VNIVERSITAT DE VALÈNCIA

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

Influencia de los galactooligosacáridos sobre la biodisponibilidad y fermentación colónica de esteroles

DOCTORADO EN CIENCIAS DE LA ALIMENTACIÓN

Presentada por:

Virginia Blanco Morales

Dirigida por:

Dra. Guadalupe García Llatas

Dra. Amparo Alegría Torán

Burjassot, diciembre 2020

Las Dras. Guadalupe García Llatas y Amparo Alegría Torán, Profesora Titular y Catedrática, respectivamente, del Área de Nutrición y Bromatología del Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal, de la Universitat de València

CERTIFICAN QUE:

La graduada en Nutrición Humana y Dietética Dña. Virginia Blanco Morales ha realizado bajo su dirección el trabajo que lleva por título **"Influencia de los galactooligosacáridos sobre la biodisponibilidad y fermentación colónica de esteroles"**. El trabajo ha dado lugar a cinco artículos y un capítulo de libro en los cuales firma como primer autor:

1. *Safe intake of a plant-sterol enriched beverage with milk fat globule membrane: Bioaccessibility of sterol oxides during storage. Journal of Food Composition & Analysis (2018), 68, 111-117.*
   Índice de impacto JCR (2017): 2,956 (32/133) Q1. Food Science & Technology.

2. *The impact of galactooligosaccharides on the bioaccessibility of sterols in a plant sterol-enriched beverage: Adaptation of the harmonized INFOGEST digestion method. Food & Function (2018), 9, 2080-2089.*
   Índice de impacto JCR (2018): 3,241 (31/135) Q1. Food Science & Technology.

3. *In vitro colonic fermentation of a plant sterol-enriched beverage in a dynamic-colonic gastrointestinal digester. LWT-Food Science & Technology, en revisión.*
   Índice de impacto JCR (2019): 4,006 (28/139) Q1. Food Science & Technology.

4. *Impact of a plant sterol- and galactooligosaccharide-enriched beverage on colonic metabolism and gut microbiota composition using an in vitro dynamic model. Journal of Agricultural & Food Chemistry (2020), 68, 1884-1895.*
   Índice de impacto JCR (2019): 4,192 (21/139) Q1. Food Science & Technology.

5. *Influence of GOS on the positive effect of plant sterol-enriched beverages on cardiovascular risk and their colon metabolism: A randomized, double-blind crossover trial. Clinical Nutrition, en revisión.*
   Índice de impacto JCR (2019): 6,360 (9/89) Q1. Nutrition & Dietetics.

6. *Sterol digestion in plant sterol-enriched foods: Bioaccessibility and fermentation. In: Bioaccessibility and digestibility of lipids from foods. Springer (2021), doi: 10.1007/978-3-030-56909-9 (pendiente de publicación).*

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

Y para que conste a los efectos oportunos,

En Burjassot, 16 de diciembre de 2020

Fdo: Guadalupe García Llatas

Fdo: Amparo Alegría Torán

Las Dras. Guadalupe García Llatas y Amparo Alegría Torán, Profesora Titular y Catedrática, respectivamente, del Área de Nutrición y Bromatología del Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal, de la Universitat de València, en calidad de directoras de la tesis doctoral de Dña. Virginia Blanco Morales

INFORMAN QUE:

La graduada en Nutrición Humana y Dietética Dña. Virginia Blanco Morales es co-autora de los artículos que se muestran a continuación y en los que se indica expresamente que debe ser considerada como primera autora de los trabajos. A continuación, se detalla su contribución a los mismos:

- "*Safe intake of a plant-sterol enriched beverage with milk fat globule membrane: Bioaccessibility of sterol oxides during storage*"

Dña. Virginia Blanco Morales participó en la determinación de productos de oxidación de esteroles en las fracciones bioaccesibles correspondientes a la bebida objeto de estudio a distintos tiempos de almacenamiento.

La redacción completa de las distintas secciones del artículo (introducción, material y métodos, resultados y discusión y bibliografía), incluyendo el diseño de las figuras y los gráficos, y la posterior revisión realizada en base a lo indicado por los recensores para la publicación del trabajo se realizó a partes iguales entre ambos co-autores.

Por su parte, Dña. Andrea Álvarez-Sala Martín, como se indicó en la tesis doctoral defendida en la Universitat de València en 2018 y que lleva por título "Biodisponibilidad y efectos biológicos de esteroles en bebidas funcionales", contribuyó a: "la parte experimental, evaluación de resultados y discusión, así como a la redacción del artículo".

- "*The impact of galactooligosaccharides on the bioaccessibility of sterols in a plant sterol-enriched beverage: Adaptation of the harmonized INFOGEST digestion method*"

En este trabajo, Dña. Virginia Blanco Morales llevó a cabo la determinación de esteroles (colesterol, campesterol, campestanol, estigmasterol, β-sitosterol y sitostanol) por cromatografía de gases acoplada a un detector de ionización de llama en las bebidas a base de zumo de frutas y leche enriquecidas con esteroles vegetales con o sin adición de galactooligosacáridos. Además, contribuyó en la aplicación del método de digestión gastrointestinal micelar y de las dos modificaciones propuestas para el método Infogest a las bebidas objeto de estudio, y determinó el

contenido de esteroles en las correspondientes fracciones bioaccesibles por cromatografía de gases acoplada a un detector de ionización de llama.

Por su parte, D. Gabriel López García evaluó la actividad enzimática de las enzimas digestivas (etapas salivar, gástrica e intestinal) del método Infogest y determinó el contenido de sales biliares en los extractos biliares utilizados en la etapa intestinal. Además, llevó a cabo la aplicación del método estandarizado Infogest a las bebidas objeto de estudio y la determinación del contenido de esteroles en sus fracciones bioaccesibles por cromatografía de gases acoplada a un detector de ionización de llama.

El análisis estadístico de los datos, la redacción completa de las distintas secciones del artículo (introducción, material y métodos, resultados y discusión y bibliografía), incluyendo el diseño de las figuras y los gráficos, y la posterior corrección realizada en base a lo indicado por los recensores para la publicación del trabajo se realizó a partes iguales entre ambos co-autores.

Finalmente, indicar que este trabajo ha sido utilizado en la tesis doctoral defendida en la Universitat de València en 2020 por D. Gabriel López García y que lleva por título “Bioactividad en dianas terapéuticas sistémicas e intestinales de una bebida funcional conteniendo β-criptoxantina, esteroles vegetales y galactooligosacáridos”.

Y para que conste a los efectos oportunos,

En Burjassot, 16 de diciembre de 2020

Fdo: Guadalupe García Llatas

Fdo: Amparo Alegría Torán

Las Dras. Guadalupe García Llatas y Amparo Alegría Torán, Profesora Titular y Catedrática, respectivamente, del Área de Nutrición y Bromatología del Departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal, de la Universitat de València, en calidad de directoras de la tesis doctoral de Dña. Virginia Blanco Morales

INFORMAN QUE:

La presente Tesis Doctoral se ha realizado en el grupo Bionutest (GIUV2013-067) y se enmarca en los proyectos AGL2012-39503-C02-01 y AGL2015-68006-C2-1-R financiados por el Ministerio de Economía y Competitividad (MINECO) y el Fondo Europeo de Desarrollo Regional (FEDER).

Dña. Virginia Blanco Morales ha disfrutado de un contrato de tipo "Investigador no doctor" (Ref.CPI-17-025) a cargo del proyecto AGL2015-68006-C2-1-R financiado por MINECO-FEDER desde mayo de 2017 hasta octubre de 2019.

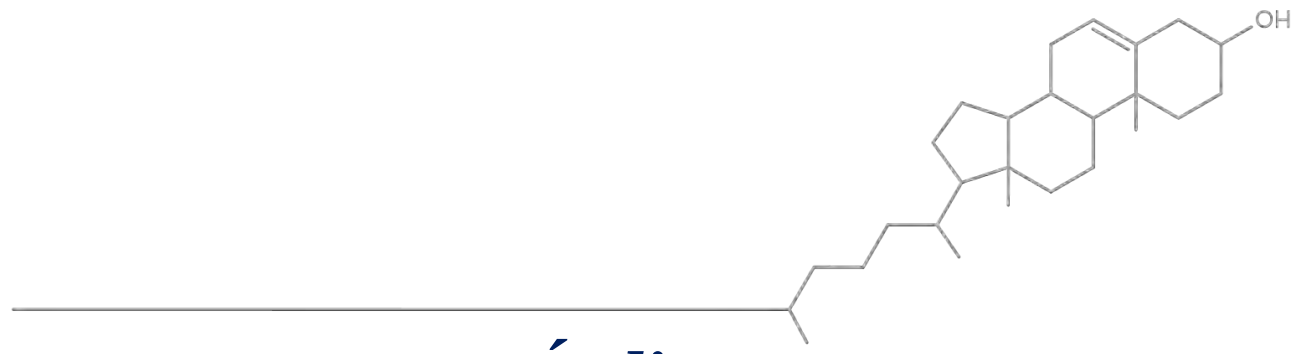
En Burjassot, 16 de diciembre de 2020

Fdo: Guadalupe García Llatas

Fdo: Amparo Alegría Torán

“La creación intelectual es el más misterioso y solitario de los oficios humanos”

Gabriel García Márquez



Índices

ÍNDICE GENERAL

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ÍNDICE DE TABLAS

ABREVIATURAS

ABCG5/8: transportadores Casete de unión Adenosina Trifosfato G5 y 8

ABCB1a/b: transportadores Casete de unión Adenosina Trifosfato B1 a y b

ABCA1: transportadores Casete de unión Adenosina Trifosfato A1

ABCG1: transportadores Casete de unión Adenosina Trifosfato G1

ACAT2: acil-coenzima A: colesterol aciltransferasa 2

AGCC: ácidos grasos de cadena corta

c-LDL: colesterol transportado en lipoproteínas de baja densidad

c-HDL: colesterol transportado en lipoproteínas de alta densidad

c-VLDL: colesterol transportado en lipoproteínas de muy baja densidad

COPs: productos de oxidación de colesterol

DGM: *Dinamic Gastric Model*

E-FA: ésteres de fitostanoles

E-FE: ésteres de fitosteroles

EV: esteroles vegetales

FSG: fluido sintético gástrico

FSI: fluido sintético intestinal

FSO: fluido sintético oral

GOS: galactooligosacáridos

HGS: *Human Gastric Simulator*

ISAPP: Asociación Científica Internacional de Probióticos y Prebióticos

MFGM: membrana del glóbulo graso de la leche

MTP: proteína microsomal de transferencia de triglicéridos

NPC1L1: proteína transportadora Niemann-Pick C1-like1

PolyFerms: *Polyfermentor Intestinal Model*

POPs: productos de oxidación de esteroles vegetales

R1: reactor colon ascendente

R2: reactor colon transversal

R3: reactor colon descendente

SHIME: *Simulator of the Human Intestinal Microbial Ecosystem*

SIMGI: *SIMulator Gastro-Intestinal*

SOPs: productos de oxidación de esteroles

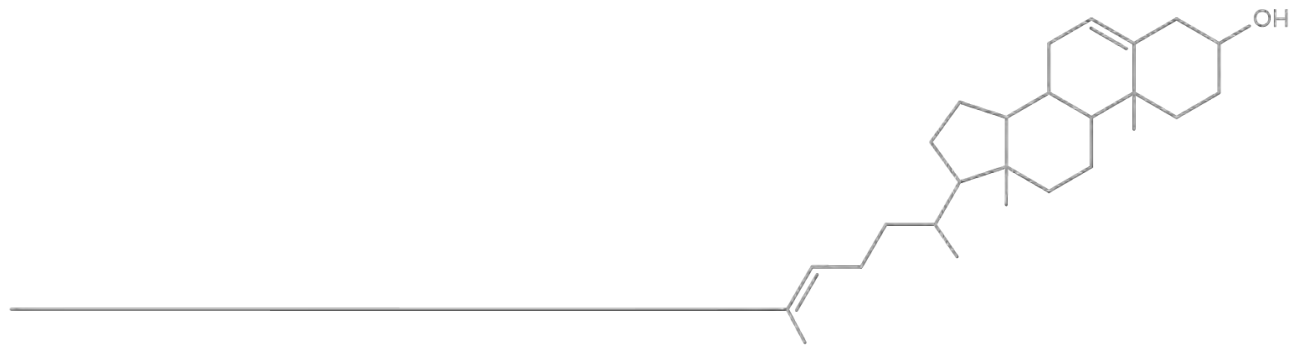
TICE: vía de excreción transintestinal de colesterol

TIM-1: *TNO Gastro-intestinal Model*

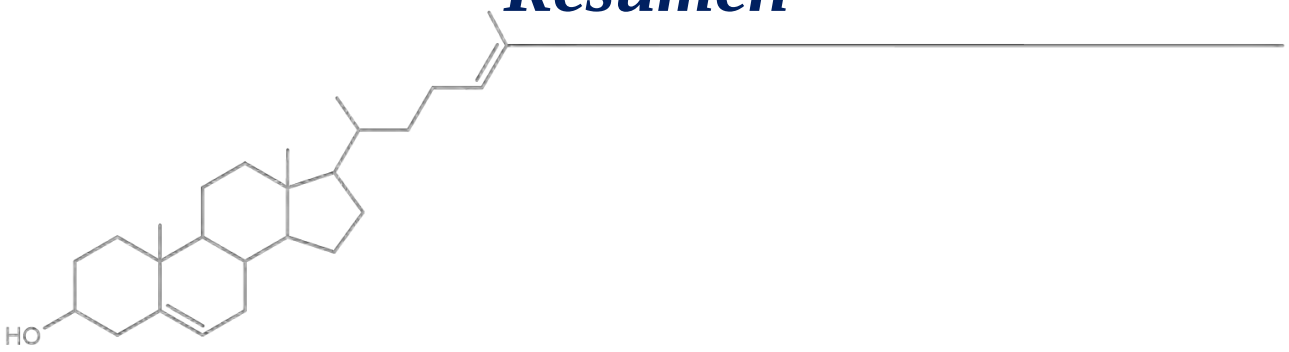
TIM-2: *TNO Gastro-Intestinal Model of the colon*

TNFα: factor de necrosis tumoral α

UE: Unión Europea



Resumen



Resumen

El enriquecimiento de alimentos con esteroles vegetales (EV) es una práctica común en el desarrollo de alimentos funcionales orientados a reducir el riesgo de enfermedades cardiovasculares debido a su conocido efecto hipocolesterolemiante. Las bebidas a base de zumo de frutas y leche son una matriz idónea para el enriquecimiento con estos compuestos bioactivos debido a su perfil nutricional saludable. Además, su efectividad se ha demostrado en poblaciones diana como mujeres postmenopáusicas con hipercolesterolemia moderada (población de interés al presentar un mayor riesgo de enfermedad cardiovascular). La adición de galactooligosacáridos (GOS) a estas bebidas podría mejorar su funcionalidad debido al efecto beneficioso que presentan sobre la microbiota intestinal, estimulando de manera selectiva el crecimiento de *Bifidobacterium* y/o *Lactobacillus spp.*, así como potenciar el efecto hipocolesterolemiante. Sin embargo, la adición de fibra podría reducir la bioaccesibilidad de los esteroles y modular su metabolismo colónico.

El objetivo general de la presente Tesis Doctoral es evaluar el efecto de los GOS sobre la biodisponibilidad y fermentación colónica de los esteroles en bebidas a base de zumo de frutas y leche enriquecidas con EV mediante ensayos *in vitro* e *in vivo*.

El contenido y bioaccesibilidad de los productos de oxidación de esteroles determinados en una bebida enriquecida con EV (2 g/250 mL) no se modifican durante el almacenamiento (20-25ºC, 6 meses). Además, el porcentaje de oxidación de los esteroles es bajo: 0,03 % para el β-sitosterol y 1,2 % para el colesterol.

Los resultados obtenidos tras una digestión gastrointestinal micelar simulada indican que la adición de GOS (2,3 o 4,5 g/250 mL) a bebidas enriquecidas con EV (2,5 g/250 mL) no afecta a la bioaccesibilidad del colesterol (78-88%) ni a la de los EV totales (~37%). Este ensayo permite seleccionar la bebida con mayor contenido de GOS para posteriores estudios de fermentación colónica (*in vitro* e *in vivo*) y biodisponibilidad (*in vivo*).

Por otro lado, la aplicación del método de digestión gastrointestinal Infogest a la bebida enriquecida con EV sin adición de GOS no permite la cuantificación del colesterol en la fracción bioaccesible correspondiente. Se demuestra así la necesidad de adaptar el método a la determinación de la bioaccesibilidad de esteroles y se propone el cambio de origen de las sales biliares utilizadas (bovinas en lugar de porcinas) o la reducción de su concentración (de 10 mM a 1,4 mM).

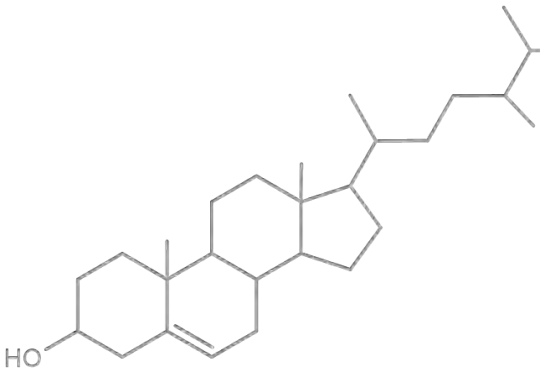
Además, se observa que la presencia de GOS en la bebida enriquecida con EV favorece la metabolización de los esteroles evaluada mediante un sistema *in vitro* dinámico multicompartimental. Se demuestra que la formación de metabolitos de los esteroles (coprostanol, etilcoprostanol y

metilcoprostanol) es dependiente del tramo colónico, ya que solo tiene lugar en los reactores del colon transversal y descendente. De la misma manera, la producción de ácidos orgánicos durante la fermentación colónica de las bebidas es mayor en estos reactores y se potencia en presencia del prebiótico. La mayor actividad metabólica observada en los tramos del colon transversal y descendente con respecto al colon ascendente coincide con una mayor riqueza y diversidad de especies microbianas, así como con una mayor similitud en la composición de la microbiota.

Por último, en mujeres postmenopáusicas con hipercolesterolemia moderada se observa que la presencia de GOS (4,3 g/250 mL) no influye en el efecto hipocolesterolemiante de las bebidas enriquecidas con EV (2 g/250 mL). Además, la adición del prebiótico no modifica la biodisponibilidad y la fermentación colónica de los esteroles. Se confirma que, en presencia de altas concentraciones de EV, se produce una mayor excreción fecal de colesterol y se favorece su metabolización a través de rutas indirectas.

Por lo tanto, los resultados obtenidos indican que la adición de GOS a bebidas a base de zumo de frutas y leche enriquecidas con EV no modifican la bioaccesibilidad/biodisponibilidad de los esteroles, ni el efecto hipocolesterolemiante de los EV. A pesar de que el metabolismo de los esteroles se ve favorecido por la presencia de GOS en ensayos *in vitro*, estos resultados no se reflejan en el estudio *in vivo*.

Antecedentes Bibliográficos



1. Introducción

Las enfermedades cardiovasculares son la principal causa de mortalidad en el mundo actual a pesar de los avances alcanzados en los procedimientos quirúrgicos y el tratamiento farmacológico. Por ese motivo, es de interés dirigir los esfuerzos a la prevención primaria, disminuyendo los factores de riesgo asociados a su desarrollo entre los cuales se encuentran el estilo de vida (tabaquismo, sedentarismo) obesidad, la hipertensión arterial, la diabetes y la hiperlipidemia, entre otros (WHO, 2017; Scolaro et al., 2018).

En este contexto, la inclusión de alimentos funcionales en la dieta puede contribuir a una prevención temprana de la enfermedad, reducir la dosis de medicamentos o mejorar la respuesta del paciente al tratamiento (Scolaro et al., 2018). Los alimentos funcionales se definen como "alimentos naturales o procesados que contienen compuestos biológicamente activos conocidos o desconocidos que, en cantidades definidas, efectivas y no tóxicas, proporcionan un beneficio clínicamente probado y documentado para la prevención, control o tratamiento de enfermedades crónicas" (Martirosyan & Singh, 2015). Los alimentos funcionales pueden ser específicamente diseñados mediante el enriquecimiento con compuestos bioactivos, que se consideran su fuente de efectividad (Granado-Lorencio & Hernández-Alvarez, 2016).

El enriquecimiento de alimentos con esteroles vegetales (EV), compuestos bioactivos de origen vegetal, se considera una herramienta útil para atenuar el riesgo de enfermedad cardiovascular debido a su conocido efecto hipocolesterolemiante. La Asociación Europea de Aterosclerosis propone el consumo de este tipo de alimentos en individuos que presentan hipercolesterolemia con riesgo cardiovascular intermedio o bajo que no son aptos para el tratamiento farmacológico (Gylling et al., 2014). En trabajos previos del grupo de investigación, se ha demostrado que las bebidas a base de zumo de frutas y leche son una matriz idónea para alcanzar la cantidad recomendada de EV asociada a un efecto hipocolesterolemiante (1,5-3 g/día) en mujeres postmenopáusicas con hipercolesterolemia moderada no tratadas farmacológicamente (Alvarez-Sala et al., 2018). Este tipo de bebidas son de gran interés debido a que presentan un perfil nutricional saludable derivado del bajo contenido en grasa y por la presencia de compuestos bioactivos proporcionados por el zumo de frutas (vitamina C, polifenoles, carotenoides).

Los galactooligosacáridos (GOS) son polisacáridos no fermentables por la microbiota intestinal y se consideran compuestos bioactivos debido a los efectos beneficiosos asociados a su ingesta (efectos protectores frente al cáncer de colon, así como el aumento de la proliferación de bacterias inmunogénicas tales como bifidofacterias y lactobacilos) (Sangwan et al., 2011; Slavin, 2013). La

información acerca del efecto de los GOS sobre el perfil lipídico sérico, sin embargo, es todavía escasa y poco concluyente y se desconoce el efecto de su combinación con EV en el contexto de una matriz alimentaria.

2. Esteroles vegetales

2.1 Estructura y características

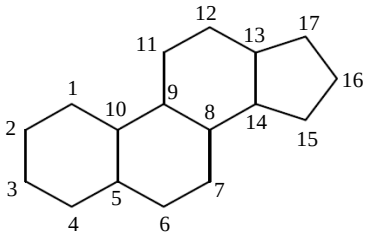
Los EV son compuestos lipídicos presentes en alimentos de origen vegetal y pertenecientes a la familia de compuestos orgánicos con estructura de triterpeno. Están formados por una estructura tetracíclica de ciclopentano[α]-perhidrofenantreno, con un grupo hidroxilo en posición C-3 y una cadena lateral de entre 8 y 10 átomos de carbono en posición C-17 (García-Llatas & Rodríguez-Estrada, 2011).

Los EV son estructuralmente similares al colesterol (principal esterol de origen animal) a excepción de la cadena lateral, donde tienen un grupo etilo o metilo extra en el carbono C-24 y puede presentar insaturaciones. Se subdividen a su vez en fitosteroles y fitostanoles, en función de la presencia o ausencia de un doble enlace en C-5, respectivamente (Moreau et al., 2018). Se han descrito más de 250 tipos de EV en especies vegetales, siendo el β-sitosterol, campesterol y estigmasterol los más abundantes. En la figura 1 se muestran las estructuras químicas de los principales esteroles de origen animal y vegetal. Los EV pueden encontrarse en los alimentos en forma libre o conjugada mediante la esterificación del grupo hidroxilo del C-3 con ácidos grasos (esteril ésteres), ácidos fenólicos (esteril ésteres de ácido hidroxicinámico) o carbohidratos (esteril glucósidos y esteril glucósidos acilados) (García-Llatas & Rodríguez-Estrada, 2011; Moreau et al., 2018).

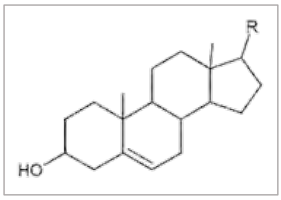
2.2 Fuentes e ingestas dietéticas

Las principales fuentes alimentarias de EV son los aceites vegetales seguidos de frutos secos, legumbres, cereales y, por último, hortalizas y frutas (Moreau et al., 2018). En la figura 2 se muestra el contenido promedio de EV aportado por alimentos pertenecientes a estos grupos. El aporte dietético de EV no solo depende de su contenido en alimentos sino también de la frecuencia de consumo, factor a tener en cuenta para estimar su ingesta dietética.

Figura 1. Estructura de los principales esteroles de origen animal y vegetal



Esteroles animales



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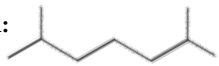
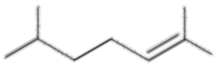
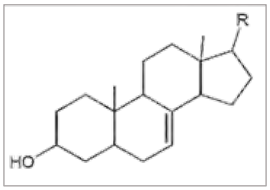


Colesterol



Desmosterol



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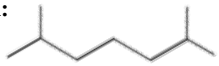
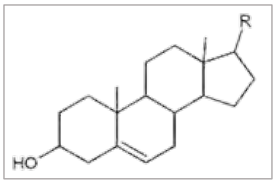


Latosterol

Esteroles vegetales

- Fitosteroles



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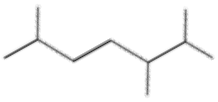
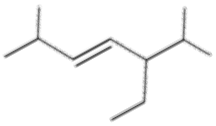
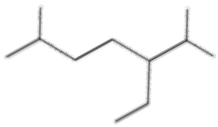


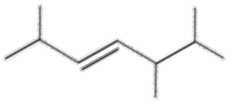
Campesterol



Estigmasterol

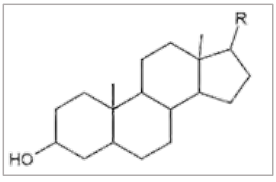


β-Sitosterol



Brasicasterol

- Fitostanoles



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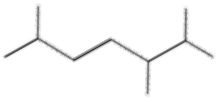
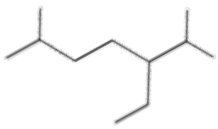
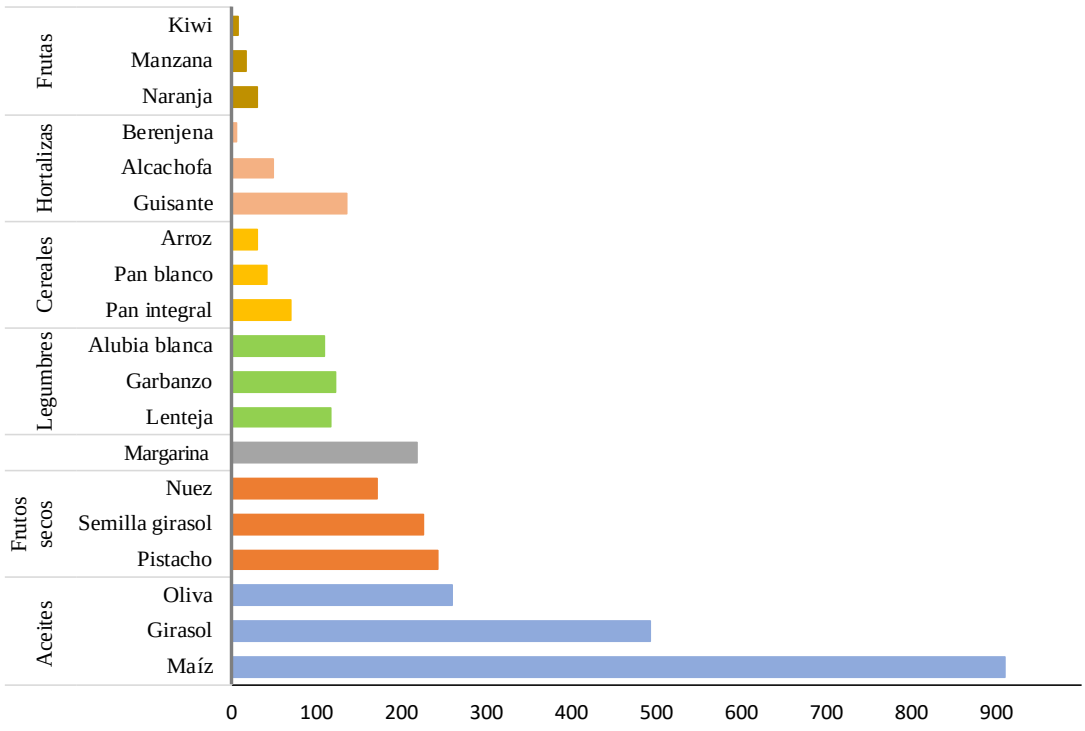


Campestanol



Sitostanol

Figura 2. Contenido de esteroles vegetales en alimentos (mg/100 g)



Adaptada de García-Llatas et al. (2015a)

Se estima que la ingesta de EV a partir de fuentes alimentarias en la población europea oscila entre 211 y 325 mg/día (García-Llatas et al., 2015a), alcanzando hasta 600 mg/día en dietas vegetarianas (Klingberg et al., 2008) y 896-1047 mg/día en dietas veganas (Racette et al., 2015). En un estudio realizado en la Comunidad Valenciana mediante cuestionarios de frecuencia de consumo, se indica que la ingesta de EV oscila entre 274-282 mg/día (García-Llatas et al., 2015a). En este estudio se observa que legumbres (30-35%), hortalizas (19-20%), frutas (11-18%) y aceites y grasas vegetales (7-16%) son los alimentos que más contribuyen a estas ingestas en la población valenciana.

El enriquecimiento de alimentos con EV es una práctica cada vez más común en la industria alimentaria. Ello se debe a que su ingesta a partir de los alimentos no es suficiente para alcanzar las dosis con efecto hipocolesterolemiante que se describe más adelante (apartado 2.4).

El primer producto en el que se autoriza el enriquecimiento con EV son las grasas amarillas de untar (margarinas y materias grasa para untar) (Decisión 2000/500/CE). Sin embargo, dado que el enriquecimiento con EV tiene como finalidad la disminución o el mantenimiento de las concentraciones sanguíneas de colesterol con el objetivo de mejorar la salud del consumidor, se ha autorizado su adición a otras matrices alimentarias con mejor perfil lipídico. Entre otras, destacan los productos tipo leche semidesnatada y leche desnatada, bebidas a base de zumo de frutas y leche,

bebidas de frutas, cereales y soja, y derivados lácteos como leche fermentada tipo yogur y queso, en los que se ha reducido la grasa y/o proteína de la leche o se ha sustituido total o parcialmente por grasa y/o proteína de origen vegetal (Decisión 2004/333/CE, 2004/334/CE, 2004/335/CE, 2004/336/CE y 2004/845/CE). El enriquecimiento con EV también está autorizado en pan de centeno (Decisión 2006/58/CE y 2006/59/CE), aceite como nuevo ingrediente (Decisión 2007/343/CE) y bebidas de arroz (Decisión 2008/36/CE).

En este marco legal, se especifica que los EV (libres o esterificados con ácidos grasos de grado alimentario) utilizados para el enriquecimiento no deben superar los siguientes porcentajes de abundancia en su composición: β-sitosterol (80%), sitostanol (15%), campesterol (40%), campestanol (5%), estigmasterol (30%), brasicasterol (3%), otros esteroles y estanoles (3%).

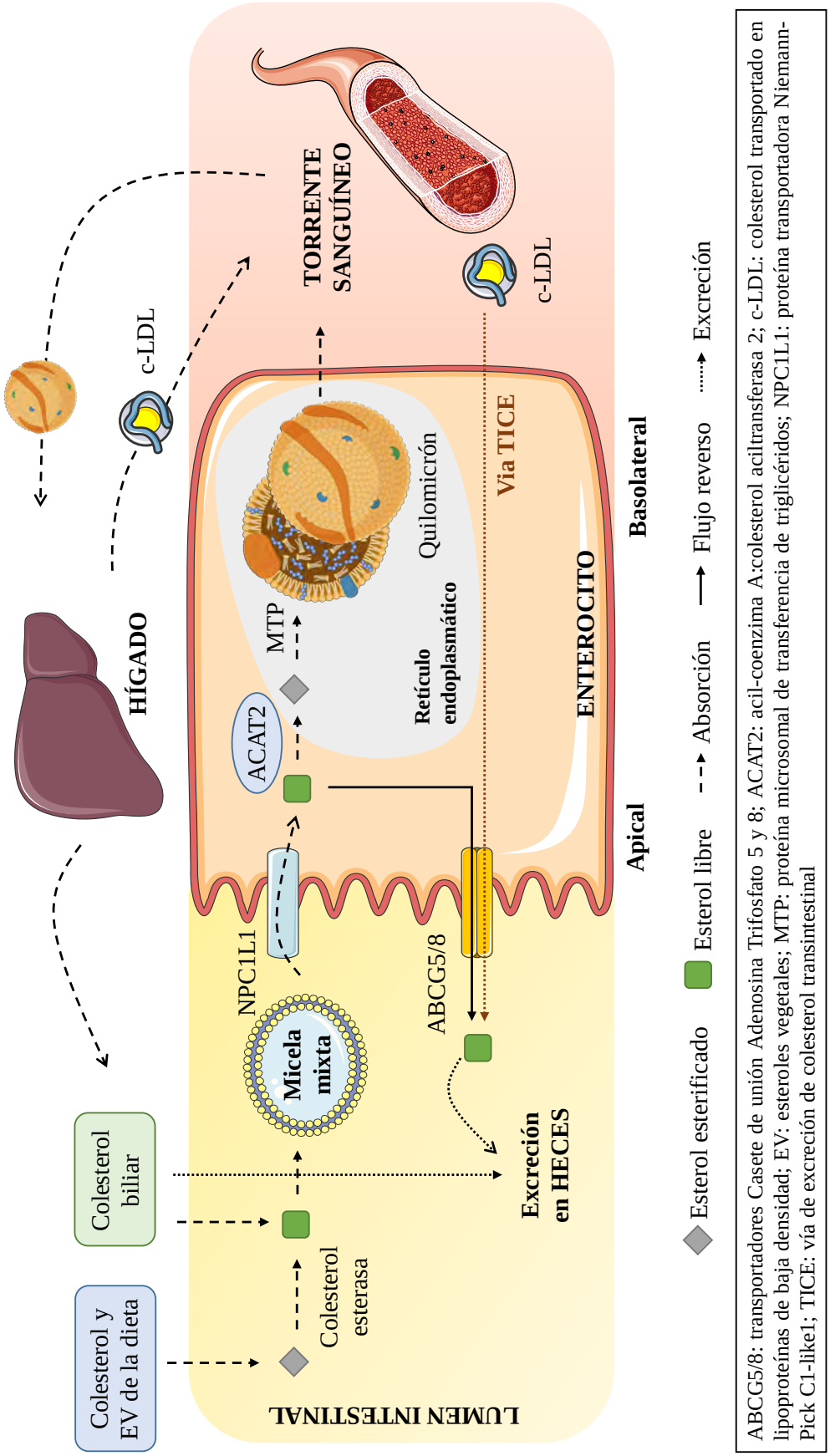
2.3 Metabolismo

La absorción de esteroles (colesterol y EV) tiene lugar en el intestino delgado en un proceso de varias etapas que se puede resumir con la entrada de los mismos al enterocito, en forma micelar, y su posterior transporte al sistema circulatorio (ver figura 3). Debido a la similitud estructural, el colesterol y los EV tienen mecanismos de absorción similares.

En el intestino, los esteroles de la dieta y el colesterol endógeno, secretado por la bilis, son digeridos por las enzimas pancreáticas. La enzima carboxil éster hidrolasa (colesterol esterasa) hidroliza los esteroles esterificados dando lugar a esteroles libres y ácidos grasos. Debido a su naturaleza hidrofóbica, los esteroles son solubilizados mediante su incorporación a las micelas mixtas para permitir su absorción, que transportan los esteroles hasta la membrana del borde en cepillo de los enterocitos, donde se produce la absorción mediante difusión facilitada a través de la proteína transportadora Niemann-Pick C1-like1 (NPC1L1) (Smet et al., 2012).

La absorción de esteroles, en menor medida, también puede llevarse a cabo por difusión pasiva en la membrana del borde en cepillo del enterocito (Nakano et al., 2019). La mayor parte del colesterol dentro del enterocito es transferido al retículo endoplasmático para ser esterificado de nuevo por la acción de la acil-coenzima A: colesterol aciltransferasa 2 (ACAT2), y es incorporado a los quilomicrones en un proceso mediado por la proteína microsomal de transferencia de triglicéridos (MTP). En el caso de los EV, solo una pequeña parte se incorpora a los quilomicrones en forma esterificada debido a la baja afinidad por la enzima ACAT2. Los quilomicrones son excretados por exocitosis a través de la membrana basolateral del enterocito y transportados por el sistema linfático hasta el torrente sanguíneo (García-Llatas & Rodríguez-Estrada, 2011).

Figura 3. Absorción y excreción intestinal de los esteroles



ABCG5/8: transportadores Casete de unión Adenosina Trifosfato 5 y 8; ACAT2: acil-coenzima A:colesterol aciltransferasa 2; c-LDL: colesterol transportado en lipoproteínas de baja densidad; EV: esteroles vegetales; MTP: proteína microsomal de transferencia de triglicéridos; NPC1L1: proteína transportadora Niemann-Pick C1-like1; TICE: vía de excreción de colesterol transintestinal

Los quilomicrones se distribuyen vía sanguínea hasta los hepatocitos y los esteroles que transportan son incorporados en su mayor parte (65-70%) a lipoproteínas de baja densidad (LDL) (Gylling et al., 2014; de Boer et al., 2018).

Aproximadamente el 50% del colesterol obtenido a partir de la dieta se absorbe a nivel intestinal, aunque esta absorción presenta una gran variabilidad interindividual (20-80%) (de Boer et al., 2018). En el caso de los EV la tasa de absorción intestinal es menor: el campesterol (9,4-14,8%) se absorbe del orden de tres veces más que el β-sitosterol (3,1-4,5%) y estigmasterol (~4%), mientras que la absorción de estanoles (campestanol y sitostanol) es inferior al 2% (García-Llatas & Rodríguez-Estrada, 2011). Como consecuencia, las concentraciones sanguíneas de EV son bajas, oscilando entre 7-24 μmol/L (0,3-1,0 mg/dL) para los fitosteroles y 0,05-0,3 μmol/L (0,002-0,012 mg/dL) para los fitostanoles. Estas concentraciones son del orden de 500 y 10.000 veces inferiores, respectivamente, a las del colesterol (Gylling et al., 2014).

Los esteroles son excretados principalmente a través de la bilis y, posteriormente, a través de las heces. Además, los esteroles libres presentes en el enterocito que no han sido incorporados a quilomicrones y, por tanto, tampoco al torrente sanguíneo, se excretan a través de un flujo reverso al lumen intestinal mediado por los transportadores Casete de unión Adenosina Trifosfato G5 y 8 (ABCG5/8) (García-Llatas & Rodríguez-Estrada, 2011; de Boer et al., 2018).

Recientemente, se ha descrito un mecanismo adicional de excreción, conocida como vía de excreción transintestinal de colesterol (TICE) (de Boer et al., 2018). Aunque los mecanismos implicados en esta vía no son completamente conocidos se ha sugerido que el enterocito es capaz de captar el colesterol transportado en lipoproteínas del torrente sanguíneo desde la membrana basolateral y desplazarlo hasta el lado apical, donde es excretado de nuevo a la luz intestinal a través de los transportadores ABCG5/8. Debido a que la disrupción de los genes ABCG5/8 no implica la inhibición de la vía TICE, se ha sugerido la participación de otros transportadores, como los transportadores Casete de unión Adenosina Trifosfato B1 a y b (ABCB1a/b). Se ha estimado que esta vía contribuye en un 35% a la excreción de esteroles fecales neutros (Nakano et al., 2019).

El efecto hipocolesterolemiante (ver apartado 2.4) asociado a la ingesta de EV se ha explicado por varios mecanismos de competición con respecto a las distintas etapas de la absorción intestinal del colesterol: i) en la incorporación en las micelas mixtas, ii) por el transportador NPC1L1 para atravesar la membrana del enterocito, iii) por la actividad de la enzima ACAT2 (aunque la esterificación de los EV se produce en menor medida, puede desplazar al colesterol y evitar su reesterificación), y iv) por la incorporación en los quilomicrones (Smet et al., 2012; García-Llatas & Rodríguez-Estrada, 2011).

Recientemente se ha propuesto que, además, la presencia de EV en el enterocito podría generar un aumento de la actividad y expresión del transportador ABCG5/8, acelerando el eflujo de colesterol al lumen intestinal (Nakano et al., 2019).

2.4 Funcionalidad

Efecto hipocolesterolemiante

Los EV tienen autorizadas dos declaraciones de propiedades saludables por parte de la Unión Europea (UE). En la primera, relativa a propiedades saludables distintas a la reducción de riesgo de enfermedad y salud de los niños (Reglamento 432/2012/UE), se indica que una ingesta diaria mínima de 0,8 g de EV contribuye a mantener niveles normales de colesterol sanguíneo en población adulta sana. En la segunda declaración autorizada, en este caso de reducción de riesgo de enfermedad, se informa que la ingesta diaria de 1,5-3 g de EV tiene un efecto beneficioso sobre la reducción del colesterol sanguíneo en sujetos con hipercolesterolemia moderada (Reglamentos 983/2009/UE y 384/2010/UE). En posteriores modificaciones (Reglamento 686/2014/UE), se señala que la magnitud del efecto podrá especificarse para los alimentos incluidos en las categorías de grasas amarillas para untar, productos lácteos, mayonesa y aliños para ensaladas. Además, cuando se haga referencia a la magnitud del efecto, se debe comunicar al consumidor que la ingesta de 1,5 a 2,4 g o de 2,5 a 3 g de EV suponen una disminución del colesterol sanguíneo del 7 al 10% o del 10 al 12,5%, respectivamente, tras 2-3 semanas de consumo.

Diversos estudios clínicos han demostrado el efecto hipocolesterolemiante resultante de la ingesta de alimentos enriquecidos con EV, observando una relación dosis-dependiente entre ingestas de 0,6 a 3,3 g de EV/día y reducciones del 6 al 12% de los niveles de c-LDL (Gylling et al., 2014; Ras et al., 2014). La magnitud de estas reducciones puede verse afectada por diversos factores como la frecuencia y el momento de la ingesta de los EV (solo o acompañado de una comida), la matriz alimentaria utilizada (sólida o líquida) y por características propias del estudio y de sus participantes (diseño del estudio y duración, número de participantes o valores basales del perfil lipídico de los sujetos) (Moreau et al., 2018; Trautwein et al., 2018). Trabajos previos de nuestro grupo de investigación, realizado en mujeres postmenopáusicas, han demostrado un efecto reductor de los niveles de colesterol total y c-LDL de 5-7%, tras la ingesta de bebidas a base de zumos de frutas y leche enriquecidas con EV (1,5g/día) y β-criptoxantina (0,75 mg/día) durante 4 semanas, hecho no observado en bebidas similares enriquecidas con solo uno de los compuestos bioactivos (Granado-Lorencio et al., 2014). En un estudio posterior doble ciego, cruzado en el mismo tipo de población, la ingesta regular (durante cuatro semanas) de una bebida similar, enriquecida con 2 g de EV y 1 mg

de β-criptoxantina y modificada por la adición de grasa láctea y concentrado de proteínas del suero enriquecido en membrana del glóbulo graso de la leche (MFGM), reduce significativamente los niveles séricos de colesterol total (3%) y c-LDL (5%) (Alvarez-Sala et al., 2018).

Otros efectos beneficiosos

Además de mejorar el perfil lipídico sanguíneo, el consumo de EV se ha asociado con otros efectos beneficiosos sobre la salud como el anticancerígeno y antiinflamatorio.

El efecto protector de los EV frente al cáncer ha sido estudiado principalmente mediante líneas celulares tumorales y modelos animales y, por lo tanto, es necesario su confirmación con un mayor número de estudios clínicos (Shahzad et al., 2017). En concreto, en modelos celulares y animales se ha demostrado que los EV reducen el crecimiento de distintos tipos de células tumorales como las de mama, próstata, pulmón, hígado, estómago y ovario (Ramprasath & Awad, 2015). Se han propuesto varios mecanismos de acción antiproliferativa de los EV como son la reducción de mediadores pro-inflamatorios como las prostaglandinas o la expresión de agentes proliferativos como la β-catenina y el antígeno nuclear de células en proliferación. Así mismo, se ha observado que los EV pueden inhibir el crecimiento, invasión y metástasis de las células cancerígenas. También se ha sugerido que pueden actuar como agentes citostáticos induciendo arrestos en el ciclo celular y apoptosis, así como reducir el proceso de angiogénesis o la producción de especies reactivas de oxígeno debido a su efecto antioxidante (Ramprasath & Awad, 2015; Shahzad et al., 2017). Recientemente, en nuestro grupo de investigación, se ha demostrado el efecto antiproliferativo de los EV (de manera individual o combinada) en células Caco-2 no diferenciadas con patrones de β-sitosterol, campesterol y estigmasterol a concentraciones colónicas similares a las observadas tras la digestión *in vitro* de una bebida enriquecida con EV (López-García et al., 2017). Además, se ha observado que los EV presentan un efecto apoptótico en células tumorales de mama, colon y cuello de útero mediante el tratamiento con un ingrediente fuente de EV (*tall oil*) o con patrón de β-sitosterol a concentraciones similares a los niveles séricos obtenidos tras el consumo de la bebida mencionada (Alvarez-Sala et al., 2019).

Por otro lado, en estudios clínicos, se ha descrito que los EV tienen un efecto modulador del mecanismo inflamatorio relacionado con aterosclerosis e hiperlipidemia (Plat et al., 2019). La inflamación es un factor de riesgo en la enfermedad cardiovascular por su participación en el proceso de aterogénesis (formación y ruptura de las placas de ateroma y trombosis). El proceso inflamatorio puede evidenciarse por el incremento de marcadores proinflamatorios como la proteína C-reactiva y determinados tipos de citoquinas (Othman & Moghadasian, 2011). Aunque diversos estudios han

demostrado que la ingesta de EV puede inducir una reducción en marcadores de inflamación, los resultados son inconsistentes (Othman & Moghadasian, 2011; Rocha et al., 2016). En células Caco-2 diferenciadas, se ha observado el efecto antiinflamatorio de la fracción bioaccesible procedente de la digestión gastrointestinal *in vitro* de una bebida a base de zumo de frutas y leche enriquecida con EV (1 g/100 mL) presenta propiedades antiinflamatorias al reducir los niveles de IL-8 (40%) e IL-6 (50%) mediante la inhibición de la translocación al núcleo del factor de transcripción nuclear κB (16%) en comparación con las células control (López-García et al., 2020). Así mismo, en un estudio con mujeres postmenopáusicas se ha observado que la ingesta de 2 g de EV en una bebida similar durante 6 semanas produce un incremento del 22,5% de la citoquina antiinflamatoria IL-10 y una reducción del 6,7% de la citoquina proinflamatoria IL-1β (Alvarez-Sala et al., 2018).

3. Óxidos de esteroles

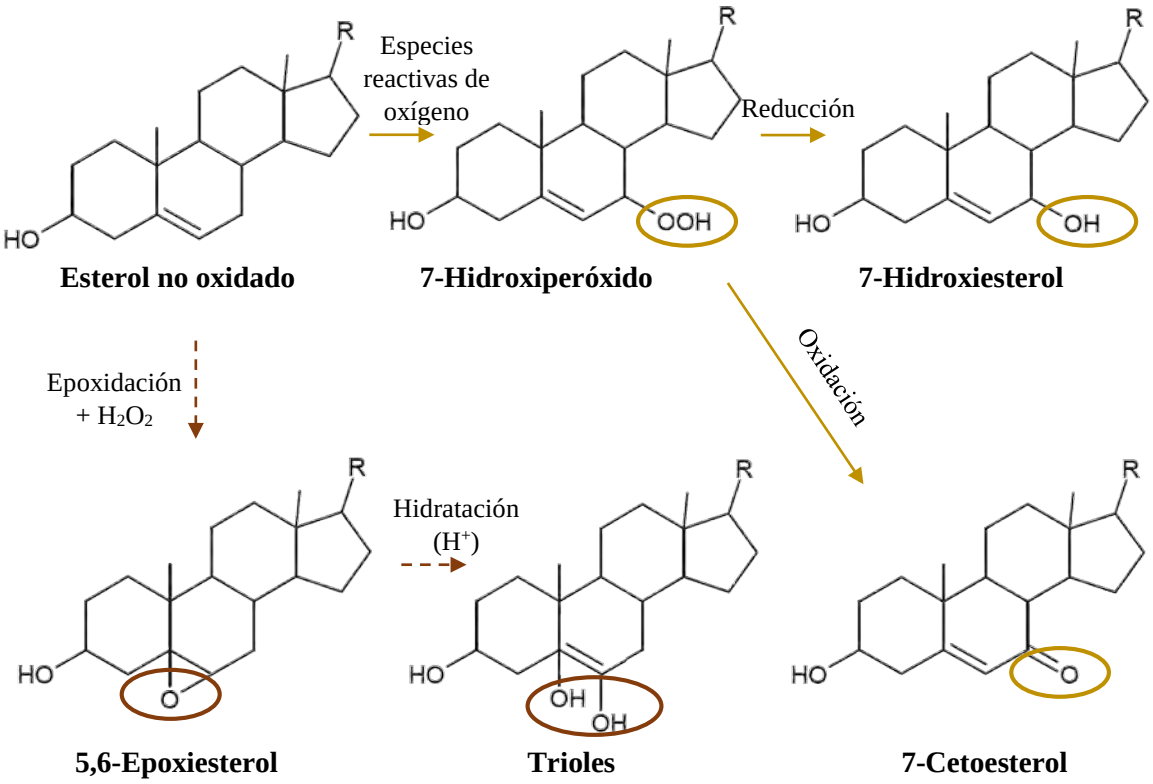
3.1 Formación

Los esteroles son susceptibles de oxidarse dando lugar a productos de oxidación de colesterol (COPs) o de EV (POPs), conocidos en conjunto como SOPs (productos de oxidación de esteroles). La formación de COPs se ha descrito ampliamente, pudiéndose producir por oxidación enzimática o por autooxidación (principal mecanismo de oxidación no enzimático). Sin embargo, el conocimiento relativo a la formación de POPs no es tan amplio, aunque la información disponible sugiere que los EV son menos susceptibles que el colesterol como sustrato para la oxidación enzimática y, por tanto, la principal vía de formación de estos es la autooxidación (Hovenkamp et al., 2008; Kulig et al., 2016).

La reacción inicial de la autooxidación de los esteroles implica la sustracción del hidrógeno del C-7 del anillo esteroideo, seguido de una reacción con especies reactivas de oxígeno (oxígeno triplete, hidroperóxidos, catión dioxígeno o radical hidroxi), formándose así radicales 3β-hidroxi-5-en-7-peroxilos. La extracción de una molécula de hidrógeno de estos radicales da lugar a 7-hidroperóxidos, que son moléculas más estables. Durante el calentamiento y/o almacenamiento, los 7-hidroperóxidos pueden descomponerse en 7α/7β-hidroxiesteroles o 7-cetoesteroles mediante una reacción de reducción u oxidación, respectivamente. Otros productos secundarios de la oxidación de esteroles son los epímeros 5α,6α/5β,6β-epoxiesteroles, formados a través de una interacción biomolecular en la que participan el radical hidroperóxido y moléculas de esterol no oxidadas. La hidratación de los epóxidos en medio ácido da lugar a su conversión en trioles (3β,5α,6β-triol) (García-Llatas & Rodríguez-Estrada, 2011; Brzeska et al., 2016). Estos procesos se muestran en la figura 4.

El proceso de autooxidación de los esteroles puede tener lugar en el propio organismo o en los alimentos durante su almacenamiento o procesado térmico (Lin et al., 2016).

Figura 4. Formación de óxidos de esteroles por autooxidación



Adaptada de García-Llatas & Rodríguez-Estrada (2011)

3.2 Fuentes e ingestas dietéticas

El contenido de COPs en distintos grupos de alimentos se ha recopilado en varios trabajos. En productos cárnicos, las concentraciones de COPs oscilan entre 0,1 y 18,7 µg/g en carne de vacuno y mortadela, respectivamente, mientras que en pescado se han descrito contenidos de 19,4 µg/g en sardinas y 33,6 µg/g en anchoas. En leche (entera o desnatada) y mantequilla los contenidos de COPs oscilan entre 1,1-7,7 µg/g y 13,7-27,3 µg/g, respectivamente y, en huevos y ovoproductos se han descrito contenidos mayores: desde 3,3-3,8 µg/g en huevos pasteurizados hasta 29,0-294,3 µg/g en huevo en polvo (Otaegui-Arrazola et al., 2010; Rodríguez-Estrada et al., 2014; Brzeska et al., 2016).

Sin embargo, los estudios sobre el contenido de POPs en alimentos son escasos (especialmente en alimentos no enriquecidos con EV) debido a ciertas limitaciones como la falta de patrones comerciales, la existencia de un amplio número de compuestos con estructuras similares y a su presencia como componentes traza en los alimentos pudiendo interferir la matriz en su análisis

(García-Llatas & Rodriguez-Estrada, 2011). En la tabla 1 se muestra el contenido de POPs en distintas matrices alimentarias enriquecidas con EV.

Tabla 1. Productos de oxidación de esteroles vegetales en alimentos enriquecidos

Alimento	Formato adicionado	EV (g/100 g)	POPs (mg/100 g)	Ratio de oxidación (%)
Aceite de colza	Libres		ND-2,3	<0,01-0,03
	E-FA	8,0	4,3-8,0	0,05-0,10
	E-FE		3,9	0,05
Bebida de fruta	Libres	0,7-1,3	0,5-0,7	0,05-0,07
	E-FE	1,4	0,8	0,006
Bebida a base de zumo de fruta y leche	Libres	0,7-1,4	0,2-0,8	0,03-0,06
	E-FE	1,9	0,8	0,04
Chocolate negro	E-FE	7,3	6,9	0,09
Grasas amarillas para untar	Libres	8,0	2,3	0,03
	E-FA	8,0	3,6	0,05
	E-FE	6,0-8,0	4,0-6,8	0,05-0,09
Leche	Libres	0,5-7	0,2-1,4	0,02-0,05
	E-FA	0,5	0,02	<0,01
	E-FE	0,3-0,5	0,2	0,04-0,07
Mantequilla	Libres		2,0	0,03
	E-FA	8,0	0,4	0,01
	E-FE		3,4	0,04
Margarina	E-FA	14,7	0,54	<0,01
	E-FE	7,5-13,3	0,1-37	<0,01-0,49

E-FA: ésteres de fitostanoles; E-FE: ésteres de fitosteroles; EV: esteroles vegetales; ND: no detectado; POPs: productos de oxidación de esteroles vegetales. Ratio de oxidación: (contenido total de POPs/contenido total de EV) x 100.

Adaptada de Scholz et al. (2015) y Lin et al. (2016)

En trabajos previos de nuestro grupo de investigación, en bebidas enriquecidas con 0,8 g de EV/100 mL se determinan sólo óxidos de β-sitosterol, por ser el esterol mayoritario. El 7-cetositosterol es el óxido más abundante, seguido por 7β-hidroxi- y β-epoxisitosterol en bebidas con zumo de mandarina como fuente de β-criptoxantina, o por sitostanotriol en ausencia de zumo de mandarina (Alemany et al., 2013a). En bebidas similares (González-Larena et al., 2015), se observa una abundancia relativa distinta para los óxidos de β-sitosterol, siendo mayoritarios los derivados β-epoxi, 7β-hidroxi y 7-ceto mayoritarios, seguidos de α-epoxi, triol y 7α-hidroxi. Además, se determinan óxidos de campesterol, siendo los derivados β-epoxi los más abundantes, seguidos por derivados α-epoxi y 7-ceto. Las diferencias entre los POPs determinados en ambas bebidas se podrían justificar por los distintos tratamientos térmicos llevados a cabo durante el proceso de producción. En

cuanto a los COPs, Alemany et al. (2013a) observa una mayor abundancia del derivado 7-ceto. Además, en ambos casos (POPs y COPs), existe una mayor presencia del isómero 7β-hidroxi con respecto a 7α-hidroxi, de acuerdo a la mayor estabilidad del epímero β desde un punto de vista termodinámico.

El contenido de SOPs en los alimentos depende de su composición, el procesado industrial, las condiciones de almacenamiento y los procedimientos culinarios utilizados, ya que existen determinadas condiciones relacionadas con estos procesos que son claves en la oxidación de los esteroles: aumento de la temperatura, presencia de oxígeno, exposición a la luz, matriz lipídica circundante y presencia de antioxidantes y agua en el alimento (Barriuso et al., 2017).

La intensidad y el tiempo de calentamiento o procesado térmico al que se someten los alimentos son factores a tener en cuenta en la formación de SOPs. El calentamiento es un inductor de oxidación lipídica ya que reduce la energía de activación para la abstracción de hidrógeno que conduce a la formación de radicales libres, responsables de iniciar la reacción de autooxidación (Otaegui-Arrazola et al., 2010; Barriuso et al., 2017). En alimentos adicionados con ésteres de fitosteroles (E-FE) (la forma más común de enriquecimiento), se observa que el contenido medio de POPs oscila entre 3,6 mg/100 g en alimentos no calentados y almacenados hasta valores de 38,1 mg/100 g en alimentos sometidos a un tratamiento térmico casero (sartén y horno a temperaturas de 140-200ºC durante 5-30 min) (Lin et al., 2016).

Durante el almacenamiento de los alimentos, la presencia de oxígeno y la exposición a la luz pueden favorecer la degradación de los esteroles. Sin embargo, se observa que las temperaturas de almacenamiento (que suelen oscilar entre los 4 y 38°C) no afectan a la formación de POPs, siempre y cuando la adición de EV proceda de E-FE o ésteres de fitostanoles (E-FA) (Lin et al., 2016). En bebidas a base de zumo de frutas y leche enriquecidas con EV libres, se ha demostrado que la temperatura de almacenamiento (4, 24 o 37ºC durante 6 meses) no afecta a los contenidos de POPs. Sin embargo, el tiempo de almacenamiento sí que supone un incremento en estos contenidos (González-Larena et al., 2015).

En cuanto a la implicación del grado de saturación de la matriz lipídica en la oxidación de los esteroles, existen dos hipótesis contrapuestas al respecto. Aunque se defiende el papel protector de los lípidos circundantes en la matriz frente al proceso de oxidación (compitiendo por el oxígeno con los esteroles y reduciendo así su autooxidación), también se indica que un grado de insaturación elevado puede promover la oxidación de los esteroles por la generación de radicales y especies oxigenadas derivadas de la oxidación lipídica. Debido a que ambas tendencias han sido demostradas

experimentalmente, se sugiere que existe un equilibrio entre ambos mecanismos (Barriuso et al., 2017).

La presencia de antioxidantes (de forma natural o adicionados) también se señala como un factor protector frente a la formación de SOPs durante el procesado, cocinado y almacenamiento de los alimentos (Otaegui-Arrazola et al., 2010; Barriuso et al., 2017). En los estudios realizados por nuestro grupo de investigación (Alemany et al., 2013a; González-Larena et al., 2015), se observa un mayor contenido de SOPs en las muestras de leche enriquecidas con EV con respecto a bebidas a base de zumo de frutas y leche, probablemente debido a una menor capacidad antioxidante.

En alimentos enriquecidos, la cantidad y tipo de EV adicionados (libres o esterificados) también influye en el grado de oxidación. De los estudios recopilados por Lin et al. (2016) se observa una correlación positiva entre el contenido inicial de EV y el contenido de POPs en el alimento. Además, se observa distinta resistencia térmica en función del tipo de EV (E-FA > E-FE > EV libres, con ratios de oxidación de 0,06, 0,48, y 0,90%, respectivamente).

Con respecto a las ingestas, se estima en 3 mg/día de COPs teniendo en cuenta que el 1% del colesterol ingerido es oxidado y que la ingesta media de colesterol es de 300 mg/día en una dieta occidental (Hovenkamp et al., 2008). La ingesta de POPs, en base a un consumo de 3 g/día de EV, se estima en 1,2-2,9 mg/día en alimentos no tratados térmicamente y en 3,5-29,6 mg/día en aquellos sometidos a tratamiento térmico. Sin embargo, este estudio solo considera grasas amarillas para untar, leche y chocolate negro enriquecidos con EV (Scholz et al., 2015). En alimentos enriquecidos con E-FE no tratados térmicamente y almacenados, la ingesta de POPs (asumiendo un consumo de 0,75-3 g/día de EV) se estima en 2,9-11,4 mg/día y para alimentos enriquecidos con EV libres, E-FE y E-FA sometidos a tratamiento térmico en 20-78, 12-48 y 0,8-3 mg/día (Lin et al., 2016), respectivamente.

3.3 Metabolismo

Los SOPs presentes en el organismo, ya sea a través de su incorporación mediante la dieta o excretados por el hígado tras la oxidación enzimática o no enzimática de esteroles, pueden ser absorbidos a nivel intestinal. Al igual que sus análogos no oxidados, los SOPs son esterificados en el interior del enterocito mediante su reacción con la enzima ACAT, incorporados a los quilomicrones y posteriormente a lipoproteínas de alta (HDL) y muy baja densidad (VLDL) y LDL para su transporte a las distintas células del organismo (Otaegui-Arrazola et al., 2010; Kulig et al., 2016). Una vez en el enterocito, los SOPs pueden ser excretados de nuevo al intestino a través de los transportadores

ABCG5/G8, de manera similar a los esteroles no oxidados (Hovenkamp et al., 2008). Debido a su hidrofobicidad, los SOPs son eliminados de las células a través de transportadores específicos de membrana como los transportadores Casete de unión Adenosina Trifosfato A1 (ABCA1) y G1 (ABCG1) localizados principalmente en macrófagos y en hígado. En el hígado, los SOPs pueden ser metabolizados o degradados hasta metabolitos solubles que pueden ser eliminados por las células (Otaegui-Arrazola et al., 2010).

Los COPs presentan una menor absorción que el colesterol, debido a su menor solubilidad en las micelas mixtas, menor susceptibilidad a la esterificación en el interior de los enterocitos o incluso por los efectos citotóxicos que producen en las células de la mucosa (Otaegui-Arrazola et al., 2010). En cambio, la absorción de POPs es mayor que la de los EV no oxidados, observándose diferencias en el grado de absorción en relación al esterol del que derivan y al tipo de oxidación que presentan. Se observa que los óxidos de campesterol son absorbidos en mayor medida que los correspondientes a β-sitosterol, sugiriéndose que a mayor longitud de la cadena lateral del esterol menor es su absorción. En cuanto al tipo de oxidación, los derivados 7α/7β-hidroxi presentan una mejor absorción en comparación con otros óxidos. La mayor abundancia de derivados 7β-hidroxi de los EV con respecto a 7α-hidroxi sugiere una menor eficiencia en su eliminación o la posible conversión de los 7α-hidroxi en ácidos biliares, como ocurre en el caso del 7α-hidroxicolesterol. Por otra parte, los derivados epoxi se absorben mejor que los 7-ceto, mientras que los derivados trioles son metabolizados y excretados de manera más lenta (García-Llatas & Rodríguez-Estrada, 2011; O'Callaghan et al., 2014; Barriuso et al., 2017).

3.4 Efectos biológicos

El estudio del efecto de los SOPs en la iniciación y progresión de diversas patologías ha ganado interés en las últimas décadas, por su implicación en el desarrollo de aterosclerosis. En concreto, la actividad biológica y patológica de los COPs se ha estudiado ampliamente en modelos *in vitro* e *in vivo*, mientras que la información relativa al efecto de los POPs sobre el organismo no es tan amplia (Brzeska et al., 2016).

Se ha demostrado que los SOPs presentan efectos citotóxicos en modelos de líneas celulares. Sin embargo, se requiere de mayores concentraciones de POPs (> 60 μM) para alcanzar efectos tóxicos similares a los observados para COPs. Los derivados 7β-hidroxi y 7-ceto de EV muestran un mayor potencial citotóxico, mientras que los derivados oxidados del β-sitosterol generan una mayor inducción a la apoptosis celular que aquellos derivados de campesterol y estigmasterol (Ryan et al., 2009; Scholz et al., 2015). La actividad citotóxica de los COPs deriva principalmente de su capacidad

para inducir apoptosis. Se han sugerido diversas rutas citotóxicas como la sobreproducción intracelular de especies reactivas de oxígeno, la modificación del potencial de membrana mitocondrial, la alteración metabólica de la poliamina y la perturbación de los niveles intracelulares de calcio (Kulig et al., 2016; Maldonado-Pereira et al., 2018). En una revisión reciente se describe concretamente el efecto del 7-cetocolesterol y el 7-hidroxicolesterol como inductores de estrés oxidativo. La alteración del balance redox conlleva a la muerte celular principalmente por vías apoptóticas, la inducción de procesos de inflamación a través de la secreción de citoquinas y de una mayor expresión de moléculas de adhesión (Vejux et al., 2020). En cuanto a la participación de los POPs en el proceso inflamatorio, la información existente es poco concluyente. En cultivos celulares expuestos a 7β-hidroxisitosterol (Vejux et al., 2012) o en ratones que ingieren una dieta enriquecida en POPs (Plat et al., 2014) no se observa efectos sobre la secreción de citoquinas proinflamatorias. Sin embargo, en células Caco-2, tras la exposición a 7-cetoestigmasterol se observa un incremento en la secreción de factor de necrosis tumoral α (TNFα) y de la citoquina proinflamatoria IL-8 (Alemany et al., 2013b).

Se ha indicado que los COPs presentan un efecto aterogénico y están involucrados en varios pasos clave de este proceso como la disfunción de las células endoteliales (por aumento de la permeabilidad), la adhesión y transmigración de monocitos (mediante la expresión de moléculas de adhesión celular, quimiocinas y citoquinas), la generación de células espumosas (por diferenciación de monocitos a macrófagos), la inflamación y formación de la capa fibrótica, y la apoptosis vascular y degradación de la matriz extracelular (Otaegui-Arrazola et al., 2010; Testa et al., 2018). La ingesta dietética habitual de POPs no supone un incremento en el tamaño de la lesión aterosclerótica en ratones, sin embargo, otras evidencias como la disminución en la funcionalidad de la aorta observada en hámsters y ratas sugieren que pueden presentar un potencial efecto aterogénico (O'Callaghan et al., 2014).

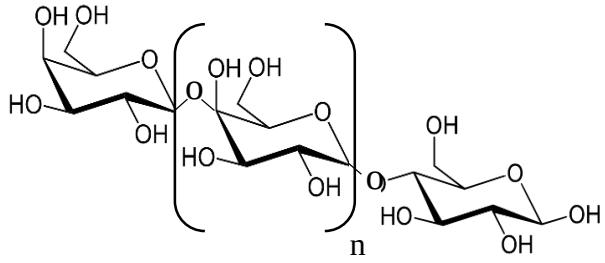
4. Galactooligosacáridos

4.1 Estructura y características

Los GOS son oligosacáridos no digeribles formados por la unión de 2 a 5 monómeros de galactosa y una glucosa terminal mediante enlaces o-glicosídicos (ver figura 5) (Sangwan et al., 2011). Estos compuestos se sintetizan generalmente a nivel industrial por la acción de β-galactosidasas que, además de tener actividad hidrolítica, presentan actividad de transglicosilación (catalizan la transferencia de residuos de azúcar desde sustratos donadores de glicosilo a los aceptores, formando así un nuevo enlace glicosídico). Durante el proceso de transglicosilación, dos residuos de ácido

glutámico o aspártico de la enzima actúan uno como catalizador ácido-base y el otro como nucleófilo. Cuando se utiliza lactosa como sustrato para la formación de GOS, el residuo nucleófilo ataca el sustrato en el centro anomérico (formándose un intermediario galactosil-enzima covalente), mientras que el residuo ácido-base facilita la liberación del grupo saliente (glucosa). Después, el residuo de base ácida activa la molécula aceptora, que ataca al intermediario galactosil-enzima. Si el aceptor es agua, se produce una reacción de hidrólisis y se libera galactosa. Sin embargo, si la lactosa o glucosa actúan como aceptor, se produce la transgalactosilación y los residuos de galactosilo se transfieren desde la enzima a los azúcares para la formación de GOS (Saqib et al., 2017; Lu et al., 2020).

Figura 5. Estructura de los galactooligosacáridos



El rendimiento de la síntesis de GOS puede favorecerse mediante el incremento de la concentración de sustrato y aceptores en la reacción (obteniéndose así GOS con un grado de polimerización más alto), disminuyendo la actividad del agua para evitar que actúe como molécula aceptora del complejo galactosil y derive en la liberación de galactosa o desplazando el equilibrio de la reacción, mediante la eliminación del producto final del medio (Saqib et al., 2017; Davani-Davari et al., 2019).

Las β-galactosidasas pueden proceder de una amplia variedad de microorganismos (bacterias, arqueas, levaduras, hongos), generalmente de *Aspergillus oryzae* o *Strepto coccus thermophilus*, que generan enlaces o-glicosídicos β1-6, *Bacillus circulans* o *Cryptococcus laurentii*, que dan lugar a enlaces del tipo β1-4 o *Bifidobacterium bifidum* con enlaces del tipo β1-3. Por ello, los productos de GOS comercializados pueden diferir en la pureza (oscilando entre el 48-70% (p/p) e incluso alcanzando el 90-92%), el grado de polimerización y el tipo de enlaces de la cadena de oligosacáridos en función de la fuente de enzima utilizada para la síntesis (Lamsal, 2012; Davani-Davari et al., 2019; Lu et al., 2020).

La relevancia de los GOS en la industria alimentaria deriva de sus propiedades tecnológicas ya que son totalmente solubles en agua y su adición no afecta a la viscosidad, textura o sabor del

producto. Así mismo, presentan una gran estabilidad a altas temperaturas, incluso en medios ácidos, debido a los enlaces o-glicosídicos, permaneciendo inalterables a temperaturas por encima de 120 ºC a pH 7 y estables a pH 2 durante meses a temperatura ambiente. Además, tienen un índice glucémico y un valor calórico bajo ya que no son hidrolizados por las enzimas pancreáticas y el jugo gástrico del intestino delgado. Estas características hacen que los GOS sean un ingrediente idóneo para la adición a una amplia gama de productos alimentarios incluyendo matrices con un alto contenido de agua (leche y sopas) o matrices ácidas (zumos de fruta y leches fermentadas) (Sangwan et al., 2011; Lamsal, 2012).

4.2 Metabolismo

Los GOS no son hidrolizados por las enzimas digestivas del tracto gastrointestinal humano, ya que éstas presentan una mayor especificidad por los enlaces α-glicosídicos. Además, las β-galactosidasas localizadas en la membrana del borde en cepillo del intestino delgado humano que podrían hidrolizarlos, generalmente son escasas o tiene una actividad deficiente. Por lo tanto, se estima que el 90% de los GOS ingeridos alcanzan intactos el colon, donde son utilizados como sustrato por la microbiota intestinal con actividad β-galactosidasa (Sako et al., 1999; Van Loo et al., 1999). Esta falta de digestibilidad es la que confiere a los GOS su conocido efecto prebiótico. La Asociación Científica Internacional de Probióticos y Prebióticos (ISAPP) define el término prebiótico como aquel "sustrato que es selectivamente utilizado por los microorganismos del hospedador confiriendo un beneficio para la salud" (Gibson et al., 2017).

El efecto prebiótico de los GOS se pone en evidencia en diversos estudios por su actividad en el crecimiento de especies microbianas beneficiosas como *Lactobacillus* y/o *Bifidobacterium* spp. (So et al., 2018). La modulación de la composición de la microbiota intestinal por la acción de los GOS se aborda con más detalle en el apartado 7.3 de la presente Tesis Doctoral.

Los principales metabolitos generados durante la fermentación colónica de los GOS son ácidos grasos de cadena corta (AGCC) (ácidos acético, butírico y propiónico mayoritariamente), ácido láctico y gases (hidrógeno, metano y dióxido de carbono) (Bruno-Barcena & Azcarate-Peril, 2015). La producción de AGCC se ha relacionado con efectos beneficiosos para la salud. El ácido acético parece participar en la lipogénesis y la colesterogénesis *de novo* en el hígado y, regula el equilibrio energético y el apetito mediante la estimulación de la secreción de leptina en los adipocitos (Morrison & Preston, 2016). El ácido butírico es la principal fuente de energía para el colonocito y sus funciones están asociadas principalmente con la integridad intestinal. Los ácidos butírico y propiónico se han relacionado con funciones de modulación del sistema inmunitario y la respuesta inflamatoria (Koh et

al., 2016; Morrison & Preston, 2016). Además, la producción de AGCC supone una disminución del pH colónico que puede inhibir la actividad de enzimas bacterianas potencialmente patógenas (nitroreductasas, azoreductasas y β-glucoronidasas), favorecer la absorción de minerales y reducir la solubilidad de los ácidos biliares (Slavin, 2013; Bruno-Barcena & Azcarate-Peril, 2015).

Por otro lado, se ha observado que prebióticos con un mayor grado de polimerización (>10) son fermentados de manera más lenta y presentan una menor producción de AGCC que aquellos con un grado de polimerización bajo o medio (<10) (Hernot et al., 2009). Además, el tipo de enlace o-glicosídico de los GOS también afecta a su digestibilidad, siendo los enlaces de tipo β1-6 más resistentes a la hidrólisis durante la digestión *in vitro* que los enlaces β1-4 y β1-3 (Ferreira-Lazarte et al., 2019). Esta mayor resistencia puede relacionarse con un mayor efecto prebiótico observado durante la fermentación *in vitro* de GOS con enlaces β1-6 *vs.* β1-4 (Li et al., 2015).

4.3 Funcionalidad

Existen evidencias de que los GOS pueden ejercer efectos beneficiosos sobre la salud intestinal, la función inmunológica y neurológica, así como sobre marcadores de riesgo cardiovascular (Davani-Davari et al., 2019; Guarino et al., 2020).

Estudios en roedores sugieren que la ingesta de GOS podría ayudar a la prevención del cáncer colorrectal al reducir la incidencia de biomarcadores como la aparición de criptas aberrantes, ralentizar la proliferación de células tumorales, y reducir el tamaño y la multiplicidad de tumores colónicos (Wijnands et al., 2001; Qamar et al., 2017; Fernández et al., 2018). También se ha sugerido que el consumo de prebióticos (mezcla de GOS y fructooligosacáridos en ratio 9:1) puede prevenir enfermedades intestinales como la enterocolitis necrotizante en recién nacidos al promover el vaciado gástrico (Indrio et al., 2009a y 2009b). En estudios clínicos, se ha demostrado la efectividad de la ingesta de GOS (2,75 y 7 g/día durante 2-12 semanas) en la mejora de los síntomas producidos por el síndrome del intestino irritable u otras afecciones gastrointestinales como hinchazón, dolor abdominal o flatulencia (Silk et al., 2009; Vulevic et al., 2018).

A nivel del sistema inmune, se ha observado que la ingesta de GOS a dosis de 5,5 g/día durante 10 semanas en población anciana incrementa los niveles sanguíneos de las citoquinas antiinflamatorias IL-10 e IL-8, las concentraciones de proteína C reactiva y mejora la función de las células *natural killer*, mientras que disminuye los niveles de citoquinas proinflamatorias IL-1β, IL-6 y TNFα (Vulevic et al., 2008 y 2015).

El efecto de los GOS sobre el sistema neurológico también ha sido estudiado en estudios con ratas, observándose una mayor expresión en el factor neurotrófico derivado del cerebro, neurotransmisores y proteínas sinápticas que podrían ejercer una acción ansiolítica o mejorar el rendimiento cognitivo (Savignac et al., 2013; Williams et al., 2016). En individuos sanos se ha demostrado que la suplementación con GOS (5,5 g/día durante 3 semanas) aumenta los niveles de cortisol en saliva y modula de manera selectiva la atención a los estímulos emocionales (Schmidt et al., 2015).

En relación a la reducción del riesgo de enfermedad cardiovascular, el efecto de los GOS sobre el perfil lipídico sérico se ha estudiado mediante modelos murinos y estudios en humanos. En ratones sanos (Cheng et al., 2018) o con síndrome metabólico inducido por una dieta alta en grasas (Dai et al., 2017) y en ratas con hipercolesterolemia (Hashmi et al., 2016) o dislipemia (Chen et al., 2019) se observa una mejora del perfil lipídico (colesterol total, triglicéridos, c-HDL, c-LDL y c-VLDL) tras el consumo de 5,4-54 g/día de GOS durante 3-8 semanas. En cambio, en estudios clínicos en humanos, la ingesta de 5,5 g/día de GOS durante 10 semanas no modifica los niveles de colesterol y c-HDL sanguíneos en adultos sanos (Vulevic et al., 2008). Cuando se evalúa la misma dosis y tipo de GOS en adultos con síndrome metabólico (5,5 g de β-GOS/día), se detecta una disminución significativa de los niveles de colesterol total, triglicéridos y de la relación colesterol total/c-HDL en periodos de tratamiento más largos (6 *vs*. 12 semanas) (Vulevic et al., 2013). Este hecho sugiere que el período de tratamiento en la población humana podría ser un factor condicionante del efecto hipocolesterolémico de los GOS y, por lo tanto, probablemente tendría que extenderse. Aunque la modulación del metabolismo lipídico por parte de los GOS no se ha estudiado en profundidad, se han sugerido diversos mecanismos relacionados con su acción sobre la composición de la microbiota intestinal como la inhibición de la absorción de lípidos debido a una mayor producción de AGCC, la inhibición de la síntesis de colesterol en el hígado a través de la formación de ácido propiónico o una mayor excreción de colesterol en ácidos biliares debido a una mayor metabolización de éstos por parte de la microbiota (Vulevic et al., 2013; Chen et al., 2019).

5. Métodos de evaluación de biodisponibilidad y fermentación colónica

El término biodisponibilidad se define como la fracción de un nutriente o compuesto bioactivo ingerido que está disponible para su utilización en las funciones fisiológicas del organismo o para ser almacenado (Fernández-García et al., 2009; Guerra et al., 2012). Este concepto incluye la digestibilidad y solubilidad del compuesto en el tracto gastrointestinal, su absorción por parte de las células intestinales y el transporte a la circulación, así como su incorporación desde la circulación a la diana funcional (Alegría et al., 2015). A su vez, la biodisponibilidad incluye los términos

bioaccesibilidad (fracción de un compuesto que se libera de la matriz alimentaria en el tracto gastrointestinal y está disponible para la absorción intestinal) y bioactividad (que incluye los procesos relacionados con el transporte y la llegada a la diana de acción del compuesto, las interacciones con biomoléculas, su metabolismo o biotransformación, la generación de biomarcadores y la respuesta fisiológica producida) (Fernández-García et al., 2009; Cilla et al., 2017).

Los estudios *in vivo* constituyen los métodos de referencia para la determinación de la biodisponibilidad de un nutriente o compuesto bioactivo. Sin embargo, desventajas como las limitaciones en el diseño experimental, la dificultad en la interpretación de los datos, el alto coste del equipamiento y del personal cualificado, las restricciones éticas y la alta variabilidad interindividual, han dado lugar al desarrollo de métodos *in vitro* de digestión gastrointestinal y/o fermentación colónica simulada que pueden utilizarse como herramientas predictivas (Alegría et al., 2015).

A continuación, se describen los principales sistemas *in vitro* para el estudio de la bioaccesibilidad y fermentación colónica, así como una descripción de las principales metodologías *in vivo* para el estudio de la biodisponibilidad, dedicando mayor atención a aquellos utilizados en la presente Tesis Doctoral.

5.1 Métodos *in vitro*

Diversos métodos de digestión gastrointestinal han sido desarrollados para la simulación del proceso digestivo de los alimentos. Estos sistemas abarcan desde modelos estáticos sencillos hasta sistemas dinámicos más complejos, de uno o varios compartimentos, que pueden incluir la simulación de diversas etapas de la digestión y, en algunos casos, la fermentación colónica (Lucas-González et al., 2018).

Modelos estáticos

Los métodos de digestión gastrointestinal estáticos son técnicas simples que reproducen las condiciones fisiológicas de temperatura, pH, agitación o, composición enzimática y química del tracto gastrointestinal humano. Suelen constar de dos o tres etapas que simulan las fases de la digestión salivar, gástrica e intestinal. Tras la digestión, la cantidad de compuesto soluble presente en el sobrenadante obtenido por centrifugación o filtración puede ser utilizada como una medida de la bioaccesibilidad del mismo (Alegría et al., 2015).

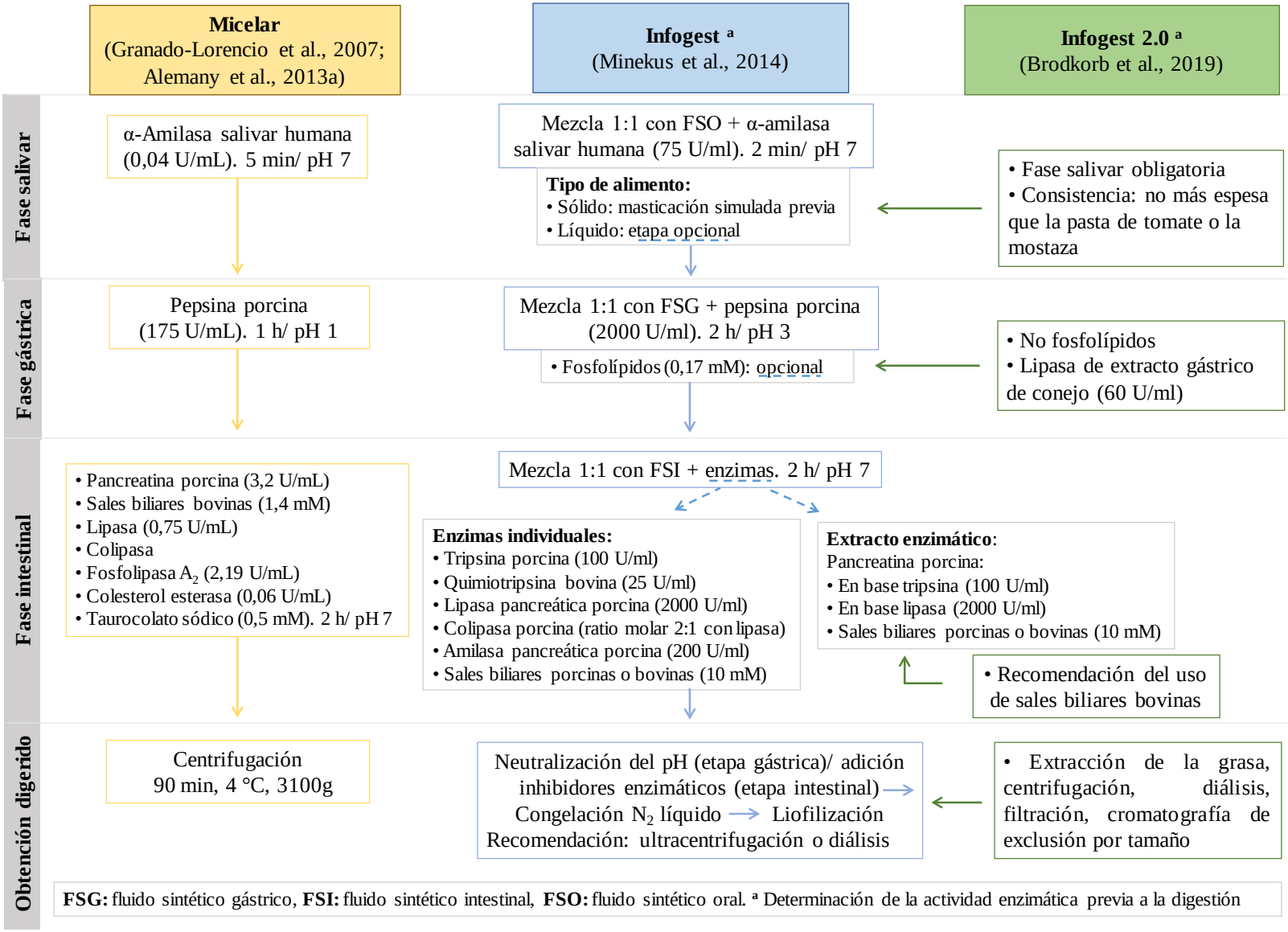
En estos métodos, la etapa salivar suele presentar una duración de segundos o minutos y se lleva a cabo generalmente mediante la adición de α-amilasa salivar a pH neutro (6,5-7,5). La etapa gástrica (1-3 h) se simula mediante la adición de HCl y/o pepsina (con un ajuste de pH a 1-2) y la etapa intestinal (1-5 h) con la incorporación de pancreatina o enzimas pancreáticas individuales (lipasa, amilasa, ribonucleasa y proteasa) con o sin adición de ácidos biliares como emulsionantes (neutralizando el pH a 6,5-7,5 con NaOH o $NaHCO_3$) (Alminger et al., 2014; Alegría et al., 2015).

La elección de enzimas y los parámetros de la digestión son condicionados por el objetivo del estudio. Un ejemplo de ello es la aplicación a compuestos lipofílicos de un método de digestión gastrointestinal micelar en tres etapas, que incluye la adición de enzimas claves del metabolismo lipídico durante la etapa intestinal (lipasa pancreática, colipasa, colesterol esterasa y fosfolipasa A_2) y taurocolato de sodio para favorecer la micelarización y permitir evaluar la bioaccesibilidad de carotenoides (Granado-Lorencio et al., 2007) y EV (Alemany et al., 2013a). Las características de esta digestión se muestran en la figura 6.

Sin embargo, la existencia de una gran diversidad de metodologías de digestión gastrointestinal *in vitro* dificulta la comparación de resultados entre diferentes grupos de trabajo. Por este motivo, dentro del marco de la acción Europea COST Infogest se ha desarrollado un método de digestión basado en parámetros fisiológicos, con el objetivo de armonizar las condiciones aplicadas a los modelos de digestión estáticos (Minekus et al., 2014) que recientemente ha sido revisado (Infogest 2.0) (Brodkorb et al., 2019).

El método Infogest (ver figura 6) estandariza la composición iónica de los fluidos sintéticos adicionados en la fase oral (FSO), gástrica (FSG) e intestinal (FSI), las condiciones de pH y tiempo de las tres etapas digestivas y la adición de enzimas digestivas en base a su actividad enzimática y de extractos biliares en función de su contenido en ácidos biliares (detallándose la metodología para estas determinaciones como paso previo a la digestión) (Minekus et al., 2014). La etapa salivar inicialmente se consideraba necesaria en alimentos sólidos y opcional en líquidos y semisólidos, especialmente en alimentos que contienen almidón (ya que una pequeña parte puede ser hidrolizado a pesar de la brevedad de esta etapa). En la última revisión se ha considerado obligatoria y se han dado directrices de la textura que se debe alcanzar al finalizar esta etapa (una pasta de consistencia no más espesa que la pasta de tomate o la mostaza).

Figura 6. Comparación de métodos de digestión gastrointestinal micelar, Infogest e Infogest 2.0



En la fase gástrica se incluye la adición de HCl y pepsina. Actualmente, respecto a la propuesta inicial que en la que no se adicionaba lipasa gástrica humana (por falta de disponibilidad comercial y por no considerarse adecuada la de origen fúngico), se ha incorporado la adición de lipasa de extracto gástrico de conejo debido a que desencadena la acción posterior de la lipasa pancreática sobre sustratos lipídicos (reajustando la concentración/actividad de pepsina debido a un aporte extra procedente del extracto). En ausencia de fosfolípidos u otras moléculas que puedan actuar como surfactantes/tensioactivos en la matriz alimentaria, se recomienda la adición de fosfolípidos para favorecer la emulsificación.

En la fase intestinal se neutraliza el pH mediante la adición de NaOH. La adición de enzimas digestivas en esta etapa se realiza mediante la incorporación de pancreatina de origen porcino (determinando su actividad en base a tripsina o lipasa en función de la naturaleza proteica o lipídica del alimento, respectivamente) o mediante enzimas pancreáticas individuales. La adición de sales biliares se contempla mediante la incorporación de extracto biliar de origen porcino o, preferentemente, bovino (debido a una mayor similitud en su composición con respecto a la bilis humana o por bilis fresca porcina).

El protocolo aconseja la conservación del digerido mediante su inmersión en nitrógeno líquido, con previa neutralización del pH en la etapa gástrica (si esta es el objetivo del estudio) o la adición de inhibidores de proteasa. El Infogest 2.0 ha proporcionado nuevos procedimientos para la obtención del digerido y la finalización de las etapas de la digestión (mediante inhibidores de amilasas, lipasas y proteasas fundamentalmente) (ver figura 6) y ha modificado algunos protocolos para la determinación de la actividad enzimática.

Desde su publicación, el método Infogest se ha aplicado a una amplia variedad de alimentos con el objetivo de evaluar la digestibilidad y bioaccesibilidad de compuestos lipofílicos y, para ello se han propuesto algunas modificaciones del método original (ver tabla 2). En este sentido, dado que el método Infogest no estandariza la actividad de enzimas involucradas en el metabolismo lipídico necesarias para la hidrólisis de los enlaces éster como la colesterol esterasa, se ha propuesto la inclusión de esta enzima con el objetivo de favorecer la incorporación de los carotenoides a las micelas mixtas durante la etapa intestinal (Estevez-Santiago et al., 2016). Otras modificaciones incluyen la adición de una mayor concentración de sales biliares para favorecer la micelarización de estos compuestos (Rodrigues et al., 2016; Petry & Mercadante, 2019) o la modificación de la composición de electrolitos de los fluidos sintéticos simulados (Eriksen et al., 2016 y 2017) (ver tabla 2).

Tabla 2. Aplicaciones y modificaciones del método de digestión Infogest para la determinación de compuestos lipofílicos en alimentos

Alimento	Compuesto estudiado	Protocolo digestión	Obtención digerido	Referencia
Empanada de cerdo - Con o sin extracto de fibra dietética obtenida como productos de la extracción de zumo de limón, uva o granada o de la producción de helados de limón o chufa	Colesterol	α-amilasa Pepsina porcina Pancreatina porcina [a] Bilis fresca	Centrifugación 4000 rpm, 20 min, 4ºC	López-Marcos et al., 2015
Naranja, mandarina, melocotón, melón, níspero y pimiento rojo	Carotenoides (β-criptoxantina y α/β-caroteno)	α-amilasa Pepsina porcina Pancreatina porcina [a] Sales biliares bovinas y ovinas [b]	Centrifugación 10000 g, 10 min	Estévez-Santiago et al., 2016
Espinacas y ensalada	Carotenoides (luteína y β-caroteno)	α-amilasa Pepsina porcina Pancreatina porcina Sales biliares bovinas y ovinas [c]	Centrifugación (x2) 4495 g, 10 min	Eriksen et al., 2016
Murici (*Byrsonima crassifolia*)	Carotenoides libres y esterificados	α-amilasa Pepsina porcina Pancreatina porcina Sales biliares bovinas y ovinas [d]	Centrifugación 20000 g, 5 min, 4ºC	Rodrigues et al., 2016
Espinacas (hoja entera o procesada en forma de puré) Con o sin adición de grasa (aceite de oliva refinado, aceite de cacahuete o mantequilla)	Carotenoides (luteína y β-caroteno)	α-amilasa Pepsina porcina Pancreatina porcina Sales biliares bovinas y ovinas [c]	Centrifugación (x2) 4495 g, 10 min, ≤ 5ºC	Eriksen et al., 2017
Bayas de Goji y espinacas Con o sin adición de grasa (aceite de coco)	Carotenoides (zeaxantina, zeaxantina dipalmitato y luteína)	α-amilasa Pepsina porcina Pancreatina porcina Bilis	Centrifugación 75000 g, 60 min, 10ºC	Hempel et al., 2017

Tabla 2. Continuación

Alimento	Compuesto estudiado	Protocolo digestión	Obtención digerido	Referencia
Uva, papaya, mango, espinaca, zanahoria, tomate, pasta de tomate, salmón, yema de huevo y ensalada con o sin huevo duro o salmón	Carotenoides	α-amilasa Pepsina porcina Pancreatina porcina Sales biliares bovinas y ovinas [d]	Centrifugación 20000 g, 5 min, 4ºC	Rodrigues et al., 2017
Chocolate negro - Diferentes contenidos de cacao con o sin adición de EV	EV	α-amilasa Pepsina porcina Pancreatina porcina Sales biliares[e]	-	Tolve et al., 2018
Caqui	Carotenoides	α-amilasa Pepsina porcina Pancreatina porcina Sales biliares [c]	Centrifugación 20000 g, 5 min, 4ºC	Cano et al., 2019
Pulpa de mandarina y aceite de soja	Carotenoides libres y esterificados	α-amilasa Pepsina porcina Pancreatina porcina (en base a tripsina) Sales biliares bovinas y ovinas [d]	Centrifugación 20000 g, 10 min, 4ºC	Petry & Mercadante, 2019
Snack de granola y pudin enriquecidos en EV - Con o sin adición de grasa	EV	α-amilasa Pepsina porcina Lipasa fúngica Pancreatina (en base a α-amilasa) Sales biliares porcinas	Centrifugación 10000 g, 30 min, 4ºC	Ubeyitogullari & Ciftci, 2019

Tabla 2. Continuación

Alimento	Compuesto estudiado	Protocolo digestión	Obtención digerido	Referencia
Higo chumbo	Betalaínas y compuestos fenólicos	α-amilasa Pepsina porcina Pancreatina porcina Sales biliares [c]	-	Gómez-Maqueo et al., 2020a
Lúcumo (*Puteria lucuma*)	Carotenoides libres y esterificados	α-amilasa Pepsina porcina Pancreatina porcina Sales biliares [c]	Centrifugación 20000 g, 10 min, 4ºC	Gómez-Maqueo et al., 2020b

EV: esteroles vegetales. [a] En base a tripsina; [b] Adaptado por Estévez-Santiago et al., 2016: adición de colesterol esterasa (3 U/mL); [c] Adaptado por Eriksen et al., 2016 y 2017: modificación de la concentración de electrolitos de los fluidos sintéticos; [d] Adaptado por Rodrigues et al., 2016: aumento del contenido de sales biliares; [e] Adaptado por Tolve et al., 2018: modificación de la duración de las etapas salivar (3 min), gástrica (1 h) e intestinal (1 h).

Aunque los modelos de digestión estáticos son herramientas rápidas, simples, económicas y presentan una gran reproducibilidad por el control de las variables experimentales, no simulan los aspectos dinámicos del proceso de digestión ni las interacciones fisiológicas que tienen lugar. Por ello, se utilizan como herramienta de cribado o categorización para evaluar la influencia de las condiciones de la digestión sobre compuestos de interés, determinar el efecto de la estructura o composición del alimento o del procesado en la bioaccesibilidad, así como para determinar interacciones entre nutrientes con el objetivo de mejorar la formulación y el diseño de los productos alimentarios (Alegría et al., 2015; Cilla et al., 2017).

En el caso de la simulación de la etapa de fermentación colónica, métodos estáticos como el "*cultivo por lote*" permiten determinar el efecto de un sustrato, compuesto o alimento (en algunos casos sometidos a una etapa previa de digestión) sobre la microbiota intestinal y viceversa. Este método consiste en la adición de cultivos (cepas específicas o microbiota intestinal o fecal de origen humano o animal) en recipientes junto con el sustrato a fermentar y el medio de cultivo en condiciones de anaerobiosis. Este tipo de fermentación es el método más simple para estudiar el efecto de determinados sustratos sobre la composición de la microbiota y evaluar modificaciones de la actividad metabólica mediante el análisis de la formación de AGCC y otros metabolitos. Es una herramienta rápida de cribado y requiere de poco volumen de sustrato. Sin embargo, los ensayos generalmente se limitan a una duración de 24-48 h debido a que los cambios en la disponibilidad del sustrato, el pH y el potencial redox pueden dar como resultado la proliferación de poblaciones microbianas no representativas a lo largo del tiempo. Además, la acumulación de metabolitos puede inhibir la actividad de la microbiota (Marzorati et al., 2014; Venema & van den Abbeele, 2013).

Modelos dinámicos

Los modelos de digestión gastrointestinal dinámicos se desarrollan con el objetivo de obtener una mayor aproximación a la situación *in vivo*. En general, en estos modelos las secreciones digestivas se adicionan de manera progresiva mediante una tasa de secreción constante o de manera previamente programada para que cambie en función del tiempo o en respuesta a otros parámetros como el volumen de llenado. La monitorización del pH durante la digestión permite la acidificación progresiva mediante la adición de HCl en la etapa gástrica y/o su neutralización mediante $NaHCO_3$ en la etapa intestinal. Estos sistemas pueden simular otros parámetros como los movimientos peristálticos, el vaciado gástrico, el tiempo de tránsito gastrointestinal o, en caso de que se incluya la etapa de fermentación colónica, cambios en la diversidad o abundancia de la microbiota intestinal y la formación de metabolitos (Guerra et al., 2012; Thuenemann, 2015).

Los modelos dinámicos se dividen a su vez en aquellos que constan de un único reactor (monocompartimentales) y los formados por varios reactores interconectados (multicompartimentales). Entre los primeros destacan el "*Dinamic Gastric Model*" (DGM) y el "*Human Gastric Simulator*" (HGS) que simulan la digestión gástrica. Sin embargo, el DGM se caracteriza por la diferenciación del fondo y antro del estómago (en el primero se adicionan las secreciones gástricas y en el último se simula las contracciones peristálticas) (Dupont et al., 2019).

El sistema DIDGI es un modelo multicompartimental de los más básicos. Se compone de dos reactores que simulan el estómago e intestino delgado y mediante una membrana de teflón (de 2 mm de poro) situada entre la bomba que conecta ambos compartimentos se simula el efecto de tamizado del píloro en humanos. El sistema "*TNO Gastro-intestinal Model*" (TIM-1) también reproduce el tracto superior gastrointestinal, sin embargo, incorpora tres reactores diferentes para representar los tramos del duodeno, yeyuno e íleon. El mezclado en cada compartimento se obtiene mediante cambios en la presión en las paredes flexibles que conforman los reactores. Además, incorpora una membrana de diálisis para retirar los compuestos hidrosolubles y un sistema de filtración para los compuestos liposolubles (Marzorati et al., 2014; Dupont et al., 2019).

Los modelos "*TNO Gastro-Intestinal Model of the colon*" (TIM-2) y "*Polyfermentor Intestinal Model*" (PolyFerms), reproducen únicamente las condiciones del colon proximal. El TIM-2 requiere de un corto periodo de estabilización de la población microbiana tras la inoculación con microbiota procedente de heces (16 h) y presenta una membrana de diálisis que simula la absorción de metabolitos (previniendo su acumulación y la posible inhibición de la actividad microbiana) (Venema & Van den Abbeele, 2013; Venema, 2015). Por otra parte, el PolyFerms consta de un compartimento que contiene microbiota fecal inmovilizada en perlas de gel que se transfiere a distintos reactores donde se lleva a cabo la fermentación en paralelo. De esta manera, permite comparar los efectos de distintos tratamientos sobre una misma población microbiana (Tanner et al., 2014).

Ente los modelos multicompartimentales más complejos destacan el "*Simulator of the Human Intestinal Microbial Ecosystem*" (SHIME) y el "*SIMulator Gastro-Intestinal*" (SIMGI), que comprenden todo el tracto gastrointestinal. En general, estos sistemas requieren de un periodo de estabilización de la microbiota más largo (aproximadamente de 2 semanas) (Barroso et al., 2015; Dupont et al., 2019).

En la tabla 3 se resumen las características de los principales modelos de digestión gastrointestinal y/o fermentación colónica dinámicos descritos.

Tabla 3. Características de los principales sistemas de digestión gastrointestinal y/o fermentación colónica dinámicos

	Monocompartimentales		***Multicompartimentales***					
Características	DGM	HGS	DIDGI	TIM-1	TIM-2	PolyFerms	SHIME	SIMGI
Simulación de:								
• Estómago	Sí	Sí	Sí	Sí	No	No	Sí	Sí
- Fondo y antro	Sí	No	No	No	-	-	No	No
• Intestino delgado	No	No	Sí	Sí	No	No	Sí	Sí
- Duodeno, yeyuno e íleon	-	-	No	Sí	-	-	No	No
- Absorción intestinal	-	-	No	Sí	-	-	No	No
• Colon	No	No	No	No	Sí	Sí	Sí	Sí
- Ascendente, transversal y descendente	-	-	-	-	No	No	Sí	Sí
- Inóculo fecal	-	-	-	-	En suspensión	Inmovilizada	En suspensión	En suspensión
- Absorción colónica	-	-	-	-	Sí		No	No
• Movimientos peristálticos	Sí	Sí	No [a]	Sí	Sí	No [a]	No [a]	No [a]
• Anaerobiosis (flujo de N_2)	No	No	Sí	Sí	Sí	Sí	Sí	Sí
• Estudios de larga duración	-	-	-	-	No	Sí	Sí	Sí

DGM: "*Dinamic Gastric Model*"; HGS: "*Human Gastric Simulator*"; SHIME: "*Simulator of the Human Intestinal Microbial Ecosystem*"; SIMGI: "*SIMulator Gastro-Intestinal*"; PolyFerms: "*Polyfermentor Intestinal Model*"; TIM-1: "*TNO Gastro-intestinal Model*"; TIM-2: "*TNO Gastro-Intestinal Model of the colon*". [a] Agitación continua

Aunque los modelos de digestión dinámicos proporcionan unas condiciones más similares a las fisiológicas que los modelos estáticos, son más caros y existe una mayor dificultad para acceder a ellos. Por ello, se han desarrollado modelos intermedios como los sistemas semidinámicos con el objetivo de ser aplicados por un mayor número de laboratorios. En este sentido, recientemente, se ha publicado un método estandarizado de este tipo basado en el método estático Infogest (Mulet-Cabero et al., 2020). Este método incluye: i) la adición de FSO en función del peso seco del alimento a digerir con una ratio 1:1; ii) la acidificación mediante HCl y adición de enzimas gástricas de forma gradual (el FSG es ajustado a pH 7 en lugar de 3); iii) la simulación del vaciado gástrico mediante la toma de alícuotas en función del contenido calórico del alimento (obtenido de la medida directa o mediante cálculos teóricos); y iv) el aumento de la actividad de las enzimas adicionadas a los FSO, FSG y FSI, así como de la concentración de sales biliares. Este modelo permite el estudio de las cinéticas de digestión de los compuestos, así como determinar los cambios estructurales que ocurren durante la fase gástrica y que afectan a la absorción.

5.2 Métodos *in vivo*

Como se ha comentado anteriormente, los estudios *in vivo* son los métodos de referencia para el estudio de la biodisponibilidad de un nutriente o compuesto bioactivo. Aunque se han utilizado diversos modelos animales (monos, roedores, cerdos) para estudios de biodisponibilidad, las diferencias existentes a nivel fisiológico y metabólico dificultan en algunos casos la extrapolación de resultados a humanos (Cardoso et al., 2015).

Entre las metodologías *in vivo* utilizadas para la estimación de la biodisponibilidad se encuentran los estudios de balances (químicos y con radioisótopos o isótopos estables) y la determinación de la concentración del compuesto de interés en tejidos o fluidos biológicos (Guerra et al., 2012; Cardoso et al., 2015). Los estudios de balances químicos evalúan la diferencia ente la cantidad ingerida de un compuesto y la excretada (heces y orina) con el objetivo de determinar la absorción del mismoEn el caso de que existan concentraciones endógenas del compuesto de interés, es necesario utilizar técnicas de balance con radioisótopos que, aunque presentan una gran precisión y son métodos relativamente económicos, tiene un riesgo potencial por la radiación ionizante. Otra opción es la utilización de técnicas de balance con isótopos estables (presenta una mayor seguridad para la aplicación en humanos, pero su coste es mayor). En cuanto al análisis de tejidos o fluidos, la determinación de compuestos bioactivos en plasma o suero ha sido ampliamente utilizada como una medida de la biodisponibilidad de los mismos (Cilla et al., 2017). En el caso de estudios sobre fermentación colónica tiene gran importancia, junto con los posibles metabolitos del compuesto

formados, evaluar los cambios en la microbiota intestinal por efecto de distintos componentes dietéticos. En este contexto, los estudios *in vivo* requieren de procedimientos invasivos para el acceso a la toma de muestra en el intestino grueso y, por ello, se limitan a la determinación de metabolitos y al análisis de la composición de la microbiota en muestras fecales (Payne et al., 2012).

6. Biodisponibilidad de esteroles y sus óxidos

La bioactividad de los alimentos enriquecidos con EV se relaciona con su biodisponibilidad, la cual depende de la matriz alimentaria y del origen del ingrediente utilizado como fuente de EV. Como se menciona en el apartado anterior, los métodos de digestión gastrointestinal *in vitro* pueden utilizarse como modelos predictivos con el fin de mejorar el diseño de los alimentos funcionales. Estos sistemas permiten determinar los factores que afectan a la solubilidad de los EV con el objetivo de optimizar sus efectos beneficiosos (Cilla et al., 2019). Además, los métodos de digestión gastrointestinal simulada son herramientas útiles para evaluar la solubilidad de los SOPs que se pueden formar durante el proceso de fabricación o almacenamiento de productos enriquecidos con EV, para determinar los factores que están implicados en su formación y minimizarlos (Barriuso et al., 2017).

A continuación, se describen los principales estudios que han evaluado la bioaccesibilidad de esteroles y SOPs en alimentos enriquecidos mediante la aplicación de digestiones gastrointestinales simuladas, así como los principales estudios de biodisponibilidad de esteroles (ensayos clínicos).

6.1 Estudios *in vitro*

Diversos estudios han evaluado la bioaccesibilidad de los esteroles presentes en alimentos enriquecidos con EV como leches fermentadas (Vaghini et al., 2016), leche desnatada o bebidas a base de zumo de frutas (Alemany et al., 2013a), bebidas a base de zumo de frutas y leche (Alemany et al., 2013a; Alvarez-Sala et al., 2016), chocolate negro (Tolve et al., 2018), *snacks* de granola y pudin (Ubeyitogullari & Ciftci, 2019) y productos de maíz fermentados similares al yogur (Gies et al., 2020).

Los resultados de la bioaccesibilidad de esteroles obtenidos tras la aplicación de la digestión gastrointestinal micelar simulada en diversas bebidas se resumen en la tabla 4.

Tabla 4. Bioaccesibilidad de esteroles (%) en bebidas enriquecidas con esteroles vegetales

	Leches fermentadas (Vaghini et al., 2016)				**Leche desnatada** Alemany et al., 2013a	**Bebida de frutas [a]** Alemany et al., 2013a
Contenido EV	**2,9**	**2,0**	**1,6**	**1,5**	**0,8**	**0,8**
Campesterol	-	18,94 ± 1,48	19,46 ± 0,38	9,01 ± 0,44	3,75 ± 0,15	4,88 ± 0,16
Campestanol	12,54 ± 0,59	-	-	-	3,47 ± 0,35	4,27 ± 0,35
Estigmasterol	-	-	-	8,74 ± 0,52	2,95 ± 0,44	3,04 ± 0,17
β-Sitosterol	6,31 ± 0,30	17,17 ± 1,49	16,88 ± 0,64	8,97 ± 0,48	2,51 ± 0,07	2,93 ± 0,10
Sitostanol	11,35 ± 0,96	13,57 ± 1,24	13,42 ± 1,18	-	2,71 ± 0,12	3,22 ± 0,16
EV totales	11,28 ± 0,85	16,84 ± 1,35	16,64 ± 0,64	8,90 ± 0,40	2,62 ± 0,08	3,10 ± 0,11
Colesterol	-	-	-	-	79,05 ± 1,09	-

Bebidas a base de zumo de frutas y leche (0,8 g EV/100 mL)					
	Alemany et al., 2013a		Álvarez-Sala et al., 2016		
		[a]	**[a]**	**+ aceite de oliva y lecitina de soja [a]**	**+ grasa láctea y concentrado de proteínas séricas enriquecido en MFGM [a]**
Campesterol	8,31 ± 0,65	6,66 ± 0,27	9,03 ± 0,59	26,37 ± 3,94	33,86 ± 3,83
Campestanol	6,82 ± 0,57	5,79 ± 0,55	27,75 ± 2,76	31,41 ± 4,74	36,92 ± 3,84
Estigmasterol	5,96 ± 0,60	4,02 ± 0,11	5,72 ± 0,60	19,37 ± 0,98	32,64 ± 3,28
β-Sitosterol	6,42 ± 0,50	4,15 ± 0,04	8,45 ± 0,81	28,14 ± 3,53	30,72 ± 2,61
Sitostanol	6,05 ± 0,71	4,41 ± 0,10	9,79 ± 1,02	29,94 ± 3,45	34,37 ± 2,90
EV totales	6,48 ± 0,52	4,35 ± 0,06	8,73 ± 0,81	28,19 ± 3,58	31,39 ± 2,70
Colesterol	53,34 ± 4,85	98,96 ± 3,30	57,60 ± 3,37	53,50 ± 4,23	64,18 ± 6,64

Bioaccesibilidad (%) calculada como (contenido de esterol en la fracción bioaccesible x 100)/(contenido de esterol en la bebida). Contenido de EV expresado por 100 mL de bebida excepto para Vaghini et al., 2016 (g/100 g). EV: esteroles vegetales; MFGM: membrana del glóbulo graso de la leche. **[a]** Con zumo de mandarina procedente de concentrado como fuente de β-criptoxantina

En general, en leches fermentadas y leche desnatada se observa una mayor bioaccesibilidad de EV totales a mayores dosis de enriquecimiento (8,9-16,8% con 1,5-2,9 g EV/100 g *vs*. 2,6% con 0,8 g EV/100 mL) (Alemany et al., 2013a; Vaghini et al., 2016) (tabla 4). Sin embargo, la bioaccesibilidad más alta en los productos de leche fermentada podría deberse a un efecto de la propia matriz ya que en este tipo de productos se ha indicado que la alteración del MFGM por acción ácida o microbiana podría favorecer la adsorción de los esteroles (Baldi & Pinotti, 2008). Así mismo, otros componentes de la matriz alimentaria podrían reducir la solubilidad de los EV, ya que leches fermentadas enriquecidas con un contenido similar de EV (1,5-1,6 g/100 g) muestran una menor bioaccesibilidad en presencia de contenidos elevados de carbohidratos y fibra (8,9 *vs.* 16,6%) (Vaghini et al., 2016).

La bioaccesibilidad de los EV también se relaciona con el ingrediente utilizado como fuente de EV. En leches fermentadas se observa que muestras con un perfil de EV igual o similar (β-sitosterol (80%) > sitostanol (12%) > campesterol (5-7%)) presentan una solubilidad semejante tanto de los esteroles individuales (campesterol > β-sitosterol > sitostanol) como de los EV totales (≈17%) (ver tabla 4, bebidas con 1,6 y 2,0 g EV/100 g). Sin embargo, en leches fermentadas con un enriquecimiento similar o más alto (1,5 o 2,9 g EV/100 g) pero utilizando distinto ingrediente de EV (β-sitosterol (45%) > estigmasterol (33%) > campesterol (22%), o sitostanol (85%) > campestanol (11%) > β-sitosterol (4%)) se observan cambios en la bioaccesibilidad individual de los EV (campesterol > β-sitosterol > estigmasterol, o campestanol > sitostanol > β-sitosterol) y una bioaccesibilidad menor de los EV totales (9 y 11%, respectivamente) (Vaghini et al., 2016). En este sentido, al comparar la bebida a base de zumo de frutas con la leche desnatada, ambas enriquecidas con la misma cantidad de EV (0,8 g/100 ml) procedentes de la misma fuente (*tall oil*), se observa una bioaccesibilidad similar de EV individuales (campesterol > campestanol > β-sitosterol ≈ sitostanol ≈ stigmasterol) y totales (aproximadamente 3%) (Alemany et al., 2013a) (ver tabla 4).

En cuanto al efecto de la matriz, las bebidas a base de zumo de frutas y leche presentan una bioaccesibilidad de EV totales mayor (4,4-8,7%) con respecto a aquellas que sólo contienen zumo de frutas (3,1%) enriquecidas con la misma cantidad de EV (0,8 g/100 mL) (ver tabla 4). Por lo tanto, la presencia de grasa láctea, la cual se ha demostrado que es un sistema de liberación efectivo para microconstituyentes lipofílicos (Baldi & Pinotti, 2008), podría favorecer la extracción y estabilidad de los EV en la matriz alimentaria, mejorando su dispersión en la fase acuosa de la digestión. En cambio, la incorporación de zumo de mandarina como fuente de β-criptoxantina en bebidas a base de zumo de frutas y leche reduce ligeramente la bioaccesibilidad de los EV totales (6,5 *vs.* 4,4%), probablemente debido a una competición entre los EV y la β-criptoxantina por su incorporación en las micelas mixtas (Alemany et al., 2013a). En relación a la bioaccesibilidad de los EV individuales,

en estas bebidas (con o sin zumo de mandarina) se observa una mayor solubilidad de campesterol/campestanol con respecto a β-sitosterol/sitostanol. Los autores sugieren que la presencia de una cadena lateral más corta en la posición 24 de campesterol/campestanol (grupo metil *vs*. grupo etil de β-sitosterol/sitostanol) y la menor afinidad del primero por las micelas mixtas favorece su solubilidad en la fase acuosa. Sin embargo, en un estudio posterior con una bebida similar a base de zumo de mandarina y leche (Alvarez-Sala et al., 2016) se obtiene una bioaccesibilidad de EV totales dos veces mayor que la observada por Alemany et al. (2013a). Los autores sugieren que la mayor contribución de campesterol a la bioaccesibilidad (27,8% *vs*. 5,8%) puede explicar la mayor bioaccesibilidad de EV totales (ver tabla 4).

El efecto de la adición de grasas y emulsionantes de origen vegetal (aceite de oliva virgen extra y lecitina de soja) o animal (grasa láctea y concentrado de proteínas séricas enriquecido con MFGM) sobre la bioaccesibilidad de EV en bebidas a base de zumo de frutas y leche se muestra en la tabla 4. El aumento del contenido de grasa de las bebidas (2,4% *vs*. 1,1%) aumenta la bioaccesibilidad de EV entre 6,5-7,2 veces. Es probable que la adición de grasa promueva la formación de micelas mixtas durante la digestión gastrointestinal, aumentando así la solubilidad de los EV. No se observa un efecto del origen de la grasa sobre la bioaccesibilidad de los EV totales. Sin embargo, en presencia de grasa de origen vegetal, el β-sitosterol y el sitostanol muestran una mayor solubilidad con respecto al campesterol mientras que en presencia de grasa de origen animal el sitostanol es el esterol menos soluble (Alvarez-Sala et al., 2016).

En todas las bebidas enriquecidas con EV mencionadas anteriormente se observa una alta solubilidad del colesterol en la fase acuosa micelar y su bioaccesibilidad es significativamente mayor que la de los EV (entre 1,9 y 30,2 veces) (ver tabla 4). Sin embargo, la mayor hidrofobicidad de los EV con cadenas laterales saturadas (campesterol, campestanol, β-sitosterol y sitostanol) con respecto al colesterol, implica una mejor transferencia a las micelas mixtas, disminuyendo así la incorporación de colesterol al considerar alimentos enriquecidos con EV (Moran-Valero et al., 2012). Además, la bioaccesibilidad del colesterol parece disminuir en presencia de zumo de mandarina, mientras que la de los EV aumenta, probablemente debido a la competición molecular con el colesterol por la incorporación en las micelas (Cilla et al., 2019).

Recientemente, se ha aplicado el método de digestión Infogest a matrices alimentarias enriquecidas con EV como chocolate negro (Tolve et al., 2018) y *sancks* de granola y pudin (Ubeyitogullari & Ciftci, 2019). La bioaccesibilidad de los EV en chocolate negro con diferente contenido de cacao (64, 72 y 85%) y diferentes dosis de enriquecimiento de EV (5, 10 y 15%) oscila

entre 6,5 y 8,4%. Estos valores son del mismo orden que los observados en bebidas de leche fermentada (Vaghini et al., 2016) pero menor a las obtenidas en bebidas a base de zumo de frutas y leche con adición de grasa láctea y MFGM (Álvarez-Sala et al., 2016).

En *snacks* de granola y pudins, se observa una mejora de la bioaccesibilidad de los EV añadidos como nanopartículas impregnadas en aerogeles nanoporosos de almidón *vs*. EV sin encapsular (53-92% vs. 16-32% y 19% *vs*. 2%, respectivamente) (Ubeyitogullari & Ciftci, 2019). Este hecho sugiere que la composición más compleja de los *snacks* de granola (mayor contenido de proteínas y emulsionantes) podría mejorar la solubilización de los EV en la fase acuosa durante la digestión *in vitro*. En este estudio también se evalúa el efecto de distintos contenidos de grasa de los *snacks* (sin contenido graso (0%), con contenidos bajos (7%) y normales (24%)) y se observa que la bioaccesibilidad de los EV varía entre contenidos bajos y normales de grasa (≈90% en EV nanoencapsulados y ≈30% en EV sin encapsular) pero disminuye significativamente en ausencia de la misma (53% en EV nanoencapsulados y 16% en EV sin encapsular). De manera similar a lo observado en bebidas a base de zumo de frutas y leche comentadas anteriormente (Alvarez-Sala et al., 2016), la presencia de grasa en los *snacks* parece mejorar la bioaccesibilidad de los EV. Sin embargo, la ausencia de diferencias en la bioaccesibilidad al comparar contenidos bajos y normales de grasa sugiere que se requiere de un umbral de contenido de lípidos para maximizar la capacidad de solubilización de las micelas mixtas (Ubeyitogullari & Ciftci, 2019).

En productos de maíz fermentado enriquecidos con EV (1,7 g/100 g) y carotenoides (1,6 g/100 g) (Gies et al., 2020), la bioaccesibilidad del β-sitosterol es de 1,6%, el valor observado más bajo para este esterol en comparación con el resto de matrices enriquecidas en EV previamente citadas. Los autores sugieren que, al igual que se ha mencionado anteriormente en bebidas a base de zumo de frutas y leche que contienen β-criptoxantina (Alemany et al., 2013a), la presencia de carotenoides limita la incorporación de β-sitosterol en las micelas mixtas. La solubilidad del colesterol se reduce un 50% con la incorporación de EV. La digestión gastrointestinal *in vitro* llevada a cabo en este estudio, se basa en la metodología de Reboul et al. (2006) y Dhuique-Mayer et al. (2016), validada con estudios en humanos como modelo fiable para estudiar el comportamiento de los carotenoides durante la digestión *in vitro* (Etcheverry et al., 2012). Esta consta de una etapa gástrica con adición de pepsina y una etapa intestinal con adición de pancreatina de origen porcino, extracto de bilis porcina y colesterol esterasa.

El efecto de la adición de fibra sobre la solubilidad de los esteroles en alimentos enriquecidos con EV no ha sido estudiado hasta el momento. En cambio, se ha demostrado la capacidad de adsorción

de colesterol por parte de fibra dietética procedente de soja (Liu et al., 2016a), cacao (Nsor-Atindana et al., 2012), bambú (Luo et al., 2017; Wu et al., 2020) y té (Guo et al., 2016) en modelos *in vitro* (tubos de ensayo) (entre 2-10 y 5-20 mg de colesterol/g de fibra a pH 2 y 7, respectivamente, simulando las condiciones gástrica e intestinal). En modelos de digestión gastrointestinal *in vitro*, la adición de goma guar a yogur líquido (3 y 6%, p/v) (Minekus et al., 2005), de fibra dietética a empanadas de cerdo (10%, p/p) (López-Marcos et al., 2015) o de higo chumbo (*Opuntia ficus-indica*) a pasta (10-30%, p/p) (Attanzio et al., 2019) reduce la bioaccesibilidad del colesterol entre 1,1 y 22 veces. Se observa que esta reducción es más efectiva a cantidades similares de fibra soluble e insoluble (López-Marcos et al., 2015). En cambio, la adición de pectinas (fibra soluble) y celulosa y quitosano (fibra insoluble) en empanadas de ternera (0,5%, p/p) no modifica la solubilidad del colesterol (Hur et al., 2009). Por lo tanto, dada la similitud estructural y fisicoquímica de los GOS con otros tipos de fibra soluble que han demostrado presentar efectos negativos sobre la absorción de los esteroles, sería de interés el estudio de las posibles interacciones entre la presencia de GOS y la bioaccesibilidad de los esteroles.

Los estudios dirigidos a evaluar el efecto de la digestión gastrointestinal simulada sobre la formación y bioaccesibilidad de SOPs en alimentos son escasos y, hasta el momento, en alimentos enriquecidos con EV solo se ha realizado en bebidas a base de zumo de frutas y/o leche (Alemany et al., 2013a). Se observa que los COPs presentan una menor bioaccesibilidad que el colesterol (2,2-17% *vs*. 53-99%), mientras que la bioaccesibilidad de los POPs es mayor que la de sus correspondientes EV (19,1–49,3% *vs*. 2,2–6,5%). Además, se observa un efecto de la matriz alimentaria con respecto a la bioaccesibilidad de los SOPs, ya que la bioaccesibilidad de los POPs es significativamente inferior en la bebida a base de leche y, en el caso de los COPs, en aquella formulada con zumo y leche. En todas las muestras, los óxidos más bioaccesibles son el 7β-hidroxicolesterol (6,8-20,7%) en el caso de los COPs y sitostanotriol (62,7-176,6%) para los POPs, este último probablemente debido a la transformación de epoxiesteroles a trioles como consecuencia de las condiciones ácidas del fluido gástrico simulado (Maerker et al., 1988). Debido a la tendencia creciente del consumo de alimentos enriquecidos con EV por parte de la población, la determinación de SOPs en este tipo de productos es de especial interés para el desarrollo de matrices que minimicen su formación.

El contenido de este apartado se ha publicado en: **Blanco-Morales, V.***, Garcia-Llatas, G., Cilla, A. (2021). *Sterol digestion in plant sterol-enriched foods: Bioaccessibility and fermentation. En: Bioaccessibility and digestibility of lipids from foods.* Springer, doi: 10.1007/978-3-030-56909-9 (pendiente de publicación) (ver en Anexo I).

6.2 Ensayos clínicos

En un metaanálisis en el que se incluyen 41 estudios de intervención en humanos (con una media de duración de 28 días) se indica que una dosis media de enriquecimiento de 1,6 g de EV/día (en su mayoría en margarina baja en grasa) produce unos incrementos medios de los niveles séricos de β-sitosterol y campesterol de 2,24 µmol/L (31%) y 5,00 µmol/L (37%), respectivamente, comparado con el control (Ras et al., 2013). Cuando se corrigen estas concentraciones con las de colesterol total, los incrementos medios de β-sitosterol y campesterol sanguíneos son de 0,59 µmol/mmol colesterol total (41,7%) y 1,34 µmol/mmol (60,8%). Además, se observa una relación dosis-respuesta entre los cambios de las concentraciones plasmáticas de EV y la dosis de EV ingeridas (0,3-3,2 g/día). A dosis >3 g/día se estabilizan los incrementos plasmáticos de EV. Por otro lado, la fuente de EV utilizada para el enriquecimiento también influye en la magnitud del efecto. De esta manera, la adición de EV derivados de *tall oil* (~5-10% campesterol y 75-80% β-sitosterol) producen incrementos menores en las concentraciones plasmáticas de campesterol y mayores en las de β-sitosterol que EV procedentes de aceite de soja (20-30% campesterol y 45-50% β-sitosterol). Por último, cuando las concentraciones séricas basales de EV son más altas se observan mayores incrementos en las concentraciones de β-sitosterol y campesterol tras la ingesta de alimentos enriquecidos con EV, probablemente debido a que estas concentraciones basales se relacionan con sujetos con una mayor eficiencia en la absorción de colesterol/EV.

En estudios previos del grupo de investigación, en mujeres postmenopáusicas, se observa que la ingesta de bebidas a base de zumo de frutas y leche enriquecidas con EV (1,5 g/día) con o sin adición de β-criptoxantina (0,75 mg/día) durante 4 semanas incrementa significativamente las concentraciones séricas de β-sitosterol (49,4-64,3%) y campesterol (10,8-12,7%), mientras que no se aprecian variaciones en las concentraciones de estigmasterol (García-Llatas et al., 2015b). En cuanto a los precursores séricos de colesterol, se observan diferencias significativas en las concentraciones de desmosterol tan solo en la bebida sin β-criptoxantina, mientras que las concentraciones de latosterol no varían en ninguna de las intervenciones (Granado-Lorencio et al., 2014). En un estudio posterior en la misma población diana, el consumo de una bebida de composición similar (con β-criptoxantina y adición de grasa láctea y MFGM) enriquecida con EV (2 g/día) durante 6 semanas produce un incremento de los niveles séricos de β-sitosterol (32,5%), campesterol (43,1%) y latosterol (11,2%), sin variaciones en las concentraciones de estigmasterol y desmosterol (Alvarez-Sala et al., 2018). Los autores sugieren que las diferencias observadas con respecto a los trabajos del mismo grupo mencionados anteriormente (Granado-Lorencio et al., 2014; García-Llatas et al., 2015b) pueden deberse a diferencias en la dosis de EV (2 *vs*. 1,5 g/día), el porcentaje de campesterol (7% *vs*.

5,5%), el contenido de grasa de las bebidas (1,9 *vs*. 1,3%) y la duración del estudio de intervención (6 *vs*. 4 semanas).

Hasta el momento, no se ha evaluado el posible impacto de los GOS sobre la absorción de los EV y por tanto se desconoce el efecto que puedan tener sobre la biodisponibilidad de los mismos.

7. Microbiota intestinal

7.1 Composición e influencia de la dieta

La infancia es la etapa de la vida durante la cual tiene lugar el establecimiento y la colonización de la microbiota intestinal. Diversos factores previos al parto (dieta materna, enfermedades, uso de antibióticos), durante (edad gestacional, tipo de parto) y después del mismo (método de lactancia, dieta, enfermedades, uso de antibióticos) condicionan la composición de la microbiota intestinal en esta etapa (Li et al., 2016; Vandenplas et al., 2020). Se establece que alrededor de los 3 años de edad la microbiota alcanza un equilibrio, es más diversa y relativamente estable, y se asemeja a la que se presenta posteriormente en la edad adulta (Yatsunenko et al., 2012).

La composición de la microbiota intestinal en adultos está dominada por los filos Firmicutes y Bacteroidetes, que representan el 90% de la población microbiana. El filo Firmicutes se compone principalmente de los géneros *Clostridium* (95%), *Lactobacillus*, *Bacillus*, *Enterococcus* y *Ruminococcus*, mientras que el filo Bacteroidetes incluye predominantemente los géneros *Bacteroides* y *Prevotella*. Otros filos minoritarios son Actinobacteria (representado principalmente por el género *Bifidobacterium*), Proteobacteria, Fusobacteria y Verrucomicrobia (Arumugam et al., 2011; Rinninella et al., 2019).

Así mismo, la composición de la microbiota varía a lo largo del tracto gastrointestinal en función de las condiciones fisiológicas características de cada región. En el intestino delgado el crecimiento de bacterias se limita a anaerobios facultativos que tienen la habilidad de adherirse al mucus para sobrevivir a las condiciones de acidez, presencia de oxígeno y tiempos de tránsito cortos característicos de este tramo. En cambio, las condiciones colónicas (ratios de flujo más lentos y pH neutro-medio ácido) permiten el desarrollo de una comunidad bacteriana más densa (10^{11}-10^{12} células/mL) y diversa, dominada por anaerobios estrictos que son capaces de utilizar los carbohidratos complejos que no son digeridos en el intestino delgado (Donaldson et al., 2016; Thursby & Juge, 2017).

A pesar de esta variabilidad, se han establecido tres enterotipos, que se caracterizan por la presencia de tres géneros dominantes: *Bacteroides* (enterotipo I), *Prevotella* (enterotipo II) o *Ruminococcus* (enterotipo III). En estos enterotipos la microbiota utiliza diferentes rutas para generar energía a partir de los sustratos fermentables disponibles en el colon. Las bacterias pertenecientes al enterotipo I obtienen energía principalmente de la fermentación de carbohidratos y proteínas, mientras que las que forman parte de los enterotipos II y III comprenden especies capaces de degradar mucinas presentes en la capa mucosa del intestino. No se observa ninguna correlación entre los distintos enterotipos y las características de los individuos (nacionalidad, género, edad o índice de masa corporal), por lo que se desconoce los factores ambientales y/o genéticos que causan esta agrupación (Arumugam et al., 2011).

Se ha observado una correlación entre los patrones dietéticos/hábitos alimentarios y los distintos enterotipos en poblaciones de diferentes áreas geográficas. En este sentido, el consumo de una dieta occidental basada en un contenido alto en proteína de origen animal y grasa se correlaciona con una prevalencia del enterotipo *Bacteroides*, mientras que hábitos alimentarios caracterizados por un alto consumo de carbohidratos y fibra, y bajo en proteína animal y grasa se relaciona con el enterotipo *Prevotella*, tanto en niños (De Filippo et al., 2010; Nakayama et al., 2017) como en adultos (Wu et al., 2011). En este sentido, diversos estudios han demostrado una asociación entre este último enterotipo y sujetos vegetarianos y veganos (Wu et al., 2011; de Moraes et al., 2017).

La modificación de la dieta a corto plazo en sujetos sanos que consumen una dieta alta en grasa y baja en fibra *vs*. dieta baja en grasa y alta en fibra durante 4-10 días supone cambios significativos y rápidos (24 h) en la composición de la microbiota. Predominantemente los filos Bacteroidetes y Actinobacteria se asocian de manera positiva con la ingesta de grasa y de manera negativa con la ingesta de fibra, mientras que Firmicutes y Proteobacteria muestran la asociación opuesta. Sin embargo, la magnitud de estos cambios no es suficiente para alterar los enterotipos asociados con la ingesta de proteína/grasa y carbohidratos (Wu et al., 2011; David et al., 2014).

Mientras que la composición de la microbiota intestinal es característica y única para cada sujeto, las funciones asociadas a la misma, de las cuales el hospedador puede beneficiarse, están muy preservadas entre individuos. Entre ellas, la microbiota presenta funciones metabólicas participando en la extracción, síntesis y absorción de nutrientes y metabolitos (ácidos biliares, lípidos, aminoácidos, vitaminas y AGCC), funciones protectoras frente a la colonización de bacterias patógenas inhibiendo su crecimiento por un mecanismo de competición mediante el consumo de los nutrientes disponibles en el medio o por la producción de bacteriocinas, así como funciones

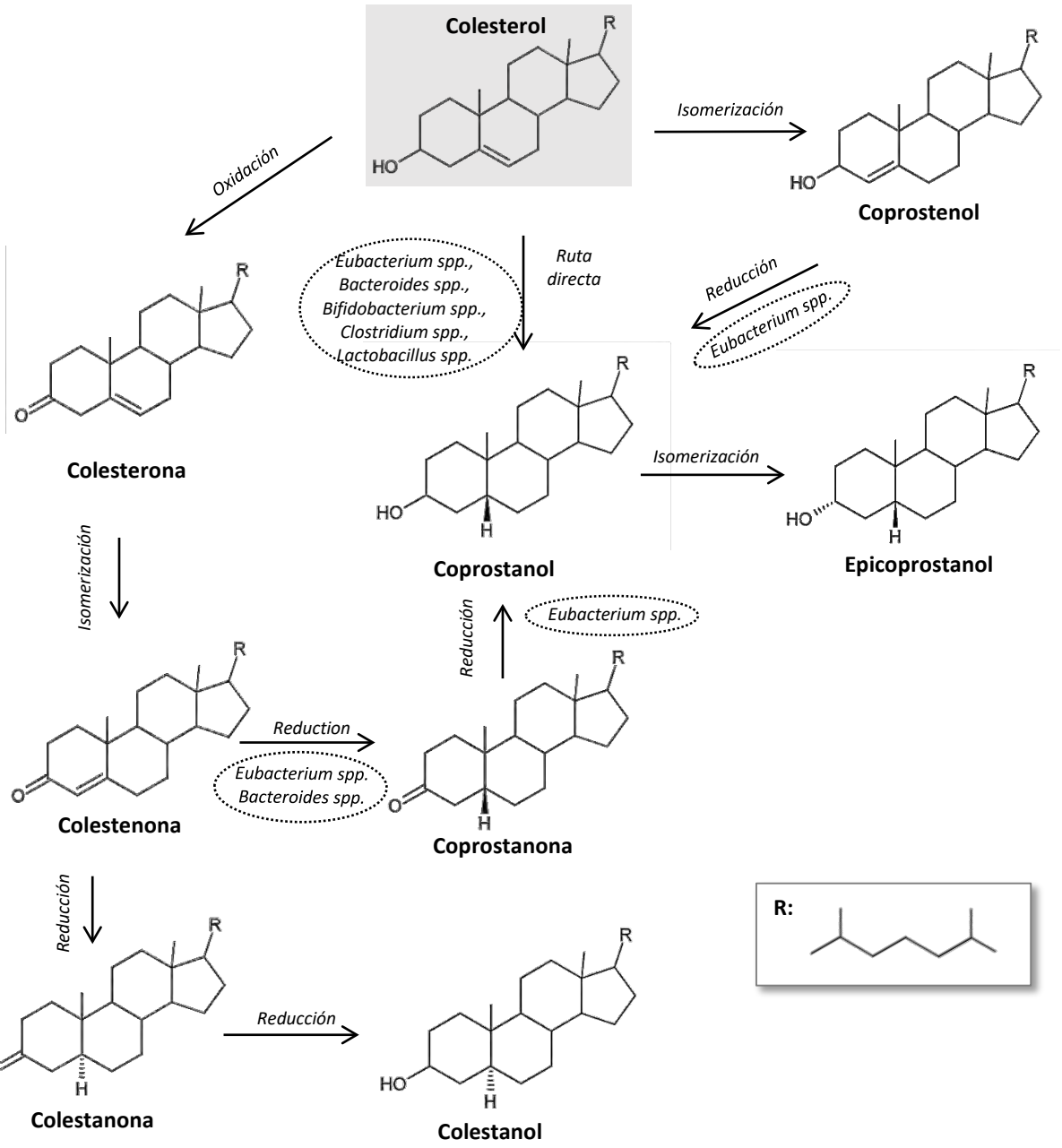
estructurales que permiten el mantenimiento de la integridad del epitelio intestinal (Rinninella et al., 2019).

7.2 Interacción microbiota y esteroles

La microbiota puede actuar sobre los esteroles que alcanzan el colon, y mediante reacciones de hidrolización, hidrogenación y deshidrogenación dar lugar a metabolitos de esteroles. La biotransformación del colesterol por parte de la microbiota se ha estudiado ampliamente mediante ensayos *in vitro* de fermentación con microbiota procedente de heces de origen humano o animal (ratas, palomas, pollos) y mediante cultivos puros con microorganismos entéricos (*Eubacterium spp.*, *Clostridium perfringens, Bifidobacterium spp., Enterobacter aerogenes, Escherichia coli, Bacteroides spp.*) (Cuevas-Tena et al., 2018a). La microbiota colónica metaboliza el colesterol principalmente a coprostanol y, en menor medida, a colestanol. Se sugiere que la formación de coprostanol sigue varias rutas: una vía directa a través de la reducción del colesterol o vías indirectas por medio de la formación de metabolitos intermedios (colesterona, colestenona, coprostanona ó coprostenol). Además, se ha indicado que el coprostanol puede transformarse en menor medida en epicoprostanol (ver figura 7) (Gérard, 2014; Cuevas-Tena et al., 2018a; Kriaa et al., 2019).

La biotransformación del colesterol en metabolitos por acción de la microbiota se relaciona principalmente con la presencia de bacterias reductoras del colesterol. Los géneros *Eubacterium* y *Bacteroides* son los responsables de las rutas directas e indirectas del metabolismo del colesterol, mientras que *Bifidobacterium*, *Clostridium* y *Lactobacillus* solo se relacionan con la vía directa (ver figura 7) (Cuevas-Tena et al., 2018a; Kriaa et al., 2019). Otras familias como *Lachnospiraceae* y *Ruminococcaceae* se proponen como bacterias coprostanoligenicas, aunque no se asocian con las rutas mencionadas anteriormente (Kriaa et al., 2019).

Figura 7. Rutas de biotransformación del colesterol por la microbiota intestinal

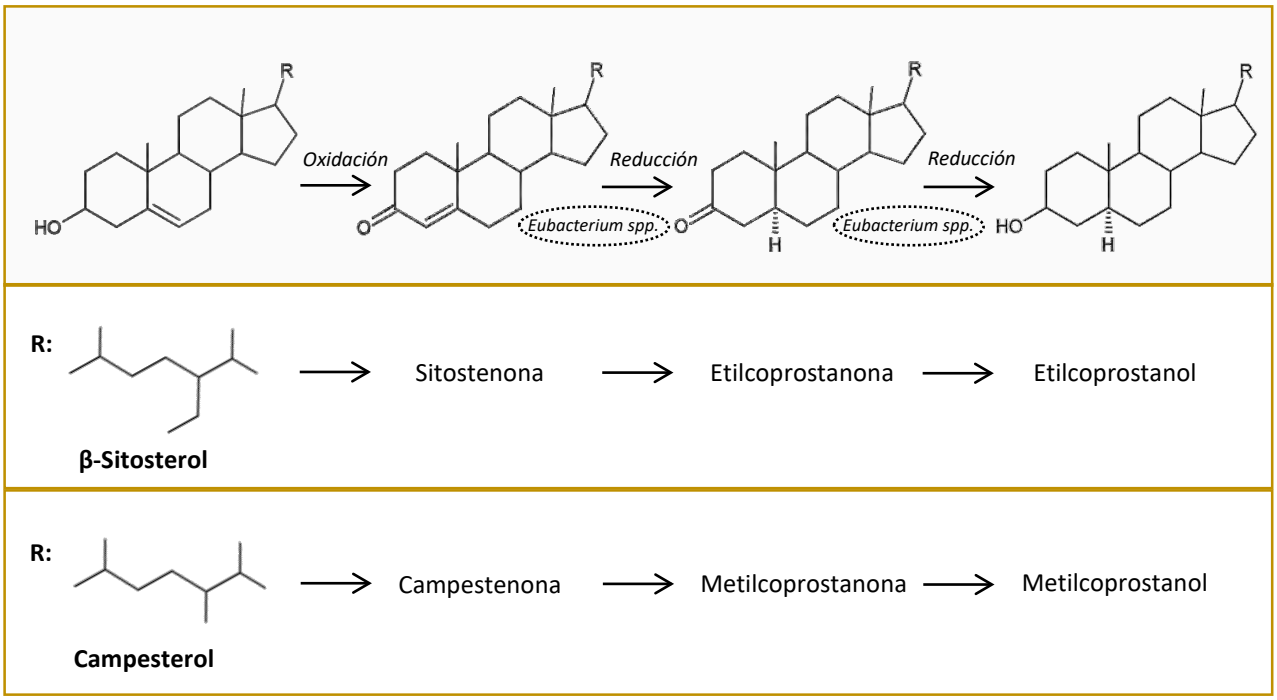


Adaptado de Cuevas-Tena et al. (2018ª) y Kriaa et al. (2019)

De forma similar al colesterol, los EV se metabolizan por acción de la microbiota, a través de la formación de diferentes metabolitos intermediarios, como fitostanonas y fitostenonas, para dar lugar finalmente a la formación de fitostanoles (Wong, 2014). El sitosterol y campesterol se transforman principalmente en etil- y metil-coprostanol y en etil- y metil-coprostanona, respectivamente (ver figura 8). El estigmasterol puede convertirse en etilcoprostenol y etilcoprostanona. Sin embargo, las

rutas de degradación de los EV no se han dilucidado completamente y, hasta la fecha, *Eubacterium spp*. es la única especie asociada directamente con su degradación (Eneroth et al., 1964; Eyssen et al., 1973; Miettinen, 1982; Cuevas-Tena et al., 2018a).

Figura 8. Rutas de biotransformación de los esteroles vegetales por la microbiota intestinal



Adaptado de Cuevas-Tena et al., 2018a

Ensayos de fermentación por lote realizados en nuestro grupo de investigación (Cuevas-Tena et al., 2018b) del residuo de una bebida a base de zumo de frutas y leche enriquecida con EV obtenido tras una digestión simulada gastrointestinal, resulta en una disminución del contenido de esteroles neutros: β-sitosterol (8 y 14%), sitostanol (21 y 16%), campesterol (19 y 39%), campestanol (17 y 15%), estigmasterol (17 y 19%) y brasicasterol (10 y 1%) a las 24 y 48 h de fermentación, respectivamente. Se observan incrementos de etilcoprostanol (18 y 50%), etilcoprostanona (16 y 39%), metilcoprostanona (63 y 21%) y estigmastenol (13 y 16%). Además, se determina una disminución de la abundancia de *Flavonifractor plautii* y *Allobaculum spp.* (de la familia *Erysipelotrichaceae*) y una proliferación de *Eubacterium hallii*, así como de algunos miembros no caracterizados de la familia *Bacteroidetes* S-24. Sin embargo, los cambios en la composición de la microbiota se asocian en mayor medida con el tiempo de fermentación y no tanto con la presencia de EV, probablemente debido a la disminución del pH que conlleva la acumulación de los productos de fermentación (Cuevas-Tena et al., 2018b).

Por otra parte, tras la fermentación de una dosis de ~2 g de EV/día procedente de dos fuentes diferentes (ingrediente de EV o una mezcla de patrones comerciales de EV) durante 72 h mediante el sistema TIM-2, se observa una menor concentración de metabolitos de colesterol y EV (coprostanol, coprostanona, colestanol, etilcoprostanol y metilcoprostanona) utilizando microbiota de sujetos delgados *vs*. sujetos obesos, lo que sugiere la existencia de una actividad microbiana diferente para estas dos poblaciones (Cuevas-Tena et al., 2019a). En sujetos delgados, el aumento de colestanol junto con la disminución de coprostanol y coprostanona sugiere una modificación de la principal vía de degradación del colesterol a coprostanol. El aumento de coprostanol y colestanol utilizando microbiota de sujetos obesos, indica que el colesterol se degrada por ambas vías. Sin embargo, la ausencia de control de la dieta (consumo real de esteroles) o de información sobre los patrones de conversión de los sujetos (altos o bajos convertidores de esteroles) dificulta la correlación de este efecto con la presencia de EV. Por otro lado, se produce un aumento de los contenidos de β-sitosterol, sitostanol, campesterol y campestanol tras la fermentación con microbiota de sujetos delgados y obesos. Además, se observa una disminución con respecto al control del contenido de etilcoprostanol (32-35% en delgados y 87-88% en obesos) con incrementos de etilcoprostanona (21 y 46 veces en delgados y 33 y 35 veces en obesos) y metilcoprostanona (8 y 25 veces en delgados). La ausencia de metabolización de β-sitosterol a etilcoprostanol sugiere una saturación de la actividad de la microbiota en presencia de una dosis enriquecida con EV.

En ambos trabajos (Cuevas-Tena et al., 2018b; Cuevas-Tena et al., 2019a), se indica una preferencia por parte de la microbiota intestinal en la utilización de los EV como sustrato cuando están presentes en una mayor proporción que el colesterol.

Del análisis de la composición de la microbiota durante la fermentación se determina un aumento de los géneros *Catenibacterium* y *Coprococcus* en presencia de EV con microbiota de sujetos delgados (ambos pertenecientes a la familia *Erysipelotrichaceae* y, por lo tanto, en línea con lo observado en la fermentación por lote comentada anteriormente). En cambio, en los ensayos con microbiota de sujetos obesos, la presencia de EV conlleva un aumento del género *Clostridium* (Cuevas-Tena et al., 2019a).

Los estudios en humanos que evalúan la metabolización de los esteroles por parte de la microbiota tras la ingesta de alimentos enriquecidos con EV y su efecto sobre la composición de la misma son escasos. En mujeres sanas de mediana edad (40-60 años) con diferentes hábitos dietéticos (omnívoras o vegetarianas) se observa que el esterol mayoritario en heces es el coprostanol, siendo sus contenidos mayores en el caso de las mujeres omnívoras. No se detectan diferencias en el contenido fecal de

esteroles de origen animal, pero sí en los EV, siendo el contenido de β-sitosterol en heces mayor en mujeres vegetarianas (Olejníková et al., 2017). En cambio, la ingesta durante 3-4 semanas de una margarina enriquecida con EV (8,6 g/día) por sujetos sanos favorece la excreción de colesterol (reflejo del impacto de los EV sobre su absorción) y disminuye la excreción de coprostanol y coprostanona. La excreción de EV y metabolitos tras el consumo de la margarina enriquecida también se incrementa (aproximadamente 17 veces), mostrando un patrón de excreción fecal similar a la composición de EV de la margarina (β-sitosterol > campesterol > estigmasterol) (Weststrate et al., 1999). Del mismo modo, tras la ingesta de una bebida a base de zumo de frutas y leche enriquecida con EV (2 g/día) por mujeres postmenopáusicas durante 6 semanas, se modifica el perfil de excreción de esteroles (Cuevas-Tena et al., 2019b). Se observa un aumento de la excreción de colesterol (65%), coprostanona (80%), colestanol + metilcoprostanol (42%) y latosterol (9%), siendo el aumento de colestanol + metilcoprostanol significativamente mayor con respecto al placebo. Se sugiere la metabolización del colesterol a través de su ruta indirecta debido al incremento en la excreción de coprostanona, así como la falta de capacidad de la microbiota para transformar los esteroles animales de la manera habitual (vía directa) debido a la presencia de altas concentraciones de EV. Se indica un aumento estadísticamente significativo de los incrementos netos de EV (β-sitosterol, sitostanol, campesterol, campestanol y estigmasterol) y sus metabolitos (etilcoprostanol, etilcoprostanona, metilcoprostanona y estigmastenol) en heces con respecto al placebo. Estos resultados sugieren, al igual que en los estudios *in vitro* comentados anteriormente (Cuevas-Tena et al., 2018b y 2019a) la preferencia de los EV como sustrato para la microbiota. Además, se indica una modulación de actividad metabólica de la microbiota intestinal en presencia de una alta dosis de EV al disminuir los porcentajes de conversión de colesterol y β-sitosterol tras la ingesta de la bebida enriquecida.

El efecto de los EV sobre la composición de la microbiota intestinal en humanos tan solo se ha determinado en un estudio tras el consumo de una margarina enriquecida en E-FA (3 g/día) durante 3 semanas. Sin embargo, no se observa modificaciones en la composición ni diversidad de la microbiota, siendo ésta similar a la observada en el periodo control (Baumgartner et al., 2017).

El contenido de este apartado se ha publicado en: **Blanco-Morales, V.***, Garcia-Llatas, G., Cilla, A. (2021). *Sterol digestion in plant sterol-enriched foods: Bioaccessibility and fermentation. En: Bioaccessibility and digestibility of lipids from foods.* Springer, doi: 10.1007/978-3-030-56909-9 (pendiente de publicación) (ver en Anexo I).

7.3 Interacción microbiota y galactooligosacáridos

El efecto de los GOS sobre la composición de la microbiota intestinal ha sido estudiado ampliamente mediante estudios *in vitro* (fermentación por lote o sistemas dinámicos de fermentación) y estudios en humanos. El efecto prebiótico de los GOS se ha demostrado en estudios *in vitro* utilizando microbiota fecal procedente de adultos (sanos o con patologías) o población anciana, en sistemas estáticos y dinámicos (ver tabla 5). El efecto bifidogénico de los GOS se asocia de manera inversa con su grado de polimerización y se observa que GOS con enlaces β1-6 aumentan la población de bifidobacterias en mayor grado que aquellos con enlaces β1-4 ya que esta estructura proporciona posiciones más susceptibles a ser atacadas por las β-galactosidasas producidas por las bacterias colónicas (Li et al., 2015). Aunque la reducción de las impurezas de los ingredientes de GOS utilizados podría mejorar su efectividad como prebióticos, no se observan diferencias en el efecto bifidogénico de GOS con distintas purezas (65 *vs*. 52%) (Grimaldi et al., 2016). Por otra parte, la fermentación de α-GOS en el sistema PolyFerms no induce ningún efecto sobre las bifidobacterias (Poeker et al., 2018), mientras que en un estudio de fermentación en placa se demuestra un efecto bifidogénico y una modulación del perfil microbiano similar tras la utilización de α- y β-GOS como sustrato (Fehlbaum et al., 2018).

Más allá del tipo y la dosis de GOS, del sistema de fermentación y de las técnicas analíticas utilizadas para determinar la composición de la microbiota intestinal, el inóculo fecal empleado determina en gran medida la modulación microbiana observada tras la fermentación del prebiótico. Diversos estudios demuestran que los cambios en la composición de la microbiota se asocian fuertemente con el origen de la misma (Aguirre et al., 2014; Poeker et al., 2018; Nogacka et al., 2020).

En general, la población de lactobacilos también se ve incrementada tras la fermentación *in vitro* de GOS (ver tabla 5). En sistemas dinámicos de tres reactores, este incremento se establece en el reactor correspondiente al colon ascendente, lo que sugiere una dependencia de las condiciones colónicas para la utilización de los GOS por las bacterias (Walton et al., 2012; Costabile et al., 2015b).

Tabla 5. Estudios *in vitro* sobre el efecto de los galactooligosacáridos sobre la microbiota

Tipo de GOS	Método, tiempo de fermentación	Dosis de GOS	Inóculo fecal (*n*)	Principales resultados	Referencia
Sistemas de fermentación estáticos[a]					
GOS purificados: -Enlaces β1-4 (de *Bacillus circulans*) -Enlaces β1-6 (de *Aspergillus oryzae*)	Por lote, 24h	0,1	Adultos sanos (*3*)	↑ Bifidobacteria, lactobacilos, bacterias totales No efecto: Bacteroides y Clostridia	Li et al., 2015
Bimuno® GOS (sirope) (p/p): -52% β-GOS, 8% lactosa, 22% glucosa, 16,5% galactosa	Por lote, 24 h	0,033/0,05/0,1	Adultos sanos (*3*)	↑ Bifidobacteria (dosis 0,05 y 0,033 g/L) ↑ Lactobacilos (todas las dosis) ↓ *Bacteroides-Prevotella* (dosis 0,1 y 0,033 g/L)	Grimaldi et al., 2016
-65% β-GOS, 10,1% lactosa, 22% glucosa, 1,8% galactosa				↑ Bifidobacteria (dosis 0,05 y 0,033 g/L) ↑ Lactobacilos (dosis 0,1 y 0,033 g/L) ↓ *Bacteroides-Prevotella* (dosis 0,1 y 0,05 g/L)	
Bimuno® β-GOS	Por lote, 48h	~10	Ancianos (60-75 años) (*3*)	↑ Bifidobacteria, Lactobacilli-enterococci, *Eubacterium rectale-Clostridium coccoides* ↓ *Clostridium histolyticum* No efecto: bacterias totales	Liu et al., 2016b
α-GOS (≥95%) β-GOS (90%)	Fermentación en placa, 24 h	0,5/2/4/8/12	Adultos sanos (*6*)	↑ Actinobacteria (*Bifidobacterium spp*.), *Lactobacillus* ↓ Firmicutes, Proteobacteria	Fehlbaum et al., 2018
GOS	Por lote, 24 h	10	Adultos sanos (*16*)	↑ *Bifidobacterium* (*B. bifidum, B.infantis, B.longum*) No efecto: *Clostridium, Escherichia*	Perdijk et al., 2019
GOS (≥57%)	Por lote, 24 h	0,1	Adultos sanos (*10*)	↑ *Bifidobacterium, Bacteroides (Prevotella_9), Megamonas* ↓ Firmicutes *(Blautia, Eubacterium hallii, Fusicatenibacter, Fecalibacterium)*	Chen et al., 2020

Tabla 5. Continuación

Tipo de GOS	Método, tiempo de fermentación	Dosis de GOS	Inóculo fecal (*n*)	Principales resultados	Referencia
Bimuno® β-GOS (79,7%)	Por lote, 24 h	~0.04	Adultos delgados (*9*)	↑ *Bifidobacterium* (*B. longum, B. adolescentis*), *Faecalibacterium*, ↓ *Bifidobacterium breve* No efecto: *Bacteroides*	Nogacka et al., 2020
			Adultos obesidad mórbida (*9*)	↑ *Bifidobacterium longum, Bacteroides, Faecalibacterium* ↓ *Bifidobacterium (B. animalis lactis, B. crudilactis)*	
GOS (≥90%)	Por lote, 48 h	10	Adultos sanos (*3*)	↑ Actinobacteria (*Bifidobacterium*), Proteobacteria, *Enterococcaceae*, *Bacteroidaceae*, ↓ Firmicutes (*Ruminococcus 2*, *Ruminococcaceae UCG-014*, *Streptococcus*, *Romboutsia*, *Blautia*), *Klebsiella*, *Alistipes* No efecto: *Lactobacillus*	Wei et al., 2020
***Sistemas de fermentación dinámicos*[b]**					
Vivinal® GOS (98,5% oligosacáridos, 1,0% lactosa, 0,3% glucosa, 0,2% galactosa)	TIM-2, 3 días	10	Adultos sanos (*8*)	↑ *Bifidobacterium* (*B. bifidum*, *B. catenulatum*), *Lactobacillus* (*L. gasseri*, *L. salivarius*), *Enterobacteriaceae*, *Klebsiella* ↓ *Bacteroides*, *Eubacterium halli*, *Prevotella*, *Lactococcus*	Maathuis et al., 2012
Zumo de naranja con Vivinal® GOS (sirope)(59% GOS, 21% lactosa, 19% glucosa, 1% galactosa)	Sistema de tres reactores	8	Adultos sanos (50-81 años) (*3*)	**R1:** ↑ Bifibobacteria, *Lactobacillus* **R2:** ↑ Bifibobacteria, ↓ *Escherichia coli* **R3:** ↑ Bifibobacteria	Walton et al., 2012
GOS (97%)	TIM-2, 3 días	7,5	Adultos delgados (*4*)	↑ Actinobacteria (*Bifidobacterium)*, Firmicutes (*Streptococcus*), Bacteroidetes (*Bacteroides spp*., *Parabacteroides spp*.) ↓ Proteobacteria (*Parasutterella spp*.)	Aguirre et al., 2014
			Adultos obesos (*4*)	↑ Actinobacteria (*Bifidobacterium*), Firmicutes (*Dorea spp*.), Proteobacteria ↓ Bacteroidetes (*Parabacteroides spp*.)	

Tabla 5. Continuación

Tipo de GOS	Método, tiempo de fermentación	Dosis de GOS	Inóculo fecal (*n*)	Principales resultados	Referencia
Zumo de naranja con Bimuno® β-GOS (48% GOS, 22% lactosa, 18% glucosa, 12% galactosa)	Sistema de tres reactores, 16 días	2,75	Adultos con síndrome metabólico (*3*)[c]	**R1:** ↑ *Bifidobacterium spp., Roseburia-Eubacterium-rectale,* ↓*Clostridium hystolyticum* **R2:** ↑ *Bifidobacterium spp., Roseburia-Eubacterium-rectale* **R3:** ↑ *Bifidobacterium spp.*	Costabile et al., 2015a
Pan con Bimuno® β- GOS (48% GOS, 22% lactosa, 18% glucosa, 12% galactosa)	Sistema de tres reactores, 16 días	2,75	Adultos con síndrome metabólico (*3*)[c]	**R1:** ↑ *Bifidobacterium spp., Lactobacillus-Enterococcus* **R2:** ↑ *Bifidobacterium spp., Clostridium* IX **R3:** ↑ *Bifidobacterium spp.*	Costabile et al., 2015b
β-GOS (58%) + 4,78 g grasa	Sistema de tres reactores, 53 días	1,06	Ancianos (>60 años) (*3*)[c]	**R1, R2 y R3:** ↑ Bifidobacteria, Lactobacilli-enterococci, *Eubacterium rectale-Clostridium coccoides, Bacillus spp.*, bacterias totales ↓ *Clostridium hystolyticum, Bacteroides-Prevotella spp.*	Liu et al., 2017a
α-GOS	PolyFerms, 7 días	9	Sujeto 1[d]	↑ *Ruminococcaceae*, *Bacteroidaceae* ↓ *Lachnospiraceae*, *Eubacteriaceae*	Poeker et al., 2018
			Sujeto 2[d]	↑ *Lachnospiraceae*, *Blautia*, *Eubacterium rectale*	

n: número de sujetos. [a] Dosis expresada como g de GOS/L medio de cultivo. [a] Dosis expresada como g de GOS/día. [c] Utiliza un donante diferente para el inóculo fecal de cada una de las tres réplicas de fermentación. [d] Sujeto 1 *vs*. Sujeto 2: *Bacteroidaceae* (17 *vs*. 8%), *Verrucomicrobiaceae* (6 *vs*. <0%), *Methanobacteriaceae* (9 *vs*. 2%), *Prevotellaceae* (0,1 *vs*. 17%), *Lachnospiraceae* (7 *vs*. 13%). R1: reactor colon ascendente, R2: reactor colon transversal, R3: reactor colon descendente.

Estudios en humanos (recopilados en la tabla 6), han demostrado que la ingesta de GOS durante un corto periodo de tratamiento (1 semana) es suficiente para alcanzar un efecto bifidogénico (Bouhnik et al., 2004; Depeint et al., 2008).

En adultos sanos se observa la necesidad de una dosis mínima o umbral de 2,5 g de GOS al día para alcanzar cambios significativos en los recuentos de bifidobacterias tras 3 semanas de ingesta (Tannock et al., 2004; Davis et al., 2010 y 2011). En este grupo de población, se ha indicado que el efecto bifidogénico de los GOS es dosis-respuesta a concentraciones entre 2,5 y 18 g/día (Bouhnik et al., 2004; Depeint et al., 2008; Davis et al., 2010 y 2011). El efecto prebiótico de los GOS se relaciona también con el numero basal de bifidobacterias de los sujetos, observándose que cuanto mayor es el número inicial de las mismas, cabe esperar un mayor efecto bifidogénico. Así mismo, se determina que tan solo el 50% de los participantes muestra un aumento de bifidobacterias en heces, sugiriéndose que la variabilidad del efecto se debe a la presencia o ausencia de cepas específicas de *Bifidobacterium spp.* capaces de usar el prebiótico como sustrato (Davis et al., 2010).

En población anciana, la ingesta de 5,5 g de GOS/día durante 10 semanas produce un aumento de la población de bifidobacterias (Vulevic et al., 2008 y 2015). Sin embargo, este efecto no se observa tras una ingesta a mayor dosis (8 g/día) durante 3 semanas (Maneerat et al., 2013), destacando la importancia de la duración del periodo de tratamiento sobre el efecto bifidogénico.

La presencia de patologías como trastornos intestinales (Silk et al., 2009; Huaman et al., 2018), sobrepeso (Vulevic et al., 2013; Morel et al., 2015; Canfora et al., 2017; Krumbeck et al., 2018) e intolerancia a la lactosa (Azcarate-Peril et al., 2017) no afecta al efecto bifidogénico esperado tras el consumo de GOS a dosis entre 1,37-18 g/día durante 2-12 semanas. Sin embargo, en adultos con sobrepeso (Vulevic et al., 2013) este efecto es menor al indicado en estudios llevados a cabo con población sana a la misma dosis (5,5 g/día) y durante el mismo periodo de tratamiento (10 semanas) (Vulevic et al., 2008).

Tabla 6. Estudios sobre el efecto de la ingesta de galactooligosacáridos sobre la microbiota intestinal en humanos

GOS/alimento	Duración (semanas)	Dosis (g/día)	Tipo de estudio	Sujetos (*n*, edad)	Principales resultados	Referencia
GOS en leche en polvo reconstituída	4	2,4	DC, AL, PC	Adultos sanos (*10*, 20-60)	↑ *Bifidobacterium*, *Lactobacillus*	Gopal et al., 2003
GOS (>95%) en polvo	1	2,5/5/7,5/10	DC, AL, PC, PL	Adultos sanos (*8*, 18-54)	↑ *Bifidobacterium* No efecto: *Lactobacillus*, *Bacteroides*, *Enterobacteria*, anaerobios totales	Bouhnik et al., 2004
GOS Oligovite en galletas de chocolate	3	2,5	DC, C	Adultos sanos (*15*)	No efecto: bifidobacteria, lactobacilo, enterobacteria (fermentadoras de lactosa y totales), *Enterococci*	Tannock et al., 2004
Vivinal® β-GOS[a] en 15 g leche en polvo	1	7	DC, AL, C	Adultos sanos (*29*, 19-55)	↑ *Bifidobacterium* ↓ *Bacteroides-Prevotella* No efecto: *Lactobacillus-Enterococcus*, *Clostridium perfringens-hystolyticum*	Depeint et al., 2008
β-GOS[b] en 15 g leche en polvo	1	3,6/7		Adultos sanos (*30*, 21-59)	↑ *Bifidobacterium* ↑ Clostridium perfringens-hystolyticum (solo dosis 3,6 g/día) No efecto: *Lactobacillus-Enterococcus*, *Bacteroides-Prevotella*	
Bimuno® β-GOS en polvo (48% GOS p/p)	10	5,5	DC, AL, PC, C	Ancianos (*44*, 64-79)	↑ *Bifidobacterium spp.*, *Lactobacillus-Enterococcus spp.*, *Clostridium coccoides-Eubacterium rectale* ↓ *Bacteroides spp., Clostridium histolyticum, Escherchia coli, Desulfovibrio spp.*	Vulevic et al., 2008
β-GOS[b] en polvo	12	3,5/7	CS, AL, C	Adultos con síndrome de intestino irritable (*44*, 20-79)	↑ *Bifidobacterium* (ambas dosis) ↓ *Clostridium histolyticum, Bacteroides-Prevotella* (dosis 7 g/día) ↑ *Clostridium coccoides-Eubacterium rectale* (dosis 3,5 g/día)	Silk et al., 2009
Purimune™ GOS[c] en caramelos masticables	3	2,5/5/10	CS	Adultos sanos (*18*, 19-50)	↑ *Bifidobacterium* (a dosis de 5 y 10 g/día) No efecto: *Bacteroides*, enterococci, enterobacterias fermentadoras de lactosa	Davis et al., 2010
					↑ *Bifidobacterium*, *Fecalibacterium prausnitzii* (a dosis de 5 y 10 g/día) ↓ *Bacteroidaceae* (*Bacteroides*) (a dosis de 5 y 10 g/día)	Davis et al., 2011

Tabla 6. Continuación

GOS/alimento	Duración (semanas)	Dosis (g/día)	Tipo de estudio	Sujetos (*n*, edad)	Principales resultados	Referencia
Vivinal® GOS[d] (sirope) en zumo de naranja	3	8	DC, AL, PC, C	Adultos sanos (*37*, >50)	↑ Bifidobacteria	Walton et al., 2012
Danisco GOS (32,5 %)	3	8	DC, AL, PC, C	Ancianos (*37*, >60)	No efecto	Maneerat et al., 2013
Bimuno® β-GOS en polvo	12	5,5	DC, AL, PC, C	Adultos con sobrepeso (síndrome metabólico) (*45*, 18-65)	↑ Bifidobacteria ↓ Bacteroides, *Clostridium histolyticum*, *Desulfovibrio spp.* No efecto: *Lactobacillus-Enterococcus spp*., *Clostridium coccoides-Eubacterium rectale*, *Atopobium*, *Eubacterium cylindroides*, *Eubacterium hallii*, *β-Proteobacteria*, *Clostridium IX*, *Faecalibacterium prausnitzii*, bacterias toles	Vulevic et al., 2013
Vivinal® GOS (sirope) (59% GOS) en batido	3	5/10	DC, AL, C	Mujeres adolescentes (*20*, 10-13)	↑ Bifidobacteria	Whisner et al., 2013
α-GOS en té	2	6/12/18	DC, AL, PC, P	Adultos sobrepeso (*88*, 18-60)	↑ Bifidobacteria	Morel et al., 2015
Bimuno® β-GOS en polvo (48% GOS)	10	5,5	DC, AL, PC, C	Ancianos (*40*, 65-80)	↑ *Bifidobacterium spp*., *Bacteroides spp*., *Atopobium* No efecto: *Lactobacillus/Enterococcus spp*., *Faecalibacterium prausnitzii*, *Roseburia-Eubacterium rectale*, *Clostridium coccoides- Eubacterium rectale*, *Clostridium histolyticum*, *Escherchia coli*, *Desulfovibrio spp*., bacterias totales	Vulevic et al., 2015
GOS (>95%)	5	Dosis escaladas cada 5 días (1,5 a 15 g/día)	DC, AL, PC, P	Intolerantes lactosa (*62*)	↑ *Bifidobacterium*, *Faecalibacterium*, *Lactobacillus*	Azcarate-Peril et al., 2017
Vivinal® GOS[e] en yogur líquido bajo en grasa	12	15	DC, PC, P	Adultos sobrepeso/obesidad pre-diabéticos (*44*, 45-70)	↑ *Bifidobacterium*, *Prevotella oralis*, *Prevotella melaninogenica*, *Bacteroides stercoris*, *Sutterella wadsworthia*	Canfora et al., 2017

Tabla 6. Continuación

GOS/alimento	Duración (semanas)	Dosis (g/día)	Tipo de estudio	Sujetos (*n*, edad)	Principales resultados	Referencia
GOS (95%) en yogur	2	16	DC, AL, C	Adultos sanos (*35*, 18-65)	↑ *Bifidobacterium* ↓ *Ruminococcus, Dehalobacterium, Synergistes, Holdemania*	Liu et al., 2017b
Bimuno® GOS	4	1,37	DC, AL, P	Adultos con trastornos intestinales funcionales (*40*, 18-80)	↑ *Bifidobacterium* ↓ *Bilophila wadsworthia*	Huaman et al., 2018
Vivinal® GOS[f] en polvo	3	5	DC, AL, P, PC	Adultos obesos (*16*, 18-65)	↑ *Bifidobacterium* ↓ *Lachnobacterium*	Krumbeck et al., 2018

n: número de sujetos. AL: aleatorizado; C: cruzado; CS: ciego simple; DC: doble ciego; P: paralelo; PC: placebo-control. [a] 57% GOS, 23% lactosa, 22% glucosa, 0,8% galactosa; [b] 48% GOS, 22% lactosa, 18% glucosa, 12% galactosa; [c] 91,8% GOS, 7% lactosa, <1% glucosa, <0,5% galactosa; [d] 59% GOS, 21% lactosa, 19% glucosa, 1% galactosa; [e] 69% GOS, 23% lactosa, 5% monosacáridos (glucosa y galactosa); [f] 72,5% GOS, 22,8% lactosa, 4,7% monosacáridos (glucosa y galactosa)

De los estudios tanto *in vitro* como *in vivo* se demuestra que los GOS son altamente específicos promoviendo el crecimiento de bifidobacterias, mientras que no presentan un efecto consistente en el incremento o disminución de otras especies bacterianas. En general, el efecto prebiótico parece tener lugar a expensas de grupos bacterianos menos beneficiosos como Bacteroides, *Escherchia coli*, *Clostridium hystolyticum* y *Desulfovibrio spp.*, probablemente debido a las propiedades inhibitorias de las bifidobacterias (Vulevic et al., 2008; Walton et al., 2012; Vulevic et al., 2013; Costabile et al., 2015a; Vulevic et al., 2015; Liu et al., 2016b).

El efecto sobre los AGCC tras la fermentación colónica de los GOS se ha estudiado principalmente mediante estudios *in vitro* mencionados anteriormente (ver tabla 5), observándose un incremento generalizado de la producción de AGCC, en concreto de ácidos acético y butírico. La población de bifidobacterias se reconoce como el principal grupo bacteriano productor de acético, mientras que las especies pertenecientes al grupo *Eubacterium rectale-Clostridium coccoides* o *Roseburia* pueden utilizar este ácido para la producción de butírico (Walton et al., 2012; Costabile et al., 2015a y 2015b; Liu et al., 2016b). De la misma manera, la fermentación de GOS puede estimular la producción de ácido láctico (Maathuis et al., 2012; Li et al., 2015), y éste a su vez, puede ser utilizado por bacterias butirogénicas como *Eubacterium halii* y *Faecalibacterium prausnitzii* (Perdijk et al., 2019). De manera similar a lo observado en el efecto prebiótico de los GOS, el incremento producido en las concentraciones de AGCC totales presenta una relación dosis-dependiente (Grimaldi et al., 2016; Fehlbaum et al., 2018).

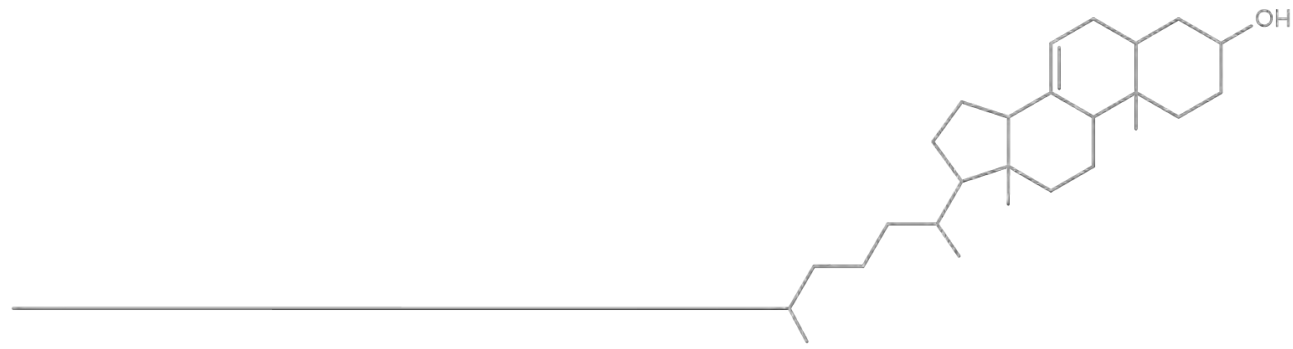
La disminución de la producción de ácidos grasos de cadena ramificada como isovalérico e isobutírico sugiere que, en presencia de GOS, la fermentación proteolítica (que genera productos finales, como amonio y aminas, capaces de alterar el equilibrio de la microbiota intestinal) se reduce a favor de una fermentación de tipo sacarolítica (Liu et al., 2016b).

El efecto de los GOS sobre las concentraciones de propiónico es controvertido, incrementando en algunos estudios (Fehlbaum et al., 2018; Chen et al., 2020) y disminuyendo en otros (Walton et al., 2012; Costabile et al., 2015b; Grimaldi et al., 2016). *Bacteroides-Prevotella* se identifica como el principal grupo bacteriano productor de propiónico y, por lo tanto, la modificación ejercida por la fermentación de los GOS sobre la composición de la microbiota presenta una importancia relevante en la formación de este AGCC (Li et al., 2015). En este sentido, el efecto del origen de la microbiota sobre la producción de AGCC se ha determinado mediante la fermentación de GOS en estudios por lote (Nogacka et al., 2020) y estudios con sistemas dinámicos (Aguirre et al., 2014; Poeker et al., 2018). Inóculos fecales procedentes de dos sujetos muestran una respuesta diferente en la producción

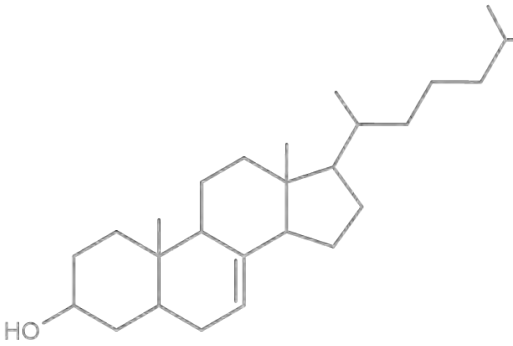
de butírico o propiónico tras la fermentación de los GOS, con porcentajes de incremento que varían de 2-96% y 3-40%, respectivamente (Poeker et al., 2018). Diferentes respuestas en la producción de AGCC se observan también al comparar la fermentación de los GOS en presencia de microbiota procedente de sujetos delgados *vs*. obesos. Aunque la presencia de GOS estimula la producción colónica de AGCC totales, en un estudio de fermentación por lote, los ensayos con microbiota de sujetos obesos no muestran un aumento de las concentraciones de butírico, sí observada en sujetos delgados (Nogacka et al., 2020). Sin embargo, en un estudio utilizando el sistema TIM-2, al comparar los ensayos con microbiota de sujetos delgados *vs*. obsesos se observa una menor producción de AGCC y una acumulación de ácido láctico en estos últimos (indicativa de una rápida fermentación del sustrato en comparación con el ensayo con microbiota de sujetos delgados) (Aguirre et al., 2014).

El estudio del efecto de la ingesta de GOS sobre la producción de AGCC es escaso en trabajos en humanos. En adultos sanos (Liu et al., 2017b) o con sobrepeso (Canfora et al., 2017), la ingesta diaria durante 2-12 semanas de 15-16 g de GOS al día no modifica el contenido fecal de AGCC, aunque se observa que las concentraciones de butírico tienden a disminuir.

No se conocen estudios que hayan evaluado la influencia de los GOS sobre la metabolización colónica de los EV mediante ensayos *in vitro* o tras la ingesta regular simultánea de GOS y EV *in vivo*.



Objetivos



El objetivo general de la presente Tesis Doctoral es evaluar el efecto de la presencia de galactooligosacáridos sobre la biodisponibilidad, fermentación colónica y efecto hipocolesterolemiante de los esteroles en bebidas a base de zumo de frutas y leche enriquecidas con esteroles vegetales mediante ensayos *in vitro* y/o *in vivo*.

Los objetivos específicos son:

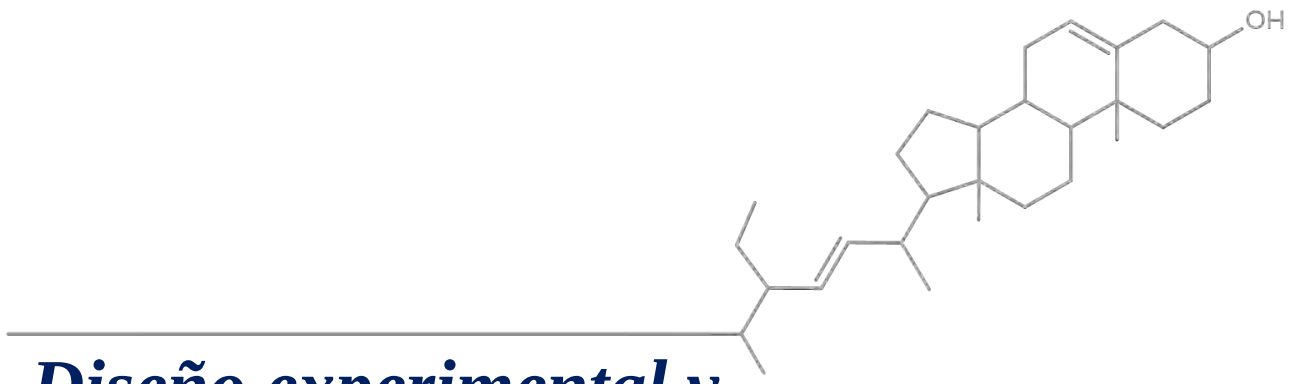
Objetivo 1: Evaluar el contenido, ingesta y bioaccesibilidad de los productos de oxidación de los esteroles en una bebida enriquecida con esteroles vegetales a lo largo de su vida útil.

Objetivo 2: Estimar la influencia de los galactooligosacáridos sobre la bioaccesibilidad de los esteroles en las bebidas mediante una digestión gastrointestinal micelar simulada.

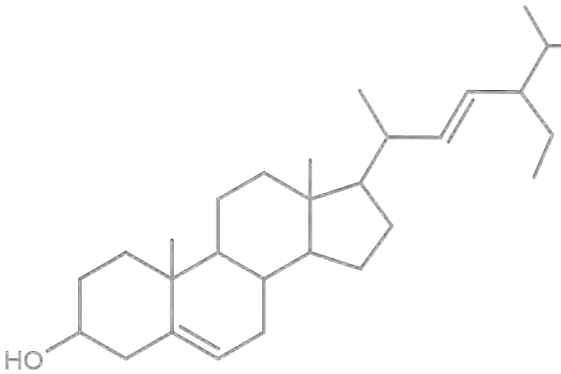
Objetivo 3: Adaptar el método de digestión gastrointestinal armonizado desarrollado dentro del marco de la acción Europea COST Infogest a la determinación de esteroles en bebidas.

Objetivo 4: Estudiar la biotransformación de esteroles y su interacción con la microbiota, en presencia y ausencia de galactooligosacáridos, durante la fermentación colónica dinámica *in vitro* de bebidas.

Objetivo 5: Determinar el efecto de la presencia de galactooligosacáridos sobre la biodisponibilidad, fermentación colónica y efecto hipocolesterolemiante de los esteroles, en mujeres postmenopáusicas con hipercolesterolemia moderada tras la ingesta regular de bebidas enriquecidas con esteroles vegetales con o sin galactooligosacáridos.

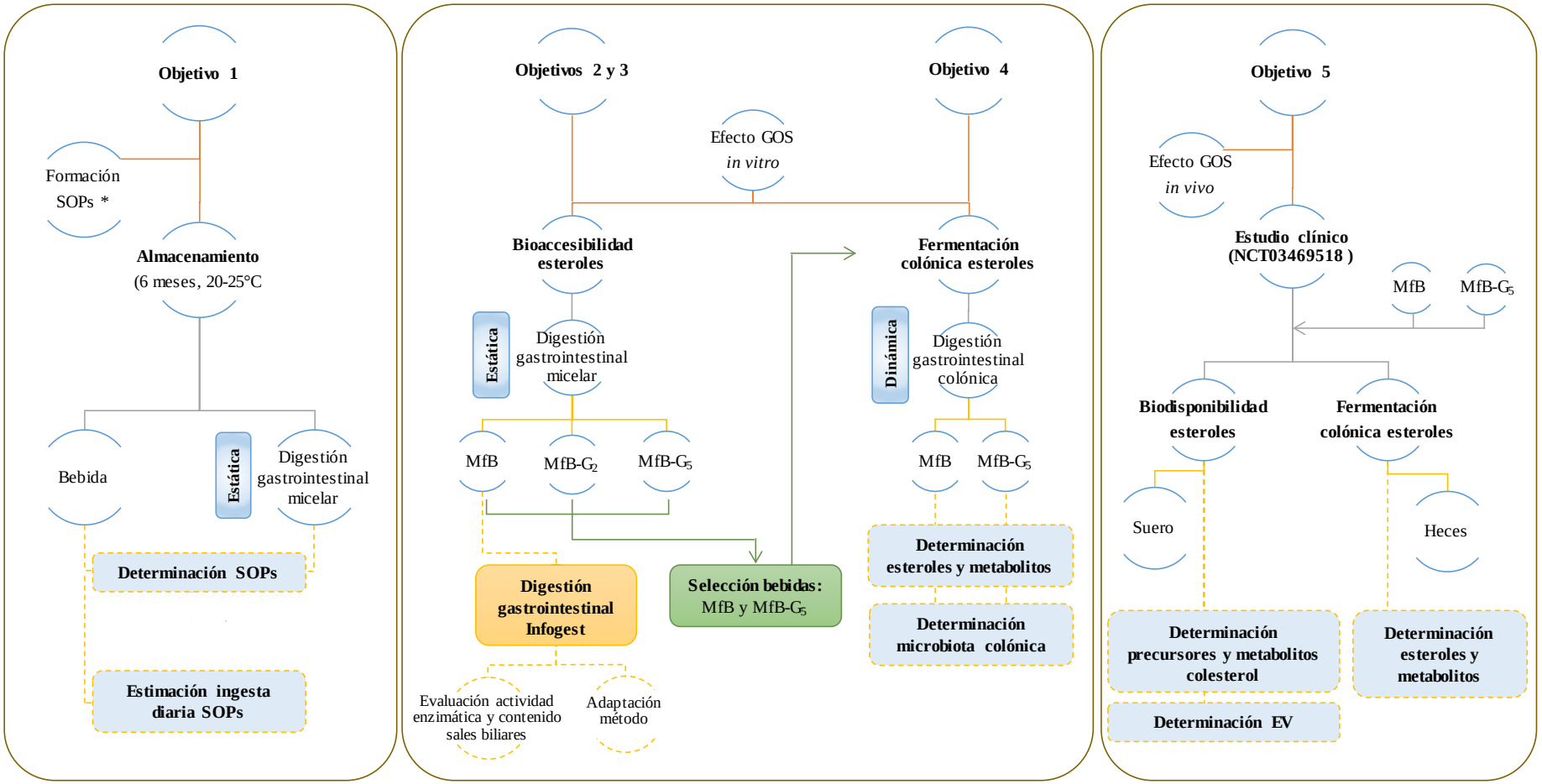


Diseño experimental y difusión de resultados



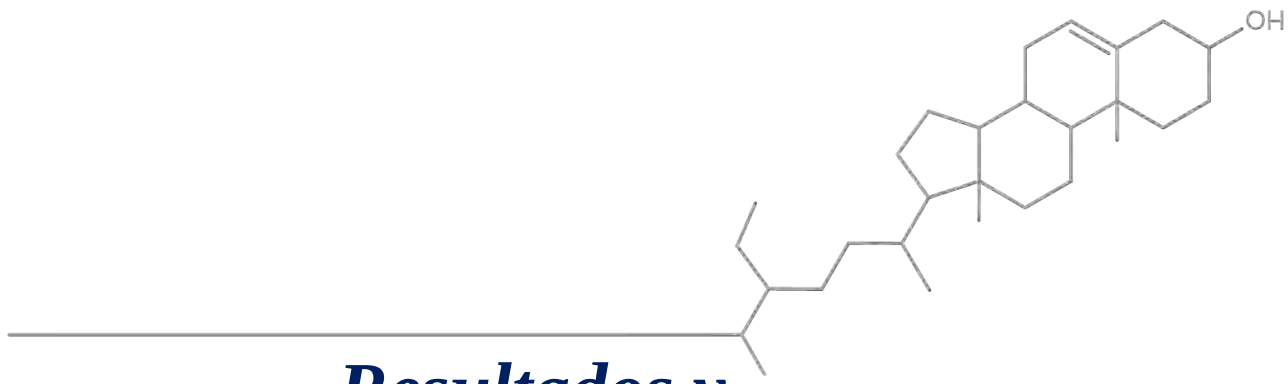
El diseño experimental realizado en la presente Tesis Doctoral para abordar los objetivos planteados y la difusión de resultados se muestran en las figuras 9 y 10, respectivamente.

Figura 9. Descripción del plan de trabajo seguido para abordar los objetivos planteados.

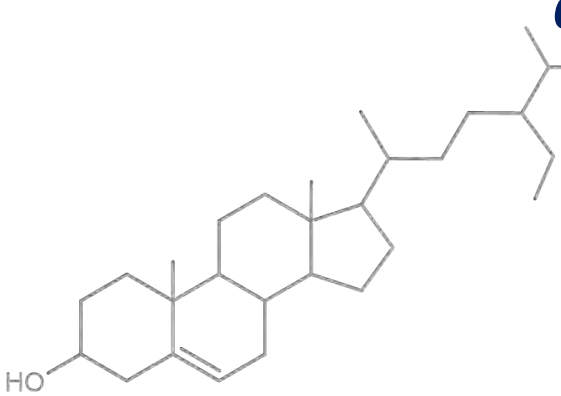


Abreviaturas. EV: esteroles vegetales, SOPs: productos de oxidación de esteroles, MfB: bebida a base de zumo de frutas y leche enriquecida con EV (2,5 g/250 mL en estudios *in vitro y* 2 g/250 mL en estudios *in vivo*), MfB-G_2: MfB con 2,3 g GOS/250 mL, MfB-G_5: MfB con 4,5 g GOS/250 mL en estudios *in vitro* y 4,3 g GOS/250 mL en estudios *in vivo*. ***** Ensayos realizados con una bebida a base de zumo de frutas y leche enriquecida con EV (2 g/250 mL).

Figura 10. Difusión de los resultados.



Resultados y discusión



A continuación, se muestra el resumen de los resultados y discusión, de acuerdo con los objetivos descritos anteriormente y las publicaciones adjuntas (aceptadas en el Anexo I, en revisión en el Anexo II y comunicaciones a congresos en el Anexo III). La metodología y bibliografía utilizada en cada estudio se describe en los correspondientes artículos.

Objetivo 1: Evaluar el contenido, ingesta y bioaccesibilidad de los productos de oxidación de los esteroles en una bebida enriquecida con esteroles vegetales a lo largo de su vida útil.

Para abordar este objetivo, se utiliza una bebida a base de zumo de frutas y leche que contiene leche desnatada, grasa láctea, concentrado de proteínas séricas enriquecido con MFGM, zumo de mandarina, puré de plátano y zumo de uva, con la adición de EV libres microencapsulados en polvo (2 g/250 mL bebida) procedentes de *tall oil*.

- *Contenido de productos de oxidación de esteroles en la bebida*

Durante el periodo de almacenamiento de la bebida (0-6 meses, 20-25˚C) se detectan los mismos COPs y POPs: 7α- y 7β-hidroxi, α- y β-epoxi, triol y 7-ceto. Solo se identifican POPs derivados del β-sitosterol, de manera similar a lo observado en estudios previos del grupo de investigación realizados en bebidas enriquecidas con EV en las que el β-sitosterol es el esterol mayoritario (Alemany-Costa et al., 2012; Alemany et al., 2013a).

El contenido total de POPs durante el almacenamiento no presenta diferencias estadísticamente significativas ($p > 0,05$), oscilando entre 187 y 204 µg/100 g bebida a 0 y 6 meses, respectivamente. Estos contenidos son inferiores a los indicados por Alemany-Costa et al. (2012) (580-830 µg/100 g) y González-Larena et al. (2015) (243 µg/100 g), pero superiores a los observados por Alemany et al. (2013a) (74 µg/100 g) en bebidas similares. En comparación con estos estudios, la bebida utilizada en este trabajo presenta un contenido cuatro veces mayor de grasa saturada (0,4 *vs*. 1,6 g/100 mL) debido a la adición de grasa láctea y concentrado de proteínas séricas enriquecido con MFGM. Por lo tanto, el tipo y el contenido de grasa no parece influir sobre el contenido de POPs en estas matrices.

El β-epoxisitosterol es el óxido mayoritario tras la elaboración de la bebida (0 meses) seguido del α-epoxisitosterol y 7β-hidroxisitosterol, mientras que el 7α-hidroxisitosterol es el minoritario. Estos resultados coinciden con un trabajo previo (González-Larena et al. 2015) en bebidas similares almacenadas a 24°C durante 6 meses. Sin embargo, en este tipo de bebidas, otros estudios observan una mayor abundancia de los derivados 7β-hidroxi, seguido por 7β-epoxi y 7-cetositosterol (Alemany-Costa et al., 2012) o 7-cetositosterol (Alemany et al., 2013a). Las diferencias entre estudios

se pueden atribuir a factores como el perfil de POPs presente en el ingrediente fuente de EV, así como a las condiciones de fabricación (escala de laboratorio o industrial).

Se observa un ligero aumento estadísticamente significativo ($p < 0,05$) en los contenidos de sitostanotriol solo tras 6 meses de almacenamiento. Sin embargo, los porcentajes relativos de cada óxido de β-sitosterol con respecto al contenido de POPs total se mantienen en unas proporciones constantes durante los 6 meses de almacenamiento de la bebida: β-epoxi (33-36%), 7β-hidroxi (18-19%), α-epoxi (15-17%), 7-ceto (13-15%), triol (10-11%) y 7α-hidroxi (5-6%).

Por otro lado, el contenido total de COPs oscila entre 99-103 μg/100 g de bebida, sin diferencias estadísticamente significativas ($p > 0,05$) durante el periodo de almacenamiento. En el único estudio en el que se determina el contenido de COPs en bebidas a base de zumo de frutas y leche (Alemany et al., 2013a), se obtienen valores mayores a los determinados en el presente trabajo (201 μg/100 g), a pesar de contener una menor cantidad de colesterol (1,4 *vs*. 8,2 mg/100 g de bebida). Colestanotriol y 7-cetocolesterol son los COPs más abundantes en la bebida (cada uno representa el 25-27% del total de COPs), mientras que los contenidos más bajos corresponden a 7α- y 7β-hidroxicolesterol (5-8%). Este orden de abundancia es similar al observado por Alemany et al. (2013a), a excepción de que estos autores no observan contenidos de colestanotriol o α- y β-epoxicolesterol. En nuestro estudio, solo se detecta un ligero aumento significativo de los contenidos de 7α- y 7β-hidroxicolesterol a partir de los 3 meses de almacenamiento (alcanzando el 7-10% del total de COPs).

Con respecto a los porcentajes de oxidación del β-sitosterol oscilan entre 0,027-0,029%, siendo mayores que los indicados por Alemany et al. (2013a) a 0 meses de almacenamiento (0,013%), pero menores o similares a los determinados en bebidas enriquecidas con EV tras su fabricación (0,07%) (Alemany-Costa et al., 2012) o durante su almacenamiento a temperatura ambiente (0,03-0,06) (0-6 meses) (González-Larena et al., 2015). Por lo tanto, se confirma que la formulación y elaboración de las bebidas del presente estudio (que incluye la adición de grasa láctea y MFGM), no implica una mayor oxidación de los EV.

El colesterol presenta mayores porcentajes de oxidación que el β-sitosterol, acorde a lo observado previamente en bebidas similares (Alemany et al., 2013a). Sin embargo, los porcentajes de oxidación tras la elaboración de la bebida (0 meses) son menores que los indicados por estos autores (1,2% *vs*. 14,9%), a pesar del mayor contenido de colesterol (8,2 *vs*. 1,4 mg/100 g bebida). Este hecho puede deberse a la distinta actividad superficial de los esteroles, que puede afectar a su susceptibilidad frente a la oxidación (Cercaci et al., 2007). En este sentido, el colesterol presenta un mayor grado de actividad superficial que el β-sitosterol y, por lo tanto, en las emulsiones se halla a más concentración

en las interfaces aceite-agua, donde el estrés oxidativo es mayor. Además, el ingrediente de EV empleado en este trabajo se encuentra microencapsulado y este formato podría ejercer cierta protección frente a la oxidación.

- *Contribución de la bebida a la ingesta diaria de productos de oxidación de esteroles*

La ingesta diaria de POPs a partir de la bebida, teniendo en cuenta el contenido de óxidos de β-sitosterol determinado y considerando que el tamaño de ración diaria corresponde a 250 g de bebida, es de 0,47 y 0,51 mg tras la elaboración y 6 meses de almacenamiento, respectivamente. Estos valores están dentro del intervalo indicado en leches pasteurizadas enriquecidas con EV (0,1-3,5 mg de POPs/día) (Scholz et al., 2015). El mayor enriquecimiento con EV de la bebida analizada en el presente estudio con respecto al trabajo anteriormente mencionado (0,8% *vs*. 0,3-0,5%) no implica un mayor contenido de POPs, aunque se asocia positivamente el contenido absoluto de POPs en los alimentos con su contenido de EV (Lin et al., 2016).

Por otra parte, teniendo en cuenta el porcentaje de oxidación y la ingesta de EV a partir del consumo de 250 g de bebida (2,14 g de EV), la ingesta estimada de POPs a través del consumo de la bebida es de 0,5 mg/día. Esta estimación se encuentra por debajo de lo observado en alimentos no tratados térmicamente (Lin et al., 2016) pero se incluye en el intervalo indicado por Scholz et al. (2015) para ratios de oxidación mínimos (0,1%).

A su vez, la ingesta de COPs aportada por ración de bebida, calculada de acuerdo a los contenidos determinados durante el almacenamiento de la bebida o teniendo en cuenta el porcentaje de oxidación y el contenido de colesterol (20,4 mg/250 g bebida), es de 0,25 mg/día. Estos valores (que se calculan a partir de un solo alimento) son mucho más bajos que los observados en trabajos previos (1,8-11,5 mg/día), ya que en estos estudios se considera una dieta o comida que incluye alimentos ricos en colesterol (Emanuel et al., 1991) y alimentos fritos en grasa de vaca (van den Bovenkamp et al., 1988; Lake & Scholes, 1997).

- *Bioaccesibilidad de productos de oxidación de esteroles en la bebida*

Se determina la bioaccesibilidad de los esteroles tras la aplicación de una digestión gastrointestinal micelar simulada basada en tres etapas (salivar, gástrica e intestinal) a la bebida objeto de estudio.

Los contenidos totales de POPs y COPs en la fracción bioaccesible de la bebida no se modifican significativamente a lo largo del periodo de almacenamiento, con valores en los intervalos de 85,5-

94,4 y 59,9-79,3 µg/100 g bebida, respectivamente. Sin embargo, se observa un ligero incremento de los derivados 7α-hidroxi de β-sitosterol y colesterol, y de 7β-hidroxicolesterol durante el almacenamiento. Estos valores son mayores que los indicados en fracciones bioaccesibles de bebidas similares tras aplicar el mismo método de digestión (32,7 µg POPs y 5,6 µg COPs/100 g bebida) (Alemany et al., 2013a).

La bioaccesibilidad de POPs y COPs totales, durante los 6 meses de almacenamiento, oscila entre 45-49% y 58-80%, respectivamente, sin diferencias estadísticamente significativas ($p > 0,05$) en el tiempo. La mayor bioaccesibilidad de los COPs con respecto a los POPs puede deberse a la mayor solubilidad micelar del colesterol con respecto al β-sitosterol, hecho demostrado, con anterioridad, en bebidas similares a las del presente estudio (Alvarez-Sala et al., 2016) y, por lo tanto, cabe esperar el mismo comportamiento para los correspondientes óxidos. Entre los POPs, los derivados triol, α-epoxi y 7β-hidroxisitosterol presentan los valores más altos de bioaccesibilidad (49-62%), mientras que los derivados 7α- y 7β-hidroxicolesterol muestran las bioaccesibilidades más altas para COPs (81-99%), acorde con los resultados obtenidos por Alemany et al., 2013a.

Los contenidos totales de POPs y COPs en las fracciones bioaccesibles (expresados como micromolaridad) oscilan entre 1,97-2,17 µM y 1,48-1,96 y 1,48-1,96 µM, respectivamente. Estos valores son muy inferiores a las concentraciones con las que se asocian efectos citotóxicos en células Caco-2 (desde 60 µM para POPs y desde 30 µM para COPs) (Ryan et al., 2009; Alemany-Costa et al., 2012; Laparra et al., 2015). Sin embargo, no se puede descartar que la ingesta continuada de estos óxidos pueda conducir a enfermedades degenerativas (Kulig et al. al., 2016).

Los resultados obtenidos en este estudio se han publicado en: Alvarez-Sala, A.*, **Blanco-Morales, V.***, Cilla, A., Garcia-Llatas, G., Sánchez-Siles, L.M., Barberá, R., & Largada M.J. (2018). *Safe intake of a plant-sterol enriched beverage with milk fat globule membrane: Bioaccessibility of sterol oxides during storage.* Journal of Food Composition & Analysis, 68, 111-117. *Estos autores han contribuido por igual y deben ser considerados como primeros autores (ver publicación en Anexo I).

Objetivo 2: Estimar la influencia de los galactooligosacáridos sobre la bioaccesibilidad de los esteroles en las bebidas objeto de estudio mediante una digestión gastrointestinal micelar simulada.

Con el propósito de abordar este objetivo, se elaboran tres bebidas a base de zumo de frutas y leche enriquecidas con EV (2,5 g/250 mL): MfB (sin adición de GOS) y MfB-G2 y MfB-G5 (con adición de 2,3 y 4,5 g GOS/250 mL, respectivamente). Todas las bebidas se fabrican bajo las mismas

condiciones conteniendo leche desnatada, grasa láctea, concentrado de proteínas séricas enriquecido con MFGM, concentrado de zumo de mandarina, puré de plátano y EV libres microencapsulados en polvo, procedentes de *tall oil*. La muestra MfB presenta una composición similar a la utilizada en el Objetivo 1. La diferencia en la dosis de EV se debe a su fabricación en tiempos diferentes correspondiendo con dos proyectos I+D+I consecutivos. El efecto de los GOS sobre la bioaccesibilidad de los esteroles se determina mediante la aplicación de una digestión gastrointestinal micelar simulada en tres etapas (salivar, gástrica e intestinal), utilizada en trabajos previos del grupo de investigación, a las tres bebidas objeto de estudio.

- *Efecto de la adición de galactooligosacáridos sobre la bioaccesibilidad de esteroles en las bebidas*

El contenido de colesterol en las bebidas oscila entre 13,07 y 13,58 mg/100 g bebida. Este contenido se encuentra por encima de lo indicado previamente en bebidas similares a base de zumo de frutas y leche enriquecidas con EV con o sin zumo de mandarina (1,4 y 2,0 mg/100 g) (Alemany et al., 2013a). El enriquecimiento con grasa láctea y MFGM de las bebidas objeto de estudio puede explicar este hecho, ya que el MFGM puede considerarse como fuente de colesterol al contener alrededor de 300 mg colesterol/100 g grasa láctea (Yao et al., 2016). El contenido total de EV en las tres bebidas oscila entre 846-931 mg/100 g bebida. El orden de abundancia y los porcentajes relativos de cada EV es similar en todas las bebidas: β-sitosterol ≈ 80%, sitostanol ≈ 12%, campesterol ≈ 7%, campestanol ≈ 1% y estigmasterol ≈ 0.7%. Estos resultados se encuentran dentro del límite especificado en la legislación relativo a bebidas a base de zumo de frutas y leche enriquecidas con fitosteroles/fitostanoles (Decision 2004/336) y acordes a los valores indicados en trabajos previos del grupo de investigación en bebidas similares (González-Larena et al., 2012; Alemany et al., 2013a; Alvarez-Sala et al., 2016).

Tras la aplicación de la digestión gastrointestinal simulada a las bebidas, el contenido de colesterol y EV totales en las fracciones bioaccesibles oscila entre 10-12 mg y 319-346 mg/100 g bebida, respectivamente, siendo ambos contenidos mayores en la fracción bioaccesible correspondiente a la MfG-G_5 (con mayor contenido en GOS). El orden de abundancia de los EV en las fracciones bioaccesibles no varía entre las bebidas (β-sitosterol > sitostanol > campesterol > campestanol > estigmasterol).

La bioaccesibilidad del colesterol es similar en todas las muestras (≈80%), a excepción de la MfG-G_5, en la que se observa un ligero aumento (85%). No existen diferencias estadísticamente significativas ($p > 0,05$) en la bioaccesibilidad de los EV individuales entre bebidas, a excepción de

campesterol, que presenta una menor bioaccesibilidad en la MfG-G_5 (43% *vs*. 41%). Del mismo modo, la bioaccesibilidad de los EV totales es también similar en las tres bebidas (37%), siendo el orden campestanol (≈43%) > sitostanol–campesterol (≈40%) > β-sitosterol (≈37%) > estigmasterol (≈33%). Estos resultados demuestran que la adición de GOS a bebidas a base de zumo de frutas y leche no afecta a la solubilidad de los esteroles.

Hasta el momento, el efecto de la fibra sobre la bioaccesibilidad de los EV no se ha estudiado, aunque, debido a su similitud estructural con el colesterol, se puede esperar un efecto similar. En este contexto, se observa que la adición de goma guar parcialmente hidrolizada a concentraciones de 3% y 6% en una bebida de yogur con 3% de aceite de girasol y 4% de yema de huevo reduce la bioaccesibilidad del colesterol entre 8 y 22 veces, respectivamente, en un modelo gastrointestinal multicompartimental (Minekus et al., 2005). En empanadas de cerdo adicionadas con un 10% de varios extractos ricos en fibra, la solubilidad del colesterol en la fase oleosa disminuye (10-31%) tras la digestión gastrointestinal simulada Infogest (López-Marcos et al., 2015). Sin embargo, además del contenido de fibra, también se debe tener en cuenta la ratio colesterol/fibra. En los estudios mencionados anteriormente, ratios entre 0,0005-0,01 se asocian con la disminución de la solubilidad o bioaccesibilidad del colesterol. En el presente trabajo, las bebidas MfB-G_2 y MfB-G_5 presentan una ratio esterol/GOS de 0,98 y 0,6, respectivamente, que es superior al utilizado por los autores anteriormente mencionados. Por lo tanto, el enriquecimiento con EV probablemente provoque que el contenido de fibra presente en la bebida no sea suficiente para producir modificaciones significativas en la bioaccesibilidad de los esteroles.

Objetivo 3: Adaptar el método de digestión gastrointestinal armonizado desarrollado dentro del marco de la acción Europea COST Infogest a la determinación de esteroles en la bebida objeto de estudio.

Dado que la presencia de GOS no afecta a la bioaccesibilidad de los esteroles, según lo indicado anteriormente en el objetivo 2, la aplicación y adaptación del método de digestión gastrointestinal Infogest a la determinación de la bioaccesibilidad de esteroles se lleva a cabo con la MfB (sin adición de GOS).

➢ *Aplicación del método de digestión gastrointestinal Infogest a la determinación de la bioaccesibilidad de esteroles*

Por primera vez, se aplica el método de digestión gastrointestinal Infogest con el fin de evaluar la bioaccesibilidad de esteroles y compararla con el método de digestión micelar.

La actividad enzimática de las enzimas y el contenido de sales biliares utilizadas en el método Infogest se determina siguiendo el protocolo descrito por Minekus et al. (2014). La α-amilasa salivar humana presenta una actividad enzimática de 62,2 ± 3,5 U/mg polvo, similar a lo determinado por Rodrigues et al. (2016) (79 U/mg polvo). La actividad de la pepsina porcina es de 3326 ± 172 U/mg polvo y se encuentra dentro de los valores medios especificados para esta enzima en el ensayo inter-laboratorio llevado a cabo por equipo de trabajo de Infogest (2976 ± 591 U/mg polvo) (Egger et al., 2016). En cuanto a las enzimas implicadas en la fase intestinal de la digestión, aunque en el método Infogest se indica la posibilidad de utilizar tanto pancreatina como enzimas intestinales individuales, esta última opción no es posible ya que la actividad de la lipasa pancreática no es suficiente para alcanzar las 2000 U/mL de mezcla intestinal final requerido por el método (la enzima presenta una baja solubilidad en el FSI). Por lo tanto, en este estudio, la etapa intestinal se lleva a cabo mediante la adición de pancreatina en base a su actividad en tripsina para alcanzar 100 U TAME/mL digerido intestinal. La actividad obtenida para esta enzima es inferior a la indicada por Rodrigues et al. (2016) (7,1 ± 0,8 *vs*. 17 U TAME/mL polvo), probablemente debido a la utilización de diferentes lotes de pancreatina.

Al aplicar el método de digestión Infogest, el colesterol de la fracción bioaccesible no puede ser cuantificado en la bebida y, por tanto, su bioaccesibilidad ya que el contenido de este esterol en los blancos de digestión es del mismo orden que el de la fracción bioaccesible de la bebida sin digerir. Estos resultados sugieren que el aporte de colesterol por las sales biliares porcinas es alto y puede saturar las micelas, reduciendo la solubilización del colesterol. Además, la presencia de EV en la fracción bioaccesible puede contribuir a una menor incorporación del colesterol en la micela como resultado de un mecanismo de competición. Sin embargo, en fracciones bioaccesibles obtenidas tras la aplicación del método Infogest a empanadas de cerdo, la cuantificación del contenido de colesterol es posible (López-Marcos et al., 2015). Esto puede deberse a un mayor contenido inicial de colesterol en la muestra o al uso de sales biliares frescas en lugar de extracto biliar en polvo.

Por otro lado, el contenido de EV totales en la fracción bioaccesible de la MfB obtenida mediante el método Infogest es un 61% menor que el obtenido mediante el método micelar (128 *vs*. 330 mg/100 g bebida, respectivamente). La bioaccesibilidad de los EV individuales con el método Infogest es

similar en todos los EV analizados, siendo el orden sitostanol (16%) > β-sitosterol (14%) > campesterol–campestanol–estigmasterol (13%). El perfil de solubilidad difiere del obtenido mediante la digestión micelar, en la cual campestanol y campesterol son los esteroles con mayor bioaccesibilidad, seguidos por el β-sitosterol. En cambio, en ambos métodos de digestión se observa que el estigmasterol presenta la bioaccesibilidad más baja. Asimismo, la bioaccesibilidad de EV totales en el método Infogest es menor que la obtenida en el método micelar (14% *vs*. 37%). Las diferencias de solubilidad de los EV observadas entre ambos métodos de digestión pueden deberse a la mayor concentración de sales biliares utilizada en el método Infogest (4 *vs*. 0,7 mg/mL digerido), que podrían inhibir la actividad de la lipasa pancreática (enzima clave del metabolismo lipídico) al desplazar la enzima e impedir su contacto con el sustrato (Li et al., 2011). Además, el diferente origen de las sales biliares utilizadas en cada digestión (bovino en el método micelar y porcino en el Infogest) puede influir en la solubilidad micelar de los esteroles debido a su distinta polaridad (Armstrong & Carey, 1987; Matsuoka et al., 2008). Estos últimos hechos llevan a plantear una adaptación del método Infogest cuyas principales modificaciones y resultados se describen a continuación.

- *Adaptación del método de digestión gastrointestinal Infogest a la determinación de la bioaccesibilidad de esteroles*

Con el fin de adaptar el método Infogest para permitir la cuantificación de colesterol en la fracción bioaccesible de la MfB se proponen dos alternativas: reducir la concentración de sales biliares utilizadas en el método Infogest, equiparándolo a las concentraciones utilizadas en el método de digestión micelar (1,4 mM en lugar de 10 mM) que permita poder comparar ambos métodos (opción 1) o cambiar el origen de las sales biliares utilizadas (bovinas en lugar de porcinas) manteniendo la concentración indicada en el método Infogest (10 mM) que se aproxima más a las concentraciones fisiológicas (opción 2).

Ambas modificaciones permiten la cuantificación del colesterol en las fracciones bioaccesibles, con contenidos de 8 y 5 mg/100 g bebida y bioaccesibilidades de 62% y 38% para las opciones 1 y 2, respectivamente. La reducción de la concentración de sales biliares de origen porcino produce un incremento de la solubilidad de EV individuales (18,25%) y totales (22%) con respecto a los resultados obtenidos con el método Infogest (13-16% y 14%, respectivamente). Sin embargo, con el cambio de origen de sales biliares no se observan diferencias estadísticamente significativas ($p > 0,05$), a excepción del estigmasterol, que disminuye un 24%. Por lo tanto, aunque el cambio de origen de las sales biliares utilizadas permite la cuantificación del colesterol, solo la disminución de la concentración de las mismas favorece la bioaccesibilidad de los esteroles.

En cualquier caso, la bioaccesibilidad del colesterol y de los EV con ambas opciones del método Infogest son menores que las obtenidas con el método de digestión micelar. Estos resultados sugieren que otros factores involucrados en la digestión, aparte de las sales biliares, pueden influir en la solubilidad de los esteroles. En este sentido, la mayor solubilidad observada en el método micelar con respecto al método Infogest puede deberse a la adición de enzimas clave del metabolismo lipídico como la colesterol esterasa, fosfolipasa A2, lipasa y co-lipasa durante la fase intestinal (enzimas que se correlacionan con una mejor micelarización de carotenoides y esteroles como el colesterol y el β-sitosterol) (Richmond et al., 2001; Nik et al., 2011; Estevez-Santiago et al., 2016).

Además, se observan diferencias entre las dos modificaciones del método Infogest, en cuanto al orden de solubilidad de los esteroles individuales. Al reducir la concentración de sales biliares, el orden de solubilidad es colesterol (62%) > campestanol (25%) > campesterol (25%) > sitostanol (24%) > β-sitosterol (22%) > estigmasterol (18%). En cambio, al cambiar el origen de las sales biliares, el orden de solubilidad de los esteroles es el siguiente: colesterol (38%) > sitostanol (16%) > campesterol (15%) > β-sitosterol (14%) > campestanol (13%) > estigmasterol (10%). De esta manera, cuando se utiliza una misma concentración de sales biliares, el método micelar y el método Infogest muestras un perfil de solubilidad idéntico. Asimismo, cuando se utilizan altas concentraciones de sales biliares (10 mM), se obtienen perfiles de solubilidad similares, independientemente del origen de las mismas (porcinas o bovinas). Estos resultados sugieren que el perfil de solubilidad de los esteroles se relaciona estrechamente con el contenido de sales biliares presentes en el digerido, independientemente del método de digestión o el origen de las sales biliares utilizadas.

Los resultados derivados de los objetivos 2 y 3 han sido publicados en: **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. *Estos autores han contribuido por igual y deben ser considerados como primeros autores (ver publicación en el Anexo I).

Objetivo 4: Estudiar la biotransformación de esteroles y su interacción con la microbiota, en presencia y ausencia de galactooligosacáridos, durante la fermentación colónica dinámica *in vitro* de las bebidas objeto de estudio.

Para el desarrollo de este objetivo se aplica una digestión gastrointestinal dinámica, utilizando un sistema que consta de cinco reactores que simulan el estómago, intestino delgado, colon ascendente,

transversal y descendente, a dos bebidas enriquecidas con EV (2,5 g/250 mL), con o sin adición de GOS (4,5 g/250 mL) (MfB y MfB-G_5, respectivamente, descritas previamente en el objetivo 2). Se toman muestras de líquidos de fermentación correspondientes a los reactores del colon ascendente, transversal y descendente tras 8 h de digestión (tiempo que dura la digestión gástrica e intestinal como control del contenido de esteroles aportados por la bebida) y una vez al día durante la semana que dura el ensayo (desde 24 a 168 h).

- *Influencia de los galactooligosacáridos en la composición de la microbiota*

El análisis de la microbiota por compartimentos mediante el índice de diversidad Shannon muestra un aumento estadísticamente significativo ($p < 0,001$) de la riqueza y diversidad de especies microbianas en los líquidos de fermentación procedentes de los tramos distales del colon (transversal y descendente) con respecto a los del colon ascendente. En el ensayo con la bebida sin GOS, este aumento es progresivo (descendente < transversal), mientras que en presencia de GOS no se observan diferencias entre los reactores del colon transversal y descendente. En ambas bebidas, el análisis de coordenadas principales (PCoA) muestra una agrupación separada para las muestras procedentes del colon ascendente (que explica el 82 y 88% de la variación total de los datos en las bebidas sin y con GOS, respectivamente), mientras que los compartimentos del colon distal presentan una comunidad microbiana más parecida. En este sentido, el estudio del proceso de colonización por parte de la microbiota en un modelo SHIME demuestra que las diferentes condiciones de los reactores (menor pH y mayor concentración de sales biliares en el colon ascendente con respecto al transversal y descendente), pueden justificar el desarrollo de distintas comunidades microbianas entre compartimentos (Van den Abbeele et al., 2010).

La composición de la microbiota a nivel de género (expresada como abundancia relativa) difiere entre los ensayos de fermentación de las bebidas. En los líquidos de fermentación procedentes del colon ascendente del experimento sin GOS el género *Klebsiella* es el mayoritario, con abundancias entre 44 y 67% y contenidos máximos a las 48 h. El género *Megasphaera* disminuye tras 24 h de fermentación (7%), incrementando posteriormente hasta alcanzar una abundancia del 28%. El género *Mitsuokella* incrementa desde 11 a 34% en 24 h de fermentación y, posteriormente, presenta una tendencia a disminuir. Los géneros *Stenotrophomonas*, *Dialister*, *Ochrobactrum*, *Sutterella* y los géneros no clasificados de las familias Lachnospiraceae y Enterobacteriaceae muestran una abundancia menor al 6% durante todo el ensayo de fermentación. La abundancia del género *Bifidobacterium* oscila entre 0,1 y 0,5%, con la excepción de la muestra a 24 h, donde se determina una abundancia de 3,2%. En cambio, en los líquidos de fermentación procedentes del colon

ascendente del ensayo con GOS predomina el género *Clostridium*, que presenta una evolución irregular con abundancias máximas a las 48 h (88 %) y mínimas a las 168 h (52 %). El género no clasificado de la familia Lachnospiraceae y el género *Ochrobactrum* también sigue una tendencia irregular, alcanzando sus contenidos máximos a 168 h (los cambios en su abundancia oscilan desde 5 a 12% y desde 3 a 5%, respectivamente). El género *Turicibacter* disminuye de manera progresiva desde 15 a 0,1%. Los géneros *Delftia* y *Pediococcus* tienden a incrementar mientras que *Stenotrophomonas, Pseudomanas, Enterococcus* y el género no clasificado de la familia Clostridiaceae, en general, disminuyen durante la fermentación, mostrando todos ellos abundancias menores a 7%. En este sentido, estudios previos de fermentación colónica *in vitro* de EV observan incrementos de algunos miembros del filo Firmicutes (al que pertenecen los géneros *Megasphaera* y *Mitsuokella* determinados en la bebida sin GOS y *Clostridium, Turicibacter* y la familia Lachnospiraceae observados en presencia de GOS). En concreto, en ensayos de fermentación por lote se determina incrementos de *Eubacterium hallii* (Cuevas-Tena et al., 2018b), mientras que en el modelo TIM-2 se produce el crecimiento de los géneros *Coprococcus* cuando se emplea un inóculo fecal procedente de sujetos delgados y *Clostridium* cuando el inóculo procede de sujetos obesos (Cuevas-Tena et al., 2019a). Además, durante la fermentación colónica de matrices similares como el zumo de naranja pasteurizado o fresco en un sistema SHIME se observa una modulación de este mismo filo (incrementos de *Lactobacillus spp*., *Enterococcus spp*. o *Clostridium spp*.) (Duque et al., 2016). De manera similar a lo observado en el presente trabajo, la fermentación de GOS mediante un sistema multicompartimental da lugar a una disminución del grupo *Clostridium histolycum* (Liu et al., 2017).

Se observa una mayor similitud en la composición de la microbiota de los reactores transversal y descendente entre los ensayos de fermentación, con una predominancia del género *Bacteroides* en ambas bebidas. En este sentido, en fermentaciones por lote se indica que el incremento de pH desde 5,5 (colon ascendente) hasta 6,5-6,7 (colon descendente) favorece el crecimiento del género *Bacteroides* (alcanzando niveles de hasta el 80% del total de eubacterias) (Walker et al., 2005; Duncan et al., 2009). En el caso del ensayo con la bebida sin GOS, el género *Bacteroides* es el más abundante al comienzo de la fermentación (51% en el transversal y 34% en el descendente), disminuyendo (144-168 h) hasta abundancias de 27-30% y 18-26%, respectivamente. Al contrario, *Klebsiella* presenta abundancias minoritarias a las 8 h de fermentación (8% en el transversal y 7% en el descendente) e incrementa hasta alcanzar abundancias de 27-40% y 30-35%, respectivamente, a 144-168 h de fermentación. El género *Akkermansia* oscila entre 6 y 13% en los compartimentos distales, mostrando una evolución irregular en el colon transversal y una tendencia a disminuir en el colon descendente. El género no clasificado perteneciente a la familia Lachnospiraceae presenta

abundancias entre 3 y 5% en ambos compartimentos, con la excepción de un incremento a las 24 h de fermentación en el colon transversal (9%). Los géneros *Parabacteroides* y Rikenellaceae no clasificado muestran una tendencia a disminuir a partir de las 24 h en los compartimentos distales (de 7 a 4% y de 4 a 0,4% en el transversal y de 11 a 5% y de 17 a 3% en el descendente, respectivamente). La abundancia de los géneros restantes (*Acidaminococcus, Mitsuokella* y *Desulfovibrio*) no supera el 6% y su evolución es irregular. De manera similar, durante la fermentación en presencia de GOS, el género *Bacteroides* es el mayoritario en los reactores del colon distal y tiende a disminuir durante la fermentación de la bebida (a partir de las 72 h), oscilando sus contenidos entre 70 y 44% en el colon transversal y entre 65 y 41% en el descendente. Destacar que en este ensayo se observa un marcado incremento del género *Parabacteroides* a las 96 h en el colon transversal y a las 120 h en el descendente, manteniendo una abundancia relativamente estable con el tiempo. El género no clasificado perteneciente a la familia Synergistaceae tiende a incrementar a partir de las 24 h en el colon transversal (desde 5,8 a 14,4%) y de las 48 h en el colon descendente (desde 5,5 a 25,5%). Además, el género no clasificado de la familia Lachnospiraceae presenta un incremento a las 24 h en el colon transversal (desde 4,2 a 9,1%), mientras que en el colon descendente su abundancia máxima se determina a 48 y 72 h de fermentación (desde 3,4 a 7,3%), disminuyendo después en ambos casos. Los géneros restantes (*Sutterella, Bilophila, Oscillospira, Akkermansia* y los géneros no clasificados de las familias Rikenellaceae y Clostridiales) muestran una evolución irregular, con abundancias menores al 5% en todos los casos.

El género *Bacteroides* se relaciona predominantemente con la proteólisis en el intestino humano (Macfarlane et al., 1986), por lo que la disminución observada durante la fermentación de ambas bebidas en los reactores del colon transversal y descendente puede asociarse con el menor contenido de proteína presente en estos compartimentos.

En el presente estudio, los géneros pertenecientes al filo Bacteroidetes tienden a disminuir durante la fermentación (*Bacteroides* y el género no clasificado perteneciente a la familia Rikenellaceae en ambas bebidas y *Parabacteroides* en la bebida sin GOS). Sin embargo, en estudios previos, se observa que la fermentación de EV da lugar al incremento de especies bacterianas pertenecientes a este filo tanto en cultivos por lote (Cuevas-Tena et al., 2018b) como en el sistema TIM-2 utilizando un inóculo fecal procedente de sujetos obesos (Cuevas-Tena et al., 2019a). En este sentido, algunos de los cambios producidos en la composición de la microbiota en el presente estudio podrían estar relacionados con otros compuestos bioactivos presentes en la matriz alimentaria como los polifenoles. De hecho, la fermentación de polifenoles procedentes de una mezcla de vino tinto y extractos de zumo

de uva en un modelo SHIME produce una disminución del género *Bacteroides* (Kemperman et al., 2013).

En estudios previos, la fermentación de GOS a distintas concentraciones (1-10g/día) en diferentes sistemas de fermentación multicompartimentales (Maathuis et al., 2012; Walton et al., 2012; Grimaldi et al., 2017; Liu et al., 2017) produce un incremento de los géneros *Bifidobacterium* y *Lactobacillus*. Sin embargo, este efecto no se observa en el presente estudio, lo que sugiere que la combinación de ambos compuestos bioactivos (EV y GOS) puede modificar la función prebiótica de los GOS.

- *Efecto de los galactooligosacáridos en el metabolismo colónico de los esteroles*

Los contenidos de desmosterol, brasicasterol, latosterol y metilcoprostanona no son cuantificables en los líquidos de fermentación procedentes de ambas bebidas, así como el coprostanol en los procedentes de la bebida sin GOS.

En el colon ascendente, los contenidos de colesterol, en presencia o ausencia de GOS, presentan un marcado aumento hasta las 48 h, a partir de lo cual se observa una evolución irregular. En el colon transversal, el contenido de colesterol durante la fermentación de la bebida sin GOS incrementa hasta las 120 h y, posteriormente, disminuye; sin embargo, en los líquidos de fermentación procedentes de la bebida con GOS, el contenido máximo de colesterol se detecta a las 72 h, estabilizándose a partir de ese momento. En el caso del colon descendente, se observa un incremento gradual de los contenidos de colesterol en ausencia de GOS, con contenidos máximos a partir de las 120 h de fermentación, mientras que en presencia del prebiótico los contenidos se mantienen relativamente estables a partir de las 72 h de estudio (7-9 mg/compartimento).

Durante la fermentación de la bebida sin GOS se observa un incremento progresivo de los contenidos de β-sitosterol y sitostanol, alcanzando sus niveles máximos entre 96 y 144 h, en función del reactor. Sin embargo, en presencia de GOS, las cantidades máximas de ambos esteroles se detectan antes en cada reactor (entre 48 y 96 h), manteniéndose después estables.

Una tendencia de acumulación similar se observa para el resto de EV determinados (campesterol, campestanol y estigmasterol), alcanzando en general sus máximos contenidos a 120 h en todos los reactores para ambas bebidas.

El patrón de metabolismo de los esteroles difiere sustancialmente entre las bebidas. No se observan metabolitos de los esteroles en ningún compartimento tras la fermentación colónica de la bebida sin

GOS. De forma similar, no se detecta metabolización del colesterol tras 48 h de fermentación colónica del residuo de digestión gastrointestinal obtenido de una matriz similar (bebida a base de zumo de frutas y leche enriquecida con EV) (Cuevas-Tena et al., 2018b). Sin embargo, a diferencia de nuestro estudio, se observa una preferencia por parte de la microbiota sobre la metabolización de los EV (los contenidos de campesterol disminuyen un 39%, mientras que los de etilcoprostanol incrementan un 50%). Aunque el contenido de EV (~0,1 mg/mL) es inferior al de este trabajo (0,6-1 mg/mL), el uso de un método de fermentación estático podría favorecer el metabolismo de los EV. Este hecho se puede constatar al comparar la metabolización de polifenoles mediante el uso de un sistema gastrointestinal multicompartimental dinámico (Ekbatan et al., 2016) *vs*. fermentación por lote (Parkar et al., 2013). Debido a la mayor biotransformación determinada en éste último, los autores sugieren que la ausencia de un flujo continuo durante la fermentación puede implicar una mayor metabolización (Ekbatan et al., 2016). Por otra parte, la fermentación de ~2 g de EV (procedentes de un ingrediente fuente de EV o de una mezcla de patrones comerciales) en el sistema TIM-2 durante 72 h, utilizando un inóculo fecal procedente de sujetos delgados, da lugar a la biotransformación de colesterol y de los EV (Cuevas-Tena et al., 2019a). En concreto, se observa una disminución de los contenidos de coprostanol del 41-61%, mientras que los contenidos de coprostanona y colestanol aumentan con respecto a los valores iniciales (109-136% y 27-29%, respectivamente). En cuanto al metabolismo de los EV, solo se refleja mediante un aumento de la producción de metilcoprostanona a 72 h (incrementando 8 veces en el ensayo con el ingrediente de EV y 25 veces con la mezcla de patrones). En cambio, cuando el ensayo se lleva a cabo con un inóculo fecal procedente de sujetos obesos, no se produce metabolización. Más allá de las diferencias entre la actividad microbiana de sujetos delgados y obesos en cuanto al metabolismo de esteroles, distintos factores podrían justificar la discrepancia entre los resultados obtenidos en el ensayo llevado a cabo con el inóculo fecal de sujetos delgados con respecto a los del presente trabajo. En concreto, el sistema TIM-2 incluye una membrana de diálisis que simula la absorción de agua y metabolitos, previniendo su acumulación y, por lo tanto, una posible inhibición de la actividad de la microbiota (Venema & Van den Abbeele, 2013). Además, la ausencia de flujo entre compartimentos podría permitir una mayor detección de metabolitos aunque se lleven a cabo tiempos de fermentación menores, al igual que se observa en las fermentaciones por lote. Por otra parte, los EV utilizados en el estudio del TIM-2 (en una concentración aproximadamente dos veces superior a la del presente trabajo) se realiza mediante la incorporación en el sistema de un ingrediente fuente de EV o una mezcla de patrones comercializados, lo que podría facilitar su biotransformación. Este hecho se observa durante la fermentación dinámica *in vitro* de polifenoles, en la cual se determina una mayor cantidad de metabolitos en los compartimentos del colon proximal cuando se utilizan polifenoles puros *vs*. incorporados en una

matriz alimentaria (vino tinto, zumo de uva y té negro) (Ekbatan et al., 2016). Los autores sugieren que la matriz podría ofrecer cierta protección frente a la actividad de la microbiota.

A diferencia de lo indicado para la bebida sin GOS, sí que se observa la formación de metabolitos derivados de los principales esteroles en los reactores correspondientes al colon distal durante la fermentación de la bebida con GOS. En el colon transversal, la detección de coprostanol se produce a las 72 h, coincidiendo con la estabilización de los contenidos de colesterol. Las ratios coprostanol/colesterol incrementan durante el tiempo de fermentación desde 0,2 (72 h) a 0,7 (120-168 h) en el colon transversal y desde 0,9 (72 h) a 1,3 (120-168 h) en el colon descendente. Se detecta etilcoprostanol desde las 48 y 24 h de fermentación con aumentos significativos hasta las 120 y 144 h en el colon transversal y descendente, respectivamente. Este hecho se refleja en las ratios etilcoprostanol/β-sitosterol, que incrementan durante la fermentación desde 0,01 (48 h) a 0,46 (desde 120 h en adelante) y desde 0,2 (24 h) a 0,7 (144 h), respectivamente. Además, la fermentación de la bebida con GOS da lugar a la formación de sitostenona (metabolito de β-sitosterol) a 144 h en el colon transversal (9,4 ± 0,3 mg/compartimento) y, desde 72 h en adelante, en el colon descendente (valor medio de 7,7 ± 0,3 mg/compartimento). La producción de metilcoprostanol (metabolito de campesterol) se detecta desde las 72 y 48 h en el colon transversal y descendente, incrementando hasta las 120 y 144 h, respectivamente. Esta tendencia se demuestra con incrementos en las ratios durante la fermentación: desde 0,08 (72 h) a 0,26 (120 h) en el colon transversal y desde 0,2 (48 h) a 0,5 (168 h) en el colon descendente. Los contenidos de etilcoprostenol (del estigmasterol) se detectan desde las 96 h en el colon transversal y desde las 72 h en el descendente, manteniéndose constante hasta el final del ensayo de fermentación, mientras que las ratios incrementan desde 0,5 (96 h) a 0,6 (120 h) en el colon transversal y disminuyen desde 1,0 (72 h) a 0,8 (168 h) en el descendente.

La presencia de coprostanol, etilcoprostanol y metilcoprostanol se ha descrito previamente en estudios *in vitro* a través de la formación de diferentes metabolitos intermediarios como fitostanonas y fitostenonas (Cuevas-Tena et al., 2018a). Además, se ha indicado una ruta de formación directa para el coprostanol (Gérard, 2014). En el presente estudio, la ausencia de metabolitos intermediarios sugiere una rápida bioconversión de los esteroles o, en el caso del colesterol, que la ruta predominante es la vía de conversión directa a coprostanol. En el ensayo con la bebida que contiene GOS, la metabolización de los esteroles tiene lugar exclusivamente en los tramos distales del colon, de acuerdo con el análisis de la composición de la microbiota (anteriormente descrito), en el que se determina una mayor similitud entre la comunidad bacteriana presente en los compartimentos distales frente a la correspondiente al tramo proximal. En este sentido, *Bacteroides* (en el colon transversal y descendente de ambos ensayos de fermentación) y *Bifidobacterium* (en el colon ascendente de la

bebida sin GOS) son los únicos géneros identificados como bacterias reductoras de colesterol (Gérard, 2014; Cuevas-Tena et al., 2018a) observados en este trabajo. Además, en ensayos *in vitro* la familia Lachnospiraceae (presente en todos los reactores del colon de ambos ensayos de fermentación) también se sugiere como bacteria coprostanoligénica sin asociación con las rutas directas o indirectas del metabolismo del colesterol (Antharam et al., 2016; Kriaa et al., 2019). Dado que, aún en presencia de bacterias coprostanoligénicas, la biotransformación del colesterol solo se produce durante la fermentación de la bebida con GOS, es probable que otras especies microbianas (quizás potenciadas por la presencia de GOS) estén involucradas en la ruta de metabolización del colesterol. Del mismo modo, *Eubacterium spp.* es la única especie asociada con la ruta de biotransformación de EV (Eyssen et al., 1973; Cuevas-Tena et al., 2018a). La ausencia de *Eubacterium spp*. en ambos ensayos y, en concreto, en la fermentación de la bebida con GOS (donde se observa metabolización de los EV), sugiere la participación de otras especies microbianas.

- *Impacto de los galactooligosacáridos en la producción de ácidos orgánicos*

Tras la fermentación colónica de ambas bebidas se produce un aumento significativo ($p < 0,05$) de la producción total de ácidos orgánicos en todos los compartimentos del colon. En ambos ensayos (sin y con GOS), la concentración de ácidos orgánicos en el colon ascendente (28-57 mM y 47-82 mM, respectivamente) es menor que en el transversal (55-87 y 65-129 mM) y descendente (44-64 y 47-133 mM). Sin embargo, en ausencia de GOS los incrementos (168h *vs* 8h) son mayores en el colon ascendente (102%) *vs*. transversal (58%) y descendente (47%), mientras que en presencia del prebiótico se observa lo contrario (incrementos de 53, 97 y 180%, respectivamente).

En el colon ascendente, el ácido isobutírico es el ácido orgánico mayoritario para ambas bebidas, con concentraciones 1,5 y 1,9 veces mayores a las 168 h de fermentación con respecto a los valores iniciales. Esta tendencia se mantiene en el reactor del colon transversal en el ensayo sin GOS (con incrementos de 1,7 veces los valores iniciales), mientras que en presencia de GOS se produce una marcada disminución del contenido de isobutírico (de 14 mM a 8 h de fermentación hasta 3 mM a las 168 h). En ambos ensayos (sin y con GOS), las concentraciones observadas en el colon descendente son minoritarias (0,7-1,3 mM y 0,3-2,4 mM, respectivamente). El incremento mencionado en el colon ascendente sugiere un proceso de fermentación proteolítica durante la fermentación de ambas bebidas, de acuerdo con una mayor presencia de proteínas procedentes de la bebida en este reactor. El aumento de isobutírico se ha asociado con efectos perjudiciales para el hospedador, sin embargo, su impacto todavía se desconoce y, recientemente, se ha indicado que puede ser usado como fuente

de energía por las células epiteliales intestinales cuando el contenido de butirato es escaso (Oliphant & Allen-Vercoe, 2019).

El acético es otro de los ácidos orgánicos más abundantes en todos los reactores colónicos en ambos ensayos de fermentación. En el colon ascendente, durante la fermentación la bebida sin GOS, su concentración aumenta a las 24 h y después disminuye, mientras que en el colon transversal y descendente los contenidos máximos se observan entre las 72 y 168 h de fermentación. Sin embargo, los incrementos, con respecto a los contenidos a las 8 h, son mayores en el colon ascendente (2 veces) *vs.* transversal y descendente (1,4 veces). En presencia de GOS, los contenidos de ácido acético incrementan significativamente en todos los compartimentos del colon (1,5 veces en el ascendente, 2,3 veces en el transversal y 2,6 veces en el descendente, con respecto a 8 h).

En el colon ascendente, el contenido de ácido butírico aumenta progresivamente durante la fermentación de la bebida sin GOS (incrementos a las 168 h de 3 veces el contenido inicial), mientras que se mantiene relativamente estable en presencia de GOS (5,2-6,2 mM). En el colon transversal y descendente, los contenidos aumentan de manera progresiva, alcanzando su máxima concentración en ambas bebidas (MfB y MfB-G_5) a las 168 h de fermentación (incrementos de 2 y 4 veces los valores iniciales, respectivamente). En la bebida sin GOS, la mayor producción de butírico en el colon ascendente con respecto al colon distal, junto con la disminución del contenido de acético en este reactor sugiere la utilización por parte de la microbiota de éste último para la formación de butírico. Además, la producción en el colon proximal se ve favorecida por valores de pH más ácidos (Walker et al., 2005; Kettle et al., 2015), a pesar de la mayor abundancia de bacterias productoras de butírico (especies pertenecientes a la familia Lachnospiraceae) (Flint et al., 2015) en los tramos distales en ambos ensayos.

El contenido de ácido propiónico en la fermentación de la bebida sin GOS presenta un marcado incremento en el colon ascendente (16 veces los valores iniciales), mientras que en la bebida con GOS no se observa contenidos de propiónico en este reactor. En cambio, en el colon transversal y descendente, se produce un aumento significativo ($p < 0,05$) de las concentraciones de este ácido orgánico (incrementos al final de la fermentación con respecto a los valores iniciales de 1,5-2,0 y 2,5-2,6 veces para la bebida sin y con GOS, respectivamente), de acuerdo con una mayor presencia en estos reactores de bacterias productoras de propiónico (especies perteneciente a la familia Bacteroidetes) (Flint et al., 2015).

En el ensayo en ausencia de GOS, el contenido de ácido láctico se mantiene estable en todos los reactores del colon (0,3-0,8 mM), a excepción de la muestra procedente del colon ascendente a las 24

h de fermentación, que alcanza contenidos de 17 mM. La mayor producción de butírico y propiónico observada en el colon ascendente puede estar relacionada con la utilización de este ácido láctico. En cambio, en presencia de GOS se produce un incremento significativo ($p < 0,05$) de ácido láctico a las 24 h de fermentación en el colon ascendente y los contenidos se mantienen relativamente estables hasta el final de la fermentación (14-16 mM). Esta acumulación de ácido láctico indica una rápida fermentación del sustrato (Morrison & Preston, 2016), de acuerdo con las condiciones de exceso de carbohidratos presentes en este compartimento como resultado de la adición de GOS. En cambio, en el colon transversal y descendente, los contenidos de ácido láctico son bajos y se mantienen estables (0,27 mM), lo que sugiere que el láctico producido en el colon ascendente puede ser utilizado en el colon distal para la producción de butírico y propiónico (que muestran sus incrementos más altos en estos compartimentos).

Finalmente, los ácidos isovalérico y valérico son minoritarios en todos los compartimentos del colon (0,5-3,8 y 1,0-3,6 mM, respectivamente, para la bebida sin GOS y 0,5-2,7 y 0,3-1,3 mM, respectivamente, para la bebida con GOS). Solo se observa formación de ácido fórmico en el colon ascendente, en la muestra correspondiente a 168 h en el ensayo con la bebida sin GOS (1,95 ± 0,24 mM) y durante toda la fermentación en el ensayo en presencia del prebiótico (2,1-4,2 mM).

La producción de ácidos orgánicos en presencia de GOS o EV se ha estudiado previamente mediante estudios *in vitro*, con resultados similares en relación a los incrementos en los contenidos de ácido acético y butírico (Maathuis et al., 2012; Walton et al., 2012; Grimaldi et al., 2017; Liu et al., 2017; Cuevas-Tena et al., 2019a; Li et al., 2019a y b), así como de propiónico y láctico (Maathuis et al., 2012; Cuevas-Tena et al., 2019a; Li et al., 2019b) en presencia de ambos compuestos.

Los resultados correspondientes al ensayo con la bebida con GOS se han publicado en: **Blanco-Morales, V.**, Garcia-Llatas, G., Yebra, M.J., Sentandreu, V., Lagarda, M.J. & Alegría, A. (2020). *Impact of a plant sterol- and galactooligosaccharide-enriched beverage on colonic metabolism and gut microbiota composition using an in vitro dynamic model*. Journal of Agricultural & Food Chemistry, 68, 1884-1895 (ver publicación en Anexo I).

Los resultados correspondientes al ensayo con la bebida sin GOS están en proceso de revisión en: **Blanco-Morales, V.**, Garcia-Llatas, G., Yebra, M.J., Sentandreu, V., & Alegría, A. *In vitro colonic fermentation of a plant sterol-enriched beverage in a dynamic-colonic gastrointestinal digester*. LWT-Food Science & Technology (ver publicación en Anexo II).

Objetivo 5: Determinar el efecto de la presencia de galactooligosacáridos sobre la biodisponibilidad, fermentación colónica y efecto hipocolesterolemiante de los esteroles, en mujeres postmenopáusicas con hipercolesterolemia moderada tras la ingesta regular de bebidas enriquecidas con esteroles vegetales con o sin galactooligosacáridos.

Este objetivo se lleva a cabo mediante la realización de un estudio clínico aleatorizado, doble ciego, cruzado en mujeres postmenopáusicas con hipercolesterolemia moderada (200-239 mg/dL) y edades comprendidas entre 45 y 67 años (estudio número NCT03469518, ClinicalTrials.gov). Para ello, se fabrican específicamente, bajo las mismas condiciones, dos bebidas a base de zumo de frutas y leche enriquecidas con EV (2 g/250 mL) sin o con adición de GOS (4,3 g/250 mL), de composición similar a la descrita en el objetivo 2 (MfB y MfB-G_5, respectivamente). Los sujetos participantes consumen diariamente durante 6 semanas una de las dos bebidas (dosis de 250 mL) y tras un periodo de lavado de 4 semanas, ingieren la bebida alternativa durante otras 6 semanas más. Antes y después de cada período de intervención se toman muestras de suero para evaluar la influencia de los GOS sobre la biodisponibilidad de los esteroles y los marcadores lipídicos sanguíneos, y de heces para determinar su influencia en la metabolización de los esteroles.

- *Efecto de los galactooligosacáridos en la biodisponibilidad de esteroles y el efecto hipocolesterolemiante de los esteroles vegetales*

Las concentraciones séricas de EV son normalizadas con los contenidos de colesterol para cada individuo con el fin de eliminar la variabilidad interindividual de lipoproteínas. Las concentraciones normalizadas de campesterol y β-sitosterol incrementan significativamente ($p < 0,05$) tras la ingesta regular de las bebidas (13,6-23,5% y 35,7-38,8%, respectivamente), considerándose de este modo marcadores de la ingesta dietética de EV. Sin embargo, el estigmasterol (esterol minoritario de la bebida), tan solo incrementa tras la ingesta de la bebida sin GOS. En este estudio, los niveles séricos de EV no se pueden considerar marcadores de la absorción de colesterol ya que la ingesta dietética de los mismos aumenta debido a la intervención (Miettinen et al., 2011). No se observa diferencias significativas en los porcentajes de cambio de los EV entre ambas bebidas (con o sin GOS), lo que sugiere que la presencia del prebiótico no tiene efecto sobre la biodisponibilidad de los EV. Tras la ingesta de las bebidas se producen incrementos de los contenidos séricos de colestanol (6,0-7,2%), desmosterol (8,9-12,5%) y latosterol (10,0-11,1%), considerados marcadores del metabolismo del colesterol, sin diferencias significativas entre bebidas. Estos resultados son similares a los indicados en un estudio clínico previo en el que la ingesta de una bebida similar enriquecida con EV produce aumentos significativos en los contenidos de desmosterol, latosterol, campesterol y β-sitosterol

(Alvarez-Sala et al., 2018). Si bien en el presente estudio no se observa un cambio significativo en el contenido de estigmasterol con respecto al ensayo clínico antes mencionado, los incrementos absolutos (0,03 *vs.* 0,00 µg / mL) no se consideran relevantes debido a su baja concentración en las bebidas y su baja absorción.

Los contenidos de colesterol total sérico disminuyen ($p < 0,05$) tras los períodos de intervención con ambas bebidas, reflejándose en un incremento significativo de los contenidos de desmosterol y latosterol (marcadores de la síntesis de colesterol). Sin embargo, esta disminución no se refleja en los contenidos colestanol (marcador de la absorción de colesterol). De acuerdo con nuestros resultados, tras la ingesta de margarina enriquecida con EV se observa un incremento de los marcadores de síntesis de colesterol en mujeres postmenopáusicas con hipercolesterolemia moderada, aunque también se determina una disminución de los niveles de colestanol (Gylling et al., 2006). Estas diferencias, en parte se puede atribuir a diferentes tiempos de intervención utilizados (6 meses *vs*. 6 semanas) y al distinto tipo y dosis de EV (3 g E-FA *vs*. 2 g de EV libres).

La adición de GOS a las bebidas no modifica el efecto hipocolesterolemiante de las mismas, ya que su consumo regular produce una disminución similar ($p > 0,05$) en los niveles de colesterol total (4,7-5,1%) y c-LDL (7,6-9,0%). Tras el consumo de ambas bebidas no se observa ningún cambio significativo en los niveles de c-HDL. De acuerdo con estos resultados, en un estudio doble ciego, cruzado, placebo-control, llevado a cabo en adultos sanos, no se produce ninguna modificación de los niveles de colesterol total y c-HDL plasmáticos tras la ingesta de GOS a una dosis similar a la utilizada en este trabajo (5,5 *vs*. 4,3 g/día, respectivamente) durante 10 semanas (Vulevic et al., 2008). Sin embargo, cuando se evalúan periodos de tratamiento más largos (6 *vs*. 12 semanas), la ingesta del mismo tipo y dosis de GOS en adultos con síndrome metabólico solo produce una disminución significativa de la ratio colesterol total/c-HDL tras 12 semanas de tratamiento (Vulevic et al., 2013). Por lo tanto, la duración del periodo de tratamiento podría ser un factor condicionante del efecto prebiótico y, en el caso de nuestro estudio (llevado a cabo durante 6 semanas), podría ser una limitación. Sin embargo, la realización de ensayos clínicos con una duración mayor aumenta el riesgo de introducir cambios en el estilo de vida (dieta, actividad física, etc) o el abandono del estudio por parte de los participantes.

Por otra parte, se ha indicado que concentraciones basales de c-LDL mayores se relacionan con mayores reducciones absolutas de c-LDL (Demonty et al., 2009). Esta asociación se observa en los estudios clínicos llevados a cabo previamente con bebidas enriquecida con EV (proporcionando 1,5-2 g EV/día): 129,4±28,5 mg/dL con 5,1% de reducción (Álvarez-Sala et al., 2018) y 146,0±31,8

mg/dL con 7% (Granado-Lorencio et al., 2014), así como en el presente estudio (138,9-142 mg/dL con una reducción de 7,6-9%).

- *Efecto de los galactooligosacáridos en la metabolización colónica de los esteroles*

El contenido total de esteroles animales en heces tras la ingesta de las bebidas oscila entre 13,29-27,91 mg/g heces liofilizadas. Estos contenidos son similares a los observados en un estudio clínico previo de nuestro grupo de investigación tras la ingesta de una bebida similar enriquecida con EV (13,9-30,10 mg/g heces liofilizadas) (Cuevas-Tena et al., 2019b). Sin embargo, no se observan diferencias estadísticamente significativas ($p > 0,05$) en el contenido total de esteroles animales fecales tras la ingesta de ambas bebidas con respecto a los contenidos previos al tratamiento, ni tampoco entre los cambios absolutos (diferencia entre los contenidos tras el tratamiento y los contenidos basales) de ambas bebidas.

Tras la ingesta de las bebidas sin y con GOS, se observan incrementos significativos ($p < 0,05$) en la excreción de colesterol (65% y 71%), coprostanona (58% y 87%) y colestanol+metilcoprostanol (54% y 76%), con respecto a los niveles basales. Además, se obtiene una disminución significativa del contenido de coprostanol (24% y 14%), mientras que los contenidos de latosterol no sufren ninguna modificación. Aunque en presencia de GOS los cambios mencionados son más pronunciados, no existen diferencias en los cambios absolutos de los esteroles animales individuales entre ambas bebidas.

La mayor excreción de colesterol observada en heces ya se indica en un estudio previo de nuestro grupo de investigación tras la ingesta de una cantidad similar de EV (2 g/día) (Cuevas-Tena et al., 2019b) y puede justificarse debido a la conocida interacción de los EV en la absorción de colesterol. Los resultados confirman que, en presencia de altas concentraciones de EV, el metabolismo del colesterol por parte de la microbiota se lleva a cabo a través de una ruta indirecta que resulta en una mayor excreción de coprostanona y una menor excreción de coprostanol o bien una ruta alternativa en la que se forma colestanol mediante la reducción de colestenona y colestanona (Cuevas-Tena et al., 2018). De la misma manera, en sujetos normolipídicos, la ingesta de margarina enriquecida con EV (8,6 g/día) durante 28 días reduce la conversión de colesterol a coprostanol (Weststrate et al., 1999).

A diferencia de los esteroles de origen animal, se observa un incremento significativo ($p < 0,05$) de los EV totales con respecto a los valores basales (23,21-55,94 *vs*. 4,96-7,58 mg/g heces liofilizadas) tras la ingesta de ambas bebidas, hecho que refleja la adherencia de los participantes al

estudio. Sin embargo, no se observa influencia de los GOS en los cambios absolutos. Este hecho también ocurre en los esteroles individuales, en los que se observa un incremento en la excreción de etilcoprostanol y metilcoprostanona.

La ausencia de efecto de los GOS sobre la metabolización de los esteroles también se detecta al representar la media del cambio absoluto de los esteroles tras el consumo de las bebidas con o sin GOS. En ambos casos, la respuesta de los sujetos es similar y se observan pocos valores atípicos, a pesar de no controlar el aporte de colesterol y EV de la dieta, lo que constituye una limitación del estudio. Otra limitación del presente trabajo es la falta de análisis de la composición de la microbiota, que podría aportar información valiosa acerca de la ausencia de efecto de los GOS sobre el metabolismo de los esteroles. En este sentido, ciertos autores han indicado la necesidad de una dosis mínima de 5 g de GOS/día (ligeramente inferior a la usada en el presente trabajo) para alcanzar incrementos significativos en el recuento de bifidobacterias (Tannock et al., 2004; Davis et al., 2010 y 2011). Los distintos ingredientes de GOS, la forma de incorporación, el diseño experimental de los estudios, así como los métodos de análisis de la composición de la microbiota utilizados son factores que pueden influir en las variaciones observadas en el efecto bifidogénico esperado (Davis et al., 2010). Además, mientras que los GOS son altamente específicos promoviendo el crecimiento de bifidobacterias, no presentan un efecto consistente en la promoción o disminución de otras especies bacterianas (Davis et al., 2011). Por lo tanto, la ausencia de efecto de los GOS sobre la metabolización de los esteroles puede deberse a la falta de modulación por parte del prebiótico de las bacterias coprostanoligénicas responsables de la metabolización del colesterol a coprostanol (*Bifidobacterium*, *Bacteroides* o *Eubacterium spp.*), así como de aquellas especies asociadas con las rutas de biotransformación de los EV (*Eubacterium spp.*) (Cuevas-Tena et al., 2018b).

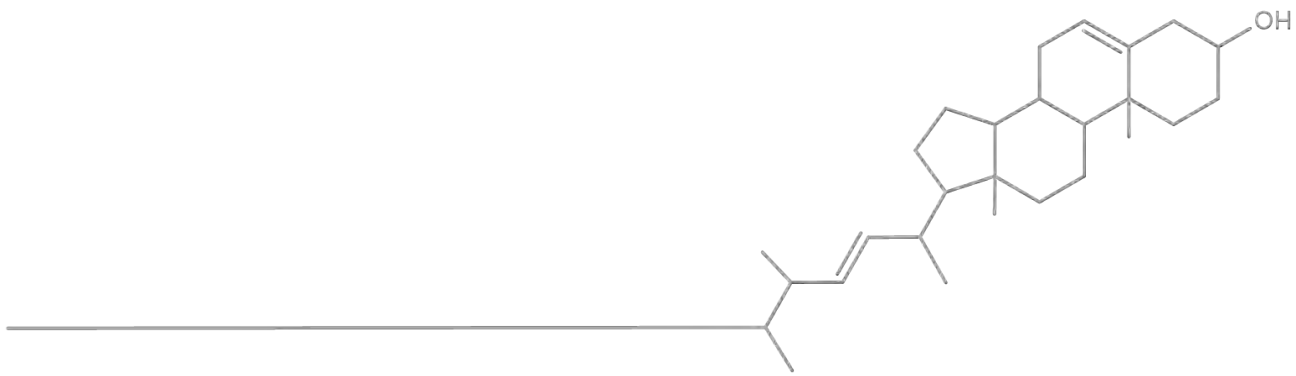
Con el objetivo de determinar el efecto de la ingesta de la bebida enriquecida en GOS sobre la biontransformación de los esteroles se calculan los porcentajes de conversión del colesterol ((coprostanol + coprostanona) x 100/(colesterol + coprostanol + coprostanona)) y los EV individuales β-sitosterol (etilcoprostanol x 100/(β-sitosterol + sitostanol + etilcoprostanol)), estigmasterol (etilcoprostenol x 100/(estigmasterol + estilcoprostenol)) y campesterol (metilcoprostanona x 100/(campesterol + campestanol + metilcoprostanona)), y se clasifica a los sujetos como bajos y altos convertidores de acuerdo a Wilkin & Hackman (1974) (porcentajes de conversión <50% o >50%, respectivamente). En general, los sujetos son predominantemente altos convertidores de colesterol (n = 39 o 40, según la bebida) y β-sitosterol (n = 26), y bajos convertidores de estigmasterol y campesterol, independientemente de la bebida ingerida. Solo en cinco mujeres se produce una reducción simultánea de la conversión de todos estos esteroles. Estos resultados coinciden

parcialmente con los resultados del estudio clínico previo mencionado anteriormente (Cuevas-Tena et al., 2019b), en el cual se determina un menor número de altos convertidores de colesterol (29) y β-sitosterol (17) y un mayor número de altos convertidores de estigmasterol (27 *vs*. 13).

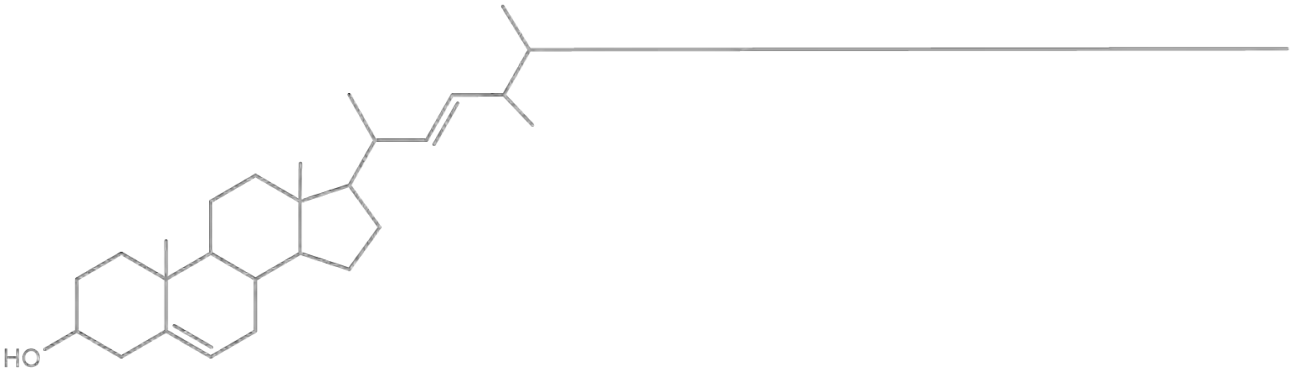
La ingesta de la bebida enriquecida con GOS induce ligeros cambios en la capacidad metabólica colónica de las mujeres. Se observa una disminución significativa (≥10%) en el porcentaje de conversión de colesterol y estigmasterol en 12 mujeres y de β-sitosterol en 16, en comparación con la bebida sin adición de GOS. La disminución del porcentaje de conversión del colesterol tras la ingesta de una bebida similar enriquecida con EV se ha asociado con la mayor ingesta de EV, que pueden reducir o bloquear la producción de metabolitos de colesterol (Cuevas-Tena et al., 2019b). La reducción de la conversión de colesterol es de interés ya que los metabolitos de colesterol se asocian con una actividad procarcinógena y con un incremento del riesgo de cáncer de colon (Cuevas-Tena et al., 2018a). A su vez, la diferente respuesta a la presencia de GOS sobre el metabolismo colónico de los esteroles puede deberse a las distintas propiedades filogenéticas de la microbiota de cada individuo. La composición de la microbiota puede estratificarse en tres grupos principales (enterotipos), que presentan diferencias funcionales con respecto al modo de obtención de energía de los sustratos disponibles en el colon. En este sentido, los sujetos pertenecientes al enterotipo 1 o *Bacteroides* obtienen energía principalmente de la fermentación de carbohidratos y proteínas, mientras que los enterotipos 2 y 3 (*Prevotella* y *Ruminococcus*, respectivamente) presentan una mayor eficiencia en la degradación de mucinas (Arumugam et al., 2011).

La alta eficiencia de conversión del colesterol a coprostanol se relaciona con una mejora del perfil lipídico sérico, ya que por un lado se evita el paso de colesterol desde el intestino a la sangre y, por otro, el coprostanol presenta una baja absorción intestinal (Beitz et al., 1999). En este sentido, se sugiere que la producción de coprostanol podría modular los niveles sanguíneos de colesterol al observarse una correlación inversa entre los niveles de colesterol séricos y la ratio coprostanol/colesterol fecales (Sekimoto et al., 1983). Sin embargo, en el presente estudio no se observa esta correlación para ninguna de las bebidas, ni tampoco entre los niveles de colesterol sérico y los porcentajes de conversión de colesterol en heces.

Los resultados correspondientes al objetivo 4 están en proceso de revisión en: **Blanco-Morales, V.**, Silvestre, R.A., Hernández-Álvarez, E., Donoso-Navarro, E., Alegría, A., Garcia-Llatas, A. *Influence of GOS on the positive effect of plant sterol-enriched beverages on cardiovascular risk and their colon metabolism: A randomized, double-blind crossover trial*. Clinical Nutrition (ver publicación en Anexo II).



Conclusiones



Del estudio sobre la formación de óxidos de esteroles en una bebida enriquecida con esteroles vegetales (2g/250mL) a lo largo de su vida útil se deduce que:

1ª. Se identifican los óxidos 7α- y 7β-hidroxi, α- y β-epoxi, triol y 7-ceto derivados del colesterol y β-sitosterol. En general, su contenido total y el porcentaje relativo no se modifican durante el almacenamiento. La adición de grasa láctea y concentrado de proteínas del suero enriquecido en membrana del glóbulo graso de la leche a las bebidas no aumenta la oxidación de los esteroles, siendo el porcentaje de oxidación del β-sitosterol menor que el del colesterol (0,03% *vs*. 1,2%).

2ª. La ingesta estimada de los productos de oxidación de β-sitosterol por ración de bebida es mayor que la de los óxidos de colesterol (0,5 *vs*. 0,25 mg/día). Estos valores, muy inferiores a los asociados con efectos citotóxicos, demuestran que la ingesta de la bebida es segura para el consumidor a lo largo de su vida útil.

3ª. La bioaccesibilidad de los productos de oxidación totales de los esteroles no se modifica durante el almacenamiento, siendo mayor para los procedentes del colesterol (58-80%) que para los del β-sitosterol (45-49%).

De los estudios *in vitro* estáticos para la estimación de la bioaccesibilidad de los esteroles se concluye que:

4ª. La bioaccesibilidad del colesterol (80-85%) y los esteroles vegetales (~37%), evaluada mediante una digestión gastrointestinal simular micelar, no se modifica por la adición de distintas dosis de galactooligosacáridos (2,3 y 4,5 g/250 mL) a bebidas enriquecidas con esteroles vegetales (2,5 g/250 mL), probablemente debido a la alta relación esteroles/galactooligosacáridos (0,6-1). Estos resultados demuestran la idoneidad del enriquecimiento conjunto de estos compuestos bioactivos.

5ª. La adaptación del método Infogest requiere la reducción de la concentración de sales biliares de origen porcino (1,4 mM) o utilizar sales biliares de origen bovino a la concentración propuesta inicialmente (10 mM). La bioaccesibilidad del colesterol (38-62%) y de los esteroles vegetales totales (14-22%) disminuye con estas modificaciones con respecto al método micelar, si bien menor concentración de sales biliares mejora dicha bioaccesibilidad.

Del estudio relativo a la digestión gastrointestinal y fermentación colónica de las bebidas con y sin galactooligosacáridos utilizando un sistema *in vitro* multicompartimental dinámico se extraen las siguientes conclusiones:

6ª. La composición de la microbiota intestinal presenta un comportamiento dependiente del tramo colónico, mostrando una mayor riqueza y diversidad de especies microbianas en los reactores del colon transversal y descendente. Independientemente de la bebida, los reactores distales muestran una prevalencia del género *Bacteroides*, que tiende a disminuir durante la fermentación. Sin embargo, existen diferencias en los géneros identificados en el colon ascendente con una mayor abundancia de *Klebsiella* (con tendencia a disminuir) y *Megasphaera* (con tendencia a aumentar) en la bebida sin galactooligosacáridos y de *Clostridium* en la bebida con galactooligosacáridos. La presencia de galactooligosacáridos en este ensayo no da lugar a un efecto bifidogénico.

7ª. Se observa formación de coprostanol, a partir del colesterol, y etilcoprostanol, sitostenona, metilcoprostanol y etilcoprostenol, a partir de esteroles vegetales, en los reactores del colon distal únicamente durante la fermentación de la bebida conteniendo galactooligosacáridos. Las ratios de biotransformación del colesterol son mayores que las de los esteroles vegetales tanto en el reactor del colon transversal (0,2-0,7 *vs*. 0,01-0,6) como en el descendente (0,9-1,3 *vs*. 0,2-0,8). Además, la ausencia de metabolitos intermediarios indica una rápida metabolización de los esteroles por la microbiota, predominando, en el caso del colesterol, la ruta directa a coprostanol.

8ª. La fermentación de ambas bebidas produce un incremento en la producción de ácidos orgánicos totales en todos los reactores del colon, viéndose favorecida en presencia de galactooligosacáridos. En general, los ácidos isobutírico (en el colon ascendente) y acético y butírico (en todos los reactores del colon) son los mayoritarios. En presencia de galactooligosacáridos, tiene lugar una mayor producción de ácido láctico en el colon ascendente indicativa de una rápida fermentación del sustrato.

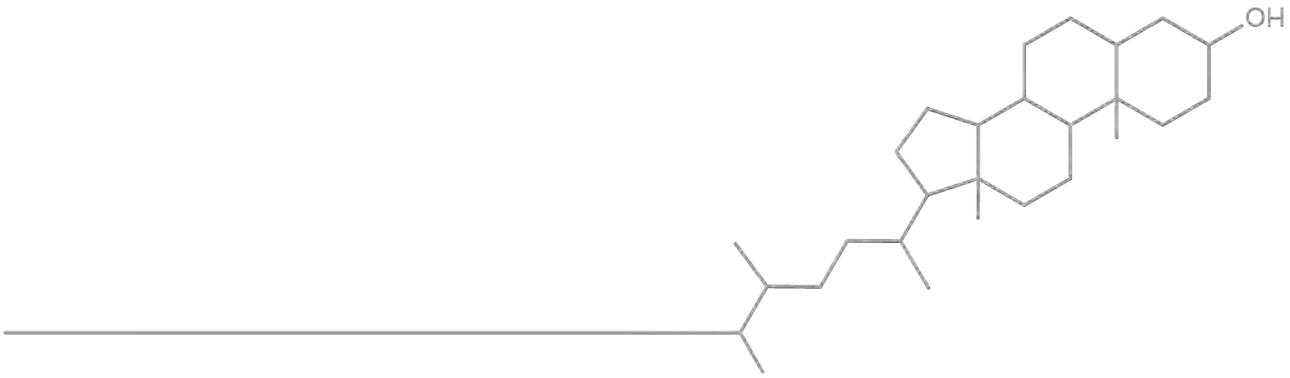
Del estudio clínico en mujeres postmenopáusicas que ingieren regularmente bebidas enriquecidas con esteroles vegetales (2 g/250 mL) con y sin galactooligosacáridos (4,3 g/250 mL) se concluye que:

9ª. La presencia de galactooligosacáridos no modifica el efecto hipocolesterolemiante de las bebidas. En ambos casos, se observan disminuciones de la concentración sérica de colesterol total (~5%) y de colesterol-LDL (7,6-9%), sin modificación de los niveles de colesterol-HDL. El efecto hipocolesterolemiante se refleja en un incremento de los marcadores séricos de síntesis del colesterol (desmosterol y latosterol), pero no en los marcadores de absorción (colestanol).

10ª. La biodisponibilidad de los esteroles no se modifica por la presencia de galactooligosacáridos, observándose en ambas bebidas incrementos similares en las concentraciones séricas de campesterol (14-24%) y β-sitosterol (36-39%).

11ª. Se confirma que la ingesta de esteroles vegetales a partir de alimentos enriquecidos modula la excreción fecal de esteroles y metabolitos. Se produce una mayor excreción de colesterol y disminuye la de su principal metabolito, el coprostanol, mientras que la metabolización a coprostanona y colestanol, a través de rutas indirectas, se ve favorecida. Se incrementa la excreción de los esteroles vegetales, así como la de sus metabolitos etilcoprostanol y metilcoprostanona.

12ª. La adición de galactooligosacáridos a la bebida enriquecida con esteroles vegetales no modifica la metabolización colónica de los esteroles, aunque en determinados sujetos induce disminuciones en el porcentaje de conversión de colesterol. Probablemente la dosis ingerida no afecta a las bacterias coprostanoligénicas responsables de la biotransformación del colesterol.



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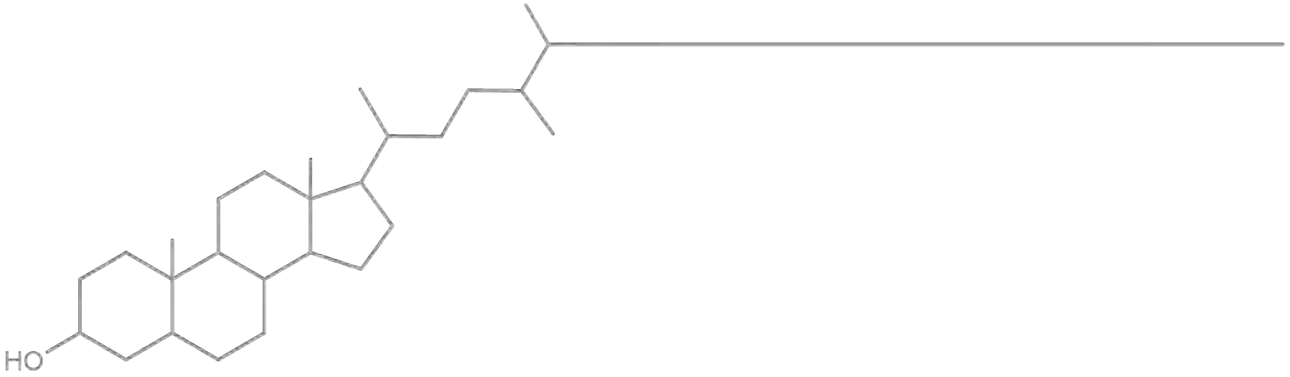

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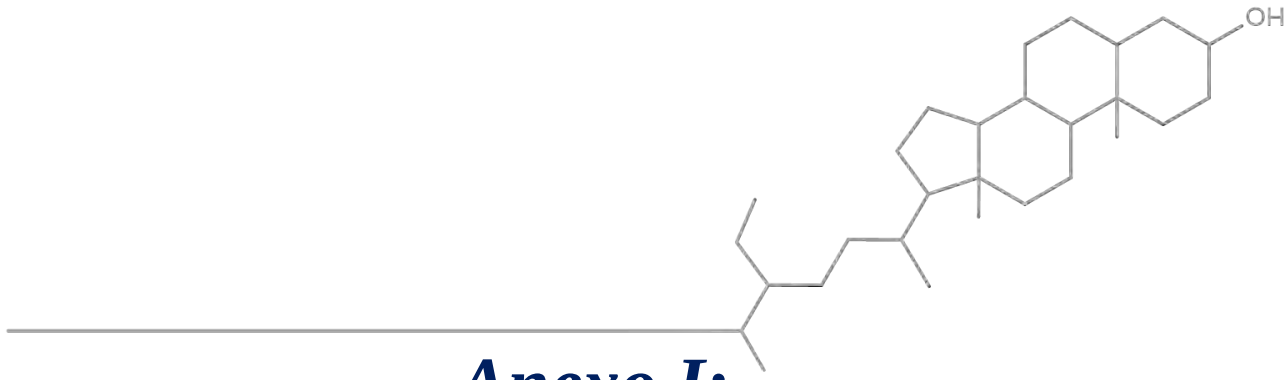
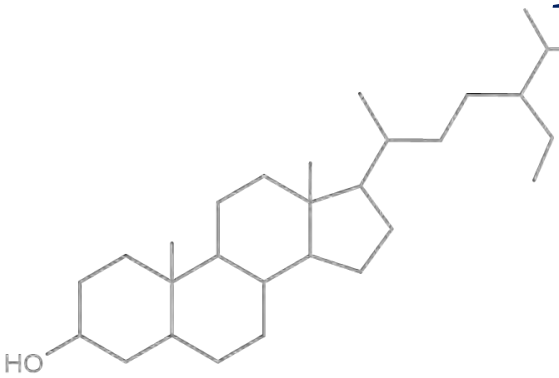


Anexo I: Publicaciones



Chapter 11
Sterol Digestion in Plant Sterol-Enriched Foods: Bioaccessibility and Fermentation

AUT

Virginia Blanco-Morales, Guadalupe Garcia-Llatas, and Antonio Cilla

11.1 Introduction

Sterols comprise the major portion of the unsaponifiable fraction of most edible fats and oils being minor components of human dietary lipids. In general, sterols derive from squalene consisting of a tetracyclic cyclopenta[α]-phenanthrene structure with a flexible side chain with 8–10 carbons at C-17 and a hydroxyl group at C-3. They can be classified according to their origin as animal sterols or plant sterols (PS). Cholesterol is the predominant sterol in foods from animal origin followed by desmosterol, whereas plant membranes contain several types of PS. More than 200 different types of PS have been reported in plant species, where the most common are β-sitosterol, campesterol and stigmasterol, called phytosterols, that are structurally similar to cholesterol, but include a methyl or ethyl group at C-24. Structures of cholesterol and common phytosterols are shown in Fig. 11.1. In turn, saturated PS, referred as phytostanols, have no double bond in the ring structure [27, 48].

The addition of PS to food matrices is an increasingly common practice in the food industry, with the aim to prevent cardiovascular disease through their cholesterol-lowering activity at dosages of 1.5–3 g PS/day [27]. Moreover, a great deal of scientific evidence supports their additional beneficial effects on human health and, consequently, their use for the formulation of functional foods is increasing. On this basis, it is important to consider that the beneficial effects attributed to these bioactive compounds are not only related to the intake levels but also to their bioavailability. In this sense, *in vitro* methods to simulate gastrointestinal digestion allow determining sterol (cholesterol and PS) bioaccessibility and, thus recognize factors that can affect their solubility with the aim to optimize their beneficial effects improving the design of functional foods [14]. Moreover, *in vitro* gastrointestinal

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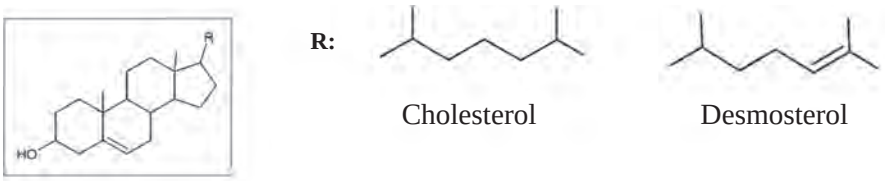
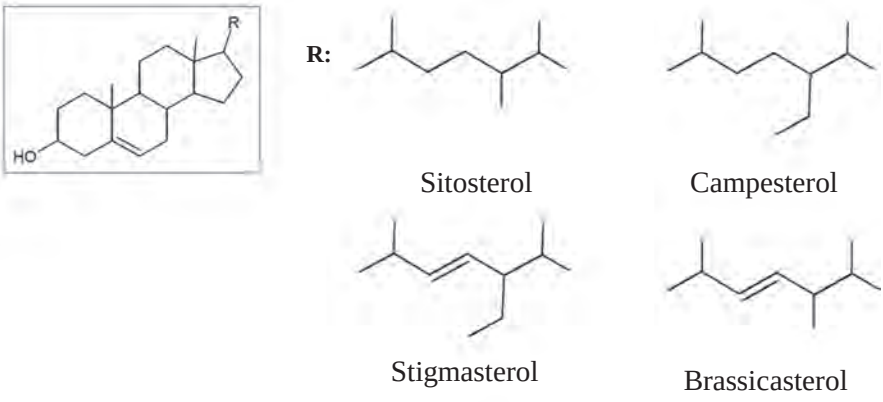
M. M.-L Grundy, P. J. Wilde (eds.), *Bioaccessibility and Digestibility of Lipids from Food*, https://doi.org/10.1007/978-3-030-56909-9_11

Animal sterols



Plant sterols

- *Phytosterols*



- *Phytostanols*

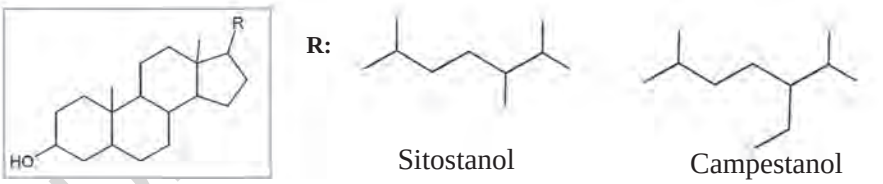


Fig. 11.1 Structure of main animal and plant sterols

digestion methods are useful tools to evaluate the solubility of sterol oxidation products (oxysterols), potentially harmful substances that can be formed during the manufacture or storage process of PS-enriched products, in order to elucidate factors that are implicated in the formation of oxysterols and minimize them [8]. In addition, sterols are also susceptible to the fermentation by gut microbiota, that lead to the formation of metabolites and, in turn, to modifications of the microbiota population. However, studies that evaluate sterols fermentation from PS-enriched products are still scarce [17].

The following chapter reviews the information available about sterol and oxysterol bioaccessibility in PS-enriched foods and the factors that could modify their solubility (contents and source of PS, food matrix composition, or digestion methodology carried out). Furthermore, current information on sterol fermentation has also been addressed, describing sterol metabolites, the microbiota species implicated in their formation and the changes in microbiota composition that can be generated by high PS concentrations (derived from PS-enriched formats).

11.2 General Concepts: Bioaccessibility and Bioavailability

In the field of nutrition research, the ambiguous application of the concepts bioavailability and bioaccessibility is common. Although used indistinctly and subject to alternative definitions, they are not synonymous terms. Thus, it is important to ascertain and be explicit about which definition is being considered [53].

On the one hand, bioavailability is defined as the fraction of an ingested component available for use in normal physiological functions and is determined by *in vivo* assays [34]. Bioavailability encompasses three main steps: (i) digestibility and solubility of the element in the gastrointestinal tract, (ii) absorption of the element by the intestinal cells and transport into the circulation, and (iii) incorporation from the circulation to the functional entity [53, 63]. In addition, in the literature, there are numerous examples of researchers who only measure the uptake or absorption with cell cultures (i.e. Caco-2 cells) in *in vitro* assays, yet refer to their analysis as bioavailability [24].

On the other hand, bioaccessibility has been given two alternative definitions, as reviewed by Cardoso et al. [13]: (a) the fraction of a compound that is released from its food matrix in the gastrointestinal tract and thus becomes available for intestinal absorption (typically based on *in vitro* procedures). Hence, this definition does not include absorption across the intestinal wall or any metabolic processing and depends only on digestion and release from the food matrix [24]; or a more stringent and much less widely used definition: (b) the fraction of a compound that is released from its food matrix in the gastrointestinal tract and thus becomes available for intestinal absorption (i.e. it enters the bloodstream), including the entire sequence of events that takes place during the digestive transformation of food into material that can be assimilated by the body, absorption/assimilation into the cells of the intestinal epithelium, and, finally, pre-systemic metabolism (both intestinal and hepatic) [26]. Thus, from this last standpoint, bioavailability includes bioaccessibility.

Bioaccessibility, as a first step and prerequisite of bioavailability, is an important concept when nutritional guidance is planned or for tailor-made food products to meet explicit requirements (i.e. high bioaccessibility and bioavailability of plant sterols and stanols to decrease plasmatic total cholesterol and low-density lipoprotein cholesterol levels in mildly hypercholesterolemic subjects). In this sense, people wishing to decrease their energy intake would be aligned with foods with a reduced macronutrient digestion and absorption. On the contrary, for individuals

who suffer from malnutrition or present higher energy requirements, nutrient-rich foods are recommended for their high bioaccessibility, subsequent bioavailability and potential biological effects. For this reason, fully understanding how the food matrix acts within the gastrointestinal tract during digestion and how this affects nutrient bioaccessibility is critical [33]. The determination of the bioaccessibility of sterols enables the comparison of individual sterols in the same and different samples and the identification of which sterols are more available in the intestinal tract for later absorption [35].

Based on these considerations, authors would like to make clear that for this book chapter review, the term bioaccessibility will only be used for assays accounting for *in vitro* digestion (excluding absorption and pre-systemic metabolism), and bioavailability considering both, the *in vitro* (uptake and transport in cell cultures) and *in vivo* (animal and human) perspectives. Therefore, it is convenient to stick to the terminology mainly used by many of the authors referenced.

11.3 Fate of Sterols and Oxysterols During Digestion

Dietary sterols from animal or plant origin, and oxidized or not, are minor components present in fat of the human diet which belong to the unsaponifiable fraction [56]. They are assumed to follow the fate of lipids in the gastrointestinal tract, consisting of several sequential steps, including physicochemical and enzymatic events, such as: their release by breakdown from the food matrix, then their dispersion/emulsification into lipid droplets, followed by their enzymatic hydrolysis by specific lipases at the emulsion-water interface, ensued by their solubilization into intestinal mixed micelles formed by bile salts and phospholipids, then their diffusion across the unstirred water layer, and finally their permeation/absorption by passive diffusion or active transport involving specific proteins in the enterocyte brush border membrane [1, 11, 44, 57]. In the particular research field of lipophilic molecules, the bioaccessibility endpoint is considered after their incorporation into an absorbable form (intestinal mixed micelles), which delivers digested lipids to the aqueous-enterocyte membrane for subsequent absorption [44]. Bioavailability, in turn, can be ascribed to their absorption into the enterocytes and/or their location in the vascular channels of lymph or blood [53].

Regarding the specific fate of sterols in the gastrointestinal tract, first they are released from the food matrix, a fact that mainly depends on two key issues such as type/form of sterol (which affect their hydrophobicity and extraction efficiency) and type of food matrix [31]. Next, in the intestinal lumen, esterified sterols locate into the core of emulsified fat droplets whereas free sterols partition between fat droplets and micelles. Then, in the duodenum, sterol esters are mainly hydrolyzed to free sterols by pancreatic cholesterol esterase, although the implication of pancreatic lipase could not be ruled out [50], and subsequently transferred to mixed micelles (transfer efficiency dependent on sterols form and degree of enzymatic hydrolysis), which provide a vehicle for absorption into enterocytes [31]. The intestinal

absorption of sterols is mediated by the Niemann-Pick C1-like 1 (NPC1L1) protein from the brush border membrane into the intestinal mucosa [65] although a slight portion is absorbed by passive diffusion [59]. This enzyme would exhibit a different response to sterols and stanols of different shape and size (i.e. cholesterol uptake being 4 times higher than that of sitosterol since cholesterol has a 7% lower molecular weight) [64]. Once within the enterocyte, sterols are re-esterified by Acyl CoA cholesterol Acyl Transferase (ACAT) before their incorporation in chylomicrons for transport through the lymph due to the action of the microsomal triglyceride transfer protein (MTP). Nevertheless, due to the low affinity of ACAT for plant sterols, only a small part of them is esterified, which explains their low absorption (<5%) compared to cholesterol (40%). In addition, two ATP-binding cassette proteins (ABCG5/ABCG8) have been shown to reduce absorption of plant sterols by pumping them back into the intestinal lumen [36, 56]. The main route for sterols excretion is the bile pathway; however, a small fraction of plant sterols can be excreted through the skin [59], and more recently, it has been described that cholesterol can also be excreted from the bloodstream to the intestinal lumen via a transintestinal efflux pathway (TICE) [20]. Subsequently, all sterols effluxed from enterocytes to the lumen reach the large intestine, where they are subjected to microbial transformation with the successive kinetic reaction steps: sterol ➔ stenone ➔ stanone ➔ stanol. Then, this "chemically-inert" stanol product would ultimately be excreted in feces [17, 64]. This topic is be expanded below (see Sect. 11.6).

All together the complex gastrointestinal process of lipids, and specifically of dietary sterols, is influenced by an array of factors which can promote or hamper their bioaccessibility and bioavailability as previously reported [11, 31, 64]. Such factors mainly include: (i) molecular size and type of the sterols (i.e. sterol/stanol, oxidized or not), (ii) the food matrix in which the sterol is present or incorporated (i.e. presence and nature of dietary lipids, dietary fiber), (iii) competitive interaction with other compounds (i.e phytostanols apparently inhibit phytosterols absorption), and (iv) genetic factors (i.e. phytosterolemia, where mutations in ABCG5/ABCG8 proteins cause an increase in the net absorption of phytosterols by decreasing their excretion back to the intestinal lumen). Some of these factors are related in the following sections.

11.4 Bioaccessibility of Sterols

The effectiveness of PS-enriched foods is closely related to their bioavailability, which depends on the food matrix and the composition of the ingredients used as PS source. *In vitro* gastrointestinal digestion methods can be used as predictive models to evaluate PS bioaccessibility in order to improve the design of functional foods and to evaluate the factors that enhance the incorporation of PS into mixed micelles, and thus leading to a greater displacement of cholesterol from them [2, 28].

Although they are scarce, different studies have evaluated sterol bioaccessibility in PS-enriched products such as commercially available fermented milk beverages [61],

milk beverages [3], fruit beverages with or without milk [3, 4, 9], dark chocolates [58], granola bars and puddings [60], and fermented maize yogurt-like products [30]. The studies on beverages were carried out by our research group and the results of PS bioaccessibility obtained are summarized in Table 11.1. In that research, the same methodology for *in vitro* gastrointestinal digestion was used and, thus, allows the comparison of results, making the evaluation of food matrix effects possible. This gastrointestinal digestion, named micellar digestion, includes three-steps (salivary, gastric and intestinal digestion), and was specifically developed for lipophilic compounds by the addition of key lipid metabolism enzymes during the intestinal phase (pancreatic lipase, colipase, cholesterol esterase and phospholipase A2) and sodium taurocholate to improve generation of a micellar medium in which sterols are incorporated [32].

In general, a larger and higher bioaccessibility range for total PS is observed in milk-based beverages with higher contents of PS (8.9–16.8% containing 1.5–2.9 g PS/100 g fermented milk) [61] versus lower contents (2.6% with 0.8 g PS/100 mL milk-based beverage) [3] (Table 11.1). In addition, to the different level of PS enrichment, it should be pointed out that the alteration of milk fat globule membranes (MFGM) by acid or microbial action in fermented products may result in different adsorption of sterols compared to native membranes, thus becoming an effective delivery system for PS [7]. However, other components of the food matrix may reduce PS solubility, since lower bioaccessibility is observed in presence of higher carbohydrates and fibre contents (8.9 versus 16.6%) in beverages with similar PS enrichment (1.5–1.6 g/100 g) [61].

Other factors such as the source of the PS ingredient can also influence ~~the~~ bioaccessibility. Determination of PS contents in beverages shows that samples with 1.6 and 2.0 g PS/100 g beverage report the same or similar source of PS (β-sitosterol (80%) > sitostanol (12%) > campesterol (5–7%)). After the application of the simulated digestion, similar solubility of individual sterols (campesterol > β-sitosterol > sitostanol) and of total PS was obtained (~17%). In turn, beverages with a similar or higher enrichment in PS (1.5 or 2.9 g PS/100 g) but with a different profile of the PS ingredient (β-sitosterol (45%) > stigmasterol (33%) > campesterol (22%), or sitostanol (85%) > campestanol (11%) > β-sitosterol (4%)) show changes in individual PS solubility (campesterol > β-sitosterol > stigmasterol, or campestanol > sitostanol > β-sitosterol) and a lower total PS bioaccessibility (9 and 11%, respectively) [61] (see Table 11.1). In fact, when comparing the fruit beverage (containing banana puree, grape juice from concentrate and mandarin fruit juice from concentrate rich in β-cryptoxanthin) with the skimmed milk-based beverage, both enriched with the same content and source of PS (0.8 g/100 mL from tall oil), similar bioaccessibility of total (approximately 3%) and individual PS (campesterol > campestanol > β-sitosterol ≈ sitostanol ≈ stigmasterol) was observed [3].

Currently, incorporating PS into low-fat foods such as milk-based fruit beverages, which can be enriched with PS [15], is a convenient way to obtain the daily recommended amount of PS in subjects with moderate hypercholesterolemia given the presence of antioxidant compounds, such as vitamin C, polyphenols, and

Table 11.1 Sterol bioaccessibility (%) in plant sterol-enriched beverages

Fermented milk beverages[a]					Skimmed milk-based beverage[b]	Fruit beverage*[b]
PS content	**2.9**	**2.0**	**1.6**	**1.5**	**0.8**	**0.8**
Campesterol	–	18.94 ± 1.48	19.46 ± 0.38	9.01 ± 0.44	3.75 ± 0.15	4.88 ± 0.16
Campestanol	12.54 ± 0.59	–	–	–	3.47 ± 0.35	4.27 ± 0.35
Stigmasterol	–	–	–	8.74 ± 0.52	2.95 ± 0.44	3.04 ± 0.17
β-Sitosterol	6.31 ± 0.30	17.17 ± 1.49	16.88 ± 0.64	8.97 ± 0.48	2.51 ± 0.07	2.93 ± 0.10
Sitostanol	11.35 ± 0.96	13.57 ± 1.24	13.42 ± 1.18	–	2.71 ± 0.12	3.22 ± 0.16
Cholesterol		–	–	–	79.05 ± 1.09	–
Total PS	11.28 ± 0.85	16.84 ± 1.35	16.64 ± 0.64	8.90 ± 0.40	2.62 ± 0.08	3.10 ± 0.11

PS-enriched milk-based fruit beverages								
PS content	**0.8**[b]	**0.8***[b]	**0.8***[c]	**+ Olive oil and soy lecithin***[c]	**+ Milk fat and whey protein concentrate enriched with MFGM***			
					0.8[c]	**1**[d]	**1****[d]	**1*****[d]
Campesterol	8.31 ± 0.65	6.66 ± 0.27	9.03 ± 0.59	26.37 ± 3.94	33.86 ± 3.83	39.55 ± 1.36	39.75 ± 0.70	38.90 ± 0.63
Campestanol	6.82 ± 0.57	5.79 ± 0.55	27.75 ± 2.76	31.41 ± 4.74	36.92 ± 3.84	43.68 ± 0.78	43.16 ± 0.83	41.53 ± 0.97
Stigmasterol	5.96 ± 0.60	4.02 ± 0.11	5.72 ± 0.60	19.37 ± 0.98	32.64 ± 3.28	33.55 ± 1.55	33.52 ± 1.40	33.96 ± 0.66
β-Sitosterol	6.42 ± 0.50	4.15 ± 0.04	8.45 ± 0.81	28.14 ± 3.53	30.72 ± 2.61	36.68 ± 1.60	37.19 ± 0.65	36.80 ± 0.62
Sitostanol	6.05 ± 0.71	4.41 ± 0.10	9.79 ± 1.02	29.94 ± 3.45	34.37 ± 2.90	39.28 ± 1.37	39.74 ± 0.64	38.22 ± 0.68
Cholesterol	53.34 ± 4.85	98.96 ± 3.3	57.60 ± 3.37	53.50 ± 4.23	64.18 ± 6.64	80.61 ± 3.44	78.45 ± 0.39	84.87 ± 0.88
Total PS	6.48 ± 0.52	4.35 ± 0.06	8.73 ± 0.81	28.19 ± 3.58	31.39 ± 2.70	37.20 ± 1.54	37.67 ± 0.65	37.12 ± 0.63

Bioaccessibility (%) calculated as (sterol content in bioaccessible fraction × 100)/(sterol content in beverage). Content of PS expressed per 100 mL of beverage except for [61] (g/100 g)

GOS galactooligosaccharides, *MFGM* milk fat globule membrane, *PS* plant sterols

[a]Vaghini et al. [61]

[b]Alemany et al. [3]

[c]Alvarez-Sala et al. [4]

[d]Blanco-Morales et al. [9]

*With mandarin juice from concentrate as source of β-cryptoxanthin; **With 1 g GOS/100 mL; ***With 2.5 g GOS/100 mL

carotenoids. In this context, our research group has evaluated PS bioaccessibility in milk-based fruit beverages with different matrix modifications [3, 4, 9] using the same PS-ingredient from tall oil (Table 11.1). As it can be observed, beverages containing milk and fruit juice showed an increase in total PS bioaccessibility (4.4–8.7%) with respect to those containing only fruit juice (2.6%) at the same level of PS-enrichment (0.8 g/100 mL). The presence of milk fat, that has been demonstrated to be an effective delivery system for lipophilic microconstituents [7], could favor the extraction and stability of PS in the food matrix, improving their dispersion in the aqueous phase of the digestion. Moreover, the incorporation of mandarin fruit juice source of β-cryptoxanthin to milk-based fruit beverages slightly reduces total PS bioaccessibility (6.5 versus 4.4%), probably due to a competition between PS and β-cryptoxanthin for its incorporation into mixed micelles [3]. Regarding solubility of individual PS in this kind of beverages (with or without mandarin juice), a larger value for campesterol/campestanol with respect to β-sitosterol/sitostanol is observed. Authors suggest that the shorter side-chain group at position 24 of campesterol/campestanol (methyl) (versus sitosterol/sitostanol (ethyl)) and the lower affinity of the first for the bile salt micelles favours their aqueous solubility. It is remarkable that in beverages containing mandarin juice, Alvarez-Sala et al. [4] reported a twofold higher total PS bioaccessibility than the one observed by Alemany et al. [3]. The larger contribution of campesterol to the bioaccessibility (27.8% versus 5.8%) explains the highest total PS bioaccessibility (Table 11.1).

One step forward in our research line was the addition of fat and emulsifiers to the PS-enriched milk-based fruit beverages (containing mandarin juice from concentrate), such as olive oil and soy lecithin [4] or milk fat and whey protein concentrate enriched with MFGM [4, 9] in order to assess the influence on PS bioaccessibility (see Table 11.1). At doses of PS enrichment of 0.8 g/100 mL, the increase in fat content (2.4% versus 1.1%) from vegetable or animal source, leads to a 6.5- or 7.2-fold increase in PS bioaccessibility, respectively [4]. Thus, the addition of fat is likely to promote the formation of mixed micelles during gastrointestinal digestion, increasing thereby PS solubility, with no influence of the origin of the lipid source. However, the bioaccessibility of individual PS is influenced by the fat origin, since β-sitosterol and sitostanol showed higher solubility than campesterol in presence of olive oil and lecithin. At doses of 1.0 g PS/100 mL [9], the increment in PS bioaccessibility observed with the presence of milk fat and MFGM in the beverages with respect to the non-fat enriched milk-based fruit beverages is even higher (8.6-fold), showing them to be suitable ingredients for improving the bioaccessibility of PS.

Regarding cholesterol, it shows a high solubility in the mixed micellar aqueous phase, and its bioaccessibility is, therefore, significantly greater than that of PS in all of the above-mentioned PS-enriched products (between 1.9- and 30.2-fold) (Table 11.1). Nevertheless, the higher hydrophobicity of PS with saturated lateral chains (campesterol, campestanol, β-sitosterol, sitostanol) with respect to cholesterol, implies a better transference to the mixed micelles, thus decreasing cholesterol incorporation when considering PS-enriched foods [47]. Additionally, cholesterol bioaccessibility seems to decrease in the presence of mandarin juice, whereas PS increase probably due to molecular competition with cholesterol incorporation into the micelles [14].

Finally, the impact of the addition of galactooligosaccharides (GOS), at different concentrations (1 and 2.5 g/100 mL), to PS-enriched beverages with milk fat and MFGM on sterol bioaccessibility has been evaluated [9]. The addition of GOS does not modify individual or total PS bioaccessibility (approximately 37%), although a slight increase on cholesterol bioaccessibility was observed in the beverage with higher GOS content (from 81% to 85%). In the same study, for the first time, the static harmonized *in vitro* gastrointestinal digestion methodology of the COST Action INFOGEST [46] was applied in order to evaluate sterol bioaccessibility. In a first trial, the Infogest digestion method carried out with porcine bile salts at a concentration of 10 mM for the intestinal digestion phase, did not allow cholesterol quantification. Probably, the extra contribution of cholesterol from the bile salts led to saturation of micelles, and therefore reduced cholesterol solubilization. In contrast, PS bioaccessibility could be determined, with percentages for total PS 2.6-fold lower than the one observed for the same beverage with micellar digestion (14 versus 37z%). Also, the profile of PS solubility was altered with respect to those obtained with our micellar digestion, with sitostanol (16%) and β-sitosterol (14%) showing the greatest bioaccessibility, followed by campesterol, campestanol and stigmasterol (13%). These results, thus, demonstrate that PS bioaccessibility is also influenced by the conditions of the applied gastrointestinal digestion method. Specifically, the higher content of bile salts proposed by Infogest with respect to micellar digestion (10 mM versus 1.4 mM), could inhibit key enzymes of lipid metabolism such as pancreatic lipase, thus diminished PS solubility. Moreover, the different origin of the bile salts (porcine versus bovine), could influence sterol micellar solubility due to their different polarity.

Therefore, in order to allow cholesterol bioaccessibility determination, we assayed two modifications of the Infogest digestion conditions [9]: the reduction of bile salts concentration from 10 mM to 1.4 mM and the change of bile salts origin from porcine to bovine, but maintaining the 10 mM concentration). Both modifications made cholesterol (62 and 38%) and PS bioaccessibility determination (22 and 14%) possible, being lower with respect to the same beverage following a/the micellar digestion protocol (79–80% for cholesterol and approximately 37% for PS). This is probably due to the presence of key enzymes in the micellar digestion protocol that improve lipophilic solubilization such as cholesterol esterase (which mediates cholesterol re-esterification promoting their incorporation in mixed micelles), phospholipase A2 (which favours the release of free fatty acids and lysophospholipid with surfactant properties and could improve cholesterol absorption efficiency) and lipase and co-lipase (that could enhance droplets lipid digestion, increasing sterol micellarization). The modifications proposed for the Infogest method also affected the PS individual solubility profile. In the case of the reduction of porcine bile salts concentration to 1.4 mM, the order of solubility was campestanol (25%) > campesterol (25%) > sitostanol (24%) > β-sitosterol (22%) > stigmasterol (18%), similar to that observed in micellar digestion using bovine bile salts at the same concentration. By contrast, when the bile salts origin was changed, the use of bovine bile salts resulted in the following order of solubility: sitostanol (16%) > campesterol (15%) > β-sitosterol (14%) > campestanol (13%) > stigmasterol (10%), similar to

that observed in Infogest digestion using porcine bile salts at the same concentration (10 mM). This indicates that sterol solubility is closely related to the bile salts content beyond of the gastrointestinal *in vitro* digestion method or the bile salt origin used.

Recently, the Infogest digestion method has been applied in order to evaluate PS bioaccessibility in other PS-enriched food matrices such as dark chocolate [58] and granola bars and pudding [60]. PS bioaccessibility in dark chocolate with different cocoa (64, 72, and 85%) and microencapsulated PS contents (5, 10, and 15%) ranged from 6.5% to 8.4%. This bioaccessibility is in the range of those observed in commercially available fermented milk beverages [61] although much lower than those obtained in optimized matrix composed by the milk-based fruit beverage with milk fat and MFGM [4, 9]. On the other hand, the bioaccessibility of PS from granola bars enriched with low-crystallinity phytosterol nanoparticles impregnated in nanoporous starch aerogels (PS-NSA) or crude PS (1 g PS per 2 bars) was evaluated in different formulations, including non- (0%), low- (7%), and regular-fat content (24%) [60]. PS-NSA improved PS bioaccessibility compared to crude PS by threefold in all granola bar formulations. Low- and regular-fat granola bars showed similar PS bioaccessibility compared to crude PS (~30%) or PS-NSA (~90%). However, when non-fat granola bars were digested, bioaccessibility decreased to 16% for crude PS and to 52.7% for PS-NSA. Similar to the commented above for PS-enriched milk-based fruit beverages [4], the presence of fat in granola bars seems to improve PS bioaccessibility, although no differences were observed in PS bioaccessibility between low- and regular-fat granola bars. Authors suggest that there is a threshold lipid content required to maximize the solubilization capacity of the mixed micelles. Additionally, in the same study, the effect of the addition of crude PS versus PS-NSA on bioaccessibility in a non-fat pudding formulation was evaluated. Similar to the granola bars, the inclusion of PS in nanoparticles improved PS solubility (19% versus 2%). However, the bioaccessibility observed in pudding was lower than in granola bars, probably due to the more complex ingredients (oats, almonds, coconut, egg whites and gum arabic), including a larger content of proteins and emulsifiers that might improve the solubilization of PS in the aqueous phase during the *in vitro* digestion.

Finally, fermented maize yogurt-like products containing dispersible PS (1.7 g/100 g) and carotenoids (1.6 g/100 g) showed the lowest β-sitosterol bioaccessibility (1.6%) observed in all the aforementioned PS-enriched products [30]. The *in vitro* gastrointestinal digestion carried out, based on Reboul et al. [54] and Dhuique-Mayer et al. [21] differs from the micellar and Infogest ones since it is composed of only two digestion steps (gastric and intestinal phases). It was validated against human studies and considered to be a reliable model for carotenoid behaviour during *in vitro* digestion [24]. It included pepsin in the gastric digestion phase, and porcine pancreatin, porcine bile extract and cholesterol esterase in the intestinal step. Authors suggest that, similar to what occurred in milk-based fruit beverages containing β-cryptoxanthin mentioned above [3], the presence of carotenoids limits the incorporation of β-sitosterol into mixed micelles. With respect to cholesterol, the incorporation of PS resulted in a reduction in its solubility of 50%.

11.5 Bioaccessibility of Oxysterols from Foods

Sterols are susceptible to oxidation generating different compounds know as oxysterols, which comprise cholesterol and phytosterol oxidation products (COPs and POPs, respectively). Although information available about POPs formation is scarce compared to COPs, autoxidation is considered the main mechanism of sterol oxidation in foods [42, 51]. The process of autoxidation comprises an initial abstraction of the C-7 hydrogen from the sterol steroid ring, followed by a several reactions with reactive oxygen species which finally leads to the formation of 3β-hydroxy-5-en-7-peroxyls. After the abstraction of a hydrogen, more stable 7-hydroperoxydes molecules are formed. During heating and/or storage conditions, 7-hydroperoxydes can decompose into 7α/7β-hydroxysterols or 7-ketosterols by reduction or oxidation reactions, respectively. Other secondary products from sterol oxidation are 5α,6α/5β,6β-epoxysterols, formed from the interaction between non-sterol oxidized molecules and a hydroperoxy radical. Hydration of epoxysterols in an acidic environment leads to triol derivate formation [12, 27]. All these reactions that make up the autoxidation of sterols are summarized in Fig. 11.2. Autoxidation can be initiated, respectively, influenced by several factors such as heat, light exposure, oxygen or presence of lipids and water, which means that food composition, industrial processing, storage conditions and culinary procedures are factors to be taken into account in oxysterol formation in foods [8].

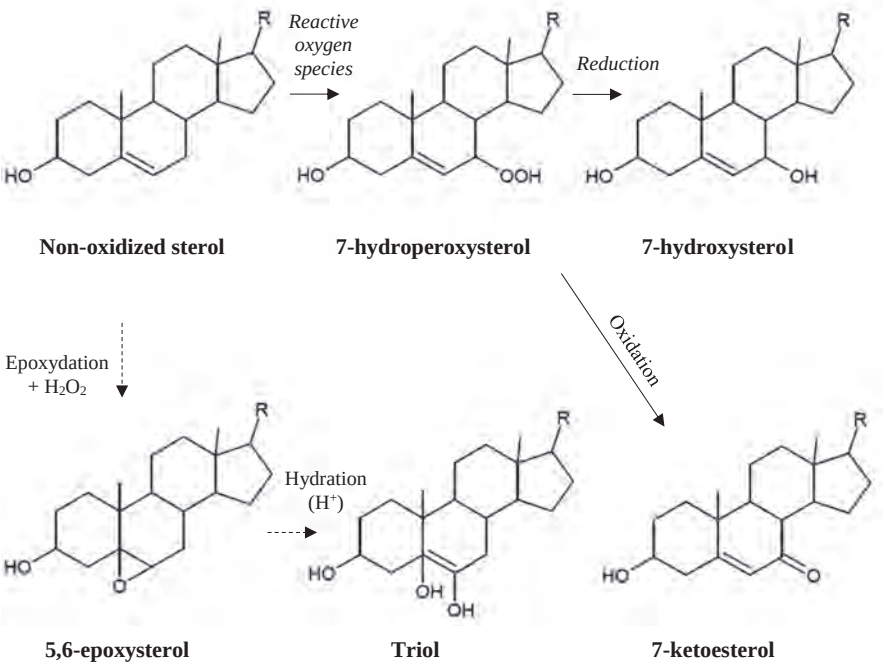


Fig. 11.2 Formation of oxysterols by autoxidation

Very few studies addressing the effect of gastrointestinal digestion on oxysterols have been performed on foods. Hur et al. [37] assessed the influence of different cooking methods (oven, pan-frying, boiling, microwave heating) on COP formation in pork patties during *in vitro* digestion. Although total COP contents in the cooked samples before digestion were not significantly different, the simulated gastrointestinal digestion exerted a significant increase in microwave-cooked patties, together with a higher lipid oxidation (measured by TBARS) compared to the other cooking methods. Moreover, the COP formation during an *in vitro* digestion of biopolymer (chitosan, pectin, onion powder, or green tea powder) encapsulated-beef patties cooked in microwave showed that the encapsulation produced less cholesterol oxidation after the intestinal step of the digestion versus control (non-encapsulated) [38]. In both studies, total COP contents included 7α- and 7β-hydroxy, 7-keto-, triol, 20α-hydroxy, and 25-hydroxycholesterol, although individual concentrations were not detailed, not even initial (raw) amounts in the samples. However, results indicated that cholesterol from meat samples can be oxidized when exposed to digestive enzymes and bile salts, increasing COP production during digestion, which is promoted by cooking methods such as microwave heating and can be inhibited by biopolymer encapsulation of the food products.

The bioaccessibility of COPs and POPs from PS-enriched milk and/or fruit beverages evidenced a lower bioaccessibility of COPs relative to cholesterol (2.2–17% versus 53–99%), whereas those for POPs were much higher than those of their corresponding phytosterols (19.1–49.3% versus 2.2–6.5%) [3], in concordance with the absorption trend in animals. Also, a matrix effect regarding bioaccessibility of oxysterols was detected, since bioaccessibility of POPs was significantly lower in the PS-enriched milk beverage, and that of COPs in fruit and milk-based beverages. In all samples, 7β-hydroxycholesterol (6.8–20.7%) for COPs and sitostanetriol (62.7–176.6%) for POPs were the most bioaccessible oxides, the latter probably due to the transformation of epoxy derivatives into triol derivatives, because of the acidic conditions of a simulated gastric juice, reported by Maerker et al. [41].

The bioaccessibility of oxysterols in PS-enriched milk-based fruit beverages with milk fat and MFGM does not increase during storage (0, 3 and 6 months) at room temperature, which demonstrates that sterols are stable in beverages of this kind [5]. In this study, total POP bioaccessibility (45.4–49%) was lower than that of COPs (58.1–80%), which is in contrast with values reported by Alemany et al. [3]. However, this behaviour is comparable to the one observed for the corresponding non-oxidized sterols determined in the beverages (β-sitosterol presents lower bioaccessibility than cholesterol) [3, 4, 9]. Regarding individual oxysterols, triol (56.3–62.0%), α-epoxy (49.3–60.6%) and 7β-hydroxysitosterol (56.2–59.0%) and 7α/7β-hydroxycholesterol (80.5–121.0 and 82.0–93.3%) show the highest bioaccessibility.

11.6 Fermentation of Sterols

Intestinal microbiota is able to act upon the sterol molecules that reach the colon, generating sterol metabolites by means of hydrolyzation, hydrogenation and dehydrogenation reactions. In this sense, microbial cholesterol degradation has been widely studied by *in vitro* assays of fermentation carried out with microbiota (from human, rat, pigeon or chicken faeces) or by pure culture experiments of enteric microorganisms (*Eubacterium spp*., *Clostridium perfringens*, *Bifidobacterium spp*., *Enterobacter aerogenes*, *Escherichia coli*, *Bacteroides spp*.) [17]. Information available indicates that cholesterol is mainly converted into coprostanol and, in to a lesser extent into cholestanol. Coprostanol formation has been suggested to follow two routes: a direct pathway through cholesterol reduction or an indirect by means of intermediate metabolite formation (cholestenone, cholesterone, coprostanone, coprostenol). Moreover, it has been suggested that coprostanol may transform slightly into epicoprostanol [17, 29, 39] (Fig. 11.3).

Cholesterol degradation into metabolites by the microbiota is related mainly to the abundance of cholesterol-reducing bacteria. *Eubacterium* and *Bacteroides* genus have been reported to be responsible for the direct as well as indirect routes for cholesterol metabolism, whereas *Bifidobacterium* is only implicated in the direct pathway [17, 43, 49, 52, 55]. Other bacteria groups such as genus *Clostridium* and *Lactobacillus* and *Lachnospiraceae* and *Rumunococcaceae* families have been suggested as coprostanoligenic bacteria with no association with the aforementioned routes [6, 16, 39, 40].

Similar to cholesterol, the biotransformation of PS has been described through the formation of different metabolites such as phytostanone and phytostenone intermediates, and ultimately phytostanols [64]. Sitosterol and campesterol are mainly transformed into ethyl- and methyl-coprostanol and subsequently into ethyl- and methyl-coprostanone, respectively. It also been suggested that stigmasterol could be converted into ethylcoprostenol and ethylcoprostanone. However, PS biotransformation routes have not been fully elucidated, and *Eubacterium spp*. are, thus far, the only species associated with PS degradation [17, 23, 25, 45].

In order to shed light on the sterol biotransformation by gut microbiota, a fermentation study by batch culture of a beverage residue obtained after the simulated gastrointestinal digestion of a PS-enriched milk-based fruit beverage that provided ~1.5 mg PS was carried out by our research group [18]. Results showed a decrease of neutral sterol contents of β-sitosterol (8 and 14%), sitostanol (21 and 16%), campesterol (19 and 39%), campestanol (17 and 15%), stigmasterol (17 and 19%) and brassicasterol (10 and 1%) at 24 and 48 h of fermentation, respectively. Correlated with these diminutions, increments of ethylcoprostanol (18 and 50%), ethylcoprostanone (16 and 39%), methylcoprostanone (63 and 21%) and stigmastenol (13 and 16%) were observed. The absence of cholesterol metabolites suggests a preference of PS as substrate by the action of the gut microbiota. Moreover, PS fermentation diminished the abundance of *Flavonifractor plautii* and *Allobaculum spp*. (from the *Erysipelotrichaceae* family), while it promoted *Eubacterium hallii*

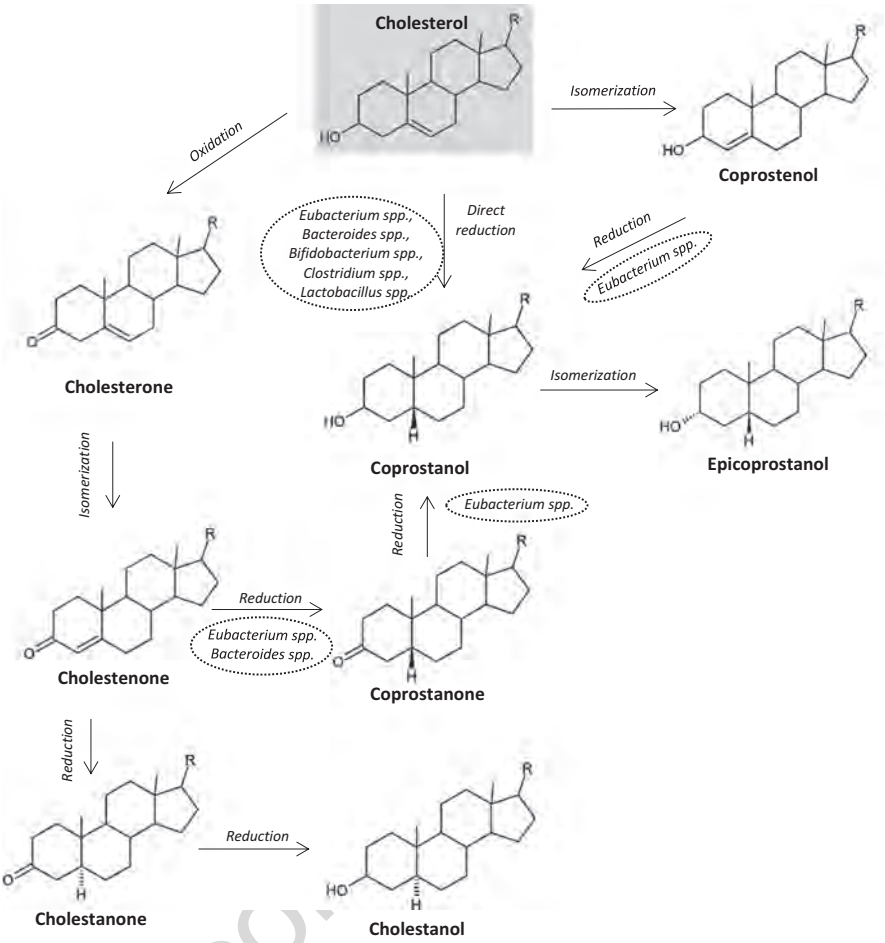


Fig. 11.3 Biotransformation pathways of cholesterol by gut microbiota [17, 39]

and some members of the uncharacterized S-24 family of *Bacteroidetes*. However, it has been noted that differences in microbiota composition were more strongly associated to time than PS presence. The accumulation of anaerobic fermentation products could result in a lowering of the pH that probably leads to microbial changes [22, 62].

As far as we are aware, there are only two studies, carried out by our research group, conducted by means of *in vitro* dynamic fermentation systems in order to evaluate PS biotransformation by gut microbiota [10, 19]. Cuevas-Tena et al. [19] evaluated interactions between PS and microbiota using a TIM-2 system with a PS-enriched dose (~2 g/day) from two sources (an ingredient source of PS or a commercial standard mixture of PS) using microbiota from lean and obese populations. The authors observed a lower concentration of animal and PS metabolites (coprostanol, coprostanone, cholestanol, ethylcoprostanol and methylcoprostanone) with

microbiota from lean than obese subjects, suggesting a different microbial activity. The increase of cholestanol with the concomitant decrease of coprostanol and coprostanone observed during the fermentation in presence of PS for microbiota from lean subjects suggest a modification of the main pathway of cholesterol biotransformation into coprostanol. In the case of experiments carried out with microbiota from obese population, the increase of both metabolites, coprostanol and cholesterol, indicates that cholesterol is biotransformed by two different routes. However, the fact that the diet was uncontrolled (real sterol intake) or the conversion patterns of the donors (high or low sterol converters) make it difficult to only correlate this effect to the presence of PS. An increase of β-sitosterol, sitostanol, campesterol and campestanol was observed for lean and obese microbiota assays in presence of PS. Also, a drop of ethylcoprostanol contents (32–35% for lean and 87–88 for obese) with increments of ethylcoprostanone (21- and 46-fold for lean and 33- and 35-fold for obese) and methylcoprostanone (8- and 25-fold for lean) were observed, with respect to control. We linked the absence of β-sitosterol metabolism to ethylcoprostanol by a saturation of the microbiota activity in presence of a PS-enriched dose. Similar to what was observed in a batch culture PS fermentation study [18], gut microbiota seems to prefer PS as a substrate when they are present in a higher proportion with respect to cholesterol. Furthermore, microbiota analysis showed that *Catenibacterium* and *Coprococcus* genera increase in the presence of PS in experiments with lean microbiota. Both of them belong to Erysipelotrichaceae family, and thus, these results are in disagreement with those obtained in the batch culture fermentation study [18]. Regarding obese microbiota experiments, *Clostridium* genus increase when PS was added.

Recently, we published novel results related to the study of PS biotransformation using a food matrix (PS- and GOS-enriched milk-based fruit beverage) by means of an *in vitro* gastrointestinal and colonic fermentation system [10]. In general, neutral sterols determined in the assay (cholesterol, β-sitosterol, sitostanol, campesterol, campestanol and stigmasterol) showed a trend of accumulation in all colon compartments (corresponding to the ascending, transverse and descending colon). Formation of coprostanol, ethylcoprostanol, sitostenone, methylcoprostanol and ethylcoprostenol was observed only in the distal colon compartments during the fermentation of the beverage, demonstrating that sterol metabolism is colon-compartment-dependent. Indeed, higher conversion ratios of the main sterols were detected in the descending versus transverse colon for cholesterol (0.9–1.3 versus 0.2–0.7), β-sitosterol (0.02–0.7 versus 0.01–0.46), campesterol (0.2–0.5 versus 0.08–0.26) and stigmasterol (0.8–1.0 versus 0.5–0.6). In contrast with our previous assays abovementioned [18, 19], higher ratios for cholesterol biotransformation than PS were observed, suggesting that the presence of GOS enhances its metabolism. Moreover, the absence of sterol intermediate metabolites suggests their rapid bioconversion or, in the case of cholesterol, that the direct pathway of conversion to coprostanol was predominant. In contrast, the digestion and fermentation of the PS-enriched milk-based fruit beverage (without addition of GOS) showed an absence of biotransformation of both PS and cholesterol (Blanco-Morales et al., unpublished results), thus evidencing an important role of GOS in this process.

Furthermore, results of the analysis of microbiota composition in the distal colon compartments showed to be similar and, also, different from the one present in the ascending compartment, supporting the similarities regarding sterol metabolism observed in these compartments. We identified *Bacteroides* as the only coprostanoligenic bacteria present in the colon compartments with cholesterol metabolism and also suggested the involvement of other bacteria species in the PS metabolization aside from *Eubacterium* spp., due to the PS metabolism observed in absence of these bacteria [10]. In the case of the non-GOS-added beverage, the fermentation also modulated certain members of the microbiota composition: in general, *Bifidobacterium* (within 24 h) and Megasphaera genera were stimulated, and *Mitsuokella* was reduced in the ascending colon, while the growth of *Klebsiella* genus was promoted, *Akkermansia* was maintained and Bacteroidetes phylum decreased in the distal compartments.

It has to be pointed out that discrepancies between the results obtained in these above-mentioned fermentation studies could be due to the different conditions applied. In this sense, the use of static methods (batch culture) [18] could have improved the PS metabolism since the absence of a continuous flow, such as in dynamic systems [10, 19], could imply a faster biotransformation. When comparing dynamic fermentation studies, the TIM-2 model [19] includes a dialysis membrane that simulates the absorption of water and metabolites preventing their accumulation and, therefore, a possible inhibition of microbial activity. Moreover, the use of PS included in a food matrix [10] versus provided by a PS-ingredient source or commercial standard mixture [19] could hamper their biotransformation due to the protection provided by the food matrix against the microbiota activity.

11.7 Conclusions

Dietary sterols are minor components present in fat of the human diet. Similar to other lipophilic compounds, in the gastrointestinal tract they are subjected to several sequential reactions (physicochemical and enzymatic) to obtain an absorbable form for subsequent absorption. Bioaccessibility studies, in which sterol incorporation into intestinal mixed micelles are determined, are useful to identify the factors that can promote or hamper sterol solubility and, subsequently, design optimal formulations in the case of functional foods.

Considering the studies related to the bioaccessibility of PS in PS-enriched foods, it can be concluded that their solubility is influenced by several conditions derived not only from the PS enrichment (dosage or source of ingredient) but also from food matrix composition. Furthermore, the presence of compounds such as carbohydrates or carotenoids can limit PS bioaccessibility, while fat and emulsifiers can improve it, with PS-enriched milk-based fruit beverages containing milk fat and MGFM, representing the matrix that offers the highest PS bioaccessibility among the ones reported in literature. However, the use of different *in vitro* gastrointestinal

digestion methodologies results in a limiting factor when comparing PS solubility between different studies, therefore it is of interest to standardize and optimize digestion methods focused on lipophilic compounds such as PS.

Furthermore, studies on bioaccessibility of oxysterols could be used as a predictive model of their rate of absorption, although information related to the behaviour of oxysterols during *in vitro* digestion is rather limited, to the effect of the food matrix on their bioaccessibility, and taking into account that an *in vivo* oxide formation can occur as well.

Finally, the non-absorbed sterols are susceptible to subsequent transformations once they reach the colon for their excretion. Although there are studies that determine sterol excretion, information about the microbiota population related to the degradation of sterols and the pathways involved are scarce, in particular, concerning PS biotransformation from enriched products. In studies contemplating *in vitro* fermentation assays in the presence of high concentrations of PS, it has been demonstrated that: (i) microbiota origin (from lean or obese subjects) can influence sterol biotransformation, (ii) other bacterial species are implicated in PS biotransformation, aside from *Eubacterium spp*., (iii) the presence of PS can modulate microbiota composition, (iv) the sterol metabolism is higher in the distal parts of the colon, and that (v) the food matrix in which PS are included can modulate the behaviour of sterol fermentation (the presence of fibre improves the metabolism of cholesterol versus PS).

Considering all the above facts on the fate of sterols during digestion and fermentation, more studies are needed in this field to provide further information and evidence to improve functional food products and to take advantage of their beneficial effects on health.

Acknowledgments Authors would like to thank the Spanish Ministry of Science and Innovation and the European Regional Development Fund (FEDER) (PID2019-104167RB-I00 project). Thanks are due to Prof. Amparo Alegría for her critical remarks of this chapter.

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Anexo I

Autorización para la publicación en la Tesis Doctoral:

Safe intake of a plant sterol-enriched beverage with milk fat globule membrane: Bioaccessibility of sterol oxides during storage

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Publication: Journal of Food Composition and Analysis

Publisher: Elsevier

Date: May 2018

Journal of Food Composition and Analysis 68 (2018) 111–117

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original research article

Safe intake of a plant sterol-enriched beverage with milk fat globule membrane: Bioaccessibility of sterol oxides during storage

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ARTICLE INFO

Keywords:
Functional beverages
Simulated gastrointestinal digestion
Oxysterols
Sterol oxidation products
MFGM
Bioaccessibility
Food analysis
Food composition

ABSTRACT

Sterols in foods are susceptible to oxidation to form oxysterols. It is interesting for consumer health to know real intake and the possible associated adverse effects associated to oxysterols. This study measured oxysterol formation and bioaccessibility (BA) in a plant sterol (PS)-enriched milk-based fruit beverage with milk fat globule membrane (MFGM) added at 0, 3 and 6 months of storage at room temperature. The same cholesterol (COPs) and phytosterol oxidation products (POPs) (exclusively from β-sitosterol) (7α/β-hydroxy, α/β-epoxy, triol and 7-keto) were detected in the beverage and its bioaccessible fraction. Total COPs and POPs contents were maintained during storage, and their BA ranged between 58 and 80% and 45–49%, respectively, without significant differences throughout storage. β-Sitosterol showed a lower mean oxidation percentage (0.028%) than cholesterol (1.24%), but the estimated POPs intake (0.5 mg/day) was two-fold higher than that of COPs (0.25 mg/day) from 250 g of beverage. These results show that the presence of milk fat and MFGM in the formulation of this beverage did not imply an increase in the contents of oxysterols and their BA. Thus, the beverage is suitable as a PS-enriched food matrix for the length of its shelf-life, and its consumption appears to be safe for consumers.

1. Introduction

Plant sterols (PS) are well known for their ability to reduce low-density lipoprotein (LDL)-cholesterol concentrations. A recent meta-analysis (Ras et al., 2014) has shown that an average intake of 2.1 g PS/day (3 g PS/day being the maximum allowed dose) gradually reduces LDL-cholesterol by an average of 6–12%. In addition, antiinflammatory and anticarcinogenic properties (against cancer of the breast, prostate, lung, stomach and ovary) have also been proposed as further beneficial effects of the consumption of PS (Othman and Moghadasian, 2011; Bin Sayeed and Ameen, 2015; Ramprasath and Awad, 2015; Shahzad et al., 2017).

In this regard, several foods currently can be enriched with PS due to their cholesterol-lowering effect, since the estimated daily intake of PS from the Western diet usually does not exceed 440 mg PS. Only in the case of vegans can an intake of 1 g PS/day be reached (García-Llatas and Rodríguez-Estrada, 2011; Klingberg et al., 2012; Ras et al., 2015). Such levels fall short of the effective PS doses.

Sterols present in food (cholesterol and PS) are susceptible to oxidation. The oxidized products formed are respectively known as cholesterol oxidation products (COPs) and plant sterol oxidation products (POPs). Overall, COPs and POPs are referred to as sterol oxidation products (SOPs) or oxysterols (García-Llatas and Rodríguez-Estrada, 2011; Brzeska et al., 2016). Milk-based fruit beverages, where the addition of PS has been approved (Commission Decision, 2004), are a good option for obtaining the recommended daily amount of PS in subjects with moderate hypercholesterolemia, and are postulated as a good vehicle for preventing the possible formation of SOPs, thanks to the presence of fruits (natural sources of antioxidants) (González-Larena et al., 2015).

The effects upon the body of consuming COPs have been intensively studied, though less information is available in the case of POPs (Hovenkamp et al., 2008; Otaegui-Arrazola et al., 2010; García-Llatas and Rodríguez-Estrada, 2011; Olkkonen et al., 2015; Brzeska et al., 2016). Recently, Kulig et al. (2016) have reviewed the biological importance of COPs in the human organism and their association to chronic diseases such as atherosclerosis, neurodegenerative disorders or cancer. Given the structural similarity between PS and cholesterol, it

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http://dx.doi.org/10.1016/j.jfca.2017.03.011
Received 30 November 2016; Received in revised form 3 March 2017; Accepted 21 March 2017
Available online 24 March 2017
0889-1575/

can be assumed that their oxidation products have similar effects in the human body, though unclear and contradictory information has been published in this regard. The biological actions associated to the consumption of POPs include effects on cholesterol metabolism, atherosclerosis and inflammation processes and cytotoxicity (García-Llatas and Rodríguez-Estrada, 2011; Vanmierlo et al., 2013; O'Callaghan et al., 2014).

Regarding oxysterol occurrence in foods, it has been found that the intensity and time of heat treatment employed are key factors affecting the oxidation of sterols, as well as food composition.

The effect of the food lipid matrix, temperature, free or esterified PS (with different degree of unsaturated fatty acids) upon sterol oxidation remains subject to controversy, and has been addressed by a number of recent reviews (Otaegui-Arrazola et al., 2010; Barriuso et al., 2016a). Soupas et al. (2004) indicated that for temperatures above 140 °C, unsaturated lipid matrices result in a lesser PS oxidation rate, since unsaturated lipid matrices are more readily oxidized, thus protecting sterols, while PS in saturated lipid matrices at higher temperatures facilitate sterol reactivity. Similar observations have been made by Soupas et al. (2007) and Barriuso et al. (2016b). However, for temperatures under 140 °C, PS oxidation is highest in unsaturated matrices. Since the mechanism underlying this effect is not clear, sterols could react more rapidly in lipid matrices where oxidation occurs more easily (Soupas et al., 2004; Otaegui-Arrazola et al., 2010). Moreover, the interaction between PS and lipids depends on whether or not PS are esterified: if PS are esterified with fatty acids, the vicinity of the radicals generated is close to the oxidation points of PS, while if the fatty acids are unsaturated, further oxidation of PS may result (Barriuso et al., 2016a). On the other hand, free PS undergo less interaction with the lipid matrix, and the more unsaturated the fatty acids surrounding PS happen to be, the more protected the sterols can be against oxidation. However, highly unsaturated lipids (such as DHA) might not exert this protective effect, due to rapid degradation of the fatty acid and the generation of a high proportion of oxidizing species (Barriuso et al., 2016a). Xu et al. (2011) also reported that polyunsaturated fatty acids possibly may compete for oxygen with sterols – the latter oxidizing first – though the effect of fatty acids upon sterol oxidation is time-dependent and is most unlikely related to their degree of unsaturation. In this regard, Soupas et al. (2007) indicate that at 160 °C, a saturated lipid matrix (butter oil) increases free PS oxidation *versus* an unsaturated lipid matrix (liquid margarine or rapeseed oil) to a greater extent (2- to 3-fold higher) than in the case of esterified PS. Furthermore, sterol oxidation is favored in oil-water emulsions, since they allow more interactions with the aqueous phase and promote the presence of free fatty acids, which accelerate oxidation of the sterols (Cercaci et al., 2007; Pignoli et al., 2009). In designing PS-enriched beverages, it could be of interest to evaluate sterol bioaccessibility (BA), defined as the maximum sterol content available for absorption, as a previous step for *in vivo* studies (Garcia-Llatas et al., 2015). In this sense, we have observed that the presence of milk fat globule membrane (MFGM), a natural emulsifier, and a fat content of 2.4% provided by milk fat, improve sterol BA in milk-based fruit beverages enriched with PS (Alvarez-Sala et al., 2016). Accordingly, the same behavior could be expected referred to the BA of SOPs, which could also be favored in this kind of beverage. To the best of our knowledge, only one study to date has assessed the BA of SOPs in a similar PS-enriched milk-based fruit beverage, though not containing MFGM in its formulation (Alemany et al., 2013). Several beneficial effects from MFGM compounds (phospholipids and gangliosides) present in bovine milk, such as improved blood lipid profiles (Vesper et al., 1999), the lowering of blood cholesterol, and the prevention of coronary heart disease (Rueda, 2014), could counteract the possible atherosclerotic effect of SOPs. Therefore, the aim of the present study was to evaluate the formation of SOPs and their BA during storage in milk-based fruit beverages enriched with PS and containing MFGM.

2. Material and methods

2.1. Chemicals and reagents

The internal standard (IS) used was 5α-cholest-5-en-3β,19-diol (19-hydroxycholesterol) (purity 98%). Other standards of COPs were cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol) (purity 98%), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol) (purity 97%), 5β,6β-epoxycholestan-3β-ol (β-epoxycholesterol) (purity 90%) and cholestane-3β,5α,6β-triol (cholestanetriol) (purity 95%), all acquired from Steraloids (Newport, RI, USA). 5α,6α-Epoxycholestan-3β-ol (α-epoxycholesterol) (purity 80%), and 5-cholesten-3β-ol-7-one (7-ketocholesterol) (purity 90%) were from Sigma Chemical Co. (St. Louis, MO, USA).

Trimethylchlorosilane (TMCS) was purchased from Fluka (Buchs, Switzerland). Ammonium chloride, anhydrous sodium sulfate, chloroform, ethanol, hydrochloric acid (purity 37%), methanol, potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium bicarbonate and urea were supplied by Merck (Whitehouse Station, NJ, USA). Sodium hydroxide was from Panreac (Barcelona, Spain). Uric acid was purchased from Prolabo (Sacramento, CA, USA). Diethyl ether, *n*-hexane, potassium hydroxide and 2-propanol were from Scharlau (Barcelona, Spain). Anhydrous pyridine, α-amylase from human saliva, bovine bile, bovine serum albumin (BSA), butylhydroxytoluene (BHT), calcium chloride dehydrate, cholesterol esterase from bovine pancreas, colipase from porcine pancreas, glucose, glucosamine hydrochloride, glucuronic acid, hexamethyldisilazane (HMDS), lipase from human pancreas, magnesium chloride, mucin from porcine stomach type II, pancreatin from porcine pancreas, pepsin from porcine stomach, phospholipase A2 from porcine pancreas, potassium thiocyanate, sodium dihydrogen phosphate, sodium taurocholate, and tris(hydroxymethyl)aminomethane were from Sigma Chemical Co. (St. Louis, MO, USA). All reagents were of analytical grade. Silica solid-phase extraction (Si-SPE) cartridges (Supelclean LC-Si, 500 mg/3 mL) were purchased from Supelco (Bellefonte, PA, USA). The syringe-driven Millex-FH filters (1 mL, 0.45 μm) were purchased from Millipore, and ultrapure water was obtained by means of a Millipore Q water purification system (Milford, MA, USA).

2.2. Sample

A beverage containing skimmed milk, milk fat, whey protein concentrate enriched with MFGM (Lacprodan® MFGM-10 from Arla Foods Ingredients) (50%), mandarin juice (48%), banana puree (1%) and grape juice (1%) with the addition of microencapsulated free microcrystalline PS (Lipohytol® ME Dispersible from Lipofoods) (2 g PS/250 mL beverage) from tall oil in powder was elaborated. The beverage was prepared by the Hero Global Technology Center (Alcantarilla, Murcia, Spain) specifically for this study (product not commercially available). This sample is one of those used in the study of Alvarez-Sala et al. (2016). Energy and nutritional information per 100 mL of beverage was: energy (kJ/kcal) 263/65.3; protein (g) 3.1; carbohydrates (g) 8.9; fat without considering PS (g) 1.6; fiber (g) 1.5; PS (g) 0.8. The mean sterol contents (mg/100 g of beverage) were: β-sitosterol 704, sitostanol 102, campesterol 34.7, campestanol 9.46, cholesterol 8.15, and stigmasterol 5.19 (Alvarez-Sala et al., 2016).

The beverage was analyzed just after manufacture (time 0) and after 3 and 6 months of storage at room temperature (20–25 °C). The storage time of up to 6 months is the common and usual turnover period for products of this kind at sales points.

2.3. Determination of sterol oxidation products

2.3.1. Beverage

Lipids were extracted according to the procedure described by Alvarez-Sala et al. (2016) To 5 g of beverage (providing approximately

40 mg of PS), 25 mL of a chloroform:methanol (1:1, v/v) mixture with 0.05% BHT (as antioxidant to avoid SOPs artifact formation during saponification) was added and subsequently homogenized (Polytron PT 2000, Kinematica AC, Switzerland) for 3 min at 19,800 rpm. After adding 12.5 mL of chloroform and mixing again with the Polytron, the sample was filtered (Whatman no. 1.90 mm) through a Buchner funnel, and 20 mL of potassium chloride 1 M solution was added to the filtrate and kept at 4 °C overnight. After the organic fraction was separated, the chloroform phase was concentrated in a rotary evaporator and taken to dryness under a nitrogen stream.

For saponification, the procedure described by González-Larena et al. (2015) was used. The lipid fraction was dissolved in 10 mL of hexane:isopropanol (4:1), and 5 mL was taken, with the addition of 10 μg of 19-hydroxycholesterol as IS. Cold saponification was performed at room temperature with 10 mL of 1 N methanolic potassium hydroxide, in darkness and under continuous agitation in a shaker at 150 rpm during 18–20 h. Afterwards, the unsaponifiable material was extracted with diethyl ether, dried under nitrogen and dissolved in 10 mL of hexane:isopropanol (4:1). Five mL of the unsaponifiable material was purified by Si-SPE. The SOPs were finally eluted with acetone (10 mL) and then subjected to derivatization with HMDS:TMCS in anhydrous pyridine (2:1:5, v/v/v) (25 min, 40 °C). The trimethylsilyl ether (TMSE) derivatives were solubilized in *n*-hexane, filtered and evaporated under a nitrogen stream. Afterwards, the TMSE derivatives obtained were dissolved in 40 μL of hexane, and 1 μL was analyzed by gas chromatography-tandem mass spectrometry (GC–MS/MS) for identification purposes, and by gas chromatography-flame ionization detection (GC-FID) for quantification purposes (González-Larena et al., 2011, 2015).

2.3.2. Bioaccessible fraction

Simulated gastrointestinal digestion was applied to 20 g of beverage according to Vaghini et al. (2016) and Alvarez-Sala et al. (2016). This process includes three phases – salivary, gastric and intestinal – with the formation of mixed micelles to obtain the aqueous-micellar fraction (supernatants) regarded as the bioaccessible fraction (BF) (*i.e.*, the soluble fraction that is released in the gastrointestinal tract and is available for absorption) of the digested beverages.

To 10 mL of BF, 5 μg of 19-hydroxycholesterol was added as IS, and the mixture was subjected to cold saponification (based on Alemany et al., 2013) with 20 mL of 2 N methanolic potassium hydroxide (0.05% BHT) in darkness and under continuous agitation in an orbital shaker at room temperature (150 rpm, 18–20 h). Afterwards, the unsaponifiable material was extracted with diethyl ether, and the totality of this fraction was purified by Si-SPE. Derivatization and SOPs determination were carried out as described for the beverages.

With the aim of eliminating any possible presence of cholesterol and its oxides (since it has been reported that crude bile extract used in the simulated digestion process can contain traces of these compounds) (Alemany et al., 2013), digestions of blanks (composed of 20 g of water, in triplicate) were performed to subtract these compounds from the BF.

The BA of SOPs (*i.e.*, the percentage of SOPs available for absorption with respect to the total SOPs content in beverage before digestion) was calculated as: [SOPs content in BF (μg SOPs/100 g beverage)/total SOP content in undigested beverage (μg SOPs/100 g beverage)] × 100.

2.3.3. Identification and quantification

Identification of COPs was performed based on the resulting fragmentation patterns and chromatographic retention times of commercial standards obtained by GC–MS/MS, while the identification of POPs was established based on the fragmentation patterns described in previous studies of the group (González-Larena et al., 2011, 2015). Due to the structural similarity between COPs and POPs, and since POP standards are not commercially available, the quantification of SOPs contained in the beverage and BF using GC-FID was performed by employing calibration curves obtained with COP standards. In this sense, calibration curves were prepared with 7α- and 7β-hydroxy, α- and β-epoxy, triol and 7-ketocholesterol at increasing concentrations, containing 5 μg of IS (19-hydroxycholesterol), with derivatization and analysis by GC-FID under the same conditions as for the beverages and BF. The calibration equations obtained are shown in Table 1.

Table 1
Calibration curves for COP standards obtained by CG-FID.

COP standards	Linearity range (μg)	Calibration equation	Correlation coefficient (r)
7α-Hydroxy	0.008–0.122	y = 1.2794x − 0.0007	0.9999
7β-Hydroxy	0.025–0.245	y = 1.3735x − 0.0067	0.9998
α-Epoxy	0.099–0.694	y = 0.9219x − 0.0027	0.9971
β-Epoxy	0.102–0.713	y = 0.7082x + 0.0016	0.9990
Triol	0.051–0.303	y = 2.9507x − 0.1519	0.9928
7-Keto	0.008–0.281	y = 1.084x − 0.0047	0.9965

y = $Area_{COP}/Area_{IS}$ and x = μg of COP/μg of IS. COP: Cholesterol oxidation products.

2.3.4. Statistical analysis

For the study of the formation of SOPs during storage, one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test, was applied to determine statistically significant differences in the same compound and in the same kind of sample (beverage, BF, or BA) as a function of storage time (0, 3 or 6 months).

The analysis of all samples was performed in triplicate. A significance level of $p < 0.05$ was adopted for all comparisons, and the Statgraphics® Centurion XVI.I statistical package (Statpoint Technologies Inc., VA, USA) was used throughout.

3. Results and discussion

3.1. Beverage

A GC–FID chromatogram of the SOPs present in the beverage analyzed after 6 months of storage is shown in Fig. 1. In the course of the storage period (0–6 months), the same oxides of cholesterol and β-sitosterol were detected (7α- and 7β-hydroxy, α- and β-epoxy, triol and 7-keto). Only POPs corresponding to β-sitosterol were identified, since β-sitosterol was the most abundant PS in the beverage, in agreement with previous studies in similar PS-enriched beverages (Alemany-Costa et al., 2012; Alemany et al., 2013) where β-sitosterol oxides were the only oxysterols detected. In addition, in another stability study involving similar functional beverages, β-sitosterol and campesterol oxides were identified – the latter being less abundant (González-Larena et al., 2015).

Table 2 reports the contents of SOPs in the beverage. The total mean contents of POPs ranged from 187 (t = 0) to 204 μg/100 g of beverage (t = 6 months), with no statistically significant differences during storage. The contents of POPs just after elaboration were lower than those found by Alemany-Costa et al. (2012) (580–830 μg/100 g) and González-Larena et al. (2015) (243 μg/100 g), but higher than those reported by Alemany et al. (2013) (74 μg/100 g) in PS-enriched milk-based fruit beverages. In the present study, formulation of the beverage comprised the addition of milk fat and whey proteins concentrate enriched with MFGM, yielding a 4-fold higher saturated fat content compared with the abovementioned previous studies (1.6 *vs.* 0.4 g/100 mL). Therefore, fat type and content do not seem to influence the POP content in these types of beverages.

Regarding the individual POP contents of the beverage analyzed just after elaboration, the prevalent oxide was β-epoxysitosterol followed by α-epoxysitosterol and 7β-hydroxysitosterol, with 7α-hydroxysitosterol being the least prevalent. In milk-based fruit beverages stored at 24 °C, González-Larena et al. (2015), in agreement with our own study, found α/β-epoxysitosterols to be the most abundant oxysterols. However, in previous studies on milk based fruit beverages, 7β-hydroxy followed by

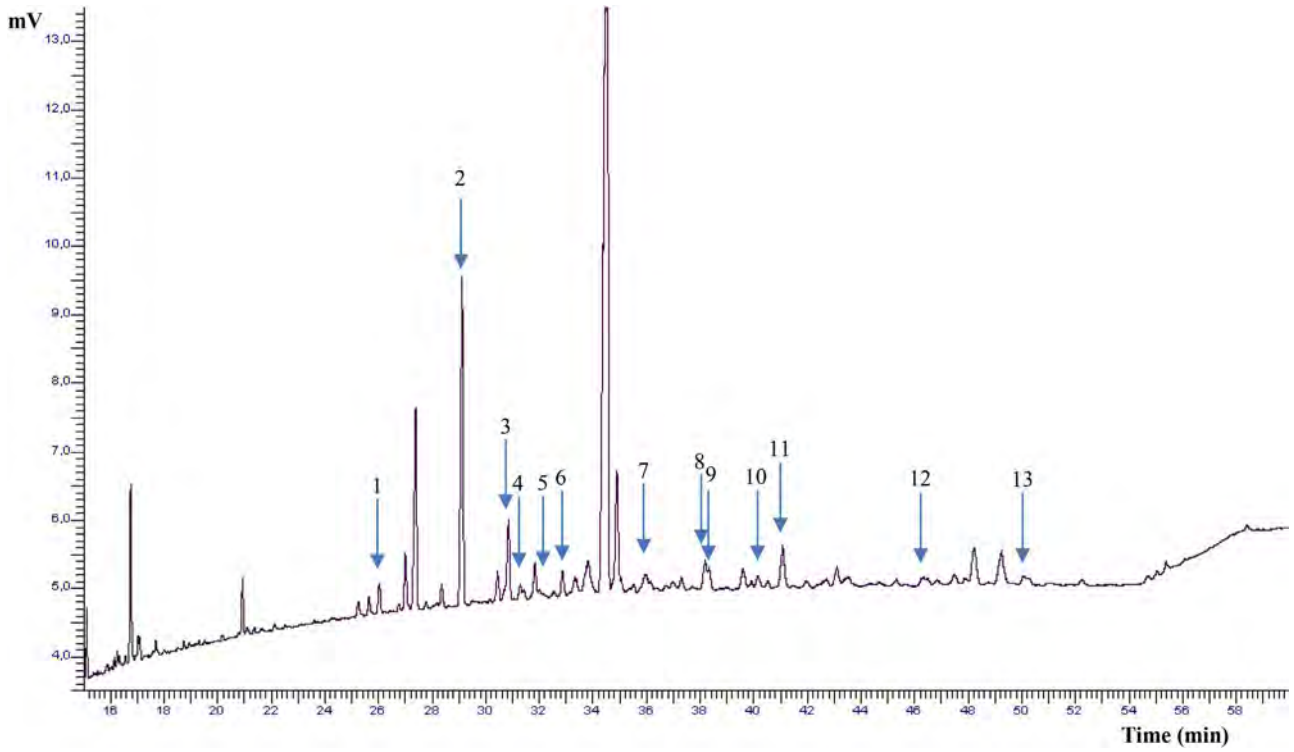

Fig. 1. Sterol oxidation products in beverage obtained by GC–FID.1: 7α-Hydroxycholesterol; 2: 19-hydroxycholesterol (IS); 3: 7β-hydroxycholesterol; 4: α-epoxycholesterol; 5: β-epoxycholesterol; 6: 7α-hydroxysitosterol; 7: cholestanetriol; 8: 7β-hydroxysitosterol; 9: 7-ketocholesterol; 10: α-epoxysitosterol; 11: β-epoxysitosterol; 12: sitostanetriol; 13: 7-ketositosterol.

β-epoxy and 7-ketositosterol (Alemany-Costa et al., 2012) or 7-ketositosterol (Alemany et al., 2013) were the most abundant POPs. The differences observed could be attributed to several factors such as the POPs profile present in the ingredient source of PS, and the manufacturing conditions involved (laboratory or industrial scale). Only a slight significant increase was recorded for sitostanetriol after 6 months of storage.

Throughout the 6 months of storage, the relative percentages of each β-sitosterol oxide with respect to the total POPs content reflected a tendency to maintain constant proportions for all the oxides: β-epoxy

Table 2
Sterol oxidation products contents in beverage and bioaccessible fraction (μg/100 g beverage) after manufacture and during storage.

		β-Sitosterol oxides (POPs)			Cholesterol oxides (COPs)		
	Month	Beverage	BF	BA	Beverage	BF	BA
7α-Hydroxy	0	11.85 ± 3.26^{a}	2.78 ± 0.44^{a}	23.48 ± 3.69^{a}	5.57 ± 0.24^{a}	4.92 ± 0.41^{a}	88.30 ± 7.32^{a}
	3	12.21 ± 4.29^{a}	3.90 ± 0.11^{b}	31.94 ± 0.88^{a}	7.47 ± 0.62^{b}	6.01 ± 0.30^{a}	80.50 ± 4.04^{a}
	6	11.18 ± 1.52^{a}	3.39 ± 0.17^{a,b}	30.32 ± 1.52^{a}	7.48 ± 0.37^{b}	9.06 ± 0.97^{b}	121.04 ± 12.97^{b}
7β-Hydroxy	0	33.96 ± 2.06^{a}	19.65 ± 0.78^{a}	57.86 ± 2.30^{a}	8.22 ± 0.65^{a}	7.67 ± 0.62^{a}	93.32 ± 7.52^{a,b}
	3	34.59 ± 3.29^{a}	19.45 ± 2.45^{a}	56.24 ± 7.08^{a}	10.35 ± 0.51^{b}	8.49 ± 0.65^{a,b}	81.99 ± 6.31^{a}
	6	38.86 ± 0.33^{a}	22.92 ± 3.92^{a}	58.98 ± 10.10^{a}	9.84 ± 0.30^{b}	9.74 ± 0.52^{b}	99.00 ± 5.27^{b}
α-Epoxi	0	32.11 ± 4.49^{a}	15.82 ± 2.26^{a}	49.27 ± 7.05^{a}	13.28 ± 2.63^{a}	8.21 ± 1.24^{a}	61.80 ± 9.31^{a}
	3	34.11 ± 4.70^{a}	20.66 ± 1.71^{a}	60.55 ± 5.01^{a}	15.07 ± 2.71^{a}	5.65 ± 0.95^{a}	37.49 ± 6.33^{a}
	6	30.00 ± 1.72^{a}	18.58 ± 4.46^{a}	58.02 ± 12.51^{a}	11.21 ± 1.37^{a}	5.89 ± 1.96^{a}	52.54 ± 17.45^{a}
β-Epoxi	0	62.15 ± 5.90^{a}	27.85 ± 0.03^{a}	44.82 ± 0.05^{a}	12.44 ± 4.72^{a}	12.20 ± 2.97^{a}	98.11 ± 23.88^{a}
	3	72.05 ± 1.11^{a}	28.77 ± 0.21^{a}	39.94 ± 0.29^{b}	15.72 ± 0.79^{a}	11.05 ± 0.49^{a}	70.30 ± 3.09^{a}
	6	72.50 ± 6.19^{a}	29.72 ± 1.04^{a}	41.00 ± 1.43^{b}	15.06 ± 3.57^{a}	11.93 ± 2.14^{a}	79.22 ± 14.18^{a}
Triol	0	18.80 ± 1.58^{a}	11.65 ± 0.43^{a}	61.97 ± 2.30^{a}	27.58 ± 1.69^{a}	19.09 ± 0.17^{a}	69.21 ± 0.62^{a}
	3	18.98 ± 0.88^{a}	11.59 ± 0.21^{a}	61.05 ± 1.09^{a}	27.04 ± 0.80^{a}	18.58 ± 2.50^{a}	68.71 ± 9.24^{a}
	6	21.96 ± 0.71^{b}	12.38 ± 0.81^{a}	56.34 ± 3.68^{a}	29.91 ± 1.93^{a}	19.53 ± 2.72^{a}	65.29 ± 9.10^{a}
7-Keto	0	28.30 ± 8.60^{a}	7.76 ± 1.82^{a}	27.44 ± 6.44^{a}	27.15 ± 6.15^{a}	10.11 ± 1.92^{a}	37.23 ± 7.09^{a}
	3	24.86 ± 3.08^{a}	8.06 ± 2.13^{a}	32.44 ± 8.59^{a}	27.43 ± 4.85^{a}	10.12 ± 1.85^{a}	36.90 ± 6.73^{a}
	6	26.32 ± 0.83^{a}	7.32 ± 0.98^{a}	27.81 ± 3.71^{a}	25.61 ± 3.91^{a}	18.37 ± 5.75^{a}	71.71 ± 22.45^{a}
Total	0	187.16 ± 12.92^{a}	85.52 ± 2.06^{a}	45.69 ± 1.10^{a}	99.84 ± 2.96^{a}	62.66 ± 5.32^{a,b}	62.77 ± 5.33^{a,b}
	3	192.53 ± 3.86^{a}	94.35 ± 3.20^{a}	49.00 ± 1.66^{a}	103.09 ± 4.56^{a}	59.90 ± 4.69^{a}	58.11 ± 4.55^{a}
	6	204.07 ± 4.44^{a}	92.54 ± 12.43^{a}	45.35 ± 6.09^{a}	99.12 ± 5.96^{a}	79.31 ± 5.93^{b}	80.02 ± 5.98^{b}

Values are expressed as mean ± standard deviation of three replicates. BF: Bioaccessible fraction content. BA: Bioaccessibility, calculated as [oxides content in BF (μg/100 g beverage)/ oxides content in undigested beverage (μg/100 g beverage)] × 100. Different superscript letters indicated significant differences ($\rho < 0.05$) in the same compound and in the same kind of sample (beverage or BF or BA) in the different months of storage (within columns, a–b).

(33–36%), 7β-hydroxy (18–19%), α-epoxy (15–17%), 7-keto (13–15%), triol (10–11%), and 7α-hydroxy (5–6%). In contrast, González-Larena et al. (2015) after 6 months of beverage storage, recorded no changes in sitostanetriol, with an increase in α/β-epoxysitosterols and a decrease in 7β-hydroxysitosterol and 7-ketositosterol.

Total amounts of COPs ranged between 99.1–103 μg/100 g of beverage, with no statistically significant changes during the storage period (Table 2). Only one study (Alemany et al., 2013) reported contents of COPs in milk-based fruit beverages, obtaining higher amounts (201 μg/100 g of beverage) than in our study, despite the lower cholesterol content in those beverages compared to the present study (1.4 *versus* 8.2 mg/100 g beverage).

Cholestanetriol and 7-ketocholesterol were the main COPs (each representing 25–27% of total COPs), while the lowest contents corresponded to 7α/β hydroxycholesterol (5–8%). This order of abundance is in agreement with the study reported by Alemany et al. (2013), where 7-ketocholesterol was the main COP and 7α/β-hydroxycholesterol were present in minor amounts. In that study, no cholestanetriol or α/β-epoxycholesterol was found. During the storage period of our beverage, only a slight significant rise in 7α/β-hydroxycholesterol was observed from three months onwards, increasing to 7–10% of total COPs.

The sterol oxidation percentages in the beverage analyzed are shown in Table 3. The oxidation percentages of β-sitosterol ranged from 0.027–0.029%, which are higher than those previously reported by Alemany et al. (2013) (0.013%) at time zero, but lower than or similar to those reported for PS-enriched milk-based fruit beverages at time zero (0.07%) (Alemany-Costa et al., 2012) or during storage at room temperature (0.03–0.06%) (0–6 months) (González-Larena et al., 2015). Thus, it can be affirmed that formulation and elaboration of the beverages of the present study, including milk fat and MFGM, do not imply greater PS oxidation.

Cholesterol, in agreement with the study of Alemany et al. (2013), presented higher rates of oxidation than β-sitosterol (Table 3). However, the percentages in our beverage after elaboration (t = 0) were lower than those reported by the aforementioned authors (1.2% *versus* 14.9%), despite the fact that our beverage had a greater cholesterol content (8.2 *versus* 1.4 mg/100 g beverage). This is in agreement with the study by Nielsen et al. (1996), where higher saturated fat and cholesterol contents implied lower cholesterol oxidation in dairy spread and butter stored at 20 °C. In contrast, at higher temperatures (180 °C), Ansorena et al. (2013) reported the opposite effect in food lipid models in the presence of triacylglycerols. In our study, the higher oxidation percentage of cholesterol *versus* PS (β-sitosterol) (see Table 3) can be ascribed to the distinct surface activity presented by the sterols, which can differently affect their susceptibility to oxidation (Cercaci et al., 2007). In this regard, cholesterol has a higher degree of surface activity than β-sitosterol – a fact that is linked to its greater capacity to reduce interfacial tension than this phytosterol, and thus cholesterol is more concentrated at oil-water interfaces in the emulsions where oxidative stress is high. Moreover, the PS ingredient employed in the formulation of our beverage was in a microencapsulated form that can afford protection against oxidation.

Table 3
Oxidation percentage of plant sterols and cholesterol in beverage.

	Sterol content (mg/100 g beverage)[a]	Month	Oxidation (%)
Plant sterols		0	0.022
	856.29 ± 84.41	3	0.022
		6	0.023
β–Sitosterol		0	0.027
	704.72 ± 70.60	3	0.027
		6	0.029
Cholesterol		0	1.225
	8.15 ± 0.41	3	1.265
		6	1.216

[a] Values from Alvarez-Sala et al. (2016). Oxidation (%) calculated as: [(total oxides content/100 g beverage)/(total sterol content/100 g beverage)] × 100.

3.2. Contribution of the plant sterol-enriched beverage to daily sterol oxidation product intake

Regarding the daily intake of SOPs from the diet, it is important to underscore that: i) the existing data are scarce, due to the fact that most studies consider a specific meal and not the total diet; ii) the diets or meals in these studies normally do not include PS-enriched foods; iii) the analytical methodology used to determine COPs and mainly POPs is complex and difficult, thereby making proper determination of these compounds in foods difficult; and iv) in some cases the levels of dietary SOPs are calculated based on theoretical oxidation percentages. Therefore, our calculated contribution to daily SOPs intake in the context of the abovementioned diets should be regarded as an approximation to the real situation.

Daily POPs intake has been estimated by Lin et al. (2016) based on PS intake from PS-enriched food (0.75–3 g PS/day) multiplied by the oxidation rate of phytosterols in these foods. For non-heated and stored free PS-enriched foods, the estimated daily POPs intakes are in the range of 2.9–11.4 mg/day. For pan-fried foods the estimated daily POPs intakes are 3.0, 47.7 or 78.3 mg/day, depending on the phytosterol forms added (free PS, plant sterol esters or plant stanol esters, respectively) and daily PS intakes. Lower POPs intakes from PS-enriched foods have been reported by Scholz et al. (2015) using two approaches: i) from POPs contents in these foods and the assumption that the upper daily PS intake is of 3 g from these foods (POP intake from non-heated and heated foods ranging between 1.2–2.9 and 3.5–29.6 mg/day, respectively); and ii) based on data referred to the dietary intake of PS from different surveys and the assumption in foods of a minimum (0.1%) and maximum oxidation rate (1%) (mean intakes of POPs were 0.35–2.45 and 3.5–24.5 mg/day, respectively).

For the contents of β-sitosterol oxides (POPs) in the PS-enriched beverage of this study (see Table 2), and given that the daily serving size/portion corresponds to 250 g of beverage, the daily POP intake provided was 0.47 mg just after elaboration and 0.51 mg after 6 months of storage. These values fall within the indicated interval reported by Scholz et al. (2015) for pasteurized milks (0.1–3.5 mg POPs/day). Although it has been indicated that the absolute POP contents of foods are positively associated to their phytosterol contents (Lin et al., 2016), greater PS enrichment in the beverage analyzed in the present study (0.8%) *versus* (0.3–0.5%) in the abovementioned review does not imply higher POP contents.

On the other hand, taking into account the percentage PS oxidation ratios (see Table 3) and the PS intake from the consumption of 250 g of beverage (2.14 g PS), the estimated POP intake is 0.5 mg/day from the beverage through storage. These POPs intakes are lower than those indicated by Lin et al. (2016) for non-heated foods, and are included in the range indicated by Scholz et al. (2015) for minimum oxidation rates (0.1%). It must be pointed out that the oxidation percentage of the PS-enriched beverage analyzed is comprised between 0.022–0.023% as a function of time storage (see Table 3).

Regarding COPs, intakes between 1.8–3.05 mg/day from three diets (consumed in The Netherlands and including all raw food, fried/baked or grilled food, or fried/baked or grilled food plus fruits and vegetables) have been reported by van de Bovenkamp et al. (1988). Subsequently, COPs intakes of 11.5 mg from a test meal (scrambled eggs containing egg powder, butter and whole milk) (Emanuel et al., 1991) and 2.5 mg from a typical fish and chips meal (Lake and Scholes, 1997) have been reported. In our case, the COPs intake provided by 250 g of beverage calculated similarly to POPs intake according to the COPs content during storage (see Table 2) or taking into account the cholesterol oxidation percentage and content (20.4 mg/250 g beverage) (see

Table 3), was 0.25 mg/day. These values are far lower than in the previously mentioned studies, since they were calculated from a single food, whereas the other studies considered a diet or meal including cholesterol-rich foods and food deep-fried in beef fat.

3.3. Bioaccessible fraction and bioaccessibility

The SOPs contents in BF, expressed as μg/100 g of beverage, and their corresponding BA (%) are shown in Table 2. The total β-sitosterol and cholesterol oxidation product contents in the BF were 85.5–94.4 and 59.9–79.3 μg/100 g of beverage, respectively, without significant changes during the storage period. However, for individual SOPs a slight increase during storage was observed for 7α-hydroxy derivatives of β-sitosterol and cholesterol, and 7β-hydroxycholesterol.

In the only study that has assessed the effect of simulated gastrointestinal digestion upon the SOPs contents in PS-enriched milk fruit-based beverages (Alemany et al., 2013), the POPs (32.7 μg/100 g of beverage) or COPs (5.6 μg/100 g of beverage) contents in the BF were lower than in the present study as expected, due to the lower oxysterol contents reported for the non-digested beverages (38.6 μg/100 g of beverage) in the study by Alemany et al. (2013) compared to our own study (148 μg/100 g of beverage).

Regarding the BA values, the BA of total POPs ranged between 45.4–49% during the 6 months of storage, being higher for COPs (58.1–80%), with no statistically significant differences through storage. This difference in BA could be attributed to the greater micellar solubility of cholesterol compared to β-sitosterol described in several studies using model systems or food products under different micellarization conditions, namely three kinds of micellar solutions including bile salts (Ikeda et al., 1989), in sodium taurocholate and sodium glycodeoxycholate solutions (Armstrong and Carey, 1987), in bile salt micelles (Matsuoka et al., 2010), from a simplified system (bile salt solutions) compared to a more complex system (model intestinal solution including bile salts, phospholipids and fatty acids) (Matsuoka et al., 2012), and from *in vitro* gastrointestinal digestion of functional beverages (Alvarez-Sala et al., 2016). By analogy, the same behavior can be expected for the corresponding oxides of cholesterol and β-sitosterol.

Regarding the individual BA of the oxides, triol, α-epoxy and 7β-hydroxysitosterol were the oxides with the highest values for POPs, and 7α- and 7β-hydroxycholesterol for COPs – this being similar to the results reported by Alemany et al. (2013).

The total POP and COP contents in the BF – expressed as micromolarity considering the mean molecular weights for the β-sitosterol and cholesterol oxides – ranged between 1.97–2.17 μM and 1.48–1.96 μM, respectively. Compared to the cytotoxic effects indicated in Caco-2 cells (a validated model of intestinal epithelial cells), for POPs (from 60 μM) and COPs (from 30 μM) (Ryan et al., 2009; Alemany-Costa et al., 2012; Laparra et al., 2015), the values found in the present study were far lower than those concentrations that may be deleterious for health, though it cannot be ruled out that continued ingestion of these oxides may lead to degenerative diseases (Kulig et al., 2016).

4. Conclusions

The analyzed beverage, where only cholesterol and β-sitosterol oxidation products were detected, offers sterols stability at room temperature throughout the studied storage period, since no modification of total SOP contents and their BA were observed – only minor changes in some individual SOPs being recorded. The presence of MFGM and the higher fat milk contents in the beverage of this study did not imply greater SOPs formation compared with similar milk-based fruit beverages containing no MFGM. Possibly the presence of saturated fat profiles added to the incorporation of free PS in microencapsulated form would not favor sterol oxidation. In addition, the low estimated intake of POPs (0.5 mg/day) and COPs (0.25 mg/day) from the ingestion of 250 g of beverage (daily portion), and the low presence of SOPs in BF (1.5–2.2 μM) – far from cytotoxic effects reported in cell cultures – demonstrated that milk-based fruit beverages with milk fat and whey protein concentrate enriched with MFGM would be a suitable matrix for the formulation of PS-enriched beverages, with a view to minimizing possible oxysterol formation during the shelf-life of the product. Therefore, the intake of this functional beverage with the purpose of achieving the recommended PS-intakes for obtaining a hypocholesterolemic effect may be considered safe.

Contributions

Andrea Alvarez-Sala and Virginia Blanco-Morales contributed with the analytical assays and to data acquisition and interpretation. Antonio Cilla, Guadalupe Garcia-Llatas, Reyes Barberá and María Jesús Lagarda contributed with the conception and design of the study, and interpretation of data. Luis Manuel Sánchez-Siles collaborated in the formulation, elaboration and provision of the beverage of this study. All authors have collaborated in the drafting and revision of the article, and have approved the final version of the manuscript for submission.

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Funding

This study was financed by the *Ministerio de Economía y Competitividad* and FEDER (Spain) (AGL2012-39503-C02-01). Andrea Álvarez-Sala holds an ACIF/2015/251 grant from the *Generalitat Valenciana* (Spain).

Acknowledgement

We thank Raquel López from Hero España, S.A., for preparing the beverages tested in the present study.

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Anexo I

Autorización para la publicación en la Tesis Doctoral:

The impact of galactooligosaccharides on the bioaccessibility of sterols in a plant sterol-enriched beverage: adaptation of the harmonized INFOGEST digestion method

V. Blanco-Morales, G. López-García, A. Cilla, G. Garcia-Llatas, R. Barberá, M. J. Lagarda, L. M. Sánchez-Siles and A. Alegría, *Food Funct.*, 2018, **9**, 2080
DOI: 10.1039/C8FO00155C

Food & Function

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2018, **9**, 2080

The impact of galactooligosaccharides on the bioaccessibility of sterols in a plant sterol-enriched beverage: adaptation of the harmonized INFOGEST digestion method

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The effect of the addition of galactooligosaccharides (GOS) on sterol bioaccessibility in three plant sterol (PS)-enriched milk-based fruit beverages (without GOS addition (MfB) and with 2.5 g (MfB-G_2) and 5.0 g (MfB-G_5) GOS per 250 mL) was evaluated after micellar gastrointestinal digestion. Cholesterol bioaccessibility was very similar among beverages, though a slight significant increase (from 80% to 85%) was observed by the addition of 5.0 g GOS. The addition of GOS did not affect total PS bioaccessibility (≈37%). Based on the results obtained after micellar digestion, it has been demonstrated that these beverages could be a suitable food matrix for simultaneous enrichment with PS and GOS. The harmonized *in vitro* digestion model INFOGEST was applied to the MfB beverage, but the cholesterol content could not be quantified due to its contribution of bile salts. Hence, it was proposed: (i) a change in porcine bile salt concentration from 10 mM to 1.4 mM (in order to compare with micellar digestion); or (ii) a change of bile salt origin (bovine instead of porcine), maintaining physiological concentration (10 mM, INFOGEST condition). Both options allowed cholesterol quantification, with bioaccessibilities of 62% (reduction of bile salts) and 38% (replacement of the bile salt source), whereas plant sterol bioaccessibilities were 22% and 14%, respectively. Therefore, the change of bile salt origin maintaining INFOGEST concentration is proposed as a method to evaluate sterol (cholesterol and PS) bioaccessibility in these beverages, demonstrating the need for the selection of appropriate conditions of the INFOGEST harmonized method according to the food matrix and compounds to be determined.

Received 24th January 2018,
Accepted 1st March 2018

DOI: 10.1039/c8fo00155c

rsc.li/food-function

1. Introduction

Different clinical trials have demonstrated that the intake of plant sterols (PS) in amounts of 1.5–3 g day^{-1} through fortified foods reduces plasma low-density lipoprotein cholesterol concentrations (5–15%) – this in turn having been associated with a lower risk of cardiovascular disease.[1,2] Other potential beneficial effects, such as antiinflammatory,[3] antiproliferative[4] and antioxidant actions,[5] have been attributed to PS consumption. In recent years, galactooligosaccharides (GOS) have gained attention in the food industry due to their selective effect on the microbiota and the improvement of human health.[6] In this sense, the consumption of 2.6–5.5 g day^{-1} of GOS leads to increased resistance to infection and diarrheal disease in healthy volunteers,[7,8] and exerts a positive effect on the immune system in overweight adults[9] and elderly persons.[10] *In vivo* studies have demonstrated that the consumption of GOS has significant positive effects on lipid profiles, including a decrease in serum cholesterol, triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and very low-density lipoprotein cholesterol.[11,12] In this context, the synergistic effect derived from the PS and GOS enrichment of functional beverages would be an interesting strategy that could contribute to reduce and prevent cardiovascular risk and inflammatory diseases – this possibility representing a novelty in this field of research.

In vitro studies have reported that addition of soluble fiber[13] to low-fat yogurt drink reduces cholesterol bioaccessibility. Likewise, the enrichment of pork patties with fiber-rich extracts (agroindustrial coproducts derived from juice and

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tiger nut beverage production)[14] displays the same effect, although the specific underlying mechanisms are poorly understood. In this regard, added GOS could interact with PS, reducing the bioaccessibility of the latter due to their structural similarity to cholesterol. *In vitro* digestion models are a good tool for evaluating possible interactions between food matrixes and the compound of interest.[15] A static harmonized method development of the COST Action INFOGEST has been proposed in order to consolidate conditions for the simulated digestion of food and establish consensus for a gastrointestinal *in vitro* digestion model.[16] To date, the INFOGEST method has been applied to evaluate the bioaccessibility of different lipophilic compounds, such as cholesterol in pork patties[14] and carotenoids in vegetables[17] and fruit.[18–21]

Plant sterol-enriched milk-based fruit beverages containing the milk fat globule membrane (MFGM) have been shown to be a good choice for securing optimum PS bioaccessibility and potential health effects.[22] In the present study, milk-based fruit beverages with PS and different GOS concentrations were developed. The objectives of this study were (i) to evaluate the possible impact of GOS on PS bioaccessibility using a micellar gastrointestinal digestion method previously employed by our research group in similar beverages;[22,23] and (ii) for the first time to apply and adapt the harmonized INFOGEST gastrointestinal digestion method[16] to assess sterol bioaccessibility in a fruit-based drink food matrix.

2. Materials and methods

2.1. Reagents

The standards used were 5β-cholestan-3α-ol (epicoprostanol) (purity 97%) as an internal standard (IS), 5-cholesten-3β-ol (cholesterol) (purity 99%), 24α-ethyl-5α-cholestan-3β-ol (stigmastanol) (purity 97%), 5,22-cholestadien-24-ethyl-3β-ol (stigmasterol) (purity 97%) and 5-cholesten-24β-ethyl-3β-ol (β-sitosterol) (purity 99%) purchased from Sigma Chemical Co. (St Louis. MO, United States). 24α-Methyl-5-cholesten-3β-ol (campesterol) (purity 96%) was acquired from Chengdu Biopurify Phytochemicals Ltd (Sichuan, China). The derivatization reagents used were anhydrous pyridine from Acros Organics (Geel, Belgium), hexamethyldisilazane (HMDS) from Sigma Chemical Co. (St Louis. MO, United States) and trimethylchlorosilane (TMCS) from Carlo Erba (Rodano, Italy). α-Amylase from human saliva (E.C 3.2.1.1), ammonium carbonate, bile salts from bovine and porcine pancreas, bovine serum albumin (BSA), butylhydroxytoluene, calcium chloride dihydrate, cholesterol esterase from porcine pancreas (E.C 3.1.1.13), colipase from porcine pancreas, glucosamine hydrochloride, glucose, glucuronic acid, lipase from porcine pancreas type II (E.C 3.1.1.3), magnesium chloride, mucin from porcine stomach type II, pancreatin from porcine pancreas, pepsin from porcine gastric mucosa (E.C 3.4.23.1), phospholipase A2 from porcine pancreas (E.C 3.1.1.4), potassium thiocyanate, sodium dihydrogen phosphate and sodium taurocholate hydrate were purchased from Sigma Chemical Co. (St Louis, MO, United States). Ammonium chloride, anhydrous sodium sulfate, hydrochloric acid (purity 37%), magnesium chloride hexahydrate, methanol, potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium hydroxide, tris(hydroxymethyl)aminomethane and urea were provided by Merck (Whitehouse Station, NJ, United States). Ethanol, sodium bicarbonate and sodium hydroxide were obtained from Panreac (Barcelona, Spain). Uric acid was from Prolabo (Sacramento, CA, United States), whereas chloroform, diethyl ether, *n*-hexane, potassium hydroxide and 2-propanol were supplied by Scharlau (Barcelona, Spain). All reagents were of analytical grade. Water was purified using a Milli-Q system (Milford, MA, United States). For enzymatic activity assays, reagents were according to Minekus *et al.*[24]

2.2. Samples

Three PS-enriched milk-based fruit beverages, elaborated by the Hero Global Technology Center (Alcantarilla, Murcia, Spain), were used in the present study: MfB, without GOS; and MfB-G_2 and MfB-G_5 with the addition of GOS (2.5 and 5 g per 250 mL beverage, respectively). The concentrations have been chosen based on EFSA scientific opinion, where it is raised that the intake of 2.6 g GOS day^{-1} would "help a healthy immune system in ageing population and energizes immunity boosting bacteria".[25] The beverages contained skimmed milk, milk fat, whey protein concentrate enriched with MFGM, mandarin juice from concentrate, banana pure and microencapsulated free microcrystalline PS (2.5 g PS per 250 mL beverage) from tall oil in a powder form. Briefly, skimmed milk powder was dissolved in water and then GOS syrup was added in MfB-G_2 and MfB-G_5 beverages. The products were pasteurized at 90 °C for 30 s by using an indirect heat exchanger, cooled to 20 °C, and filled aseptically in 250 mL tetra bricks. The process and conditions were identical for all beverages.

2.3. Micellar gastrointestinal *in vitro* digestion

Micellar gastrointestinal *in vitro* digestion was carried out in the three PS-enriched beverages according to Alvarez-Sala *et al.*,[22] with the exception of lipase origin (human lipase has been discontinued by the manufacturer). Briefly, 20 g of each beverage was taken and salivary solution (α-amylase, uric acid and mucin, as well as organic compounds and electrolytes) was added (9 mL, pH 6.5 ± 0.2). The mixture was incubated in a shaking water bath for 5 min at 37 °C and 95 orbits per minute (opm). Then, gastric solution containing BSA, pepsin from porcine gastric mucosa and mucin was added (13.5 mL, pH 1.07 ± 0.07). The mixture was adjusted to pH 1 and incubated in the same shaking water bath (1 h, 37 °C, 95 opm). Then, for the intestinal phase, 25 mL duodenal solution (pH 7.8 ± 0.2) and 9 mL bile solution (pH 8.0 ± 0.2) that included BSA, pancreatin from porcine pancreas and bile salts from bovine pancreas (1.4 mM in the final mixture) were added. After adjusting the pH of the mixture (7.0 ± 0.2), pancreatic lipase and colipase, phospholipase A2, cholesterol esterase and sodium taurocholate were added. The final mixture was incubated in a shaking water bath (2 h, 37 °C, 95 opm) and

subsequently centrifuged (90 min, 4 °C, 3100*g*). The supernatant was taken and considered as the bioaccessible fraction (BF) of the beverages. Blanks of digestion (n = 3) were performed using 20 g ultrapure water, and cholesterol and PS contents determined in their BF were subtracted from the corresponding ones found in the beverage BF.

2.4. INFOGEST gastrointestinal *in vitro* digestion

A harmonized *in vitro* gastrointestinal digestion method developed in the COST action INFOGEST network[16] was applied. The digestive fluids (simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)) were prepared according Minekus *et al.*[16] Briefly, 5 g sample was taken and mixed with 3.5 mL SSF, shaking for one minute. Then, 0.5 mL α-amylase solution, 25 μL of 0.3 M calcium chloride and 975 μL ultrapure water were added to produce a final volume of 10 mL. The mixture was placed in a shaker bath for 2 min at 37 °C and 95 opm. Upon completion of the oral phase, 7.5 mL SGF, 1.6 mL pepsin solution, and 5 μL of 0.3 M calcium chloride were added and mixed for one minute. The pH of the mixture was adjusted to 3 and ultrapure water was added up to a volume of 20 mL. The gastric mixture was placed again in a shaker bath for 2 h under the same conditions. To simulate the intestinal conditions, 11 mL SIF, 5 mL pancreatin solution (based on trypsin activity), 40 μL of 0.3 M calcium chloride, and 2.5 mL bile salt solution of porcine origin (65 mg mL^{-1} solution) were added, and the final mixture was agitated for one minute. The intestinal mixtures were adjusted to pH 7 and ultrapure water was added to a final volume of 40 mL. Finally, they were incubated in a shaker bath for 2 h at 95 opm. The digest obtained was centrifuged (90 min, 4 °C, 3100*g*), and the supernatant corresponding to BF was collected. Besides this, in relation to intestinal conditions, two additional alternatives were assayed to adapt the methodology, since original INFOGEST conditions do not allow cholesterol quantification: (i) reduction of bile salt concentration (1.4 mM), maintaining the bile salt origin (porcine) used in the method (option 1) (in order to compare with the micellar digestion method); or (ii) modification of the bile salt origin (from porcine to bovine, both allowed in the INFOGEST method), maintaining the original bile salt concentration (10 mM) (option 2) (more similar to physiological levels ranging 5–15 mM).[26]

For improved understanding, Fig. 1 provides an overview of the digestion protocols and conditions employed in the study.

2.5. Determination of GOS

The GOS content in the MfB-G_2 and MfB-G_5 beverages was confirmed following the AOAC method 2001.02,[27] and similar contents in the beverages and BFs were obtained (2.3 ± 0.06 and 4.5 ± 0.06 g per 250 mL, respectively) (n = 6).

2.6. Determination of sterols

Beverages. The methodology used by Alvarez-Sala *et al.*[22] was applied: 5 g beverage (providing approximately 40 mg PS) was taken and the lipid fraction was extracted with chloroform : methanol (1 : 1, v/v) with 0.05% butylhydroxytoluene at 60 °C (Oven Haraeus Instruments, Germany). Then, chloroform was added and the sample was filtered (Whatman no. 1.90 mm). 1 M potassium chloride solution was added to the filtrate and kept at 4 °C overnight. The chloroform phase was concentrated using a rotary evaporator (BÜCHI Labortechnik, Switzerland) and dried under a nitrogen stream. The lipid fraction extracted was dissolved in 10 mL of hexane : isopropanol (4 : 1) (v/v), and 0.5 mL was taken. After the addition of IS (200 μg), the samples were subjected to hot saponification (2 mL of 1 M potassium hydroxide, ethanolic solution (90%)) and subsequent extraction of unsaponifiable fractions with diethyl ether.

Bioaccessible fractions. An amount equivalent to 2.5 g BF from micellar gastrointestinal digestion or 5 g BF from INFOGEST digestion was taken, and IS (200 μg) was added.

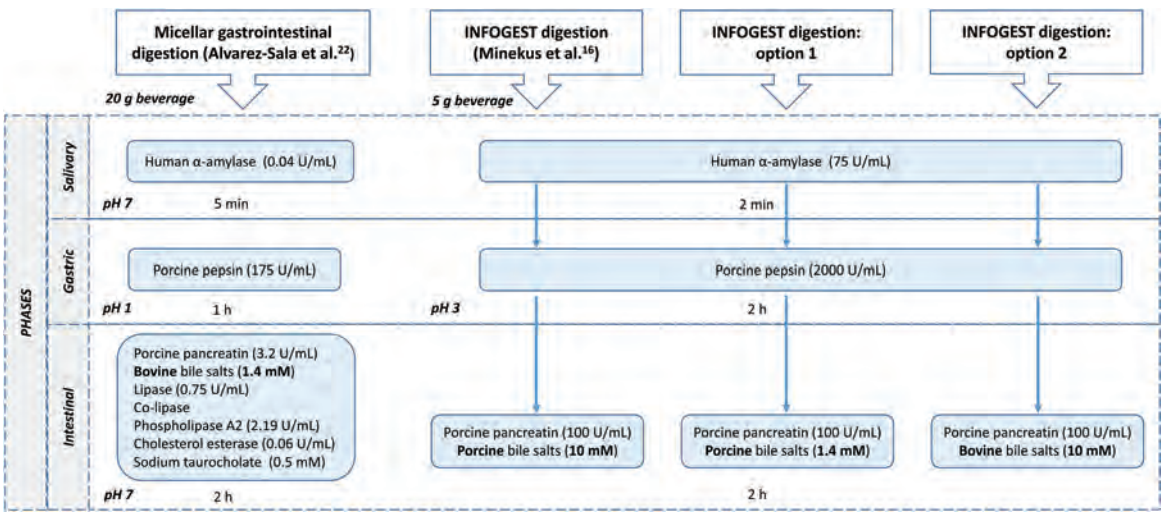

Fig. 1 Schematic representation of *in vitro* digestion protocols assayed in the present study (enzymatic activity expressed as U/mL of each specific phase determined according to Minekus *et al.* (2014b)).

Hot saponification of BF with 10 mL of 2 M potassium hydroxide ethanolic (90%) solution and unsaponifiable fraction extraction with diethyl ether were carried out.[22]

The unsaponifiable fractions from beverages and BFs were derivatized and injected into a gas chromatography-flame ionization detector (GC-FID) under the same conditions indicated by Alemany-Costa *et al.*[28]

2.7. Statistical analysis

One-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test, was used to determine statistically significant differences ($p < 0.05$) between different beverages (MfB, MfB-G_2 and MfB-G_5) or different conditions of the INFOGEST method applied for the same sterol in the beverage, BF or bioaccessibility. The Statgraphics® Centurion XVI.I statistical package (Statpoint Technologies Inc., VA, United States) was used for the statistical analysis. Digestions were performed in two independent days in triplicate ($n = 6$).

Table 1 Sterol contents in plant sterol-enriched milk-based beverages (mg per 100 g beverage). The relative percentage of plant sterol contents provided within parenthesis

Sterols	MfB	MfB-G_2	MfB-G_5
Cholesterol	13.07 ± 0.63	13.09 ± 0.84	13.58 ± 0.30
Campesterol	55.19 ± 2.01 (6.7)	52.87 ± 3.89 (6.7)	57.77 ± 0.78 (6.7)
Campestanol	8.92 ± 0.24 (1.2)	8.36 ± 0.75 (1.2)	9.17 ± 0.16 (1.2)
Stigmasterol	5.65 ± 0.20 (0.7)	5.39 ± 0.41 (0.7)	5.91 ± 0.09 (0.7)
β-Sitosterol	716.98 ± 25.47 (79.7)	684.57 ± 52.51 (79.7)	753.00 ± 8.69 (79.7)
Sitostanol	100.03 ± 3.82 (11.8)	94.88 ± 7.78 (11.7)	105.32 ± 1.24 (11.8)
Total PS	**886.77 ± 31.73**	**846.07 ± 65.30**	**931.17 ± 10.89**

Data expressed as mean ± standard deviation of three replicates. No statistical significant differences ($p > 0.05$) were observed. MfB: PS-enriched milk-based fruit beverage without GOS; MfB-G_2: PS-enriched milk-based fruit beverage with 2.5 g GOS per 250 mL; MfB-G_5: PS-enriched milk-based fruit beverage with 5.0 g GOS per 250 mL.

3. Results and discussion

3.1. Sterol content in beverages

Sterol contents in beverages (MfB, MfB-G_2 and MfB-G_5) are shown in Table 1. The cholesterol content (approximately 13 mg per 100 g beverage) was higher than that reported by Alemany *et al.*[23] in two similar milk-based fruit beverages with or without added mandarin (1.4 and 2.0 mg per 100 g beverage), due to the higher enrichment of milk fat and MFGM. The latter is a source of cholesterol containing around 300 mg per 100 g milk fat.[29] In this regard, in similar beverages, Alvarez-Sala *et al.*[22] indicated that the addition of milk fat and MFGM to milk-based fruit beverages increases the cholesterol content 3.5- to 4-fold compared to beverages without fat addition or with equivalent amounts of fat from olive oil, respectively.

The total PS content in the three beverages ranged from 846 to 931 mg per 100 g beverage (see Table 1). The order of abundance and the relative percentages of each PS with respect to the total PS content were similar in all three beverages: β-sitosterol ≈ 80%, sitostanol ≈ 12%, campesterol ≈ 7%, campestanol ≈ 1% and stigmasterol ≈ 0.7%. These results are in agreement with the specified legislation for milk-based fruit drinks with added phytosterols/phytostanols[30] and with the relative PS abundance reported in previous studies by our research group in beverages with similar profiles (PS-enriched milk-based fruit) and enriched with the same source of PS (tall oil).[22,23,31]

3.2. Micellar gastrointestinal digestion: the impact of GOS addition on sterol bioaccessibility

Table 2 summarizes sterol contents in BFs and their bioaccessibility. Cholesterol in BFs ranged between 10 and 12 mg per 100 g beverage, while the mean total PS contents ranged from 319 to 346 mg per 100 g beverage. Cholesterol and total PS contents were higher in BFs from the beverage with the highest GOS content (MfB-G_5). The order of abundance of PS in BFs was similar for all beverages (β-sitosterol > sitostanol > campesterol > campestanol > stigmasterol). Cholesterol bioaccessibility was similar for all analyzed beverages (≈80%),

Table 2 Sterol contents in plant sterol-enriched milk-based beverages and in bioaccessible fractions (mg per 100 g beverage) and their bioaccessibility after micellar gastrointestinal digestion

	MfB		MfB-G_2		MfB-G_5	
Sterol	BF	Bioaccessibility	BF	Bioaccessibility	BF	Bioaccessibility
Cholesterol	10.54 ± 0.45^a	80.61 ± 3.44^a	10.27 ± 0.05^a	78.45 ± 0.39^a	11.53 ± 0.12^b	84.87 ± 0.88^b
Campesterol	21.81 ± 0.75^a	39.55 ± 1.36^a	21.02 ± 0.37^b	39.75 ± 0.70^a	22.48 ± 0.36^a	38.90 ± 0.63^a
Campestanol	3.90 ± 0.07^a	43.68 ± 0.78^a	3.61 ± 0.07^b	43.16 ± 0.83^a	3.81 ± 0.09^a	41.53 ± 0.97^b
Stigmasterol	1.90 ± 0.09^a	33.55 ± 1.55^a	1.81 ± 0.08^a	33.52 ± 1.40^a	2.01 ± 0.04^b	33.96 ± 0.66^a
β-Sitosterol	262.97 ± 11.44^a	36.68 ± 1.60^a	254.56 ± 4.43^a	37.19 ± 0.65^a	277.14 ± 4.69^b	36.80 ± 0.62^a
Sitostanol	39.29 ± 1.37^a	39.28 ± 1.37^a	37.70 ± 0.61^b	39.74 ± 0.64^a	40.26 ± 0.71^a	38.22 ± 0.68^a
Total PS	**329.86 ± 13.64^a**	**37.20 ± 1.54^a**	**318.69 ± 5.53^a**	**37.67 ± 0.65^a**	**345.68 ± 5.86^b**	**37.12 ± 0.63^a**

Data expressed as mean ± standard deviation of six replicates. MfB: PS-enriched milk-based fruit beverage without GOS; MfB-G_2: PS-enriched milk-based fruit beverage with 2.5 g GOS per 250 mL; MfB-G_5: PS-enriched milk-based fruit beverage with 5 g GOS per 250 mL. BF: bioaccessible fraction; bioaccessibility calculated as: (sterol content in BF/sterol content in beverage) × 100. Different superscripts letters denote statistically significant differences ($\rho < 0.05$), in the same sterol in the BF or bioaccessibility, between beverages (MfB, MfB-G_2 and MfB-G_5).

though a slight increase was observed in MfB-G_5 (85%). No statistically significant differences ($p > 0.05$) were observed in the bioaccessibility of individual PS between beverages with or without GOS, except for campestanol, whose bioaccessibility was slightly lower in MfB-G_5 (43% *vs.* 41%). Bioaccessibility of total PS was similar in all three beverages (37%), the order being campestanol (≈43%) > sitostanol–campesterol (≈40%) > β-sitosterol (≈37%) > stigmasterol (≈33%). These results therefore indicate that GOS addition to milk-based fruit beverages does not affect sterol solubility.

As far as we know, no studies have evaluated the effect of fiber on PS solubility or bioaccessibility, though in view of the structural similarity with cholesterol, a similar effect could be expected. In this sense, some *in vitro* studies have demonstrated the cholesterol adsorption capacity of different soluble and insoluble fibers. López-Marcos *et al.*[14] observed that the addition of different fiber-rich extracts (from lemon, grapefruit, pomegranate, lemon albedo and tiger nut) to sunflower oil (added with cholesterol) reduced cholesterol solubility between 23 and 70%. However, no relationship was found between the dietary fiber content or the proportion of insoluble/soluble dietary fiber within fiber-rich extracts and cholesterol solubility. The authors suggested that the influence of other factors such as the type and amount of bioactive compounds (polyphenols) cannot be ruled out. Likewise, Luo *et al.*[32] demonstrated the cholesterol binding capacity of dietary fibers (total, soluble and insoluble) from bamboo shoot shell in fresh egg yolk. Soluble fiber showed the highest cholesterol binding capacity (10 mg g^{-1} fiber), followed by total (5 mg g^{-1} fiber) and insoluble fiber (2 mg g^{-1} fiber).

In the context of *in vitro* gastrointestinal digestions, Minekus *et al.*[13] found that partially hydrolyzed guar gum addition at a concentration of 3% and 6% to a yogurt drink with 3% sunflower oil and 4% egg yolk reduced cholesterol bioaccessibility 8- and 22-fold, respectively, in a multi-compartmental gastrointestinal tract model. In pork patties added with 10% of various fiber-rich extracts,[14] cholesterol solubility in the oil phase was reduced (10–31%) after INFOGEST gastrointestinal digestion. On the other hand, in a recent study,[33] different concentrations of mandarin fiber (0.5, 1, 1.5 and 2 g per 100 g aqueous phase) were added to nanoemulsions containing β-carotene, and effective enhancement of the bioaccessibility of this lipophilic compound was observed on adding up to 1 g of mandarin fiber per 100 g. Above this concentration, the bioaccessibility of β-carotene was reduced two-fold. The authors suggested that high concentrations of soluble fiber could produce interactions with bile salts and lipase, making contact with the micelles and small intestine difficult, and producing a significant increase in viscosity that complicates the transport of digestive enzymes.

However, the cholesterol–fiber ratio – and not only fiber content – must be taken into account. The estimated lipophilic compound-fiber ratio in these studies[13,14,33] were 0.0005–0.01, which was associated with the impairment of their solubility or bioaccessibility. In the present study, MfB-G_2 and MfB-G_5 showed a sterol-GOS ratio of 0.98 and 0.6, respectively, which is higher than that used by other authors. Therefore, PS enrichment probably causes fiber content present in the beverages to not be enough to produce significant modifications in sterol bioaccessibility.

3.3. INFOGEST gastrointestinal digestion: application to sterol bioaccessibility

Because the presence of GOS does not affect sterol bioaccessibility, as mentioned above, MfB was used to compare the micellar[16] and INFOGEST[22] gastrointestinal methods. The addition of enzymes to gastrointestinal digestion based on their activity is one of the mean features of the harmonized method, and allows the comparison of studies. The enzymatic activity must be determined following the protocols described in the ESI.†[24] Table 3 shows the enzymatic activity of the enzymes used in the INFOGEST digestion and bile salt contents present in bile extracts used in both gastrointestinal digestions. Studies on gastrointestinal *in vitro* digestion describing the specific activity of enzymes and bile salt contents are scarce. In our study, human salivary α-amylase showed an enzymatic activity similar to that reported by Rodrigues *et al.*[19] (79 U mg^{-1} powder) and stated in the specifications of the manufacturer (Sigma-Aldrich) (77 U mg^{-1} powder). The pepsin activity obtained was in agreement with a recent inter-laboratory trial within the INFOGEST network,[34] which found the average pepsin activity to be 2976 ± 591 U mg^{-1} powder. Regarding enzymes implied in the intestinal phase, most studies use pancreatin instead of individual intestinal enzymes, since it is widely employed in the literature and is less costly than individual enzymes. It is important to underscore that it was not possible to use individual enzymes, since pancreatic lipase activity was not enough to achieve 2000 U mL^{-1} in the intestinal final mixture required by the INFOGEST method – this enzyme being poorly dissolved in SIF. Moreover, it was difficult and expensive to carry out the assays used to measure the enzymatic activity described by Minekus *et al.*[24] In the present study, pancreatin was added to the digestion based on its trypsin activity in order to achieve 100 U TAME mL^{-1} of intestinal digestion. Rodrigues *et al.*[19] indicate that pancreatin exhibits 17 U TAME mg^{-1} of powder,

Table 3 Determination of the enzymatic activity and content of bile salts used under different conditions of the INFOGEST digestion

Enzymes	Enzymatic activity (U mg^{-1} powder)
Bovine pancreatic chymotrypsin (E.C 3.4.21.1)	67.2 ± 7.4
Human salivary α-amylase (E.C 3.2.1.1)	62.2 ± 3.5
Pancreatin from porcine pancreas	7.1 ± 0.8
Porcine pancreatic α-amylase (E.C 3.2.1.1)	7.5 ± 0.8
Porcine pancreatic trypsin (E.C 3.4.21.4)	201 ± 17
Porcine pancreatic lipase (E.C 3.1.1.3)	50.0 ± 1.1
Porcine pepsin (E.C 3.4.23.1)	3326 ± 172
Porcine bile extract	2.5 ± 0.2[a]
Bovine bile extract	2.0 ± 0.2[a]

Data expressed as mean ± standard deviation of three or six replicates in two independent assays. [a] μmol mg^{-1} powder.

Table 4 Comparison of the sterol contents present in the bioaccessible fraction (mg per 100 g beverage) of the MfB beverage and their *in vitro* bioaccessibility assayed under different INFOGEST conditions

Sterol	INFOGEST method		Option 1		Option 2	
	BF	Bioaccessibility	BF	Bioaccessibility	BF	Bioaccessibility
Cholesterol	NQ	NQ	8.05 ± 0.16[a]	61.54 ± 1.26[a]	4.93 ± 0.17[b]	37.68 ± 1.31[b]
Campesterol	7.20 ± 1.09[a]	13.05 ± 1.98[a]	13.56 ± 0.96[b]	24.57 ± 1.74[b]	8.09 ± 0.39[a]	14.66 ± 0.71[a]
Campestanol	1.13 ± 0.19[a]	12.70 ± 2.11[a]	2.25 ± 0.19[b]	25.23 ± 2.07[b]	1.13 ± 0.05[a]	12.62 ± 0.58[a]
Stigmasterol	0.72 ± 0.06[a]	12.75 ± 1.04[a]	1.03 ± 0.09[b]	18.24 ± 1.66[b]	0.55 ± 0.07[c]	9.69 ± 1.31[c]
β-Sitosterol	102.56 ± 10.73[a]	14.30 ± 1.50[a]	155.54 ± 12.47[b]	21.69 ± 1.74[b]	98.15 ± 5.25[a]	13.69 ± 0.73[a]
Sitostanol	16.25 ± 1.85[a]	16.25 ± 1.85[a]	24.18 ± 1.78[b]	24.17 ± 1.78[b]	15.82 ± 0.66[a]	15.82 ± 0.66[a]
Total PS	**127.71 ± 13.79[a]**	**14.40 ± 1.55[a]**	**196.56 ± 15.46[b]**	**22.17 ± 1.74[b]**	**123.74 ± 6.37[a]**	**13.95 ± 0.72[a]**

Data expressed as mean ± standard deviation of six replicates. INFOGEST method: bile salts porcine (10 mM); option 1: bile salts porcine (1.4 mM); option 2: bile salts bovine (10 mM). BF: bioaccessible fraction; bioaccessibility calculated as (sterol content in BF/sterol content in beverage) × 100. NQ: not quantified. Different superscripts letters denote statistically significant differences ($\rho < 0.05$), in the same sterol in the BF or bioaccessibility, between different conditions of INFOGEST method applied (INFOGEST method, option 1 and option 2).

which is 2.4-fold higher than our results (7.1 U TAME mg^{-1} of powder), probably due to the different pancreatin batches used.

Sterol contents in the BF obtained after INFOGEST gastrointestinal digestion and the corresponding bioaccessibility values are shown in Table 4. In contrast to micellar gastrointestinal digestion, cholesterol could not be quantified in the BF using the INFOGEST method because the cholesterol content in the blanks of digestion was of the same order as that obtained from the BF of the MfB beverage (Fig. 2A and B). The results obtained suggest that high cholesterol contribution by bile salts could saturate the micelles, reducing cholesterol solubilization. It has been reported, using *in vitro* micellar models, that cholesterol solubility with 50–52 mM of bile salts (glycochenodeoxycholate, glycocholate, glycodeoxycholate or taurocholate) under steady state conditions ranges from 0.45–3.2 mM – the cholesterol/bile salt ratio being 0.01–0.06.[35–37] Under INFOGEST method conditions, a similar cholesterol/bile salt ratio was observed (0.01), though the presence of PS in the BF fraction possibly could result in competition with cholesterol, contributing to impede cholesterol incorporation into the micelle.[38,39] In contrast to our results, López-Marcos *et al.*[14] were able to quantify cholesterol content

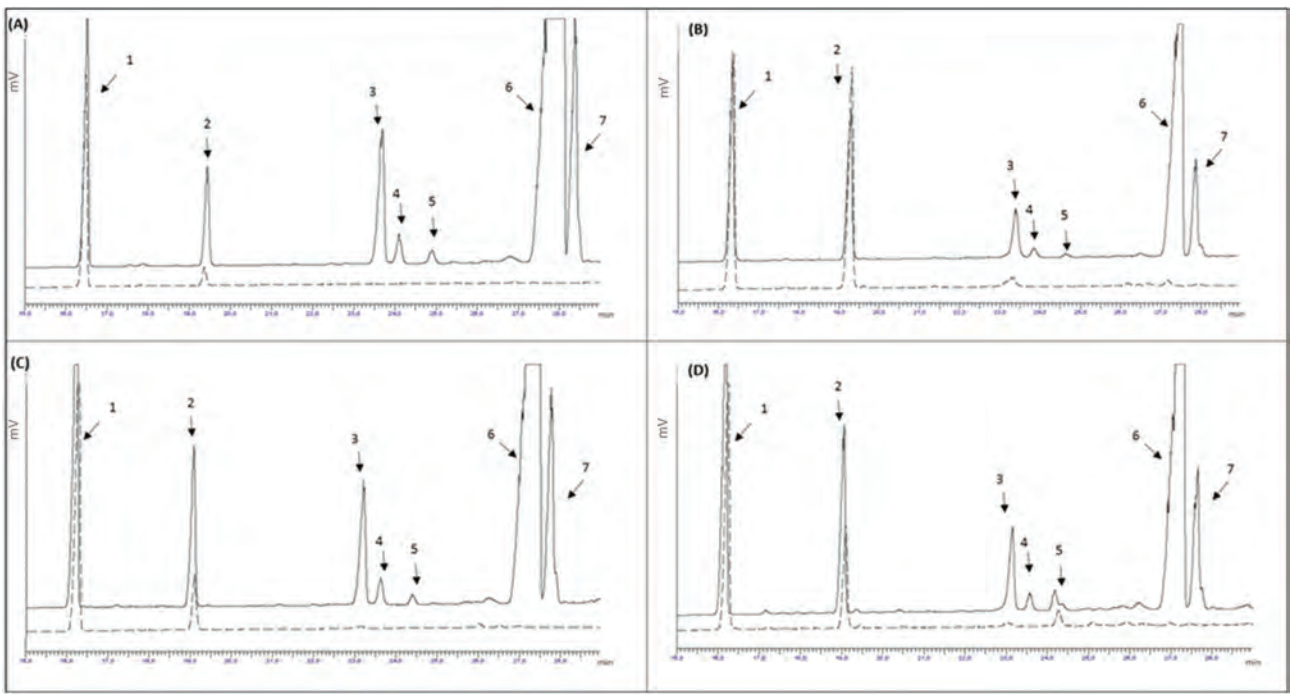

Fig. 2 Representative chromatograms obtained by GC–FID after applying different gastrointestinal digestion methods to a milk-based fruit beverage (MfB): (A) micellar (bovine bile salts at 1.4 mM); (B) INFOGEST (porcine bile salts at 10 mM); (C) INFOGEST-option 1 (porcine bile salts at 1.4 mM); and (D) INFOGEST-option 2 (bovine bile salts at 10 mM). Continuous line: sterols present in the MfB; discontinuous line: the respective blank of digestion. 1: epicoprostanol (IS), 2: cholesterol, 3: campesterol, 4: campestanol, 5: stigmasterol, 6: β-sitosterol, and 7: sitostanol.

in BFs after applying the gastrointestinal INFOGEST method to pork patties. This could be explained by a higher initial cholesterol content in the sample or the use of fresh bile instead of powdered bile extract.

The total PS content in the BF (128 mg per 100 g beverage) obtained by the INFOGEST method was 61% lower than that obtained from the same beverage using the micellar gastrointestinal method (330 mg per 100 g beverage) (see Table 2). Individual PS bioaccessibility by the INFOGEST method was similar in all PS analyzed – the order being sitostanol (16%) > β-sitosterol (14%) > campesterol–campestanol–stigmasterol (13%). The solubility profile differs from that obtained in micellar gastrointestinal digestion, where campestanol and campesterol exhibited the greatest bioaccessibility, followed by sitostanol and β-sitosterol. However, stigmasterol was the sterol with the lowest bioaccessibility in both gastrointestinal digestion methods. Total PS bioaccessibility by the INFOGEST method was lower (14%) than that obtained by the micellar digestion method (37%). Discrepancies in PS solubility between micellar and INFOGEST digestion could be due to the differences in digestion protocols (see Fig. 1), in particular, the higher concentration of bile salts used in the INFOGEST method (4 *vs.* 0.7 mg mL^{-1} digest). It has been reported that bile salt concentrations of 2.5–20 mg mL^{-1} inhibit pancreatic lipase – a key enzyme in lipid metabolism – because bile salts displace the enzyme and hinder its contact with the substrate.[40] Moreover, the origin of the bile salt extract largely determines the composition of bile acids. Based on the information provided by the manufacturer, the bovine bile salts contained a high percentage of taurocholic and glycolic acids, while the porcine salts were rich in glycodeoxycholic and taurodeoxycholic acids. The different polarity of the bile salts greatly influences sterol micellar solubility.[35,36] In this sense, due to the lack of knowledge of the exact composition of the bile salts, it is not possible to explain the differential effects they may exert on sterol bioaccessibility in the two digestion methods.

3.4. Adaptation of INFOGEST gastrointestinal digestion

The results obtained demonstrate the need to adapt the INFOGEST method for application to food matrixes containing lipophilic bioactive compounds such as sterols. The alternatives proposed comprised reduction of the concentration of bile salts used in the INFOGEST method to the same concentration used in micellar digestion (1.4 mM) (option 1) (in order to compare both gastrointestinal digestion methods), or a change in bile salt origin (bovine instead of porcine), keeping the concentration constant (10 mM) (option 2) (closer to physiological concentrations). Table 4 shows sterol content and bioaccessibility after the two options of the harmonized INFOGEST digestion. Both INFOGEST methods allowed cholesterol quantification in BF, its content being 8 and 5 mg per 100 g of beverage, with bioaccessibilities of 62% and 38% for options 1 and 2, respectively (see Fig. 2C and D). Regarding PS, the reduction of bile salt concentration (option 1) increased the solubility of individual (43–99%) and total PS (54%) with respect to the INFOGEST method carried out with porcine bile salts. However, no statistically significant differences ($p > 0.05$) were observed with the change of bile salt origin (option 2), with the exception of stigmasterol, which decreased 24%. Therefore, although the change in bile salt origin allowed cholesterol quantification (option 2), only a decrease in bile salt concentration favored sterol bioaccessibility (option 1).

The cholesterol and PS bioaccessibilities obtained by the INFOGEST methods (options 1 and 2) were lower than those obtained with micellar digestion, which indicates that other specific factors of the digestion method, apart from bile salts, must be considered. One possible factor could be the absence in the INFOGEST methods of cholesterol esterase, an enzyme that mediates cholesterol de-esterification as a prior step to its inclusion in the micelle. Estévez-Santiago *et al.*[18] indicated that the addition of cholesterol esterase (3.1 U mL^{-1}) in the INFOGEST method is necessary for xanthophyll bioaccessibility determination, since the esterified form is the main form found in fruit and vegetables. For this reason, esterified cholesterol from milk was probably not hydrolyzed during the intestinal phase and incorporated into the bile salt micelle, thereby reducing cholesterol content in the BF. The addition of phospholipase A2 into micellar digestion could also have a positive impact on sterol bioaccessibility, since this enzyme favors free fatty acid and lysophospholipid release – these being molecules with surfactant properties that improve cholesterol absorption efficiency.[41] Moreover, the addition of lipase and co-lipase to the intestinal phase (micellar digestion) would favor lipid digestion of the droplets, which has been positively correlated to increased β-sitosterol micellarization.[42]

Regarding the order of solubility of the individual sterols, differences were observed between the two options. On reducing the bile salt concentration, the sterol order was cholesterol (62%) > campestanol (25%) > campesterol (25%) > sitostanol (24%) > β-sitosterol (22%) > stigmasterol (18%). In contrast, the change in the origin of the bile salts yielded the following sterol order: cholesterol (38%) > sitostanol (16%) > campesterol (15%) > β-sitosterol (14%) > campestanol (13%) > stigmasterol (10%). Interestingly, when an equal bile salt concentration was used in both the micellar and INFOGEST methods (option 1), an identical solubility profile was observed. Similarly, when the INFOGEST method was used with a high concentration of bile salts (10 mM) from porcine and bovine origin (option 2), similar profiles were observed. Therefore, our results demonstrate that the sterol solubility profile is closely related to the bile salt content present in the digest, independently of the gastrointestinal *in vitro* digestion method and bile salt origin used.

4. Conclusions

The addition of GOS to PS-enriched milk-based fruit beverages at concentrations of up to 5 g GOS per 250 mL did not affect sterol (cholesterol and PS) bioaccessibility, evaluated by micellar gastrointestinal digestion. Therefore, this kind of beverage

could be a suitable food matrix for simultaneous enrichment with PS and GOS, and be a good strategy for the prevention of cardiovascular and inflammatory bowel diseases. Further, *in vivo* bioavailability studies are currently performed in our research group.

INFOGEST method application carried out with porcine bile salts did not allow cholesterol quantification in the BF due to the high cholesterol contribution from bile salts. Therefore, the change of bile salt origin from porcine to bovine maintaining the INFOGEST method condition (closer to physiological parameters) was proposed for sterol determination (cholesterol and PS) and the evaluation of their bioaccessibility. This study may contribute to improve the INFOGEST method for assays on the bioaccessibility of lipophilic compounds.

Abbreviations

BF	Bioaccessible fraction
BSA	Bovine serum albumin
GOS	Galactooligosaccharides
HMDS	Hexamethyldisilazane
IS	Internal standard
MFGM	Milk fat globule membrane
TAME	Nα-p-tosyl-Ll-arginine methyl ester
PS	Plant sterols
MfB	PS-enriched milk-based fruit beverage without GOS
MfB-G2	PS-enriched milk-based fruit beverage with 2.5 g GOS per 250 mL
MfB-G5	PS-enriched milk-based fruit beverage with 5 g GOS per 250 mL
TMCS	Trimethylchlorosilane

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

This study was financed by the *Ministerio de Economía y Competitividad* and FEDER (Spain) (AGL2015-68006-C2-1-R). Gabriel López-García holds an ACIF/2016/449 grant from the *Generalitat Valenciana* (Spain). Virginia Blanco holds a research contract under the project AGL2015-68006-C2-1-R (Ref. CPI-17-025).

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Anexo I

Autorización para la publicación en la Tesis Doctoral:

Impact of a Plant Sterol- and Galactooligosaccharide-Enriched Beverage on Colonic Metabolism and Gut Microbiota Composition Using an In Vitro Dynamic Model

Author: Virginia Blanco-Morales, Guadalupe Garcia-Llatas, María J. Yebra, et al

Publication: Journal of Agricultural and Food Chemistry

Publisher: American Chemical Society

Date: Feb 1, 2020

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2020, 68, 1884−1895

pubs.acs.org/JAFC

Impact of a Plant Sterol- and Galactooligosaccharide-Enriched Beverage on Colonic Metabolism and Gut Microbiota Composition Using an *In Vitro* Dynamic Model

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Supporting Information

ABSTRACT: A beverage enriched with plant sterols (1 g/100 mL) and galactooligosaccharides (1.8 g/100 mL) was subjected to a dynamic gastrointestinal and colonic fermentation process to evaluate the effect on sterol metabolism, organic acid production, and microbiota composition. Production of sterol metabolites (coprostanol, methylcoprostanol, ethylcoprostenol, ethylcoprostanol, and sitostenone) was observed in the transverse colon (TC) and descending colon (DC) vessels in general, from 24 and 48 h, respectively. Microbial activity was assessed through the production of organic acids, mainly acetate in all colon vessels, lactate in the AC, and butyrate and propionate in the TC and DC. A higher diversity in the microbial community was found in the TC and DC, in accordance with a higher sterol metabolism and organic acid production. Although the prebiotic effect of galactooligosaccharides was not detected, changes in microbiota composition (an increase in the *Parabacteroides* genus and the Synergistaceae and Lachnospiraceae families) indicated an enhancement of sterol metabolism.

KEYWORDS: *dynamic colonic fermentation, sterol metabolites, short-chain fatty acids, lactate, 16S rRNA gene sequencing*

INTRODUCTION

Diet is considered a modulator of the gut microbiota composition and function and could, therefore, influence the health of the host.[1] The major metabolites generated during colonic fermentation of non-digestible carbohydrates by the microbiota are short-chain fatty acids (SCFAs, such as acetate, propionate, and butyrate) that have been related to beneficial effects, including promotion of gut integrity and regulation of glucose homeostasis, lipid metabolism, appetite, immune system, and inflammatory response.[2]

In vitro fermentation studies in batch[3−5] or dynamic[6−9] models, using fecal inocula from healthy subjects, have demonstrated that galactooligosaccharides (GOS) stimulate the growth of *Bifidobacterium* and *Lactobacillus* species and imply an increase on SCFA production.

Likewise, plant sterols (PS) used for food enrichment as a result of their cholesterol-lowering effect[10] present a low absorption (0.1−15%) and reach the colon,[11] where they are subjected to the action of the gut microbiota, generating sterol metabolites, as in the case of cholesterol.[12] Milk-based fruit beverages (MfB), which can be enriched with PS,[13] are healthy foods as a result of their low fat content and the presence of antioxidant compounds, such as vitamin C, polyphenols, and carotenoids. In previous studies of our research group, a MfB enriched with PS had a cholesterol-lowering and anti-inflammatory effect in postmenopausal women with mild hypercholesterolemia.[14] As far as we are aware, only two studies carried out by our research group have reported the use of *in vitro* fermentation systems to evaluate the biotransformation of sterols (cholesterol and PS): first, applying batch-culture fermentation to a residue from the simulated gastrointestinal digestion of a MfB enriched with PS[15] and in a TIM-2 model feeding the system with different formats of PS (ingredient or standard).[16] In both studies, a preference for PS rather than for cholesterol as a substrate by the microbiota was demonstrated (production of ethylcoprostanol in batch culture and methylcoprostanone in TIM-2 was observed). In addition, the presence of PS during the batch-culture fermentation led to a decrease in Erysipelotrichaceae species and an increment of *Eubacterium hallii*,[15] whereas in the TIM-2 model, the presence of PS increased the prevalence of some genera belonging to the Firmicutes phylum using microbiota of lean or obese subjects. With regard to SCFA production, an increase of mainly acetate and butyrate was only reported in the fermentation with the lean microbiota.[16]

Special Issue: Food Bioactives and Health

Received: July 29, 2019
Revised: September 14, 2019
Accepted: September 16, 2019
Published: September 16, 2019

DOI: 10.1021/acs.jafc.9b04796
J. Agric. Food Chem. 2020, 68, 1884−1895

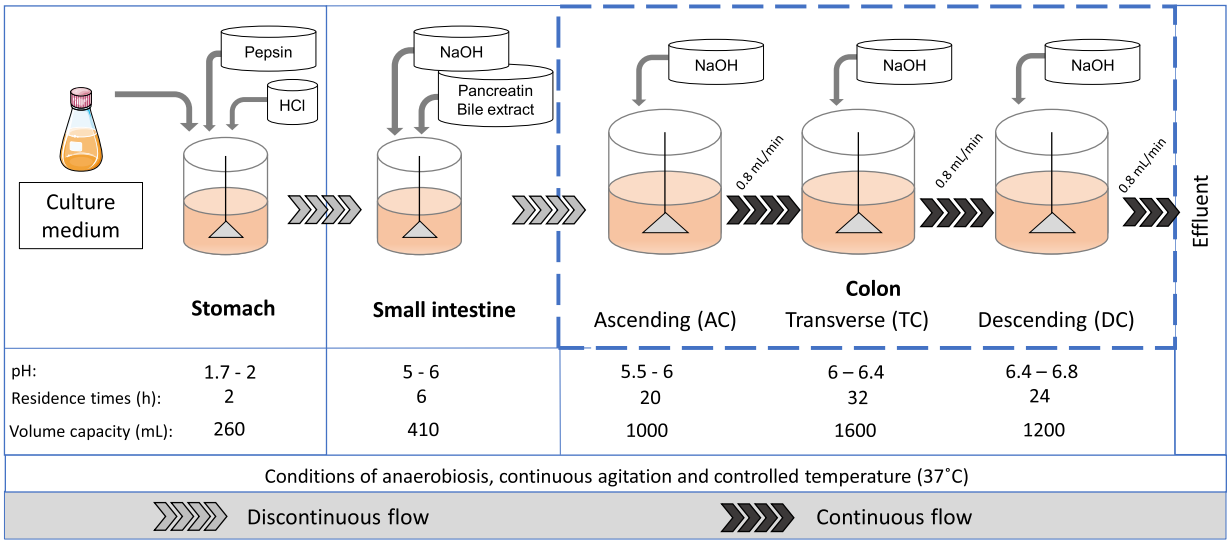

Figure 1. Schematic representation of the D−CGD.

It has recently been demonstrated that the addition of GOS to a similar beverage enriched with PS had no effect on the bioaccessibility of sterols.[17] Thus, this combined enrichment with GOS and PS could improve the beneficial effects previously mentioned for both bioactive compounds and could also modulate microbial genera implied in sterol metabolism. In addition, the presence of GOS does not affect organoleptic characteristics and shows high stability against low pH and high temperature.[18] Given the lack of studies on the metabolism of sterols present in food matrices using dynamic fermentation systems and the absence of literature regarding the evaluation of the presence of GOS on this biotransformation, the present study was carried out with the aim of shedding light on this issue. Thus, we have evaluated, for the first time, the impact of the dynamic *in vitro* fermentation of a food matrix (MfB) enriched with PS and GOS on sterol metabolism, organic acid production, and microbiota composition.

MATERIALS AND METHODS

Reagents. The sterol standards used were cholestane (≥97%) as the internal standard (IS), coprostanol (98%), cholesterol (99%), desmosterol (84%), stigmastanol (97%), stigmasterol (97%), β-sitosterol (99%), and lathosterol (99%); all of them were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Campesterol (96%) was provided by Chengdu Biopurify Phytochemicals, Ltd. (Sichuan, China), and brassicasterol (98%) and coprostanone (98%) were provided by Steraloids, Inc. (Newport, RI, U.S.A.). The derivatization reagents used were anhydrous pyridine from Acros Organics (Geel, Belgium) and *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) [1% trimethylchorosilane (TMCS)] from Sigma Chemical Co. (St. Louis, MO, U.S.A.). For determination of organic acids, butyrate, isobutyrate, lactate, propionate, and valerate were provided from Sigma Chemical Co. (St. Louis, MO, U.S.A.), acetate was provided from Janssen Chimica (Beerse, Belgium), formate was provided from Panreac (Barcelona, Spain), and isovalerate and phosphoric acid were provided from Fluka (Buch, Switzerland). Butylhydroxytoluene (BHT), pancreatin from porcine pancreas (EC 232.468.9), and pepsin from porcine gastric mucosa (E.C 3.4.23.1) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Sodium hydroxide and thioglycolate were provided by Merck (Whitehouse Station, NJ, U.S.A.). Dehydrated fresh bile (Difco Oxgall) was purchased from BD Biosciences (Oxford, U.K.). Water was purified using a Milli-Q system (Milford, MA, U.S.A.).

Experimental Beverage. A PS-enriched (1%, w/v) MfB with the addition of GOS (1.8%, w/v) was elaborated by the Hero Global Technology Center (Alcantarilla, Murcia, Spain) according to the manufacturing process described by Blanco-Morales et al.[17] This beverage contained skimmed milk with the addition of milk fat and whey protein concentrate enriched with milk fat globule membrane (MFGM, 49%), mandarin juice from concentrate (45%), banana puree (4%), microencapsulated free microcrystalline PS from tall oil (Lipophytol 146 ME Dispersible, Lipofoods), and GOS syrup (Vivinal GOS from Friesland Campina Ingredients). The energy and nutritional information per 100 mL was energy (kJ, kcal), 359.0/85.8; protein (g), 3.0; carbohydrates (g), 12.2; fat (g), 2.8; and fiber (g), 0.5. The cholesterol content was 13.6 mg/100 g of beverage, and the PS relative abundance was β-sitosterol, ≈80% (753 mg/100 g); sitostanol, ≈12% (105 mg/100 g); campesterol, ≈7% (58 mg/100 g); campestanol, ≈1% (9.2 mg/100 g); and stigmasterol, ≈0.7% (5.9 mg/100 g).[17]

Dynamic Gastrointestinal and Colonic Fermentation Model. The dynamic−colonic gastrointestinal digester (D−CGD) was developed by AINIA Technology Center (Valencia, Spain) based on the one developed and validated by Molly et al.[19,20] The equipment consists of five vessels that simulate the stomach, small intestine, ascending colon (AC), transverse colon (TC), and descending colon (DC), whose pH values, residence times, temperature (37 °C), and volume capacities were controlled by a software specifically developed by the aforementioned center (Figure 1). All vessels were connected by peristaltic pumps working semi-continuously in the stomach and small intestine and continuously in the colon stretch. The gastric digestion step was simulated by the addition of 60 mL of a 0.03% (w/v) pepsin solution (2100 units/mg) to the stomach vessel, whereas digestion on the small intestine was simulated by the addition of 240 mL of a solution containing pancreatin (0.9 g/L), $NaHCO_3$ (12 g/L) and oxgall dehydrated fresh bile (6 g/L) in distilled water. Anaerobiosis was maintained by flushing gaseous N_2 for 15 min twice a day.

A pool with the feces from five healthy volunteers who, in the previous month, had not received any antibiotic, hormonal, anabolic, or hypocholesterolemic treatments and had not consumed enriched foods or supplements with vitamins, carotenoids, probiotics/prebiotics, phytoestrogens or PS, herbal products, or foods high in fiber and who had not followed weight loss diets, suffered acute inflammation or diseases of the gastrointestinal tract, taken chronic medication, or were vegan or vegetarian was used. A fecal solution at 20% (w/v) was prepared with regenerated thioglycolate, inoculated in the colon vessels (50, 80, and 60 mL for AC, TC, and DC, respectively), and filled with culture medium up to a total volume of 1000, 1600, and 1200 mL, respectively. The culture medium,

DOI: 10.1021/acs.jafc.9b04796
J. Agric. Food Chem. 2020, 68, 1884−1895

elaborated according to Molly et al.,[19,20] provided all of the necessary nutritional components to simulate the conditions of the human colon and allowed for the growth of the intestinal microbiota.

An 11 day period was required to allow for the stabilization of the human fecal microbiota in the colonic vessels, during which 200 mL of culture medium was added to the stomach vessel 3 times a day. Samples of the fermentation liquids (FL) from the AC, TC, and DC vessels corresponding to time 0 were taken at the end of the microbiota stabilization period. Because the beverage provided substrates (sugars and proteins) for the microbiota, a mixture (1:1, v/v)[21] of the beverage and a modified culture medium was added to the system once per day. The modified culture medium was elaborated without carbohydrates and taking into account the content of proteins provided by the beverage. The other two additions were performed only with culture medium. The maintenance of the microbial population after the stabilization (time 0) and during treatment (72 h) periods was checked by plate counts of total anaerobic bacteria (on Schaedler agar under anaerobic incubation, 37 °C/72 h). No relevant differences were observed among both periods (6.5 versus 6.9 log CFU/mL for the AC, 7.4 versus 8.7 log CFU/mL for the TC, and 7.9 versus 8.3 log CFU/mL for the DC). The 8 h samples from all colon vessels were taken when the small intestinal contents had been poured into the AC (constituting the control of sterols provided by the beverage without fermentation). However, the following samples (from the AC, TC, and DC) were collected once a day, 15 min before the contents of the small intestine was transferred to the AC (corresponding to 24, 48, 72, 96, 120, 144, and 168 h). All collected FL were aliquoted and stored at −20 °C until sterols were analyzed.

Sample Preparation. As a result of the complexity of the composition of the FL, different sample preparations were evaluated: (i) filtration with a Millex-FH filter unit of 0.45 μm (Millipore, Milford, MA, U.S.A.), (ii) sonication (30 min) with subsequent centrifugation at 18 °C for 10 min at 4000 rpm (Eppendorf centrifuge 5810R, Hamburg, Germany) to obtain the supernatant phase of the FL, and (iii) sampling under continuous stirring. Taking into account a previous screening applying the methodology of Cuevas-Tena et al.,[22] the FL from the TC compartment at 120 h of fermentation (from now, experimental sample) was selected as a representative sample for these assays as a result of its larger quantity and diversity of sterol metabolites. Each sample preparation was assayed in duplicate, and the sterol content was determined in 150 μL of FL according to the methodology described in the following section.

Determination of Sterols by Gas Chromatography−Mass Spectrometry (GC−MS). Contents of sterols and metabolites, from now on sterols, in the samples of FL were determined in triplicate, applying the methodology validated by Cuevas-Tena et al.,[22] and applied to FL by our research group.[15,16] The amount of sterols quantified at 0 h was subtracted from the amounts measured at each sampling point. Different volumes of FL (25 μL for the analysis of ethylcoprostanol, campesterol, β-sitosterol, and sitostanol and 150 μL for coprostanol, cholesterol, methylcoprostanol, ethylcoprostenol, campestanol, stigmasterol, and sitostenone) were taken, and 20 μg of 5α-cholestane (IS) and 5 μL of 0.02% (w/v) BHT ethanolic solution were added. Then, hot saponification with 1 mL of 0.75 M potassium hydroxide (ethanolic solution) was carried out at 65 °C for 1 h, and subsequently, the unsaponifiable fraction was extracted with 3 × 2 mL of *n*-hexane. After evaporation until dryness under N_2, the unsaponifiable fractions were derivatized with 200 μL of 10:3 (v/v) BSTFA (1% TMCS)/pyridine at 65 °C for 1 h. The trimethylsilyl ether derivatives obtained were dissolved and filtered (Millex-FH filter unit, 0.45 μm Millipore, Milford, MA) with 3 × 1 mL of *n*-hexane, evaporated, and dissolved in 50 μL of *n*-hexane. A total of 1 μL of derivatized samples was injected into a GC−MS system (Thermo Science Trace GC-Ultra with ion trap ITQ 900, Waltham, MA, U.S.A.) under the same conditions as those reported by Cuevas-Tena et al.[22] Moreover, two extraction methods of the unsaponifiable fraction (with hexane, described above, and with diethyl ether, used by Alvarez-Sala et al.[23]) were applied to the saponified experimental sample (150 μL) taken under continuous stirring.

For the identification of sterols, relative retention times and the ion fragmentation patterns obtained from commercial standards, literature,[22,24−26] and databases[27,28] were used (characteristic ions are shown in Table S1 of the Supporting Information). Calibration curves for the quantification of sterols were performed with standards with the addition of IS (20 μg) (Table 1). Coprostanol curves were used for coprostanol, methylcoprostanol, ethylcoprostenol, and ethylcoprostanol quantification, stigmastanol for campestanol and sitostanol, and coprostanone for sitostenone. Moreover, the limit of detection (LOD) and limit of quantitation (LOQ) of sterols were determined from the background response of 0 h samples (n = 6) in each colon compartment (see Table 2).

Table 1. Calibration Curves with Sterol Standards Obtained by GC−MS

sterol	range (μg)	calibration equation[a]	linear correlation coefficient
coprostanol	0.002−0.42	$y = 0.0539x - 0.0181$	0.9973
coprostanone	0.50−8.00	$y = 0.0211x - 0.0150$	0.9921
cholesterol	0.50−6.03	$y = 0.0366x - 0.0175$	0.9927
desmosterol	0.04−0.84	$y = 0.0152x - 0.0008$	0.9930
brassicasterol	0.10−1.57	$y = 0.0851x - 0.0141$	0.9803
lathosterol	0.10−1.19	$y = 0.0744x - 0.0076$	0.9958
campesterol	0.25−4.00	$y = 0.0289x - 0.0100$	0.9959
stigmasterol	0.25−2.01	$y = 0.0317x - 0.0061$	0.9872
β-sitosterol[b]	0.50−5.00	$y = 0.0321x - 0.0121$	0.9931
	5.0−20.09	$y = 0.0589x - 0.1794$	0.9952
stigmastanol	0.10−4.07	$y = 0.0831x - 0.0258$	0.9813

[a] y = sterol area/internal standard area, and x = micrograms of sterol. [b] Two sets of calibration curves at different ranges of concentrations were prepared as a result of the great differences in the contents present in the sampling points.

Organic Acid Analysis. The FL (1 mL) from 8, 24, 72, 120, and 168 h sample points was centrifuged (12 000 rpm/4 °C/5 min, AccuSpin Micro 17R, Fisher Scientific), and 100 μL of the supernatant was dissolved up to 1 mL with 0.1% (w/v) phosphoric acid (pH 2.4). Then, the mixture was filtered [Millex 0.45 μm filter unit, polytetrafluoroethylene (PTFE), Millipore, Burlington, MA, U.S.A.], and 20 μL were injected in high-performance liquid chromatography (HPLC, Jasco Corporation, Japan) with an ultraviolet (UV) detector at 210 nm. System conditions were specified by Sarmiento-Rubiano et al.[29] The determined organic acids were SCFA (acetate, butyrate, propionate, and valerate), branched-chain fatty acids (isobutyrate and isovalerate), and intermediate metabolites (lactate and formate).

Microbiota Composition: DNA Extraction, 16S rRNA, Gene Sequencing, and Bioinformatics. Total DNA from FL samples (0.25 g) were isolated using the DNeasy PowerSoil Kit (Qiagen, Carlsbad, CA, U.S.A.), according to the instructions of the manufacturer. Then, the variable V3 and V4 regions of the 16S rDNA gene were amplified following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Code 15044223 Revision A). Gene-specific primers 515F/806R containing Illumina adapter overhang nucleotide sequences were selected according to Klindworth et al.[30] After 16S rDNA gene amplification, the multiplexing step was performed using Nextera XT Index Kit. DNA libraries were sequenced on the MiSeq platform using a paired-end 2 × 300 bp Reagent Kit (Illumina, San Diego, CA, U.S.A.). Raw reads generated from the MiSeq run were checked for quality and adapter trimmed using cutadapt 1.8.3[31] and FastQC version 0.11.5.[32] Resulting reads were analyzed using QIIME software version 1.9.1,[33] including forward and reverse read joining, quality filtering, chimera removal, and taxonomic annotation. Reads were clustered into 97% identity using an open-reference operational taxonomic unit (OTU) picking protocol against the 13_8 revision of

DOI: 10.1021/acs.jafc.9b04796
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Table 2. LOD and LOQ in Each Colon Compartment

		LOD[a]		LOQ[b]	
sterol	compartment	ng in assay	mg/compartment	ng in assay	mg/compartment
coprostanol	AC	5.2	0.03	17.3	0.12
	TC	2.6	0.03	8.6	0.09
	DC	50.4	0.40	168.0	1.34
cholesterol	AC	253.1	1.69	843.5	5.62
	TC	59.4	0.63	197.9	2.11
	DC	18.5	0.15	61.5	0.49
methylcoprostanol	AC	7.0	0.05	23.5	0.16
	TC	1.5	0.02	5.0	0.05
	DC	6.6	0.05	22.0	0.18
desmosterol	AC	7.8	0.05	26.1	0.17
	TC	2.0	0.02	6.5	0.07
	DC	4.7	0.04	15.5	0.12
brassicasterol	AC	2.1	0.01	7.0	0.05
	TC	1.0	0.01	3.3	0.04
	DC	1.0	0.01	3.2	0.03
lathosterol	AC	0.9	0.01	3.0	0.02
	TC	0.7	0.01	2.5	0.03
	DC	1.2	0.01	4.2	0.03
methylcoprostanone	AC	3.7	0.02	12.3	0.08
	TC	1.6	0.02	5.2	0.06
	DC	4.4	0.04	14.7	0.12
ethylcoprostenol	AC	3.0	0.02	10.1	0.07
	TC	3.3	0.04	11.1	0.12
	DC	3.0	0.02	9.9	0.08
ethylcoprostanol	AC	6.0	0.04	19.9	0.13
	TC	3.8	0.04	12.6	0.13
	DC	3.8	0.03	12.7	0.10
campesterol	AC	9.1	0.06	30.2	0.20
	TC	16.6	0.18	55.3	0.59
	DC	3.7	0.03	12.3	0.10
campestanol	AC	10.3	0.07	34.3	0.23
	TC	8.4	0.09	27.9	0.30
	DC	4.2	0.03	14.0	0.11
stigmasterol	AC	37.6	0.25	125.2	0.83
	TC	42.2	0.45	140.6	1.50
	DC	51.4	0.41	171.4	1.37
β-sitosterol	AC	30.2	0.20	100.6	0.67
	TC	53.8	0.57	179.2	1.91
	DC	54.4	0.44	181.4	1.45
sitostanol	AC	2.4	0.02	8.1	0.05
	TC	10.5	0.11	35.1	0.37
	DC	7.4	0.06	24.6	0.20
sitostenone	AC	11.5	0.08	38.4	0.26
	TC	11.3	0.12	37.5	0.40
	DC	11.8	0.09	39.3	0.31

[a]LOD = 3SD/slope of the calibration curve for each sterol. [b]LOQ = 10SD/slope of the calibration curve for each sterol.

the Greengenes database.[34] Taxonomic assignments was made using the RDP Naive Bayesian classifier. Chimeric sequences were removed from the reads using the Usearch6.1 algorithm. A biome table was constructed for downstream analyses, and OTUs represented by 10 or less sequences were removed. Calypso version 8.48[35] was applied to calculate α and β diversities, differences in community composition, and result visualization. For α diversity, the Shannon index was applied and statistical comparisons were performed using the analysis of variance (ANOVA) test at the genus level ($p < 0.001$). To analyze β diversity, Bray−Curtis dissimilarities were calculated using normalized data (total sum normalization combined with square root transformation) and a principal coordinate analysis (PCoA) plot was used to visualize differences among compartments.

Statistical Analysis. Statistically significant differences ($p < 0.05$) in the sterol content in the different sample preparations and sterol or organic acid contents in FL at the different fermentation times were assessed applying a one-way ANOVA, followed by Tukey's post hoc test. The Statgraphics Centurion XVI.I statistical package (Statpoint Technologies, Inc., Warrenton, VA, U.S.A.) was used.

RESULTS

Assays of Sample Preparation and Unsaponifiable Extraction. With regard to sample preparation, a great loss of sterols was observed after filtration and sonication + centrifugation compared to sampling under continuous stirring

DOI: 10.1021/acs.jafc.9b04796
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Table 3. Assays of Sample Preparation and Method for Unsaponifiable Extraction[a]

	filtered	sonication + centrifugation	sampled in continuous stirring	
	unsaponifiable extraction			
sterol	with hexane[22]	with hexane[22]	with hexane[22]	with diethyl ether[23]
coprostanol		4.15 ± 0.03 x	14.68 ± 2.01 y	13.77 ± 1.55 y
cholesterol	*b*	*b*	20.54 ± 2.97 x	24.34 ± 2.17 x
methylcoprostanol		4.58 ± 0.04 x	29.97 ± 4.57 y	23.63 ± 1.32 y
ethylcoprostenol			4.46 ± 0.23 x	4.72 ± 0.11 x
ethylcoprostanol	*b*	18.44 ± 0.64	*c*	*c*
campesterol	4.91 ± 0.09 x	7.37 ± 0.07 y	*c*	*c*
campestanol	*b*	*b*	13.03 ± 1.70 x	9.84 ± 0.80 x
stigmasterol			8.14 ± 1.12 x	7.54 ± 1.04 x
β-sitosterol	*b*	68.40 ± 0.82	*c*	*c*
sitostanol	5.47 ± 0.15 x	9.74 ± 0.27 y	*c*	*c*
sitostenone			8.70 ± 0.36	*b*

[a]Values were expressed as the mean ± SD (n = 2) in mg/compartment. Assays were performed with FL (150 μL) from the TC at 120 h of beverage fermentation. [b]Value was below the lowest point of the quantification curve. [c]Value was above the highest point of the quantification curve (see Table 1). Different letters (x and y) denote statistically significant differences (p < 0.05) in the same sterol in each sample preparation.

(Table 3). In the latter, sterols present in the beverage as well as the metabolites coprostanol, methylcoprostanol, and ethylcoprostenol could be determined. It should be indicated that ethylcoprostanol, campesterol, *β*-sitosterol, and sitostanol were present in amounts above the highest point of the calibration curve. Although no significant differences in sterol contents were observed between the evaluated methods using sampling under continuous stirring, sitostenone could only be determined by means of unsaponifiable extraction with hexane (method by Cuevas-Tena et al.)[22] (see Table 3). Therefore and taking into account that the extraction by Cuevas-Tena et al.[22] is less time-consuming and requires a lower volume of solvents compared to that by Alvarez-Sala et al.,[23] sampling under continuous stirring and using hexane for unsaponifiable extraction was chosen as the best option for sterol determination.

The accuracy of this method was tested, in triplicate, with 100 μL (or 25 μL in the case of *β*-sitosterol) of the experimental sample. The samples were spiked with standards of the main sterols at levels similar to their contents in FL; recoveries (%) were 116 ± 3 for cholesterol, 109 ± 3 for campesterol, 90 ± 17 for *β*-sitosterol, and 105 ± 3 for sitostanol. The method is of good accuracy because the suitable recovery range proposed by AOAC International for analyte concentrations of μg/g is 80−115%.[36]

Sterol Metabolism. The evolution of sterols throughout the fermentation experiment from the AC, TC, and DC is shown in Figure 2. In the Supporting Information, the mean sterol contents ± standard deviation (SD) values (Tables S2−S4 of the Supporting Information) as well as a representative chromatogram of the sterols present in a sample from the fermentation of the beverage (Figure S1 of the Supporting Information) are shown. Non-quantifiable contents of desmosterol, brassicasterol, lathosterol, and methylcoprostanone (values below the LOQ indicated in Table 2) were observed in all colon vessels during beverage fermentation (data not shown).

Cholesterol contents along the fermentation in the AC presented a marked increase until 48 h, with an irregular evolution thereafter (Figure 2a). In the TC, the maximum amount of cholesterol was detected at 72 h (when coprostanol started to be detected), reaching a plateau. The coprostanol/cholesterol ratios increased along fermentation times from 0.2 (72 h) to 0.7 (for 120, 144, and 168 h). In the case of the DC, cholesterol was detected at 72 h and kept relatively stable along the entire assay (7−9 mg/compartment). In this compartment, the evolution of the coprostanol contents is similar to that in the TC with slightly higher coprostanol/cholesterol ratios (from 0.9 at 72 h to 1.3 at 120 h and onward).

A progressive increase in the *β*-sitosterol (Figure 2b) and sitostanol contents (Figure 2c) was observed during fermentation, reaching maximum levels between 48 and 96 h and remaining stable afterward. Ethylcoprostanol (Figure 2b) was only detected in the TC and DC from 48 and 24 h, with a significant increase (p < 0.05) until 120 and 144 h, respectively. This fact is reflected in the ethylcoprostanol/*β*-sitosterol ratios, which increased during the fermentation from 0.01 (48 h) to 0.46 (from 120 h onward) in the TC and from 0.2 (24 h) to 0.7 (144 h) in the DC (Figure 2b). Moreover, sitostenone (another *β*-sitosterol metabolite) was only detected at 144 h in the TC (9.4 ± 0.3 mg/compartment) and from 72 h onward in the DC (mean value of 7.7 ± 0.3 mg/compartment) (see Tables S3 and S4 of the Supporting Information).

In the case of the other PS (campesterol, campestanol, and stigmasterol) (panels d, e, and f of Figure 2, respectively), a similar trend of accumulation was observed, reaching, in general, their maximum amounts in all vessels between 96 and 120 h. With regard to metabolites, a production of methylcoprostanol (from campesterol) was detected from 72 and 48 h in the TC and DC, increasing until 120 and 144 h, respectively (Figure 2d). This tendency is also observed in the increments of the ratios throughout fermentation: from 0.08 (72 h) to 0.26 (120 h) in the TC and from 0.2 (48 h) to 0.5 (168 h) in the DC. Additionally, the presence of ethylcoprostenol (from stigmasterol) was detected from 96 h in the TC and from 72 h in the DC, remaining constant until the end of the fermentation assay, whereas the ratios increased from 0.5 (96 h) to 0.6 (120 h) in the TC and decreased from 1.0 (72 h) to 0.8 (168 h) in the DC (see Tables S3 and S4 of the Supporting Information).

Analysis of Organic Acids. Contents of organic acids through the fermentation in all colon compartments (the AC, TC, and DC) are shown in Figure 3. Moreover, the mean organic acid contents are shown in Table S5 of the Supporting Information. Results showed that the fermentation of the

DOI: 10.1021/acs.jafc.9b04796
J. Agric. Food Chem. 2020, 68, 1884−1895

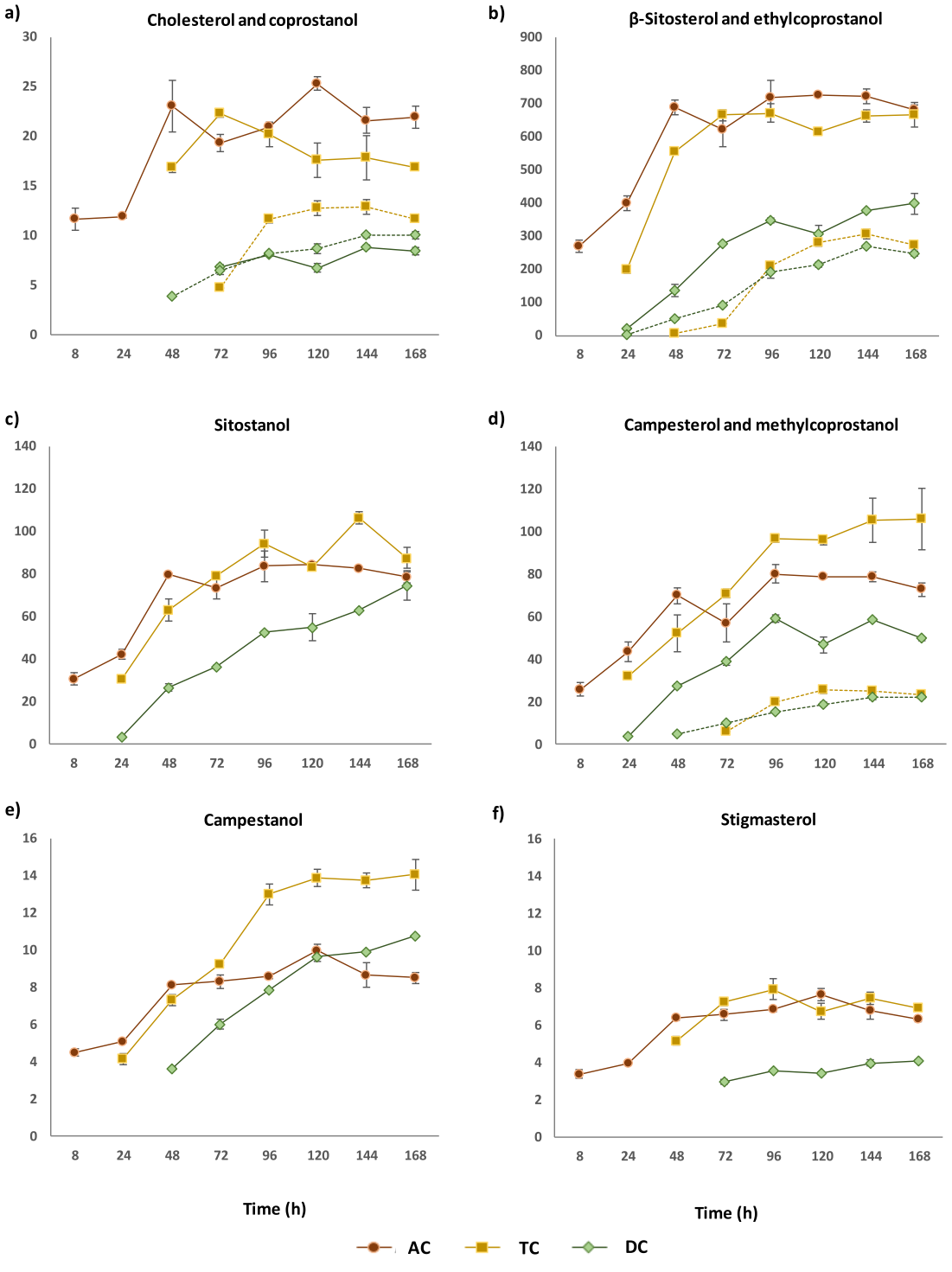

Figure 2. Evolution of sterol (continuous line) and metabolite (dashed line) contents (mg/compartment) in the AC, TC, and DC.

beverage led to a significant increment in total organic acid production in all colon compartments (Figure 3) from 72 h in the AC and from 24 h in the TC and DC. Higher concentrations of organic acids were determined in the distal compartments with respect to the AC, and increments at 168 h with respect to initial values (8 h) were also higher (1.5-fold for the AC, 2.0-fold for the TC, and 2.8-fold for the DC).

The organic acid profile was compartment-dependent, differing the AC with respect to the TC and DC (isobutyrate > acetate > lactate > butyrate versus acetate > propionate > butyrate > isobutyrate, respectively) (Figure 3). In the AC, a significant increase in lactate contents at 24 h of fermentation was observed and maintained relatively stable thereafter. Lactate contents in the TC and DC remained stable during the fermentation (0.27 mM) (see Table S5 of the Supporting Information). Isobutyrate contents showed a significant increase after 72 h of fermentation in the AC, whereas in the TC and DC, low contents were detected (0.3−3.2 mM). Acetate contents in all colon compartments increased significantly, 1.5-fold in the AC, 2.3-fold in the TC, and 2.6-

DOI: 10.1021/acs.jafc.9b04796
J. Agric. Food Chem. 2020, 68, 1884−1895

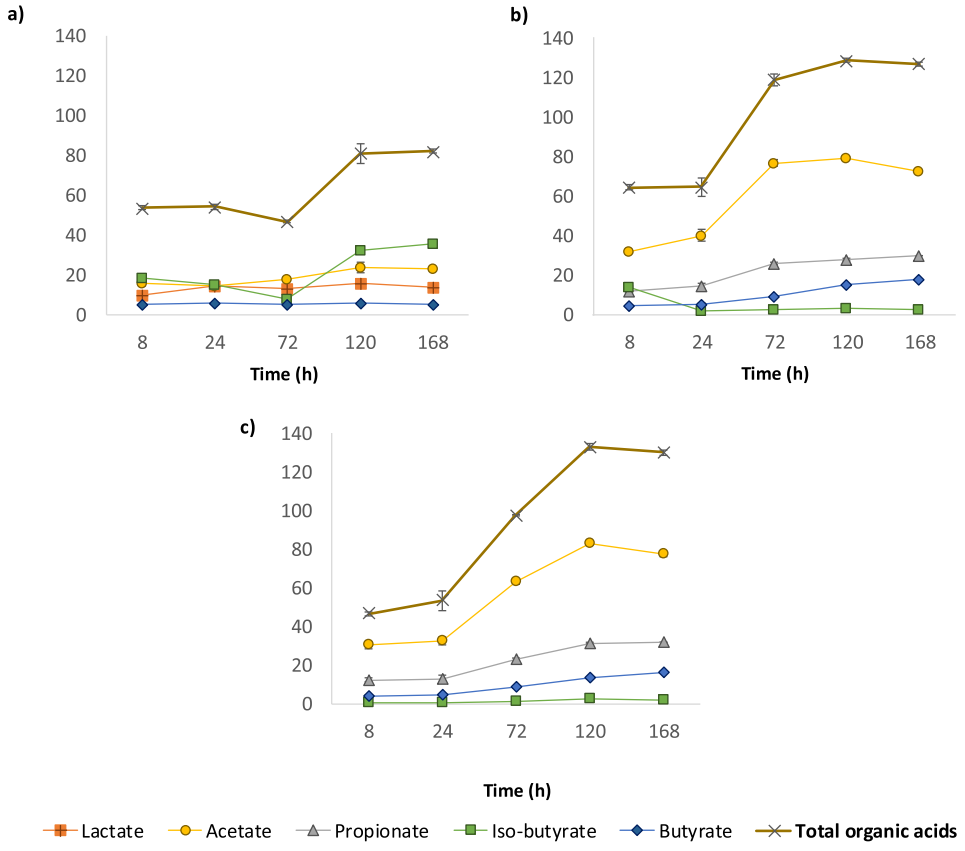

Figure 3. Total and individual organic acid contents (mM) in the (a) AC, (b) TC, and (c) DC.

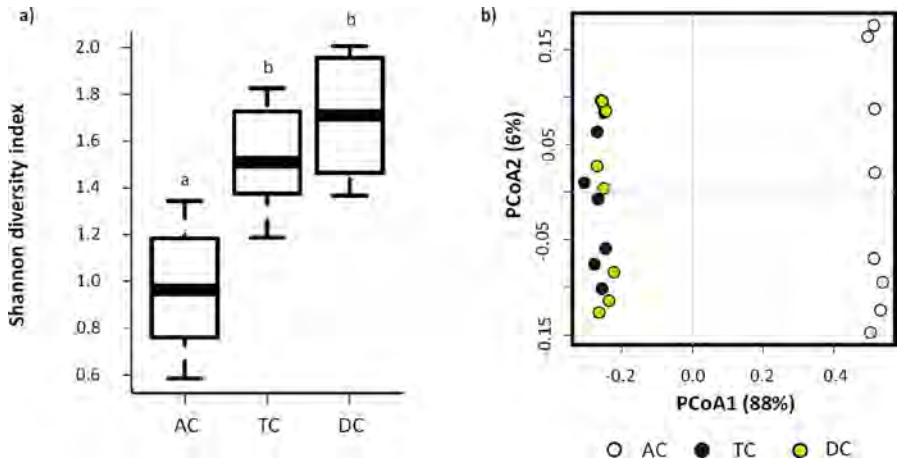

Figure 4. (a) Shannon diversity index and (b) PCoA score plot of microbiota composition based on colon compartment (AC, TC, and DC).

fold in the DC, at the end of the fermentation with respect to 8 h. Relatively stable amounts of butyrate were detected in the AC, ranging between 5.2 and 6.2 mM, whereas a similar progressive increase were observed in the TC and DC (increments of 3.8- and 4.0-fold, respectively). Although no contents of propionate were observed in the AC, a significant increase was observed in the TC (from 24 h) and DC (from 72 h). However, its contents remained stable in these compartments from 120 h, showing increments at 168 h, with respect to initial values, of 2.5- and 2.6-fold, respectively. Finally, isovalerate and valerate were a minority in all colon compartments (0.5−2.7 and 0.3−1.3 mM, respectively), while formate was only detected in the AC (2.1−4.2 mM) (Table S5 of the Supporting Information).

Microbiota Composition. The Shannon diversity index (Figure 4a) showed no differences between the TC and DC compartments, although it significantly increased ($p < 0.001$) with respect to the AC, thus demonstrating a higher species richness and evenness in the distal compartments. With regard to PCoA score plot (Figure 4b), the AC compartment was grouped in a separate cluster than the TC and DC, which is indicative that the different conditions of the compartments led to a distinctive microbial community. The relative abundance of the genera during the fermentation of the beverage is shown in Figure 5. In the AC, the *Clostridium*

DOI: 10.1021/acs.jafc.9b04796
J. Agric. Food Chem. 2020, 68, 1884−1895

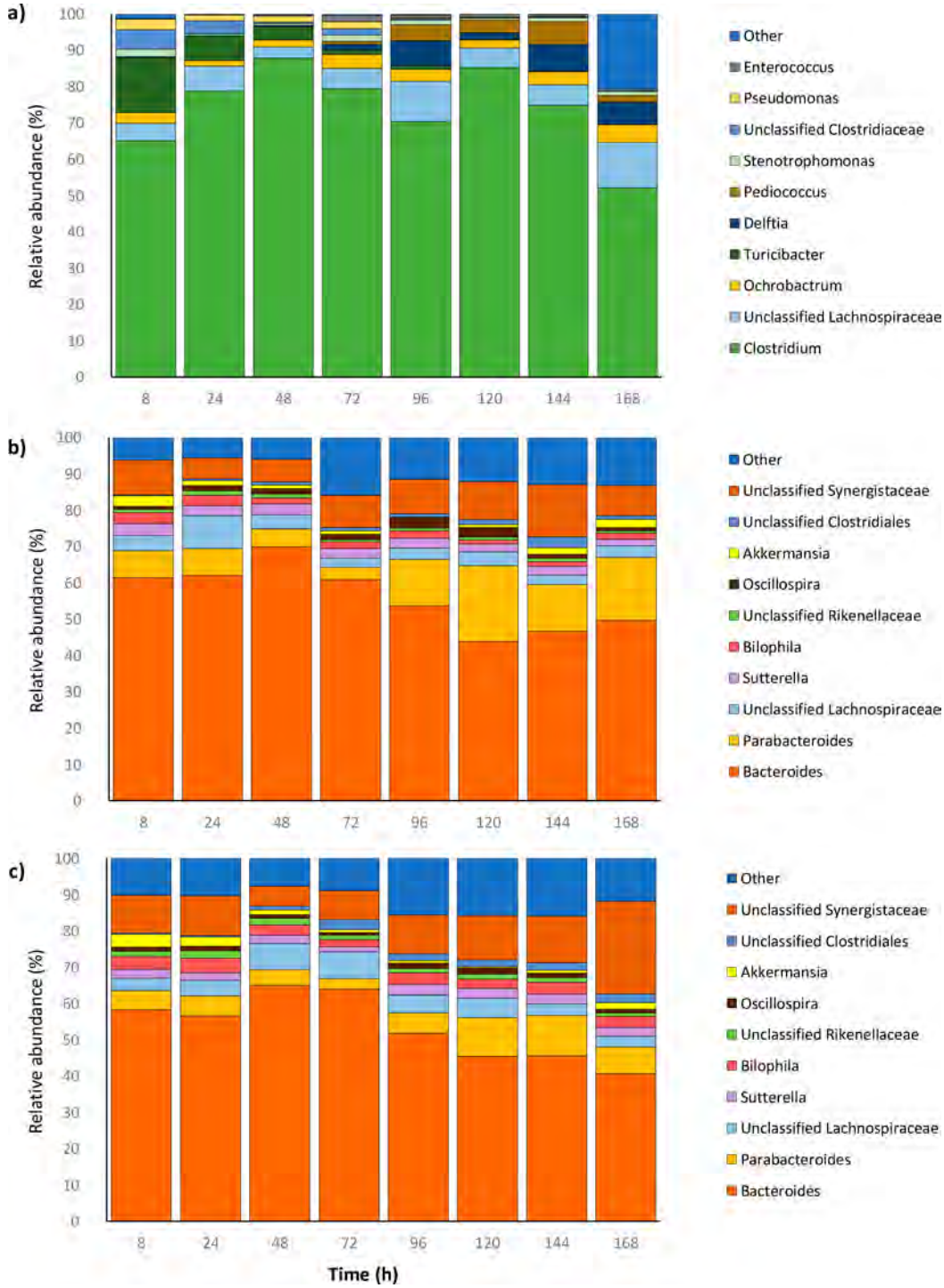

Figure 5. Changes in microbiota composition at the genus level (relative abundance) in the (a) AC, (b) TC, and (c) DC.

genus is the most abundant during all of the experiments, showing a maximum of abundance at 48 h (88%) and a minimum at 168 h (52%) with an irregular evolution (Figure 5a). The unclassified genus of the family Lachnospiraceae and the *Ochrobactrum* genus also presented an irregular trend, reaching their maximum contents at 168 h (changes in abundance were from 5 to 12% and from 3 to 5%, respectively). A progressive decrease of the *Turicibacter* genus over time was observed, from 15 to 0.1%. Other genera, such as *Delftia* and *Pediococcus*, increased with respect to their initial values (0.2 and 0%) during fermentation, presenting maximum contents at 144 h (7 and 6%, respectively). Finally, *Stenotrophomonas*, an unclassified genus of the family Clostridiaceae, *Pseudomonas*, and *Enterococcus* genera tended, in general, to decrease during fermentation, showing abundances lower than 5%.

In contrast, in the TC and DC (panels b and c of Figure 5, respectively), the *Bacteroides* genus showed a trend to decrease during the fermentation of the beverage from 72 h, with their content ranging between 70 and 44% for the TC and 65 and 41% for the DC. A marked increase in the abundance of the *Parabacteroides* genus was observed at 96 h in the TC and 120

DOI: 10.1021/acs.jafc.9b04796
J. Agric. Food Chem. 2020, 68, 1884−1895

h in the DC, with their abundances relatively maintained over time. Unclassified genus belonging to the Synergistaceae family tended to increase from 24 h in the TC (from 5.8 to 14.4%) and 48 h in the DC (from 5.5 to 25.5%). In addition, the unclassified genus of the family Lachnospiraceae presented an increase at 24 h in the TC (from 4.2 to 9.1%), whereas in the DC, the maximum abundance was observed at 48 and 72 h of fermentation (from 3.4 to 7.3%), decreasing in both cases thereafter. The remaining genera (*Sutterella*, *Bilophila*, unclassified Rikenellaceae, *Oscillospira*, *Akkermansia* and unclassified Clostridiales and Synergistaceae) showed an irregular evolution and, in any case, exceeded 5% of abundance.

DISCUSSION

In this study, for the first time, the metabolism of sterols present in a food matrix (MfB) enriched with PS and GOS, which has demonstrated beneficial systemic biological effects, has been assessed using a five-vessel dynamic simulator, which includes a complete process of gastric digestion and colonic fermentation. Moreover, the effect of the fermentation of the beverage upon organic acid production and microbiota composition was also evaluated to estimate other potential effects in the colonic tract.

Sterol metabolism was only observed in the distal colon compartments (the TC and DC) during the fermentation of the beverage. The presence of coprostanol, methylcoprostanol, and ethylcoprostanol has been described in other *in vitro* studies through the formation of different metabolites, such as phytostanone and phytostenone intermediates.[12] Besides, a direct pathway has been suggested for coprostanol formation.[37] In the present study, the non-detection of sterol intermediate metabolites suggests their rapid bioconversion or, in the case of cholesterol, that the dominant route for its degradation was the direct pathway of conversion to coprostanol. In contrast to our results, a recent study[15] reported the absence of cholesterol metabolism during the batch fermentation of a residue from the gastrointestinal digestion of a PS-enriched beverage during 48 h. Only a decrease in campesterol and an increment in ethylcoprostanol contents (39 and 50%, respectively) at 48 h of fermentation were statistically significant, suggesting that gut microbiota prefers PS as substrate. On the other hand, the fermentation of approximately 2 g of PS (from an ingredient source of PS or a commercial standard mixture of PS) during 72 h using a TIM-2 system and fecal inoculum from lean subjects[16] resulted in a drop of coprostanol contents (41−61%) and an increase in coprostanone (109−136%) and cholestanol (27−29%) contents with respect to the initial values. Moreover, production of methylcoprostanone was observed from 24 to 72 h (8- and 25-fold increases in the PS ingredient or commercial standard mixture of PS, respectively). In contrast, a lack of both cholesterol and PS metabolism was detected when the assay was carried out with fecal inoculum from obese subjects. Moreover, it is noteworthy that higher conversion ratios of the main sterols were detected in the DC versus TC, cholesterol > stigmasterol > β-sitosterol > campesterol, considering the maximum ratios.

With regard to organic acid production, acetate increased in all colon compartments, while butyrate and propionate only increased in the TC and DC. In fact, higher organic acid production was detected in the DC and TC versus AC. The similar microbiota in the TC and DC support the above-mentioned results (for organic acids and also sterol metabolism), showing more similarity and a more diverse community with respect to the AC. This is in accordance with the results reported in a simulator of the human intestinal microbial ecosystem (SHIME) model,[38] whose authors suggested that the lower species richness observed in the AC was probably due to the lower pH and the higher concentration of bile salts. Moreover, the different pH conditions of the vessels favor the selective growth of microbial populations. Similar to our results, in batch culture models,[39,40] higher *Bacteroides* levels (up to 80% of total eubacteria) were observed with the increase of pH from 5.5 (AC) to 6.5−6.7 (DC), suggesting that a mild acidic pH might limit the *Bacteroides* population in the proximal colon. In our study, a predominance of *Bacteroides* was observed in the colon compartments with cholesterol metabolism (the TC and DC), although with a decreasing tendency during the fermentation of the beverage. The genera *Bacteroides* and *Eubacterium* have only been reported, in the studies carried out several years ago with isolated cultures, to be cholesterol-reducing bacteria related to the direct as well as indirect routes for cholesterol metabolism.[12,41−43] An absence of *Eubacterium* spp., the only species associated with the PS biotransformation pathway,[12,44] was observed in the present study, being suggestive of the involvement of other bacterial species in the PS metabolization pathway.

Production of organic acids has been studied in the presence of GOS or PS using *in vitro* studies. According to our results, in the presence of both compounds an increase in acetate and butyrate contents[6−9,16,45,46] and also propionate and lactate was observed.[8,16,46] Butyrate- and propionate-producing bacteria (species belonging to Lachnospiraceae and Bacteroidetes)[47] are most representative in the TC and DC. Moreover, accumulation of lactate was only observed in the AC, being indicative of a faster fermentation of the substrate,[48] and is in accordance with the carbohydrate-excess conditions of this compartment as a result of the presence of GOS from the beverage. In the TC and DC, lactate contents were low and remained stable, suggesting that lactate from the AC could be used in the distal compartments for the production of butyrate and propionate (showing their highest increments in this compartments).

Furthermore, to estimate other potential effects of the combined addition of PS and GOS, changes in microbiota composition were also evaluated. Increments of *Bifidobacterium* and *Lactobacillus* have been reported during the fermentation of GOS in different models (three-stage continuous culture systems[6,7,9] and TIM-2 model[8]) using concentrations that ranged from 1 to 10 g/day. However, in the present study, these effects were not observed, thus suggesting that the combination of both bioactive compounds (PS and GOS) could influence the prebiotic function of GOS, besides improving the fast biotransformation of PS, as mentioned above. In our work, the *Clostridium* genus presented an increasing tendency in the AC, which has also been reported after the fermentation of a PS ingredient in the TIM-2 model with microbiota of obese subjects.[16] However, after GOS fermentation, decreases in the *Clostridium histolyticum* group were observed.[7] On the other hand, the *Bacteroides* genus tended to decrease, while *Parabacteroides* tended to increase in the TC and DC (both belonging to the Bacteroidetes phylum). Fermentation of PS in batch culture is related to the increase of some members of the uncharacterized S-24 family of Bacteroidetes,[15] and in a study carried out with hamsters,

DOI: 10.1021/acs.jafc.9b04796
J. Agric. Food Chem. 2020, 68, 1884−1895

the addition of PS to a high-fat diet showed an increase of *Bacteroides*.[45] However, *in vitro* fermentation of GOS has shown the decrease of *Bacteroides*−*Prevotella* spp.[7,8] In our study, unclassified Lachnospiraceae genera increased in the AC and at short fermentation times in the TC and DC. Similarly, increments of different species belonging to this family have been reported after the fermentation of PS in batch culture (*E. hallii*),[15] in a TIM-2 model carried out with microbiota from lean subjects (*Coprococcus*),[16] in an *in vivo* study with hamsters (*Coprococcus*),[45] and also after GOS fermentation (*Eubacterium rectale*−*Clostridium coccoides* group).[6,7] Thus, in general, changes in microbiota composition seem to be more influenced by the presence of PS than GOS.

Therefore, the results of the current study provide valuable novel insights into the impact of the fermentation of a complex food matrix (MfB) with GOS on sterol metabolism. It has been demonstrated that sterol metabolism is colon-compartment-dependent and that the presence of GOS enhances their rapid biotransformation (with higher ratios for cholesterol than PS). Likewise, organic acid production was higher in the distal compartments, in accordance with a higher diversity of the microbial community. In addition, effects on microbiota composition during beverage fermentation could be determined by the higher PS presence, showing an increase in the Bacteroidetes phylum and Lachnospiraceae family (related to PS fermentation). However, because *in vitro* fermentation models are only an approximation of the *in vivo* situation, the results of this study should be considered a first stage for future human trials, taking into account the interindividual variability in gut microbiota composition.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b04796.

GC−MS representative chromatogram (Figure S1), characteristic MS ions (Table S1), and sterol and organic acid contents (Tables S2−S5) (PDF)

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Funding

This study was financed by the Spanish Ministry of Economy and Competitiveness (MINECO) and the European Regional Development Fund (FEDER, AGL2015-68006-C2-1-R). Virginia Blanco-Morales holds a research contract under the aforementioned project (CPI-17-025).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful to Hero España, S.A. for manufacturing the beverage tested in the present study and AINIA Technology Center (Valencia, Spain) for their contribution, in particular to Dra. Blanca Viadel (Department of Biotechnology, AINIA, Spain). The authors also thank Prof. Dieter Lütjohann for his advice concerning mass spectra interpretation.

■ ABBREVIATIONS USED

AC, ascending colon; BHT, butylhydroxytoluene; DC, descending colon; D−CGD, dynamic−colonic gastrointestinal digester; FL, fermentation liquid; GC−MS, gas chromatography−mass spectrometry; GOS, galactooligosaccharide; IS, internal standard; LOD, limit of detection; LOQ, limit of quantitation; MFGM, milk fat globule membrane; MfB, milk-based fruit beverage; BSTFA, *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide; PS, plant sterol; SCFA, short-chain fatty acid; TC, transverse colon; TMCS, trimethylchlorosilane

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DOI: 10.1021/acs.jafc.9b04796
J. Agric. Food Chem. 2020, 68, 1884−1895

Anexo II:
Artículos en revisión

In vitro colonic fermentation of a plant sterol-enriched beverage in a dynamic-colonic gastrointestinal digester

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In vitro colonic fermentation of a plant sterol-enriched beverage in a dynamic-colonic gastrointestinal digester

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research paper
Keywords:	dynamic colonic fermentation; gut microbiota; milk-based fruit beverage; sterols; organic acids
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ABSTRACT

This study aims to evaluate the impact of a plant sterol-enriched milk-based fruit beverage on the sterol metabolism, organic acid production and microbiota composition by means of a dynamic gastrointestinal and colonic fermentation model. After one week of fermentation, an absence of sterol metabolites was reported, in accordance with the lack of microbiota related to their metabolism. Increments of acetate, butyrate and propionate were observed in all colon vessels and production of lactate in the first hours of fermentation was reported in the ascending colon. Additionally, results showed that the fermentation of the beverage also modulated certain members of the microbiota: *Bifidobacterium* (within 24 hours) and *Megasphaera* genera were stimulated, and *Mitsuokella* was reduced in the ascending colon, while the growth of *Klebsiella* genus was promoted and Bacteroidetes phylum decreased in the distal compartments. These results show that the beverage modulates the microbial community and activity, which could be physiologically relevant to the host.

Keywords: dynamic colonic fermentation, gut microbiota, milk-based fruit beverage, sterols, organic acids

1. Introduction

Milk-based fruit beverages (MfB), which present a healthy nutritional profile due to the low-fat content and the bioactive compounds provided by the fruit juice, have previously been used in studies by our research group as a matrix for plant sterol (PS) enrichment showing a cholesterol-lowering, anti-inflammatory and reduction of osteoporosis risk effect in a target population of postmenopausal women with mild hypercholesterolemia (Álvarez-Sala et al., 2018; Granado-Lorencio et al., 2014). Other intestinal beneficial effects such as protection against oxidative damage and pro-inflammatory mediators were also observed in differentiated Caco-2 cells (López-García, Cilla, Barberá, & Alegría, 2019 and 2017).

However, although PS are widely used by the food industry, studies on the metabolism of these bioactive compounds by the microbiota are scarce. Due to their low absorption (4-16%), PS reach the colon where they are susceptible to the action of the microbiota, resulting in the formation of PS metabolites (similarly to non-absorbed cholesterol) (Cuevas-Tena, Alegría, & Lagarda, 2018a). Previous studies reported a preference for PS as substrate by the microbiota rather than for cholesterol as observed in *in vitro* fermentation models (Cuevas-Tena, Alegría, Lagarda, & Venema, 2019; Cuevas-Tena et al., 2018b).

It is well documented that diet modulates microbiota composition and function, and reciprocally digestion and absorption of food components are influenced by the microbial community, therefore, playing an important role in the health of the host (Danneskiold-Samsøe et al., 2019). In this context, *in vitro* dynamic gastrointestinal and fermentation systems are a suitable option to evaluate reciprocal interactions between diet and microbiota composition and activity, since they mimic physiological conditions of temperature, pH and residence times and present the advantage of having colon compartment-specific separation (Moon, Li, Bang, & Han, 2016). These systems have usually been used to evaluate the fermentation of food components such as polyphenols, fibers, etc (Wang et al.,

2019; Ekbatan et al., 2016; Dueñas et al., 2015). Only two studies have used multi-compartmental systems to evaluate the colonic fermentation of food matrices similar to MfB: orange juice (Duque, Monteiro, Adorno, Sakamoto, & Sivieri, 2016) and a PS-enriched MfB (Blanco-Morales et al., 2020), being the latter the only study in which sterol metabolism has been evaluated. However, this study, carried out by our research group, used a combined enrichment of PS and galactooligosaccharides (GOS), and, therefore, the modulation exerted by the presence of the prebiotic did not allow the observation of possible effects of the PS-enriched MfB. Thus, the aim was to evaluate the effect of dynamic *in vitro* colonic fermentation of a PS-enriched MfB on the PS metabolism, organic acid production and microbiota composition. With the present study, we aim to shed light on the interactions between a food matrix containing PS and the microbial community in view of a potential health effect of this beverage besides the aforementioned ones.

2. Material and methods

2.1 Reagents

Pancreatin from porcine pancreas (EC 232.468.9) and pepsin from porcine gastric mucosa (E.C 3.4.23.1) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium hydroxide and thioglycolate were provided by Merck (Whitehouse Station, NJ, U.S.A), whereas dehydrated fresh bile (Difco™ Oxgall) was from BD Biosciences (Oxford, U.K).

2.2 Beverage

The beverage was elaborated by the Hero Global Technology Center (Alcantarilla, Murcia, Spain) and its manufacturing process is described by Blanco-Morales et al., 2018. The PS-enriched MfB contained (w/w) skimmed milk with the addition of 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 PS from tall oil (1%) (Lipophytol® 146 ME Dispersible, Lipofoods, Barcelona, Spain).

The energy and nutritional information of the beverage is shown in Table 1. Cholesterol content of the beverage was 13.1 mg/100 g, and the PS relative abundance was 79.7% for β-sitosterol (717 mg/100 g), 11.8% for sitostanol (100 mg/100 g), 6.2% for campesterol (55 mg/100 g), 1.2% for campestanol (8.9 mg/100 g) and 0.7% for stigmasterol (5.7 mg/100 g) (Blanco-Morales et al., 2018).

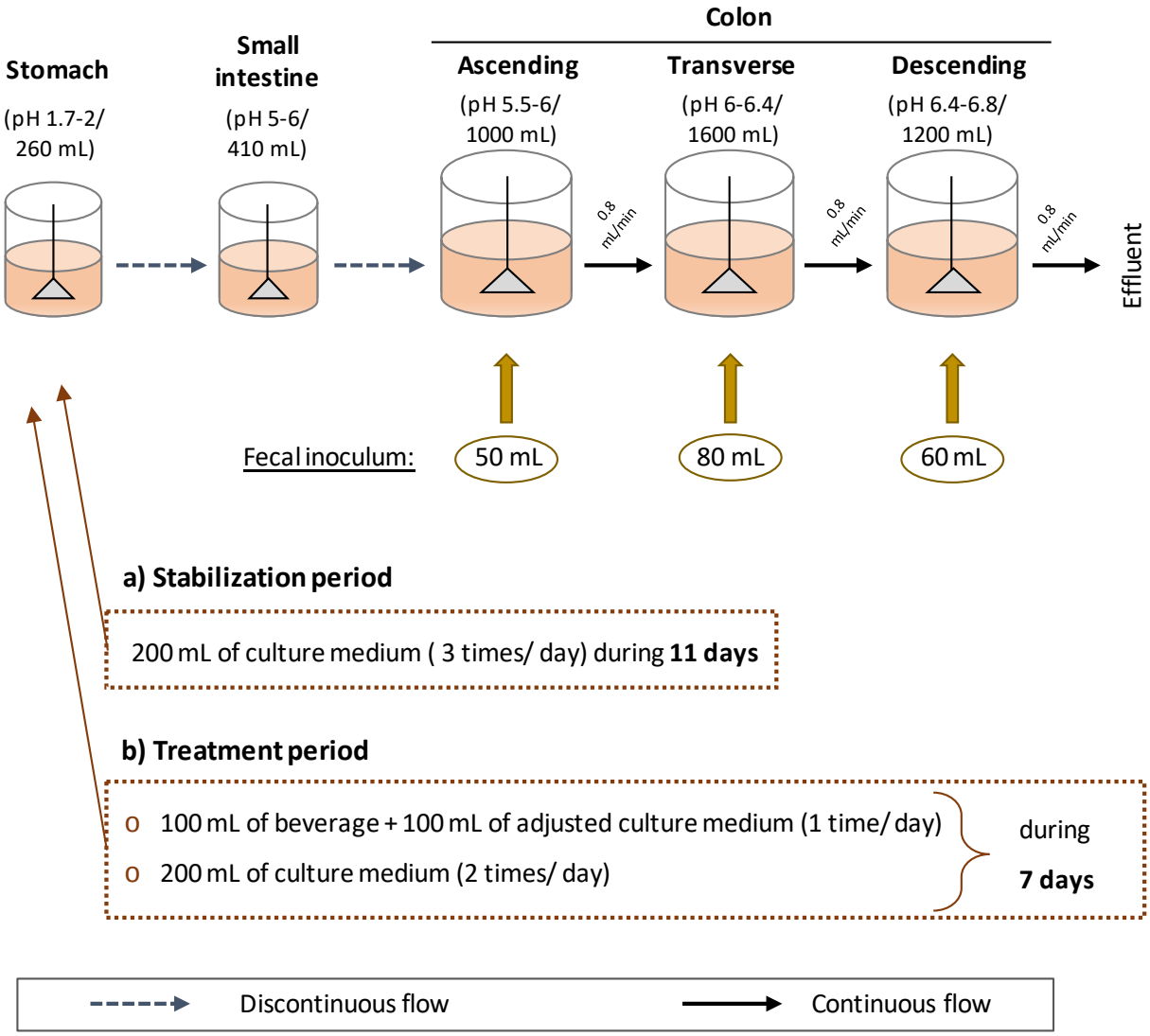
Table 1. Nutritional composition of the plant sterol-enriched milk-based fruit beverage.

Contents per 100 mL	
Energy (KJ, Kcal)	292.0, 69.9
Protein (g)	3.0
Carbohydrates (g)	9.0
Fat (g)	2.4
Fiber (g)	<0.5

2.3 Dynamic-Colonic Gastrointestinal Digester

The fermentation assay was carried out in the AINIA Technology Center (Valencia, Spain) by using a dynamic-colonic gastrointestinal digester (D-CGD) (see Figure 1). The setup of the D-CGD as well as the methodology carried out for the PS-enriched MfB fermentation was previously described by Blanco-Morales et al. (2020). Briefly, stomach conditions were simulated by the addition of 60 mL of pepsin solution at 0.03% (w/v) (2100 U/mg) for 2 h, whereas the passage of food in the small intestine was simulated by the addition of 240 mL of a solution elaborated with pancreatin (0.9 g/L), $NaHCO_3$ (12 g/L) and oxgall dehydrated fresh bile (6 g/L) in distilled water for 6 h. To simulate colonic fermentation, the ascending colon (AC), transverse colon (TC) and descending colon (DC) vessels were inoculated with a fecal solution elaborated at 20% (w/v) from a pool of feces diluted with regenerated thioglycolate. These fecal samples were obtained from 5 healthy subjects who followed the inclusion criteria (Blanco-Morales et al., 2020).

Figure 1. The setup of the dynamic-colonic gastrointestinal digester (D-CGD) during the stabilization and the treatment period.



The fecal inoculum was stabilized for 11 days (stabilization period), adding to the stomach vessel 200 mL of culture medium three times a day (elaborated according to Molly, Woestyne, Smet, & Verstraete (1994) and Molly, Woestyne, & Verstraete (1993)). After the stabilization period, the reactor was fed daily during 7 days (treatment period) with a mixture of 100 mL of beverage and 100 mL of an adjusted culture medium taking into account the content of carbohydrates and proteins provided by the beverage. The other two daily additions consisted only of culture medium without modification (200 mL). Samples of fermentation liquids from the three colon compartments were

collected on a daily basis during the treatment period (15 minutes before the contents of the small intestine were transferred to the AC) and stored at -20˚C. Moreover, samples at time 0 (immediately after the stabilization period) and at time 8 h (when the small intestine contents had been poured into the AC) were taken to subtract the sterol contents provided by the culture medium from the amounts measured at each sampling point and to control the sterols provided by the beverage without fermentation, respectively.

Plate counts of total anaerobic bacteria were checked after the stabilization period (time 0) and during beverage fermentation (time 72 h of the treatment period on Schaedler agar (anaerobic conditions, 37˚C, 72 h) by duplicate and no relevant differences were observed (7.6 *vs*. 8.0 log CFU/mL for the AC, 8.6 *vs*. 8.3 log CFU/mL for the TC and 7.9 *vs*. 7.9 log CFU/mL for the DC).

2.4 Sterol analysis

The sterol content was determined by gas chromatography-mass spectrometry (GC-MS), following the methodology previously optimized and applied to samples of fermentation liquids from the D-CGD (Blanco-Morales et al., 2020).

2.5 *Determination of organic acids*

This determination was carried out in a HPLC (Jasco Corporation, Japan) with a UV detector (210 nm) following the methodology previously applied by Blanco-Morales et al. (2020).

2.6 DNA extraction, 16S rRNA, gene sequencing and bioinformatics analysis

Microbiota composition was analyzed following the methodology used by Blanco-Morales et al. (2020). Briefly, the genomic DNA from samples of fermentation liquids was extracted using the DNeasy PowerSoil Kit (Qiagen, Carlsbad, CA) and the variable V3 and V4 regions of the 16S rDNA gene were amplified. Then, the MiSeq platform was used to sequence DNA libraries using a paired-

end 2 × 300-bp reagent kit (Illumina, San Diego, CA, USA). Forward and reverse reads joining, quality filtering, chimera removal and taxonomic annotation were analyzed using QIIME software version 1.9.1. Sample from TC at 96 h of fermentation was discarded due to the low reads obtained, being then the minimum sequencing depth 28,000 reads. Reads were assigned to operational taxonomic units (OTUs) picking protocol against the Greengenes database. Alpha and beta diversity analysis was performed as previously described by Blanco-Morales et al. (2020).

2.7 Statistical analysis

Significance of sterol and organic acids contents at different fermentation times in each colon compartment was evaluated using the Statgraphics® Centurion XVI.I statistical package (Statpoint Technologies Inc., Warrenton, VA, U.S.A), applying a one-way ANOVA ($p < 0.05$) following by Tukey's *post hoc* test.

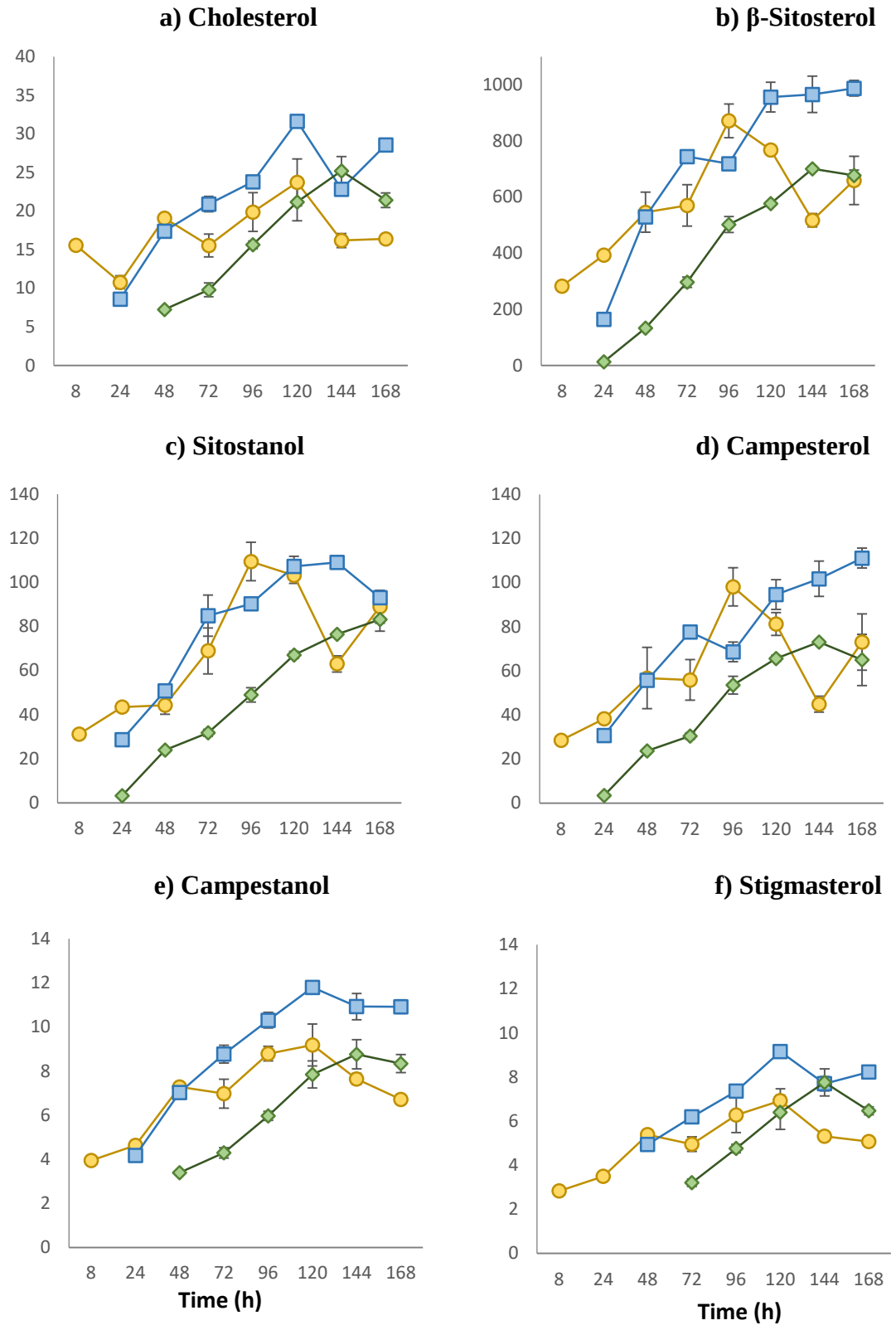
3. Results

3.1 Sterol evolution

To analyze the effect of colonic fermentation, sterol contents in fermentation liquids from the three colon compartments were determined daily during one week (Figure 2). The mean sterol contents ± standard deviation values and a representative chromatogram of this determination are shown in the Supplementary Table 1 and Supplementary Figure 1. Non-quantifiable contents of coprostanol, desmosterol, brassicasterol, lathosterol, and methylcoprostanone were observed in all colon vessels during beverage fermentation.

Cholesterol contents in the AC presented an irregular evolution, with a trend to increase between 72 and 120 h. In the TC, cholesterol increased until 120 h and decreased afterwards. In the case of the DC, a gradual increase of the cholesterol contents was observed, with maximum contents as from 120 h of fermentation reaching a plateau onwards (Figure 2a).

Figure 2. Evolution of the sterol contents (mg/compartment) during the beverage fermentation in the ascending (AC), transverse (TC) and descending colon (DC).



With regard to PS, β-sitosterol, sitostanol and campesterol contents increased progressively until 96 h during beverage fermentation in the AC. In the TC and DC, the maximum amounts were detected between 120 and 168 h (Figure 2b-d). Campesterol and stigmasterol were the minor PS detected during the fermentation and presented their maximum contents in all colon vessels at 120 h (Figure 2e and f, respectively). In the AC and TC contents decreased thereafter, while in the DC reached a plateau until the end of the fermentation.

Contents below of the limit of quantitation (mg/compartment) for coprostanol (0.12, 0.09 and 1.34), desmosterol (0.17, 0.07 and 0.12), brassicasterol (0.05, 0.04 and 0.03), lathosterol (0.02, 0.03 and 0.03) and methylcoprostanone (0.08, 0.06 and 0.12) were observed in the AC, TC and DC, respectively. In general, these metabolites could be detected from 8-24 h in the AC, 24-48 h in the TC and 48-72 h in the DC.

3.2 Evolution of organic acid production

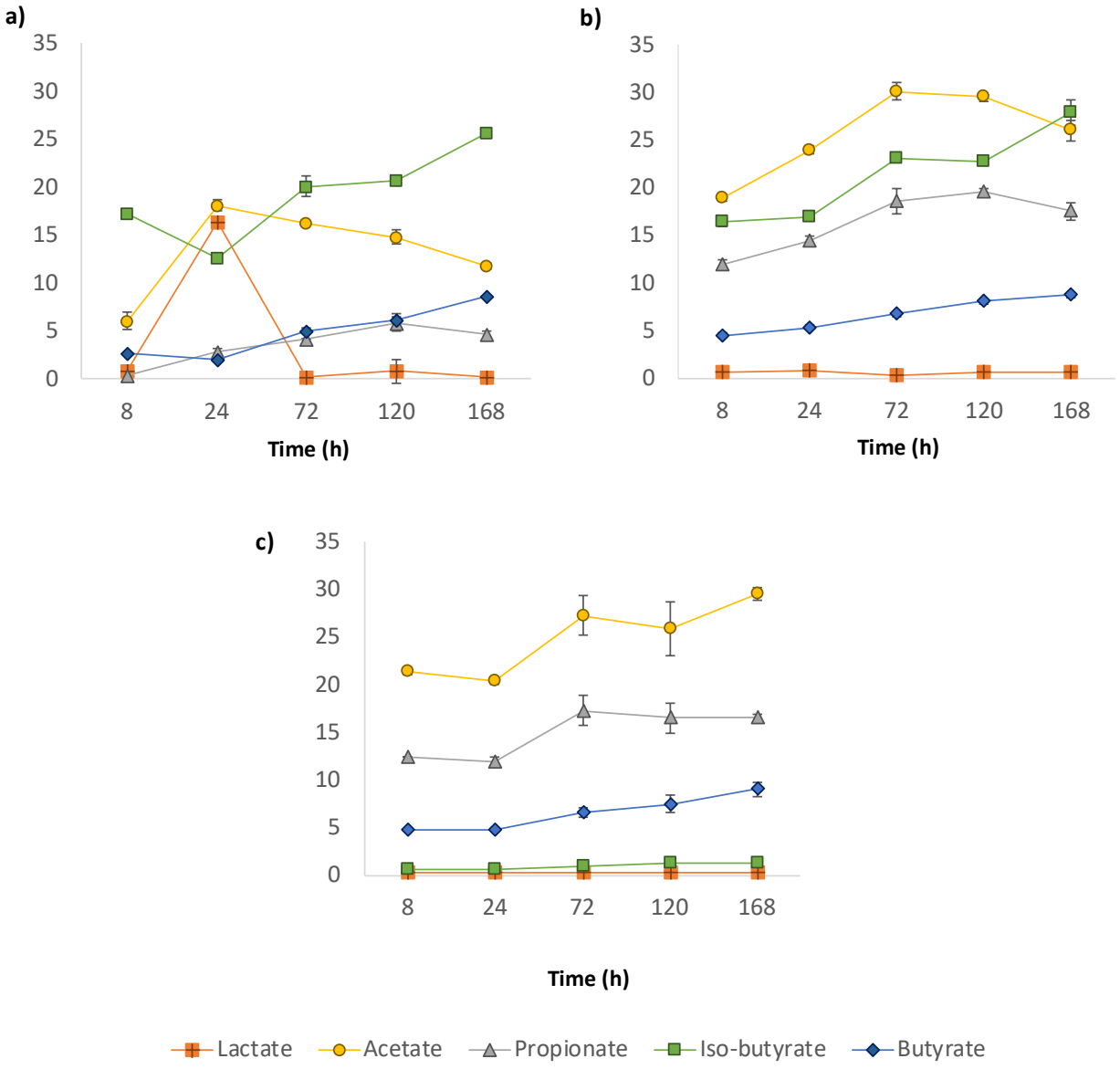
In the AC, contents of total organic acids were lower (28-57 mM) than in the TC (55-87 mM) and DC (44-64 mM) (see Supplementary Table 2). However, increments at 168 h with respect to 8 h were higher in the AC (2-fold) and similar in TC and DC (1.6- and 1.5-fold, respectively). In the distal regions of the colon total organic acid contents reached a plateau at 72 h.

The production of individual organic acids through the fermentation from all colon compartments are shown in Figure 3).

Iso-butyrate was one of the most abundant organic acid in the AC and TC presenting a progressively increase with increments at 168 h with respect to the initial values of 1.5- and 1.7-fold, respectively, while in the DC their contents were of the lowest (0.7 to 1.3 mM). Acetate was another of the most abundant organic acids in all colon compartments. Its contents in the AC increased at 24 h and decreased thereafter, meanwhile in the TC and DC the maximum contents were observed

between 72 and 168h. Nevertheless, increments with respect to contents at 8 h were higher in the AC (2-fold) *vs*. TC and DC (1.4-fold).

Figure 3. Contents of individual organic acids (mM) during the beverage fermentation in the ascending (a), transverse (b) and descending colon (c).



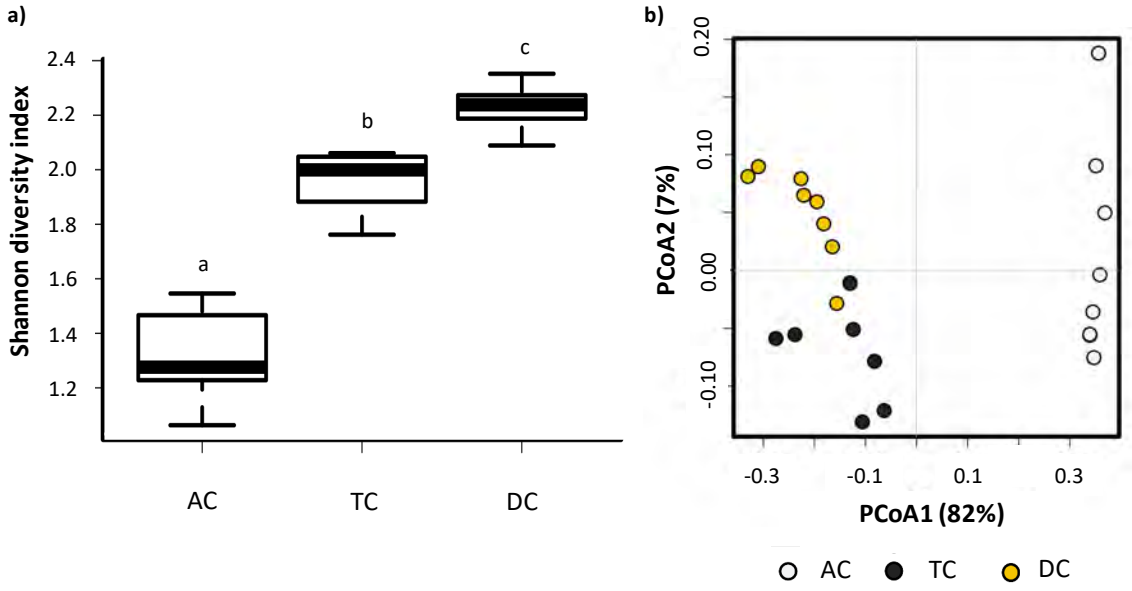
Propionate y butyrate presented a similar profile with a tend to increase, reaching their maximum contents, in general, between 120 and 168 h. Increments at 168 h were highest in the AC (16-fold *vs*. 1.5- and 2-fold for propionate and 3-fold *vs*. 2- and 2-fold for butyrate). Lactate contents remained

relatively stable in all colon vessels during the fermentation (0.3-0.8 mM), with the exception to sample from 24 h in the AC (Figure 3a), where reached contents of 17 mM. Regarding to minor organic acids (not included in Figure 3), iso-valerate and valerate were detected in all colon vessels and presented a trend to increase during the fermentation (between 0.5 to 3.8 mM and 1.0 to 3.6mM, respectively), while formate was only detected in the AC at 168 h (1.95 ± 0.24 mM).

3.3 Microbiota composition

To compare the microbial community between compartments, the Shannon diversity index and a PCoA score plot were obtained (Figure 4a and 4b, respectively). Results shown a progressive significantly increase ($p < 0.0001$) in species richness and evenness from the AC to the DC (Figure 4a). The PCoA analysis shown a separate cluster in which the AC is grouped (explaining 82% of the total variation of the data), while the TC and DC shown a microbial community more closely (Figure 4b).

Figure 4. Analysis of alpha (a) and beta (b) diversities of the microbiota composition based on colon compartment (ascending colon, AC; transverse colon, TC and descending colon, DC).

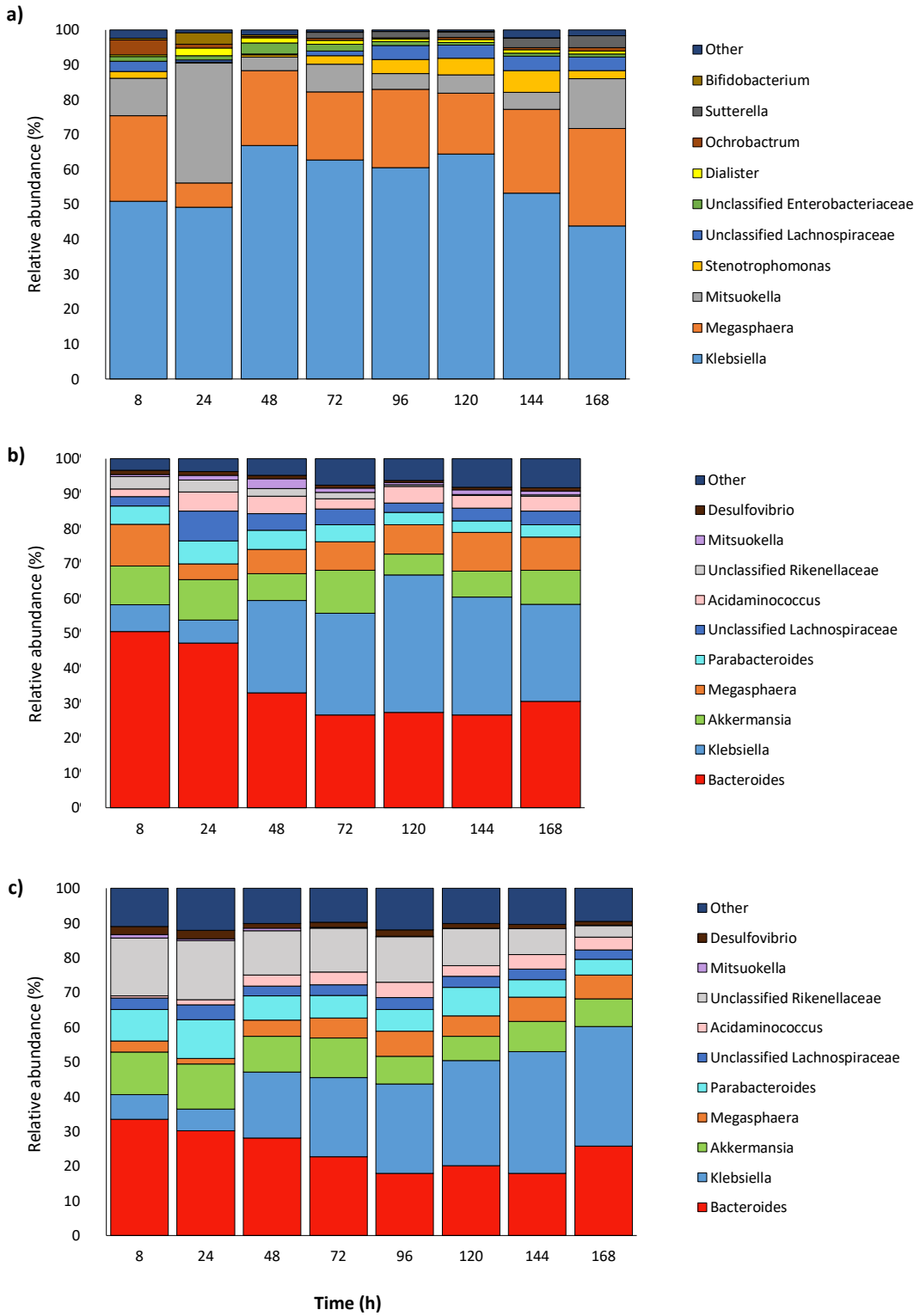


Different letters indicate significant differences ($p < 0.001$)

Abundances of the most important genera during fermentation of the beverage were also studied as shown in Figure 5. *Klebsiella* was the most abundant genus in the AC during the fermentation, presenting abundances between 44 and 67%, with maximum contents at 48 h. The genus *Megasphaera* decreased after 24 h of fermentation (abundance of 7%), increasing subsequently until reaching an abundance of 28%. Abundance of the genus *Mitsuokella* increased from 11 to 34% in 24 h of fermentation, however presented a trend to decrease onwards. The genera *Stenotrophomonas*, unclassified Lachnospiraceae and *Enterobacteriaceae*, *Dialister*, *Ochrobactrum*, *Sutterella* and *Bifidobacterium* did not show abundances greater than 6% during the fermentation. *Stenotrophomonas*, unclassified Lachnospiraceae and *Sutterella* genera tended to increase, in general after 24 and 48h, while unclassified *Enterobacteriaceae*, *Dialister* and *Ochrobactrum* tended to decrease. Abundance of *Bifidobacterium* ranged between 0.1 and 0.5 %, with the exception of the 24 h sample, where an abundance of 3.2% was determined.

Microbiota composition in the TC and DC showed a similar profile, although differences in the abundances of each genus can be observed in function of the compartment. In the distal colon compartments *Bacteroides* genus was the most abundant at the beginning of the fermentation (51% in the TC and 34% in the DC), decreasing thereafter until reach abundances of 27-30% and 18-26%, respectively, at the end of the fermentation (144-168 h). In contrast, *Klebsiella* presented minor abundances at 8 h of fermentation (8% in the TC and 7% in the DC) and increased as from 48 h reaching abundances of 27-40% and 30-35%, respectively, at 144-168 h of fermentation. Abundance of *Akkermansia* genus ranged between 6 and 13% in the distal compartments, showing an irregular evolution in the TC and a tendency to decrease in the DC. Abundance of *Megasphaera* genus decreased at 24 h from 12 to 5% in the TC and from 3 to 2% in the DC, remaining thereafter relatively stable (7-11% and 5-7%, respectively). Unclassified genus belonging to Lachnospiraceae family presented abundances between 3 and 5% in both compartments with the exception of an increase reported in the TC at 24 h of fermentation (9% of abundance).

Figure 5. Evolution of microbiota abundances at genus level in the ascending colon (a), transverse colon (b) and descending colon (c).



Genus *Parabacteroides* and unclassified Rikenellaceae shown a tendency to decrease as from 24 h in the distal compartments (from 7 to 4% and 4 to 0.4% in the TC and from 11 to 5% and 17 to 3%

in the DC, respectively). The remaining genera (*Acidaminococcus*, *Mitsuokella* and *Desulfovibrio*) not presented abundances higher that 6% and shown an irregular evolution.

4. Discussion

Dynamic *in vitro* fermentation models are promising tools allowing the evaluation of the changes bioactive compounds, isolated or present in food matrices, undergo when passing through the gastrointestinal tract. Furthermore, they shed light on the reciprocal interaction between these compounds and the microbiota (Moon et al., 2016). In this study, the fermentation of the beverage tested showed no sterol metabolism in the three colon compartments. Consistent with our results, recent research showed a lack of cholesterol metabolism when batch fermentation of a residue from the gastrointestinal digestion of a similar matrix (PS-enriched MfB) was carried out during 48 h. However, a preference for PS as substrate for the microbiota was observed (campesterol contents decreased by 39%, while ethylcoprostanol increased by 50% after 48 h) (Cuevas-Tena et al., 2018b). Although the PS content (~ 0.1 mg/mL) was lower than the one used in the present study (0.6 – 1 mg/mL), the use of a static method could have improved the PS metabolism. This fact was also observed when the polyphenol metabolism by a dynamic multi-reactor gastrointestinal simulator under gastrointestinal and culture medium conditions, similar to the one used in this study (Ekbatan et al., 2016) and in batch culture assays (Parkar, Trower, & Stevenson, 2013) were compared, suggesting that the absence of a continuous flow implies a faster biotransformation. The metabolism of sterols (from a PS-ingredient source or commercial standard mixture) was also evaluated in a TIM-2 system during 72 h (Cuevas-Tena et al., 2019). When fermentation was carried out with microbiota from lean subjects, both PS sources led to cholesterol and PS biotransformation. A decrease of coprostanol contents (41-61%) and an increase of coprostanone and cholestanol (109-136% and 27-29%, respectively) were observed compared to the initial values. With regard to PS metabolism, it was only reflected by the increase of methylcoprostanone at 72 h of fermentation originated from the PS-ingredient (8-fold) and the commercial PS-standard mixture (25-fold). In contrast, when the

fermentation assay was carried out with fecal inoculum from obese subjects, cholesterol and PS were not metabolized. Besides the differences between lean and obese microbiota activity with regard to sterol metabolism, it has to be highlighted that the TIM-2 model includes a dialysis membrane that simulates the absorption of water and metabolites preventing their accumulation and, therefore, a possible inhibition of microbial activity (Venema & Van den Abbeele, 2013). Moreover, the PS in the TIM-2 study (at an amount approximately 2-fold higher than the one in the present work) was provided by a PS-ingredient source or commercial standard mixture, which could facilitate their biotransformation. This fact has also been observed in dynamic *in vitro* fermentation studies of polyphenols, where higher amounts of metabolites in the proximal colon vessels were observed when pure polyphenols were used, compared with those polyphenols included in a food matrix (red wine, grape juice and black tea) (Ekbatan et al., 2016). Authors suggested that the food matrix could protect from the microbiota activity. These remarkable differences are likely to justify the discrepancy between the results obtained in the present work when compared with the assays carried out in TIM-2 with fecal inoculum from lean subjects.

In addition, in a recent study published by our research group, fermentation of a similar PS-enriched MfB in presence of GOS and carried out in a D-CGD showed sterol metabolism in the TC and DC from 24 and 48 h, respectively (Blanco-Morales et al., 2020). In this sense, ratios with respect to the sterol precursor were higher for coprostanol (from cholesterol) than for ethylcoprostanol and sitostenone (from β-sitosterol), methylcoprostanol (from campesterol) and ethylcoprostenol (from stigmasterol). Microbiota modulation by GOS addition was also suggested to exert enhanced sterol metabolism. In the present study, microbiota composition observed during beverage fermentation might also play a pivotal role in the absence of sterol metabolism. Different pathways for sterol biotransformation (direct or indirect) have been described through the formation of phytostanone and phytostenone intermediates, with *Bacteroides* and *Bifidobacterium* being the only cholesterol-reducing bacteria observed in the present study (Cuevas-Tena et al., 2018a; Gérard, 2014). However,

Bacteroides genus was present only in the TC and DC and tended to decrease during fermentation, while *Bifidobacterium* was only observed in the AC, being below 3%; facts that could explain the low cholesterol metabolism detected. The Lachnospiraceae family has also been suggested as coprostanoligenic bacteria in *in vitro* assays with no association to the direct or indirect routes of cholesterol metabolism (Kriaa et al., 2019; Antharam et al., 2016). In this context, an unclassified genus of the Lachnospiraceae family was observed in all colon vessels, but with an abundance below 8%. The presence of both coprostanoligenic bacteria could explain the variation of cholesterol content in the AC and the detection of coprostanol. However, the limited abundance of these bacteria could justify the fact that coprostanol amount was not detected at quantification levels. With respect to PS, only *Eubacterium spp*. has been associated with their biotransformation pathway (Cuevas-Tena et al., 2018a). However, the absence of this genus could justify the fact that no PS metabolites were observed.

The absence of sterol metabolism in the present study could also be related to a low bioaccessibility of the sterols caused by interactions with food components (such as carbohydrates, fiber and fat) present in the matrix. Although different studies have reported that the presence of fiber reduce cholesterol bioaccessibility (López-Marcos, Bailina, Viuda-Martos, Pérez-Alvarez, & Fernández-López, 2015; Minekus et al., 2005), studies on interactions with respect to PS bioaccessibility are scarce. In relation with PS-enriched beverages, our research group demonstrated that fermented milk beverages with higher carbohydrates and fiber contents showed lower PS bioaccessibility (Vaghini, Cilla, Garcia-Llatas, & Lagarda, 2016), whereas the addition of GOS to PS-enriched milk-based fruit beverages did not affect it (Blanco-Morales et al., 2018). On the other hand, we also reported that the presence of fat (from animal or vegetable sources) increased PS bioaccessibility in PS-enriched beverages, suggesting that it could improve mixed micelles formation during *in vitro* gastrointestinal digestion (Álvarez-Sala et al., 2016). Thus, although studies of *in vitro* digestion and colonic fermentation with food matrix are complex and it is difficult to make a solid conclusion, they are

necessary considering all the possible interactions that could occur in physiological conditions and could limit the bioaccessibility and biological activity of bioactive compounds.

Regarding organic acids, although the highest total contents were observed in the distal colon regions (the TC and DC), the highest increments were reported in the AC. In this sense, acetate tends to decrease in the AC after 24 h of fermentation (suggestive of it being used by the microbiota for the formation of butyrate) and to increase in the TC and DC. In fact, butyrate production in the AC was higher than in the TC and DC. Curiously, butyrate-producing bacteria (species belonging to the Lachnospiraceae family) (Flint, Duncan, Scott, & Louis, 2015) were more abundant in these latter compartments. Therefore, the enhanced production of butyrate in the proximal colon is likely to be favored by more acidic pH values (Kettle, Louis, Holtrop, Duncan, & Flint, 2015; Walker, Duncan, Leitch, Child, & Flint, 2005). The increase of iso-butyrate in the AC and TC suggest a proteolytic fermentation process in these compartments, since higher protein contents, provided by the beverage or the culture medium, can be present compared to DC. Although the increment of branched-chain fatty acids (iso-butyrate and iso-valerate) has been associated with harmful effects in the host, their impact is until unknown and recently it has been observed that iso-butyrate can be used as a fuel source by host intestinal epithelial cells when butyrate is scarce (Oliphant & Allen-Vercoe, 2019). *Bacteroides*, the predominant bacterial specie involved in proteolysis in the human gut, is only present in the TC and DC with a trend to decrease, according to a lower amount of protein in these compartments (Macfarlane, Cummings, & Allison, 1986). The presence of other genera considered proteolytic such as *Sutterella* and unclassified *Enterobacteriaceae* family (belonging Proteobacteria phylum) in the AC and *Desulfovibrio* and *Acidaminococcus* in the TC could explain the activity observed in these compartments (Diether & Willing, 2019; Dallas et al., 2017). Propionate-producing bacteria such as *Bacteroides*, *Rikinellaceae* and *Akkermansia* (Louis & Flint, 2017) were also more abundant in the TC and DC, although generally tended to decrease. The higher production of butyrate and propionate in the AC is more likely to be related to the contents of lactate during fermentation.

A maximum lactate content was observed in the AC at 24 h of fermentation, in line with the optimal conditions (pH 5.9) reported for its formation (Belenguer et al., 2007) and with the highest abundance of *Bifidobacterium* (the genus involved in its production). The fast decrease of lactate produced after 24 h is suggestive of its metabolism to butyrate and propionate (Morrison & Preston, 2016). Even though the Lachnospiraceae family was identified in the present work and some species belonging to this family contribute to the conversion of lactate into butyrate, the high abundance of *Megasphaera* genus (converter of lactate into propionate) in the AC after 24 h of fermentation supports the elevated production of propionate in this compartment (Flint, Duncan, & Louis, 2017). In agreement with our results, increments of acetate, butyrate, propionate and lactate were also observed during *in vitro* colonic fermentation in the presence of PS (Cuevas-Tena et al., 2019). Moreover, the presence of juice from concentrate in our beverage might also have improved the production of SCFAs. In this sense, in a study carried out with a SHIME model, fermentation of fresh juice increased acetate and butyrate contents, while fermentation of pasteurized juice increased acetate, propionate and butyrate in the TC and DC (Duque et al., 2016).

Microbiota composition was also evaluated during one-week fermentation of the PS-enriched MfB in order to estimate other potential effects. Comparison of microbial communities between compartments showed a progressive increase of species richness from the AC towards the DC, with a more similar microbiota composition in the distal compartments (TC and DC). This behavior was also observed in other *in vitro* dynamic fermentation studies (Blanco-Morales et al., 2020; Cha et al., 2018), having been justified as a consequence of the conditions present in the proximal compartment (low pH together with high concentration of bile salts) (Van den Abbeele et al., 2010). With regard to the evolution of the abundances in the microbiota, in our work *Megasphaera* genus (belonging to Firmicutes phylum) tended to increase after 24 h of fermentation in the AC. Similarly, after *in vitro* colonic fermentation of PS, increments in some members belonging to this phylum were observed in batch culture (*Eubacterium hallii*) (Cuevas-Tena et al., 2018b) and in a TIM-2 model using fecal

inoculum from lean (*Coprococcus*) or obese subjects (*Clostridium*) (Cuevas-Tena et al., 2019). During the fermentation of fresh or pasteurized orange juice in a SHIME model (Duque et al., 2016), Firmicutes phylum also was modulated (increase of *Lactobacillus spp.*, *Enterococcus spp.* or *Clostridium spp*.). Moreover, fermentation of fresh juice also increased *Bifidobacterium spp.* in the distal compartments. In contrast, we only observed *Bifidobacterium* genus in the AC at short fermentation times. The lack of effect on *Lactobacillus* and *Bifidobacterium* genera in presence of a high concentration of PS was previously reported even when GOS was present (Blanco-Morales et al., 2020). On the other hand, Bacteroidetes phylum tended to decrease in the TC and DC (*Bacteroides*, *Parabacteroides* and *Rikenellaceae* genera). However, fermentation of PS has been reported to lead to increments of some members of the uncharacterized S-24 family of Bacteroidetes in batch culture (Cuevas-Tena et al., 2018b) and of the kingdom Bacteria in a TIM-2 model when microbiota inoculum from obese subjects was used (Cuevas-Tena et al., 2019). In this sense, some of the changes produced in the microbiota composition in the present study could be related to other bioactive compounds present in the food matrix such as polyphenols. Our research group reported a total soluble polyphenol content of 223 mg gallic acid equivalent/100 mL (López-García et al., 2017) in a similar PS-enriched MfB to the one used in the present work, and Kemperman et al. (2013) observed an increase of *Klebsiella* and *Akkermansia* genera and a decrease of *Bacteroides* after the fermentation of polyphenols (from a mixture of red wine and grape juice extracts) in a SHIME model. This is in line with the findings in the present study and could explain the marked increase of *Klebsiella* genus in the TC and DC and also the maintenance of *Akkermansia* in the distal colon compartments. Moreover, the abundance of *Akkermansia muciniphila* (a mucin-utilizing specialist) has been related to beneficial effects to the host reducing the development of some metabolic diseases (Cani & de Vos, 2017).

Finally, a limitation of our study is the lack of a dialysis process during the intestinal digestion that could remove the digested macromolecules. In addition, the promotion of bile acid secretion by the presence of high PS contents has not been considered either.

5. Conclusions

In the present study, the absence of metabolites (neither from cholesterol or from PS) indicated the lack of microbiota involved in their biotransformation pathways. However, the increase in SCFA contents after fermentation of the beverage implies a potential beneficial effect. In addition, fermentation of the beverage had a positive impact on beneficial bacteria, stimulating the growth of *Bifidobacterium* in the AC at short fermentation times, as well as the maintenance of *Akkermansia* in the distal compartments. Changes observed in the composition of the microbiota suggest a multiple effect of the different compounds present in the matrix, evidencing complex diet-microbiota interactions.

Abbreviations

AC: ascending colon; D-CGD: dynamic-colonic gastrointestinal digester; DC: descending colon; GC-MS: gas chromatography-mass spectrometry; GOS: galactooligosaccharides; MfB: milk-based fruit beverage; OTUs: operational taxonomic units; PS: plant sterols; SCFA: short-chain fatty acids; TC: transverse colon.

Acknowledgements

We thank Hero España S. A., for manufacturing the beverage evaluated in the present study and to AINIA Technology Center (Valencia, Spain) for carrying out the fermentation assays, in particular to Dr. Blanca Viadel (Department of Biotechnology, AINIA, Spain). The authors also thank Prof. M. J. Lagarda for her support and invaluable comments and advice.

Funding

This study was financed by the Spanish Ministry of Economy and Competitiveness (MINECO) and the European Regional Development Fund (FEDER) (AGL2015-68006-C2-1-R). Virginia Blanco-Morales holds a research contract under the aforementioned project (CPI-17-025).

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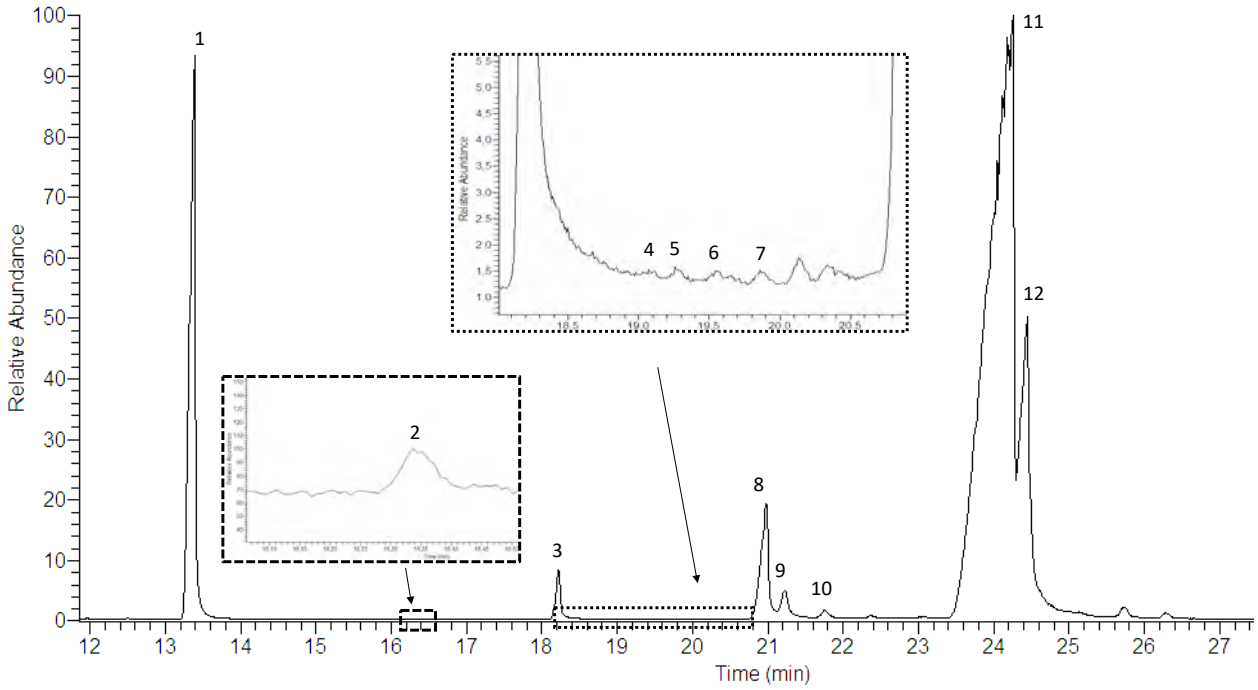
Supplementary Figure 1. Chromatogram obtained by GC-MS of sterol determination in fermentation liquid from transverse colon after 168 h of the beverage fermentation.



1: 5α-cholestane (IS), 2: coprostanol, 3: cholesterol, 4: desmosterol, 5: brassicasterol, 6: lathosterol, 7: methylcoprostanone, 8: campesterol, 9: campestanol, 10: stigmasterol, 11: β-sitosterol, and 12: sitostanol.

Supplementary Table 1. Contents of sterols (mg/ compartment) in fermentation liquids from the ascending colon (AC), transverse colon (TC) and descending colon (DC) along one-week dynamic colonic fermentation[1].

		Fermentation time (h)							
		8	24	48	72	96	120	144	168
Cholesterol	AC	15.6 ± 0.2 ab	10.8 ± 0.8 a	19.1 ± 0.5 bc	15.6 ± 1.5 ab	19.9 ± 2.5 bc	23.7 ± 3.0 c	16.2 ± 0.9 ab	16.4 ± 0.1 ab
	TC	-	8.6 ± 0.1 a	17.4 ± 0.3 b	20.9 ± 1.0 c	23.8 ± 0.1 d	31.6 ± 0.6 e	22.8 ± 0.1 d	28.6 ± 0.2 f
	DC	NQ	NQ	7.3 ± 0.2 a	9.8 ± 0.9 a	15.6 ± 0.5 b	21.2 ± 2.5 c	25.2 ± 1.9 c	21.4 ± 1.0 c
Campesterol	AC	28.6 ± 1.1 a	38.3 ± 0.8 a	56.7 ± 13.9 abc	55.9 ± 9.2 abc	98.1 ± 8. 7 d	81.2 ± 5.2 cd	44.9 ± 3.6 ab	73.0 ± 12.8 bcd
	TC	-	30.7 ± 0.5 a	55.7 ± 1.0 b	77.6 ± 1.4 cd	68.6 ± 4.5 bc	94.6 ± 6.7 de	101.8 ± 8.0 e	111.1 ± 4.5 e
	DC	-	3.5 ± 0.04 a	23.7 ± 0.8 b	30.5 ± 1.4 b	53.5 ± 4.1 c	65.6 ± 1.4 cd	73.0 ± 0.9 d	64.9 ± 11.7 cd
Campestanol	AC	3.9 ± 0.03 a	4.6 ± 0.1 a	7.3 ± 0.2 bc	7.0 ± 0.7 b	8.8 ± 0.3 cd	9.2 ± 1.0 d	7.7 ± 0.3 bcd	6.7 ± 0.2 b
	TC	-	4.2 ± 0.01 a	7.0 ± 0.2 b	8.8 ± 0.4 c	10.3 ± 0.4 d	11.8 ± 0.1 e	10.9 ± 0.6 de	10.9 ± 0.3 de
	DC	-	NQ	3.4 ± 0.01 a	4.3 ± 0.2 ab	6.0 ± 0.2 b	7.9 ± 0.6 c	8.8 ± 0.7 c	8.3 ± 0.4 c
Stigmasterol	AC	2.8 ±0.1 a	3.5 ± 0.2 ab	5.4 ± 0.1 c	5.0 ± 0.3 bc	6.3 ± 0.8 cd	6.9 ± 0.5 d	5.3 ± 0.2 c	5.1 ± 0.1 c
	TC	-	NQ	4.9 ± 0.2 a	6.2 ± 0.2 b	7.4 ± 0.1 c	9.2 ± 0.2 d	7.7 ± 0.3 ce	8.2 ± 0.2 e
	DC	-	NQ	NQ	3.2 ± 0.2 a	4.8 ± 0.2 ab	6.4 ± 0.8 bc	7.8 ± 0.6 c	6.5 ± 0.2 bc
β-Sitosterol	AC	282.9 ± 5.1 a	393.1 ± 9.1 ab	546.2 ± 71.5 bc	570.6 ± 73.6 bcd	871.9 ± 60.2 e	767.8 ± 14.4 de	517.3 ± 23.6 bc	659.2 ± 86.0 cd
	TC	-	165.0 ± 5.3 a	529.9 ± 6.1 b	744.6 ± 11.4 c	719.0 ± 0.5 c	956.7 ± 52.9 d	966.2 ± 65.1 d	987.7 ± 27.8 d
	DC	NQ	13.7 ± 0.6 a	133.8 ± 5.9 b	296.8 ± 18.6 c	502.4 ± 28.7 d	576.8 ± 7.4 e	701.0 ± 8.9 f	677.1 ± 19.4 f
Sitostanol	AC	31.2 ± 1. 7 a	43.4 ± 0.2 ab	44.3 ± 4.2 ab	68.9 ± 10.4 cd	109.4 ± 8.7 e	103.1 ± 3.6 e	63.0 ± 3.7 bc	88.9 ± 7.5 de
	TC	NQ	28.6 ± 0.6 a	50.8 ± 0.03 b	84.9 ± 9.3 c	90.2 ± 1.7 c	107.2 ± 4.6 d	109.0 ± 0.1 d	93.0 ± 2.7 cd
	DC	NQ	3.2 ± 0.04 a	24.0 ±0.04 b	31.8 ± 1.6 b	48.9 ± 3.3 c	67.1 ± 1.6 d	76.4 ± 0.4 de	83.2 ± 5.3 e

NQ: non-quantifiable. Different letters denote statistically significant differences ($p < 0.05$) in the same sterol between different fermentation times.

[1] Data expressed as mean ± standard deviation (n = 3).

Supplementary Table 2. Organic acids contents (mM) present in fermentation liquids from the ascending colon (AC), transverse colon (TC) and descending colon (DC) along one-week dynamic colonic fermentation[1].

		Fermentation time (h)				
		8	24	72	120	168
Lactate	AC	0.84 ± 0.00 a	16.46 ± 0.43 b	0.27 ± 0.00 c	0.84 ± 0.00 a	0.27 ± 0.00 ac
	TC	0.56 ± 0.00 a	0.84 ± 0.00 b	0.27 ± 0.00 c	0.56 ± 0.00 a	0.56 ± 0.00 a
	DC	0.27 ± 0.00 a	0.27 ± 0.00 a	0.27 ± 0.00 a	0.27 ± 0.00 a	0.27 ± 0.00 a
Acetate	AC	6.05 ± 0.93 a	18.09 ± 0.61 b	16.25 ± 0.00 c	14.82 ± 0.71 c	11.76 ± 0.35 d
	TC	18.90 ± 0.35 a	24.00 ± 0.35 b	30.12 ± 0.93 c	29.51 ± 0.35 c	26.04 ± 1.06 d
	DC	21.35 ± 0.35 a	20.33 ± 0.35 a	27.27 ± 2.12 b	25.84 ± 2.83 b	29.51 ± 0.71 b
Propionate	AC	0.29 ± 0.00 a	2.83 ± 0.44 b	4.09 ± 0.00 bc	5.87 ± 0.88 d	4.60 ± 0.44 cd
	TC	11.95 ± 0.44 a	14.48 ± 0.44 b	18.54 ± 1.32 c	19.55 ± 0.44 c	17.52 ± 0.88 c
	DC	12.46 ± 0.00 a	11.95 ± 0.44 a	17.27 ± 1.58 b	16.51 ± 1.58 b	16.51 ± 0.44 b
Iso-butyrate	AC	17.24 ± 0.19 a	12.55 ± 0.38 b	20.07 ± 1.05 c	20.73 ± 0.38 c	25.63 ± 0.19 d
	TC	16.48 ± 0.50 a	16.91 ± 0.19 a	23.13 ± 0.19 b	22.69 ± 0.19 b	27.92 ± 1.32 c
	DC	0.67 ± 0.00 a	0.67 ± 0.00 a	1.00 ± 0.00 b	1.22 ± 0.19 bc	1.33 ± 0.00 c
Butyrate	AC	2.60 ± 0.00 a	2.03 ± 0.25 b	5.04 ± 0.25 c	6.19 ± 0.25 d	8.63 ± 0.00 e
	TC	4.47 ± 0.25 a	5.33 ± 0.25 b	6.76 ± 0.25 c	8.05 ± 0.25 d	8.77 ± 0.25 e
	DC	4.76 ± 0.00 a	4.76 ± 0.00 a	6.62 ± 0.50 b	7.48 ± 0.90 b	9.06 ± 0.74 c
Iso-valerate	AC	0.46 ± 0.00 a	0.46 ± 0.00 a	0.46 ± 0.00 a	1.29 ± 0.00 b	1.29 ± 0.00 b
	TC	0.46 ± 0.00 a	1.01 ± 0.24 ab	1.84 ± 0.24 c	1.42 ± 0.24 bc	1.49 ± 0.29 c
	DC	1.70 ± 0.00 a	1.70 ± 0.00 a	2.67 ± 0.24 b	3.49 ± 0.48 c	3.77 ± 0.00 c
Valerate	AC	0.96 ± 0.00 a	0.96 ± 0.00 a	2.05 ± 0.19 b	2.27 ± 0.00 bc	2.49 ± 0.19 c
	TC	1.61 ± 0.00 a	2.16 ± 0.19 b	3.15 ± 0.19 c	3.26 ± 0.00 c	3.26 ± 0.00 c
	DC	2.27 ± 0.00 a	1.94 ± 0.00 a	3.04 ±0.38 b	3.48 ± 0.50 b	3.59 ± 0.00 b
Total organic acids	AC	28.03 ± 0.44 a	53.36 ± 1.21 b	47.46 ± 0.07 c	52.00 ± 1.92 b	56.53 ± 0.30 d
	TC	54.94 ± 0.66 a	64.73 ± 0.51 b	83.81 ± 2.95 c	85.04 ± 0.77 c	86.58 ± 4.59 c
	DC	43.48 ± 0.35 a	41.65 ± 0.97 a	58.13 ± 4.69 b	58.29 ± 5.36 b	64.03 ± 1.29 b

Different letters denote statistically significant differences ($p < 0.05$) in the same organic acid between different fermentation times.

[1] Data expressed as mean ± standard deviation (n = 3).

Influence of GOS on the positive effect of plant sterol-enriched beverages on cardiovascular risk and their colon metabolism: A randomized, double-blind crossover trial

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Clinical Nutrition

Influence of GOS on the positive effect of plant sterol-enriched beverages on cardiovascular risk and their colon metabolism: A randomized, double-blind crossover trial

--Manuscript Draft--

Manuscript Number:	YCLNU-D-20-01929
Article Type:	Randomized Control Trials
Keywords:	Cholesterol; feces; galactooligosaccharides; milk-based fruit beverage; Plant sterols; serum
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Abstract

Background & aim: Hypocholesterolemic effect of plant sterols (PS) has been widely confirmed by *in vivo* studies, as well as galactooligosaccharides (GOS) beneficial intestinal effects. However, studies about the interaction of GOS intake on serum lipid profile are scarce and no information is available about their effect on sterol bioavailability or colonic metabolism. Therefore, in the present study, the impact of GOS addition to a PS-enriched milk-based fruit beverage on hypocholesterolemic effect and on bioavailability and colonic metabolization of sterols was evaluated in post-menopausal women with mild hypercholesterolemia.

Methods: A randomized, double-blind crossover trial was undertaken in 42 post-menopausal women, aged 45-67 years, who intake daily a PS-enriched (2 g PS/250 mL) or PS-GOS-enriched beverage (2 g PS/250 mL and 4.3 g GOS/250 mL) for 6 weeks. After a 4-week whashout period, women were crossover to the alternative treatment for another 6 weeks. Sterols and their metabolites contents were determined before and after of each intervention period in serum and feces samples by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS), respectively.

Results: The presence of GOS did not modify the hypocholesterolemic effect of the PS-enriched beverage (total- and LDL-cholesterol reductions, with no significant variations in HDL-cholesterol serum levels) or the sterol bioavailability (increments of serum markers of dietary PS intake and of cholesterol synthesis). Furthermore, the consumption of both PS-enriched beverages led to an increase of sterol and metabolites excretion (with the exception of coprostanol, main cholesterol metabolite, that decrease), without influence of GOS presence.

Conclusions: This clinical trial demonstrates the suitability of the simultaneous enrichment with PS and GOS in milk-based fruit beverages, considering their hypocholesterolemic effect. Although

no influence of GOS on sterol bioavailability nor their colonic biotransformation was observed, slight changes on sterol conversion capacities were found.

Clinical Trial Registry Number: NCT03469518 listed on the NIH website: ClinicalTrials.gov. The full trial protocol is available upon request to the corresponding author.

Clinical Trial Registry Name: Effect of β-cryptoxanthin (β-Cx), Plant Sterols and Galactooligosaccharides on Systemic and Gastrointestinal Markers.

Keywords: cholesterol, feces, galactooligosaccharides, milk-based fruit beverage, plant sterols, serum

1. Introduction

Cardiovascular disease (CVD) is the main cause of death in Western countries, with 17.5 million deaths worldwide in 2012, representing 31% of all global deaths. The risk factors for CVD include factors such as high blood cholesterol and LDL-cholesterol, among others [1]. These parameters could be increased in post-menopausal women favoring atherosclerotic processes and increasing cardiovascular risk processes [2,3]. According to the European Atherosclerosis Society Consensus Panel, individuals with high hypercholesterolemia at intermediate or low cardiovascular risk who are not qualified for drug treatment ought to consider the consumption of plant sterol (PS)-enriched foods [4]. The hypocholesterolemic effect of PS has been demonstrated and is obtained with a daily intake of 1.5 to 3 g, recognized as a health claim related to the reduction of a disease risk [5–7].

In this context, the enrichment of milk-based fruit beverages with PS, a matrix that meets the healthy food profile, is allowed in the European Community [8]. In previous works of our group, a positive synergistic effect of β-criptoxanthin (β-Cx) and PS added to milk-based fruit beverages, without [9] or with milk fat and milk fat globule membrane (MFGM) [10], has been demonstrated in cardiovascular risk in post-menopausal women with moderate hypercholesterolemia not pharmacologically treated. In addition, the bioavailability of sterols has been evaluated in these clinical studies by determining the serum concentrations of PS and cholesterol precursors [10,11].

On the other hand, non-absorbed PS (β-sitosterol, campesterol and stigmasterol) can be transformed, in the same way as cholesterol, into their corresponding metabolites (ethyl and methylcoprostanol or ethylcoprostanol; subsequently to ethyl and methylcoprostanone or ethylcoprostanone) [12,13].

There is a great deal of information on the contents of sterols and their metabolites in human feces after the intake of Western and vegetarian diets [13], although, as far as we are aware, only two studies have assessed the influence of the intake of high doses of PS, from enriched foods, on the excretion

of cholesterol and its metabolites. In normolipidic subjects, whose diet has been enriched or not with a margarine containing PS (8.6 g/day), there is no increase in the concentrations of neutral sterols and the metabolism of cholesterol to coprostanol is reduced [14]. In a previous study carried out by our research group in post-menopausal women with moderate hypercholesterolemia who ingested milk-based fruit beverages containing β-Cx enriched or not with PS (2g/day) [15], no differences in the excretion of cholesterol after the intake of the PS-enriched beverage compared to placebo were observed, although an increase in coprostanone and ethylcoprostanone was observed.

Galactooligosaccharides (GOS) are soluble and more stable components than probiotics (under acidic pH and high temperature conditions) and can be easily incorporated into milk-based fruit beverages without changing their viscosity, texture or flavour. Moreover, GOS could enhance the already demonstrated hypocholesterolemic effect of PS-enriched beverages and have beneficial effects at the intestinal level [16]. As far as we are aware, information regarding the effect of GOS on the serum lipid profile is scarce and unconclusive involving murine models [17–19] or humans [20,21]. Moreover, it has been confirmed that the addition GOS to this type of beverages does not affect the bioaccessibility of total PS after simulated gastrointestinal digestion [22], which remains to be confirmed by *in vivo* studies in order to assure their functionality. As far as we know, there are no data available on the effect of the presence of GOS in these beverages on the bioavailability of PS, nor has the hypocholesterolemic effect in humans, derived from their simultaneous intake with GOS, been assessed.

Therefore, the objective of this work is to evaluate whether the regular consumption of GOS in a PS-enriched milk-based fruit beverage by post-menopausal women modifies the *in vivo* bioavailability of PS and the hypocholesterolemic effect/markers of cardiovascular risk, as well as the colonic metabolization of sterols. The measurement of primary (serum levels of sterols) and secondary (serum lipid and sterol fecal profile) out comes were used for this aim.

2. Materials and Methods

2.1 Samples.

Two PS-enriched skimmed milk-based fruit beverages (2 g PS/250 mL) enriched with or without GOS (4.3 g/250mL) were manufactured under the same conditions, specifically for this study. Both beverages had similar ingredients: skimmed milk with the addition of milk fat and whey protein concentrate enriched with MFGM (49%), mandarin juice from concentrate (45%), banana puree (4%), microencapsulated free microcrystalline PS from tall oil (Lipophytol® 146 ME Dispersible, Lipofoods) and GOS syrup (Vivinal® GOS from Friesland Campina Ingredients). The energy and nutritional information, per 100 mL, for the GOS-PS-enriched and PS-enriched beverages were, respectively: energy (kcal): 78 or 80; fat (g): 2 or 2.2; carbohydrates (g) 11.3 and 15.6; protein (g) 2.7 and 2.7 and fibre (g) <0.5. PS contents in both beverages were analyzed by gas chromatography-flame ionization detection (GC-FID) described elsewhere [23]. Relative percentages of PS were: β-sitosterol: 81.0 %, sitostanol: 11.2%, campesterol: 6.1%, campestanol: 1.0% and stigmasterol: 0.7 %.

2.2 Clinical study/Intervention study

A single and combined randomized, double-blind, crossover trial was carried out in post-menopausal women with mild hypercholesterolemia (200-239 mg/dL) according to the guidelines of the American Heart Association [24] (ClinicalTrials.gov number NCT03469518). The clinical study took place in the Vitamins Unit of the Department of Clinical Biochemistry of Hospital Universitario Puerta de Hierro-Majadahonda (Madrid, Spain). The study protocol was approved by the Clinical Research Ethics Committee of the aforementioned hospital, and all participants gave their written consent.

The inclusion criteria were as follows: age (45-65 years), body mass index (BMI)<35 Kg/m^2, amenorrhoea over 12 months, non-dieting and non-intake of vitamin D, calcium and ω-3 fatty acids

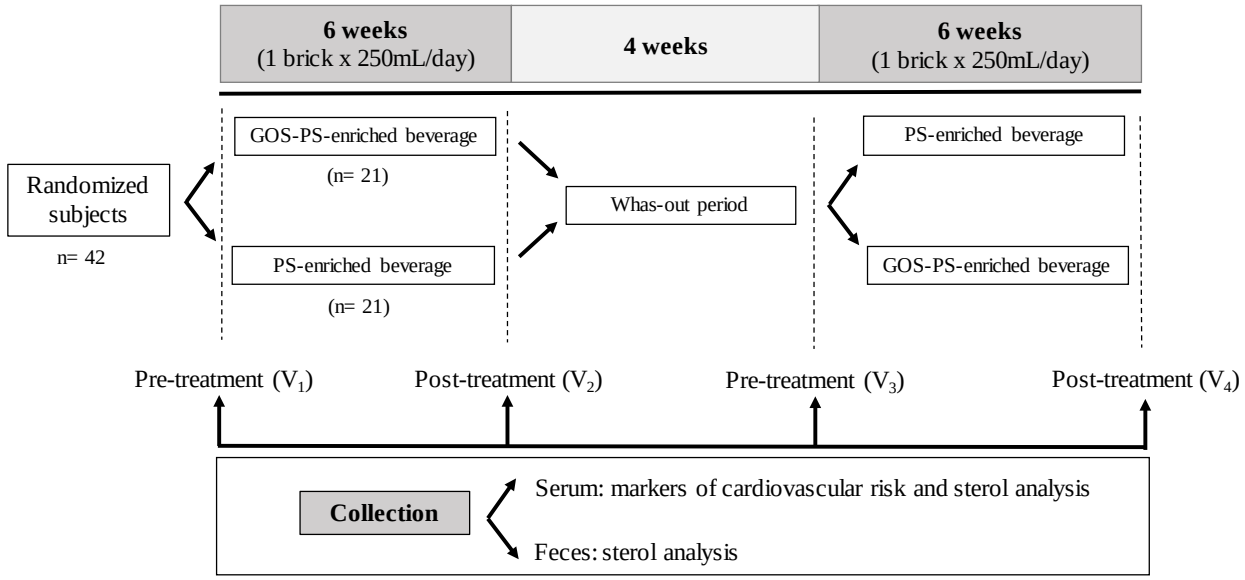
and PS or vitamin-enriched foods or supplements or other dietary bioactive components. Exclusion criteria were: use of vitamins, hormone replacement therapy, fibrates, statins and a weight-loss diet, as well as acute inflammation, chronic medication and infection or intercurrent illness capable of affecting the bioavailability or status of the compounds of interest.

A total of 42 healthy post-menopausal women were finally included in the study and were sequentially numbered from 1 to 42. The sample size was calculated considering the total PS and cholesterol results obtained in a previous clinical trial (ClinicalTrials.gov number NCT01074723). Taken from previous assumption, we chose the most conservative option to ensure the detection of a 7% decrease in cholesterol levels in mildly hypercholesterolemic subjects (e.g., 15 mg/dL) with a type I error of 0.05 and a statistical power of 80%. Moreover, allowing for a 45% rate of the Western population likely to present polymorphisms implicated in the cholesterol absorption process, and assuming a drop-out rate of 10%, the final required sample size was stipulated to comprise 42 persons.

The two beverages, of the same colour and taste, were filled in 250-mL cartons being indistinguishable, but with different anonymous labelling (A or B). During the 6-week intervention period, 21 subjects consumed the PS-enriched beverage, while 21 subjects consumed the PS-enriched beverages with GOS, both on a daily basis. After a 4-week wash-out period, the type of beverage to be consumed during another 6-week period was changed in-between groups. A diagram of the clinical trial is shown in Figure 1.

The volunteers were allocated either intervention at random order, using a computer-generated pseudo-random numbers table. A member of the research team (not involved in subject selection) requested each subject to randomly select one of a series of opaque sealed envelopes containing identification of the type of beverage.

Figure 1. Overview of the study



The details of group assignment were kept in a sealed envelope that was opened at the end of the complete experimental period. Neither the subjects nor the rest of the research team knew about subject assignment during the experimental period. The participants were provided with a list of foods and beverages rich in β-Cx that were to be avoided, and were asked not to change their usual diet or physical activity. They were also instructed to record any side effects during the study by means of a Food Frequency Questionnaire (FFQ) [15] that they completed at the end of each intervention period. Compliance was confirmed at the end of each intervention period by requesting the number of non-ingested cartons.

2.3. Blood and feces sampling

Sample collection was performed before and after each 6-week treatment period (see Figure 1), when a centralized service assigned a 7-digit identification number to each subject (following the usual practice for all hospital patients), and a member of the research team supervised that samples from each subject were collected in sterile plastic containers and stored at -20ºC until analysis. Only the nurse or laboratory technicians knew the assigned number of the sample, and were unaware of which treatment was received by the participants.

2.4. Markers of cardiovascular risk

To confirm mild hypercholesterolemia (basal level) and to evaluate the effect of the PS-enriched beverages (with or without GOS) on the lipid profile, total and HDL-cholesterol were determined by an automated routine method (Advia 2400 Clinical Chemistry System, Siemens Healthineers) [9,10]. Periodically, these analyses are subjected to External Quality Assurance Program of the Spanish Society of Medicine of the SEQC-ML Laboratory, which have implemented a quality management system in accordance with the UNE-EN ISO 9001 standard certified by AENOR. The Friedewald equation was used to estimate the LDL-cholesterol concentration [25].

2.5 Serum biomarkers: sterol analysis

PS (campesterol, stigmasterol and β-sitosterol) and cholesterol precursors (demosterol and lathosterol) and metabolite (cholestanol) in serum samples were analyzed by GC-FID following a previously validated methodology [26].

2.6 Fecal sterols and their metabolites

This determination was carried out by gas chromatography-mass spectrometry (GC-MS) following the methodology validated by Cuevas-Tena et al. (2017) [27].

2.7 Statistical analysis

Comparison of the total cholesterol, HDL- and LDL-cholesterol levels was performed by paired t-test for parametric variables. A value of $p<0.05$ will be considered statistically significant and Medcalc program (MedCalc® Version 11.4.2.0) was used.

To confirm the use of nonparametric test, the normal distribution of sterol and metabolite contents in serum and feces was evaluated using the Shapiro-Wilk test. A Wilcoxon test was used in order to detect statistically significant differences in serum sterol (cholesterol precursors and metabolite and PS) and in fecal sterol contents between pre-treatment and post-treatment and in changes values

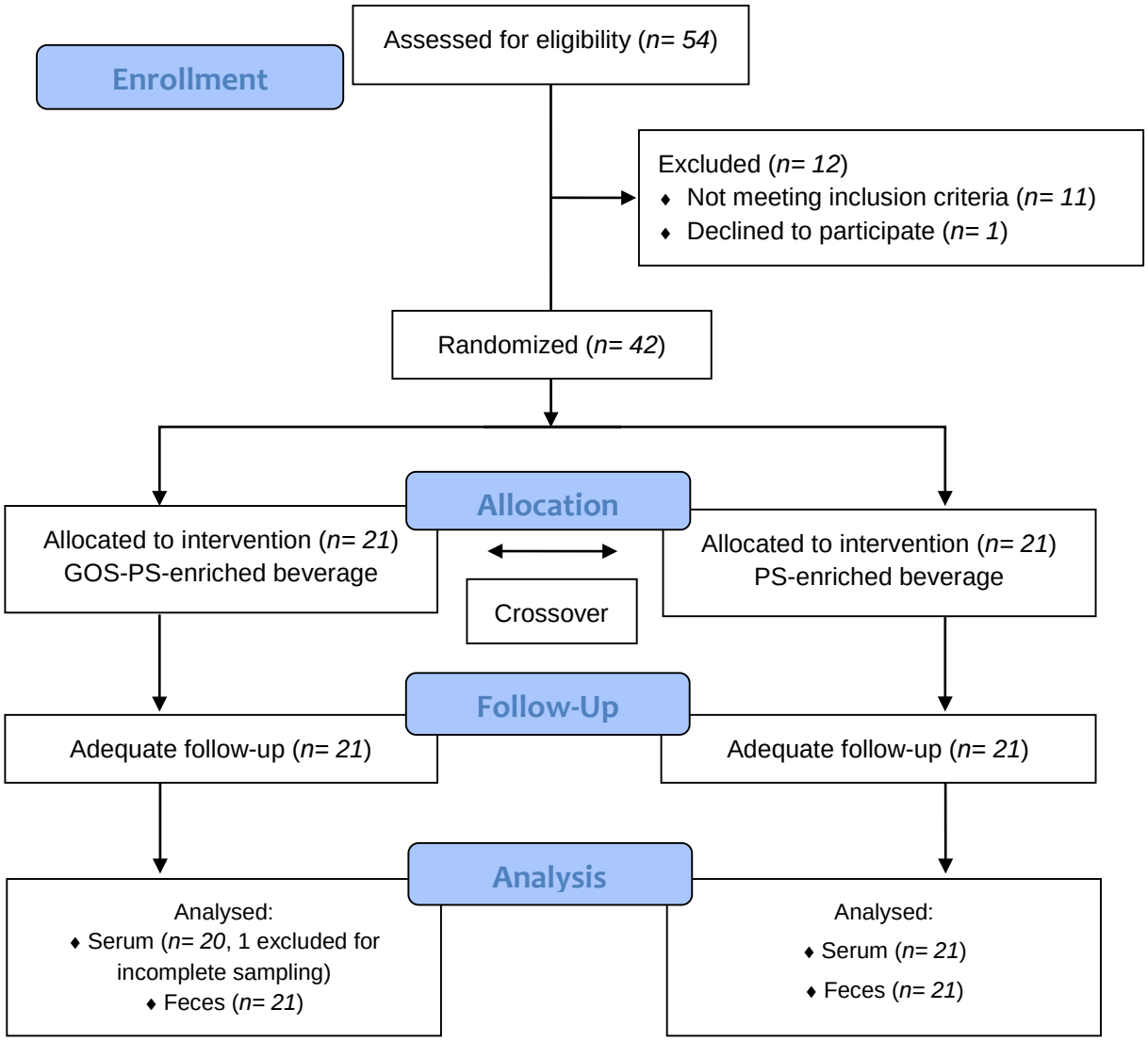
(absolute and percentage) between beverages (PS-enriched or GOS-PS-enriched). Univariate correlations between serum cholesterol levels and fecal cholesterol/coprostanol ratio or fecal cholesterol percentage of conversion after the intake of both beverages were investigated using the Spearman coefficient. In all cases, a level of $p < 0.05$ was used as the criterion for statistical significance and the Statgraphics Centurion XVI.I statistical package was used. The analysis of all samples was performed in triplicate.

3. Results and Discussion

3.1. Progress of the study

Participant flow is shown in Figure 2, which started in March 2017 and was completed in June 2017. 54 post-menopausal women were contacted for participation and interviewed in order to confirm that they met the inclusion criteria (enrollment). 12 were excluded, and 42 participated and were randomly assigned. In the analysis phase of sterols in serum samples, a subject was excluded due to incomplete sampling in the second intervention period. The women who finally participated in the study had an average age of 58.4±4.1 years (range 45-67), presented untreated mild hypercholesterolemia (229.8±25.2 g/dL) with a BMI of 24.5±3.2 kg/m^2. At the end of the study, the participants reported not having detected any differences in the organoleptic properties of the two beverages in the FFQ; neither did they express any body weight changes.

Figure 2. Participant flow



3.2. Impact of GOS on sterol bioavailability and hypocholesterolemic effect

In Table 1, the serum sterol contents and change values (absolute and percentages) are shown. The values have been normalized with total cholesterol levels in order to avoid the interindividual variations of the lipoprotein levels.

Table 1. Sterols response in serum upon regular consumption of the beverages (n=41) (Mean, Confidence Intervals 95%)

Sterols	Pre-treatmet		Post-treatment (6 weeks)		Change			
	μg mL^{-1}	(μmol $mmol^{-1}$ cholesterol)	μg mL^{-1}	(μmol $mmol^{-1}$ cholesterol)	Absolute (μg mL^{-1})	Absolute (μmol $mmol^{-1}$ cholesterol)	(%) (μg mL^{-1})	(%) (μmol $mmol^{-1}$ cholesterol)
PS-enriched beverage								
Cholestanol	6.68 a	2.87 a	6.74 a	3.03 b	0.06 y	0.16 y	2.42 y	7.18 y
	(6.27,7.09)	(2.69, 3.05)	(6.39,7.09)	(2.87, 3.19)	(-0.27,0.39)	(0.01,0.31)	(-2.59,7.43)	(1.75,12.61)
Desmosterol	1.90 a	0.84 a	1.96 a	0.91 b	0.07 y	0.07 y	4.03 y	8.88 y
	(1.77,2.03)	(0.79, 0.89)	(1.81,2.11)	(0.84, 0.98)	(-0.02,0.16)	(0.03,0.11)	(-0.34,8.4)	(4.61,13.15)
Lathosterol	3.41 a	1.49 a	3.51 a	1.60 b	0.10 y	0.11 y	6.10 y	11.12 y
	(2.95,3.87)	(1.29, 1.69)	(3.1,3.92)	(1.43, 1.77)	(-0.1,0.3)	(0.03,0.19)	(0.13,12.07)	(4.93,17.31)
Total animal	12.28 a	5.29 a	12.53 a	5.64 b	0.25 y	0.35 y	2.61 y	7.35 y
	(11.53,13.03)	(4.97,5.61)	(11.75,13.31)	(5.31,5.97)	(-0.23,0.73)	(0.20,0.50)	(-1.23,6.45)	(3.28,11.42)
Campesterol	4.10 a	1.72 a	4.59 b	2.02 b	0.51 y	0.30 y	18.54 y	23.50 y
	(3.61,4.58)	(1.53, 1.91)	(4.13,5.05)	(1.83, 2.21)	(0.15,0.87)	(0.15,0.45)	(6.65,30.43)	(11.09,35.91)
Stigmasterol	0.37 a	0.15 a	0.40 a	0.17 b	0.03 y	0.02 y	15.53 y	21.03 y
	(0.31,0.43)	(0.13, 0.17)	(0.34,0.46)	(0.15, 0.19)	(-0.01,0.07)	(0.01,0.04)	(1.33,29.73)	(6.48,35.58)
β-Sitosterol	4.39 a	1.79 a	5.63 b	2.40 b	1.24 y	0.61 y	32.48 y	38.75 y
	(3.96,4.82)	(1.62, 1.96)	(5.12,6.14)	(2.20, 2.60)	(0.82,1.66)	(0.44,0.78)	(22.01,42.95)	(27.77,49.73)
Total PS	8.79 a	3.62 a	10.46 b	4.51 b	1.67 y	0.89 y	22.81 y	28.73 y
	(7.92,9.66)	(3.28,3.96)	(9.54,11.38)	(4.15,4.87)	(0.97,2.37)	(0.61,1.17)	(13.29,32.33)	(18.69,38.77)
GOS-PS-enriched beverage								
Cholestanol	6.67 a	2.90 a	6.73 a	3.06 b	0.06 y	0.16 y	1.05 y	6.04 y
	(6.31,7.03)	(2.73, 3.07)	(6.33,7.13)	(2.88, 3.24)	(-0.16,0.28)	(0.05,0.27)	(-2.26,4.36)	(2.09,9.99)
Desmosterol	1.92 a	0.84 a	1.99 a	0.92 b	0.07 y	0.08 y	6.76 y	12.50 y
	(1.76,2.08)	(0.78, 0.90)	(1.85,2.13)	(0.86, 0.98)	(-0.03,0.17)	(0.03,0.13)	(-0.98,14.5)	(4.35,20.65)
Lathosterol	3.43 a	1.50 a	3.50 a	1.61 b	0.06 y	0.11 y	4.37 y	9.97 y
	(3.00,3.86)	(1.32, 1.68)	(3.07,3.93)	(1.42, 1.80)	(-0.12,0.24)	(0.03,0.19)	(-1.74,10.48)	(3.52,16.42)
Total animal	12.30 a	5.35 a	12.51 a	5.70 b	0.21 y	0.35 y	1.80 y	6.79 y
	(11.62,12.98)	(5.07,5.63)	(11.81,13.21)	(5.40,6.00)	(-0.06,0.48)	(0.20,0.50)	(-0.52,4.12)	(3.91,9.67)
Campesterol	4.23 a	1.78 a	4.51 b	2.00 b	0.29 y	0.22 y	8.03 y	13.58 y
	(3.74,4.73)	(1.59, 1.97)	(4.01,5.00)	(1.79, 2.21)	(0.03,0.55)	(0.12,0.32)	(1.79,14.27)	(7.54,19.62)
Stigmasterol	0.38 a	0.16 a	0.37 a	0.16 a	-0.01 y	0.002 y	3.98 y	10.13 y
	(0.32,0.44)	(0.14, 0.18)	(0.31,0.43)	(0.14, 0.18)	(-0.05,0.03)	(-0.01,0.02)	(-10.36,18.32)	(-5.67,25.93)
β-Sitosterol	4.65 a	1.89 a	5.84 b	2.51 b	1.19 y	0.62 y	28.88 y	35.73 y
	(4.12,5.18)	(1.68, 2.10)	(5.24,6.44)	(2.26, 2.76)	(0.83,1.55)	(0.47,0.77)	(21.39,36.37)	(27.99,43.47)
Total PS	9.19 a	3.78 a	10.61 b	4.67 b	1.43 y	0.83 y	17.33 y	23.57 y
	(8.21,10.17)	(3.40,4.16)	(9.54,11.68)	(4.23, 5.11)	(0.83,2.03)	(0.58,1.08)	(11.01,23.65)	(17.10,30.04)

Analyses were made in triplicate. Different superscript letters denote significant differences ($p < 0.05$) in the same kind of beverage (PS-enriched or GOS-PS-enriched beverage) among pre-treatment and post-treatment values (within lines) (a,b), or in different beverages among changes (absolute or expressed as percentage) (within columns) (y,z). Absolute change = post-treatment level minus pre-treatment level. Change (%) = Absolute change × 100/pre-treatment level. Total PS: sum of campesterol, stigmasterol and β-sitosterol.

No significant differences in the percentages of change of PS levels were detected after the consumption of either beverage (without and with GOS addition), suggesting no effect of the presence of GOS on PS bioavailability. The regular intake of the beverages significantly increased normalized concentrations of campesterol (13.6-23.5%) and β-sitosterol (35.7-38.8%) as markers of dietary PS intake, whereas stigmasterol (minor PS in the beverage) only increased in the PS-enriched beverage. In addition, in this study, serum levels of PS cannot be considered as markers of cholesterol absorption since the intake of dietary PS increased due to the intervention [28].

Similarly to the PS contents, no differences of cholestanol, desmosterol and lathosterol (cholesterol metabolism markers) were observed between treatments, showing increases of 6.0-7.2%, 8.9-12.5% and 10.0-11.1%, respectively, after the intervention. On comparing our results with a previous clinical study [10], similar significant increases of desmosterol, lathosterol, campesterol and β-sitosterol were obtained with a similar PS-enriched beverage. Although in the present study, there was a significant change in stigmasterol contents with respect to the aforementioned clinical trial, the absolute increments (in μg/mL, 0.03 *vs.* 0.00) cannot be considered relevant since its low concentration in the beverages as well as its low absorption.

Contents of total, HDL-, and LDL-cholesterol at pre-treatment and post-treatment are reported in Table 2. The decrease ($p < 0.05$) in total cholesterol levels obtained during the intervention was not reflected in a drop of cholestanol (cholesterol absorption marker) but in a significant increase in desmosterol and lathosterol (cholesterol synthesis markers) (Table 1). In agreement with our results, an increase in serum cholesterol synthesis markers in post-menopausal women with mild hypercholesterolemia who intake PS-enriched margarine has been observed [29], although they also reported a decreased in cholestanol levels. Perhaps the higher intervention times (6 months vs. 6 weeks), and the different type and dose of plant sterols (3 g of plant stanol ester vs. 2 g of free phytosterols) could partly justify these differences.

Table 2. Serum lipid profile response upon regular consumption of the beverages (n=42). Results are expressed as Mean±SD

	PS-enriched beverage		GOS-PS-enriched beverage	
(mg/dL)	**Pre-treatment**	**Post-treatment**	**Pre-treatment**	**Post-treatment**
Total cholesterol	229.8±25.2[a]	219.1±22.9[b,x]	227.7±25.4[a]	216.2±23.8[b,x]
LDL-cholesterol	142.0±21.1[a]	129.2±22.5[b,x]	138.9±21.5[a]	128.3±18.8[b,x]
HDL-cholesterol	70.1±14.8[a]	71.9±14.8[a,x]	71.4±17.7[a]	71.0±19.0[a,x]

Different superscript letters denote significant differences ($p < 0.05$) in the same kind of beverage (PS-enriched or GOS-PS-enriched) between pre-treatment and post-treatment values (a,b) and in the post-treatment values between beverages (x). Reference range (mg/dL): total cholesterol (150-200); LDL-cholesterol (70-160); HDL-cholesterol (35-75).

As it can be observed, the addition of GOS to these beverages did not modify the hypocholesterolemic effect since their consumption exerted similar ($p > 0.05$) decreases in total (4.7-5.1%) and LDL-cholesterol (7.6-9.0%) without significant changes in HDL-cholesterol. Accordingly, a double-blind, cross-controlled, placebo-controlled study carried out with healthy adults showed that the intake of GOS (in powder form) at a similar dose of our study (5.5 vs. 4.3 g/day, respectively) during 10 weeks, did not modify cholesterol and HDL plasma cholesterol [20]. However, when longer treatment periods are assessed (6 *vs.* 12 weeks) using the same type and dose of GOS, a significant decrease of the total cholesterol/HDL-cholesterol ratio in adults with metabolic syndrome was only detected after 12 weeks [21]. Furthermore, studies carried out in doses of GOS both equivalent and much higher (5.4-54 g/day, assuming a 65 kg of body weight) to those used in humans during 6-8 weeks reported an improvement of the lipid-related serum parameters (triglycerides, total, HDL-, LDL-, and VLDL-cholesterol) in high-fat-diet-induced metabolic syndrome mice [18] and dyslipemia rats [19], and in healthy rats with a dose-response effect [17]. Thus, the studies related to the effect of GOS on serum lipid profile in humans cannot be as conclusive as in murine models. In addition, the treatment period in human population could be a factor conditioning the hypocholesterolemic effect of the GOS and, thus, would likely have to be extended. Therefore, the 6-week treatment

period of our study may be a limitation, although longer intervention periods could increase the risk of lifestyle changes (diet, physical activity, etc.) as well as favour the withdrawal of the trial.

On the other hand, it has been stated that higher baseline LDL-cholesterol concentrations result in greater absolute LDL-cholesterol reductions [30]. In this regard, we have observed this association among the previous clinical trials carried out with PS-enriched beverages (providing 1.5-2 g PS/day): 129.4±28.5 mg/dL with a 5.1% of reduction [10] and 146.0±31.8 mg/dL with a 7% [9], as well as in the present study (138.9-142 mg/dL with a reduction between 7.6-9%).

3.3. Impact of GOS on colonic sterol metabolization

Table 3 show fecal animal contents for the two sampling points (pre-treatment and post-treatment), as well as the absolute change from basal values after regular consumption of the PS or GOS-PS-enriched beverages.

The total fecal animal sterol contens ranged 13.29 – 27.10 mg/g freeze-dry feces, with coprostanol as the main metabolites representing 53-54% of total sterols of post-treatment animal origin. No statistically significant differences ($p<0.05$) were observed after the intake of the beverages (neither with respect to the baseline/pre-treatment value or between absolute change for both beverages). These results were according to those obtained in a previous study of our research group (13.9-30.10 mg/g freeze-dry feces) [15].

After intake of PS- or GOS-PS-enriched beverages, a significant increase (post-treament *vs*. pre-treatment) in the excretion of cholesterol (65% and 71%), coprostanone (58% and 87%) and cholestanol + methylcoprostanol (54% and 76%) was observed, with a significant decrease in coprostanol (24% and 14%), without modification of the latosterol content. It should be noted that although in the presence of GOS these changes are more pronounced, there are no differences between the absolute changes of individual animal sterols for both beverages (see Table 3).

The increase in cholesterol excretion observed in feces is a fact already indicated in a previous study by our group after ingesting a beverage enriched in PS (2g/day) similar to that administered in this study [15] and can be justified by the known interaction of PS in the absorption of cholesterol.

In addition, this work confirms again that in the presence of large amounts of PS, the metabolism of cholesterol by the microbiota occurs through an incomplete indirect route that results in greater excretion of coprostanone and less coprostanol or another pathway in which cholestanol is formed by reduction of cholestenone and cholestanone [31]. The intake of margarine containing PS (8.6 g/day) by normolipidic subjects during 28 days also reduces the metabolism of cholesterol to coprostanol [14].

Unlike total animal sterols, significant increases ($p<0.05$) are observed in total PS after the intake of both beverages with respect pre-treatment values (23.31-55.94 *vs*. 4.96-7.58), a fact that reflects the adherence of the women to the study, although no influence of the GOS is observed in the absolute changes (see Table 4). These facts also occur in the individual sterols, increasing mainly of ethylcoprostanol (post-treatment: 45-48% of the total PS, derived from β-sitosterol) and methylcoprostanone (from campesterol).

Table 3. Fecal animal sterols contents (mg/g freeze-dry feces) after regular consumption of the beverages (n=42) (Median, Percentile 25-75%)

Sterol	Pre-treatment	Post-treatment (6 weeks)	p value	Absolute change	p value	Conversion percentages	
						Low converters	High converters
PS-enriched beverage							
Cholesterol	2.19[a] (1.48; 2.76)	3.94[b] (1.99; 5.58)	2×10^{-4}	1.43 (0.04; 3.09)[y]		30.3-36.0 (2)	51.3-93.8 (40)
Coprostanol	13.38[a] (9.62; 18.71)	10.68[b] (6.74; 15.89)	8×10^{-4}	-3.16 (-5.35; -0.47)[y]			
Coprostanone	0.93[a] (0.40; 2.28)	1.67[b] (0.96; 3.11)	2×10^{-3}	0.54 (-0.27; 1.22)[y]			
Cholestanol + methylcoprostanol[c]	1.15[a] (0.93; 1.42)	1.77[b] (1.23; 2.76)	3×10^{-6}	0.62 (0.16; 1.41)[y]			
Lathosterol	0.09[a] (0.07; 0.12)	0.09[a] (0.07; 0.13)	0.40	0.01 (-0.01; 0.02)[y]			
Total animal sterols	19.28[a] (13.49; 26.24)	20.05[a] (13.29; 27.10)	0.65	-0.01 (-3.33; 5.40)[y]			
GOS-PS-enriched beverage							
Cholesterol	1.90[a] (1.49; 3.03)	3.99[b] (2.33; 6.05)	7×10^{-6}	1.35 (0.43; 3.79)[y]	0.38	2.1-44.1 (3)	51.0-94.3 (39)
Coprostanol	14.45[a] (10.96; 18.64)	12.07[b] (7.06; 15.26)	4×10^{-3}	-2.05 (-7.51; 0.70)[y]	0.96		
Coprostanone	0.87[a] (0.47; 2.14)	2.34[b] (1.15; 3.13)	7×10^{-4}	0.76 (-0.17; 2.24)[y]	0.45		
Cholestanol + methylcoprostanol[c]	1.02[a] (0.90; 1.35)	1.84[b] (1.33; 2.37)	7×10^{-7}	0.78 (0.21; 1.29)[y]	0.48		
Lathosterol	0.09[a] (0.07; 0.12)	0.09[a] (0.07; 0.15)	0.11	0.01 (-0.02; 0.03)[y]	0.50		
Total animal sterols	19.58[a] (16.30; 25.90)	22.49[a] (17.66; 27.91)	0.65	1.59 (-3.21; 5.84)[y]	0.67		

Absolute change: post-treatment level minus pre-treatment level. Different superscript letters denote significant differences ($p < 0.05$) in the same kind of beverage (PS-enriched or GOS-PS-enriched) among pre-treatment and post-treatment values (within lines) (a,b), or in different beverages among absolute changes (within columns) (y,z). Cholesterol conversion percentage: [coprostanol + coprostanone/(cholesterol + coprostanol + coprostanone)] x 100. Low and high converters were defined according to Wilkins & Hackman (1974) [35] considering that low converters have a sterol conversion rate of <50% and high converters of >50%. Number of subjects corresponding to each group are indicated between parentheses.

[c] The applied method does not allow the separation of these compounds.

Table 4. Fecal plant sterols contents (mg/g freeze-dry feces) after regular consumption of the beverages (n=42) (Median, Percentile 25-75%)

Sterol	Pre-treatment	Post-treatment (6 weeks)	p value	Absolute change	p value	Conversion percentages	
						Low converters	High converters
PS-enriched beverage							
β-Sitosterol	0.74^{a} (0.64; 0.99)	11.21^{b} (2.29; 22.33)	2×10^{-7}	8.29^{y} (1.49; 17.27)		9.4-49.5 (15)	50.0-87.8 (26)
Sitostanol	0.57^{a} (0.46; 0.66)	3.33^{b} (1.99; 5.74)	5×10^{-8}	2.84^{y} (1.34; 4.91)			
Ethylcoprostanol	3.97^{a} (2.70; 5.17)	17.49^{b} (7.36; 28.66)	8×10^{-8}	12.95^{y} (2.65; 20.66)			
Campesterol	0.32^{a} (0.24; 0.43)	1.57^{b} (0.62; 2.40)	2×10^{-7}	1.09^{y} (0.34; 2.03)		0.5-44.3 (42)	
Campestanol	0.38^{a} (0.31; 0.50)	0.86^{b} (0.58; 1.31)	4×10^{-8}	0.46^{y} (0.20; 0.80)			
Methylcoprostanone	0.05^{a} (0.03; 0.11)	0.24^{b} (0.07; 0.43)	2×10^{-6}	0.15^{y} (0.03; 0.40)			
Stigmasterol	0.06^{a} (0.04; 0.08)	0.16^{b} (0.08; 0.26)	1×10^{-5}	0.07^{y} (0.00; 0.19)		0.0002-48.6 (30)	50.9-90.3 (12)
Ethylcoprostenol	0.11^{a} (0.08; 0.13)	0.10^{a} (0.08; 0.13)	0.93	0.002^{y} (-0.02; 0.02)			
Total PS	6.77^{a} (4.96; 7.58)	36.49^{b} (23.27; 53.89)	2×10^{-10}	29.05^{y} (11.77; 44.55)			
GOS-PS-enriched beverage							
β-Sitosterol	0.76^{a} (0.56; 1.13)	12.02^{b} (2.68; 20.36)	3×10^{-8}	10.79^{y} (2.14; 19.30)	0.79	1.5-45.1 (15)	51.2-85.8 (26)
Sitostanol	0.56^{a} (0.50; 0.73)	3.90^{b} (1.98; 5.42)	3×10^{-8}	3.16^{y} (1.27; 4.80)	1.00		
Ethylcoprostanol	3.47^{a} (2.47; 4.58)	17.40^{b} (8.65; 27.32)	5×10^{-8}	14.47^{y} (4.91; 21.56)	0.88		
Campesterol	0.31^{a} (0.23; 0.42)	1.60^{b} (0.87; 2.55)	1×10^{-10}	1.41^{y} (0.47; 2.11)	0.64	0.7-49.0 (42)	
Campestanol	0.36^{a} (0.26; 0.46)	0.83^{b} (0.59; 1.23)	8×10^{-8}	0.44^{y} (0.23; 0.82)	0.61		
Methylcoprostanone	0.05^{a} (0.03; 0.08)	0.25^{b} (0.12; 0.50)	1×10^{-6}	0.18^{y} (0.03; 0.45)	0.36		
Stigmasterol	0.06^{a} (0.05; 0.09)	0.18^{b} (0.09; 0.30)	8×10^{-8}	0.09^{y} (0.02; 0.23)	0.67	0.0002-47.9 (29)	51.9-83.7 (13)
Ethylcoprostenol	0.10^{a} (0.09; 0.12)	0.11^{a} (0.09; 0.13)	0.37	0.01^{y} (-0.01; 0.02)	0.25		
Total PS	6.01^{a} (5.01; 7.26)	38.99^{b} (23.21; 55.94)	4×10^{-8}	32.54^{y} (18.07; 49.28)	0.60		

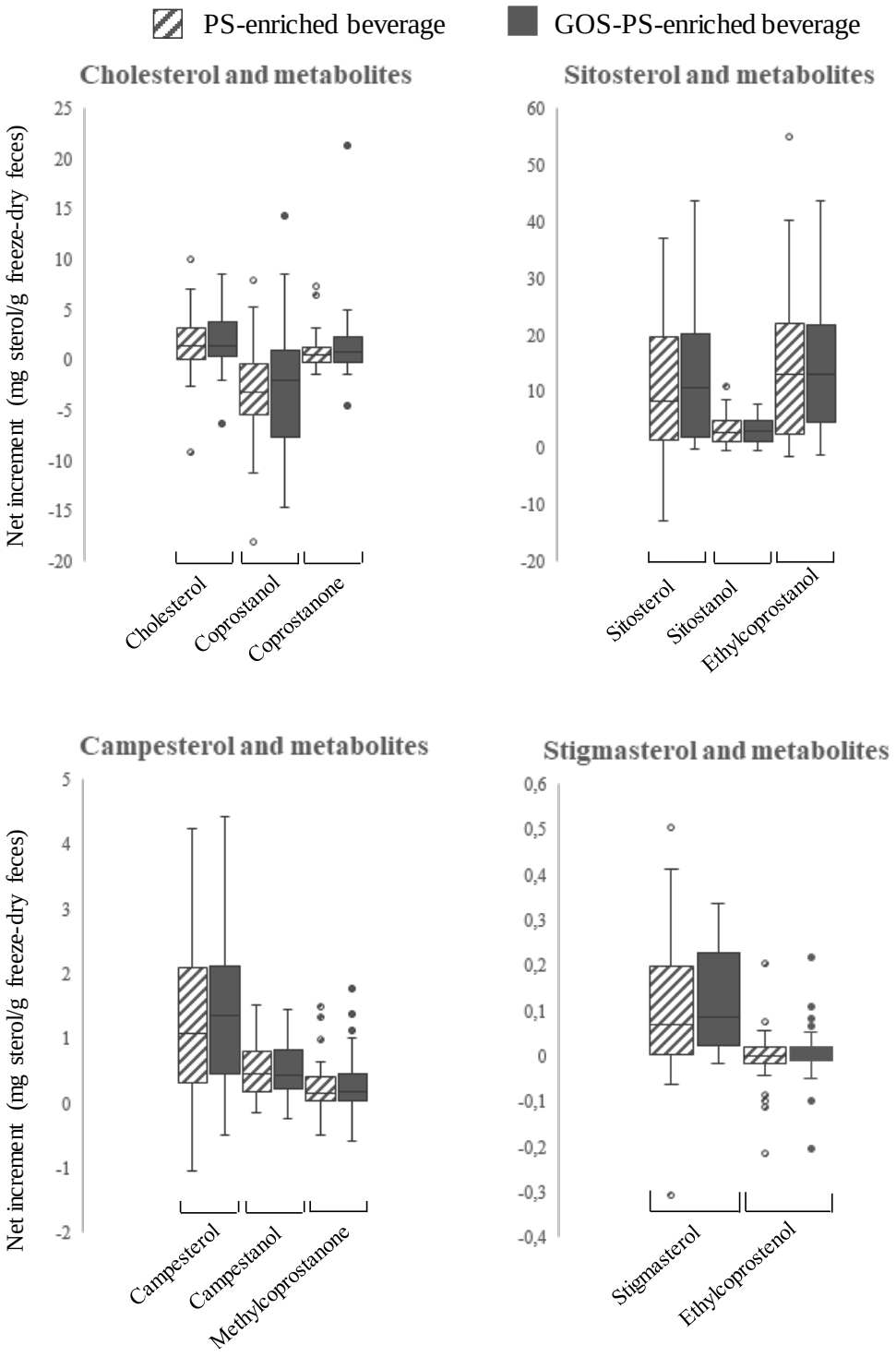
Absolute change: post-treatment level minus pre-treatment level. Different superscript letters denote significant differences ($p < 0.05$) in the same kind of beverage (PS-enriched or GOS-PS-enriched) among pre-treatment and post-treatment values (within lines) (a,b), or in different beverages among absolute change (within columns) (y,z). β-Sitosterol conversion percentage: [ethylcoprostanol/(β-sitosterol + sitostanol + ethylcoprostanol)] x 100; Campesterol conversion percentage: [methylcoprostanone/(campesterol + campestanol + methylcoprostanone)] x 100; Stigmasterol conversion percentage: [ethylcoprostenol/(stigmasterol + ethylcoprostenol)] x 100. Low and high converters were defined according to Wilkins & Hackman (1974) [35] considering that low converters have a sterol conversion rate of <50% and high converters of >50%. Number of subjects corresponding to each group are indicated between parentheses.

No influence of GOS on colonic fermentation of cholesterol and PS has been observed and this fact well displayed on the boxes which represent the mean of the absolute change after consumption of PS- or GOS-PS-enriched beverages (see Figure 3). Although the intake of cholesterol and PS from the diet was not controlled, which constitutes a limitation of the study, in both cases the response of the women was very similar, observing very few outliers. Other limitation of our study was the lack of a microbiota analysis, which would have provided valuable information about the absence of GOS effect on sterol metabolism. Although the bifidogenic effect of GOS is widely reported in *in vivo* studies [16], certain investigations have been shown a minimum dosage of 5 g GOS per day (slightly higher to the one used in the present study) to achieve significant increases of bifidobacteria counts [32–34]. Differences on GOS ingredient, delivery vehicle, experimental design and microbiota methods of analysis used could also influence the variations in the expected bifidogenic effect [33]. Moreover, although GOS are highly specific promoting the growth of bifidobacteria, they do not have a consistent effect on the increase or decrease of other bacterial species [34]. In the present study, the absence of GOS effect on sterol metabolism could be explaining by a lack of modulation of coprostanoligenic bacteria (responsible of cholesterol-to-coprostanol reduction) which includes *Bifidobacterium*, *Bacteroides* or *Eubacterium* species, being moreover this last one the only species associated with the PS biotransformation pathway [13].

In order to know if the intake of the beverages enriched in GOS modifies the biotransformation/colonic fermentation of cholesterol and individual PS (β-sitosterol, stigmasterol and campesterol), conversion percentages have been calculated and women have been classified as low or high converters according to Wilkin & Hackman (1974) [35] (see Tables 3 and 4). Independently of the beverages ingested, in general women are predominat high converters of cholesterol (n=39 or 40) and β-sitosterol (n= 26) However, all subjects are low converters of campesterol and, in general also of stigmasterol. These results are only partially coincident with a previous study [15] which a lower number of high cholesterol converters (29) and β-sitosterol (17) and a higher number of high stigmasterol converters were detected (27 *vs*. 13). Note that it is known that the efficiency of microbial

cholesterol-to-coprostanol conversion in human populations (membres of genus *Eubacterium* and strains of *Bifidobacterium, Lactobacillus and Peptostreptococcus)* is majority of high converters [36].

Figure 3. Sterol response in feces upon regular consumption of beverages (n = 42). Boxes represent the mean of the absolute change (post-treatment – pre-treatment values). Points in each box represent outlier values



Intake of the GOS enriched beverage induces slight changes in the colonic metabolic capacity of women (see Supplementary Figure 1). The conversion capacity of cholesterol and stigmasterol in 12 women are reduced by 10 %, and of β-sitosterol in 16. Only in five of them is the metabolism of all these sterols reduced simultaneously. A decrease of cholesterol conversion percentage after the intake of a similar PS-enriched beverage has been previously reported [15], associated with the increase of PS consumption that could reduce or block cholesterol metabolite production. Reduction of cholesterol conversion is the interest since cholesterol metabolites has been associated with pro-carcinogenic action and could increase the risk of colon cancer [13]. Different response to GOS presence on sterol colonic metabolism could also be derived by different phylogenetic propierties of the microbiota of each woman. It has been reported that microbiota composition can be stratified in to three main groups (enterotypes), which present functional differences with respect to the obtention way of energy from the substrates available in the colon. In this sense, subjects belonging to Enterotype 1 or *Bacteroides* have been showed to obtain energy primaly from the fermentation of carbohydrates and proteins, whereas Enterotype 2 and 3 (*Prevotella* and *Ruminococcus*, respectively) are more efficient as degrading mucin [37].

A high efficiency of conversion from cholesterol to coprostanol has been related with an improved lipid serum profile, due to the passage of cholesterol from the intestine into the blood is avoided and for the poorly intestinal absoption of coprostanol [38]. In this sense, Sekimoto et al. [39] observed an inverse correlation between serum cholesterol leves and fecal coprostanol/cholesterol ratio, suggesting that coprostanol production could be modulate cholesterol blood levels. However, in the present study, this correlation has not been observed for any beverages, nor between serum cholesterol levels and cholesterol conversion percentages in feces.

4. Conclusions

In the clinical trials carried out with the regular intake of two PS-enriched beverages with or without GOS, it has been found that the GOS do not modify the bioavailability of PS, nor its colonic metabolism and do not enhance its blood cholesterol lowering effect. Probably longer intervention

times (over 12 weeks) are required to verify any beneficial effect of the same. Even though many trials were performed, the results are at times contradictory and, therefore, prebiotics supplementation and its relationship with blood lipid levels warrant further research.

In future studies, factors that limit this work should be controlled, such as individuals genotype and lifestyle (physical exercise), cholesterol and PS content of the diet, and the changes exerted upon microbiota composition.

Statements of authorship

AA was the principal investigator. AA, GG-L and RAS contributed to the study design and the writing of the study protocol. RAS, EH-A and ED-N conducted the subject enrollment of the clinical trial and the sample collection. VB-M was in charge of the samples analysis and data collection. AA, GG-L, RAS and VB-M carried out the data analysis and wrote the manuscript. All authors have read and approved the final manuscript.

Sources of support

This study is part of the National Project AGL2015-68006-C2-1-R, supported by the Spanish Ministry of Economy and Competitiveness (MINECO) and the European Regional Development Fund (FEDER). Virginia Blanco-Morales holds a research contract under the afromentioned project (Ref. CPI-17-025).

Conflict of interest

The author declare that they have no conflict of interest.

Acknowledgements

We thank Hero España, S.A. for manufacturing the beverages used in the present clinical trial. The authors also thank Dr. María Cuevas-Tena for her support and invaluable advice.

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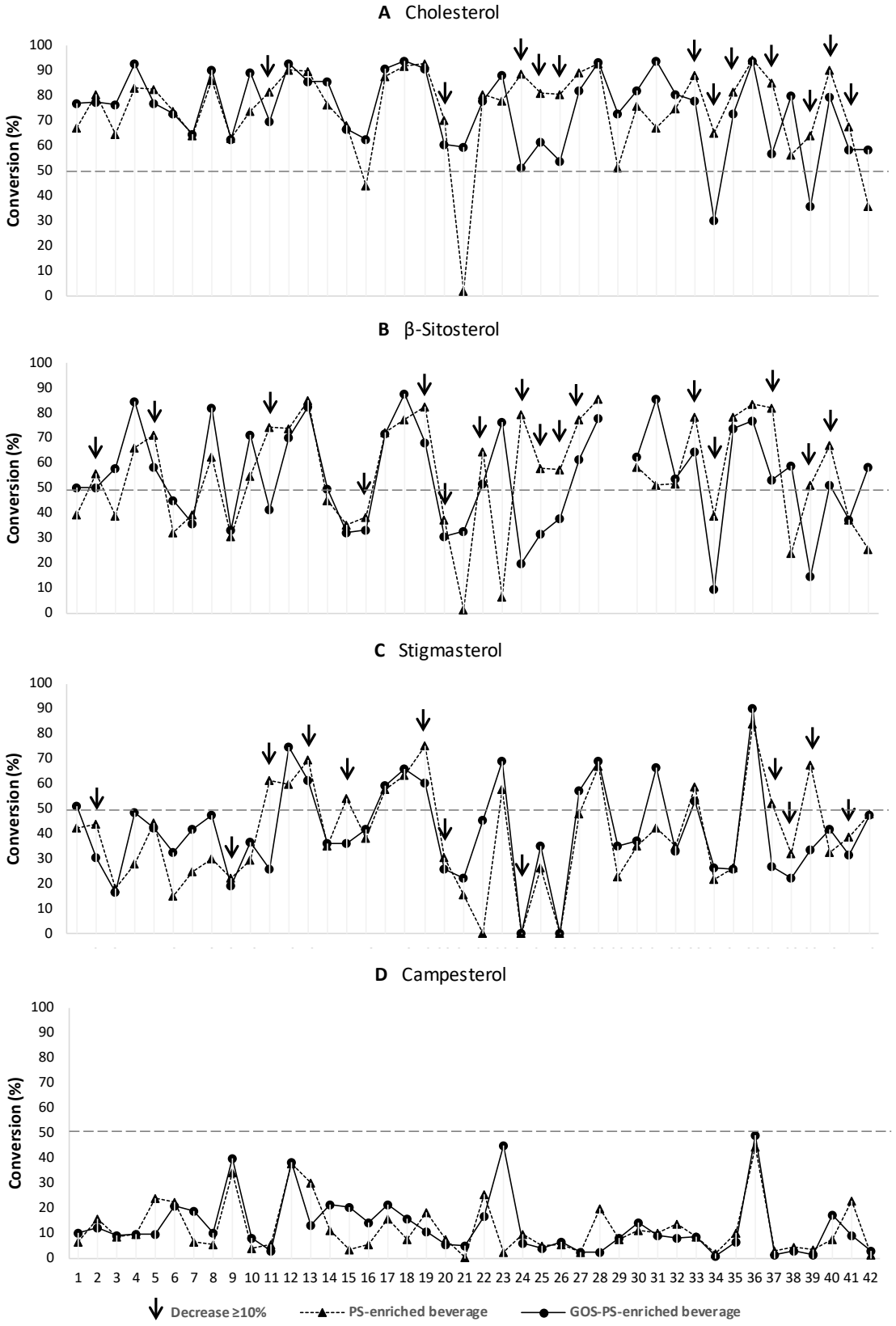
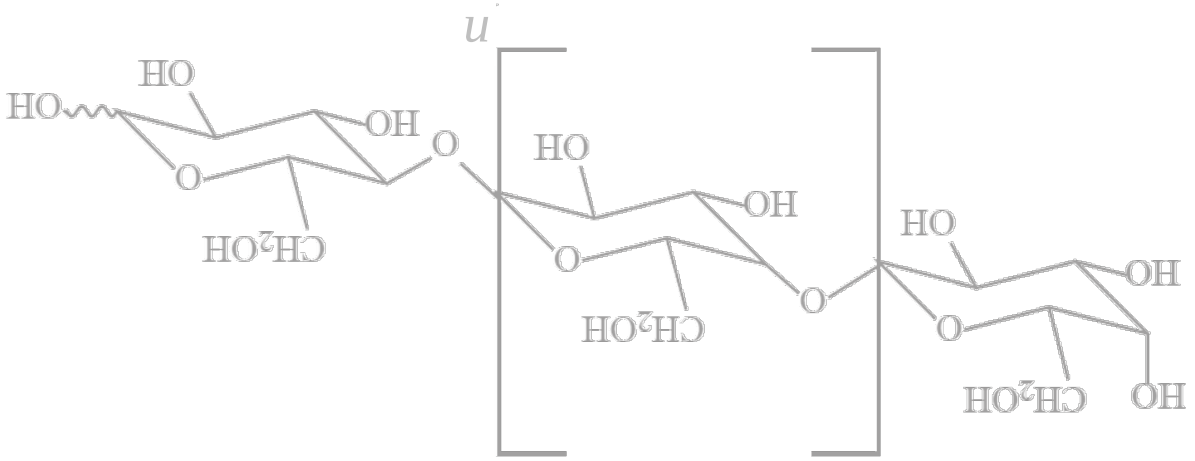
Supplementary data: Figure 1. Sterol conversion percentages after beverages consumption (*n = 42*)

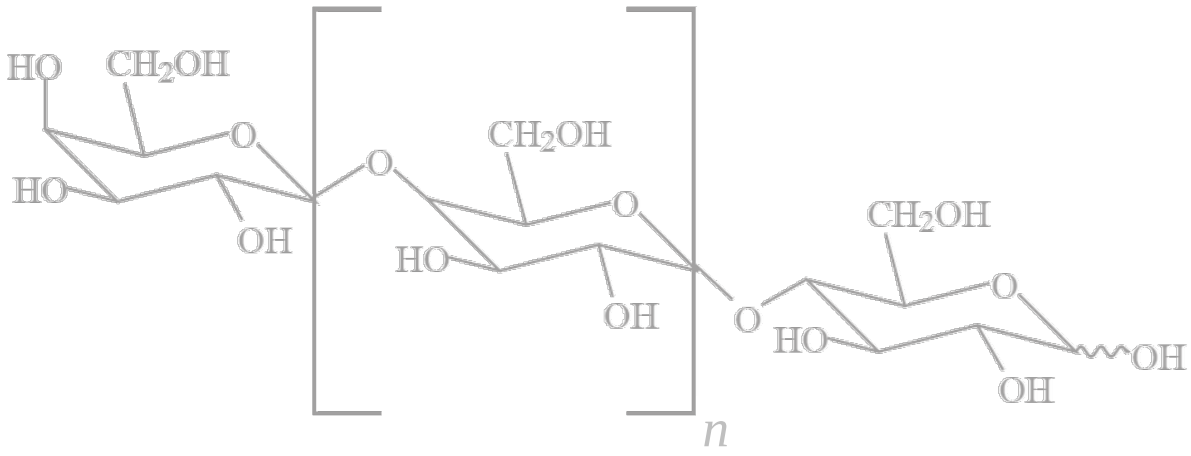


Abscissa axis represents the 42 postmenopausal women from the clinical study, whereas ordinate axis represents the sterol conversion percentage. Cholesterol conversion percentage: [coprostanol + coprostanone/(cholesterol + coprostanol + coprostanone)] x 100. β-Sitosterol conversion percentage: [ethylcoprostanol/(β-sitosterol + sitostanol + ethylcoprostanol)] x 100; Stigmasterol conversion percentage: [ethylcoprostenol/(stigmasterol + ethylcoprostenol)] x 100; Campesterol conversion percentage:

[methylcoprostanone/(campesterol + campestanol + methylcoprostanone)] x 100. To classify low and high converters, a dashed line was located on each graph indicating the threshold of 50% (according to Wilkins & Hackman, 1974). Black arrows indicate a significant decrease of sterol conversion percentage (≥10%) after PS-GOS-enriched beverage intake compared to PS-enriched beverage.



Anexo III: Comunicaciones a congresos



Autores: Blanco-Morales, V., López-García, G., Cilla, A., Garcia-Llatas, G., Barberá, R., Lagarda, M.J., Alegría, A.

Título: Métodos *in vitro* para la evaluación de la bioacesibilidad de esteroles: armonización de métodos. Aplicación a bebidas de zumo de frutas y leche.

Tipo de participación: Póster y Comunicación oral

Congreso: IV Congreso de estudiantes de Nutrición Humana y Dietética

Lugar de celebración: Valencia (España)

Año: 2017

Autores: López-García, G., **Blanco-Morales, V.**, Cilla, A., García-Llatas, G., Barberá, R., Lagarda, M.J., Alegría, A.

Título: Bioaccessibility of plant sterols in plant sterol enriched milk-based fruit beverages: comparison of two *in vitro* methods

Congreso: 5th International Conference on Food Digestion

Tipo de participación: Póster

Lugar de celebración: Rennes (Francia)

Año: 2017

Autores: Blanco-Morales, V., López-García, G., Cilla, A., Garcia-Llatas, G., Barberá, R., Lagarda, M.J., Alegría, A.

Título: Plant sterol enriched milk-based fruit beverages with or without galactooligosaccharides: Plant sterol stability and bioaccessibility.

Tipo de participación: Póster

Congreso: 31st EFFOST International Conference

Lugar de celebración: Sitges (España)

Año: 2017

Autores: Blanco-Morales, V., Garcia-Llatas, G., Lagarda, M. J., Alegría, A.

Título: Composición de la microbiota intestinal tras la fermentación *in vitro* de una bebida funcional, rica en esteroles vegetales, con o sin adición de galactooligosacáridos

Tipo de participación: Póster

Congreso: V Congreso de estudiantes de Nutrición Humana y Dietética

Lugar de celebración: Valencia (España)

Año: 2018

Autores: Blanco-Morales, V., Garcia-Llatas, G., Lagarda, M. J., Alegría, A.

Título: Colonic fermentation of sterols: optimization of a GC-MS method

Tipo de participación: Póster

Congreso: V National and IV International Student Congress of Food Science and Technology

Lugar de celebración: Valencia (España)

Año: 2018

Autores: Blanco-Morales, V., Garcia-Llatas, G., Lagarda, M. J., Alegría, A.

Título: Impact of galactooligosaccharides on colonic metabolism of sterols by a dynamic gastrointestinal model

Tipo de participación: Póster

Congreso: ENOR 8th Symposium

Lugar de celebración: Bolonia (Italia)

Año: 2018

Autores: Blanco-Morales, V., Garcia-Llatas, G., Lagarda, M. J., Alegría, A.

Título: Gut microbiota modulation in presence of galactooligosaccharides: evaluation by *in vitro* dynamic colonic fermentation

Tipo de participación: Póster

Congreso: 2nd International Conference on Food Bioactives and Health

Lugar de celebración: Lisboa (Portugal)

Año: 2018

Autores: Blanco-Morales, V., Garcia-Llatas, G., Yebra, M. J., Lagarda, M. J., Alegría, A.

Título: Evaluation of microbiota metabolism in presence of galactooligosaccharides by an *in vitro* dynamic fermentation

Tipo de participación: Póster

Congreso: VI International Student Congress of Food Science and Technology

Lugar de celebración: Valencia (España)

Año: 2019

Autores: Blanco-Morales, V., Garcia-Llatas, Sentandreu, V., G., Lagarda, M. J., Alegría, A.

Título: Changes in microbiota composition induced by the presence of galactooligosaccharides in a functional beverage: an *in vitro* dynamic colonic fermentation study

Tipo de participación: Póster

Congreso: 6th International Conference on Food Digestion

Lugar de celebración: Granada (España)

Año: 2019

Autores: Blanco-Morales, V., Alegría, A., Garcia-Llatas, G.

Título: Influencia de la adición de galactooligosacáridos a una bebida enriquecida en esteroles vegetales sobre la metabolización colónica del colesterol: estudio clínico.

Tipo de participación: Póster

Congreso: VII Congreso de estudiantes de Nutrición Humana y Dietética

Lugar de celebración: Valencia (España)

Año: 2020

Autores: Blanco-Morales, V., Alegría, A., Garcia-Llatas, G.

Título: The influence of galactooligosaccharide addition to a plant sterol-enriched beverage upon plant sterol colonic metabolization: a clinical trial.

Tipo de participación: Póster

Congreso: 1st International Electronic Conference on Food Science and Functional Foods

Lugar de celebración: Online

Año: 2020

Autores: Blanco-Morales, V., Silvestre, R.A., Alegría, A., Garcia-Llatas, G.

Título: The impact of galactooligosaccharides on the bioavailability of sterols: a randomized, crossover, double-blind clinical trial.

Tipo de participación: Póster

Congreso: 1st International Electronic Conference on Food Science and Functional Foods

Lugar de celebración: Online

Año: 2020
