VNIVERSITAT DE VALÈNCIA
Facultad de Farmacia

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

INFLUENCIA DE LA FERMENTACIÓN COLÓNICA EN LOS ESTEROLES DE LA DIETA

INFLUENCE OF COLONIC FERMENTATION ON DIETARY STEROLS

DOCTORADO EN CIENCIAS DE LA ALIMENTACIÓN
TESIS DOCTORAL INTERNACIONAL

Presentada por:
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Burjassot, septiembre 2018

VNIVERSITAT DE VALÈNCIA Facultat de Farmàcia

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CERTIFICAN QUE:

La graduada en Nutrición Humana y Dietética Dña. Maria Cuevas Tena ha realizado bajo su dirección el trabajo que lleva por título **"Influencia de la fermentación colónica en los esteroles de la dieta"**. El trabajo ha dado lugar a cinco artículos.

1. *Determination of fecal sterols following a diet with and without plant sterols. Lipids, (2017), 52, 817-884.*
   Índice de impacto JCR (2017): 1,936 (61/81) Q4. Nutrición y Dietética.

2. *Plant sterols and human gut microbiota relationship: An in vitro colonic fermentation study. Journal of Functional Foods, (2018), 44, 322-329.*
   Índice de impacto JCR (2017): 3,470 (16/133) Q1. Ciencia y Tecnología de los Alimentos.

3. *Impact of colonic fermentation on sterols after the intake of a plant sterol-enriched beverage: A randomized, double-blind crossover trial. Clinical Nutrition, (2018), doi.org/10.1016/j.clnu.2018.08.012.*
   Índice de impacto JCR (2017): 5,496 (8/81) Q1. Nutrición y Dietética.

4. *Relationship dietary sterols and gut microbiota: A review. European Journal of Lipid Science and Technology. (Aceptado 2018).*
   Índice de impacto JCR (2017): 2,200 (48/133) Q2. Ciencia y Tecnología de los Alimentos.

5. *Impact of high dose plant sterols on gut microbiota from lean and obese subjects using TIM-2 in vitro fermentation model. Journal of Functional Foods. (En recensión, 2018).*

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

Y para que conste a los efectos oportunos,

En Burjassot, 28 de septiembre de 2018

Fdo. Amparo Alegría Torán

Fdo. Mª Jesús Lagarda Blanch

La presente tesis doctoral internacional se ha realizado en el grupo de investigación *Bionutest* (03/003) y se enmarca en el proyecto AGL2012-39503-C02-01 "Esteroles vegetales, metabolitos y óxidos en bebidas de zumo de frutas y leche: efecto del tamaño de partícula y emulsionantes sobre la biodisponibilidad. Efectos biológicos" financiado por el Ministerio de Economía y Competitividad (MINECO) y el Fondo Europeo de Desarrollo Regional (FEDER).

La doctoranda Maria Cuevas Tena, ha disfrutado de una ayuda predoctoral para la Formación de Doctores (BES-2013-062705) del Ministerio de Economía y Competitividad (MINECO), en el periodo entre diciembre de 2013 y diciembre de 2017 (Resolución de 4 de diciembre de 2013).

Dña. Maria Cuevas Tena ha disfrutado de una ayuda a la movilidad predoctoral para la realización de estancias breves en centros de I+D españoles y extranjeros del Ministerio de Economía y Competitividad:

- Estancia Internacional (EEBB-I-16-11870) (Resolución 30 diciembre de 2015) realizada en *Healthy Eating & Food Innovation Departament of Maastricht University* (Campus Venlo), The Netherlands, en el periodo entre agosto y diciembre de 2016.

Maastricht University
Campus Venlo

En Burjassot, 28 de septiembre de 2018

Fdo. Amparo Alegría Torán

Fdo. Mª Jesús Lagarda Blanch

Sin duda este es el apartado de la tesis más fácil de escribir, quizás sea porque Consuelo y Toni (mis padres) me enseñaron desde pequeña a ser una persona agradecida.

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tipos que mentalmente no envejece, que tiene una filosofía de vida tal que así… "Vive y Deja Vivir". Gracias *iaio* por tus chistes malos pero graciosos y por decirme lo guapa y hermosa que estoy cada vez que nos vemos. Mi abuela, nació antes de tiempo, una mujer progre, que te transmitía su ambición sana y que en sus últimos días me preguntaba si ya sabía algo sobre la resolución de la beca. *Iaia* gracias a ti quiero ser mejor cada día. Gracias también a mis tíos Fidel y Emi, a mi prima Viqui, a mi primo Mat, a Helen (mi cuñy) y a mi sobrino Mario, ese pequeñín que me ha robado el corazón. No puedo olvidarme de mi fan número 1, mi tía Malu, siempre dándome ánimos, alegrándose por cada artículo aceptado y recordándome cada día lo afortunada que soy por estar haciendo lo que me apasiona, investigar.

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No te rindas que la vida es eso,

continuar el viaje,

perseguir tus sueños.

Mario Benedetti

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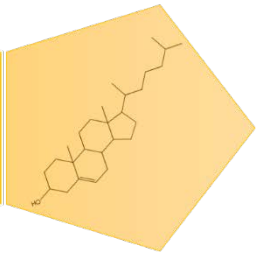

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ABREVIATURAS / ABBREVIATIONS

ABCG5/ABCG8/ABCB1a/ABCB1b: trasportadores casete de unión adenosina trifosfato

ACAT: acyl-coenzima A aciltransferasa

ADN: ácido desoxirribonucleico

AGCC: ácidos grasos de cadena corta

AOAC: *Association of Official Analytical Chemists*

AS: *animal sterols*

β-Cx: *β-cryptoxanthin*

BMI: *body mass index*

bp: *base pairs*

BR: *beverage residue*

CG-EM: cromatografía de gases acoplada a espectrometría de masas

CRP: proteína C reactiva

CVML: células vasculares del musculo liso

EA: esteroles animales

EV: esteroles vegetales

GC/MS: *gas chromatography with mass spectrometry*

GI: gastrointestinal

HDL: lipoproteínas de alta densidad

IMC: índice de masa corporal

KEGG: Kyoto encyclopedia of genes and genomes;

LDA: *linear discriminant analysis*

LDL: lipoproteínas de baja densidad

LEfSe: *linear discriminant analysis effect size*

LOD: *limit of detection*

LOQ: *limit of quantitation*

MO: microorganismos

MTP: *microsomal triglyceride transfer protein*

NPC1L1: niemann-Pick C1-like 1

OMS: Organización Mundial de la Salud

OTU: *operational taxonomic unit*

PARP: poli(ADP-ribosa)-polimerasa

PCNA: antígeno nuclear celular proliferante

PCoA: *principal coordinate analysis*

PICRUSt: *Phylogenetic Investigation of Communities by Reconstruction of Unobserved States*

PS: *plant sterols*

QIIME: *quantitative insights into microbial ecology*

QM: quilomicrones

rLDL: receptor para LDL

RSD: *relative standard deviation*

SCFA: *short chain fatty acids*

SHIME: *simulator of the human intestinal microbial ecosystem*

SIEM: medio estándar de eflujo ileal

SIMGI: *SIMulator Gastro-Intestinal*

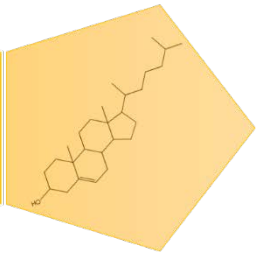
TICE: vía de excreción transintestinal de colesterol

TIM-2: modelo del tracto intestinal-2/ *TNO Intestinal Model-2*

TNO: Organización de Investigación Científica Aplicada de los Países Bajos

VLDL: lipoproteínas de muy baja densidad

Resumen/
Abstract



Resumen

La adición de esteroles vegetales (EV) está permitida a varios alimentos por su efecto hipocolesterolemiante, con el fin de "mantener normales los niveles de colesterol sanguíneo" y "prevención de patologías cardiovasculares". Los EV presentan una baja absorción (2-3%), mientras que los del colesterol oscilan entre el 30 y el 60%. Los esteroles no absorbidos llegan al colon y son transformados por la microbiota. Es conocida la biotransformación microbiana del colesterol a coprostanol y en menor medida a coprostanona y colestanol. Los EV también son metabolizados por la microbiota a fitostanonas, fitostenonas y fitostanoles.

El objetivo de la presente tesis doctoral es evaluar la influencia de la fermentación colónica en los esteroles de la dieta y el impacto de estos sobre la microbiota intestinal mediante estudios *in vivo* y/o *in vitro*.

Se pone a punto y valida un método para la determinación de esteroles y sus metabolitos en heces por cromatografía de gases acoplada a espectrometría de masas. Se realizan ensayos de fermentación colónica *in vitro* aplicando altas concentraciones de EV a modelo estático y dinámico (TIM-2). Se estudia el impacto de la microbiota intestinal sobre los esteroles tras la ingesta de una bebida a base de zumo y leche enriquecida en EV (2g/día), mediante un estudio clínico aleatorizado, doble ciego y cruzado, en el que participan 40 voluntarias postmenopáusicas con hipercolesterolemia moderada que ingieren la bebida diariamente durante 6 semanas.

El método validado muestra una buena linealidad (r > 0,96), alta sensibilidad (LD: 0,10-3,88 µg/g heces liofilizadas y LC: 0,34-12,94 µg/g), precisión (intra e inter-día (CV%) 0,9-9,2 y 2,1-11,3, respectivamente) y exactitud (80-119%). Los estudios *in vivo* e *in vitro* muestran que, las concentraciones altas de EV modifican la biotransformación de los esteroles, ya que se ha observado una reducción en el metabolismo de los esteroles animales, principalmente en la

concentración de coprostanol y un aumento en el metabolismo microbiano de los EV, observándose un aumento de las concentraciones colónicas de etilcoprostanol y etilcoprostanona. La producción de ácidos grasos de cadena corta, es similar o en algunos casos mayor a altas concentraciones de EV, no modificándose la actividad metabólica microbiana de los carbohidratos.

Los EV a concentraciones altas se correlacionan con una disminución de la proporción especies de la familia *Erysipelotrichaceae* y aumento de *Eubacterium hallii* y especies del género *Catenibactrerium, Coprococcus* y *Clostridium.* Además, la producción de metilcoprostanona se correlaciona negativamente con el orden *Clostridiales*, con los géneros *Peptostreptococcus* y *Methanobrevibacter* y positivamente con *Bacteroides*.

Abstract

The addition of plant sterols (PS) to several foods is added due to its hypocholesterolemic effect, in order to "maintain normal blood cholesterol levels" and "prevention of cardiovascular diseases". PS present low absorption (2-3%), while the absorption of cholesterol range between 30 and 60%. Unabsorbed sterols reach the colon and are transformed by the microbiota. The microbial biotransformation pathway of cholesterol to coprostanol, coprostanone and cholestanol is widely known. PS are also metabolized by the microbiota to phytostanones, phytostenones and phytostanols.

The objective of the present doctoral thesis is to evaluate the influence of colonic fermentation on dietary sterols and the impact of these on the intestinal microbiota through *in vivo* and/or *in vitro* studies.

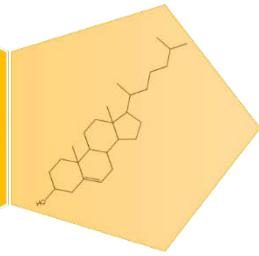
A method to determination of sterols and its metabolites in feces by gas chromatography with mass spectrometry is developed and validated. *In vitro* colonic fermentation assays are performed using high concentrations of PS in static and dynamic models (TIM-2). The impact of gut microbiota on sterol after the intake a PS-enriched milk based fruit beverage (2g/day) is studied through a randomized, double-blind crossover trial involving 40 postmenopausal women with moderate hypercholesterolemia, who ingest the beverage daily for 6 weeks.

The validated method shows good linearity ($r > 0.96$), high sensitivity (LOD: 0.10-3.88 μg/g freeze-dry feces and LOQ: 0.34-12.94 μg/g), precision (intra and inter-day (RSD%) 0.9-9.2 and 2.1-11.3, respectively) and accuracy (80-119%).

In vivo and *in vitro* studies show that high concentrations of PS modify the biotransformation of sterols, since a reduction in the metabolism of animal sterols has been observed, mainly in the concentration of coprostanol. In addition, an increase in the microbial metabolism of PS, with an increase in colonic concentrations of ethylcoprostanol and ethylcoprostanone has been found. In the

presence of high concentrations of PS, the production of short chain fatty acids is similar or in some cases greater than control, therefore the microbial metabolic activity of carbohydrates is not modified. The high concentrations of PS are correlated with a decrease in the proportion of species of *Erysipelotrichaceae* family and with an increase of *Eubacterium hallii* and species of the genus *Catenibactrerium*, *Coprococcus* and *Clostridium*. In addition, the production of methylcoprostanone are negatively correlated with the *Clostridiales* order, with the genera *Peptostreptococcus* and *Methanobrevibacter*, and positively with *Bacteroides*.

Objetivos/ Objectives



Objetivos

El objetivo principal de la tesis es evaluar la influencia de la fermentación colónica sobre los esteroles de la dieta y el impacto de estos sobre la microbiota colónica.

Como objetivos secundarios se propone:

- Validar una metodología para la determinación de esteroles fecales en líquidos de fermentación y heces por cromatografía de gases acoplada a espectrometría de masas.
- Estudiar la interacción entre esteroles y microbiota mediante modelos de fermentación colónica *in vitro* estático y dinámico.
- Estimar la influencia del consumo regular de una bebida a base de zumo de frutas y leche rica en β-criptoxantina y enriquecida en EV en el contenido de esteroles y sus metabolitos en heces de mujeres postmenopáusicas con hipercolesterolemia moderada, mediante un estudio de intervención clínica.
- Evaluar la posible relación entre los ensayos *in vivo* e *in vitro* en cuanto a la metabolización de los esteroles.

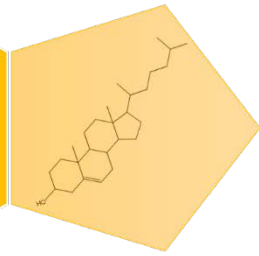
Objectives

The main objective of the present doctoral thesis is to evaluate the influence of colonic fermentation on dietary sterols and the impact of these on the colonic microbiota.

As secondary objectives are proposed:

- To validate a method for the determination of fecal sterol in fermentation liquids and feces by gas chromatography with mass spectrometry.
- To study the interaction between sterols and microbiota through *in vitro* static and dynamic colonic fermentation models.
- To estimate the influence of regular consumption of a PS-enriched skimmed milk based fruit beverage containing β-cryptoxanthin in the content of sterols and its metabolites in feces from postmenopausal women with moderate hypercholesterolemia, through a clinical trial.
- To evaluate the relationship between *in vivo* and *in vitro* assays in relation to the sterols biotransformation.

Plan de trabajo/ Work plan



Plan de trabajo

Para alcanzar los objetivos mencionados se propone el siguiente plan de trabajo:

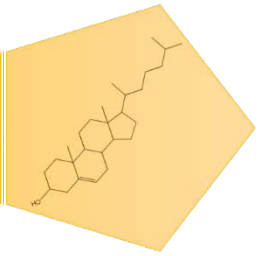
- Revisión de los antecedentes bibliográficos relativos a la determinación de esteroles y sus metabolitos en heces, su biotransformación mediante la microbiota colónica e impacto de los mismos sobre las poblaciones bacterianas del colon y *viceversa*.
- Puesta a punto y validación de un método que permita la determinación de esteroles fecales por cromatografía de gases acoplada a espectrometría de masas (CG-EM).
- Estudio de la interacción entre esteroles y microbiota colónica mediante un modelo estático de fermentación *in vitro*, en presencia de concentraciones altas de EV presentes en un residuo obtenido tras digestión gastrointestinal *in vitro* de una bebida a base de zumo de frutas y leche, utilizando microbiota fecal de individuos sanos.
- Estudio de la interacción entre esteroles y microbiota colónica mediante un modelo dinámico (TIM-2) de fermentación *in vitro* en presencia de concentraciones altas de EV presentes en un ingrediente alimentario y un estándar, utilizando microbiota fecal de individuos delgados u obesos.
- Evaluación del impacto de la microbiota colónica sobre los esteroles dietéticos mediante la determinación de esteroles y sus metabolitos en heces de mujeres postmenopáusicas tras el consumo regular de una bebida a base de zumo de frutas y leche enriquecida con EV (estudio de intervención clínica).
- Evaluación de los resultados obtenidos.
- Redacción de la tesis doctoral.

Work plan

To achieve the aforementioned objectives, the following work plan is proposed:

- Literature review on the determination of sterols and their metabolites in feces, its microbial biotransformation and their impact on the colonic bacterial populations and *viceversa*.
- Development and validation of a method that allows the determination of fecal sterols by gas chromatography with mass spectrometry (GC-MS).
- Study of the interaction between sterols and colonic microbiota through a static *in vitro* fermentation model, in presence of high concentrations of PS from a residue obtained after *in vitro* gastrointestinal digestion of a PS-enriched milk-based fruit beverage, using fecal microbiota of healthy individuals.
- Study of the interaction between sterols and colonic microbiota through a dynamic *in vitro* fermentation model (TIM-2), in presence of high concentrations of PS from a food ingredient and a standard, using fecal microbiota from lean or obese individuals.
- To evaluate the colonic microbiota impact on dietary sterols through the sterols and their metabolites determination in feces from postmenopausal women after regular consumption of a PS enriched skimmed milk based fruit beverage containing β-cryptoxanthin (clinical trial).
- To evaluate the results obtained.
- Drafting of the doctoral thesis.

Antecedentes bibliográficos



1. Esteroles

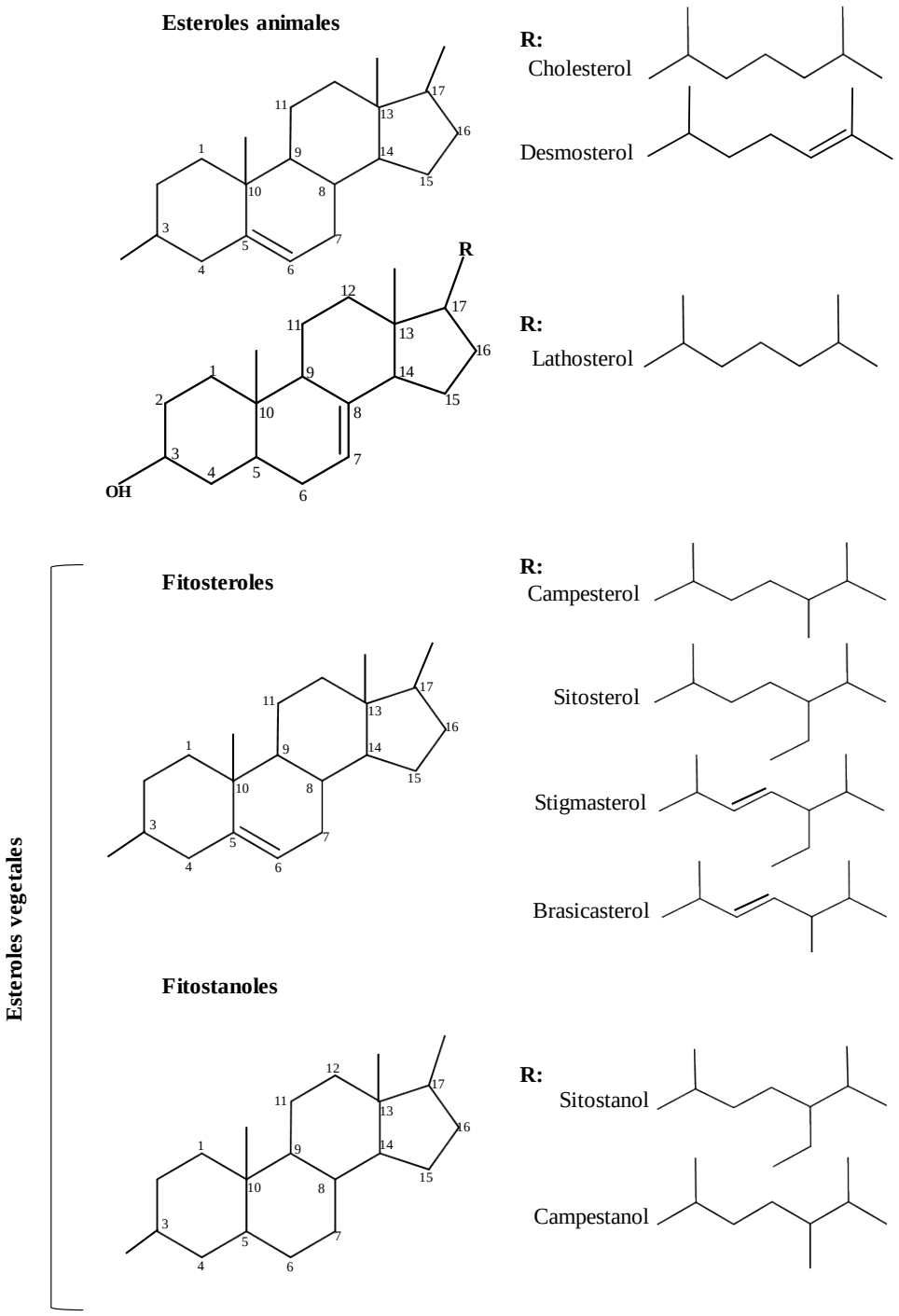
1.1. Estructura

Atendiendo a su origen, los esteroles se pueden clasificar en animales (EA) y EV. El principal EA es el colesterol, que predomina en las membranas celulares de los animales, mientras que los principales EV, por orden de abundancia son el sitosterol, campesterol y estigmasterol, que a su vez se encuentran en las membranas celulares de los tejidos vegetales.

Todos ellos derivan del escualeno y presentan una estructura tetracíclica ciclopentanoperhidrato-fenantreno, con un grupo hidroxilo en el carbono 3 y una cadena lateral en el carbono 17, compuesta por 8-10 átomos de carbono (Figura 1) (García-Llatas & Rodríguez-Estrada, 2011).

La estructura de los EV es similar a la del colesterol, excepto por la presencia de un grupo metilo (campesterol) o etilo (sitosterol) en el carbono 24 de la cadena lateral y en algunos casos, por el doble enlace adicional de la cadena lateral (estigmasterol). Los fitosteroles presentan un doble enlace en el carbono 5, mientras que los fitostanoles son menos abundantes y saturados (Figura 1) (García-Llatas & Rodríguez-Estrada, 2011; Von Bergmann et al., 2005).

Figura 1. Estructuras químicas de colesterol y diferentes esteroles vegetales.



Fuente: Adaptada de Von Bergmann et al. (2005).

1.2. Fuentes e ingestas

Los esteroles son componentes minoritarios en la dieta humana y representan un alto porcentaje de la fracción insaponificable de las grasas de los alimentos (García-Llatas & Rodríguez-Estrada, 2011).

Existen dos fuentes de colesterol para el organismo humano: exógena, a través de la dieta occidental que aporta 300-500mg/día (aproximadamente el 55% del colesterol total consumido procede de la carne y el pescado; los huevos aportan un 25% y los productos lácteos un 20% (Grundy, 2016)) y endógena, por síntesis que representa aproximadamente 800mg/día (Pujol, 2014; Ros, 2006).

Por su parte, los EV no son sintetizados en el organismo y son aportados por la dieta (Rozner & Garti, 2006). Los EV están presentes en los alimentos de forma libre, esterificados, glucosilados o acetilados glucosilados. Las principales fuentes dietéticas son aceites, frutos secos, cereales, hortalizas y frutas (ver Tabla 1).

Se ha estimado que los contenidos totales de EV ingeridos diariamente por la población española son de 276mg/día (Jiménez-Escrig et al., 2006), similares a los de Finlandia (271 mg/día) (Valsta et al., 2004), Holanda (284 mg/día) (Normén et al., 2001) e Inglaterra (296 mg/día) (Klinberg et al., 2008).

Tabla 1. Contenidos de esteroles vegetales por grupo de alimentos.

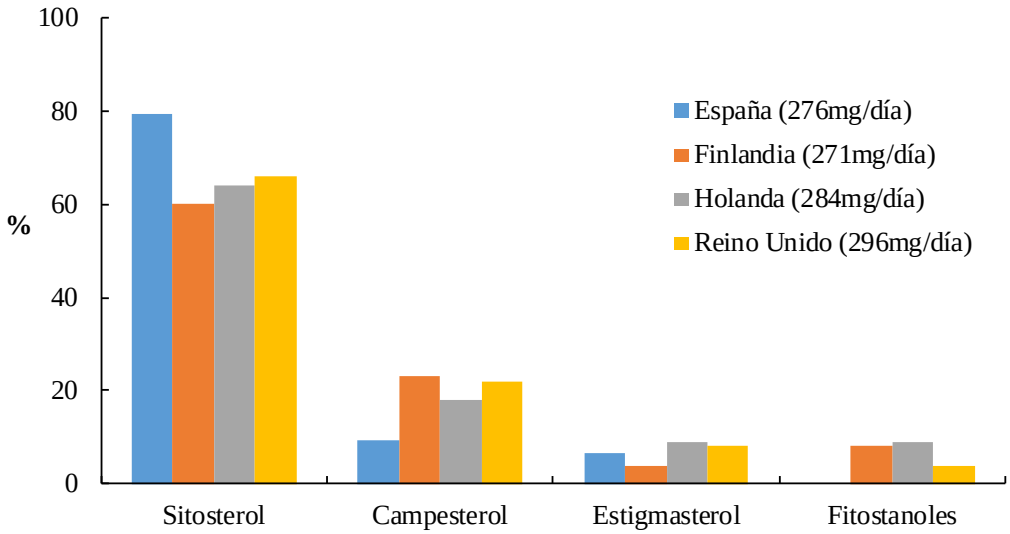
Alimento	**mg/100 g alimento**
Aceites	
Maíz	968
Oliva	260
Palma	49
Frutos secos	
Pistachos	242
Pipas girasol	230
Nueces	131
Cereales	
Germen de trigo	364
Centeno	100
Trigo	61
Hortalizas	
Alcachofa	49
Espinacas	16
Calabacín	2
Frutas	
Uva blanca	33
Manzana	12
Limón	3

Fuente: Plumb et al., 2011; Klingberg et al., 2008; Jiménez-Escrig et al., 2006; Lagarda et al., 2006; Abidi, 2001; Piironen et al., 2000.

En la dieta española los aceites vegetales representan la primera fuente de EV con un 40% de la ingesta total de EV, mientras que en la dieta finlandesa y holandesa solo representan el 9,5 y 2%, respectivamente, hecho relacionado con el mayor consumo de margarinas y grasas vegetales untables (17-24% de la ingesta total de EV) en las dietas del norte de Europa. Además de los aceites, la ingesta de EV en España es a través de cereales (30%), frutas (12%), legumbres (9%), hortalizas (7%) y frutos secos (2%) (Jiménez-Escrig et al., 2006). En la

Figura 2 puede observarse como el orden de abundancia de los distintos EV de la dieta es similar en todos los países europeos indicados.

Figura 2. Porcentaje relativo de cada uno de los esteroles vegetales con respecto a la ingesta total diaria.



Fuente: Klingberg et al., 2008; Jiménez-Escrig et al., 2006; Valsta et al., 2004; Normén et al., 2001

Las ingestas de EV son más altas en las dietas vegetarianas (500 y 1000mg EV/día) (Vuoristo & Miettinen, 1994) y/o mediante el consumo de alimentos enriquecidos en EV, donde las cantidades ingeridas pueden llegar hasta los 3g/día (EFSA, 2008).

Actualmente, existen distintos alimentos para los que la Comisión Europea regula el enriquecimiento en EV (Tabla 2) los cuales se estima que son consumidos por el 10-15% de la población (EFSA, 2008).

Tabla 2. Decisiones de la Comisión Europea por las que se autoriza la comercialización de algunos alimentos con fitosteroles y fitostanoles vegetales añadidos como nuevo alimento o nuevo ingrediente alimentario con arreglo al Reglamento (CE) nº 258/97 del Parlamento Europeo y del Consejo.

Decisión de la Comisión Europea	**Alimento**	**Forma de adición de los EV**	**Solicitante (País)**
2000/500/CE	• Grasas amarillas para untar	Ésteres de fitosterol	Unilever (Países Bajos)
2004/333/CE	• Grasas amarillas para untar • Aliños para ensaladas que contengan mayonesa • Bebidas tipo leche, como productos tipo leche semidestanda y leche destanada, bebidas de frutas, cereales y soja, y productos tipo leche fermentada, como productos tipo yogur y queso	Fitosteroles o fitostanoles	Archer Daniels Midland Company (ADM) (Países Bajos)
2004/334/CE	• Grasas amarillas para untar • Productos tipo leche, como los productos tipo leche semidestanda y leche destanada, y productos tipo yogur, en los que se haya reducido la grasa de la leche o se haya sustituido total o parcialmente por grasa vegetal • Salsas aromáticas	Fitosteroles o fitostanoles	Pharmaconsult Oy Ltd. (antes MultiBene Health Oy Ltd.) (Finlandia)
2004/335/CE	• Productos tipo leche, como los productos de tipo leche semidesnatada y leche desnatada, los productos tipo yogur y los productos tipo leche/yogur en los que la grasa de la leche se haya sustituido total o parcialmente por grasa vegetal.	Ésteres de fitosterol	Unilever (Reino Unido)

Tabla 2. Continuación.

Decisión de la Comisión Europea	Alimento	Forma de adición de los EV	Solicitante (País)
2004/336/CE	• Grasas amarillas para untar • Bebidas de fruta a base de leche, productos de tipo yogur y productos tipo queso (contenido graso ≤ 12g/100g producto), en los que la grasa y/o la proteína de la leche se hayan reducido o se haya sustituido total o parcialmente por grasa y/o proteína de origen vegetal	Fitosteroles o fitostanoles	Teriaka Ltd. (Finlandia)
2004/845/CE	• Productos tipo leche, como los productos de tipo leche semidesnatada y leche desnatada, en los que la grasa de la leche se haya sustituido total o parcialmente por grasa vegetal	Fitosteroles o fitostanoles	Forbes Medi-Tech Inc. (antes Novartis) (Bélgica)
2006/58/CE y 2006/59/CE	• Pan de centeno con harina que contiene ≥ 50% de centeno (harina integral de centeno, granos de centeno enteros o fragmentados y copos de centeno) y ≤ 30% de trigo: con ≤ 4% de azúcar añadido pero sin grasa añadida.	Fitosteroles o fitostanoles	Pharmaconsult Oy Ltd. (antes MultiBene Health Oy Ltd) (Finlandia) y Karl Fazer Ltd. (Finlandia)
2007/343/CE	• Aceite enriquecido en EV, para su uso como ingrediente en alimentos como: Grasas para untar; Productos lácteos, como los productos a base de leche semidesnatada y leche desnatada, con posible adición de frutas o cereales, productos a base de leche fermentada, como los productos tipo yogur y queso; Bebidas de soja; Salsas aromáticas y aliños de ensalada, incluida la mayonesa.	Fitosteroles o fitostanoles	Ezymotec (Países Bajos)
2008/36/CE	• Bebidas de arroz	Fitosteroles o fitostanoles	Teriaka Ltd. Paulig Group (Finlandia)

1.3. Metabolismo y Excreción

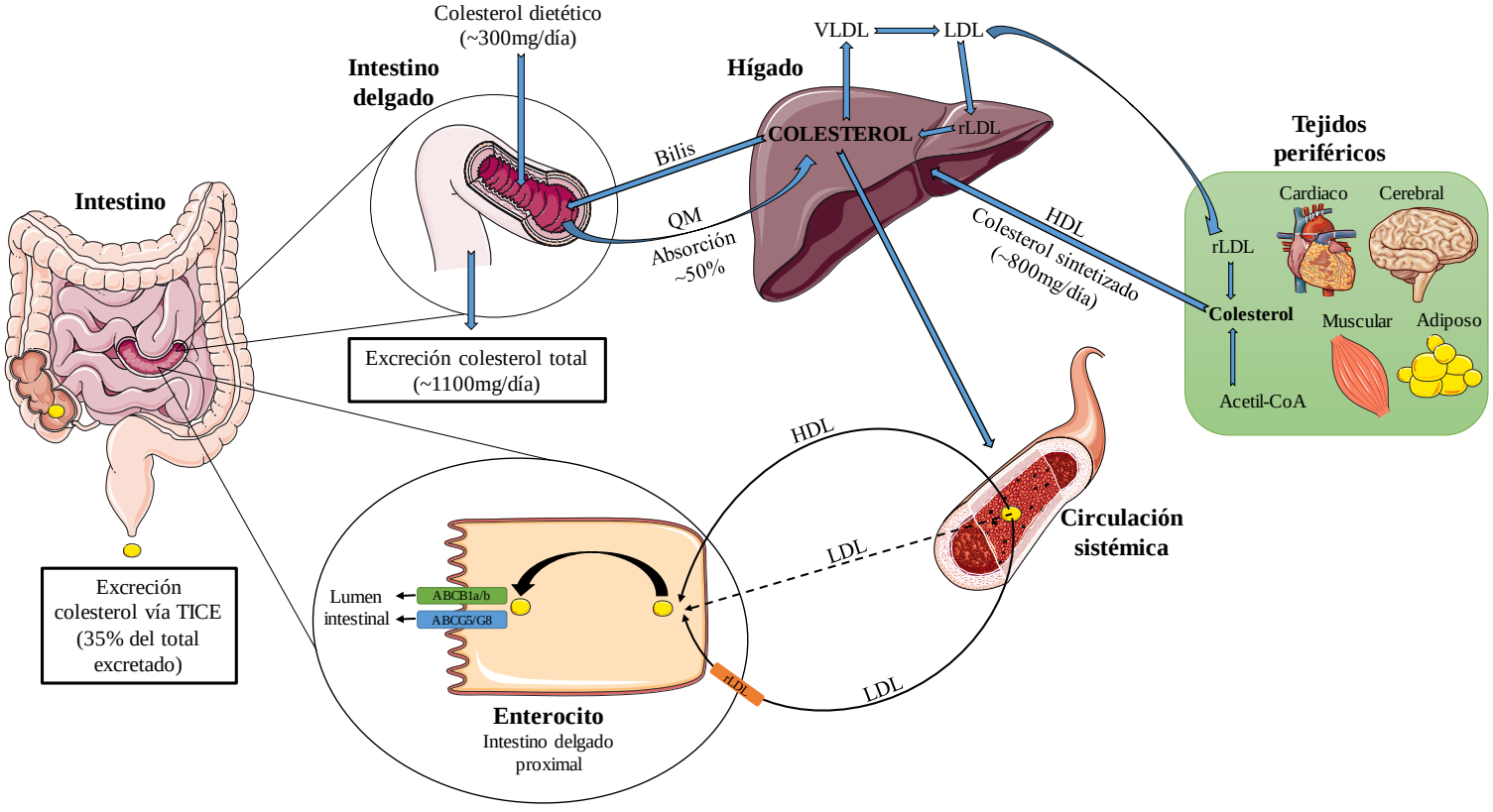
El 50% del colesterol dietético es absorbido (De Boer et al., 2018) aunque su absorción varía sustancialmente entre individuos (20-80%) (Stellaard & Lutjohann et al., 2015; Bosner et al., 1999; Heinemann et al., 1993).

Un paso previo para la absorción de los esteroles es su incorporación a las micelas mixtas ayudados por las sales biliares. Previamente, si se encuentran esterificados son liberados por la acción de esterasas. Una vez liberados, los esteroles se incorporan a los enterocitos a través de la membrana del borde en cepillo, principalmente en la región proximal del intestino delgado (De Boer et al., 2018; Ros, 2006; Rozner & Garti, 2006).

La transferencia del colesterol desde las micelas mixtas a los enterocitos esta mediada por el transportador *Niemann-Pick C1-Like 1* (NPC1L1) localizado en el borde en cepillo de la membrana de los enterocitos (De Boer et al., 2018; Pujol, 2014; Turley, 2008; Ros, 2006) (ver Figura 3), si bien este mecanismo no está del todo elucidado. En el enterocito es esterificado por la Acyl-Coenzima A Aciltransferasa (ACAT) e incorporado a los quilomicrones (QM) junto con una pequeña porción de colesterol libre, triglicéridos y apolipoproteina B48. En la configuración de los QM es imprescindible la acción de la enzima *Microsomal Triglyceride Transfer Protein* (MTP).

Los QM transportan el colesterol al hígado, donde ejerce importantes efectos reguladores sobre la homeostasis del colesterol (Figura 3), de esta manera la suma del colesterol sintetizado por los tejidos (~800mg) y el aportado por la dieta (~300mg), debe ser igual al colesterol excretado diariamente por heces (~1100mg) (De Boer et al., 2018; Ros, 2006).

Figura 3. Homeostasis del colesterol y vía de excreción transintestinal (TICE).



Fuente: Adaptada de Le May et al. (2013) y Ros (2006); HDL lipoproteínas de alta densidad; rLDL: receptor para las lipoproteínas de baja densidad (LDL); QM: quilomicrones; VLDL: lipoproteínas de muy baja densidad.

La principal vía de eliminación del colesterol, bien establecida, es la hepatobiliar o también llamada vía de transporte reverso del colesterol, que consiste en el flujo de colesterol desde los tejidos periféricos al hígado mediante Lipoproteínas de Alta Densidad (HDL) (Figura 3) y su posterior secreción a la luz intestinal mediante la bilis (Pujol, 2014).

Los esteroles que no han sido esterificados en el interior de los enterocitos son secretados a la luz intestinal mediante los transportadores Casete de unión Adenosina Trifosfato (ABC: ABCG5 y ABCG8) situados en el borde de cepillo (De Boer et al., 2018; García-Llatas & Rodriguez-Estrada, 2011) y son eliminados por las heces.

Se ha sugerido otro mecanismo de eliminación de colesterol, la vía de excreción transintestinal (TICE). Esta tiene lugar en el intestino delgado proximal, el cual tiene capacidad de secretar activamente el colesterol del torrente sanguíneo hasta la luz intestinal (Pujol, 2014).

Aunque actualmente los mecanismos relativos a la vía TICE no se conocen completamente, se ha sugerido que consta de cuatro pasos importantes: (1) síntesis de colesterol, muy probablemente mediada por el hígado, y su secreción al torrente sanguíneo, (2) transporte del colesterol al enterocito y su captación en el lado basolateral, (3) translocación del colesterol desde el lado basolateral al apical del enterocito, y finalmente (4) excreción del colesterol en la luz intestinal (ver Figura 3) (De Boer et al., 2018). Aunque, aún no se sabe bien qué proteínas están implicadas en la vía TICE, varios estudios han señalado que ABCG5 y ABCG8 tienen un papel importante en esta vía mediando la excreción del colesterol desde los enterocitos a la luz intestinal (De Boer et al., 2017; Brufau et al., 2011; Van der Veen et al., 2009). Sin embargo, se ha observado que el flujo de colesterol a través de la vía TICE no está completamente inhibido en ratones que carecen de la función de los

transportadores ABCG5 y ABCG8 en los enterocitos. En este sentido, se ha propuesto que ABCB1a y ABCB1b podrían estar implicados. Queda por demostrar si estos transportadores pueden facilitar un alto flujo de colesterol desde el enterocito hacia la luz intestinal del mismo modo que ABCG5 y ABCG8 (Le May et al., 2013).

En este contexto, en un estudio reciente en el que han participado 15 sujetos sanos se ha demostrado que, en condiciones basales, alrededor del 65% de la excreción diaria de esteroles neutros deriva de la bilis (vía hepatobiliar), y el resto (35%) deriva de la vía TICE (Jakulj et al., 2016). Además, se ha sugerido que un posible mecanismo de acción de los EV en la disminución del colesterol sea mediante la vía TICE (Pujol, 2014).

Los EV, de mayor hidrofobicidad que el colesterol, compiten por la incorporación en las micelas mixtas reduciendo la incorporación de éste (Coreta-Gomes et al., 2016). La tasa de absorción intestinal de los EV totales es tan solo del 2-3% (Garía-Llatas & Rodriguez-Estrada, 2011; Trautwein & Demonty, 2007), siendo diferente para cada uno de estos esteroles: campesterol (9.4–16%), sitosterol (4.2–6.0%) y stigmasterol (4.8%) (Von Bergmann et al., 2005; Piironen et al., 2000; Heinemann et al., 1993). En la transferencia de los EV al enterocito también intervienen los mismos transportadores que para el colesterol. Sin embargo, se ha demostrado una baja afinidad de la enzima ACAT por los EV (Sato et al., 1995). Por tanto, solo una pequeña parte de los EV absorbidos se esterifica en los enterocitos y se incorpora a los QM. Este hecho podría explicar la baja absorción de los EV y su baja concentración en el torrente sanguíneo (Sato et al., 1995; Field & Mathur, 1983).

1.4. Funcionalidad

El colesterol es precursor de ácidos biliares, hormonas esteroideas y vitamina D. Sin embargo, un desequilibrio en su homeostasis, está asociado con el desarrollo de patologías cardiovasculares como la aterosclerosis (Lecerf & Lorgeril, 2011).

Ha sido ampliamente demostrado el efecto hipocolesterolemiante de los EV (6 - 12 %) (Ras et al., 2014). Al ser estructuralmente similares al colesterol compiten por su incorporación a las micelas mixtas, lo que reduce el contenido de colesterol dietético y biliar en el interior de las micelas y, por lo tanto, disminuye su transporte hacia la membrana del enterocito (Trautwein and Demonty, 2007). Además, los EV aumentan la expresión de los transportadores entéricos ABC, lo cual también contribuye a una menor absorción del colesterol. Como consecuencia, la síntesis de colesterol y la actividad de los receptores LDL aumentan, produciendo una disminución de la concentración del colesterol LDL sérico (De Jong et al., 2003).

Existen declaraciones de propiedades de salud relativas a la reducción del riesgo de enfermedad para los fitoesteroles (esteroles extraídos de plantas, libres o esterificados con ácidos grasos para uso alimentario), ésteres de fitoestanol y fitoesteroles/esteroles de fitoestanol, ya que se ha demostrado que los fitoesteroles y ésteres de fitoestanol disminuyen/reducen el colesterol sanguíneo, el cual en tasas elevadas constituye un factor de riesgo en el desarrollo de cardiopatías coronarias (Reglamento 2009/983/CE y Reglamento 2010/384/CE). En 2014 las condiciones de uso de estas dos declaraciones se modifican: “el efecto beneficioso de los fitoesteroles y los ésteres de fitoestanol se obtiene con una ingesta diaria de 1,5 a 3 g de fitoesteroles/fitoestanoles. Solo podrá hacerse referencia a la magnitud del efecto para los alimentos incluidos en las siguientes categorías: grasas

amarillas para untar, productos lácteos, mayonesa y aliños para ensaladas. Cuando se haga referencia a la magnitud del efecto, deberá comunicarse al consumidor la variación del 7 al 10% para los alimentos que aportan una ingesta diaria de 1,5 a 2,4g de fitosteroles/fitoestanoles o la variación del 10 al 12,5 % para los alimentos que una ingesta diaria de 2,5 a 3 g de fitoesteroles/fitoestanoles, así como el periodo a partir del cual se obtiene el efecto: "de dos a tres semanas" (Reglamento 2014/686/CE). La ingesta de EV en alimentos enriquecidos no debe superar los 3g/día, ya que varios estudios han demostrado que altas dosis de los mismos disminuyen significativamente la concentración sérica de carotenoides y vitamina E (Trautwein & Demonty, 2007).

Además, para los fitosteroles y fitoestanoles existe una declaración de propiedad saludable distinta de las relativas a la reducción del riesgo de enfermedad y al desarrollo y la salud de los niños, en la cual se indica que contribuyen a mantener normales los niveles de colesterol sanguíneo. El efecto beneficioso se obtiene con una ingesta diaria mínima de 0,8g de fitoesteroles o fitoestanoles (Reglamento 2012/432/CE).

Se ha comprobado que la ingesta de altas dosis de EV ejerce un efecto sobre el desarrollo y regresión de la aterosclerosis (De Jong et al., 2003). Estudios con modelos animales de aterosclerosis han mostrado que, los EV tienen claros efectos protectores, previniendo el desarrollo de la placa de ateroma o reducción del tamaño de la lesión, e inhiben la formación y progresión de la lesión e incluso la regresión de las lesiones existentes (Gylling et al., 2014; Trautwein & Demonty, 2007; De Jong et al., 2003). Mediante ensayos *in vitro* se conoce que, los EV estimulan la liberación de prostaciclinas (PGI_2) de las células vasculares del musculo liso (CVML), sugiriendo que pueden prevenir la hiperproliferación de CVML, que a su vez

podría desempeñar un papel beneficioso contra el desarrollo de aterosclerosis (Awad et al., 2001). En estudios *in vitro* con macrófagos han observado una reducción en la liberación de prostaglandinas, posiblemente ofreciendo protección contra el desarrollo de ateromas, a través de la agregación plaquetaria o la vasodilatación de los vasos sanguíneos (Awad et al., 2004). Sin embargo, en estudios clínicos no se han demostrado los posibles beneficios que los EV podrían tener en el desarrollo de la aterosclerosis (Trautwein & Demonty, 2007). Además, existe una correlación entre los EV séricos y las lesiones ateroscleróticas, es decir, posibles depósitos de EV en el tejido cardiovascular podrían estar involucrados en el desarrollo de aterosclerosis (Helske et al., 2008; Miettinen et al., 2005). Sin embargo, en base a la evidencia actual, no hay indicios de que la suplementación dietética con esteroles/estanoles vegetales esté asociada con beneficios o daños en la función vascular (Gylling et al., 2014).

Por otra parte, se ha sugerido que los EV, especialmente β-sitosterol, podrían tener actividad antiinflamatoria. En estudios con humanos, los datos sobre los efectos del consumo de alimentos enriquecidos con EV en los marcadores inflamatorios son escasos y contradictorios (Trautwein & Demonty, 2007). Se ha observado que la ingesta de zumo de naranja enriquecido con EV (2g/día) durante 8 días, reduce significativamente (12%) las concentraciones séricas de la proteína C reactiva (CRP), la cual se utiliza como biomarcador de inflamación, tanto en sujetos sanos (Devaraj et al., 2006) como en sujetos con hipercolesterolemia o hiperlipemia (Solà et al., 2012; Athyros et al., 2011; Bitzur et al., 2010). Sin embargo, predominan los estudios que no observan cambios en la concentración de CRP en suero (AbuMweis et al., 2006).

Además, en sujetos hiperlipidémicos tras el consumo, durante tres semanas, de capsulas de aceite de pescado (ricas en ácidos grasos poliinsaturados) junto con margarina enriquecida en EV (2 g/día), se ha observado un descenso en las concentraciones séricas de interleuquina-6 (IL-6) (11%) y factor de necrosis tumoral-α (TNF-α) (10%) (Micallef & Garg, 2009). Por otra parte, Devaraj et al. (2011) en un estudio con sujetos sanos observan una reducción significativa de IL-6 (46%) e IL-1β (43%) tras la ingesta durante 8 semanas de un zumo de naranja bajo en calorías y enriquecido en EV (2 g/día). Además, tras la ingesta de una bebida a base de naranja, también baja en calorías observan una reducción de IL-6 (27%). Para el resto de citoquinas estudiadas (TNF-α, IL-10 o IL-8) no indican cambios. Sin embargo, otros estudios no observan diferencias significativas en las concentraciones séricas de TNF-α, IL-6 y IL-8 en sujetos con hipercolesterolemia que ingieren alimentos enriquecidos (margarina o leche desnatada) con EV (2g/día) (Kunces et al., 2013; Hallikainen et al., 2006).

Los EV podrían ejercer un efecto preventivo frente a diferentes tipos de cáncer (mama, próstata, ovario, leucemia, pulmón, colon, etc.), debido a su capacidad para inhibir numerosas vías implicadas en la carcinogénesis (Sayeed & Ameen, 2015). Dado que, la presente tesis está relacionada con interacciones microbiota-esteroles en el tracto colónico, la relación entre los EV y el riesgo de cáncer de colon cobra mayor relevancia; sin embrago, esta asociación aún es controvertida.

En algunos estudios *in vivo* con ratas, la suplementación de la dieta con β-sitosterol (0.2% (p/p), durante 28 semanas) se ha asociado con una disminución en la proliferación de células epiteliales cancerosas (Deschner et al., 1982) y una menor formación de tumores de colon (Raicht et al., 1980). Además, en un estudio con ratas en las que se induce cáncer de colon con

carcinógeno 1,2 Dimethyldrazine, la ingesta de una dieta suplementada con β-sitosterol (20mg/kg, una vez a la semana durante 4 semanas) reduce la actividad enzimática de bacterias intestinales consideradas características en la carcinogénesis de colon (Arul et al., 2012). Sin embargo, en ratas tratadas con metil-nitroso-urea (agente carcinógeno) y siguiendo una dieta suplementada con EV (24mg/rata/día, durante 26 semanas) tienen una frecuencia tumoral (8/20) similar a aquellas no suplementadas (11/20) (Quilliot et al., 2001).

Además, estudios con células tumorales de cáncer de colon (HT-29) han mostrado el efecto inhibidor de β-sitosterol (16µmol/L) en el crecimiento de tumores cuando se suplementa el medio con este esterol durante 5 días (Awad et al., 1996). En células HCT-116 se ha observado que el β-sitosterol (7.5-20µmol/L), tras 48h de incubación, inhibe el crecimiento entre un 50% y un 75% e induce apoptosis a través de la activación de eventos proapoptóticos (caspasa-3 y -9, escisión de Poli-(ADP-ribosa)-Polimerasa (PARP) y citocromo C) relacionados con la ruta intrínseca de la apoptosis celular (Choi et al., 2003). También se ha indicado que, tras 24h de incubación, β-sitosterol el (15, 30, 60, 120 y 240µM) induce fragmentación del ácido desoxirribonucleico (ADN) en células COLO 320 DM, incluso a concentraciones de 15µM, reduce la generación de especies reactivas de oxígeno (especialmente a 120 y 240µM) y disminuye la expresión de β-catenina y Antígeno Nuclear Celular Proliferante (PCNA), los cuales están involucrados en la proliferación celular y resistencia a apoptosis (Baskar et al., 2010). Concentraciones colónicas de EV (β-sitosterol 115µM; campesterol 11µM; estigmasterol 6µM y combinados 132µM), estos ejercen efectos anti-proliferativos en células Caco-2 tras 24, 48 y 72h de incubación. A nivel individual estos EV actúan como agentes citostáticos, inhibiendo el

crecimiento celular a través de la inducción de la detención en la fase G0 / G1 del ciclo celular, sin activación de la vía apoptótica. Sin embargo, la mezcla de EV muestra un mayor efecto antiproliferativo, ya que es capaz de producir muerte celular por necrosis (López-García et al., 2017).

En este contexto, un reciente estudio caso-control en población china, se ha indicado que la ingesta de EV (202mg/día, β-sitosterol: 63%, campesterol: 15, estigmasterol: 12%, β-sitostanol: 9%, y campestanol: 2%) está directamente asociada con una reducción del 50% en el riesgo de cáncer de colon, mientras que la ingesta de estigmasterol muestra una correlación opuesta (Huang et al., 2017). Sin embargo, en otro estudio epidemiológico llevado a cabo en los Países Bajos, se ha observado que una ingesta de 307mg/día (hombres) o 263mg/día (mujeres) de EV (β-sitosterol, campesterol, stigmasterol, β-sitostanol y campestanol), no se relaciona con una disminución del riesgo de cáncer de colon, incluso se observa una asociación positiva con campesterol y estigmasterol (Normén et al., 2001).

2. Microbiota intestinal

El intestino grueso humano esta colonizado por una compleja comunidad de microorganismos (MO), compuesta principalmente por bacterias anaerobias estrictas (Bernalier-Donadille, 2010; Kleessen et al., 2000), que difieren para cada individuo humano y cambian a lo largo de la vida (Lozupone et al., 2012). Además, factores como la dieta (Macfarlane & Dillon, 2007), el pH debido principalmente a la concentración de sales biliares (Walter & Ley, 2001) y posibles patologías del hospedador (Manichanh et al., 2006) afectan a la composición y diversidad de la comunidad microbiana. Todos estos aspectos se desarrollan a continuación.

2.1. Composición y diversidad

En los seres humanos, los MO se comienzan a adquirir durante el parto mediante liberación vaginal (Reid et al., 2011; Dominguez-Bello et al., 2010) y durante el proceso de amamantamiento (Robinson et al., 2010).

Existen una variedad de factores, incluido el método del parto (vaginal *versus* cesárea), la lactancia y el destete, que influyen en la microbiota infantil (Nicholson et al., 2012). De hecho, la microbiota intestinal de los niños nacidos por vía vaginal ha mostrado una mayor abundancia de *Lactobacillus*, *Prevotella* y *Atopobium*, mientras que los nacidos mediante cesárea tienen una comunidad microbiana similar a la piel materna, siendo *Staphylococci* un miembro dominante (Ravel et al., 2011).

El ambiente en el útero no es tan estéril como se pensó inicialmente. Bacterias como *Enterococcus fecalis*, *Staphylococcus epidermidis* y *Escherichia coli* han sido aisladas del meconio (primeras deposiciones fecales) de recién nacidos sanos (Jiménez et al., 2008). Durante las primeras semanas de vida la microbiota se diversifica para formar una compleja comunidad microbiana compuesta principalmente por MO anaerobios (Mitsou et al., 2008). En la edad adulta, la microbiota intestinal se vuelve más estable, aunque se ha sugerido que los adolescentes tienen una mayor abundancia de *Bifidobacterias* y *Clostridios* que los adultos (Agans et al., 2011).

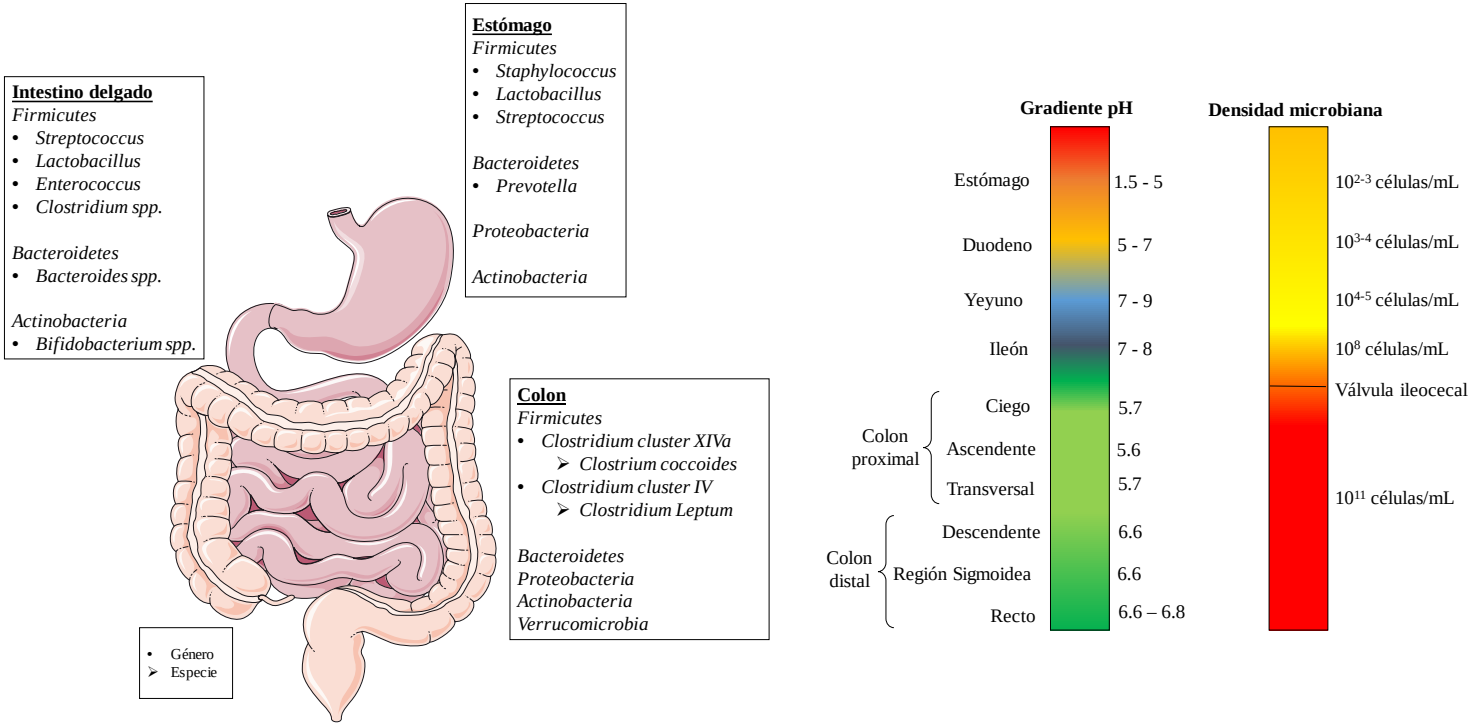
El medio gastrointestinal humano es muy diferente en las distintas regiones del tracto digestivo, creando ambientes específicos para diferentes especies o conjuntos bacterianos funcionales y variando la densidad bacteriana (Portune, et al., 2017). En la Figura 4 se muestran las comunidades microbianas características en cada región del tracto gastrointestinal y el efecto del pH sobre las poblaciones microbianas.

El estómago se caracteriza por una comunidad microbiana ácido-tolerante diversamente distribuida de forma desigual, ya que las poblaciones de estos MO dependen en gran medida del pH gástrico (1.5-5) y oscilan entre 10^2 y 10^3 células/mL de lumen estomacal (Riedel et al., 2014).

En el intestino delgado, el crecimiento microbiano se ve obstaculizado por los cortos tiempos de retención de los alimentos, los péptidos antimicrobianos producidos por las células *Paneth* y las sales biliares (Walter & Ley, 2011). Sin embargo, las condiciones de crecimiento para las poblaciones microbianas mejoran hacía el final del intestino delgado. El número de MO aumenta de aproximadamente de 10^3/mL en el duodeno hasta 10^8/mL en el íleon terminal.

En el colon, el pH intestinal varía significativamente a lo largo de las diferentes regiones del colon (Figura 4) (Evans et al., 1988). Esta variación está determinada tanto por las secreciones del hospedador (principalmente debido a ácidos biliares) como por los productos generados tras la fermentación microbiana (Duncan, et al., 2009). En el colon proximal (ciego, región ascendente y descendente) el pH (5.6-5.7) es más bajo que en el íleon (7-8) debido a la presencia de ácidos grasos de cadena corta (AGCC), producidos durante la fermentación bacteriana de carbohidratos no digeribles. Más adelante, en el colon distal (descendente, región sigmoidea y recto) se produce un aumento del pH (6.6-6.8) como consecuencia de la absorción de AGCC y la secreción de bicarbonato por parte de la mucosa (Wilson, 2009).

Figura 4. Comunidades microbianas características del tracto gastrointestinal y efecto del gradiente de pH sobre sus poblaciones microbianas.



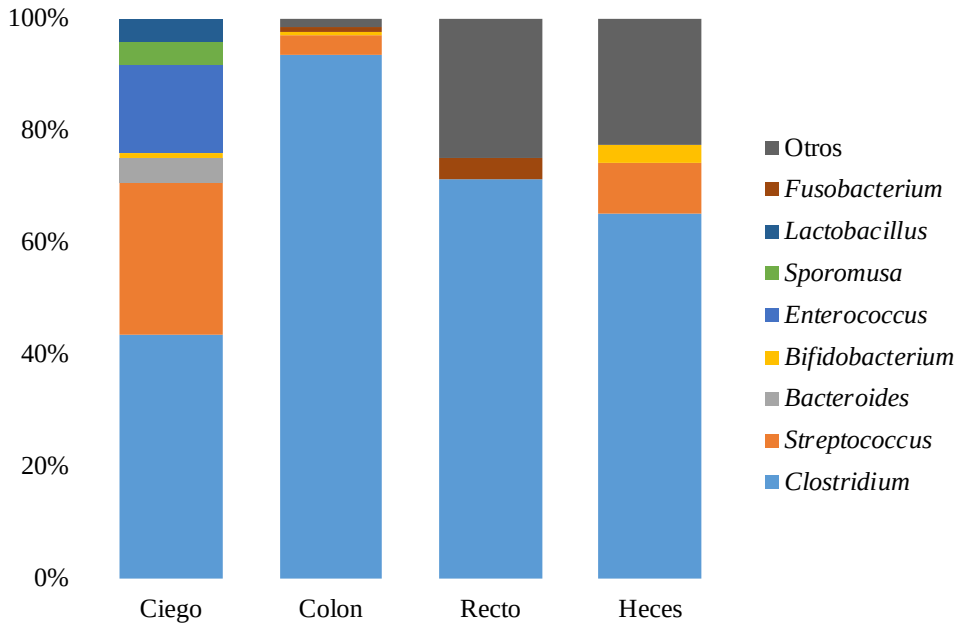
Fuente: Adaptada de Riedel et al. (2014); Walter & Ley (2011).

El colon está dominado principalmente por una compleja microbiota anaerobia. De hecho, el estudio de Eckburg et al. (2005) reveló una diversidad asombrosamente alta, con más de 800 especies o filotipos de casi 200 géneros. Los análisis realizados sugieren que el 60-80% de las bacterias colónicas pertenecen al filo *Firmicutes*, *Bacteroidetes* comprende el 25% (Flint, 2006), *Verrucomicrobia* el 1-2% (Zoetendal et al., 2006) y *Proteoacteria* generalmente representa el componente minoritario de la microbiota (Duncan et al., 2007).

Más recientemente, Arumugam et al. (2011) han propuesto el concepto de tres enterotipos humanos generales basados en la microbiota intestinal. Los tres enterotipos se asignan en función de las abundancias relativas de los siguientes géneros: *Bacteroides* (enterotipo 1), *Prevotella* (enterotipo 2) y *Ruminococcus* (enterotipo 3). En términos de función, algunos de estos géneros se han relacionado con la preferencia en la metabolización de nutrientes: *Bacteroides* los carbohidratos, *Prevotella* las mucinas y *Ruminococcus* las mucinas y los azúcares.

Todavía se conoce poco sobre la composición/diversidad microbiana intestinal, ya que su caracterización presenta algunas limitaciones (Sproule-Willoughby et al., 2010). Además, se ha sugerido que las bacterias fecales no son representativas de las poblaciones de la mucosa intestinal (Eckburg et al., 2005; Zoetendal et al., 2002) ya que la microbiota intestinal crece sobre la mucosa formando biofilms (Sproule-Willoughby et al., 2010). Este hecho ha provocado que aumenten los estudios sobre la composición y el significado ecológico de estos biofilms del colon humano. En la Figura 5 se muestran algunos estudios en los que se ha caracterizado la comunidad microbiana de la mucosa en algunas regiones colónicas y en heces humanas.

Figura 5. Diversidad microbiana (% relativo) a nivel de género en la mucosa de algunas regiones del tracto colónico y en heces humanas de individuos sanos.



Ciego: Hayashi et al., 2005 (n=3); **Colon:** Delgado et al., 2006; Lucke et al., 2006; Wang et al., 2005; Prindiville et al., 2004; Hold et al., 2002 (n=17); **Recto:** Wang et al., 2005 (n=1); **Heces:** Delgado et al., 2006; Hayashi et al., 2005; Hayashi et al., 2002; Suau et al., 1999 (n=9).

En este sentido, Zoetendal et al. (2002) han evaluado las poblaciones en la mucosa del colon ascendente, transversal y descendente de seis individuos sanos. Indican que la microbiota es específica para cada individuo, que los MO están uniformemente distribuidos a lo largo del colon y que la composición de la microbiota procedente de la mucosa colónica es diferente de aquellas que proceden de la materia fecal.

Eckburg et al. (2005) en un estudio de secuenciación genómica a partir de muestras fecales y de mucosa intestinal (ciego, colon ascendente, transversal, descendente sigmoideo y recto) procedente de tres donantes sanos, detectan variabilidad entre las poblaciones microbianas de los donantes, siendo

Firmicutes el filo más abundante, seguido por *Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria* y *Verrucomicrobia*. Además, si bien la diversidad microbiana de las muestras de la mucosa es similar para todos los sujetos, difiere en el caso de las muestras fecales.

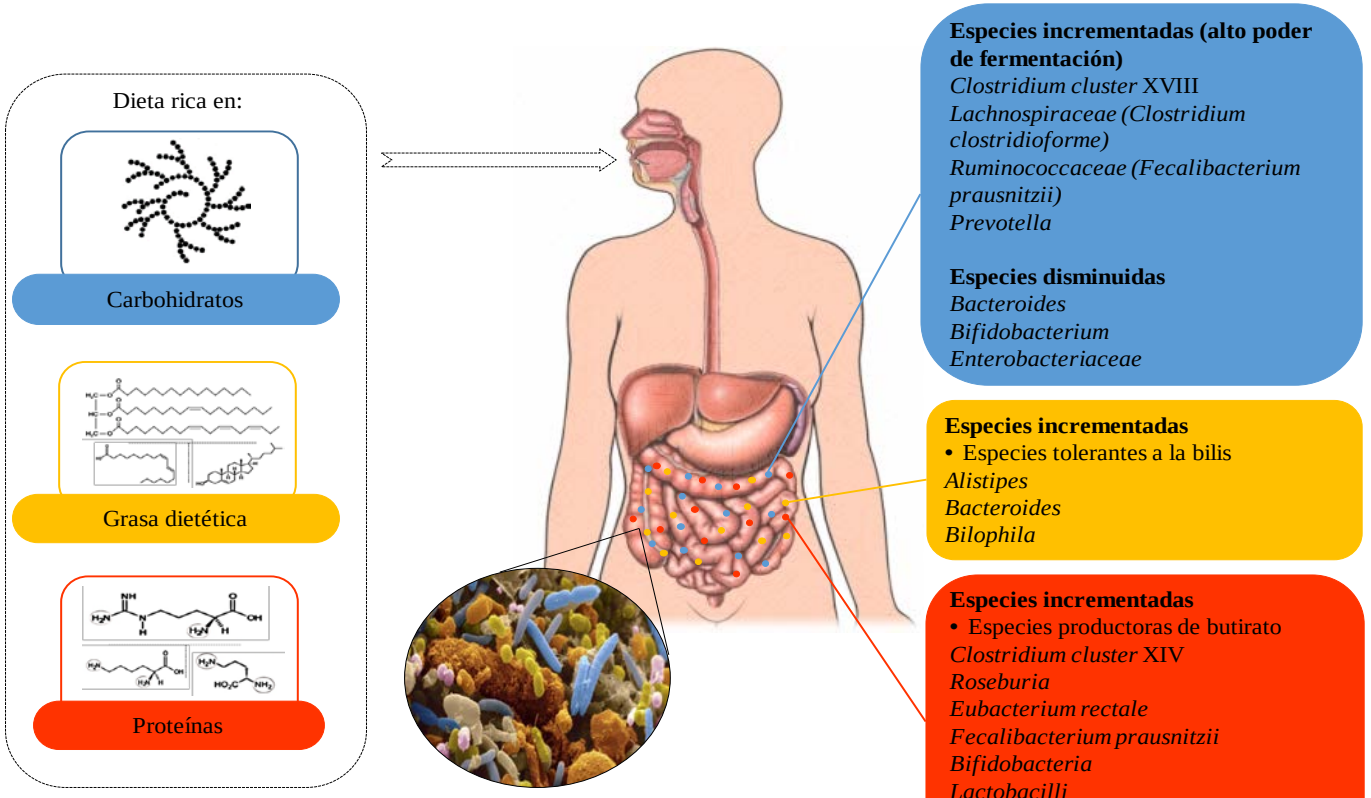
A pesar de estos hallazgos y dado que las técnicas para el muestreo de mucosa intestinal son invasivas, la mayoría de los estudios sobre la composición y diversidad de la microbiota intestinal humana, se han centrado principalmente en el análisis de la microbiota fecal (Kleessen et al., 2000).

Por último, el envejecimiento se asocia con funciones fisiológicas alteradas, incluida la función del sistema inmunitario, que afectan a la composición de la microbiota intestinal. Los cambios de composición, incluyen un aumento en el número total de anaerobios facultativos. Concretamente, la relación *Bacteroidetes-Firmicutes*, que es mayor en los primeros 20 años de vida y disminuye con la edad (Nicholson et al., 2012). Además, en personas mayores de 60 años, se ha observado una marcada disminución en *Bifidobacterias* relacionada con la menor actividad del sistema inmune del hospedador (Mariat et al., 2009).

2.2. Influencia de la dieta

Como se muestra en la Figura 6, carbohidratos, proteínas o grasas influyen específicamente en la composición de la microbiota intestinal (Alou et al., 2016; Conlon & Bird, 2014; Walker et al., 2011).

Figura 6. Influencia de los macronutrientes de la dieta sobre las especies microbianas intestinales.



Fuente: Adaptada de Alou et al., 2016.

El consumo de fibra dietética favorece el aumento de las especies productoras de butirato (*Roseburia, Blautia, Eubacterium rectale* y *Faecalibacterium prausnitzii*) que fermentan estas fibras principalmente en el colon distal, también de las pertenecientes al filo *Actinobacteria* (*Bifidobacteria, Lactobacilli*) y puede provocar también variaciones en la proporción de *Bacteroidetes* dependiendo del tipo de fibra dietética, mientras que una dieta alta en proteínas estimula el aumento de especies con actividades proteolíticas tales como *Bacteroides* (Chung et al., 2016; Dominianni et al., 2015; Graf et al., 2015; Maukonen & Saarela, 2015).

Una dieta alta en grasas estimula la producción de ácidos biliares que, a su vez, estimulan el crecimiento de especies con capacidad para metabolizarlos (*Eubacterium* and *Clostridium*) y puede inducir la pérdida de algunas especies como *Bifidobacterium* debido a la actividad antimicrobiana de estos (Graf et al., 2015; Maukonen & Saarela, 2015). Además, en un estudio realizado en 10 individuos sanos, la ingesta a largo plazo de dietas ricas en grasas animales se asocia con un aumento en la abundancia de *Bacteroides* (Wu et al., 2011) y en el consumo a corto plazo (5 días) de dietas basadas exclusivamente en alimentos de origen animal (carnes, huevos y quesos) en 10 individuos, también produce un aumento en la abundancia de *Bacteroides*, así como *Alistipes* y *Bilophila*, junto con una disminución en ciertos grupos pertenecientes al filo *Firmicutes* (*Roseburia, Eubacterium rectale, Ruminococcus bromii*) (David et al., 2014).

Turnbaugh et al, (2006) evalúan el efecto de la dieta sobre la microbiota colónica en 12 individuos con obesidad, a los cuales se les restringe la grasa y los hidratos de carbono de la dieta durante un 1 año. Las proporciones relativas de *Firmicutes* y *Bacteroidetes* para los individuos obesos cambian gradualmente con el tiempo y se aproximaron a los valores encontrados en

individuos delgados (control). Además, se observa un incremento en la abundancia relativa de *Bacteroidetes* que esta correlacionado con la disminución de la masa corporal en los individuos obesos.

Se ha demostrado que la abundancia de *Erysipelotrichaceae* (*Firmicutes*) se altera como respuesta a cambios en la ingesta de grasa dietética. Tras inducir obesidad a ratones alimentados con una dieta "occidental" (alta en grasas saturadas e insaturadas principalmente de origen animal), se observa un aumento en la proliferación de esta familia, mientras que cuando se instaura la dieta habitual (pienso), su abundancia relativa disminuye (Turnbaugh et al., 2008). En ratones "humanizados" (ratones libres de MO que albergan microbiota fecal humana), se ha observado que la proliferación de *Erysipelotrichaceae* de origen humano aumenta siguiendo una dieta rica en grasas (Turnbaugh et al., 2009).

2.3. Microbiota y enfermedad

Se ha sugerido que la microbiota intestinal juega un papel importante en el desarrollo de un sistema inmune equilibrado (Von Martels et al., 2017). Se ha indicado que varias enfermedades comunes están asociadas con la "disbiosis" de la microbiota intestinal (desequilibrio entre bacterias "beneficiosas" con propiedades antiinflamatorias y agentes patógenos con propiedades proinflamatorias). La enfermedad inflamatoria intestinal, como el síndrome de Crohn o/y colitis ulcerosa, la diabetes tipo 2 y la obesidad, se asocian con una disminución de la diversidad de la microbiota intestinal (Hartstra et al., 2015; Schippa & Conte, 2014).

Por otra parte, el sobrepeso y la obesidad se definen como una acumulación anormal o excesiva de grasa o hipertrofia general del tejido adiposo en el cuerpo. El índice de masa corporal (IMC) (kg/m^2) es utilizado

frecuentemente para clasificar el sobrepeso y la obesidad en adultos. La Organización Mundial de la Salud (OMS) define el sobrepeso como un IMC igual o superior a 25, y la obesidad como un IMC igual o superior a 30 (OMS, 2017).

Existen evidencias de la microbiota intestinal como un importante factor ambiental que contribuye a la obesidad por alterar la obtención y el almacenamiento de la energía del hospedador (Bäckhed et al., 2004), Así, algunos estudios en humanos (Turnbaugh et al., 2009; Ley et al., 2006) y en animales (Turnbaugh et al., 2006; Ley et al., 2005; Bäckhed et al., 2004) han asociado la disbiosis microbiana intestinal con la obesidad.

En general, los individuos obesos muestran una disminución de la diversidad bacteriana (Mishra et al., 2016; Kasai et al., 2015; Lau et al., 2015; Turnbaugh et al., 2009) caracterizada por un incremento en la ratio *Firmicutes-Bacteroidetes* (Boulangé et al., 2016; Gerard, 2016; Kasai et al., 2015; Lau et al., 2015; Lecomte et al., 2015). Además, se ha constatado que esta relación disminuye después de la pérdida de peso inducida por intervención dietética (Ley et al., 2006).

Un aumento en la abundancia de *Firmicutes* se ha asociado con un aumento de los dos principales AGCC: butirato, que es el principal suministro de energía para los colonocitos y que se correlaciona negativamente con la permeabilidad intestinal y el acetato, sustrato que interviene en la síntesis de colesterol hepático y la lipogénesis de *novo* (Lau et al., 2015; Lecomte et al., 2015; Turnbaugh et al., 2006), la cual produce un aumento en la adiposidad y el peso corporal del individuo (Janssen & Kersten, 2015).

Estos hallazgos apoyan el concepto de que el microbioma intestinal debe considerarse como un conjunto de factores genéticos que, junto con el

genotipo del hospedador y el estilo de vida (consumo y gasto de energía), contribuyen a la fisiopatología de la obesidad (Turnbaugh et al., 2006).

Por otro lado, en individuos hipercolesterolémicos (n=30) se ha observado una disminución de la diversidad microbiana frente a sujetos control (n=27), sugiriendo que la microbiota intestinal podría influir en el desarrollo de hipercolesterolemia (Rebolledo et al., 2017).

3. Modelos de fermentación colónica *in vitro*

La microbiota colónica posee numerosas funciones fisiológicas que repercuten en la nutrición y salud del hospedador (Bernalier-Donadille, 2010). Suministra energía al huésped a través de procesos fermentativos anaeróbicos (Duncan et al., 2007) siendo su principal fuente de energía los carbohidratos, seguido de las proteinas (Portune et al., 2017).

Esta función metabólica está relacionada con la naturaleza de los sustratos disponibles, que bien pueden ser exógenos (principalmente polisacáridos vegetales) o endógenos (Bernalier-Donadille, 2010), como la producción endógena de mucus, a partir del cual se sintetizan AGCC (Bassotti & Battaglia, 2017).

Mientras que existen bastantes estudios sobre los MO que metabolizan los hidratos de carbono no digeribles y péptidos o aminoácidos, se conoce muy poco sobre la degradación de la grasa dietética *in vivo* a nivel intestinal, así como las especies bacterianas que metabolizan los lípidos (Portune et al., 2017). En la Tabla 3 se muestran algunos metabolitos producidos por la acción de la microbiota intestinal y sus efectos biológicos en el hospedador.

Tabla 3. Metabolitos producidos por la microbiota intestinal y sus funciones fisiológicas (Adaptada de Postler & Ghosh (2017); Nicholson et al. (2012)).

Metabolitos	**Bacterias implicadas**	**Funciones biológicas**
AGCC: acetato, propionato, butirato, isobutirato, 2-metilpropionato, valérico, isovalérico, hexanoico	*Bacteroidetes y Firmicutes*: Clostridiales IV y XIVa, incluyendo especies de *Eubacterium*, *Roseburia*, *Fecalibacterium* y *Coprococcus*	Disminuyen el pH colónico inhibiendo el crecimiento de patógenos, estimulan la reabsorción de agua y sodio, participan en la síntesis de colesterol y proporcionan energía a las células epiteliales del colon, efecto antiinflamatorio, inhiben la producción de citoquinas proinflamatorias, promueven la producción de anticuerpos.
Ácidos biliares: cólico, hyocólico, chenodesoxicólico, desoxicólico, litocólico, ursodesoxicólico, hyodesoxicólico	*Lactobacillus, Bifidobacteria, Enterobacter, Bacteroides, Clostridium*	Facilitar la absorción de la grasa dietética y las vitaminas liposolubles, mantienen las funciones de la barrera intestinal, regular la homeostasis de triglicéridos, colesterol, glucosa y energía, inhibir la producción de citoquinas proinflamatorias.
Lípidos: ácidos grasos conjugados, lipopolisacáridos, peptidoglicanos, acilgliceroles, esfingomielina, cholesterol, fosfatidilcolina, fosfoetanolaminas, trigliceridos	*Bifidobacterium, Roseburia, Lactobacillus, Klebsiella, Enterobacter, Citrobacter, Clostridium*	Impactan sobre la permeabilidad intestinal, regulan la homeostasis de la glucosa. Lipopolisacáridos: inducen inflamación sistémica crónica. Ácidos grasos conjugados: mejoran la hiperinsulinemia, el sistema inmune y alteran los perfiles de lipoproteínas. Colesterol: base para la producción de esteroles y ácidos biliares.
Metabolitos de la colina: metilamina, dimetilamina, trimetilamina, trimetilamina-*N*-oxido, dimetilglicina, betaina	*Fecalibacterium prausnitzii*, *Bifidobacterium*	Modular el metabolismo de lípidos y la homeostasis de la glucosa. Involucrado en la enfermedad de "hígado graso no alcohólico", la obesidad inducida por la dieta, diabetes y enfermedad cardiovascular.

Tabla 3. Continuación.

Metabolitos	Bacterias implicadas	Funciones biológicas
Derivados del indol: N-acetiltriptofano, indolacetato, indolacetilglicina, indol, indoxil sulfato, indol-3-propionato, melatonina, melatonina-6-sulfato, serotonina, 5-hidroxiindol	*Clostridium sporogenes*, *Escherichia coli*	Protege contra lesiones inducidas por el estrés en el tracto GI, modula la expresión de genes proinflamatorios, aumenta la expresión de genes antiinflamatorios, fortalece las propiedades de barrera de las células epiteliales. Implicado en patologías GI, eje cerebro-intestino y algunas afecciones neurológicas.
Poliaminas: putrescina, cadaverina, espermidina, espermina	*Campylobacter jejuni*, *Clostridium saccharolyticum*	Mejora el desarrollo y mantenimiento de la mucosa intestinal y las células inmunes residentes. Ejercen efectos genotóxicos sobre el huésped, efectos antiinflamatorios y antitumorales. Posibles marcadores tumorales.
Vitaminas: vitamina K, B12, biotina, folato, tiamina, riboflavina, piridoxina	*Bifidobacterium*	Proporcionan fuentes endógenas complementarias de vitaminas, fortalecen la función inmune, ejercen efectos epigenéticos para regular la proliferación celular.
Otros: lactato, formiato, metanol, etanol, succinato, lisina, glucosa, urea, creatina, creatinina, endocanabinoides, etc…	*Bacteroides*, *Pseudobutyrivibrio*, *Ruminoccus, Fecalibacterium*, *Subdoligranulum*, *Bifidobacterium, atopobium*, *Firmicutes, Lactobacillus*	Síntesis o utilización directa o indirecta de compuestos o modulación de vías vinculadas, incluido el sistema endocannabinoide.

AGCC: ácidos grasos de cadena corta; GI: gastrointestinal.

El estudio de los procesos metabólicos microbianos y el efecto de los componentes dietéticos sobre la microbiota intestinal, pueden realizarse mediante estudios *in vivo* o *in vitro*.

La realización de estudios en humanos es compleja debido a las consideraciones sociales y éticas de los procedimientos invasivos necesarios para acceder al intestino grueso. Además, la variabilidad genética, fisiológica y dietética dificulta la evaluación *in vivo* de las interrelaciones entre la comunidad microbiana y los componentes dietéticos. Por ello, estos estudios se limitan principalmente al análisis de microbiota y metabolitos en muestras fecales. Aunque los estudios en animales se utilizan como sustituto de los estudios en humanos, la relevancia de los datos obtenidos está siendo cuestionada debido a las diferencias fisiológicas (Payne et al., 2012).

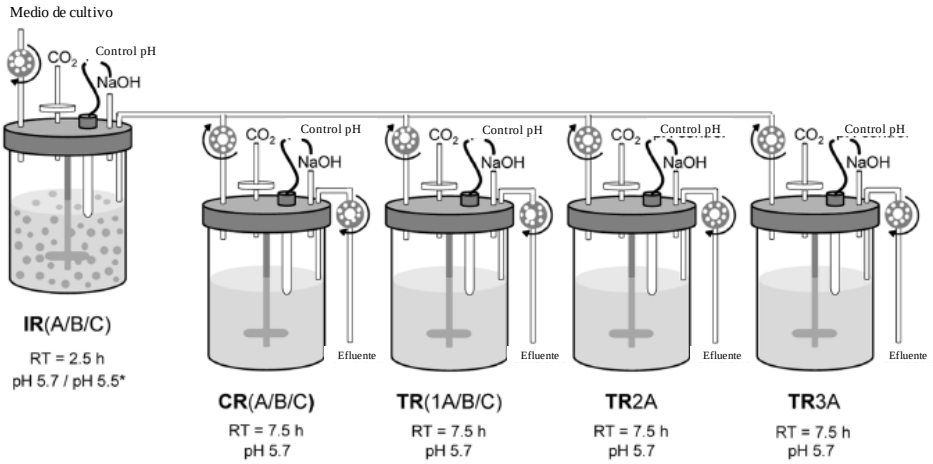
En el caso de los estudios *in vitro*, existen modelos que simulan únicamente el tracto colónico, como el sistema estático de "*cultivo por lote*". Se aplica en ensayos iniciales, o previos, por su fácil configuración y los pequeños volúmenes de muestra que requiere. Sin embargo, se produce acumulación de metabolitos microbianos que pueden inhibir la actividad microbiana y tanto el pH, como el potencial redox, no pueden ser controlados. Su aplicación se limita a ensayos para evaluar la capacidad metabólica microbiana y la determinación de metabolitos (Verhoeckx et al., 2015; Venema & Van der Abbeele, 2013; Payne et al., 2012; Gibson & Fuller, 2000).

Otros sistemas que simulan las condiciones del colon son los modelos continuos o semicontinuos de una sola etapa. La mayoría de ellos se han adaptado a partir del sistema descrito por Miller & Wolin, (1981) que, a su vez, fue adaptado de un sistema ideado para su aplicación en estudios de microecología del rumen. Se trata de sistemas dinámicos que permiten

reproducir con mayor exactitud las condiciones de pH, temperatura (37ºC) y anaerobiosis del colon proximal. A diferencia de los anteriores, estos se emplean para estudios a largo plazo sobre la reposición de sustrato y/o eliminación de productos. Sin embargo, presentan una limitación importante, ya que las condiciones del colon distal no se reproducen de forma adecuada (Venema & Van der Abbeele, 2013; Payne et al., 2012; Miller & Wolin, 1981).

Uno de los sistemas más conocido dentro de este grupo es el modelo "*Polyfermentor Intestinal (PolyFerms)*" (Figura 7) desarrollado por el grupo de investigación liderado por el Profesor Christophe Lacroix (*ETH Zürich University*).

Figura 7. Configuración y diseño del modelo *Polyfermentor Intestinal (PolyFerms)*



Control (CR); reactores para ensayos (TR); reactores de inóculo (IR); tiempo de retención (RT).

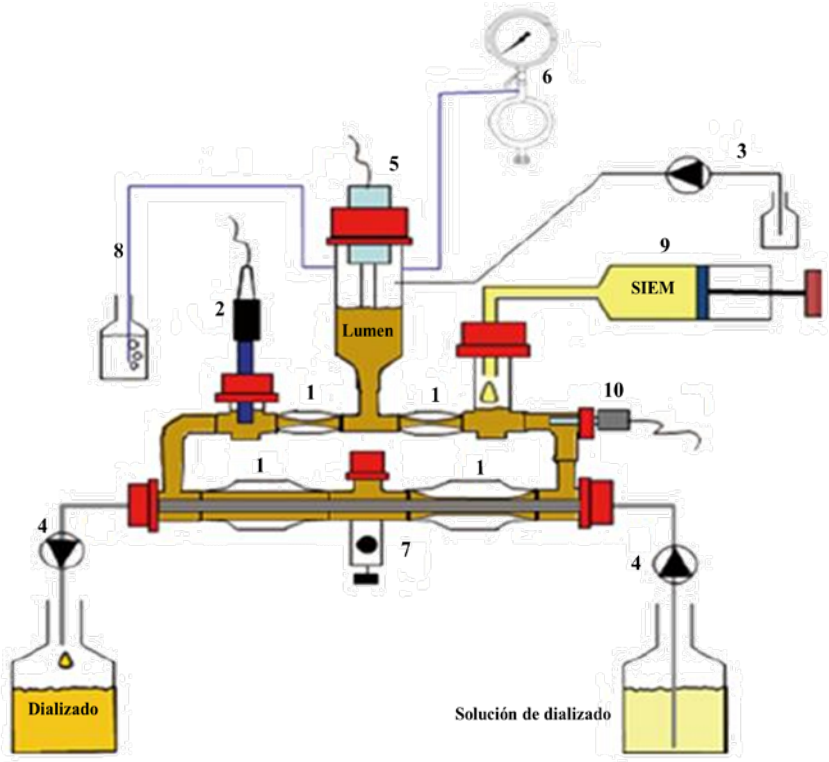
Fuente: Adaptada de Berner et al. (2013).

Este sistema está diseñado para comparar los efectos de diferentes tratamientos sobre la misma microbiota intestinal. Se compone de un primer compartimento que contiene la microbiota fecal inmovilizada en perlas de

gel. Mediante un flujo constante y continuo, el inoculo fecal se va añadiendo al reactor donde se lleva a cabo la fermentación. Debido a la alta densidad de la microbiota presente en las perlas de gel y a su liberación progresiva, se trata de modelos que pueden ser aplicados en estudios a largo plazo. Una de las ventajas de este sistema es que permite llevar a cabo varios ensayos de forma paralela. Sin embargo, el tiempo de los ensayos es largo, 54 días incluyendo la estabilización de la microbiota, el proceso de fermentación y la limpieza del sistema (Tanner et al., 2014; Berner et al., 2013).

Otro sistema perteneciente al grupo de los modelos continuos de una sola etapa es, el "*Tract Intestinal Model 2 (TIM-2)*" (Figura 8) desarrollado por la Organización de Investigación Científica Aplicada de los Países Bajos (TNO) hace unos 15 años (Minekus et al., 1999). Se trata de un sistema automatizado y controlado mediante software que consta de cuatro compartimentos de vidrio, individualmente interconectados mediante membranas flexibles y a diferencia del sistema anterior, éste permite ejecutar hasta diez unidades de forma paralela en un corto periodo de tiempo (estabilización microbiana: 16h, ensayo de fermentación: 24-72h). Además, simula los movimientos peristálticos y para evitar la acumulación de metabolitos microbianos que provocarían la inhibición o muerte de la comunidad microbiana, el sistema está equipado con un sistema de dializado (Venema, 2015; Venema & Van der Abbeele, 2013).

Figura 8. Modelo de fermentación colónica *in vitro* TIM-2.



1: compartimentos peristálticos; 2: electrodo de pH; 3: bomba alcalina; 4: circuito de diálisis / dializado con membrana de fibra hueca; 5: sensor nivel de volumen; 6: entrada N_2; 7: puerto de muestreo; 8: salida de gas; 9: contenedor del medio estándar de eflujo ileal (SIEM) (mezcla carbohidratos); 10 sensor temperatura.

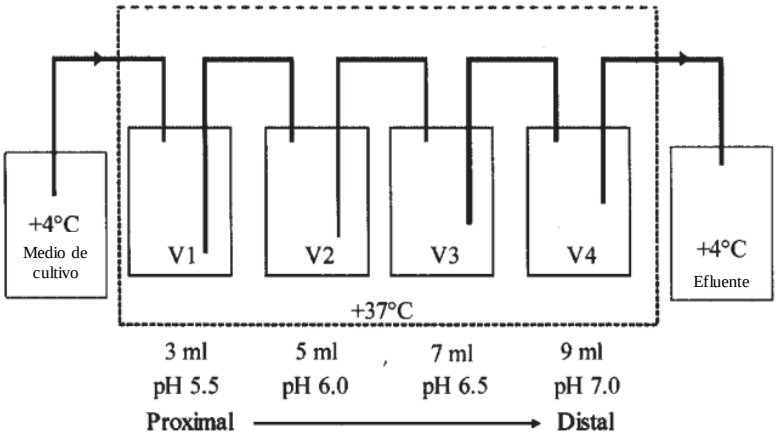
Fuente: Adaptada de Minekus et al. (1999).

Debido a las limitaciones de los modelos anteriores, se han validado otros complejos modelos dinámicos multietapa que simulan las tres regiones del colon (ascendente, transversal y descendente), mediante varios compartimentos interconectados que operan también bajo condiciones controladas. Un claro ejemplo es el sistema "*Lacroix Model*", que del mismo modo que en el sistema *PolyFerms*, la microbiota está inmovilizada mediante

perlas de gel, pero la duración del ensayo es más larga (27 días) (Venema & Van der Abbeele, 2013; Cinquin et al., 2006; Cinquin et al., 2004).

En cambio, el sistema "*EnteroMix*" (Figura 9) consta de cuatro compartimentos que, además de simular las tres regiones del colon mencionadas anteriormente, también imita las condiciones de la región sigmoidea y recto. Permite ejecutar cuatro unidades simultáneamente en un corto periodo de tiempo (estabilización microbiana: 15h, ensayo de fermentación: 24-48h) (Venema & Van der Abbeele, 2013; Makivuokko et al., 2005).

Figura 9. Modelo "*EnteroMix*".



V1: ascendente; V2: transversal; V3: descendente; V4: distal

Fuente: Adaptada de Makivuokko et al. (2005).

También existen modelos continuos que comprenden todo el tracto gastrointestinal, es decir, que simulan, de forma dinámica, las condiciones del estómago, intestino delgado y colon. Los más conocidos son el "*Simulator of the Human Intestinal Microbial Ecosystem (SHIME)*" y "*SIMulator Gastro-Intestinal (SIMGI)*" Estos constan de cinco reactores interconectados que representan las condiciones del estómago, intestino delgado y las tres

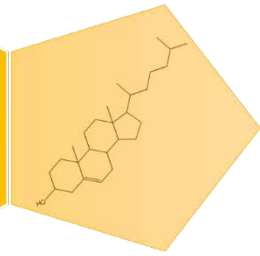
regiones del colon, y por lo general, la duración de los ensayos suele ser larga, de aproximadamente 7 semanas (Barroso et al., 2015; Van de Wiele et al., 2015; Prodigest & Ghent University, 2010; Van den Abbeele et al., 2010).

Por lo general, las poblaciones bacterianas utilizadas en estos modelos están formando parte de una suspensión fecal líquida. Este hecho puede ser un factor limitante debido a que las poblaciones bacterianas están en forma libre, lo que implica un tiempo de fermentación prolongado de aproximadamente cuatro semanas (Venema & Van der Abbeele, 2013; Payne et al., 2012; Macfarlane et al., 1998; Gibson & Fuller, 2000). Además, tal y como se ha mencionado anteriormente, las bacterias fecales no son representativas de las poblaciones de la mucosa intestinal. Por esta razón, han comenzado a desarrollarse nuevos modelos donde se utiliza mucosa intestinal y microbiota colónica formando biofilms (Sproule-Willoughby et al., 2010). Es el caso del sistema dinámico "*Mucus-Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME)*" que, como los anteriores, simula secuencialmente todo el tracto gastrointestinal y permite que la microbiota se adhiera a la capa de mucus formando una barrera o *biofilm* semejante a la que se da en condiciones fisiológicas normales (Prodigest & Ghent University, 2010; Van den Abbeele et al., 2009).

4. Esteroles y microbiota intestinal

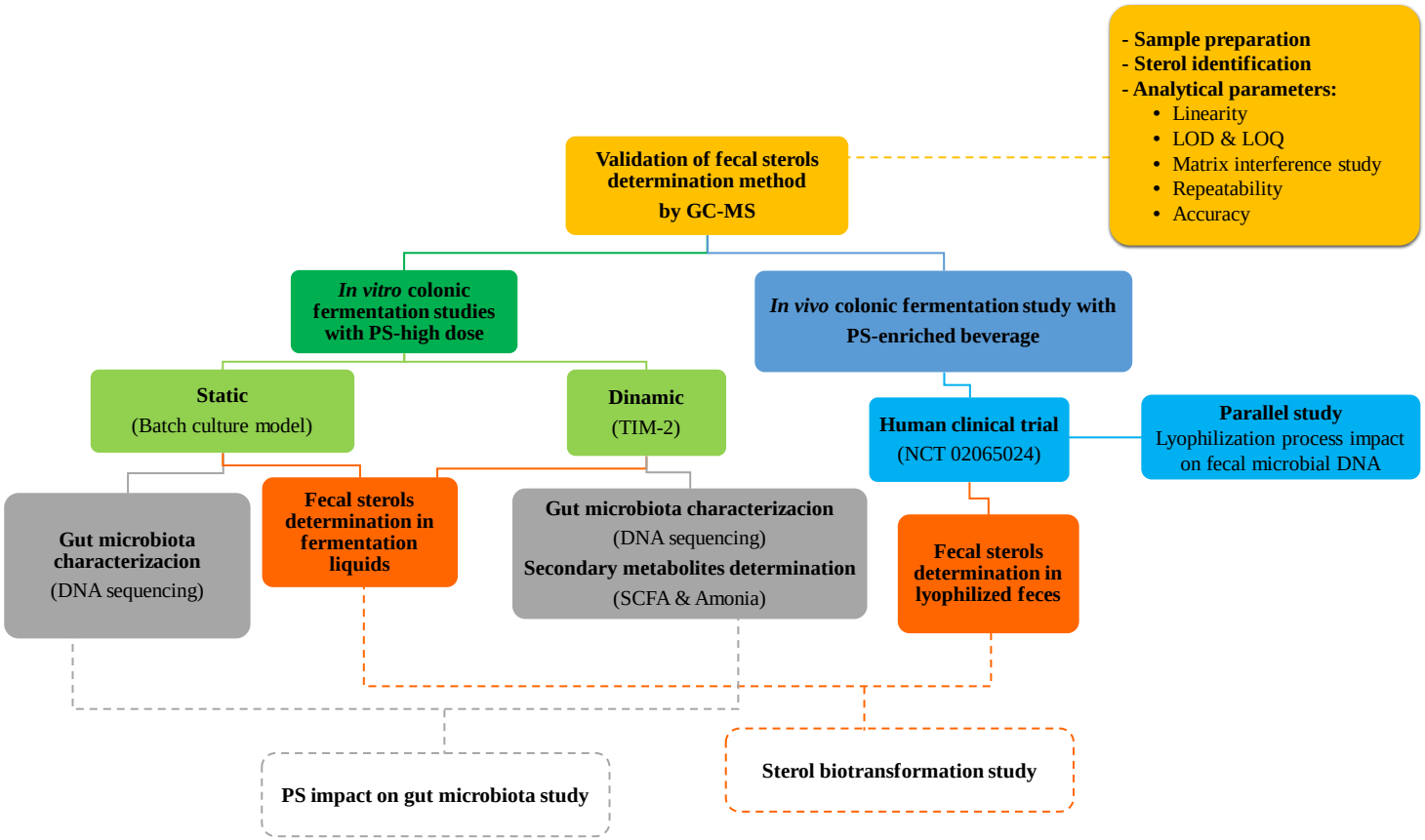
Se ha revisado la información relativa a la a la determinación de esteroles y sus metabolitos en heces, su biotransformación mediante la microbiota colónica e impacto de los mismos sobre las poblaciones bacterianas del colon y *viceversa* incluyéndola en una publicación de revisión aceptada en *European Journal of Lipid science and Technology* que puede consultarse en el anexo II.

Results & Discussion



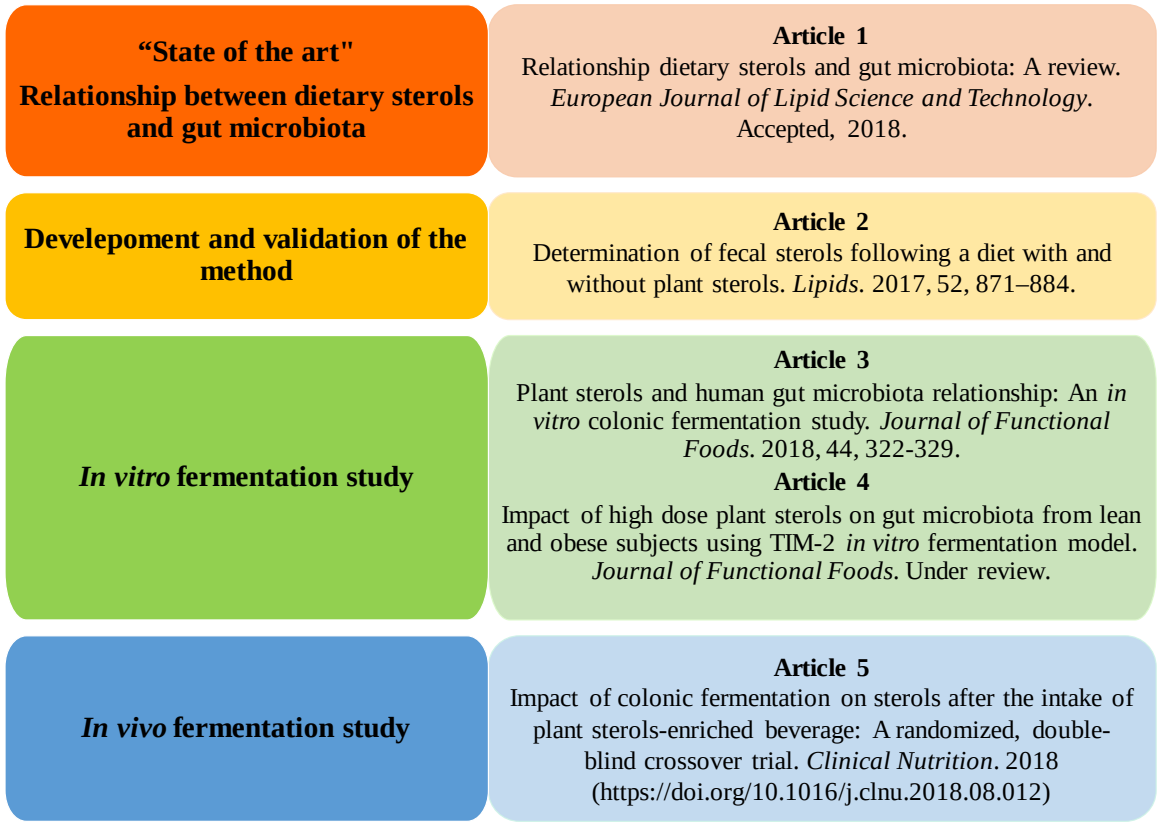
En las figuras 10 y 11 se detallan el esquema del diseño experimental realizado en la presente Tesis Doctoral y la difusión de resultados mediante artículos, respectivamente.

Figure 10. Experimental design scheme.



DNA: deoxyribonucleic acid; LOD: limit of detection; LOQ: limit of quantitation; SCFA: short chain fatty acids; TIM-2: TNO intestinal model -2.

Figure 11. Experimental design development. Relationship with the results dissemination.



I. *Determination of fecal sterols following a diet with and without plant sterols*

In the literature, there are studies that determine sterols and their metabolites in fresh human feces after Western diet intake (Shah et al., 2007; Arca et al., 1983; Korpela, 1982; McNamara et al., 1981; Huang et al., 1976; Gustafsson & Sjövall, 1969; Miettinen et al., 1965; Eneroth et al., 1964) or lyophilized (Lubinus et al., 2013; Keller & Jahreis, 2004; Batta et al., 2002; Weststrate et al., 1999; Batta et al., 1999; Glatz et al., 1985). Not all the aforementioned studies applied validated methods. In some cases, precision and accuracy values are given for both cholesterol and their metabolites (Glatz et al., 1985; Korpela, 1982) and for plant sterols (PS) (campesterol, β-sitosterol, and stigmasterol) after the intake of a normal diet (Korpela, 1982). Only the studies conducted by Wu et al. (2009) have validated a method for the determination of fecal sterols and their metabolites in feces and water samples.

Since few studies have validated the methodology used in determining fecal sterols and their metabolites, and considering the interest in determining these compounds on the basis of the findings of clinical intervention studies with PS-enriched foods, a methodology for determination sterols and their metabolites in feces with high and low contents of PS using gas chromatography and mass spectrometry (CG/MS) has been developed and validated (see annex I), to later study the influence of colonic fermentation on dietary sterols by *in vitro* and *in vivo* models.

The proposed method has been applied to lyophilized feces, since these samples have several advantages from the point of view of storage, safety and sampling. In addition, the present method requires less (two- to fivefold) sample compared with others authors (Wu et al., 2009; Czubayco et al., 1991; Van

Faassen et al., 1987; Glatz et al., 1985; Arca et al., 1983; Korpela, 1982; McNamara et al., 1981; Huang et al., 1976; Miettinen et al., 1965; Eneroth et al., 1964).

In order to obtain a more environmentally friendly method, the volume of solvent was reduced. In this context, sample homogenization was carried out with water, and the solvent volumes (10mL n-hexane, approximately) used for unsaponifiable extraction and derivatization were between 2- and 50-fold lower compared with those used by other authors (Wu et al., 2009; Glatz et al., 1985; Arca et al., 1983; Korpela, 1982; McNamara et al., 1981; Huang et al., 1976; Gustafsson & Sjövall, 1969; Miettinen et al., 1965; Eneroth et al., 1964). Besides, the optimal volume of feces solution (between 25 and 500µL) has also been evaluated. In feces from subjects consuming a Western diet, the determination of major sterols (coprostanone, cholestanol+methylcoprostanol, cholesterol, ethylcoprostanol and sitosterol) was performed using aliquots of 100µL, while aliquots of 500µL were used for the rest of sterols. However, in those individuals who ingested the PS-enriched beverage, a large content of PS and their metabolites was observed in feces. For this reason, a lower sample volume (100µL and in some cases even 25µL was necessary).

The method developed allows the identification of 21 sterols (brassicasterol, campestanol, campesterol, coprostanol, coprostanone, cholestane, cholestanol, cholestanone, cholesterol, desmosterol, epicoprostanol, ethylcoprostanol, ethylcoprostanone, ethylcoprostenol, lathosterol, methylcoprostanol, methylcoprostanone, sitostanol, sitosterol, stigmastenol and stigmasterol) and the quantification of 17 of them.

However, the method did not allow separate cholestanol and methylcoprostanol because they co-eluted and in the case of coprostanol/epicoprostanol when their ratios was >1600, these sterols

overlapped, and it proved difficult to quantify. For ethylcoprostanol and campesterol their separation also depended on the ratio between them (<25), in coincidence with the observations of other authors (Czubayco et al., 1991; Korpela, 1982), which they could not separate ethylcoprostanol and campesterol.

A complete validation has also been performed, taking into account all analytical parameters. Besides, the concentration ranges used were wide in order to allow quantification of sterols and their metabolites present in feces from subjects with a PS-high intake.

The present study is the only publication offering detection and quantitation limits for determining cholestanone, desmosterol, brassicasterol, lathosterol, methylcoprostanone, ethylcoprostenol, ethylcoprostanone, stigmastenol, campestanol and sitostanol by GC/MS. The limit of detection (LOD) and limit of quantitation (LOQ) values covered a broad range between 8–291 and 25–970 ng/mL, respectively—the lowest corresponding to cholestanol and the highest to ethylcoprostanone. This method is more sensitive than others published using the same technique (Shah et al., 2007; Keller & Jahreis, 2004).

Intra-assay values (Relative Standard Deviation (RSD%)) for total sterol contents varied from 0.9 to 9.2%, and the range of inter-assay values was 2.1–11.3%. This values were in agreement with the criteria of the Association of Official Analytical Chemists (AOAC, 2002), which indicate that for analytes present at concentrations of μg/g, values up to 15% are acceptable. Besides, the intra- and inter-assay precision results were similar to those reported by other authors such as Glatz et al. (1985) (intra- and interassay precision 1.1–2.6% and 0.6–4.0%, respectively) for cholesterol and its metabolites (not specified) and PS (not specified), and Keller & Jahreis (2004) (intra- and inter-assay precision 2.7–9% and 4.2–11.2%, respectively) for cholesterol and its metabolites (coprostanol, coprostanone and cholestanol). However, the precision values obtained were

lower than those reported by Wu et al. (2009) for coprostanol (11.3%) and cholestanol (19.6%), and similar for cholesterol (8%).

Recoveries were usually above 80% for all sterols and were in accordance with the intervals proposed by the AOAC (AOAC, 2002) (80–115%) for analyte concentrations of μg/g, which confirmed the good accuracy of the method. Regarding cholesterol and its metabolites, other authors have evaluated accuracy in feces, obtaining good recoveries (Wu et al., 2009; Korpela, 1982; Eneroth et al., 1964). As far as we know, only Korpela (1982) reported PS recovery in feces referred to campesterol (85.4%), stigmasterol (96%) and sitosterol (81.1%). Notably, the present study for the first time evaluates accuracy referred to desmosterol, brassicasterol, lathosterol and sitostanol. Furthermore, the described method affords high sensitivity, precision and accuracy in application to feces from subjects who consume a normal diet and a diet supplemented with PS.

The developed method was applied to samples obtained after *in vitro* colonic fermentation assay with PS-high concentration using a static (section II) and other dynamic model (section III). Feces from hypercholesterolemic postmenopausal women (n=40), who have participated in a clinical study with a PS-enriched milk based fruit beverage (section IV), were also analyzed by this method.

II. *Plant sterols and human gut microbiota relationship: An in vitro colonic fermentation study*

Sterols biotransformation has been widely studied by *in vitro* assays using microbial inocula from human feces (Snog-Kjaer et al., 1956; Rosenfeld et al., 1954; Dam, 1934a; Dam, 1934b) or pure cultures of human enteric microorganisms such as *Eubacterium* spp. (Ren et al., 1996; Eyssen & Parmentier, 1974; Eyssen, et al., 1973), *Clostridium perfringens*, *Bifidobacterium* spp., *Escherichia coli*, or *Enterobacter aerogenes* (Snog-Kjaer et al., 1956) and *Bacteroides* sp. (Gerard et al., 2007) in order to study cholesterol metabolites. In the case of PS, only one study has been carried out with *Eubacterium* sp. (Eyssen et al., 1973). Besides, *in vivo* studies with subjects following a vegan diet have found only slight changes in the bacterial anaerobic genera (*Bifidobacterium*, *Bacteroides* and *Clostridium*) (Van Faassen et al., 1987) and after consumption of plant stanol ester-enriched margarines (3.0g/day), have not been observed significant changes in microbiota composition (Baumgartner et al., 2017).

Therefore, an *in vitro* fermentation study using a beverage residue (BR) obtained after *in vitro* simulated gastrointestinal digestion of a PS-enriched milk based fruit beverage was carried out to evaluate the transformation of PS by gut microbiota and the impact of PS on colonic bacterial population (see annex I).

It should be noted that, the assays, in parallel, were composed of a sample (culture medium enriched with PS and feces inoculum) and two controls: 1) culture medium with feces inoculum and 2) culture medium with BR.

In this sense and considering the sterols amounts in PS sample compared with the sum of two controls, there was a decrease in the contents of sitosterol,

sitostanol, campesterol, campestanol, stigmasterol and brassicasterol during colonic fermentation, with no differences between 24 and 48h. Statistically significant differences ($p < 0.05$) were observed between 24 and 48h of fermentation referred to the mean contents of ethylcoprostanol in the PS sample.

The reductions at 24h were more pronounced for sitostanol (21%) than for sitosterol (8%), while similar reductions were observed after 48h (16% *vs.* 14%). However, in the case of campesterol and campestanol, similar reductions were observed at 24h (19% *vs*. 17%), with a more marked reduction after 48h (39% *vs*. 15%) which showed statistically significant difference ($p < 0.05$). In the case of stigmasterol, the reductions at 24 and 48h were similar (17% and 19%). The difference in the percentage reductions of brassicasterol at 24 and 48h (10% *vs.* 1%) were influenced by the low contents of this compound. This decrease in sterols with respect to unfermented sample (control 1; culture medium + BR) was correlated to an increase in ethylcoprostanol (18% and 50%), ethylcoprostanone (16% and 39%), methylcoprostanone (63% and 21%) and stigmastenol (13% and 16%) at 24 and 48h, respectively.

Unlike in the case of PS, there was no formation of cholesterol metabolites after 24 and 48h of fermentation, although the feces donors were high converters (Wilkins & Hackman, 1974) (cholesterol conversion percentage at 24 (82%) and 48h (84%)). This fact could suggests that bacterial groups would prefer to use PS as a substrate because they are more closely related to cholesterol. This is in concordance with the study carried out by Weststrate et al. (1999), where the intake of a margarine enriched with PS (8.6g/day) in adults did not result in an increase in the concentrations of neutral sterols, and reduced the amount of cholesterol metabolized in the form of coprostanol.

Agree also with results obtained in this study, in previous studies in rats following a diet with sitosterol (0.8%, w/w) supplementation, has been seen a

reduction in cholesterol biotransformation into coprostanol compared to a diet containing cholesterol (1.2%, w/w) (Cohen et al., 1974). In addition, it is known that a reduction of dietary fat modifies the composition of fecal neutral sterol metabolites and the rate of cholesterol metabolism. In healthy volunteers who consume diets with less than 30g of fat/day, a rapid decrease of approximately 70% in fecal bile acid levels within a week has been reported, together with a less pronounced decrease in fecal neutral sterols - though a 40% decrease is reached at the end of the study (Hill, 1971). The metabolic rate of fecal cholesterol (coprostanol+coprostanone/cholesterol ratio) has been observed to be lower in individuals consuming vegetarian diets than in Western diets (1.25–1.46 *vs*. 2.17–2.49) (Hill & Aries, 1971).

As mentioned above, these trials were also carried out to evaluate the impact of PS high doses on the colonic bacterial species. In this sense, we obtained similar results for all the alpha diversity descriptors analyzed in culture medium with feces inoculum (control 1) and PS samples. Comparisons at *phylum* and family level in order to distinguish microbial species potentially affected by the presence of PS showed no differential abundances in phyla distribution when control 1 and sample were compared, however at family level we found that PS sample decreases the abundance of the *Erysipelotrichaceae* family of *Firmicutes* (LDA=3.19; $p < 0.0250$), even when time (24 h and 48 h fermentation) was included as covariate.

The operational taxonomic unit (OTU) abundance was similarly assessed in control 1 and PS sample. Combination of treatment and time variables did not produce any significant change in any OTU, however, when we only compared samples per treatment (control 1 *vs*. PS sample, thus combining 24 and 48h samples), we found differential abundances in 14 OTUs, of which OTU27, OTU103, OTU107, OTU128, OTU214, OTU394 and OTU536 where associated

with control samples, whereas OTU38, OTU51, OTU52, OTU92, OTU104, OTU157 and OTU208 where associated with PS samples ($p \leq 0.05$, LDA score ≥ 2.5).

Similarly, the abundances of some members of the uncharacterized S-24 family of *Bacteroidetes* were also promoted by PS presence in the fermentation assay (OTU51 and OTU52). In contrast, proportions of the flavonoid-converting bacterium *Flavonifractor plautii* (OTU103), as well as proportions of *Allobaculum* spp. (OTU27), a member of the *Erysipelotrichaceae* family, were significantly attenuated.

The results indicate that microbiota profiles are more strongly associated to time than PS condition. Besides, we found that only time explains significant changes in the microbial profiles observed for all samples (pseudo-F test=20.7; $p \leq 0.0030$).

Although major changes in microbial communities were associated to fermentation time, probably because the accumulation of anaerobic fermentation products strongly lowered the pH, we were able to distinguish a particular microbial group modulated by the presence of PS (sample) during the fermentation assay. In effect, our results indicated that PS influenced members of the *Erysipelotrichaceae* family of *Firmicutes*, with a reduction in the proportion of these bacteria. There is evidence on the role of *Erysipelotrichaceae* in human disease related to metabolic disorders (Kaakoush, 2015). In fact, previous studies have confirmed the association between this bacterial family and lipidemic profiles. For example, a series of species belonging to the family *Erysipelotrichaceae* in diet-induced obese animals has been found (Turnbaugh, et al., 2008). In this regard, high levels of *Erysipelotrichaceae* have been observed in obese human individuals (Zhang et al., 2009). Otherwise, hypercholesterolemic hamsters following treatment with grain sorghum lipid

extract containing PS have shown a decrease in *Erysipelotrichaceae* (Martinez et al., 2009). In another study carried out in hamsters, Martinez et al. (2013) found PS esters intake to induce shifts in the fecal microbiota, reducing several bacterial taxa of the *Erysipelotrichaceae* family. Besides, the abundance of these taxa displayed remarkably high correlations with host cholesterol metabolites. In addition, a recent analysis has indicated that *Erysipelotrichaceae* species also could be associated with the digestion of dietetic protein and fat components in dogs (Bermingham et al., 2017).

A detailed analysis was conducted in order to disclose single microbial species or phylotypes potentially also affected by presence of Ps during the *in vitro* fermentation assay. More specific evidence was obtaining when microbial abundances were assessed at OTUs level, showing that species belonging the genus *Allobaculum* (of the *Erysipelotrichaceae* family) were one of the main phylotypes whose abundances were negatively affected by PS. The proportions of *Flavonifractor plautii* species were modulated by PS in a way similar to the *Allobaculum* species. Besides, induced an important increase in the abundance of *Eubacterium hallii*, a recognized butyrate producer belonging to the *Lachnospiraceae* family, butyrate being considered a healthy microbial metabolite (Hamer et al., 2008).

Other authors have attempted to evaluate the influence of Ps upon the gut microbiota (Van Faassen et al., 1987), who only found slight changes in the anaerobic bacterial genera (*Bifidobacterium*, *Bacteroides* and *Clostridium*) in subjects with a vegan diet, which is rich in PS. In a clinical trial (Baumgartner et al., 2017), where 13 healthy subjects received three weeks of control or plant stanol ester enriched margarine (3 g plant stanols /day), no differences in gut microbiota composition were recorded between the two target groups.

III. *Impact of high dose plant sterols on gut microbiota from lean and obese subjects using a dynamic (TIM-2) in vitro fermentation model.*

Overweight and obesity constitute the main risk factors for some chronic disorders, including cardiovascular disease and cancer. It is well known that PS could have a hypocholesterolemic effect and colon cancer. However, there are scare data on PS-gut microbiota interaction. We evaluated the influence of PS (~2g/day) from food ingredient and a standard upon gut microbiota and vice-versa, using a validated *in vitro* model of the large intestine (TIM-2), with feces from lean or obese subjects to evaluate the transformation of PS by gut microbiota and the impact of PS on colonic bacterial population (see annex III).

The PS source used for the microbial fermentation in the *in vitro* system were a commercial food ingredient with free microcrystalline PS from tall oil in powder form and a commercial standard of β-sitosterol (≥ 70%). Besides, as control assay a standard ileal efflux medium (SIEM) composed mainly to carbohydrates was also used. The microbiota inoculum was obtained from feces of healthy volunteers who were selected according to their body mass index (BMI), in order to carried out fermentation experiments with lean and obese microbiota.

The concentrations of fecal animal sterols (AS) in the *in vitro* fermentation at time zero represented 64 and 73% of total sterols in feces from lean and obese subjects, respectively. The mainly sterol were cholesterol (28% for lean and 13% for obese), and its metabolites coprostanol (26% and 35%), coprostanone (7% and 21%) and cholestanol (2% for both).

PS concentrations in fermentation liquid at time zero represented 36 and 27% of total sterols from lean and obese feces, respectively. The abundance order was

sitosterol > campesterol > sitostanol and campestanol. The following metabolites were identified: ethylcoprostanol, ethylcoprostanone and methylcoprostanone. The most relevant statistically significant differences ($p < 0.05$) among the experiments (ingredient, standard or control) using microbiota of lean subjects, are observed for cholesterol at 24h of fermentation where its concentration follows the order; ingredient > standard > control. In the cases in which there are statistically significant differences ($p < 0.05$) between the experiments (ingredient, standard or control) using obese microbiota, in general, the contents of coprostanol (24, 48 and 72h) and cholestanol (24 and 72h) follow the order; ingredient> standard> control.

The cholesterol concentration decreased in control (77 and 45%), ingredient (20 and 69%) and standard (32 and 26%) experiments after 72h of fermentation when using lean and obese microbiota, respectively. It should be noted that all metabolites (coprostanol, coprostanone and cholestanol) were higher in obese than in lean microbiota assays. In addition, the coprostanol concentration after 72h of fermentation decreased in control (13% for lean and 52% for obese), ingredient (41 and 55%) and standard (61 and 60%). This fact suggests that the colonic microbiota from lean and obese subjects would prefer PS as a substrate, because they were present in greater proportion than cholesterol. These results agree with another *in vitro* static colon fermentation study of our research group, in which lower microbial metabolism for cholesterol was found (Cuevas-Tena et al., 2018a). In addition, the results obtained also are in concordance with other *in vivo* studies carried out by Weststrate et al. (1999), where after the intake of margarine enriched with PS (8.6g/day) by healthy subjects, no increase in the concentrations of neutral sterols was found and the amount of cholesterol metabolized into coprostanol was reduced. Cuevas-Tena et al. (2018b) evaluated the impact of intake of a PS-enriched milk based fruit beverage (2g PS/day) by

postmenopausal women on fecal sterols excretion, and found a lower tendency in the production of coprostanol in the presence of high doses of PS. In a previous study in rats, following a diet with sitosterol (0.8%, w/w) also a decrease in the coprostanol production compared to a diet containing cholesterol (1.2%, w/w) was observed (Cohen, Raicht & Mosbach, 1974).

However, coprostanone concentration at final period of fermentation (72h) increased in experiments with ingredient (136%) and standard (109%) when lean microbiota was used. In the assays with obese microbiota, the coprostanone concentration decreased from t0 to 24h (control: 64%; ingredient: 76%; standard: 83%) and then the concentration remained more or less equal until 72h for all experiments. Cuevas-Tena et al. (2018b), in agree with our results, found a significant increase in coprostanone (0.52mg/g freeze-dry feces) after intake a high doses of PS compared with the basal period and they conclude that this fact is probably due to saturation of the gut microbiota. In the case of cholestanol, at the final period of fermentation using lean microbiota, their concentration increased in experiments with ingredient (27%) and standard (29%), while in the assays with obese microbiota, its concentration decreased in presence of ingredient (30%) and standard (40%).

The sitosterol concentration in assays using microbiota of lean and obese subjects was the same (without significant differences, $p < 0.05$) between control, ingredient and standard at time zero. However, given that sitosterol is the major sterol in the ingredient and standard, a significant increase in its respective experiments compared to control was observed for each fermentation time (24-72h). The same was observed for sitostanol, campesterol and campestanol with ingredient and standard.

Ethylcoprostanol, the main metabolite of sitosterol, showed a higher concentration in experiments with obese microbiota (2 fold more) compared to

lean (time zero). Its concentration remained similar throughout fermentation in control experiment using lean microbiota and decreased significantly in the ingredient and standard experiments when using lean (ingredient: 32% and standard: 35%) and obese microbiota (control: 30%, ingredient: 88% and standard: 87%), contrary to the increment obtained by other authors (Weststrate et al., 1999; Cuevas-Tena et al., 2018a; Cuevas-Tena et al., 2018b).

Conversely, ethylcoprostanone showed similar concentrations in control, ingredient and standard using lean and obese microbiota at time zero. However, a significant increase of this metabolite was observed in ingredient (46 and 35 fold more) and standard (21 and 33 fold more) with respect to control when microbiota of lean and obese subjects was used, respectively. This suggests that the capacity of the gut microbiota was not sufficient to transform sitosterol into ethylcoprostanol due to the large amounts of PS present (Cohen et al., 1974), causing a lower production of ethylcoprostanol.

In the case of methylcoprostanone (campesterol metabolite), an increase of its concentration was observed from 24 to 72h in ingredient (8 fold more) and standard (25 fold more) using lean microbiota. When obese microbiota was used, the methylcoprostanone concentration remained similar from time zero to 48h in all experiments, but after 72h a decrease was observed in control (43%) and ingredient (33%) and it was increased for standard (11%). There was only a significant increase between control and standard after 24h of fermentation. Throughout the experiments with lean microbiota, campesterol metabolites were not detected in the control.

On the other hand, SCFA are produced in the proximal colon through the fermentation of mainly indigestible carbohydrates. As far as we know, this is the first time that microbial SCFA production is reported after *in vitro* fermentations assays with high doses PS. The most abundant acids were acetate, butyrate,

propionate and succinate. Total cumulative SCFA after fermentation (72h) with control, ingredient and standard ranged between 54 and 66 mmol using lean microbiota and from 30 to 46 mmol using obese microbiota. There were significant increases ($p < 0.05$) of total SCFA production in the experiments using lean microbiota with ingredient (net increment: 7.05 and 11.90 mmol) and standard (4.00 and 8.50 mmol) compared to control after 48h and 72h, respectively.

The highest acetate production was found in experiments with ingredient and standard using lean microbiota. Specifically, a significant increase ($p < 0.05$) of this SCFA after 24 and 48h of fermentation occurred in the assays with ingredient and standard compared to control, being highest in the presence of ingredient. It has been suggest that acetate stimulates cholesterol and long chain fatty acids synthesis in the liver (Delzenne, et al., 2011) and a study using mice showed that acetate from colonic fermentation can cross the blood-brain barrier and supress appetite in the hypothalamus (Frost et al., 2014).

In the case of butyrate, a slight increase in its production was observed for ingredient and standard compared to control throughout the fermentation experiment using lean microbiota. Butyrate is another main SCFA with higher production in assays with microbiota from lean subjects than obese. However, a significant increase ($p < 0.05$) was only found after 24h of fermentation with standard. Butyrate functions as the major energy source for colonic epithelial cells (Koenen et al., 2016). In addition, this SCFA is considered to be especially important, as it may also play a major role in the prevention of colon cancer (Mortensen & Clausen, 1996; Perrin et al., 2001; Leonel & Alvarez-Leite, 2012) and other colonic diseases (Hamer et al., 2008). Besides, butyrate protects against diet-induced obesity and suppresses food intake (Lin et al., 2012).

For propionate and succinate, greater production was observed when ingredient and standard were used, but no significant differences were found between these two and control. Propionate was also shown to be produced at greater amounts in experiments with microbiota from lean subjects. In the case of this SCFA, it has been suggested that it reduces liponeogenesis, cholesterol synthesis and recently has been demonstrated to be involved in the activation of G-protein.coupled receptors (GPR-41 and GPR-43) releasing satiety hormones, thus reducing food intake (Kimura et al., 2011).

In general, the experiments with microbiota of obese subjects showed a lower production of SCFA compared with lean microbiota experiments. Respect to control, a greater production of acetate and butyrate was observed, but there were no significant differences between control, ingredient and standard. The succinate and propionate production was similar for all three experiments, and no significant differences were found. These acids serve as intermediates in the metabolism of SCFA and generally they do not accumulate in the colon (Gibson et al., 2004).

Lactate production was low for both lean and obese microbiota, although its production in ingredient and standard was slightly higher in both microbiotas. On the other hand, lactate production only showed a significant increase ($p < 0.05$) in ingredient (net increment: 0.50 mmol) after 24h of fermentation using lean microbiota. In this context, lactate only accumulates when there is a fast fermentation of a substrate. If substrates are fermented slowly, lactate is mostly converted into butyrate (Morrison et al., 2006).

Regarding to intestinal microbiota characterization, sequencing of the V3-V4 region of 16S rDNA gene was performed and reads were analysed using QIIME. Principal Coordinate Analysis (PCoA) and Linear discriminant analysis effect size (LEfSe) were performed to determine operational taxonomic units (OTUs)

that were modulated by the interventions. Neither weighted nor unweighted PCoA showed specific clustering by substrate (not shown). Yet LEfSe showed some OTUs that were specific for the treatments. For the lean microbiota, LEfSe only showed OTUs that were more prevalent upon addition of ingredient, which belonged to the genus *Catenibacterium*. When ingredient and standard were combined (indicated as Soy) and compared to control, a few more discriminative OTUs were observed. Apart from *Catenibacterium*, *Coprococcus* was increased upon addition of PS, while *Collinsella* and *Slackia* of the *Coriobacteriaceae* family were increased in control. For the obese microbiota, the genus *Clostridium* was increased for ingredient, while a few OTUs (including *Christensenellaceae* and other OTUs in the order *Clostridiales*) were higher in control. Also here, when ingredient and standard were combined (indicated as Soy) and compared to control, more discriminative OTUs were observed. Regarding to the increase of the *Erysipelotrichaceae* family members, these results were not coincident with those found in a previous study by our group (Cuevas-Tena et al., 2018a). In a clinical trial (Baumgartner et al., 2017), where 13 healthy subjects received during three weeks a control or plant stanol ester enriched margarine (3g/day plant stanols), no differences in gut microbiota composition were recorded between the two target groups. However, van Faassen et al. (1987) found slight changes for *Bifidobacterium*, *Bacteroides* and *Clostridium* genera in subjects with a vegan diet, which was rich in PS.

Using Spearman correlation with false-discovery rate correction, the presence of OTUs was correlated to microbial metabolites produced (correlations between rho > 0.666 or -0.666 < rho). For the lean microbiota positive correlations between the genus *Dorea* and propionate acid, and between an uncharacterized OTU in the order *Clostridiales* and methylcoprostanone was observed. In addition, negative correlations were found between propionic acid and the genus

Atopobium and *Peptostreptococcus*. In the case of experiments with obese microbiota, there were positive correlations between the genus *Klebsiella* and butyric acid and methylcoprostanone. The latter metabolite (methylcoprostanone) has also been shown to be positively correlated with the genus Methanobrevibacter. Other positive correlations recorded were between propionic acid and the *Rikenellaceae* family, and between butyrate acid and the *Ruminococcaceae* family. Finally, a negative correlation was observed between the genus *Bacteroides* and methylcoprostanone.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict the microbial pathways contributing to the metagenomes of the samples identified using 16S rRNA sequencing, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and the data was visualized using STAMP software. Using Welsh's two-sided t-test, pathways predicted for each test product were compared to the other experiments. For the lean microbiota three pathways were significantly different. For the obese microbiota a total of 47 pathways were predicted to be differentially present, including those for flavone and flavonol biosynthesis, flavonoid biosynthesis.

IV. *Impact of colonic fermentation on sterols after the intake of plant sterols-enriched beverage: A randomized, double-blind crossover trial*

PS-enriched foods have a health statement referred to the reduction of hypercholesterolemia, and can be of use in mildly hypercholesterolemic patients. In addition, an association has been reported between postmenopause women and higher serum levels of total cholesterol and low-density lipoprotein cholesterol (LDL-C), which could increase the risk of cardiovascular disease (Choi et al., 2015; Derby et al., 2009). Considering also that an increase in PS intake could play a preventive role in relation to colon cancer risk, the impact of the daily consumption of PS-enriched milk based fruit beverage (2g PS/day) on fecal excretion of sterols and their microbial conversion in postmenopausal women with mild hypercholesterolemia has been evaluated in the context of an *in vivo* colonic fermentation clinical trial (see annex I).

This clinical study was carried out to evaluate the impact of high PS intake upon excreted fecal sterols and their microbial conversion in postmenopausal women with mild hypercholesterolemia.

This clinical trial was a single and combined randomized, double-blind, crossover trial carried out with two beverages: a PS-enriched skimmed milk based fruit beverage containing β-cryptoxanthin (β-Cx) and 2g of PS/250 mL (active beverage), and a skimmed milk based fruit beverage (placebo beverage), in postmenopausal women with mild hypercholesterolemia (ClinicalTrials.gov number NCT 02065024).

During the trial period, 40 women were selected and randomly distributed into two groups: 20 women (group A) consumed the active beverage (1 brick x 250 mL/day) and the other 20 women (group B) consumed the placebo beverage (1 brick x 250 mL/day) for 6 weeks. After a four-week washout period, the type of

beverage was exchanged and consumed for another 6 weeks. Finally, 36 women completed the study.

Fecal sitosterol together with its metabolites and fecal AS with PS were identified as the most adequate predictors in the binary logistic regression analysis for establishing subject adherence in the trial. In addition, individual fecal sterol PS (sitosterol, campesterol and stigmasterol present in the active beverage) were directly and strongly correlated to total fecal PS after active beverage intake.

Generally, the postmenopausal women showed a higher net increment in PS excretion after active beverage intake compared to placebo. Furthemore, the net increment of excretion of neutral sterols (those present in the active beverage) showed fewer outliers after active beverage intake. This suggests that both groups, A and B, responded homogeneously to active beverage intake, independently of the timing of ingestion.

Total AS contents, expressed as medians, were found to be 19.82 and 18.81 mg/g freeze-dried feces at basal and 21.58 and 18.64 mg/g freeze-dried feces after placebo and active beverage intake (final), respectively. These contents were similar to those reported by other authors after diets not enriched with PS (19.4-28.5 mg/g freeze-dried feces) (Cuevas-Tena et al., 2017; Keller & Jahreis, 2004; Batta et al., 1999; Weststrate et al., 1999; Korpela & Adlercreutz, 1985; Korpela, 1982; Wilkins & Hackman, 1974).

After placebo intake, only cholesterol content showed a significant increase (34%). However, after active beverage intake, significant increases in cholesterol (65%), coprostanone (80%), cholestanol + methylcoprostanol (42%), and lathosterol (9%) were observed. In other study with humans following a diet with PS-enriched margarine (8.6 g PS/day) (Weststrate et al., 1999), an increase in fecal cholesterol content has also been evidenced (net cholesterol excretion 20.7

mg/g freeze-dried feces). However, this marked fact was not observed in our study, probably due to a lesser PS intake (2.0 g/day). The same authors have reported a decrease in the excretion not only of coprostanol but also of coprostanone (9 and 1.5 mg/g freeze-dried feces, respectively) after PS-enriched food intake (Weststrate et al., 1999). Besides, epidemiological studies with subjects following vegetarian and low-animal fat diets have reported a decrease in coprostanol (from 12.2 to 2.3 mg/g freeze-dried feces) and coprostanone (between 2.3 and 0.3 mg/g freeze-dried feces), compared with high-animal fat (Western) diets (Reddy et al., 1998; Korpela et al., 1988; Van Faassen et al., 1987; Korpela & Adlercreutz, 1985; Reddy et al., 1975; Hill & Aries, 1971; Hill et al., 1971). In our study, this fact could be due to the diet effect in the women, since the real sterol intake had not been controlled – this constituting a limitation of the study. Besides, the significant increase in coprostanone after active beverage intake could also suggest that the gut microbiota metabolized cholesterol through an indirect pathway, which was interrupted in this step, causing a lower production of coprostanol. This also suggests that the capacity of the gut microbiota was not sufficient to transform AS in the usual manner, due to the large amounts of PS present (Cohen et al., 1974). In fact, it has been suggested that the efficiency of cholesterol conversion is related to microbial density. In this regard, a coprostanol/cholesterol ratio of ≥ 15 has been associated to high levels of coprostanoligenic bacteria (108 cells/g) and to nearly complete cholesterol conversion (Veiga et al., 2005). In this context, we found lower coprostanol/cholesterol ratios in postmenopausal women after active beverage intake compared with placebo (3.2 *vs.* 6.2). In coincidence with our findings, other authors have recorded lower ratios after the intake of PS-enriched margarine compared to the controls (0.35 *vs.* 2.6) (Weststrate et al., 1999) in vegetarian subjects compared to omnivorous individuals (1.62 *vs.* 4.38) (Korpela

& Adlercreutz, 1985), and in low converters *versus* high converters (0.13 *vs.* 7.6) (Wilkins & Hackman, 1974). In concordance with other authors (Midtvedt et al., 1990; Korpela & Adlercreutz, 1985;Wilkins & Hackman, 1974), we found a low coprostanol/cholesterol ratio (<0.9) after active beverage intake *versus* placebo (8%) in 20% of the postmenopausal women. Besides, it has also been suggested that low cholesterol conversion could be due to a lack of mucosal receptors for coprostanoligenic bacteria (Midtvedt et al., 1990) or to the inhibition of these bacteria by other components of the gut microbiota (Sadzikowski et al., 1977).

Regarding fecal PS contents, a statistically significant increase in total and individual PS (except ethylcoprostenol and brassicasterol) was recorded after active beverage intake. Furthermore, statistically significant differences ($p < 0.05$) in net increment between placebo and active beverage intake were observed for all PS except ethylcoprostenol and brassicasterol. The fecal contents of PS and their metabolites (6.50-11.64 mg/g freeze-dried feces) were similar in two basal periods and in final period for placebo, in concordance with other authors (5.50-10.20 mg/g freeze-dried feces) (Olejnikova et al., 2017; Cuevas-Tena et al., 2017; Batta et al., 1999;Wilkins & Hackman, 1974). However, active beverage intake produced greater (nearly 5-fold) PS excretion with respect to placebo, in concordance with one of the aforementioned studies after PS-enriched margarine consumption (nearly 17-fold) (Weststrate et al., 1999). Besides, it has been found a significant increase (nearly 3-fold) in ethylcoprostanol excretion after active beverage intake. These results suggest that the gut microbiota preferentially uses PS as substrate, because they were present in greater proportion with respect to cholesterol. In addition, the relative percentages of fecal AS and PS with respect to total fecal sterols in the two basal periods and in the final period for placebo were consistent with the results of other authors (~70 and ~30%, respectively) (Olejnikova et al., 2017; Cuevas-Tena et al., 2017; Batta

et al., 2002; Batta et al., 1999; Weststrate et al., 1999; Wilkins & Hackman, 1974). However, these relative percentages were reversed (~30 and ~70%, respectively) after active beverage intake.

As far as we know, this is the first time that microbial conversion percentages corresponding to cholesterol, sitosterol and stigmasterol are reported after a PS-enriched food intervention in postmenopausal women with mild hypercholesterolemia, describing the effect of high PS intake upon their conversion percentage rates.

The number of women found to be high converters after placebo and active beverage intake were: cholesterol 33 and 29; sitosterol 29 and 17; and finally stigmasterol 18 and 27, respectively. However, in the case of campesterol, all women were low converters after placebo and active beverage intake.

We consider it interesting to note the effect of the active beverage upon the decrease in conversion percentage. Based on the conversion percentage frequency distribution used by Wilkins and Hackman (1974), the $\geq$ 10% reduction in the conversion percentage of the sterols stands out.

In this context, active beverage intake produced a significant decrease (between 11% and 50%) in cholesterol conversion in 16 women, though 12 of them remained as high converters and 4 became low converters. Regarding, sitosterol, 24 women showed a significant decrease (between 15% and 61%) after active beverage intake, and 7 of them remained as high converters and 4 low converters, while 13 women changed from high to low converters. However, in the case of stigmasterol, the intake of active beverage only produced a significant decrease (between 14% and 67%) in 6 women, of which only 3 remained high converters. In general, many women (n=31) did not change their conversion percentage referred to campesterol after active beverage intake.

Most subjects in our study were high cholesterol converters. They showed an average conversion percentage of 82.3 ± 11.7 after placebo versus 75.4 ± 13.3 after active beverage intake, in the same way as in subjects following a Western diet (75-89%) (Benno et al., 2005; Korpela & Adlercreutz, 1985; Korpela, 1982; Wilkins & Hackman, 1974) and in vegetarians (66%) (Korpela & Adlercreutz, 1985). Low converters showed percentages of 23.5 ± 20.9 and 4.5 ± 5.2 after active and placebo intake, respectively, according to the values reported in other studies (1.0-43.0) among subjects following a Western diet (Korpela, 1982; Wilkins & Hackman, 1974).

However, there are no consensus-based data regarding the thresholds that classify high and low converters. While Wilkins and Hackman (1974) reported that high converters present sterol conversion percentage of ≥50%, Midtvedt et al. (1990) reported a value of ≥40%. Most authors consider the threshold to be ≥50% (Keller & Jahreis, 2004; Korpela, 1982; Perogambros et al., 1982; Wilkins & Hackman, 1974).

On the other hand, in 16 subjects, active beverage intake produced a significant decrease in fecal cholesterol conversion percentage compared to placebo, a condition that has been reported in humans after PS-enriched margarine intake (Weststrate et al., 1999). However, this decrease has not been observed in vegetarian *versus* omnivorous diets (66.3 ± 7.9 *vs.* 75.3 ± 6.3) (Korpela & Adlercreutz, 1985). In this sense, it has been suggested that an increase in PS intake could reduce or block fecal cholesterol conversion, thereby resulting in a lesser production of cholesterol metabolites, which are associated to pro-carcinogenic action and could increase the risk of colon cancer (Bradford & Awad, 2007; Weststrate et al., 1999; Reddy & Wynder, 1977). Furthermore, a previous study by our group found PS at human colonic concentrations to exhibit

antiproliferative effects against colon cancer cells (Caco-2 cells) (López-García et al., 2017).

Most subjects were also high sitosterol converters after placebo, in agreement with the only study that reports data in this regard (23 high and 8 low converters) (Wilkins & Hackman, 1974), though fewer than half of them were high converters after active beverage intake. The high converters showed an average in fecal sitosterol conversion percentage of 52.7 ± 9.6 after active beverage *versus* 75.9 ± 11.3 after placebo intake, while the values for the low converters were 26.9 ± 15.1 and 34.5 ± 8.5, respectively. After active beverage intake, the fecal sitosterol conversion percentage decreased in 24 subjects. In this regard, the sitosterol/sitosterol metabolites ratio (in 22 subjects) after active beverage intake was higher than in the placebo group (expressed as median, 0.5 versus 0.3). Indeed, the positive correlation between fecal PS metabolites and total PS was greater after placebo than after active beverage intake. These observations would suggest that the gut microbiota was unable to metabolize the PS from active beverage, due to the abundant presence of these sterols.

A limitation of our study was the lack of a microbiota analysis, which did not form part of the objectives of the clinical study. Such an analysis would have provided valuable information about the impact of high PS intake upon the intestinal microbial community, allowing it to be correlated to the fecal sterol conversion percentage found in our study. Nevertheless, the present study is interesting, since only one publication has evaluated the effect of a PS-enriched food upon the fecal sterol profile – this fact representing a limitation for the discussion of results.

V. *Impact of the lyophilization process on microbial DNA from human fecal samples*

Fecal samples are essential in some human metabolomics studies to investigate the relationship between gut microbiota and food constituents. Generally, in this assays the number of fecal samples is usually high and feces are highly complex from the point of view of stability and safety. In order to determine the microbial profile after the *in vivo* fermentation trial, a preliminar study evaluating the impact of the lyophilization process on the bacterial DNA from human feces was carried out for future research.

Samples

Feces from three healthy subjects, two following a Western diet (1 and 2) and one following vegetarian diet (3) were used in order to evaluate the impact of lyophilization in bacterial DNA extraction. Fecal samples were collected in sterile plastic containers, a portion of at least 3 g was taken to be lyophilized (Sentry 2.0, Virtis SP Scientific). The fresh sample were stored at -20ºC and the lyophilized sample was homogenized (by crushing) in a glass mortar and stored in glass Petri dishes at -20ºC until analysis.

Fecal DNA isolation, PCR sequencing and data analysis

DNA extraction from 150 mg of fresh and 95 mg of lyophilized feces was performed using the Stool Total RNA Purification Kit (Norgen Biotek Corp. Thorold, Ontario, Canada) with some modifications over the manufacturer's instructions. Briefly, it was included an enzymatic pre-treatment step with lysozyme (0.05 mg enzyme/100 mg feces) from Sigma-Aldrich and mutanolysin (40U enzyme/100 mg feces) from Sigma-Aldrich, for 60 minutes at 37ºC. Also,

it was included an incubation step with 2 µL of RNAse A per sample (Epicentre) for 30 minutes at 37ºC before the column washing step and a purification step using phenol: chloroform: isoamyl alcohol (25:24:1). For DNA recovery, it was used Magnetic Beads AMPure XP (Life Technologies, Carlsbad, California, USA) and the following elution step was performed with Tris-HCl buffer 10 mM (pH 8.5).

The V4-V5 hypervariable regions from bacterial 16S rRNA gene were amplified using 20 ng of DNA and 25 PCR cycles at 95ºC for 20s, 40ºC for 30s, and 72ºC for 20s. Phusion High-Fidelity Taq Polymerase (Thermo Scientific) and the 6-mer barcoded primers S-D-Bact-0563-a-S-15 (AYTGGGYDTAAAGNG) and S-D-Bact-0907-a-A-20 (CCGTCAATTYMTTTRAGTTT), which target a wide range of bacterial 16S rRNA genes (Klindworth et al. 2012), were used during PCR. Dual barcoded PCR products, consisted of ~380bp, were purified from triplicate reactions by using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and quantified through Qubit 3.0 and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). Samples were multiplexed by combining equimolar quantities of amplicon DNA (100 ng/sample) and sent to Eurofins Genomics GmbH (Ebersberg, Germany) to perform Illumina MiSeq high-throughput sequencing with 2x300 PE configuration. DNA raw data was obtained in fastq files. Pair ends with quality filtering were assembled using Flash software (Magoc and Salzberg 2011). Sample de-multiplexing was carried out using sequence information from respective DNA barcodes and Mothur v1.36.1 suite of analysis (Schloss et al. 2009). After assembly and barcodes/primers removal, the sequences were processed for chimera removal using Uchime algorithm (Edgar et al. 2011) and SILVA reference set of 16S sequences (Quast et al. 2013). Diversity indexes were calculated with Mothur using default parameters and average method in the clustering step. Alpha diversity parameters

such as the Chao's richness and Shannon evenness and reciprocal Simpson index were computed using a high quality and a normalized subset of 15,000 sequences per sample, randomly selected after shuffling (10,000X) of respective and original datasets. Taxonomy assessment was performed using the RDP classifier v2.12 (Wang et al. 2007) to analyze differential abundance in phylum and family proportions. The Operational Taxonomic Unit (OTUs)-picking approach was performed with the normalized subset of 15,000 sequences and the clustering algorithm implemented in USEARCH v8.0.1623 (Edgar 2010).

Results & discussion

Size sample description

After lyophilization, percentages of wet in samples were 77.5, 64.7 and 72.0, respectively. Lewis et al. (2016) not detected differences appreciable in the relative abundances of taxa from DNA extracted in high and low mass sample. Therefore, due to the difficulty to re-hydrate high weight samples, we have preferred to use a lower mass to lyophilized samples (95 mg) near to equivalent mass to fresh samples (150 mg).

Best taxonomic assignments from lyophilized samples were obtained, which were directly correlated with the lowest proportion of chimeric sequences found in these samples (see Table 4) similarly to fresh sample. Since the level of chimeric sequences depends exclusively on the PCR process, the DNA obtained from the lyophilized samples would seem to improve the amplification process by decreasing the likelihood of such devices. This fact could be due to a hypothetical greater stability and quality of isolated DNA from lyophilized samples. In this sense, in cow feces by PCR-RFLP analysis were found improvement in DNA recovery (1.5 - 2 fold) from lyophilized feces. Besides,

agarose gel electrophoresis of the community DNA extracted showed a high-quality of DNA (Ruiz & Rubio, 2009).

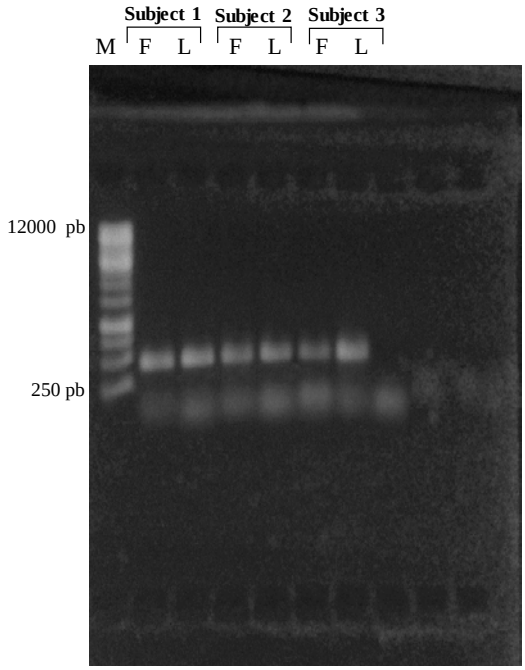
Table 4. Statistics of the sequences obtained in fresh (F) and lyophilized feces (L).

Statistics analysis	**Subjects**					
	1		**2**		**3**	
	F	**L**	**F**	**L**	**F**	**L**
Total sequences	80739	36273	48708	61404	23133	37897
Non-chimeric sequences	78034 (97%)	35622 (98%)	47854 (98%)	60730 (99%)	21616 (93%)	35452 (94%)
Analyzed sequences	17000	17000	17000	17000	17000	17000
Assigned phylum sequences	16778 (99%)	16895 (99%)	15079 (89%)	16044 (94%)	15519 (91%)	16136 (95%)
Assigned family sequences	16634 (98%)	16825 (99%)	14663 (86%)	15647 (92%)	13585 (80%)	14478 (85%)
Assigned genera sequences	14888 (88%)	15452 (91%)	12951 (76%)	13539 (80%)	5908 (35%)	6304 (37%)

Samples 1 and 2: subjects following a Western diet; sample 3: subject following a vegetarian diet

On the other hand, Figure 12 shows the agarose gel electrophoresis (1%), in which it was observed that all samples had similar base pairs (bp) after DNA extraction and PCR. This fact suggest that the method of extraction and DNA amplification applied on both samples fresh and lyophilized provided similar content of genetic material.

Figure 12. Agarose gel electrophoresis (1%) of the microbial DNA from human fecal microbiota. M: marker; F: fresh samples; L: lyophilized. Numbers on the left of the figure are size in base pairs



Samples 1 and 2: subjects following a Western diet; sample 3: subject following a vegetarian diet

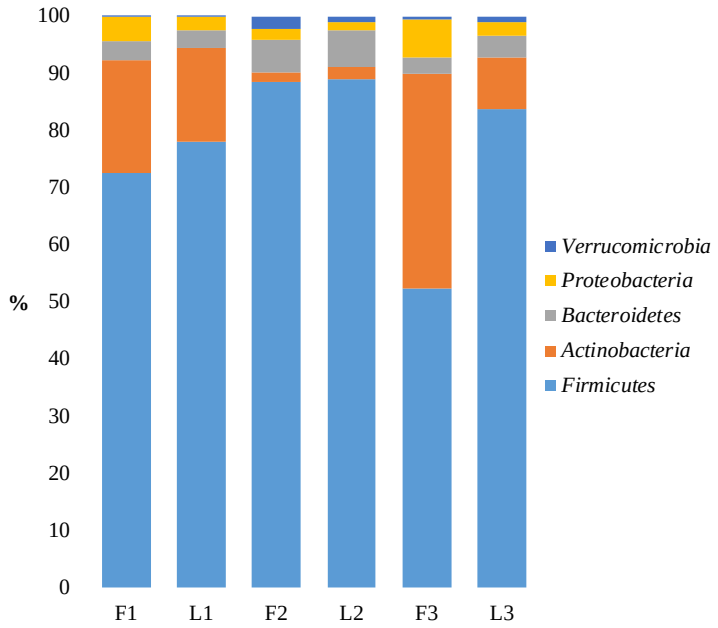
Effect of lyophilisation on microbiota

In order to identify the major bacterial groups potentially affected after lyophilization process the taxonomic assignment of the sequences was performed at three different levels.

First, an analysis of bacterial phylum, identifying sequences from 5 of the main bacterial group was carried out. As can be seen in Figure 13, in order from most to least proportion, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia* were identified. Globally, *Actinobacteria* seems to be under-represented in lyophilized feces when compared with paired

fresh samples. A great specificity in the proportions of the main bacterial groups for individuals following a western diet (1 and 2) was observed, which presented a pattern of fecal microbiota that was remained even after the lyophilization process. Note that no major changes are evident except in the lyophilized sample of vegetarian subject (3) in which a notable decrease in the proportion of sequences associated with *Actinobacteria* phylum was observed.

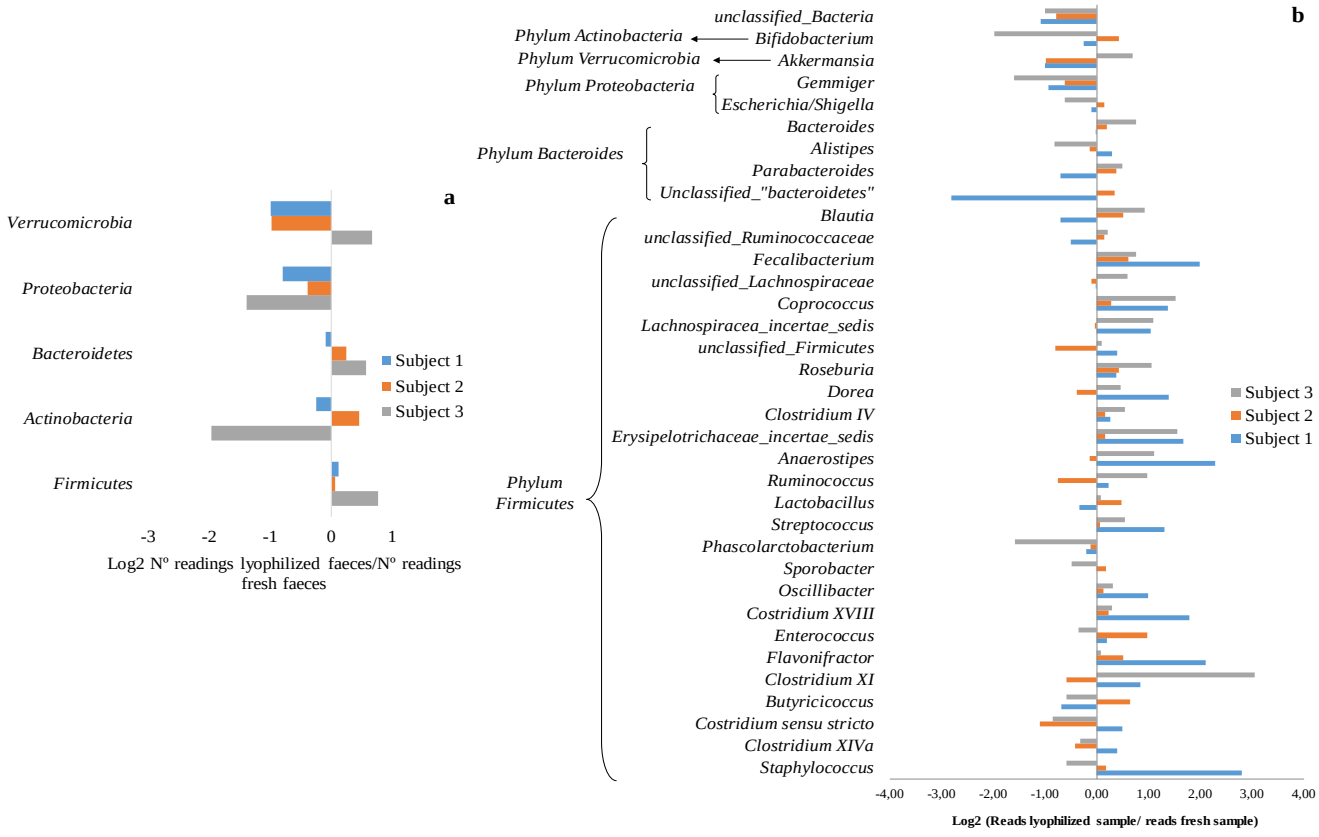
Figure 13. Taxonomic allocation: Percentage ratio of sequences assigned to each group (Phylum). F: fresh feces, L: Lyophilized feces.



Samples 1 and 2: subjects following a Western diet; sample 3: subject following a vegetarian diet

In order to measure variations in the pattern of the main taxonomic groups, independently of the sample donor subject, an analysis of ratios was carried out (Log 2: reads number of lyophilized sample/reads number of fresh sample) (see Figure 14a).

Figure 14. Variations in each group depending on the individual and lyophilization process (*phylum*) (a) and percentage proportion of sequences assigned to genus level (b).



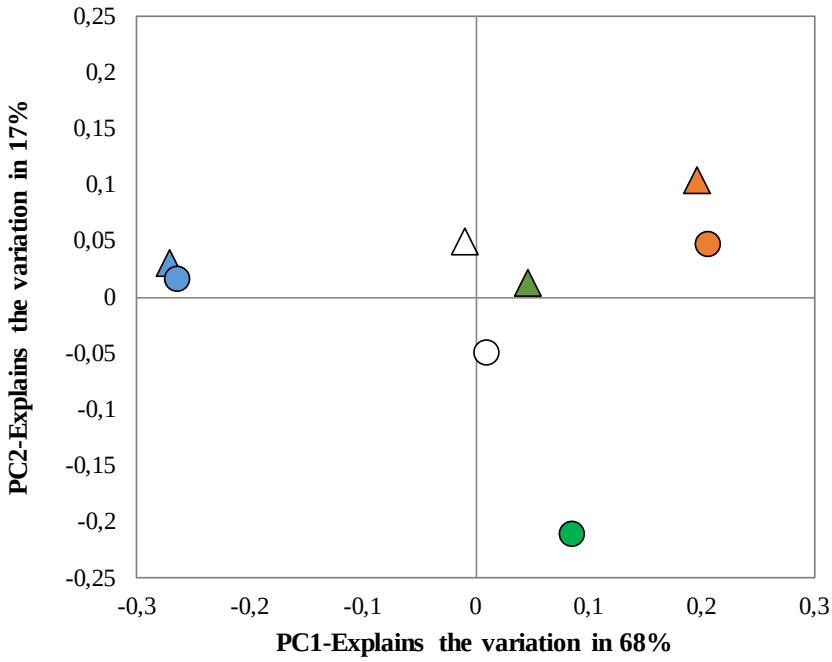
Samples 1 and 2: subjects following a Western diet; sample 3: subject following a vegetarian diet

The variation has been considered significant when there was a decrease or increase of more than two times (< -1 or > 1) in the concentration of sequences associated with the bacterial group. In this sense, no large variations were observed in the proportions of the main bacterial groups, except in *Actinobacteria* and *Proteobacteria* from vegetarian subject, a pattern that suggests a specific effect of lyophilization in this sample. The *Proteobacteria* group was the only one that showed a decrease in sequences for all analyzed subjects (-0.85 on average), and the vegetarian subject sample presented a higher coefficient of variation in proportions after the comparison of treatments (Subject 1: 0.22; Subject 2: 0.33; Subject 3: 1.70).

In order to determine with greater specificity if the lyophilization process affected the abundance of the bacterial groups positively or negatively, an analysis has been performed at the genus level, obtaining the results showed in Figure 14b. A great consistency between treatments (fresh *versus* lyophilized feces) was found, with drastic changes observed only in some categories such as *Bifidobacterium*, *Gemmiger*, *Phascolartobacterium* and "Unclassified *Bacteroidetes*", which abundances decreased more twice in the lyophilized samples. Conversely, it was observed that the *Faecalibacterium*, *Coprococcus*, *Erysipelotrichaceae_insertae_cedis*, *Anaerostipes*, *Clostridium* XVIII, *Flavonifractor*, *Clostridium* XI y *Staphylococcus* groups showed an increase (more than twice) in the proportion of their sequences. The loss of microbiota associated with the Actinobacteria phylum in vegetarian subject (3) seems to be affecting specifically the genus *Bidifobacterium*, while the loss of *Proteobacteria* affects concretely the members of *Gemmiger* genus (see Figure 14). In this sense, gram-negative Bacteroides was over-represented in lyophilized from infant fecal and gram-positive *Bifidobacteria* and *Bacilli* were under-represented. This fact it has been attribute to differences in cell wall structure (Lewis et al. 2016).

Finally, in order to quantify the variations between treatments (fresh *versus* lyophilized) and subjects, a beta-diversity analysis at OTUs level was carried out (Figure 15). After establishing the distances based on the analysis of coordinates (UNIFRAC) using the number of OTUs and their respective abundances, it has been possible to differentiate the samples in two different main components that explain more than 85% of the variation observed (Figure 15).

Figure 15. Principal coordinates analysis (PCoA) based on genetic distances UNIFRAC. The circles represent fresh feces, while triangles represent lyophilized feces. The different plot corresponds to: orange = subject 1, green= subject 2 and blue = subject 3. The white circle and triangle represent the centroids used to measure the Euclidean distances.

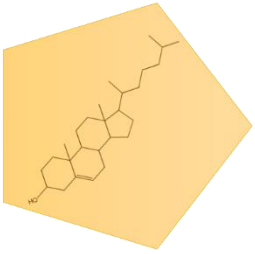


Samples 1 and 2: subjects following a Western diet; sample 3: subject following a vegetarian diet

The calculation of the average distances between the individuals indicated that the fresh and lyophilized samples were closer when they came from the same donor (distance = 0.10), while when classifying the samples by treatment their relation was lower ("Fresh" samples: 0.23, "Lyophilized" samples: 0.18).

Therefore, the results indicate that the lyophilization process had a minimal impact on the microbial species and their respective genetic material detected by massive sequencing. The variability observed and associated with the lyophilization process does not seem to affect all the samples in the same way. In addition, the variability associated with the lyophilization process is not greater than that observed among samples from different individuals. In fact, the variability between samples from different individuals tends to be minimized.

Conclusiones



Conclusiones

1- El método propuesto para la determinación de esteroles fecales por cromatografía de gases acoplada a espectrometría de masas presenta na buena sensibilidad, precisión y exactitud para la determinación de colesterol, esteroles vegetales y sus metabolitos en un amplio intervalo de concentraciones, llegando a determinarse un total de 17 esteroles fecales. Supone el uso de un menor volumen de disolvente y un tiempo de preparación de la muestra más corto respecto a otras metodologías indicadas en la bibliografía.

El estudio de la interacción entre esteroles y microbiota colónica mediante un modelo estático de fermentación *in vitro*, durante 24 y 48 horas, en presencia de cantidades altas de esteroles vegetales en bebidas a base de zumo de frutas y leche permite concluir que:

2- Los esteroles vegetales son preferentemente biotransformados por la microbiota intestinal en comparación al colesterol, especialmente sitosterol a etilcoprostanol y etilcoprostanona; campesterol a metilcoprostanona y estigmasterol a stigmastenol.

3- En presencia de esteroles vegetales a concentraciones altas, se observa un aumento de la abundancia de *Eubacterium halii,* especie beneficiosa para el colonocito por ser productora de butirato y una disminución de la familia *Erysipelotrichaceae* asociada con desordenes metabólicos (perfil lipídico y obesidad).

El estudio de la interacción entre esteroles y microbiota colónica mediante un modelo de fermentación dinámico (TIM-2) *in vitro* durante 72 horas, aplicado a un ingrediente alimentario fuente de esteroles vegetales y a un estándar a concentraciones altas de los mismos (2g/día), utilizando la microbiota de sujetos delgados u obesos, ha dado lugar a las siguientes conclusiones:

4- Las concentraciones de coprostanol, coprostanona, colestanol, etilcoprostanol y metilcoprostanona al inicio de la fermentación son mayores en los ensayos realizados con microbiota de individuos obesos, respecto a la de delgados, si bien las concentraciones de colesterol, sitosterol, sitostanol, campesterol y campestanol son similares.

5- En los ensayos con cantidades altas de esteroles vegetales y microbiota de individuos delgados, se observa, a lo largo de todo el periodo de fermentación (tiempo 0 hasta 72h), una disminución en las concentraciones de colesterol y coprostanol y un aumento de coprostanona y colestanol. Este hecho podría deberse a una saturación en el metabolismo de la microbiota. En los ensayos con microbiota de individuos obesos las concentraciones del colesterol y sus metabolitos.

6- Tras una fermentación de 72 horas con altas cantidades de esteroles vegetales, las concentraciones de etilcoprostanol disminuyen de forma más acusada en los ensayos con microbiota de individuos obesos y las de etil- y metilcoprostanona aumentan, independientemente de la microbiota utilizada. Este hecho, como en el caso de los esteroles animales, podría deberse a la saturación del metabolismo microbiano.

7- La producción total de ácidos grasos de cadena corta es mayor cuando se utiliza la microbiota de individuos delgados. En presencia de cantidades altas de esteroles vegetales, la producción de ácidos grasos de cadena corta es similar o, en el caso de acetato y butirato mayor frente a control independientemente de la procedencia de la microbiota fecal. Este hecho confirma que la suplementación de la dieta con altas cantidades de esteroles vegetales no modifica la actividad metabólica microbiana de los carbohidratos.

8- En los ensayos con microbiota de individuos delgados, en presencia de concentraciones altas de esteroles vegetales, a diferencia del estudio estático de fermentación, se observa un aumento de la proporción de los géneros *Catenibacterium* y *Coprococcus* pertenecientes a la familia *Erysipelotrichaceae*, mientras que con microbiota de obesos aumenta el género *Clostridium*.

Del consumo regular de una bebida a base de zumo de frutas y leche enriquecida esteroles vegetales (2g/día) durante seis semanas por mujeres voluntarias postmenopáusicas, se concluye que:

9- No se modifica la excreción fecal de colesterol. Su principal metabolito, coprostanol, tiende a disminuir y se observa un aumento de coprostanona, probablemente debido a la saturación de la microbiota intestinal. En relación a los esteroles vegetales, el etilcoprostanol y etilcoprostanona aumentan significativamente.

10- Se modifica el perfil de conversión microbiana del colesterol y sitosterol, disminuyendo los individuos altos convertidores, mientras que aumentan los altos convertidores para el estigmasterol.

Los ensayos *in vitro* e *in vivo* realizados sugieren una misma tendencia en la metabolización microbiana de los esteroles, en presencia de cantidades altas de esteroles vegetales, con disminución en la biotransformación del colesterol y preferencia de la microbiota intestinal por los esteroles vegetales como sustratos.

Futuros estudios *in vivo* que evalúen la influencia de concentraciones altas de esteroles vegetales sobre la microbiota intestinal, serían necesarios para clarificar los resultados obtenidos mediante los modelos de fermentación *in vitro*.

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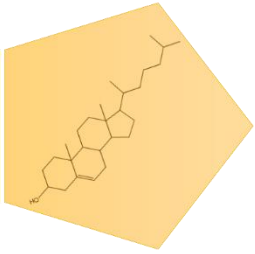

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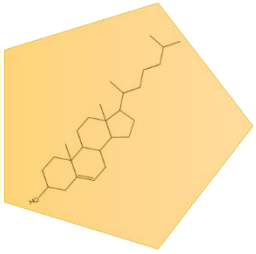
Anexo/ Annex I



Determination of fecal sterols following a diet with and without plant sterols

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ORIGINAL ARTICLE

Determination of Fecal Sterols Following a Diet with and without Plant Sterols

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Abstract The aim of this study was to develop a method for neutral fecal sterols determination in subjects receiving a normal diet with or without a plant sterols-enriched beverage using gas chromatography–mass spectrometry (GC/MS). Sample preparation conditions (homogenization of lyophilized feces with water) were evaluated. Sterol determination required direct hot saponification, unsaponifiable extraction with hexane, and the formation of trimethylsilyl (TMS) ether derivatives. The method allows the quantification of cholesterol, plant sterols and their metabolites (coprostanol, coprostanone, cholestanol, cholestanone, methylcoprostanol, methylcoprostanone, ethylcoprostenol, stigmastenol, ethylcoprostanol and ethylcoprostanone). Good linearity was obtained ($r > 0.96$) and interference was only observed for coprostanone, where the standard addition method proved necessary for quantification. The limits of detection (LOD) ranged from 0.10 to 3.88 μg/g dry feces and the limits of quantitation (LOQ) from 0.34 to 12.94 μg/g dry feces. Intra- and inter-assay precision (RSD %) were 0.9–9.2 and 2.1–11.3, respectively. Accuracy, expressed as percentage recovery (80–119%) was obtained for all determined sterols.

Keywords Plant sterols · Cholesterol · Feces · Gas chromatography · Validation method

Abbreviations

PS	Plant sterols
GC/MS	Gas chromatography–mass spectrometry
RSD	Relative standard deviation
LOD	Limit of detection
LOQ	Limit of quantitation
IS	Internal standard
BHT	Butylhydroxytoluene
BSTFA	*N*,*O*-Bis(trimethylsilyl) trifluoroacetamide
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
Rrt	Relative retention time
Rt	Retention time
NIST	National Institute of Standards and Technology
SD	Standard deviation
AOAC	Association of Official Analytical Chemists
Brassicasterol	5,22-Cholestadien-24β-methyl-3β-ol
Campestanol	24α-Methyl-5β-cholestan-3β-ol
Campesterol	24α-Methyl-5-cholesten-3β-ol
Coprostanol	5β-Cholestan-3β-ol
Coprostanone	5β-Cholestan-3-one
Cholestane	5α-Cholestane
Cholestanol	5α-Cholestan-3β-ol
Cholestanone	5α-Cholestan-3-one
Cholestenone	4-Cholesten-3-one
Cholesterol	5-Cholesten-3β-ol
Cholesterone	5-Cholesten-3-one
Desmosterol	5,24-Cholestadien-3β-ol
Epicoprostanol	5β-Cholestan-3α-ol
Ethylcoprostanol	24β-Ethyl-5β-cholestan-3β-ol

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Ethylcoprostanone	24β-Ethyl-5β-cholestan-3-one
Ethylcoprostenol	24β-Ethyl-5β-cholestan-3α-ol
Lathosterol	5α-Cholest-7-en-3β-ol
β-Sitosterol	5-Stigmansten-3β-ol
Methylcoprostanol	24α-Methyl-5β-cholestan-3β-ol
Methylcoprostanone	24α-Methyl-5β-cholestan-3-one
Stigmastanol	3β-5α-Stigmastan-3-ol
Stigmastenol	5α-Stigmast-7-en-3β-ol
Stigmasterol	Stigmastan-5,22-dien-3β-ol

Introduction

Plant sterol (PS) intake varies between ≤60 and 500 mg/day [1]. Foods enriched with these compounds for their cholesterol-lowering effect could increase this intake. Cholesterol and PS have absorption rates of 30–60% and 2–3%, respectively, when ingested in a normal diet [2, 3]. Absorption levels for campesterol (9.4–14.8%) are approximately three times higher than for β-sitosterol (3.1–4.5%) and stigmasterol (~4%). Absorption of campestanol and sitostanol has been shown to be even lower (0.1–2%) [3].

Neutral sterols are eliminated with feces as a complex mixture of metabolites formed by the action of the intestinal microbiota [4]. The cholesterol biotransformation mechanisms have been well established. In this regard, a direct route involves microbial degradation to coprostanol [5]; another route involves cholesterone, cholestenone and coprostanone as intermediates [6, 7]; and finally a third route involving conversion to cholestanol has been described in which cholesterone, cholestenone and cholestanone act as intermediates [4, 5, 8, 9].

Plant sterols are also biotransformed in the gut into phytostenone and phytostanone intermediaries, and subsequently can be reduced to phytostanol [4]. Some of these sterol metabolites have been detected in human feces [10–18]. Figure 1 shows sterol and some of the metabolites.

The incidence of colon cancer is mainly associated with the daily intake of fat, and it has been further suggested that such fat influences the composition of the fecal microbiota, which may be involved in the pathogenesis of colon cancer. Transformation of cholesterol in the large intestine could increase the colon cancer risk due to the formation of several metabolites such as coprostanol, coprostanone, cholestanone and cholestanol [19]. Some animal and epidemiological studies suggest that PS may in fact lower the colon cancer risk, and various mechanisms have been proposed to explain the potential anticancer properties of PS [20, 21]. In addition, the determination of cholesterol, PS and their metabolites in feces is used for the diagnosis of lipid disorders such as homozygous familial hypercholesterolemia [22].

Most methods focus on determining sterols and fecal metabolites after normal intakes of PS [11–13, 15–18, 23]. Feces from subjects that consume fat-rich foods (butter or corn oil) [10], PS-enriched foods such as margarine (6.8 g PS/day) [14] or skimmed milk yoghurt beverage (2 g/day) [24] have been analyzed in relation to neutral sterols and their metabolites.

Not all the aforementioned studies applied validated methods. In some cases, precision and accuracy values are given for both cholesterol and their metabolites [11, 25], and for PS (campesterol, β-sitosterol, stigmasterol) after the intake of a normal diet [11]. Only the studies conducted by Wu *et al.* [18] have validated a method for the determination of fecal sterols and their metabolites in feces and water samples.

Since few studies have validated the methodology used in determining fecal sterols and their metabolites, and considering the interest in determining these compounds on the basis of the findings of clinical intervention studies with PS-enriched foods, the present study was designed to validate a convenient procedure for determining fecal sterols and their metabolites by GC/MS, following the consumption of a normal diet and a PS-enriched diet.

Materials and Methods

Standards and Reagents

Cholestane (5α-cholestane, ≥97%) was used as internal standard (IS). Coprostanol (5β-cholestan-3β-ol, 98%), cholestanol (5α-cholestan-3β-ol, ≥95%), cholesterol (5-cholesten-3β-ol, 99%), desmosterol (5,24-cholestadien-3β-ol, 84%), epicoprostanol (5β-cholestan-3α-ol, ≥95%), lathosterol (5α-cholest-7-en-3β-ol, 99%), stigmastanol (stigmastan-3β-ol, ≥95%), stigmasterol (stigmasta-5,22-dien-3β-ol, 95%), β-sitosterol (5-stigmasten-3β-ol, 98%), butylhydroxytoluene (BHT, purity 99%), *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), and potassium hydroxide (KOH) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Brassicasterol (5, 22-cholestandien-24β-methyl-3β-ol, 98%), campesterol (24α-methyl-5-cholesten-3β-ol, ≥98%) and coprostanone (5β-cholestan-3-one, 98%) were obtained from Steraloids Inc. (Newport, RI, USA).

n-Hexane was purchased from Scharlau Chemie, S.A. (Barcelona, Spain), and absolute ethanol and 2-propanol were from Merck & Co., Inc. (Whitehouse Station, NJ, USA). Pyridine extra-dry was from Fluka (Buch, Switzerland).

All standards used were of GC grade, and all reagents were of analytical grade. Millipore MilliQ deionized water (Millipore Ibérica S.A., Barcelona, Spain) was used.

All sterol standard solutions of 1 mg/mL were prepared and stored frozen (−20 °C) until use. Working solutions

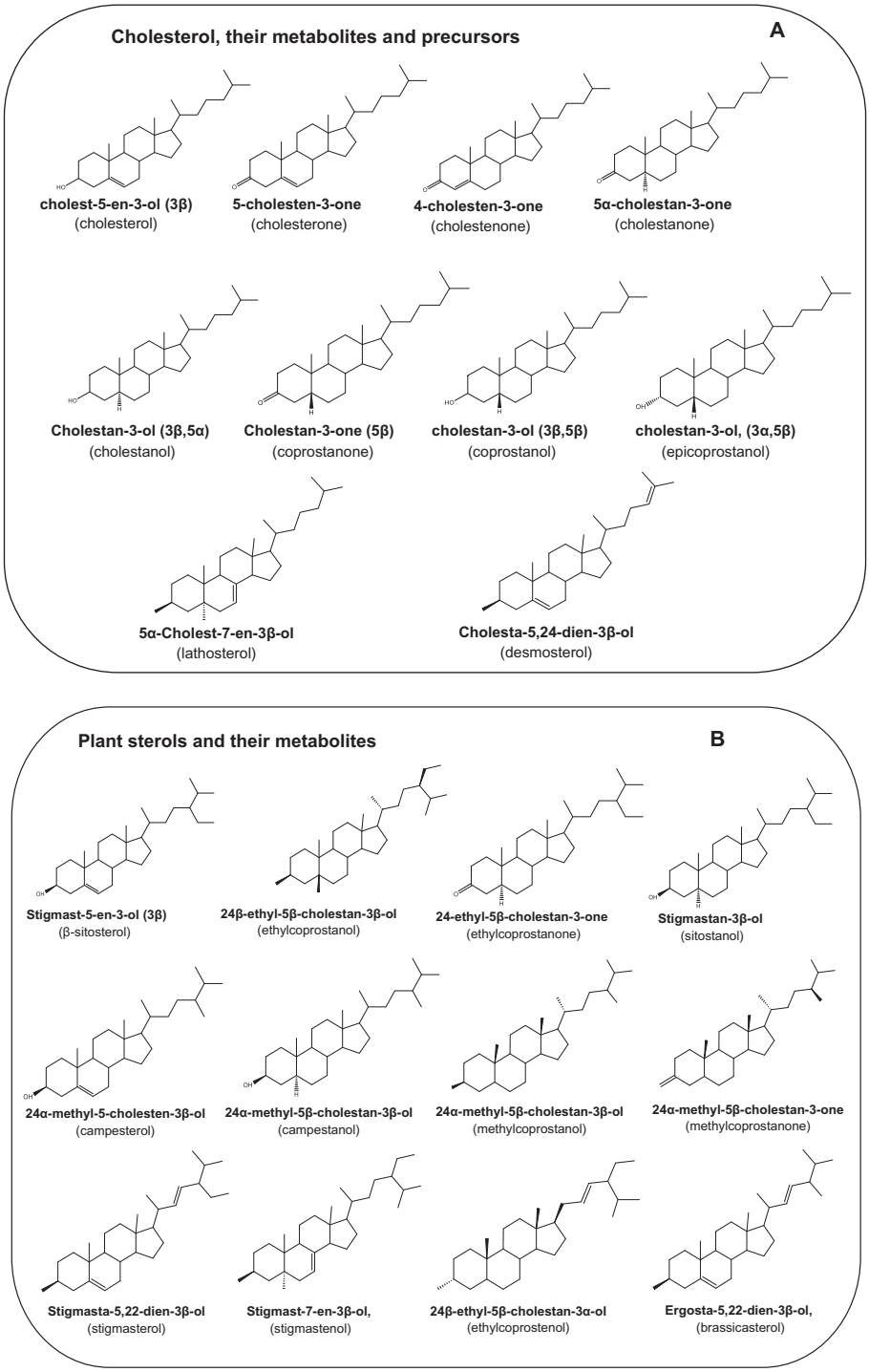

Fig. 1 Structures of sterols identified in feces

of 5α-cholestane, cholesterol, cholestanol, coprostanol, coprostanone (100 μg/mL) and epicoprostanol (10 μg/mL) were prepared in ethanol. Campesterol, sitosterol, stigmastanol, stigmasterol (100 μg/mL), brassicasterol (20 μg/mL), desmosterol and lathosterol (10 μg/mL) were prepared in hexane:2-propanol 3:2 (v/v).

All glass test tubes and vials were scrupulously cleaned, rinsed with distilled water, and placed in an Heraeus Thermicon K1253A oven at 450ºC for 8 h to remove contamination. The Pyrex glass tubes with a conical bottom, employed for derivatization, were previously silanized with derivatization solution in order to avoid the adsorption of TMS ether derivatives.

Sample Collection and Dilution

Fresh feces were obtained from four healthy postmenopausal women with moderate hypercholesterolemia participating in a clinical trial involving a total of 40 volunteers (ClinicalTrials.gov number NCT 02065024). The subjects consumed a low PS diet along with a beverage with or without PS (microencapsulated free PS: 2 g/day). Relative percentages in beverages were sitosterol 81.1%, sitostanol 11.9%, campesterol 4.1%, campestanol 1.2%, stigmasterol 0.7% and also cholesterol 1.1%, which was characterized according to Alvarez-Sala *et al.* [26]. The participants were asked not to change their usual diet and physical activity, to record any side effects during the study, and to fill a semiquantitative Food Frequency Questionnaire (FFQ) at the end of each intervention period to check compliance with these criteria.

Fecal samples were collected in sterile plastic containers and lyophilized (Sentry 2.0, Virtis SP Scientific). Then, the samples were homogenized (by crushing) in a glass mortar and stored at −20 °C in glass Petri dishes until analysis.

An amount of lyophilized feces (~30 mg) equivalent to approximately 125 mg of fresh feces was weighed, homogenized with 5 mL of MilliQ water in a volumetric flask, sonicated for 20 min with or without 2 h at room temperature, and a filtration step with a cellulose filter (Whatman™ 125 mm, GE Healthcare®) was also used.

Sterols Determination

Sterols were extracted in triplicate according to the serum method of García-Llatas *et al.* [27], with slight modifications. Aliquots of 100 and 500 μL of feces solution (according to section "Sample Collection and Dilution") were added with IS (20 μg of 5α-cholestane) and 5 μL of BHT 0.02% (w/v). Saponification was carried out in Pyrex glass tubes fitted with screw stoppers and containing 1 mL of ethanolic KOH 0.71 M at 65 °C for 1 h using a block heater (Stuart Scientific, Staffordshire, UK). After reaching room temperature, the unsaponifiable fraction was extracted. To this end, 0.5 mL of MilliQ water and 2 mL of hexane were added, followed by stirring for 30 s at 2500 rpm in an MS2 IKA vortex (Staufen, Germany). Then, the sample was centrifuged to facilitate phase separation at 18 °C for 10 min at 3600 rpm using an Eppendorf® centrifuge 5810R (Hamburg, Germany). Extraction with hexane was performed twice, and all hexane phases were collected in a 10-ml Pyrex glass tube with a conical bottom (Vidra Foc, Barcelona, Spain), followed by evaporation to dryness under nitrogen. Two hundred μL of derivatization reagent (BSTFA + 1% TMCS: pyridine, 10:3, v/v, prepared weekly) was added. The oxygen was removed with nitrogen for 30 s and was then heated at 65 °C during 1 h. The sample was evaporated under nitrogen, and the TMS ether derivatives obtained were dissolved with 3 mL of hexane, filtered (Millex-FH filter unit, 0.45 μm Millipore, Milford, MA, USA), evaporated under nitrogen, and dissolved in 40 μL of hexane.

For identification and quantification of sterols, we used a GC/MS system (Thermo Science Trace® GC-Ultra with ion trap ITQ 900, Waltham, MA, USA) with an Xcalibur data processor, and equipped with a CP-Sil8 CB low bleed/MS (50 m × 25 mm × 0.25 μm) capillary column (Agilent Technologies®, CA, USA). One μL of derivatized sample was injected using a PTV Splitless port (initial temperature 80 °C, with a heating rate of 14.5 °C/s to 280 °C during 5 min). The carrier gas was hydrogen operating at a constant flow of 1 ml/min. The oven program was: 150 °C holding for 3 min, 30 °C/min to 280 °C, and holding for 28 min; 10 °C/min to 295 °C and holding for 10 min. The transfer line temperature was 310 °C, and the ion source temperature was 250 °C. The mass spectrometer operated in the electron impact ionization mode at −70 eV, and a mass range from 50 to 650 *m/z* was scanned.

Identification of sterols was carried out by comparing the relative retention time (Rrt) and mass spectra with those of the commercial sterol standards and the mass spectra library (NIST v.2). Calibration curves were used for the quantification of fecal sterols and their metabolites. Cholestanol curves were used for cholestanol + methylcoprostanol quantitation; coprostanol curves were used to quantify ethylcoprostenol and ethylcoprostanol; and coprostanone curves were used to quantify cholestanone, methylcoprostanone and ethylcoprostanone.

Results

Sample Preparation

The influence of filtration and homogenization on lyophilized feces with water was evaluated. The results obtained by applying the method with an aliquot of 100 μL are shown in Table 1. After filtration, a great loss of sterols

and metabolites (30–84%) was observed. In the unfiltered sample we detected the presence of particles in suspension, hindering pipetting and resulting in low precision (between 1.2 and 47.5 RSD%). This was improved when the sample was allowed to stand for 2 h at room temperature (from 0.1 to 12.4 RSD%), yielding good recoveries (see analytical parameters section).

We also evaluated the optimal volume of feces solution (25, 100, 500 and 1000 µL) for derivatizing and determining these components by GC/MS. Table 2 shows the results obtained with different volumes of sample from subjects following a normal diet or after the intake of a diet with PS-enriched beverage (2 g/day). In feces from subjects consuming a normal diet, the determination of coprostanone, cholestanol + methylcoprostanol, cholesterol, ethylcoprostanol and sitosterol was performed using aliquots of 100 µL, while aliquots of 500 µL were used for the rest of sterols. However, in those individuals who ingested the PS-enriched beverage, a large content of PS and their metabolites was observed in the feces. For this reason, a lower sample volume (100 µL and in some cases even 25 µL, Table 2) was necessary for their determination. Since sitosterol and sitostanol were the major sterols in the PS-enriched beverage, ethylcoprostanol was the metabolite with the greatest presence after beverage intake.

In addition, the volume of derivatizing reagent (400 and 200 µL) was evaluated. The fecal sterols contents were similar (data not shown) using both volumes. Furthermore, 400 µL of reagent caused a larger number of residual peaks due to excess derivatization. Finally, 200 µL was selected as the optimum volume.

Sterol Identification

Figure 2 shows a chromatogram corresponding to the sterols and metabolites identified in a fecal sample from subjects that ingested a normal diet (A); in another sample after the intake of a PS-enriched beverage (B); and in plant sterol standards (C). The spectra obtained from the fecal samples were compared with those of standards and with the mass spectra library for identification by GC/MS.

Table S1 shows the retention time (Rt), relative retention time (Rrt) and characteristic ions of the sterols and metabolites identified by GC/MS [28–44].

The total analysis time was 27 min, continuing until a total of 47 min for column cleaning. In the first 20 min we identified cholesterol and 5 of its metabolites (coprostanol, epicoprostanol, coprostanone, cholestanol and cholestanone). In addition, lathosterol and desmosterol as cholesterol precursors were identified. With regard to PS, we identified brassicasterol, campesterol and their metabolites methylcoprostanol and methylcoprostanone, stigmasterol and its metabolite ethylcoprostenol, sitosterol, campestanol, stigmastanol, sitostanol, ethylcoprostanone and ethylcoprostanol as sitosterol metabolites.

Table 1 Sample preparation. Influence of filtration and homogenization upon fecal sterols content (mg/g lyophilized feces)

Sterol	Unfiltered	Filtered	Unfiltered (after 2 h homogenization)
Coprostanol	7.90 ± 1.15	2.54 ± 0.04	4.01 ± 0.003
Coprostanone	2.40 ± 0.31	0.98 ± 0.60	0.72 ± 0.01
Cholesterol	11.50 ± 1.90	4.37 ± 0.1	5.97 ± 0.51
Cholestanol + methylcoprostanol	0.47 ± 0.03	0.24 ± 0.03	0.25 ± 0.01
Brassicasterol	0.21 ± 0.01	0.10 ± 0.004	0.11 ± 0.002
Lathosterol	0.19 ± 0.01	0.10 ± 0.01	0.10 ± 0.004
Methylcoprostanone	0.05 ± 0.01	0.02 ± 0.01	[a]
Ethylcoprostenol	0.28 ± 0.003	0.21 ± 0.005	0.21 ± 0.001
Ethylcoprostanol	1.85 ± 0.20	0.60 ± 0.02	1.62 ± 0.04
Campesterol	0.77 ± 0.10	0.23 ± 0.06	0.21 ± 0.02
Campestanol	0.17 ± 0.03	[a]	[a]
Stigmasterol	0.43 ± 0.02	0.21 ± 0.02	0.20 ± 0.01
Stigmastenol	0.30 ± 0.04	[a]	[a]
Sitosterol	2.15 ± 0.14	0.92 ± 0.11	1.82 ± 0.01
Sitostanol	0.47 ± 0.22	[a]	0.23 ± 0.03
Ethylcoprostanone	0.96 ± 0.20	0.21 ± 0.01	0.35 ± 0.02

Mean ± SD, $n = 3$

[a] Value below the lowest point of the quantification curve

Table 2 Selection of volume of sample. Fecal sterol contents (mg/g lyophilized feces) with and without intake of PS-enriched beverage (2g/day)

Sterol	Without beverage		With beverage		
	100 µL	500 µL	25 µL	100 µL	500 µL
Coprostanol	9.40 ± 0.35	▲	▼	14.90 ± 0.40	▲
Coprostanone	2.34 ± 0.20	▲	▼	5.00 ± 0.09	▲
Cholesterol	2.34 ± 0.09	▲	▼	4.60 ± 0.20	▲
Cholestanol + methyl-coprostanol	▼	0.38 ± 0.002	▼	1.85 ± 0.02	▲
Cholestanone	▼	0.05 ± 0.002	▼	0.20 ± 0.005	0.23 ± 0.05
Brassicasterol	▼	0.25 ± 0.003	▼	0.41 ± 0.01	0.23 ± 0.02
Lathosterol	▼	0.12 ± 0.001	▼	0.18 ± 0.001	0.20 ± 0.02
Methylcoprostanone	▼	▲	▼	0.68 ± 0.02	0.50 ± 0.07
Ethylcoprostenol	▼	0.33 ± 0.02	▼	▲	0.13 ± 0.01
Ethylcoprostanol	3.23 ± 0.12	▲	7.00 ± 0.48	▲	▲
Campesterol	▼	0.31 ± 0.02	▼	3.58 ± 0.14	▲
Campestanol	▼	0.14 ± 0.001	▼	1.57 ± 0.01	1.48 ± 0.05
Stigmasterol	▼	0.19 ± 0.01	▼	0.62 ± 0.01	0.51 ± 0.07
Stigmastenol	▼	0.44 ± 0.03	▼	5.26 ± 0.22	▲
Sitosterol	1.82 ± 0.07	▲	4.79 ± 0.15	▲	▲
Sitostanol	▼	0.02 ± 0.01	0.40 ± 0.12	▲	▲
Ethylcoprostanone	▼	1.37 ± 0.01	▼	2.22 ± 0.04	▲

Mean ± SD, $n = 3$; ▼ = non-quantifiable value, below quantification limit; ▲ = non-quantifiable value, above the highest point in the curve

Cholestanol and methylcoprostanol co-eluted and could not be resolved. Coprostanol/epicoprostanol and ethylcoprostanol/campesterol eluted at separate Rt (Table S1). However, we found that if their ratios are >1600 and >25, respectively, these sterols overlap, and it proves difficult to quantify minority sterols.

Analytical Parameters

The matrix interference study was carried out by the standard addition method applied to extract from human feces. One calibration curve for each sterol quantified with or without the addition of matrix in 50% proportion (standard addition method) was analyzed. The slopes of the regression equations were compared by a *t* test ($p < 0.05$), and the confidence intervals of the slopes with or without matrix are shown in Table 3.

The absence of interferences was observed, except for coprostanone. In this case, and in order to confirm whether the same interference occurred in different fecal matrixes, the assay was carried out with three fecal samples. No statistically significant differences ($p < 0.05$) were found (Fig. 3) for three matrix curves. Therefore, only one standard curve added with feces was necessary to quantify this metabolite.

Calibration standards were converted to TMS ether derivatives and determined by GC/MS to evaluate the linearity of the responses of the instrument over the concentration range of interest. The linear range and calibration curves for the different sterols studied are summarized in Table 3. For coprostanone we performed a matrix curve (Fig. 3, feces 3) to quantify the coprostanone, cholestanone, methylcoprostanone and ethylcoprostanone contents in fecal samples (shown in Table 3).

The assayed concentration ranges showed good linearity, since all correlation coefficients obtained were >0.96. The greatest sensitivity, defined from the signal-concentration relationships given by the slope, was found to correspond to cholestanol, while desmosterol was the sterol with the lowest sensitivity.

The limits of detection (LOD) and quantitation (LOQ) of the method were determined from the background response of six blanks performed on two different days. They were calculated as three and 10 times (for LOD and LOQ, respectively) the standard deviation (SD) of the response against the slope of the calibration curve for each sterol [45].

The LOD and LOQ values covered a broad range between 8–291 and 25–970 ng/mL, respectively—the lowest corresponding to cholestanol and the highest to ethylcoprostanone (Table 4).

Repeatability (intra-assay precision) was evaluated with 6 aliquots of the sample analyzed on the same day. In order to estimate reproducibility (inter-assay precision), the same samples were analyzed on two different days. The results were expressed with the relative standard deviation (RSD). Intra-assay values (RSD%) for total sterol contents varied

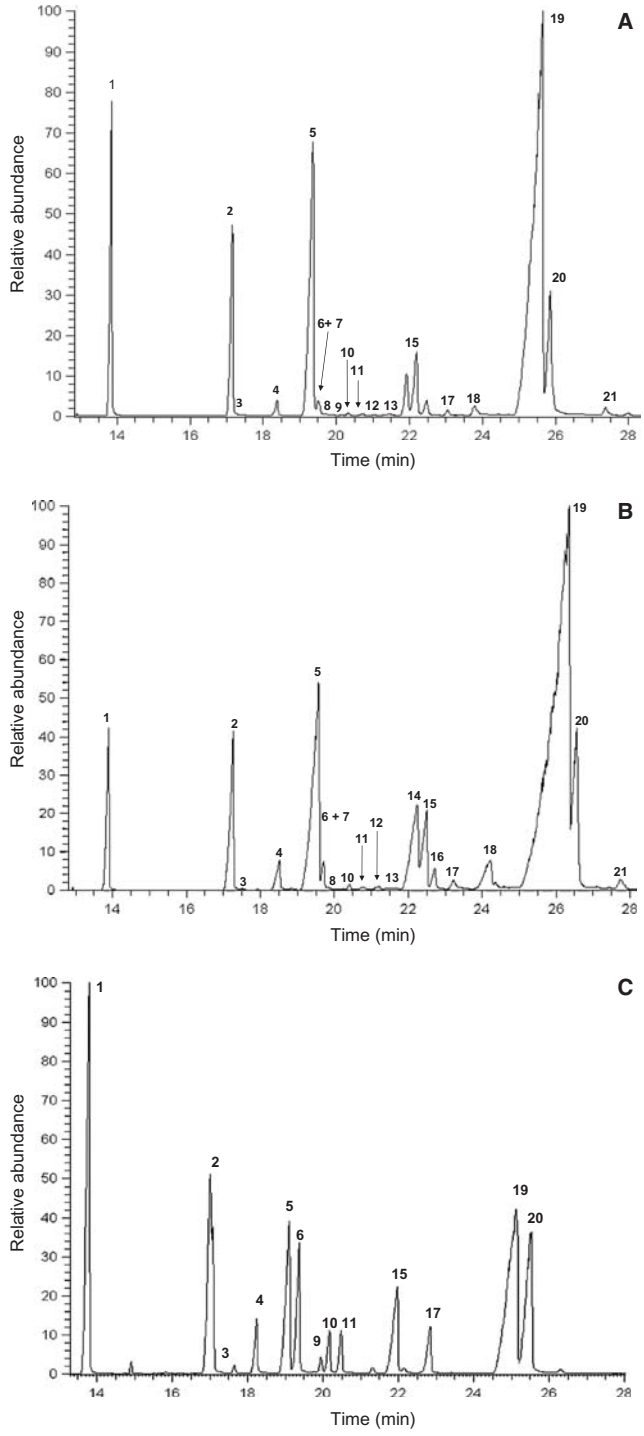

Fig. 2 Chromatogram of sterols and metabolites identified in standard and in a fecal sample. **a** Faecal sample without PS-enriched beverage intake; **b** faecal sample with PS-enriched beverage intake; **c** sterols standard; *1* 5α-cholestane (IS); *2* coprostanol; *3* epicoprostanol; *4* coprostanone; *5* cholesterol; *6* cholestanol; *7* methylcoprostanol (formed from campesterol); *8* cholestanone; *9* desmosterol; *10* brassicasterol; *11* lathosterol; *12* methylcoprostanone (formed from campesterol); *13* ethylcoprostenol (formed from stigmasterol); *14* ethylcoprostanol (formed from β-sitosterol); *15* campesterol; *16* campestanol; *17* stigmasterol; *18* stigmastenol; *19* sitosterol; *20* sitostanol; *21* ethylcoprostanone (formed from sitosterol)

Table 3 Sterols determination: matrix interferences assay and linearity

Sterols	Matrix interference assay		Linearity			
	Confidence interval of slope (95%)		Range (µg in assay)	Range (µg/mL in assay)	*r*	Calibration curve
	Without matrix	With matrix				
Coprostanol	0.067–0.085	0.075–0.079	0.1–24	2.45–588	0.985	$y = 0.076x-0.047$
Coprostanone	0.012–0.016	0.016–0.041	0.5–20	12.25–490	0.999	$y = 0.0302x$[a]
Epicoprostanol	–	–	0.1–2.0	2.38–4.28	0.990	$y = 0.041x-0.002$
Cholesterol	0.054–0.077	0.062–0.066	0.5–20	12.25–495	0.992	$y = 0.079x-0.021$
Cholestanol	0.072–0.110	0.081–0.100	0.2–8.0	4.75–190	0.996	$y = 0.137x-0.049$
Desmosterol	–	–	0.05–2.0	1.25–50	0.968	$y = 0.028x-0.004$
Brassicasterol	0.060–0.067	0.062–0.074	0.1–2.0	2.45–49	0.976	$y = 0.106x-0.023$
Lathosterol	0.052–0.110	0.074–0.090	0.1–2.0	2.48–49.5	0.994	$y = 0.083x-0.011$
Campesterol	0.056–0.057	0.055–0.071	0.2–16	4.75–3.92	0.997	$y = 0.053x+0.002$
Stigmasterol	0.040–0.050	0.042–0.054	0.2–8.0	4.75–194	0.995	$y = 0.042x-0.014$
Sitosterol	0.056–0.071	0.051–0.075	0.2–20	4.75–475	0.991	$y = 0.074x-0.053$
Sitostanol	0.060–0.080	0.073–0.099	0.1–16	2.38–380	0.992	$y = 0.055x+0.022$

Matrix interference referred to coprostanone

Confidence interval of slopes (95%)		
Samples	Without matrix	With matrix
Faeces 1	0.012–0.016	0.026–0.031
Faeces 2		0.016–0.041
Faeces 3		0.029–0.030

r Linear correlation coefficient, *y* sterol area/IS area, *x* µg sterol, – no detected sterols in matrix used for this assay

[a] Standard curve modified by fecal matrix

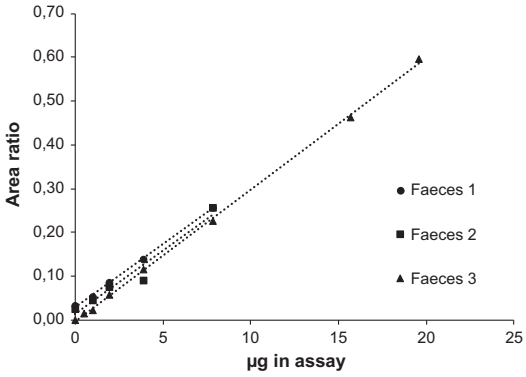

Fig. 3 Matrix interference assay referred to coprostanone. Standard curves added to three different feces

from 0.9 to 9.2%, and the range of inter-assay values was 2.1–11.3% (Table 4).

The accuracy of the method was established from apparent recovery assays (Table 5). Four aliquots of feces were spiked with sterol standards at levels similar to those found in feces (sample added). In addition, the sterol standards at levels spiked in the samples were analyzed to confirm the quantities added. The recoveries of cholestanol plus methylcoprostanol were carried out with addition of cholestanol standard.

Application

Table 6 shows the total contents of cholesterol, PS and their metabolites in individuals enrolled in a clinical trial while consuming a normal diet or a normal diet with a PS-enriched beverage (2 g/day). These are preliminary data corresponding to four postmenopausal women out of a total of 40 subjects. Firstly, we applied the method to feces from subjects after the consumption of a normal diet (feces 1 and 2), using a consumption questionnaire to confirm that there were no substantial changes in dietary habits resulting in significant changes in the intake of cholesterol and PS during the study. Besides, the method was also applied to feces from subjects consuming a normal diet with a PS-enriched beverage (2 g/day) (feces 3 and 4). The total animal sterol and PS contents, and individual and total relative percentages, are also indicated.

Using the analyzed validated method, we were able to quantify lathosterol (a cholesterol precursor), stigmastenol

Table 4 Precision, limits of detection and quantitation

Sterol	LOD			LOQ			Precision (%RSD)	
	ng in assay	ng/ml	μg/g dry faeces	ng in assay	ng/ml	μg/g dry faeces	Intra-assay ($n = 6$)	Inter-assay
Coprostanol	1.6	40	2.67	5.4	134	8.92	0.9	4.0
Epicoprostanol	4.4	110	1.46	14.7	367	4.88	–	–
Coprostanone	3.0	75	1.00	10	250	3.33	2.4	3.7
Cholesterol	1.2	30	2.00	4	100	6.63	2.7	8.2
Cholestanol + methylcoprostanol	0.3	8	0.10	1.0	25	0.34	1.3	5.6
Cholestanone	3.2	81	1.10	10.8	270	3.60	6.2	–
Desmosterol	3.3	83	1.11	11.1	278	3.70	6.9	9.4
Brassicasterol	0.6	15	0.20	2.00	50	0.67	1.7	5.3
Lathosterol	1.3	32	0.42	4.2	106	1.41	1.3	2.9
Methylcoprostanone	0.7	18	0.24	2.4	61	0.81	9.2	11.3
Ethylcoprostenol	0.6	14	0.20	1.8	46	0.62	3.7	3.9
Ethylcoprostanol	7.7	193	12.88	25.8	644	43.0	3.4	7.4
Campesterol	0.6	14	0.18	1.8	45	0.61	8.6	8.1
Campestanol	0.4	9	0.12	1.2	30	0.40	3.0	5.7
Stigmasterol	2.5	63	0.85	8.5	212	2.82	1.3	2.1
Stigmastenol	1.0	25	0.33	3.3	83	1.10	3.2	3.1
Sitosterol	0.5	13	0.90	1.8	45	3.00	3.7	3.2
Sitostanol	0.7	18	0.24	2.4	60	0.80	2.7	3.2
Ethylcoprostanone	11.6	291	3.88	38.8	970	12.94	1.7	9.1

LOD Limit of detection, *LOQ* limit of quantitation

Table 5 Fecal sterols content (mg/g dry faeces) and recovery (%) (expressed as means ± SD) ($n = 4$)

Sterol	Present	Added	Found	Recovery (%)
Coprostanol	13.12 ± 0.22	18.46 ± 0.52	34.27 ± 2.54	105 ± 12
Coprostanone	5.66 ± 0.31	6.27 ± 0.23	10.52 ± 0.42	81 ± 3
Cholesterol	7.57 ± 0.17	4.87 ± 0.10	11.38 ± 0.65	85 ± 4
Cholestanol	1.25 ± 0.07[a]	1.20 ± 0.04[b]	2.57 ± 0.09[a]	110 ± 7
Desmosterol	0.07 ± 0.003	0.11 ± 0.01	0.15 ± 0.01	80 ± 9
Brassicasterol	0.11 ± 0.002	0.42 ± 0.004	0.61 ± 0.02	119 ± 5
Lathosterol	0.05 ± 0.001	0.18 ± 0.01	0.24 ± 0.004	103 ± 2
Campesterol	0.23 ± 0.02	1.05 ± 0.01	1.22 ± 0.07	94 ± 7
Stigmasterol	0.23 ± 0.003	0.31 ± 0.001	0.43 ± 0.01	99 ± 4
Sitosterol	1.15 ± 0.09	1.25 ± 0.06	2.34 ± 0.02	94 ± 2
Sitostanol	0.47 ± 0.01	1.13 ± 0.06	1.71 ± 0.03	110 ± 3

Recovery (%) = (found − present) × 100/Added

[a]Cholestanol + methylcoprostanol

[b]Cholestanol

and ethylcoprostenol (from stigmasterol), ethylcoprostanone (from sitosterol) and methylcoprostanone (from campesterol) in feces.

The order of abundance of cholesterol and its metabolites present in feces 1 and 2 (after consumption of a normal diet) was coprostanol > coprostanone > cholestanol > cholestanone. However, their contents in feces 3 and 4 decreased notoriously, and the order of abundance moreover varied—the cholesterol content being greater than that of coprostanone. Furthermore, it was seen that the difference between the coprostanol and cholesterol contents was smaller in these samples.

Conversion percentages of cholesterol (see Table S2) were calculated without taking cholestanol into account, because it overlaps with methylcoprostanol. The subjects with a normal diet showed a higher cholesterol conversion rate, in contrast to the subjects consuming a PS-enriched diet, who presented greater total animal sterol contents despite having lower contents of cholesterol (Table S2). Therefore, there was less conversion of cholesterol into coprostanol and coprostanone after ingestion of the PS-enriched beverage, probably due to interaction between PS and cholesterol in the context of their biotransformation mediated by the microbiota. In relation to PS, we found wide conversion ranges for sitosterol (24–80%), stigmasterol (9–86%) and campesterol (3–24%).

Table 6 Sterols contents in feces (mg/g lyophilized feces). Values expressed as mean ± standard deviation of three replicates

Sterol	Faeces 1[a]	Faeces 2[a]	Faeces 3[b]	Faeces 4[b]	Literature data[c]
Animal sterols					
Cholesterol	1.84 ± 0.02	1.37 ± 0.06	6.24 ± 0.21	4.35 ± 0.30	1.2–28 [11, 13–17, 46–48]
Coprostanol	20.94 ± 0.07	23.51 ± 0.71	7.40 ± 0.01	10.43 ± 1.00	3.01–27.41 [11, 13–17, 46–49]
Coprostanone	5.62 ± 0.17	5.00 ± 0.40	2.11 ± 0.07	2.25 ± 0.25	nd–3.7 [11, 13, 15–17, 46–49]
Cholestanol + methylcoprostanol	1.18 ± 0.01	1.15 ± 0.02	0.71 ± 0.03	1.20 ± 0.004	0.22–1.54 [11, 16, 17, 49]
Cholestanone	0.12 ± 0.004	0.09 ± 0.005	–	0.12 ± 0.005	nd–1.22 [14, 49]
Lathosterol	0.14 ± 0.01	0.15 ± 0.0003	0.17 ± 0.004	0.11 ± 0.002	–
Total animal sterols	29.8	31.3	16.6	18.5	11.7–34.6 [11, 13–17, 46–49]
Plant sterols					
Sitosterol	1.67 ± 0.001	1.77 ± 0.02	29.00 ± 1.30	13.62 ± 1.15	0.4–54 [13–15, 17]
Sitostanol	0.07 ± 0.007	0.05 ± 0.004	7.70 ± 0.40	5.67 ± 0.44	0.46–3.5 [14]
Ethylcoprostanol	5.18 ± 0.25	4.10 ± 0.16	8.90 ± 0.15	16.32 ± 1.53	0.6–1.3 [13–15, 17]
Ethylcoprostanone	1.38 ± 0.13	1.53 ± 0.06	2.80 ± 0.12	1.88 ± 0.21	–
Stigmasterol	0.17 ± 0.006	0.16 ± 0.002	0.60 ± 0.01	0.43 ± 0.02	0.16–24
Stigmastenol	0.17 ± 0.06	0.42 ± 0.008	2.00 ± 0.05	2.65 ± 0.16	–
Ethylcoprostenol	0.22 ± 0.001	–	0.23 ± 0.01	–	–
Campesterol	0.30 ± 0.04	0.27 ± 0.04	3.90 ± 0.21	1.77 ± 0.006	0.2–28 [13–15, 17]
Campestanol	0.34 ± 0.04	0.25 ± 0.007	1.21 ± 0.05	1.00 ± 0.04	0.75–2.9 [14]
Methylcoprostanone	0.21 ± 0.02	–	0.18 ± 0.006	0.34 ± 0.03	–
Brassicasterol	0.37 ± 0.006	0.38 ± 0.0002	–	0.32 ± 0.01	0.03–1.8 [14]
Total plant sterols	10.1	8.9	56.5	44.0	11.6–17.6 [13–15, 17]
Total sterols	39.9	40.2	73.1	62.5	21.8–190 [11, 13–17, 46–49]

[a] Feces from normal-diet subjects

[b] Feces from PS-enriched diet (2 g/day) subjects

[c] Minimum and maximum values

Discussion

We have developed a method that reduces solvents volume and the amount of reagents used by other authors. Other advantages of the proposed method are a smaller sample size and faster analysis. In addition, the method allows the determination of a large number of sterols. In the literature, direct saponification applied to fresh [6, 10–12, 17, 23, 50–53] or lyophilized [13–16, 24, 25] human feces to determine sterols and their metabolites has been described. The proposed method has been applied to lyophilized feces, since these samples have several advantages from the point of view of storage, safety and sampling. In diluting feces without filtration, we observed the presence of particles in suspension that hindering pipetting. Consequently, the samples diluted in water [11, 16, 23, 52] were sonicated and allowed to stand for 2 h at room temperature without filtration, followed by the observation of a decrease in variability.

In addition, the present method requires less (two- to five-fold) sample compared with others authors [6, 10–12, 18, 23, 25, 50–52]. In order to obtain a more environmentally friendly method, the volume of solvent was reduced. In this context, sample homogenization was carried out with water [11, 16, 23, 52], and the solvent volumes (10 mL *n*-hexane, approximately) used for saponification, unsaponifiable extraction and derivatization were between 2- and 50-fold lower compared with those used by other authors [6, 10–12, 18, 23, 25, 50, 53].

One milliliter of sample resulted in a saturated GC/MS signal, and chromatograms with distorted peaks were observed. In addition, an unnecessary increase in sample dirtied the ion trap, and for this reason the 1 mL volume was discarded. Therefore, the volume of the aliquots (between 25 and 500 µL) was adapted according to the abundance of the sterols.

The method developed allows the identification of 21 sterols and the quantification of 17. In comparison, other authors have only focused on the identification of cholesterol and its fecal metabolites [11, 16, 47, 48]. With regard to cholesterol precursors, lathosterol was previously identified in feces [54], and both this metabolite and desmosterol, as a cholesterol precursor, were identified in some biological samples such as serum [27]. As far as we know, this is the

first time that desmosterol and lathosterol have been identified and quantified in human feces.

Ethylcoprostanol and campesterol separation depends on the ratio between them, in coincidence with the observations of other authors such as Korpela [11] using GC–MS and Czubayco *et al.* [52] working with GC-FID, who could not separate ethylcoprostanol and campesterol.

It should be noted that complete validation has been performed, taking into account all the analytical parameters. Besides, the concentration ranges used were wide in order to allow quantification of sterols and their metabolites present in feces from subjects with a high intake of cholesterol and especially of PS.

Our results referred to as LOD and LOQ indicate that the proposed method is more sensitive than in the case of the method of Keller and Jahreis [16] (0.9–2.40 μg/mL for cholesterol, coprostanol, coprostanone and cholestanol), probably due to the non-derivatization of samples. Shah *et al.* [17] reported LOD values between 9 and 27 μg/mL (for coprostanol, cholesterol, cholestanol, campesterol, stigmasterol, sitosterol and ethylcoprostanol), which are higher than those found in our study. The present study is the only publication offering detection and quantitation limits for cholestanone, desmosterol, brassicasterol, lathosterol, methylcoprostanone, ethylcoprostenol, ethylcoprostanone, stigmastenol, campestanol and sitostanol.

The precision values obtained were in agreement with the criteria of the Association of Official Analytical Chemists [55], which indicate that for analytes present at concentrations of μg/g, values of up to 15% are acceptable. Our results referred to precision showed RSD values below 10% (Table 4), indicative of optimal precision. Our intra- and inter-assay precision results were similar to those reported by other authors such as Glatz *et al.* [25] (intra- and inter-assay precision 1.1–2.6% and 0.6–4.0%, respectively) for cholesterol and its derivatives (not specified) and PS (not specified), and Keller and Jahreis [16] (intra- and inter-assay precision 2.7–9% and 4.2–11.2%, respectively) for cholesterol and its metabolites (coprostanol, coprostanone and cholestanol). However, the precision values obtained were lower than those reported by Wu *et al.* [18] for coprostanol (11.3%) and cholestanol (19.6%), and similar to that reported for cholesterol (8%).

Recoveries were usually above 80% (shown in Table 5), and were in accordance with the intervals proposed by the AOAC [55] (80–115%) for analyte concentrations of μg/g—confirming that the method has good accuracy. Regarding cholesterol and its metabolites, other authors have evaluated accuracy in feces, obtaining good recoveries [10, 11, 18]. As far as we know, only Korpela [11] reported PS recovery in feces referred to campesterol (85.4%), stigmasterol (96%) and sitosterol (81.1%). Notably, the present study for the first time evaluates accuracy referred to desmosterol, brassicasterol, lathosterol and sitostanol.

Feces from individuals who ingest a normal diet have a total sterol content of approximately 40 mg/g, with a predominance of cholesterol and its metabolites. Therefore, methods for the determination of sterols in feces have been partially [11, 16, 25] or fully validated [18] primarily for cholesterol and its metabolites. However, feces from diets that have incorporated PS-enriched foods have greater sterol and metabolite contents in feces. In this context, previous studies that validate the determination of fecal sterols after large PS intake have not been found.

The excretion of sterols and their metabolites in subjects with a normal diet (feces 1 and 2) was consistent with the data published in the literature [11, 13–17], except as refers to coprostanone and ethylcoprostanol, whose values were found to be higher. Cholesterol is mainly transformed into coprostanol (30–68%) [13–16], followed in decreasing order by coprostanone (6.8%) [14, 16] and cholestanol (9.8%) [16]—though other minority metabolites have also been identified, such as cholestanone (0.6%) [14], cholesterone and cholestenone [13]. The order of abundance of cholesterol and its metabolites in fecal samples (Table 6) was in concordance with the observations of other studies [11, 14–16, 48].

The PS profile in feces 3 and 4 resembles the composition of PS-enriched beverages. This has also been reported by Weststrate *et al.* [14] in subjects consuming margarine enriched with PS esters (8.6 g PS/day), and by Lubinus *et al.* [24] in feces from subjects ingesting three different fermented milks enriched with PS esters (2 g PS/day). Weststrate *et al.* [14] reported that PS-enriched margarine intake significantly increased fecal neutral sterol excretion from about 40 mg/g to about 190 mg/g dry weight of feces, i.e., nearly fivefold. In this context, we have also found increases in total sterol content (from 39.9 to 73.1 mg/g dry weight of feces, i.e., 1.8-fold) in feces after the intake of a PS-enriched beverage. This increase was smaller because the mentioned beverage provided a lower daily intake (2 g/day).

It has also been noted that cholesterol excretion in subjects who ingested PS-enriched beverages was higher (from three- to nearly fivefold), probably due to the impact of PS upon cholesterol absorption in the small intestine. In this sense, the conversion of cholesterol into its metabolites has been considered to be of interest in studies on neutral sterols in feces and colon carcinogenesis [11, 47]. An increase in PS intake could reduce or block the biotransformation of cholesterol, thereby resulting in a lesser production of coprostanol, coprostanone, cholestanone and cholestanol—compounds that increase the risk of colon cancer [19, 47]. Animal and epidemiological studies suggest that the colon cancer risk

is lowered by the consumption of PS, due to the presence of lower concentrations of these metabolites in the colon [14, 19, 20].

Plant sterols are also transformed by the microbiota through an effect similar to that occurring for cholesterol [4, 10]. Accordingly, sitosterol and campesterol are mainly transformed into ethyl- and methyl-coprostanol, and then into ethyl- and methyl-coprostanone, respectively. In the case of stigmasterol, the microbial metabolic route has not been fully clarified, though the evidence suggests that it might be similar to the mechanisms commented above (i.e., transformation into ethylcoprostenol and ethylcoprostanone). An increase in these metabolites has been recorded after ingestion of the PS-enriched beverage, in accordance to the results obtained by Weststrate *et al.* [14].

We have developed this method to assess the effect of consumption of a high dose of PS on neutral sterol excretion. Certain animal sterols have been suggested to play a role in the development of colon cancer in humans [19–21]. Therefore, it was considered interesting to apply this method to the determination of sterols and their metabolites in feces from subjects with normal PS intakes and in feces from subjects consuming PS-enriched foods. In this sense, the literature describes that the population is characterized by low and high converters of cholesterol—most subjects showing high conversion of cholesterol (>50%) [16]. Wilkins and Hackman [46] determined fecal sterols (animal and plant) in healthy individuals, finding low- (cholesterol 10%, sitosterol 4%) and high-conversion subjects (cholesterol 88%, sitosterol 83%), and suggested that this situation is due not only to the composition of the microbiota but also to the physical state of the cholesterol molecule in the lumen associated to differences in transit between individuals. In omnivorous and vegetarian subjects, Korpela and Aldercreutz [48] recorded similar cholesterol conversion percentages of 75 and 66%, respectively, and reported that the higher incidence of colon cancer observed in omnivores *versus* vegetarians cannot be attributed to increased conversion of cholesterol into its metabolites. On the other hand, Perogambros *et al.* [47] suggested that there is still no clear relationship between fecal sterol metabolites and colon cancer risk, with the identification of low- and high-conversion subjects after the determination of fecal sterols in both healthy individuals and cancer patients. Thus, the wide conversion range in PS (sitosterol, campesterol and stigmasterol) would indicate that subjects could be high or low converters, in the same way as has been described for sitosterol (4–83%) [46]. However, this possibility needs to be confirmed with a more representative number of subjects.

Conclusions

The method has been validated and is suitable for determining sterols and their metabolites over a wide range of concentrations. Because the proposed method has advantages such as the use of lower solvent volumes than indicated in other methodologies, with a relatively simple and relatively short sample preparation phase, we propose this method for determining sterols and their metabolites in feces containing high and low PS concentrations. Using small amounts of sample and solvent, we have been able to determine 17 fecal sterols, thus underscoring the cost-effectiveness of the method. Furthermore, the described method affords high sensitivity, precision and accuracy in application to feces from subjects who consume a normal diet and a diet supplemented with PS.

This study reports contents of plant sterols and their metabolites after the ingestion of a PS-enriched beverage. These preliminary data should be confirmed by studies involving a greater number of subjects, and considering the existence of high converters not only of cholesterol but also of sitosterol and stigmasterol.

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Plant sterols and human gut microbiota relationship: an *in vitro* colonic fermentation study

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ABSTRACT

Due to the preventive effect that plant sterols could have in relation to colon cancer and the scarce information available on plant sterols-gut microbiota interaction, we evaluate the sterols influence upon gut microbiota and *viceversa*. *In vitro* colonic fermentation using a residue from the *in vitro* digestion of a plant sterol-enriched beverage were used. Faecal sterols by GC–MS, and gut microbiota using DNA sequencing were determined. A higher plant sterols metabolism and lower for cholesterol in presence of plant sterols was occurred. Neutral plant sterols decreased and its metabolites increased during fermentation times. The global changes in microbial communities were associated to fermentation time regardless the sterol supplementation. Notwithstanding, plant sterols decreased the proportion of *Erysipelotrichaceae* species and increased the abundance of phylotypes associated with *Eubacterium hallii.* The study confirms a higher plant sterols metabolism against cholesterol by gut microbiota. Plant sterols could help to increase the beneficial species abundance.

1. Introduction

The habitual plant sterols (Ps) intake in the diet (400–600 mg/day) does not reach the established levels with hypocholesterolemic properties (Gylling et al., 2014). The European Food Safety Authority has concluded that the daily intake of 2–2.4 g of Ps added to fat-based foods and low-fat foods such as milk and yoghurt can lower serum LDL-cholesterol by an average of 9%. However, the magnitude of the cholesterol-lowering effect may differ in other food matrices (EFSA, 2008). In addition, Ps and stanol esters at a daily intake of 3 g (range 2.6–3.4 g), with sterols/stanols in matrices such as yellow fat spreads, dairy products, mayonnaise and salad dressings, lower LDL-cholesterol by 11.3% (EFSA, 2012). Therefore, the mentioned levels can only be achieved by incorporating Ps-enriched foods to the usual diet.

The absorption of these bioactive compounds is low (2–3%) (Garcia-Llatas & Rodriguez-Estrada, 2011) and reach the colon. However, the faecal contents of Ps and its metabolites are low respect to cholesterol in individuals with a normal/Western diet (Batta et al., 1999; Batta et al., 2002; Keller & Jahreis, 2004; Korpela, 1982; Shah et al., 2007; Wu, Hu, Yue, Yang & Zhang, 2009) although the faecal Ps contents increase after the consumption of Ps-enriched foods (Lubinus, Barnsteiner, Skurk, Hauner, & Engel, 2013; Weststrate, Ayesh, Bauer-Plank, & Drewitt, 1999).

Cholesterol and Ps can be biotransformed by colonic bacteria (Keller & Jahreis, 2004). In the human large intestine, cholesterol transforms into 5β-cholesterol and to a lesser degree into 5α-cholesterol through the action of the microbiota (Huang, Rodriguez, Woodward & Nichols, 1976), with the production mainly of coprostanol (Dam, 1934a). It has been suggested that there are two routes in the microbial degradation of cholesterol to coprostanol and another route to cholestanol. The first indirect pathway is characterized by cholesterol oxidation and transformation into 5-cholesten-3-one (cholesterone), followed by isomerization to form 4-cholesten-3-one (cholestenone), after which dehydrogenation leads to coprostanone with subsequent reduction of the 3-keto group to yield coprostanol (Huang et al., 1976; Ren, Li, Schwabacher, Young & Beitz, 1996)- and these latter compounds can transform slightly into epicoprostanol (Rosenheim & Webster, 1943). In this sense, in a study with rat faeces it has been seen that oxidation of cholesterol into cholestenone probably involves removal of the 3α-hydrogen as a rate-limiting step (forming cholesterone), followed by isomerization of the Δ5-double bond to a Δ4-double bond via a mechanism involving partial transfer of hydrogen from the 4β-position to the 6

Abbreviations: Ps, plant sterols; BR, beverage residue; IS, internal standard; OTUs, operational taxonomic units; TMSE, trimethylsilyl ether

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position (Bjorkhem, Wrange & Gustafsson, 1973). Besides, it has been isolated and characterizated *Bacteroides* sp. Strain D8 of human intestinal origin, which has a strong capacity to reduce cholesterol to coprostanol by aforementioned pathway (Gerard et al., 2007). In the second pathway, coprostanol forms through direct reduction of the double bond (in position 5–6) of cholesterol. Bjorkhem & Gustafsson (1971) indicated that different microorganisms catalyse the cholesterol conversion into coprostanol through the first and second pathways. Wong (2014) proposed a third pathway where 4-cholesten-3-one (cholestenone) transforms into 5α-cholestan-3-one (cholestanone) and then into cholestanol.

In a way similar to cholesterol, Ps are also transformed by the gut microbiota into phytostenone and phytostanone intermediates, and subsequently into phytostanols (Eneroth, Hellström & Ryhage, 1965; Eyssen, Parmentier, Compernolle, Pauw, & Piessens-Denef, 1973; Miettinen, 1982). In this regard, sitosterol is transformed into ethylcoprostanol and ethylcoprostanone similarly to campesterol, which is transformed into methylcoprostanol and methylcoprostanone (Eneroth, Hellström & Ryhage, 1965). Sterols and its metabolites have been detected in human faeces after the intake of a western diet (Arca, Montali, Ciocca, Angelico & Cantafora, 1983; Batta et al., 1999; Batta et al., 2002; Eneroth, Hellström & Ryhage, 1964; Huang et al., 1976; Keller & Jahreis, 2004; Shah et al., 2007; Olejníková et al., 2017). Sterols biotransformation has been widely studied in *in vitro* assays using microbial inocula from human faeces (Dam, 1934a; Dam, 1934b; Rosenfeld, Fukushima, Hellman & Gallagher, 1954; Snog-Kjaer, Prange & Dam, 1956) or pure cultures of human enteric microorganisms such as *Eubacterium* spp. (Eyssen, et al., 1973; Eyssen & Parmentier, 1974; Ren et al., 1996), *Clostridium perfringens*, *Bifidobacterium* spp., *Escherichia coli*, or *Enterobacter aerogene* (Snog-Kjaer et al., 1956) and *Bacteroides* sp. (Gerard et al., 2007). In this context, it has been proposed that there are high (> 50% sterol conversion percentage) and low (< 50% sterol conversion percentage) sterols converters (Midtvedt et al., 1990; Veiga et al., 2005; Wilkins & Hackman, 1974).

Studies on neutral sterols in faeces and colon carcinogenesis has evidenced the implication of cholesterol metabolites (Hill & Aries, 1971; Korpela, 1982; Perogambros, Papavassiliou & Legakis, 1982; Reddy, Martin & Wynder, 1977). Perogambros et al. (1982) and Reddy, Mastromarino, & Wynder (1977) have evidenced that an increase in Ps intake could reduce the excretion of cholesterol metabolites. Besides, animal and epidemiological studies have associated the consumption of Ps and vegetarian diets with the low incidence of colon cancer, respectively, due to the lesser presence of cholesterol metabolites in the colon (Arul, Al Numair, Al Saif & Savarimuthu, 2012; Deschner, Cohen & Raicht, 1982; Hill et al., 1971; Hill & Aries, 1971; Raitch, Cohen, Fazzini & Sarwal, 1980). *In vivo* studies in subjects following a vegan diet have found only slight changes in the bacterial anaerobic genera (*Bifidobacterium, Bacteroides and Clostridium*) (van Faassen et al., 1987). However, after the consumption of plant stanol ester-enriched margarines (3.0 g/day), significant changes in microbiota composition have not been observed (Baumgartner et al., 2017).

As far as we know, after Ps-enriched foods intake, only two studies (margarine and fermented milk) evaluate the excretion of dietary sterols (Lubinus, Barnsteiner, Skurk, Hauner, & Engel, 2013; Weststrate, et al., 1999) and only one study carried out with margarine evaluate the changes in the microbial population (Baumgartner et al., 2017). The aim of this study was evaluate the transformation of Ps by gut microbiota and the impact of Ps on colonic bacterial population, through an *in vitro* fermentation assay using a residue obtained after *in vitro* simulated gastrointestinal digestion of a Ps-enriched milk based fruit beverage.

2. Materials and methods

2.1. In vitro colonic fermentation

In order to evaluate the influence of Ps upon the human gut microbiota, we conducted an *in vitro* colonic fermentation study using a beverage residue (BR) obtained after simulated gastrointestinal digestion of a Ps-enriched milk based fruit beverage (2g/250 mL) (Alvarez-Sala et al., 2016). The BR constitutes the non-bioaccessible fraction (non-soluble or non-absorbable), which allows better reproducing *in vivo* conditions relative to the concentrations of sterols that would reach the colon within a food matrix. This non-bioaccesible fraction were stored at −20 °C until *in vitro* fermentation assays. In addition, in accordance with the method of Cuevas-Tena, Alegría & Lagarda (2017), the BR was characterized (total sterols mg/g residue: 46.9 mg, of which 80.2% corresponded to sitosterol, 11.6% to sitostanol, 5.4% to campesterol, 1.3% to campestanol, 1.0% to stigmasterol and 0.5% to cholesterol). The starter material for BR fermentation consisted of pooled human faeces prepared with equal amounts from 5 healthy donors with an age of 32.4 ± 14.8 years. The donors had not been treated with antibiotics or statins, and had not consumed Ps-enriched foods or similar supplements during three months prior to faeces collection. Human faeces from donors were collected in tightly closed sterile plastic containers under anaerobiosis and processed in < 12 h (Sanz et al., 2007) and the fermentation starter material (faeces inoculum) consisted of 6 g of pooled faeces suspended in 24 mL PBS and homogenized during 1 min in a Stomacher system (Saulnier, Gibson & Kolida, 2008). An amount of pooled faces was lyophilized to determine the fresh/freeze-dry conversion ratio. In addition, a small aliquot of the homogenized starter material was stored at −20 °C, and after the respective DNA isolation method to study microbial communities together the fermented samples, it was used as reference of the initial microbiota composition.

Beverage residue fermentation assaying was performed in laminar flow hood by three independent assays using 1.5 mL of started material and 15 mL of sterile Brain & Hearth (BH) culture medium (Scharlau Chemie, S.A., Barcelona, Spain) supplemented with 33 mg BR (~1.5 mg Ps). This amount of Ps used for *in vitro* colonic fermentation corresponded to the usual faecal Ps concentration, since the minimum and maximum levels of faecal sitosterol after the intake of normal diets with or without enriched foods is between 1.5 and 54 mg sitosterol/g of lyophilized faeces, respectively (Weststrate et al., 1999). In addition, taking into account that the fresh faeces concentration in assay was 0.025 g/mL of liquid fermentation, it was assumed that the concentration of freeze-dry faeces within the assay was 0.005 g of freeze-dry faeces/mL of fermentation liquid. Therefore, the theoretical amount of sitosterol in the assay would be 0.11–4.1 mg. The BH culture medium was tested before the addition of BR, and we found no traceable Ps content in it – thus defining BR as the sole source of Ps. Two controls were evaluated: 1. – Culture medium with faeces inoculum; and 2. – Culture medium with BR. The fermentation process was carried out in 50-mL sterile tubes incubated in an anaerobic chamber (DG250 don Whitley Scientific®, Yorkshire, UK) at 37 °C for 24 and 48 h (Saulnier, et al., 2008). Aliquots of the respective treatments (Ps sample and controls) over time (24 and 48 h) were recovered and stored at −20 °C until sterols quantification by GC–MS and DNA processing for the determination of microbiota composition.

2.2. Sterols and metabolites determination by GC–MS

Fermentation assays (Ps sample and controls 1 and 2) in aliquots of 0.1 and 0.5 mL with 20 μg of internal standard (IS) were saponified with ethanolic potassium hydroxide (0.71 M) at 65 °C during one hour according to Cuevas-Tena, et al. (2017). Then, the unsaponifiable fraction was extracted three times with n-hexane and centrifuged (2608*g*/18 °C/10 min); all n-hexane fractions were combined in conical tubes (of

capacity 10 mL) and evaporated to dryness under nitrogen. Then, 200 μL of derivatizing reagent (BSTFA + 1% TMCS:pyridine, 10:3 v/v prepared weekly) was added for the formation of trimethylsilylether (TMSE) derivatives at 65 °C during one hour. In order to eliminate the interferences of the derivatizing reagent, the sample was re-dissolved with 1 mL of n-hexane and filtered (three times) through Millex® FH filters previously activated with 1 mL of hexane. The samples were subsequently evaporated to dryness under nitrogen and redissolved in 40 μL of n-hexane. The Pyrex glass tubes with a conical bottom, employed for derivatization, were previously silanized with derivatization solution in order to avoid the adsorption of TMSE derivatives (Guardiola, Codony, Rafecas & Botella, 1995).

Identification and quantification were performed by GC–MS with ion trap ITQ, GC Thermo Science Trace® GC-Ultra, using an Xcalibur data processor, equipped with precolumn VSD Tubing (2m × 320 μm) (SGE Analytical Science, Milton Keynes, UK) and a capillary column CP-Sil8 CB low bleed/MS (50 m × 25 mm × 0.25 μm film thickness) (Agilent Technologies®, CA, USA) with hydrogen (1 mL/min) as carrier gas.

One μL of derivatized samples was injected in PTV splitless mode (initial temperature 80 °C, with a heating rate of 14.5 °C/s to 280 °C during 5 min). The oven temperature was 150 °C, and after 3 min the temperature was raised to 280 °C at 30 °C/min and then to 295 °C at 10 °C/min for 10 min. The mass spectrometer operated in the electron impact ionization mode at −70 eV, and a mass range from 50 to 650 m/z was scanned.

Standards used were: cholestane (5α-cholestane, purity ≥ 97%) as IS, cholestanol (5α-cholestan-3β-ol, ≥95%), cholesterol (5-cholesten-3β-ol, 99%), coprostanol (5β-cholestan-3β-ol, 98%), cysteine, desmosterol (5,24-cholestadien-3β-ol, 84%), epicoprostanol (5β-cholestan-3α-ol, ≥95%), lathosterol (5α-cholest-7-en-3β-ol, 99%), stigmastanol (3β-5α-stigmastan-3-ol, ≥95%), stigmasterol (stigmasta-5,22-dien-3β-ol, 95%), and β-sitosterol (5-stigmasten-3β-ol, 98%), purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Brassicasterol (5,22-cholestandien-24β-methyl-3β-ol, 98%), campesterol (24α-methyl-5-cholesten-3β-ol, ≥98%), and coprostanone (5β-cholestan-3-one, 98%) were obtained from Steraloids Inc. (Newport, RI, USA). Individual standard solutions with ethanol at 100 μg/mL for cholestane, cholesterol, coprostanol or coprostanone, and at 10 μg/mL for cholestanol and epicoprostanol, were prepared. Campesterol, sitosterol, stigmastanol or stigmasterol (100 μg/mL), brassicasterol (20 μg/mL), desmosterol (10 μg/mL) or lathosterol (10 μg/mL) were prepared with n-hexane:2-propanol 3:2 (v/v). All glass test tubes and vials were scrupulously cleaned, rinsed with distilled water, and introduced in a Heraeus Thermicon K1253A oven at 450 °C during 8 h to remove contamination.

Changes in sterols abundance and its resultant metabolites after 24 and 48 h of colonic fermentation respect controls (control 1 + control 2) were evaluated by Student t-test ($\alpha = 0.05$) using Statgraphics Centurion XVI.I package.

2.3. Faecal DNA isolation, PCR, sequencing and data analysis

The isolation of DNA from the fermentation assays was carried out using the Stool Total RNA Purification Kit (Norgen Biotek Corp. Thorold, Ontario, Canada), with some modifications with respect to the instructions of the manufacturer. Briefly, we included an enzymatic pretreatment step with lysozyme (0.05 mg enzyme/100 mg faeces) (Sigma-Aldrich) and mutanolysin (40 U enzyme/100 mg faeces) (Sigma-Aldrich) for 60 min at 37 °C. An incubation step was also included with 2 μL of RNAse A per sample (Epicentre) for 30 min at 37 °C before the column washing step, together with a purification step using phenol: chloroform: isoamyl alcohol (25:24:1). For DNA recovery, we used Magnetic Beads AMPure XP (Life Technologies, Carlsbad, CA, USA), and the subsequent elution step was performed with Tris-HCl buffer 10 mM (pH 8.5).

The V4-V5 hypervariable regions from bacterial 16S rRNA gene were amplified using 20 ng of DNA and 25 PCR cycles at 95 °C for 20 s, 40 °C for 30 s, and 72 °C for 20 s. Phusion High-Fidelity Taq Polymerase (Thermo Scientific) and the 6-mer barcoded primers S-D-Bact-0563-a-S-15 (AYTGGGYDTAAAGNG) and S-D-Bact-0907-a-A-20 (CCGTCAATTYMTTTRAGTTT), which target a wide range of bacterial 16S rRNA genes (Klindworth et al., 2012), were used during PCR. Dual barcoded PCR products consisted of ~380 bp, were purified from triplicate reactions by using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), and then quantified through Qubit 3.0 and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). Samples were multiplexed by combining equimolar quantities of amplicon DNA (100 ng per sample) and sent to Eurofins Genomics GmbH (Ebersberg, Germany) to perform Illumina MiSeq high-throughput sequencing with 2 × 300 PE configuration. Raw DNA data were obtained in fastq files. Pair ends with quality filtering were assembled using Flash software (Magoc & Salzberg, 2011). Sample de-multiplexing was carried out using sequence information from respective DNA barcodes and the Mothur v1.36.1 analysis suite (Schloss et al., 2009). After assembly, de-multiplexing, and barcodes/primers removal, we obtained more than 870 k reads to analyze be processed for chimera removal using the Uchime algorithm (Edgar, Haas, Clemente, Quince & Knight, 2011) and SILVA reference set of 16S sequences (Quast et al., 2013). After the chimera detection step, we removed approximately the 12.5% of reads in average retaining more than 760 k reads (63.5 k reads per samples in average) for further processing steps. Diversity indexes were calculated with Mothur using default parameters and average method in the clustering step. Consequently, alpha diversity parameters such as the Chao's richness and Shannon evenness and reciprocal Simpson index were computed using a high quality and a normalized subset of 15,000 sequences per sample, randomly selected after shuffling (10,000×) of respective and original datasets. Taxonomy assessment was performed using the RDP classifier v2.12 (Wang, Garrity, Tiedje & Cole, 2007) to analyze differential abundance in phylum and family proportions. The Operational Taxonomic Units (OTUs)-picking approach was adopted with the normalised subset of 15,000 sequences and the clustering algorithm implemented in USEARCH v8.0.1623 (Edgar, 2010). Non-parametric Kruskal-Wallis and Wilcox Rank statistical tests implemented in LEfSe server (Segata et al., 2011) were used to assess differences in the control 1 and Ps-containing samples, while the Mann-Whitney-Wilcox test implemented in R v3.2.3 (http://cran.r-project.org) was used to evaluate differences in alpha diversity parameters. Additionally, fermentation mode (Ps or control 1) and time (24 h, 48 h) condition information were used as categorical variables in beta diversity approaches based on the Bray-Curtis dissimilarity index and Permanova analysis through QIIME analysis suite (Caporaso et al., 2010).

3. Results

3.1. Influence of colonic fermentation upon sterols

Table 1 shows the animal origin sterol contents in faeces used in the fermentation assay (control 1, 24 and 48 h), which represent > 67% of total sterols. The predominant sterols were cholesterol (10–12%) and its metabolites coprostanol (41–44%), coprostanone (9–11%) and cholestanol (> 3%).

Ps and its metabolites represented > 29% of total sterols (see Table 2), the order of abundance being: sitosterol > stigmasterol > brassicasterol > campesterol. As sitosterol metabolites we identified sitostanol (> 2%) - part of which can come from diet - ethylcoprostanol (> 10%) and ethylcoprostanone (> 2%). Other Ps, such as campesterol (< 1%), campestanol (> 2%), stigmasterol (> 2%) and brassicasterol (1.8%) showed to be lower.

In addition, Tables 1 and 2 show the mean contents (mg sterol/g BR) of sterols and metabolites present in the sample after 24 and 48 h of colonic fermentation and for each fermentation time the net increments

Table 1
Animal sterol contents (mg sterol/g BR) in residue from plant sterol-enriched milk based fruit beverage (BR) after *in vitro* colonic fermentation assay at 24 and 48 h.

Sterol	24 h				48 h			
	Control 1	Control 2	Sample	Net increment (mg/g)	Control 1	Control 2	Sample	Net increment (mg/g)
Cholesterol	9.70 ± 0.28	0.31 ± 0.02	7.50 ± 0.13	−2.51 ± 0.13	7.32 ± 0.46	0.32 ± 0.04	7.30 ± 0.16	−0.35 ± 0.15
Coprostanol	33.9 ± 2.02	–	27.72 ± 0.90	−6.18 ± 0.87	32.1 ± 1.85	–	30.80 ± 0.81	−1.31 ± 0.81
Coprostanone	8.88 ± 0.22	–	6.08 ± 0.30	−2.80 ± 0.29	6.67 ± 0.51	–	6.55 ± 0.18	−0.14 ± 0.18
Cholestanol + methylcoprostanol	2.60 ± 0.05	–	2.36 ± 0.13	−0.24 ± 0.13	2.40 ± 0.04	–	2.55 ± 0.01	−0.15 ± 0.01
Lathosterol	0.76 ± 0.01	–	0.71 ± 0.05	0.05 ± 0.05	0.72 ± 0.01	–	0.72 ± 0.01	0.00 ± 0.01

Data are the means of three independent analyses ± standard deviations (n = 3). Control 1: culture medium + faeces inoculum; control 2: culture medium + BR; sample: culture medium + faeces inoculum + BR. Net increment: sample – (control 1 + control 2).

were calculated as the difference between [sample - (control 1 + 2)] (see Tables 1 and 2).

There was a decrease in the contents of sitosterol, sitostanol, campesterol, campestanol, stigmasterol and brassicasterol during colonic fermentation (Table 2), with no differences between 24 and 48 h. Statistically significant differences ($p < 0.05$) were observed between 24 and 48 h of fermentation referred to the mean contents of ethylcoprostanol in the sample. The reductions at 24 h (see Table 2) were more pronounced for sitostanol (21%) than for sitosterol (8%), while similar reductions were observed after 48 h (16% vs 14%). However, in the case of campesterol and campestanol, similar reductions were observed at 24 h (19% vs 17%), with a more marked reduction for campesterol at 48 h (39% vs 15%) which showed statistically significant difference ($p < 0.05$). In the case of stigmasterol, the reductions at 24 and 48 h were similar (17% and 19%). The difference in the percentage reductions of brassicasterol at 24 and 48 h (10% vs 1%) were influenced by the low contents of this compound. This decrease in sterols with respect to unfermented sample (control 2) was correlated to an increase in ethylcoprostanol (18% and 50%), ethylcoprostanone (16% and 39%), methylcoprostanone (63% and 21%) and stigmastenol (13% and 16%) at 24 and 48 h, respectively (see Table 2). Unlike in the case of Ps, there was no formation of cholesterol metabolites after 24 and 48 h of fermentation (see Table 1), although the faeces donors were high converters (cholesterol conversion percentage at 24 (82%) and 48 h 84%, control 1) (Wilkins & Hackman, 1974).

3.2. Plant sterol influence upon the gut microbiota

After processing and obtaining almost 200 k high quality DNA readings for taxonomy analyses regarding the impact of Ps upon the gut microbiota, we performed an initial analysis to determine whether Ps significantly alter alpha diversity parameters such as richness (Chao1 index), evenness (Shannon Evenness), or diversity as a whole (Reciprocal Simpson's index). We obtained similar results for all the alpha diversity descriptors analyzed in control 1 (faeces inoculums and culture medium) and Ps samples. Comparisons at phylum and family level in order to distinguish microbial species potentially affected by the presence of Ps showed no differential abundances in phyla distribution when control 1 and sterol-containing samples were compared, however at family level we found that sterol supplement decreases the abundance of the *Erysipelotrichaceae* family of Firmicutes (LDA = 3.19; $p < 0.0250$), even when time (24 h and 48 h fermentation) was included as covariate (Fig. 1).

The OTU abundance was similarly assessed in control 1 and Ps samples. Combination of treatment and time variables did not produce any significant change in any OTU, however, when we only compared samples per treatment (control 1 vs sterol, thus combining 24 and 48 h samples), we found differential abundances in 14 OTUs, of which OTU27, OTU103, OTU107, OTU128, OTU214, OTU394 and OTU536 where associated with control samples, whereas OTU38, OTU51, OTU52, OTU92, OTU104, OTU157 and OTU208 where associated with Ps samples ($p \leq 0.05$, LDA score ≥ 2.5). We attempted to establish the respective identifications of such OTUs through Blast-based searches against the non-redundant 16S database at NCBI (https://blast.ncbi.nlm.nih.gov/), but only retrieved confident identifications for OTU92 and OTU103 respectively matching with *Eubacterium hallii* (alignment length = 100%, identity = 98%) and *Flavonifractor plautii* species (alingment length = 100%, identity 100%). Similarly, the abundances of some members of the uncharacterized S-24 family of *Bacteroidetes* were also promoted by Ps presence in the fermentation assay (OTU51 and OTU52). In contrast, proportions of the flavonoid-converting bacterium *Flavonifractor plautii* (OTU103), as well as proportions of *Allobaculum spp*. (OTU27), a member of the *Erysipelotrichaceae* family, were significantly attenuated. The respective abundance of OTUs with certain taxonomy identifications and those with the top LDA scores (≥ 3.0 for OTU27, OTU38, OTU51 and OTU52) are shown in Fig. 2.

Finally, we sought to determine whether Ps could drastically change the structure of the microbial community of starter material during the

Table 2
Plant sterol contents (mg sterol/g BR) in residue from plant sterol-enriched milk based fruit beverage (BR) after *in vitro* colonic fermentation assay at 24 and 48 h.

Sterol	24 h				48 h			
	Control 1	Control 2	Sample	Net increment (mg/g)	Control 1	Control 2	Sample	Net increment (mg/g)
Sitosterol	5.16 ± 0.10	32.15 ± 4.40	34.28 ± 2.20	−3.03 ± 2.20	4.66 ± 0.17	30.61 ± 1.06	30.28 ± 0.31	−5.00 ± 0.31
Sitostanol	2.10 ± 0.01	4.76 ± 0.53	5.45 ± 0.16	−1.41 ± 0.16	2.02 ± 0.02	4.32 ± 0.16	5.34 ± 0.11	−1.00 ± 0.11
Ethylcoprostanol	8.53 ± 0.30	–	10.10 ± 0.40	1.55 ± 0.40	7.90 ± 0.32	–	11.86 ± 0.42*	4.00 ± 0.42
Ethylcoprostanone	1.50 ± 0.04	–	1.74 ± 0.18	0.24 ± 0.18	1.10 ± 0.04	–	1.53 ± 0.16	0.43 ± 0.16
Campesterol	0.70 ± 0.02	2.92 ± 0.20	2.92 ± 0.26	−0.70 ± 0.26	0.50 ± 0.03	3.90 ± 0.24	2.72 ± 0.26	−1.72 ± 0.26
Campestanol	2.21 ± 0.01	0.68 ± 0.03	2.40 ± 0.10	−0.50 ± 0.09	2.11 ± 0.03	0.75 ± 0.04	2.45 ± 0.06*	−0.42 ± 0.06
Methylcoprostanone	0.12 ± 0.02	–	0.20 ± 0.02	0.08 ± 0.02	0.12 ± 0.01	–	0.15 ± 0.01	0.03 ± 0.01
Stigmasterol	1.73 ± 0.03	0.55 ± 0.02	1.90 ± 0.10	−0.40 ± 0.10	1.70 ± 0.03	0.60 ± 0.04	1.87 ± 0.01	−0.43 ± 0.01
Stigmastenol	2.26 ± 0.03	–	2.56 ± 0.07	0.30 ± 0.07	2.20 ± 0.03	–	2.55 ± 0.06	0.35 ± 0.06
Brassicasterol	1.38 ± 0.02	–	1.25 ± 0.08	−0.13 ± 0.08	1.32 ± 0.02	–	1.31 ± 0.01	−0.01 ± 0.01

Data are the means of three independent analyses ± standard deviations (n = 3). Control 1: culture medium + faeces inoculum; control 2: culture medium + BR; sample: culture medium + faeces inoculum + BR. Net increment: sample – (control 1 + control 2); *statistical significant difference between controls (1 + 2) and sample. Student *t*-test ($p < 0.05$).

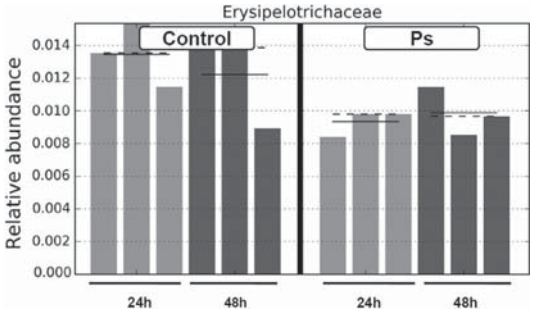

Fig. 1. Relative abundance of DNA reads assigned to the *Erysipelotrichaceae* family. Distribution of reads in control 1 (faeces inoculum and culture medium) and plant sterols (Ps) groups is shown respectively at left and right of the plot with sub-stratification by fermentation time (light grey = 24 h, dark grey = 48 h). Black solid lines indicate average of distribution whereas black dashed lines indicate median values. Linear discrimination analysis (LDA).

in vitro fermentation process. For this purpose we used the OTUs abundance information and beta diversity descriptors such as the Bray-Curtis dissimilarity index. When a principal coordinate analysis (PCoA) was achieved with the obtained Bray-Curtis dissimilarity index, we observed that PC1 and PC2 dimensions explain more than 80% of the variability, and that PC1 clearly indicates that microbiota profiles are more strongly associated to time than Ps condition (Fig. 3). By using permutation analysis (Permanova) and categorical variables such as Ps condition (control 1 vs Ps-sample) and time (24 h vs 48 h), we found that only time explains significant changes in the microbial profiles observed for all samples (pseudo-F test = 20.7; $p \leq 0.0030$).

4. Discussion

The relative percentages of animal sterols, sitosterol and ethylcoprostanol in faeces inoculum (control 1) were consistent with the data reported in the literature (Batta et al., 1999; Batta et al., 2002; Keller & Jahreis, 2004; Shah et al., 2007; Weststrate et al., 1999; Wilkins & Hackman, 1974). Slight differences were observed on comparing the relative percentages of campesterol and campestanol with those reported by other authors (campesterol: 1.7–7.8%; campestanol: 2.6–7.4%) (Batta et al., 1999; Batta et al., 2002; Keller & Jahreis, 2004; Weststrate et al., 1999). The stigmasterol contents were similar to those reported by Shah et al. (2007) and Weststrate et al. (1999), and lower than those obtained by Batta et al. (1999) (4.8%). Our relative percentage of brassicasterol was higher than the only value found in the literature corresponding to human faces (0.1%) (Weststrate et al., 1999). In the case of lathosterol (as cholesterol precursor, 0.9%), methylcoprostanone (campesterol metabolite, 0.2%), stigmastenol (stigmasterol metabolite 3%), sitostanol (> 2%) and ethylcoprostanone (sitosterol metabolite, > 2%) we found no data from other studies.

The fact that cholesterol metabolites do not increase in the sample after fermentation for 24 or 48 h (Table 1), suggests that bacterial groups would prefer to use Ps as a substrate because they are more closely related to cholesterol. This is in concordance with the study carried out by Weststrate et al. (1999), where the intake of a margarine enriched with Ps (8.6 g/100 g) in adults did not result in an increase in the concentrations of neutral sterols, and reduced the amount of cholesterol metabolized in the form of coprostanol.

Agree with results obtained in this study, in previous studies in rats following a diet with sitosterol (0.8%, w/w) supplementation, has been seen a reduction in cholesterol biotransformation into coprostanol compared to a diet containing cholesterol (1.2%, w/w) (Cohen, Raicht & Mosbach, 1974). In addition, it is known that a reduction of dietary fat modifies the composition of faecal neutral sterol metabolites and the rate of cholesterol metabolism. In healthy volunteers who consume diets with less than 30 g of fat/day, a rapid decrease of approximately 70% in faecal bile acid levels within a week has been reported, together with a less pronounced decrease in faecal neutral sterols - though a 40% decrease is reached at the end of the study (Hill, 1971). The metabolic rate of faecal cholesterol (coprostanol + coprostanone/cholesterol ratio) has been observed to be lower in individuals consuming vegetarian diets than in western diets (1.25–1.46 vs 2.17–2.49) (Hill & Aries, 1971). In contrast, an intense conversion of cholesterol (88%) into coprostanol and coprostanone has been detected in individuals who ingest a western diet rich in animal fat, though a small number of subjects exhibited lesser cholesterol conversion (10%). These authors thus indicated that there are both high (> 50%) and low converters (< 50%). If the conversion rate is associated to colon cancer, then these two population groups must exhibit different risk levels (Wilkins & Hackman, 1974).

Plant-rich diets are associated with a low incidence of colon cancer, as it has been shown that the excretion of animal-derived neutral sterols and its microbial degradation products is lower in individuals with a vegetarian diet (Korpela & Adlercreutz, 1985; Korpela, Adlercreutz & Turunen, 1988). On the other hand, van Faassen et al. (1987) determined bile acids, neutral sterols and bacterial groups in faeces after the intake of mixed western, lacto-ovo-vegetarian or vegan diets. They found a lower concentration of faecal cholesterol after the intake of such diets, but did not report significant differences in the rate of metabolization to coprostanol of each of them with respect to the mixed western diet.

Some *in vivo* studies in rats suggest that dietary supplementation with Ps reduces the activity of some characteristic intestinal bacteria in carcinogenesis (Arul et al., 2012), the proliferation of cancerous epithelial cells (Deschner et al., 1982), and the development of tumours (Raicht, Cohen, Fazzini, Sarwal, & Takahashi, 1980).

However, other studies in rats have found that supplementation with β-sitosterol (Quilliot et al., 2001) or stigmasterol (Andriamiarina, Laraki, Pelletier & Debry, 1989) results in an increase in cholesterol and coprostanol excretion. In epidemiological studies with mainly vegan diets (Hill & Aries, 1971; Hill et al., 1971), and in a murine study involving a diet supplemented with β-sitosterol (Cohen et al., 1974), a lower concentration of cholesterol and its metabolites was found in faeces. As far as we know, it has not been established whether the microbial metabolites of Ps exert the same pro-carcinogenic effect as the cholesterol metabolites.

Although major changes in microbial communities were associated to fermentation time, probably because the accumulation of anaerobic fermentation products strongly lowered the pH, we were able to distinguish a particular microbial group modulated by the presence of Ps in the BR used to supplement the culture media during the fermentation assay. In effect, our results indicate that Ps influences members of the *Erysipelotrichaceae* family of Firmicutes, with a reduction in the proportion of these bacteria in BR-containing medium. There is evidence on the role of *Erysipelotrichaceae* in human disease related to metabolic disorders (Kaakoush, 2015). In fact, previous studies have confirmed the association between this bacterial family and lipidemic profiles. For example, a series of species belonging to the family *Erysipelotrichaceae* in diet-induced obese animals has been found (Turnbaugh, Backhed, Fulton & Gordon, 2008). In this regard, high levels of *Erysipelotrichaceae* have been observed in obese human individuals (Zhang et al., 2009). Otherwise, hypercholesterolemic hamsters following treatment with grain sorghum lipid extract have shown a decrease in *Erysipelotrichaceae* (Martinez et al., 2009). In another study carried out in hamsters, Martinez et al. (2013) found Ps esters intake to induce shifts in the faecal microbiota, reducing several bacterial taxa of the *Erysipelotrichaceae* family. Besides, the abundance of these taxa displayed remarkably high correlations with host cholesterol metabolites. In addition, a recent analysis has indicated that *Erysipelotrichaceae* species also could be associated with the digestion of dietetic protein and fat

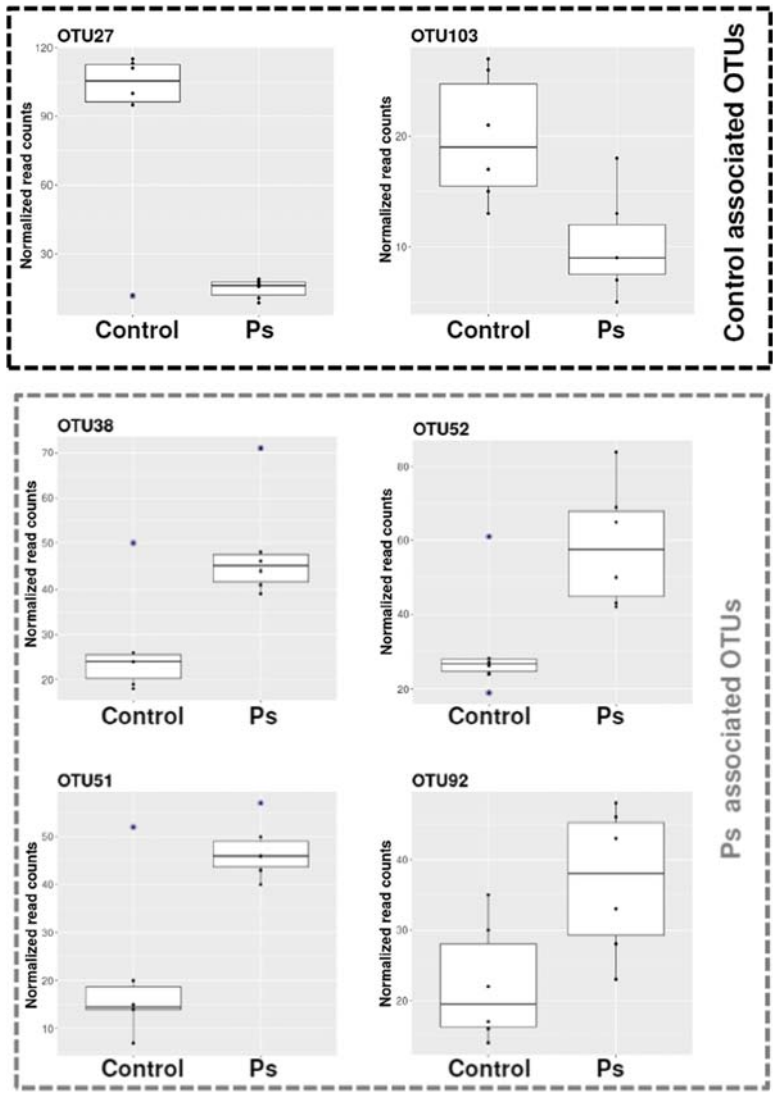

Fig. 2. Gut microbial phylotypes affected by plant sterols (Ps). The abundance distribution per group of the Operational Taxonomic Units (OTUs) with top LDA scores (LDA ≥ 3.00 for OTU27, OTU51, OTU52, OTU38) and with certain taxonomy identification at species level (OTU92 = *Eubacterium hallii* and OTU103 = *Flavonifractor plautii*) are shown in boxplot fashion. OTUs are distinguished as "control associated" and "Ps associated" according to the patterns of abundance in both groups analyzed.

components in dogs (Bermingham, Maclean, Thomas, Cave & Young, 2017).

A detailed analysis was conducted in order to disclose single microbial species or phylotypes potentially also affected by presence of Ps during the *in vitro* fermentation assay. More specific evidence was obtaining when microbial abundances were assessed at OTUs level, showing that species belonging the genus *Allobaculum* (of the *Erysipelotrichaceae* family) were one of the main phylotypes whose abundances were negatively affected by Ps. The proportions of *Flavonifractor plautii* species were modulated by Ps in a way similar to the *Allobaculum* species.

Besides, induced an important increase in the abundance of *Eubacterium hallii*, a recognized butyrate producer belonging to the *Lachnospiraceae* family, butyrate being considered a healthy microbial metabolite (Hamer et al., 2008).

Other authors have attempted to evaluate the influence of Ps upon the gut microbiota, such as van Faassen et al. (1987), who only found slight changes in the anaerobic bacterial genera (*Bifidobacterium, Bacteroides and Clostridium*) in subjects with a vegan diet, which is rich in Ps. In a clinical trial (Baumgartner et al., 2017), where 13 healthy subjects received three weeks of control or plant stanol ester enriched margarine (3g/day plant stanols), no differences in gut microbiota composition were recorded between the two target groups.

5. Conclusions

A low rate of cholesterol conversion was observed in the presence of Ps, which means a lesser presence of cholesterol metabolites. The present study confirms a higher metabolism on Ps against cholesterol by gut microbiota, especially on sitosterol and stigmasterol, since a

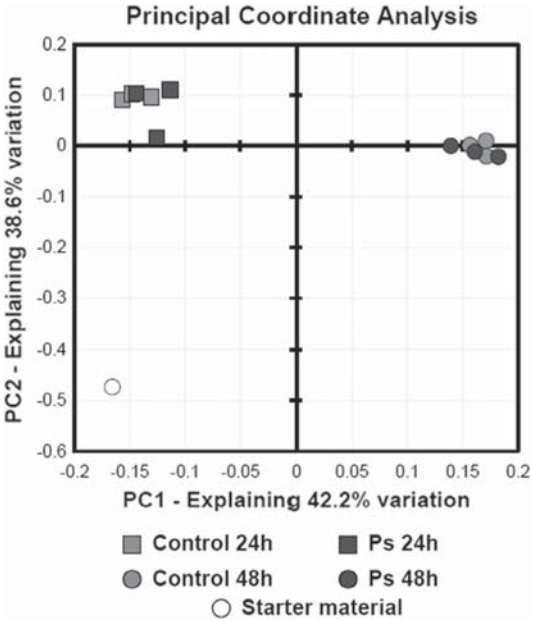

Fig. 3. Principal coordinate analysis (PCoA) of control and plant sterols (Ps) samples. The Operational Taxonomic Units abundance and the Bray-Curtis dissimilarity index were used to perform multidimensional analysis based on PCoA. The PC1 and PC2 dimensions, explaining the largest proportion of variability, were plotted and categorical variables as Ps condition and time for all samples are indicated respectively below the graph.

decrease of them along with an increase of its metabolites was observed. Besides, Ps could help to increase the abundance of beneficial species as *Eubacterium halii* and decrease the members of *Erysipelotrichaceae* family, which has been related to some metabolic disorders. However, futures studies are therefore needed to determine whether Ps and gut microbiota relationship are a direct or indirect effect of the Ps intake, and whether this modulation pattern can be replicated in *in vivo* studies based on controlled dietetic interventions.

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Conflict of interest

The authors declare that they have no conflict of interest.

Availability of data and material

The raw sequences for all samples analyzed in the present study can be freely accessed through the MG-RAST server (Meyer et al., 2008) upon accession number mgp84404.

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Impact of colonic fermentation on sterols after the intake of a plant sterol-enriched beverage: A randomized, double-blind crossover trial

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Randomized Control Trials

Impact of colonic fermentation on sterols after the intake of a plant sterol-enriched beverage: A randomized, double-blind crossover trial

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SUMMARY

Background: Cholesterol microbial transformation has been widely studied using *in vitro* fermentation assays, but less information is available on the biotransformation of plant sterols (PS). The excretion percentage of animal sterols (AS) (67–73%) is considerably greater than that of PS (27–33%) in feces from healthy humans following a Western diet. However, a lower content of AS in feces from subjects following a vegetarian, vegan or low-fat animal diet has been seen when compared to omnivorous subjects. Although only one human study has reported fecal sterol excretion after the consumption of PS-enriched food (8.6 g PS/day), it was found that the target group showed an increase in the excretion of cholesterol and a 57% decrease in its metabolites compared to the control group.

Objective: Evaluation of the impact of a PS-enriched milk based fruit beverage intake on fecal sterol excretion and the microbial conversion of sterols in postmenopausal women with mild hypercholesterolemia.

Methods: Forty postmenopausal women participated in a randomized, double-blind, crossover study with two beverages, with a PS-enriched (2 g PS/day) or without. The women were divided in two groups: 20 women consumed the PS-enriched beverage and the other 20 women consumed a placebo (without PS) beverage for 6 weeks. After a four-week washout period, the type of beverage was exchanged and consumed for another 6 weeks.

Feces were collected at the start (0 and 10 weeks) and end of each intervention period (6 and 16 weeks), and fecal sterols were determined by capillary gas chromatography with mass spectrometry.

Results: The intake of the PS-enriched beverage modified the fecal sterol excretion profile. A significant increase mainly in PS and their metabolites *versus* the placebo intervention period was observed. Although the same effect was not observed in the case of AS, a tendency towards increased cholesterol and decreased coprostanol (the main metabolite of cholesterol) was recorded after PS-enriched beverage intake *versus* placebo. Furthermore, the PS-enriched beverage also modified the microbial conversion of sterols. In this context, an important decrease in the conversion percentage of cholesterol in 16 women (between 11% and 50%) and of sitosterol in 24 women (between 15% and 61%) was observed.

Conclusions: The results obtained suggest that the microbiota could preferably use PS as a substrate, when present in a greater proportion compared with cholesterol. Besides, a lower sitosterol and cholesterol conversion trend would mean that intake of the PS-enriched beverage could modulate the metabolic activity of the gut microbiota. Therefore, further studies on the impact of PS-enriched foods upon gut microbiota modulation are needed.

Clinical Trial Registry Number: NCT 02065024 listed on the NIH website: ClinicalTrials.gov.

Abbreviations: PS, plant sterols; AS, animal sterols; TMSE, trimethylsilyl ethers; ROC, receiver operating characteristic.

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Clinical Trial Registry Name: Food Matrix and Genetic Variability as Determinants of Bioavailability and Biological Effects of Beta-cryptoxanthin and Phytosterols (foodmagenpol).
The full trial protocol is available upon request to the corresponding author.

1. Introduction

The daily dietary intake of plant sterols (PS) (160–400 mg/day) [1] does not reach the established levels to have a hypocholesterolemic effect (1.5–3.0 g/day) [2]. Therefore, several foods (yellow fat spreads, salad dressings, milk type products, fermented milk products, soya drinks, cheese type products, rye bread and rice drinks) may be enriched with PS for this purpose and can also be used in patients with mild hypercholesterolemia.

There is an association between postmenopausal women and higher serum levels of total cholesterol and low-density lipoprotein cholesterol (LDL-C), which could increase the risk of cardiovascular disease [3,4]. In fact, in a previous clinical study (NCT01074723) of our group with postmenopausal women [5], β-critptoxanthin (β-Cx) improved the cholesterol lowering effect of PS when they were supplied simultaneously from a PS-enriched milk-based fruit beverage rich in β-Cx. This combination may also be beneficial in reducing the risk of osteoporosis, suggesting a synergistic effect. The effect observed, however, implies a moderate reduction of c-LDL and total cholesterol, on average 5–7%, which is in the range of other studies using different food matrices.

Fifty percent of dietary cholesterol is absorbed [6], although its absorption varies substantially between individuals (20–80%) [7]. However, the intestinal absorption rate of total PS is only 2–3% [8]. Non-absorbed sterols reach the colon and can be biotransformed by the gut microbiota. Although cholesterol biotransformation mediated by gut microbiota has been widely studied using *in vitro* assays (Wong, 2014) [9] and some studies have suggested that cholesterol metabolites could act as carcinogenic compounds [10–13], less information is available on the biotransformation of PS and its possible effects (in the case of high intake levels) upon cholesterol metabolization within the colon. Sterols and their corresponding microbial metabolites have been detected in feces from healthy humans following a Western diet [14–18] - the percentage of animal sterols (AS) (67–73%) being considerably greater than PS (27–33%). The AS excretion in subjects following a vegetarian [18–21], vegan [22] or low-animal fat diet [10,23,24] is lower (between 10 and 50%) than in omnivorous subjects. Besides, compared with healthy individuals, greater excretion of cholesterol and its metabolites has been found in feces from subjects with colon cancer (74–92%) [11,20,25] or with precancerous conditions such as adenomatous polyposis (80%) [11] or ulcerative colitis (83%) [26]. These authors attributed the increased excretion of cholesterol metabolites to diet and microbial conversion. However, other investigators have found no differences in AS excretion between patients with colon cancer [13] or familial polyposis [27,28] and healthy subjects.

As far as we know, only one human study has reported fecal sterol excretion after PS-enriched food intake (margarine, 8.6 g PS/day) [29] – the mentioned content exceeding the values established by the European Commission. Therefore, the aim of the present study was to evaluate the impact of the daily consumption of a PS-enriched milk based fruit beverage (2 g PS/day) on fecal sterol excretion and its microbial conversion in postmenopausal women with mild hypercholesterolemia through a clinical trial.

2. Material and methods

2.1. Clinical study

This clinical trial was a single and combined randomized, double-blind, crossover trial carried out with two beverages: a PS-enriched skimmed milk based fruit beverage containing β-Cx and 2 g of PS/250 mL (active beverage), and a skimmed milk based fruit beverage (placebo beverage), in postmenopausal women with mild hypercholesterolemia (ClinicalTrials.gov number NCT 02065024).

The inclusion criteria were: age 45–65 years, amenorrhea for over 12 months, and mild hypercholesterolemia (200–239 mg/dL) according to the guidelines of the American Heart Association [30]. Non-dieting and non-intake of vitamin D, calcium, ω-3 fatty acids and PS or vitamin-enriched foods, supplements or other dietary bioactive components were also considered as inclusion criteria.

Body mass index (BMI) > 35 kg/m^2, the use of vitamins, antibiotics, hormone replacement therapy, fibrates or statins, as well as acute inflammation, chronic medication and infection or intercurrent illness capable of affecting the bioavailability or status of the compounds of interest were regarded as exclusion criteria.

The nutritional composition of the two beverages (active and placebo) is shown in Table 1. It should be noted that although both beverages had a similar composition (50% skimmed milk/50% fruit juice), the placebo beverage was formulated with grape juice from concentrate, and banana puree, while the active beverage was elaborated mainly with mandarin juice from concentrate (as a β-Cx source) and a lower proportion of grape and banana. Free microcrystalline PS from tall oil in powder form (Lipohytol® 146 ME Dispersible, Lipofoods) was added only to the active beverage. The manufacturing conditions were similar for both beverages, which had the same appearance, but with different anonymous labeling (A or B). Plant sterols from the active beverage were characterized (sitosterol: 79%, sitostanol: 12%, campesterol: 7%, stigmasterol: 0.8% and campestanol: 1%) [31], and their stability along a 6-months period (intervention period) was confirmed.

The clinical study took place in the Vitamins Unit of the Department of Clinical Biochemistry of Hospital Universitario Puerta de Hierro-Majadahonda (Madrid, Spain).

Fifty postmenopausal women were contacted for participation and interviewed in order to confirm that they met the inclusion criteria (enrollment). A total of 40 apparently healthy postmenopausal women were finally included in the study and were sequentially numbered from 1 to 40. The sample size was calculated taking into account the results referred to total PS and cholesterol

Table 1
Energy and nutritional composition per 100 mL of active and placebo beverages used in the clinical trial intervention.

	Active	Placebo
Energy (Kcal)	65.3	64.0
Protein (g)	3.1	2.6
Carbohydrate (g)	8.9	10
Fat without PS (g)	1.6	1.5
Fiber (g)	1.5	1.5

Information provided by the manufacturer (Hero España, S.A).

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obtained in a previous clinical trial (ClinicalTrials.gov number NCT01074723). Taken from previous assumption, we chose the more 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%. Furthermore, taking into account that 45% of the Western population may present polymorphisms implicated in the cholesterol absorption process, and assuming a drop-out rate of 10%, the final required sample size was considered to comprise 40 subjects.

During the trial period, 40 women were selected and randomly distributed into two groups: 20 women consumed the active beverage (1 brick x 250 mL/day) and the other 20 women consumed the placebo beverage (1 brick x 250 mL/day) for 6 weeks. After a four-week washout period, the type of beverage was exchanged and consumed for another 6 weeks.

The volunteers were allocated to receive either intervention in random order by 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. After opening the selected envelope, the investigator recorded which type of beverage (active or placebo) should be assigned to each subject, and prepared a pack with enough tetra-bricks to cover the first experimental period (6 weeks). This investigator also ensured that each subject was assigned to the other study group (placebo or active) following the corresponding washout period. The details of group assignment were kept in a sealed envelope that was opened at the end of the complete experimental period. Neither the subject 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, and to complete a semi-quantitative Food Frequency Questionnaire (FFQ) (Supplementary Table 1) at the end of each intervention period, in which the women recorded the number of food portions per week. In addition, the subjects reported no differences in the organoleptic properties of the two beverages in the FFQ. However, the FFQ was not validated, constituting a limitation of the study.

Study compliance was assessed by means of an adherence questionnaire. Each participant completed this questionnaire after the active and placebo periods, reporting the number of non-ingested tetra-bricks.

Feces collection was performed before and after each 6-week treatment period. At this time, a centralized service assigned an identification number (7 digits) 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 the sterile plastic containers and stored at −20 °C until analysis. In order to confirm mild hypercholesterolemia in the women, serum total cholesterol levels were measured by a routine quality controlled method using an Advia 2400 Clinical Chemistry system (Siemens Healthineers). Only phlebotomists or laboratory technicians knew the assigned number of the sample, and were unaware of which treatment was received by the patients.

The women of this study had a mean age of 55.7 ± 3.4 years (range 50–65), with a mean body mass index of 24.6 ± 4.7 kg/m^2 [32], and presented untreated mild hypercholesterolemia (220 ± 27.8 mg/dL). In addition, in Table 2, the serum cholesterol profile after each intervention period (placebo and active) is shown.

An overview of the clinical trial is provided in Fig. 1. The study protocol was approved by the Clinical Research Ethics Committee of Hospital Universitario Puerta de Hierro-Majadahonda (Madrid, Spain), and all subjects gave written consent to participate in the study.

Table 2
Serum cholesterol profile response upon regular consumption of the beverages (n = 36). Results are expressed as Mean ± SD.

mg/dL	Placebo beverage		PS-enriched beverage	
	Basal	Final	Basal	Final
Total cholesterol	219.7 ± 24.5^a	221.8 ± 25.4^a	219.2 ± 28.0^a	212.6 ± 25.5^b
LDL-cholesterol	129.6 ± 27.5^a	131.5 ± 23.6^a	128.6 ± 29.0^b	121.3 ± 24.4^b
HDL-cholesterol	71.9 ± 18.7^a	70.1 ± 17.3^a	71.9 ± 17.3^a	71.9 ± 20.4^a

Different superscript letters denote significant differences ($p < 0.05$) in the same type of beverage (with PS-enriched or placebo) among basal and final values (within lines) (a,b). Reference range (mg/dL): total cholesterol (150–200); LDL-cholesterol (70–160); HDL-cholesterol (35–75). The statistical analysis was done by applying a t-test for paired samples using the SPSS program.

2.2. Analyses performed

2.2.1. Fecal samples

Fresh fecal samples were collected before (V_1 and V_3) and after (V_2 and V_4) each intervention period (see Fig. 1). The samples were then stored at −20 °C and subsequently freeze-dried (Sentry 2.0, Virtis SP Scientific) and crushed in a glass mortar and stored at −20 °C until analysis.

2.2.2. Sterol analysis

Fecal sterols and their metabolites as secondary outcome within the clinical trial were determined according to Cuevas-Tena et al. (2017) [33]. Briefly, approximately 30 mg of freeze-dried feces were dispersed in 5 mL of Milli-Q water, sonicated (20 min) and allowed to stand for two hours at room temperature. The analysis was performed in triplicate using 5α-cholestane (20 μg) as internal standard in aliquots of 100 and 500 μL. The saponification step was carried out with 1 mL of ethanolic potassium hydroxide solution 0.71 M (65 °C/1 h) using a block heater. The unsaponifiable fraction was extracted with 0.5 mL of Milli-Q water and 2 mL of n-hexane (centrifuged at 18 °C/10 min/3600 rpm). The n-hexane extraction step was performed twice under the same conditions as described above. The organic extracts were evaporated to dryness under nitrogen. In order to obtain the trimethylsilyl ether (TMSE) derivatives, 200 μL of BSTFA + 1% TMCS:pyridine 10:3 (v/v) were added (65 °C/1 h). The TMSE derivatives obtained were dissolved with 3 mL of hexane, filtered (Millex-FH filter unit, 0.45 μm Millipore, Milford, MA, USA), evaporated under nitrogen, and dissolved in 40 μL of hexane. One μL of this solution was injected into a GC/MS system (Thermo Science Trace® GC-Ultra with ion trap ITQ 900, Waltham, MA, USA) with a CP-Sil8 CB low bleed/MS (50 m × 25 mm x 0.25 μm) column (Agilent Technologies®, CA, USA). Hydrogen was used as carrier gas, operating at a constant flow of 1 mL/min. The mass spectrometer operated at −70 eV, and a mass range from 50 to 650 m/z was scanned.

3. Statistical analysis

To confirm the use of a nonparametric test, the normal distribution of neutral sterol content and net increment was evaluated using the Shapiro–Wilk test. The two-sample Wilcoxon test was used to detect significant differences in fecal sterol contents between the basal and final intervention period and in net increments between placebo and active beverage intake. Univariate correlations between excreted contents after active beverage or placebo intake were investigated using the Spearman coefficient. In all cases, $p < 0.05$ was used as the criterion for statistical significance. The statistical analyses were performed using the Statgraphics Centurion XVI.I statistical package. It should be noted that most excretion values for cholestanone were below the limit of

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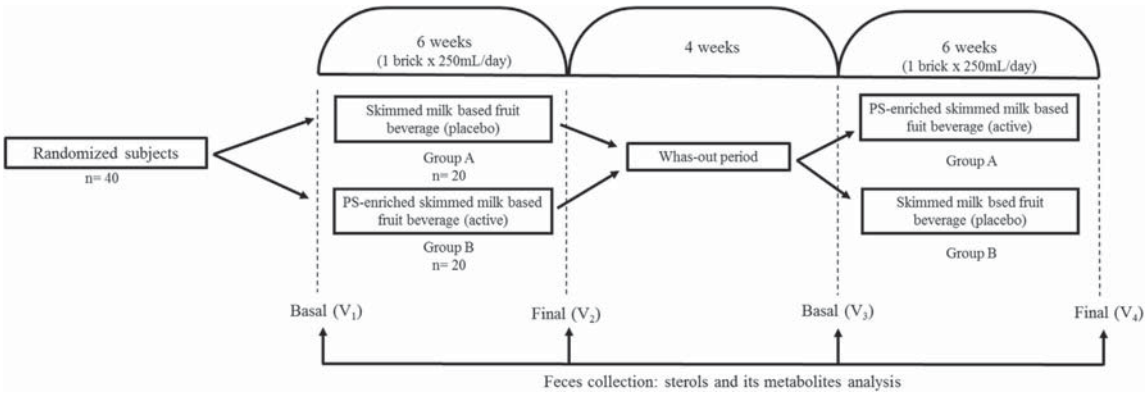

Fig. 1. Overview of the study.

quantification (0.0036 mg/g); limits of detection (0.0011 mg/g) were therefore used.

We used a binary logistic regression analysis for paired data to determine which fecal sterol grouping discriminates best between active or placebo beverage intake. Logistic regression for paired data was necessary due to the crossover design of the trial. These analyzes were performed using the statistical package R V3.2.2 (http://cran.r-project.org). The goodness of fit of the resulting models was tested according to the area under the curve (AUC) of the receiver operating characteristic (ROC).

4. Results

4.1. Participant enrollment

The participant flow of the randomized, double-blind crossover trial is shown in Fig. 2, starting in March 2014 and ending in August 2015. Fifty postmenopausal women with mild hypercholesterolemia (age range 50–65 years) were enrolled. Of these, 10 were excluded and 40 were randomized to participate in the intervention study (from April to July 2014). Thirty-six women finally completed the study.

4.2. Subject adherence

In both intervention periods, active and placebo (6 weeks), one tetra-brick beverage was ingested per day. Thus, the tetra-bricks consumed in each period totaled 42. According to the registry of non-ingested tetra-bricks, 35 women consumed ≥38 tetra-bricks of active beverage and ≥39 tetra-bricks of placebo beverage during the intervention periods, and only one woman consumed 35 or 36 tetra-bricks of active and placebo beverage, respectively.

Binary logistic regression analysis was used to diagnose active or placebo beverage intake. For this purpose, ROC curves were plotted to calculate the sensitivity and specificity of animal and plant fecal sterols as predictors (cut-off points). Figure 3 shows the ROC curves with several cut-off points corresponding to cholesterol and its metabolites (area under the curve [AUC]: 0.60, 95%CI: 0.46–0.73, Fig. 3A), which were not found to be of use in the study adherence determination. Some PS and their metabolites such as sitosterol (Fig. 3B), campesterol (Fig. 3C) and stigmasterol (Fig. 3D) showed high sensitivity and specificity (AUC: 0.92, 95%CI: 0.84–0.99; AUC: 0.87, 95%CI: 0.78–0.97; and AUC: 0.82, 95%CI: 0.71–0.93, respectively). In addition, neutral sterols such as cholesterol, sitosterol, campesterol and stigmasterol (Fig. 3E), and total AS together with total PS (Fig. 3F) were also used as cut-off points, and were seen to be highly sensitive and specific (AUC: 0.88, 95%CI: 0.79–0.97; and AUC: 0.91, 95%CI: 0.83–0.99, respectively) in diagnosing active or placebo beverage intake.

4.3. Excretion of fecal sterols

Figure 4 shows the net excretion increments for AS and PS after active and placebo beverage intake. Generally, the postmenopausal women showed a higher net increment in PS excretion after active beverage intake compared to placebo. After active beverage intake, fewer outliers in the net increment in excretion of neutral sterols (cholesterol, sitosterol, sitostanol, campesterol and stigmasterol) were observed (1, 1, 1, 1 and 0 outliers, respectively) compared to placebo (7, 8, 5, 8 and 2 outliers, respectively). Conversely, the outliers in the net increment in excretion of sterol metabolites such as coprostanol, coprostanone and methylcoprostanone proved more numerous after active beverage intake (3, 5 and 5 outliers, respectively) *versus* placebo (0, 3 and 3 outliers, respectively). However, the outliers of ethylcoprostanol were more numerous after placebo intake (2 outliers) *versus* active beverage (0 outliers). For the rest of the sterol metabolites, the number of outliers remained the same after both beverages.

Fecal AS contents at basal and after active and placebo beverage (final), and their net increments, are shown in Table 3. Due to the non-normal distribution of the data, the total AS contents, expressed as medians, were found to be 19.82 and 18.81 mg/g freeze-dried feces at basal and 21.58 and 18.64 mg/g freeze-dried feces after placebo and active beverage intake (final), respectively.

After placebo intake, only the cholesterol content showed a significant increase (34%). However, after active beverage intake, significant increases in cholesterol (65%), coprostanone (80%), cholestanol + methylcoprostanol (42%), and lathosterol (9%) were observed.

Cholestanol + methylcoprostanol increased significantly after active beverage intake compared to placebo. In the case of cholestanone, the statistically significant difference proved irrelevant, as the values obtained were below the detection or quantitation limits.

Fecal PS contents after the intervention and their net increments are shown in Table 4. A statistically significant increase in total and individual PS (except ethylcoprostenol and brassicasterol) was recorded after active beverage intake (final). Furthermore, statistically significant differences ($p < 0.05$) in net increment between placebo and active beverage intake were observed for all PS except for ethylcoprostenol and brassicasterol.

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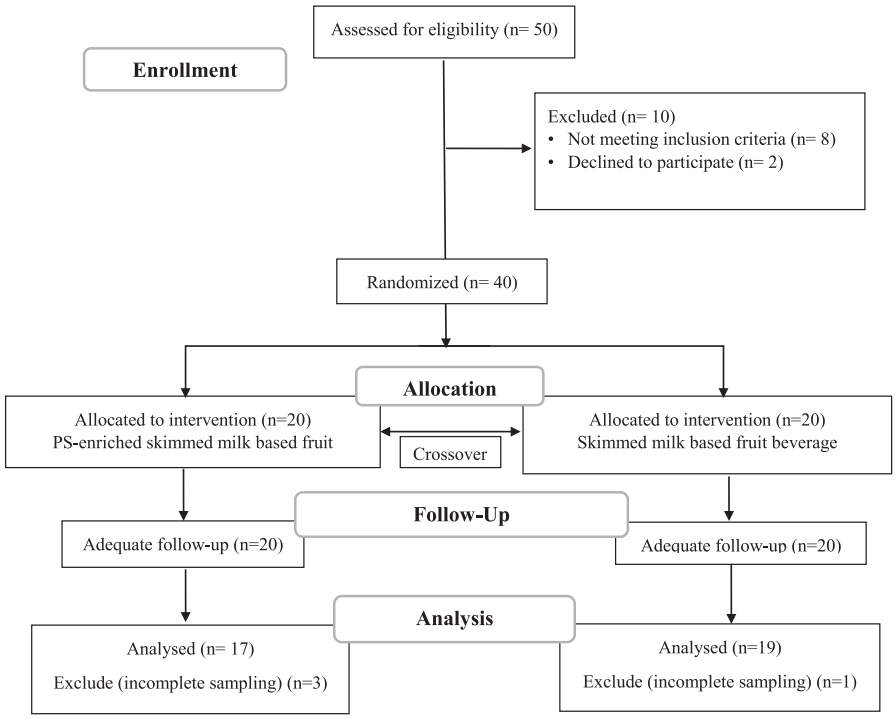

Fig. 2. Flow diagram of the progress through the phases of a randomized double-blind crossover trial (intervention allocation, follow-up, and data analysis).

4.4. Conversion of sterols

Figure 5 shows the conversion percentages of different neutral sterols (cholesterol, sitosterol, and stigmasterol) in all subjects after active and placebo beverage intake. To calculate this percentage, the following equation was used: [metabolites/(neutral sterol + metabolites)] x 100. In order to classify women as low or high converters, we considered that a low converter presents a sterol conversion rate of <50%, while a high converter presents a sterol conversion rate of ≥50% [34]. Hence, the number of women found to be high converters after placebo and active beverage intake were: cholesterol 33 and 29 (Fig. 5A); sitosterol 29 and 17 (Fig. 5B); and finally stigmasterol 18 and 27 (Fig. 5C), respectively. However, in the case of campesterol, all women were low converters after placebo and active beverage intake (data not shown).

We consider it interesting to note the effect of the active beverage upon the decrease in conversion percentage. Based on the conversion percentage frequency distribution used by Wilkins and Hackman (1974) [34], the ≥10% reduction in the conversion percentage of the sterols stands out (Fig. 5). In this context, active beverage intake produced a significant decrease (between 11% and 50%) in cholesterol conversion in 16 women (Fig. 5A, marked with arrows), although 12 of whom remained high converters and 4 became low converters. Regarding sitosterol, 24 women showed a significant decrease (between 15% and 61%) after active beverage intake (Fig. 5B, marked with arrows), and 7 of whom remained high converters and 4 low converters, while 13 women changed from high to low converters. However, in the case of stigmasterol, the intake of the active beverage only produced a significant decrease (between 14% and 67%) in 6 women (Fig. 5C, marked with arrows), of whom only 3 remained as high converters. In general, most women (n = 31) did not change their conversion percentage with regard to campesterol after active beverage intake.

4.5. Correlation of sterols

The Spearman correlation coefficient (ρ) was used to evaluate statistically significant associations referred to fecal AS, PS contents and their corresponding metabolites, after active or placebo beverage intake. In this sense, only after active beverage intake, was a statistically significant correlation found between fecal sitosterol (ρ: 0.632, p = 0.0002), campesterol (ρ: 0.428, p = 0.0112) and stigmasterol (ρ: 0.370, p = 0.0282) (sterols present in active beverage) and total fecal PS. In addition, a positive and strong correlation was observed between total fecal PS and total sterols after active beverage intake (ρ: 0.926, p = 0.0000), the correlation being weaker in the case of the placebo (ρ: 0.654, p = 0.0001). However, a weak correlation was observed between total fecal AS and total fecal sterols after active beverage intake (ρ: 0.643, p = 0.0001), with a strong correlation after placebo intake (ρ: 0.944, p = 0.0000).

It should be noted that the inverse relationship observed between fecal cholesterol and its metabolites after both active (ρ: −0.592, p = 0.0005) and placebo beverage intake (ρ: −0.335, p = 0.0471), was more intense and significant after consumption of the active beverage. Furthermore, negative and strong correlations were observed for fecal sitosterol, stigmasterol and campesterol with fecal cholesterol metabolites after active beverage intake (ρ: −0.519, p = 0.0021; ρ: −0.643, p = 0.0001; and ρ: −0.620, p = 0.0002, respectively) *versus* placebo (only for sitosterol ρ: −0.361, p = 0.0325; and stigmasterol ρ: −0.506, p = 0.0027).

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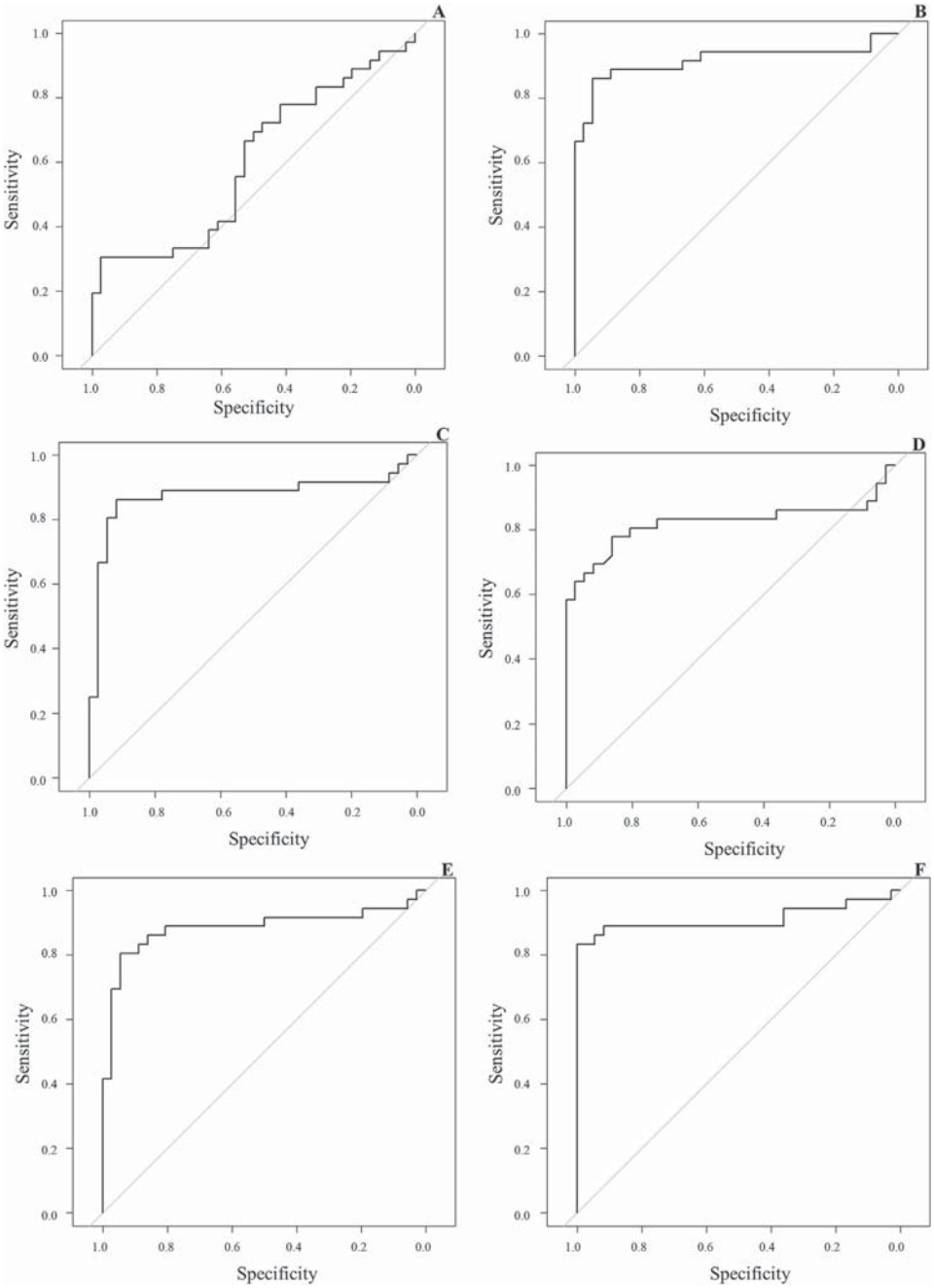

Fig. 3. Receiver Operating Characteristic (ROC) curves to predict beverage intake (active or placebo) in the intervention clinical trial. Active: PS-enriched skimmed milk based fruit beverage. Placebo: skimmed milk based fruit beverage. Group: A: cholesterol + coprostanol + coprostanone; B: sitosterol + sitostanol + ethylcoprostanol + ethylcoprostanone; C: campesterol + campestanol + methylcoprostanone; D: stigmasterol + stigmastenol + ethylcoprostenol; E: cholesterol + sitosterol + campesterol + stigmasterol; F: total animal sterols + total plant sterols.

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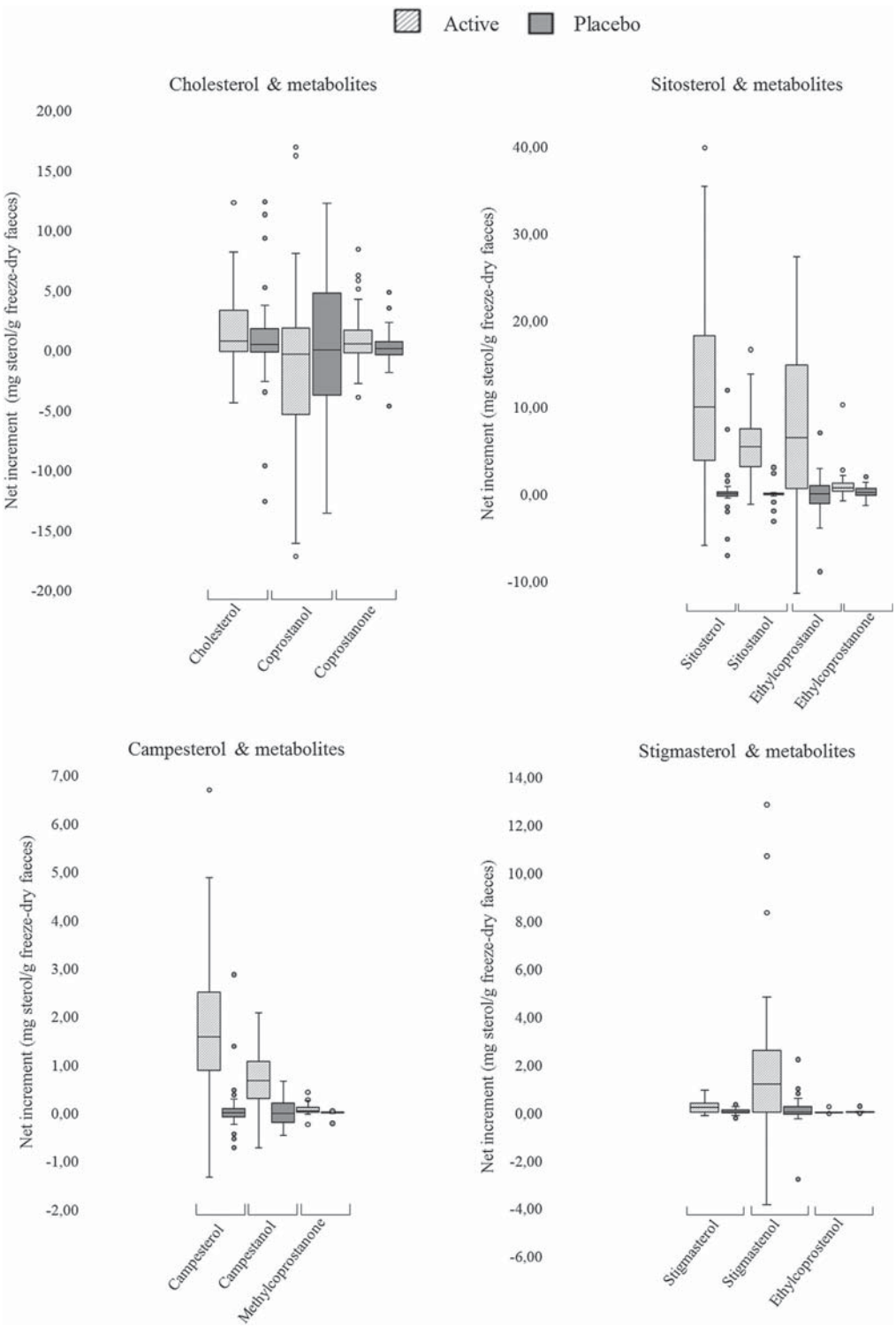

Fig. 4. Sterols and their metabolites response in feces upon regular consumption of active and placebo beverages (n = 36). Active: PS-enriched skimmed milk based fruit beverage. Placebo: skimmed milk based fruit beverage. Boxes represent the mean of the net increment: final − basal (n = 36). Points in each box represent outlier values.

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Table 3
Fecal animal sterols contents (mg/g freeze-dry feces) after placebo and active beverages intake.

Sterol	Basal	Final	p value	Net increment	p value
Placebo					
Cholesterol	1.51[a] (0.79; 3.02)	2.02[b] (1.12; 4.25)	0.03	0.43 (−0.14, 1.71)	
Coprostanol	12.74[a] (7.77, 22.02)	12.50[a] (9.51, 21.21)	1.00	0.00 (−3.72, 4.74)	
Coprostanone	1.40[a] (0.55, 2.85)	1.23[a] (0.60, 3.04)	0.49	0.10 (−0.38, 0.66)	
Cholestanol + methylcoprostanol[c]	0.55[a] (0.46, 0.81)	0.58[a] (0.40, 0.80)	0.61	−0.01 (−0.16, 0.10)	
Cholestanone[d]	0.0011[a] (0.0011, 0.095)	0.0011[a] (0.0011, 0.10)	0.93	0.00 (0.00, 0.02)	
Lathosterol	0.11[a] (0.09, 0.15)	0.12[a] (0.09, 0.16)	1.00	0.00 (−0.02, 0.03)	
Total animal sterols	19.82[a] (13.89, 28.50)	21.58[a] (14.47, 30.10)	0.61	−1.10 (−3.34, 6.45)	
Active					
Cholesterol	2.30[a] (1.70, 4.20)	3.80[b] (1.87, 7.24)	0.01	0.76 (−0.12, 3.20)	0.16
Coprostanol	12.87[a] (7.03, 19.05)	9.90[a] (6.39, 16.26)	0.13	−0.35 (−5.30, 1.52)	0.11
Coprostanone	1.24[a] (0.61, 3.00)	2.23[b] (0.83, 4.54)	0.04	0.52 (−0.26, 1.65)	0.14
Cholestanol + methylcoprostanol[c]	0.67[a] (0.44, 0.85)	0.95[b] (0.62, 1.53)	5×10^{-4}	0.36* (0.005, 0.71)	6×10^{-4}
Cholestanone[d]	0.0011[a] (0.0011, 0.090)	0.0011[b] (0.0011, 0.22)	0.01	0.00* (0.00, 0.10)	6×10^{-4}
Lathosterol	0.11[a] (0.09, 0.17)	0.12[b] (0.09, 0.17)	0.04	0.01 (−0.01, 0.05)	0.32
Total animal sterols	18.81[a] (14.18; 25.20)	18.64[a] (15.92; 28.51)	0.24	1.44 (−4.76; 7.38)	1.00

Placebo: skimmed milk based fruit beverage intake. Active: PS-enriched skimmed milk based fruit beverage intake. Net increment: final − basal. Values are expressed as median (n = 36). Percentile: 25–75% is indicated between parentheses.
Different lowercase letters (a, b) indicate statistically significant differences ($p < 0.05$) in the excretion to each sterol (mg sterol/g freeze-dry feces) between basal and final samples for each type of period (active or placebo).
*Indicate statistically significant differences ($p < 0.05$) in the net increment excretion of each sterol (mg sterol/g freeze-dry feces) between placebo and active period.
[c] The applied method does not allow the separation of these compounds.
[d] In those women who had cholestanone contents lower than the limit of detection (0.0011 mg/g) or quantitation (0.0036 mg/g); the limit of detection were used for statistical treatment.

However, negative correlations between fecal cholesterol and PS metabolites were similar after active (ρ: −0.397, p = 0.0187) and placebo beverage intake (ρ: −0.442, p = 0.0089). In addition, similar positive correlations between fecal cholesterol metabolites and PS metabolites after active (ρ: 0.702, p = 0.0000) and placebo beverage intake (ρ: 0.711, p = 0.0000) were observed. Finally, the positive correlation observed between fecal PS metabolites and total PS was greater after placebo (ρ: 0.873, p = 0.0000) than after active beverage intake (ρ: 0.589, p = 0.0005).

5. Discussion

This clinical study was carried out to evaluate the impact of high PS intake on excreted fecal sterols and their microbial conversion in postmenopausal women with mild hypercholesterolemia. Fecal sitosterol together with its metabolites (Fig. 3B) and fecal AS with PS (Fig. 3F) were identified as the most adequate predictors in the binary logistic regression analysis (ROC curves) for establishing subject adherence in the trial. In addition, individual fecal sterol PS

Table 4
Fecal plant sterols contents (mg/g freeze-dry feces) after placebo and active beverages intake.

Plant sterol	Basal	Final	p value	Net increment	p value
Placebo					
Sitosterol	1.21[a] (0.93; 2.19)	1.63[a] (1.08; 2.34)	1.00	−0.03 (−0.26; 0.21)	
Sitostanol	0.14[a] (0.07; 0.24)	0.13[a] (0.03; 0.21)	0.71	0.00 (−0.09; 0.05)	
Ethylcoprostanol	4.00[a] (3.06; 5.85)	4.42[a] (3.05; 5.22)	0.73	−0.02 (−1.14; 0.92)	
Ethylcoprostanone	0.96[a] (0.57; 1.45)	1.13[a] (0.75; 1.66)	0.49	0.15 (−0.20; 0.62)	
Campesterol	0.22[a] (0.04; 0.36)	0.24[a] (0.12; 0.33)	0.85	0.00 (−0.085; 0.07)	
Campestanol	0.37[a] (0.20; 0.58)	0.41[a] (0.13; 0.55)	0.47	−0.02 (−0.20; 0.19)	
Methylcoprostanone	0.01[a] (0.004; 0.03)	0.01[a] (0.004; 0.03)	0.90	0.00 (−0.01; 0.01)	
Stigmasterol	0.17[a] (0.09; 0.24)	0.18[a] (0.13; 0.26)	0.15	0.01 (−0.03; 0.08)	
Stigmastenol	0.21[a] (0.0003; 0.51)	0.23[a] (0.0003; 0.74)	0.23	0.00 (0.00; 0.01)	
Ethylcoprostenol	0.06[a] (0.04; 0.06)	0.06[a] (0.05; 0.07)	0.26	0.00 (0.00; 0.01)	
Brassicasterol	0.25[a] (0.15; 0.34)	0.22[a] (0.15; 0.34)	1.00	0.00 (−0.08; 0.05)	
Total plant sterols	8.73[a] (6.50; 10.94)	9.20[a] (7.11; 10.28)	0.86	0.10 (−1.33; 1.67)	
Active					
Sitosterol	1.83[a] (1.06; 2.68)	11.82[b] (5.87; 21.03)	4×10^{-6}	10.00* (4.12; 17.73)	7×10^{-7}
Sitostanol	0.11[a] (0.0002; 0.35)	5.75[b] (4.31; 7.89)	6×10^{-7}	5.42* (3.34; 7.38)	4×10^{-7}
Ethylcoprostanol	4.62[a] (2.64; 5.36)	11.42[b] (6.74; 20.63)	2×10^{-5}	6.40* (0.77; 14.72)	2×10^{-5}
Ethylcoprostanone	1.03[a] (0.73; 1.46)	1.85[b] (1.40; 2.23)	1×10^{-5}	0.66* (0.27; 1.20)	2×10^{-3}
Campesterol	0.31[a] (0.17; 0.50)	1.88[b] (1.06; 3.35)	2×10^{-6}	1.57* (0.91; 2.44)	5×10^{-6}
Campestanol	0.32[a] (0.15; 0.63)	1.01[b] (0.74; 1.40)	5×10^{-6}	0.67* (0.31; 1.06)	5×10^{-6}
Methylcoprostanone	0.01[a] (0.0004; 0.04)	0.05[b] (0.02; 0.13)	2×10^{-4}	0.04* (0.02; 0.10)	5×10^{-4}
Stigmasterol	0.18[a] (0.10; 0.28)	0.39[b] (0.22; 0.60)	3×10^{-5}	0.20* (−0.005; 0.36)	7×10^{-4}
Stigmastenol	0.10[a] (0.0003; 0.46)	1.58[b] (0.38; 3.57)	4×10^{-5}	1.17* (0.00; 2.53)	6×10^{-5}
Ethylcoprostenol	0.06[a] (0.05; 0.06)	0.06[a] (0.05; 0.07)	0.10	0.00 (0.00; 0.01)	0.52
Brassicasterol	0.25[a] (0.18; 0.38)	0.25[a] (0.18; 0.35)	0.88	0.00 (−0.07; 0.07)	1.00
Total plant sterols	8.58[a] (6.75; 11.64)	43.86[b] (33.51; 55.72)	1×10^{-6}	33.29* (22.19; 47.62)	2×10^{-6}

Placebo: skimmed milk based fruit beverage intake. Active: PS-enriched skimmed milk based fruit beverage intake. Net increment: final − basal. Values are expressed as median (n = 36). Percentile: 25–75% is indicated between parentheses.
Different lowercase letters (a, b) indicate statistically significant differences ($p < 0.05$) in the excretion to each sterol (mg sterol/g freeze-dry feces) between basal and final samples for each type of period (active or placebo).
*Indicate statistically significant differences ($p < 0.05$) in the net increment excretion of each sterol (mg sterol/g freeze-dry feces) between placebo and active period.

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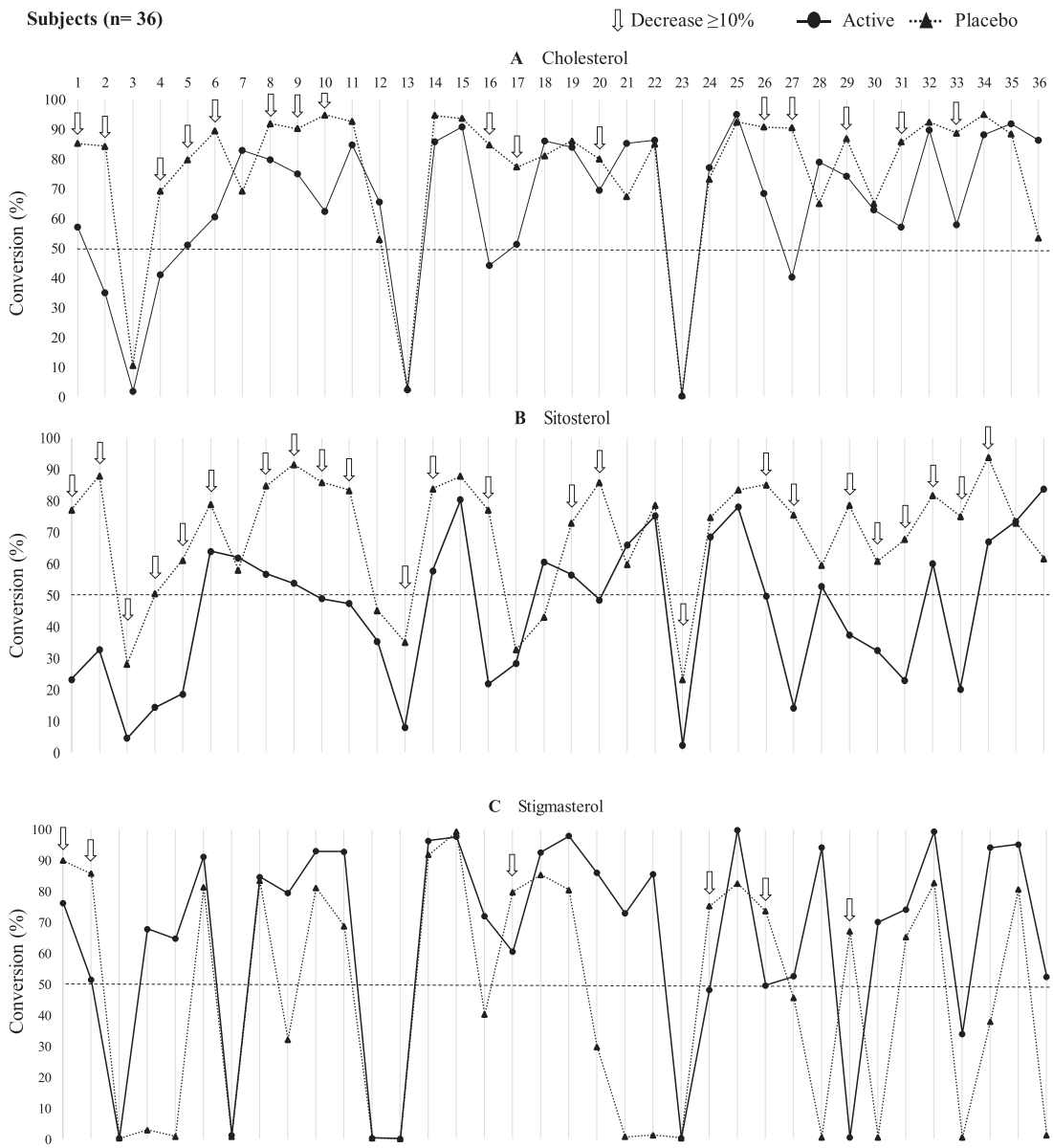

Fig. 5. Conversion percentages of cholesterol, sitosterol and stigmasterol from intervention clinical trial with post-menopausal women after active and placebo beverage intake (n = 36). Abscissa axis represents the 36 post-menopausal women who participated in the clinical study; Ordinate axis shows the sterol conversion percentage, which was calculated according to the following equation: [metabolites/(neutral sterols + metabolites)] x 100. A: cholesterol conversión [coprostanol + coprostanone/(cholesterol + comprostanol + coprostanone)]; B: sitosterol conversion [ethylcoprostanol + ethylcoprostanone/(sitosterol + sitotanol + ethylcoprostanol + ethylcoprostanone)]; C: stigmasterol conversion [stigmastenol + ethylcoprostenol/(stigmasterol + stigmastenol + ethylcoprostenol)]. Solid line with a circle as marker: conversion after active beverage; Dashed line with a triangle as marker: conversion after placebo. The dashed line located on each graph shows the threshold at 50% to define the low and high converters according to Wilkins & Hackman, (1974) [27]. The arrow indicates the significant decrease (≥10%) after active beverage intake compared to placebo.

(sitosterol, campesterol and stigmasterol present in the active beverage) were directly and strongly correlated to total fecal PS after active beverage intake.

Furthermore, the net increment of excretion of neutral sterols (those present in the active beverage) showed fewer outliers after active beverage intake (Fig. 4). This suggests that both groups, A and B (Fig. 1), responded homogeneously to active beverage intake, independently of the timing of ingestion. However, the net increment in ethylcoprostanol excretion showed a more homogeneous response after active beverage intake, perhaps due to the high

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sitosterol content present in the latter (Fig. 4B). The heterogeneous response referred to the net increment excretion of neutral sterols after placebo beverage intake was possibly the result of variability in dietary habits among the women [10,21,22,24], which was not controlled and has emerged as an instrumental factor in the configuration of the gut microbiota [22,24,35,36]. In this context, the response of fecal sterol metabolites after active beverage intake was more heterogeneous, especially with regard to AS (Fig. 4A) and methylcoprostanone (Fig. 4C), due to interindividual microbial variability. In fact, each individual human is known to harbor specific bacteria [37], and the long-term intake of high-animal fat diets is associated to changes in the gut microbiota [38,39].

The total fecal AS contents found in our study (13.9–30.10 mg/g freeze-dried feces) (Table 3) were similar to those reported by other authors after diets not enriched with PS (19.4–28.5 mg/g freeze-dried feces) [12,16,19,29,33,34,40]. In humans with PS-enriched margarine intake (8.6 g PS/day) [29], an increase has been evidenced in fecal cholesterol content (net cholesterol excretion 20.7 mg/g freeze-dried feces). However, this fact was not observed in our study, probably due to a lower PS intake (2.0 g/day).

The fecal contents in PS and their metabolites (6.50–11.64 mg/g freeze-dried feces) were similar in the two basal periods and in the final period for placebo, in concordance with other authors (5.50–10.20 mg/g freeze-dried feces) [18,33,34,40]. However, active beverage intake produced greater (nearly 5-fold) PS excretion with respect to placebo (Table 4), in agreement with one of the aforementioned studies after PS-enriched margarine consumption (nearly 17-fold) [29]. We also found a significant increase (nearly 3-fold) in ethylcoprostanol excretion after active beverage intake. These results suggest that the gut microbiota preferentially uses PS as substrate, as they were present in a greater proportion with respect to cholesterol. In addition, the relative percentages of fecal AS and PS with respect to total fecal sterols in the two basal periods and in the final period for placebo were consistent with the results of other authors (~70 and ~30%, respectively) [15,18,29,33,34,40]. However, these relative percentages were reversed (~30 and ~70%, respectively) after active beverage intake. Similarly, while the positive correlation between total fecal PS/total sterols was greater after active beverage intake, the correlation between total fecal AS/total sterols was greater after placebo.

An inverse correlation between fecal cholesterol and its metabolites was observed that proved greater after active beverage intake than after placebo consumption. In this context, a significant net increment in the excretion of coprostanone, and a slight and nonsignificant decrease in the excretion of coprostanol, were recorded after active beverage intake. However, other authors reported a decrease in the excretion not only of coprostanol but also of coprostanone (9 and 1.5 mg/g freeze-dried feces, respectively) after PS-enriched food intake [29]. Besides, epidemiological studies with subjects following vegetarian and low-animal fat diets have reported a decrease in coprostanol (from 12.2 to 2.3 mg/g freeze-dried feces) and coprostanone (between 2.3 and 0.3 mg/g freeze-dried feces), compared with high-animal fat (Western) diets [10,19–24]. In our study, this fact could be due to the diet effect in the women, since the real sterol intake had not been controlled – this constituting a limitation of the study. Besides, the significant increase in coprostanone after active beverage intake could also suggest that the gut microbiota metabolized cholesterol through an indirect pathway, which was interrupted during this step, causing a lower production of coprostanol. This also suggests that the capacity of the gut microbiota was not sufficient to transform AS in the usual manner, due to the large amounts of PS present [41]. In fact, it has been suggested that the efficiency of cholesterol conversion is related to microbial density. In this regard, a coprostanol/cholesterol ratio of $\geq$15 has been associated to high levels of coprostanoligenic bacteria (10^8 cells/g) and to nearly complete cholesterol conversion [42]. In this context, we found lower coprostanol/cholesterol ratios in postmenopausal women after active beverage intake compared with placebo (3.2 *versus* 6.2). In coincidence with our findings, other authors have recorded lower ratios after the intake of PS-enriched margarine compared to the controls (0.35 *versus* 2.6) [29], in vegetarian subjects compared to omnivorous individuals (1.62 *versus* 4.38) [19], and in low converters *versus* high converters (0.13 *versus* 7.6) [34]. In concordance with other authors [19,34,43], we found a low coprostanol/cholesterol ratio (<0.9) after active beverage intake *versus* placebo (8%) in 20% of the postmenopausal women. Besides, it has also been suggested that low cholesterol conversion could be due to a lack of mucosal receptors for coprostanoligenic bacteria [43] or to the inhibition of these bacteria by other components of the gut microbiota [44].

As far as we know, this is the first time that microbial conversion percentages corresponding to cholesterol, sitosterol and stigmasterol are reported after a PS-enriched food intervention in postmenopausal women with mild hypercholesterolemia, describing the effect of high PS intake upon their conversion percentage rates. Most subjects in our study were high cholesterol converters. They showed an average conversion percentage of 82.3 ± 11.7 after placebo *versus* 75.4 ± 13.3 after active beverage intake, in the same way as in subjects following a Western diet (75–89%) [12,19,34,45] and in vegetarians (66%) [19]. Low converters showed percentages of 23.5 ± 20.9 and 4.5 ± 5.2 after active and placebo intake, respectively, according to the values reported in other studies (1.0–43.0) among subjects following a Western diet [12,34].

However, there are no consensus-based data regarding the thresholds that classify high and low converters. While Wilkins and Hackman (1974) [34] reported that high converters present a sterol conversion percentage of $\geq$50%, Midtvedt et al. (1990) [43] reported a value of $\geq$40%. Most authors consider the threshold to be $\geq$ 50% [12,13,16,19].

On the other hand, in 16 subjects, active beverage intake produced a significant decrease in the fecal cholesterol conversion percentage compared to placebo – a condition that has been reported in humans after PS-enriched margarine intake [29]. However, this decrease has not been observed in vegetarian *versus* omnivorous diets (66.3 ± 7.9 *versus* 75.3 ± 6.3) [19]. In this sense, it has been suggested that an increase in PS intake could reduce or block fecal cholesterol conversion, thereby resulting in a lower production of cholesterol metabolites, which are associated to procarcinogenic action and could increase the risk of colon cancer [11,29,46]. Furthermore, a previous study by our group found PS at human colonic concentrations to exhibit antiproliferative effects against colon cancer cells (Caco-2 cells) [47].

Most subjects were high sitosterol converters after placebo, in agreement with the only study that reports data in this regard (23 high and 8 low converters) [34], although fewer than half of whom were high converters after active beverage intake. The high converters showed an average in fecal sitosterol conversion percentage of 52.7 ± 9.6 after active beverage *versus* 75.9 ± 11.3 after placebo intake, while the values for the low converters were 26.9 ± 15.1 and 34.5 ± 8.5, respectively. After active beverage intake, the fecal sitosterol conversion percentage decreased in 24 subjects. Thus, the sitosterol/sitosterol metabolites ratio (in 22 subjects) after active beverage intake was higher than in the placebo group (expressed as median, 0.5 *versus* 0.3). Indeed, the positive correlation between fecal PS metabolites and total PS was greater after placebo than after active beverage intake. These observations would suggest that the gut microbiota was unable to metabolize the PS from active beverage, due to the abundant presence of these sterols.

A limitation of our study was the lack of a microbiota analysis, which did not form part of the objectives of the clinical study. Such

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an analysis would have provided valuable information about the impact of high PS intake upon the intestinal microbial community, allowing it to be correlated to the fecal sterol conversion percentage found in our study. Nevertheless, the present study is of interest, since only one publication has evaluated the effect of a PS-enriched food upon the fecal sterol profile – this fact represents a limitation for the discussion of the results.

6. Conclusions

In the present intervention study focusing on the intake of a PS-enriched skimmed milk based fruit beverage (2 g/day over a 6-week period), which had a hypocholesterolemic effect, a decrease in fecal cholesterol metabolites was observed. These metabolites could be associated to pro-carcinogenic action. The PS-enriched beverage did not modify the fecal excretion of cholesterol, although an increase in coprostanone was observed, probably due to saturation of the gut microbiota. Furthermore, an expected significant increase in PS excretion - especially ethyl-coprostanol - was recorded. Besides, intake of the PS-enriched beverage modified the microbial converter profile with regard to cholesterol and sitosterol (decreasing the number of high converters), and stigmasterol (increasing the number of high converters).

Therefore, further studies on the effect of PS-enriched foods could have in the microbial metabolism of dietary sterols and on the impact of PS-enriched foods upon the gut microbiota composition and diversity are needed.

Statements of authorship

MJL was the main researcher. MJL and AA contributed to the study design and the writing of the study protocol. MC-T was in charge of sample analysis and data collection. MJL, AA and MC-T carried out the data analysis and writing the manuscript. JDB provided statistical advice and carried out the binary logistic regression for paired data analysis. All authors have read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.08.012.

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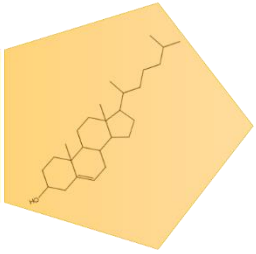
Anexo/ Annex II



Relationship dietary sterols and gut microbiota: A review

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Additional Keywords (select from list):	

Abstract

Cholesterol intestinal absorption differs markedly from that of plant sterols; whereas the cholesterol absorption rate is high (30-60%) for total plant sterols it is low (2-3%). Non-absorbed sterols reach the colon, where the microbiota interacts with them.

Non-absorbed cholesterol biotransformation has been widely studied by *in vitro* fermentation assays using gut microbiota from human feces and pure cultures of enteric microorganisms. A great variety of sterols and its metabolites have been detected, which allowed establishing two pathways for cholesterol microbial degradation to coprostanol. However, biotransformation studies of plant sterols are scarce and its microbial transformation pathway remains to be fully clarified.

Furthermore, the sterol contents in feces are highly variable among individuals, due to specific microbiota and especially dietary factors. However, no standardized methodology has been developed for sterols and their metabolites determination in feces.

Studies on sterol excretion values, microbial transformation and sterol determination methodology have been reviewed. Given that, cholesterol metabolites could contribute to the development of colon cancer and that information about of plant sterols biotransformation is scarce, this review contribute to improve the knowledge of the sterol implication in the gut microbiota and of PS impact in the colonic microbiota metabolization of cholesterol.

1. Introduction

Cholesterol and plant sterols (PS) are compounds found in the unsaponifiable portion of animal and plant lipids, respectively, and can be present as esterified forms. Both types of compounds have similar structures derived from squalene, comprising a tetracyclic cyclopenta[α]-phenanthrene structure (hydrophobic region) with a hydroxyl group at C-3 (polar region). In general, sterols have a double bond between carbon 5 and 6 and a flexible iso-octyl hydrocarbon side chain with 8–10 carbons at C-17. In the case of PS, there is an extra hydrophobic carbon chain (methyl or ethyl group) at the C-24 position. Besides, the compounds are classified into sterols and stanols, according to the presence or absence of a double bond at the Δ5 position [1-3]

Cholesterol is absorbed in a proportion of between 30-60%, while absorption for total PS is lower (2-3%) [2, 3]. However, the hypocholesterolemic effect of PS is well known, since intakes of 2-3 g a day of PS reduce the absorption cholesterol level by an average of 12% [4]. The unabsorbed sterols reach the colon where they can be biotransformed by the microbiota. Although cholesterol biotransformation mediated by the microbiota has been widely studied and some studies have suggested that cholesterol metabolites could act as carcinogenic compounds [5-8], less information are available on the biotransformation of PS and its effects (in the case of high intake levels) upon cholesterol metabolization in the colon. A review is therefore made of the existing information on sterol biotransformation by the microbiota and its impact upon the latter. In addition, since there is no standardized method for the determination of sterols and their metabolites in feces, a compilation is made of the techniques that have been used to date. Given the complexity of the analysis, evaluation is made of each of the

steps, including sample preparation, and the existing degree of analytical validation.

2. The relationship between dietary sterols and gut microbiota

The human large intestine harbors a great variety of microorganism with an estimated more than 400-500 microbial species. Most of the bacteria living in the colon are obligate anaerobic, including *Bacteroides, Eubacterium, Bifidobacterium, Clostridium, Fusobacterium, Lactobacilli Ruminococcus, Peptococcus* and *Peptostreptococcus* genera [9-12], which have important metabolic and immune functions, with a marked effect upon the nutritional and health status of the host [13, 14].

Transformation by the microbiota of non-absorbed food compounds, including cholesterol and PS, has been revealed as a key factor for several biological effects, due to the metabolites produced [15], and for energy supply to the host through anaerobic fermentative processes [16]. However, the consequences on health of the intestinal cholesterol and PS metabolism by gut microbiota are currently unexplored [17]. Although cholesterol metabolites (coprostanol, coprostanone and cholestanol) are associated to pro-carcinogenic action and could increase the risk of colon cancer [5-8].

2.1. Dietary sterols biotransformation by gut microbiota

Sterols biotransformation by the gut microbiota has been widely studied by *in vitro* assays using microbiota from human [18-24], rat [25-27], pigeon and chicken feces [24]. In addition, pure cultures of enteric microorganisms such as *Eubacterium* sp. [28-34], *Clostridium perfringens*, *Bifidobacterium* sp., *Enterobacter aerogenes* [35], *Escherichia coli* [35, 36], *Bacteroides* sp. [37] have

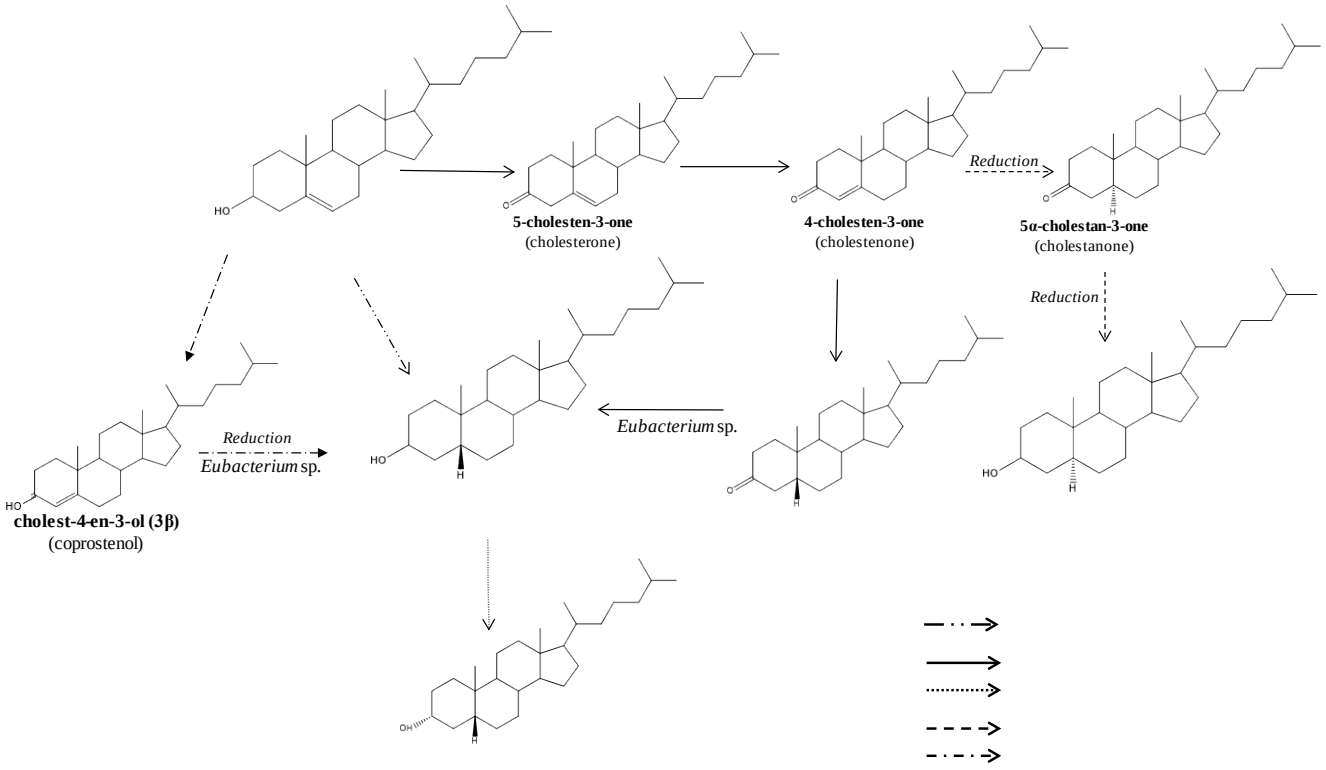
been used in order to study cholesterol metabolites. In the case of PS, only one study has been carried out with *Eubacterium* sp. [28].

It has been suggested that the bacteria are able to hydrolyzed, hydrogenated, dehydrogenated and transformed sterols into secondary products [38]. The first cholesterol biotransformation studies were carried out in the 1930s, with the identification of coprostanol as the main metabolite [18, 19]. Several routes of microbial cholesterol degradation into coprostanol and another route to cholestanol have been proposed (as summarize in Fig. 1). Over the next two decades, other 5α and 5β derivatives were identified [20, 22]. The former include direct coprostanol formation through cholesterol double bond (in position 5-6) reduction (Fig. 1, route I). The latter route, based on studies in rat feces, shows cholesterol oxidation to cholestenone to probably involve removal of the 3α-hydrogen as a rate-limiting step in cholesterone formation, followed by isomerization of the Δ5-double bond to a Δ4-double bond by partial transfer of hydrogen from the 4β-position to the 6 position. Finally, hydrogenation into coprostanone, with subsequent reduction of the 3-keto group to coprostanol takes place (Fig. 1, route II) [27, 34, 41]. Furthermore, it has been suggested that coprostanol may transform slightly into epicoprostanol (Fig. 1, route III) [20]. Another pathway in which cholestenone is converted to cholestanone through reduction (in position 4-5) and then to cholestanol (Fig. 1, route IV) has also been proposed [40]. In addition, coprostenol has been identified as an isomer of cholesterol, which by reduction (in position 4-5) is transformed into coprostanol (Fig. 1, route V) [28, 32].

Since the 1970s there has been controversy regarding which microorganisms metabolize cholesterol. In this regard, it has been suggested that different microorganisms catalyze cholesterol biotransformation into coprostanol through

routes I and II (Fig. 1), though it has not been specified which of them [26]. Some authors have demonstrated that Eubacterium sp. is able to convert cholesterol into coprostanol, and cholestenone and coprostanone into coprostanol [28, 30].

Figure 1. Cholesterol transformation path into by gut microbiota.



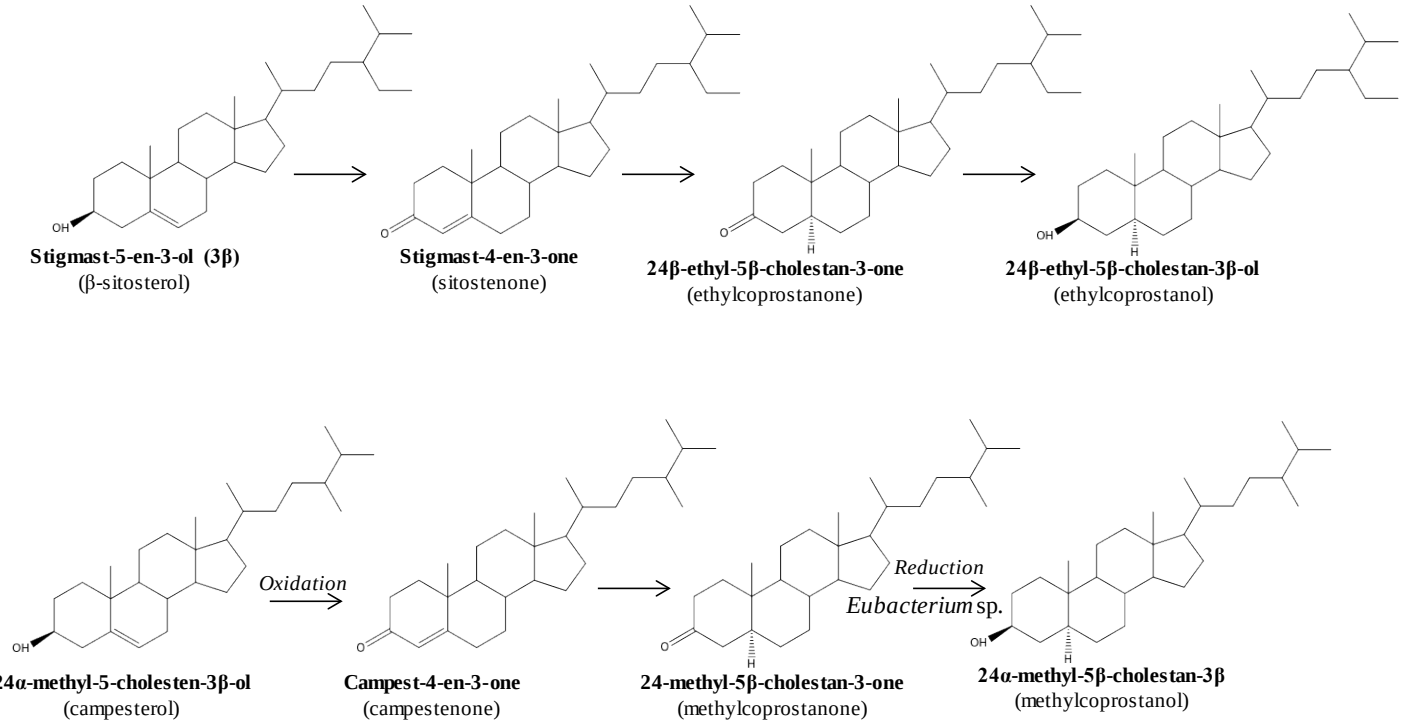
Adapted from [22, 24, 28, 32, 34, 40]

Besides, it has been seen that *Eubacterium* sp. are able to degrade two cholesterol metabolites (coprostenol (cholest-4-en-3β-ol) and cholestenone) into coprostanol [28, 32]. In contrast, Ren et al. (1996) [34], using pure culture of *Eubacterium coprostanoligenes* found no production of intermediary metabolites such as cholesterone and cholestenone, which could imply that the conversion of cholesterol into coprostanol occurs through route I (Fig. 1). Besides, it has also been found that *Bacteroides thetaiomicrom* produces a larger amount of coprostanol than *Bifidobacterium* sp. and *Clostridium* sp. [42].

In turn, it has been suggested that *Bifidobacterium* sp. could be responsible for the chemical conversion of cholesterol to coprostanol [24], and that *Bacteroides* sp. has a strong capacity to reduce cholesterol to coprostanol, with the occasional observation of cholestenone and coprostanone - thus suggesting an indirect pathway for cholesterol conversion [37]. In addition, it has been suggested that *Escherichia coli* is able to produces cholesterol metabolites and androstane metabolites [36].

On the other hand, phytosterols are also transformed by the gut microbiota into phytostanone and phytostenone intermediates, and subsequently into phytostanols [40] (see Fig. 2). In this regard, sitosterol and campesterol are mainly transformed into ethyl- and methyl-coprostanol, and then into ethyl- and methyl-coprostanone, respectively [43]. Although the stigmasterol microbial degradation pathway has not been fully clarified, it has been suggested that ethylcoprostenol and ethylcoprostenone are its degradation products [44].

Figure 2. Sitosterol transformation path into ethylcoprostanol and campesterol into methylcoprostanol by gut microbiota.



Adapted from [40]

PS and its metabolites mentioned above have been detected in human feces (see section 3 below) and its biotransformation route is similar to that of cholesterol [40, 43] (see Fig. 2). Rosenheim and Webster (1941) [45], and Coleman and Baumann (1957) [25] demonstrated the conversion of β-sitosterol into ethylcoprostanol through *in vivo* and *in vitro* studies, respectively. In addition, biotransformation studies using pure culture of *Eubacterium* sp. (isolated from rat feces) on β-sitosterol, campesterol and stigmasterol showed that this microorganism reduce the 5,6-double bond of these sterols, exclusively yielding 5β-saturated derivatives (phytostanones) (reduction ratio 95% for all sterols) [28].

Cholesterol conversion is considered to be of interest since cholesterol metabolites have procarcinogenic action and could increase the risk of colon cancer [46-48]. In fact, high levels of cholesterol and its metabolites were found in feces from subjects with colon cancer [6, 46, 49], adenomatous polyposis [46] and ulcerative colitis [50] - attributing this fact to sterol intake with the diet and to microbial conversion. Some authors have suggested that an increase in PS intake could reduce or block the biotransformation of cholesterol, thereby resulting in a lower production of coprostanol, coprostanone, cholestanone and cholestanol. Besides, it has been suggested that an increase in PS intake could reduce or block fecal cholesterol conversion, thereby resulting in a lesser production of cholesterol metabolites, which are associated to pro-carcinogenic action and could increase the risk of colon cancer [46-48]. Specifically, dietary β-sitosterol supplementation in rats has been associated with a decrease in cancerous epithelial cell proliferation [51], enzymatic activity of characteristic colon carcinogenesis intestinal bacteria [52], and colon tumor formation [53]. However, other murine studies have documented an increase in cholesterol and

coprostanol excretion after β-sitosterol [54] or stigmasterol supplementation [55].

On the other hand, it has been suggested that the majority of population may present high or low conversion of sterols – though most subjects appear to be high converters [38, 56, 57]. High converters are those that show a sterol conversion rate ≥ 50% and the low converters < 50% [56]. In this sense, Wilkins and Hackman (1974) [56] determined fecal sterols (of animal and plant origin) in healthy individuals, with the identification of low- (cholesterol: 10%, sitosterol: 4%) and high-converter subjects (cholesterol: 88%, sitosterol: 83%). The authors suggested that this situation is due not only to the composition of the microbiota but also to the physical state of the cholesterol molecule in the lumen, associated to different transit time between individuals. In fact, some investigators have suggested that the cholesterol conversion rate is associated with microbial density [37, 57], mucosal receptors for coprostanoligenic fecal bacteria [58] or inhibition of these bacteria by other gut microbiota members [31].

2.2 Impact of dietary sterols on gut microbiota

Dietary habits have become an instrumental factor in gut microbiota definition and configuration [59-62]. The fraction of the dietary fat that reaches the colon could be partially metabolized by gut microbiota, although the most amount of nutrient that reach the colon is constituted by undigested carbohydrates and protein [63]. However, the long-term intake of high-fat animal diets is associated with changes in the gut microbiota [64, 65]. Specifically, it has been seen that *lactobacilli* and *enterococci* counts (colony forming units [cfu]) are higher in feces from subjects consuming mixed and ovolactovegetarian diets than in those consuming a vegan diet [66]. In the case of vegans, slight changes in the

anaerobic bacterial genera (*Bifidobacterium, Bacteroides and Clostridium*) have been observed compared to omnivorous subjects [60]. However, the available data seems to be controversial, since no differences in the gut microbiota of vegetarian and omnivorous Slovak subjects have been found [67], and the levels remained stable after a high cholesterol diet in a murine study [68]. Other authors have also evaluate the influence of PS upon the gut microbiota in the context of a clinical trial in which 13 healthy subjects received during three weeks a control or plant stanol ester-enriched margarine (3g a day of plant stanols) – no differences being observed in gut microbiota composition compare to control group [69].

3. Contents of sterols and their metabolites in feces

In human feces, a complex mixture of sterols and their metabolites can be found as a result of the diet, digestive processes and gut microbiota action [70]. Tables 1 and 2 summarize the animal and plant fecal sterol contents, respectively, determined in some studies after the consumption of a western diet. The total fecal sterols content is between 10 and 44 mg/g in freeze-dry feces and from 0.4 to 3.5 mg/g in fresh feces. Total animal sterols (AS) excretion (from 7-29 mg/g freeze-dry feces) is higher than PS (3-6 mg/g freeze-dry feces). Taking into account the data from tables 1 and 2, AS represent 65-77% of total sterols and the PS represent the 23-35%.

The most abundant AS in feces is coprostanol (45-89%), followed by cholesterol (9-56%) and coprostanone (3-15%) [26, 38, 39, 77, 79]. Other minor cholesterol metabolites were cholestanol (2-7%), cholestanone (0.3-5%) and cholestenone (0.9%) (see Table 1).

Table 1. Cholesterol and its metabolites content (mg/g freeze-dry feces) after western diet.

Cholestanol	Cholestanone	Cholesterol	Coprostanol	Coprostanone	Epicoprostanol	Total animal sterols	References
-	-	0.7 ± 0.20	0.8 ± 0.1	0.1 ± 0.0	-	1.6	[5[a]]
-	-	1.3 ± 1.1	1.2 ± 0.6	0.1 ± 0.1	-	2.6	[71[a]]
-	-	2.8 ± 1.9	21.3 ± 8.6	2.0 ± 1.9	-	26.1	[56]
-	-	2.7 ± 0.4	14.2 ± 1.9	2.2 ± 0.5	-	19.1	[57[a]]
0.4 ± 0.2	-	3.9 ± 3.2	14.9 ± 7.7	1.4 ± 1.2	-	20.6	[7]
-	-	1.9 ± 0.4	19.2 ± 3.9	0.7 ± 0.1	-	21.8	[8]
-	-	4.4 ± 0.9	19.3 ± 3.4	2.2 ± 0.5	-	25.9	[72]
-	-	5.1 ± 1.5	8.4 ± 1.9	0.8 ± 0.2		14.3	[72[b]]
-	-	0.8	3.7	-	-	4.5	[60]
-	-	0.4	1.4	-	-	1.8	[60[c]]
-	-	4.8 ± 2.5	14.3 ± 2.5	1.1 ± 0.2	-	20.2	[73]
-	-	1.0 ± 0.2	10.3 ± 3.0	0.8 ± 0.3		12.2	[73[c]]
1.5 ± 0.8	1.2 ±2.0	3.5 ± 1.1	17.3 ± 8.6	2.6 ± 1	-	26.1	[74]
-	-	-	20.6	-	-	20.6	[49]
-	-	-	8.4	-	-	8.4	[49[c]]
-	-	11.6 ± 7.1	9.3 ± 5.6	-	-	20.9	[75]
-	0.2 ± 0.04	6.1 ± 1.2	18.0 ± 3.1	2.4 ± 0.5	0.03 ± 0.01	26.7	[47]
-	-	1.2 ± 0.05	5.9 ± 0.3	-	-	7.1	[76]
0.4 ± 0.2	-	2.7 ± 3.0	15.0 ± 9.4	1.3 ± 0.9	-	19.4	[38]
0.1 ± 0.04	-	1.2 ± 0.6	2.0 ± 0.6	-	$2.6x10^{-5}$ ± $2.3x10^{-5}$	3.3	[70*]
-	-	0.4	$6.3x10^{-4}$	-	$1.6x10^{-4}$	0.4	[77*]
-	0.1 ± 0.004	1.8 ± 0.02	20.9 ± 0.1	5.6 ± 0.2	-	28.4	[78]
-	-	2.8 ± 1.0	6.3 ± 1.6	1.8 ± 1.2	-	10.9	[67]

a high-fat vegetable oils diets; b vegetarian diet; c vegan diet; *Fresh feces.

Table 2. Plant sterols and its metabolites content (mg/g freeze-dry feces) after western diet.

Sterols	**Wilkins & Hackman, 1974** [56]	**Batta et al., 1999** [75]	**Weststrate et al., 1999** [47]	**Batta et al., 2002** [76]	**Shah et al., 2007** [70]*****	**Cuevas-Tena et al., 2017** [78]	**Olejníková et al., 2017** [67]
Brassicasterol	-	-	0.03 ± 0.02	-	-	0.4 ± 0.006	-
Campestanol	-	-	0.8 ± 0.07	-	-	0.3 ± 0.04	-
Campesterol	-	2.5 ± 1.1	0.5 ± 0.1	0.2 ± 0.01	0.1 ± 0.08	0.3 ± 0.04	-
Clerosterol	-	-	0.1 ± 0.07	-	-	-	-
Ethylcoprostanol	4.8 ± 1.8	0.6 ± 0.2	0.4 ± 0.06	1.3 ± 0.1	0.01 ± 0.004	5.2 ± 0.2	1.7 ± 0.44
Ethylcoprostanone	-	-	-	-	-	1.4 ± 0.1	-
Ethylcoprostenol	-	-	-	-	-	0.2 ± 0.001	-
Methylcoprostanol	2.2 ± 0.8	1.0 ± 0.2	-	0.7 ± 0.05	-	-	0.2 ± 0.10
Methylcoprostanone	-	-	-	-	-	0.2 ± 0.02	1.6 ± 0.40
Stigmastenol	-	-	-	-	-	0.2 ± 0.06	0.3 ± 0.11
Stigmasterol	-	1.3 ± 0.4	0.2 ± 0.06	-	0.006 ± 0.003	0.2 ± 0.006	-
Sitostanol	-	-	-	-	-	0.1 ± 0.007	-
Sitosterol	0.9 ± 0.4	3.5 ± 1.2	1.6 ± 0.40	0.4 ± 0.02	0.1 ± 0.103	1.7 ± 0.001	1.7 ± 0.64
5α-sitostan-3β-ol	-	-	0.4 ± 0.04	-	-	-	-
5α-stigmastan-3β-ol	-	-	0.03 ± 0.03	-	-	-	-
5β-sitostan-3β-ol	-	-	4.0 ± 0.5	-	-	-	-
Total plant sterols	7.9	8.9	8.1	2.6	0.2	10.0	5.5

*Fresh feces

PS excretion is less due to its lower intake compare to cholesterol intake. Although a substantial fraction of cholesterol is absorbed (30-60%) [2, 3], individual PS absorption is lower (range 4-16%; campesterol: 9.4-16%, campestanol: 12.5%, β-sitosterol: 4.2-6.0% and stigmasterol: 4.8%) [3, 80-82]. The major PS in feces were sitosterol (10-46%) and campesterol (2-46%), though their metabolites such as ethylcoprostanol (4-61%) and methylcoprostanol (4-28%) respectively, were also found to be present (see Table 2). This variability in excretion for both AS and PS is mainly due to the influence of diet and to the inter-individual variability, the specific gut microbiota and health condition.

In humans, the reduction of animal fat intake (less than 30 g fat a day) during four weeks resulted in a 40% decrease in AS excretion over the time [83]. In addition, cholesterol metabolites excretion decreased of 10% in population groups with diets consisting largely of fruit and vegetables with very little fat or animal components (from Japan, Uganda and India), compared to population groups with high-fat animal diets (from the United Kingdom and the United States) [5, 71]. In this context, it has been observed that in subjects following a commercial liquid diet (*Vivasorb*®) containing 1.3 g essential fatty acids during 10 days, the excretion of AS metabolites (4-10%) was lower than during the control period (55-75%), mainly due to the effect of the liquid diet, which accelerated the intestinal transit time [42]. It has also been observed that AS metabolites excretion is significantly elevated in subjects during the consumption of a high-meat diet (420 mg of cholesterol a day) compared to a non-meat diet (320 mg cholesterol a day) (25.2 versus 16.4 mg/g freeze-dry feces) [59]. Some authors have studied the effect of vegetarian, lacto-ovo vegetarian and omnivorous diets upon fecal sterols excretion (see Table 1). In this regard, it has been found that omnivorous subjects excrete two-fold more AS (especially

coprostanol) than vegetarian subjects [49, 72, 73] or ovo-lacto vegetarian subjects [60]. Besides, through a clinical trial it has been seen that a diet with fiber-supplemented foods (10 g wheat bran, oat fiber, or cellulose for 5 weeks) decreases AS metabolites compare to control diets (cellulose: 14 mg/g freeze-dry feces, wheat bran: 15 mg/g freeze-dry feces, non-supplemented: 22 mg/g freeze-dry feces). The exception was an oat fiber diet, which resulted in the same excretion as non-supplemented diets (23 mg/g freeze-dry feces) [74].

On the other hand, it has been seen that PS-enriched foods or dietary supplementation with PS modify sterols excretion and microbial conversion. In this sense, Weststrate et al. (1999) [47] investigated the impact of PS-enriched margarine (8.6 g PS a day) upon fecal sterol concentrations in humans, finding an increase in fecal neutral sterols (AS and PS) compared to the control group (190 versus 40 mg/g freeze-dried feces), without increase in their metabolites. In addition, a murine study involving a β-sitosterol-supplemented diet (0.8% w/w) found higher fecal cholesterol contents compared with the control group (20.9 versus 9.4 mg/day, respectively), and greater sitosterol and ethylcoprostanol excretion with respect to the control group (196 *versus* 11.8 mg/day and 24.5 *versus* 6.2 mg/day, respectively) [84].

On the other hand, the sterols content and profile in feces would also depend of the action of human gut microbiota on them [12]. In fact, the efficiency of sterols conversion is related to microbial density [57]. Besides, it has been seen that gut microbiota activity differs in each stage of life. In this sense, the microbial conversion of cholesterol to coprostanol usually begins during the second half of the first year of life [84]. In this context, Huang et al. (1976) [41] reported that the cholesterol excretion rate is low in four-year-old infants and adults with respect to breastfed infants (6 months of age) (27%, 25% and 94% of

AS with respect to total fecal sterols, respectively), because in the latter population group intake is richer in cholesterol and the gut microbiota density is comparatively lower. In addition, the authors found that coprostanol (62.6%, 65.8% and 3.8%), epicoprostanol (2.4%, 2.8% and 0.2%) and coprostanone (2.1%, 3.2% and 0.9%) excretion increased in infants and adults.

Furthermore, it has been suggested that dietary sterol excretion can be modified in the context of certain colon disorders. Specifically, greater total contents of cholesterol and its metabolites have been found in feces from subjects with colon cancer (28-36 mg/g freeze-dry feces) [46, 50, 73] adenomatous polyposis (30 mg/g freeze-dry feces) [46] or ulcerative colitis (41 mg/g freeze-dry feces) [6], compared with healthy subjects (17-20mg/g freeze-dry feces). This increase in AS has been attributed to diet and intestinal microbial activity. However, other authors have observed no significant differences in total AS excretion between patients with colon cancer (19 mg/g freeze-dry feces) [8] or familial polyposis (15 mg/g freeze-dry feces) [86] *versus* healthy subjects (18-22 mg/g freeze-dry feces).

4. Determination of dietary sterols and metabolites in feces

The analysis of sterols and their metabolites on human feces has generally been carried out by gas chromatography, and sample preparation involves four stages: (1) fat extraction, (2) saponification, (3) unsaponifiable extraction and (4) derivatization. Table 3 summarizes the sample preparation that has been applied for the determination of sterols and their metabolites in feces. Fresh feces were initially used for fecal sterol determination. However, freeze-dry feces are currently the most widely used option, since these samples offer several advantages in terms of conservation, safety and sampling [78].

Table 3. Sterols determination in feces by gas chromatography: Sample preparation.

Feces homogenization	**Fat extraction/ Purification**	**Saponification**	**Extraction unsaponifiable /Purification**	**Derivatization**	**References**
Fresh + Cl_3CH:MetOH (1:1)/ 5'	Soxhlet Cl_3CH:MetOH (1:1) 48h	2N KOH + MetOH/ reflux 1h	PE (x3) + wash (50% EtOH)/ Na_2SO_4 / dryness (N_2)	HMDS:DMF (1:1)	[43]
Fresh + water/ 3'	-	NaOH + 90% EtOH + SS (cholesterol-7α-H^3)/ reflux 1h	H_2O:PE (1:5)/ centrifugation 5' (1000 xg) (x3)/ dryness (N_2)/ PE/ TLC florisil: transfer quantitavely PE/ EE:Hp (55:45)/ rhodamine-UV light/ extraction EE (x4)/ dryness (N_2) 40ºC	EAC + IS (5α-cholestane)/ dryness (N_2)/ HMDS:DMF:TMCS (40:40:1)	[41[a], 79, 87-90]
Fresh Cl_3CH:MetOH (1:1) (x2) + EtOH/ vacuum filtration	-	-	PE: EtOH (30:70) PE phase: analysis free sterols EtOH phase: column (Amberlyst-15 and Sephadex LH-20)	Pyridine:HMDS+TMCS (5:1) 1h/ dryness (N_2)/ Hx	[91]
Freeze-dry	-	NaOH + 90% EtOH 2h (80ºC)/ cool/ filter/ wash NaOH 50% EtOH (x2)/ H_2O	PE (x3) TLC silica gel Hx:$CHCl_3$:acetone (50:45:5)/ dryness (N_2)/ PE	-	[5[a], 56]
Fresh	-	1M NaOH + 90% EtOH/ reflux 1h/ SS [4-^{14}C]Cholesterol	Hx (x6)/ dryness (N_2)/ Hx TLC silica gel: DEE:Hp (55:45)/ 1,2 dichlorofluorescein in MetOH/ extraction $CHCl_3$/ dryness (N_2)/ EAC + IS (5α-cholestane)/ dryness (N_2)	Pyridine:HMDS:TMCS (9:3:1) 30-45'	[59[a]]

Feces homogenization	Fat extraction/ Purification	Saponification	Extraction unsaponifiable /Purification	Derivatization	References
Fresh + IS (5α-cholestane)	-	1M KOH MetOH 1h (65ºC)/ mixing every 10'/ cool/ H_2O	Hx/ shake 1'/ centrifuged 10' (x5)/ dryness (N_2)/ Hx/ dryness (N_2) Underivatized sample: EAC (over CaH_2)/ sonication	HMDS:DMF:TMCS (40:40:1)/ sonicated/ 30'/ dryness (N_2)/ EAC/ sonication	[92]
Fresh + water/ omni-mixer	Soxhlet EtOH 48h	2N KOH 2h/ H_2O	PE (x4) TLC silica acid: Hx:EAC (8:2) TLC alumina/ $AgNO_3$:Cl_3CH:PE:acetone (60:30:30)/ extraction EE/ IS (5α-cholestane)	-	[93]
Fresh + H_2O/ omni-mixer/ Cl_3CH:MetOH (1:1)/ vacuum filtration/ dryness	TLC Lipidex-5000: MetOH:H_2O:Cl_3CH (9:1:5) 2h/ free sterols extraction MetOH:H_2O:Cl_3CH (9:1:5)/ wash/ esterified sterols extraction MetOH:Cl_3CH (1:1)	Esterified sterols dryness/ KOH + MetOH 1h (60ºC)/ mixing every 15'/ cool	TLC Lipidex-5000 (free sterols): water/ EtOH:PE (3:7) (x2)/ wash PE (x3-4)/ oxosterols extraction PE/ hydroxylated sterols extraction Cl_3CH	Hydroxylated sterols/ pyridine:HDMS:TMCS (9:3:1) 30' (60ºC)/ dryness/ Hx + IS (5α-cholestane)	[7]
Freeze-dry	-	5M NaOH + MetOH + boiling chips/ reflux 2h (80ºC)	PE/ centrifuge 5'(1000 xg) (x3) TLC florisil: EE:Hp (55:45)/ extraction EE	DMF:BST (2:1) 30' (80ºC)	[94[a]]
Freeze-dry	-	MetOH + heptadecanoic acid and nor-deoxycholic acid/ 2.5M NaOH 2h (120ºC)	HCl (pH 1)/ 6mL $CHCl_3$:MetOH (2:1) (x3)/ N_2 (40ºC)	$CHCl_3$ + DMAP + acetic anhydre/ N_2 (40ºC)/ $CHCl_3$/ water (x2)/ magnesium sulphate/ N_2	[95[b]]

Feces homogenization	Fat extraction/ Purification	Saponification	Extraction unsaponifiable /Purification	Derivatization	References
Fresh + IS (5α-cholestane)	-	1N NaOH/ MetOH 76%	PE/ acetic acid pH 4/ evaporation	Trifluoracetylated	[60ª]
Fresh + water + IS (5α-cholestane)	-	Macro-method: 1N NaOH + 90% EtOH 1h (67ºC) Micro-method: The same steps as macro-method, but with 90% less solvent	H_2O + Cx (x3) / N_2	Pyridine:HMDS:TCS (9:3:1) 30'/ dryness (N_2)/ decane/ centrifugation 10' (2000 rpm)	[96ª]
Fresh + water (1:1) + IS (5α-cholestane)	-	1N NaOH + 90% EtOH/ 1h (67ºC)	Hx (x3)/ N_2	Pyridine:HMDS:TCS (3:2:1) 30' (60ºC)/ dryness (N_2)/ Hx	[82]
Freeze-dry + IS (nor-cholic acid)	-	-	-	Butil ester formation: n-butanol + HCl 4h (60ºC)/ Pyridine:HMDS:TMCS (9:3:1) 30' (55ºC)/ dryness (N_2)/ Hx/ centrifugation	[75ª]
Freeze-dry + IS (5α-cholestane)	-	NaOH extraction: 1N NaOH 1h (90ºC)	Hx (x4)/ evaporate	Pyridine:HMDS:TMCS (9:3:1) 30' (55ºC)/ dryness (N_2)/ Hx	
	Soxhlet: filtration/ 1% ammonia EtOH 16h/ evaporate	-			
	Folch: Cl_3CH:MetOH (2:1) 30' (65ºC)/ centrifugation/ Cl_3CH:MetOH (2:1) (x3)	-			

Feces homogenization	Fat extraction/ Purification	Saponification	Extraction unsaponifiable /Purification	Derivatization	References
Freeze-dry + IS (5α-cholestane)	-	NaOH + MetOH	PE	BSTFA/ Hx	[47[a]]
Freeze-dry + IS (nor-cholic acid)	-	-	-	Butil ester formation: n-butanol + HCl 4h (60ºC)/ Pyridine:HMDS:TMCS (9:3:1) 30' (55ºC)/ dryness (N_2)/ Hx /centrifugation	[76[b]]
Freeze-dry + IS (5α-cholestane)	Soxhlet: extraction: filtration/ 1% ammonia EtOH 16h/ evaporate	0.5N NaOH 1h (90ºC)	Hx (x4)/ evaporate	Pyridine:HMDS:TMCS (9:3:1) 30' (55ºC)/ dryness (N_2)/ Hx	
Freeze-dry	pH~3/ Cl_3CH:MetOH (2:1)	1N KOH	EE/ N_2 dryness/ acetone/ TLC PE:EE (85:15)	-	[97]
Freeze-dry + H_2O + IS (5α-cholestane)	-	NaOH + 90% EtOH 1h (70ºC)	H_2O + Cx (x4)/ dryness (N_2)/ decane	-	[38[a], 67]
Fresh + IS (5α-cholestane)/ filtration/ freeze-dry (12h)	Soxhlet: DEE (x5)	KOH + 10% MetOH 120' (100ºC)/ 6M HCl/ freeze-dry (liquid N_2)	EtOH/ centrifuge 3' (3000 rpm) (x3)/ freeze-dry (liquid N_2)	Methoxyamine hydrochloride 30' (60ºC)/ BSTFA 60' (100ºC)/ centrifugation 5' (3000 rpm)	[70]
Dry + SS (cholesterol-d_6)	-	-	DCM/ sonication 20' (30ºC) (x3)/ Na_2SO_4/ DCM/ Hx:T/ clean up: alumina TLC florisil: MetOH + DCM + Hx:T (95:5)/ extraction Hx:T (95:5) + MetOH/ dryness (N_2)/ DCM	BSTFA + 1% TCMS + IS (5α-cholestane)/ vortex 10"	[77]

Feces homogenization	Fat extraction/ Purification	Saponification	Extraction unsaponifiable /Purification	Derivatization	References
Freeze-dry + IS_1 (5α-cholestane) + IS_2 (cholesteryl palmitate) + IS_3 (cholesteryl cinnamate)	-	KOH + 90% EtOH 1h (80ºC)/ 6M HCl	Hx:MTBE (3:2)/ filtration/ dryness (N_2)	BSTFA+1%TMCS:pyridine 20' (80ºC)/ dryness (N_2)/ Hx:MTBE (3:2)	[98]
Freeze-dry + H_2O/ sonication 20'/ 2h	-	IS (5α-cholestane) + 0.71M KOH + EtOH + 0.02% BHT/ 1h (65ºC)	H_2O + Hx/ vortex 30''/ centrifugation 10' (3600 rpm/ 18ºC) (x3)/ dryness (N_2)/	BSTFA+1%TMCS:pyridine (10:3) 1h (65ºC)/ dryness (N_2)/ Hx/ filtration (x3)/ dryness (N_2)/ Hx	[78]

BHT: butylhydroxytoluene; **BST**: bis-silyl-trifluoroacetamide; **BSTFA**: *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; **Cx:** ciclohexane; **DCM**: dichloromethane; **DEE**: diethyl ether; **DMAP**: dimethylaminopyridine; **DMF**: dimethylformamide; **EAC:** ethylacetate; **EE:** ethyl ether; **EtOH**: ethanol; **HMDS**: hexamethyldisilizane; **Hp:** heptane; **Hx:** hexane; **IS**: internal standard; **MetOH:** methanol; **MTEB**: methyl-*tert*-buthyl ether; **PE:** petroleum ether; **SS**: surrogate standard; **T:** toluene; **TCS**: trichlorosilane; **TLC:** thin Layer Chromatography; **TMCS**: trimethylchlorosilane; **UV:** ultraviolet; [a] neutral sterols and bile acids simultaneous determination; [b] neutral sterols, fatty acids and bile acids simultaneous determination

Generally, both fresh and freeze-dry feces are homogenized with organic solvents (e.g. chloroform (Cl_3CH): methanol (MetOH)) (Table 3). In a previous study carried out by our group, freeze-dry feces were homogenized with water to obtain a more environmentally friendly method. Besides, due to the presence of particles in suspension that hindered pipetting, the influence of filtration was evaluated. We concluded that subjecting the samples to ultrasound (20 min) and keeping the dispersion (feces + water) at room temperature for two hours was better than the filtration process, which caused a low precision (between 1.2 and 47.5 RSD%) [78]. Other authors have attempted to improve feces homogenization with water by applying only agitation [87, 88, 90, 96], or using an Omni-Mixer [7, 93].

4.1. Internal standard

Quantification requires the inclusion of an internal standard (IS) prior to the saponification step. The most widely used IS is 5α-cholestane (Table 3), though exceptionally other options such as nor-cholic acid [75, 76] are also used. Cholesteryl acetate and cholesteryl cinnamate have been used in determining esterified forms [98]. Besides, some authors use a surrogate standard (SS), this is a non-target analyte that has chemical properties similar to those of the analyte of interest. A known concentration of SS is added at the start of the analysis, together with the sample, in order to evaluate the analyte response during the measurement [99]. Some authors use cholesterol-7α-H_3 [41, 79, 87-90] and cholesterol-d_6 [77] as SS to determine extraction efficiency before starting the analysis, and prior to the derivatization step they also introduce the IS to correct analyte concentrations during analysis (see Table 3).

In this sense, Miettinen et al. (1965) [87] described a method for isolating and separating fecal neutral steroids by thin layer chromatography (TLC), with quantification using gas chromatography (GC). They reported that the use of two standards - one (cholesterol-7α-H^3) to correct losses through the multistep isolation (TLC) procedure and another (5α-cholestane) for conversion of GC peak areas into weights of neutral steroids - vastly improved the accuracy of the procedure.

4.2. Fat extraction and saponification

Many authors have carried out saponification without previous fat extraction or purification, using mainly sodium hydroxide or potassium hydroxide with MetOH or ethanol (EtOH) as saponifying reagents (see Table 3). The temperature used during saponification was between 60-100ºC, and the time employed ranged from 30 minutes to two hours. Eneroth et al. (1964) [43] compared two different saponification strategies using reflux (1 h at 60ºC) and nitrogen (1 h at 60ºC), with the obtainment of similar results for cholesterol and some of its metabolites. Miettinen et al. (1965) [87] in turn evaluated the influence of saponification in feces from subjects after the oral administration of cholesterol-C^{14} using cholesterol-7α-H^3 as SS and NaOH 1N during one hour under reflux with different EtOH concentrations (2%, 50%, 63% and 90%). They then performed three extractions with petroleum ether (PE), followed by another saponification step during 24 hours with 63% EtOH. The authors reported that one-hour reflux extraction with 63% EtOH was sufficient to obtained optimum recoveries (101%). Results showed that saponification was effective, since less than 0.2% of the sterols were found as non-hydrolyzed sterol esters. Besides, the authors

indicated that at a lower concentration of EtOH, sterols were retained in the aqueous phase due to the solubilizing effects of bile salts and soaps.

This saponification method has been applied in feces by other authors with different purposes, such as evaluation of the cellulose effect on dietary sterols excretion in humans [88]; analysis of the influence of age upon neutral sterol patterns in healthy human subjects [41, 90]; development of a method for the identification of human and rat neutral fecal steroids [89]; and improvement of the method for determining human fecal neutral sterols [79]. A method based on that of Miettinen et al. (1965) [87], allowing the evaluation of daily neutral and acid sterols excretion with small feces sample (1 g), has been proposed. The authors used sitostanol as a fecal flow marker instead of employing a homogenized sample of daily-excreted feces. Besides, they reduced the saponification reagent volume and 90% of the ciclohexane (Cx) as organic solvent used in the extraction step [96].

Before saponification, some authors have applied a prior fat extraction using Soxhlet [43, 70, 75, 76, 93] or Folch extraction [75], while others have purified the sample by means of a chromatographic column [7]. Batta et al. (1999) [75] proposed a simple method for quantifying neutral (coprostanol, cholesterol, methylcoprostanol, ethylcoprostanol, campesterol, stigmasterol and sitosterol) and acid fecal sterols without a prior isolation step. This method was compared against other rigorous solvent extraction methods such as Soxhlet and Folch (Table 3), and showed similar reproducibility and accuracy. An advantage of the simplified proposed method is the complete hydrolysis of sterol esters to produce the corresponding derivative compounds.

4.3. Unsaponifiable extraction or purification

The unsaponifiable fraction is extracted using solvents such as hexane (Hx), EtOH, ethyl ether (EE), dichloromethane (DCM) and Cx. These solvents have replaced the initially used PE. Solvent mixtures such as Hx:Cl_3CH, DCM:MetOH and Hx:EE (see Table 3) are often used. Since the 1960s and 1970s, many authors have carried out determination studies based on the method of Miettinen et al. (1965) [87], involving a TLC step after saponification. In the 1980s, the introduction of capillary columns to separate steroids allowed suppression of the TLC stage [44, 94]. In fact, this advance allowed simpler, shorter and more precise determinations. On the other hand, since the 1990s there has been a change in unsaponifiable extraction solvents, with the introduction of greener solvents such as Hx and Cx.

4.4. Derivatization

Several derivatization reagents has been used in order to improve the volatility, peak shape, and response factors of sterols. Although acetylation is also used for derivatization, employing trifluoroacetylated [60] or acetic anhydride with dimethylaminopyridine (DMAP), this practice was more common in the past [100]. Silylation is currently the most common derivatization method [100, 101]. In this context, the prevalent silylation reagent used is hexamethyldisilizane (HMDS) with dimethylformamide (DMF) and trimethylchlorosilane (TMCS) (Table 3). Besides, other mixtures such as pyridine, HMDS and TMCS or trichlorosilane (TCS) and pyridine with N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) + 1% TMCS are also employed. In the latter mixture, TMCS acts as a catalyst to increase the silylating power,

improve the reactivity of hindered hydroxyl groups or enhance reaction rates [101].

On the other hand, pyridine began to be used for the derivatization of bile acids [102], since silylation of the hydroxyl groups on carbons 3, 6, 7, and 12 occurred rapidly and quantitatively [87]. In application to neutral fecal sterols it has been used since the 1980s [7]. Despite the advantages of the derivatization procedure, some authors do not include this step in fecal sterols determination [5, 38, 56, 93, 97].

The derivatization temperatures and times were between 55-80ºC and 15-60 min, respectively. However, from the 1960s to the 1980s, derivatization reactions using silylation mixtures containing HMDS with TMCS were generally completed in 5-15 minutes at room temperature [100]. Since the 1990s, the application of heating (55-100ºC) during the derivatization step has been used. In this sense, Wu et al. (2009) [77] optimized the injection-port derivatization parameters such as temperature (from 200ºC to 300ºC), purge-off time (0.2-2.0 min in splitless mode) and derivatization reagent volume (from 10 µl to 200 µl). Finally, 300ºC was chosen as the optimized injection-port temperature; GC-mass spectrometry (MS) responses increased when the purge-off time was 1.5 min; and abundance increased with 50 µl of BSTFA, 1% TCMS. In order to increase reaction efficiency and prevent hydrolysis of the products, the type of organic solvent was also optimized. DCM provided the highest derivatization efficiency, followed by ethylacetate (EAC), tert-butyl methyl ether, Hx and acetonitrile as the option yielding the poorest derivatization efficiency.

4.5. Identification and quantification

Gas chromatography with flame ionization detector (FID) or MS has been the methodology most commonly used for the identification and quantification of sterols and their metabolites in feces (Table 4). Currently, MS has displaced FID. Studies were made between the 1960s and 1980s using MS [7, 43, 91, 92].

Table 4. Sterol analysis in feces by gas chromatography: Instrumental conditions and analytical parameters.

Column	Injector (°C)	Oven (°C)	Detector (°C)/ carrier gas	Validation	Reference
U-tube column, packed with acid-washed, acetone-washed and silanized Gas Chrom P (100-140 mesh) coated with 0.5-4% QF-1 and 1% SE-30 (4 m x 2 mm).	290	250	MS/ He	Recovery: 80-109%	[43]
6-ft glass U-tubes, packed with silanized acid-washed Gas Chrom P (100-120 mesh) coated with 1-2% DC-560 and SE-30 (ID 4 mm)	300	240	FID (290)/ N_2	Recovery: 97%	[87]
3% SE-30, 2m x 3.5mm (GLC)	260	250	Ar	-	[91]
1% SE-30 and 3% QF-1 columns (GC-MS)	-	210	MS/ -		
4 ft glass U-tube, packed with Distoport S (80-100 mesh) coated with 3.8% of SE-30 (ID 4 mm)	300	230	FID/ He	-	[88]
3% SE-30 column	-	223	-	-	[5]
6-ft coiled glass column, ID 0.25 inch, Gas Chrom (Q 100-200 mesh) coated with 3% QF-1	300	223	FID (260)/ N_2	-	[56]
2% SE-30 + 1% SP-2401	250	220	FID (260)	Recovery: 95%	[57]
Double-looped U-shaped glass column packed with 1% SP-1000, Gas Chrom (100-200 mesh)	-	245	FID/ N_2 & MS/ N_2	Recovery: 73-96% RSD: 2 - 10% LOD: 3 µg/g fecal slurry	[92]

Column	Injector (°C)	Oven (°C)	Detector (°C)/ carrier gas	Validation	Reference
6 ft x 2mm U-shaped glass column packed with 3% OV-225, Gas Chrom Q (100-120 mesh)	260	230	FID (250)/ N_2	-	[41]
Stainless steel column (1.50m x 3.2mm) coated with 2% QF 1	220	200	FID (240)/ N_2	-	[93]
180cm U-tubes, ID 4 mm, packed with silanized acid-washed Gas Chrom P (100-120 mesh) coated with 1% DC-560 and SE-30	50	215-265	FID (40)/ N_2	-	[89]
Packed column 1% SE-30 (46 m x 0.3 mm ID)	-	170 (20°C/min) to up 265	FID/ N_2	-	[44]
Packed column 1% SE-30	-	170 (20°C/min) to up 280	MS/ H_2	Recovery: 81-99% RSD intra-day: 3.7% ± 2.9% RSD inter-day: 7.9% ± 3.9%	[7]
Glass column (1.8 m x 2 mm), packed with 3% SP-2401 on 100/120 Supelcoport	250	220	FID (270)/ N_2	Linearity: >0.99 Recovery: 97%	[79]
6-ft glass U-tubes, ID 4 mm, packed with silanized acid-washed Gas Chrom P (100-120 mesh) coated with 1-2% DC-560 and SE-30	300	240	FID (290)/ N_2	-	[90]
CP-Sil-5 CB (25m x 0.22 mm), 100% dimethylsiloxane	250	200˚C (15°C/min) to up 260 held 20’	FID/ H_2	Recovery: 88-104% RSD intra-day: 1.1-2.6% RSD inter-day: 0.6-4.0%	[94]
15m DB5 polimethyl, 5% phenyl soloxane (0.32 mm ID x 0.25 µm)	300	50 (50°C/min) to up 200, (4°C/min) to up 285, (2°C/min) to up 300	FID/ He	RSD intra-day: 2.9-33.3%	[95]

Column	Injector (°C)	Oven (°C)	Detector (°C)/ carrier gas	Validation	Reference
Capillary glass column (4mm CP Sil 5), 25 m x 0.45 mm ID	Solid injector (235)	224	FID (260)/He	-	[60]
50-m fused-silica (CS-FS-OV-101, 0.32 mm ID)	-	150 held 3', (10°C/min) to up 270	FID/ H_2	-	[96]
SE-30 (25 m x 0.32 mm x 0.25 µm) + pre-column (2 m, 0.53 mm ID)	-	150 held 3', (30°C/min) to up 250, (5°C/min) to up 280 held 5'	FID/ H_2	-	[81]
CP-Sil-5 CB (25 m x 0.22 mm), 100% dimethylsiloxane	260	100 held 2', (35°C/min) to up 278	FID (290)/ He & MS/ He	-	[75]
Column coated with unipolar stationary phase	-	-	FID	-	[47]
CP-Sil-5 CB (25m x 0.22 mm), 100% dimethylsiloxane	260	100 held 2', (35°C/min) up to 278	FID & MS/ He	-	[76]
2% SE-30, Gas Chrom Q	-	-	FID	-	[97]
Fused-silica capillary column (50m x 0.2 mm x 0.2µm)	280 Split (1:50)	150 held 5', (40°C/min) to up 240, (1°C/min) to up 280 held 10'	MS/ He	Linearity: >0.99 RSD intra-day: 2.7-8% RSD inter-day: 4.2-11.2% LOD: 0.9-2.4µg/ml	[38]
HP-5ms fused silica (30m x 0.25mm x 0.25µm)	Splitless	120 held 2', (5°C/min) to up 250, (3°C/min) to up 300 held 10'	MS/ He	-	[70]
HP-5MS (30m x 0.25mm x 0.25µm), 5% phenyl + 95% dimethylsiloxane and (20 m x 0.25 mm x 0.25 µm) as guard column	Splitles	70 held 2', (20°C/min) to up 270 held 1', (5°C/min) to up 300 held 13'	MS/ He	Linearity: >0.99 Recovery: 88-97% RSD: 8.0-19.6% LOD: 2-15µg/ml	[77]

Column	Injector (°C)	Oven (°C)	Detector (°C)/ carrier gas	Validation	Reference
Crossbond trifluoropropylmethylpolysiloxane Rtx-200MS (30 m x 0.25 mm x 0.1 µm)	280 Split	100 held 2', (15°C/min) up to 310 held 2', (1.5°C/min) up to 340 held 3'	FID (360)/ H_2 & MS/ He	Linearity: >0.99 Recovery: 92-99%	[98]
CP-Sil8 CB low bleed/ MS (50m x 25mm x 0.25µm) + pre-column VSD Tubing (2m x 320 µm)	Splitles 80 (14.5°C/sec) to up 280	150 held 3', (30°C/min) to up 280, (10°C/min) to up 290 heald 28'	MS/ H_2	Linearity: >0.96 Recovery: 80-119% RSD intra-day: 0.9-9.2% RSD inter-day: 2.1-11.3% LOD: 0.10-3.88µg/ml LOQ: 0.34-12.94µg/ml	[78]
Fused-silica capillary column (50m x 0.2 mm x 0.2µm)	280 Split (1:50)	150 held 5', (40°C/min) to up 240, (1°C/min) to up 280 held 10'	MS/ He	-	[67]

FID: flame ionization detector; **ID**: inside diameter; **LOD**: limit of detection; **LOQ**: limit of quantitation; **MS:** mass spectroscopy; **RSD**: relative standard deviation.

Selected ion monitoring (SIM) has frequently been used in this latter technique [38, 77]. Other authors preferred to combine different techniques such as TLC with GC-FID [79, 87, 97] or GC-FID with GC-MS [75, 76, 91].

At present, capillary columns have displaced the packed columns used above. The most commonly used capillary column characteristics were 30 m in length x 0.25 mm internal diameter (ID) x 0.25 µm thickness size. On the other hand, nitrogen was the most commonly used carrier gas in GC-FID when a packed column was used, *versus* hydrogen and helium in the case of capillary columns. Helium was used with GC-MS, though in some cases hydrogen has also been used [7, 78]. Table 4 summarizes the validation analytical parameters for sterols and their metabolites in feces. In the 1970s and 1980s, some authors [7, 79, 92, 94] reported incomplete validation parameters in the determination of sterols and their metabolites in feces. At present, few studies carry out complete validation (selectivity and linearity, precision, accuracy and detection and quantitation limits) [38, 77, 78].

5. Conclusions

Cholesterol biotransformation has been extensively studied, and it has been seen that a broad of bacteria, such as *Eubacterium* (routes I, II and V), *Bacteroides* (routes I and II) and *Bifidobacterium* (route I) are implicated. In the case of PS, although its main microbial metabolites are known, there are few studies on the microbiota responsible of their metabolization. While five possible biotransformation routes for cholesterol have been established, for PS, studies on their microbial transformation are scarce. It has been suggested that cholesterol metabolites act as pro-carcinogens in high-fat animal diets, while the presence of such metabolites decreases in diets rich in vegetables (vegetarian and vegan) or

with PS supplementation diets. However, the reason for this decrease in cholesterol metabolites is still unknown. Further studies are needed on the influence of the dietary supply of PS upon the incidence of colon cancer.

In the other hand, the technique of choice for the determination of sterols and their metabolites is GC/MS. The complexity of sample preparation, which includes several steps, would require future studies to develop, to validate and standardize a method for such determinations in feces – few data are available for PS analysis. This review contribute to improve the knowledge of the sterol implication in the gut microbiota and of PS impact in the colonic microbiota metabolization of cholesterol in healthy subjects and in the context of concrete diseases (colon cancer, adenomatous polyps and ulcerative colitis).

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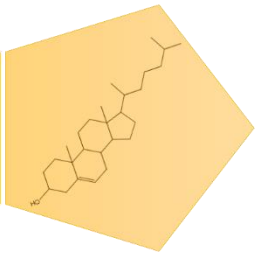
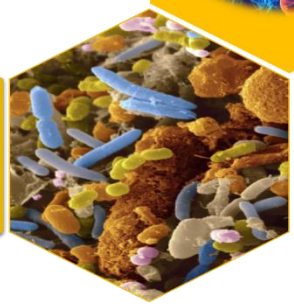
Anexo/ Annex III





Impact of high dose plant sterols on gut microbiota from lean and obese subjects using TIM-2 *in vitro* fermentation model.

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Abstract

Overweight and obesity constitute the main risk factors for some chronic disorders, including cardiovascular disease and cancer. It is well known that plant sterols could have a hypocholesterolemic effect and colon cancer. However, there are scare data on PS-gut microbiota interaction. We evaluated the influence of PS (~2g/day) upon gut microbiota and *vice-versa,* using a validated *in vitro* model of the large intestine (TIM-2), with feces from lean or obese subjects. Faecal sterols, short chain fatty acids and microbiota composition were determined by GC-MS, IEC, and 16S-sequencing (V3-V4 regions), respectively. PS feeding led to a decrease in the concentration of coprostanol and ethylcoprostanol (more pronounced with lean microbiota). Besides, plant sterols increased the production of acetate and butyrate. Methylcoprostanone production for lean microbiota was negatively correlated with an OTU in the *Clostridiales*-order. For obese microbiota a negative-correlation with *Peptostreptococcus* and *Methanobrevibacter*, and a positive-correlation with *Bacteroides* were observed.

1. Introduction

The daily dietary intake of plant sterols (PS) (160–400 mg/day) (Lagarda, García-Llatas & Farré, 2006; Gylling et al., 2014) does not reach the established levels to have a hypocholesterolemic effect (1.5–3.0 g/day) (Commission Regulation (EU), 2014). Therefore, the mentioned levels can only be achieved by incorporating PS-enriched foods to the usual diet (such as, yellow fat spreads, salad dressings, milk type products, fermented milk products, soya drinks, cheese type products, rye bread and rice drinks).

Dietary cholesterol is absorbed 50% (de Boer, Kuipers & Groen, 2018), although its absorption varies substantially between individuals (20–80%) (Stellaard & Lütjohann, 2015). However, the intestinal absorption rate of total PS

is only 2–3% (García-Llatas & Rodríguez-Estrada, 2011). Non-absorbed sterols reach the colon and can be biotransformed by the gut microbiota. Although cholesterol biotransformation mediated by gut microbiota has been widely studied using *in vitro* assays (Wong, 2014), less information is available on the biotransformation of PS and its possible impact (in the case of high intake levels) on colonic microbial metabolization of other substrates.

There is an important association between colonic microbiota and host, and the microbiota has been acknowledged as a metabolic organ (Bäckhed et al., 2004). A well-balanced intestinal microbiota has been shown to be important for the health of the host (e.g. increased colonization resistance, stimulation of the immune system, support in digestion and synthesis of nutrients, production of butyrate for enterocytes) (Portune, Benítez-Páez, Del Pulgar, Cerrudo & Sanz, 2017). The metabolic function of the microbiota highly depends on the type of substrates available to be fermented. The major fermentation processes in the colon are saccharolytic and proteolytic fermentation. However, there are many more dietary components that make it to the colon and are fermented, but there are few studies on the lipolytic fermentation process, and specifically on PS (Bernalier-Donadille, 2010).

On the other hand, overweight and obesity constitute the main risk factors for a wide range of chronic disorders, including diabetes (with a 44% increased risk in obese subjects), cardiovascular disease (23%), and cancer (41%) (Bray, 2004; WHO, 2018). Some studies in humans (Turnbaugh et al., 2009; Ley, Turnbaugh, Klein & Gordon, 2006) and animals (Turnbaugh et al., 2006; Ley et al., 2005; Bäckhed et al., 2004) have associated the intestinal microbial dysbiosis with obesity. In general, obese individuals show a decrease in bacterial diversity (Mishra, Dubey & Ghosh, 2016; Kasai et al., 2015; Lau, Carvalho, Pina-Vaz, Barbosa & Freitas, 2015; Turnbaugh et al., 2009) characterized by an increase in

the *Firmicutes-Bacteroidetes* ratio (Boulangé, Neves, Chilloux, Nicholson & Dumas, 2016; Gerard, 2016; Kasai et al., 2015; Lau et al., 2015; Lecomte et al., 2015).

In the present study, the validated dynamic *in vitro* proximal colon model developed by The Netherlands Organization for Applied Scientific Research (TNO) was used to evaluate the effect of high doses of PS on composition and metabolic functions of microbiota from lean and obese subjects.

Given that there are no studies on the interaction between high doses of PS and microbiota from obese subjects and this is the first time that data on the production of short chain fatty acids (SCFA) after *in vitro* fermentation with high doses of PS are reported, this work contributes to the understanding whether the addition of PS to foods could modify the profile of colonic microbiota to that in lean subjects.

2. Material & Methods

2.1. Test compounds

The PS source used for the microbial fermentation in the *in vitro* system were a commercial food ingredient (Lipophytol® 146ME Dispersible) with free microcrystalline PS from tall oil in powder form (Lipofoods SLU, Barcelona, Spain) and a commercial standard of β-sitosterol ≥70% obtained from Sigma-Aldrich® (Munich, Germany). These PS sources were previously analyzed as decribed by González-Larena et al. (2011) and the PS present in both ingredient and standard are show in Table 1.

PS from the ingredient were micro-encapsulated with maltodextrin, esters of sucrose and inulin, thus it was necessary to carry out an acidification (pH 2, 37ºC with a water bath) for 2 hours on the same day prior to introduction of the test compounds in the system, to degrade the carbohydrates capsule.

Table 1. Plant sterols present (g/100g) in the test compounds.

Sterols	Ingredient	Standard
Sitosterol	43.4 ± 1.2	80.7 ± 1.0
Sitostanol	5.3 ± 0.6	6.1 ± 0.1
Campesterol	3.3 ± 0.1	8.7 ± 1.0
Stigmasterol	0.8 ± 0.01	-
Campestanol	0.1 ± 0.02	-

It was considered appropriate to apply this acidification also to the standard and control, so tests compounds were treated under the same conditions. Table 2 shows a summary of the amount of tests compounds and the reagents used to carry out the acidification in glass jars (100mL). After acidification the pH was adjusted to 5.8, the final volume was 30mL, which was collected with a sterile plastic syringe.

Table 2. Reagents used for the acidification process applied to the test compounds.

Test compounds	Amount assessed (g)	NaCl 0.85% (mL)	Tween 80 (g)	HCl 1M (µL)
Ingredient	4			160
Standard	2.5	20	0.5	140
Control	-			120

2.2. Microbiota: source, collection and processing

Homogenates of human feces were made from subgroups of healthy volunteers who were selected according to their body mass index (BMI). The group of participants had not used prebiotics or probiotics in the 2 weeks prior to the donation and had not taken antibiotics for at least 3 months.

Lean participants: five donors (3 women and 2 men) with a mean age of 27.2 ± 4.3, an average weight of 61.6 ± 3.9 and a mean BMI of 21.7 ± 0.8. Obese participants: thirteen donors (7 women and 6 men) with a mean age of 55.1 ± 9.8, an average weight of 100.0 ± 12.9 and mean BMI of 33.2 ± 3.5.

All the donors collected a fresh fecal sample in a gastight bag and placed it immediately into a plastic jar containing an anaerobic strip (AnaeroGenTM, Cambridge, UK). Samples were transported in a period not longer than 5h to the laboratory, were homogenized and mixed under strict anaerobic conditions (Sheldom Lab-Bactron IV, Cornelius, OR, USA) to create a standardized microbiota stock according to Aguirre et al. (2014). Mixing was done with a dialysate solution (content per litre: 2.5g $K_2HPO_4 \bullet 3H_2O$, 4.5g NaCl, 0.005g $FeSO_4 \bullet 7H_2O$, 0.5g $MgSO_4 \bullet H_2O$, 0.45g $CaCl_2 \bullet 2H_2O$, 0.05g ox bile, 0.4g cysteine hydrochloride; pH 5.8), with 1mL of a vitamins mixture (content per litre: 1mg menadiona, 2mg D-biotin, 0.5mg vitamin B12, 10mg pantothenate, 5mg nicotinamide, 5mg *p*-animobenzoic acid and 4mg thiamine), and glycerol (14% w/w) as cryoprotective agent. Fecal suspension was aliquoted (35mL), frozen in liquid nitrogen, and stored at -80ºC until inoculation in TNO *in vitro* Intestinal Model of the colon (TIM-2). Before being introduced into the system, the inoculum was thawed during 1h in a 37ºC in a water bath (Aguirre et al., 2015). Subsequently, in the anaerobic chamber the aliquot (35mL) of fecal suspension was diluted with dialysate (1: 1, v/v) and 60 mL transferred to a sterile plastic syringe. This was used as the inoculum of the TIM-2 unit.

2.3. Standard ileal efflux medium (SIEM)

The microbiota was adapted to the system conditions with the standard ileal efflux medium (SIEM), which simulates material passing the ileocecal valve in humans, or in other words material reaching the colon. SIEM contained the following components: 100g CHO medium (per litre: 12g pectine, 12g xylan, 12g arabinogalactan, 12g amylopectine, 100g starch), 25g TBCO 6.25x (per litre: 270g Tween 80, 375g bactopepton, 375g casein, 6.25g ox-bile), 2g $MgSO_4$ (50g/L), 2g cysteine (20g/L), 0.2mL vitamin mixture (mentioned above), 4mL salts solution (4.7g $K_2HPO_4 \bullet 3H_2O$, 8.4g NaCl, 0.8g $CaCl_2 \bullet 2H_2O$, 0.009g $FeSO_4 \bullet 7H_2O$, 0.02g haemin) and antifoam B emulsion (Sigma-Aldrich®, Munich, Germany). The pH was adjusted to 5.8.

2.4. TIM-2 fermentation assays

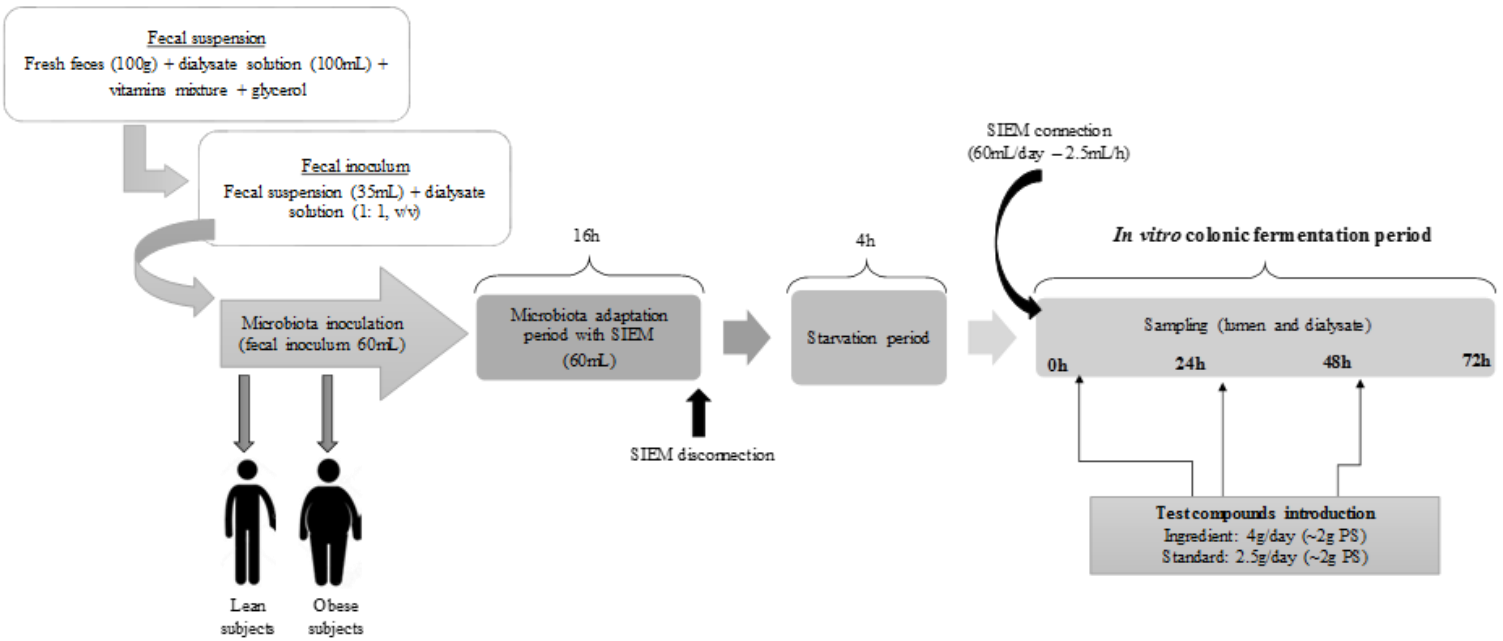
The TIM-2 system is a validated dynamic, computer-controlled model which simulates the human proximal colon, mimicking body temperature (37ºC), lumen pH (5.8), absorption of water and microbial metabolites through a semipermeable membrane inside de model, mixing and transporting the intestinal contents with peristaltic movements, anaerobic conditions by flushing with gaseous N_2, and using an microbiota from human origin (Maathuis, Hoffman, Evans, Sanders & Venema, 2009; Venema Vermunt & Brink, 2005).

All experiments (control, ingredient and standard) were performed in duplicate. At the start of each experiment, the model was inoculated with approximately 60mL of the standardized microbiota from lean or obese donors (see 2.2) and 60mL of dialysate (see 2.2). SIEM was fed at a rate of 2.5 ml/h. The microbiota was allowed to adapt to the model conditions and SIEM for 16h and after that, a 4h starvation period allowed the bacteria to ferment all available carbohydrates in the system prior to the addition of the test compounds (Fig. 1). After the

starvation period, samples of lumen and dialysate were collected at time-point zero (t0), and SIEM was fed in all units of the system (feeding rate: 60mL/day – 2.5mL/h). Samples of acidified ingredient, standard and control (see 2.1) collected in the syringe were introduced through the sampling port each day.

After 24, 48 and 72h, samples of the lumen and dialysate were collected, as is shown in Fig. 1. A total lumen sample of 30mL was removed from the system to simulate passage of material from the proximal to the distal colon.

Figure1. Experimental design



SIEM: Standard Ileal Efflux Medium; PS: Plant Sterol

2.5. Analytical methods

2.5.1. Fecal sterols

Fecal sterols and their metabolites were determined according to Cuevas-Tena, Alegría & Lagarda, (2017). Briefly, the samples were diluted with MilliQ water (1:100) and volumes from 25µL to 200µL were needed for those samples with large amounts of PS (ingredient and standard 24, 48 or 72h), and volumes between 1 and 3mL were taken mainly for the analysis of samples at t0 and control. The analysis was performed in triplicate using 5α-cholestane (20µg) as internal standard. The saponification step was carried out with 1mL of ethanolic potassium hydroxide solution 0.71 M (65ºC/1h) using a block heater. The unsaponifiable fraction was extracted with 0.5mL of Milli-Q water and 2mL of n-hexane (centrifuged at 18ºC/10 min/3600 rpm). The n-hexane extraction step was performed twice under the same conditions as described above. The organic extracts were evaporated to dryness under nitrogen. In order to obtain the trimethylsilyl ether (TMSE) derivatives, 200 µL of BSTFA + 1% TMCS:pyridine 10:3 (v/v) were added (65ºC/1h). The TMSE derivatives obtained were dissolved with 3mL of n-hexane, filtered (Millex-FH filter unit, 0.45 µm Millipore, Milford, MA, USA), evaporated under nitrogen, and dissolved in 40µL of n-hexane. One µL of this solution was injected into a GC/MS system (Thermo Science Trace® GC-Ultra with ion trap ITQ 900, Waltham, MA, USA) with a CP-Sil8 CB low bleed/MS (50 m x 25 mm x 0.25 µm) column (Agilent Technologies®, CA, USA). Hydrogen was used as carrier gas, operating at a constant flow of 1 ml/min. The mass spectrometer operated at -70 eV, and a mass range from 50-650 m/z was scanned.

2.5.2. SCFA, lactate and succinate

Both lumen (1.5mL) and dialysate (2mL) were centrifuged at 14000 rpm for 10 minutes, filtered through a 0.45μm PFTE filter, and diluted in the mobile phase (1.5mM aqueous sulfuric acid). Ten microliters were loaded onto the column with the help of an automatic sampler 730 (Metrohm, Herisa, Switzerland). The acids were eluted according to their pKa. The analysis was carried out by ion exclusion chromatography (IEC) using an 883 chromatograph (IC, Metrohm,) equipped with a Transgenomic IC Sep ICE-ION-300 column (30cm x 7.8mm x 7μm) and a MetroSep RP2 Guard. A column flow of 0.4mL/min with a column temperature of 65ºC was used. The acids were detected using suppressed conductivity detection. The company Brightlabs (Venlo, The Netherlands) carried out these analyses.

2.5.3. Gut microbiota

The isolation of genomic DNA from the fecal samples (3mL lumen) was performed using standard molecular biology kits from ZYMO Research provided by BaseClear (Leiden, The Netherlands). The PCR amplification of the 16S rRNA gene (V3 and V4 regions), the barcoding and the library preparation were carried out by BaseClear. The sequencing was carried out using the Illumina MiSeq system and later the sequences were converted into FASTQ files using BCL2FASTQ pipeline version 1.8.3. The quality cut was applied based on the quality level of Phred (Phred quality score). Quantitative Insights Into Microbial Ecology (QIIME) software package (1.9.0) was used for microbial analyses (Caporaso et al., 2010). The sequences were classified using Greengenes (version 13.8) as a reference 16S rRNA gene database. Linear discriminant analysis effect size (LEfSe) (Segata et al., 2011) was used to find biomarkers between groups using relative abundances from the operational taxonomic unit (OTU) tables

generated in QIIME. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013) was used to predict the gene families contributing to the metagenomes of the samples identified using 16S rRNA sequencing. This data was visualized using statistical analysis of taxonomic and functional profiles (STAMP) (Parks, Tyson, Hugenholtz, & Beiko, 2014).

2.6. Statistical analysis

Changes in fecal sterols, SCFA and lactate after *in vitro* colonic fermentation assays during 3 days were evaluated by ANOVA and means were compared by an LSD test (confidence level 95.0%) using Statgraphics Centurion XVI.I package. The software package R (3.5.0) (R Core Team, 2013) was used to determine correlations between OTUs and metabolites. Statistical analyses were performed with RStudio. Spearman correlation was calculated between the relative abundance of OTUs and continuous variables (fecal sterols and SCFA). Multiple comparison was corrected using the false discovery rate (FDR), and q-values (adjusted p-values) were considered significantly different at <0.05. Correlation between OTUs and metabolites are indicated by the rho-value.

3. Results

3.1. Microbiota effect on sterols

3.1.1. Fecal sterols

The concentrations of fecal animal sterols (AS) in the *in vitro* fermentation assays with lean and obese microbiota are show in Table 3.

Table 3. Cholesterol and metabolites concentration (µg/mL fermentation liquid) after *in vitro* colonic fermentation. Data are means of two independent *in vitro* assays with standard deviations (n = 2). For each fermentation time (0, 24, 48 and 72h), different letter indicate statistical differences (confidence level 95.0%) between experiments (control *vs.* ingredient *vs.* standard) for lean (a-c) and obese (x-z) microbiota.

	Lean			Obese		
	Control	**Ingredient**	**Standard**	**Control**	**Ingredient**	**Standard**
Cholesterol						
0h	123.10 ± 41.19a	133.19 ± 27.30a	159.16 ± 62.78a	106.55 ± 31.76x	241.61 ± 98.13x	106.90 ± 45.98x
24h	43.76 ± 4.95a	173.24 ± 22.55b	93.99 ± 6.43c	52.81 ± 9.00x	132.18 ± 2.16y	112.73 ± 3.58z
48h	39,22 ± 23,60a	93.23 ± 17.39a	63.08 ± 15.52a	46.93 ± 17.01x	102.40 ± 11.06y	116.26 ± 14.79y
72h	27.39 ± 11.34a	106.46 ± 15.62b	75.42 ± 6.19b	57.70 ± 2.72x	74.90 ± 5.51xy	78.98 ± 7.29y
Coprostanol						
0h	116.55 ± 35.33a	112.13 ± 13.05a	124.17 ± 49.60a	283.68 ± 88.07x	500.74 ± 139.79x	447.81 ± 209.37x
24h	127.68 ± 27.75a	115.49 ± 1.10ab	66.25 ± 0.14b	102.04 ± 3.61x	241.15 ± 28.50y	163.13 ± 16.47z
48h	39.29 ± 14.26a	59.79 ± 16.78a	46.18 ± 4.69a	111.60 ± 38.52x	277.18 ± 2.05y	228.79 ± 30.00y
72h	100.63 ± 11.57a	66.66 ± 13.17b	48.34 ± 1.92b	134.28 ± 6.70x	225.93 ± 11.72y	178.89 ± 1.07z

	Lean			Obese		
	Control	**Ingredient**	**Standard**	**Control**	**Ingredient**	**Standard**
Coprostanone						
0h	31.43 ± 1.35a	31.97 ± 1.11a	36.81 ± 2.90a	169.11 ± 1.10x	248.61 ± 63.94x	330.42 ± 123.95x
24h	51.84 ± 0.91a	39.30 ± 1.15b	39,78 ± 2,60b	61.26 ± 4.03x	60.73 ± 7.84x	55.02 ± 0.28x
48h	40.60 ± 3.36a	39.54 ± 0.16a	45,44 ± 0,82a	72.91 ± 26.50x	53.37 ± 2.81x	65.30 ± 11.78x
72h	74.29 ± 42.17a	75.64 ± 0.19a	76.99 ± 0.33a	61.11 ± 5.82x	54.06 ± 3.10x	50.98 ± 2.13x
Cholestanol						
0h	7.55 ± 2.40a	8.02 ± 1.29a	7.16 ± 0.93a	14.66 ± 2.24x	23.90 ± 5.18x	21.48 ± 4.37x
24h	8.52 ± 1.07a	9.61 ± 1.25a	7.01 ± 0.57a	8.16 ± 0.49x	16.39 ± 1.86y	12.90 ± 0.71y
48h	6.29 ± 2.06a	7.38 ± 0.20a	0.01 ± 0.00a	8.67 ± 2.45x	14.44 ± 6.08x	17.13 ± 1.53x
72h	7.28 ± 0.42a	10.15 ± 0.09b	9.21 ± 0.39b	10.55 ± 0.35x	16.64 ± 0.40y	13.04 ± 0.50y

In control at time zero, these represented 64 and 73% of total sterols in feces from lean and obese subjects, respectively. The mainly sterol were cholesterol (28% for lean and 13% for obese), and its metabolites coprostanol (26% and 35%), coprostanone (7% and 21%) and cholestanol (2% for both).

PS concentrations in fermentation liquid using lean and obese microbiota are shown in Table 4 (sitosterol and metabolites) and Table 5 (campesterol and metabolites). In control at time zero, PS represented 36 and 27% of total sterols from lean and obese feces, respectively. The abundance order was sitosterol > campesterol > sitostanol and campestanol. The following metabolites were identified: ethylcoprostanol, ethylcoprostanone (Table 4) and methylcoprostanone (Table 5). In addition, Table 3 - 5 show the mean concentrations (µg/mL) of sterols and metabolites present in control, ingredient and standard experiments, during the fermentation period of 72h.

The most relevant statistically significant differences ($p < 0.05$) among the experiments (ingredient, standard or control) using microbiota of lean subjects, are observed for cholesterol at 24h of fermentation where its concentration follows the order; ingredient > standard > control. In the cases in which there are statistically significant differences ($p < 0.05$) between the experiments (ingredient, standard or control) using obese microbiota, in general, the contents of coprostanol (24, 48 and 72h) and cholestanol (24 and 72h) follow the order; ingredient> standard> control (Table 3).

Table 4. Sitosterol and metabolites concentration (µg/mL fermentation liquid) after *in vitro* colonic fermentation. Data are means of two independent *in vitro* assays with standard deviations (n = 2). For each fermentation time (0, 24, 48 and 72h), different letter indicate statistical differences (confidence level 95.0%) between experiments (control *vs.* ingredient *vs.* standard) for lean (a-c) and obese (x-z) microbiota.

	Lean			Obese		
	Control	**Ingredient**	**Standard**	**Control**	**Ingredient**	**Standard**
Sitosterol						
0	36.42 ± 2.66a	35.32 ± 0.36a	59.25 ± 20.22a	36.36 ± 3.68x	56.96 ± 25.96x	42.63 ± 8.93x
24	45.82 ± 3.03a	15760.71 ± 135.88b	7658.03 ± 1533.56c	41.32 ± 2.04x	11381.96 ± 1442.39y	8579.77 ± 119.52z
48	55.25 ± 5.87a	18976.32 ± 295.95b	18552.76 ± 1087.75b	42.33 ± 1.32x	20492.36 ± 1868.53y	17403.47 ± 8733.12y
72	52.60 ± 12.89a	49928.70 ± 3292.11b	63621.02 ± 8662.05b	50.98 ± 1.85x	34394.99 ± 3844.29y	32691.71 ± 7755.71y
Sitostanol						
0	14.79 ± 0.75a	14.34 ± 0.34a	18.32 ± 3.46a	14.91 ± 0.71x	17.61 ± 3.84x	15.56 ± 1,05x
24	21.30 ± 0.71a	2116.15 ± 43.97b	1022.49 ± 232.23c	20.58 ± 0.47x	1761.98 ± 157.18y	1218.25 ± 162.51z
48	25.31 ± 5.23a	4458.65 ± 163.33b	4825.53 ± 539.55b	21.02 ± 0.52x	3016.77 ± 236.29y	2664.78 ± 1126.86y
72	21.86 ± 0.86a	6908.59 ± 1436.32b	6021.77 ± 2071.15b	21.96 ± 0.23x	5816.29 ± 748.20y	6022.08 ± 2060.30y

	Lean			Obese		
	Control	**Ingredient**	**Standard**	**Control**	**Ingredient**	**Standard**
Ethylcoprostanol						
0	18.99 ± 3.91a	20.08 ± 3.16a	23.10 ± 4.36a	35.89 ± 7.98x	62.28 ± 19.31x	57.06 ± 22,22x
24	25.56 ± 0.37a	7.80 ± 0,47b	9.31 ± 1.64b	16.22 ± 1.95x	10.19 ± 0.54y	7.96 ± 1,17y
48	15.06 ± 3.03a	7.09 ± 0,02b	7.36 ± 0.03b	18.13 ± 3.13x	7.93 ± 0.29y	8.01 ± 0,13y
72	24.76 ± 3.90a	13.50 ± 0,18b	14.95 ± 0.76b	25.11 ± 0.35x	7.26 ± 0.15y	7.12 ± 0,06y
Ethylcoprostanone						
0	29.41 ± 1.99a	26.29 ± 1.42a	32.24 ± 2.35a	33.71 ± 0.59x	41.08 ± 5.85x	35.87 ± 0.66x
24	39.57 ± 1.18a	743.23 ± 0.33b	341.56 ± 42.28c	39.57 ± 0.95x	805.40 ± 76.11y	484.37 ± 75.17y
48	79.64 ± 56.30a	1046.41 ± 221.89b	1708.19 ± 332.70b	38.93 ± 1.34x	1555.47 ± 113.58y	1243.34 ± 183.81y
72	41.80 ± 2.50a	1252.41 ± 60.04b	732.94 ± 140.88b	40.53 ± 0.79x	1469.70 ± 132.60y	1217.45 ± 234.81y

Table 5. Campesterol and metabolites concentration (µg/mL fermentation liquid) after *in vitro* colonic fermentation. Data are means of two independent *in vitro* assays with standard deviations (n = 2). For each fermentation time (0, 24, 48 and 72h), different letter indicate statistical differences (confidence level 95.0%) between experiments (control *vs.* ingredient *vs.* standard) for lean (a-c) and obese (x-z) microbiota.

	Lean			Obese		
	Control	**Ingredient**	**Standard**	**Control**	**Ingredient**	**Standard**
Campesterol						
0	45.58 ± 1.17a	47.04 ± 2.46a	48.33 ± 2.26a	45.83 ± 1.10x	51.52 ± 4,70x	46.60 ± 2.40x
24	62.68 ± 0.60a	1649.61 ± 37.06b	925.10 ± 58.97c	62.55 ± 0.36x	1329.05 ± 200,85y	961.23 ± 27.22y
48	62.61 ± 3.72a	3372.85 ± 27.13b	3894.42 ± 115.94c	62.47 ± 0.91x	2392.91 ± 116,94y	2257.26 ± 590.48y
72	62.12 ± 0.66a	5008.40 ± 1067.39b	4466.94 ± 1575.89b	62.99 ± 0.15x	4039.39 ± 532,52y	4344.57 ± 1434.51y
Campestanol						
0	14.40 ± 0.39a	15.12 ± 0.72a	15.47 ± 1.04a	16.34 ± 1.00x	18.73 ± 1.62x	18.48 ± 2.16x
24	21.19 ± 0.68a	255.41 ± 30.14b	160.74 ± 5.41c	20.37 ± 0.03x	284.58 ± 19.52y	252.49 ± 3.45y
48	20.43 ± 1.23a	678.00 ± 8.10b	729.36 ± 6.84c	20.63 ± 0.18x	552.08 ± 10.13y	523.47 ± 66.91y
72	22.94 ± 1.24a	825.99 ± 55.15b	773.76 ± 174.62b	20.69 ± 0.27x	761.35 ± 66.75y	793.61 ± 184.24y
Methylcoprostanone						
0	-	-	-	32.30 ± 2.60x	34.88 ± 1.99x	38.78 ± 1.94x
24	-	9.96 ± 2.85a	8.53 ± 0.03a	37.30 ± 0.62x	42.15 ± 1.75xy	43.54 ± 2.63y
48	-	49.58 ± 7.52a	49.99 ± 0.09a	37.18 ± 1.20x	44.04 ± 0.82x	48.87 ± 6.27x
72	-	87.59 ± 5.45a	226.08 ± 76.98a	18.37 ± 25.97x	23.35 ± 33.01x	42.94 ± 0.30x

The cholesterol concentration decreased in control (77 and 45%), ingredient (20 and 69%) and standard (32 and 26%) experiments after 72h of fermentation when using lean and obese microbiota, respectively. It should be noted that all metabolites (coprostanol, coprostanone and cholestanol) were higher in obese than in lean microbiota. In addition, the coprostanol concentration after 72h of fermentation decreased in control (13% for lean and 52% for obese), ingredient (41 and 55%) and standard (61 and 60%). However, coprostanone concentration at final period of fermentation (72h) increased in experiments with ingredient (136%) and standard (109%) when lean microbiota was used. In the assays with obese microbiota, the coprostanone concentration decreased from t0 to 24h (control: 64%; ingredient: 76%; standard: 83%) and then the concentration remained more or less equal until 72h for all experiments. In the case of cholestanol, at the final period of fermentation using lean microbiota, their concentration increased in experiments with ingredient (27%) and standard (29%), while in the assays with obese microbiota, its concentration decreased in presence of ingredient (30%) and standard (40%).

The sitosterol concentration in assays using microbiota of lean and obese subjects was the same between control, ingredient and standard at t0 (Table 4). However, given that sitosterol is the major sterol in the ingredient and standard, a significant increase in its respective experiments compared to control was observed for each fermentation time (24-72h). The same was observed for sitostanol (Table 4), campesterol and campestanol (Table 5) with ingredient and standard.

Ethylcoprostanol, the main metabolite of sitosterol, showed a higher concentration in experiments with obese microbiota (2 fold more) compared to lean (t0) (Table 4). Its concentration remained similar throughout fermentation in control experiment using lean microbiota and decreased significantly in the

rest of experiments when using lean (ingredient: 32% and standard: 35%) and obese microbiota (control: 30%, ingredient: 88% and standard: 87%). Conversely, ethylcoprostanone showed similar concentrations in all experiments (control, ingredient and standard) using lean and obese microbiota at t0 (Table 4). However, a significant increase of this metabolite was observed in experiments with ingredient (46 and 35 fold more) and standard (21 and 33 fold more) with respect to control when microbiota of lean and obese subjects was used, respectively.

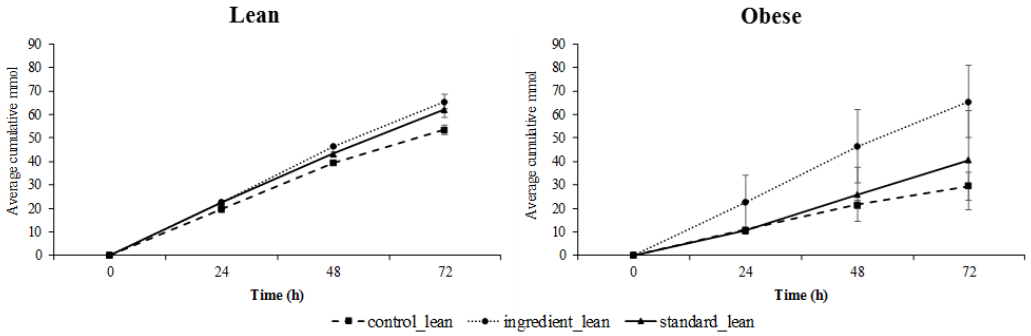
In the case of methylcoprostanone (campesterol metabolite), an increase of its concentration was observed from 24 to 72h in experiments with ingredient (8 fold more) and standard (25 fold more) (Table 5) using lean microbiota. When obese microbiota was used, the methylcoprostanone concentration remained similar from t0 to 48h in all experiments, but after 72h a decrease was observed in control (43%) and ingredient (33%) experiments and it was increased for standard (11%). There was only a significant increase between control and standard after 24h of fermentation. Throughout the experiments with lean microbiota, campesterol metabolites were not detected in the SIEM (control) experiment.

3.2. *Production of Microbial Metabolites*

3.2.1. SCFA, succinate and lactate

The average cumulative amount of SCFA produced during the fermentation time (0, 24, 48 and 72h) of control, ingredient and standard using microbiota from lean or obese subjects, are shown for total SCFA (Fig. 2) and the individual SCFA (Fig. 3).

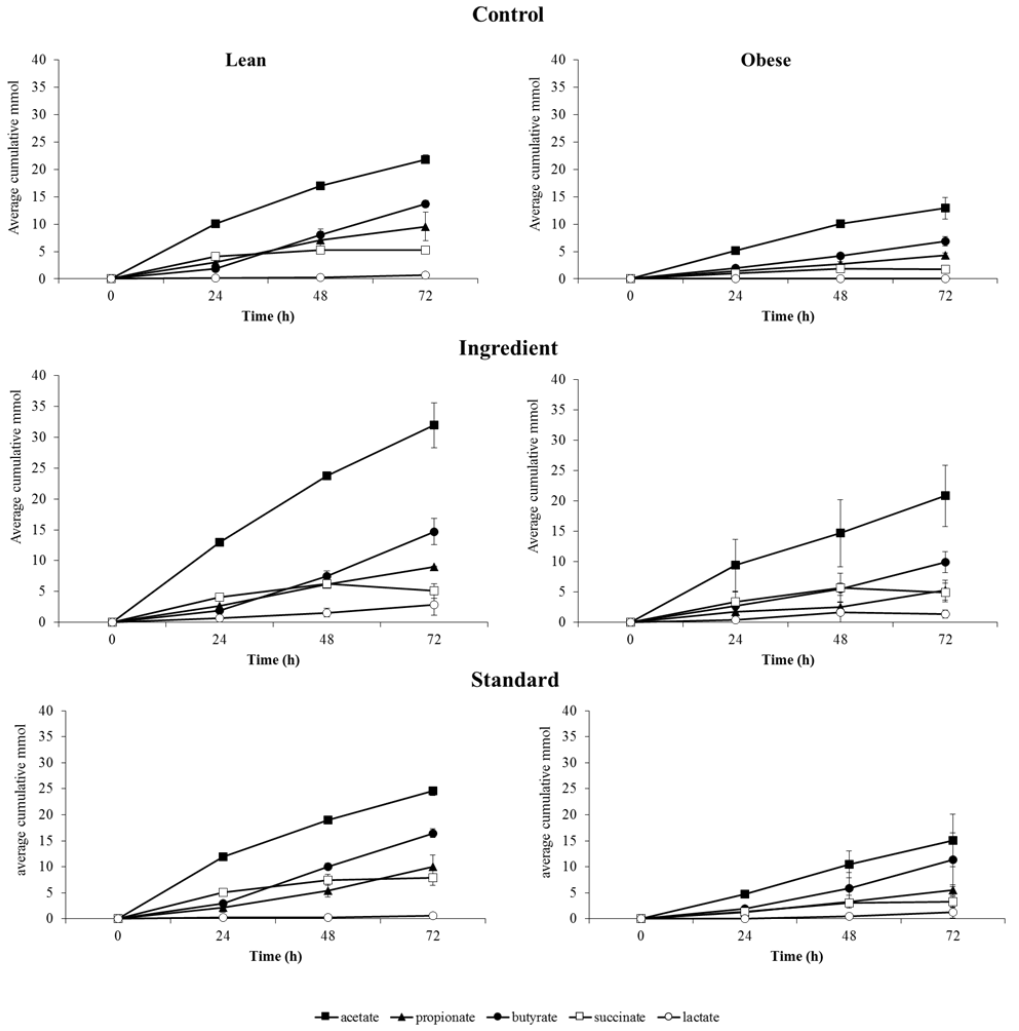
Figure 2. Average cumulative production of the sum of some short chain fatty acids (acetate, butyrate and propionate) and organic acids succinate and lactate (mmol) in the experiments with control, ingredient and standard using lean and obese microbiota. The concentrations at t0 were artificially set to zero.



The most abundant acids were acetate, butyrate, propionate and succinate. Total cumulative SCFA after fermentation (72h) with control, ingredient and standard ranged between 54 and 66 mmol using lean microbiota and from 30 to 46 mmol using obese microbiota (Fig. 2). There were significant increases ($p < 0.05$) of total SCFA production in the experiments using lean microbiota with ingredient (net increment: 7.05 and 11.90 mmol) and standard (4.00 and 8.50 mmol) compared to control after 48h and 72h, respectively.

The highest acetate production was found in experiments with ingredient and standard using lean microbiota (Fig. 3). Specifically, a significant increase ($p < 0.05$) of this SCFA after 24 and 48h of fermentation occurred in the assays with ingredient and standard compared to control, being highest in the presence of ingredient. In the case of butyrate, a slight increase in its production was observed for ingredient and standard compared to control throughout the fermentation experiment using lean microbiota. However, a significant increase ($p < 0.05$) was only found after 24h of fermentation with standard. For propionate and succinate, greater production was observed when ingredient and standard were used, but no significant differences were found between these two and control.

Figure 3. Average cumulative production of the short chain fatty acids acetate, propionate and butyrate and the organic acids succinate and lactate (mmol) in the experiments with control, ingredient and standard using lean and obese microbiota. The concentrations at t0 was artificially set to zero.



In the experiments with microbiota of obese subjects, a lower production of SCFA is observed compared with lean microbiota experiments. Compared to control, a greater production of acetate and butyrate was observed, but there were

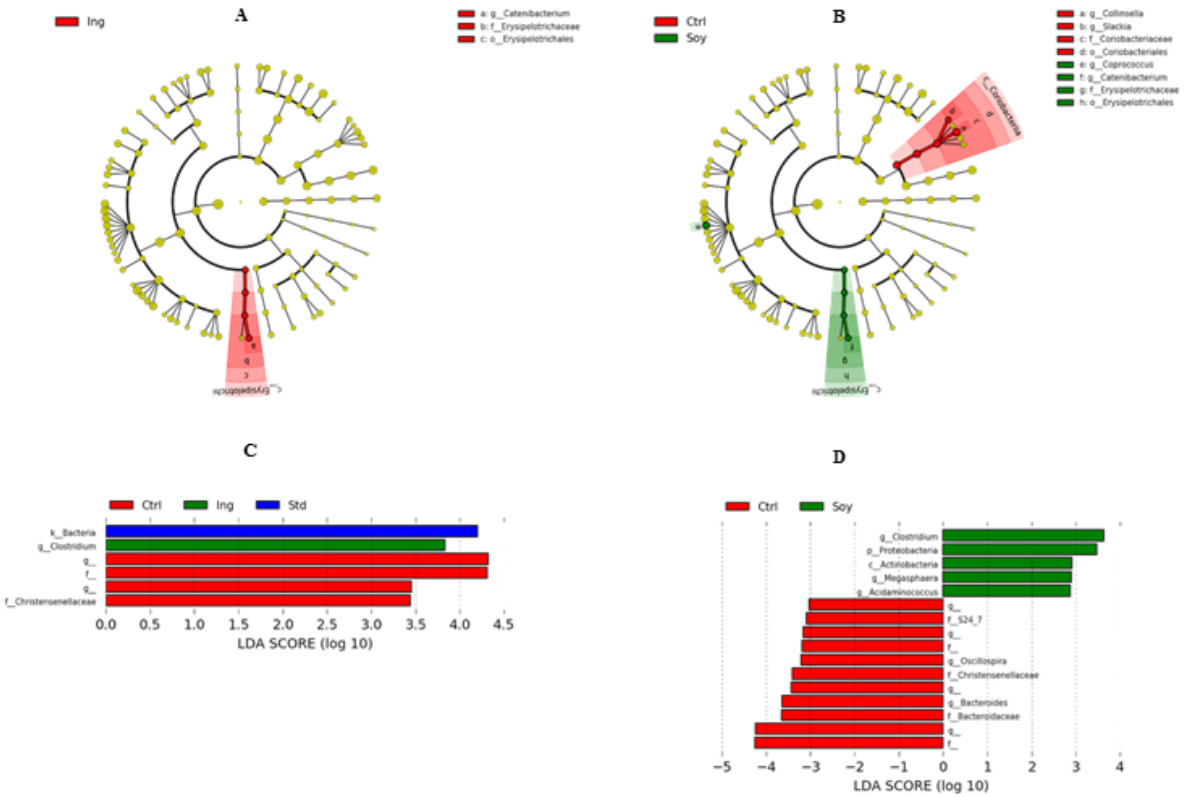
no significant differences between the three experiments (control, ingredient and standard). The succinate and propionate production was similar for all three experiments, and no significant differences were found (Fig. 3).

Lactate production was low for both lean and obese microbiota, although its production in the experiments with ingredient and standard was slightly higher in both microbiotas (Fig. 3). On the other hand, lactate production only showed a significant increase ($p < 0.05$) in the experiments with ingredient (net increment: 0.50 mmol) after 24h of fermentation using lean microbiota.

3.3. Plant sterol modulation of the microbiota

Sequencing of the V3-V4 region of 16S rDNA gene was performed and reads were analysed using QIIME. Principal Coordinate Analysis (PCoA) and Linear discriminant analysis effect size (LEfSe) were performed to determine operational taxonomic units (OTUs) that were modulated by the interventions. Neither weighted nor unweighted PCoA showed specific clustering by substrate (not shown). Yet LEfSe showed some OTUs that were specific for the treatments. For the lean microbiota, LEfSe only showed OTUs that were more prevalent upon addition of ingredient (Fig. 4A), which belonged to the genus *Catenibacterium*. When ingredient and standard were combined (indicated as Soy) and compared to control, a few more discriminative OTUs were observed (Fig. 4B). Apart from *Catenibacterium*, *Coprococcus* was increased upon addition of PS, while *Collinsella* and *Slackia* of the *Coriobacteriaceae* family were increased in control.

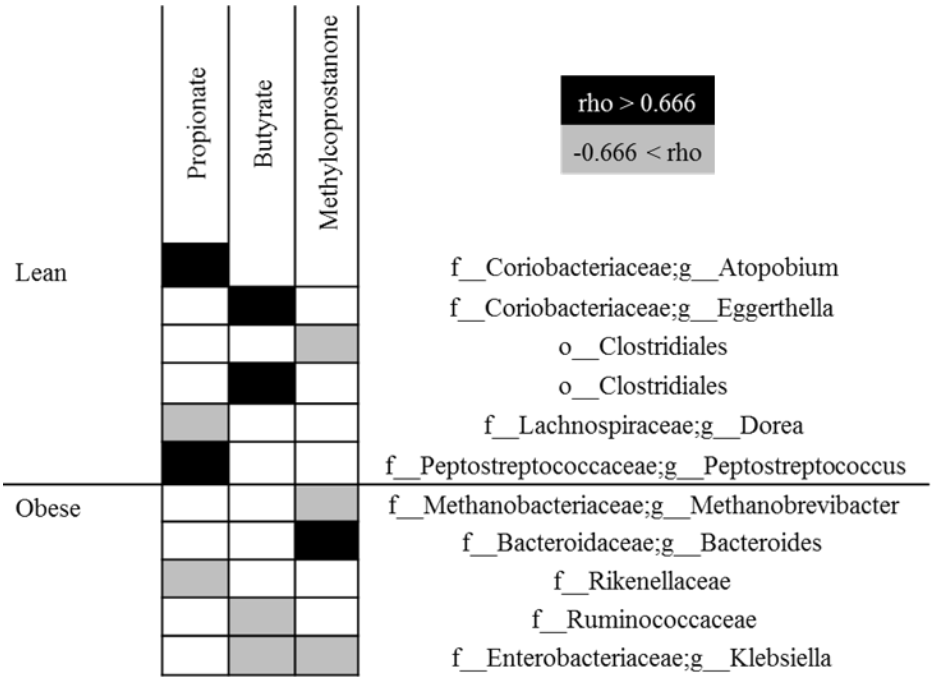
Figure 4. Linear discriminant analysis effect size for microbiota composition of the lean (A, B) and obese (C, D) microbiota.



For the obese microbiota, the genus *Clostridium* was increased for ingredient, while a few OTUs (including *Christensenellaceae* and other OTUs in the order *Clostridiales*) were higher in control (Fig. 4C). Also here, when ingredient and standard were combined (indicated as Soy) and compared to control, more discriminative OTUs were observed (Fig. 4D).

Using Spearman correlation with false-discovery rate correction, the presence of OTUs was correlated to microbial metabolites produced (Fig. 5, only shown for strong correlations with a rho > 0.666 or -0.666 < rho).

Figure 5. Spearman correlation with false-discovery rate (FDR) correction between OTUs and some microbial metabolites produced (short chain fatty acids and fecal sterols).



For the lean microbiota positive correlations between the genus *Dorea* and propionate acid, and between an uncharacterized OTU in the order *Clostridiales*

and methylcoprostanone was observed. In addition, negative correlations were found between propionic acid and the genus *Atopobium* and *Peptostreptococcus*. In the case of experiments with obese microbiota, there were positive correlations between the genus *Klebsiella* and butyric acid and methylcoprostanone.

The latter metabolite (methylcoprostanone) has also been shown to be positively correlated with the genus *Methanobrevibacter*. Other positive correlations recorded were between propionic acid and the *Rikenellaceae* family, and between butyrate acid and the *Ruminococcaceae* family. Finally, a negative correlation was observed between the genus *Bacteroides* and methylcoprostanone (Fig. 5).

PICRUSt was used to predict the microbial pathways contributing to the metagenomes of the samples identified using 16S rRNA sequencing, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and the data was visualized using STAMP. Using Welsh's two-sided t-test, pathways predicted for each test product were compared to the other experiments. For the lean microbiota three pathways were significantly different (Table 6A).

Table 6. Pathways significantly different between control, ingredient and standard after projecting the OTU abundance on the pathways in KEGG (Kyoto Encyclopedia of Genes and Genomes) for the lean (A) and obese (B) microbiota.

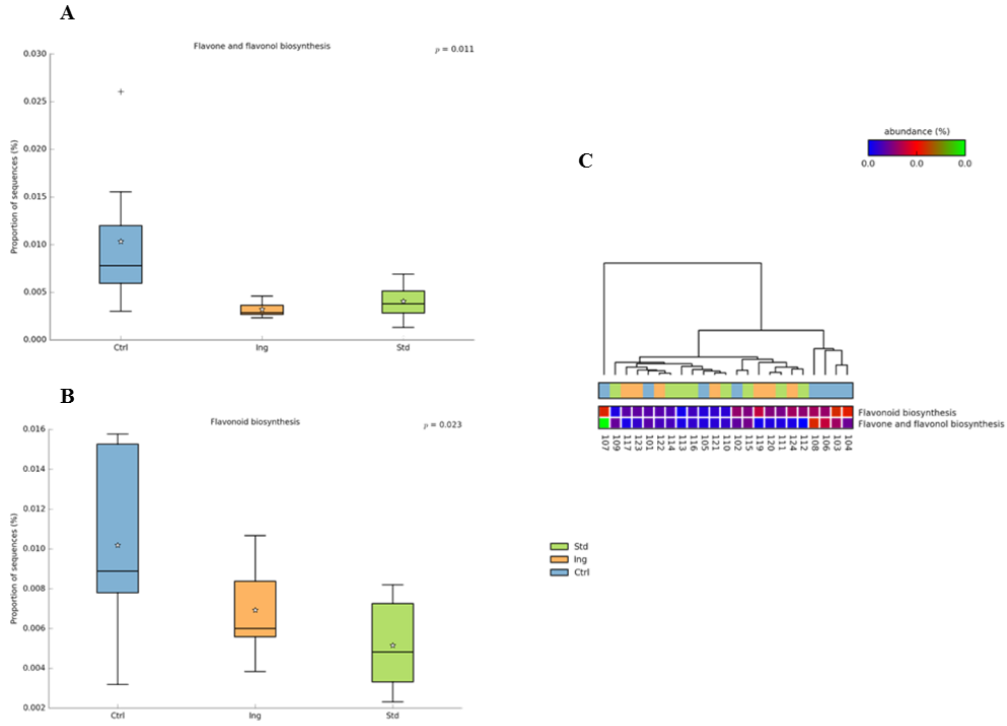
Observations Ids	**p-values**
Lean (A)	
Phenylpropanoid biosynthesis	0.03
Starch and sucrose metabolism	0.04
Transcription related proteins	0.03
Obese (B)	
Alpha-Linolenic acid metabolism	0.01
Alzheimer's disease	0.02
Amytrophic lateral sclerosis (ALS)	0.02
Apoptosis	0.01
Baterial toxins	0.00
Benzoate degradation	0.04
Beta-Alanine metabolism	0.01

Observations Ids	p-values
Obese (B)	
Butanoate metabolism	0.03
Carbohydrate metabolism	0.04
Chaperones and folding catalysts	0.004
Chromosome	0.05
D-Alanine metabolism	0.02
Dioxin degradation	0.05
Drug metabolism – other enzymes	0.03
Electron transfer carriers	0.01
Fatty acid biosynthesis	0.02
Flavanone and flavonol biosynthesis	0.01
Flavonoid biosynthesis	0.04
Galactose metabolism	0.01
Glyoxylate and dicarboxylate metabolism	0.02
Inositol phosphate metabolism	0.05
Isoquinoline alkaloid biosynthesis	0.003
Lipoic acid metabolism	0.03
Membrane and intracellular structural molecules	0.02
Novobiocin biosynthesis	0.04
One carbon pool by folate	0.001
Other glycan degradation	0.02
Other ion-coupled transporters	0.04
Other transporters	0.03
Others	0.02
Pentose and glucoronate interconversions	0.02
Pentose phosphate pathway	0.01
Phosphonate and phosphinate metabolism	0.02
Polycyclic aromatic hydrocarbon degradation	0.01
Prenytransferases	0.02
Pyruvate metabolism	0.01
Riboflavin metabolism	0.00
Ribosome biogenesis	0.02
RNA degradation	0.05
Sphingolipid metabolism	0.01
Terpenoid backbone biosynthesis	0.01
Transcription machinery	0.01
Transcription related proteins	0.02
Transporters	0.03
Tuberculosis	0.03
Vibrio cholerate pathogenic cycle	0.03

For the obese microbiota a total of 47 pathways were predicted to be differentially present (Table 6B), including those for flavone and flavonol biosynthesis (Fig. 6A), flavonoid biosynthesis (Fig.6B). The box-plots in Fig. 6

shows the predicted pathway presence and the heatmap shows the abundance of the pathways in the individual samples (Fig. 6C).

Figure 6. Boxplot of flavone and flavonol biosynthesis (A) and flavonoid biosynthesis (B) pathways and heatmap (C) predicted to be differentially present in the different experiments by using PICRUSt, visualised using STAMP.



4. Discussion

The fact that high concentrations of cholesterol metabolites were not produced during *in vitro* fermentation trials using the TIM-2 model (Fig. 2), suggests that the colonic microbiota from lean and obese subjects would prefer PS as a substrate, because they were present in greater proportion than cholesterol. These results agree with another *in vitro* static colon fermentation study of our research

group, in which lower microbial metabolism for cholesterol was found (Cuevas-Tena et al., 2018a). In addition, the results obtained also are in concordance with other *in vivo* studies carried out by Weststrate, Ayesh, Bauer-Plank & Drewitt, (1999), where after the intake of margarine enriched with PS (8.6g/day) by healthy subjects, no increase in the concentrations of neutral sterols was found and the amount of cholesterol metabolized into coprostanol was reduced. Cuevas-Tena, Bermúdez, Silvestre, Alegría & Lagarda, (2018b) evaluated the impact of intake of a PS-enriched milk based fruit beverage (2g PS/day) by postmenopausal women on fecal sterols excretion, and found a lower tendency in the production of coprostanol in the presence of high doses of PS. In a previous study in rats, following a diet with sitosterol (0.8%, w/w) also a decrease in the coprostanol production compared to a diet containing cholesterol (1.2%, w/w) was observed (Cohen, Raicht & Mosbach, 1974).

However, in our study an increase in the coprostanone production was observed only in experiments with lean microbiota and when the medium was supplemented with ingredient or standard compared to control, where the concentration remained similar throughout the assay. Cuevas-Tena et al. (2018b), in agree with our results, found a significant increase in coprostanone (0.52mg/g freeze-dry feces) after intake a high doses of PS compared with the basal period and they conclude that this fact is probably due to saturation of the gut microbiota.

It was expected that the concentration of neutral sterols (sitosterol, sitostanol, campesterol and campestanol) present in the ingredient and standard would increase throughout experiments with lean and obese microbiota (Tables 4 and 5). PS are also transformed by the microbiota through biochemical reactions similar to those occurring for cholesterol (Wong, 2014; Keller & Jahreis, 2004). A decrease in ethylcoprostanol was observed after ingestion of the high dose PS,

contrary to the results obtained by other authors (Weststrate et al., 1999; Cuevas-Tena et al., 2018a; Cuevas-Tena et al., 2018b). However, a considerable increase in the production of ethylcoprostanone was observed in both experiments (lean and obese microbiota) in the presence of ingredient or standard. This suggests that the capacity of the gut microbiota was not sufficient to transform sitosterol into ethylcoprostanol due to the large amounts of PS present (Cohen Raicht & Mosbach, 1974), causing a lower production of ethylcoprostanol. Similarly, an increase in methylcoprostanone has also been observed in experiments with lean microbiota mainly after 72h with ingredient and standard (Table 5).

SCFA are produced in the proximal colon through the fermentation of mainly indigestible carbohydrates. As far as we know, this is the first time that microbial SCFA production is reported after *in vitro* fermentations assays with high doses PS. In this sense, a greater production of SCFA was observed in experiments with microbiota from lean subjects *versus* obese. These SCFA are beneficial to the host because they can be used as an energy source by the cells of the body (Maathuis et al., 2009), they also promote the production of mucin and gastrointestinal peptide, they can be utilized by colonocytes, and those that are not used by the epithelium are absorbed and reach other organs (liver and muscle) (Verbeke et al., 2015)

Acetate was the main SCFA produced independently of the type of microbiota (lean or obese) and substrates (control, ingredient or standard) used. However, this SCFA showed a significantly higher production in the presence of high doses of PS using lean microbiota. It has been suggest that acetate stimulates cholesterol and long chain fatty acids synthesis in the liver (Delzenne, Neyrinck, Bäckhed & Cani, 2011) and a study using mice showed that acetate from colonic fermentation can cross the blood-brain barrier and supress appetite in the hypothalamus (Frost et al., 2014). Butyrate is another main SCFA with higher

production in experiments with microbiota from lean subjects than obese. Butyrate functions as the major energy source for colonic epithelial cells (Koenen, Rubio, Mueller & Venema, 2016). In addition, this SCFA is considered to be especially important, as it may also play a major role in the prevention of colon cancer (Mortensen & Clausen, 1996; Perrin et al., 2001; Leonel & Alvarez-Leite, 2012) and other colonic diseases (Hamer et al., 2008). Besides, butyrate protects against diet-induced obesity and suppresses food intake (Lin et al., 2012). Propionate was also shown to be produced at greater amounts in experiments with microbiota from lean subjects. In the case of this SCFA, it has been suggested that it reduces liponeogenesis, cholesterol synthesis and recently has been demonstrated to be involved in the activation of G-protein.coupled receptors (GPR-41 and GPR-43) releasing satiety hormones, thus reducing food intake (Kimura et al., 2011). Succinate and lactate showed to be the minority compounds and a greater production was observed in the experiments with microbiota from lean subjects *versus* obese. These acids serve as intermediates in the metabolism of SCFA and generally they do not accumulate in the colon (Gibson, Probert, Van Loo, Rastall & Roberfroid, 2004). However, a significant increase in lactate production was observed in the presence of ingredient (lean microbiota experiment). In this context, lactate only accumulates when there is a fast fermentation of a substrate. If substrates are fermented slowly, lactate is mostly converted into butyrate (Morrison et al., 2006). Besides, our results indicate that PS influences *Catenibacterium* and *Coprococcus* genera (*Erysipelotrichaceae* family) in experiments with lean microbiota and *Clostridium* genus in obese microbiota, with an increase in the proportion of these bacteria when PS was added to the fermentation medium. Regarding to the increase of the *Erysipelotrichaceae* family members, these results were not coincident with those found in a previous study by our group (Cuevas-Tena et

al., 2018a). In a clinical trial (Baumgartner et al., 2017), where 13 healthy subjects received during three weeks a control or plant stanol ester enriched margarine (3g/day plant stanols), no differences in gut microbiota composition were recorded between the two target groups. However, van Faassen et al. (1987) found slight changes for *Bifidobacterium*, *Bacteroides* and *Clostridium* genera in subjects with a vegan diet, which was rich in PS.

5. Conclusions

The concentration of neutral faecal sterols (cholesterol, sitosterol, sitostanol, campesterol and campestanol) in all experiments at t0 were similar between experiments with microbiota from lean and obese subjects. However, the concentration of its metabolites (coprostanol, coprostanone, cholestanol, ethylcoprostanol and methylcoprostanone) was higher in experiments with microbiota of obese *versus* lean, suggesting a different microbial activity between these two population groups. In the presence of high doses of PS and throughout the fermentation period (time 0 compared to 72h), a decrease in cholesterol and coprostanol concentrations and an increase in coprostanone and cholestanol were observed in assays with microbiota of lean subjects. Besides, the PS metabolites, ethylcoprostanol decreased and ethyl- and methylcoprostanone increased. This fact, as in the case of AS, could be due to the saturation of the microbiota. In addition, they produced a decrease in the concentration of coprostanol and ethylcoprostanol (main cholesterol and sitosterol metabolites, respectively), being more pronounced in experiments with lean microbiota. The total SCFA production was different between experiments with microbiota from lean and obese subjects, being higher when lean microbiota was used. This fact would support the hypothesis that the microbial profile of these population groups is different, which was corroborated by PCoA in QIIME (not shown). In the

presence of high doses of PS it was observed that the production of SCFA is similar or in some cases (acetate and butyrate) greater than control experiments in both lean and obese microbiota experiments. Therefore, it could be confirmed that the supplementation of the diet with high doses of PS would not modify the carbohydrates metabolic activity of the microbiota, and therefore SCFA continues to be produced. The supplementation of the fermentation medium with high amounts of PS increased the proportion of the genera *Catenibacterium* and *Coprococcus* in the experiments with lean microbiota and the genus *Clostridium* in the experiments with obese microbiota. Besides, the production of methylcoprostanone (microbial metabolite of campesterol) was negatively correlated with members of the *Clostridiales* order in the experiments with lean microbiota and in experiments with obese microbiota, this metabolite showed a negative correlation with *Peptostreptococcus* and *Methanobrevibacter*, and a positive correlation with *Bacteroides*.

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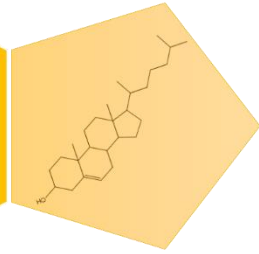
Anexo/ Annex IV



Comunicaciones a congresos

Cuevas-Tena M., Sanz Y., Alegría A and Lagarda MJ. Identification of sterols metabolites produced by action of the intestinal microbiota. XVIII Conferencia de Nutrición Práctica y IX Congreso Internacional de Nutrición, Alimentación y Dietética. Madrid, Spain (19 – 21 Marzo, 2014). Póster.

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María Cuevas-Tena, Carlota Bussolo de Souza, Antonio Diogo Silva Vieira, Amparo Alegría, María Jesús Lagarda, Koen Venema. Impact of plant sterols on intestinal microbiota from lean and obese subjects using TIM-2 *in vitro* model. The 7th Congress of European Microbiologists FEMS 2017. Valencia, Spain (9-13 Julio, 2017). Póster.

FE DE ERRATAS

Tesis Doctoral Internacional, "Influencia de la fermentación colónica en los esteroles de la dieta / *Influence of colonic fermentation on dietary sterols*" realizada por Dña. Maria Cuevas Tena.

Página 29:

En la línea 5, la palabra "que" se debe cambiar por la palabra "con".

Página 49:

En la línea 18 tras las palabras "peristálticos y" se deben incluir las siguientes palabras "absorción colónica".

Página 91:

En la leyenda de la Figura 15, la palabra "green" debe ser cambiada por la palabra "blue" y la palabra "blue" por la palabra "green".

Página 95:

En la línea 3, la palabra "na" debe ser cambiada por la palabra "una".

Página 96:

En la línea 16, la frase "En los ensayos con microbiota de individuos obesos las concentraciones de colesterol y sus metabolitos" se debe eliminar.

Página 192:

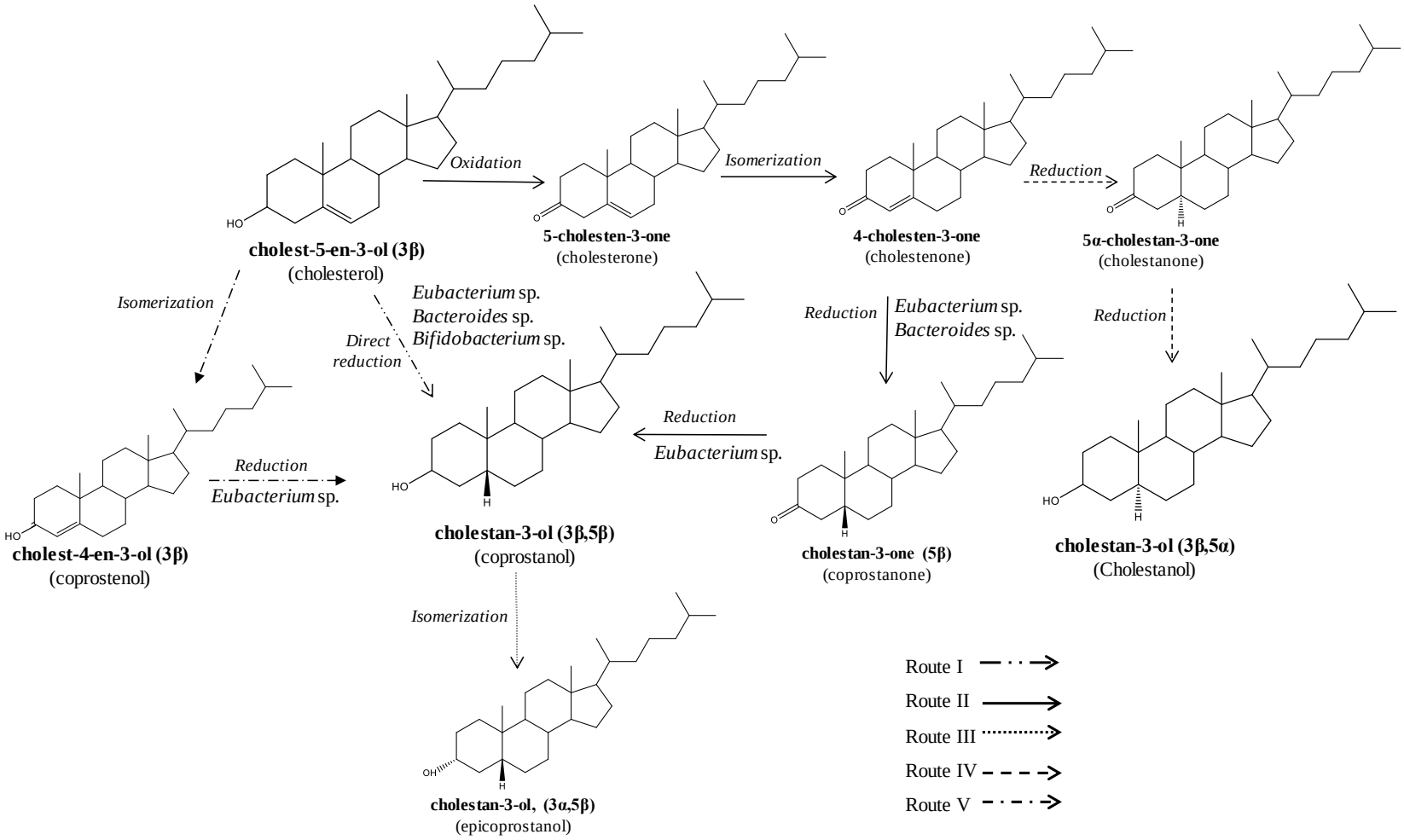
En la leyenda de la Figura 1, se ha eliminado la palabra "into". Por tanto, la leyenda resulta del siguiente modo: "*Figure 1. Cholesterol transformation path by gut microbiota*". Además, la imagen está incompleta, ya que se produjo una incompatibilidad entre el documento en formato Word y el procesador de documentos Adobe Acrobat DC. A continuación, se adjunta la leyenda y la figura correcta.

Página 194:

La "*Figure 2. Sitosterol transformation path into ethylcoprostanol and campesterol into methylcoprostanol by gut microbiota*" está incompleta, ya que se produjo una

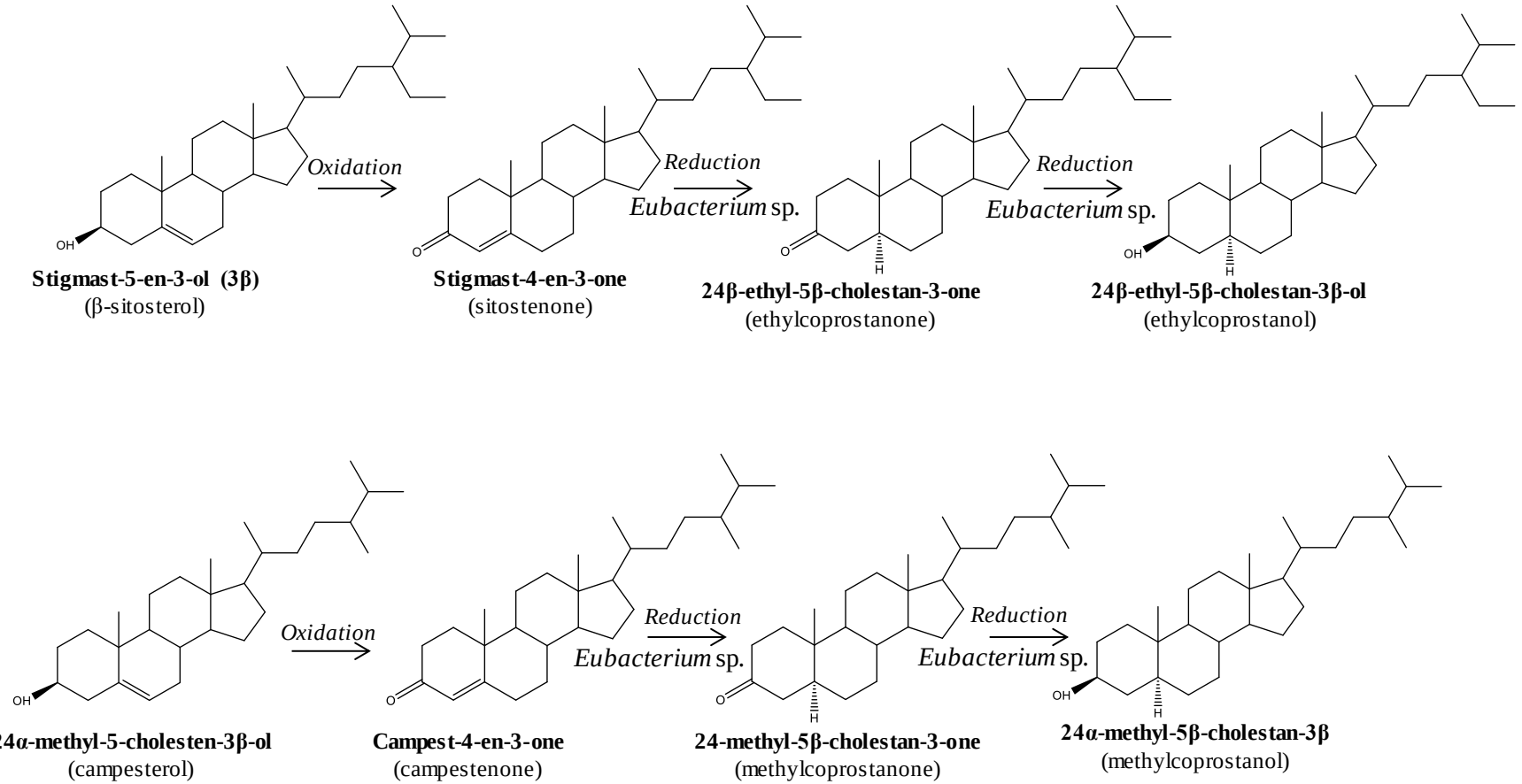
incompatibilidad entre el documento en formato Word y el procesador de documentos Adobe Acrobat DC. A continuación, se adjunta la figura correcta.

Página 192: Figure 1. Cholesterol transformation path by gut microbiota.



Adapted from [22, 24, 28, 32, 34, 40]

Página 194: Figure 2. Sitosterol transformation path into ethylcoprostanol and campesterol into methylcoprostanol by gut microbiota.



Adapted from [40]