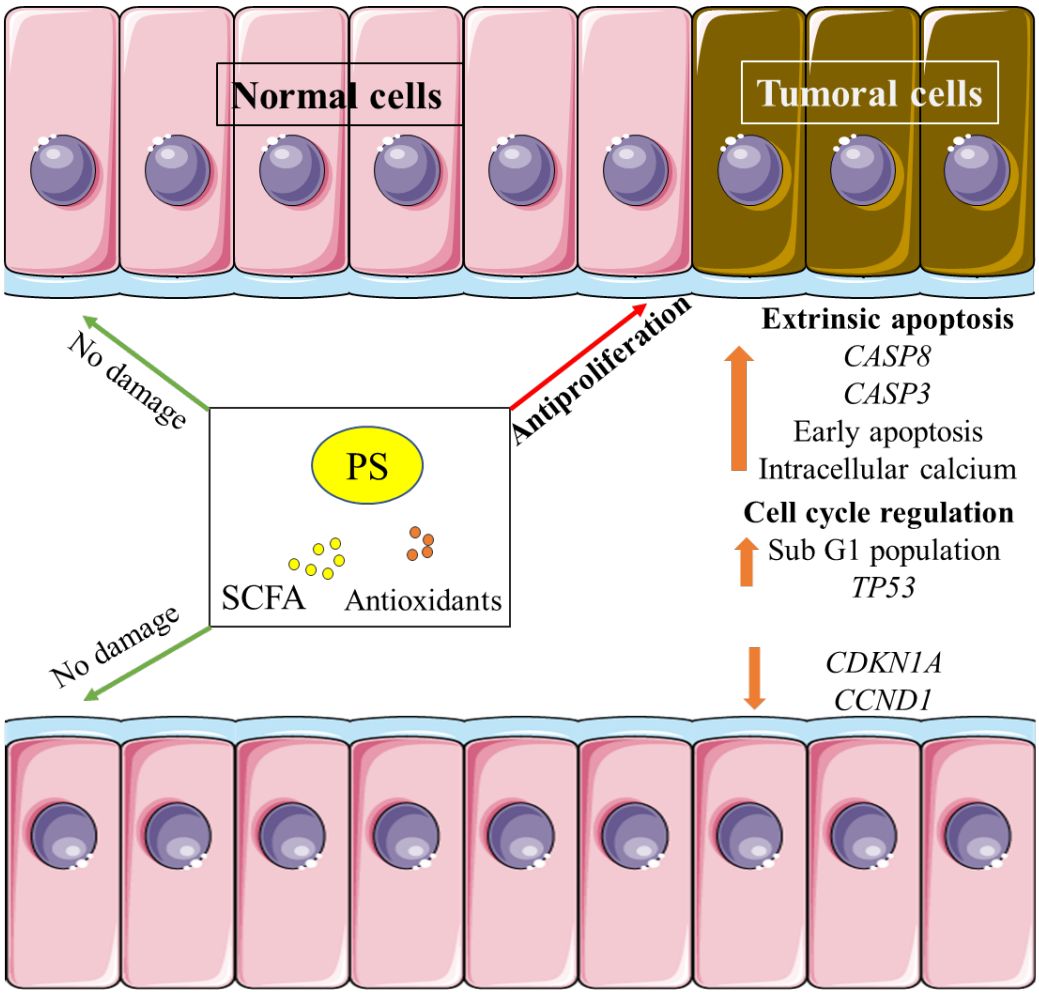
key role in the mechanism of action of butyrate. Through this pathway, it is possible to induce apoptosis in HT-29 colon cancer cells (Hinnebusch et al., 2002). Therefore, butyrate could prevent the appearance of tumor cells and also prevent tumor progression (secondary prevention) (Scharlau et al., 2009). In addition to the effect of butyrate, acetate and propionate have also shown a potential antiproliferative effect on CRC (Xiong et al., 2022). In this sense, the production of these two SCFAs from fiber by probiotic bacteria inhibited cell proliferation and promoted cell death in CRC cells (CRL-2577). Furthermore, these compounds produced cell cycle arrest, showing a clear chemopreventive effect (Casanova et al., 2018). This highlights the relevance of the probiotic activity of colonic bacteria. Their activity is crucial to produce compounds with antiproliferative effect such as SCFA and to prevent CRC.

In vitro studies evaluating the effect of FL obtained from fiber-rich foods like cereals and nuts on tumoral cells (HT-29 and LT-97) have shown the cytotoxic effect of butyrate (Schlörmann et al., 2012, 2020, 2022; Caicedo-López et al., 2021). This fact reinforces the hypothesis that the production of SCFAs by the microbiota from fermentable fiber is a major cause of the chemopreventive effect of this type of food. In our study, SCFA production (and butyrate specifically) was significantly higher in FL0 than in FLPS. For this reason, FL0 may have shown a higher increase in the Sub G1 population in the cell cycle, a higher GSH reduction, and higher intracellular calcium levels.

Lastly, the TAC of the samples may be relevant to the effect they have on the colon. Given the differences observed in the antioxidant capacity assays (TPC and ORAC), it seems likely that it is relevant to the effect at the colonic level. The slightly higher antioxidant capacity of FLPSs could exert a beneficial effect on the colon tissue. It is not clear to what extent antioxidant compounds can prevent CRC, but there is evidence that has linked some antioxidants to a lower risk of CRC. As oxidative stress is a main contributor to a malfunction of the DNA mismatch repair mechanism, antioxidant compounds could reduce this malfunction, reducing the oxidative environment. Nevertheless, the influence of long-term antioxidant intake on CRC remains unclear (Stone et al., 2014). The global effect of the samples in the study is summarized in **Figure 25**.

Even though the FL produced a greater antiproliferative effect compared to the digestion and fermentation blanks, the latter also exerted cell damage compared to the control. This damage could be related to the bile acid content of both blanks. Bile acids,

Figure 25. Effect of digestion and fermentation products from PS-enriched wholemeal rye bread upon normal and tumoral colon cells



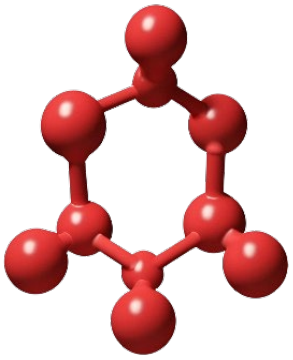
PS: plant sterols; SCFA: short chain fatty acids.

mainly secondary ones, have been shown to be able to inhibit cell proliferation at the colonic level. In fact, physiological concentrations of deoxycholic acid inhibit colonic cell proliferation through cell cycle arrest and apoptosis. The generation of ROS in the tumoral cells has been proposed as one of the possible factors responsible for this effect (Zeng et al., 2019). This could explain the cytotoxic effect that our digestion blanks produced on Caco-2 cells. In fact, Tamargo and colleagues observed this toxic effect of the gastrointestinal digestion and colonic fermentation blanks on HCT-116 and HT-29 colon cancer cells (Tamargo et al., 2023). Since the digestion model used is the same as ours, the blanks may be similar. The damage could be related to the bile acid content of both blanks, which is related with CRC risk, as observed in a crossover trial where rye bread was able to prevent the formation of potentially negative secondary bile acids such

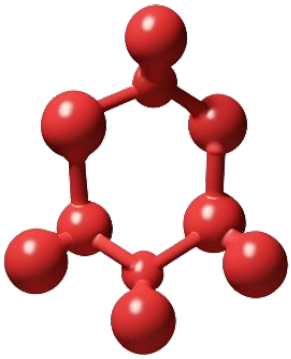
as lithocholic acid, while beneficial ones such as ursodeoxycholic acid were increased (Gråsten et al., 2000). This could explain the higher toxic effect of blanks in some assays compared with FLs.

These results have been shared in one publication:

Miedes, D., Cilla, A., & Alegría, A. (2023). Chemopreventive effect of an *in vitro* digested and fermented plant sterol-enriched wholemeal rye bread in colon cancer cells. *Foods*, *13*, 112.



Chapter III:
Effect of plant sterols and lifestyle on cardiometabolic parameters in statin-treated hypercholesterolemic individuals: A randomized placebo-controlled trial



1. Materials and methods

1.1. Plant sterol-enriched food supplement

A powdered food supplement enriched with 2 g of PS and an analogous placebo supplement without PS were manufactured (NutraResearch 2011 SL, Hospitalet de Llobregat, Spain). For this purpose, free microencapsulated PS powder from tall oil (Lipophytol® P Dispersible, Lipofoods, Barcelona, Spain) was added to elaborate the PS-enriched food supplement. The content of PS in the ingredient was determined according to Faubel et al. (2024). The proportion of each individual PS was, from largest to smallest, β-sitosterol (69.9%), sitostanol (18.3%), campesterol (7.6%), campestanol (2.3%), Δ7-stigmastenol (0.6%), stigmasterol (0.6%), Δ7-avenasterol (0.4%), Δ5,24-stigmastadienol (0.3%), and Δ5-avenasterol (0.1%). As excipients, mannitol, citric acid, magnesium stearate, hydrated silica, sucralose, and lemon flavoring were used for both food supplements (enriched and placebo). The manufacturing conditions were similar for them, having the same appearance, but with different anonymous labeling.

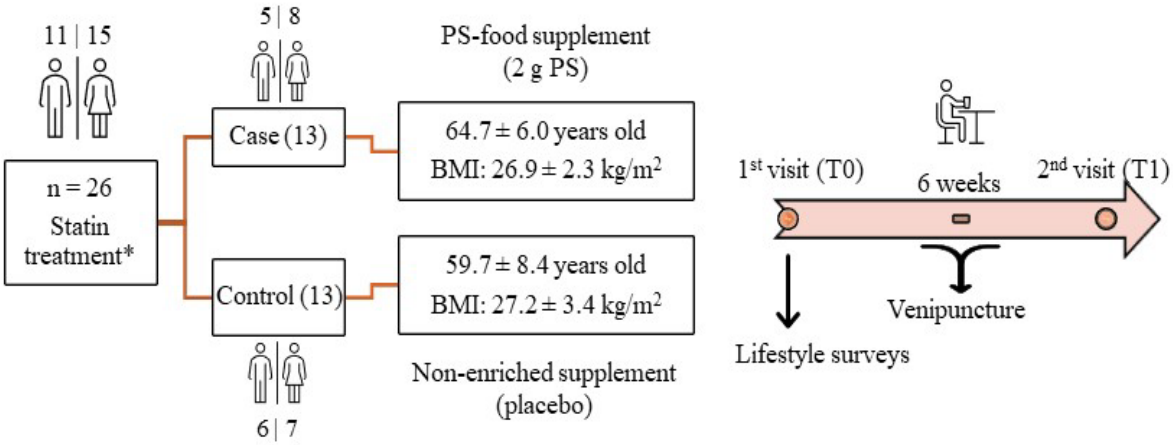
In a test for flavoring selection, 4 volunteers were chosen. They tested 3 different flavors (lemon, orange, and berries) of both enriched and non-enriched supplements, and were told to judge the smell, taste, and solubility. The volunteers selected the lemon flavor as the most pleasant.

1.2. Study design

A parallel double-blind, placebo-controlled clinical trial was designed (see **Figure 26**). Twenty-six subjects were contacted, interviewed, and selected (between September 2023 to May 2024) to participate in the study, and if they complied with the inclusion criteria, were sequentially numbered. All had to be subjects with mild hypercholesterolemia diagnosed according to the guidelines of the European Society of Cardiology/European Atherosclerosis Society (Mach et al., 2020) (LDL-c ≥ 160 mg/dL at the time of diagnosis) under treatment with statins of low-to-moderate intensity (atorvastatin: 10–20 mg; simvastatin: 20–40 mg; rosuvastatin: 5–10 mg; pitavastatin: 1–4 mg) prior to the selection process and an age range of 18–70 years. The exclusion criteria were smokers; previous episodes of CVD; diabetes mellitus; subjects in secondary prevention; liver or renal disease; uncontrolled hypothyroidism; consumption of PS-enriched foods or supplements; other analytical abnormalities or previous illnesses; or treatment with lipid-lowering drugs other than the aforementioned ones. The participants (n = 26) were

randomly divided into 2 groups (Random Allocation Software 2.0). Case subjects took a daily food supplement enriched with 2 g of PS; while control subjects took a daily food supplement without enrichment. Both groups of subjects were told to take it before the main meal for 6 weeks.

Figure 26. Flow chart of the study characteristics and the 6-week intervention



BMI: Body mass index; PS: Plant sterols. * atorvastatin: 10–20 mg, simvastatin: 20–40 mg, rosuvastatin: 5–10 mg, or pitavastatin: 1–4 mg.

The adherence to the treatment (number of non-ingested sticks and changes in the suggested schedule of intake), sensorial (ease of taking and acceptance), and adverse effects (bloating, fullness, indigestion, altered bowel habit, and/or any other indicated by the participants) were evaluated with a questionnaire conducted at the end of the study.

The study design and protocol were made according to the Declaration of Helsinki and were approved by the Ethics Committee of the Hospital Clínico Universitario de Valencia (approval number 2023/069) and registered in the Clinical Trials database (ClinicalTrials.gov ID: NCT05901246). All the participants received and signed informed consent.

The informed consent is included in **Annex II: Supplementary materials**.

1.3. Blood samples collection

Each participant was subjected to 2 blood extractions, one before starting the treatment (T0) and one after the 6-week intervention (T1). The venipuncture was performed after overnight (10–12 h) fasting in the Endocrinology and Nutrition department of the

Hospital Clínico Universitario de Valencia. A single tube was taken for biochemical determination and 3 K_2EDTA tubes were taken for eryptosis, adhesion to the endothelium and cholesterol oxidation products (COPs) assays. For this purpose, immediately after blood extraction, erythrocytes were isolated from the plasma (the buffy coat was discarded) by centrifugation (180 *g*/10 min/4 °C). The supernatant (plasma) was transferred to cryogenic vials and immediately stored at -80ºC until the analysis. On the other hand, the pellet (erythrocytes) was washed 3 times with PBS to fully isolate the red blood cells (RBCs). A solution of 0.4% *v*/*v* hematocrit in Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM HEPES, 5 mM glucose, and 1 mM $CaCl_2$) (0.4% RBC) was prepared for the subsequent assays, as described by Cilla et al. (2021).

1.4. Lifestyle surveys

A qualitative differentiation regarding diet and exercise was done by using validated surveys, both only before the beginning of the intervention. The adherence to the Mediterranean Diet was assessed for each participant using the Mediterranean Diet Adherence Screener (MEDAS) questionnaire (Schröder et al., 2011). It contains 14 questions, and each participant obtains a numeric value (low adherence < 7; moderate adherence > 7 and < 10; high adherence > 10). The level of physical activity was evaluated using the International Physical Activity Questionnaire-short form (IPAQ-short) (Craig et al., 2003). It contains 7 questions, divides activity into different intensities and classifies patients depending on the metabolic equivalent (MET), between low, moderate and high activity.

The surveys used are included in **Annex II: Supplementary materials**.

1.5. Determination of biochemical parameters

One blood sample aliquot was used for the determination of the biochemical cardiometabolic parameters. Glucose, TC, triglycerides, HDL-c, Apo A1, Apo B, high sensitive C reactive protein (hs CRP), and insulin were measured using standardized and validated methods as previously described (Martinez-Hervas et al., 2008). The LDL-c was calculated by Friedewald's formula. The homeostasis model assessment (HOMA) index was calculated using the following formula: (Glucose × 0.0555 × insulin)/22.5.

1.6. Evaluation of the externalization of phosphatidylserine (EPHS) and forward scatter (FSC)

In order to determine the degree of evolution of the eryptotic event, the EPHS to the outer layer of RBCs was measured (Tesoriere et al., 2014). For this purpose, 35 µL of 0.4% RBC were mixed with 100 µL of PBS, centrifuged (145 *g*/5 min/25 °C) to wash the sample, and once the supernatant was discarded, the pellet was suspended in 100 µL binding buffer prior to annexin V-FITC addition (5 µL). The samples were incubated in darkness for 15 min. Then, 400 µL of binding buffer was added and fluorescence was measured by flow cytometry (λ_{exc} = 488 nm and λ_{em} = 527/532 nm; FACS Verse, BD Biosciences). The cell volume was measured using the geometric mean of the FSC. At least 1×10^4 events were analyzed for each sample.

1.7. Intracellular reduced glutathione

The content of GSH in the erythrocytes was determined by flow cytometry using CMFDA, a specific dye that binds nonprotein cytosolic thiol (Cilla et al., 2021). Briefly, 55 µL of 0.4% RBC were mixed with 445 µL of PBS and incubated (40 min, 37 °C, 5% CO_2, and 95% RH). After centrifugation (145 g/5 min/25 °C), the pellet was suspended in 500 µL of PBS and fluorescence was measured by flow cytometry (λ_{exc} = 488 nm and λ_{em} = 527/532 nm). At least 1×10^4 events were analyzed for each sample.

1.8. Assessment of the adhesion to the endothelium

Human umbilical vein endothelial cells (HUVECs) were freshly isolated from umbilical cords provided by healthy female donors from the Hospital Clínico Universitario de Valencia, under informed consent approved by the hospital's ethics committee (approval number 2021/038), through collagenase type I treatment. Briefly, the umbilical cord veins were rinsed of blood products with warm PBS, after which the vein was filled with collagenase (1 mg/mL) for 17 min at 37 °C. The cords were then gently massaged to ensure the detachment of endothelial cells from the vessel wall. The digest was collected, centrifuged, and pelleted once more. The pellet was suspended in endothelial cell growth medium (EGM-2) in T25 culture flasks, where the cells were cultured until confluence.

The erythrocyte–endothelium cell adhesion assay was performed by using the parallel-plate flow chamber technique (Cilla et al., 2021; Kaliyaperumal et al., 2019). Passage 1

from these primary cultures was subsequently employed. After reaching confluence, the primary cultures were detached with trypsin and transferred to fibronectin (5 μg/mL)-coated 25 mm plastic coverslips on a 6-well plate until confluence (approximately 48 h). RBCs (5×10^6 cells/mL) were re-suspended in Dulbecco's PBS containing 20×10^{-3} M HEPES and 0.1% human serum albumin. To perform the adhesion assays, the coverslip containing confluent HUVEC monolayer was placed in the flow chamber. RBC suspensions were drawn across the monolayer at a flow rate of 0.37 mL/min (approximately shear stress of 0.7 dyne/cm^2) for a period of 3 min, after which the flow was stopped for 1 min and re-started for another 2 min (total time recorded: 6 min). The flow chamber was on a microscope (Nikon Eclipse TE 2000-S, Nikon, Amstleveen, the Netherlands) connected to a video camera (Sony Exware HAD, Koeln, Germany) where images were recorded. Adhesion was measured by counting the number of cells that maintained stable contact with the HUVEC monolayer for 30 s.

1.9. GC-MS determination of cholesterol and cholesterol oxidation products (COPs)

1.5 mL of plasma were subjected to cold saponification (Menéndez-Carreño et al., 2008). Briefly, the IS for cholesterol (betulinol, 143 μg) and COPs (19-hydroxycholesterol (19-HC), 12.7 μg) were added to a round bottom tube. 10 mL of 2 N KOH in methanol were added to each tube and samples were maintained in agitation for 20 h in a shaker at room temperature (for preventing by-products or artifacts formation). The unsaponifiable fraction was extracted with diethyl ether and then suspended with hexane and isopropanol (4:1, v/v). The whole extract was divided into 2 tubes, 10% for cholesterol determination and 90% for COPs determination. The latter was subjected to purification by solid phase extraction (SPE-NH2 with 500 mg solid phase, Phenomenex, Torrance, CA, USA) (Rose-Sallin et al., 1995). The cartridges were activated with 3 mL of hexane and the sample was added after suspension in hexane and ethyl acetate (95:5, v/v). The cartridges were washed with hexane and ethyl acetate (95:5, v/v) and then with the same solvents at a different ratio (9:1, v/v). COPs were eluted with acetone, dried in a rotary evaporator, suspended with 1 mL hexane and isopropanol (4:1, v/v) and stored at -20ºC. Prior to chromatographic analysis, the extracts were derivatized with 600 μL pyridine:HMDS:TMCS (5:2:1, v/v/v) for 30 min at 40ºC. The trimethylsilylether derivatives were dissolved in 50 μL hexane and 1 μL was injected into a GC Shimadzu QP 2010 Plus (split-splitless injector) coupled to a mass spectrometric detector. Fast GC/MS analysis was carried out using a fused-silica capillary column Restek RTX-5 (10

m x 0.1 mm x 0.1 μm film thickness) and helium as a carrier gas (linear velocity 43 cm/s). Chromatographic conditions used were described by Cardenia et al. (2012). The acquisition (mass spectra) was done in full scan mode (total ion current, TIC), whilst the integration was done with single ion monitoring (SIM), using the higher-abundance characteristic ions. The mass-to-charge ratio for the characteristic ions of each COP were: 19-HC (353 m/z), α-epoxy-cholesterol (α-EC) and β-epoxy-cholesterol (β-EC) (384 m/z), cholestanetriol (triol) (403 m/z), 7α-hydroxy-cholesterol (7α-HC), 7β-hydroxy-cholesterol (7β-HC) and 27-hydroxy-cholesterol (27-HC) (456 m/z), 7-ketocholesterol (7-KC) (472 m/z), 24-hydroxy-cholesterol (24-HC) (145 m/z), and 25-hydroxy-cholesterol (25-HC) (131 m/z). Quantification was performed using calibration curves for each compound comparing them with the IS (19-HC). Regarding cholesterol determination, the fraction (1/9) was directly derivatized under the same conditions and injected into the GC-MS with the same equipment and conditions. Quantification was carried out using a calibration curve for cholesterol compared to its IS (betulinol).

1.10. Statistical analysis

Data are presented as mean ± standard deviation for each group. Shapiro–Wilk, D'Agostino, and Pearson normality tests were performed, and the possible presence of outliers was verified. Unpaired Student's *t* test or Wilcoxon signed-rank test were used to determine statistically significant differences between the treatments ($p < 0.05$). Correlations between different variables were evaluated using the Pearson correlation coefficient, the Chi-square test, and Fisher's exact test. GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, CA, USA) was used for all the analysis.

2. Results

2.1. Lifestyle surveys

Table 17 shows the results of the MEDAS questionnaire biased by sex, counting the number of positive answers for each question and the number of participants classified for each adherence category. No participant had low adherence to the Mediterranean Diet, which allows us to affirm that the studied population had generally good dietary habits. No statistically significant differences ($p > 0.05$) between men and women were observed in the answers to each question and in the proportions of high and moderate adherence. Nevertheless, a slight trend to consume more fruits, vegetables and olive oil was noted among women participants. Likewise, **Table 18** shows the results of the IPAQ-short

questionnaire, dividing all participants into the 3 categories and biased by sex. Most of the subjects were classified as low physical activity (54%), while only 3 of them (11.5%) were considered as high physical activity. No statistically significant differences (p>0.05) were observed dividing by sex in the proportion of each activity level.

Table 17. MEDAS questionnaire positive answers for each item expressed by sex. Below, levels of adherence to the Mediterranean Diet following the questionnaire criteria. All subjects were under statin treatment for mild hypercholesterolemia

	Total (n=26)	**Men (n=11)**	**Women (n=15)**	
Olive oil consumption	26 (100)	11 (100)	15 (100)	$p > 0.99$
Olive oil (≥4 /day)	23 (88.5)	8 (72.7)	15 (100)	$p = 0.06$
Vegetables (≥2 / day)	12 (46.2)	4 (36.4)	8 (53.3)	$p = 0.45$
Fruits (≥3 / day)	14 (53.8)	5 (45.5)	9 (60.0)	$p = 0.69$
Red/processed meat (<1 / day)	26 (100)	11 (100)	15 (100)	$p > 0.99$
Butter/cream (<1 / day)	26 (100)	11 (100)	15 (100)	$p > 0.99$
Sweet drinks (<1 / day)	26 (100)	11 (100)	15 (100)	$p > 0.99$
Wine (≥3 / week)	2 (7.7)	1 (9.1)	1 (6.7)	$p > 0.99$
Legumes (≥3 / week)	10 (38.5)	5 (45.5)	5 (33.3)	$p = 0.69$
Seafood (≥3 / week)	14 (53.8)	8 (72.7)	6 (40.0)	$p = 0.13$
Desserts (<3 / week)	21 (80.8)	9 (81.8)	12 (80.0)	$p > 0.99$
Nuts (≥1 / week)	23 (88.5)	10 (90.9)	13 (86.7)	$p > 0.99$
White > red meat	26 (100)	11 (100)	15 (100)	$p > 0.99$
Sofrito (≥2 / week)	17 (65.4)	7 (63.6)	10 (66.7)	$p > 0.99$
Adherence to the Mediterranean Diet*				
Moderate	19 (73.1)	7 (63.6)	12 (80.0)	$p = 0.41$
High	7 (26.9)	4 (36.4)	3 (20.0)	

Data are expressed as number of subjects and percentage (in brackets). *No patient had low adherence to the Mediterranean Diet.

Table 18. Levels of physical activity and distribution among participants following IPAQ-short criteria. All subjects were under statin treatment for mild hypercholesterolemia

	Low	**Moderate**	**High**
Total (n=26)	14 (53.8)	9 (34.6)	3 (11.5)
Men (n=11)	6 (54.5)	3 (27.3)	2 (18.2)
Women (n=15)	8 (53.3)	6 (40.0)	1 (6.7)

Data are expressed as number of subjects and percentage (in brackets).

2.2. Biochemical parameters

The results of the biochemical analysis before and after the treatment for both groups are shown in **Table 19**. In the control group, a statistically significant decrease ($p < 0.05$) was observed in the average contents of glucose (4.6%) and LDL-c (9.2%) after the intervention. In the case group, a significant increase ($p < 0.05$) in Apo A1 (6.4%) was observed.

Table 19. Biochemical parameters before (T0) and after (T1) the treatment with placebo (control) or plant sterol-supplement (case)

	Control T0 (n = 13)	**Control T1 (n = 13)**	**Case T0 (n = 13)**	**Case T1 (n = 13)**
Glucose (mg/dL)	97.9 ± 10.0	93.7 ± 12.6 *	95.5 ± 9.8	93.9 ± 8.4
Insulin (µU/mL)	9.0 ± 4.5	8.0 ± 3.1	10.0 ± 5.3	9.8 ± 5.4
HOMA index	2.3 ± 1.3	1.9 ± 1.1	2.4 ± 1.4	2.3 ± 1.4
TC (mg/dL)	173.1 ± 25.9	165.2 ± 27.9	172.2 ± 28.2	168.1 ± 34.8
HDL-c (mg/dL)	53.8 ± 13.1	55.0 ± 13.2	55.5 ± 8.8	56.2 ± 10.4
LDL-c (mg/dL)	101.3 ± 22.7	92.0 ± 24.6 *	98.6 ± 25.7	94.9 ± 28.7
TG (mg/dL)	89.9 ± 24.9	91.6 ± 31.9	90.8 ± 32.9	84.6 ± 25.9
Apo A1 (mg/dL)	172.1 ± 30.1	177.1 ± 31.8	163.7 ± 19.4	174.1 ± 21.2 *
Apo B (mg/dL)	86.9 ± 15.2	84.2 ± 17.2	78.6 ± 18.6	83.6 ± 18.8
hsCRP	2.7 ± 2.2	2.1 ± 1.1	1.5 ± 1.0	1.8 ± 0.8

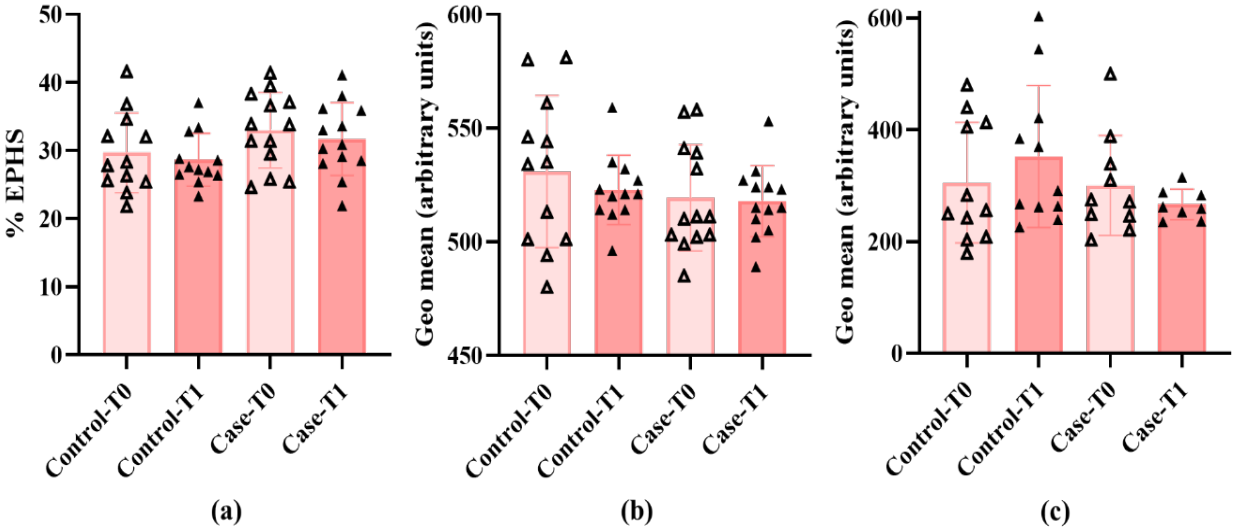
Data are expressed as mean ± standard deviation. * Indicates statistically significant differences ($p < 0.05$) compared to the basal levels from the same group. Apo: apolipoprotein; hsCRP: high sensitive C reactive protein; HDL-c: HDL cholesterol; HOMA: homeostasis model assessment; LDL-c: LDL cholesterol; TC: total cholesterol; TG: triglycerides.

2.3. Eryptosis and redox status

Data on the EPHS, FSC and intracellular GSH levels are shown in **Figure 27**. Regarding EPHS, the results show that no differences between the groups were observed. Neither the placebo (control) nor PS supplement (case) groups varied statistically ($p > 0.05$) after the 6-week treatment (control: T0, 29.7 ± 5.9 and T1, 28.7 ± 3.9% EPHS; case: T0, 32.9 ± 5.6 and T1, 31.7 ± 5.3% EPHS) (**Figure 27a**). The same output was observed for FSC since both treatments produced no differences in cell size after the 6-week period ($p > 0.05$) (control: T0, 531 ± 33 and T1, 523 ± 15; case: T0, 519 ± 23 and T1, 518 ± 16 geometric mean) (**Figure 27b**).

Regarding the redox status of the erythrocytes, after withdrawing the outliers detected in the GSH measurement, no difference was observed in both groups after the treatment period ($p > 0.05$) (control: T0, 305 ± 108 and T1, 352 ± 127; case: T0, 300 ± 89 and T1, 266 ± 27 geometric mean) (**Figure 27c**).

Figure 27. Eryptosis and redox status before (T0) and after (T1) the treatment with placebo (control) or plant sterol-supplement (case) (**a**) Percentage of cells expressing phosphatidylserine externalization (EPHS); (**b**) Relative size of cells (forward scatter, FSC); (**c**) Levels of reduced glutathione (GSH)



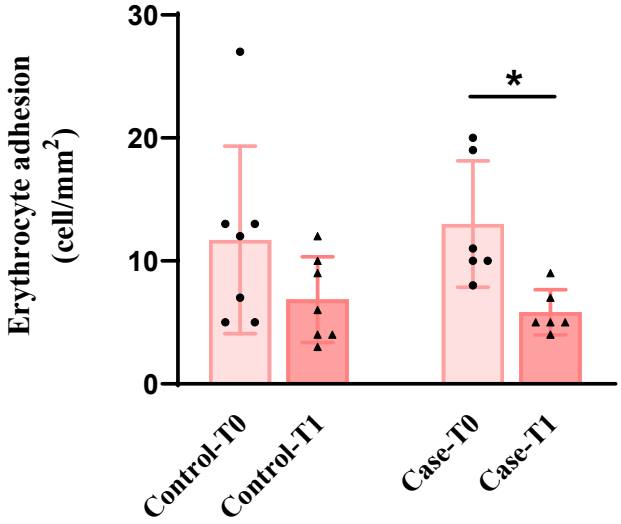
Results are expressed as mean ± standard deviation (n = 3). No statistically significant differences were observed ($p > 0.05$).

Considering these results, we decided to stratify them into homogenous groups based on sex, age (cut-off point > 60 years), body mass index (cut-off point > 27 kg/m^2), and adherence to the treatment (maximum five unused portions). None of them showed statistically significant differences between groups ($p > 0.05$), confirming that the distribution of data was homogenous.

2.4. Adhesion to the endothelium

Subjects assigned either to the control or to the case group presented an adhesion of their erythrocytes to the vascular endothelium of 11.7 ± 7.6 cells/mm^2 and 13.0 ± 5.1 cells/mm^2, respectively, at T0. After the 6-week treatment, there was a significant ($p < 0.05$) reduction in this parameter in those patients that received the PS food supplement, but not in the ones to whom placebo was administered (**Figure 28**).

Figure 28. Adhesion of erythrocytes of hypercholesterolemic patients to the vascular endothelium before (T0) and after (T1) the treatment with placebo (control) or plant sterol-supplement (case)



Results are expressed as mean ± standard deviation. * Indicates statistically significant differences between the basal and final measurement ($p < 0.05$).

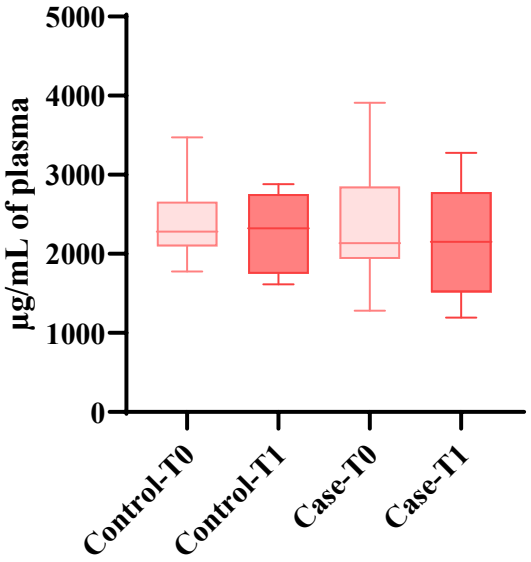
2.5. Cholesterol oxidation products

The cholesterol profile determined by Fast GC/MS was analyzed. Baseline cholesterol levels (T0) were similar in both groups (2409 ± 500 in control and 2425 ± 799 µg/mL in case subjects) (**Figure 29**). After the treatment (T1), there were no statistically significant differences ($p > 0.05$), even though both showed a slight non-significant trend to decrease (2276 ± 480 in control and 2169 ± 735 µg/mL in case subjects, representing a 5.5% and 10.6% decrease, respectively).

Up to 6 different COPs were detected with the GC-MS analysis in the plasma samples. The levels of COPs are summarized in **Figure 30**. Total COPs T0 levels in plasma from control subjects were 1.41 ± 0.35 µg/mL, whereas levels after the 6-week period (T1) were 1.12 ± 0.26 µg/mL, showing no statistically significant differences ($p > 0.05$). Regarding the case group, T0 levels were 1.42 ± 0.33 µg/mL and T1 levels were 1.47 ± 0.48 µg/mL, again, with no statistically significant differences ($p > 0.05$). The oxidation ratio (OR) was calculated for each subject (total COPs level/cholesterol level × 100). There was no statistically significant difference ($p > 0.05$) between control T0 and T1 subjects for OR (0.060 ± 0.020% and 0.050 ± 0.016%, respectively, non-significant

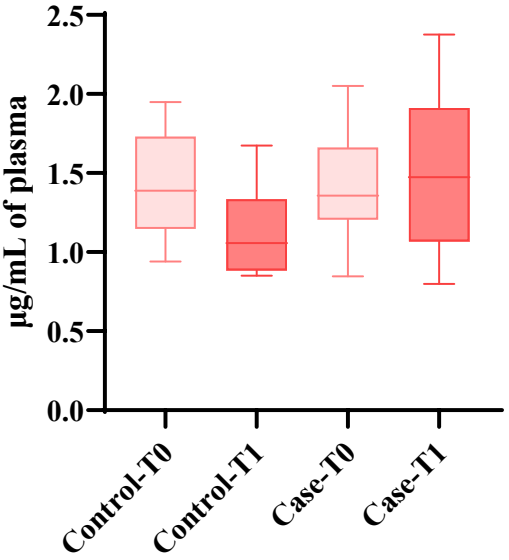
decrease of 16.7%), and between case T0 and T1 subjects (0.066 ± 0.030 and 0.078 ± 0.042%, respectively, non-significant increase of 18.2%).

Figure 29. Plasma cholesterol levels of statin-treated hypercholesterolemic subjects before (T0) and after (T1) the 6-week treatment with placebo (control) or plant sterol-supplement (2g/day) (case)



Results are expressed as quartiles. No statistically significant differences were observed ($p > 0.05$)

Figure 30. Plasma cholesterol oxidation products levels of statin-treated hypercholesterolemic subjects before (T0) and after (T1) the 6-week treatment with placebo (control) or plant sterol-supplement (2g/day) (case)



Results are expressed as quartiles. No statistically significant differences were observed ($p > 0.05$)

Regarding individual COPs levels (see **Table 20**), β-EC and 7-KC were the most abundant. All groups showed a similar amount of these two COPs (0.39-0.48 μg/mL for β-EC, 0.42-0.43 μg/mL for 7-KC) apart from control T1, which was notably lower (0.29 and 0.26 μg/mL for β-EC and 7-KC, respectively). This is in accordance with the lower trend of total COPs level observed for this specific group. On the other hand, the least abundant COPs were 7α-HC and triol, with a similar magnitude (0.11-0.13, and 0.11-0.14 μg/mL μg/mL, respectively) in the groups. 7β-HC (0.14-0.22 μg/mL), and α-EC (0.21-0.26 μg/mL) showed an intermediate magnitude.

Table 20. Individual cholesterol oxidation products levels (μg/mL) of statin-treated hypercholesterolemic subjects before (T0) and after (T1) the 6-week treatment with placebo (control) or plant sterol-supplement (2 g/day) (case)

	Control			**Case**		
	T0	**T1**	**Change (%)**	**T0**	**T1**	**Change (%)**
7α-HC	0.13 ± 0.04 (0.08 - 0.23)	0.11 ± 0.03 (0.09 - 0.16)	-15.4	0.12 ± 0.02 (0.09 - 0.17)	0.11 ± 0.02 (0.08 - 0.16)	-8.3
7β-HC	0.21 ± 0.11 (0.09 - 0.25)	0.14 ± 0.04 (0.09 - 0.21)	-33.3	0.18 ± 0.05 (0.10 - 0.24)	0.22 ± 0.10 (0.12 - 0.48)	22.2
β-EC	0.48 ± 0.28 (0.17 - 0.87)	0.29 ± 0.20 (0.09 - 0.84)	-39.6	0.41 ± 0.14 (0.21 - 0.76)	0.39 ± 0.16 (0.18 - 0.70)	-4.9
α-EC	0.26 ± 0.17 (0.07 - 0.38)	0.24 ± 0.11 (0.08 - 0.45)	-7.7	0.22 ± 0.04 (0.16 - 0.28)	0.21 ± 0.08 (0.08 - 0.37)	-4.5
Triol	0.14 ± 0.06 (0.00 - 0.19)	0.12 ± 0.03 (0.09 - 0.20)	-14.3	0.14 ± 0.06 (0.00 - 0.24)	0.11 ± 0.07 (0.00 - 0.23)	-21.4
7-KC	0.42 ± 0.28 (0.20 - 0.43)	0.26 ± 0.04 (0.20 - 0.32)	-38.1	0.42 ± 0.28 (0.08 - 1.21)	0.43 ± 0.21 (0.19 - 0.92)	2.4

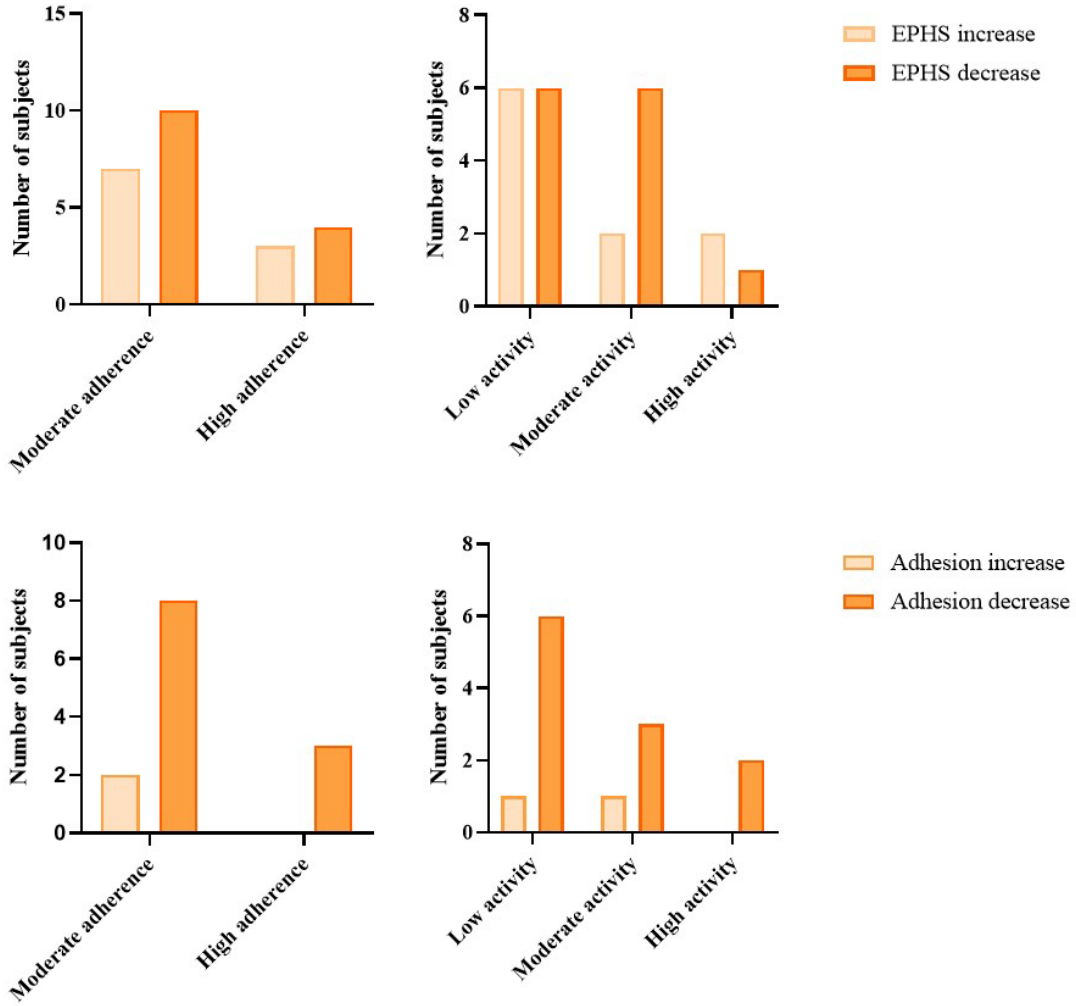
Results are expressed as mean ± standard deviation (minimum - maximum). Change (%) = (Final minus basal content) x 100/basal. 7α-hydroxy-cholesterol (7α-HC), 7β-hydroxy-cholesterol (7β-HC), β-epoxy-cholesterol (β-EC), α-epoxy-cholesterol (α-EC), cholestanetriol (triol), and 7-ketocholesterol (7-KC).

2.6. Correlations between variables

The possible correlation between a qualitative variable and the main quantitative outputs (EPHS and RBC adhesion to the endothelium) was analyzed. Neither the adherence to the Mediterranean Diet nor the level of physical activity were able to predict

the output of both parameters, showing no correlation ($p > 0.05$) (**Figure 31**). Nevertheless, the adhesion to the vascular endothelium and Apo A1 levels were considered for searching for any correlation, the variables being potentially linked with cardiovascular health. No correlation was observed between both variables at T0 (r = 0.156, $p = 0.739$ in the control subjects, and r = 0.230, $p = 0.660$ in the case subjects). At T1, however, the trend observed was the opposite in the control (r = 0.482, $p = 0.518$) vs. case subjects (r = −0.799, $p = 0.410$), showing a slight inverse correlation between adhesion to the endothelium levels and Apo A1 ones only after the treatment with the PS supplement. None of the lifestyle variables were associated with the quantitative value of cholesterol or COPs levels ($p > 0.05$).

Figure 31. (**a**) Correlation between adherence to the Mediterranean Diet and the change in EPHS; (**b**) correlation between the level of physical activity and the change in EPHS. (**c**) Correlation between adherence to the Mediterranean Diet and the change in adhesion to the endothelium; (**d**) correlation between the level of physical activity and the change in adhesion to the endothelium



EPHS: externalization of phosphatidylserine.

3. Discussion

3.1. Eryptosis and cardiometabolic parameters

No reduction in TC or LDL-c was observed in the PS-food supplement group. The subjects included in this group were under statin treatment and consequently had relatively low baseline TC (172.2 mg/dL) and LDL-c (98.6 mg/dL) concentrations. Several randomized clinical trials (RCTs) have been carried out in hypercholesterolemic patients receiving statin therapy (atorvastatin, simvastatin, rosuvastatin, lovastatin, pravastatin, orpravastatin, or cerivastatin, in variable doses) and PS. The subjects received mostly 2 to 3 g per day of PS for at least 4 weeks to simulate a chronic intake. The only one combining statin treatment (40 mg of atorvastatin), and a PS food supplement demonstrated that the PS addition (2 g/day) was able to reduce 7.7% of TC and 6.5% of LDL-c compared to the statin alone (Malina et al., 2015). This is a relatively low change when compared to the reduction stated in the regulations of the European Union for PS-enriched foods (up to 12.5% with 2.5–3 g/day of PS) (Regulation 686/2014/EU). Subjects that respond better to the statin treatment might benefit less from the activity of PS, since both have completely different mechanisms of action (statins only reduce the synthesis whereas PS only reduces the absorption). However, it must be pointed out that the study conducted by Malina and colleagues recruited 86 participants, enough to observe those slight differences (Malina et al., 2015) in comparison with our study which presented a relatively small number of participants as a main limitation. Additionally, that RCT included subjects that were not under pharmacologic treatment at the beginning of the study, gave them statins and after that, the PS-food supplement.

Furthermore, previous RCTs from our group using similar PS ingredients also saw slight decreases in TC and LDL-c. In that case, the treatment was a PS-enriched milk-based beverage (2 g/day), and the duration of the treatment (6 weeks) was the same. The participants were not under pharmacologic treatment, resulting in notably higher baseline TC and LDL-c levels. Even so, the reduction in TC was only of 2.9 and 4.7 %, and the reduction in LDL-c of 5.1 and 9.0% (Alvarez-Sala et al., 2018c; Blanco-Morales et al., 2022). Basal levels affect the potential reduction produced by the PS. In this sense, higher baseline levels are significantly related to a higher decrease in TC and LDL-c (Demonty et al., 2009; Naumann et al., 2008). Compared with these previous studies, the baseline levels of TC and LDL-c were ~ 25% and ~ 30% lower in our study.

Notwithstanding the previous observations, after treatment with PS-enriched supplement (case group), a significant increase ($p < 0.05$) in apolipoprotein ApoA-1 occurs. This protein is the main component of HDL lipoproteins, a known CVD protective indicator. The HDL-c concentration itself may not correctly indicate the cholesterol efflux (the main function of these particles), but ApoA-1 does (Kalayci et al., 2022). Indeed, ApoA-1 itself removes cholesterol from the atheroma plaque through lecithin-cholesterol acyltransferase activation. In addition, this protein can improve the atherosclerotic environment even more due to its anti-inflammatory effect. In keeping with this, ApoA-1 may increase nitric oxide synthesis, producing vasorelaxation (Primer et al., 2020). Furthermore, this protein is capable of inhibiting the expression of CD11b, a protein from leukocytes that triggers inflammatory response, and is directly related to the adhesion of these cells to the vascular endothelium (Murphy et al., 2011). This could be interesting as an important finding in our study is the fact that a reduction in the adhesion to the endothelium of RBC in the case group was observed. Thus, it is easy to hypothesize that the increase in Apo-1 could be inhibiting not only the adhesion molecules of leukocytes—such as CD11b—but also of those of RBC or endothelium. The inverse correlation observed ($r = -0.799$) after the treatment with the PS supplement between ApoA-1 levels and RBC adhered to the endothelium suggests this mechanism of action. Although PSs do not directly affect the eryptotic status of the RBC (no changes in EPHS), they could exert an effect on the CXCL16/SR-PSOX or other endothelium receptors, explaining the reduced adhesion to the endothelium.

EPHS may increase with the intake of cholesterol-lowering drugs like statins. Both groups of treatment were compounded by statin-treated subjects, so this effect cannot be observed. But, as Cilla and colleagues found in their study, hypercholesterolemic subjects have higher EPHS levels than normocholesterolemic ones. In addition, basal endothelium cell adhesion levels for hypercholesterolemic patients were 11.7 ± 1.8 cells/mm^2 (in line with our present results and significantly higher than that obtained in healthy donors, 5.5 ± 0.8 cells/mm^2) (Cilla et al., 2021). The hypothesis of the present work was that if statins are unable to reduce the EPHS despite their cholesterol-lowering activity, PS may reduce both. Again, as no significant reduction in TC was observed (only a trend), it is plausible that no effect was observed regarding any of the eryptosis parameters determined. Nevertheless, our previous *ex vivo* study evaluating the PS effect on eryptosis-related parameters showed positive effects (Alvarez-Sala et al., 2018a), but that study was

conducted with blood from eight healthy volunteers. That *ex vivo* evaluation could not perfectly mimic the biological situation, since it did not consider the digestive process, interactions with the food matrix (bioaccessibility), and interindividual absorptive capacity (bioavailability). RBCs were stressed after blood extraction and then treated with PS, whereas in the present study, the oxidative stress was physiological. In addition, the PS concentration used by the authors was compatible with the chronic consumption of a PS-enriched beverage, not a food supplement, which could vary the serum concentration and, therefore, the biological activity.

No changes were observed after stratifying by sex, age, or body mass index (BMI) in terms of the eryptotic parameters. On the other hand, regarding potential correlations between variables, the no-change situation in EPHS remained whether the subjects were classified regarding physical activity or adherence to the Mediterranean Diet. In addition, no correlation was found between diet or physical activity and the outcome of adhesion to the endothelium.

It is worth noting that the small sample size (13 participants per group) could be responsible for the difficulty of detecting statistically significant changes. A higher sample would be necessary to confirm that the PS food supplement possesses a synergic hypocholesterolemic effect with low-to-moderate intensity statins and an effect on eryptosis. Nevertheless, in the present study, the broad and rigorous exclusion criteria, as well as the inclusion criteria, made it extremely difficult to enroll volunteers for the RCT.

Taking this into account, additional studies with a larger and more diverse population would be necessary to explore this topic in greater depth. These studies could provide a better understanding of the effects of the joint use of PSs and statins on cardiovascular health parameters. In this sense, using enriched foods could be a good option, since it is easier to include them in the regular diet and some of them have good nutritional profiles.

3.2. Cholesterol oxidation products

The intake of the PS-food supplement for 6 weeks did not decrease cholesterol nor COPs levels compared to the intake of the placebo supplement. The small sample, the high interindividual variability and the fact that subjects were under pharmacologic treatment could explain these results.

No reduction in plasma cholesterol (assessed by GC-MS) was observed in the group receiving the PS supplement, which consisted of individuals already undergoing statin therapy. Statin users that respond well to their treatment may experience less benefit from PS, as its effects are primarily related to inhibiting intestinal cholesterol absorption (Gylling et al., 2014).

As previously stated, other RCTs from our group observed slight decreases in plasma cholesterol (2.9-4.7%) (Alvarez-Sala et al., 2018c; Blanco-Morales et al., 2022). Although the conditions (regarding PS) were like the present study, some differences must be pointed out. These studies used a PS-enriched milk-based beverage with the same dose as the present study (2 g PS/daily), and the treatment duration was the same as our study. In addition, participants were not pharmacologically treated, so the potential cholesterol reduction could have been greater. Taking this into account, the results obtained in the present study could be plausible with this ingredient and conditions. On the other hand, the cholesterol value in this study was not correlated to greater or lesser physical activity or adherence to the Mediterranean diet, so differences in lifestyle cannot explain the results obtained. Even so, it is worth noting that, compared to the mean of the adult Spanish population (based on the ENSE survey), the participants of this study showed lower physical activity levels (i.e. 54% of subjects with low activity in our study vs. only 35% in general population) (Ministerio de Sanidad, Consumo y Bienestar Social, 2017).

COPs were not reduced after the treatment with the PS-food supplement, likely because plasma cholesterol levels were maintained rather than decreased. Being molecules that derived from the oxidation of cholesterol, their formation is closely tied to the availability of cholesterol in the bloodstream. This is confirmed when subjects are given pharmacologic treatment for hypercholesterolemia, reducing their COPs concentration (4β-hydroxycholesterol, 4β-HC) but without modifying the 4β-HC /cholesterol ratio (Björkhem-Bergman et al., 2016). Thus, if plasma cholesterol concentrations are not significantly reduced, there may be a continued substrate availability for oxidation, preventing a decrease in COPs.

COPs levels are highly variable even in a homogeneous population. Menéndez-Carreño et al. (2011) observed a wide range of COPs levels (0.09 to 2.05 μg/mL, mean 0.75 μg/mL) in 88 subjects. Subjects with cardiometabolic risk factors such as hypertension or diabetes showed slightly higher levels, and hypercholesterolemia was directly associated with an increased COPs concentration (by 23% vs.

normocholesterolemic subjects). The levels observed in the present study fit into this range, even though the mean is higher, which is probably ascribable to the fact that all participants suffer hypercholesterolemia. This correlation between cholesterol and COPs levels could explain why COPs levels were not modified in our study.

In this sense, 7α–HC was detected in the groups at similar levels (0.11-0.13 μg/mL). This COP is known for being produced in the liver, as it is an intermediate of bile production, and therefore, of relevance for cholesterol efflux (Hahn et al., 1995; Olkkonen et al., 2017). Hence, the correlation between similar levels of 7α-HC at T0 and T1 in both groups and similar levels of plasma cholesterol could mean that no change in efflux is occurring, and that cholesterol metabolism is not affected by the PS supplement intake.

The comparison of total COPs levels with literature is difficult due to the lack of homogeneity on individual COP determinations. Some studies with healthy population present low total levels of COPs (0.215-0.615 μg/mL), but none of them included α- and β-EC, which are major oxysterols in plasma, or cholestanetriol (Guardiola et al., 2007; Larsson et al., 2007; Narayanaswamy et al., 2015; Pataj et al., 2016; Schött & Lütjohann, 2015). The studies that detected these COPs showed slightly higher total levels (0.325-0.70 μg/mL) (Guardiola et al., 2007; Menéndez-Carreño et al., 2011). Nevertheless, even considering the lack of homogeneity, a high interindividual biological variability is highlighted in this determination. This fact, combined with hypercholesterolemia, could explain the higher levels observed in the present study.

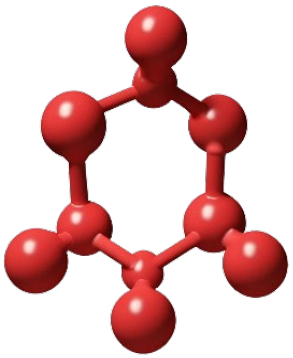
On the other hand, no side-chain COPs (24-HC, 25-HC and 27-HC) were detected in the plasma from the studied subjects. The absence of these specific COPs is particularly noteworthy, as their formation is often indicative of altered sterol metabolism or elevated oxidative stress, both critical factors when evaluating the systemic impact of PS supplementation. Dumolt & Rideout (2017) demonstrated that dietary PS intake can inhibit the activity of CYP27A1 (sterol 27-hydroxylase), the enzyme responsible for converting cholesterol to 27-HC; this inhibition is thought to be related to the reduced availability of cholesterol as a substrate for enzymatic oxidation. A reduction in circulating 27-HC may mitigate oxysterol-mediated inflammation and vascular injury; however, this remains a theoretical proposition in the absence of direct clinical evidence. In contrast, the potential effects of PS intake on 24-HC and 25-HC remain poorly characterized. The studies by Weingärtner et al. (2008) and Scholle et al. (2009) report that statin therapy decreases cholesterol biosynthesis, thereby potentially limiting

substrate availability for the formation of all oxysterols. Nevertheless, any additive or synergistic effect of PS supplementation on the concentrations of 24-HC and 25-HC has yet to be elucidated. In summary, PS supplementation appears to reduce 27-HC levels via CYP27A1 inhibition, whereas its influence on 24-HC and 25-HC warrants further investigation. While the cardiovascular benefits of combined statin and PS therapy (particularly LDL-C reduction) are well established, the specific impact on oxysterol profiles remains an underexplored aspect (Weingärtner et al., 2008).

These results have been shared in two publications:

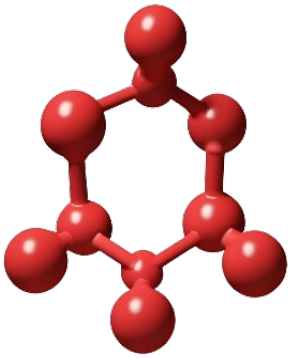
Miedes, D., Ortega-Luna, R., Broseta, S., Martínez-Hervás, S., Álvarez-Ribelles, Á., Collado-Díaz, V., Cilla, A., & Alegría, A. (2024). Impact of a plant sterol food supplement on eryptotic and associated cardiometabolic parameters: A randomized placebo-controlled trial in statin-treated patients. *Foods*, *13*, 4108.

Miedes, D., Mercatante, D., Broseta, S., Cilla, A., Rodriguez-Estrada, M.T., & Alegría, A. Effect of plant sterols on cholesterol oxidation products in statin-treated hypercholesterolemic individuals: A randomized clinical trial. *Under review, Journal of Food Science.*



Conclusiones

Conclusions



De los estudios realizados para evaluar la influencia de la digestión gastrointestinal armonizada (INFOGEST 2.0) y adaptada a las condiciones fisiológicas del anciano, sobre la bioaccesibilidad de los esteroles vegetales, se deduce:

1ª. El pan integral de centeno enriquecido contiene 2,18 g esteroles vegetales/100g de pan y su perfil es similar al ingrediente fuente de estos, sin modificaciones significativas entre diferentes horneados, lo que permite su uso para ensayos de bioaccesibilidad y funcionalidad *in vitro*.

2ª. La bebida de fruta a base de leche enriquecida con esteroles vegetales (0,91g/100g de bebida) presenta una bioaccesibilidad superior tras ser digerida bajo las condiciones gastrointestinales del anciano respecto al adulto (14,9 *vs*. 8,0 %), principalmente por la menor actividad de las enzimas lipolíticas debido a la utilización de un pH subóptimo para lipasa gástrica y a la mayor duración de la digestión (3 *vs*. 2 h).

3ª. Se produce una disminución de la bioaccesibilidad de los esteroles vegetales en el pan integral de centeno digerido bajo las condiciones del anciano respecto al adulto (11,2 *vs*. 20,3 %) debido a una menor liberación de los esteroles de la matriz sólida y más baja solubilización de los mismos, probablemente por la menor concentración de enzimas y sales biliares.

De la evaluación de la actividad antiproliferativa de un pan integral de centeno enriquecido con esteroles vegetales tras su digestión y fermentación colónica en células de cáncer colorrectal (Caco-2), se deduce:

4ª. Los líquidos de fermentación procedentes del pan integral de centeno, enriquecido o no con esteroles vegetales, producen un efecto citotóxico selectivo para células Caco-2 mediante la inducción de apoptosis extrínseca (*CASP8*), así como la regulación del ciclo celular y una modificación en la transcripción de genes relacionados (reducción de *CCND1* y *CDKN1A* y aumento de, *CASP3* y *TP53*).

5ª. Los líquidos de fermentación del pan enriquecido presentan un efecto antiproliferativo más pronunciado en la viabilidad celular y la apoptosis, sin embargo, los del pan sin enriquecer inducen mayor efecto antiproliferativo a nivel de regulación del ciclo celular, niveles de glutatión reducido y de calcio intracelular.

6ª. Los líquidos de fermentación del pan enriquecido incrementan en mayor medida la transcripción de los genes *TP53* y *CASP8*, mientras que los procedentes del pan sin enriquecer aumentan más la transcripción del gen *CASP3* y menos la del gen *CCND1*.

7ª. La acción antiproliferativa observada en modelos celulares de cáncer de colon sugiere que el pan integral de centeno enriquecido o no con esteroles vegetales podría tener un efecto protector a nivel gastrointestinal, debido a la sinergia entre los ácidos grasos de cadena corta procedentes de la fermentación de la fibra, los polifenoles totales, la capacidad antioxidante total y de los propios esteroles vegetales.

De la evaluación del efecto de la ingesta de un complemento alimenticio en polvo, con 2 g de esteroles vegetales, durante 6 semanas y del estilo de vida sobre parámetros cardiometabólicos de pacientes hipercolesterolémicos tratados con estatinas, se concluye:

8ª. No se observa modificación del perfil lipídico (colesterol total, LDL y triglicéridos), de los óxidos de colesterol plasmáticos totales e individuales, ni de otros parámetros como la glucosa, insulina o proteína C-reactiva, que se mantienen en los niveles basales.

9ª. Los marcadores de eriptosis (externalización de fosfatidilserina, niveles de glutatión intracelular y *forward scatter*) no mejoran en sujetos con hipercolesterolemia tratados con estatinas tras la ingesta del complemento con esteroles vegetales.

10ª. La población estudiada presenta buena adherencia a la dieta Mediterránea y baja actividad física. No se observan correlaciones significativas entre el nivel de actividad física o la adherencia a la dieta Mediterránea con la externalización de la fosfatidilserina, la adhesión al endotelio o la variación en el nivel de óxidos de colesterol.

11ª. Como efectos positivos del tratamiento, señalar que se produce un aumento de ApoA1 y, como consecuencia, una reducción de la adhesión de los eritrocitos al endotelio. Ambos factores son fundamentales para reducir la aterosclerosis y disminuir el riesgo cardiovascular, por lo que el consumo de este complemento alimenticio podría ser de interés en sujetos con hipercolesterolemia moderada, tratada o no con estatinas.

Estos resultados clínicos beneficiosos deberían ser confirmados en estudios con mayor tamaño muestral, lo cual ha podido limitar la significación estadística en el perfil lipídico, marcadores de eriptosis y niveles plasmáticos de óxidos de colesterol, así como las posibles correlaciones con el estilo de vida.

From the studies carried out to evaluate the influence of harmonized gastrointestinal digestion (INFOGEST 2.0) and adapted to the physiological conditions of the elderly on the bioaccessibility of plant sterols, it can be deduced:

1st. The enriched wholemeal rye bread contains 2.18 g plant sterols/100g of bread and its profile is similar to their source ingredient without significant modifications between different baking, which allows its use for bioaccessibility and *in vitro* functionality assays.

2nd. The milk-based fruit beverage enriched with plant sterols (0.91g/100g of drink) presents a higher bioaccessibility after being digested under the gastrointestinal conditions of the elderly compared to the adult (14.9 vs. 8.0 %), mainly due to the lower activity of lipolytic enzymes because of the use of a suboptimal pH for gastric lipase and to the longer duration of digestion (3 vs. 2 h).

3rd. There is a decrease in the bioaccessibility of plant sterols in the wholemeal rye bread digested under the conditions of the elderly compared to the adult (11.2 vs. 20.3 %) due to a lower release of the sterols from the solid matrix and lower solubilization of the sterols probably due to the lower concentration of enzymes and bile salts.

From the evaluation of the antiproliferative activity of a wholemeal rye bread enriched with plant sterols after digestion and colonic fermentation in colorectal cancer cells (Caco-2), it follows:

4th. The fermentation liquids from wholemeal rye bread, enriched or not with plant sterols, produce a selective cytotoxic effect on Caco-2 cells through the induction of extrinsic apoptosis (*CASP8*), as well as the regulation of the cell cycle and a modification in the transcription of related genes (reduction of *CCND1* and *CDKN1A* and increase of *CASP3* and *TP53*).

5th. The fermentation liquids of the enriched bread present a more pronounced antiproliferative effect on cell viability and apoptosis; however, those of the non-enriched bread induce a greater antiproliferative effect regarding cell cycle regulation, reduced glutathione levels and intracellular calcium.

6th. The fermentation liquids from enriched bread increase to a greater extent the transcription of the *TP53* and *CASP8* genes, while those from non-enriched bread increase more the transcription of the *CASP3* gene and less the *CCND1* gene.

7th. The antiproliferative action observed in colon cancer cell models suggests that wholemeal rye bread enriched with plant sterols could have a protective effect at the gastrointestinal level, due to the synergy between the short chain fatty acids from the fermentation of the fiber, the polyphenol content, the total antioxidant capacity, and the plant sterols themselves.

From the evaluation of the effect of the intake of a powdered food supplement containing 2 g of plant sterols for 6 weeks and lifestyle on cardiometabolic parameters in hypercholesterolemic patients treated with statins, it is concluded:

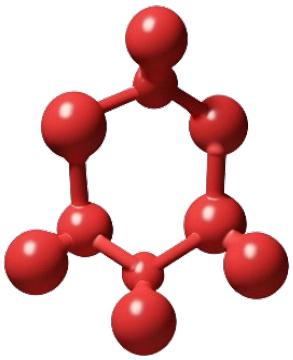
8th. No modification is observed in the lipid profile (total cholesterol, LDL and triglycerides), in total and individual plasma cholesterol oxides, or in other parameters such as glucose, insulin or C-reactive protein, which are maintained at baseline levels.

9th. Eryptosis markers (phosphatidylserine externalization, intracellular glutathione levels and forward scatter) do not improve in subjects with hypercholesterolemia treated with statins after supplementation with plant sterols.

10th. The population studied had good adherence to the Mediterranean diet, and low level of physical activity. However, no significant correlations were observed between the level of physical activity or adherence to the Mediterranean diet with the externalization of phosphatidylserine, adhesion to the endothelium or the variation in the level of cholesterol oxides.

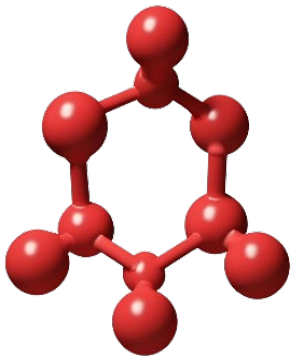
11th. The positive effects of the treatment include an increase in ApoA1 and, consequently, a reduction in the adhesion of erythrocytes to the endothelium. Both factors are fundamental for reducing atherosclerosis and decreasing cardiovascular risk, so that the consumption of this food supplement could be of interest in subjects with moderate hypercholesterolemia, whether or not treated with statins.

These beneficial clinical results should be confirmed in studies with a larger sample size, which may have limited the statistical significance in the lipid profile, markers of erythrocytosis and plasma levels of cholesterol oxides, as well as possible correlations with lifestyle.



Bibliografía

References



Aalaei, K., Khakimov, B., De Gobba, C., & Ahrné, L. (2021). Gastric digestion of milk proteins in adult and elderly: Effect of high-pressure processing. *Foods*, *10*, 786.

Acuff, R. V., Cai, D. J., Dong, Z. P., & Bell, D. (2007). The lipid lowering effect of plant sterol ester capsules in hypercholesterolemic subjects. *Lipids in Health and Disease*, *6*, 1-10.

Al Mamun Bhuyan, A., Nüßle, S., Cao, H., Zhang, S., & Lang, F. (2017). Simvastatin, a novel stimulator of eryptosis, the suicidal erythrocyte death. *Cellular Physiology and Biochemistry*, *43*, 492–506.

Alghareeb, S. A., Alfhili, M. A., & Fatima, S. (2023). Molecular mechanisms and pathophysiological significance of eryptosis. *International Journal of Molecular Sciences*, *24*, 5079.

Alvarez-Sala, A., Garcia-Llatas, G., Cilla, A., Barbera, R., Sánchez-Siles, L. M., & Lagarda, M. J. (2016). Impact of lipid components and emulsifiers on plant sterols bioaccessibility from milk-based fruit beverages. *Journal of Agricultural and Food Chemistry*, *64*, 5686-5691.

Álvarez-Sala, A., Ávila-Gálvez, M. Á., Cilla, A., Barberá, R., Garcia-Llatas, G., Espín, J. C., & González-Sarrías, A. (2018b). Physiological concentrations of phytosterols enhance the apoptotic effects of 5-fluorouracil in colon cancer cells. *Journal of Functional Foods*, *49*, 52-60.

Alvarez-Sala, A., Blanco-Morales, V., Cilla, A., Silvestre, R. Á., Hernández-Álvarez, E., Granado-Lorencio, F., ... & Garcia-Llatas, G. (2018c). A positive impact on the serum lipid profile and cytokines after the consumption of a plant sterol-enriched beverage with a milk fat globule membrane: A clinical study. *Food & Function*, *9*, 5209-5219.

Alvarez-Sala, A., López-García, G., Attanzio, A., Tesoriere, L., Cilla, A., Barberá, R., & Alegría, A. (2018a). Effects of plant sterols or β-cryptoxanthin at physiological serum concentrations on suicidal erythrocyte death. *Journal of Agricultural and Food Chemistry*, *66*, 1157–1166.

Åman, P., Andersson, A. A. M., Rakha, A., & Andersson, R. (2010). Rye, a healthy cereal full of dietary fiber. *Cereal Foods World*, *55*, 231-234.

Amara, S., Bourlieu, C., Humbert, L., Rainteau, D., & Carrière, F. (2019). Variations in gastrointestinal lipases, pH and bile acid levels with food intake, age and diseases: Possible impact on oral lipid-based drug delivery systems. *Advanced Drug Delivery Reviews*, *142*, 3-15.

Anagnostis, P., Kotsis, V., Banach, M., & Mikhailidis, D. P. (2023). Could lowering phytosterol absorption as part of lipid-lowering therapy have a beneficial effect on residual risk? *Metabolites*, *13*, 145.

Andrade, I., Santos, L., & Ramos, F. (2015). "Add-on" effect of phytosterols-enriched fermented milk on lipids and markers of cholesterol metabolism in statin-treated elderly patients. *Steroids*, *99*, 293–298.

Assad-Bustillos, M., Palier, J., Rabesona, H., Choiset, Y., Della Valle, G., & Feron, G. (2020). Role of the bolus degree of structure on the protein digestibility during *in vitro* digestion of a pea protein-fortified sponge cake chewed by elderly. *Journal of Texture Studies*, *51*, 134-143.

Aune, D., Keum, N., Giovannucci, E., Fadnes, L. T., Boffetta, P., Greenwood, D. C., ... & Norat, T. (2016). Whole grain consumption and risk of cardiovascular disease, cancer, and all cause and cause specific mortality: Systematic review and dose-response meta-analysis of prospective studies. *BMJ*, *353*.

Bañuls, C., Martínez-Triguero, M. L., López-Ruiz, A., Morillas, C., Lacomba, R., Víctor, V. M., ... & Hernández-Mijares, A. (2010). Evaluation of cardiovascular risk and oxidative stress parameters in hypercholesterolemic subjects on a standard healthy diet including low-fat milk enriched with plant sterols. *The Journal of Nutritional Biochemistry*, *21*, 881-886.

Behera, S. K., Mishra, S., Mohapatra, M. (2021). Physicochemical study of dietary fiber methylcellulose and human intestinal bile salt micellar aggregates. *Colloid and Interface Science Communications*, *44*, 100493.

Bingham, S. A., Day, N. E., Luben, R., Ferrari, P., Slimani, N., Norat, T., ... & Riboli, E. (2003). Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): An observational study. *Lancet*, *361*, 1496–1501.

Björkhem-Bergman, L., Nylén, H., Eriksson, M., Parini, P., & Diczfalusy, U. (2016). Effect of statin treatment on plasma 4β-hydroxycholesterol concentrations. *Basic & Clinical Pharmacology & Toxicology*, *118*, 499-502.

Blair, S. N., Capuzzi, D. M., Gottlieb, S. O., Nguyen, T., Morgan, J. M., & Cater, N. B. (2000). Incremental reduction of serum total cholesterol and low-density lipoprotein cholesterol with the addition of plant stanol ester-containing spread to statin therapy. *The American Journal of Cardiology*, *86*, 46–52.

Blanco-Morales, V., García-Llatas, G., & Cilla, A. (2021a). Sterol digestion in plant sterol-enriched foods: Bioaccessibility and fermentation. En Grundy, M. M.-L., & Wilde, P. J. (Eds.), Bioaccessibility and digestibility of lipids from food (pp. 205-224). Springer eBooks.

Blanco-Morales, V., Garcia-Llatas, G., Yebra, M. J., Sentandreu, V., & Alegría, A. (2021b). *In vitro* colonic fermentation of a plant sterol-enriched beverage in a dynamic-colonic gastrointestinal digester. *LWT*, *145*, 111273.

Blanco-Morales, V., López-García, G., Cilla, A., Garcia-Llatas, G., Barberá, R., Lagarda, M. J., Sánchez-Siles, L. M., & Alegría, A. (2018). The impact of galactooligosaccharides on the bioaccessibility of sterols in a plant sterol-enriched beverage: Adaptation of the harmonized INFOGEST digestion method. *Food & Function*, *9*, 2080–2089.

Blanco-Morales, V., Mercatante, D., Rodriguez-Estrada, M. T., & Garcia-Llatas, G. (2024). Current and new insights on delivery systems for plant sterols in food. En Lizard, G. (Ed.), Implication of oxysterols and phytosterols in aging and human diseases (pp. 403-435). Springer.

Blanco-Morales, V., Silvestre, R. D. L. A., Hernández-Álvarez, E., Donoso-Navarro, E., Alegría, A., & Garcia-Llatas, G. (2022). Influence of galactooligosaccharides on the positive effect of plant sterol-enriched beverages on cardiovascular risk and sterol colon metabolism. *Journal of Agricultural and Food Chemistry*, *70*, 532-542.

Bohn, T., Carriere, F., Day, L., Deglaire, A., Egger, L., Freitas, D., … & Dupont, D. (2018). Correlation between *in vitro* and *in vivo* data on food digestion. What can we predict with static *in vitro* digestion models? *Critical Reviews in Food Science and Nutrition*, *58*, 2239–2261.

Boyd, A. P., Talbert, J. N., & Acevedo, N. C. (2021). Effect of agitation and added cholesterol esterase on bioaccessibility of phytosterols in a standardized *in vitro* digestion model. *LWT*, *150*, 112051.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., ... & Recio, I. (2019). INFOGEST static *in vitro* simulation of gastrointestinal food digestion. *Nature Protocols*, *14*, 991–1014.

Caicedo-Lopez, L. H., Cuellar-Nuñez, M. L., Luzardo-Ocampo, I., Campos-Vega, R., & Lóarca-Piña, G. (2021). Colonic metabolites from digested *Moringa oleifera* leaves induced HT-29 cell death via apoptosis, necrosis, and autophagy. *International Journal of Food Sciences and Nutrition*, *72*, 485-498.

Cao, X., Zhao, F., Lin, Z., Sun, X., Zeng, X., Liu, H., ... & Zhou, G. (2024). *In vitro* digestion mimicking conditions in adults and elderly reveals digestive characteristics of pork from different cooking ways. *Food Research International*, *183*, 114204.

Cardenia, V., Rodriguez-Estrada, M. T., Baldacci, E., Savioli, S., & Lercker, G. (2012). Analysis of cholesterol oxidation products by fast gas chromatography/mass spectrometry. *Journal of Separation Science*, *35*, 424-430.

Casanova, M. R., Azevedo-Silva, J., Rodrigues, L. R., & Preto, A. (2018). Colorectal cancer cells increase the production of short chain fatty acids by *Propionibacterium freudenreichii* impacting on cancer cells survival. *Frontiers in Nutrition*, *5*, 44.

Castro-Cabezas, M., de Vries, J. H., Van Oostrom, A. J., Iestra, J., & van Staveren, W. A. (2006). Effects of a stanol-enriched diet on plasma cholesterol and triglycerides in patients treated with statins. *Journal of the American Dietetic Association*, *106*, 1564–1569.

Cater, N. B., Garcia-Garcia, A. B., Vega, G. L., & Grundy, S. M. (2005). Responsiveness of plasma lipids and lipoproteins to plant stanol esters. *The American Journal of Cardiology*, *96*, 23D–28D.

Chau, I., & Cunningham, D. (2002). Chemotherapy in colorectal cancer: New options and new challenges. *British Medical Bulletin*, *64*, 159-180.

Chen, L., Xiu, R., Wang, H., Wang, L., Wu, G., Liang, J. & Han, X. (2019). Simultaneous quantification of ten oxysterols based on LC–MS/MS and its application in atherosclerosis human serum samples. *Chromatographia*, *82*, 553-564.

Cheung, C. L., Ho, D. K. C., Sing, C. W., Tsoi, M. F., Cheng, V. K. F., Lee, G. K. Y., ... & Cheung, B. M. (2017). Randomized controlled trial of the effect of phytosterols-enriched low-fat milk on lipid profile in Chinese. *Scientific Reports*, *7*, 41084.

Cilla, A., Alegría, A., Attanzio, A., Garcia-Llatas, G., Tesoriere, L., & Livrea, M. A. (2017). Dietary phytochemicals in the protection against oxysterol-induced damage. *Chemistry and Physics of Lipids*, *207*, 192–205.

Cilla, A., Attanzio, A., Barberá, R., Tesoriere, L., & Livrea, M. A. (2015). Anti-proliferative effect of main dietary phytosterols and β-cryptoxanthin alone or combined in human colon cancer Caco-2 cells through cytosolic Ca+2 – and oxidative stress induced apoptosis. *Journal of Functional Foods*, *12*, 282-293.

Cilla, A., López-García, G., Collado-Díaz, V., Amparo Blanch-Ruiz, M., Garcia-Llatas, G., Barberá, R., ... & Martínez-Hervás, S. (2021). Hypercholesterolemic patients have higher eryptosis and erythrocyte adhesion to human endothelium independently of statin therapy. *International Journal of Clinical Practice*, *75*, e14771.

Cilla, A., López-García, G., Barberá, R., Frazzitta, A., Restivo, I., Tesoriere, L., & Attanzio, A. (2022). Antiproliferative effects of bioaccessible fractions of honeys from Sicilian black honeybee (*Apis mellifera ssp. sicula*) on human colorectal carcinoma cells. *International Journal of Food Science and Technology*, *57*, 2636-2645.

Clifton, P. M., Noakes, M., Sullivan, D., Erichsen, N., Ross, D., Annison, G., ... & Nestel, P. (2004). Cholesterol-lowering effects of plant sterol esters differ in milk, yoghurt, bread and cereal. *European Journal of Clinical Nutrition*, *58*, 503-509.

Craig, C. L., Marshall, A. L., Sjöström, M., Bauman, A. E., Booth, M. L., Ainsworth, B. E., ... & Oja, P. (2003). International physical activity questionnaire: 12-country reliability and validity. *Medicine and Science in Sports and Exercise*, *35*, 1381-1395.

Cuevas-Tena, M., Alegría, A., & Lagarda, M. J. (2018). Relationship between dietary sterols and gut microbiota: A review. *European Journal of Lipid Science and Technology*, *120*, 1800054.

Daly, T. J., Aherne, S. A., O'Connor, T. P., & O'Brien, N. M. (2009). Lack of genoprotective effect of phytosterols and conjugated linoleic acids on Caco-2 cells. *Food and Chemical Toxicology*, *47*, 1791–1796.

Darzynkiewicz, Z., Bedner, E., & Smolewski, P. (2001). Flow cytometry in analysis of cell cycle and apoptosis. *Seminars in Hematology*, *38*, 179-193.

de Boer, J. F., Kuipers, F., & Groen, A. K. (2018). Cholesterol transport revisited: A new turbo mechanism to drive cholesterol excretion. *Trends in Endocrinology & Metabolism*, *29*, 123-133.

De Jong, A., Plat, J., Bast, A., Godschalk, R. W., Basu, S., & Mensink, R. P. (2008). Effects of plant sterol and stanol ester consumption on lipid metabolism, antioxidant status and markers of oxidative stress, endothelial function and low-grade inflammation in patients on current statin treatment. *European Journal of Clinical Nutrition*, *62*, 263–273.

Decisión 2000/500/CE. Comisión de 24 de julio de 2000 relativa a la autorización de la comercialización de las «grasas amarillas de untar con ésteres de fitosterol» como nuevo alimento o nuevo ingrediente alimentario con arreglo al Reglamento (CE) nº 258/97 del Parlamento Europeo y del Consejo. Diario Oficial de la Unión Europea, L200, 59-60.

Decisión 2004/333/CE. Comisión de 31 de marzo de 2004 relativa a la autorización de comercialización de grasas amarillas para untar, aliños para ensaladas, productos de tipo leche, productos tipo leche fermentada, bebidas de soja y productos tipo queso con fitosteroles/fitostanoles añadidos como nuevo alimento o nuevo ingrediente alimentario, con arreglo al Reglamento (CE) no 258/97 del Parlamento Europeo y del Consejo. Diario Oficial de la Unión Europea, L105, 40-42.

Decisión 2004/334/CE. Comisión de 31 de marzo de 2004 relativa a la autorización de comercialización de grasas amarillas para untar, productos tipo leche, productos tipo yogur y salsas aromáticas con fitosteroles/fitostanoles añadidos como nuevo alimento o nuevo ingrediente alimentario, con arreglo al Reglamento (CE) no 258/97 del Parlamento Europeo y del Consejo. Diario Oficial de la Unión Europea, L105, 43-45.

Decisión 2004/335/CE. Comisión de 31 de marzo de 2004 relativa a la autorización de comercialización de productos tipo leche y productos tipo yogur con ésteres de fitosterol

añadidos como nuevo ingrediente alimentario, con arreglo al Reglamento (CE) no 258/97 del Parlamento Europeo y del Consejo. Diario Oficial de la Unión Europea, L105, 46-48.

Decisión 2004/336/CE. Comisión de 31 de marzo de 2004 relativa a la autorización de comercialización de grasas amarillas para untar, bebidas de frutas a base de leche, productos tipo yogur y productos tipo queso, con fitosteroles/fitostanoles añadidos como nuevo alimento o nuevo ingrediente alimentario, con arreglo al Reglamento (CE) no 258/97 del Parlamento Europeo y del Consejo. Diario Oficial de la Unión Europea, L105, 49-51.

Decisión 2004/845/CE. Comisión de 12 noviembre de 2004 relativa a la autorización de la comercialización de bebidas a base de leche con fitosteroles/ fitostanoles añadidos como nuevos alimentos o nuevos ingredientes alimentarios con arreglo al Reglamento (CE) no 258/97 del Parlamento Europeo y del Consejo. Diario Oficial de la Unión Europea, L366, 14-16.

Decisión 2006/58/CE. Comisión de 24 enero de 2006 por la que se autoriza la comercialización de pan de centeno con fitosteroles/fitostanoles añadidos como nuevos alimentos nuevos ingredientes de conformidad con el Reglamento (CE) no 258/97 del Parlamento Europeo y del Consejo. Diario Oficial de la Unión Europea, L31, 18-20.

Decisión 2006/59/CE. Comisión de 24 enero de 2006 relativa a la autorización de comercialización de pan de centeno con fitosteroles/fitostanoles añadidos como nuevos alimentos nuevos ingredientes de conformidad con el Reglamento (CE) no 258/97 del Parlamento Europeo y del Consejo. Diario Oficial de la Unión Europea, L31, 21-23.

Decisión 2007/343/CE. Comisión de 15 mayo de 2007 por la que se autoriza la comercialización de aceite enriquecido con fitoesteroles o fitoestanoles como nuevo ingrediente alimentario con arreglo al Reglamento (CE) no 258/97 del Parlamento Europeo y del Consejo. Diario Oficial de la Unión Europea, L129, 63-66.

Decisión 2008/36/CE. Comisión 10 de enero de 2008 por la que se autoriza la comercialización de bebidas de arroz con fitosteroles o fitostanoles añadidos como nuevo alimento con arreglo al Reglamento (CE) no 258/97 del Parlamento Europeo y del Consejo. Diario Oficial de la Unión Europea, L8, 15-17.

Demonty, I., Ras, R. T., van der Knaap, H. C., Duchateau, G. S., Meijer, L., Zock, P. L., ... & Trautwein, E. A. (2009). Continuous dose-response relationship of the LDL-cholesterol–lowering effect of phytosterol intake. *The Journal of Nutrition*, *139*, 271-284.

Devaraj, S., Jialal, I., & Vega-López, S. (2004). Plant sterol-fortified orange juice effectively lowers cholesterol levels in mildly hypercholesterolemic healthy individuals. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *24*, e25-e28.

Directiva 2002/46/CE del Parlamento Europeo y del Consejo, de 10 de junio de 2002, relativa a la aproximación de las legislaciones de los Estados miembros en materia de complementos alimenticios. Diario Oficial de las Comunidades Europeas, L183, 51-56.

Domenech, M., Casas, R., Ruiz-León, A. M., Sobrino, J., Ros, E., & Estruch, R. (2019). Effects of a novel nutraceutical combination (Aquilea Colesterol®) on the lipid profile and inflammatory biomarkers: A randomized control trial. *Nutrients*, *11*, 949.

Doornbos, A. M. E., Meynen, E. M., Duchateau, G. S. M. J. E., Van der Knaap, H. C. M., & Trautwein, E. A. (2006). Intake occasion affects the serum cholesterol lowering of a plant sterol-enriched single-dose yoghurt drink in mildly hypercholesterolaemic subjects. *European Journal of Clinical Nutrition*, *60*, 325-333.

Drira, M., Jabeur, H., Marrakchi, F., & Bouaziz, M. (2018). Delta-7-stigmastenol: Quantification and isomeric formation during chemical refining of olive pomace oil and optimization of the neutralization step. *European Food Research and Technology*, *244*, 2231-2241.

Dumolt, J. H., & Rideout, T. C. (2017). The lipid-lowering effects and associated mechanisms of dietary phytosterol supplementation. *Current Pharmaceutical Design*, *23*, 5077-5085.

Dupont, D., Alric, M., Blanquet-Diot, S., Bornhorst, G., Cueva, C., Deglaire, A., ... & Van den Abbeele, P. (2019). Can dynamic *in vitro* digestion systems mimic the physiological reality? *Critical Reviews in Food Science and Nutrition*, *59*, 1546–1562.

Earnest, C. P., Mikus, C. R., Lemieux, I., Arsenault, B. J., & Church, T. S. (2007). Examination of encapsulated phytosterol ester supplementation on lipid indices associated with cardiovascular disease. *Nutrition*, *23*, 625-633.

Ebert, M. N., Beyer-Sehlmeyer, G., Liegibel, U. M., Kautenburger, T., Becker, T. W., & Pool-Zobel, B. L. (2001). Butyrate induces glutathione S-transferase in human colon cells and protects from genetic damage by 4-hydroxy-2-nonenal. *Nutrition and Cancer*, *41*, 156–164.

EFSA Panel on Dietetic Products, Nutrition and Allergies. (2010). Scientific opinion on dietary reference values for carbohydrates and dietary fibre. *EFSA Journal*, *8*, 1462.

Escurriol, V., Marí-Dell'Olmo, M., Rohlfs, I., Borrell, C., Chirlaque, M. D., Buckland, G., ... & Ros, E. (2009). Plant sterol intake and education level in the Spanish EPIC cohort. *Nutrition*, *25*, 769–773.

Fang, M., Xia, F., Chen, Y., Shen, Y., Ma, L., You, C., Tao, C., & Hu, X. (2022). Role of eryptosis in hemorrhagic stroke. *Frontiers in Molecular Neuroscience*, *15*, 932931.

Faubel, N., Blanco-Morales, V., Sentandreu, V., Barberá, R., & Garcia-Llatas, G. (2025). Modulation of microbiota composition and markers of gut health after *in vitro* dynamic colonic fermentation of plant sterol-enriched wholemeal rye bread. *Food Research International*, *201*, 115570.

Faubel, N., Makran, M., Barberá, R., Garcia-Llatas, G., Giardina, I. C., Tesoriere, L., ... & Cilla, A. (2024). Anti-inflammatory activity of plant sterols in a co-culture model of intestinal inflammation: Focus on food-matrix effect. *Food & Function*, *15*, 6502-6511.

Faubel, N., Makran, M., Cilla, A., Alegría, A., Barberá, R., & Garcia-Llatas, G. (2022). Bioaccessibility of plant sterols in wholemeal rye bread using the INFOGEST protocol: Influence of oral phase and enzymes of lipid metabolism. *Journal of Agricultural and Food Chemistry*, *70*, 13223-13232.

Feldman, M., Cryer, B., McArthur, K. E., Huet, B. A., & Lee, E. (1996). Effects of aging and gastritis on gastric acid and pepsin secretion in humans: A prospective study. *Gastroenterology*, *110*,1043–1052.

Feng, S., Belwal, T., Li, L., Limwachiranon, J., Liu, X., & Luo, Z. (2020). Phytosterols and their derivatives: Potential health-promoting uses against lipid metabolism and associated diseases, mechanism, and safety issues. *Comprehensive Reviews in Food Science and Food Safety*, *19*, 1243–1267.

Ferguson, J. J., Wolska, A., Remaley, A. T., Stojanovski, E., MacDonald-Wicks, L., & Garg, M. L. (2019). Bread enriched with phytosterols with or without curcumin modulates lipoprotein profiles in hypercholesterolaemic individuals. A randomised controlled trial. *Food & Function*, *10*, 2515-2527.

Floride, E., Föller, M., Ritter, M., & Lang, F. (2008). Caffeine inhibits suicidal erythrocyte death. *Cellular Physiology and Biochemistry*, *22*, 253–260.

Fogh, J., & Trempe, G. (1975). New human tumor cell lines. En Fogh, J (Ed.), *Human tumor cells in vitro* (pp. 115-141). Springer International Publishing.

Fontané, L., Pedro-Botet, J., Garcia-Ribera, S., Climent, E., Muns, M. D., Ballesta, S., ... & Benaiges, D. (2023). Use of phytosterol-fortified foods to improve LDL cholesterol levels: A systematic review and meta-analysis. *Nutrition, Metabolism, and Cardiovascular Diseases*, *33*, 1472–1480.

Frostegård J. (2013). Immune mechanisms in atherosclerosis, especially in diabetes type 2. *Frontiers in Endocrinology*, *4*, 162.

Fuentes, F., Lopez-Miranda, J., Garcia, A., Perez-Martinez, P., Moreno, J., Cofan, M., ... & Perez-Jimenez, F. (2008). Basal plasma concentrations of plant sterols can predict LDL-C response to sitosterol in patients with familial hypercholesterolemia. *European Journal of Clinical Nutrition*, *62*, 495–501.

Fumeron, F., Bard, J. M., & Lecerf, J. M. (2017). Interindividual variability in the cholesterol-lowering effect of supplementation with plant sterols or stanols. *Nutrition Reviews, 75*, 134–145.

Gallego, M., Barat, J. M., Grau, R., & Talens, P. (2022). Compositional, structural design and nutritional aspects of texture-modified foods for the elderly. *Trends in Food Science & Technology*, *119*, 152-163.

Gallego, M., Grau, R., & Talens, P. (2023). Behaviour of texture-modified meats using proteolytic enzymes during gastrointestinal digestion simulating elderly alterations. *Meat Science*, *206*, 109341.

Gao, Y., Xun, R., Xia, J., Xia, H., & Sun, G. (2023). Effects of phytosterol supplementation on lipid profiles in patients with hypercholesterolemia: A systematic

review and meta-analysis of randomized controlled trials. *Food & Function*, *14*, 2969–2997.

Garcia-Llatas, G., Alegría, A., Barberá, R., & Cilla, A. (2021a). Current methodologies for phytosterol analysis in foods. *Microchemical Journal*, *168*, 106377.

Garcia-Llatas, G., Mejía, E., Cilla, A., Alegría, A., & Lagarda, M.J. (2015). Estimation of plant sterol intake in a resident population of the valencian community (Spain). En Guiné, R. (Ed.) Phytosterols. Food sources and chemistry of plant sterols and stanols. (1st ed.) Nova Science Publishers, 185-207.

Garcia-Llatas, G., Mercatante, D., López-García, G., & Rodriguez-Estrada, M.T. (2021b). Oxysterols - how much do we know about food occurrence, dietary intake and absorption? *Current Opinion in Food Science*, *37*, 1-9.

Gargiulo, S., Testa, G., Gamba, P., Staurenghi, E., Poli, G., & Leonarduzzi, G. (2017). Oxysterols and 4-hydroxy-2-nonenal contribute to atherosclerotic plaque destabilization. *Free Radical Biology and Medicine*, *111*, 140–150.

Gerlier, D., & Thomasset, N. (1986). Use of MTT colorimetric assay to measure cell activation. *Journal of Immunological Methods*, *94*, 57-63.

Glei, M., Fischer, S., Lamberty, J., Ludwig, D., Lorkowski, S., & Schlörmann, W. (2018). Chemopreventive potential of *in vitro* fermented raw and roasted hazelnuts in lt97 colon adenoma cells. *Anticancer Research*, *38*, 83–93.

Glei, M., Ludwig, D., Lamberty, J., Fischer, S., Lorkowski, S., & Schlörmann, W. (2017). Chemopreventive potential of raw and roasted pistachios regarding colon carcinogenesis. *Nutrients*, *9*, 1368.

Glei, M., Zetzmann, S., Lorkowski, S., Dawczynski, C., & Schlörmann, W. (2021). Chemopreventive effects of raw and roasted oat flakes after *in vitro* fermentation with human faecal microbiota. *International Journal of Food Sciences and Nutrition*, *72*, 57–69.

Goldberg, A. C., Ostlund, R. E., Jr, Bateman, J. H., Schimmoeller, L., McPherson, T. B., & Spilburg, C. A. (2006). Effect of plant stanol tablets on low-density lipoprotein cholesterol lowering in patients on statin drugs. *The American Journal of Cardiology*, *97*, 376–379.

González-Larena, M., Garcia-Llatas, G., Clemente, G., Barberá, R., & Lagarda, M. J. (2015). Plant sterol oxides in functional beverages: Influence of matrix and storage. *Food Chemistry*, *173*, 881-889.

González-Larena, M., García-Llatas, G., Vidal, M. C., Sánchez-Siles, L. M., Barberá, R., & Lagarda, M. J. (2011). Stability of plant sterols in ingredients used in functional foods. *Journal of Agricultural and Food Chemistry*, *59*, 3624-3631.

González-Rodríguez, L. G., Perea Sánchez, J. M., Aranceta-Bartrina, J., Gil, Á., González-Gross, M., Serra-Majem, L., Varela-Moreiras, G., & Ortega, R. M. (2017). Intake and dietary food sources of fibre in spain: Differences with regard to the prevalence of excess body weight and abdominal obesity in adults of the ANIBES study. *Nutrients*, *9*, 326.

Granado-Lorencio, F., Lagarda, M. J., Garcia-López, F. J., Sánchez-Siles, L. M., Blanco-Navarro, I., Alegría, A., ... & Barberá, R. (2014). Effect of β-cryptoxanthin plus phytosterols on cardiovascular risk and bone turnover markers in post-menopausal women: A randomized crossover trial. *Nutrition, Metabolism and Cardiovascular Diseases*, *24*, 1090-1096.

Gråsten, S. M., Juntunen, K. S., Poutanen, K. S., Gylling, H. K., Miettinen, T. A., & Mykkänen, H. M. (2000). Rye bread improves bowel function and decreases the concentrations of some compounds that are putative colon cancer risk markers in middle-aged women and men. *The Journal of Nutrition*, *130*, 2215–2221.

Grundy, M. M. L., Moughan, P. J., & Wilde, P. J. (2024). Bioaccessibility and associated concepts: Need for a consensus. *Trends in Food Science & Technology*, *145*, 104373.

Guardiola, F., Tres, A., Codony, R., Addis, P. B., Bergmann, S. D., & Zavoral, J. H. (2007). Lack of effect of oral supplementation with antioxidants on cholesterol oxidation product concentration of human plasma, as revealed by an improved gas chromatography method. *Analytical and Bioanalytical Chemistry*, *389*, 277-289.

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., & Alric, M. (2012). Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends in Biotechnology*, *30*, 591–600.

Gylling, H., & Miettinen, T. A. (1996). Effects of inhibiting cholesterol absorption and synthesis on cholesterol and lipoprotein metabolism in hypercholesterolemic non-insulin-dependent diabetic men. *Journal of Lipid Research*, *37*, 1776–1785.

Gylling, H., Plat, J., Turley, S., Ginsberg, H. N., Ellegård, L., Jessup, W., ... & Chapman, M. J. (2014). Plant sterols and plant stanols in the management of dyslipidaemia and prevention of cardiovascular disease. *Atherosclerosis*, *232*, 346-360.

Hahn, C., Reichel, C., & Von Bergmann, K. (1995). Serum concentration of 7 alpha-hydroxycholesterol as an indicator of bile acid synthesis in humans. *Journal of Lipid Research*, *36*, 2059-2066.

Hakala, P., Lampi, A. M., Ollilainen, V., Werner, U., Murkovic, M., Wähälä, K., ... & Piironen, V. (2002). Steryl phenolic acid esters in cereals and their milling fractions. *Journal of Agricultural and Food Chemistry*, *50*, 5300-5307.

Hallikainen, M., Kurl, S., Laakso, M., Miettinen, T. A., & Gylling, H. (2011). Plant stanol esters lower LDL cholesterol level in statin-treated subjects with type 1 diabetes by interfering the absorption and synthesis of cholesterol. *Atherosclerosis*, *217*, 473–478.

Han, S., Jiao, J., Xu, J., Zimmermann, D., Actis-Goretta, L., Guan, L., ... & Qin, L. (2016). Effects of plant stanol or sterol-enriched diets on lipid profiles in patients treated with statins: Systematic review and meta-analysis. *Scientific Reports*, *6*, 31337.

Hansel, B., Nicolle, C., Lalanne, F., Tondu, F., Lassel, T., Donazzolo, Y., ... & Bruckert, E. (2007). Effect of low-fat, fermented milk enriched with plant sterols on serum lipid profile and oxidative stress in moderate hypercholesterolemia. *The American Journal of Clinical Nutrition*, *86*, 790-796.

He, W. S., Zhu, H., & Chen, Z. Y. (2018). Plant sterols: Chemical and enzymatic structural modifications and effects on their cholesterol-lowering activity. *Journal of Agricultural and Food Chemistry*, *66*, 3047-3062.

Hernández-Mijares, A., Banuls, C., Rocha, M., Morillas, C., Martínez-Triguero, M. L., Víctor, V. M., ... & Lagarda, M. J. (2010). Effects of phytosterol ester-enriched low-fat milk on serum lipoprotein profile in mildly hypercholesterolaemic patients are not related to dietary cholesterol or saturated fat intake. *British Journal of Nutrition*, *104*, 1018-1025.

Hernández-Olivas, E., Munoz-Pina, S., Andres, A., & Heredia, A. (2020a). Impact of elderly gastrointestinal alterations on *in vitro* digestion of salmon, sardine, sea bass and hake: Proteolysis, lipolysis and bioaccessibility of calcium and vitamins. *Food Chemistry*, *326*, 127024.

Hernández-Olivas, E., Muñoz-Pina, S., Andrés, A., & Heredia, A. (2023). The impact of age-related digestive disorders on *in vitro* digestibility of macronutrients and bioaccessibility of minor components of chia seeds. *Food Research International*, *169*, 112874.

Hernández-Olivas, E., Muñoz-Pina, S., Sánchez-García, J., Andrés, A., & Heredia, A. (2020b). Understanding the role of food matrix on the digestibility of dairy products under elderly gastrointestinal conditions. *Food Research International*, *137*, 109454.

Hernández-Olivas, E., Muñoz-Pina, S., Andrés, A., & Heredia, A. (2021a). Age-related gastrointestinal alterations of legumes and cereal grains digestibility. *Food Bioscience*, *41*, 101027.

Hernández-Olivas, E., Muñoz-Pina, S., Andrés, A., & Heredia, A. (2021b). Impact of cooking preparation on *in vitro* digestion of eggs simulating some gastrointestinal alterations in elders. *Journal of Agricultural and Food Chemistry*, *69*, 4402-4411.

Hinnebusch, B. F., Meng, S., Wu, J. T., Archer, S. Y., & Hodin, R. A. (2002). The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. *The Journal of Nutrition*, *132*, 1012-1017.

Huang, J., Xu, M., Fang, Y. J., Lu, M. S., Pan, Z. Z., Huang, W. Q., ... & Zhang, C. X. (2017). Association between phytosterol intake and colorectal cancer risk: A case–control study. *British Journal of Nutrition*, *117*, 839-850.

Iddir, M., Porras Yaruro, J. F., Cocco, E., Hardy, E. M., Appenzeller, B. M., Guignard, C., ... & Bohn, T. (2021). Impact of protein-enriched plant food items on the bioaccessibility and cellular uptake of carotenoids. *Antioxidants*, *10*, 1005.

Ikeda, I. (2015). Factors affecting intestinal absorption of cholesterol and plant sterols and stanols. *Journal of Oleo Science*, *64*, 9–18.

International Agency for Research on Cancer (IARC), World Health Organization. (2023). Disponible: https://www.iarc.who.int/cancer-type/colorectal-cancer/. Acceso: 15 de marzo de 2024.

Jang, J. Y., Kim, D., & Kim, N. D. (2023). Pathogenesis, intervention, and current status of drug development for sarcopenia: A review. *Biomedicines*, *11*, 1635.

Jiménez-Escrig, A., Santos-Hidalgo, A. B., & Saura-Calixto, F. (2006). Common sources and estimated intake of plant sterols in the Spanish diet. *Journal of Agricultural and Food Chemistry*, *54*, 3462–3471.

Johnston, N., Dettmar, P. W., Bishwokarma, B., Lively, M. O., & Koufman, J. A. (2007). Activity/stability of human pepsin: Implications for reflux attributed laryngeal disease. *The Laryngoscope*, *117*, 1036-1039.

Kalayci, A., Gibson, C. M., Ridker, P. M., Wright, S. D., Kingwell, B. A., Korjian, S., ... & Libby, P. (2022). ApoA-I infusion therapies following acute coronary syndrome: Past, present, and future. *Current Atherosclerosis Reports*, *24*, 585-597.

Kaliyaperumal, R., Deng, X., Meiselman, H. J., Song, H., Dalan, R., Leow, M. K. S., & Neu, B. (2019). Depletion interaction forces contribute to erythrocyte-endothelial adhesion in diabetes. *Biochemical and Biophysical Research Communications*, *516*, 144-148.

Kamal-Eldin, A., Lærke, H. N., Knudsen, K. E. B., Lampi, A. M., Piironen, V., Adlercreutz, H., ... & Åman, P. (2009). Physical, microscopic and chemical characterisation of industrial rye and wheat brans from the Nordic countries. *Food & Nutrition Research*, *53*, 1912.

Kelly, E. R., Plat, J., Mensink, R. P., & Berendschot, T. T. (2011). Effects of long term plant sterol and -stanol consumption on the retinal vasculature: A randomized controlled trial in statin users. *Atherosclerosis*, *214*, 225–230.

Khan, A., Carmona, R., & Traube, M. (2014). Dysphagia in the elderly. *Clinics in Geriatric Medicine*, *30,* 43-53.

Khandelwal, S., Demonty, I., Jeemon, P., Lakshmy, R., Mukherjee, R., Gupta, R., ... & Reddy, K. S. (2009). Independent and interactive effects of plant sterols and fish oil n-3

long-chain polyunsaturated fatty acids on the plasma lipid profile of mildly hyperlipidaemic Indian adults. *British Journal of Nutrition*, *102*, 722-732.

Kondrashina, A., Arranz, E., Cilla, A., Faria, M. A., Santos-Hernández, M., Miralles, B., ... & Giblin, L. (2023). Coupling *in vitro* food digestion with *in vitro* epithelial absorption; recommendations for biocompatibility. *Critical Reviews in Food Science and Nutrition*, *64*, 9618-9636.

Kulig, W., Cwiklik, L., Jurkiewicz, P., Rog, T., & Vattulainen, I. (2016). Cholesterol oxidation products and their biological importance. *Chemistry and Physics of Lipids*, *199*, 144–160.

Kumar, K., & Afonso, L. (2019). Cardiovascular risk prediction in the elderly. *Journal of Geriatric Cardiology*, *16*, 414–420.

Kumar, A., Chinnathambi, S., Kumar, M., & Pandian, G. N. (2023). Food intake and colorectal cancer. *Nutrition and Cancer*, *75*, 1710–1742.

Lang, F., Abed, M., Lang, E., & Föller, M. (2014). Oxidative stress and suicidal erythrocyte death. *Antioxidants & Redox Signaling*, *21*, 138–153.

Lang, F., Lang, E., & Föller, M. (2012). Physiology and pathophysiology of eryptosis. *Transfusion Medicine and Hemotherapy*, *39*, 308–314.

Larsson, H., Böttiger, Y., Iuliano, L., & Diczfalusy, U. (2007). *In vivo* interconversion of 7β-hydroxycholesterol and 7-ketocholesterol, potential surrogate markers for oxidative stress. *Free Radical Biology and Medicine*, *43*, 695-701.

Laugier, R., Bernard, J. P., Berthezene, P., & Dupuy, P. (1991). Changes in pancreatic exocrine secretion with age: Pancreatic exocrine secretion does decrease in the elderly. *Digestion*, *50*, 202–211.

Lea, T. (2015). Caco-2 cell line. En Verhoeckx K. et al. (Eds.), *The impact of food bioactives on health: In vitro and ex vivo models* (pp. 103-111). Springer International Publishing.

Lee, S. Y., Kang, H. J., Kang, J. H., Jang, H. W., Kim, B. K., & Hur, S. J. (2021). Analysis of *in vitro* digestion using human gut microbiota in adult and elderly individuals. *Food Chemistry*, *362*, 130228.

Le Goff, M., Le Ferrec, E., Mayer, C., Mimouni, V., Lagadic-Gossmann, D., Schoefs, B., & Ulmann, L. (2019). Microalgal carotenoids and phytosterols regulate biochemical mechanisms involved in human health and disease prevention. *Biochimie*, *167*, 106-118.

Lestiani, L., Chandra, D. N., Laitinen, K., Ambarwati, F. D., Kuusisto, P., & Lukito, W. (2018). Double-blind randomized placebo controlled trial demonstrating serum cholesterol lowering efficacy of a smoothie drink with added plant stanol esters in an Indonesian population. *Cholesterol*, *2018*, 4857473.

Li, L., Baima, C., Jiang, J., Liu, Z., Wang, J., Chen, X. D., & Wu, P. (2022a). *In vitro* gastric digestion and emptying of tsampa under simulated elderly and young adult digestive conditions using a dynamic stomach system. *Journal of Food Engineering*, *327*, 111054.

Li, X., Xin, Y., Mo, Y., Marozik, P., He, T., & Guo, H. (2022b). The bioavailability and biological activities of phytosterols as modulators of cholesterol metabolism. *Molecules*, *27*, 523.

Lin, Y., Knol, D., & Trautwein, E. A. (2016). Phytosterol oxidation products (POP) in foods with added phytosterols and estimation of their daily intake: A literature review. *European Journal of Lipid Science and Technology*, *118*, 1423-1438.

Lin, Y., Koppenol, W. P., Knol, D., Vermeer, M. A., Hiemstra, H., Friedrichs, S., ... & Trautwein, E. A. (2019). Serum concentration of plant sterol oxidation products (POP) compared to cholesterol oxidation products (COP) after intake of oxidized plant sterols: A randomised, placebo-controlled, double-blind dose–response pilot study. *Nutrients*, *11*, 2319.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, *25*, 402-408.

López-García, G., Cilla, A., Barbera, R., & Alegria, A. (2017). Antiproliferative effect of plant sterols at colonic concentrations on Caco-2 cells. *Journal of Functional Foods*, *39*, 84-90.

López-Marcos, M. C., Bailina, C., Viuda-Martos, M., Pérez-Alvarez, J. A., & Fernández-López, J. (2015). Effects of various fibre-rich extracts on cholesterol binding capacity during *in vitro* digestion of pork patties. *Food & Function*, *6*, 3473-3478.

Mach, F., Baigent, C., Catapano, A. L., Koskinas, K. C., Casula, M., Badimon, L., ... & Wiklund, O. (2020). 2019 ESC/EAS Guidelines for the management of dyslipidaemias: Lipid modification to reduce cardiovascular risk: The Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS). *European Heart Journal*, *41*, 111-188.

Mackie, A., Mulet-Cabero, A. I., & Torcello-Gómez, A. (2020). Simulating human digestion: Developing our knowledge to create healthier and more sustainable foods. *Food & Function*, *11*, 9397-9431.

Mahmud, H., Qadri, S. M., Föller, M., & Lang, F. (2010). Inhibition of suicidal erythrocyte death by vitamin C. *Nutrition*, *26*, 671–676.

Maiyo, F., Moodley, R., & Singh, M. (2016). Phytochemistry, cytotoxicity and apoptosis studies of b-sitosterol-3-o-glucoside and β -amyrin from prunus africana. *African Journal of Traditional, Complementary, and Alternative Medicines*, *13*, 105–112.

Makharia, G., Gibson, P. R., Bai, J. C., Karakan, T., Lee, Y. Y., Collins, L., ... & Le Mair, A. (2022). World gastroenterology organisation global guidelines: Diet and the gut. *Journal of Clinical Gastroenterology*, *56*, 1-15.

Makran, M., Barberá, R., & Cilla, A. (2021). Gene-diet interaction in plasma lipid response to plant sterols and stanols: A review of clinical trials. *Journal of Functional Foods*, *87*, 104751.

Makran, M., Cilla, A., Haros, C. M., & Garcia-Llatas, G. (2023b). Enrichment of wholemeal rye bread with plant sterols: Rheological analysis, optimization of the production, nutritional profile and starch digestibility. *Foods*, *12*, 93.

Makran, M., Faubel, N., López-García, G., Cilla, A., Barberá, R., Alegría, A., & Garcia-Llatas, G. (2022). Sterol bioaccessibility in a plant sterol-enriched beverage using the INFOGEST digestion method: Influence of gastric lipase, bile salts and cholesterol esterase. *Food Chemistry*, *382*, 132305.

Makran, M., Garcia-Llatas, G., Alegría, A., & Cilla, A. (2023a). Ethylcoprostanol modulates colorectal cancer cell proliferation and mitigates cytotoxicity of cholesterol metabolites in non-tumor colon cells. *Food & Function*, *14*, 10829–10840.

Malina, D. M., Fonseca, F. A., Barbosa, S. A., Kasmas, S. H., Machado, V. A., França, C. N., ... & Izar, M. C. (2015). Additive effects of plant sterols supplementation in addition to different lipid-lowering regimens. *Journal of Clinical Lipidology*, *9*, 542–552.

Mangiola, F., Nicoletti, A., Gasbarrini, A., & Ponziani, F. R. (2018). Gut microbiota and aging. *European Review for Medical and Pharmacological Sciences*, *22*, 7404–7413.

Mannarino, E., Pirro, M., Cortese, C., Lupattelli, G., Siepi, D., Mezzetti, A., ... & Notarbartolo, A. (2009). Effects of a phytosterol-enriched dairy product on lipids, sterols and 8-isoprostane in hypercholesterolemic patients: A multicenter Italian study. *Nutrition, Metabolism and Cardiovascular Diseases*, *19*, 84-90.

Martinez-Hervas, S., Fandos, M., Real, J. T., Espinosa, O., Chaves, F. J., Saez, G. T., ... & Ascaso, J. F. (2008). Insulin resistance and oxidative stress in familial combined hyperlipidemia. *Atherosclerosis*, *199*, 384-389.

Matsuoka, K., Nakazawa, T., Nakamura, A., Honda, C., Endo, K., & Tsukada, M. (2008). Study of thermodynamic parameters for solubilization of plant sterol and stanol in bile salt micelles. *Chemistry and Physics of Lipids*, *154*, 87-93.

Matthews, G. M., Howarth, G. S., & Butler, R. N. (2012). Short-chain fatty acids induce apoptosis in colon cancer cells associated with changes to intracellular redox state and glucose metabolism. *Chemotherapy*, *58*, 102–109.

McClements, D. J., & Li, Y. (2010). Review of *in vitro* digestion models for rapid screening of emulsion-based systems. *Food & Function*, *1*, 32-59.

McKenney, J. M., Jenks, B. H., Shneyvas, E., Brooks, J. R., Shenoy, S. F., Cook, C. M., & Maki, K. C. (2014). A softgel dietary supplement containing esterified plant sterols and stanols improves the blood lipid profile of adults with primary hypercholesterolemia: A randomized, double-blind, placebo-controlled replication study. *Journal of the Academy of Nutrition and Dietetics*, *114*, 244-249.

Melchior, S., Moretton, M., Alongi, M., Calligaris, S., Nicoli, M. C., & Anese, M. (2023). Comparison of protein *in vitro* digestibility under adult and elderly conditions: The case study of wheat, pea, rice, and whey proteins. *Food Research International*, *163*, 112147.

Ménard, O., Lesmes, U., Shani-Levi, C. S., Calahorra, A. A., Lavoisier, A., Morzel, M., ... & Dupont, D. (2023). Static *in vitro* digestion model adapted to the general older adult population: An INFOGEST international consensus. *Food & Function*, *14*, 4569–4582.

Menéndez-Carreño, M., García-Herreros, C., Astiasarán, I., & Ansorena, D. (2008). Validation of a gas chromatography–mass spectrometry method for the analysis of sterol oxidation products in serum. *Journal of Chromatography B*, *864*, 61-68.

Menéndez-Carreño, M., Varo, N., Mugueta, C., Restituto, P., Ansorena, D., & Astiasarán, I. (2011). Correlation between serum content of the main COPs (cholesterol oxidation products) from autoxidation and cardiovascular risk factors. *Nutricion Hospitalaria*, *26*, 144-151.

Messner, B., & Bernhard, D. (2014). Smoking and cardiovascular disease: Mechanisms of endothelial dysfunction and early atherogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *34*, 509–515.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T. O. R. S. T. E. N., Bourlieu, C., ... & Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food - an international consensus. *Food & Function*, *5*, 1113–1124.

Minekus, M., Jelier, M., Xiao, J. Z., Kondo, S., Iwatsuki, K., Kokubo, S., ... & Havenaar, R. (2005). Effect of partially hydrolyzed guar gum (PHGG) on the bioaccessibility of fat and cholesterol. *Bioscience, Biotechnology, and Biochemistry*, *69*, 932-938.

Ministerio de Sanidad, Consumo y Bienestar Social. (2017). Encuesta Nacional de Salud ENSE: 2- Actividad física, descanso y ocio. Disponible: https://www.sanidad.gob.es/va/estadEstudios/estadisticas/encuestaNacional/encuestaNac2017/ACTIVIDAD_FISICA.pdf Acceso 15 diciembre de 2024.

Mokhtar, F. B. A., Plat, J., & Mensink, R. P. (2022). Genetic variation and intestinal cholesterol absorption in humans: A systematic review and a gene network analysis. *Progress in Lipid Research*, *86*, 101164.

Moretton, M., Alongi, M., Melchior, S., & Anese, M. (2023). Adult and elderly *in vitro* digestibility patterns of proteins and carbohydrates as affected by different commercial bread types. *Food Research International*, *167*, 112732.

Moretton, M., Casertano, M., Pellegrini, N., Anese, M., Fogliano, V., & Capuano, E. (2024). Effect of high pressure homogenization on *in vitro* digestibility and colon fermentability of pea protein-rich bread designed for elderly consumers. *Food & Function*, *15*, 10459-10471.

Murphy, N., Norat, T., Ferrari, P., Jenab, M., Bueno-de-Mesquita, B., Skeie, G., ... & Riboli, E. (2012). Dietary fibre intake and risks of cancers of the colon and rectum in the European prospective investigation into cancer and nutrition (EPIC). *PloS One*, *7*, e39361.

Murphy, A. J., Woollard, K. J., Suhartoyo, A., Stirzaker, R. A., Shaw, J., Sviridov, D., & Chin-Dusting, J. P. (2011). Neutrophil activation is attenuated by high-density lipoprotein and apolipoprotein A-I in *in vitro* and *in vivo* models of inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*, 1333-1341.

Musa-Veloso, K., Poon, T. H., Elliot, J. A., & Chung, C. (2011). A comparison of the LDL-cholesterol lowering efficacy of plant stanols and plant sterols over a continuous dose range: Results of a meta-analysis of randomized, placebo-controlled trials. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *85*, 9-28.

Muscaritoli, M., Anker, S. D., Argilés, J., Aversa, Z., Bauer, J. M., Biolo, ... & Sieber, C. C. (2010). Consensus definition of sarcopenia, cachexia, and pre-cachexia: Joint document elaborated by Special Interest Groups (SIG) ‘‘cachexia-anorexia in chronic wasting diseases’’ and ‘‘nutrition in geriatrics’’. *Clinical Nutrition, 29,* 154-159.

Nagler, R. M., & Hershkovich, O. (2005). Relationships between age, drugs, oral sensorial complaints and salivary profile. *Archives of Oral Biology*, *50*, 7–16.

Nakano, T., Inoue, I., & Murakoshi, T. (2019). A newly integrated model for intestinal cholesterol absorption and efflux reappraises how plant sterol intake reduces circulating cholesterol levels. *Nutrients*, *11*, 310.

Narayanaswamy, R., Iyer, V., Khare, P., Bodziak, M. L., Badgett, D., Zivadinov, R., ... & Browne, R. W. (2015). Simultaneous determination of oxysterols, cholesterol and 25-hydroxy-vitamin D3 in human plasma by LC-UV-MS. *PLoS One*, *10*, e0123771.

Naumann, E., Plat, J., Kester, A. D., & Mensink, R. P. (2008). The baseline serum lipoprotein profile is related to plant stanol induced changes in serum lipoprotein

cholesterol and triacylglycerol concentrations. *Journal of the American College of Nutrition*, *27*, 117-126.

Niittynen, L. H., Jauhiainen, T. A., Poussa, T. A., & Korpela, R. (2008). Effects of yoghurt enriched with free plant sterols on the levels of serum lipids and plant sterols in moderately hypercholesterolaemic subjects on a high-fat diet. *International Journal of Food Sciences and Nutrition*, *59*, 357-367.

National Institute of Standards and Technology (NIST) Database of organic chemistry compounds: Chemistry WebBook. (2015). Disponible: https://webbook.nist.gov/chemistry Acceso: 20 de enero de 2022.

Norata, G. D., & Banach, M. (2024). The impact of red yeast rice extract use on the occurrence of muscle symptoms and liver dysfunction: An update from the adverse event reporting systems and available meta-analyses. *Nutrients*, *16*, 444.

Nordlund, E., Katina, K., Mykkänen, H., & Poutanen, K. (2016). Distinct characteristics of rye and wheat breads impact on their *in vitro* gastric disintegration and *in vivo* glucose and insulin responses. *Foods*, *5*, 24.

Ogawa, K., Hirose, S., Nagaoka, S., & Yanase, E. (2016). Interaction between tea polyphenols and bile acid inhibits micellar cholesterol solubility. *Journal of Agricultural and Food Chemistry*, *64*, 204-209.

Olkkonen, V. M., Gylling, H., & Ikonen, E. (2017). Plant sterols, cholesterol precursors and oxysterols: Minute concentrations—Major physiological effects. *The Journal of Steroid Biochemistry and Molecular Biology*, *169*, 4-9.

Osadnik, T., Goławski, M., Lewandowski, P., Morze, J., Osadnik, K., Pawlas, N., ... & Banach, M. (2022). A network meta-analysis on the comparative effect of nutraceuticals on lipid profile in adults. *Pharmacological Research*, *183*, 106402.

Ottestad, I., Ose, L., Wennersberg, M. H., Granlund, L., Kirkhus, B., & Retterstøl, K. (2013). Phytosterol capsules and serum cholesterol in hypercholesterolemia: A randomized controlled trial. *Atherosclerosis*, *228*, 421-425.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry*, *49*, 4619-4626.

Párraga-Martínez, I., López-Torres-Hidalgo, J. D., del Campo-del Campo, J. M., Galdón-Blesa, M. P., Precioso-Yáñez, J. C., Rabanales-Sotos, J., ... & Lloret-Callejo, Á. (2015). Efecto a largo plazo de los estanoles vegetales en el perfil lipídico de pacientes con hipercolesterolemia. Ensayo clínico aleatorizado. *Revista Española de Cardiología*, *68*, 665-671.

Pasquier, B., Armand, M., Castelain, C., Guillon, F., Borel, P., Lafont, H., & Lairon, D. (1996). Emulsification and lipolysis of triacylglycerols are altered by viscous soluble dietary fibres in acidic gastric medium *in vitro*. *Biochemical Journal*, *314*, 269-275.

Pataj, Z., Liebisch, G., Schmitz, G., & Matysik, S. (2016). Quantification of oxysterols in human plasma and red blood cells by liquid chromatography high-resolution tandem mass spectrometry. *Journal of Chromatography a*, *1439*, 82-88.

Peters, U., Sinha, R., Chatterjee, N., Subar, A. F., Ziegler, R. G., Kulldorff, M., ... & Hayes, R. B. (2003). Dietary fibre and colorectal adenoma in a colorectal cancer early detection programme. *Lancet*, *361*, 1491–1495.

Petry, F. C., & Mercadante, A. Z. (2019). Bile amount affects both the degree of micellarization and the hydrolysis extent of carotenoid esters during *in vitro* digestion. *Food & Function*, *10*, 8250–8262.

Peyron, M. A., Sayd, T., Sicard, J., Mirade, P. S., Pinguet, J., Chambon, C., & Santé-Lhoutellier, V. (2021). Deciphering the protein digestion of meat products for the elderly by *in vitro* food oral processing and gastric dynamic digestion, peptidome analysis and modeling. *Food & Function*, *12*, 7283-7297.

Piironen, V., Toivo, J., & Lampi, A. M. (2002). Plant sterols in cereals and cereal products. *Cereal Chemistry*, *79*, 148-154.

Pinto, M., Robine-Leon, S., Appay, M. D., Kedinger, M., Triadou, N., Dussaulx, E., ... & Zweibaum, A. (1983). Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biology of the Cell*, *47*, 323-330.

Pinzón-Díaz, C. E., Calderón-Salinas, J. V., Rosas-Flores, M. M., Hernández, G., López-Betancourt, A., & Quintanar-Escorza, M. A. (2018). Eryptosis and oxidative damage in hypertensive and dyslipidemic patients. *Molecular and Cellular Biochemistry*, *440*, 105–113.

Plana, N., Nicolle, C., Ferre, R., Camps, J., Cos, R., Villoria, J., & Masana, L. (2008). Plant sterol-enriched fermented milk enhances the attainment of LDL-cholesterol goal in hypercholesterolemic subjects. *European Journal of Nutrition*, *47*, 32-39.

Planche, C., Ratel, J., Mercier, F., Zhang, C., Angénieux, M., Blinet, P., ... & Engel, E. (2022). *In vitro* assessment of polychlorinated biphenyl bioaccessibility in meat: Influence of fat content, cooking level and consumer age on consumer uptake. *Food Chemistry*, *374*, 131623.

Plat, J., Brufau, G., Dallinga-Thie, G. M., Dasselaar, M., & Mensink, R. P. (2009). A plant stanol yogurt drink alone or combined with a low-dose statin lowers serum triacylglycerol and non-HDL cholesterol in metabolic syndrome patients. *The Journal of Nutrition*, *139*, 1143–1149.

Ponz de Leon, M., Iori, R., Barbolini, G., Pompei, G., Zaniol, P., & Carulli, N. (1982). Influence of small-bowel transit time on dietary cholesterol absorption in human beings. *New England Journal of Medicine*, *307*, 102-103.

Prasadi, V. P., & Joye, I. J. (2020). Dietary fibre from whole grains and their benefits on metabolic health. *Nutrients*, *12*, 3045.

Pretorius, E., du Plooy, J. N., & Bester, J. (2016). A Comprehensive review on eryptosis. *Cellular Physiology and Biochemistry*, *39*, 1977–2000.

Primer, K. R., Psaltis, P. J., Tan, J. T., & Bursill, C. A. (2020). The role of high-density lipoproteins in endothelial cell metabolism and diabetes-impaired angiogenesis. *International Journal of Molecular Sciences*, *21*, 3633.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, *53*, 4290-4302.

Putaala, H., Mäkivuokko, H., Tiihonen, K., & Rautonen, N. (2011). Simulated colon fiber metabolome regulates genes involved in cell cycle, apoptosis, and energy metabolism in human colon cancer cells. *Molecular and Cellular Biochemistry*, *357*, 235-245.

Qian, E. W., Ge, D. T., & Kong, S. K. (2012). Salidroside protects human erythrocytes against hydrogen peroxide-induced apoptosis. *Journal of Natural Products*, *75*, 531–537.

Qiu, Z., Shi, Y., Zheng, Y., Shi, W., Zhang, L., Yin, M., & Wang, X. (2025). Comparison of *in vitro* digestive characteristics of proteins from different sources in simulated elderly gastrointestinal conditions. *Food Chemistry*, *463*, 141299.

Racette, S. B., Lin, X., Ma, L., & Ostlund Jr, R. E. (2015). Natural dietary phytosterols. *Journal of AOAC International*, *98*, 679-684.

Rana, R. B., Jilani, K., Shahid, M., Riaz, M., Ranjha, M. H., Bibi, I., Asghar, A., & Irfan, M. (2019). Atorvastatin induced erythrocytes membrane blebbing. *Dose-Response*, *17*, 1559325819869076.

Ras, R. T., Geleijnse, J. M., & Trautwein, E. A. (2014). LDL-cholesterol-lowering effect of plant sterols and stanols across different dose ranges: A meta-analysis of randomised controlled studies. *British Journal of Nutrition*, *112*, 214-219.

Real Decreto 1487/2009, de 26 de septiembre, relativo a los complementos alimenticios, que incorpora al ordenamiento jurídico interno la Directiva 2002/46/CE del Parlamento Europeo y del Consejo, de 10 de junio de 2002, relativa a la aproximación de las legislaciones de los Estados miembros en materia de complementos alimenticios. Boletín Oficial del Estado, BOEA-244, 85370-85378.

Real Decreto 130/2018, de 16 de marzo, por el que se modifica el Real Decreto 1487/2009, de 26 de septiembre, relativo a los complementos alimenticios. Boletín Oficial del Estado, BOEA-75, 33335-33342.

Reglamento 983/2009/UE. De la Comisión, de 21 de octubre de 2009, sobre la autorización o la denegación de autorización de determinadas declaraciones de propiedades saludables en los alimentos relativas a la reducción del riesgo de enfermedad y al desarrollo y la salud de los niños. Diario Oficial de la Unión Europea, L277, 3-12.

Reglamento 376/2010/UE. De la Comisión de 3 de mayo de 2010, por el que se modifica el Reglamento (CE) nº 983/2009, sobre la autorización o la denegación de autorización de determinadas declaraciones de propiedades saludables en los alimentos relativas a la reducción del riesgo de enfermedad y al desarrollo y la salud de los niños. Diario Oficial de la Unión Europea, L111, 3-4.

Reglamento 384/2010/UE. De la Comisión de 5 de mayo de 2010 sobre la autorización o denegación de autorización de determinadas declaraciones de propiedades saludables

en los alimentos relativas a la reducción del riesgo de enfermedad y al desarrollo y la salud de los niños. Diario Oficial de la Unión Europea, L113, 6-10.

Reglamento 432/2012/UE. De la Comisión de 16 de mayo de 2012 por el que se establece una lista de declaraciones autorizadas de propiedades saludables de los alimentos distintas de las relativas a la reducción del riesgo de enfermedad y al desarrollo y la salud de los niños. Diario Oficial de la Unión Europea, L13, 1-40.

Reglamento 686/2014/UE. De la Comisión de 20 de junio de 2014 por el que se modifican los Reglamentos (CE) no 983/2009 y (UE) no 384/2010 en lo que se refiere a las condiciones de uso de determinadas declaraciones de propiedades saludables al efecto de los fitoesteroles y fitoestanoles en la reducción del colesterol LDL en la sangre. Diario Oficial de la Unión Europea, L182, 27-30.

Reglamento 648/2023/UE. De la Comisión de 20 de marzo de 2023 por el que se autoriza una declaración de propiedades saludables en los alimentos relativa a la reducción del riesgo de enfermedad. Diario Oficial de la Unión Europea, L81, 8-12.

Reglamento 2041/2024/UE. De la Comisión de 29 de julio de 2024 por el que se modifica el Reglamento (UE) n.o 432/2012 en lo que respecta a la declaración de propiedades saludables sobre la monacolina K procedente del arroz fermentado con levadura roja. Diario Oficial de la Unión Europea.

Rémond, D., Shahar, D. R., Gille, D., Pinto, P., Kachal, J., Peyron, M. A., ... & Vergères, G. (2015). Understanding the gastrointestinal tract of the elderly to develop dietary solutions that prevent malnutrition. *Oncotarget*, *6*, 13858–13898.

Restivo, I., Attanzio, A., Giardina, I. C., Di Gaudio, F., Tesoriere, L., & Allegra, M. (2022b). Cigarette smoke extract induces p38 MAPK-initiated, fas-mediated eryptosis. *International Journal of Molecular Sciences*, *23*, 14730.

Restivo, I., Attanzio, A., Tesoriere, L., & Allegra, M. (2021). Suicidal erythrocyte death in metabolic syndrome. *Antioxidants*, *10*, 154.

Restivo, I., Attanzio, A., Tesoriere, L., Allegra, M., Garcia-Llatas, G., & Cilla, A. (2022a). Anti-eryptotic activity of food-derived phytochemicals and natural compounds. *International Journal of Molecular Sciences*, *23*, 3019.

Restivo, I., Attanzio, A., Tesoriere, L., Allegra, M., Garcia-Llatas, G., & Cilla, A. (2023). A mixture of dietary plant sterols at nutritional relevant serum concentration inhibits extrinsic pathway of eryptosis induced by cigarette smoke extract. *International Journal of Molecular Sciences*, *24*, 1264.

Romano, A., Giosafatto, C. V. L., Al-Asmar, A., Masi, P., Aponte, M., & Mariniello, L. (2019). Grass pea (*Lathyrus sativus*) flour: Microstructure, physico-chemical properties and *in vitro* digestion. *European Food Research and Technology*, *245*, 191-198.

Rose-Sallin, C., Huggett, A. C., Bosset, J. O., Tabacchi, R., & Fay, L. B. (1995). Quantification of cholesterol oxidation products in milk powders using [2H7] cholesterol to monitor cholesterol autoxidation artifacts. *Journal of Agricultural and Food Chemistry*, *43*, 935-941.

Roussi, S., Winter, A., Gosse, F., Werner, D., Zhang, X., Marchioni, E., ... & Raul, F. (2005). Different apoptotic mechanisms are involved in the antiproliferative effects of 7β-hydroxysitosterol and 7β-hydroxycholesterol in human colon cancer cells. *Cell Death & Differentiation*, *12*, 128-135.

Salehi, B., Quispe, C., Sharifi-Rad, J., Cruz-Martins, N., Nigam, M., Mishra, A. P., ... & Koch, W. (2021). Phytosterols: From preclinical evidence to potential clinical applications. *Frontiers in Pharmacology*, *11*, 599959.

Scharlau, D., Borowicki, A., Habermann, N., Hofmann, T., Klenow, S., Miene, C., ... & Glei, M. (2009). Mechanisms of primary cancer prevention by butyrate and other products formed during gut flora-mediated fermentation of dietary fibre. *Mutation Research/Reviews in Mutation Research*, *682*, 39-53.

Schlörmann, W., Atanasov, J., Lorkowski, S., Dawczynski, C., & Glei, M. (2020). Study on chemopreventive effects of raw and roasted β-glucan-rich waxy winter barley using an *in vitro* human colon digestion model. *Food & Function*, *11*, 2626–2638.

Schlörmann, W., Atanasov, J., Lorkowski, S., Dawczynski, C., & Glei, M. (2021). Thermal processing has no impact on chemopreventive effects of oat and barley kernels in LT97 colon adenoma cells. *Nutrition and Cancer*, *73*, 2708–2719.

Schlörmann, W., Bockwoldt, J. A., Hübner, S. M., Wittwer, E., Reiners, S., Lorkowski, S., ... & Glei, M. (2022). Use of the β-glucan-producing lactic acid bacteria strains

Levilactobacillus brevis and *Pediococcus claussenii* for sourdough fermentation-chemical characterization and chemopreventive potential of in situ-enriched wheat and rye sourdoughs and breads. *Nutrients*, *14*, 1510.

Schlörmann, W., Hiller, B., Jahns, F., Zöger, R., Hennemeier, I., Wilhelm, A., Lindhauer, M. G., & Glei, M. (2012). Chemopreventive effects of *in vitro* digested and fermented bread in human colon cells. *European Journal of Nutrition*, *51*, 827–839.

Schlörmann, W., Lamberty, J., Lorkowski, S., Ludwig, D., Mothes, H., Saupe, C., & Glei, M. (2017). Chemopreventive potential of *in vitro* fermented nuts in LT97 colon adenoma and primary epithelial colon cells. *Molecular Carcinogenesis*, *56*, 1461–1471.

Scholle, J. M., Baker, W. L., Talati, R., & Coleman, C. I. (2009). The effect of adding plant sterols or stanols to statin therapy in hypercholesterolemic patients: Systematic review and meta-analysis. *Journal of the American College of Nutrition*, *28*, 517-524.

Schött, H. F., & Lütjohann, D. (2015). Validation of an isotope dilution gas chromatography–mass spectrometry method for combined analysis of oxysterols and oxyphytosterols in serum samples. *Steroids*, *99*, 139-150.

Schröder, H., Fitó, M., Estruch, R., Martínez-González, M. A., Corella, D., Salas-Salvadó, J., ... & Covas, M. I. (2011). A short screener is valid for assessing Mediterranean diet adherence among older Spanish men and women. *The Journal of Nutrition*, *141*, 1140-1145.

Shani-Levi, C., Alvito, P., Andrés, A., Assunção, R., Barberá, R., Blanquet-Diot, S., ... & Lesmes, U. (2017). Extending *in vitro* digestion models to specific human populations: Perspectives, practical tools and bio-relevant information. *Trends in Food Science & Technology*, *60*, 52–63.

Shin, E. C., Pegg, R. B., Phillips, R. D., & Eitenmiller, R. R. (2010). Commercial peanut (*Arachis hypogaea L.*) cultivars in the United States: Phytosterol composition. *Journal of Agricultural and Food Chemistry*, *58*, 9137-9146.

Simons, L. A. (2002). Additive effect of plant sterol-ester margarine and cerivastatin in lowering low-density lipoprotein cholesterol in primary hypercholesterolemia. *The American Journal of Cardiology*, *90*, 737–740.

Singh, J., Metrani, R., Shivanagoudra, S. R., Jayaprakasha, G. K., & Patil, B. S. (2019). Review on bile acids: Effects of the gut microbiome, interactions with dietary fiber, and alterations in the bioaccessibility of bioactive compounds. *Journal of Agricultural and Food Chemistry*, *67*, 9124-9138.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture*, *16*, 144-158.

Söderholm, P. P., Alfthan, G., Koskela, A. H., Adlercreutz, H., & Tikkanen, M. J. (2012). The effect of high-fiber rye bread enriched with nonesterified plant sterols on major serum lipids and apolipoproteins in normocholesterolemic individuals. *Nutrition, Metabolism and Cardiovascular Diseases*, *22*, 575-582.

Soenen, S., Rayner, C. K., Jones, K. L., & Horowitz, M. (2016). The ageing gastrointestinal tract. *Current Opinion in Clinical Nutrition and Metabolic Care*, *19*, 12-8.

Stone, W. L., Krishnan, K., Campbell, S. E., & Palau, V. E. (2014). The role of antioxidants and pro-oxidants in colon cancer. *World Journal of Gastrointestinal Oncology*, *6*, 55.

Sukmaniah, S., Bardosono, S., Oetoro, S., Permadhi, I., & Lestarina, L. (2008). The effects of phytosterol in low fat milk on serum lipid levels among mild-moderately hypercholesterolemic subjects. *Medical Journal of Indonesia*, *17*, 5-12.

Tamargo, A., Gil-Sánchez, I., Miralles, B., Martín, D., Rodríguez García-Risco, M., Fornari, T., ... & Cueva, C. (2017). Simulador gastrointestinal dinámico (simgi®): una herramienta potencialmente útil en nutrición clínica. *Nutrición Hospitalaria*, *34*, 1489-1496.

Tamargo, A., Cueva, C., Taladrid, D., Khoo, C., Moreno-Arribas, M. V., Bartolomé, B., & de Llano, D. G. (2022). Simulated gastrointestinal digestion of cranberry polyphenols under dynamic conditions. Impact on antiadhesive activity against uropathogenic bacteria. *Food Chemistry*, *368*, 130871.

Tamargo, A., de Llano, D. G., Cueva, C., Del Hierro, J. N., Martin, D., Molinero, N., ... & Moreno-Arribas, M. V. (2023). Deciphering the interactions between lipids and red

wine polyphenols through the gastrointestinal tract. *Food Research International*, *165*, 112524.

Tesoriere, L., Attanzio, A., Allegra, M., Cilla, A., Gentile, C., & Livrea, M. A. (2014). Oxysterol mixture in hypercholesterolemia-relevant proportion causes oxidative stress-dependent eryptosis. *Cellular Physiology and Biochemistry*, *34*, 1075–1089.

Tesoriere, L., Attanzio, A., Allegra, M., & Livrea, M. A. (2015). Dietary indicaxanthin from cactus pear (*Opuntia ficus-indica* L. Mill) fruit prevents eryptosis induced by oxysterols in a hypercholesterolaemia-relevant proportion and adhesion of human erythrocytes to endothelial cell layers. *British Journal of Nutrition*, *114*, 368-375.

Trautwein, E. A., Vermeer, M. A., Hiemstra, H., & Ras, R. T. (2018). LDL-cholesterol lowering of plant sterols and stanols—which factors influence their efficacy? *Nutrients*, *10*, 1262.

Vejux, A., Abed-Vieillard, D., Hajji, K., Zarrouk, A., Mackrill, J. J., Ghosh, S., ... & Lizard, G. (2020). 7-Ketocholesterol and 7β-hydroxycholesterol: *In vitro* and animal models used to characterize their activities and to identify molecules preventing their toxicity. *Biochemical Pharmacology*, *173*, 113648.

Wang, D. Q. H. (2007). Regulation of intestinal cholesterol absorption. *Annual Review of Physiology*, *69*, 221-248.

Wang, Y., Shi, J., Xu, Y. J., Tan, C. P., & Liu, Y. (2024). The digestion fates of lipids with different unsaturated levels in people with different age groups. *Food Chemistry*, *438*, 137400.

Wong, A. (2014). Chemical and microbiological considerations of phytosterols and their relative efficacies in functional foods for the lowering of serum cholesterol levels in humans: A review. *Journal of Functional Foods*, *6*, 60-72.

Woodgate, D., Chan, C. H., & Conquer, J. A. (2006). Cholesterol-lowering ability of a phytostanol softgel supplement in adults with mild to moderate hypercholesterolemia. *Lipids*, *41*, 127-132.

World Health Organisation (WHO). (2021). Cardiovascular diseases (CVDs). Disponible: https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) Acceso: 20 de mayo de 2024.

World Health Organisation (WHO). (2022). Cancer. Disponible: https://www.who.int/news-room/fact-sheets/detail/cancer Acceso: 20 de mayo de 2024.

Weingärtner, O., Lütjohann, D., Ji, S., Weisshoff, N., List, F., Sudhop, T., ... & Laufs, U. (2008). Vascular effects of diet supplementation with plant sterols. *Journal of the American College of Cardiology*, *51*, 1553-1561.

Xiong, R. G., Zhou, D. D., Wu, S. X., Huang, S. Y., Saimaiti, A., Yang, Z. J., ... & Li, H. B. (2022). Health benefits and side effects of short-chain fatty acids. *Foods*, *11*, 2863.

Xue, Z., Gao, X., Jia, Y., Wang, Y., Lu, Y., Zhang, M., Panichayupakaranant, P., & Chen, H. (2020). Structure characterization of high molecular weight soluble dietary fiber from mushroom *Lentinula edodes* (Berk.) Pegler and its interaction mechanism with pancreatic lipase and bile salts. *International Journal of Biological Macromolecules*, *153*, 1281–1290.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., ... & Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *nature*, *486*, 222-227.

Yorulmaz, A., Velioglu, Y. S., Tekin, A., Simsek, A., Drover, J. C., & Ates, J. (2009). Phytosterols in 17 Turkish hazelnut (Corylus avellana L.) cultivars. *European Journal of Lipid Science and Technology*, *111*, 402-408.

Zeng, H., Umar, S., Rust, B., Lazarova, D., & Bordonaro, M. (2019). Secondary bile acids and short chain fatty acids in the colon: A focus on colonic microbiome, cell proliferation, inflammation, and cancer. *International Journal of Molecular Sciences*, *20*, 1214.

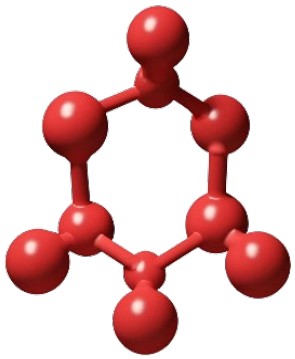
Zhang, R., Liu, W., Zeng, J., Meng, J., Jiang, H., Wang, J., & Xing, D. (2022). Niemann-Pick C1-Like 1 inhibitors for reducing cholesterol absorption. *European Journal of Medicinal Chemistry*, *230*, 114111.

Zhang, P., Iqbal, S., Deng, R., Duan, X., Han, H., Chen, X. D., & Wu, P. (2023a). Impact of elderly gastrointestinal alterations on gastric emptying and enzymatic hydrolysis of skim milk: An *in vitro* study using a dynamic stomach system. *Food Chemistry*, *402*, 134365.

Zhang, J., Liu, D., Xie, Y., Yuan, J., Wang, K., Tao, X., ... & Zhou, P. (2023b). Gastrointestinal digestibility of micellar casein dispersions: Effects of caprine vs bovine origin, and partial colloidal calcium depletion using *in vitro* digestion models for the adults and elderly. *Food Chemistry*, *416*, 135865.

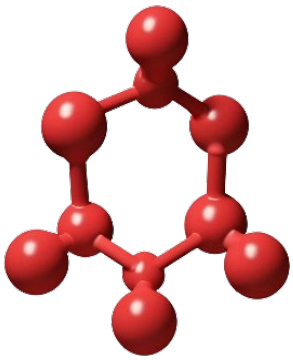
Zhao, H., Zhang, X., Wang, M., Lin, Y., & Zhou, S. (2021). Stigmasterol simultaneously induces apoptosis and protective autophagy by inhibiting Akt/mTOR pathway in gastric cancer cells. *Frontiers in Oncology*, *11*, 629008.

Zhu, Z., Guan, X., Liu, N., Zhu, X., Dai, S., Xiong, D., & Li, X. (2023). Association between dietary factors and colorectal serrated polyps: A systematic review and meta-analysis. *Frontiers in Nutrition*, *10*, 1187539.



Anexos

Annexes



Anexo I: Artículos publicados / ***Annex I: Published papers***

Trends in Food Science & Technology 129 (2022) 283–295

Contents lists available at ScienceDirect

Trends in Food Science & Technology

journal homepage: www.elsevier.com/locate/tifs

Understanding the influence of simulated elderly gastrointestinal conditions on nutrient digestibility and functional properties

Mussa Makran [1], Diego Miedes [1], Antonio Cilla [*], Reyes Barberá, Guadalupe Garcia-Llatas, Amparo Alegría

Nutrition and Food Science Area, Faculty of Pharmacy, University of Valencia, Av. Vicente Andr´es Estell´es s/n, 46100, Burjassot, Spain

ARTICLE INFO

Keywords:
Bioaccessibility
Bioactivity
In vitro digestion
Mastication
Macronutrient
Micronutrient

ABSTRACT

Background: The increase in the senior population has enhanced the interest in evaluating the food digestibility in that age group, using simulated digestion methods.
Scope and approach: The effects of gastrointestinal changes on food digestion in the elderly were reviewed. Articles with an elderly-adapted simulated gastrointestinal digestion were selected (28 of 136 screened).
Key findings and conclusions: The changes in oral physiology affect food disintegration, with consequences in perception and food digestion. Under elderly conditions (*vs.* adult), proteolysis tends to be diminished or delayed, but the lack of harmonization of methodology does not allow conclusive results to be drawn. An increase in lipid digestibility was observed, although the contribution of gastric lipase remains unknown. A lower lactose release and changes in starch hydrolysis dependent on the food matrix and system model (static or dynamic) were related. Oral phase adaptation and the inclusion of non-glycolytic enzymes should be considered in future studies. The micronutrient bioaccessibility (Ca, vitamins A, D and E) was reduced under elderly conditions. These conditions may modify the bioactivity of digesta release (enzymatic inhibitory properties and, antioxidant capacity), depending on the bioactive compound. Besides, there are scarce studies carried out on the bioaccessibility of bioactive compounds (capsaicin, plant sterols, polyphenols). All these findings could shed additional light on the development of foods adapted to the requirements of older people, reducing the incidence of malnutrition, and be useful in the design of dietary recommendations in this population group, although they need to be confirmed by further *in vivo* studies.

1. Introduction

According to the latest available data, elderly people (>65 years old) represent a fifth of the total population (20.6%) in the European Union, almost three times more than 10 years ago (2020 *vs.* 2010) (Eurostat, 2021). Older people suffer from a physiological decline, with gastrointestinal alterations derived from the ageing process. In this sense, relevant changes in oral physiology occur, such as dentition worsening and salivation reduction, accompanied by an increase in the ion and salivary amylase concentration (Khan et al., 2014; Nagler & Hershkovich, 2005). On the other hand, the ageing process slows down gastric emptying, reduces the secretion of gastric lipase and pepsin, and alkalizes the gastric environment (Holt, 2007; Moreau et al., 1988; R´emond et al., 2015; Salles, 2007). At small intestinal level, a decline in peristaltic activity and a reduction in the secretion of pancreatic enzymes and bile salts is produced (Holt & Balint, 1993; Laugier et al., 1991; Soenen et al., 2016). On the other hand, colonic health and microbiota alter as we age. In this sense, a reduction in colonic motility occurs, that predisposes to constipation (Salles, 2007). Likewise, there are changes in the colonic microbiota, among which the decrease in microbial diversity and in the ratio of Firmicutes/Bacteroidetes, reduction in *Bifidobacterium* and *Lactobacillus*, as well as of butyrate-producing species (i.e., *Eubacterium*), the increase in potentially pathogenic species (i.e., *Escherichia*), and changes in microbiome involved in the metabolism of cobalamin and folate stand out (Lee et al., 2021b; Mangiola et al., 2018; Yatsunenko et al., 2012). These gastrointestinal changes play an etiological role in age-related malnutrition (de Groot et al., 2000), which is associated with serious consequences (increased hospital stay, decreased motor capacity, worsening of quality of life or increased mortality risk) (Agarwal et al., 2013).

* Corresponding author.
E-mail address: antonio.cilla@uv.es (A. Cilla).
[1] These authors have contributed equally and must be considered as first authors.

https://doi.org/10.1016/j.tifs.2022.10.005
Received 30 May 2022; Received in revised form 30 September 2022; Accepted 5 October 2022
Available online 11 October 2022

Food & Function

ROYAL SOCIETY OF CHEMISTRY

PAPER

Cite this: *Food Funct.*, 2022, 13, 4478

Elderly gastrointestinal conditions increase sterol bioaccessibility in a plant sterol-enriched beverage: adaptation of the INFOGEST method

Diego Miedes,† Mussa Makran,† Reyes Barberá, Antonio Cilla, * Amparo Alegría and Guadalupe Garcia-Llatas

Received 23rd December 2021,
Accepted 14th March 2022

DOI: 10.1039/d1fo04375g

rsc.li/food-function

Elderly people suffer from a higher cardiovascular risk. Thus, the fortification of foods with plant sterols (PSs), which have a cholesterol-lowering function, could be of great interest for this target group. To date, no studies have analyzed how the gastrointestinal conditions of the elderly affect PS bioaccessibility. Therefore, this study evaluated the impact of the adaptation of the gastric phase alone and in combination with the intestinal phase on sterol bioaccessibility. For this purpose, the standardized INFOGEST 2.0 method previously adapted for sterol bioaccessibility evaluation in healthy adults was applied to PS-enriched milk-based fruit beverages, examining changes in enzyme activity, incubation time, agitation and pH, based on elderly physiology. The results suggest that the specific gastrointestinal conditions of the elderly could increase absorption of PSs, since their bioaccessibility (%) in a PS-enriched milk-based fruit beverage was significantly increased compared with that in adults (14.95 ± 0.33 *vs.* 7.96 ± 0.26), also indicating that these conditions increase the bioaccessibility of the beverage's own cholesterol (61.25 ± 2.91 *vs.* 20.86 ± 2.79). These data support the recommendation of foods of this type for the elderly who can benefit from the increase in bioaccessibility of PSs to have an improved potential cholesterol lowering effect, thus decreasing their risk of cardiovascular disease. However, the performance of subsequent *in vivo* tests to confirm these results is necessary.

1. Introduction

The growing elderly population worldwide has led to a higher prevalence of and mortality due to cardiovascular diseases such as acute myocardial infarction and stroke.[1,2]

Plant sterols (PSs) are of great interest due to their hypocholesterolemic effect[3] at daily intakes ranging from 1.5 to 3 g day^{-1}, although other functions such as antiproliferative,[4] anti-inflammatory[5] and immunomodulatory[6] effects have also been described. Cholesterol and PSs are hydrolyzed from possible esterified forms by pancreatic enzymes in the small intestine, such as cholesterol esterase, although the implication of pancreatic lipase cannot be discarded. The first step for their intestinal absorption is their incorporation into mixed micelles with other lipophilic compounds. Subsequently, sterols are transported from the micelles to the enterocyte by facilitated diffusion through the Niemann–Pick protein C1-like (NPC1L1). In the enterocyte, sterols are re-esterified by enzyme acyl-coenzyme-A cholesterol acyltransferase 2 (ACAT2). Nevertheless, due to the low affinity of ACAT for PSs, only a small part of them is esterified, which explains their low absorption (<5%) compared with cholesterol (40%). Afterwards, they are introduced into the chylomicrons by microsomal triglyceride transport protein (MTP) and are distributed into the bloodstream.[7]

The usual western diet does not provide the amounts of PSs with cholesterol-lowering action required and a significant interindividual variation has been reported.[8] In Europe, milk-based fruit beverages are among the permitted foods for PS enrichment.[9] This type of food has a good nutritional profile, including a low fat content and the presence of antioxidant compounds such as polyphenols, carotenoids and vitamins.[10]

In the elderly population, several factors, such as salivation being reduced by up to 3-fold,[11] tooth deficiency, gastric lipase (GL) secretion being reduced by up to 5-fold,[12] slow gastric emptying and peristalsis, hypochlorhydria, mainly due to a higher prevalence of atrophic gastritis,[13] and reduced secretion of intestinal enzymes, by up to 75% in pancreatic enzymes,[14] influence the specific conditions that aging causes in the digestion of food and, more specifically, in the bioaccessibility

Nutrition and Food Science Area, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain.
E-mail: Antonio.cilla@uv.es
†These authors have contributed equally and must be considered as first authors.

Food & Function

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2023, 14, 6012

Aging-related gastrointestinal conditions decrease the bioaccessibility of plant sterols in enriched wholemeal rye bread: *in vitro* static digestion

Diego Miedes, Mussa Makran, Antonio Cilla, * Reyes Barberá, Guadalupe Garcia-Llatas and Amparo Alegría

Received 20th February 2023,
Accepted 17th May 2023

DOI: 10.1039/d3fo00710c

rsc.li/food-function

The prevention of cardiovascular disease using foods fortified with plant sterols (PS), with a hypocholesterolemic effect, is important for the elderly population. This study aimed at identifying the different PS present in PS-enriched wholemeal rye bread (WRB) and in the ingredient source of PS, to evaluate their bioaccessibility in WRB by simulated static digestion. The gastrointestinal conditions of the elderly were adapted, and the results were compared with the adult population. Nine PS were identified, and a total amount of 2.18 g/100 g WRB was determined. Bioaccessibility was reduced in the elderly model with gastrointestinal adaptation *vs.* the adult model (11.2 *vs.* 20.3%), but no differences were observed when adapting only the gastric phase. Even though there was lower bioaccessibility of PS in the elderly, they could benefit from the consumption of WRB as it has a good nutritional profile. Further investigation including *in vivo* assays is needed to strengthen the results.

1. Introduction

In 2019, the elderly population (people over 65 years old) was 703 million persons, but it is expected to double to 1.5 billion in 2050.[1] This fact implies that a greater effort must be devoted to preventing chronic non-communicable diseases, which the elderly population has a higher risk of suffering from. Cardiovascular diseases stand out among these pathologies, being the world's biggest cause of death (up to 27% of total deaths in 2019).[2] One of the most important risk factors for these diseases is hypercholesterolemia.

The intake of 1.5 to 3 g day^{-1} of plant sterols (PS) has a hypocholesterolemic effect. It can reach between 7 and 12.5% reduction in plasma cholesterol in yellow fat spreads, dairy products, mayonnaise and salad dressings.[3] Since it is very difficult to achieve this daily intake with the usual diet, fortification of foods with these compounds may be promising. Among the foods for which the legislation allows their addition is rye bread.[4] Wholemeal rye bread stands out for being rich in fiber. Compared to wheat bread, it contains up to twice as much β-glucan, which has been shown to have a hypocholesterolemic effect that could complement that provided by PS. This effect seems to be independent of the origin of the β-glucan.[5] Also, arabinoxylans, the main component of rye fiber, have shown a hypocholesterolemic effect due to their capacity to increase the viscosity of the food bolus. In fact, fibers with high viscosity, together with their bile salt sequestration activity, could have the same effect. Therefore, rye fiber as a whole product could have a greater hypocholesterolemic effect,[6] being a beneficial matrix for the dietary prevention of cardiovascular diseases. For this reason, it could be an alternative in the diet to traditionally consumed white wheat bread.

Aging leads to distinct changes in the functioning of the gastrointestinal tract. There is a decrease in saliva secretion and a worsening of chewing force.[7,8] An increased prevalence of hypochlorhydria due to atrophic gastritis is observed in elders. Furthermore, there is a reduction in gastric and intestinal enzyme and bile salt secretion and a general slowing down of digestion.[8–11] These changes are expected to influence the digestibility of foods, their different nutrients, and bioactive compounds such as PS, so when adapting simulated digestion methods, they should be considered.

In vitro static digestion assays are economical in terms of time and money and simple to develop. They serve as a preliminary test for more expensive studies such as dynamic digestion trials or *in vivo* studies and therefore allow the screening of a huge number of matrices and parameters. The INFOGEST method was created in consensus to standardize *in vitro* static assays and allow comparison between them.[12] The method has been subsequently updated,[13] including the use of gastric lipase (GL).

To date, numerous *in vitro* studies have been carried out for determining the differences between the elderly and adult

Nutrition and Food Science Area, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain.
E-mail: Antonio.cilla@uv.es

foods

MDPI

Article

Chemopreventive Effect of an In Vitro Digested and Fermented Plant Sterol-Enriched Wholemeal Rye Bread in Colon Cancer Cells

Diego Miedes, Antonio Cilla * and Amparo Alegría

Nutrition and Food Science Area, Faculty of Pharmacy, University of Valencia, Av. Vicente Andrés Estellés s/n, 46100 Burjassot, Spain; diego.miedes@uv.es (D.M.); amparo.alegria@uv.es (A.A.)
* Correspondence: antonio.cilla@uv.es; Tel.: +34-(9635)-44229

Abstract: Diet is crucial for the prevention of colorectal cancer. Whole grains are the source of beneficial compounds for this, such as fiber. The enrichment of wholemeal rye bread with plant sterols (PSs) could increase its beneficial effects. This study aimed to assess the potential antiproliferative effect of this enriched food on colon adenocarcinoma cells (Caco-2) compared with a non-enriched one. After a human oral chewing, simulated semi-dynamic gastrointestinal digestion and colonic fermentation in a simgi® system, fermentation liquids (FLs) obtained were used as treatment for cells. Cytotoxicity assay showed that samples diluted 1/5 (*v*/*v*) with DMEM are not toxic for non-tumoral cells, whereas they damage tumoral cells. Samples with PS (FLPS) produced a higher chemopreventive effect (vs. blank) in MTT and apoptosis assays, as well as higher gene expression of *TP53* and *Casp8*. Nevertheless, FL0 (without PS) produced a higher chemopreventive effect in a cell cycle and reduced glutathione and calcium assays, besides producing higher gene expression of *Casp3* and lower *CCND1*. The distinct antiproliferative effect of both FLs is attributed to differences in PSs, short chain fatty acids (lower concentration in FLPS vs. FL0) and antioxidant compounds. These results may support wholemeal rye bread consumption as a way of reducing the risk of colorectal cancer development, although further research would be needed.

Keywords: plant sterols; antiproliferative; colonic fermentation; short chain fatty acids; Caco-2 cells; rye

Citation: Miedes, D.; Cilla, A.; Alegría, A. Chemopreventive Effect of an In Vitro Digested and Fermented Plant Sterol-Enriched Wholemeal Rye Bread in Colon Cancer Cells. *Foods* **2024**, *13*, 112. https://doi.org/10.3390/foods13010112

Academic Editor: Weiqun Wang

Received: 10 November 2023
Revised: 21 December 2023
Accepted: 27 December 2023
Published: 28 December 2023

1. Introduction

In 2020, colorectal cancer (CRC) produced almost 1 million deaths per year worldwide, representing the second cause of cancer death [1]. One of the most important risk factors for this disease is diet, which can interfere with colonic microbiota, and increase or reduce the risk of tumor formation. The intake of fruits and vegetables, rich in bioactive compounds (fiber, plant sterols (PSs), phenolic compounds, flavonoids, and sulfur compounds), is related with a lower risk of CRC development [2,3].

In many cases, prior to exerting their health beneficial effects, bioactive compounds orally ingested are released from the food matrix and/or transformed during digestion through the gastrointestinal tract. In this sense, simulated gastrointestinal digestion methods are widely used in order to evaluate the bioaccessibility and bioactivity of complex food matrices containing bioactive compounds since they can mimic human digestion conditions. The dynamic ones allow us to reproduce the human digestive conditions (pH gradient, peristalsis, or enzyme secretion) more accurately than static methods. Among the dynamic gastrointestinal digestion methods, the simgi® model consists of a multi-compartmental and continuous system, that includes five reactors (for the stomach, small intestine, and ascending, transverse, and descending colon) [4].

Dietary fiber is known for its protective effect, with many epidemiological studies supporting its preventive effect on CRC [5–7]. Fiber intakes superior to 20 g/day are related

foods

MDPI

Article

Impact of a Plant Sterol Food Supplement on Eryptotic and Associated Cardiometabolic Parameters: A Randomized Placebo-Controlled Trial in Statin-Treated Patients

Diego Miedes [1], Raquel Ortega-Luna [2], Sonia Broseta [1], Sergio Martínez-Hervás [3,4,5,6], Ángeles Álvarez-Ribelles [2,7], Víctor Collado-Díaz [2], Antonio Cilla [1,*] and Amparo Alegría [1]

[1] Nutrition and Food Science Area, Faculty of Pharmacy and Food Sciences, University of Valencia, 46100 Valencia, Spain; diego.miedes@uv.es (D.M.); sonia.broseta@uv.es (S.B.); amparo.alegria@uv.es (A.A.)
[2] Department of Pharmacology, Faculty of Medicine, University of Valencia, 46010 Valencia, Spain; raquel.ortega-luna@uv.es (R.O.-L.); angeles.alvarez@uv.es (Á.Á.-R.); victor.collado@uv.es (V.C.-D.)
[3] Endocrinology and Nutrition Department, Hospital Clínico Universitario, 46010 Valencia, Spain; sergio.martinez@uv.es
[4] Department of Medicine, University of Valencia, 46010 Valencia, Spain
[5] INCLIVA Institute of Health Research, 46010 Valencia, Spain
[6] CIBER Diabetes and Associated Metabolic Diseases (CIBERDEM), 28029 Madrid, Spain
[7] CIBER Enfermedades Hepáticas y Digestivas (CIBERehd), 28029 Madrid, Spain
* Correspondence: antonio.cilla@uv.es; Tel.: +34-(9635)-44229

Abstract: Eryptotic erythrocytes are prone to adhere to the vascular endothelium, provoking atherosclerosis. As statins do not prevent eryptosis compounds with anti-eryptotic effects could help treated hypercholesterolemic subjects in decreasing cardiovascular disease risk. Plant sterols (PSs) have shown this anti-eryptotic effect ex vivo, along with their cholesterol-lowering activity. A parallel double-blind placebo-controlled randomized trial was conducted using a PS-food supplement (2 g of PS/day) (case, n = 13) or a placebo supplement (control, n = 13) in statin-treated hypercholesterolemic subjects. Blood samples were extracted before (T0) and after (T1) a 6-week treatment, and erythrocytes were isolated for biochemical determination, phosphatidylserine externalization (EPHS), cell size and reduced glutathione (GSH) analyses, and endothelium adhesion evaluation. A reduction in glucose (4.3%) and LDL cholesterol (9.2%) was observed only in the control group, whereas in the case group, an increase in ApoA1 (6.4%) was observed. Neither EPHS, cell size nor GSH were modified by the treatment with any of the supplements, whilst endothelium adhesion was reduced (55.1%) only in the case group. These results suggest that the PS supplement may improve some cardiovascular health parameters in the target population even though eryptosis status is not modified by this treatment.

Keywords: hypercholesterolemia; erythrocytes; endothelium adhesion; atherosclerosis; phytosterols

Citation: Miedes, D.; Ortega-Luna, R.; Broseta, S.; Martínez-Hervás, S.; Álvarez-Ribelles, Á.; Collado-Díaz, V.; Cilla, A.; Alegría, A. Impact of a Plant Sterol Food Supplement on Eryptotic and Associated Cardiometabolic Parameters: A Randomized Placebo-Controlled Trial in Statin-Treated Patients. *Foods* **2024**, *13*, 4108. https://doi.org/10.3390/foods13244108

Academic Editor: Zhuohong Xie

Received: 18 November 2024
Revised: 12 December 2024
Accepted: 17 December 2024
Published: 19 December 2024

1. Introduction

The suicidal death of erythrocytes, also known as eryptosis, is a normal recycling process of the blood. Oxidative stress, osmotic shock, and energy depletion are responsible for targeting eryptosis. Likewise, some drugs and xenobiotics can effectively activate the process as well. The eryptotic event is characterized by intracellular calcium increase, cell shrinkage, ceramide accumulation, and, mainly, the externalization of phosphatidylserine (EPHS) to the outer layer of the cell membrane [1]. The disrupted membrane of red blood cells (RBCs) is prone to adhere to the vascular endothelium and platelets through the CXCL16/SR-PSOX receptor [2,3]. In this sense, an increased eryptosis leads to higher adhesion to the endothelium, an increase in the inflammatory status, and thus, atherosclerosis, which is strongly related to worse cardiovascular (CV) health [4]. Additionally, eryptosis has been associated with metabolic syndrome, another risk factor of CV disease [5].

En revision / *Under review*

Authors: Miedes, D., Mercatante, D., Broseta, S., Cilla, A., Rodriguez-Estrada, M.T., & Alegría, A.

Title: Effect of plant sterols on cholesterol oxidation products in statin-treated hypercholesterolemic individuals: A randomized clinical trial

Journal: *Journal of Food Science*

Anexo II: Materiales suplementarios / *Annex II: Supplementary materials*

Consentimiento informado / *Informed consent*

GENERALITAT VALENCIANA
CONSELLERIA DE SANIDAD UNIVERSAL Y SALUD PÚBLICA

Hospital Clínic Universitari

DEPARTAMENT CLÍNIC MALVA-ROSA

F-CE-GEva-2C V1 (25.04.2019)

HOJA DE INFORMACIÓN AL PACIENTE

TÍTULO DEL ESTUDIO: Efecto antieriptótico de la ingesta regular de un complemento alimenticio con esteroles vegetales en sujetos con hipercolesterolemia tratados con estatinas	
CÓDIGO DEL ESTUDIO	
PROMOTOR	Grupo Bionutest. Área de Nutrición y Bromatología. Facultad de Farmacia. Universitat de València en colaboración con el Servicio de Endocrinología y Nutrición del Hospital Clínico Universitario de Valencia
INVESTIGADOR PRINCIPAL	Amparo Asunción Alegría Torán y Sergio Martínez Hervás
SERVICIO	Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal.
CENTRO	Servicio de Endocrinología y Nutrición. Hospital Clínico Universitario de Valencia (INCLIVA)

Nos dirigimos a usted para informarle sobre un estudio de investigación en el que se le invita a participar. El estudio ha sido aprobado por el Comité de Ética de la Investigación de su centro, de acuerdo a la legislación vigente, Ley 14/2007, de 3 de julio, de Investigación biomédica. Nuestra intención es que usted reciba la información correcta y suficiente para que pueda decidir si acepta o no participar en este estudio. Lea esta hoja de información con atención y nosotros le aclararemos las dudas que le puedan surgir. Además, puede consultar con las personas que considere oportuno.

Así mismo, podrá solicitar cualquier explicación que desee sobre cualquier aspecto del estudio y sus implicaciones a lo largo del mismo contactando con el investigador principal del proyecto, la Dra. AMPARO ASUNCIÓN ALEGRÍA TORÁN en el teléfono 963544907 y el Dr. SERGIO MARTINEZ HERVÁS en el teléfono 961973860

1. Participación voluntaria

Le invitamos a participar en el estudio porque ha sido diagnosticado de **hipercolesterolemia.**

Debe saber que su participación en este estudio es voluntaria y que puede decidir NO participar. Si decide participar, puede cambiar su decisión y retirar el consentimiento en cualquier momento, sin que por ello se altere la relación con su médico ni se produzca perjuicio alguno en su atención sanitaria.

2. Justificación y Objetivo del estudio

El desarrollo de arteriosclerosis es el principal responsable de las enfermedades cardiovasculares, que son la principal causa de muerte a nivel mundial. La hipercolesterolemia es uno de los principales factores de riesgo, asociado a arteriosclerosis. El mecanismo exacto para su desarrollo no es totalmente conocido, y uno de los factores involucrados es la eriptosis (muerte programada de eritrocitos). Ésta se relaciona con procesos de adhesión a la pared vascular que conlleva el desarrollo de enfermedad cardiovascular y se ha sugerido como buen marcador de riesgo cardiovascular.

El objetivo de este proyecto es evaluar el potencial efecto de prevención sobre la eriptosis (factor involucrado en la arterosclerosis) tras la ingesta diaria de un complemento alimenticio que contiene esteroles vegetales en pacientes con hipercolesterolemia con tratamiento con estatinas, de forma que en un futuro se puedan desarrollar medidas nutricionales junto a las terapéuticas que permitan reducir el desarrollo de arteriosclerosis y, por tanto, supondrán un beneficio para la población.

3. Descripción del estudio

El estudio se llevará a cabo con 48 pacientes con hipercolesterolemia tratados con estatinas, con edades entre 18 y 65 años, que no tengan antecedentes de enfermedades cardiovasculares. Durante seis semanas, ingerirán diariamente un complemento alimenticio con esteroles vegetales (2 g/día) o sin ellos (placebo), según al grupo al que sean asignados (control o estudio).

Se realizarán dos extracciones de sangre (al inicio y fin del estudio) para el análisis de diferentes parámetros.

4. Actividades del estudio

Su participación en este estudio, que dura 6 semanas, incluirá 2 visitas a la consulta del Dr. Martínez del Servicio de Endocrinología y Nutrición del Hospital Clínico Universitario (Valencia), con una duración aproximada a la habitual.

En la primera visita (coincidiendo con una habitual) se le informará en que consiste el estudio, se firmará el consentimiento informado, se le solicitará permiso para la extracción y análisis de una muestra de sangre, se le realizará una encuesta sobre hábitos alimentarios y de ejercicio, y se le proporcionarán los sobres a ingerir diariamente durante 6 semanas. Trascurridas las seis semanas se programará una visita extraordinaria, se solicitará una segunda extracción y se analizará la muestra de sangre, y se proporcionará el cuestionario de adherencia, donde se informará del número de sobres no ingeridos.

La participación en el presente proyecto no supone ninguna alteración del tratamiento que esté llevando (si lo tiene) y todo tratamiento que se le pueda poner a partir de los estudios clínico-bioquímicos que se le realicen será siempre bajo criterio médico, en base a la práctica clínica habitual.

5. Riesgos y molestias derivados de su participación en el estudio

El riesgo previsible de su participación únicamente será el mínimo riesgo que conlleva la extracción de las muestras de sangre, que incluye molestias, dolor, enrojecimiento o hinchazón y/o pequeños hematomas en el lugar del brazo donde se ha producido la extracción.

6. Posibles beneficios

Es muy posible que usted no obtenga ningún beneficio para su salud por participar en este estudio, pero podrá ayudar a conocer mejor su enfermedad y mejorar el pronóstico y el tratamiento de futuros pacientes.

7. Protección de datos personales

El investigador/promotor y el centro son responsables respectivamente del tratamiento de sus datos y se comprometen a cumplir con la normativa de protección de datos en vigor, la Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y Garantía de los Derechos Digitales y el Reglamento (UE) 2016/679 del Parlamento europeo y del Consejo de 27 de abril de 2016 de Protección de Datos (RGPD).

Los datos recogidos para el estudio estarán identificados mediante un código, de manera que no incluya información que pueda identificarle, y sólo su médico del estudio/colaboradores podrá

relacionar dichos datos con usted y con su historia clínica. Por lo tanto, su identidad no será revelada a persona alguna salvo excepciones en caso de urgencia médica o requerimiento legal.

El acceso a su información personal identificada quedará restringido al médico del estudio/colaboradores, autoridades competentes, al Comité de Ética de la Investigación y personal autorizado por el promotor (monitores del estudio, auditores), cuando lo precisen para comprobar los datos y procedimientos del estudio, pero siempre manteniendo la confidencialidad de los mismos de acuerdo a la legislación vigente.

De acuerdo a lo que establece la legislación de protección de datos, usted puede ejercer los derechos de acceso, modificación, oposición y cancelación de datos, para lo cual deberá dirigirse a su médico del estudio. Si usted decide retirar el consentimiento para participar en este estudio, ningún dato nuevo será añadido a la base de datos, pero sí se utilizarán los que ya se hayan recogido.

Además, puede limitar el tratamiento de datos que sean incorrectos, solicitar una copia o que se trasladen a un tercero (portabilidad) los datos que usted ha facilitado para el estudio. Para ejercitar sus derechos, diríjase al investigador principal del estudio o al Delegado/a de Protección de Datos del centro/institución en dpd@gva.es. Así mismo tiene derecho a dirigirse a la Agencia de Protección de Datos si no quedara satisfecho.

Los datos codificados pueden ser transmitidos a terceros y a otros países, pero en ningún caso contendrán información que le pueda identificar directamente, como nombre y apellidos, iniciales, dirección, nº de la seguridad social, etc. En el caso de que se produzca esta cesión, será para los mismos fines del estudio descrito o para su uso en publicaciones científicas, pero siempre manteniendo la confidencialidad de los mismos de acuerdo a la legislación vigente.

8. INFORMACION RELATIVA A MUESTRAS BIOLÓGICAS

Su participación en este estudio conlleva la obtención y utilización de muestras biológicas con fines de investigación, para lo que se observará la Ley 14/2007 de investigación biomédica y el Real Decreto 1716/2011 de Biobancos, normativas que garantizan el respeto a los derechos que le asisten.

Al firmar este documento, revisado y evaluado favorablemente por el Comité de Ética de Investigación de su centro, usted acepta que se utilicen sus muestras para las finalidades del presente estudio.

Se van a obtener muestras de sangre para el análisis de los efectos biológicos: Evaluación de la eriptosis, adhesión de eritrocitos eriptóticos al endotelio vascular, evaluación del estado redox, concentración plasmática de óxidos de colesterol y parámetros bioquímicos y hematológicos.

8.1 Procedimientos de obtención de muestras, molestias y posibles riesgos

Las muestras serán obtenidas durante el seguimiento habitual de su enfermedad o proceso.

Se realizarán dos extracciones de sangre (antes y al finalizar el período de seis semanas de ingesta del complemento alimenticio). En cada extracción se tomarán 4 tubos (1 tubo de sangre (10ml), 3 tubos K_2EDTA (6mL) y la cantidad extraída en cada análisis será aproximadamente 28ml de sangre).

Para la mayoría de las personas, las punciones con agujas para la extracción de sangre no suponen ningún problema. Sin embargo, en ocasiones, pueden provocar hemorragias, hematomas, molestias, infecciones y/o dolor en el punto de extracción de sangre. También puede sentirse mareado.

Las muestras estarán asociadas a un código que solo podrá ser relacionado con su identidad por personal autorizado, de la misma manera que se ha explicado previamente con los datos obtenidos durante el estudio.

Los datos que se deriven de la utilización de estas muestras se tratarán del mismo modo que el resto de los datos que se obtengan durante este estudio en cuanto a la protección de datos.

Las muestras y los datos asociados se mantendrán bajo las condiciones de seguridad adecuadas y se garantiza que los sujetos no podrán ser identificados a través de medios considerados razonables por personas distintas a las autorizadas.

Es posible que sea necesario algún dato o muestras adicionales. En ese caso, su médico se pondrá en contacto con usted para solicitarle de nuevo su colaboración. Se le informará de los motivos y se le solicitará de nuevo su consentimiento.

8.2 Beneficios esperados

No se espera un beneficio directo por su participación en el estudio. No obstante, los conocimientos obtenidos gracias a los estudios llevados a cabo a partir de sus muestras y de muchas otras pueden ayudar al avance científico.

No percibirá ningún beneficio económico por la donación de las muestras y la cesión de los datos proporcionados, ni tendrá derechos sobre posibles beneficios comerciales de los descubrimientos que puedan conseguirse como resultado de la investigación efectuada.

8.3. Lugar de análisis y almacenamiento de muestras

Durante el desarrollo del estudio sus muestras pueden ser analizadas en dos departamentos de la Universitat de València: Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal de la Facultad de Farmacia (Grupo Bionutest) y de Farmacología de la Facultad de Medicina, así como en el Departamento Ciencias de la Agricultura y de la Alimentación de Alma Mater Studiorum de la Universidad de Bolonia (Unibo).

Durante este proceso el responsable de las muestras será el promotor/investigador del estudio.

8.4. Implicaciones de la información obtenida al analizar las muestras

En el caso de que usted lo solicite, se le podrá facilitar información acerca de los estudios generales del presente estudio.

En el caso de que en este estudio se obtengan datos que pudieran ser clínica o genéticamente relevantes para usted, e interesar a su salud o a la de su familia, podrá solicitar que le sean comunicados por su médico del estudio.

No obstante, si usted manifiesta su negativa ser informado, pero según criterio del médico responsable, la información obtenida sea necesaria para evitar un grave perjuicio para su salud o la de sus familiares biológicos, se informará a un familiar próximo o a un representante, previa consulta al Comité de Ética Asistencial del centro. La comunicación de esta información se llevará a cabo por profesionales que le podrán explicar adecuadamente su relevancia y las opciones que se pudieran plantear. En caso de información genética clínicamente relevante podrá recibir el preceptivo consejo genético.

8.5. Uso futuro de las muestras

Una vez finalizado el estudio, las muestras sobrantes serán destruidas

GENERALITAT VALENCIANA
CONSELLERIA DE SANIDAD UNIVERSAL Y SALUD PÚBLICA

Hospital Clínic Universitari

DEPARTAMENT CLÍNIC MALVA-ROSA

F-CE-GEva-2C V1 (25.04.2019)

CONSENTIMIENTO INFORMADO

TÍTULO DEL ESTUDIO: Efecto antieriptótico de la ingesta regular de un complemento alimenticio con esteroles vegetales en sujetos con hipercolesterolemia tratados con estatinas	
CÓDIGO DEL ESTUDIO	
PROMOTOR	Grupo Bionutest. Área de Nutrición y Bromatología Facultad de Farmacia. Universitat de València en colaboración con el Servicio de Endocrinología y Nutrición del Hospital Clínico Universitario de Valencia
INVESTIGADOR PRINCIPAL	Amparo Asunción Alegría Torán y Sergio Martínez Hervás
SERVICIO	Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal.
CENTRO	Servicio de Endocrinología y Nutrición. Hospital Clínico Universitario de Valencia (INCLIVA)

Yo, _____ <<nombre y apellidos del participante>> (Nombre de puño y letra por el paciente)

He leído la hoja de información que se me ha entregado sobre el estudio.

He podido hacer preguntas sobre el estudio.

He recibido suficiente información sobre el estudio.

He hablado con _____<<nombre del investigador>>(Nombre de puño y letra por el paciente)

Comprendo que mi participación es voluntaria.

Comprendo que puedo retirarme del estudio:

- Cuando quiera.
- Sin tener que dar explicaciones.
- Sin que esto repercuta en mis cuidados médicos.

Presto libremente mi conformidad para participar en el estudio.

Consiento al uso y tratamiento de mis datos personales para esta investigación en las condiciones explicadas en esta hoja de información.

Uso de Muestras

Consiento ~~el~~ uso de las muestras y de los datos asociados para esta investigación en las condiciones explicadas en esta hoja de información.

☐ SI ☐ NO

Deseo que el médico del estudio me comunique la información derivada de la investigación (genética o no genética, a matizar dependiendo del caso) que pueda ser relevante y aplicable para mi salud o la de mis familiares:

☐ SI ☐ NO Teléfono o e-mail de contacto _____

Consiento a ser contactado en el caso de necesitar más información o muestras biológicas adicionales.

☐ SI ☐ NO Teléfono o e-mail de contacto _____

Recibiré una copia firmada y fechada de este documento de consentimiento informado

Firma del participante

Fecha: ____/____/____

Firma del investigador

Fecha: ____/____/____

(firma y fecha de puño y letra por el paciente)

GENERALITAT VALENCIANA
CONSELLERIA DE SANIDAD UNIVERSAL Y SALUD PÚBLICA

Hospital Clínic Universitari

DEPARTAMENT CLÍNIC MALVA-ROSA

F-CE-GEva-2C V1 (25.04.2019)

CONSENTIMIENTO INFORMADO REPRESENTANTE LEGAL

TÍTULO DEL ESTUDIO: Efecto antieriptótico de la ingesta regular de un complemento alimenticio con esteroles vegetales en sujetos con hipercolesterolemia tratados con estatinas	
CÓDIGO DEL ESTUDIO	
PROMOTOR	Grupo Bionutest. Área de Nutrición y Bromatología. Facultad de Farmacia. Universitat de València en colaboración con el Servicio de Endocrinología y Nutrición del Hospital Clínico Universitario de Valencia
INVESTIGADOR PRINCIPAL	Amparo Asunción Alegría Torán y Sergio Martínez Hervás
SERVICIO	Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal.
CENTRO	Servicio de Endocrinología y Nutrición. Hospital Clínico Universitario de Valencia (INCLIVA)

Yo, _____<<nombre y apellidos del representante>>, (Nombre de puño y letra por el representante) en calidad de _____ <<indicar parentesco>>, de _____<<nombre y apellidos del participante>> (Nombre de puño y letra por el representante)

He leído la hoja de información que se me ha entregado sobre el estudio.

He podido hacer preguntas sobre el estudio.

He recibido suficiente información sobre el estudio.

He hablado con _____<<nombre del investigador>> (Nombre de puño y letra por el representante)

Comprendo que su participación es voluntaria.

Comprendo que puede retirarse del estudio:

- Cuando quiera.
- Sin tener que dar explicaciones.
- Sin que esto repercuta en sus cuidados médicos.

Presto libremente mi conformidad para su participación en el estudio

Consiento al uso y tratamiento de sus datos personales para esta investigación en las condiciones explicadas en esta hoja de información.

Uso de Muestras

Consiento del uso de las muestras y de los datos asociados para esta investigación en las condiciones explicadas en esta hoja de información.

☐ SI ☐ NO

Deseo que el médico del estudio me comunique la información derivada de la investigación (genética o no genética, a matizar dependiendo del caso) que pueda ser relevante y aplicable para mi salud o la de mis familiares:

☐ SI ☐ NO Teléfono o e-mail de contacto _____

Consiento a ser contactado en el caso de necesitar más información o muestras biológicas adicionales.

☐ SI ☐ NO Teléfono o e-mail de contacto _____

Recibirá una copia firmada y fechada de este documento de consentimiento informado

Firma del representante legal, familiar o persona vinculada de hecho

Firma del investigador

Fecha: ____/____/____ Fecha: ____/____/____

(Firma y fecha de puño y letra por el representante)

GENERALITAT VALENCIANA
CONSELLERIA DE SANIDAD UNIVERSAL Y SALUD PÚBLICA

Hospital Clínic Universitari

DEPARTAMENT CLÍNIC MALVA-ROSA

F-CE-GEva-2C V1 (25.04.2019)

CONSENTIMIENTO INFORMADO ANTE TESTIGOS

TÍTULO DEL ESTUDIO: Efecto antieriptótico de la ingesta regular de un complemento alimenticio con esteroles vegetales en sujetos con hipercolesterolemia tratados con estatinas	
CÓDIGO DEL ESTUDIO	
PROMOTOR	Grupo Bionutest. Área de Nutrición y Bromatología. Facultad de Farmacia. Universitat de València en colaboración con el Servicio de Endocrinología y Nutrición del Hospital Clínico Universitario de Valencia
INVESTIGADOR PRINCIPAL	Amparo Asunción Alegría Torán y Sergio Martínez Hervás
SERVICIO	Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal.
CENTRO	Servicio de Endocrinología y Nutrición. Hospital Clínico Universitario de Valencia (INCLIVA)

Yo, _____<<nombre y apellidos del testigo>>,

(Nombre de puño y letra por el testigo)

como testigo, afirmo que en mi presencia se ha informado a D/Dª _____

_____ <<nombre y apellidos del participante>>

(Nombre de puño y letra por el testigo)

y se ha leído la hoja de información que se le ha entregado sobre el estudio, de modo que:

Ha podido hacer preguntas sobre el estudio.
Ha recibido suficiente información sobre el estudio.
Ha hablado con _____ <<nombre del investigador>>

(Nombre de puño y letra por el testigo)

Comprende que su participación es voluntaria.
Comprende que puede retirarse del estudio:
- Cuando quiera.
- Sin tener que dar explicaciones.
- Sin que esto repercuta en sus cuidados médicos.

Presta libremente su conformidad para su participación en el estudio.
Consiente al uso y tratamiento de sus datos personales para esta investigación en las condiciones explicadas en esta hoja de información.

Uso de Muestras

Consiente el uso de las muestras y de los datos asociados para esta investigación en las condiciones explicadas en esta hoja de información.

☐ SI ☐ NO

Desea que el médico del estudio me comunique la información derivada de la investigación (genética o no genética, a matizar dependiendo del caso) que pueda ser relevante y aplicable para mi salud o la de mis familiares:

☐ SI ☐ NO Teléfono o e-mail de contacto _____

Consiente a ser contactado en el caso de necesitar más información o muestras biológicas adicionales.

☐ SI ☐ NO Teléfono o e-mail de contacto _____

Recibirá una copia firmada y fechada de este documento de consentimiento informado

Firma del testigo

Fecha: ____/____/____

(Firma y fecha de puño y letra por el testigo

Firma del investigador

Fecha: ____/____/____

Cuestionarios de hábitos de vida / *Lifestyle surveys*

Los datos personales que usted facilita se tratarán de acuerdo al punto 7 del informe de consentimiento (protección de datos personales) de acuerdo a la Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y Garantía de los Derechos Digitales y el Reglamento (UE) 2016/679 del Parlamento europeo y del Consejo de 27 de abril de 2016 de Protección de Datos (RGPD).

Nombre y apellidos: _____

SIP: _____

Sexo: Hombre [ ] Mujer [ ]

Edad: _____

Peso (kg): _____

Talla (m): _____

Nº paciente: _____

Fecha inicio: _____

Cuestionario de adherencia a la Dieta Mediterránea

Pregunta	Valoración	Puntos
1. ¿Usa el aceite de oliva como principal grasa para cocinar?	Sí = 1 punto	
2. ¿Cuánto aceite de oliva consume en total al día? (incluyendo el usado para freír, comidas fuera de casa, ensaladas, etc.)	2 o más cucharadas = 1 punto	
3. ¿Cuántas raciones de verduras u hortalizas consume al día? (1 ración = 200 g. Las guarniciones o acompañamientos = ½ ración)	2 o más (al menos 1 de ellas en ensalada o crudas) = 1 punto	
4. ¿Cuántas piezas de fruta (incluyendo zumo natural) consume al día?	3 o más = 1 punto	
5. ¿Cuántas raciones de carnes rojas, hamburguesas, salchichas o embutidos consume al día? (1 ración = 100-150 g)	Menos de 1 = 1 punto	
6. ¿Cuántas raciones de mantequilla, margarina o nata consume al día? (porción individual = 12 g)	Menos de 1 = 1 Punto	
7. ¿Cuántas bebidas carbonatadas y/o azucaradas consume al día? (refrescos, colas, tónicas, bitter)	Menos de 1 = 1 Punto	
8. ¿Bebe vino? ¿Cuánto consume a la semana?	3 o más vasos = 1 Punto	
9. ¿Cuántas raciones de legumbres consume a la semana? (1 plato o ración = 150 g)	3 o más = 1 punto	
10. ¿Cuántas raciones de pescado/mariscos consume a la semana? (1 plato, pieza o ración = 100-150 g de pescado o 4-5 piezas o 200 g de marisco)	3 o más = 1 punto	
11. ¿Cuántas veces consume repostería comercial a la semana? (no casera, como: galletas, flanes, dulces, bollería, pasteles)	Menos de 3 = 1 punto	
12. ¿Cuántas veces consume frutos secos a la semana (1 ración = 30 g)?	1 o más = 1 punto	
13. ¿Consume preferentemente carne de pollo, pavo o conejo en vez de ternera, cerdo, hamburguesas o salchichas? (carne de pollo, pavo o conejo: 1 pieza o ración de 100-150 g)	Sí = 1 punto	
14. ¿Cuántas veces a la semana consume los vegetales cocinados, la pasta, arroz u otros platos aderezados con salsa de tomate, ajo, cebolla o puerro elaborada a fuego lento con aceite de oliva? (sofrito)	2 o más = 1 punto	

Cuestionario de actividad física

Piense acerca de todas aquellas actividades **vigorosas** que usted realizó en los últimos 7 días. Actividades vigorosas son las que requieren un esfuerzo físico fuerte y le hacen respirar mucho más fuerte que lo normal. Piense *solamente* en esas actividades que usted hizo por lo menos 10 minutos continuos.

1. Durante los últimos 7 días, ¿Cuántos días realizó usted actividades físicas vigorosas como levantar objetos pesados, excavar, aeróbicos, o pedalear rápido en bicicleta?
- _____ días por semana
- Ninguna actividad física vigorosa (*Pase a la pregunta 3*)
2. ¿Cuánto tiempo en total usualmente le tomó realizar actividades físicas vigorosas en uno de esos días que las realizó?
- _____ horas por día
- _____ minutos por día
- No sabe/No está seguro(a)

Piense acerca de todas aquellas actividades **moderadas** que usted realizo en los últimos 7 días. Actividades moderadas son aquellas que requieren un esfuerzo físico moderado y le hace respirar algo más fuerte que lo normal. Piense *solamente* en esas actividades que usted hizo por lo menos 10 minutos continuos.

3. Durante los últimos 7 días, ¿Cuántos días hizo usted actividades físicas moderadas tal como cargar objetos livianos, pedalear en bicicleta a paso regular, o jugar dobles de tenis? No incluya caminatas.
- _____ días por semana
- Ninguna actividad física moderada (*Pase a la pregunta 5*)
4. Usualmente, ¿Cuánto tiempo dedica usted en uno de esos días haciendo actividades físicas moderadas?
- _____ horas por día
- _____ minutos por día
- No sabe/No está seguro(a)

Piense acerca del tiempo que usted dedicó a **caminar** en los últimos 7 días. Esto incluye trabajo en la casa, caminatas para ir de un sitio a otro, o cualquier otra caminata que usted hizo únicamente por recreación, deporte, ejercicio, o placer.

5. Durante los últimos 7 días, ¿Cuántos días caminó usted por al menos 10 minutos continuos?
- _____ días por semana
- No caminó (*Pase a la pregunta 7*)
6. Usualmente, ¿Cuánto tiempo gastó usted en uno de esos días caminando?
- _____ horas por día

- _____ minutos por día
- No sabe/No está seguro(a)

La última pregunta se refiere al tiempo que usted permaneció **sentado(a)** en la semana en los últimos 7 días. Incluya el tiempo sentado(a) en el trabajo, la casa, estudiando, y en su tiempo libre. Esto puede incluir tiempo sentado(a) en un escritorio, visitando amigos(as), leyendo o permanecer sentado(a) o acostado(a) mirando televisión.

7. Durante los últimos 7 días, ¿Cuánto tiempo permaneció sentado(a) en un día en la semana?
- _____ horas por día
- _____ minutos por día
- No sabe/No está seguro(a)

Anexo III: Comunicaciones a congresos / *Annex III: Congress communications*

Anexo III: Comunicaciones a congresos / *Annex III: Congress communications*

Autores: Miedes, D.; Makran, M.; Barberá, R.; Cilla, A.; Alegría, A.; Garcia-Llatas, G.

Título: Caracterización de esteroles vegetales en pan de centeno integral enriquecido

Tipo de participación: Comunicación oral

Congreso: IX Congreso Estudiantes Nutrición Humana y Dietética (ADINU)

Lugar: Valencia (España)

Año: 2022

Autores: Makran, M.; Miedes, D.; Barberá, R.; Cilla, A.; Alegría, A.; Garcia-Llatas, G.

Título: Plant sterols as functional ingredients for elderly: bioaccessibility evaluation from different matrices using the INFOGEST 2.0 simulated gastrointestinal digestion

Tipo de participación: Comunicación oral

Congreso: FORTHEM Food Science workshop

Lugar: Palermo (Italia)

Año: 2022

Autores: Miedes, D.; Makran, M.; Garcia-Llatas, G.; Barberá, R.; Cilla, A.; Alegría, A.

Título: Adaptation of the INFOGEST digestion for the elderly population to assess sterol bioaccessibility in a plant sterol-enriched wholemeal rye bread

Tipo de participación: Comunicación oral

Congreso: The 3rd International Electronic Conference on Foods: Food, Microbiome, and Health - A Celebration of the 10th Anniversary of Foods' Impact on Our Wellbeing

Lugar: online

Año: 2022

Autores: Miedes, D.; Cilla, A.; Alegría, A.

Título: Efecto antiproliferativo en células de cáncer de colon de un pan de centeno integral enriquecido con esteroles vegetales

Tipo de participación: Póster

Congreso: I Congreso de CyTAV sobre Innovación Alimentaria

Lugar: Valencia (España)

Año: 2023

Autores: Makran, M.; Miedes, D.; Cilla, A.; Barberá, R.; Garcia-Llatas, G.; Alegría, A.

Título: Efecto de los cambios en la fisiología gastrointestinal de la población anciana sobre la actividad biológica y bioaccesibilidad de compuestos bioactivos

Tipo de participación: Póster

Congreso: I Congreso de CyTAV sobre Innovación Alimentaria

Lugar: Valencia (España)

Año: 2023

Autores: Miedes, D.; Makran, M.; Cilla, A.; Barberá, R.; Garcia-Llatas, G.; Alegría, A.

Título: Efectos de la fisiología digestiva del anciano en la digestibilidad de nutrientes

Tipo de participación: Póster

Congreso: X Congreso Estudiantes Nutrición Humana y Dietética (ADINU)

Lugar: Valencia (España)

Año: 2023

Autores: Miedes, D.; Cilla, A.; Alegría, A.

Título: Chemopreventive effect of colonic fermentation supernatants of a wholemeal rye bread with/without plant sterols in colon adenocarcinoma cells

Tipo de participación: Póster

Congreso: The 4th International Conference on Food Bioactives & Health

Lugar: Praga (República Checa)

Año: 2023

Autores: Miedes, D.; Cilla, A.; Alegría, A.

Título: Modulation of apoptotic gene expression by a plant sterol-enriched rye bread digesta in Caco-2 cells

Tipo de participación: Póster

Congreso: The 37th EFFosT International Conference

Lugar: Valencia (España)

Año: 2023

Autores: Miedes, D.; Broseta, S.; Cilla, A.; Alegría, A.

Título: Evaluation of the effect of plant sterol food supplement intake on eryptotic markers in statin-treated hypercholesterolemic patients

Tipo de participación: Póster

Congreso: The 5th International Electronic Conference on Foods

Lugar: online

Año: 2024

Autores: Miedes, D.; Mercatante, D.; Broseta, S.; Cilla, A.; Rodriguez-Estrada, M.T.; Alegría, A.

Título: Assessment of cholesterol oxidation products in statin-treated subjects after a plant sterol-food supplement intake

Tipo de participación: Póster

Congreso: The 5th International Conference on Food Bioactives & Health

Lugar: Marsella (Francia)

Año: 2024
