Determinación de biomarcadores nutricionales: Desarrollo de bases de datos y estudio de la interacción de los compuestos fenólicos con la microbiota intestinal en estudios de intervención con vino tinto

María Boto Ordóñez

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MARÍA BOTO ORDÓÑEZ

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ABREVIATURAS

ATCC	*American Type Culture Collection*
AUC	Área bajo la curva
CE	*Collision Energy*
CECT	Colección Española de Cultivos Tipo
COR	Característica Operativa del Receptor
DGGE	Electroforesis en gel con gradiente de desnaturalización
DHPV	Dihidroxifenilvalerolactona
DP	*Declustering Potential*
eBASIS	*Electronic BioActive Substances Information System*
EDTA	Ácido etilendiaminotetraacético
EI	Electroionización
ESI	Fuente de ionización por electrospray
FFQ	Cuestionario de frecuencia de consumo de alimentos
GC-MS	Cromatografía de gases acoplada a un espectrómetro de masas
HCl	Ácido clorhídrico
HDL	Lipoproteínas de alta densidad
HLB	Balance hidrofílico-lipofílico
HMDB	*Human Metabolome Database*
IMC	Índice de masa corporal
INRA	Instituto Nacional de Investigaciones Agronómicas
KEGG	*Kyoto Encyclopedia of Genes and Genomes*
LC	Cromatografía líquida
LC-MS	Cromatografía líquida acoplada a un espectrómetro de masas

LDL	Lipoproteínas de baja densidad
MCX	Modo mixto catiónico
MMCD	*Madison Metabolomics Consortium Database*
MRM	*Multiple Reaction Monitoring*
MS	Espectrometría de masas
MS/MS	Espectrometría de masas en tandem
NMR	Resonancia Magnética Nuclear
PCA	Análisis de componentes principales
PCR	Reacción en cadena de la polimerasa
PIS	*Product Ion Scan*
qPCR	Reacción en cadena de la polimerasa cuantitativa
RoR	*Ruby on Rails*
SPE	Extracción en fase sólida
STD	Estándar
UPLC	Cromatografía líquida trabajando a ultra presión
UPLC-MS/MS	Cromatografía líquida trabajando a ultra presión acoplada a un espectrómetro de masas en tandem
USDA	Departamento de Agricultura de los Estados Unidos
VT	Vino tinto
VTD	Vino tinto desalcoholizado

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1.INTERÉS Y OBJETIVOS

1. INTERÉS Y OBJETIVOS

1.1 INTERÉS

El estudio de la relación entre la dieta o factores específicos de la dieta y el estado de salud requieren medidas exactas de la exposición dietética. Los tradicionales análisis de exposición alimentaria, como las encuestas o los cuestionarios de frecuencia de consumo contienen una serie de errores sistemáticos, como la limitación en la lista de alimentos considerados o estimaciones erróneas en el tamaño y la frecuencia de la ingesta, que han provocado una cierta inconsistencia en el verdadero papel de la alimentación sobre la salud humana. Como resultado de este hecho, existe un intenso esfuerzo por parte de la comunidad científica hacia la identificación de biomarcadores (Scalbert *et al.*, 2013).

Los biomarcadores, de manera generalista, se definen como una característica que puede ser objetivamente medida y evaluada como indicador de procesos biológicos normales, estados de enfermedad o respuestas farmacológicas a una intervención terapéutica (Biomarkers Definitions Working Group, 2001). Concretamente dentro de la Nutrición, los biomarcadores nutricionales, quedan definidos como indicadores bioquímicos de la ingesta o estado nutricional (a corto o largo plazo o incluso derivados de ingestas puntuales), índice del metabolismo de los nutrientes, o como un marcador del efecto sobre el organismo de la ingesta alimentaria (Potischman y Freudenheim, 2003).

Más alla de su uso para establecer la relación dieta-salud, en el campo de la alimentación pueden ser utilizados como instrumentos de validación del registro dietético (*FFQ*, recordatorio de varios días, registro de 24h), para corroborar la ingesta real con la observada y como medidas integradas del estado de un nutriente en el organismo (Potischman y Freudenheim, 2003; Jenab *et al.*, 2009; Hedrick *et al.*, 2012).

Las principales razones para su uso son su capacidad para: i) proporcionar una medida más objetiva y precisa de la ingesta, disminuyendo los errores de medida; ii) evaluar nutrientes con información limitada o imprecisa sobre su presencia en alimentos; iii) obtener una mejor medida del estado nutricional al tener en cuenta el metabolismo y la biodisponibilidad alimentaria (Zamora-Ros *et al.*, 2012).

A la hora de buscar biomarcadores, existen tres tipos de metabolitos derivados de la ingesta de alimentos. El primer grupo, son aquellos que pueden ser digeridos en boca, estómago e intestino delgado, y después ser absorbidos a nivel intestinal. El segundo grupo, son aquellos transformados por los tejidos del huésped, como el enterocito, hígado o riñón, y finalmente un tercer grupo, derivados del metabolismo de la microbiota intestinal (Scalbert *et al.*, 2013). Tradicionalmente, la búsqueda de biomarcadores de consumo se limitaba al primer grupo de compuestos presentes en biofluídos tras la ingesta de un alimento (de Vries *et al.*, 1998). Posteriormente, se realizaron numerosos estudios de biodisponibilidad, y se incorporaron aquellos derivados del metabolismo del propio individuo (Zamora-Ros *et al.*, 2006). Actualmente, es el último grupo el que está generando un creciente interés, por el amplio rango de metabolitos generados específicamente por acción de la microbiota intestinal del colon (Selma *et al.*, 2009; Bolca *et al.*, 2013; Clifford *et al.*, 2013).

La microbiota humana ha sido fuente de estudio en los últimos años por su importancia en estados de salud y enfermedad. Desde los primeros estudios realizados con ratones libres de gérmenes colonizados con microbiota, donde se observó su papel en la obesidad (Turnbaugh *et al.*, 2006), muchos han sido los estudios que han relacionado la composición bacteriana intestinal con otras enfermedades como cáncer (Schwabe y Jobin, 2013), diabetes (Burcelin *et al.*, 2013) o enfermedad cardiovascular (Wang *et al.*, 2011), confirmado la existencia de un complejo microbiota-huesped, del que dependen estados de salud y enfermedad.

En el caso de los polifenoles, su interacción a nivel digestivo puede llevarse a cabo de dos maneras: i) ejerciendo un papel prebiótico, modulando el crecimiento de bacterias o grupos de bacterias (Queipo-Ortuno *et al.*, 2012); ii) metabolizando moléculas complejas y liberando los metabolitos formados (Monagas *et al.*, 2010; Moco *et al.*, 2012). Estos últimos suponen la ampliación del rango de posibles metabolitos, y por tanto la posible identificación de nuevos biomarcadores.

En el estudio de biomarcadores coexisten aspectos importantes y factores limitantes que necesitan especial atención y desarrollo científico: i) bases de datos de utilidad en estudios metabolómicos que incluyan información del metabolismo de los alimentos y

sus metabolitos derivados; ii) identificación de nuevas aproximaciones tanto analíticas como bioestadísticas.

En el caso de las nuevas herramientas analíticas, la exploración de biomarcadores se realiza desde un enfoque dirigido y no dirigido. En este aspecto la Resonancia Magnética Nuclear (RMN) y/o la espectrometría de masas son las técnicas de elección (Scalbert *et al.*, 2013). Por su parte, las herramientas estadísticas tienen un papel clave a la hora de discriminar biomarcadores, identificando aquellos con la sensibilidad suficiente que permitan la medición a dosis nutricionales, y específicos, para el alimento o compuesto de estudio, valorando tanto biomarcadores individuales, como combinaciones de los mismos (Scalbert *et al.*, 2013).

En relación con las bases de datos, la complejidad de los componentes de los alimentos, la diversidad de los metabolitos, y el alto número de estudios sobre su metabolismo, ha generado la especial consideración de su elaboración, con el fin de que los usuarios puedan extraer y manipular de forma automatizada la gran cantidad de datos generados (Scalbert *et al.*, 2011).

Concretamente en el campo de los polifenoles y bases de datos, el conocimiento del metabolismo se encuentra distribuido en una amplia y extensa cantidad de literatura, donde estudios *in vivo* e *in vitro* han demostrado los numerosos procesos que tiene lugar en nuestro organismo (Manach *et al.*, 2004; Manach *et al.*, 2005). Adicionalmente, la reciente incorporación del estudio del metabolismo microbiano, y su demostrada importancia tanto a nivel cualitativo como cuantitativo en el estudio del metabolismo de los compuestos bioactivos de los alimentos, ha incrementado de manera muy significativa los perfiles metabólicos asociado a microbiota (Selma *et al.*, 2009; Bolca *et al.*, 2013). Toda esta información no ha sido hasta el lanzamiento del Phenol-Explorer 2.0, con la creación del módulo específico de metabolismo de polifenoles, cuando se ha recogido de manera armonizada en una base de datos.

Con todo ello, la identificación de biomarcadores necesita un arduo proceso de validación y análisis, como se refleja en el gran número de biomarcadores propuestos, pero pocos suficientemente validados y aceptados (Hedrick et al. 2012). La validación, tanto técnica (laboratorios que cumplan la normativa ISO17025, incluyendo parámetros de exactitud, precisión y repetibilidad), como biológicamente (que sea

altamente predecible y biológicamente plausible), y su aplicabilidad a una población donde administren suficiente información del proceso o ingesta para el que están concebidos (Spencer *et al.*, 2008; Jenab *et al.*, 2009), limita la identificación de nuevos marcadores, y al mismo tiempo el desarrollo de los conocidos *health claims* de la industria alimentaria (van Loveren *et al.*, 2012). El desarrollo de estos productos, de acuerdo con la legislación europea, necesita la comprobación en estudio en humanos usando biomarcadores válidos, lo que supone un gran reto en el sector a nivel europeo y mundial.

El vino es un alimento propio de nuestra cultura y patrón alimentario mediterráneo, y son numerosos los trabajo que aportan evidencia científica epidemiológica sobre los efectos saludables de un consumo moderado y responsable de vino (Renaud y de Lorgeril, 1992), y más específicamente en estudios clínicos de intervención (Chiva-Blanch *et al.*, 2012a; Chiva-Blanch *et al.*, 2012b). Sin embargo, la necesidad de disponer de biomarcadores robustos de ingesta se manifiesta tanto en dichos estudios clínicos de intervención, como en estudios epidemiológicos y en epidemiología nutricional, para poder evaluar el potencial efecto en la salud y en la disminución de riesgo de enfermedad.

Hasta ahora el estudio de biomarcadores del vino se ha focalizado en el resveratrol debido a su especificidad (Zamora-Ros *et al.*, 2006). Sin embargo, la complejidad de su composición hace que sea necesario una revisión del papel de los biomarcadores a aquellos formados en el organismo, no solo para identificar su consumo, sino también para poder valorar el papel de los mismos en sus actividades biológicas y efectos beneficiosos (Fernandez-Panchon *et al.*, 2008), en ocasiones más activos que su compuesto padre (Monagas *et al.*, 2010).

1.2 HIPÓTESIS DE TRABAJO

Hipótesis 1

La revisión sistemática del metabolismo de los polifenoles, y su posterior aplicación en la construcción de bases de datos facilitará la validación de biomarcadores y la creación de rutas metabólicas complejas tras el consumo de un alimento.

Hipótesis 2

Los cambios producidos en la microbiota intestinal tras el consumo de vino quedan reflejados en los cambios de la huella metabólica de los compuestos fenólicos derivados del catabolismo microbiano en plasma y orina.

Hipótesis 3

Los consumidores de vino presentarán una mayor concentración de polifenoles y sus metabolitos en orina y plasma, tanto aquellos derivados del metabolismo de fase II, como del metabolismo microbiano, de modo que podrá establecerse la sensibilidad y especificidad de la concentración de polifenoles y de sus metabolitos en ambas matrices para su uso como marcadores de consumo, valorando la idoneidad y posible asociación en la utilización de cada espécimen en función de la intervención dietética.

Hipótesis 4

El consumo moderado de vino tinto, vino tinto desalcoholizado y ginebra, tiene un efecto modulador en la microbiota intestinal del individuo, lo que afectará a marcadores biológicos de estado de salud.

1.3 OBJETIVOS

El objetivo principal de esta tesis ha sido la búsqueda de biomarcadores de consumo de vino tinto, incluyendo principalmente aquellos metabolitos originados por la acción de la microbiota intestinal, mediante la generación de la base de datos Phenol-Explorer y su aplicación a dos estudios clínicos con vino tinto.

De manera más concreta se han planteado los siguientes objetivos de acuerdo a cada una de las 4 hipótesis previamente planteadas:

(Objetivos a partir de la Hipótesis 1)

1. Desarrollar la primera base de datos que considere del metabolismo de los compuestos fenólicos, concretándose en la generación del módulo de metabolismo de la base de datos Phenol-Explorer.
2. Identificar y validar el perfil metabólico y las rutas metabólicas asociadas hasta el momento, derivado del consumo de vino.

(Objetivos a partir de la Hipótesis 2)

3. Evaluar las rutas metabólicas de polifenoles presentes en el vino mediante la identificación y cuantificación por UPLC-MS/MS del perfil metabólico de polifenoles más completo en un estudio clínico de intervención con vino tinto desalcoholizado.
4. Determinar los cambios en la excreción urinaria de los metabolitos fenólicos derivados del metabolismo microbiano después del consumo de vino desalcoholizado.

(Objetivos a partir de la Hipótesis 3)

5. Establecer una nueva estrategia de búsqueda de biomarcadores de consumo, concretamente de vino tinto, comparándola y evaluándola con marcadores ya descritos mediante la determinación de su especificidad y sensibilidad.
6. Evaluar la idoneidad de las diferentes muestras biológicas en humanos para la determinación de biomarcadores en estudios de intervención.

(Objetivos a partir de la Hipótesis 4)

7. Evaluar el efecto prebiótico de los polifenoles del vino tinto en determinados grupos microbianos intestinales implicado en los beneficios para la salud del individuo.

8. Estudiar los cambios bioquímicos asociados a los cambios bacterianos tras la intervención dietética con vino.

9. Evaluar la relación entre los cambios observados del perfil polifenólico urinario y la composición microbiana encontrada en heces tras el consumo de vino tinto.

2.ANTECEDENTES BIBLIOGRÁFICOS

2. ANTECEDENTES BIBLIOGRÁFICOS

A continuación se presentan los antecedentes bibliográficos de la Tesis Doctoral permitiendo situar la presente en el contexto científico, antes de abordar el desarrollo de los objetivos mencionados en relación con los tres aspectos cardinales de este trabajo:

1. Bases de datos en el estudio del metabolismo de los polifenoles**.**
2. Desarrollo y evaluación del uso de biomarcadores nutricionales.
3. Interacción entre polifenoles y microbiota intestinal.

En este apartado se presenta el capítulo publicado:

Boto-Ordóñez M, Urpi-Sarda M, Monagas M, Tulipani S, Llorach R, Rabassa-Bonet M, García-Aloy M, Queipo-Ortuño MI, Estruch R, Tinahones FJ; Bartolomé B, Andres-Lacueva C. *Phenolic acids from microbial metabolism of dietary flavan-3-ols.* Nova Science Publishers, Inc, 2012, 8, 147-172. ISBN:978-1-61942-032-8.

2.1 BASES DE DATOS EN EL ESTUDIO DEL METABOLISMO DE LOS POLIFENOLES

Tradicionalmente la composición de los alimentos se veía reducida a macronutrientes (carbohidratos, grasas, proteínas) y micronutrientes (vitaminas, minerales), y por tanto sus efectos sobre la salud humana se limitaba a estos (Willett, 1994). Sin embargo, esta clasificación se ha quedado obsoleta debido a que cada vez es más conocido que los alimentos son sistemas complejos formados por un gran número de compuestos químicos, que sin ser esenciales y/o encontrarse a baja concentración pueden influenciar la salud del organismo. Dentro de estos compuestos se encuentran los fitoquímicos (Tabla A1), y en este caso más concreto, los polifenoles.

Tabla A1: Clasificación de los fitoquímicos.

Categoría	Clase	Subclase	Ejemplo
Fenoles	Flavonoides, ácidos fenólicos, lignanos, coumarinas, fenoles, fenilpropanoides, quinonas, estilbenos, xantonas.	Antocianinas, flavanoles, flavonoles, dihidroflavonoles, flavonas, isoflavonoides, flavanonas, dihidrochalconas, ácidos hidroxibenzoicos, ácidos hidroxicinamicos, coumestanos, furanocoumarinas, alquilfenoles, metoxifenoles, benzodioxoles, curcuminoides, hidroxifenilpropenos, benzoquinonas, naftoquinonas, antraquinonas.	Cianidina, resveratrol.
Compuestos organosulfurados	Indoles, isotiocianatos, sulfatos de alicina, tioles, disulfuros, polisulfuros, tioéteres, tioésteres.	Tioacetales, tiocetales.	Aliína, dibenzotiofeno.
Compuestos nitrogenados	Aminas, glucósidos cianogénicos, glucosinolatos, purinas compuestos nitrogenados.	Benzilaminas, feniletilaminas, triptaminas, glucosinolatos alifáticos, glucosinolatos arómaticos, xantinas, alcoholes indólicos.	Efedrina, cafeína.
Alcaloides	Alcaloides piridina, betalaína, indoles, indolizidine, pirrolidina, quinolina, isoquinolina, esteroideos, tropánicos.	Betacianinas, betaxantinas, ergolinas, yohimbina, tryptolinas o β-carbolinas, morfinanos, saponinas.	Trigonelina, solanina.
Terpenoides/Isoprenoides	Monoterpenoides, sesquiterpenoides, diterpenoides, triterpenoides, tetraterpenoides.	Terpenos fenólicos, saponinas, fitoesteroles, carotenoides.	Timol, vitamina E.

Durante las últimas décadas se ha generado gran cantidad de información sobre fitoquímicos presentes en la dieta, con decenas de miles de publicaciones en este campo. Sin embargo, la diversidad en el origen, el formato, las técnicas y análisis de datos hacen la explotación de esta información muy difícil. El acceso a ella de una

manera sencilla y rápida ha puesto en valor la importancia de la creación de bases de datos, especialmente para la evaluación de estos componentes en la salud humana. Actualmente existen más de 50 bases de datos de fitoquímicos donde se recoge información de estructuras químicas, espectros, rutas metabólicas, composición de alimentos, metabolitos, propiedades biológicas y los efectos sobre la salud (Scalbert *et al.*, 2011). La mayoría de estas bases de datos se encuentran en forma electrónica, donde los usuarios pueden consultar u extraer, de manera sencilla la información presente. En el caso de los polifenoles, su distribución en alimentos se encuentra recogida en bases de datos de composición alimentaria como por ejemplo Phenol-Explorer (Neveu *et al.*, 2010) o las bases de datos de la USDA (USDA, 2007) (Tabla A2), mientras que los metabolitos generados por la ingesta se encuentran dispersos en la bibliografía y muy pocos metabolitos se encuentran en bases de datos. Phenol-Explorer es una base de datos que aglutina información sobre polifenoles en los alimentos (Neveu *et al.*, 2010; Rothwell *et al.*, 2012), metabolismo de polifenoles en estudios *in vivo* (Rothwell et al. 2012) y factores de corrección de pérdidas durante el procesado de alimentos (Rothwell *et al.*, 2013).

A continuación se detalla la clasificación y las características que debería tener una base de datos para considerarla de alta calidad científica (Scalbert *et al.*, 2011), así como las principales bases de datos que existen en el campo de los polifenoles (Tabla A2).

Tabla A2: Bases de datos usadas para el estudio de los polifenoles.

	Nombre	Contenido	Características	URL	Reference
Composición fenólica de los alimentos	Dictionary of Food Compounds	50,000 componentes naturales de los alimentos y aditivos.	Comercial y consultable.	http://dfc.chemnetbase.com/	(Buckingham, 1993)
	USDA Databases	26 agliconas flavonoides, 6 proantocianidinas, 6 isoflavonas y 3 fitoesteroles en 8463 alimentos comúnmente consumidos en los EE.UU.	Acceso libre, consultable y descargable.	http://www.ars.usda.gov/	(USDA, 2007)
	Phenol-Explorer	506 polifenoles en 455 alimentos.	Acceso libre, consultable y descargable.	http://www.phenol-explorer.eu	(Neveu *et al*. 2010)
	EuroFIR-BASIS	256 fitoquímicos en 199 alimentos.	Solo miembros, consultable.	http://ebasis.eurofir.org/	(Gry *et al*. 2007)
Identificación de metabolitos	Human Metabolome Database (HMDB)	Espectros NMR 1D y 2D, MS/MS, GC-MS.	Acceso libre, consultable y descargable.	www.hmdb.ca	(Wishart *et al*. 2009)
	METLIN Metabolite	Espectros MS/MS.	Acceso libre, consultable y descargable.	http://metlin.scripps.edu/	(Smith *et al*. 2005)
	MMCD	Espectros NMR 1D y 2D 13C y 1H.	Acceso libre, consultable y descargable.	http://mmcd.nmrfam.wisc.edu/	(Cui *et al*. 2008)
	BioMagResBank	Espectros 1H y 13C NMR (1D y 2D).	Acceso libre, consultable y descargable.	http://www.bmrb.wisc.edu/ metabolomics/	(Ulrich *et al*. 2008)
	Fiehn Metabolome	Espectros GC-MS.	Comercial y consultable.	http://www.chem.agilent.com	
	KEGG	Estructuras, rutas metabólicas.	Acceso libre, consultable y descargable.	http://www.genome.jp/kegg/pathwa y.html	(Okuda *et al*. 2008)
	Mass Bank	Espectros MS/MS y EI-MS.	Acceso libre, consultable y descargable.	http://www.massbank.jp	(Horai *et al*. 2010)
	PubChem	Estructuras, links a artículos.	Acceso libre, consultable y descargable.	http://pubchem.ncbi.nlm.nih.gov/	(Wang *et al*. 2009)
	Manchester Metabolome	Datos en GC-MS y MS/MS.	Acceso libre, consultable y descargable.	http://dbkgroup.org/MMD/	(Brown *et al*. 2009)
Metabolitos en muestras biológicas	Phenol-Explorer	375 polifenoles de 236 artículos científicos.	Acceso libre, consultable y descargable.	http://www.phenol-explorer.eu	(Rothwell *et al*. 2012)
	Human Metabolome Database (HMDB)	Distribución de metabolitos en biofluídos.	Acceso libre, consultable y descargable.	http://www.hmdb.ca	(Wishart *et al*. 2009)
Propiedades biológicas	Human Metabolome Database (HMDB)	Rutas metabólicas.	Acceso libre, consultable y descargable.	http://www.hmdb.ca	(Wishart *et al*. 2009)
	EuroFIR-BASIS	Actividades biológicas de resultados de estudios clínicos.	Solo miembros, consultable.	http://ebasis.eurofir.org/	(Gry *et al*. 2007)

2.1.1 CLASIFICACIÓN DE LAS BASES DE DATOS EN CIENCIAS DE LA VIDA

Las bases de datos usadas en ciencias de la vida se pueden clasificar de dos maneras, según es la obtención de los datos:

- **Archivísticas**: Bases de datos diseñadas para capturar grandes cantidades de datos de un cierto tipo (metabolitos, genes, composición química etc.) de manera automática, independientemente de su calidad, la redundancia y/o utilidad. Ejemplo: PubChem (Wang *et al.*, 2009), GenBank (Benson *et al.*, 2009).
- **Verificadas**: Bases de datos diseñadas para capturar datos de alta calidad, mediante la introducción y examen de los datos de manera manual por una persona entrenada. Dichas bases de datos contienen cantidades más limitadas de datos. Ejemplo: MassBank (Taguchi *et al.*, 2007), KEGG (Okuda *et al.*, 2008), HMDB (Wishart *et al.*, 2009; Wishart *et al.*, 2013).

2.1.2 CARACTERÍSTICAS DE LAS BASES DE DATOS EN CIENCIAS DE LA VIDA

Las características que tiene que cumplir una base de datos de calidad en las ciencias de la vida son las siguientes y coinciden con el acrónimo ACQUIRE, que en inglés significa: *Accessible, Comprehensive, Queryable, User-friendly, Interactive, Referenced, and Expandable,* como describió Scalbert et al. (Scalbert *et al.*, 2011):

- **Accesible** (*Accessible*): Bases de datos de fácil y libre acceso, para aumentar la visibilidad en comunidades científicas de distintos campos.
- **Completa** (*Comprehensive*)**:** Proporcionando una base de datos integral que incluya una amplia diversidad de tipos de datos, como información numérica, gráfica y de texto, de un campo o tema concreto.
- **Consultable** (*Queryable*)**:** Las bases de datos deben ser compatibles con una amplia gama de búsquedas, desde texto simple a combinaciones más complejas. Así como permitir desde búsquedas más globales a otras más específicas.
- **Fácil de usar** (*User-friendly*)**:** Una base de datos debe permitir que cualquier persona pueda acceder a la información sin ninguna dificultad. Mediante el

diseño de una interfaz en el que las consultas complejas se pueden realizar a través de los menús desplegables simples o motores de búsquedas que usen un lenguaje sencillo.

- **Interactiva** (*Interactive*)**:** Bases de datos que permitan a los usuarios consultas complejas, seleccionando las opciones de menú, ampliando puntos de vista, manipulando imágenes, accediendo a otros archivos de datos o a otros bases de datos para ver datos complementarios, ampliando la información presente en una base de datos individual.
- **Referenciada** (*Referenced*)**:** Una base de datos tiene que proveer una referencia completa y adecuada del origen de los datos, pudiendo incluir métodos, técnicas o programas utilizados para generar los datos, permitiendo su validación y la detección de errores por usuarios y autores.
- **Ampliable** (*Expandable*): Las bases de datos deben estar diseñadas de manera que puedan ser continuamente ampliadas y actualizadas, permitiendo entradas adicionales y modificaciones en relación a su diseño, uso, tipo de datos, o para evitar la obsolescencia de las mismas.

2.1.3 PRINCIPALES BASES DE DATOS EN EL ESTUDIO DEL METABOLISMO DE LOS POLIFENOLES

El estudio del metabolismo de los polifenoles requiere el conocimiento de varios aspectos a la hora de hacer una aproximación a lo que podemos obtener en un estudio clínico. Por un lado, se requiere conocer la estructura, la clasificación y las concentraciones de los polifenoles presentes en los alimentos, con bases de datos de composición alimentaria (Tabla A2). Esto nos puede dar una idea de lo que podemos encontrar en el alimento y en qué concentración. Las modificaciones que sufren los polifenoles en alimento tras su consumo, requiere el conocimiento del metabolismo con el fin de planificar el análisis en muestras biológicas, y es aquí donde se han puesto los últimos esfuerzos con el lanzamiento de Phenol-Explorer 2.0 (Rothwell *et al.*, 2012), y su posterior traspaso a la base de datos Human Metabolome Database (HMDB) (Wishart *et al.*, 2009). Una vez realizado el análisis, existen bases de datos que nos

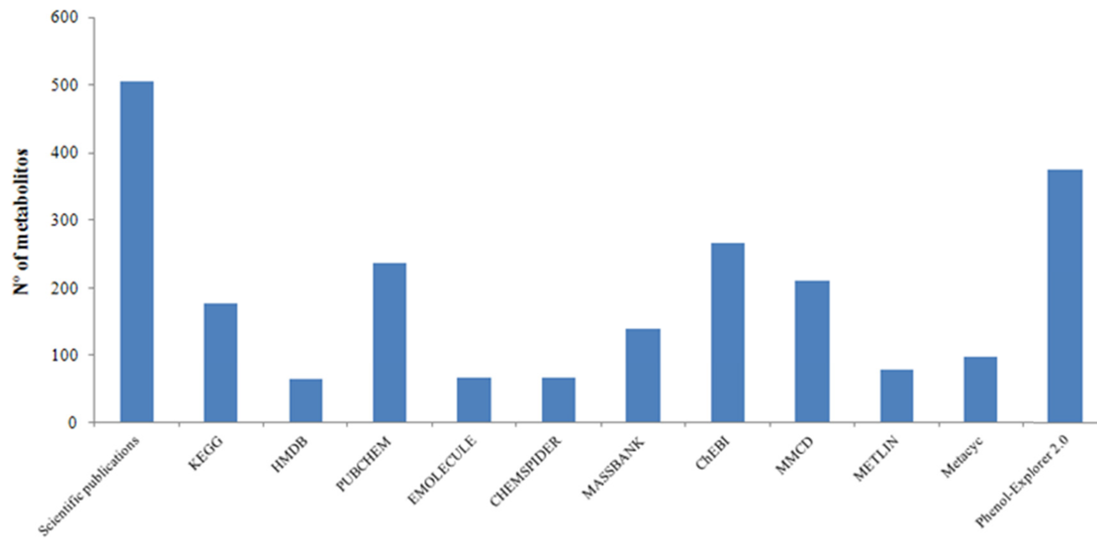
pueden ayudar a la identificación de compuestos, a través de la publicación de masas y espectros de RNM, así como la ubicación de dichos metabolitos en tejidos o datos sobre actividades biológicas.

De manera global, solo la base de datos Phenol-Explorer, cubre todos aspectos de la investigación en el metabolismo de los polifenoles (Neveu *et al.*, 2010; Rothwell *et al.*, 2012; Rothwell *et al.*, 2013). Desde la composición química de alimentos y productos alimentarios (Neveu *et al.*, 2010), las modificaciones de su procesado (Rothwell *et al.*, 2013), su clasificación y estructura, así datos cinéticos de los metabolitos fenólicos encontrados en estudios *in vivo* y sus características de diseño (Rothwell *et al.*, 2012). Todo ello a través de una interface de fácil acceso y uso para cualquier usuario (http://www.phenol-explorer.eu/).

La elaboración de la base de datos Phenol-Explorer se inicia con un primer lanzamiento que supuso la incorporación de más de 35,000 valores de 500 polifenoles encontrados en más de 400 alimentos (Neveu *et al.*, 2010) y finaliza con Phenol-Explorer 3.0, que incorpora el efecto de 35 técnicas del procesado alimentario sobre 139 polifenoles y 155 alimentos.

El segundo lanzamiento, Phenol-Explorer 2.0, resultado presentado en la presente tesis doctoral, supone la compilación de 375 metabolitos de 236 publicaciones originales (Rothwell *et al.*, 2012), el mayor contenido en una base de datos de polifenoles. La comparación con otras bases de datos donde podemos encontrar metabolitos de los polifenoles, supone el distanciamiento de Phenol-Explorer, junto con HMDB, base de datos a la que ha cedido su contenido, como la mayor base de datos de metabolitos de los polifenoles (Figura A1). El número elevado de metabolitos en otras bases de datos se debe a duplicidades, y al hecho que muchos de los metabolitos están ampliamente distribuidos, como los ácidos fenólicos, pero no siendo así para aquellos formados en el organismo, como son los conjugados sulfatados y glucuronidados.

Figura A1: Comparación del número de metabolitos de polifenoles en bases de datos previa a la incorporación de la información de Phenol-Exporer a HMDB.



2.2 DESARROLLO Y EVALUACIÓN DEL USO DE BIOMARCADORES NUTRICIONALES

Los marcadores biológicos fueron definidos inicialmente como una alteración celular, bioquímica o molecular, medida en medios biológicos como tejidos, células o fluidos (Hulka, 1990). Esta definición, ligada a procesos patológicos, fue ampliada para cubrir también la respuesta a agentes externos, como una intervención terapéutica o una exposición a algún compuesto, o el grado de susceptibilidad biológica a un tratamiento o a la toxicidad ambiental, definiéndose finalmente como una característica que puede ser objetivamente medida y evaluada como indicador de procesos biológicos normales, estados de enfermedad o respuestas farmacológicas a una intervención terapéutica (Biomarkers Definitions Working Group, 2001).

En epidemiología molecular, los biomarcadores han sido clasificados en tres categorías (Schulte, 2005):

- biomarcadores de exposición
- biomarcadores de efecto
- biomarcadores de susceptibilidad

La potencialidad de los biomarcadores para determinar los primeros eventos de una enfermedad, proporcionar nuevos conocimientos sobre mecanismos potenciales relacionados con la patogénesis, progresión de la enfermedad, pronóstico y respuesta a la terapia, junto con el crecimiento de la nuevos campos de investigación como la proteómica o la metabolómica, ha dado como resultado un gran número de nuevos biomarcadores (Scalbert *et al.*, 2013), y en el campo de la alimentación, los conocidos como biomarcadores nutricionales (Potischman y Freudenheim, 2003).

2.2.1 DEFINICIÓN Y CLASIFICACIÓN DE BIOMARCADORES NUTRICIONALES

Un biomarcador nutricional puede ser cualquier compuesto biológico que sea un indicador del estado nutricional con respecto a la ingesta dietética, o al metabolismo de cualquier componente de la dieta o un marcador del efecto sobre el organismo de la ingesta alimentaria, y que analizado en las muestras biológicas de los individuos

sirve para determinar su exposición o ingesta de dicho alimento o componente (Potischman y Freudenheim, 2003; Hedrick *et al.*, 2012).

Como se ha comentado anteriormente, los biomarcadores nutricionales deben ser indicadores específicos de la ingesta de un determinado constituyente de la dieta, que resulta de su absorción, digestión, metabolismo, y disponibilidad biológica. Entre los biomarcadores de la ingesta se distinguen además entre dos subgrupos (Zamora-Ros *et al.*, 2012):

– Biomarcadores de la ingesta de un **alimento o grupo de alimentos específicos**, que incluyen, por ejemplo, la estaquidrina o prolina betaína, biomarcador muy conocido del consumo de cítricos (Heinzmann *et al.*, 2010; Lloyd *et al.*, 2011) o los metabolitos de resveratrol como biomarcadores de consumo de vino (Zamora-Ros *et al.*, 2006).

– Biomarcadores de la ingesta de un **componente de la dieta o grupo de componentes específicos**, tales como la sacarosa y fructosa como un biomarcador del consumo de azúcar (Tasevska *et al.*, 2005) o la creatinina para el consumo de proteína animal (Cross *et al.*, 2011).

Otra clasificación también descrita para los biomarcadores de ingesta es en tres grupos (Jenab *et al.*, 2009; Zamora-Ros *et al.*, 2012) :

– Biomarcadores de **recuperación**: proporcionan una estimación del nivel de consumo total en un período de tiempo determinado, como ejemplo el nitrógeno urinario total.

– Biomarcadores **predictivos**: proporcionan una alta correlación con la ingesta pero una baja recuperación global: la sacarosa y fructosa como un biomarcador del consumo de azúcar (Tasevska *et al.*, 2005).

– Biomarcadores de **concentración**: proporcionan una medida de la cantidad de este compuesto en los tejidos, teniendo en cuenta la ingesta, la biodisponibilidad, y la regulación fisiológica del nivel de compuesto, como vitaminas, carotenoides.

2.2.2 IMPORTANCIA Y UTILIZACIÓN DE LOS BIOMARCADORES NUTRICIONALES

El principal papel del uso de biomarcadores en epidemiología nutricional es evaluar los efectos de la dieta en el riesgo de padecer enfermedades, para de una manera fiable conocer la ingesta real de nutrientes de los participantes, y poder asociarla así a sus efectos sobre la salud (Scalbert *et al.*, 2013).

El uso de biomarcadores resulta ser la medición más directa de la ingesta alimentaria respecto a los métodos tradicionales. Hechos como la variación del compuesto dentro de un mismo alimento y el lugar y su forma de cultivo, así mismo como las variaciones propias del propio individuo, hacen que sea imposible conocer la ingesta real (Zamora-Ros *et al.*, 2012). En algunos casos, el uso combinado de una evaluación dietética mediante encuestas y biomarcadores de consumo establece una estimación más exacta y real de la exposición nutricional, y puede servir para la validación de encuestas dietéticas (Zamora-Ros *et al.*, 2012).

Las principales razones para el uso de biomarcadores nutricionales son los siguientes (Potischman y Freudenheim, 2003; Spencer *et al.*, 2008; Zamora-Ros *et al.*, 2012):

- Los biomarcadores nutricionales son más precisos que las estimaciones dietéticas. Estos limitan problemas de los cuestionarios de frecuencia de consumo y otros registros dietéticos como la sobreestimación de alimentos saludables (por ejemplo frutas y verduras) e infravaloración de los menos saludables, así como la identificación de la gran gama de alimentos presentes en el mercado o la amplia variedad de técnicas culinarias.
- Los biomarcadores integran en la medida, la biodisponibilidad y el metabolismo del componente, yendo más allá de cantidad presente en el alimento.
- Las bases de datos utilizadas para analizar las estimaciones ditéticas presentan limitaciones en cuanto a número de alimentos, variabilidad en composición de los alimentos, métodos analíticos utilizados y número de componentes disponibles.

Así mismo, los biomarcadores nutricionales permiten valorar el cumplimiento de una intervención nutricional, comprobando objetivamente que los participantes realmente han seguido el tratamiento o la suplementación, en este caso dietética, de forma correcta. A pesar de ello, las encuestas dietéticas siguen siendo muy usadas en epidemiología nutricional, ya que a pesar de ser más precisos, su determinación es cara, y el número de marcadores disponibles todavía es bajo (Zamora-Ros *et al.*, 2012).

2.2.3 EVALUACIÓN DE LOS BIOMARCADORES NUTRICIONALES

El uso de un compuesto como biomarcador requiere un proceso de evaluación. Como en cualquier medida analítica que se precie un biomarcador debe cumplir los clásicos requisitos de exactitud, reproducibilidad, fiabilidad y validez (Marshall, 2003). De manera adicional se espera que un biomarcador cumpla con los siguientes preceptos (Spencer *et al.*, 2008; Scalbert *et al.*, 2013):

- Disponer de un método analítico cualitativa y cuantitativamente robusto. En el caso de los polifenoles, la baja concentración en muestras hace que la sensibilidad y especificidad de la técnica sea muy importante.
- Sensible a cambios de consumo del compuesto de interés, para tener la capacidad de discriminar consumidores de los no consumidores y específico de la ingesta del componente de interés, haciendo que cualquier variación en su concentración sea debido a una variación del consumo del compuesto.
- Que disponga de estudios cinéticos para conocer la absorción, metabolismo y excreción del componente, dando una plausibilidad biológica al componente estudiado.

Existen limitaciones en la evaluación de metabolitos o compuestos como biomarcadores debido a su tiempo de vida medio en las muestras biológicas o la gran variabilidad interindividual. El tiempo de vida media limita el tipo de muestra al tipo de información que queramos obtener. Así pues, un biomarcador a corto plazo nos informará de una ingesta aguda y un biomarcador a medio/largo plazo nos informará de una ingesta regular o habitual.

Todo ello hace que se requiera una validación de los biomarcadores en tres aspectos, i) técnicamente: incluyendo medidas como la exactitud, precisión o reproductibilidad; ii) de utilización: demostrando que la validación analítica y biológica da el suficiente apoyo para el biomarcador propuesto; iii) biológicamente: demostrando los efectos de una intervención en el biomarcador.

2.2.4 PERSPECTIVAS DE FUTURO DE LOS BIOMARCADORES NUTRICIONALES

La necesidad de nuevos biomarcadores es especialmente importante para el desarrollo de nuevos alimentos funcionales ya que de acuerdo con la legislación europea deben estar suficientemente evaluados en estudios en humanos (van Loveren *et al.*, 2012). Esta falta de buenos biomarcadores nutricionales, supone un impedimento para el desarrollo de esta industria a nivel europeo y mundial. A nivel científico es necesario el uso de biomarcadores para proporcionar medidas objetivas del estado nutricional y consumo de alimentos, una limitación de las evaluaciones dietéticas tradicionales. Para ello el desarrollo de la metabolómica en la nutrición humana, los cuestionarios de frecuencia validados y la continua expansión de las bases de datos, permitirían la identificación de los componentes específicos derivados de la dieta.

Además de esto las futuras investigaciones deberían ir encaminadas a (Raiten *et al.*, 2011; Hedrick *et al.*, 2012):

- Mejorar los biomarcadores ya existentes, mejorando las técnicas analíticas hacia unas más rentables, rápidas y menos invasivas.
- Elucidar los factores que pueden afectar a la homeostasis de un nutriente.
- Identificar interacción nutriente-nutriente y nutriente-genoma.
- Identificar combinaciones de biomarcadores que mejoren la sensibilidad y especificidad.
- Valorar la utilidad de los biomarcadores de manera individual y en la población.

2.3 INTERACCIÓN ENTRE POLIFENOLES Y MICROBIOTA INTESTINAL

Las propiedades biológicas de los polifenoles dietéticos dependen en gran medida de su biodisponibilidad, y es aquí donde la microbiota intestinal juega un importante papel tras el consumo de alimentos por su capacidad de metabolización. De la misma manera, existe la evidencia de que los polifenoles tienen la capacidad para modular la composición bacteriana y por tanto mejorar la salud o prevenir el desarrollo de ciertas enfermedades. A continuación se presenta una aproximación interacción polifenoles-microbiota en el caso concreto del vino.

2.3.1 PAPEL DE LA MICROBIOTA EN EL METABOLISMO DE POLIFENOLES DEL VINO

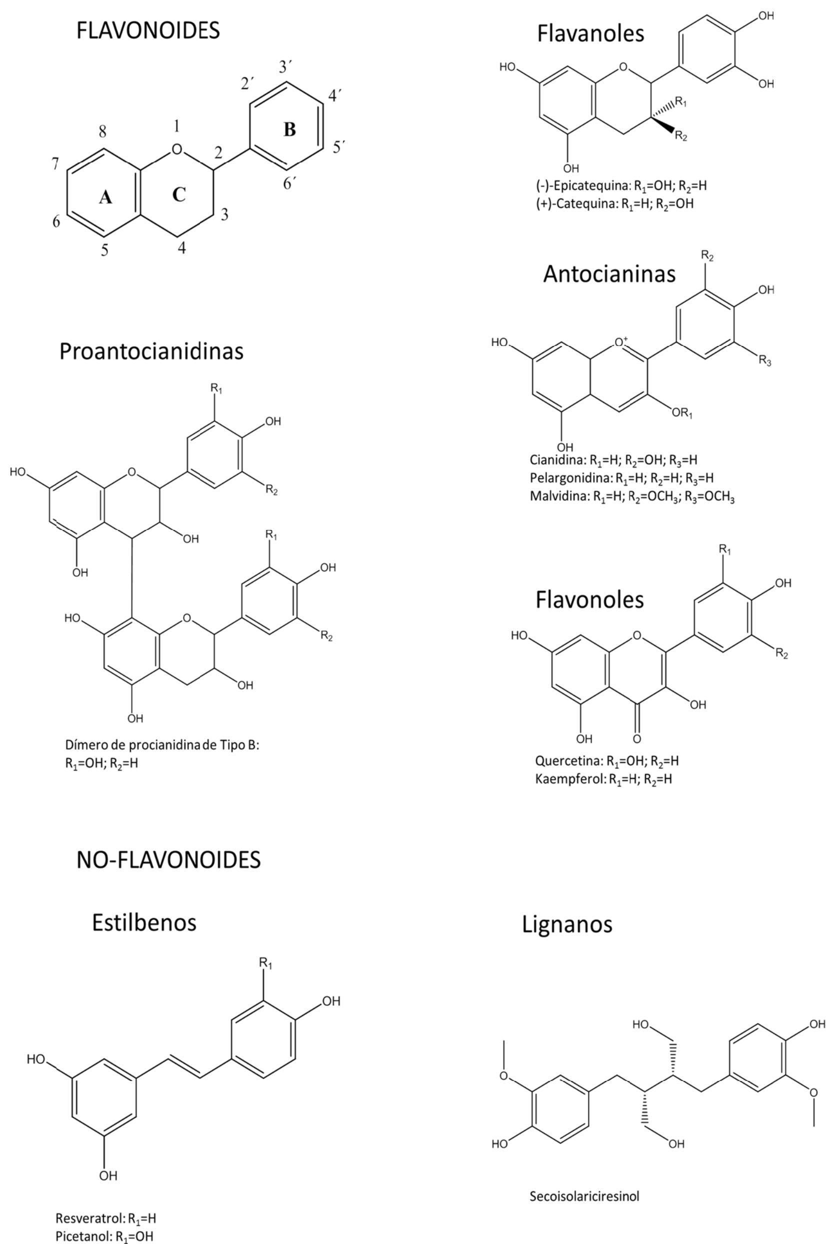
Un porcentaje superior al 90% de los polifenoles dietéticos ingeridos pueden llegar a los tramos finales del tubo digestivo donde son metabolizadas de manera extensiva por las bacterias intestinales (Clifford, 2004). Estos polifenoles, y los metabolitos generados, pueden tener un papel clave en el mantenimiento de la salud intestinal (Selma *et al.*, 2009; Bolca *et al.*, 2013). El metabolismo de los polifenoles por la microbiota está influenciado por la estructura química de los compuestos que llegan al intestino. Para los compuestos fenólicos presentes en el vino se han descrito fenómenos de hidrólisis, reducción, desmetilación, isomerización, deshidroxilación o descarboxilación en función de su estructura química (Requena *et al.*, 2010).

El principal grupo de polifenoles son los flavonoides que se caracterizan por tener una estructura base común C6-C3-C6 (Figura A2). Dentro de los flavonoides, los flavanoles o flavan-3-oles, principalmente proantocianidina y (epi)catequina, se encuentran en una concentración entre 30.78–86.53 mg/100 mL. Los siguientes en concentración son los antocianos, antocinidinas glucosiladas que están en un rango de 2.32-76.51 mg/100 mL. A mayor distancia están el resto de polifenoles, tanto flavonoides como los flavonoles o las flavanonas, como no flavonoides, estilbenos, lignanos o ácidos fenólicos (Neveu *et al.*, 2010).

Los polifenoles una vez llegados al intestino pueden sufrir fenómenos de desglicosilación, hidrólisis de ésteres y amidas y desglucuronización. A continuación la degradación microbiana de los flavonoides comienza con la rotura del anillo C,

liberando compuestos aromáticos hidroxilados derivados del anillo A y en numerosos ácidos fenólicos derivados del anillo B (Requena *et al.*, 2010). Los compuestos generados por este proceso son susceptibles de sufrir más reacciones de hidroxilación, desmetilación, hidrogenación y α-oxidación y β-oxidación, generando nuevos metabolitos (Monagas *et al.*, 2010).

Figura A2. Principales polifenoles del vino.



El metabolismo bacteriano de los flavanoles se encuentra discutido en profundidad en el capítulo de libro escrito durante la presente Tesis Doctoral y añadido a continuación.

ÁCIDOS FENÓLICOS DERIVADOS DEL METABOLISMO MICROBIANO DE LOS FLAVAN-3-OLES DIETÉTICOS

Los flavan-3-oles son polifenoles presentes en la dieta en formas monoméricas, oligoméricas y poliméricas, cuya bioactividad y efectos sobre la salud *in vivo* siguen sin estar claros debido a su complejo metabolismo. De acuerdo con el grado de polimerización, los flavan-3-oles simples pueden ser absorbidos en el intestino delgado, mientras que los oligómeros y polímeros necesitan ser biotransformados por la microbiota colónica antes de la absorción. Este paso da lugar a un gran número y variedad de ácidos fenólicos que pueden ser responsables de los efectos sobre la salud derivados del consumo de los flavan-3-oles, en lugar de las formas fenólicas originales presentes en los alimentos.

Aunque en estudios *in vitro* han revelado que algunas bacterias son capaces de catabolizar polifenoles, la identificación de las bacterias del colónicas con capacidad para catabolizar flavan-3-oles se encuentra en sus primeras etapas, no así los metabolitos generados por las mismas, que en la última década se ha logrado un gran progreso en la identificación de los ácidos fenólicos derivados del catabolismo de flavan-3-oles por la microbiota intestinal.

Sin embargo, la relación entre el consumo de alimentos ricos en flavan-3-oles, los metabolitos encontrados *in vivo* y sus efectos en la salud sigue siendo un reto difícil debido a la gran variabilidad interindividual, y otros factores tales como la diversidad estructural propia de estos polifenoles o la matriz alimentaria en la que se encuentran. Los estudios realizados con compuestos fenólicos aislados en estudios que simulan el ambiente colónico, nos pueden ayudar a identificar las bacterias que participan en el catabolismo y comprender su actividad, estableciendo un vínculo entre los metabolitos producidos y los que se encuentran en circulación *in vivo*. A pesar de la importancia biológica de los metabolitos microbianos, se desconoce su verdadero papel, y solo evidencias relacionadas con sus propiedades antioxidantes, anti-inflamatorias, anti-proliferativos y citotóxicas han sido descritas en estudios *in vitro*.

En este capítulo se pretende dar una visión general de los ácidos fenólicos formados por el catabolismo de los flavan-3-oles en el colon, incluyendo las posibles rutas

metabólicas, grupos y especies que intervienen en su catabolismo, así como las concentraciones en plasma y orina tras su consumo, y bioactividades específicas. Todos estos aspectos pueden ayudar a comprender mejor la complejidad del metabolismo microbiano a nivel colónico de los flavan-3-oles y el papel de los ácidos fenólicos en los efectos sobre la salud derivados del consumo de fuentes ricas en estos compuestos.

Toda esta información referente a los ácidos fenólicos se presenta a continuación bajo el título "*Phenolic acids from microbial metabolism of dietary flavan-3-ols*"capítulo 8 del libro "*Phenolic Acids: Composition, Applications and Health Benefits*" (Nova Science Publishers, Inc, 2012, 8, 147-172. ISBN:978-1-61942-032-8).

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Chapter VIII

Phenolic Acids from Microbial Metabolism of Dietary Flavan-3-ols

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Abstract

Flavan-3-ols are polyphenols present in the diet in monomeric, oligomeric and polymeric forms, but their bioactivity and *in vivo* health effects remain unclear due to their complex metabolism. According to the degree of polymerization, monomeric flavan-3-ols can be absorbed in the small intestine, whereas oligomers and polymers need to be biotransformed by the colonic microbiota before absorption. This latter gives rise to a wide number and variety of phenolic acids which may be responsible for the health effects derived from flavan-3-ol consumption rather than the original phenolic forms

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found in foods. Although *in vitro* studies have revealed that some bacteria are able to catabolise certain class of polyphenols, the identification of human colonic bacteria with capacity to catabolise flavan-3-ols is in its early stages. However, in the last decade a great progress has been achieved in the identification of phenolic acids derived from the catabolism of flavan-3-ols by gut microbiota. The link between consumption of flavan-3-ols food sources and those metabolites found *in vivo*, with related health effects is still a difficult challenge due to the huge variability in colonic biotransformation found among individuals, and other factors such as the own structural diversity of these polyphenols and food matrix that add a further variability in catabolism. Studies performed with isolated phenolic compounds in a colonic environment may help us to identify colonic bacteria involved in catabolism and understand their activity in the colon, and set up a link to circulating metabolites found *in vivo*. Although the biological relevance of microbial metabolites remains largely unknown, evidences related to their antioxidant, anti-inflammatory and anti-proliferative activities and cytotoxicity are starting to be accumulated. This chapter aims to give an insight into the phenolic acids formed by the colonic catabolism of dietary flavan-3-ols, including tentative metabolic pathways, potential microbial groups/species involved in their catabolism, plasma and urine concentrations found after *in vivo* consumption, and specific bioactivities. All these aspects may help us better understand the complexity of the colonic catabolism of flavan-3-ols and the role of phenolic acids in health effects derived from the consumption of flavan-3-ol rich sources.

Introduction

Flavan-3-ols are the most common group of flavonoids found in the diet, and, in view of their presence in food as functional ingredients, also one of the most commonly groups linked to health benefits. The group includes a wide number of molecules of different chemical structure and degree of polymerization that may vary depending on the source. This variation is reflected in the number of structural units, hydroxylation pattern, stereochemistry of C2 and C3 of the central ring, type interflavan linkages and possible esterification with other polyphenols (i.e. galloylation) [1]. Those chemical characteristics have a profound impact on the absorption and metabolism, and hence in their functionality in our organism. Monomers and oligomers may be absorbed at the small-intestine level, undergoing glucuronidation and methylation in the enterocyte. The portal vein drives them to the liver, where a new process of methylation, glucuronidation and glycination takes place. From the liver, new metabolites are then released to various tissues and organs, before being excreted through urine or turning back to the intestine for enterohepatic circulation. Procyanidins and some oligomeric forms are not absorbed in the small intestine and reach the colon where they are metabolized by intestinal bacteria to more simple metabolites, phenolic acids. Phenolic acids, with different patterns of hydroxylation and methylation and side-chain length, have shown diverse bioactivities and in certain cases could be more active than their precursors. These metabolites generated by microbiota are also absorbed and metabolized in the same way as monomers once they reach the liver, and they are also found in biofluids such as urine or plasma in their simple or conjugated form [2-4]. A small fraction of phenolic acids is also eliminated through the faeces and found in faecal waters. Most of the bacteria responsible for these changes in flavan-3-ols remains unknown, because of the number of species (concentrations of 10^{12} microorganisms per gram gut content) and interindividual variety,

which could be affected by age, diet, environment and phylogeny [5-7], the complex structural features of these polyphenols and their own antimicrobial activity, but the number of studies evaluating the bacterial enzymes that change flavan-3-ols into more active components is of current interest [8, 9].

Sources of Flavan-3-ols

Flavan-3-ols are widely distributed in foodstuffs such as fruits, red wine, beer and nuts, as well as in herbs, being the richest sources and the most widely studied tea and chocolate [1, 10]. Their presence in foods ranges from monomers such as (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin-3-O-gallate, (-)-epigallocatechin-3-O-gallate and (+)-gallocatechin to procyanidins (dimers, trimers or other polymers). Flavan-3-ol classification and their main food sources are described in Table 1.

Table 1. Flavan-3-ols distribution in foods

Flavan-3-ols	Food	Mean	Min-Max	
Monomers				
(+)-Catechin	Red wine	6.81 mg/100 ml	1.38- 39 mg/100 ml	[11-13]
	Black tea	2.45 mg/100 ml	0 -17.08 mg/100 ml	
	Chocolate	20.50 mg/100 mg FW	0.75-50mg/100 mg FW	
	Cocoa, powder	107.75 mg/100 g FW	61-202 mg/100 mg FW	
	Strawberry	6.36 mg/100 g FW	1.57-18.7 mg/100 mg FW	
	Red Grape	5.46 mg/100 g FW	0.82-8.94 mg/100 mg FW	
	Peach, peeled	5.47 mg/100 g FW	0.53-19.6 mg/100 mg FW	
	Apple cider	5.56 mg/100 g FW	0-58.04 mg/100 mg FW	
(-)-Epicatechin	Red wine	3.78 mg/100 ml	0-16.50 mg/100 ml	
	Green tea	7.93 mg/100 ml	0-73.89 mg/100 ml	
	Chocolate	70.36 mg/100 g FW	32.74-125 mg/100 mg FW	
	Cocoa, powder	158.30 mg/100 g FW	63-330 mg/100 mg FW	
	Red Grape	5.24 mg/100 g FW	0.7-8.64 mg/100 mg FW	
	Peach, peeled	7.97 mg/100 g FW	0.6-16.4 mg/100 mg FW	
	Cherry, sweet	7.78 mg/100 g FW	5.4-9.5 mg/100 mg FW	
	Apple Cider	28.67 mg/100 g FW	0-141mg/100 mg FW	
	Broad beans	22.51 mg/100 g FW	--	
(-)-EGC	Green tea	19.68 mg/100 ml	0.01-100 mg/100ml FW	
	Black tea	7.19 mg/100 ml	0.006-51 mg/100ml FW	
	Broad beans	14.03 mg/100 g FW	--	
	Pecan Nuts	5.60 mg/100 g FW	--	
	Plums	13.06 mg/100 g FW	--	
(-)-ECG	Green tea	7.50 mg/100 ml	0.1-64.2 mg/100 ml FW	
	Peppermint tea	9.24 mg/100 ml	3.72-14.76 mg/100 ml FW	
	Grape black	1.68 mg/100 g FW	0.17-2.81 mg/100 mg FW	
	Carob, flour	30.06 mg/100 g FW	--	
	Strawberries	0.15 mg/100 g FW	--	
Flavan-3-ols	Food	Mean	Min-Max	
(-)-EGCG	Kiwi	0.08 mg/100 g FW	0-0.2 mg/100 g FW	
	Green tea	27.16 mg/100 ml	0.57-271.4 mg/100 ml FW	
	Black tea	9.12 mg/100 ml	0-67.9 mg/100 ml FW	
	Hazelnuts	1.10 mg/100 g FW	--	
	Raspberries	0.54 mg/100 g FW	--	
	Plum	0.48 mg/100 g FW	--	
(+)-Gallocatechin	Red wine	0.08 mg/100 ml	0-0.42 mg/100 ml	

Table 1. (Continued)

Flavan-3-ols	Food	Mean	Min-Max	
	Redcurrant	1.28 mg/100 g FW	1.22-1.35 mg/100 mg FW	
	Green tea	2.26 mg/100 ml	0-15.69 mg/100 ml	
	Black tea	14.01 mg/100 ml	0-59.2 mg/100 ml	
	Broad bean	9.68 mg/100 g FW	--	
Procyanidins dimers				
B1	Red wine	4.14 mg/100 ml	2.15-14 mg/100 ml	
	Cocoa powder	112 mg/100 g FW	--	
	Peach, peeled	25.77 mg/100 g FW	0.7-68.7 mg/100 mg FW	
	Plum, fresh	8.84 mg/100 g FW	--	
	Broad bean	11.26 mg/100 g FW	--	
B2	Red wine	4.97 mg/100 ml	0.43-9 mg/100 ml	
	Cocoa powder	71.57 mg/100 g FW	13-262 mg/100 mg FW	
	Apple, cider	19.60 mg/100 g FW	5.6-87.5 mg/100 mg FW	
	Plum, fresh	5.20 mg/100 g FW	--	
	Broad bean	12.08 mg/100 g FW	--	
B3	Red wine	9.47 mg/100 ml	0-11.96 mg/100 ml	
	Barley grain	10.90 mg/100 g FW	8.8-14.2 mg/100 mg FW	
	Quince jelly	29.38 mg/100 g FW	4.3-81.9 mg/100 mg FW	
B4	Red wine	7.29 mg/100 ml	0.08-11. mg/100 mg FW	
	Custard apple	2.48 mg/100 g FW	--	
	Broad bean	18.47 mg/100 g FW	--	
B5	Plum, fresh	1.59 mg/100 g FW	--	
	Apple, dessert	0.97 mg/100 g FW	0.18-1.9 mg/100 mg FW	[11, 12, 14]
	Custard apple	0.82 mg/100 g FW	--	
B7	Red wine	0.27 mg/100 ml	--	
	Plum, fresh	4.69 mg/100 g FW	--	
	Apple dessert	3.76 mg/100 g FW	--	
	Green tea	0.63 mg/100 ml	--	
Procyanidins trimers				
C1	Chocolate	26.00 mg/100 g FW	13-44 mg/100 mg FW	
	Plum, fresh	10.01 mg/100 g FW	--	
	Apple juice	29.97 mg/100 ml	19.9-40 mg/100 mg FW	
Trimer EEC	Plum, fresh	7.73 mg/100 g FW	--	
	Apple dessert	1.37 mg/100 g FW	0.43-2.4 mg/100 mg FW	
	Custard apple	0.97 mg/100 g FW	--	
T2	Red wine	6.71 mg/100 ml	--	
C2	Beer	0.3 mg/100 ml	--	
>Trimers				
4-6 mers	Blueberries	25.7 mg/100 g FW	--	
	Cranberries	70.3 mg/100 g FW	--	
	Plums	58 mg/100 g FW	--	
7-10 mers	Blueberries	27.8 mg/100 g FW	--	
	Cranberries	62.9 mg/100 g FW	--	
	Plums	58 mg/100 g FW	--	
>10 mers	Blueberries	266.4 mg/100 g FW	--	
	Cranberries	233.5 mg/100 g FW	--	
	Plums	57.3 mg/100 g FW	--	

(-)-EGC, Epigallocatechin; (-)-ECG, Epicatechin-3-gallate; (-)-EGCG, Epigallocatechin-3-gallate; FW, Fresh Weight.

In contrast to the main classes of flavonoids which are present as glycosides, the aglycones (+)-catechin and (-)-epicatechin are present in foods such as cocoa powder (61-330 mg/100 mg FW), tea (0–74 mg/100 mL FW) red wine (0–39 mg/100 mL FW), nuts (0–4 mg/100 mg FW), beer (0 10 mg/100 mL FW) and fruits (0-58.04 mg/100 mg FW). Brewed

tea is a rich source of flavan-3-ols, with (+)-catechin and (-)-epicatechin being present in high amounts (6.8 395 mg/100 g FW) [11, 15]. (Epi)catechins are present in the majority of foods, while gallo(epi)catechins and galloyled monomers such as (+)-gallocatechin, (-)-epicatechin 3-gallate, and (-)-epigallocatechin 3-gallate are only found in certain fruits, such as plums, apples, berries, red grapes and peaches, and normally at very low concentrations (less than 1 mg/100 g FW) [13].

On the other hand, proanthocyanidins, also known as condensed tannins, are widely distributed and are the second most abundant natural phenolic compounds after lignins, in terms of their presence and distribution. They are oligomeric and polymeric flavan-3-ols that are usually found as procyanidins (i.e. oligomers and polymers constituted by (epi)-catechin).

The proportion of monomers, dimers, trimers and polymers of total flavan-3-ols ingested following an American-style diet has been estimated to account for 7.1, 11.2, 7.8 and 73.9% respectively, being apples (32.0%), followed by chocolate (17.9%) and grapes (17.8%) the major sources of procyanidins [14].In the Spanish diet, total flavan-3-ol content was found to range from 10 mg to 50 mg/100 g FW in fruits such as plums, apples and cherries. Among the beverages, the highest flavan-3-ol levels were determined in green and black teas (43.8 and 26.8 mg/100 mL of infusion, respectively), followed by wine, mainly red wine (2.66 mg/100 mL), but also rosé and white wines (2.20 and 0.2 mg/100 mL respectively). Finally, a few vegetables contained flavan-3-ols at very low concentrations (below 1.5 mg/100 g FW) [11, 13].

Bioavailability

Flavan-3-ols: Absorption and Metabolism

The bioavailability of flavan-3-ols is affected by different factors, such as the degree of polymerization and galloylation, and the matrix effect in which the compound is delivered. Therefore, beneficial effects are largely dependent on the precursor consumed.

Most polyphenols present in food are conjugated (esters, glycosides or polymers), which means that they cannot be absorbed in their native form and they must be hydrolyzed in the gut by enzymes or by the colonic microflora to become more simple components [10].

However, monomeric flavan-3-ols do not need to be hydrolyzed, and are readily absorbed in the small intestine and extensively metabolized to monoglucuronides, sulfates, methyl ethers or combined derivatives. This conjugation occurs in the small intestine and liver and the most common conjugation positions are the hydroxyl groups at C-5 and C-7 (A ring), C-3′ and C-4′ (B ring), and C-3 (C ring). The enzymes responsible for glucuronidation in the small intestine are uridine 5′-diphosphate glucuronosyl transferases (UGTs), especially UGT1, present in the luminal part of the endoplasmatic reticulum, cytosol sulfotransferases and catechol-*O*-methyltransferase (COMT) for sulfation and methylation in the liver, respectively.

The formed metabolites are released to the systemic circulation from where they may be distributed to tissues or be excreted through urine and bile. Some of the glucuronides and sulfates excreted in the liver return back to the intestine to be reabsorbed, a phenomenon known as enterohepatic circulation, which may lead to a longer presence of polyphenols within the body, thereby continuing to have beneficial effects (Figure 1) [9, 10].

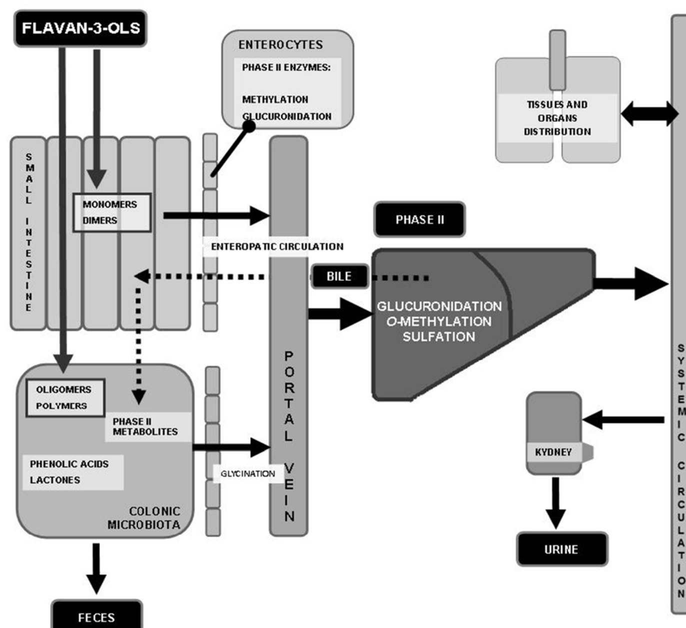

Figure 1. Schematic diagram of bioavailability of flavan-3-ols in organism after its ingestion. After ingestion, flavan-3-ols reach the small intestine where monomers and dimers could be absorbed through enterocytes and may suffer methylation and glucuronidation. Metabolites reach the liver where may be glucuronidated, methylated or sulfated before being liberated to systemic circulation for its latter distribution to organs and excretion by urine. Oligomers and polymers that are not absorbed in intestine arrive intact to colon where are metabolized by colonic microbiota releasing phenolic acids, that could be absorbed, metabolized, distributed and excreted by faeces. Metabolites reach the liver from portal vein, where may be glucuronidated, methylated or sulphated before being liberated to systemic circulation for its latter distribution to organs and excretion by urine.

Microbial Catabolism: From Flavan-3-ols to Phenolic Acids

The colon is considered to be a complex organ where metabolism of polyphenols is particularly significant due to the fact that around 90–95% of dietary polyphenols are not absorbed in the small intestine and arrive intact to colon, where they are metabolized by microbiota or excreted in their intact form [16]. In the case of flavan-3-ols, particularly proanthocyanidins, greater knowledge of biotransformation by the colonic microbiota is needed for a better understanding of the bioavailability and bioactivity described in *in vivo* studies [17, 18]. Deprez et al. first demonstrated that only 9 22% of the radioactivity of a ^{14}C-radiolabel proanthocyanidin polymer was present in the metabolite pool after *in vitro* fermentation by human microbiota, and that ethyl acetate soluble metabolites represented 2.7% of the initial radioactivity, demonstrating the extensive catabolism by intestinal bacteria [19].

More recently, in an *in vivo* study performed with ileostomy patients, ileostomy fluids were recovered after ingestion of green tea, and it was found that approximately 70% of the ingested monomeric flavan-3-ols from green tea could pass from the small to the large

intestine, including 33% corresponding to the intact parent compounds [20] .Enzymatic reactions start from microbial glycosidases and esterases, then microbial glucuronidases and sulfatases deconjugate the phase II metabolite from the enterohepatic circulation enabling re-uptake [21].

Demethylation, isomerization and fission reactions, among others, may also be achieved by the human intestinal microbiota before being absorbed, producing several derivatives of phenylvaleric, phenylpropionic, phenylacetic and benzoic acids with different patterns of hydroxylation, depending on the degree of polymerization-condensed catechins [17, 18].

Firstly, it was thought that procyanidins could be depolymerized into bioavailable monomers because of the acidic conditions in the stomach [22], but this could not been proven by *in vivo* studies [23]. However, the possible depolymerization of dimeric structures into monomeric units by the gut microbiota, firstly proposed by Groenewould et al. [24] has been recently confirmed, however it represents less than 10% in the case of procyanidin B2 [25]. In the same study 5-(2,4-dihydroxyphenyl)-2-eno-valeric acid arising exclusively from the catabolism of dimeric procyanidins by microbiota was identified, together with other derivatives from the A-ring of the upper unit [25]. Figure 2 summarizes the metabolic pathways for microbial catabolism of flavan-3-ols. In the case of galloylated monomeric flavan-3-ols (ECG and EGCG), the microbial catabolism usually starts with the rapid cleavage of the gallic acid ester moiety by microbial esterases, giving rise to gallic acid which is further decarboxylated into pyrogallol. Subsequent reactions involve the reductive cleavage reaction of the heterocyclic C-ring resulting in the formation of diphenylpropan-2-ols, which are later converted by lactonization into 5-(3′,4′-dihydroxyphenyl)- -valerolactone if the precursor is (epi)catechin, or 5-(3′,4′,5′-trihydroxyphenyl)- -valerolactone if the precursor is (epi)gallocatechin [7]. The next stage is the fission of the valerolactone ring leading to hydroxyphenylvaleric acids: 5-(3′,4′-dihydroxyphenyl) valeric acid and/or 4-hydroxy-5-(3′,4′-dihydroxyphenyl) valeric acid [7, 26].

In addition, it has been proposed that an interconversion between the 4-hydroxy-5- (3′,4′-dihydroxyphenyl) valeric acid and 5-(3′,4′-dihydroxyphenyl)- -valerolactones forms, may exist with a strong tendency to be displaced through the former molecule [25]. Hydroxyphenylvaleric acids undergo β-oxidation of the side chain resulting in 3,4-dihydroxyphenylpropionic and 3,4-dihydroxybenzoic acids through a successive loss of carbon atoms [7]. The α-oxidation of 3,4-dihydroxyphenylpropionic acid leading to phenylacetic acids has been described for monomeric and dimeric procyanidins.

However, Appeldoorn et al. suggested that this may exclusively arise from the cleavage of the upper unit of dimeric procyanidins, being 5-(3′,4′-dihydroxyphenyl)- -valerolactone derived from lower units [27]. Dehydroxylation of 3,4-dihydroxylated phenolic acids at C-4′ and, preferentially, at C-3′, results in 3 and 4-monohydroxylated phenolic acids, respectively. In the case of (epi)-gallocatechins dehydroxylation at C-5 results in the 3,4-dihydroxylated form which could be further dehydroxylation at C-4 and C-3 [9, 28].

Phenolic acids that are formed during microbial catabolism of flavan-3-ols in the gut may suffer further changes by microbial metabolism, as has been observed for caffeic and ferulic acids [18]. These findings were first described when caffeic acid was transformed either to 4-ethylcatechol via decarboxylation and reduction, or to 3-(3-hydroxyphenyl)-propionic acid after incubation with fecal microflora [29].

The same authors observed the production of metabolites in urine after ingestion of caffeic acid, when germ-free rats were infected with human microbial species [30]. Ferulic

acid was shown to be transformed to 3-(3-hydroxyphenyl)-propionic acid in rodents and ruminants [18]. In the liver and kidney, phenolic acids may undergo different reactions involving glycine conjugation, dehydrogenation, hydroxylation and methylation, releasing glycinates, monoglucuronide and monosulfate conjugates, which are later found in urine and plasma [17, 28].

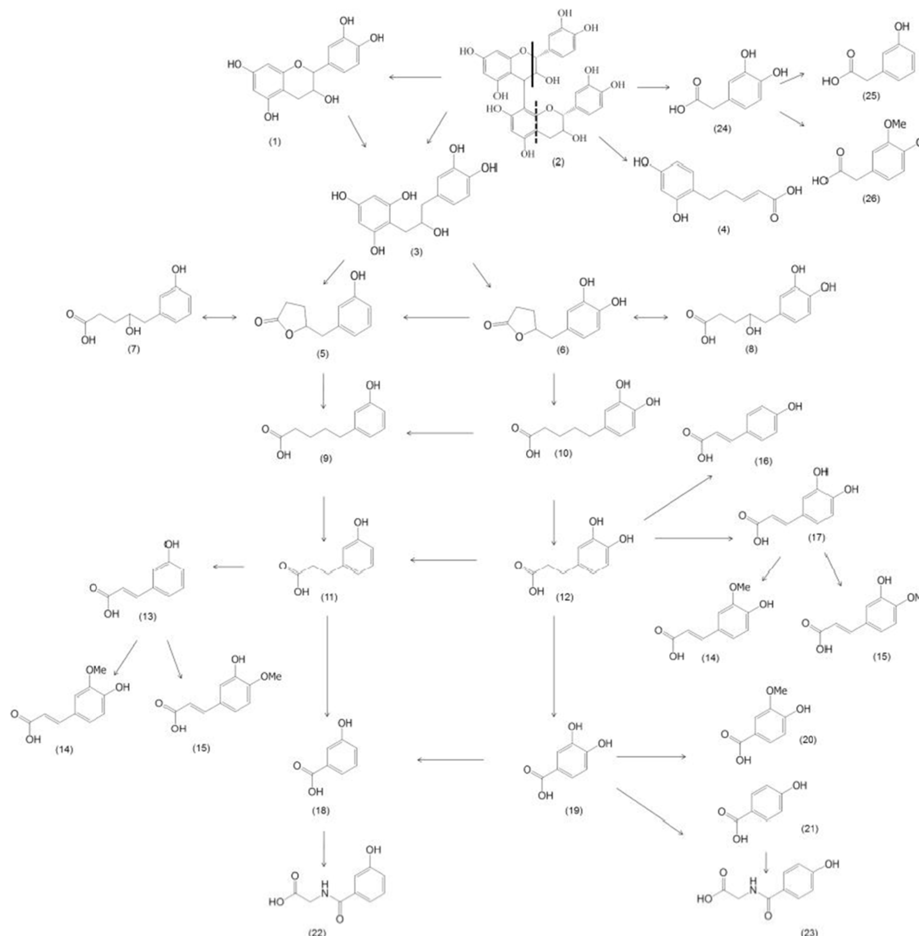

Figure 2. Metabolic pathway for microbial catabolism of flavan-3-ols. Reactions of demethylation, isomerization and fission reactions, among others, may be achieved before being absorbed, producing several phenolic acids with different patterns of hydroxylation, depending on their precursors. (1) (epi)-catechin; (2) dimeric procyanidin; (3) 1-(3´, 4´-dihydroxyphenyl)-3-(2´´,4´´,6´´-trihydroxyphenyl) propan-2-ol; (4) 5-(2,4-dihydroxy)-phenyl-2-ene-valeric acid; (5) 5-(3-hydroxyphenyl)- -valerolactone; (6) 5-(3,4-dihydroxyphenyl)-γ-valerolactone; (7) 4-hydroxy-5-(3,4-dihydroxyphenyl)-valeric acid; (8) 4-hydroxy-5-(3-hydroxyphenyl)-valeric acid; (9) 3-hydroxyphenyl valeric acid; (10) 3,4-(dihydroxyphenyl)-valeric acid; (11) 3-hydroxyphenylpropionic acid; (12) 3,4-dihydroxyphenylpropionic acid; (13) *m*-coumaric acid; (14) isoferulic acid; (15) ferulic acid; (16) *p*-coumaric acid; (17) caffeic acid; (18) 3-hydroxybenzoic acid; (21) protocatechuic acid; (20) vanillic acid; (21) 4-hydroxybenzoic acid; (22) 3-hydroxyhippuric acid; (23) 4-hydroxyhippuric acid; (24) 3,4-dihydroxyphenylacetic acid; (25) 3-hydroxyphenylacetic acid; (26) 3-methoxy-4-hydroxyphenylacetic acid.

Table 2. Degree of excretion and presence in urine and plasma of main metabolites derived from microbial metabolism of flavan-3-ol in human studies

Metabolite	Nº Subjects	Source [d]	Urine [e]	Plasma [e]	Reference
vanillic acid	10 humans	40 g cocoa powder dissolved in 250 ml whole milk or water	Identified	--	[31][a]
4-hydroxy-5- (3,4-dihydroxyphenyl) valeric acid			Identified	--	
3´-methoxy-4´-hydroxyphenyl valerolactone			Identified	--	
5-(3´,4´-dihydroxyphenyl)-γ-valerolactone sulfate			2 metabolites identified	--	
5-(3´,4´-dihydroxyphenyl)-γ-valerolactone glucuronide			2 metabolites identified	--	
3´-methoxy-4´-hydroxyphenylvalerolactone glucuronide			Identified	--	
protocatechuic acid	2 humans	10 capsules almond skin extract	Medium	High	[2][a]
vanillic acid			Medium	High	
4-hydroxybenzoic acid			Medium	High	
3-hydroxybenzoic acid			Low	ND	
4-hydroxyhippuric acid			High	Low	
p-coumaric acid			Low	Low	
caffeic acid			Medium	Medium	
ferulic acid			Low	Low	
m-coumaric acid			Low	Low	
isoferulic			Medium	Low	
3,4-dihydroxyphenylacetic acid			Medium	Medium	
4-hydroxy-3-methoxyphenylacetic acid			High	Medium	
3-hydroxyphenylacetic acid			Medium	Medium	[2][a]
phenylacetic acid			Medium	High	
3,4-dihydroxyphenylpropionic acid			Medium	High	
3-hydroxyphenylpropionic acid			Low	High	
5-(hydroxyphenyl)-γ-valerolactone			Low	ND	
5-(dihydroxyphenyl)-γ-valerolactone			High	ND	
5-(dihydroxyphenyl)-γ-valerolactone glucuronide			Medium	ND	
5-(dihydroxyphenyl)-γ-valerolactone sulfate			High	Medium	
5-hydroxymethoxyphenyl)-γ-valerolactone glucuronide			Low	Low	

Table 2. (Continued)

Metabolite	Nº Subjects	Source [d]	Urine [c]	Plasma [c]	Reference
5-(hydroxymethoxyphenyl)-γ-valerolactone sulfates			Sulf_ 1:Medium; Sulf_2: Low	ND	
protocatechuic acid	42 humans	40 g/day cocoa powder with 250 mL skimmed milk Treatment period: 4 weeks	Medium	Medium	[7][b]
vanillic acid			Medium*	Medium	
4-hydroxybenzoic acid			Medium	Medium	
3-hydroxybenzoic acid			Low	ND	
4-hydroxyhippuric acid			Medium	Low	
3-hydroxyhippuric acid			High	Low	
p-coumaric acid			Low	Low	
caffeic acid			Low	Low	
ferulic acid			Low	Low	
m-coumaric acid			Low	ND	
3,4-dihydroxyphenylacetic acid			Low	Low	
3-methoxy-4-hydroxyphenylacetic acid			Medium	ND	
3-hydroxyphenylacetic acid			Medium*	Low*	[7][b]
phenylacetic acid			Medium	Medium	
3,4-dihydroxyphenylpropionic acid		[illegible]	Low	Low	
3-hydroxyphenylpropionic acid			Low	Low	
5-(3´,4´-dihydroxyphenyl)-γ-valerolactone				Low*	
gallic acid	12 humans	200 mg of the pure flavonoids: (a) epicatechin and (b) (-)-EGCG	a) --; b)Low*	a) Low*; b)Low*	[32][b]
4-methylgallic acid			a) --; b)Low*	a) Low*; b)Low*	
3-methylgallic acid			a) --; b)Medium *	a) Low*; b)Low*	
1,3,5-trimethoxybenzene				High*; b) [illegible]	
benzoic acid			--	a) High; b) High	
3-(4-hydroxyphenyl) propionic acid			--	a) Medium; b) Medium	
3,4-dihydroxyphenylpropionic acid			--	a) Medium; b) Medium	
3,4-dihydroxybenzoic acid			--	a) Low; b) Low	
vanillic acid			--	a) Low; b) Low	
ferulic acid			--	a) Low; b) Low	
4-ethylphenol			--	a) Medium; b)High	
3-phenylpropionic acid			--	a) Low; b) Medium	
2-methoxyphenylacetic acid			--	a) Medium; b) High	

Metabolite	Nº Subjects	Source [d]	Urine [c]	Plasma [c]	Reference
4-ethylbenzoic acid			--	a) Medium; b) High	
protocatechuic acid	20 humans	4 g of black tea solids Treatment period: 1 week	Low	--	[33][b]
gallic acid			Low*	--	
vanillic acid			Medium	--	
4-hydroxybenzoic acid			Low	--	
syringic acid			Low	--	
benzoic acid			Low	--	
3-hydroxybenzoic acid			Medium *	--	
2,4-dihydroxybenzoic acid			Low	--	
3,5-dihydroxybenzoic acid			Low	--	
salicyluric acid			Low*	--	
hippuric acid			High *	--	
ferulic acid			Low	--	
3,4-dihydroxyphenylacetic acid			Low*	--	
4-hydroxyphenylacetic acid			Medium	--	
homovanillic acid			Medium	--	
3-hydroxyphenylacetic acid			Medium *	--	
2-hydroxyphenylacetic acid			Low	--	
phenylacetic acid			Low	--	
mandelic acid			Low	--	
3-hydroxybenzoic acid	11 humans	80 g chocolate	Low	--	[34][b]
4-hydroxybenzoic acid			Medium	--	
4-hydroxyhippuric acid			Medium	--	
hippuric acid			High	--	[34][b]
vanillic acid			High *	--	
ferulic acid			High *	--	
3,4-dihydroxyphenylacetic acid			Medium *	--	
3-hydroxyphenylacetic acid			High *	--	
Phenylacetic acid			Medium *	--	
3,4-dihydroxyphenylpropionic acid			Low	--	
3-hydroxyphenylpropionic acid			Low*	--	
protocatechuic acid	21 humans	40 g cocoa powder dissolved in 250 ml (a) water or (b) whole milk	a) Medium*; b) Medium*	--	[3][b]
vanillic acid			a) Medium*; b) Medium*	--	

Table 2. (Continued)

Metabolite	Nº Subjects	Source [d]	Urine [c]	Plasma [c]	Reference
4-hydroxybenzoic acid			a) Low*; b) Low*	--	
3-hydroxybenzoic acid			a) Low*; b) Low	--	
4-hydroxyhippuric acid			a) Low*; b) Low*	--	
hippuric acid			a) High*; b) High*	--	
p-coumaric acid			a) Low*; b) Low*	--	
caffeic acid			a) Low*; b) Low	--	
ferulic acid			a) Medium *; b) Low	--	
m-coumaric acid			a) Low; b) Low	--	
3,4-dihydroxyphenylacetic acid			a) Low*; b) Low	--	
3-methoxy-4-hydroxyphenylacetic acid			a) Medium*; b) Medium*	--	[3][b]
3-hydroxyphenylacetic acid			a) Medium; b) Medium	--	
phenylacetic acid			a) High*; b) High*	--	
3,4-dihydroxyphenylpropionic acid			a) Medium*; b) Medium*	--	
4-hydroxyphenylacetic acid	26 humans	Cellulose capsules with a polyphenol-rich mix of red wine and red grape juice extracts Treatment period: 4 weeks	Identified	--	[35][a]
vanillic acid			Identified	--	
hippuric acid			Identified	--	
3-hydroxyphenylacetic acid			Identified	--	
3-hydroxyhippuric acid			Identified	--	
4-hydroxyhippuric acid			Identified	--	
homovanillic acid			Identified	--	
3-(3.hydroxyphenyl)-hydroxypropionic acid			Identified	--	
syringic acid			Identified	--	
3-(3-Hydroxyphenyl) propionic acid			Identified	--	
phenylacetic acid			--	Identified	
4-hydroxybenzoic acid			--	Identified	
4-hydroxymandelic acid			Identified	--	
pyrocatechol	5 humans	300 mL of green tea	Low*	--	[36][b]
pyrogallol			Medium*	--	
4-hydroxybenzoic acid			a) Medium*; b) Medium*	--	
5-(3´,4´,5´-trihydroxyphenyl)-γ-valerolactone			a) Medium; b) Medium	--	

Metabolite	Nº Subjects	Source [d]	Urine [c]	Plasma [c]	Reference
4-hydroxyphenylacetic acid			High	--	[36][b]
3-methoxy-4-hydroxyphenylacetic acid			Medium*	--	
hippuric acid			High	--	
3-(3-hydroxyphenyl)-3-hydroxypropionic acid			Medium*	--	
5-(3´,5´-dihydroxyphenyl)-γ-valerolactone	4 humans	20 mg/kg green tea solids	High	Low	[37][a]
5-(3´,4´-dihydroxyphenyl)-γ-valerolactone			High	Identified	
5-(3´,4´,5´-trihydroxyphenyl)-γ-valerolactone			Low	Identified	
5-(3´,4´,5´-trihydroxyphenyl)-γ-valerolactone	1 humans	200 mg of pure (-)-EGCG, (-)-EGC or (-)-epicatechin	High	--	[37][a]
5-(3´,4´-dihydroxyphenyl)-γ-valerolactone			Medium	--	
5-(3´,5´-dihydroxyphenyl)-γ-valerolactone			Medium	--	
5-(3´,4´-dihydroxyphenyl)-γ-valerolactone glucuronide	3 men	200 ml reconstituted green tea (from 3 g tea solids)	Identified	--	[38][a]
5-(3´,4´-dihydroxyphenyl)-γ-valerolactone sulfate			Identified	--	
5-(3´,4´,5´-trihydroxyphenyl)-γ-valerolactone			Identified	--	
5-(3´,4´,-dihydroxyphenyl) valerolactone			Identified	--	
5-(3´,5´,-dihydroxyphenyl)-valerolactone			Identified	--	
5-(3´,5´-dihydroxyphenyl)-γ-valerolactone glucuronide			Identified	--	
5-(3´,4´,5´-trihydroxyphenyl)-γ-valerolactone sulfate			Identified	--	[38][a]
5-(4´-methoxy-3´,5´-dihydroxyphenyl) valerolactone sulfate			Identified	--	
4´-O-methyl-5-(3´,4´,5´-trihydroxyphenyl)-γ-valerolactone-methyl-sulfate-3´-sulfate			Identified	--	
5-(3´,5´-dihydroxyphenyl)-γ-valerolactone sulfate			Identified	--	
5-(3´,5´-dihydroxyphenyl)-γ-valerolactone sulfate			Identified	--	
5-(3´,4´,5´-trihydroxyphenyl)-γ-valerolactone glucuronide			Identified	--	
5-(3´,5´-dihydroxyphenyl)-γ-valerolactone glucuronide			Identified	--	

[a] Study with no statistical analysis.
[b] Study with statistical analysis. * Compound that significantly increased after the intervention
[c] Concentrations categorized in low, medium and high (classified within a same study in percentiles). When no category is given, metabolites are not quantified.
[d] If treatment period is not described, the study was carried out with a single dose of polyphenols or food source.
(-)-EGCG, (-)-Epigallocatechin gallate, (-)-EGC, (-)-

Principal Phenolic Acids Found in Plasma and Urine

The phenolic acids present in biofluids after consumption of flavan-3-ols depend on several factors, such as food source, food matrix and the compounds that are delivered in these foods. The phenolic acid concentration in plasma or urine after consumption of food rich in flavan-3-ols or pure compounds is shown in Tables 2, 3 and 4.

Human Studies

Sources of flavan-3-ol that have been used in humans studies include cocoa powder, chocolate, almond skins, black and green tea, and red wine and red grape juice. In addition, (-)-epicatechin, (-)-epigallocatechin, and (-)-epigallocatechin-3-*O*-gallate have also been tested (Table 2).

After intake of cocoa powder with milk, main phenolic acids identified in plasma were: protocatechuic, vanillic, 4-hydroxybenzoic, and phenylacetic acids. Besides these metabolites, 4-hydroxyhippuric, 3-hydroxyhippuric, 4-hydroxy-3-methoxyphenylacetic, and 3-hydroxy-phenylacetic acids, most of them arising from further liver/kidney metabolism after absorption from the colonocytes acid were also found in urine in medium concentration range, with the exception of 3-hydroxyhippuric acid which was present in high concentration [7]. Only 3-hydroxyphenylacetic and vanillic acids were significantly changed in urine and plasma after the intake of cocoa powder for one month [7].

Similarly, Rios et al. found that vanillic together with ferulic and 3-hydroxy-phenylacetic acids, as one of the main phenolic acids in urine significantly changing after a single intake of chocolate in humans. 3,4-Dihydroxyphenylacetic and phenylacetic acids (found in the medium concentration range), and 3-hydroxyphenylpropionic acid (found in the low concentration range), were also characteristic phenolic acids of the intake of chocolate flavan-3-ols [34].

However, differences in the profile of the urinary excretion of phenolic acids were found when cocoa was consumed with water instead of milk, indicating that the food matrix has a profound influence in microbial metabolism of flavan-3-ols [3].

After a single intake of almond skin polyphenols, main phenolic acids identified in plasma were: protocatechuic, vanillic, 4-hydroxybenzoic, phenylacetic, 3,4-dihydroxyphenylpropionic, and 3-hydroxyphenylpropionic acids. In urine, main metabolites include those derived from subsequent hepatic metabolism in the form of methoxylated, sulfated or glycinated derivates, including: 4-hydroxyhippuric and 4-hydroxy-3-methoxyphenyl-acetic acids. In the case of red wine and red grape juice, other phenolic acids such as 4-hydroxymandelic acid were also identified by Grün et al. [35].

The profile of phenolic acids was slightly different after the intake of pure (-)-epicatechin or (-)-epigallocatechin-3-O-gallate, since 1,3,5-trimethoxybenzene was detected in the highest concentration in both plasma and urine. In addition to this metabolite, gallic, 4-methyl-gallic, 3-methyl-gallic acids, derived from the hydrolysis of the gallic acid moiety from (-)-epigallocatechin-3-O-gallate, significantly changed after consumption of this compound in both type of biological samples [32].

Gallic acid was also found as one of the phenolic acids significantly changing after consumption of black tea solids for 1 week [33]. Other metabolites showing significant differences included the following ones, which were found in different concentration ranges: high (hippuric acid); medium (3-hydroxybenzoic and 3-hydroxyphenylacetic acids), and low (salicyluric and 3,4-dihydroxyphenylacetic acids) [33].

Pyrogallol and pyrocatechol, derived from the further decarboxylation and dehydroxylation of gallic acid, are among other metabolites derived from galloylated flavan-3-ols of tea [36].

Animal Studies

For animal studies, diets supplemented with (+)-catechin, (-)-epicatechin-3-*O*-gallate, procyanidin B3 and C2, polymeric flavan-3-ol, and wine powder have been used (Table 3 and Table 4).

The influence of the degree of polymerization (DP) on microbial phenolic acid was studied by Gonthier et al. [28]. Although some metabolites were found in the same concentration range in rats fed either (+)-catechin, dimers, trimeric or polymers, changes for some metabolites such as protocatechuic, vanillic, 4-hydroxybenzoic, 3-hydroxybenzoic, 4-hydroxyhippuric, and 3-hydroxyhippuric acids were only significant after the intake of (+)-catechin and dimeric procyanidins, indicating an influence of polymeric proanthocyanidins in the extent of microbial degradation.

Other metabolites such as 3,4-dihydroxyphenylacetic, 3-hydroxyphenylacetic, and 3,4-dihydroxyphenylpropionic acids only appear to change after the intake of procyanidin B3, whereas others (*m*-coumaric, 3-hydroxyphenylpropionic, and 3-hydroxyphenyl valeric acids) significantly changed independently of the DP [39].

The influence of different doses (low and high) of red wine powder in comparison to (+)-catechin in the formation of microbial phenolic acids was also studied in rats. The urinary levels of some metabolites such as vanillic and *p*-coumaric acids appeared to significantly increase after the intake of the high dose of red wine powder, whereas other including 3-hydroxy-benzoic, 3-hydroxy-hippuric and 3-hydroxyphenyl-propionic acids tended to increase after the intake of (+)-catechin, indicating that not only the dose but the flavan-3-ols source and structural features influence the catabolism of these compounds by gut microbiota [28].

In addition to all the phenolic acids described above, phenyl-γ-valerolactones and 4-hydroxy-5-(phenyl)-valeric acid derivatives are considered as characteristic metabolites derived from flavan-3-ols.

Although the quantification of these compounds has been limited by the lack of the appropriate standard, both humans and animal studies have a allowed great advances in the identification of tri-, di- and monohydroxylated forms of phenyl- -valerolactones with different conjugation profile (glucuronidated, sulfated and methoxylated forms) after the intake of flavan-3-ols rich sources such as cocoa, almond skins, and green tea, or after supplementation of purified compounds such as (+)-catechin and (-)-epicatechin-3-O-gallate [2, 26, 31, 36-38, 40].

Table 3. Degree of excretion and presence in urine and plasma of main metabolites derived from microbial metabolism of flavan-3-ol in animal studies

Metabolite	Nº Subjects	Source [d]	Urine [c]	Reference
protocatechuic acid	25 male Wistar rats	0.1% (w/w) catechin diet (a) procyanidin dimer B3 diet (b) trimer C2 diet (c) polymer diet (d) Treatment period: 5 days	a) Low*; b) Low*; c) Low ;d) Low	[39][b]
vanillic acid			a) Medium*; b) Medium; c) Medium; d) Medium	
4-hydroxybenzoic acid			a) Medium*;b) Medium*; c) Medium; d) Medium	
3-hydroxybenzoic acid			a) Low *;b) Low*;c, d) ND	
4-hydroxyhippuric acid			a) Medium; b) Medium*; c) Medium; d) Medium	
3-hydroxyhippuric acid			a) Medium *;b) Low*;c, d) ND	
hippuric acid			a) High; b) High; c) High; d) High	
p-coumaric acid			a) Low; b) Low; c) Low ;d) Low	
ferulic acid			a) Low; b,c,d) ND	
m-coumaric acid			a) Medium*; b) Medium*; c) Low*; d) Low*	
3,4-dihydroxyphenylacetic acid			a) Low; b) Medium*; c) Low; d) Low	
3-hydroxyphenylacetic acid			a) Low; b) Medium*; c) Medium; d) Medium	
3,4-dihydroxyphenylpropionic acid			a) Low; b) Low*; c,d, c: ND	
3-hydroxyphenylpropionic acid			a) Medium*;b) Medium*;c) Medium*;d)medium*	
3,4-dihydroxyphenylvaleric acid			a) Low*;b,c,d,) ND	
3-hydroxyphenylvaleric acid			a) Low*; b) Low*; c) Low* ;d) ND	
vanillic acid	32 male Wistar rats	(a)0.25 g/100 g wine powder, (b) 0.50 g/100 g wine powder, (c) 0.12 g/100 g catechin Treatment period: 8 days	a) Medium*; b) High*; c) Medium	[28][b]
4-hydroxybenzoic acid			a) Medium*; b) Medium*; c) Medium*	
3-hydroxybenzoic acid			a) Low*; b) Low*; c) Medium*	
4-hydroxyhippuric acid			a) Medium; b) Medium; c) Medium	
3-hydroxyhippuric acid			a) Low*; b) Low*; c) Medium*	
hippuric acid			a) High*; b) High*; c) High	
p-coumaric acid			a) Low*; b) Medium*; c) Low	
caffeic acid			a) Low*; b) Low*; c) ND	
ferulic acid			a) Low*; b) Low*; c) Low*	
3,4-dihydroxyphenylacetic acid			a) Low; b) Low*; c) Low*	
3-hydroxyphenylacetic acid			a) Medium*; b) Medium*; c) Low	
phenylacetic acid			a) High; b) High; c) High	
3,4-dihydroxyphenyl-propionic acid			a) Low*; b) Low*; c) Low*	
3-hydroxyphenyl-propionic acid			a) Medium*; b) Medium*; c) High*	
5-(4-hydroxy)-3-hydroxyphenylvaleric acid			Identified	

Metabolite	Nº Subjects	Source [d]	Urine [c]	Reference
3-hydroxyphenylvaleric acid		hyperlipidemic with catechin supplementation Treatment period: 6 weeks	Identified	
hippuric acid	48 male Wistar rats	Normolipidemic or hyperlipidemic with catechin supplementation Treatment period:6 weeks	Identified	[40][a]
4-hydroxy-hippuric acid			Identified	
methoxy-hydroxyphenyl valerolactone			Identified	
dihydroxyphenyl valerolactone glucuronide			Identified	
methoxy-hydroxyphenyl valerolactone glucuronide			Identified	
methoxy-hydroxyphenyl valerolactone-sulfate			Identified	
m-coumaric acid	6 male Wistar rats	100 mg/kg of body weight (-)-ECG	Free and conjugated: high	[26][a]
3-(3-hydroxyphenyl)-propionic acid			Free and conjugated: high	
4-hydroxy-5-(3,4-dihydroxyphenyl)-valeric acid			Free and conjugated: medium	
5-(3´,4´,-dihydroxyphenyl)-valerolactone			Free and conjugated: medium	

[a] Study with no statistical analysis.
[b] Study with statistical analysis. * Compound that significantly increased after the intervention.
[c] Concentrations categorized in low, medium and high (classified within a same study in percentiles). When no category is given, metabolites are not quantified.
[d] If treatment period is not described, the study was carried out with a single dose of polyphenols or food source.
(-)-ECG, (-)-Epicatechingallate, ND: not de

Table 4. Degree of excretion and presence in plasma of main metabolites derived from microbial metabolism of flavan-3-ol in animal studies

Metabolite	Nº Subjects	Source [d]	Plasma [c]	Reference
hippuric acid	32 male Wistar rats	0.25 g-0.50 /100 g wine powder or 0.12 g/100 g catechin Treatment period: 8 days	Main	[28][b]
3-(3-hydroxyphenyl) propionic acid	6 male Wistar rats	100 mg/kg of body weight (-)-ECG	Free and conjugated: high	[26][b]
4-hydroxy-5-(3,4-dihydroxyphenyl) valeric acid			Free and conjugated: medium	
5-(3´,4´,-dihydroxyphenyl) valerolactone			Free and conjugated: high	

[a] Study with no statistical analysis.
[b] Study with statistical analysis. * Compound that significantly increased after the intervention
[c] Concentrations categorized in low, medium and high (classified within a same study in percentiles). When no category is given metabolites are not quantified.
[d] If treatment period is not described, the study was carried out with a single dose of polyphenols or food source.
(-)-ECG, (-)-Epicatechingallate, ND: not detec

Intestinal Microbiota Involved in the Metabolism of Flavan-3-ols

The differences in microbial metabolites found in samples from *in vitro* fermentation studies or after flavan-3-ol ingestion could be partly attributed to differences in the individual microbiota, suggesting that metabolites and pathways could be different, depending on the microbiota composition [6]. Few studies have analyzed, identified and characterized bacteria with the ability to catabolize flavan-3-ols and, therefore, the bacteria involved in the catabolism of flavan-3-ols remains largely unknown. The inhibitory effects of proanthocyanidins and structural features of flavan-3-ols as complex non-planar molecules could explain the difficulties in establishing specific bacteria [9].

Most of the findings made in this field come from *in vitro* studies with selected bacteria from either the human gut or fecal suspensions [27, 41-43]. *Eubacterium spp* strain SDG-2 was found to be able to cleave the C-ring of (3R)- and (3S)-flavan-3-ols to give 1,3-diphenylpropan-2-ol derivatives, as well as to produce *p*-dehydroxylation reactions in the B-ring of (3R)-flavan-3-ols, after incubation with (-)-epicatechin, (-)-catechin, (-)-epigallocatechin and (-)-gallocatechin. The same bacteria were also found to be able to metabolize other related compounds such as taxifolin to caffeic acid and *m*-hydroxyphenylpropionic acid [44].

In a recent study, the intestinal human bacteria involved in the conversion of catechins were isolated and characterized as *Eggerthella lenta* and *Flavonifractor plautii* (formerly *Clostridium orbiscindens*). *Eggerthella lenta* rK3 reductively cleaved the heterocyclic C-ring of both (-)-epicatechin and (+)-catechin, giving rise to 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl) propan-2-ol, and *Flavonifractor plautii* aK2 further converted the former metabolite into 5-(3,4-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3,4-dihydroxyphenyl) valeric acid [8]. It was suggested that individual species catalyze only single steps in the degradation pathway, which implies an even more complex situation in metabolites such as procyanidins or other polymers.

Bioactivity of Phenolic Acids

There are few studies in humans linking phenolic acids from microbial metabolites with health effects after *in vivo* consumption of flavan-3-ols, instead most of them have been carried out on cell cultures or animal models. To get a complete understanding of the role of microbiota in the release of phenolic acids and bioactivity, further studies are needed.

Effects of Phenolic Acids on Gut Microbiota

Phenolic acids released to the medium by microbial metabolism in colon may exert a protective effect by regulation of the microbial species. Prebiotic actions have been demonstrated *in vitro* [41], in which incubation of (-)-epicatechin or (+)-catechin with fecal bacteria led to the generation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-phenyl- -valerolactone and 3-phenylpropionic acid.

The growth of selected microflora was affected, resulting in an increase in the growth of the *Clostridium coccoides–Eubacterium rectale* group, *Bifidobacterium spp.* and *Escherichia coli,* as well as a significant inhibitory effect on the growth of the *C. histolyticum* group, more significantly in the case of catechin than epicatechin [41]. Similarly, it has been reported that phenolic acids produced from the microbial degradation of flavan-3-ols such as 3-*O*-methyl gallic, gallic, caffeic, 4-hydroxyphenylpropionic, phenylpropionic and 4-hydroxyphenylacetic acids, were able to protect against the growth of several pathogenic and non-beneficial intestinal bacteria, including *Clostridium perfringens, Clostridium difficile* and *Bacteroides spp*., but to a lesser extent affected the growth of some beneficial bacteria such as *Lactobacillus spp.* and *Bifidobacterium spp* [45].

The tentative mechanisms that will explain their antimicrobial activity for flavanols have been elucidated as membrane interaction, enzyme inhibition, reactive oxygen generation and inhibition of virulence factors [21]. A link between the number and position of substitutions in the benzene ring of the phenolic acids and the saturated side-chain length and the antimicrobial activity was also described, and they were more active that their corresponding precursors. Changes in intestinal bacteria and pathogen growth can be affected by phenolic acids from the microbial degradation of dietary phenolic compounds by affecting the epithelium at the site of conversion and may also affect colonic microbiota locally [46]. In this line, an effect on cell surface structures was observed when incubating with berry extracts inhibited the growth of Gram-negative but not Gram-positive bacteria, weakening *Salmonella,* which may be specifically related to dihydroxylated forms [47, 48]. Evidence related to the *in vivo* effects of polyphenols on the intestinal microbiota is still scarce. First studies conducted in both humans and animals (pigs and chickens) have revealed an increase in *Lactobacillus* and a decrease in *Enterabacteriaceae* after the administration of monomeric flavan-3-ols from green tea [49]. Smith et al. later also showed that animals rats feed with a tannin-rich diet significantly decreased the *Clostridium leptum* cluster and increased the growth Bacteroides group [50]. Similarly, Dolara et al. found that rats feed with red wine polyphenols had significantly lower levels of *Clostridium spp*, and higher levels of *Lactobacillus spp* [51]. More recently, a significant increase in the numbers of *Bifidobacteria* and/or *Lactobacillos* together with a significant decrease in the numbers of *Bacteriodes* and *Clostridium* have been reported in rat fed blackcurrant extract powder [52]. Viveros et al. also found that birds fed grape pomace concentrate and grape seed extract had higher populations of *Escherichia coli, Lactobacillus, Enterococcus,* and *Clostridium* in the cecal digesta than the control group [53].

Anti-Inflammatory Activity

Larrosa et al. recently proved that the polyphenol metabolites, hydrocaffeic, dihydroxyphenyl acetic and hydroferulic acid, derived from colon microbiota, provided the best inhibition of prostaglandin E2 production in cancer cells of fibroblast (CCD-18) stimulated with IL-1β. The same metabolites had an anti-inflammatory effect in rodents. The effect of hydrocaffeic acid was investigated in *in vivo* experiments with rats using a dextran sodium sulfate (DSS)-induced colitis model, and it was found to be the most potent metabolite for reducing the expression of cytokines TNF-α, IL-1 , IL-8, as well as the levels of malonaldehyde and oxidative damage to DNA in the distal colon mucosa [54].In an *in vitro*

study the ability of the compounds to inhibit the release of arachidonic acid and the production of nitric oxide (NO) by lipopolysaccharide (LPS)-stimulated murine macrophages was evaluated with (-)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, which inhibited NO production by 50% at 20 μM [55].Other studies involving inflammatory molecules showed that phenolic acids presenting 4-hydroxy-3-methoxy substitution and a one-carbon side chain (vanillic acid and its derivatives) and three-carbon side chain (cinnamic, *o*-, *m*- and *p*-coumaric acid, and caffeic acid) inhibited cytokine-induced prostanoid biogenesis in human colonic fibroblasts by up to 81% in the case of vanillin and 75% in the case of coniferyl alcohol [56], and it was also shown that dihydroxylated phenolic acids incubated peripheral blood mononuclear cells (PBMC) stimulated with LPS, significantly inhibiting the production of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, but none are affected by monohydroxylated phenolic acids [57].

Effects of Phenolic Acids on Anti-Thrombotic Activity

Rechner et al. demonstrated the anti-trombotic activity of phenolic acids using a mixture of polyphenols and some derived microbial metabolism in different tests related to platelet aggregation, P-selectin expression on resting platelets, the effect on TRAP-induced platelet activation and epinephrine-stressed platelets. In those tests the most significant activity was performed by dihydrocaffeic acid (3,4-dihydroxyphenyl propionic acid), dihydroferulic acid (4-hydroxy-3-methoxyphenylpropionic acid) and 3-hydroxyphenylpropionic acid [58].

Anti-Proliferative Activity, Cytotoxicity and Inhibition of Specific Enzymes

(-)-5-(3′,4′,5′-trihydroxyphenyl)- -valerolactone and (-)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and their methoxy-derivatives were first assessed for their ability to inhibit the growth of a panel of immortalized and malignant human cell lines, the former being the more active component for human esophageal squamous cell carcinoma cells (KYSE150), human colon adenocarcinoma cells (HT-29 and HCT-116), immortalized human intestinal epithelial cells (INT-407) and an immortalized rat intestinal epithelial cell line (IEC-6) [55]. Karlsson et al. demonstrated that fecal samples containing microbial phenolic acids inhibited cyclooxygenase-2 (COX-2) protein levels in colon cancer cells (HT-29) induced with TNF- in a range between 14.7±15% and 67 ± 6% depending on the concentration and dose [59]. Similar effects have been proved for 3,4-dihydroxyphenylacetic acid and 3-(3,4-dihydroxyphenyl)-propionic acid in human adenoma cells LT97 for which a reduction in COX-2 gene and protein expression has been observed [60] .In another study in which LNCaP prostate cell line, HCT116 colonic cell line, and IEC6 normal intestinal epithelial cell line were incubated with phenolic acids from human microbial fermentations, 3,4-dihydroxyphenylacetic acid presented anti-proliferative activity of in the two former cell lines [61]. Protocatechuic acid is a very active metabolite and its cytotoxic capacity has been tested on HepG2 hepatocellular carcinoma cells by stimulating the c-Jun N-terminal kinase (JNK) and p38 subgroups of the mitogen-activated protein kinase (MAPK) family [62]. Similar pathways haven been proved in human gastric adenocarcinoma cells b [63].Finally, the

neuroprotective effects of protocatechuic acid on retenone- induced apoptosis in PC12 cells by improving apoptosis/necrosis have also been described [64].

Other Effects

Due to their phenolic nature, phenolic acids are related to other effects such as oxidative stress, antioxidant activity and modulation of lipid metabolism [9]. 3,4-Dihydroxyphenyl acetic is linked to oxidative stress [65, 66] and antioxidant activity, acting as a radical scavenging activity against DPPH in cultured rat hepatocytes [67]. Unno et al. proved that antioxidant potential measured by TEAC, of epicatechin and its bacterial metabolite 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in comparison with L-ascorbic acid. (-)-Epicatechin showed almost double the capacity, of 5-(3′,4′-dihydroxyphenyl)- -valerolactone, however the latter one showed stronger antioxidant activity than L-ascorbic acid [68].

Conclusions

Although the health benefits of flavan-3-ols have been described in numerous intervention studies, the direct relationship between the flavan-3-ol bioavailability and activities such as antioxidant, anticarcinogen, cardiopreventive, antimicrobial or neuroprotective has not yet been fully established *in vivo*. Over the past years, the recognition that the microbiota plays a fundamental role in the bioavailability of flavan-3-ols, has led to the consideration that microbial phenolic acids may be partly responsible for their *in vivo* health effects. Although great advances have been made in the determination of metabolic pathways and identification of phenolic acids in biological samples, understanding the role of these microbial metabolites in the health benefits derived from flavan-3-ols is still a very difficult task. Considering the interaction existing between flavan-3-ols and gut microbiota, more interest should be focused on microbiota-related diseases affecting the host immune system, such intestinal inflammatory diseases and infections, obesity and allergies, among others. In order to obtain greater knowledge in this field, the link between bacterial composition, phenolic acids and health benefits should be made by combining the complex information from human intervention studies with that from animals and *in vitro* mechanistic studies. These studies would reveal specific bacterial groups and phylum involved in the catabolism of flavan-3-ol as well as new potential bioactive microbial metabolites that could then be correlated with *in vivo* health effects. All this information will finally provide the means to adopt new nutritional strategies to increase the bioavailability and health effects of flavan-3-ols, for example, by redirecting or shifting the microbiota towards a more active microbial ecosystem.

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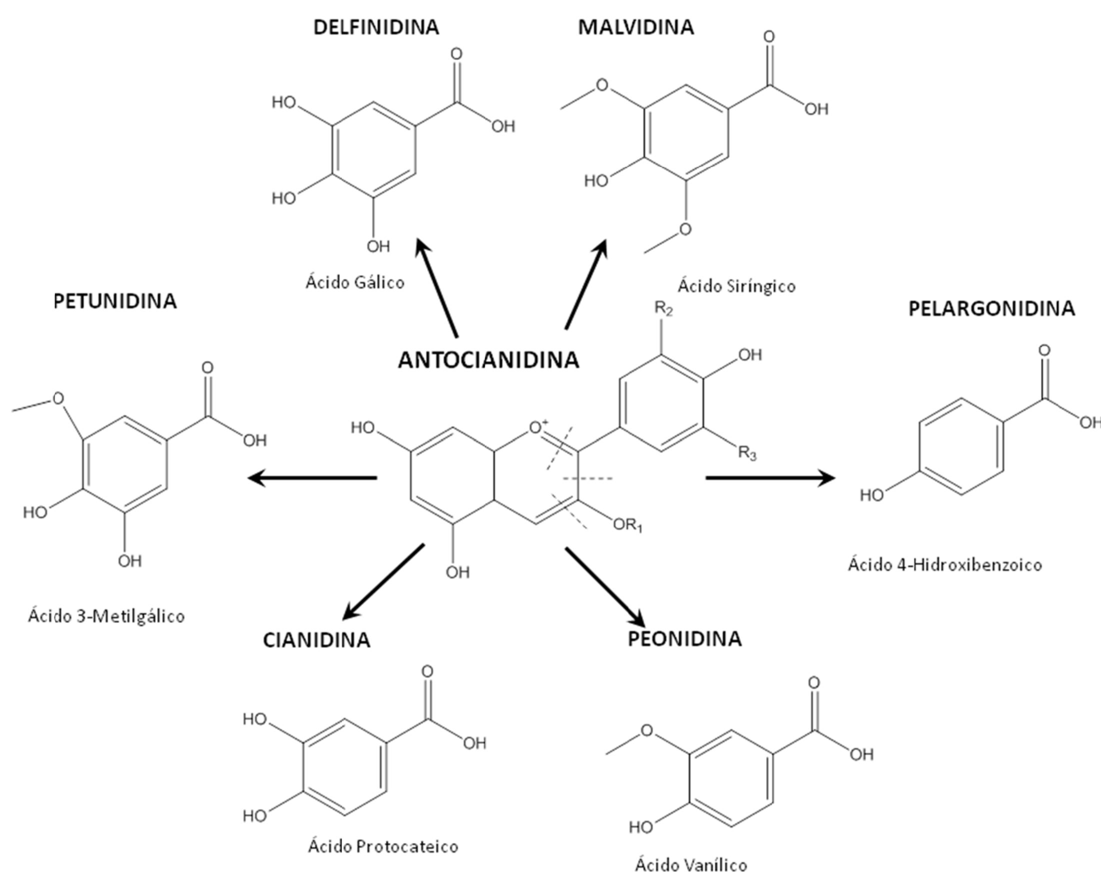
METABOLISMO MICROBIANO DE ANTOCIANOS, FLAVONOLES, ESTILBENOS Y LIGNANOS.

Tras los flavan-3-oles, el siguiente grupo en importancia son los antocianos (Figura A3). Los antocianos son los glicósidos de las antocianidinas (agliconas), compuestos que rara vez se encuentran libres en alimentos debido a su baja estabilidad. Inicialmente asociados a una baja biodisponibilidad tras su consumo (Bub *et al.*, 2001), se ha observado que un gran porcentaje llegan intactos al colon donde interaccionan con la flora bacteriana (Kahle *et al.*, 2006). La metabolización de estos compuestos a nivel intestinal se inicia con la liberación del azúcar por la desglicosilación de la microflora colónica (Aura *et al.*, 2005).

Figura A3: Estructura de una antocianidina y sus principales ácidos fenólicos derivados del metabolismo de la flora colónica por la ruptura del anillo B.



La metabolización por parte de la microbiota continua con la ruptura del anillo y la liberación de ácidos benzoicos, hidroxilados y metoxilados, dependiendo de los residuos del anillo B de la molécula de antocianina padre (Requena *et al.*, 2010).

Centrándose en el vino, los productos primarios de la degradación de la cianidina, malvidina y peonidina son los ácidos protocatéquico, siríngico y vaníllico, respectivamente (Hidalgo *et al.*, 2012). Por su parte el siríngico puede ser demetilado dando ácido gálico o continuar su degradación a pirogalol o catecol y el vaníllico dar lugar a protocateico (Alberto *et al.*, 2004).

Estos metabolitos se han identificado en estudios *in vitro*, donde el ácido vaníllico, ácido siríngico y protocatéquico fueron los principales metabolitos tras la incubación de peonidina-3-glucósido y la cianidina-3-glucósido con microflora humana (Fleschhut *et al.*, 2006) o el ácido 3-O-metilgálico, siríngico o 2,4,6-trihidroxibenzaldehído cuando se incubó extracto de uva Cabernet Sauvignon rica en malvidina-3-glucósido, petunidina-3-glucósido, delfinidina-3-glucósido y peonidina-3-glucósido en intestino de cerdo (Forester y Waterhouse, 2008). Igualmente la incubación de antocianos con *Lactobacterias* y *Bifidobacterias* dio lugar a ácidos gálico, siríngico y *p*-coumárico provenientes de la degradación de la malvidina y delfinidina glucósido (Hidalgo *et al.*, 2012).

Los metabolitos de antocianos derivados del catabolismo microbiano se han detectado aumentados en biofluídos tras consumo de vino (van Dorsten *et al.*, 2010; Jacobs *et al.*, 2012; van Dorsten *et al.*, 2012) y otros alimentos ricos en antocianinas como frambuesas (Wu *et al.*, 2009) o arándanos (Nurmi *et al.*, 2009). Esto se debe a que una vez liberados pueden ser absorbidos y pasar al torrente sanguíneo para una posterior conjugación a nivel hepático (Kay, 2006) o finalmente estos compuestos pueden ser excretados, volver al duodeno por la vía enterohepática o incorporados a tejidos (Talavera *et al.*, 2005).

Los siguientes compuestos fenólicos en concentración en el vino son los flavonoles. Su metabolismo microbiano, al igual que para los otros flavonoides**,** puede presentar un primer paso de deglicosilación por parte de la flora colónica, y una posterior ruptura del anillo C y liberación del anillo B. El principal metabolito de la quercetina es el ácido 2-(3,4-dihidroxifenil)acético, y la de la miricetina el ácido 2-(3,5-dihidroxifenil)acético,

pudiendo sufrir un proceso de deshidroxilación, pasando a ser ácidos fenólicos monohidroxilados (Aura, 2008), y continuar su metabolismo hacia ácidos benzoicos e hipúricos (Rechner *et al.*, 2004), como se ha observado tras su consumo en plasma y orina (Olthof *et al.*, 2003; Loke *et al.*, 2009) .

Dentro de los compuestos no flavonoides, existe un alto contenido en ácidos fenólicos libres, que pueden absorberse intactos o ser modificados por la flora intestinal donde reacciones de α-oxidación o β-oxidación, demetilaciones, deshidrogenación y de decarboxilaciones, generan ácidos fenólicos de una estructura química diferente a al original o sufrir metabolismo de fase II a nivel tisular (Monagas *et al.*, 2010).

Finalmente, la degradación microbiana de los no flavonoides, aunque menos estudiada, tiene su importancia debido al gran número de metabolitos generados y las actividades biológicas asociadas a algunos de ellos (Monagas *et al.*, 2010). Dentro de los no-flavonoides cabe destacar el grupo de los estilbenos y los lignanos (Figura A2).

El resveratrol pertenece al primer grupo, y su limitada distribución en pocos alimentos como son el vino, los cacahuetes y frutos rojos (Neveu *et al.*, 2010), ha hecho que se le considere como biomarcador de vino (Zamora-Ros *et al.*, 2012). Al igual que los otros fenoles, puede llegar al intestino donde se metaboliza en dihidroresveratrol (Rotches-Ribalta *et al.*, 2012), y desde hace poco tiempo también se sabe de su paso a 3,4´-dihidroxi-*trans*-estilbeno y 3,4´-dihidroxibibenzil (Bode *et al.*, 2013). Por otro lado, los metabolitos microbianos de los lignanos, el enterodiol y la enterolactona, son compuestos con capacidad fitoestrogénica generados tras reacciones de desmetilación y deshidroxilación de los lignanos presentes en los alimentos (Aura, 2008; Roncaglia *et al.*, 2011).

A pesar de todos los cambios descritos por la microbiota intestinal, pocas han sido a bacterias asociadas al catabolismo de los polifenoles y la producción de metabolitos está ligada a la presencia o ausencia de las mismas, haciendo que la variabilidad interindividual sea muy grande entre personas productoras y no productoras (Blaut y Clavel, 2007). Por lo tanto, el grado de degradación de los compuestos fenólicos no solo se ve influenciado por su ingesta, sino también por las variaciones de la flora microbiana, lo que podría explicar los diferentes efectos beneficiosos de su consumo (Bolca *et al.*, 2013).

Las bacterias asociadas con la metabolización de los principales grupos de polifenoles presentes en el vino se encuentran recogidas en la Tabla A3.

Tabla A3: Bacterias implicadas en el metabolismo de los polifenoles del vino.

Polifenoles del vino	Conversion	Bacteria	Referencias
Flavanoles	Rotura del anillo	*Eggerthella lenta K3* *Eubacterium sp.* *Flavonifractor plautii*	(Wang *et al.*, 2001; Kutschera *et al.*, 2011)
Flavonoles	Rotura del anillo, deglicosilación	*Enterococcus casseliflavus* *Clostridium orbiscindens* *Butyrivibrio sp C3* *Eubacterium ramulus*	(Krishnamurty *et al.*, 1970; Winter *et al.*, 1991; Schneider *et al.*, 1999; Schneider y Blaut, 2000)
Antocianos	Deglicosilación	*Bifidobacterium lactis* *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidophilus*	(Ávila *et al.*, 2009)
Estilbenos	Dehidroxilación	*Eggerthella lenta* *Bacteroides uniformis* *Slackia equolifaciens* *Adlercreutzia equolifaciens*	(Jung *et al.*, 2009; Bode *et al.*, 2013)
Lignanos	Dehidroxilación, demetilación, formación de enterolactona.	*Clostridium scindens* *Eggerthella lenta* *Butyribacterium methylotrophicum* *Eubacterium calanderi, Eubacterium limosum* *Peptostreptococcus productus* *Lactonifactor longoviformis* *Bifidobacterium*	(Clavel *et al.*, 2005; Clavel *et al.*, 2006a; Clavel *et al.*, 2006b; Clavel *et al.*, 2007; Roncaglia *et al.*, 2011)

2.3.2 EFECTO DE LOS POLIFENOLES DE LA DIETA Y SUS METABOLITOS EN LA MICROBIOTA INTESTINAL

El impacto de los dieta sobre la flora intestinal supone una de las principales líneas de investigación que se están llevando a cabo en la campo de la alimentación, con el fin de establecer las posibles relaciones sobre la salud (Queipo-Ortuno *et al.*, 2012). Actualmente, están aumentando los estudios que sugieren la existencia de un equilibrio en la composición bacteriana del individuo, cuya alteración puede desembocar en estados de enfermedad, como la enfermedad inflamatoria intestinal, enfermedad de Crohn, el cáncer colorrectal, la obesidad, la enfermedad cardiovascular, diabetes y alergias alimentarias, o molestias intestinales como dolor abdominal o flatulencia (Wallace *et al.*, 2011; Wang *et al.*, 2011; Duda-Chodak, 2012; Burcelin *et al.*, 2013; Manichanh *et al.*, 2013). En el caso de los polifenoles, solo un

número limitado de compuestos y bacterias se han identificado en relación a este efecto modulador, mayoritariamente *in vitro*, con lo que la profundización en este campo, supone un desafío para la comunidad científica.

A nivel intestinal el impacto de los polifenoles presentes en alimentos con la flora colónica puede ser promoviendo el desarrollo bacteriano o actuando como bacterioestático o antimicrobianos (Laparra y Sanz, 2010). Esta actividad modulatoria se ha observado para alimentos como las bayas (Nohynek *et al.*, 2006), el té (Lee *et al.*, 2006a), las olivas (Medina *et al.*, 2009) y el vino (Dolara *et al.*, 2005b).

Centrándonos en el vino, estudios *in vivo* con polifenoles presentes en su composición han demostrado un papel modulador tras su consumo. Un ejemplo de ello es incremento de *Lactobacillus* y *Bifidobacterium* y la disminución de la *E.coli* y *Enterobacteriacea* respecto al control, tras la ingesta de resveratrol en ratas con colitis ulcerosa (Larrosa *et al.*, 2009). Una dieta rica en taninos en ratas Wistar Furth supuso un incremento en *Enterobacteriacea* y *Bacteroides* y una disminución en el grupo *Clostridium leptum* (Smith y Mackie, 2004). Dolara *et al*., administró polifenoles del vino tinto a ratas F344 durante 15 semanas, y las bacterias finales fueron comparadas con un grupo control. El resultado fue un mayor número de *Bifidobacterium, Lactobacillus* spp y *Bacteroides* en el grupo de tratamiento, y *Clostridium, Propionibacterium* spp y *Bacteroides* en el grupo control (Dolara *et al.*, 2005b). En humanos, un estudio con extractos de semilla de uva implicó un aumento de especies de *Bifidobacterium* después de su ingesta, y la disminución de *Enterobacteriacea* (Yamakoshi *et al.*, 2011).

En estudios *in vitro,* el vino tinto ha demostrado tener la capacidad para inhibir el crecimiento de bacterias patógenas como *Salmonella*, *Staphylococcus aureus* o *Escherichia Coli* (Dolara *et al.*, 2005a). En otro estudio posterior, esta última bacteria, fue la más sensible a los polifenoles del vino tinto, mientras que el *Flavobacterium spp*, fue resistente durante la incubación (Vaquero *et al.*, 2007). También *in vitro*, el extracto fenólico de orujo de uva aumento *Lactobacillus acidophilus* (Hervert-Hernandez *et al.*, 2009).

La incubación de manera aislada de compuestos fenólicos presentes en vino con bacterias intestinales, ha demostrado también tener efectos sobre el crecimiento

bacteriano. La incubación con catequina, promovió el crecimiento de *Bifidobacterium* spp*, Escherichia coli y Clostridium coccoides–Eubacterium rectale,* esta última también cuando se incubó con la epicatequina*,* pero resultó tener un efecto inhibitorio para el grupo de *Clostridium hystolyticum* (Tzounis *et al*. 2008). La incubación con compuestos extraídos de la piel de la uva con 14 bacterias, resultaron ser inhibidores de bacterias patógenas como la *Salmonella*, *Escherichia coli* y la *Pseudomonas aureginosas*. Solo la *Yersinia enterocolitica*, resultó ser más resistente a las bajas concentraciones del extracto (Özkan *et al.*, 2004).

La actividad antimicrobiana ha sido también probada para las proantocianidinas de las semillas de la uva con distintos grados de polimerización sobre *Staphylococcus aureus, Staphylococcus aureus* resistente a meticilina*, Pseudomonas aeruginosa, Pneumococcus* sp*, Streptococcus Pyogenes, Klebsiella sp, Escherichia coli, Haemophilus influenzae, Staphylococcus epidermidis, Enterococcus faecalis y Enterococcus casilliflavus* resistentes a vancomicina*,* siendo los monómeros los menos efectivos (Mayer *et al.*, 2008).

En un estudio donde se probaron los compuestos fenólicos de varias bayas, incluyendo antocianinas como la mirecitina, delfinidina o cianidina glucósido y los ácidos fenólicos cafeico, ferúlico, *trans*-cinámico, cumárico y clorogénico, todos ellos tuvieron un efecto inhibitorio sobre la *Escherichia coli.* En el caso de la mirecitina, el efecto se observó para todas las especies de *Lactobacillus* probadas. Adicionalmente, los ácidos cafeico, ferúlico, cumárico y *trans*-cinámico, inhibieron la *Salmonella enterica ser. Typhimurium* (Puupponen-Pimia *et al.*, 2001). Cuando se probó el efecto sobre el crecimiento de 43 bacterias con resveratrol, a partir de 50 mg/L, se redujo el crecimiento de la mayoría de las bacterias probadas, pero no llegó a inhibir totalmente el crecimiento incluso cuando se usó a concentraciones más altas, siendo las menos resistentes la *Escherichia coli, Klebsiella pneumoniae* y *Yersinia pseudotuberculosis* (Jung *et al.*, 2009).

A pesar de los estudios que destacan el papel beneficioso de los polifenoles en la modulación de la microbiota intestinal se necesitan estudios más amplios de intervención en humanos para conocer la verdadera capacidad de los polifenoles de la dieta y sus metabolitos sobre el crecimiento bacteriano y sus potenciales actividades.

3.METODOLOGÍA

3. METODOLOGÍA

3.1 CREACIÓN, DISEÑO Y ESTUDIO DE LA PLATAFORMA DE METABOLISMO DE POLIFENOLES: MÓDULO 2 DE LA BASE DE DATOS PHENOL-EXPLORER

3.1.1 RECOPILACIÓN DE DATOS Y APLICACIÓN DE LA ACTUALIZACIÓN

El desarrollo del módulo 2.0 de metabolismo de polifenoles de la base de datos Phenol-Explorer se realizó gracias a una colaboración del grupo de investigación con los Drs Augustin Scalbert y Claudine Manach de la Unidad de Nutrición Humana del INRA (Instituto Nacional de Investigaciones Agronómicas, Clermont-Ferrand, Francia) y la Universidad de Alberta (Canadá) (Figura M1).

Figura M1: Implementación y créditos de Phenol-Explorer 2.0.

Phenol-Explorer Database on polyphenol content in foods

Welcome to Phenol-Explorer 3.0

New Release! Phenol-Explorer release 3.0 adds data on the effect of food processing on the polyphenol content of foods and offers a streamlined and enhanced interface. We hope you enjoy browsing the updated site.

Phenol-Explorer is the **first comprehensive database on polyphenol content in foods**. The database contains more than 35,000 content values for 500 different polyphenols in over 400 foods. These data are derived from the systematic collection of more than 60,000 original content values found in more than 1,300 scientific publications. Each of these publications has been critically evaluated before inclusion in the database. The whole data on the polyphenol composition of foods is available for download.

Release 2.0 of Phenol-Explorer added **comprehensive data on polyphenol metabolism**. Pharmacokinetic data on 380 metabolites identified in biofluids after the consumption of polyphenol-rich sources are presented. These data have been extracted from 236 publications and originate from 221 intervention studies in human subjects and experimental animals.

Release 3.0 of Phenol-Explorer introduces data on the **effects of food processing and cooking**. Retention factors describe changes in polyphenol content upon food transformation and can be used to estimate composition of processed foods when laboratory measurements are missing and the data are absent from food composition tables. Retention factors are available for 155 foods, 139 polyphenols, and 35 processes. They are derived from 129 publications.

A wide range of queries on foods, polyphenols and metabolites, as identified by various analytical methods, can be executed. Simple queries can be made by typing the name of a polyphenol, metabolite or food source at the top of any page. More complex queries can be performed by using the Advanced search. In this search, two or more foods or polyphenols can be selected, to easily compare for example the contents of the various polyphenols in green and black tea. Using the 'Advanced display option' it is also possible to sort results as desired and to calculate total polyphenol contents per class and subclass.

Phenol-Explorer has been developed at INRA in collaboration with AFSSA, the University of Alberta, the University of Barcelona, IARC and In Siliflo. The work has been made possible thanks to the financial support of the French government, the Institut National du Cancer (France), Unilever, Danone and Nestlé.

We wish you a fruitful polyphenol exploration!

Augustin Scalbert

Contributors

INRA INSILIFLO

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Version 2.0

The Phenol-Explorer database, release 2.0, on polyphenol metabolism in humans and experimental animals, was developed at INRA, Unité de Nutrition Humaine, in collaboration with the University of Barcelona, Group of Biomarkers and Nutritional & Food Metabolomics.

The development of Phenol-Explorer 2.0 was supported by INRA, Danone and the University of Barcelona.

Phenol-Explorer 2.0 was released in September 2011.

Cristina Andres-Lacueva and **Mireia Urpi-Sarda** supervised the work of compilation of metabolism data at the University of Barcelona.

Joseph Rothwell (INRA) and **Mireia Urpi-Sarda** (University of Barcelona) developed the structure of the Microsoft Access database for curation of the data on polyphenol metabolism.

Mireia Urpi-Sarda and **Maria Boto-Ordoñez** (University of Barcelona) compiled and evaluated over 10,000 original data on polyphenol metabolism from the scientific literature. **Rafael Llorach** and **Cristina Andres-Lacueva** (University of Barcelona) provided their expertise on polyphenol metabolism and the analysis of polyphenol metabolites in biological samples.

Joseph Rothwell defined the specifications for the web interface (Phenol-Explorer 2.0).

Mireia Urpi-Sarda, Rafael Llorach, Cristina Andres-Lacueva, Maria Boto-Ordoñez

Joe Rothwell

Augustin Scalbert

El desarrollo de este módulo 2.0 de metabolismo en Phenol-Explorer se realizó mediante una búsqueda sistemática de la literatura en estudios publicados hasta el 1 de marzo de 2011, sobre el metabolismo *in vivo* de polifenoles. Los términos de búsqueda fueron construidos a partir de una plantilla que incluyó el fluido analizado, el animal de experimentación o especie humana y las clases, subclases y los nombres de los polifenoles de interés (Figura M2). Esta búsqueda se aplicó en paralelo a tres bases de datos bibliográficas: *Cochrane Library* (a partir de 1800; www. thecochranelibrary.com), *MEDLINE PubMed* (desde 1950; www.pubmed.org) y el *ISI Web of Knowledge* (desde el 1945; webofknowledge.com). Las publicaciones recuperadas fueron compiladas y organizadas mediante EndNote (Thomson Reuters, New York, NY).

Figura M2: Estrategia de búsqueda.

Search steps	Search terms	Number of publications
1	urin* or plasma* or metabol*	>100 000
2	Dietary intake or intake* or exposure* or consum* or supplementation* or dietary supplements or supplement* or intervention study or experiment* or exp clinical trial or interven* or feasibility study or pilot study	>100 000
3	Human* or volunteer* or participant* or subject* or rat* or mouse* or mice* or rabbit* or pig* or sheep* or ewe* or dog*	>100 000
4	Polyphenol* or flavan* or flavon*or anthocyan* or isoflav* or phytoestrogen* or phyto-estrogen* or lignin* or stilbene* or chalcon* or phenolic acid* or ellagic* or coumarin* or hydroxycin-namic* or quercetin* or kaempferol* or rutin* or apigenin* or luteolin* or catechin* or epica-techin* or gallocatechin* or epigallocatechin* or procyanidin* or hesperetin* or naringenin* or cyanidin* or malvidin* or petunid* or peonid*or daidz* or genist* or glycit* or equol* or gallic* or vanillic* or chlorogenic* or tyrosol* or hydoxytyrosol* or resveratrol* or viniferin*	>100 000
5	Steps 1–4 combined	10780

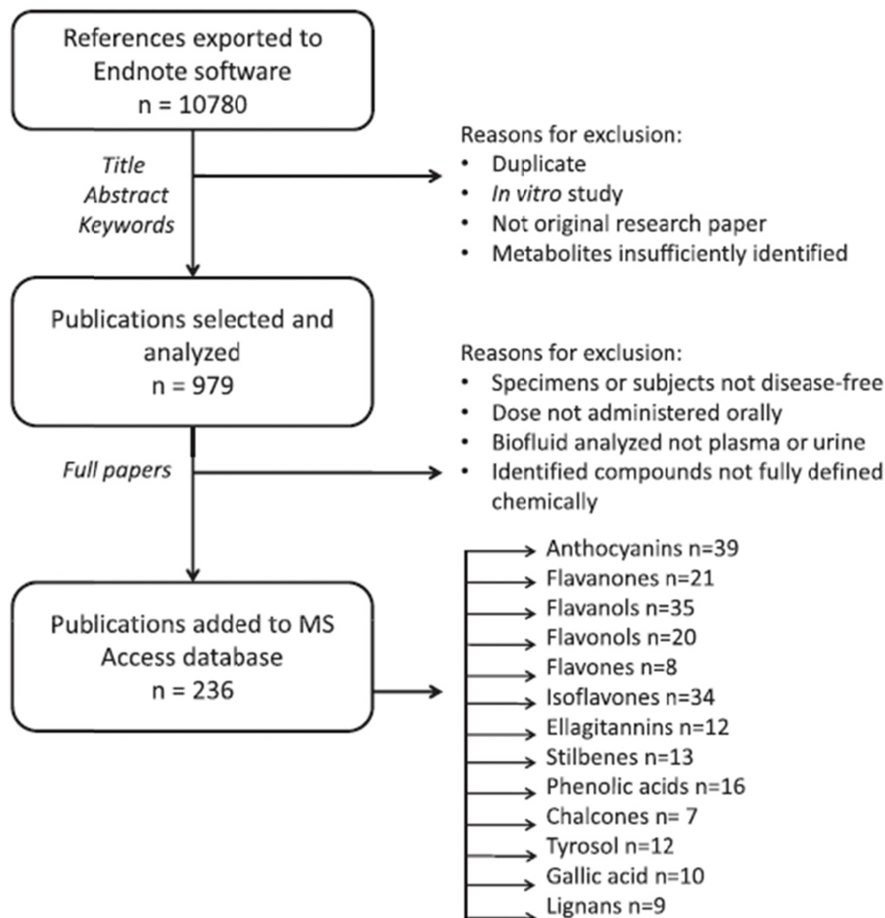
En un primer *screening* de títulos y resúmenes, las revisiones, los estudios *in vitro* y los trabajos duplicados fueron eliminados de Endnote. El resto de los artículos fueron posteriormente incluidos o excluidos de acuerdo con diversos criterios (Figura M3).

Los criterios de inclusión fueron:

- Estudios *in vivo* en humanos o animales.
- Ser estudios de intervención utilizando una dosis única o repetida de un alimento común, un alimento experimental o un suplemento oral.

- Utilizar una técnica analítica apropiada, preferiblemente espectrometría de masas, capaz de identificar de manera fiable los metabolitos.
- Detectar o cuantificar al menos un metabolito en orina o plasma.
- El uso de seres humanos o de los animales sanos.
- Presentar los datos en un formato utilizable.
- Publicados en inglés.
- Metabolitos totalmente definidos.

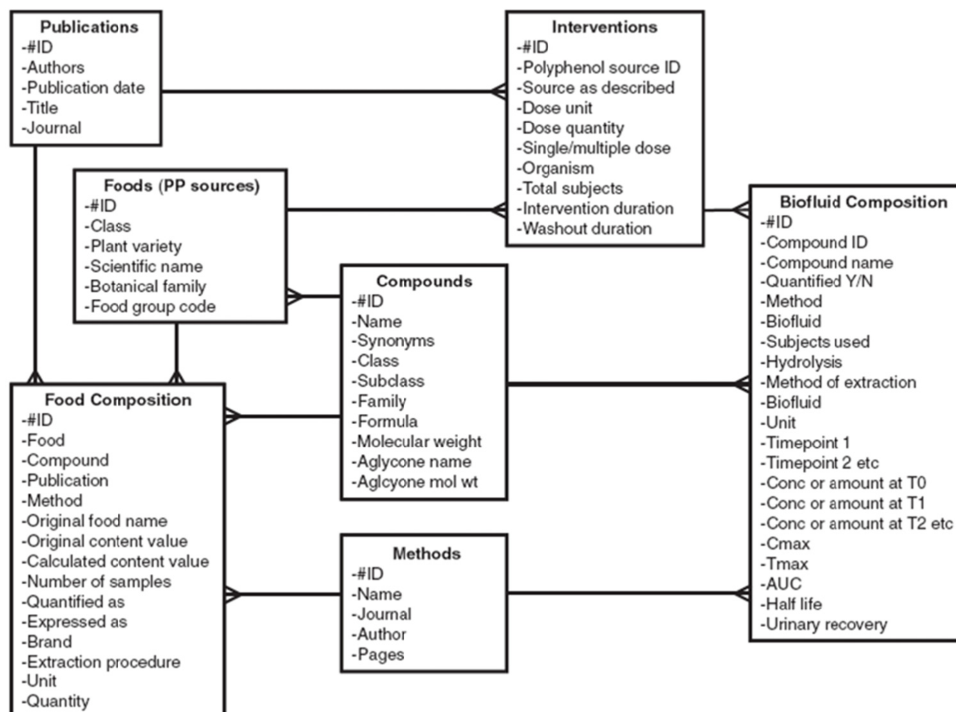
Figura M3: Esquema del proceso de revisión de literatura e inclusión de datos.



Toda esta información fue compilada en una base de datos de Microsoft Access, la cual se unió a la ya existente base de datos Phenol-Explorer versión 1.0 de la composición fenólica de los alimentos.

El diseño físico de Phenol-Explorer versión 1.0 consistía en cinco tablas principales (Neveu *et al.*, 2010) (Figura M4). Cuatro de ellas fueron tablas independientes: *Foods*, *Publications*, *Compounds* y *Methods*, cuyos registros fueron numerados, y una quinta tabla, *Food Composition*, enlazada a las anteriores. Para incorporar datos de metabolismo, se añadieron dos tablas adicionales, la tabla de *Interventions* y la de *Biofluid Composition* (Figura M4). La tabla de intervención (*Intervention*) se define como una combinación del tipo de dosis, la cantidad de la dosis y el organismo y está unida por una relación de *muchos a uno* a *Foods* and *Publications*. La tabla composición del biofluido (*Biofluid Composition*) muestra los valores de concentración de los metabolitos en plasma u orina y datos cinéticos, y se encuentra vinculada con *Interventions, Compounds* y *Methods* (Figura M4).

Figura M4: Diagrama de la incorporación de nuevas tablas de información a Phenol-Explorer.



La base de datos en Access finalmente se exporta a un Base de datos MySQL, que es capaz de trasmitirlo a la web. El sitio Phenol-Explorer se basa en el Marco Ruby on

Rails (RoR) (http://www.rubyonrails.org). RoR emplea el patrón de diseño *Modelo-Vista-Controlador* y permite la construcción de una aplicación web fiable y sensible. El servidor web Phenol-Explorer 2.0 funciona desde un servidor web Apache 2 en un sistema Linux Debian. El resultado de la incorporación de este módulo se muestra en la Figura M5 y M6.

Figura M5: Diseño de la interface y métodos de búsqueda de Phenol-Explorer 2.0.

Motor de búsqueda

Filtros

Figura M6: Datos tabulares y gráficas farmacocinéticas para plasma y orina de Phenol-Explorer 2.0.

Metabolitos tras consumo de té

Farmacocinética

Plasma

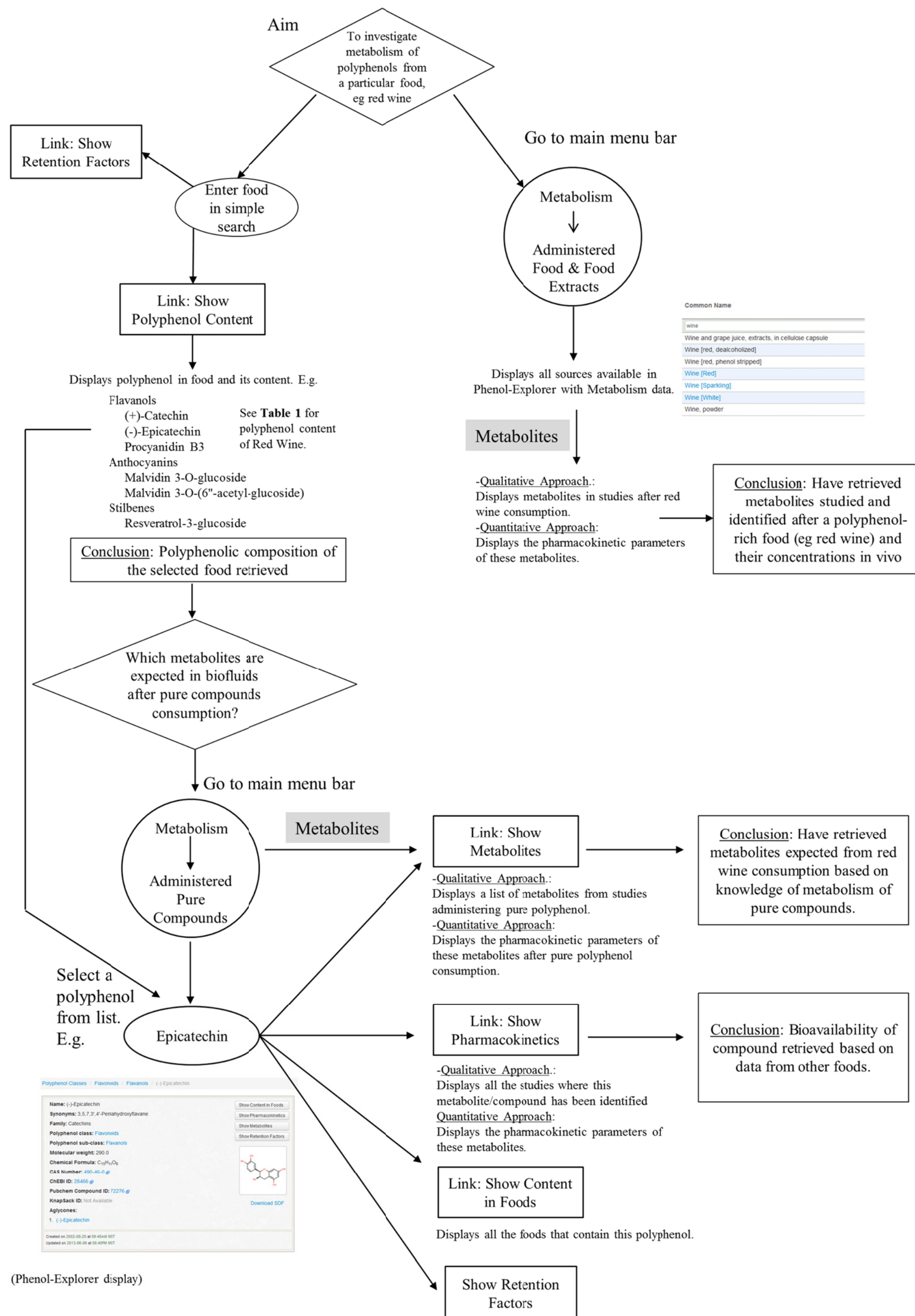
Orina

3.1.2 ESTUDIO DEL PERFIL METABÓLICO DE LOS POLIFENOLES DEL VINO A TRAVÉS DE LA BASE DE DATOS PHENOL-EXPLORER

La utilización de la base de datos creada se plasmó en el estudio del metabolismo de los polifenoles del vino y productos derivados del vino. Para esto, los datos sobre los metabolitos reportados después del consumo de los productos vitivinícolas (vino tinto, vino tinto desalcoholizado, el vino tinto sin polifenoles, cápsulas de extracto de vino, vino tinto en polvo, vino blanco y vino espumoso) y metabolitos descritos después del consumo de compuestos puros presentes en la composición del vino se recuperaron de una manera secuencial siguiendo un diagrama de flujo creado para búsquedas en la previamente desarrollada base de datos, Phenol-Explorer 2.0 (Figura M7).

Para obtener la lista de los metabolitos tras la ingesta de vino tinto y derivados, se puede seleccionar de la barra de herramientas principal *Metabolism* seguido de *Administered Food and Food Extracts*. En este punto, se muestra una imagen metabólica cualitativa y cuantitativa del metabolismo de los componentes polifenólicos de los productos de vino y derivados. Del mismo modo, la base de datos se puede utilizar para recuperar los metabolitos después del consumo de algunos polifenoles puros presentes en la composición original del vino. Hay dos posibles rutas a estos datos: 1) mediante la selección de un polifenol que aparece como un componente del vino tinto (por ejemplo, (-)-epicatequina) y seleccionando *Show Metabolites*, o 2) a través de la pestaña *Metabolism*, seguido de "*Administred pure compounds*" y luego "*Metabolites*". Adicionalmente Phenol-Explorer permite conocer los datos cinéticos de un metabolito en el enlace "*Show Pharmacokinetics".* Finalmente la pestaña "*Show Content in food*" muestra todos los alimentos que contienen este metabolito.

Figura M7: Diagrama de flujo de la estrategia de búsqueda de los metabolitos derivados del consumo de vino y productos derivados del vino.



3.2 ESTUDIO CLÍNICO ALEATORIZADO Y CRUZADO DE INTERVENCIÓN EN HUMANOS CON UN CONSUMO REGULAR DE POLIFENOLES DE VINO TINTO MEDIANTE VINO, CON Y SIN ALCOHOL, Y ALCOHOL CONTROL EN FORMA DE GINEBRA

3.2.1 DISEÑO DEL ESTUDIO CLÍNICO

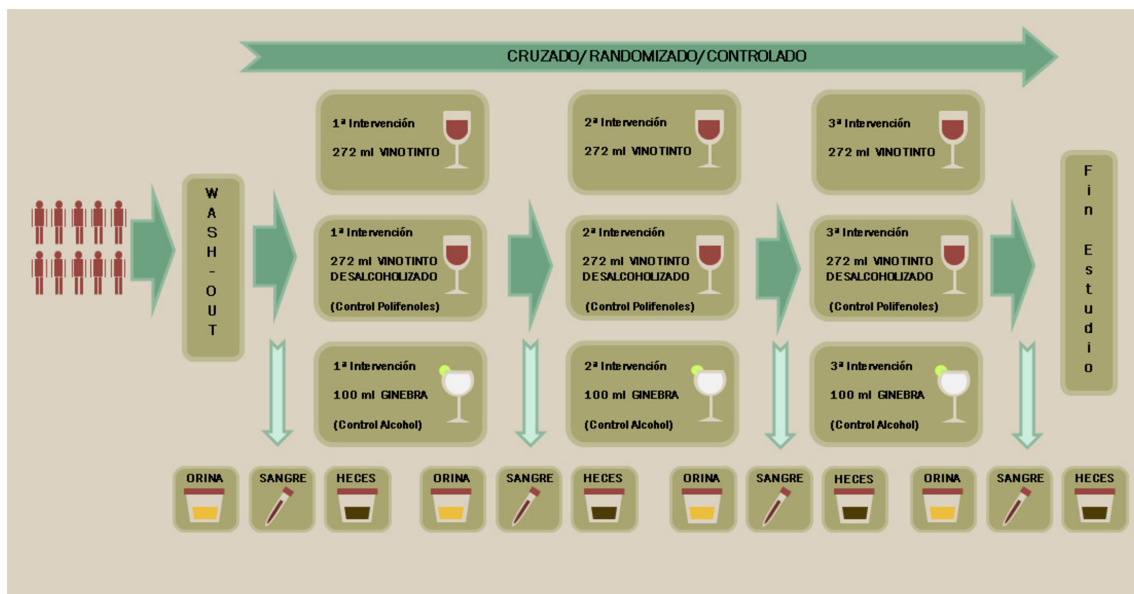
El estudio clínico se diseñó inicialmente para evaluar los efectos de los polifenoles y/o alcohol del vino tinto en la enfermedad cardiovascular (Chiva-Blanch *et al.*, 2012b) y se realizó en colaboración con el Dr Estruch del Hospital Clínic de Barcelona subvencionado a través del CICYT AGL2006-14228-C03-02. Posteriormente, este mismo diseño se aplicó a un subestudio para evaluar los cambios que producían los polifenoles y/o alcohol del vino tinto en la microbiota fecal (Queipo-Ortuno *et al.*, 2012) en colaboración con el Dr Francisco J. Tinahones del Hospital Virgen de la Victoria (Málaga) gracias a un proyecto concedido dentro del programa Ingenio-Consolider Fun-C-Food (CSD2007-063), y una beca de estancia predoctoral del Ministerio de Educación, Cultura y Deporte durante los meses de septiembre a diciembre del 2010.

El primer estudio se realizó con un total de 73 individuos con elevado riesgo cardiovascular y edades comprendidas entre los 55 y 75 años. Los 73 voluntarios se seleccionaron por ser consumidores moderados de alcohol (1-3 copas al día) y tener diabetes o 3 o más de los siguientes factores de riesgo de la enfermedad cardiovascular: adicción al tabaco, hipertensión, concentraciones de colesterol LDL plasmático ≥160 mg/dL, concentraciones de colesterol HDL plasmático ≤35 mg/dL, sobrepeso u obesidad (IMC ≥25 kg/m^2) y/o antecedentes familiares de enfermedades cardiovasculares. Los criterios de exclusión fueron el conocimiento de enfermedades cardiovasculares previas, embolia o enfermedad vascular periférica, infección del VIH, enfermedad hepática alcohólica, desnutrición y neoplasia o enfermedades infecciosas agudas. Para el presente trabajo de tesis se seleccionaron orina de 24 horas y plasma en ayunas disponible de 36 participantes. La segunda parte de este estudio realizado en Málaga, 10 individuos siguieron el mismo diseño de intervención, con la diferencia de que después de cada tratamiento se recogieron además muestras de heces.

El estudio de intervención, abierto, controlado, aleatorizado y cruzado, se llevó a cabo en tres periodos de 4 semanas (Figura M6). Tras un periodo de lavado de 2 semanas, en el que se les pidió que siguieran una dieta sin bebidas alcohólicas ni productos derivados de la uva los voluntarios consumieron 272 mL al día de vino tinto (30 g de alcohol/día) o 272 mL al día de vino tinto desalcoholizado o 100 mL de ginebra (30 g de alcohol/día) durante 4 semanas. Una vez pasadas estas cuatro semanas, la intervención se repitió dos veces más con las restantes intervenciones (Figura M8). Durante todo el estudio, a los voluntarios se les pidió que mantuviesen su dieta habitual.

Tras el período de lavado y tras cada periodo de intervención se recogieron muestras de orina de 24 horas y plasma en ayunas. En el estudio realizado en Málaga, además se recogieron muestras de heces. Las muestras fueron inmediatamente congeladas a -80ºC para su conservación.

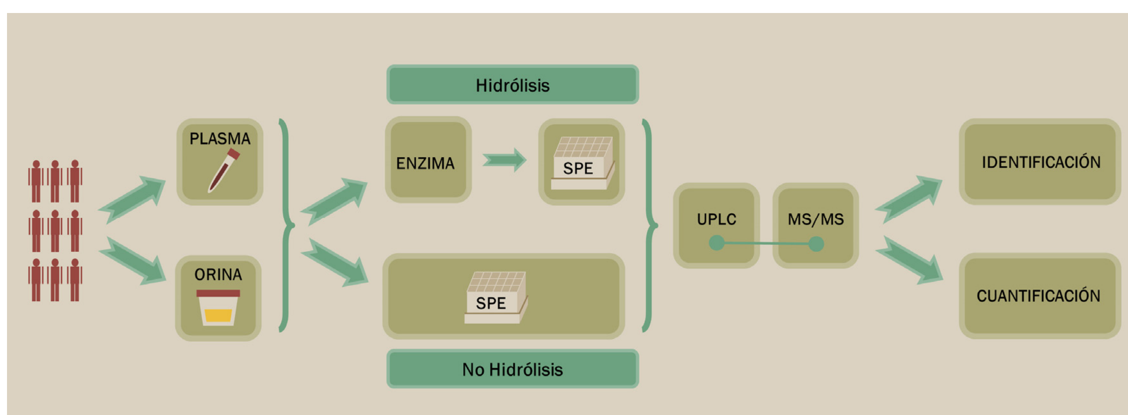
Figura M8: Diseño del estudio clínico aleatorizado, controlado y cruzado de intervención con vino tinto, vino tinto desalcoholizado y ginebra en humanos.



3.2.2 IDENTIFICACIÓN Y CUANTIFICACIÓN DE LOS COMPUESTOS FENÓLICOS DEL VINO Y SUS CORRESPONDIENTES METABOLITOS EN MUESTRAS BIOLÓGICAS POR UPLC-MS/MS.

El análisis de la identificación y cuantificación de los metabolitos de polifenoles por UPLC-MS/MS en las muestras de plasma y orina queda resumido en la Figura M9. A continuación se hace una explicación de cada proceso de manera más pormenorizada (Boto-Ordonez *et al.*, 2013c).

Figura M9: Resumen del análisis de la identificación y cuantificación de los compuestos fenólicos y sus metabolitos por UPLC-MS/MS.



3.2.2.1 PREPARACIÓN DE LAS MUESTRAS DE PLASMA Y ORINA

Debido a la complejidad de la matriz biológica de las muestras, el paso previo a su análisis resulta esencial para hacer una buena valoración cuantitativa y cualitativa de los compuestos fenólicos y sus metabolitos. Su realización es de vital importancia debido a las bajas concentraciones en las que se encuentran los metabolitos en fluidos que hacen que requieran un alto nivel de sensibilidad.

Las muestras biológicas suelen tener un elevado contenido proteico y, en algunos casos, también lipídico dificultando su posterior análisis, sobre todo cuando éste tiene lugar en un espectrómetro de masas, pudiendo dar lugar al llamado efecto matriz, que define a la disminución o supresión de la ionización de los analitos en una muestra. Para el análisis de compuestos fenólicos y sus metabolitos en muestras biológicas, se

ha llevado a cabo mediante técnicas de extracción en fase sólida (SPE), en placas de 96 pocillos, realizando simultáneamente un elevado número de muestras (Urpi-Sarda *et al.*, 2009a; Urpi-Sarda *et al.*, 2009b).

Previamente a la extracción en fase sólida las muestras de plasma se pretrataron con 20 µL de ácido orto-fosfórico, seguido de 2 minutos de vórtex, con el fin de romper las uniones de los analitos con las estructuras proteicas. Las muestras de orina de los dos estudios requirieron una centrifugación a 10,000 rpm durante 3 minutos a temperatura de refrigeración (4 ºC).

En el caso de la determinación de ácidos fenólicos, se incluyó un paso previo de hidrólisis enzimática (Urpi-Sarda *et al.*, 2009a). Para ello 1 mL de plasma u orina se incubaron con β-glucuronidasa/sulfatasa de *Helix Pomatia* a 37°C durante 45 min, después de ser acidificada con 50 µl de 0.58 mol/L de ácido acético. Pasado el tiempo de incubación, las muestras se acidificaron a pH 2 con 6 mol/L de HCl para parar la reacción.

Se siguieron 2 procedimientos para el análisis de polifenoles y sus metabolitos según si las muestras estaban hidrolizadas o no:

Extracción en fase sólida de ácidos fenólicos en plasma y orina

La extracción en fase sólida de los polifenoles y sus metabolitos en muestras hidrolizadas se realizó en placas de 96 pocillos Oasis® MCX (Waters, Milford, Massachusetts), siguiendo el método previamente desarrollado por nuestro grupo (Urpi-Sarda *et al.*, 2009a). La placa se acondicionó con metanol y 2% de ácido fórmico en agua. Las muestras hidrolizadas se cargaron entonces en la placa, se lavaron con 2% de ácido fórmico en agua, y a continuación los analitos se eluyeron con metanol. Los eluatos se evaporaron a sequedad y se reconstituyeron con 100 µL de taxifolina (1.64 µmol/L) disuelto en la fase móvil.

Extracción en fase sólida de metabolitos fenólicos conjugados en plasma y orina

El proceso de extracción en fase sólida de metabolitos fenólicos conjugados en las muestras biológicas no hidrolizadas se realizó en placas Waters Oasis® HLB de 96 pocillos (Milford, Massachusetts, Estados Unidos), siguiendo el método previamente validado y optimizado en nuestro grupo (Urpi-Sarda *et al.*, 2009b). La placa se

acondicionó con 1 mL de metanol y 1.5 mol/L de ácido fórmico en agua. Tras el acondicionamiento, los cartuchos se cargaron con 1 mL de orina o plasma. A continuación, los cartuchos se lavaron con 1 mL de agua acidificada (1.5 mol/L de ácido fórmico) y 1 mL de 5% de metanol en agua. Los analitos se eluyeron con metanol al 1% en ácido fórmico. Los eluatos se evaporaron a sequedad y se reconstituyeron con 100 µL de taxifolina (1.64 µmol/L) disuelto en la fase móvil.

3.2.2.2 ANÁLISIS INSTRUMENTAL UPLC-MS/MS

Separación cromatográfica

El análisis de los metabolitos en la orina y plasma se llevó a cabo en un cromatógrafo de ultra presión (UPLC) (Waters Acquity UPLC; Milford, Massachusetts, Estados Unidos) acoplado a espectrometría de masas en tándem (UPLC-MS/MS), adaptado de una anterior metodología validada (Urpi-Sarda *et al.*, 2009a; Urpi-Sarda *et al.*, 2009b), con el que se redujo el tiempo de análisis, costes, solventes y residuos.

La columna analítica utilizada para la separación cromatográfica fue una ACQUITY UPLC BEH C18 (Milford, MA, EE.UU.) (1.7 m, 2.1 mm x 5 mm), con prefiltro, trabajando a 40°C, a una velocidad de flujo de 0.5 mL/min y un volumen de inyección de 5 µl.

Las fases móviles usadas para el análisis fueron 0,1% de ácido fórmico en agua como fase A y 0,1% de ácido fórmico en acetonitrilo como fase B a una velocidad de flujo de 500 µL/min con las siguientes proporciones (v/v) de la fase A [t (min),% A]: (0,92), (2.5,50), (2.6,0), (3,0), (3.1,92) (3.5,92).

Espectrometría de masas

La identificación y cuantificación de los metabolitos tuvo lugar en un espectrómetro de masas API 3000 de triple cuadrupolo de Applied Biosystems (Pe Sciex, Concord, Ontario, Canada), equipado con una fuente de ionización (ESI) Turbo IonSpray, trabajando en modo negativo. Los parámetros de MS y MS/MS se encuentran resumidos en la tabla M1.

Tabla M1: Parámetros de MS y MS/MS utilizados en el análisis de muestras biológicas UPLC.

Voltaje capilar	-3700 V
Gas nebulizador, N_2	10 unidades arbitrarias
Gas cortina, N_2	12 unidades arbitrarias
Gas de colisión, N_2	5 unidades arbitrarias
Potencial de enfoque	-200 V
Potencial de entrada	-10 V
Potencial de desagrupamiento	Según compuesto
Energía de colisión	Según compuesto
Temperatura del gas de secado	400ºC
Gas auxiliar	6000 cm^3/min

Identificación y cuantificación de metabolitos

La identificación de los metabolitos se realizó comparando el tiempo de retención con aquel de los estándares disponibles o mediante experimentos de *Product Ion Scan* (PIS) cuando no se disponía de estándar comercial. Para efectos de cuantificación, los datos se recogieron con *Multiple Reaction Monitoring* (MRM), definiendo una transición de los padres y los iones de productos específicos para cada compuesto (Tabla M2). Se construyeron curvas de calibración con los estándares disponibles en orina y plasma sintéticos, y se sometieron al mismo procedimiento que las muestras. Las concentraciones de metabolitos identificados por PIS se estimaron utilizando la curva estándar del compuesto más similar y los resultados se expresaron como sus equivalentes.

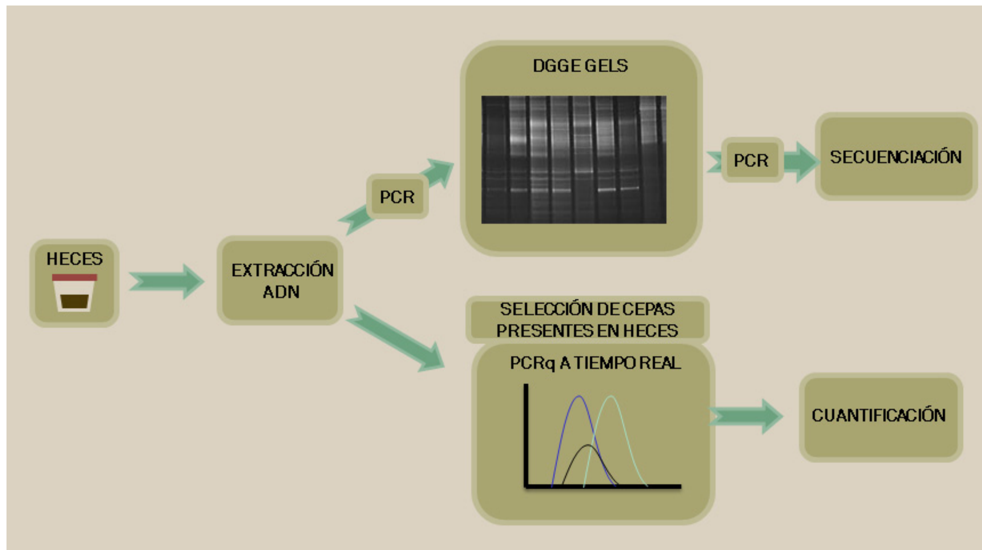
Tabla M2: Parámetros de MS y MS/MS utilizados en el análisis de muestras de plasma y orina por UPLC.

Analito	MRM	Identificado por	DP	CE	Cuantificado como
Ácido Hidroxibenzoicos					
Ácido 2,4-dihidroxibenzoico	153/109	STD[a]	-50	-20	STD
Ácido 2,6-dihidroxibenzoico	153/109	STD	-50	-20	STD
Ácido 2,5-dihidroxibenzoico	153/109	STD	-50	-20	STD
Ácido 3,5-dihidroxibenzoico	153/109	STD	-50	-20	STD
Ácido Protocatéico	153/109	STD	-50	-20	STD
Ácido Vanílico	167/152	STD	-50	-20	STD
Ácido Siríngico	197/121	STD	-50	-25	STD
Ácido 4-hidroxibenzoico	137/93	STD	-50	-16	STD
Ácido 3-hidroxibenzoico	137/93	STD	-50	-16	STD
Ácido 4-hidroxihipúrico	194/100	STD	-50	-20	STD
Ácido 3-hidroxihipúrico	194/150	PIS[b]	-50	-20	Ácido 4-hidroxihipúrico
Metabolitos del Ácido Gálico					
Ácido Gálico	169/125	STD	-40	-20	STD
Ácido 4-O-metilgálico	167/108	STD	-50	-26	STD
Ácido metilgálico	167/108	PIS	-50	-26	Ácido 4-O-metilgálico
Ácido metilgálico sulfato	263/183	PIS	-50	-25	Ácido Gálico
Metabolitos del Etilgalato					
Etilgalato	197/169	STD	-50	-25	Ácido Gálico
Etilgalato sulfato	277/197	PIS	-50	-25	Ácido Gálico
Etilgalato glucurónido 1,2	373/197	PIS	-50	-25	Epicatechin-5-*O*-glucurónido
Ácidos Hidroxifenilacéticos					
Ácido Fenilacético	135/91	STD	-30	-12	STD
Ácido 3-hidroxifenilacético	151/107	STD	-50	-12	STD
Ácido 2-hidroxifenilacético	151/107	STD	-50	-12	STD
Ácido 3,4-dihidroxifenilacético	167/123	STD	-50	-12	STD
Ácido Homovanílico	181/137	STD	-40	-10	Ácido Vanílico
Ácidos Hidroxicinámicos					
Ácido *m*-cumárico	163/119	STD	-50	-30	STD
Ácido *o*-cumárico	163/119	STD	-50	-30	STD
Ácido *p*-cumaric	163/119	STD	-50	-30	STD
Ácido Caféico	179/135	STD	-50	-21	STD
Ácido Ferúlico	193/134	STD	-50	-25	STD
Ácido Sinápico	223/164	STD	-50	-25	STD
Ácidos Hidroxifenilpropiónicos					
Ácido 3-(4-hidroxifenil)propiónico	165/121	STD	-30	-16	STD
Ácido 3-(3-hidroxifenil) propiónico	165/121	STD	-30	-16	STD
Ácido Dihidrocaféico	181/137	STD	-40	-10	STD
Flavan-3-ols					
(Epi)Catequina glucurónido 1,2,3,4	465/289	PIS	-50	-25	Epicatequina-5-*O*-glucurónido
(Epi)Catequina sulfato 1,2,3	369/289	PIS	-50	-25	(Epi)Catequina
Metil (Epi)Catequina glucurónido 1,2,3	479/303	PIS	-50	-30	Epicatequina-5-*O*-glucurónido
Metil (Epi)Catequina sulfato 1,2,3	383/303	PIS	-50	-25	(Epi)Catequina
Glicinados					
Vanilloilglicina	224/180	PIS	-50	-25	Ácido 4-hidroxihipúrico
Feruloilglicina	250/100	PIS	-50	-25	Ácido 4-hidroxihipúrico
Hidroxifenilvalerolactonas					
DHPV 1	207/163	PIS	-50	-25	(Epi)Catequina
DHPV 2	207/163	PIS	-50	-25	(Epi)Catequina
DHPV glucurónido 1,2	383/207	PIS	-50	-25	Epicatequina-5-*O*-glucurónido
DHPV sulfato 1,2	287/207	PIS	-50	-25	(Epi)Catequina
MHPV 1	221/162	PIS	-50	-25	(Epi)Catequina
MHPV glucurónido 1	397/221	PIS	-50	-25	Epicatequina-5-*O*-glucurónido
MHPV sulfato 1,2	301/221	PIS	-50	-25	(Epi)Catequina
Otros polifenoles					
Enterolactona	297/253	STD	-50	-25	STD
Pirogalol	125/69	STD	-50	-25	STD

3.2.3 ANÁLISIS MICROBIOLÓGICO DE LA FLORA FECAL HUMANA

El análisis de las muestras fecales, desde su descongelación hasta su cuantificación queda resumido en la figura M10. A continuación se detallan los procedimientos utilizados de una manera más pormenorizada.

Figura M10: Análisis de la microbiota fecal por PCR y electroforesis en gel con gradiente de desnaturalización (DGGE)



3.2.3.1 PREPARACIÓN DE LAS MUESTRAS DE HECES

El análisis de la microbiota fecal se llevó a cabo mediante purificación de ADN en heces. Para ello se extrajo el ADN de 200 mg de heces mediante el uso del QIAamp DNA stool Mini kit (Qiagen, Valencia, California, USA). El procedimiento de purificación de ADN mediante QIAamp stool Mini kit implica la digestión de las proteínas, la unión del DNA a la membrana de sílice, la limpieza de las impurezas, y finalmente su elución.

La concentración de DNA se determinó por absorbancia a 260 nm, y la pureza se estimó mediante la determinación de la relación A260/A280 con un espectrofotómetro Nanodrop (NanoDrop Technologies, Inc., Wilmington, Delaware, USA).

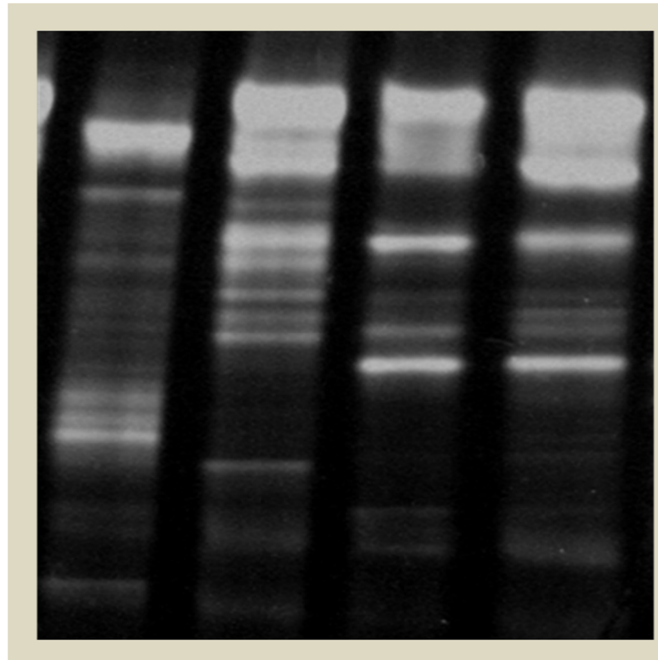
3.2.3.2 ANÁLISIS DE LA MICROBIOTA FECAL POR REACCIÓN EN CADENA DE LA POLIMERASA (PCR) ACOPLADA A ELECTROFORESIS EN GEL CON GRADIENTE DE DESNATURALIZACIÓN (DGGE)

Las muestras de heces de cada sujeto se examinaron mediante la ampliación por PCR de la región V2-V3 del ARNr 16S del gen (posiciones 339 a 539 en el gen de la bacteria *Escherichia coli*) de las bacterias presentes en las muestras de heces. Para ello se usaron los *primers* HDA1-GC (5´- **CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G**CC TAC GGG AGG CAG CAG T-3´; la abrazadera GC está en negrita) y HDA2 (5´-GTA TTA CCG CGG CTG CTG GCA C-3´), generando un producto de 200 pares de bases.

Alícuotas de 2 µL de ADN se amplificaron por PCR cuantitativa en tiempo real (qPCR) hasta un volumen de 20 µL en un instrumento 7500 Fast RT-PCR Systems mediante Fast SYBR Green Master Mix (Applied Biosystems, California, USA) y 200 nmol/L de cada uno de los *primers* (HDA1-GC y HDA2). Los ciclos de PCR fueron los siguientes: desnaturalización inicial a 95°C durante 20 segundos, 45 ciclos de amplificación, incluyendo desnaturalización a 95°C durante 3 segundos, hibridación a 55°C durante 30 segundos, y extensión a 72°C durante 1 min. El producto obtenido fue separado mediante DGGE utilizando geles de poliacrilamida al 6% con un gradiente de urea y formamida del 20 al 80% que se incrementó en la dirección de la electroforesis, con un instrumento de DCode Universal Mutation Detection System (Bio-Rad, Hercules, California, USA). La electroforesis se llevó a cabo en una solución tampón de EDTA (40 mmol Tris/L, 20 mmol ácido acético/L, y 1 mmol EDTA /L, pH 7.4) a 130 V y 60°C durante 4 horas y media.

La electroforesis se detuvo cuando un marcador colorante, xileno cianol, alcanzó el fondo del gel. Los geles fueron teñidos con bromuro de etidio (0.5 mg/L) durante 5 minutos. Tras limpiarlos con agua desionizada, se observaron por transiluminación ultravioleta y la imagen fue capturada con un software de adquisición de imágenes, Gelcapture (DNR Bio-Imaging Systems Ltd, Jerusalén, Israel) (Figura M11).

Figura M11: Banda de gel de poliacrilamida de un individuo de estudio.



Las muestras de cada sujeto después de cada período fueron analizadas en el mismo gel DGGE para evitar la posible influencia de las variaciones en las condiciones electroforéticas entre los diferentes geles.

Las similitudes entre los patrones de bandas en el perfil DGGE se calcularon en base a la presencia y ausencia de bandas y expresado como Cs (coeficiente de similitud) mediante el uso de software de BioNumerics (Applied Maths, Kortrijk, Belgium). El *Dice Similarity Coefficient* (DSC) se utilizó para calcular comparaciones por pares de los perfiles obtenidos del DGGE. Un valor de Cs 100% indica que los perfiles de DGGE son idénticos, mientras que los perfiles completamente diferentes resultan en un valor de Cs 0%. El método de agrupamiento por pares no ponderado con algoritmo de la media aritmética se utilizó para la construcción de dendrogramas.

3.2.3.3 SECUENCIACION DE LAS BANDAS DE DGGE

Una vez finalizado la electroforesis y la captación de imágenes, las bandas de interés obtenidas fueron escindidas del gel, reamplificadas y secuenciadas.

Las bandas de interés fueron extirpadas de geles de DGGE y colocadas en 40 µL de agua estéril a 4°C, para la difusión del ADN. Los *primers* HDA1/2 se utilizaron para amplificar el ADN de las bandas, con un ciclo de PCR de desnaturalización inicial a 95°C durante 20 segundos, 45 ciclos de amplificación, incluyendo desnaturalización a 95°C durante 3 segundos, hibridación a 55°C durante 15 segundos, y extensión a 72°C durante 10 segundos.

Los productos de PCR se diluyeron hasta 20 ng/µL, purificados con ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA), y secuenciados en un ABI 3130 (Applied Biosystems, California, USA) mediante el uso de la BigDye-Kit-Standard (Applied Biosystems, California, USA). Los datos de la secuencia de nucleótidos obtenida se analizaron mediante el uso de software de MicroSeqID v2.1.1 (Applied Biosystems, California, USA).

3.2.3.4 CUANTIFICACIÓN MICROBIANA POR PCR A TIEMPO REAL

Para la cuantificación se utilizaron cebadores específicos dirigidos a diferentes géneros y especies bacterianas (Tabla M3). La PCR cuantitativa en tiempo real (qPCR) se realizó con un sistema de detección de secuencia de LightCycler 2.0 PCR mediante FastStart DNA Master SYBR Green kit (Roche Diagnostics, Mannheim, Germany) por duplicado. Todas las pruebas de PCR se llevaron a cabo con un volumen final de 20 µL, que contiene 100 ng de cada preparación de ADN fecal y 200 nmol/L de cada *primer*. Los ciclos de PCR fueron los siguientes: desnaturalización inicial a 95°C durante 10 minutos, 45 ciclos de desnaturalización a 95°C durante 10 segundos, con una temperatura óptima de hibridación durante 20 segundos, y extensión a 72°C durante 1 minuto. La temperatura óptima de hibridación fue seleccionada para cada bacteria. Por último, el análisis de curva de fusión se realizó mediante enfriamiento lento de 95°C a 60°C (0.05°C por ciclo) con la medición simultánea de la intensidad de la señal SYBR Green. El análisis de punto de fusión permitió la confirmación de la especificidad de los productos de amplificación.

Se calculó la concentración de bacterias de cada muestra mediante la comparación de los valores de Ct (Ciclo Umbral) obtenidos a partir de las curvas de calibración con el

software LightCycler 4.0 (Roche Diagnostics, Mannheim, Germany). Se crearon curvas patrón mediante el uso de una dilución de 10 veces en serie de ADN de cultivos puros, correspondientes a 10^{1}-10^{10} Copias/g de heces.

Las cepas usadas en este estudio fueron obtenidas de la Colección Española De Cultivos Tipo (CECT)(*Bacteroides vulgatus* CECT 11154, *Fusobacterium varium* CECT 10560, *Enterococcus faecalis* CECT 184, *Enterobacter cloacae* CECT 194, y *Clostridium perfringens* CECT 376) y Colección Americana De Cultivos Tipo (ATCC) (*Bifidobacterium bifidum* ATCC 15696, *Lactobacillus casei* ATCC 334D-5, *Prevotella intermedia* ATCC 25611D-5, *Clostridium histolyticum* ATCC 19401, *Eggerthella lenta* ATCC 25559, *Bacteroides uniformis* ATCC 8492 y *Ruminococus productus* ATCC 27340D-5). Los datos presentados son los valores medios de la qPCR por duplicado.

Tabla M3: Primers utilizados para la PCR en tiempo real (Queipo-Ortuno *et al.*, 2012).

Grupo	Secuencia de oligonucleótidos (5´-3´)	Tamaño del Amplicón (bp)
Bacteroidetes	CATGTGGTTTAATTCGATGAT	126
	AGCTGACGACAACCATGCAG	
Bacteroides	GAGAGGAAGGTCCCCCAC	106
	CGCTACTTGGCTGGTTCAG	
Lactobacillus	GAGGCAGCAGTAGGGAATCTTC	126
	GGCCAGTTACTACCTCTATCCTTCTTC	
Fusobacterium	CCCTTCAGTGCCGCAGT	273
	GTCGCAGGATGTCAAGAC	
Firmicutes	ATGTGGTTTAATTCGAAGCA	126
	AGCTGACGACAACCATGCAC	
Actinobacteria	CGCGGCCTATCAGCTTGTTG	600
	CCGTACTCCCCAGGCGGGG	
Bifidobacterium	CTCCTGGAAACGGGTGG	550
	GGTGTTCTTCCCGATATCTACA	
Prevotella	GGTTCTGAGAGGAAGGTCCCC	121
	TCCTGCACGCTACTTGGCTG	
Enterococcus	CCCTTATTGTTAGTTGCCATCATT	144
	ACTCGTTCTTCCCATGT	
Proteobacteria	CATGACGTTACCCGCAGAAGAAG	195
	CTCTACGAGACTCAAGCTTGC	
Clostridium cluster IV	GCACAAGCAGTGGAGT	239
	CTTCCTCCGTTTTGTCAA	
Eggerthella lenta	TGGCGAACGGGTGAGTAA	1221
	AGGCCCGGGAACGTATTCAC	
Grupo Blautia coccoides–Eubacterium rectale	CGGTACCTGACTAAGAAGC	429
	AGTTTCATTCTTGCGAACG	
Grupo Clostridium histolyticum	ATGCAAGTCGAGCGA(G/T)G	120
	TATGCGGTATTAATCT(C/T)CCTTT	
Bacteroides uniformis	TCCGTTTTCCACTTATAAGA	350
	GGGTTBCCCCATTCGG	

3.2.4 ANÁLISIS ESTADÍSTICO PARA LA DETERMINACIÓN Y EVALUACIÓN DE MARCADORES DE CONSUMO DE ALIMENTOS.

El uso de biomarcadores tiene un gran potencial en el campo de la alimentación y la salud, sin embargo necesitan ser validados por métodos estadísticos robustos previamente a su uso (Buyse *et al.*, 2010). De la misma manera, las nuevas herramientas metodológicas y analíticas permiten poder identificar y discriminar de manera eficaz, aquellos metabolitos presentes en fluidos que podrían ser biomarcadores del consumo de un alimento (Scalbert *et al.*, 2013). A continuación se presentan las herramientas estadísticas utilizadas en la presente tesis doctoral para la identificación y evaluación los biomarcadores asociados al consumo de vino.

3.2.4.1 METABOANALYST

La plataforma Metaboanalyst es un paquete de herramientas online que permite el análisis y la interpretación de datos metabolómicos (Xia y Wishart, 2011). La interface web permite al usuario, procesar los datos, normalizarlos y realizar análisis univariante o multivariante, tanto de intensidades de espectros, intensidades de picos, como de concentraciones extraídas de estudios en los que se ha usado espectometría de masas o RMN.

Para la identificación de biomarcadores, la web Metaboanalyst pone a disposición del usuario las siguientes herramientas:

- Normalización de los datos: da la posibilizar de normalizar y escalar los datos de diversas formas (normalización mediante suma, media o muestra de referencia, transformación por logaritmo o raíz cuadrada y escalado por rango, pareto, autoescalado) devolviendo al usuario un gráfico con la distribución de los datos antes y después de la normalización donde su observa su comportamiento hacia una curva Gausiana.
- Análisis univariante:
    - *t-test* para muestras pareadas: Prueba para contrastar hipótesis sobre medias en una población con distribución normal.

- Análisis multivariante:
    - Análisis de componentes principales (PCA): Es una técnica usada para el análisis exploratorio de los datos, que reduce su dimensionalidad (número de variables), reteniendo aquellas características del conjunto de datos que más contribuyen a su varianza. Trata de determinar un sistema más pequeño de variables que sinteticen el sistema original.
    - Análisis de clusterización (Heatmap): Es una técnica que permite agrupar una serie de variables o individuos en un pequeño número de grupos, de forma que las variables o individuos sean muy similares dentro de cada uno, y diferentes entre los grupos formados.

3.2.4.2 REGRESIÓN POR PASOS (*STEPWISE*)

El análisis de regresión lineal es una técnica utilizada para estudiar la relación entre dos variables, una variable dependiente y una o más variables independientes o predictoras, para predecir un amplio rango de fenómenos (Doménech Massons y Granero, 2006; Martínez-González *et al.*, 2006). La capacidad de predicción puede ser imprecisa si la asociación entre las dos variables es débil, pero puede tener una finalidad predictiva en el caso de que la asociación sea fuerte. Cuando se utilizan varias variables predictoras o independientes, se trata de una regresión múltiple (Doménech Massons y Granero, 2006; Martínez-González *et al.*, 2006).

La representación gráfica de estas variables se hace a través de la ecuación de regresión mínimo cuadrática:

$$Y=\beta_{0+}\beta_1 X_1+\beta_2 X_2+....+\beta_k X_k,$$

donde la variable dependiente Y se interpreta como la combinación del conjunto de k variables independientes (X_k), un coeficiente (β_k) que indica el peso relativo de cada variable en la ecuación, así como una constante, $\beta_{0,}$ para recoger lo que las variables independientes no pueden explicar. Cuando nuestra variable dependiente se trata de una variable dicotómica, es decir, con dos posibilidades, y nuestras variables

independientes son candidatas a predecir la ocurrencia de ese fenómeno se utiliza una regresión logística.

El análisis de regresión por pasos (*stepwise*), aplicable a la regresión logística como a la regresión lineal simple, se utiliza para seleccionar las variables independientes que permitan el mejor ajuste posible. En el caso del *stepwise*, las variables se incorporan de una en una. En primer lugar se incorpora la variable que además de superar los criterios de entrada al modelo, es la que más se correlaciona con la variable dependiente, a continuación se van añadiendo las restantes, hasta que ya no queden variables que cumplan con los criterios de selección. Uno de los riesgos que puede tener este análisis es la colinealidad de variables. Esto ocurre cuando las variables independientes tienen una alta correlación entre ellas. A la hora de valorar la colinealidad, se miran los *índices de condición* que en condiciones de no colinealidad deberían presentar valores menores de 15. En el caso de que exista colinealidad se tiene que aplicar alguna de las siguientes soluciones: i) aumentar el tamaño de muestras; ii) crear indicadores múltiples combinando varias variables; iii) excluir variables redundantes; iv) utilizar una técnica de estimación sesgada.

3.2.4.3 CURVAS COR

Las curvas COR son una representación gráfica de un test de diagnóstico continuo (Figura M12), donde la 1-especificidad, nos da una idea de los falsos positivos y la sensibilidad, la proporción de verdaderos positivos. Los distintos puntos de corte se corresponden con diferentes parejas de valores de 1-especificidad y sensibilidad, conformando finalmente la curva COR.

Las curvas COR en pruebas diagnósticas permiten clasificar a los sujetos según presenten o no una característica determinada, mediante la comparación del área bajo la curva (AUC) (Figura M12). Esta área posee un valor comprendido entre 0.5 y 1, donde 1 representa un valor diagnóstico perfecto y 0.5 es una prueba con una capacidad diagnóstica aleatoria. A la hora del uso de las curvas COR como medida para evaluar un biomarcador, se puede considerar (Xia *et al.*, 2013):

- 0.9-1.0 = excelente

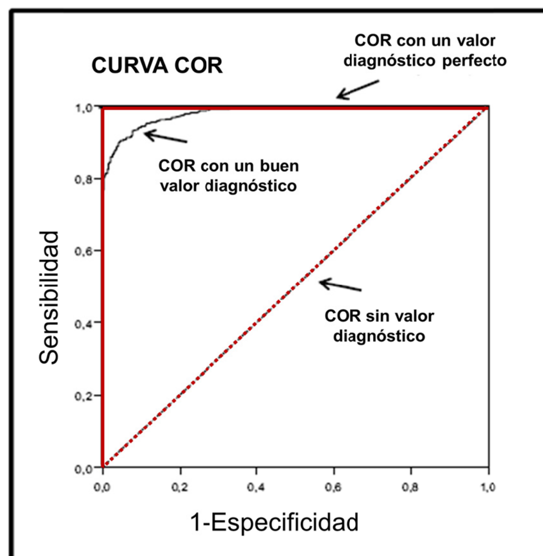
- 0.8-0.9 = bueno
- 0.7-0.8 = regular
- 0.6-0.7 = malo
- 0.5-0.6 = erróneo

Las curvas COR también nos permiten obtener un punto de corte óptimo a partir del cual los sujetos se clasifican en un resultado positivo o negativo de la manera más eficiente, y corresponde con la combinación de sensibilidad y especificidad.

Una de las maneras de obtenerlo se basa en la distancia mínima de la esquina superior izquierda a un punto de la curva COR, que se puede calcular con la fórmula (Heinzmann *et al.*, 2012), dond Sn es la Sensibilidad y Sp, la Especificidad:

$$d= \text{raíz } [(1 - Sn)^2 + (1 - Sp)^2]$$

Figura M12: Curva COR de tres pruebas con distinto poder diagnóstico.



3.2.4.3 CORRELACIONES

La correlación indica la magnitud y dirección de una relación lineal entre dos variables aleatorias cuantitativas. La correlación entre dos variables cuantitativas indica que los valores de una de ellas varían sistemáticamente respecto a los valores de la otra. Las correlaciones en ningún caso suponen causalidad (Martínez-González *et al.*, 2006).

Las correlaciones, se representan gráficamente mediante una línea de ajuste, compuesta por tres elementos: fuerza, sentido y forma. El valor de las correlaciones se mide mediante unos coeficientes de correlación, de los cuales los más conocidos son el coeficiente de Pearson, para datos que cumplen con una distribución paramétrica, y coeficiente de Spearman, para datos no paramétricos.

El coeficiente de Pearson estima la adaptación a una línea recta que defina idealmente la relación, mientras que el coeficiente de Spearman, mide asociaciones que no tienen por qué ser lineales (Martínez-González *et al.*, 2006). Los coeficientes de correlación se establecen con valores que van de +1, proporcionalidad directa o positiva, a -1, proporcionalidad inversa o negativa, no existiendo relación cuando el coeficiente es igual a 0.

Las relaciones entre las 2 variables se miden individualmente y se observan en el gráfico como puntos. El valor de r será más grande cuando los puntos estén concentrados en torno a la recta, y menor cuando estén más dispersos en el gráfico, así una $r \leq 0.30$ se considera una asociación débil, entre 0.30-0.70 moderada, y $r \geq 0.70$ una asociación fuerte (Martínez-González *et al.*, 2006).

Los coeficientes de variación puede ser r, expresando en qué grado las variables dependientes e independientes tienen el mismo orden (línea de regresión), y el r^2, que expresa la proporción de variación conjunta (varianza común).

4.RESULTADOS

4. RESULTADOS

4.1 DESAROLLO DE LA PLATAFORMA DE METABOLISMO DE COMPUESTOS POLIFENÓLICOS DE LA BASE DE DATOS PHENOL-EXPLORER

Dentro de un convenio de colaboración entre la Unidad de Nutrición Humana del INRA (Clermont-Ferrand, Francia), la Universidad de Barcelona, la Agencia Internacional de Investigación sobre el Cáncer (IARC) y la Universidad de Alberta (Canadá), se ha desarrollado el nuevo módulo de metabolismo de polifenoles en la base de datos Phenol-Explorer. La creación e implantación de la misma ha sido publicada en una de las revistas considerada por el *Science Citation Index*, con un índice de impacto de 4.200 y situada en el primer cuartil de la categoría *Mathematical & Computational Biology* (5 de 47).

Publicación 1: Joseph A. Rothwell, Mireia Urpi-Sarda, María Boto-Ordóñez, Craig Knox, Rafael Llorach, Roman Eisner, Joseph Cruz, Vanessa Neveu, David Wishart,Claudine Manach, Cristina Andres-Lacueva, Augustin Scalbert. Phenol-Explorer 2.0: a major update of the Phenol-Explorer database integrating data on polyphenol metabolism and pharmacokinetics in humans and experimental animals. *Database (Oxford)*, 2012; bas031. DOI: 10.1093/database/bas031.

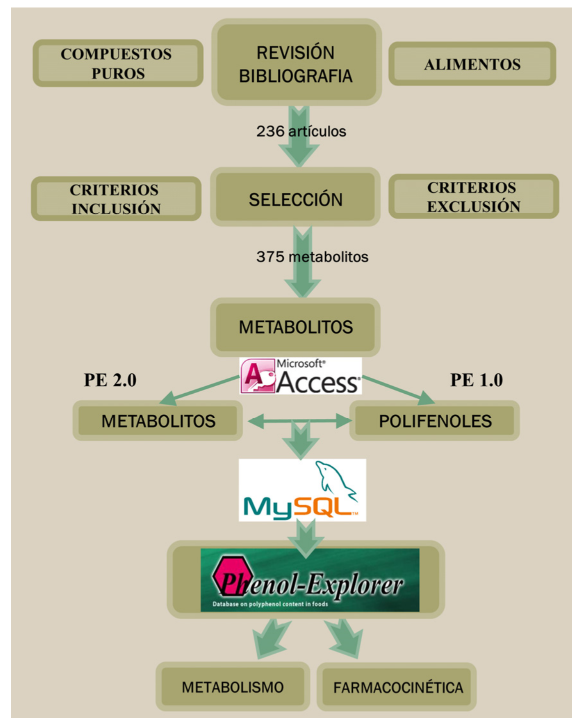
Resumen:

Phenol-Explorer, publicada *online* en 2009, es la única base de datos completa en la web sobre el contenido de polifenoles en los alimentos. Estos compuestos bioactivos presentes en alimentos reciben una considerable atención debido a su papel en la prevención de las enfermedades.

Los polifenoles, rara vez se absorben y se excretan en sus formas ingeridas debido a que son ampliamente metabolizados en el cuerpo. Hasta el momento, ninguna base de datos había recogido la información de los metabolitos de polifenoles y sus concentraciones en biofluidos después del consumo de fuentes ricas en los mismos. El

conocimiento de estos metabolitos es esencial en la planificación de los experimentos cuyo objetivo es dilucidar los efectos de los polifenoles en la salud. La versión 2.0 es la primera gran actualización de la base de datos, lo que permite la rápida recuperación de los datos sobre biotransformaciones y farmacocinética de polifenoles dietéticos. Como resultado, datos de 375 metabolitos polifenólicos identificados en orina y plasma se obtuvieron de 236 publicaciones revisadas sobre el metabolismo de los polifenoles en seres humanos y animales de experimentación, añadiendose a la base de datos por medio de un modelo relacional extendido. Los parámetros farmacocinéticos recogidos se pueden recuperar en forma tanto tabular como gráfica. La interfaz de la web se ha mejorado y ahora permite el filtrado de la información de acuerdo a varios criterios. Phenol-Explorer 2.0, se actualizará periódicamente, con el fin de ser un recurso útil y competente para los científicos en el campo de los polifenoles, ya que las bioactividades y los efectos sobre la salud son dependientes de la naturaleza y de las concentraciones de metabolitos que llegan a los tejidos diana. La base de datos Phenol-Explorer está disponible al público y se puede encontrar en línea en http://www.phenol-explorer.eu.

Figura Resultados R1: Gráfico resumen desarrollo de la plataforma del metabolismo de la base de datos Phenol-Explorer.



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Database update

Phenol-Explorer 2.0: a major update of the Phenol-Explorer database integrating data on polyphenol metabolism and pharmacokinetics in humans and experimental animals

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Phenol-Explorer, launched in 2009, is the only comprehensive web-based database on the content in foods of polyphenols, a major class of food bioactives that receive considerable attention due to their role in the prevention of diseases. Polyphenols are rarely absorbed and excreted in their ingested forms, but extensively metabolized in the body, and until now, no database has allowed the recall of identities and concentrations of polyphenol metabolites in biofluids after the consumption of polyphenol-rich sources. Knowledge of these metabolites is essential in the planning of experiments whose aim is to elucidate the effects of polyphenols on health. Release 2.0 is the first major update of the database, allowing the rapid retrieval of data on the biotransformations and pharmacokinetics of dietary polyphenols. Data on 375 polyphenol metabolites identified in urine and plasma were collected from 236 peer-reviewed publications on polyphenol metabolism in humans and experimental animals and added to the database by means of an extended relational design. Pharmacokinetic parameters have been collected and can be retrieved in both tabular and graphical form. The web interface has been enhanced and now allows the filtering of information according to various criteria. Phenol-Explorer 2.0, which will be periodically updated, should prove to be an even more useful and capable resource for polyphenol scientists because bioactivities and health effects of polyphenols are dependent on the nature and concentrations of metabolites reaching the target tissues. The Phenol-Explorer database is publicly available and can be found online at http://www.phenol-explorer.eu.

Database URL: http://www.phenol-explorer.eu

Introduction

Polyphenols are secondary plant metabolites abundant in many plant foods (1). An average dietary intake of 1–2 g/day has been reported in several Western populations with fruits and beverages such as tea, coffee, wine and fruit juices recognized as the major dietary sources (2, 3). Polyphenols have long been known to exert a range of biological activities, and their intake has been associated

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with a reduced risk of chronic diseases, such as coronary heart disease, stroke, type 2 diabetes and some cancers in various epidemiological studies and clinical trials (4–6). For this reason, they are now regarded as important components of a healthy diet and are thought to be partly responsible for the health benefits of an increased fruit and vegetable consumption (7). They may also explain the protective effects of tea against cardiovascular diseases (8, 9) or of coffee against type 2 diabetes (10). In the last 15 years, scientific and commercial interest in polyphenols has grown dramatically, and several thousand studies investigating their bioactivities, metabolism and health effects are published every year.

Since its launch in 2009, the first release of Phenol-Explorer (Phenol-Explorer 1.0) has aided such research with its ability to generate an instant estimate of the polyphenol composition of a queried food, based on data from as many quality-assessed publications as possible (11). Similarly, it was possible to search for a phenolic food component and retrieve all foods in which this polyphenol had been identified, along with average concentrations. This ease of data retrieval and comprehensive aggregation of studies have made the database a unique and essential tool for polyphenol scientists.

A detailed knowledge of the bioavailability of the various polyphenols and the nature and concentrations of the metabolites formed in the body is also essential to understand their effects on health. Most polyphenols are glycosylated in foods and are deglycosylated in the brush border of the small intestine or by colonic microbiota and re-conjugated to water-soluble glucuronides and sulfate esters and methylated in the intestine and the liver (12). Conjugated metabolites are then rapidly eliminated in the bile and urine. Polyphenols not absorbed in the small intestine are metabolized by the microbiota in the colon to a variety of low-molecular-weight phenolic acids, which may be absorbed into systemic circulation.

Knowledge of polyphenol metabolism has grown considerably during the past decade, but data are inherently difficult to search for and place into context because of the variable nature of doses, study organisms and time scales used in experimental studies. This information is still scattered in a large number of scientific publications, and its analysis often requires laborious searches, and no dedicated database has been available so far. More general databases contain scattered information on polyphenol metabolism; for example, the Human Metabolome Database (http://www.hmdb.ca) contains data on some polyphenols and gives example concentrations of the parent compound in biofluids as observed after the consumption of a few selected foods (13). Also, the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) contains information on some polyphenol metabolites (14). However, a large number of known polyphenol metabolites are missing from these databases, and the identities of phase II conjugates of a given polyphenol are not listed. Detailed pharmacokinetic data on these compounds are not available.

Here, we report the second major release of Phenol-Explorer (Phenol-Explorer 2.0), which extends the utility of the database by allowing the retrieval of information pertaining to the *in vivo* metabolism and pharmacokinetics of dietary polyphenols. The database now includes identities of all known plasma and urinary polyphenol metabolites together with detailed pharmacokinetic data. All food sources that have been reported to give rise to one of these metabolites can be retrieved. Finally, the results of these searches are accompanied by detailed pharmacokinetic data [maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), area under concentration-time curve (AUC), half-life of elimination ($T_{1/2}$), urinary recovery and plasma or urinary time courses where available. Searches are made through the existing user-friendly and intuitive web interface, and metabolism data are linked to food composition data. We believe that this new release will be the most powerful and useful resource to easily identify those polyphenol metabolites and respective concentrations most relevant for future experimental research or most useful as biomarkers of exposure for future epidemiological studies.

A limit of our current knowledge is that much of the evidence on the role of polyphenols in the prevention of chronic diseases arises from clinical trials where complex polyphenol-rich foods were administered (6). More data on the properties and effects of individual polyphenols are clearly needed, and this implies the collection and use of detailed information on food composition and polyphenol metabolism, as well as new clinical trials with pure polyphenol supplements (15).

Compilation of data and implementation of the update

A systematic search was performed for literature on polyphenol metabolism, published up until 1 March 2011. Search terms were built from a template that included the biofluid analyzed, the selected animal or human species and the names of the polyphenols and polyphenol classes of interest (Table 1), and these were applied in parallel to three databases: the Cochrane Library (from 1800; www.thecochranelibrary.com), MEDLINE Pubmed (from 1950, www.pubmed.org) and the ISI Web of Knowledge Science Citation Index Expanded (1945 to present). The publications retrieved were compiled and organized using Endnote (Thomson Reuters, New York, NY).

After the screening of titles and abstracts, reviews, *in vitro* studies and any duplicates were excluded

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Table 1. Search strategy and terms

Search steps	Search terms	Number of publications
1	urin* or plasma* or metabol*	>100 000
2	Dietary intake or intake* or exposure* or consum* or supplementation* or dietary supplements or supplement* or intervention study or experiment* or exp clinical trial or interven* or feasibility study or pilot study	>100 000
3	Human* or volunteer* or participant* or subject* or rat* or mouse* or mice* or rabbit* or pig* or sheep* or ewe* or dog*	>100 000
4	Polyphenol* or flavan* or flavon*or anthocyan* or isoflav* or phytoestrogen* or phyto-estrogen* or lignin* or stilbene* or chalcon* or phenolic acid* or ellagic* or coumarin* or hydroxycinnamic* or quercetin* or kaempferol* or rutin* or apigenin* or luteolin* or catechin* or epicatechin* or gallocatechin* or epigallocatechin* or procyanidin* or hesperetin* or naringenin* or cyanidin* or malvidin* or petunid* or peonid*or daidz* or genist* or glycit* or equol* or gallic* or vanillic* or chlorogenic* or tyrosol* or hydoxytyrosol* or resveratrol* or viniferin*	>100 000
5	Steps 1–4 combined	10 780

(Figure 1). Of the remainder, full-text articles were obtained for more detailed analyses. Articles were subsequently included or excluded according to various criteria. For inclusion, studies needed to (i) be conducted *in vivo* on humans or animals; (ii) be intervention studies using a single or repeated dose of a normal food source, an experimental food or an oral supplement; (iii) use an appropriate analytical technique, preferably mass spectrometry, capable of reliably identifying metabolites; (iv) detect or quantify at least one polyphenol metabolite in urine or plasma; (v) use disease-free humans or animals as subjects; (vi) present data in a usable format and (vii) be published in English. In addition, some articles were rejected at the compilers' discretion due to a lack of clarity or essential information or due to an excessively complex study design. Finally, only metabolites with fully defined structures were included; for example, data on quercetin 3-glucuronide would be included but not, for example, quercetin glucuronide, as quercetin may be glucuronidated at different positions on the phenolic rings.

As with Phenol-Explorer 1.0, data were compiled by means of a Microsoft Access database. The physical design of Phenol-Explorer 1.0 consisted of five main tables (4). Four of these were independent tables named Foods, Publications, Compounds and Methods, whose records were allocated autonumbers. A fifth table, Food Composition, listed each concentration value collected as a record, accompanied by codes linking to the food, publication, polyphenol quantified and method of analysis. To incorporate metabolism data, two extra tables were added (Figure 2). The Interventions table lists every separate intervention experiment, defined as a combination of the dose type, dose quantity and organism. Interventions table is linked, by many-to-one relationships, to Foods and Publications. The Biofluid Composition table lists concentration values of metabolites in plasma or urine and detailed temporal data. Biofluid Composition is linked by many-to-one relationships with Interventions, Compounds and Methods. The following is a description of each of the seven tables and their contents.

Foods

In the Phenol-Explorer 1.0 release, the table only contained foods representative of those regularly consumed in various populations. For Phenol-Explorer 2.0, experimental foods specific to a particular intervention study, such as polyphenol-rich extracts, enzymatically modified foods or pure polyphenol preparations, have been added.

Compounds

Fully defined polyphenol metabolites described in the intervention studies were added to this table. Compound classification was left unchanged from Phenol-Explorer 1.0, although an extra class to include non-phenolic breakdown products was added.

Publications

Bibliographic details for intervention studies were added to this table.

Methods

Methods of analysis for metabolites in intervention studies were classed as high performance liquid chromatography, liquid chromatography–mass spectrometry or gas chromatography–mass spectrometry with or without enzymatic hydrolysis before analysis, giving a total of six methods. Polyphenol metabolites are often analyzed after enzymatic hydrolysis to group together glucuronide and sulphate esters and simplify the chromatograms.

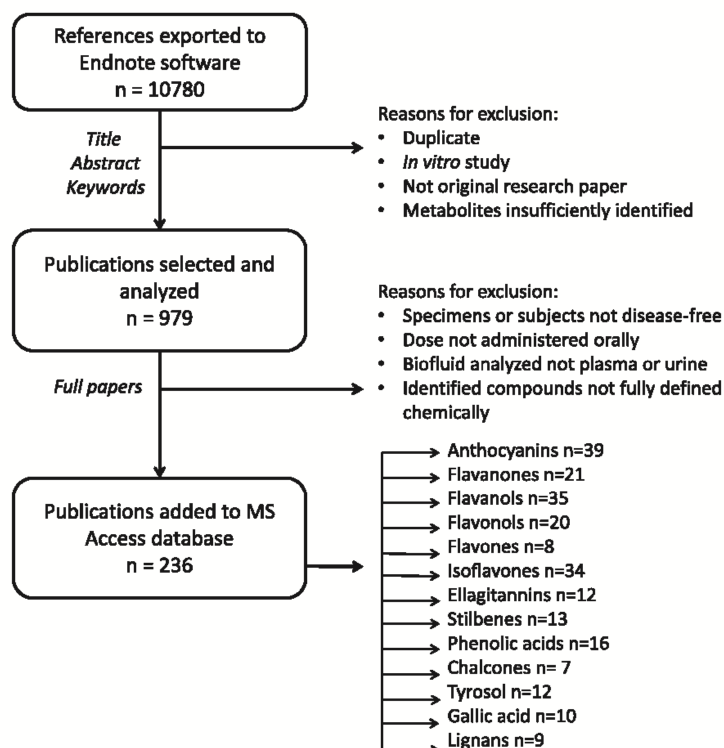

Figure 1. Scheme showing process of literature review and data inclusion.

Food composition

If the polyphenol composition of the dose was specified in the publication, it was entered in this table in the same way as for the food composition data. Polyphenol compositions of experimental foods and supplements were entered in addition to those of normal foods. Compounds that were not precisely chemically defined were omitted. Food composition data from metabolism studies were not aggregated with existing data from the first release used to produce mean polyphenol content values in foods but were kept separate for display with individual intervention studies.

Interventions

Studies carried out in each publication were split into separate interventions, each being a combination of a dose type or source, dose quantity administered and organism. All aspects of the intervention study design as described in the publication were recorded, such as information on number of subjects/animals, doses (multiple or single) and duration of intervention.

Biofluid composition

This table is analogous to the Food composition table, and each record corresponds to one metabolite detected or quantified in the intervention study. Each record contains information on the metabolite as described, a numeric identifier based on the polyphenol class and subclass, the analytical method used, details of sample preparation and any enzymatic hydrolysis, a plasma or urinary time course with up to six time points (baseline and five subsequent time points of any duration), C_{max}, T_{max}, $T_{1/2}$, AUC and urinary recovery. Biofluid composition data were extracted from the text, tables and figures of each publication.

The MS Access database was finally exported to a MySQL database, which is able to feed the web interface. The Phenol-Explorer site is built in the Ruby on Rails (RoR) framework (http://www.rubyonrails.org). RoR employs the Model-View-Controller design pattern and allows the construction of a reliable and responsive web application. The publicly accessible Phenol-Explorer 2.0 web server runs from an Apache 2 web server on a Debian Linux system.

Database, Vol. 2012, Article ID bas031, doi:10.1093/database/bas031

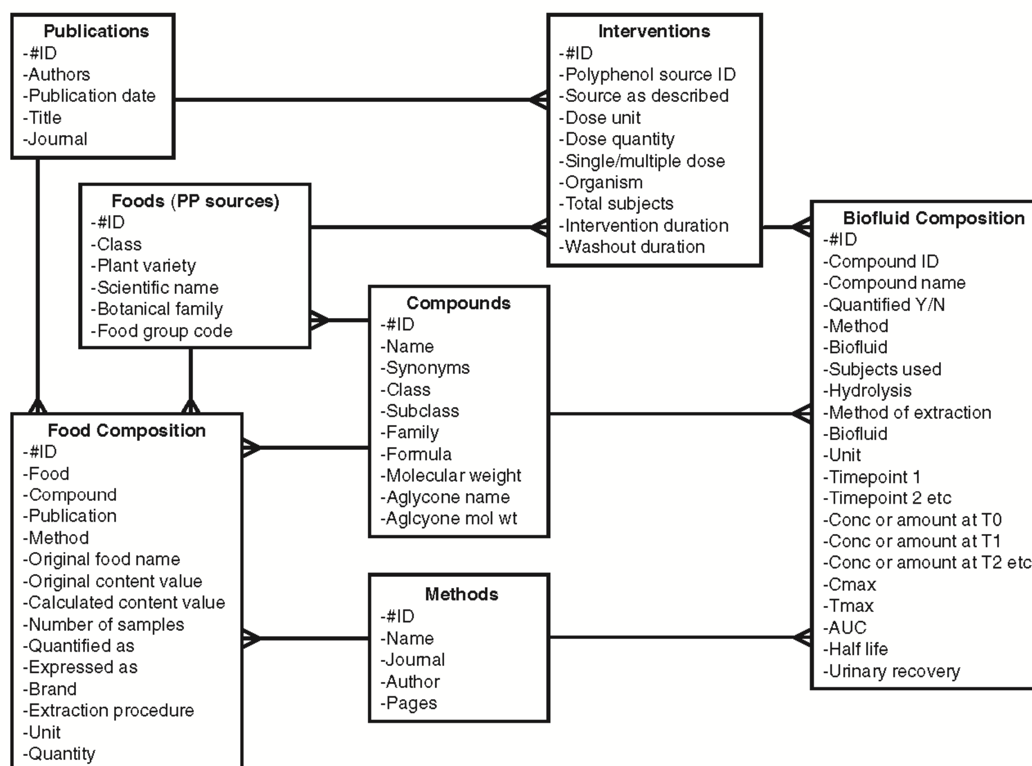

Figure 2. Entity-relationship diagram of the Phenol-Explorer 2.0 database showing new tables.

Querying and output

Metabolism information is retrieved in the same way as food composition information, although searches have been enhanced and refined. The 'simple search' has been retained from the first release and allows the entry of terms into a search box in the top-right hand corner of any page on the site. When a search term, i.e. a name of a food, polyphenol or polyphenol metabolite, is entered, a list of matching items is displayed in the form of boxes, which contain links to data. For example, if 'wine' is typed, eight matching foods and four matching food groups are displayed. These boxes are visually oriented and contain photographs of foods or chemical structures for phenolic compounds, and more detailed data are accessed by hyperlinks. In the first release, food boxes contained the links 'show polyphenol content' and 'show polyphenol distribution'. For the second release, these are now supplemented with 'show metabolites', where available. Similarly, polyphenol boxes contain the additional link 'show pharmacokinetics' in addition to 'show content in foods'. There is also a new box type for matches corresponding to polyphenol metabolites. In the same way as for polyphenols, a chemical structure is shown, accompanied by the single link 'show food sources and pharmacokinetics'. If a compound is both a food component and metabolite, it appears separately as both a 'polyphenol' and a 'polyphenol metabolite', distinguishing the routes taken to arrive at food composition or metabolism data. Therefore, after the retrieval of a food or compound or interest using the simple search, the user chooses whether to then pursue food composition or *in vivo* metabolism data.

Alternatively, data may be retrieved using links in the main toolbar, which appears at the top of every page. This toolbar allows the complete list of foods, food polyphenols or polyphenol metabolites to be browsed. The 'Metabolism' tab is new for Phenol-Explorer 2.0, and the user is able to browse the whole list of metabolites (Figure 3). This list may be filtered by polyphenol class and subclass using a scrolling box at the top of the screen. For each metabolite, the molecular weight, species in which identified, biofluid and the number of references in which detected are shown. New text boxes at the top of each column allow the further filtering of data according to

Figure 3. Phenol-Explorer 2.0 page layout and search methods.

these criteria. A clickable button links to a list of all sources (foods, food extracts or pure compounds) that have led to the detection of a given metabolite in either urine or plasma.

Upon retrieval of a food, a link leads directly to a list of all metabolites identified after consumption of that food, which is displayed in the form of a data table (Figure 4). Metabolite identified in biofluids, composition of the source, range of doses administered, number of recipients of the dose, total number of subjects, urinary and plasma pharmacokinetic data and reference information are displayed. Urinary pharmacokinetic data consist of two columns, one for mean urinary excretion and another for a graph icon, if a time course of excretion is available. Plasma pharmacokinetic data are further divided into five columns, one for each of T_{max}, C_{max}, $T_{1/2}$, AUC and another for a graph icon, if a plasma concentration time course is available. Metabolites are grouped according to whether they were detected before or after enzymatic deconjugation and by human or animal studies.

Phenol-Explorer 2.0 is, to the best of our knowledge, the first searchable database to contain urinary excretion and plasma concentration time courses for any metabolites formed in humans or experimental animals. These graphs are displayed by clicking on the graph icon corresponding to a particular polyphenol source or metabolite (Figure 4). References are displayed at the top of the page followed by the graph(s) and corresponding data in tabular format. If more than one quantity of the same dose was administered, the corresponding curves are overlaid. In addition, there is a tick box ('show related metabolites from the same publication') allowing the user to overlay comparable data from the same study, such as different metabolites after administration of the same polyphenol source or the same metabolite after administration of experimental and control foods.

Discussion

Metabolism studies were variable in their design, and certain rules for handling different doses and time scales, analytical methods and units had to be fixed for data entry. In many studies, a single dose was given, and biofluids were taken for analysis at one or more time points after this dose. In others, the same dose was repeated continuously over a certain time or incorporated as part of a standardized diet (in the case of animal studies). Output data resulting from repeated doses are clearly marked as such. The dose unit is then usually expressed in weight unit per day and the time period over which the dose was repeated clearly stated.

A limited number of conjugated polyphenol metabolites (53 glucuronides and 23 sulfate esters) have been fully identified in the literature, mainly due to the limited availability of commercial standards. Polyphenol metabolites have more often been identified as aglycones after hydrolysis by β-glucuronidase and sulfatase enzymes. Therefore, analytical data obtained with and without enzymatic deconjugation are reported separately.

Plasma and urinary pharmacokinetic data were expressed in publications in a wide range of units. The treatment of plasma data was more straightforward than that of urinary data, because the former are almost always point concentrations expressed in moles or weights per volume. Plasma concentrations expressed as weights per volume were converted to molar concentrations for easier comparison. Urinary data may also be point concentrations. Excretion values are sometimes normalized by creatinine

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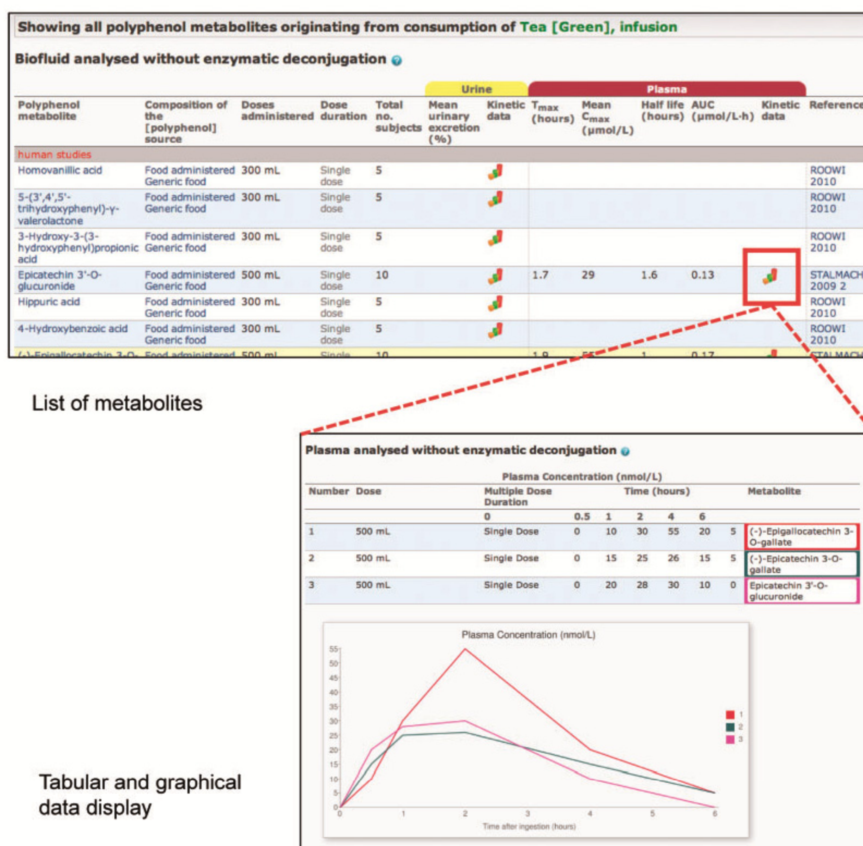

Figure 4. Tabular and graphical data output from Phenol-Explorer 2.0.

excretion but are more commonly expressed as an amount excreted, in moles or grams, during a particular time period. They may also be expressed as a percentage of the polyphenol dose in intervention studies with pure polyphenols or when the nature of the ingested polyphenol can be identified without any ambiguity. Urinary data could not be standardized to a common unit, as creatinine concentration and urine volume are most often missing from the publication. The original unit was therefore retained. To graphically represent amounts of metabolite excreted over given time periods (such as 0–2 h and 2–5 h), amounts of metabolite excreted were summed to produce cumulative urinary excretion curves.

The database allows various fundamental questions on food polyphenol metabolites to be answered. The main aim of Phenol-Explorer 1.0 was to easily retrieve the polyphenol content of a food of interest. Phenol-Explorer 2.0 now allows to retrieve all reliably identified plasma and urinary metabolites formed from a given food, food extract or pure polyphenol. Therefore, while the user could previously obtain an instant estimate of the polyphenol intake from an intervention study dose, Phenol-Explorer 2.0 enables the display of all metabolites identified in plasma and urine, along with plasma concentrations, pharmacokinetics and a minimum extent of absorption as estimated by urinary recovery. Similarly, if the researcher was interested in a particular polyphenol, they could instantly find out from Phenol-Explorer 1.0 which foods provide that polyphenol. With the second release, researchers interested in a particular polyphenol metabolite (whose bioactivity may have been postulated from *in vitro* studies) may instantly recall a list of foods known to give rise to that metabolite. This information is key in the identification of putative biomarkers of exposure specific to different dietary polyphenols and can be used to better characterize exposure in population studies (16).

In addition, every effort has been made to retain comparisons between polyphenols and sources made in a given intervention study; if the plasma time course of a metabolite of interest is recalled, a link then allows the graphical

comparison of different metabolites examined in the same study. Phenol-Explorer 2.0, as well as providing answers to general questions on polyphenol metabolism, incorporates the comparisons made within individual metabolism studies.

Phytochemical databases were recently reviewed (17), and Phenol-Explorer 2.0 is the first to include chemical information on this important class of phytochemicals as well as comprehensive information on food composition, metabolism and pharmacokinetics. Databases containing metabolism and pharmacokinetics data for certain drugs are already available (18, 19), although Phenol-Explorer is the first to contain similar data for natural or dietary compounds and to allow all retrieved information to be traced back to the original publications. It is also unique in that all intervention studies have been systematically analyzed and relevant data comprehensively curated. The database will be periodically updated to ensure that the current state of polyphenol metabolism research is adequately represented.

Conclusions

Phenol-Explorer was initially conceived to allow the retrieval of screened and aggregated compositions of polyphenols in foods, enabling definitive data to be obtained in a matter of seconds and saving the user the time and effort of manual literature searches. Similar objectives were intended for Phenol-Explorer 2.0 and its metabolism data. We believe that this update has transformed Phenol-Explorer to a more comprehensive polyphenol knowledge base, especially when food composition data are used in tandem with metabolism data. Phenol-Explorer 2.0 should greatly aid the advance of the understanding of the relations between polyphenol consumption and health by helping researchers design and interpret studies on these important dietary components.

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Conflict of interest: none declared.

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4.2 PREDICCIÓN DEL ESPACIO METABÓLICO DE LOS POLIFENOLES DEL VINO. APLICACIÓN DE LA BASE DE DATOS PHENOL-EXPLORER.

Con el objetivo de ampliar el conocimiento sobre el metabolismo derivado del consumo de vino, se ha usado la base de datos Phenol-Explorer para predecir el mayor número de metabolitos tras su consumo. Para ello se ha establecido una estrategia de búsqueda donde todos los metabolitos derivados de su consumo y de los compuestos que lo componen quedasen reflejados en una misma ruta metabólica.

Este trabajo ha sido publicado en una de las revistas considerada por el *Science Citation Index*, con un índice de impacto de 4.310 y situada en el primer cuartil de la categoría *Food Science & Technology* (4 de 124).

Publicación 2: María Boto-Ordóñez, Joseph A. Rothwell, Cristina Andres-Lacueva, Claudine Manach, Augustin Scalbert, Mireia Urpi-Sarda. Prediction of the wine polyphenol metabolic space: an application of the Phenol-Explorer database. *Mol Nutr Food Res,* 2013; Oct 9. DOI: 10.1002/mnfr.201300411.

Resumen:

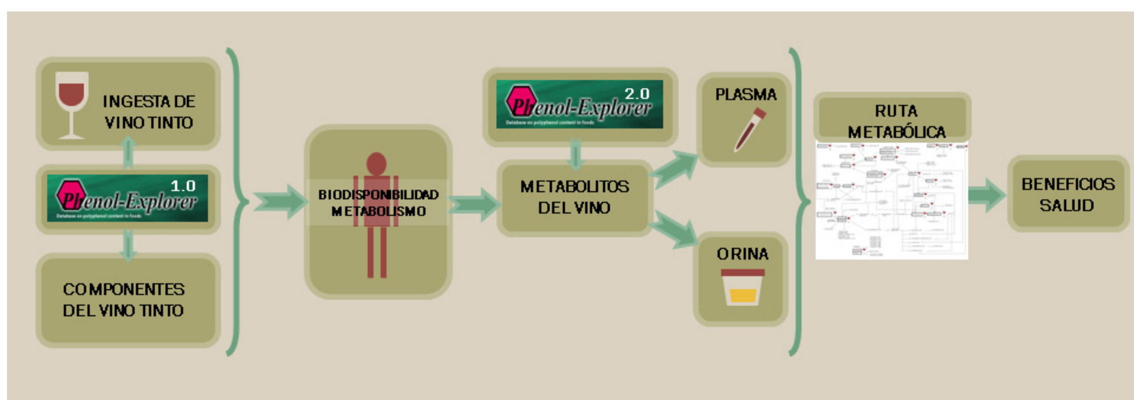
El conocimiento de los metabolitos polifenólicos *in vivo* derivados del consumo de vino tinto y productos derivados podría ser clave para entender sus beneficios para la salud. Este trabajo tuvo como objetivo predecir el espacio metabólico de los polifenoles del vino en biofluidos utilizando todos los datos disponibles recogidos en la base de datos de Phenol-Explorer.

Se desarrolló una estrategia de búsqueda en Phenol-Explorer para obtener la más amplia gama de metabolitos relacionados con el consumo de vino y productos derivados del vino. Se describieron un total de 97 metabolitos en estudios de intervención con vino y productos afines (n=37), y después del consumo de compuestos puros que se saben constituyentes del vino (n=90). Estos 97 metabolitos, derivados del metabolismo de fase II y metabolismo microbiano de varias clases de polifenoles, se encontraron en el plasma y/o orina, alguno de los cuales se les había

descrito una mayor actividad biológica que el compuesto original en estudios *in vitro*. Los metabolitos generados se relacionaron, por primera vez, en una ruta metabólica global de los polifenoles del vino *in vivo*.

La recuperación de la más amplia gama de metabolitos hasta ahora descritos y su ensamblaje como ruta metabólica podría ayudar a la identificación de posibles biomarcadores de consumo de vino, y mejorar la comprensión actual de los efectos saludables de dicho consumo.

Figura Resultados R2: Gráfico resumen del estudio de predicción del espacio metabólico de los polifenoles del vino mediante la aplicación de la base de datos Phenol-Explorer.



Mol. Nutr. Food Res. 2013, *00*, 1–12 DOI 10.1002/mnfr.201300411 1

RESEARCH ARTICLE

Prediction of the wine polyphenol metabolic space: An application of the Phenol-Explorer database

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Scope: Knowledge of in vivo polyphenol metabolites derived from the consumption of red wine could be key to understanding its health benefits. This work aimed to predict the wine polyphenol metabolic space in biofluids by using all available data compiled in the Phenol-Explorer database.

Methods and results: A search strategy was developed for Phenol-Explorer to obtain the widest range of metabolites related to wine consumption. A total of 97 metabolites have been described in intervention studies with wine and related products ($n = 37$), and after consumption of pure compounds known to be wine constituents ($n = 90$). These 97 metabolites, derived from host and microbial metabolism of several classes of polyphenols, were found in plasma and urine samples and some of them have demonstrated higher or lower biological activities than the parent compound in in vitro studies. The metabolites have been linked to generate, for the first time, a global pathway map of wine in vivo polyphenol metabolism.

Conclusion: The retrieval of the widest range of metabolites so far described and their assembly as a metabolic pathway map could aid the identification of possible biomarkers of wine consumption and improve current understanding of the health effects of wine consumption.

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Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Wine is an alcoholic beverage that is consumed worldwide. The highest consumption in 2010 was in South European countries such as Italy, France, and Luxembourg with a mean consumption per capita of 46.8 L [1]. From "The French Paradox" [2], the number of studies that have linked red wine consumption with health benefits has markedly increased. A moderate intake of red wine has been associated with a reduction in mortality rate and a lower incidence of cardiovascular diseases and other diseases [3–5], but the molecular mechanisms involved are not fully understood. As major components of wine, both polyphenols and alcohol could be responsible for these health-promoting properties [6–8]. The health effects of polyphenols depend on chemical structure, bioavailability, food matrix, and habitual diet, and are likely to vary between individuals [9]. In vivo, polyphenols usually undergo glucuronidation, sulfation, and/or methylation in the intestine and later in the liver, producing conjugated metabolites [9, 10]. This metabolism may affect the biological effects of absorbed polyphenols. The conjugated metabolites are more readily eliminated back to the intestinal lumen or

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Abbreviations: GlcUA, glucuronide; **MET**, methyl; **SULF**, sulfate; **WED**, without enzymatic deconjugation; **WP**, wine product

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via the bile, undergoing an enterohepatic cycle where conjugated metabolites may be metabolized by colonic microbiota to smaller molecules, such as phenolic acids, before being reabsorbed or eliminated through feces [10]. Metabolites have been shown to reach various target tissues where they may exert their biological effects [9,10]. Finally, better knowledge of the nature and levels of polyphenol metabolites in tissues is key to understanding the beneficial effects attributed to polyphenol-rich foods [11]. Until now, few studies have evaluated the biological activities of wine polyphenol metabolites in vitro because these compounds are usually not commercially available [12–14].

The metabolism of wine phenolic compounds has scarcely been studied and has been limited to a few in vivo studies focusing on a single component such as resveratrol or catechin [15–19]. The data and metadata associated with studies on dietary phytochemicals are complex and include data on the composition of foods, in vivo metabolism, and concentrations in biofluids, which makes the data analysis not straightforward. Database development in the field of health and nutrition has therefore become essential to exploit this information with more than 50 open-access or commercial databases related to these topics published [20]. Recently, we have developed the first user-friendly open-access web database to contain comprehensive data on polyphenol metabolites: Phenol-Explorer [21], which was first developed to contain data on polyphenols in foods [22] (www.phenol-explorer.eu).

Here, we aim to use Phenol-Explorer to predict the profile of polyphenol metabolites after red wine consumption as comprehensively as possible. By using a qualitative and quantitative approach, we aim to determine metabolites described in intervention studies after grape products intake, mainly red wine, and metabolites derived from the consumption of polyphenols known to be present in wine.

2 Materials and methods

2.1 Strategy for using Phenol-Explorer

Phenol-Explorer is the most detailed database on polyphenol metabolism data [21,22]. Phenol-Explorer contains more than 37 000 content values for 506 polyphenols in 455 foods (release 1.0), contains comprehensive data on polyphenolic metabolism with more than 370 polyphenol metabolites identified in biofluids from 236 in vivo peer-reviewed publications (release 2.0) [21], and contains data on the effects of 35 food processes on 155 foods and more than 130 polyphenols from 129 publications (release 3.0).

For this study, data on metabolites reported after consumption of wine products (WPs) (red wine, dealcoholized red wine, phenol-stripped red wine, wine extract capsules, red wine powder, white wine, and sparkling wine) and metabolites reported after consumption of pure compounds found in WPs were retrieved using a step-by-step process (Fig. 1). To retrieve the list of metabolites from wine-derived food sources and extracts, "Metabolism" would be selected from the site's main toolbar followed by "Administered Food & Food Extracts." At this point, a qualitative and quantitative metabolic picture of the metabolism of the polyphenolic components of WPs and its extracts is displayed. Similarly, the database can be used to retrieve those metabolites reported after consumption of some pure polyphenols found in red wine. There are two possible routes to these data: (i) by selecting an individual polyphenol listed as a constituent of red wine (e.g. (−)-Epicatechin) and then selecting "Show Metabolites" or (2) through the "Metabolism" tab followed by "Administered Pure Compounds" and then "Metabolites." Another additional option that Phenol-Explorer provides for a phenolic component is the link "Show Pharmacokinetics," which displays the studies in which this polyphenol has been found as a metabolite and its kinetic data. Additionally, the tab "Show content in foods" displays all the foods that contain this metabolite.

3 Results

3.1 Polyphenolic composition of red wine based on Phenol-Explorer

Knowledge of the phenolic composition of wine and WPs is essential for identifying the bioactive compounds and their metabolites that may contribute to its health effects. Table 1 summarizes Phenol-Explorer data for red wine polyphenol classes and subclasses, with their mean concentrations and their principal compounds present in red wine. A total of 75 polyphenols have been quantified in red wine, of which flavan-3-ols were found in the highest concentrations, followed by anthocyanins.

3.2 Determination of the wine polyphenol metabolic space after consumption of WPs or pure compounds

The number of metabolites reported after consumption of WPs (red wine, dealcoholized red wine, phenol-stripped red wine, wine and red grape extract capsules, red wine powder, white wine and sparkling wine) and those reported after consumption of pure compounds are represented in a Venn diagram (Fig. 2A). A total of 37 metabolites were derived from consumption of WP. A total of 90 metabolites were reported after consumption of pure compounds, known to be wine constituents, of which 30 were common to both groups. Therefore, 97 metabolites may be expected as a result of WP consumption (Fig. 2A). When these metabolites were compared with red wine constituents, 24 compounds were common to both groups while ten polyphenols were common to WP and pure compound metabolites and wine composition. In relation to biofluid, 90 metabolites were found in urine and 59 in plasma (Fig. 2B), 52 being common to both.

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Aim

To investigate metabolism of polyphenols from a particular food, eg red wine

Link: Show Retention Factors

Enter food in simple search

Link: Show Polyphenol Content

Displays polyphenol in food and its content. E.g.

Flavanols
(+)-Catechin
(-)-Epicatechin
Procyanidin B3
Anthocyanins
Malvidin 3-O-glucoside
Malvidin 3-O-(6"-acetyl-glucoside)
Stilbenes
Resveratrol-3-glucoside

See **Table 1** for polyphenol content of Red Wine.

Conclusion: Polyphenolic composition of the selected food retrieved

Go to main menu bar

Metabolism → Administered Food & Food Extracts

Displays all sources available in Phenol-Explorer with Metabolism data.

Common Name
wine
Wine and grape juice, extracts, in cellulose capsule
Wine [red, dealcoholized]
Wine [red, phenol stripped]
Wine [Red]
Wine [Sparkling]
Wine [White]
Wine, powder

Metabolites

-Qualitative Approach.: Displays metabolites in studies after red wine consumption.
-Quantitative Approach: Displays the pharmacokinetic parameters of these metabolites.

Conclusion: Have retrieved metabolites studied and identified after a polyphenol-rich food (eg red wine) and their concentrations in vivo

Which metabolites are expected in biofluids after pure compounds consumption?

Go to main menu bar

Metabolism → Administered Pure Compounds

Metabolites

Link: Show Metabolites

-Qualitative Approach.: Displays a list of metabolites from studies administering pure polyphenol.
-Quantitative Approach: Displays the pharmacokinetic parameters of these metabolites after pure polyphenol consumption.

Conclusion: Have retrieved metabolites expected from red wine consumption based on knowledge of metabolism of pure compounds.

Select a polyphenol from list. E.g.

Epicatechin

Link: Show Pharmacokinetics

-Qualitative Approach.: Displays all the studies where this metabolite/compound has been identified
Quantitative Approach: Displays the pharmacokinetic parameters of these metabolites.

Conclusion: Bioavailability of compound retrieved based on data from other foods.

Link: Show Content in Foods

Displays all the foods that contain this polyphenol.

Show Retention Factors

(Phenol-Explorer display)

Figure 1. Flow diagram of a Phenol-Explorer search to predict polyphenol metabolites after the consumption of a given food.

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Table 1. Content of polyphenols in red wine analyzed by chromatography [22]

Polyphenol class	Total (mg/100 mL)[a)]	Range (mg/100 mL)	Main polyphenols
Flavonoids			
Flavan-3-ols	40.1[b)]	30.78–86.53	Proanthocyanidin oligomers > (+)-Catechin > (−)-Epicatechin
Anthocyanins	22.3	2.32–76.51	Malvidin 3-*O*-glucoside > Malvidin 3-*O*-(6′′-acetyl-glucoside) > Malvidin 3-*O*-(6′′-*p*-coumaroyl-glucoside)
Flavonols	6.9	1.97–15.40	Quercetin 3-*O*-rhamnoside > Quercetin 3-*O*-glucoside > Quercitin
Dihydroflavonols	5.4	4.58–5.98	Dihydromyricetin 3-*O*-rhamnoside
Flavanones	0.85	0.78–0.94	Naringin
Phenolic acids			
Hydroxycinnamic acids	10.0	1.37–22.05	Caffeoyl tartaric acid > 2,5-di-*S*-glutathionyl caftaric acid
Hydroxybenzoic acids	7.01	1.49–3.76	Gallic acid
Hydroxyphenylacetic acids	0.16	0.11–0.21	4-Hydroxyphenylacetic acid
Stilbenes			
Stilbenes	3.41	0.64–13.98	Piceatannol 3-*O*-glucoside> d-Viniferin> Resveratrol 3-O-glucoside
Other polyphenols			
Tyrosols	3.65	0.64–5.43	Tyrosol
Hydroxybenzaldehydes	0.71	0.00–4.56	Syringaldehyde

a) Values are the sum of individual means of red wine polyphenols in Phenol-Explorer [22]. Contents measured by chromatography with no previous hydrolysis of the glycosides and esters.
b) Sum of catechins (determined by chromatography) and proanthocyanidin oligomers (determined by direct phase HPLC).

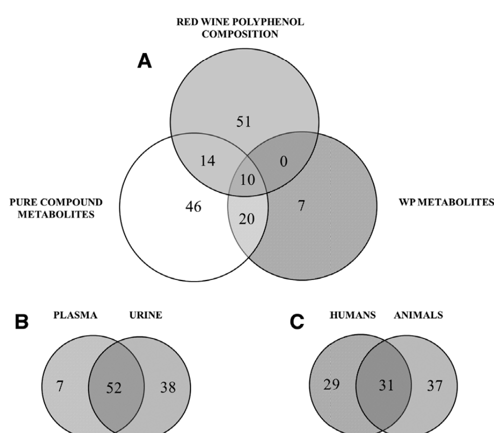

Figure 2. Venn diagrams showing the overlap between metabolites from (A) phenolic wine constituents wine products (WPs) and pure compounds metabolites; (B) plasma and urine; and (C) human and animal metabolites distribution.

Twenty-nine metabolites were found only in human studies, 37 only in animal studies, and 31 were common to both groups (Fig. 2C).

The chemical classification of the 97 reported metabolites is summarized in Table 2. 55 metabolites were aglycones, 5 were glucosides (exclusively anthocyanin metabolites), 17 were glucuronides (GlcUAs), 6 were sulfates (SULF), 16 were methylated (MET), and 3 were glycinated. They included five diconjugated metabolites (three MET-glycosides and two MET-GlcUAs). These results corresponded with the fact that more than half the total metabolites were aglycones (57%) followed by GlcUA (18%), MET (16%), SULF (6%), glycosylated (5%), and glycinated (3%) conjugates. The high percentage attributed to aglycones was due to the fact that 39% of aglycones were identified after enzymatic hydrolysis of samples while 37% of them were free aglycones in the biological samples. The 24% remaining were determined after both enzymatic and nonenzymatic sample pretreatment.

The range of maximum plasma concentration of metabolites for the main polyphenol classes present in wine is presented in Table 3. To facilitate comparison, only studies that provided maximum plasma metabolite concentrations after ingestion were used. For animal studies, plasma concentrations after WPs were not included since maximal plasma concentration values were not available in these studies. After WP consumption in humans, resveratrol conjugates were the only metabolites quantified without enzymatic deconjugation. In addition, the most abundant metabolites in hydrolyzed plasma samples were hydroxybenzoic acids followed by hydroxycinnamic acids and flavan-3-ols. Few studies examined the plasma maximum concentrations of metabolites after consumption of pure compounds in humans. Only resveratrol has been more widely studied. In animals, flavanones, flavan-3-ols, and anthocyanins accounted for most of the polyphenol metabolites, although the concentration of metabolites of these studies depended on the administered doses.

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Table 2. Number of polyphenol metabolites identified after intake of red wine, red wine products, and pure wine polyphenols by class, subclass, and type of conjugation

	TOTAL[a)]	Aglycone	Glycoside	GlcUA[b)]	SULF[b)]	MET[b)]	Glycinated
Flavonoids							
Flavanols	11	4	0	4	0	5	0
Anthocyanins	5	0	5	0	0	3	0
Flavanones	6	4	0	2	0	0	0
Flavonols	2	1	0	0	0	1	0
Phenolic acids							
Hydroxybenzoic acids	17	14	0	0	0	2	1
Hydroxycinnamic acids	15	7	0	7	0	0	1
Hydroxyphenylacetic acids	10	9	0	0	0	1	0
Hydroxyphenylpropanoic acids	8	5	0	0	0	2	1
Hydroxyphenylpentanoic acids/lactones	7	6	0	0	0	1	0
Stilbenes							
Stilbenes	12	2	0	4	6	0	0
Other polyphenols							
Alkylphenols	1	1	0	0	0	0	0
Tyrosols	1	1	0	0	0	0	0
Others (1,3,5-trimethoxybenzene, 4-ethylbenzoic acid)	2	1	0	0	0	1	0

a) Totals represent the number of metabolites within each group present in Phenol-Explorer.
b) Some compounds are subject to more than one type of metabolism reaction and the total number of compounds may not correspond to the sum of each type.
GlcUA, glucuronide; MET, methyl; SULF, sulfate.

The identities of metabolites found in biofluids after intake of WP and pure compounds have allowed the proposal of a tentative global pathway of wine polyphenol metabolism (Fig. 3). Wine polyphenols described in Phenol-Explorer were selected as starting points (in boxes). The metabolites described after WP and pure compound intake were sequentially added. The metabolic routes described were based on knowledge of phenolic metabolism from in vitro and/or in vivo studies, and references were included for each pathway (Fig. 3) prioritizing those that analyzed metabolism of pure compounds. Based on polyphenol metabolism literature, metabolites from microbial origin were indicated in italics. Nearly all the compounds from wine, with the exception of stilbenes, lead to common metabolites, mainly phenolic acids that have been previously described to come from microbial metabolism (Fig. 3).

4 Discussion

In the present study, we have exploited the Phenol-Explorer database to produce a complete profile of those polyphenolic metabolites derived from the consumption of red wine. Metabolites derived from the administration of pure compounds, known to be present in red wine, were far more numerous than those derived from the consumption of red wine itself. This is first because much greater doses of polyphenols were administered from pure compounds than from foods. Consequently, higher concentrations of these metabolites facilitated their detection. Low concentrations of phenolic metabolites were expected after consumption of foods, and there is thus a need for sensitive analytical methods usable in nutritional studies. Second, most studies measured a limited number of red wine metabolites such as catechin or resveratrol derivatives, and some analyzed only hydrolyzed biofluids that limited the information regarding the metabolic profile of polyphenols [16–18]. Other than these, only certain metabolites of phenolic acids have been characterized after intake of WP [19, 23], and the metabolism of other polyphenolic classes after WP intake remains to be studied.

To our knowledge, only one study [24] has reviewed the metabolites obtained from each major class of wine phenolic groups. In contrast to the present study, metabolites with undefined position of conjugation were included, as well as, some identified metabolites in in vitro studies, such as the identification of glucuronidated and sulfated hydroxycinnamic metabolites in Caco-2 cells model [25] or in rat hepatocyte microsomes [26]. However, more metabolites have been elucidated from the Phenol-Explorer database due to the ability to combine doses of foods and pure compounds, demonstrating that the database can efficiently reproduce results from extensive literature searches in a harmonized format.

Some of the red wine derived metabolites were also known constituents of red wine (Fig. 2A). One possibility is that WP compounds arrive intact in biofluids, although in low concentrations [10] or that aglycone is detected because of the pretreatment of samples with β-glucuronidases and sulfatases that release the GlcUA or SULF conjugates from the parent compound [27, 28] (Fig. 2, Table 2). The metabolites not found as wine components were formed through host biotransformation reactions such as methylation [29, 30] or glycination [31] and through metabolism by intestinal microbiota

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Table 3. Range of maximum plasma concentrations (μmol/L) of metabolites by intervention type, specimen, and sample pretreatment

		Wine products	Pure compounds			
		Human	Human	Animal		References
Flavonoids						
Flavan-3-ols	ED	0.02–0.08	-a)	0.15–35.0	(−)-Epicatechin > 3′-*O*-METepicatechin > (+)-Catechin>3′-*O*-METcatechin > Procyanidin dimer B2> (−)-Epicatechin 3-*O* –gallate	[17, 18, 27, 29, 30, 56–59]
	WED	-	-	2.50–5.00		
Anthocyanins	WED	-	-	0.03–15.0	Cyanidin 3-*O*-glucoside> Delphinidin 3-*O*-glucoside> 4-*O*-METdelphinidin 3-*O*-β-d-Glucoside	[60–62]
Flavanones	ED	-	-	62.4–161	Naringenin	[38, 63, 64]
	WED	-		10.8–16.3		
Flavonols	ED	-	1.48b)	0.18–0.24	Isorhamnetin > Quercetin	[65–69]
	WED	-	0.05b)	-		
Stilbenes						
Stilbenes	WED	0.02–0.45	0.16–13.9	1.1–2.6	*trans*-Resveratrol-3-*O*-SULF> *trans*-Resveratrol > *trans*-Resveratrol 3,5-diSULF > *trans*-Resveratrol-4′-*O*-GlcUA> *trans*-Resveratrol 3,4′-diSULF> *trans*-Resveratrol-3-*O*-GlcUA	[16, 70–75]
Phenolic acids						
Hydroxybenzoic acids	ED	0.17–0.18	-	-	Ethylgallate > Protocatechuic acid > Gallic acid > 4-*O*-METgallic acid	[19, 27, 31, 61, 67, 76]
	WED	-	-	1.03–2.75		
Hydroxycinnamic acids	ED	0.08–0.09	-	1.68b)	Ferulic acid > Caffeic acid	[19, 31, 67, 77]
Hydroxyphenylpropionic acids	WED	-	-	0.32b)	3-Hydroxyphenylpropionic acid	[31, 59, 67]
Hydroxyphenylvalerolactones	ED	-	-	0.56b)	5-(3′,4′,-dihydroxyphenyl)-γ-valerolactone	[59]

a) No data available.
b) Only one value.
ED, with enzymatic deconjugation; GlcUA, glucuronide; MET, methyl; SULF, sulfate; WED, without enzymatic deconjugation.

releasing phenolic acid derivatives [32]. Microbial metabolism is important since more complex polyphenols such as flavanols, anthocyanins, and flavanones are broken down to a variety of simpler phenolic acids [32–34]. For instance, flavonol and flavan-3-ols intake produced 93% and 74%, respectively, of phenolic acid metabolites, mainly hydroxyphenylacetic, hydroxycinnamic, and hydroxybenzoic acids, and the remainder were phase II metabolites (data not shown).

The type of biological sample and the time of collection used in clinical and intervention studies are of great importance since they will influence the concentration and the number of metabolites detected. In this study, more metabolites were described in urine than in plasma. Analysis of urine is generally favored in intervention studies due to ease of collection and analysis. Nevertheless, comparison between different studies to give a concentration range value is difficult due to variability of sampling methods (24 hours versus spot urine), quantification (sample preparation and use of standards), and expression of results (accumulative excretion, percentage of dose excreted, 24-h excretion, and creatinine-adjusted concentration). In recent years, interest has grown in finding the most accurate and reproducible methodology to evaluate and compare the expression of results in biological samples [35–37]. When plasma samples were analyzed,

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Figure 3. Metabolic pathway tentatively proposed for the metabolism of wine polyphenols [15, 27, 28, 31–34, 56, 59, 62–64, 67, 73, 76, 78–100].

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several factors such as the time of collection after ingestion, method of extraction, and the use of enzymatic hydrolysis must be taken into account when interpreting results. The different compositions of WP, the matrix in which they were delivered, and the interindividual variability may also affect the number and concentration of metabolites in biofluids. The range of maximum plasma concentrations of metabolites is summarized in Table 3. The results showed that few studies administered WP and these usually studied a single compound group such as catechins, resveratrol, or phenolic acids, and the metabolic profile has only been considered for resveratrol [16–19]. The study of metabolism of pure compounds revealed a complex metabolic profile whose concentration depends on administered doses. In general, the results have suggested that flavan-3-ols are more bioavailable than other wine flavonoids [9], except for one study on flavanones, where bioavailability was higher but much higher doses (270 mg/kg bw) were administered [38]. This dose is far above of the real consumption through wine, as they are minor polyphenolic components. These data suggest that more studies are needed on the bioavailability of food polyphenols to allow associations to be made between their plasma concentrations and diet-related risk factors for diseases in nutritional epidemiology studies. To our knowledge, only a few studies have evaluated and compared the biological activities of metabolites with their parent compounds due to the limited availability of standards of metabolites. Supporting information Table 1 summarizes biological activities of wine metabolites compared with their parent compounds. Antioxidant activity was the most studied and was generally higher for parent compounds than for metabolites [39–45], whereas the opposite was observed for inhibition of NADPH oxidase of MET epicatechin [45], some valerolactones [46], and anthocyanin metabolites [47]. In addition, flavan-3-ol metabolites were more active than their parent compounds in some reactions related to the regulation of lipid metabolism [48], an activity that could explain the health benefits due to changes in lipoproteins associated with high intakes of flavanol-rich foods such as cocoa or wine [7, 49]. Stilbenes metabolites had lower anti-inflammatory activity than their parent compounds. This activity has been attributed to inhibition of cyclooxygenase 1 and 2, inhibition of NF-κB, nitric oxide production [13, 50], and delipidating effect over adipocites [14], each related to cardiovascular health. Furthermore, cytotoxic properties against cancer cells have been described for resveratrol metabolites, but weaker than those of its aglycone [50, 51]. However, the metabolites were more active in cancer chemoprevention than resveratrol through the induction of quinone reductase 1 and the less effective in the inhibition of quinone reductase 2 [13, 50]. Resveratrol aglycone produced a higher activation of sirtuin 1, a protein associated with the beneficial effects of resveratrol, than its sulfate derivatives [13].

A global pathway map of polyphenolic wine metabolites is proposed in Fig. 3. This is an important point since, until now, metabolic pathway maps on polyphenol metabolism after food consumption do not exist in available resources or databases. Therefore, this is the first attempt to develop them. In this pathway map, polyphenols from wine (in boxes) may be absorbed at intestinal level and pass into the bloodstream [52]. Those compounds not absorbed arrive intact in the colon where they are hydrolyzed by the colonic microbiota to phenolic acids [27,28] and then they could be absorbed and pass into the bloodstream. Absorbed metabolites from WP and pure compounds intake could be metabolized to their GlcUA, SULF, and METesters in the enterocyte, liver, and kidney before excretion in plasma and urine, reflecting the complex metabolism in vivo. All classes of wine polyphenols except stilbenes were connected through metabolism to phenolic acids (Fig. 3), suggesting that phenolic acids are common metabolites of major substrates as stated before after ingestion of berry polyphenols [53]. As shown, some metabolites reported to be formed by microbiota were also constituents of WP (Table 1). Thus, some of these metabolites are derived from both WP and metabolism of different WP flavonoids [19, 27]. In particular, hydroxybenzoic acids were the principal phenolic acid metabolites (Table 3) and also appeared to be the main endpoint of WP polyphenol metabolism (Fig. 3). However, considering the high content of gallic acid in WP, its presence in biological samples could be not limited to formation in the organism.

The present analysis on the metabolites formed after WP consumption and their concentrations could aid the identification of possible biomarkers of consumption. Previously, resveratrol has been proposed an effective biomarker of wine intake due to its specificity and distinct metabolic route in the global metabolism of wine polyphenols [54]. Gallic acid was another proposed biomarker of wine intake. It is present in wine but is also a metabolite of other wine components. However, its relatively wide distribution in foods also makes it a possible biomarker of other polyphenol-rich foods such as tea [36, 55]. In addition, phenolic acids might not be considered as potential biomarkers of intake of wine or other polyphenol-rich foods since they are common metabolites of several classes of flavonoids [53]. Thus, further studies are needed to assess the strength of individual or panels of metabolites as biomarkers of consumption, by evaluating their sensitivity and specificity, and hence their discriminatory power.

In conclusion, the present work has allowed the proposal of a global pathway built from the polyphenol metabolic profile obtained after wine intake by using the Phenol-Explorer database. This has allowed the retrieval of the widest range of metabolites so far described and enabled analysis of the potential of different metabolites as biomarkers of wine consumption. Knowledge of the whole spectrum of metabolites from the consumption of wine will be a key to understanding its health benefits.

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Online Supplemental Material

Supplemental Table 1. Comparison of biological activity of metabolites with their parent compounds.

Biological Activity	Parent compound	Metabolites with the same activity than parent	Metabolites with higher activity than parent	Metabolites with lower activity than parent	Ref
Flavan-3-ols					
Superoxide scavenging activity	(-)-Epi			3´-*O*-MeEpi, 4´-*O*-MeEpi	[1]
Inhibition of Cu2+ induced LDL oxidation	(-)-Epi	Epi-7-*O*-GlcUa		Epi-3'-*O*- GlcUa, 4'-*O*-MeEpi-3'-*O*- GlcUa	[2]
Inhibition of NADPH oxidase	(-)-Epi		3´-*O*-MeEpi, 4´-*O*-MeEpi		[1]
Increased of luciferase activity from the Site B of ApoAI promoter construct in Hela cells	(-)-Epi		3´-*O*-MeEpi, 4´-*O*-MeEpi	Epi-3´-Sulf	[3]
Induced expression of HNF-3β mRNA	(-)-Epi			3´-*O*-MeEpi, Epi-3´-Sulf, 4´-*O*-MeEpi	[3]
Induced expression of ApoAI in HepG2 cells	(-)-Epi	3´-*O*-MeEpi	4´-*O*-MeEpi	Epi-3-Sulf	[3]
Protection from UV induced cell damage	(-)-Epi	3´-*O*-MeEpi			[4]
Antioxidant activity measured with ABTS	(-)-Epi			5-(3´,4´-DHP)V	[5]
ABTS/persulfate assay; ABTS/peroxide assay; Ferric reducing power (FRAP) assay	(-)-Epi			3´-*O*-MeEpi, 4´-*O*-MeEpi	[6]
Antioxidant activity measured with ABTS	ECG, EGC, Epi			THPVa, 5-(3´,5´-DHP)V, 5-(3´,5´- DHP)Va, 4-OH-(3´,5´-DHP)Va, 5-(3´,4´-DHP)V, 3’-(HP)Va, 5-(3´- HP)V	[7]
ABTS/persulfate assay; ABTS/peroxide assay; Ferric reducing power (FRAP) assay	(+)-Cat			3´-*O*-MeCat, 4´-*O*-MeCat	[6]
Antioxidant activity measured with FRAP	(+)-Cat		5-(3´,4´-DHP)V	5-(3´-Me-4´-HP)V	[8]
Radical scavenging test against superoxide radicals	(+)-Cat		5-(3´,4´-DHP)V	5-(3´-Me-4´-HP)V	[8]
Inhibition of the enzymatic activity of metalloproteinases	(+)-Cat		5-(3´,4´-DHP)V, 5-(3´-Me-4´-HP)V		[8]
Phenolic acids					

Antioxidant activity measured with FRAP and ABTS	Ferulic acid			Ferulic acid 4´-*O*- GlcUa	[9]
Antioxidant activity measured with FRAP and ABTS	Caffeic acid			Caffeic acid 3´-*O*- GlcUa, Caffeic acid 4´ and 3´-Sulf, Caffeic acid 4´-*O*- GlcUa	[9]
Stilbenes					
Cytotoxic properties against breast cancer cells	RV			RV-3-*O*-Sulf, RV-4´-*O*-Sulf, RV-3-4´-*O*-diSulf, RV-3,5-diSulf, RV-3,4´,5-triSulf	[10, 11]
Inhibition NO production by NO scavenging and down-regulation of iNOS expression, inhibition of NFκB induction by TNF-α and inhibition of aromatase	RV			RV-4´-Sulf, RV-3,4´-diSulf, RV-3-Sulf, RV- 3,4´,5-triSulf, RV-3,5-diSulf	[11]
Induction of quinone reductase 1	RV		RV-3-Sulf, RV-4´-Sulf, RV-3,4´-diSulf, RV-3,5-diSulf, RV-3 -Sulf, RV-3,4´,5-triSulf		[11]
Inhibition of quinone reductase 2	RV			RV-3,4´-diSulf	[12]
Antioxidant capacity by DPPH free radical scavenging	RV	RV-3-Sulf		RV-4´-Sulf, RV-3,5-diSulf, RV 3,4´-diSulf, RV-3,4´,5-triSulf	[11]
Inhibition of COX-1 and COX-2	RV			RV-3-Sulf, RV-4´-Sulf, RV-3,4´,5-triSulf, RV-3,5-diSulf, RV-3,4´-diSulf	[11, 12]
Activation SIRT 1	RV	RV-4´-O-Sulf, RV-3-O-Sulf			[12]
Delipidating effects in maturing pre-adipocytes	RV	RV-4´-*O*-GlcUa, RV-3-*O*-Sulf		RV-3-*O*-GlcUa	[13]
Delipidating effects in mature adipocytes	RV			RV-3-*O*- GlcUa, RV-4´-*O*-GlcUa, RV- 3-*O*-Sulf	[13]
Anthocyanins					
Ability to counteract H_2O_2-induced apoptotic events in a human neuronal cell line	Cy-3-O-Gl		Cyanidin, Protocatechuic acid		[14]

ABTS, 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); (+)-Cat, (+)-Catechin; Cy-3-O-Gl, Cyanidin-3-*O*-Glucoside; COX, Cyclooxygenase; DHPV, dihydroxyphenyvalerolactone; DHPVa, dihydroxyphenyvaleric acid; DMNQ, 2,3-dimethoxy-1,4-naphtoquinone; Epi, (-)-Epicatechin; EGCG, Epigallocatechin gallate; EGC, Epigallocatechin; ECG, Epicatechin gallate; FRAP, Ferric Reducing Antioxidant Power ;GlcUa, -O-Glucuronide; HP, hydroxyphenyl; MCF7, Michigan Cancer Foundation-7; Me, methyl; SIRT, Sirtuin; NO, Nitric Oxide; OH, hydroxyl; ROS, Reactive Oxygen Species; RV, *trans*-Resveratrol; Sulf, Sulfate; THP, 5-(3´,4´,5´-trihydroxyphenyl); Va, valeric acid; V, γ-valerolactone.

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4.3 ESTUDIO DEL PERFIL DE LOS METABOLITOS MICROBIANOS EN ORINA TRAS EL CONSUMO DE VINO TINTO DESALCOHOLIZADO EN HUMANOS

Con el objetivo de identificar el mayor número de metabolitos tras el consumo de vino que podrían ser utilizados como biomarcadores de consumo y responsables de los efectos beneficiosos del consumo de vino tinto desalcoholizado, se utilizó un estudio de intervención, dando lugar al perfil de metabolitos más amplio después de consumo de vino tinto desalcoholizado hasta el momento.

Los resultados de este trabajo sobre el estudio del perfil de metabolitos microbianos en orina tras el consumo regular de vino desalcoholizado, ha sido publicado en una de las revistas considerada por el *Science Citation Index*, con un índice de impacto de 2.906 y situada en el primer cuartil de la categoría *Food Science & Technology* (15 de 124).

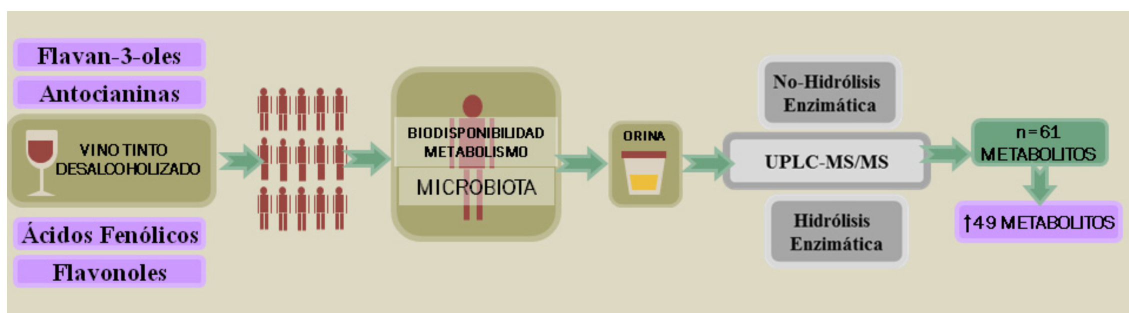
Publicación 3: María Boto-Ordóñez, Mireia Urpi-Sarda, María Isabel Queipo-Ortuño, Dolores Corella, Francisco J. Tinahones, Ramon Estruch, Cristina Andres-Lacueva. Microbial metabolomic fingerprinting in urine after regular dealcoholized red wine consumption in humans. *J Agric Food Chem,* 2013; 61: 9166-75.

Resumen:

El consumo regular de vino tinto desalcoholizado (VTD) ha demostrado beneficios en los factores de riesgo cardiovascular. El análisis de los metabolitos fenólicos formados en el organismo, especialmente aquellos que podrían provenir del metabolismo microbiano, podrían ayudar a entender estos efectos beneficiosos. El objetivo de este estudio fue determinar el más amplio perfil metabolómico de compuestos fenólicos y ácidos fenólicos microbianos (n=61) derivados de la ingesta regular de VTD en hombres con alto riesgo cardiovascular por UPLC-MS/MS utilizando una aproximación dirigida. Tras el consumo de VTD, la concentración urinaria de 49 metabolitos, incluyendo aquellos de fase II y derivados de la microbiota, aumentó en comparación con el estado basal (*P* <0.05). El mayor porcentaje de aumento fue encontrado para los

metabolitos microbianos de la degradación de antocianinas, tales como el ácido siríngico, el ácido *p*-cumárico, el ácido gálico y el pirogalol. La degradación de flavan-3-oles aumentó la concentración urinaria de hidroxifenilvalerolactonas y (epi)catequinas. Estos hallazgos proporcionaron el perfil metabolómico más completo después del consumo de vino, amplificando el espectro de los metabolitos derivados de la microbiota y su posible papel en relación con los beneficios para la salud.

Figura Resultados R3: Gráfico resumen del estudio perfil de los metabolitos microbianos en orina tras el consumo de vino desalcoholizado en humanos.



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Microbial Metabolomic Fingerprinting in Urine after Regular Dealcoholized Red Wine Consumption in Humans

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Supporting Information

ABSTRACT: The regular consumption of dealcoholized red wine (DRW) has demonstrated benefits in cardiovascular risk factors. The analysis of phenolic metabolites formed in the organism, especially those that could come from microbiota metabolism, would help to understand these benefits. The aim of this study was to determine the widest urinary metabolomic fingerprinting of phenolics and microbial-derived phenolic acids ($n = 61$) after regular intake of DRW in men at high cardiovascular risk by UPLC-MS/MS using a targeted approach. Up to 49 metabolites, including phase II and microbial phenolic metabolites, increased after DRW consumption compared to baseline ($P < 0.05$). The highest percentage of increase was found for microbial metabolites from anthocyanin degradation such as syringic, *p*-coumaric, gallic acids and pyrogallol and from flavan-3-ols degradation such as hydroxyphenylvalerolactones and (epi)catechins. These findings provide the most complete metabolic fingerprinting after wine consumption, amplifying the spectrum of microbial derived metabolites and their potential bioactivity related with health benefits.

KEYWORDS: *dealcoholized red wine, microbiota, human urine, UPLC-MS/MS, food metabolome, biomarkers, phenolic acids*

■ INTRODUCTION

Red wine consumption has been associated with the prevention of several diseases, mainly cardiovascular diseases.[1,2] These effects were not only explained by its alcoholic content[3] but also by its phenolic composition.[4,5] Moreover, there is an increasing interest in developing new products derived from red wine due to its reported beneficial effects. These newly developed products from red wine have a polyphenolic content similar to red wine but without alcohol (<1.2%, v/v), which could make them suitable to be considered a functional food after complying with regulations.[6] But before making nutritional claims, bioavailability studies are necessary in order to ensure that sufficient amounts of the compound are available at target tissues after consumption of a reasonable dose.

The regular consumption of dealcoholized red wine (DRW) used in this study has been associated with benefits for blood pressure[5] and inflammatory parameters[4] in patients at high cardiovascular risk. The health benefits of polyphenols have been classically related to those originally present in foods.[7,8] However, in the past few years, there has been an increased interest in metabolites formed in the organism, particularly those formed by the intestinal microbiota.[9,10] Moreover, biological activity of these compounds produced in the gastrointestinal tract has been proved in some cases to be more active than their parent compounds.[11] One of the critical points for phenolic transformation is the interaction between polyphenols and microbiota. This interaction has been shown in two senses. First, polyphenols that arrive at the intestine can exert a prebiotic effect, stimulating the growth or inhibition of certain bacteria.[12,13] Additionally, microbial enzymes may produce new molecules from those originally present in the food, as has been established in in vivo and in vitro studies.[14] These structures are phenolic acids formed by gut bacteria through reactions of hydrolysis, ring-cleavage, decarboxylation, demethylation, reduction, and dehydroxylation.[15] In some cases, these reactions have been linked to specific bacteria such as *Enterococcus casseliflavus*, *Butyrivibrio sp C3*, *Clostridium orbiscindensor*, and *Eubacterium ramulus* associated with deglycosylation and ring fission.[16] DRW composition com-

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prises a wide range of compounds, from simple compounds such as phenolic acids or simple flavonoids to more complex ones such as proanthocyanidins.[17] Simple components may be absorbed in the upper part of the gastrointestinal tract and pass to the bloodstream, being exposed to metabolism in the intestine, liver, and tissues. The nonabsorbed polyphenols, such as proanthocyanidins, flavan-3-ols, or anthocyanins, can be metabolized by gut microbiota, releasing an extensive number of metabolites prior to its absorption and phase II metabolism.[11] Therefore, a complete understanding of phenolic metabolism, taking into account chemical structure, bioavailability, food matrix, background diet, and individual factors, is essential for associating its effects with its consumption.[18] To our knowledge, there is only a small number of human studies in which phenolic metabolism is studied after wine intervention, and they are mainly focused on a single component and its derived metabolites such as catechin and resveratrol.[19−23] However, foods are complex systems where several phenolic classes are present, and the number of possible metabolites found in biofluids derived from all these combinations is high[24] and thus there are more metabolites that can exert their biological activity in vivo. The importance of broadening the phenolic study to metabolites formed in the organism, especially from microbiota, would help to understand the benefits derived from consumption, bearing in mind that in some cases metabolites have been proved to be more biologically active than their parent compounds.[11] In the present study, a long-term feeding trial was performed to determine changes in the urinary excretion of microbial phenolic metabolites after DRW consumption, taking into account all the phenolic classes present in wine composition and obtaining the widest phenolic metabolic profile after DRW intake in humans.

■ MATERIALS AND METHODS

Standards and Reagents. The following compounds (% purity when available) were used: 2,4-dihydroxybenzoic acid (≥97%), 2,6-dihydroxybenzoic acid (98%), 2,5-dihydroxybenzoic acid (98%), 3,5-dihydroxybenzoic acid (97%), 4-hydroxybenzoic acid (≥98%), 3-hydroxybenzoic acid (≥98%), gallic acid (≥98.5%), syringic acid (≥95%), phenylacetic acid (≥98%), 3-hydroxyphenylacetic acid (≥97%), 2-hydroxyphenylacetic acid (99%), 3,4-dihydroxyphenylacetic acid (98%), 3-(4-hydroxyphenyl)propionic acid (≥98%), 3-(3,4-dihydroxyphenyl)propionic acid or dihydrocaffeic acid (98%), *p*-coumaric acid (≥98%), *o*-coumaric acid (97%), caffeic acid (≥95%), ferulic acid (≥98%), protocatechuic acid (>97%), sinapic acid (≥98%), enterolactone (95%), pyrogallol (≥98%), ethylgallate (≥96%), (−)-epicatechin (≥98%), (+)-catechin (≥98%), and *β*-glucuronidase/sulfatase (from *Helix pomatia*) were purchased from Sigma−Aldrich (St. Louis, MO, USA). 4-Hydroxyhippuric acid (>99%) was purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). 3-(3-Hydroxyphenyl)propionic acid was purchased from Apin Chemicals Limited (Abingdon, UK). Vanillic acid, 4-*O*-methylgallic acid, *m*-coumaric acid, and taxifolin (>90%) were purchased from Extrasynthèse (Genay, France). Standard of epicatechin-5-*O*-glucuronide was chemically synthesized and characterized as previously published.[25] Liquid chromatography grade solvents methanol, acetonitrile, glacial acetic and formic acids were purchased from Scharlau Chemie, SA (Sentmenat, Spain). Hydrochloric acid was purchased from Panreac Química, SAU (Castellar del Vallès, Spain). Ultrapure water (Milli-Q) was obtained from Millipore (Bedford, MA, USA). Synthetic urine was prepared as previously described.[26]

Subjects and Study Design. In this study, the urine of 36 men (mean age of 61 ± 9) at baseline and after one month of DRW consumption was obtained from a previous clinical trial.[4] Baseline characteristics of the participants were included in Supporting Information Table 1. Subjects were first asked to follow a 2-week run-in period in which they were requested to exclude all grape-derived products and alcoholic beverages. After that, the subjects consumed 272 mL of DRW (0.42% alcohol) daily for 4 weeks during the meals. The Institutional Review Board of the hospital approved the study protocol, and all participants gave written consent before participating in the study. Urine samples (24 h) were collected at baseline and after the intervention period with DRW and immediately were stored at −80 °C until analysis. This trial has been registered in the Current Controlled Trials in London, International Standard Randomized Controlled Trial Number (ISRCTN88720134).

DRW was elaborated with the Merlot grape variety, from the Penedès appellation (Catalonia, Spain). The phenolic composition of DRW (Table 1) was analyzed throughout the study period (n = 5).[27−29] Total phenolic composition was measured by Folin−Ciocalteu.[30] Individual phenolic compounds were quantified as previously reported by Ibern-Gomez et al.[27] and Romero-Perez et al.[28] The five time points analyzed along the study period did not show significant differences in the phenolic composition (data not shown).

Table 1. Phenolic Composition (Mean ± SD) of the Dealcoholized Red Wine[a]

phenolic compound (mg/L)	DRW[b]
gallic acid[c]	73.17 ± 7.01
protocatechuic acid[c]	5.85 ± 0.51
tyrosol[c]	47.81 ± 3.90
catechin[c]	126.45 ± 13.35
epicatechin[c]	70.57 ± 8.22
procyanidins[d]	187.84 ± 15.10
trans-caftaric acid[c]	19.21 ± 1.62
trans-caffeic acid[c]	12.18 ± 0.92
trans-coutaric acid[c]	5.62 ± 0.52
2-*S*-glutathionylcaftaric[c]	10.76 ± 1.26
quercetin-3-glucuronide[c]	11.25 ± 1.42
quercetin[c]	23.82 ± 2.37
isorhamnetin[c]	2.96 ± 0.14
delphinidin-3-glucoside[c]	14.71 ± 1.62
petunidin-3-glucoside[c]	12.04 ± 1.15
peonidin-3-glucoside[c]	6.68 ± 0.57
malvidin-3-glucoside[c]	49.86 ± 4.27
malvidin-(6-acetyl)-3-glucoside[c]	10.41 ± 1.20
malvidin-(6-coumaroyl)-3-glucoside[c]	3.54 ± 0.33
trans-resveratrol[e]	2.73 ± 0.23
cis-resveratrol[e]	2.75 ± 0.15
trans-piceid[e]	10.53 ± 0.96
cis-piceid[e]	7.08 ± 0.87
total phenol (meq gallic acid/L)[f]	2694.92 ± 86.79

[a]Analyses were performed at five time points along the study in duplicate. [b]DRW, dealcoholized red wine. [c]Determined as previously described by Ibern-Gomez et al.[27] [d]Determined as previously described by Queipo-Ortuño et al.[12] [e]Analyzed following the work by Romero-Perez et al.[28] [f]Analyzed by Folin−Ciocalteu methodology.[30]

Extraction of Phenolic Acid Metabolites from Urine. Solid-phase extraction was performed using Oasis MCX 96-well plates (Waters, Milford, Massachusetts) as previously described.[26] Briefly, 1 mL of urine was subjected to enzymatic hydrolysis using *β*-glucuronidase/sulfatase from *Helix pomatia* at 37 °C for 45 min after being acidified with 50 μL of 0.58 mol/L acetic acid. Immediately afterward, samples were acidified to pH 2 with 6 mol/L HCl. The plate was conditioned with methanol and 2% formic acid in water. The hydrolyzed samples were then loaded onto the plate, washed with 2% formic acid in water, and analytes were then eluted with methanol.

Table 2. Multiple Reaction Monitoring (MRM) Transitions, Declustering Potential (DP), and Collision Energy (CE) for Each Microbial and Conjugate Metabolite Identified in This Study

analyte	MRM transitions	identified by	DP	CE	quantified as
hydroxybenzoic acids					
2,4-dihydroxybenzoic acid	153/109	STD[a]	−50	−20	STD
2,6-dihydroxybenzoic acid	153/109	STD	−50	−20	STD
2,5-dihydroxybenzoic acid	153/109	STD	−50	−20	STD
3,5-dihydroxybenzoic acid	153/109	STD	−50	−20	STD
protocatechuic acid	153/109	STD	−50	−20	STD
vanillic acid	167/152	STD	−50	−20	STD
syringic acid	197/121	STD	−50	−25	STD
4-hydroxybenzoic acid	137/93	STD	−50	−16	STD
3-hydroxybenzoic acid	137/93	STD	−50	−16	STD
4-hydroxyhippuric acid	194/100	STD	−50	−20	STD
3-hydroxyhippuric acid	194/150	PIS[b]	−50	−20	4-hydroxyhippuric acid
gallic acid metabolites					
gallic acid	169/125	STD	−40	−20	STD
4-*O*-methylgallic acid	167/108	STD	−50	−26	STD
methylgallic acid	167/108	PIS	−50	−26	4-*O*-methylgallic acid
methylgallic sulfate	263/183	PIS	−50	−25	gallic acid
ethylgallate metabolites					
ethylgallate	197/169	STD	−50	−25	gallic acid
ethylgallate sulfate	277/197	PIS	−50	−25	gallic acid
ethylgallate glucuronide 1,2	373/197	PIS	−50	−25	epicatechin-5-*O*-glucuronide
hydroxyphenylacetic acids					
phenylacetic acid	135/91	STD	−30	−12	STD
3-hydroxyphenylacetic acid	151/107	STD	−50	−12	STD
2-hydroxyphenylacetic acid	151/107	STD	−50	−12	STD
3,4-dihydroxyphenylacetic acid	167/123	STD	−50	−12	STD
homovanillic acid	181/137	STD	−40	−10	vanillic acid
hydroxycinnamic acids					
m-coumaric acid	163/119	STD	−50	−30	STD
o-coumaric acid	163/119	STD	−50	−30	STD
p-coumaric acid	163/119	STD	−50	−30	STD
caffeic acid	179/135	STD	−50	−21	STD
ferulic acid	193/134	STD	−50	−25	STD
sinapic acid	223/164	STD	−50	−25	STD
hydroxyphenylpropionic acids					
3-(4-hydroxyphenyl)propionic acid	165/121	STD	−30	−16	STD
3-(3-hydroxyphenyl)propionic acid	165/121	STD	−30	−16	STD
dihydrocaffeic acid	181/137	STD	−40	−10	STD
flavan-3-ols					
(epi)catechin glucuronide 1,2,3,4	465/289	PIS	−50	−25	epicatechin-5-*O*-glucuronide
(epi)catechin sulfate 1,2,3	369/289	PIS	−50	−25	(epi)catechin
methyl(epi)catechin glucuronide 1,2,3	479/303	PIS	−50	−30	epicatechin-5-*O*-glucuronide
methyl(epi)catechin sulfate 1,2,3	383/303	PIS	−50	−25	(epi)catechin
glycinates					
vanilloylglycine	224/180	PIS	−50	−25	4-hydroxyhippuric acid
feruloylglycine	250/100	PIS	−50	−25	4-hydroxyhippuric acid
hydroxyphenylvalerolactones					
DHPV 1	207/163	PIS	−50	−25	(epi)catechin
DHPV 2	207/163	PIS	−50	−25	(epi)catechin
DHPV glucuronide 1,2	383/207	PIS	−50	−25	epicatechin-5-*O*-glucuronide
DHPV sulfate 1,2	287/207	PIS	−50	−25	(epi)catechin
MHPV 1	221/162	PIS	−50	−25	(epi)catechin
MHPV glucuronide 1	397/221	PIS	−50	−25	epicatechin-5-*O*-glucuronide
MHPV sulfate 1,2	301/221	PIS	−50	−25	(epi)catechin
other polyphenols					
enterolactone	297/253	STD	−50	−25	STD
pyrogallol	125/69	STD	−50	−25	STD

[a]STD, standard available. [b]PIS, product ion scan.

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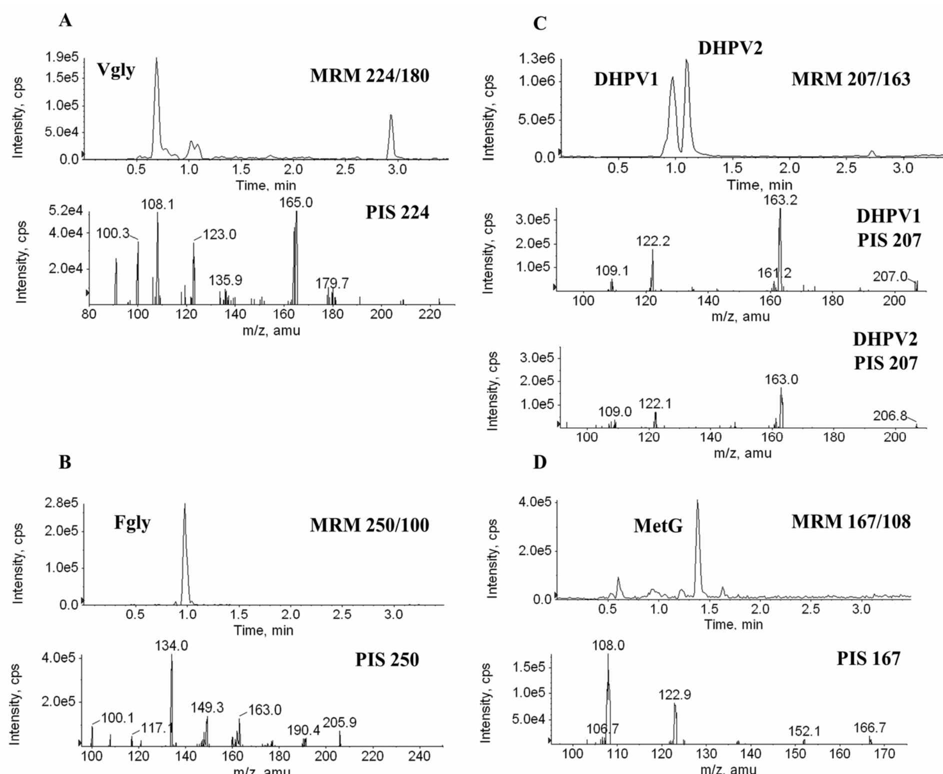

Figure 1. Multiple reaction monitoring (MRM) trace chromatograms and product ion scan (PIS) of (A) vanilloylglycine, Vgly (*m/z* 224), (B) feruloylglycine, Fgly (*m/z* 250), (C) dihydroxyphenyl-γ-valerolactone, DHPV (*m/z* 207), and (D) methylgallic acid, MetG (*m/z* 167) in hydrolyzed urine samples after DRW intake.

Eluates were evaporated to dryness and reconstituted with 100 μL of taxifolin (1.64 μmol/L) dissolved in mobile phase.

Extraction of Conjugated Phenolic Metabolites from Urine. Solid-phase extraction was performed using Oasis HLB 96-well plates (Waters, Milford, Massachusetts) as previously described.[31] Briefly, the plate was conditioned with 1 mL of methanol and 1.5 mol/L of formic acid in water. One milliliter of urine was loaded onto the cartridge plate. Then, the cartridges were washed with 1 mL of acidified water (1.5 mol/L of formic acid) and 1 mL of 5% methanol. Analytes were eluted with methanol containing 0.1% formic acid. The eluates were evaporated to dryness and reconstituted with 100 μL of taxifolin (1.64 μmol/L) dissolved in mobile phase.

UPLC-MS/MS Analysis of Conjugated and Microbial Metabolites in Urine. The analysis of metabolites in urine with or without enzymatic hydrolysis was carried out by UPLC coupled to tandem mass spectrometry (UPLC−MS/MS) adapted from a previous validated methodology.[26,31] A Waters Acquity UPLC system (Milford, MA, USA) equipped with a binary solvent manager and a refrigerated autosampler plate was used. It was coupled to an AB Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray ionizing in negative mode. The analytical column used for chromatographic separation was an Acquity UPLC BEH C18 (Milford, MA, USA) (1.7 μm, 2.1 mm × 5 mm), using a prefilter, working at 40 °C, at a flow rate of 0.5 mL/min with an injection volume of 5 μL. The linear gradient elution was carried out with 0.1% formic acid in water as phase A and 0.1% formic acid in acetonitrile as phase B at a flow rate of 500 μL/min with the following proportions (v/v) of phase A [*t* (min), %A]: (0,92), (2.5,50), (2.6,0), (3,0), (3.1,92), (3.5,92). The MS/MS parameters used were: collision cell exit potential (−15 V), focusing potential (−200 V), entrance potential (−10 V), nebulizer gas (10 arbitrary units), curtain gas (12 arbitrary units), collision gas (5 arbitrary units), auxiliary gas temperature (400 °C), auxiliary gas flow rate (6000 cm^3/min), and capillary voltage (−3700 V). Collision energy and declustering potential were optimized for each compound (Table 2). The identification of metabolites was done by comparing retention time with available standards or by product ion scan (PIS) when standards were not available. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound (Table 2), using a dwell time of 10 ms. Calibration curves were constructed with available standards in synthetic urine[26] and subjected to the same procedure as the samples. Concentrations of metabolites with no available standard were estimated using the most similar compound standard curve and results were expressed as their equivalents (Table 2). Limits of detection and limits of quantification had already been published.[26]

Statistical Analysis. The MetaboAnalyst web-based platform for data analysis[32] was used for data normalization and the evaluation of mean differences of phenolic metabolites, through a *t*-test for paired samples. Normalization was carried out by a cube root transformation and a range scaling of the data. Statistical significance was defined as $P \leq 0.05$.

■ RESULTS AND DISCUSSION

Identification of Microbial-Derived and Phase II Phenolic Metabolites in Urine. A total of 37 metabolites were determined after enzymatic hydrolysis and 24 conjugated metabolites were determined without enzymatic hydrolysis.

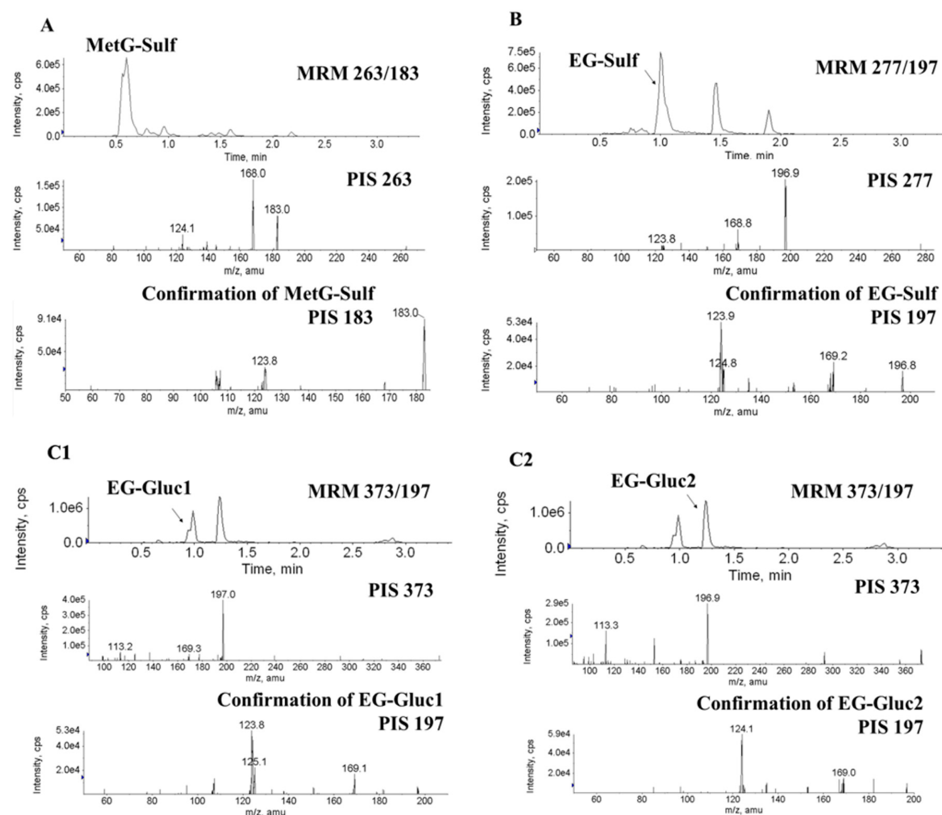

Figure 2. Multiple reaction monitoring (MRM) trace chromatograms and product ion scan (PIS) of (A) methylgallic sulfate, MetG-Sulf (*m*/*z* 263) with its confirmation through the PIS of *m*/*z* 183 in the CID-MS/MS experiments; (B) ethylgallate sulfate, EG-Sulf (*m*/*z* 277) with its confirmation through the PIS of *m*/*z* 197 in the CID-MS/MS experiments and (C1 and C2) ethylgallate glucuronide 1 and 2, EG-Gluc 1,2 (*m*/*z* 373) with its confirmation through the PIS of *m*/*z* 197 in the CID-MS/MS experiments.

From the metabolites determined after enzymatic hydrolysis, 30 were identified by comparison with the pure available standard. The other seven metabolites were tentatively identified by PIS experiments. Previously, attempts were made to identify glycinated ($n = 18$) metabolites of phenolic acids (results not shown) after DRW consumption but only three were positively identified based on their mass spectra. Feruloylglycine and vanilloylglycine were tentatively identified based on their published mass spectra.[33,34] The mass spectra and fragmentation pattern generated for vanilloylglycine (*m*/*z* 224) showed the ion *m*/*z* 180, loss of 44 amu (−COOH), *m*/*z* 165, loss of 59 amu ($-CH_2-COOH$), *m*/*z* 123, loss of 101 amu ($-CO\text{-}NH\text{-}CH_2\text{-}COOH$), and *m*/*z* 108, loss of 15 amu ($-CH_3$) and 101 amu, and *m*/*z* 100, which were coincident with the previous identifications of vanilloylglycine (Figure 1A).[33] Feruloylglycine (*m*/*z* 250) showed the fragments *m*/*z* 206, *m*/*z* 163, *m*/*z* 149, *m*/*z* 134 (loss of 101 and 15 amu), and *m*/*z* 100 reported in previous studies (Figure 1B).[34,35] 3-Hydroxyhippuric acid (*m*/*z* 194) was identified based on its mass spectra as previously published,[31,36] showing a characteristic fragment of *m*/*z* 150. In addition, two peaks of dihydroxyphenyl-γ-valerolactone (DHPV) (*m*/*z* 207) (Figure 1C) and one peak of methoxy-hydroxyphenyl-γ-valerolactone (MHPV) were identified according to the typical spectra and fragmentation pattern.[26] At MRM of 167/108, corresponding to the 4-*O*-methylgallic acid fragmentation, an additional peak was detected in samples. After studying its mass spectra, fragments coincided with those from the PIS of the 4-*O*-methylgallic acid standard (*m*/*z* 152, *m*/*z* 123, *m*/*z* 108) (Figure 1D), meaning the presence of a possible isomer, tentatively identified as 3-methylgallic acid. The concentration of some previously identified metabolites such as vanillic acid, 4-*O*-methylgallic acid, epicatechin, and MHPV were under the limit of detection of the method.

Besides the above-mentioned metabolites, conjugated metabolites derived from flavanol and microbial degradation metabolites were also investigated. Glucuronides and sulfates of (epi)catechin and methyl(epi)catechin, DHPV and MHPV, previously identified after cocoa and almond consumption,[31,37] were also found in urine. In addition, four new phenolic acid conjugates derived from methylgallic acid and ethylgallate were tentatively identified by PIS (Figure 2). The mass spectra and fragments generated by methylgallic sulfate (*m*/*z* 263) showed *m*/*z* 183, corresponding to the loss of 80 amu ($-SO_3$), and *m*/*z* 168, corresponding to the subsequent loss of 15 amu ($-CH_3$). This metabolite was confirmed through the PIS of *m*/*z* 183 in the CID-MS/MS experiments,[29] showing the fragmentation of methylgallic *m*/*z* 124, loss of 59 amu,

previously reported (Figure 2A).[38] In addition, three ethylgallate conjugates were tentatively identified. The peak at m/z 277 was identified as ethylgallate sulfate obtaining a product ion at m/z 197 (loss of 80 amu) and two peaks at m/z 373 as ethylgallate glucuronides which also showed the typical fragment of the glucuronide moiety (m/z 113 and 175) (Figure 2, C1 and C2). These conjugates were confirmed through the CID-MS/MS experiments of the product compound, ethylgallate (m/z 197), which showed its typical fragments at m/z 169 and m/z 124.[39] Additionally, more than 20 conjugated phenolic acids derived from hydroxybenzoic, hydroxyphenylacetic, hydroxycinnamic, and hydroxypropionic acids were investigated, but identification by mass spectra was not conclusive (data not shown).

Changes in Microbial-Derived and Phase II Phenolic Metabolites in Urine after DRW Consumption. The concentration of phase II and microbial-derived metabolites in urine at baseline and after consumption of DRW is presented in Tables 3 and 4. In this study, 21 phase II metabolites (Table 3) and 28 microbial metabolites (Table 4) significantly increased in urine after DRW compared to baseline.

In the present study, 11 (epi)catechin phase II metabolites (glucuronides, sulfates, and methyl conjugates) significantly increased after DRW ($P < 0.05$) with fold changes (FC) from 1.67 to 11.43 (Table 3). According to red wine composition, procyanidins are the most abundant polyphenols, followed by flavanols and anthocyanins.[17] Procyanidins are polymeric molecules that could arrive intact to the lower gastrointestinal tract and be hydrolyzed by microbiota into more simple components before absorption.[36,40,41] Procyanidins may suffer an interflavan cleavage from microbiota activity which results in catechin and epicatechin.[11] Then, (epi)catechins which were formed from procyanidins metabolism or which were present in the original food, could be subjected to C-ring-opening, giving rise to diphenylpropan-2-ol, later converted into 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone.[11] This step has been described for *Eggerthella lenta* and *Flavonifractor plautii*.[42] In this study, two glucuronide and two sulfate conjugates of DHPV and one glucuronide and one sulfate of MHPV increased after DRW intake compared to baseline ($P < 0.05$) (Table 3). Nevertheless, the origin of valerolactones is not exclusively from (epi)catechins but also from epicatechin gallate and epigallocatechin, which could release DHPV and also trihydroxyphenylvalerolactones.[11,15] This last compound was expected to be in low concentration in the urine of the participants of this study because epigallocatechins are present in low concentrations in wine,[17] thus it could not be identified in the present study. At intestinal level, valerolactone ring may suffer a break resulting in valeric acids and a possible interconversion between both forms was described but largely displaced to the former.[11] The hydroxyphenylpropionic acids analyzed in this study significantly increased after DRW intake (Table 4). They have been described from several routes of polyphenols: (i) β-oxidation of valeric acids, (ii) ring fission of the flavonol, (iii) breakdown of naringenin, and (iv) double-bond reduction of caffeic acid.[11,43] Hydroxyphenylpropionic acids could also be transformed into hydroxycinnamic acids after microbial hydrogenation and methylation in the liver.[11,40] Almost all the hydroxycinnamic acids reported in this study increased significantly after DRW intake in a fold change of 1.29−2.31, being the highest increment for *p*-coumaric, an anthocyanin microbial metabolite[44] and also derived of coutaric acid hydrolysis.[40] The metabolic origin of hydroxybenzoic acids may come from several routes of polyphenol metabolism: (i) by β-oxidation of hydroxyphenylpropionic acids and gallates which could be further glycinated into hydroxyhippuric acids,[11,36] (ii) microbial metabolism of anthocyanins,[16,44] and (iii) from quercetin metabolism.[45] In our study, nearly all the hydroxybenzoic acids increased in a significant way after DRW intake, although no significant increase was observed for protocatechuic acid. The highest increase was observed for syringic acid (2.78-fold change), while the other hydroxybenzoic acid metabolites ranged from 1.33 to 1.93 fold changes (Table 4). This high increase was due to the fact that syringic acid is the main microbial metabolite of malvidin-3-glucoside,[16,44,46] the most prevalent anthocyanin in wine.[17] The further metabolism of syringic acid such as enzymatic

Table 3. Concentrations (Mean ± SEM) of Phase II Metabolites of (Epi)catechin, Hydroxyphenylvalerolactones, and Hydroxybenzoic Acids in 24 h Urine Samples in 36 Subjects at Baseline and after DRW Intake

	urine samples (μmol, 24 h)		
metabolites	baseline	DRW	fold change
hydroxybenzoic acids			
gallic acid metabolites			
methylgallic sulfate	2.97 ± 0.74	19.94 ± 3.08*[a]	6.71
ethylgallate metabolites			
ethylgallate sulfate	2.16 ± 0.76	15.81 ± 1.64*	7.32
ethylgallate glucuronide 1	36.73 ± 6.01	114.52 ± 10.73*	3.12
ethylgallate glucuronide 2	101.74 ± 22.40	240.98 ± 24.23*	2.37
flavan-3-ols			
(epi)catechin glucuronide 1	0.46 ± 0.14	5.26 ± 2.66*	11.43
(epi)catechin glucuronide 2	0.26 ± 0.19	2.24 ± 0.95*	8.61
(epi)catechin glucuronide 3	5.35 ± 1.09	8.92 ± 1.68*	1.67
(epi)catechin glucuronide 4	3.36 ± 0.65	10.19 ± 1.93*	3.03
(epi)catechin sulfate 1	1.38 ± 0.24	5.22 ± 0.93*	3.78
(epi)catechin sulfate 2	0.98 ± 0.26	4.13 ± 0.73*	4.21
(epi)catechin sulfate 3	0.67 ± 0.18	1.34 ± 0.35	
methyl(epi)catechin glucuronide 1	2.04 ± 0.53	8.55 ± 1.92*	4.19
methyl(epi)catechin glucuronide 2	0.96 ± 0.36	3.92 ± 0.91*	4.08
methyl(epi)catechin glucuronide 3	0.75 ± 0.23	1.38 ± 0.46	
methyl(epi)catechin sulfate 1	2.37 ± 0.33	5.62 ± 0.76*	2.37
methyl(epi)catechin sulfate 2	8.37 ± 1.67	19.42 ± 2.68*	2.32
methyl(epi)catechin sulfate 3	0.13 ± 0.05	0.60 ± 0.15*	4.61
hydroxyphenylvalerolactones			
DHPV glucuronide 1	8.22 ± 1.82	31.73 ± 5.30*	3.86
DHPV glucuronide 2	62.40 ± 13.16	145.76 ± 20.32*	2.34
DHPV sulfate 1	14.70 ± 7.84	23.69 ± 6.32*	1.61
DHPV sulfate 2	512.44 ± 52.44	889.74 ± 110.51*	1.74
MHPV glucuronide 1	23.81 ± 4.43	38.43 ± 7.08*	1.61
MHPV sulfate 1	8.38 ± 1.68	12.65 ± 2.60*	1.51
MHPV sulfate 2	23.90 ± 4.89	30.49 ± 4.42	

[a]The asterisk indicates that the mean value is significantly different from the baseline concentration ($P < 0.05$).

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Table 4. Concentrations (Mean ± SEM) of Microbial Phenolic Acids Metabolites in 24 h Urine Samples in 36 Subjects at Baseline and after DRW Intake

	urine samples (μmol, 24 h)		
metabolites	baseline	DRW	fold change
hydroxybenzoic acids			
2,4-dihydroxybenzoic acid	1.57 ± 0.17	2.67 ± 0.37*[a]	1.70
2,6-dihydroxybenzoic acid	6.19 ± 0.60	8.74 ± 0.88*	1.41
2,5-dihydroxybenzoic acid	16.23 ± 1.65	27.29 ± 2.90*	1.68
3,5-dihydroxybenzoic acid	3.93 ± 0.66	7.57 ± 1.26*	1.93
protocatechuic acid	12.10 ± 1.15	14.45 ± 1.66	
syringic acid	0.73 ± 0.15	2.03 ± 0.32*	2.78
4-hydroxybenzoic acid	25.79 ± 2.21	34.30 ± 2.81*	1.33
3-hydroxybenzoic acid	3.77 ± 1.27	5.67 ± 1.57*	1.50
4-hydroxyhippuric acid	54.05 ± 5.42	72.13 ± 9.02*	1.33
3-hydroxyhippuric acid	192.30 ± 39.81	237.58 ± 54.21	
gallic acid metabolites			
gallic acid	0.85 ± 0.18	4.76 ± 0.53*	5.60
methylgallic acid	2.97 ± 0.42	4.76 ± 0.68*	1.60
ethylgallate metabolites			
ethylgallate	1.06 ± 0.37	4.97 ± 0.73*	4.69
hydroxyphenylacetic acids			
phenylacetic acid	22.15 ± 2.21	27.66 ± 3.00*	1.25
3-hydroxyphenylacetic acid	24.72 ± 3.50	56.57 ± 6.90*	2.29
2-hydroxyphenylacetic acid	5.89 ± 0.40	7.41 ± 0.54*	1.26
3,4-dihydroxyphenylacetic acid	1.61 ± 0.17	2.37 ± 0.24*	1.47
homovanillic acid	164.35 ± 13.99	215.13 ± 25.55	
hydroxycinnamic acids			
m-coumaric acid	0.54 ± 0.09	0.83 ± 0.20*	1.54
o-coumaric acid	0.07 ± 0.02	0.10 ± 0.03	
p-coumaric acid	0.64 ± 0.07	1.48 ± 0.15*	2.31
caffeic acid	5.42 ± 0.34	7.05 ± 0.55*	1.30
ferulic acid	11.80 ± 0.98	15.25 ± 0.94*	1.29
sinapic acid	0.99 ± 0.18	1.43 ± 0.20*	1.44
hydroxyphenylpropionic acids			
3-(4-hydroxyphenyl) propionic acid	287.44 ± 27.16	389.20 ± 39.36*	1.35
3-(3-hydroxyphenyl) propionic acid	6.22 ± 1.09	10.07 ± 2.05*	1.62
dihydrocaffeic acid	14.09 ± 1.39	17.29 ± 1.50*	1.23
glycinates			
vanilloylglycine	0.80 ± 0.09	1.31 ± 0.16*	1.64
feruloylglycine	9.23 ± 1.05	11.24 ± 1.31	
hydroxyphenylvalerolactones			
DHPV 1	6.73 ± 1.21	13.61 ± 2.68*	2.02
DHPV 2	18.50 ± 3.67	37.04 ± 4.31*	2.00
other polyphenols			
enterolactone	8.73 ± 1.10	14.81 ± 3.40*	1.70
pyrogallol	1.96 ± 0.43	8.08 ± 1.78*	4.12

[a]The asterisk indicates that the mean value is significantly different from the baseline concentration ($P < 0.05$).

demethylation of the B-ring could degenerate into gallic acid (5.60-fold change, Table 4), and the subsequent decarboxylation[44] could release pyrogallol (4.12-fold change, Table 4). In vitro studies have shown that the incubation of other minor wine anthocyanins such as cyanidin and peonidin with microbiota[16] released protocatechuic and vanillic acids. Delphinidin and petunidin derivatives, present in wine composition in lower concentrations than malvidin derivatives, could also suffer microbial degradation. These compounds could produce gallic and 3-methylgallic acids, respectively.[46−48] In our study, gallic and methylgallic acids showed a 5.60- and 1.60-fold significantly increase, respectively, after DRW intake (Table 4). Gallic acid metabolites have been clearly associated with wine consumption as they are present in wine composition and could also be released from anthocyanins, gallates, through the cleveage of gallic ester moiety, and syringic acid.[44,49−52] In addition, ethylgallate, which was originally present in wine,[53] also increased after DRW, along with its glucuronide and sulfate metabolites. The formation of hydroxyphenylacetic acids could come from three described routes: (i) α-oxidation of hydroxyphenylpropionic acids,[11] (ii) through the cleveage of the upper unit of dimeric procyanidins, or (iii) quercetin degradation via ring fission.[11,41,43] Participants of this study significantly increased the concentrations of mono-, dihydrolyphenylacetic, and phenylacetic acids, except for homovanillic acid, after one-month of DRW intake.

However, not only flavanols but flavonols and anthocyanins are present in DRW composition. Other minority components such as tyrosol and hydroxytyrosol can be metabolized to homovanillic alcohol, homovanillic acid, 3,4-dihydroxyphenylacetaldehyde and 3,4-dihydroxyphenylacetic acid,[54] or sinapic, which could also be transformed into syringic acid.[49] In addition, in this study, the concentration of enterolactone metabolite increased after DRW intake (Table 4). This has been described as a metabolite of lignans, which have been in wines[55] and was formed by selected strains of *Bifidobacterium* genus and *Lactonifactor longoviformis*.[16,56] Therefore, the increases of phenolic acids in urine after DRW consumption would be represented by the proportion of the phenolic compounds ingested through red wine and by the proportion of microbial transformation of different classes of wine polyphenols.

The targeted metabolism of phenolic acids after wine products intake has not been deeply studied. Intervention studies with wine and derived products studied the phase II metabolism of individual classes of polyphenols such as catechins or resveratrol.[22,29] A few other studies have implied the microbiota metabolism of wine phenolics. Cacceta et al.[23] determined 4-*O*-methylgallic acid, caffeic acid, and protocatechuic acid in plasma after intake of RW and DRW. While the two first metabolites increased significantly after wine consumption, no significant differences were observed for protocatechuic acid.[23] These results in plasma are in accordance with our study with urine samples. In addition, the similar content of urinary protocatechuic acid as well as other nonsignificant phenolic acids between baseline and after DRW intake could imply its origin from the habitual dietary pattern of participants which was maintained during the study with no differences in nutrient intake, daily intake of antioxidants, and fat intake.[4] Recently, two human intervention studies reported the gut microbial derived degradation products after the intake of extracts of grape juice during four days[57] or four weeks[58] by GC-MS. In their studies, authors found the strongest urinary markers for syringic acid, 3- and 4-hydroxyhippuric acid, pyrogallol, 3-hydroxyphenylacetic acid, 3-hydroxyphenylpropionic, and 4-hydroxymandelic acid.[57,58] These results are also in accordance to our study, except for 3-hydroxyhippuric acid, which no significant differences were observed between baseline and DRW intake period, and for 4-

hydroxymandelic acid that was not determined in the present study.

The potential prebiotic effect of rich phenolic sources such as DRW has already been reported,[12] but the role of these metabolites at intestinal level remains unknown. Some of the metabolites formed in the organism, such as hydroxyphenylpropionic or hydroxybenzoic acids, have been proved to have the ability to inhibit the growth of pathogenic bacteria and nonpathogenic bacteria[59] in in vitro studies and proposed as being responsible for phenolic health benefits in the organism.[11] However, more studies are needed to clarify this point because huge interindividual variability is described for polyphenol, probably to the high variability of bacterial species,[60] and thus beneficial effects.[18] Changes in the bacterial population may modify the metabolites that have formed, so the approach of urinary metabolism could be the key to understanding what is happening at intestinal level and linking to its biological effects.

To our knowledge, this study constitutes the most complete report of gut and microbial metabolites derived from wine consumption in humans. The numerous metabolites described to come from microbial degradation highlight the important role of intestinal bacteria in polyphenol degradation, modulating bioavailability and possible effects in the organism.

■ ASSOCIATED CONTENT

Supporting Information

Baseline characteristics of the participants. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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Supplementary Table 1. Baseline characteristics of 36 subjects[1].

	Values
Age (y)	61±9[2]
Current smokers [*n* (%)]	6 (16.6)
Sedentarism [*n* (%)]	21 (58.3)
Family history of premature CAD [*n* (%)]	27 (75)
BMI (kg/m^2)	29.5±3.8
BMI >25 kg/m^2 [*n* (%)]	33 (91.6)
WHR	0.98±0.05
Type 2 diabetes [*n* (%)]	9 (25)
Hypertension [*n* (%)]	17 (47.2)
Dyslipemia [*n* (%)]	12 (33.3)
Medications [*n* (%)]	
ACE inhibitors	17 (47.2)
Statins	12 (33.3)
Oral hypoglycemic drugs	9 (25.0)
Aspirin or antiplatelet drugs	8 (22.2)
Systolic blood pressure (mm Hg)	140±19.1
Diastolic blood pressure (mm Hg)	80±9.8
Heart rate (beats/min)	69±9
Glucose (mg/dL)	119±36
Triglycerides (mg/dL)	122±53
Total cholesterol (mg/dL)	198±34
LDL cholesterol (mg/dL)	129±29
HDL cholesterol (mg/dL)	44±10
LDL cholesterol:HDL cholesterol ratio	3.1±0.9
Folic acid (serum) (ng/mL)	9.0±3.6
Intraerythrocytary folic acid (ng/mL)	395±100
Vitamin B-12 (pg/mL)	400 ±136
Albumin (mg/mL)	44.6±3.1
ASAT (UI/L)	24.8±8.5
ALAT (UI/L)	31.9±23.5
GGT (UI/L)	33.6±20.6

[1]ACE, angiotensin-converting enzyme; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CAD, coronary artery disease; GGT, c-glutamyl transpeptidase; WHR, waist-to-hip ratio.
[2]Results are expressed as mean ±SD

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4.4 ANÁLISIS DISCRIMINATORIO DE MARCADORES DE CONSUMO EN PLASMA Y ORINA. NUEVAS ESTRATEGIAS EN EL ESTUDIO DE BIOMARCADORES.

La generación de una nueva estrategia de análisis de biomarcadores de ingesta de un alimento y su posible traslocación en plasma y orina queda reflejada en este trabajo que se encuentra en proceso de publicación en la revista *Mol Nutr Food Res*.

Publicación 4: María Boto-Ordóñez, Cristina Andres-Lacueva, María Isabel Queipo-Ortuño, Sara Tulipani, Dolores Corella, Francisco J. Tinahones, Ramon Estruch, Mireia Urpi-Sarda. Novel approach to discover and evaluate markers of dietary exposure through a targeted LC-MS metabolomic approach in plasma and urine: Application in a wine intervention trial. *En proceso de publicación.*

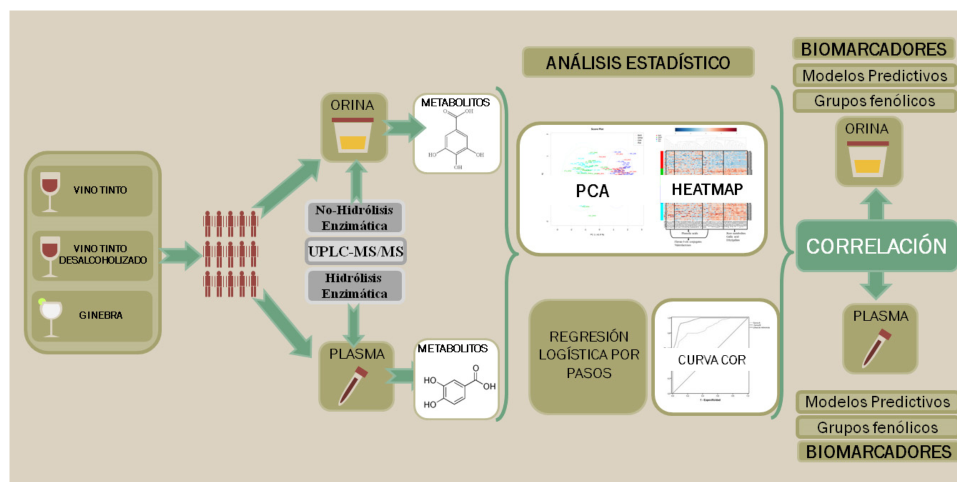
Resumen:

El descubrimiento de biomarcadores de la ingesta de alimentos es esencial para asociar la ingesta alimentaria (teniendo en cuenta su biodisponibilidad) y los factores de riesgo de enfermedades en los estudios epidemiológicos nutricionales. Debido ha este hecho, el objetivo de este estudio fue establecer un nuevo método de cribado para el descubrimiento de nuevos biomarcadores de la ingesta de alimentos. Para ello 36 voluntarios varones de alto riesgo cardiovascular fueron incluidos en un estudio clínico de intervención, aleatorizado y cruzado, donde después de un periodo de lavado, los sujetos recibieron vino tinto o ginebra (30g de alcohol/día) o vino tinto desalcoholizado durante 4 semanas. El plasma en ayunas y la orina de 24 horas se recogieron al inicio del estudio y después de cada período de intervención. Los metabolitos fenólicos microbianos y los metabolitos de fase II (> 60) fueron analizados por UPLC-MS/MS utilizando muestras hidrolizadas y no hidrolizadas.

Tras la identificación y cuantificación de los metabolitos derivados de flavanoles, antocianinas, estilbenos y ácidos fenólicos, se aplicaron técnicas multivariantes como el PCA y el análisis de cluster y después se realizó análisis de regresión logística por

pasos para crear un modelo de predicción de consumo de vino. Finalmente, estos modelos juntamente con metabolitos individuales y grupos de metabolitos se evaluaron mediante la utilización de curvas COR con sus parámetros de sensibilidad, selectividad y AUC. Tras el análisis de regresión logística, se establecieron modelos de predicción, que incluyeron principalmente metabolitos del ácido gálico y etilgalato, que obtuvieron valores de AUC para los grupos de entrenamiento y validación de más del 95% para la orina y del 80% para el plasma. Además, los metabolitos del resveratrol, etilgalato y del ácido gálico resultaron ser buenos predictores del consumo de vino (AUC> 93%) en las muestras de orina. En el plasma, los valores del AUC de los metabolitos resultaron ser más bajos (hasta 80%). Cuando se establecieron correlaciones entre los metabolitos obtenidos en plasma en ayunas y en orina de 24 horas los valores más altos se obtuvieron de los modelos de predicción (*r*=0.6, P<0.001), seguidos por los metabolitos del ácido gálico (*r*=0.5-0.6, P <0,001). Nuestro modelo de predicción, establecido a través de un análisis dirigido por UPLC-MS/MS en orina y plasma, es una herramienta prometedora para evaluar, comparar y discriminar biomarcadores de alimentos en diferentes muestras biológicas. Este estudio proporciona nuevos conocimientos sobre el descubrimiento de biomarcadores de alimentos y los mecanismos biológicos de metabolización y eliminación de metabolitos.

Figura Resultados R4: Gráfico resumen de para la identificación de biomarcadores en plasma y orina mediante el uso de herramientas estadísticas.



Novel approach to discover and evaluate markers of dietary exposure through a targeted LC-MS metabolomic approach in plasma and urine: Application in a wine intervention trial

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Keywords: Biological markers/ Food Metabolome/ Logistic Models/ Multivariate analysis/ ROC curve.

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Abbrevations used: AUC, Area Under the Curve; DHPV, dihydroxyphenyl-γ-valerolactone; DRW Dealcoholized Red Wine; MRM, Multiple Reaction Monitoring; PCA, Principal Components Analysis; PM, Prediction Model; ROC, Receiver Operating Characteristic; RW, Red Wine; SPE, solid-phase extraction; Tmax, Time to Maximum; $t_{1/2}$, Half live; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry.

ABSTRACT

Scope: The discovery of biomarkers of intake is essential to associate dietary intake (considering their bioavailability) and diet-related risk factors for diseases in nutritional epidemiological studies. The aim is to establish a new screening strategy for discovering novel potential biomarkers of food intake.

Methods and Results: Samples were collected from an intervention clinical trial where participants consumed wine or control beverage over 4-weeks. Phenolic metabolites were analyzed by UPLC-MS/MS. Logistic regression and ROC curves were performed for evaluation of wine biomarkers. Prediction models based mainly in gallic acid metabolites, obtained area under the curve (AUC) for the training and validation sets over 96% for urine and over 80% for plasma. Additionally, resveratrol, ethylgallate and gallic acid metabolites groups in urine samples also resulted in being good predictors of wine intake (AUC>87%). However, lower values were obtained in plasma samples for metabolites. The highest correlations between fasting plasma and urine were obtained for the prediction model score (r=0.6, P<0.001), followed by gallic acid metabolites (r=0.5-0.6, P<0.001).

Conclusion: Our prediction model is a promising tool for evaluating, comparing and discriminating food biomarkers in different biological samples. This study provides new insights into the discovery of food biomarkers and biological mechanisms.

1. Introduction

Biomarkers in epidemiological and clinical trials have to be indicators of exposure, and must have several characteristics, including being robust, sensitive to changes, specific to the dietary source and biologically and physiologically understandable [1]. In the food research field, biomarkers have been discussed in depth with regard to their ability to solve classical problems regarding estimating an index of quantitative exposure to individual food [1-3], and recently, identifying dietary patterns that may be related to major health benefits.

After consumption of polyphenols, beneficial health effects in the prevention of diseases have been widely analysed in *in vivo* and *in vitro* studies [4-6]. In particular, the consumption of grape-derived products such as red wine (RW) and dealcoholized RW (DRW) has been associated with a protective effect on cardiovascular diseases, possibly through their anti-inflammatory and antihypertensive activities [4,7]. These associations were first linked to phytochemicals found in foods, which could exert their biological activity. However, in recent years there has been an increased attention on the metabolites formed in the organism, especially those formed by microbiota, due to their role in the prevention of some diseases such as obesity and diabetes [8-10]. This supposes an increase in the variety of metabolites found in biofluids after consumption, and therefore the increased number of possible food biomarkers [11]. Moreover, new targeted and untargeted approaches have also increased the range of metabolites found in biofluids, allowing the use of metabolomic tools for a new approach in biomarker research. In the case of RW, resveratrol metabolites have been described as being good biomarkers of wine intake [12, 13], and also gallic acid has been suggested as a marker, due to its increased excretion after wine consumption [14]. Both compounds were determined in 24-h urine. This sample has been suggested as being better for biomarker determination, than others but since it is difficult to obtain in large epidemiological studies [1, 12-14], other samples such as fasting plasma need to be assessed to identify biomarkers.

Here, we describe for the first time a new screening strategy for the evaluation and discrimination of biomarkers of intake, specifically RW, through a targeted metabolomic approach in a clinical intervention trial where several biological samples including fasting plasma and 24-h urine samples were considered.

2. Material and methods

2.1 Subjects and study design

Thirty-six volunteers were included for the study of the targeted phenolic metabolite profile. The study was an open, randomized, crossover and controlled clinical intervention trial comprising three 4-week periods [4]. Baseline characteristics of participants and inclusion and exclusion criteria are shown in the online Supporting information and Supporting Information Table S1. After following a 15-day run-in period free of grape-derived products and alcoholic beverages, subjects were requested to consume 272 mL of RW (30 g ethanol/day), 272 mL of DRW and 100 mL of gin (30 g ethanol/day) every day for 4 weeks, following the same background diet. Fasting blood samples (n=33) and 24-h urine samples (n=36) were collected after each intervention period and immediately stored at −80 °C until analysis. The Institutional Review Board of the Hospital approved the study protocol. All participants gave written consent before participation in the study. This trial was registered in the Current Controlled Trials at the International Standard Randomized Controlled Trial Number Register, at controlled-trials.com, as ISRCTN88720134.

2.2 Chemicals and reagents

Chemical reagents and solvents used in this study are detailed in online Supporting Information.

2.3 Red wine, dealcoholized red wine and gin

The RW and DRW used in this study were made with the Merlot grape variety, from the Penedès appellation (Catalonia, Spain). No differences in phenolic composition were found between wines (online Supporting Information Table S2) [15]. Gin Xoriguer was used to ensure the same alcoholic consumption as the RW period.

2.4 Sample extraction

The targeted analyses of microbial-derived and conjugated metabolites were performed using solid-phase extraction (SPE). Oasis® MCX and HLB 96-well plates (Waters, Milford, Massachusetts) were used in hydrolyzed and non-hydrolyzed samples, respectively, as previously described [16-18]. Briefly, urine and plasma samples (1mL) were loaded onto the conditioned cartridge plate. Then, the cartridges were washed and

analytes were eluted with methanol or acidified methanol (0.1% formic acid) , respectively. Eluates from both extraction methods were evaporated to dryness. Residues were reconstituted with taxifolin 100 µL(1.64 µmol/L) mobile phase [16, 18].

2.5 UPLC-MS/MS analysis

The analysis of metabolites in urine and plasma was performed by UPLC-MS/MS equipped with a binary solvent manager and a refrigerated autosampler plate (Waters Acquity UPLC system, Milford, MA, USA), coupled to an AB Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray, in a negative electrospray ionization mode (PE Sciex). An Acquity UPLC BEH C18 (Milford, MA, USA) (1.7 µm, 2.1 mm × 5 mm), using a prefilter, working at 40 ◦C with 0.5 mL/min with an injection volume of 5 µL, was used as described before [16]. Mobile phase A (0.1% formic acid) and B (0.1% formic acid in acetonitrile) were used at a flow rate of 500 µL/min with the following proportions (v/v) of phase A [t(min),%A]: (0,92); (2.5,50); (2.6,0); (3,0); (3.1,92); (3.5,92). The MS/MS parameters used were as previously described [16, 17].

2.6 Quantitative analysis

For quantification purposes, data were collected using the Multiple Reaction Monitoring (MRM) mode with a dwell time of 10 ms. When commercial standards were not available, concentrations were quantified using the most similar compound standard curve. Results were expressed as their equivalents [16]. Quality parameters of the methodology had already been published [16, 17].

2.7 Statistical analysis

Two statistical programs for data analysis were used: the MetaboAnalyst Web-based platform [19] and IBM SPSS Statistics software program for Windows version 20 (Chicago, IL). Data normalization was performed by a cube root transformation and a range scaling of the data, retrieving an unsupervised segregation by Principal Component Analysis (PCA) and hierarchical clustering analysis. ANOVA for repeated measures was used to compare changes in phenolic metabolites in plasma and urine after intervention treatments (Bonferroni post hoc test).

Among the metabolites that displayed significantly different levels between wine interventions and baseline and gin period, a binary stepwise logistic regression analysis

was performed to assess which metabolite combination predicted the wine intervention. For this purpose, 80% of random samples of wine interventions and baseline and gin periods were used as a training set, in which the logistic regression model was calculated, and the remaining samples of each groups (20%) as a validation set. The sensitivity, specificity and AUC of the model were compared with parameters of phenolic group metabolites in the whole population through a Receiver Operating Characteristic (ROC) curve. The phenolic metabolite groups in urine and plasma are described in online Supporting Information Table S3. In addition to the metabolites analyzed in this study, resveratrol data, as it has already been described as a wine intake biomarker [12, 13], were included from previous analysis [15] to be evaluated and compared.

The optimal cut-off for the ROC curves was determined through the identification of the shortest distance to the optimal point (0,1) for which specificity and sensitivity was calculated.

To estimate the association between fasting plasma and 24-h urine in the prediction models and within the phenolic group metabolites, the Spearman correlation coefficients were calculated. Statistical significance was defined as $P \leq 0.05$.

3. Results

3.1 Urine and plasma analysis of targeted polyphenol metabolomic pattern

Nineteen individual metabolites and conjugates of (epi)catechin, methyl(epi)catechin, dihydroxyphenyl-γ-valerolactone (DHPV) and stilbenes significantly increased in urine after RW or DRW interventions compared to the baseline and gin periods (online Supporting Information Tables S4 and 5). Only ethylgallate metabolites showed a statistically significant difference between both wines' periods of intake. The plasma metabolites that increase after the wine interventions in relation to the baseline and gin periods included 10 phenolic acids such as gallic acid and DHPV and their conjugates.

The PCA differentiated easily between samples from RW and DRW interventions and samples from those in the baseline and gin period (online Supporting Information Fig. S1A). PC1 explained 41.9% of the total variance while PC2 explained 7.6% of the total variance, where the loading plot showed that gallic acid, ethylgallate and resveratrol metabolites were mainly responsible for this difference (data not shown). The clustering analysis executed by the heat map compared participants in the four intervention periods

with metabolites. It was used as a first approach to assess the possible use of phenolic groups as biomarkers of wine consumption. A progression in the strongest discriminatory signals was observed in the heat map (online Supporting Information Fig. S1B). The strongest discriminatory signals were observed for resveratrol, gallic acid and ethylgallate metabolites, followed by (epi)catechin and valerolactone metabolites, and the least discriminatory signals were for phenolic acids. These phenolic metabolite groups and the prediction model were evaluated and compared to determine the best biomarkers of wine consumption through a ROC analysis.

3.2 Discriminant analysis of food biomarkers

The applicability of a logistic regression model involving multiple metabolites was examined to find the best markers of wine consumption in hydrolyzed and non-hydrolyzed fasting plasma and 24-h urine samples from a clinical study in the training set. Metabolites were subjected to a stepwise variable selection method. The results of the model for each type of sample are shown in Table 1. Metabolites included in the models did not display multicollinearity (data not shown). All the resveratrol metabolites were excluded from this analysis since individual metabolites and both groups of resveratrol (resveratrol biomarker and microbial resveratrol metabolites) have already presented AUC over 96%. Then, a logistic regression model for predicting wine intake was established on the basis of the data for these metabolites (Table 1). The validity of the model was confirmed with the validation set, and then applied to the whole population. The results of sensitivity, specificity and AUC for the model were similar and higher than 92% and 75% for urine and fasting plasma samples, respectively, among training and validation sets, and for the whole population (Table 2). The global performance of the model for each kind of sample considering the whole population was depicted in the ROC curves (Table 2 and Fig. 1) and compared with the results obtained for the different phenolic groups described previously (Table 2 and online Supporting Information Fig. S2). In hydrolyzed urine, ethylgallate with a sensitivity of 93.06%, a specificity of 84.72% and an AUC of 92.35% resulted in being the best discriminatory metabolite, followed by gallic acid metabolites, while in non-hydrolyzed urine samples, all the selected groups resulted in being good discriminators of wine intake: ethylgallate, methylgallic and resveratrol metabolites (AUC: 93-99%) and (epi)catechin and DHPV metabolites (AUC: 76-86%). The best sensitivity and specificity were obtained for microbial resveratrol metabolites with 95.83% and

93.06%, respectively (cut-off value: 1424.19 μmol/24-h), closely followed by resveratrol biomarker. Plasma metabolites were worse indicators of wine intake and only methylgallic sulfate had an AUC over 80%, which matched the results obtained in the prediction model. The ROC curves of the prediction models resulted in being more discriminatory than the phenolic metabolite groups (Table 2 and online Supporting Information Fig. S2), with the exception of the microbial resveratrol metabolite group, which had similar values, in the non-hydrolyzed samples.

3.3 Correlations between fasting plasma and 24-h urine

Correlations of individual metabolites, phenolic metabolite groups and the prediction model between 24-h urine and fasting plasma samples were performed (Table 3). The highest correlations in both hydrolyzed and non-hydrolyzed samples were obtained for the prediction model score (r=0.565 and 0.599, P<0.001, respectively) (Fig. 2), followed by the gallic acid metabolite group (r=0.451 and 0.587, P<0.001, respectively). The group of flavan-3-ols and DHPV metabolites had lower but significant correlation values (r=0.4, P<0.001) in non-hydrolyzed samples.

4. Discussion

This is the first study in which several derived metabolites from wine intake have been systematically evaluated and trialled as a new evaluation strategy for the discovery and discrimination of food intake biomarkers. The methodology was based on a UPLC-MS/MS targeted approach analysis [16] of wine polyphenol metabolites in 24-h urine and fasting plasma samples obtained from an intervention clinical trial [4] paired to multivariate statistical methods. Up to 19 individual metabolites and conjugates of (epi)catechin, methyl(epi)catechin and DHPV resulted in being higher after RW and DRW compared with baseline and gin periods in plasma and urine (online Supplementary Table 3 and 4). No differences were observed between RW and DRW metabolites in plasma and urine except for urinary concentrations of ethylgallate, whose concentration increased after the RW period (P<0.001) (online Supporting Information Table S4). Ethylgallate is a wine compound derived from ethanol and gallic acid esterification [20], with similar concentration values in both wines (online Supporting Information Table S1). The increment observed after RW intake may be due to the fact that ethylgallate could also be formed in the organism favored by ethanol and gallate

consumption through ethyl esterification by human esterases or by microbial metabolism [21, 22]. Therefore, biomarkers of wine consumption were evaluated after both wine interventions and compared with baseline and gin periods.

The assessment of metabolites as food biomarkers depends on their specificity, sensitivity, robustness, variability and biological sense [1, 3], which means that has to be an objective measure of intake and an evaluated indicator of food intervention [3]. There is an increased interest in biomarker research, for the development of new functional foods, as well as for validation of the existing biomarkers [23]. Therefore, global metabolic approaches need to be carried out in order to evaluate the role of individual or groups of metabolites in the discrimination of selected food consumption. In addition, the type of sample matrix in which they are measured also influences biomarker evaluation [24, 25]. In the current study, we compared biomarkers obtained by a new screening strategy through stepwise logistic regression and through groups of polyphenol metabolites. These biomarkers were tested using ROC curves to evaluate their capacity to discriminate wine consumers (Table 2 and online Supporting Information Fig. S2). This approach, traditionally used in clinical diagnosis [26], has been applied for the first time in food research. All of the metabolites included in the model (Table 1)could come from the microbial degradation of several wine phenolics [11] and some of them are also present in wine composition, such as gallic acid, ethylgallate and 2,4-dihydroxybenzoic acid [16, 27, 28]. Gallic acid could also be released from several compounds present in wine, such as gallates and anthocyanins [14, 29]. 2,4-Dihydroxybenzoic acid has also been described as coming from the degradation of anthocyanins [30] and 3-hydroxyphenylacetic and p-coumaric acids, derived from procyanidins and anthocyanins, respectively [18, 31], which can be found in wine in high content [27]. However, due to the fact that wine is a food with a complex phenolic composition, it is difficult to establish whether metabolites come from a single group of polyphenols or, what is more likely, from the whole composition. Other analyzed phenolic acids were not considered in the model since they were less discriminant as most of phenolic acids may arise from several food compounds. Thus, these metabolites could be misleading if they were considered as biomarkers, as has previously been suggested after the intake of berries [9, 11, 24].

When the model was compared with the phenolic metabolite groups in urine samples, we observed that the resveratrol biomarker and microbial resveratrol metabolites had similar values to the model with AUC values of 96.5 and 98.8%, respectively. Until

now, phase II metabolites of resveratrol have been proposed as good biomarkers of wine intake [12, 13], but microbial-derived metabolites have not been evaluated before. As was discussed above, one of the characteristics of a good biomarker is being specific to food intake, thus, resveratrol is well known for its being almost exclusively distributed in grape products [32]. Other phenolic groups with good but lower AUC values than the model were ethylgallate and gallic acid metabolites (Table 2). Thus, they could also be considered as biomarkers of wine intake. Previously, some authors positively associated gallic acid and methylgallic acid with the consumption of wine [33], but, to our knowledge, no associations have been published for ethylgallate. Gallic acid has even been described as the main metabolite of ethylgallate, with longer Tmax and $t_{1/2}$ than its parent compound [34]. Both ethylgallate and gallic acid have been described in other foods such as grape products, wine and vinegar, and tea, nuts and berries [27]. Other metabolites such as (epi)catechin and valerolactones were less discriminant than the other described metabolites and the model. Although the concentrations of flavan-3-ols are high in wine, they are not exclusively found in wine since metabolites have been described after cocoa, tea or nut consumption [18, 35, 36].

The type of sample is a determinant in the evaluation of biomarkers. In this study, we compared fasting plasma with 24-h urine samples. 24-h urine has been described as the gold standard sample for biomarker evaluation [37]. It provides a better measure of total polyphenol metabolites than fasting plasma as it provides a better index of intake [1]. However, for practical reasons, 24-h urine is not an easy sample to obtain in large-scale epidemiological studies [1]. Consequently, we have assessed that fasting plasma should be considered for biomarker determination. In this study, individual and phenolic metabolite groups formed for biomarker identification were correlated between fasting plasma and 24-h urine (Table 3), along with the score obtained from the prediction model (Fig. 2). The best correlations were observed among model scores from hydrolyzed and non-hydrolyzed samples, indicating that those volunteers that were better classified as wine consumers in urine, they were also in plasma (r=0.565 and r=0.599, respectively P<0.001). In addition, gallic acid metabolites in plasma were also significantly correlated with their presence in hydrolyzed and non-hydrolyzed urine samples (r=0.451 and r=0.587, respectively, P<0.001), as well as valerolactone metabolites (r=0.348 and r=0.356, respectively, P<0.001). Ethylgallate could not be evaluated due to the low concentrations obtained in plasma since Tmax and half-lives of ethylgallate were expected to be lower than its main metabolite gallic acid [34].

Valerolactones and gallic acid microbial metabolites were selected for their important role as biomarkers in urine, and possible presence in fasting plasma due to their longer half-life [34, 36]. Although the coefficients of correlation were significant, the r values were clinically moderate (r < 0.750) [25], which were similar to previous studies that correlated urinary and plasma alkylresorcinol metabolites [25]. Correlations between 24-h urine and fasting plasma were previously described for total flavonol in a crossover trial with a low flavonoid diet or with the same diet supplemented with flavonols (r=0.624) [38], as well as for isoflavones, using spot plasma (r=0.99) [39].
These correlations could open the possibility of finding those metabolites in plasma and establishing them as biomarkers of consumption and effect, but larger studies in a free-living population are needed to confirm and generalize this statement. In addition, a problem with the fasting plasma, as suggested previously [40], could be the substantial number of concentrations lower than the limit of quantification due to the short half-lives of polyphenol metabolites.
In conclusion, this study proposes the use of a strategy that combines targeted metabolomic analysis and logistic regression in interventional studies to identify, evaluate, compare and validate biomarkers of food intake, more precisely biomarkers of wine intake. Correlations between fasting plasma and urine provide the opportunity to discriminate metabolites that could be good urinary biomarkers of consumption in urine, and that could also be found in plasma. This new approach is a promising tool that has great potential for identifying possible biomarkers to evaluate compliance in clinical studies, identifying eating patterns and making associations between polyphenol consumption and health benefits.

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Figure captions

Figure 1. ROC curves of the established prediction model (PM) for each considered sample in the whole population. (A), Hydrolyzed and non-hydrolyzed 24-h urine samples. (B), Hydrolyzed and non-hydrolyzed fasting plasma samples.

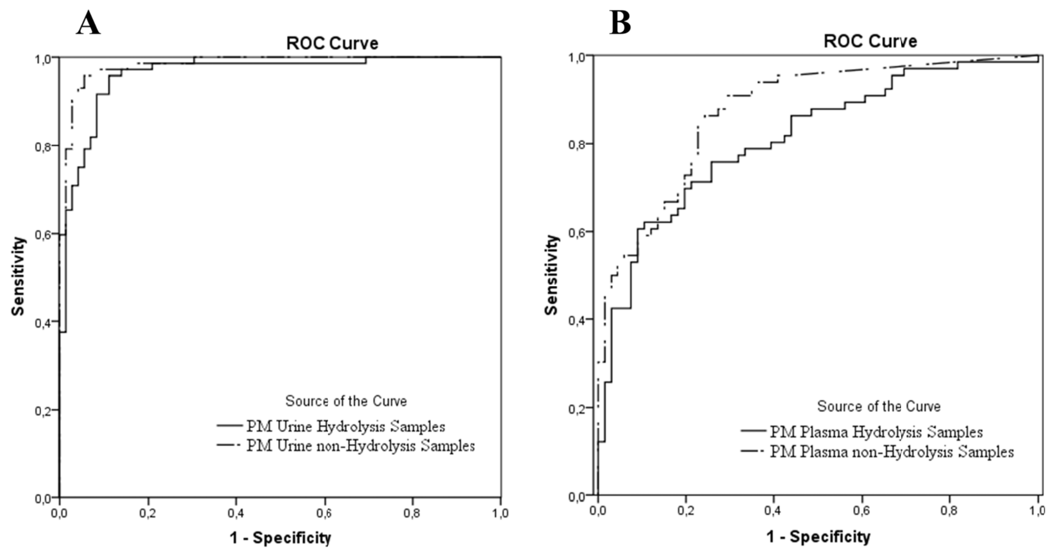


Figure 2. Spearman's correlations between fasting plasma and 24-h urine biomarker score obtained from prediction models (PM). (A), Hydrolyzed samples. (B), Non-hydrolyzed samples.

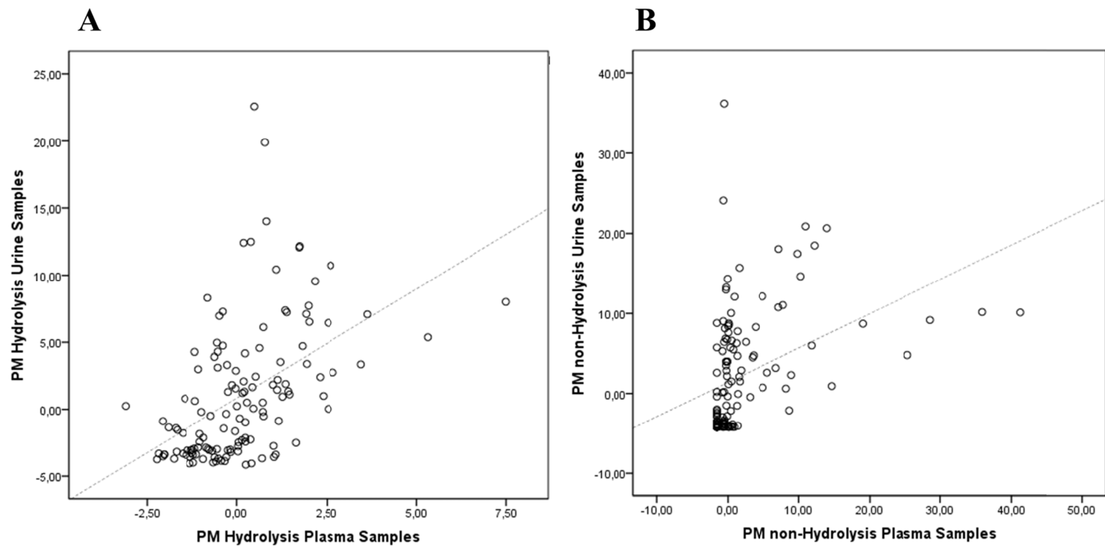


Table 1. Urine and plasma metabolites in hydrolyzed and non-hydrolyzed samples selected by the stepwise logistic regression model for discriminating wine consumers obtained from the training set.

	Coefficient	Standard error	*p* value	Coefficient CI 95%
URINE				
Hydrolyzed samples				
2,4-Dihydroxybenzoic Acid	0.60	0.23	0.007	0.16, 1.05
Gallic Acid	0.84	0.24	<0.001	0.38, 1.31
Ethylgallate	0.48	0.18	0.009	0.12, 0.83
Constant	-4.47	0.94	<0.001	-6.31, -2.63
Non-Hydrolyzed samples				
Methylgallic Acid Sulfate	0.17	0.06	0.005	0.05, 0.29
Ethylgallate Sulfate	0.41	0.10	<0.001	0.21, 0.62
Constant	-4.19	0.91	<0.001	-5.98, -2.41
PLASMA				
Hydrolyzed samples				
3-Hydroxyphenylacetic Acid	2.38	1.02	0.020	0.39, 4.38
Gallic acid	62.21	22.41	0.006	18.29, 106.12
P-Coumaric acid	40.91	15.43	0.008	10.67, 71.14
Constant	-3.09	0.79	<0.001	-4.64, -1.54
Non-Hydrolyzed samples				
Methylgallic Acid Sulfate	525.00	118.68	<0.001	292.39, 757.61
Constant	-1.63	0.35	<0.001	-2.32, -0.94

Table 2. Threshold (cut-off), sensitivity, specificity, AUC and confidence interval of phenolic metabolite group biomarkers and the prediction model.[a)]

	Threshold[b)]	Sensitivity (%)	Specificity (%)	AUC (%)	AUC 95% CI (%)
URINE					
Hydrolyzed samples					
Gallic acid Metabolites	4.89	88.89	77.80	87.75	81.97, 93.53
DHPV Metabolites	18.17	83.33	66.70	81.15	74.14, 88.17
Phenolic acid Metabolites	207.2	69.44	62.50	71.95	64.03, 80.34
Ethylgallate	0.69	93.06	84.72	92.35	87.73, 96.97
Training Set		94.74	91.23	96.24	92.73, 99.76
Validation Set		93.33	93.33	96.00	89.24, 100.0
All population		**91.66**	**91.66**	**96.14**	**93.12, 99.16**
Non-hydrolyzed samples					
(Epi)catechin Metabolites	39.84	75.00	83.33	86.32	80.38, 92.27
DHPV Metabolites	695.53	66.70	73.60	76.33	68.70, 83.96
Ethylgallate Metabolites	152.31	98.60	84.70	93.67	89.49, 97.86
Methylgallic Sulfate	5.49	87.50	86.11	93.23	89.31, 97.15
Resveratrol Biomarker	1966.05	91.67	95.83	96.45	93.38, 99.52
Microbial Resveratrol Metabolites	1424.19	95.83	93.06	98.77	97.46, 100.0
Training Set		94.74	96.49	98.68	97.13, 100.0
Validation Set		100.0	93.33	96.44	89.32, 100.0
All population		**95.83**	**94.44**	**98.40**	**96.80, 100.0**
PLASMA					
Hydrolyzed samples					
Gallic acid Metabolites	0.06	68.18	57.58	64.10	54.66, 73.53
DHPV Metabolites	0.14	68.18	62.12	68.37	59.31, 77.42
Phenolic acid Metabolites	0.24	72.73	65.15	68.02	58.88, 77.16
Training Set		74.07	76.92	80.13	71.75, 88.51
Validation Set		75.00	100.0	88.10	74.20, 100.0
All population		**75.76**	**74.24**	**81.18**	**73.86, 88.49**
Non-hydrolyzed samples					
(Epi)catechin Metabolites	0.03	69.70	75.76	76.92	68.76, 85.07
DHPV Metabolites	0.06	72.73	62.12	71.12	62.37, 79.87
Methylgallic Sulfate	0.002	84.85	77.27	87.50	81.65, 93.35
Training Set		85.19	76.92	86.89	80.13, 93.66
Validation Set		91.67	78.57	91.07	80.22, 100.0
All population		**84.85**	**77.27**	**87.50**	**81.65, 93.35**

[a)] DHPV, dihydroxyphenyl-γ-valerolactone; ROC, receiver operating characteristic.
[b)]Urine (μmol/24-h) or plasma (μmol/L)

Table 3. Spearman's correlations between fasting plasma and 24-h urine samples for individual, phenolic metabolite groups and the prediction model.

	r	*P*
Hydrolyzed samples		
Gallic acid	0.338	<0.001
3-Methylgallic acid	0.441	<0.001
Gallic acid group	0.451	<0.001
2,5-Dihydroxybenzoic acid	0.411	<0.001
Protocatechuic acid	0.174	0.046
3-(3-Hydroxyphenyl)propionic acid	0.402	<0.001
Ferulic acid	0.253	0.003
4-Hydroxyhippuric acid	0.310	<0.001
3-Hydroxyphenylacetic acid	0.444	<0.001
2-Hydroxyphenylacetic acid	0.204	0.019
Enterolactone	0.503	<0.001
p-Coumaric acid	0.370	<0.001
Phenolic acid group	0.442	<0.001
DHPV1	0.321	<0.001
DHPV2	0.336	<0.001
DHPV group	0.348	<0.001
Prediction Model Score	0.565	<0.001
Non-hydrolyzed samples		
Methylgallic acid Sulfate	0.587	<0.001
(Epi)catechin Glucuronide 3	0.342	<0.001
Methyl (epi)catechin Glucuronide 2	0.294	0.001
Flavan-3-ol group	0.382	<0.001
DHPV1 Glucuronide	0.321	<0.001
DHPV2 Glucuronide	0.342	<0.001
DHPV group	0.356	<0.001
Prediction Model Score	0.599	<0.001

Supporting Information

Subjects and study design

Inclusion and exclusion criteria

Volunteers included in this trial were moderate alcohol consumers (1–3 drinks/d) and had type 2 diabetes or ≥3 of the cardiovascular disease (CAD) risk factors (tobacco smoking, hypertension, plasma LDL cholesterol concentrations ≥160 mg/dL, plasma HDL cholesterol concentrations ≤35 mg/dL, overweight or obesity [BMI (in kg/m^2) ≥25], and/or family history of premature CAD). Exclusion criteria included documented CAD, stroke, or peripheral vascular disease, HIV infection, alcoholic liver disease, malnutrition, and neoplastic or acute infectious diseases [1].

Chemicals and reagents

2,4-Dihydroxybenzoic, 2,6-dihydroxybenzoic, 2,5-dihydroxybenzoic, 3,5-dihydroxybenzoic, 4-hydroxybenzoic, 3-hydroxybenzoic, gallic, syringic, phenylacetic, 3-hydroxyphenylacetic, 2-hydroxyphenylacetic, 3,4-dihydroxyphenylacetic, 3-(4-hydroxyphenyl)propionic, dihydrocaffeic, *p*-coumaric, *o*-coumaric, caffeic, ferulic, protocatechuic and sinapic acids and enterolactone, pyrogallol, ethylgallate, (−)-epicatechin, (+)-catechin, *β*-glucuronidase/sulfatase (from *Helix pomatia*) and blank human plasma were purchased from Sigma-Aldrich (St Louis, MO, USA). 4-Hydroxyhippuric acid was purchased from PhytoLab GmbH&Co.KG (Vestenbergsgreuth, Germany). 3-(3-Hydroxyphenyl)propionic acid was purchased from Apin Chemicals Limited (Abingdon, UK). Taxifolin, vanillic, 4-*O*-methylgallic, and *m*-coumaric acids were purchased from Extrasynthèse (Genay, France). Standard of epicatechin-5-*O*-glucuronide was chemically synthesized and characterized as previously published [2]. HPLC grade solvents methanol, acetonitrile, glacial acetic, O-phosphoric acid and formic acid were purchased from Scharlau Chemie, S.A. (Sentmenat, Spain) and acetone and ammonia (35%) and hydrochloric acid from Panreac Química, S.A.U. (Castellar del Vallès, Spain). Ultrapure water (Milli-Q) was obtained from Millipore System (Bedford, MA, USA). Synthetic urine was prepared as previously described [3].

Supporting Information Table S1. Baseline characteristics of 36 subjects.[a)]

	Values [b)]
Age (y)	61±9
Current smokers [*n* (%)]	6 (16.6)
Sedentarism [*n* (%)]	21 (58.3)
Family history of premature CAD [*n* (%)]	27 (75)
BMI (kg/m^2)	29.5±3.8
BMI >25 kg/m^2 [*n* (%)]	33 (91.6)
WHR	0.98±0.05
Type 2 diabetes [*n* (%)]	9 (25)
Hypertension [*n* (%)]	17 (47.2)
Dyslipemia [*n* (%)]	12 (33.3)
Medications [*n* (%)]	
ACE inhibitors	17 (47.2)
Statins	12 (33.3)
Oral hypoglycemic drugs	9 (25.0)
Aspirin or antiplatelet drugs	8 (22.2)
Systolic blood pressure (mm Hg)	140 ± 19.1
Diastolic blood pressure (mm Hg)	80 ± 9.8
Heart rate (beats/min)	69 ± 9
Glucose (mg/dL)	119 ± 36
Triglycerides (mg/dL)	122 ± 53
Total cholesterol (mg/dL)	198 ± 34
LDL cholesterol (mg/dL)	129 ± 29
HDL cholesterol (mg/dL)	44 ± 10
LDL cholesterol:HDL cholesterol ratio	3.1 ± 0.9
Folic acid (serum) (ng/mL)	9.0 ± 3.6
Intraerythrocytary folic acid (ng/mL)	395 ± 100
Vitamin B-12 (pg/mL)	400 ± 136
Albumin (mg/mL)	44.6 ± 3.1
ASAT (UI/L)	24.8 ± 8.5
ALAT (UI/L)	31.9 ± 23.5
GGT (UI/L)	33.6 ± 20.6

[a)]ACE, angiotensin-converting enzyme; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CAD, coronary artery disease; GGT, c-glutamyl transpeptidase; WHR, waist-to-hip ratio.
[b)]Results are expressed as mean ± SD or *n* (%)[4].

Supporting Information Table S2. Phenolic composition of red wine (RW), dealcoholized red wine (DRW) and gin during the study period.[a)]

Phenolic compound (mg/L)[b)]	**RW** [c)]	**DRW** [c)]	**Gin**
trans-Resveratrol	2.92 ± 0.36	2.73 ± 0.23	ND
cis-Resveratrol	2.79 ± 0.15	2.75 ± 0.15	ND
trans-Piceid	9.41 ± 1.12	10.53 ± 0.96	ND
cis-Piceid	7.71 ± 0.34	7.08 ± 0.87	ND
Gallic acid	68.48 ± 6.40	73.17 ± 7.01	ND
Ethylgallate	4.12 ± 1.30	3.53 ± 0.62	ND
Protocatechuic acid	5.22 ± 0.62	5.85 ± 0.51	ND
Tyrosol	43.59 ± 4.73	47.81 ± 3.90	ND
Catechin	123.51 ± 11.30	126.45 ± 13.35	ND
Epicatechin	67.86 ± 7.74	70.57 ± 8.22	ND
Procyanidins	183.98±16.14	187.84±15.10	ND
trans-Caftaric acid	18.62 ± 1.44	19.21 ± 1.62	ND
trans-Caffeic acid	11.50 ± 0.79	12.18 ± 0.92	ND
trans-Coutaric acid	5.21 ± 0.45	5.62 ± 0.52	ND
2-*S*-Glutathionylcaftaric	10.30 ± 1.00	10.76 ± 1.26	ND
Quercetin-3-glucuronide	11.88 ± 1.38	11.25 ± 1.42	ND
Quercetin	26.66 ± 0.78	23.82 ± 2.37	ND
Isorhamnetin	3.34 ± 0.27	2.96 ± 0.14	ND
Delphinidin-3-glucoside	15.25 ± 0.89	14.71 ± 1.62	ND
Petunidin-3-glucoside	12.29 ± 1.06	12.04 ± 1.15	ND
Peonidin-3-glucoside	6.78 ± 0.62	6.68 ± 0.57	ND
Malvidin-3-glucoside	48.83 ± 4.45	49.86 ± 4.27	ND
Malvidin-(6-acetyl)-3-glucoside	10.97 ± 0.96	10.41 ± 1.20	ND
Malvidin-(6-coumaroyl)-3-glucoside	4.15 ± 0.27	3.54 ± 0.33	ND
Total Phenol (mEqGA/L)[c)]	2933.35 ± 377.31	2694.92 ± 86.79	ND

[a)]DRW, dealcoholized red wine; mEqGA/L, mEq Gallic acid/L; ND, not detected; RW, red wine. No significant differences were observed between RW and DRW polyphenols (Student's t test for independent samples, $P>0.05$).
[b)]Determined as previously described by Romero-Perez, et al. [5], by Ibern-Gomez, et al. [6] and by Queipo-Ortuño et al.[7].
[c)]Values are expressed as mean ± SD (n=5 by duplicate during the study period).
[d)]Determined by Folin-Ciocalteu methodology [8].

Supporting Information Table S3. Phenolic metabolites groups considered in this study in plasma and urine samples.

Groups	**Metabolites included in each group** [a)]
Hydrolyzed samples	
Gallic acid metabolites	Gallic acid and methylgallic acid
DHPV Metabolites [b)]	DHPV1 and DHPV2
Phenolic Acid Metabolites	Significant metabolites, see Supplemental Table 4 and 5
Ethylgallate	Ethylgallate
Non-hydrolyzed samples	
(Epi)catechin Metabolites	(Epi)catechin and methyl(epi)catechin glucuronides and sulfates
DHPV Metabolites	DHPV glucuronides and sulfates
Ethylgallates	Ethylgallate sulfate and glucuronides
Methylgallic sulfate	Methylgallic sulfate
Resveratrol Biomarker [9, 10] [c)]	*trans*-resveratrol-3-*O*-glucuronide, *cis*-resveratrol-4'-*O*-glucuronide, *cis*-resveratrol-3-*O*-glucuronide, *trans*-resveratrol-4′-*O*-sulfate, *trans*-resveratrol-3-*O*-sulfate, *cis*-resveratrol-4′-*O*-sulfate, *cis*-resveratrol-3-*O*-sulfate
Resveratrol Microbial Metabolites[c)]	Dihydroresveratrol, dihydroresveratrol glucuronides, dihydroresveratrol sulfates, dihydroresveratrol sulfoglucuronide

a) Metabolites in plasma were only considered when quantifiable amounts were observed in samples.
b) DHPV, dihydroxyphenyl-γ-valerolactone.
c) Only analyzed in urine samples.

Supporting Information Table S4. Urinary concentrations of metabolites in 36 subjects at baseline and after the three intervention periods.[a)]

Metabolites	MRM	Urine samples (µmol, 24-h)[b)]				
		BAS	RW	DRW	GIN	*P* [c)]
Hydroxybenzoic acids						
4-Hydroxybenzoic acid	137/93	25.79±2.21$^{\alpha}$	29.84±3.52$^{\alpha,\beta}$	34.30±2.81$^{\beta}$	27.07±2.21$^{\alpha}$	0.006
3-Hydroxybenzoic acid	137/93	3.77±1.27$^{\alpha,\beta}$	4.11±0.89$^{\alpha,\beta}$	5.67±1.57$^{\beta}$	2.97±0.9$^{\alpha}$	0.001
2,4-Dihydroxybenzoic acid	153/109	1.57±0.17$^{\alpha}$	2.47±0.35$^{\beta}$	2.67±0.37$^{\beta}$	1.62±0.22$^{\alpha}$	<0.001
2,6-Dihydroxybenzoic acid	153/109	6.19±0.6$^{\alpha}$	8.35±0.91$^{\beta}$	8.74±0.88$^{\beta}$	6.08±0.59$^{\alpha}$	<0.001
2,5-Dihydroxybenzoic acid	153/109	16.23±1.65$^{\alpha}$	24.79±2.91$^{\beta}$	27.29±2.9$^{\beta}$	17.2±2.2$^{\alpha}$	<0.001
3,5-Dihydroxybenzoic acid	153/109	3.93±0.66$^{\alpha,\gamma}$	6.41±1.01$^{\alpha,\beta}$	7.57±1.26$^{\beta}$	3.97±0.72$^{\gamma}$	0.006
Protocatechuic acid	153/109	12.10±1.15	13.07±1.27	14.45±1.66	11.29±1.19	0.09
Syringic acid	197/121	0.73±0.15$^{\alpha}$	1.91±0.43$^{\beta}$	2.03±0.32$^{\beta}$	0.70±0.17$^{\alpha}$	<0.001
4-Hydroxyhippuric acid	194/100	54.05±5.42	58.83±4.47	72.13±9.02	53.63±5.69	0.09
3-Hydroxyhippuric acid	194/150	192.30±39.81	204.09±38.07	237.58±54.21	169.25±34.57	0.19
Gallic acid metabolites						
Gallic acid	169/125	0.85±0.18$^{\alpha}$	5.61±0.49$^{\beta}$	4.76±0.53$^{\beta}$	0.73±0.17$^{\alpha}$	<0.001
Methylgallic acid [e)]	167/108	2.97±0.42$^{\alpha}$	4.37±0.62$^{\beta}$	4.76±0.68$^{\beta}$	3.03±0.41$^{\alpha}$	<0.001
Methylgallic sulfate[d),e)]	263/183	2.97±0.74$^{\alpha}$	24.8±5.64$^{\beta}$	19.94±3.08$^{\beta}$	2.00±0.60$^{\alpha}$	<0.001
Ethylgallate metabolites						
Ethylgallate	197/169	1.06±0.37$^{\alpha}$	8.19±0.93$^{\beta}$	4.97±0.73$^{\gamma}$	0.22±0.09$^{\alpha}$	<0.001
Ethylgallate sulfate [d),e)]	277/197	2.16±0.76$^{\alpha}$	24.18±2.73$^{\beta}$	15.81±1.64$^{\gamma}$	0.36±0.14$^{\alpha}$	<0.001
Ethylgallate glucuronide 1 [d),e)]	373/197	36.73±6.01$^{\alpha}$	176.89±20.38$^{\beta}$	114.52±10.77$^{\beta}$	31.49±5.43$^{\alpha}$	<0.001
Ethylgallate glucuronide 2 [d),e)]	373/197	101.74±22.4$^{\alpha}$	366.5±37.6$^{\beta}$	240.9±24.23$^{\gamma}$	64.5±5.75$^{\alpha}$	<0.001
Hydroxyphenylacetic acids						
Phenylacetic acid	135/91	22.15±2.21$^{\alpha,\beta}$	25.49±2.40$^{\alpha,\beta}$	27.66±3.00$^{\alpha}$	21.31±2.17$^{\beta}$	0.005
3-Hydroxyphenylacetic acid	151/107	24.72±3.50$^{\alpha}$	52.27±6.76$^{\beta}$	56.57±6.9$^{\beta}$	19.74±2.51$^{\alpha}$	<0.001
2-Hydroxyphenylacetic acid	151/107	5.89±0.40$^{\alpha,\beta}$	6.48±0.55$^{\alpha,\beta}$	7.41±0.54$^{\beta}$	5.76±0.49$^{\alpha}$	0.008
3,4-Dihydroxyphenylacetic acid	167/123	1.61±0.17$^{\alpha}$	1.98±0.17$^{\alpha,\beta}$	2.37±0.24$^{\beta}$	2.12±0.32$^{\alpha,\beta}$	0.026
Homovanillic acid	181/137	164.35±13.99	185.49±21.12	215.13±25.55	166.92±23.28	0.09
Hydroxycinnamic acids						
m-Coumaric acid	163/119	0.54±0.09$^{\alpha,\beta}$	0.86±0.20$^{\alpha}$	0.83±0.20$^{\alpha,\beta}$	0.40±0.06$^{\beta}$	0.005
p-Coumaric acid	163/119	0.64±0.07$^{\alpha}$	1.75±0.35$^{\beta}$	1.48±0.15$^{\beta}$	0.55±0.08$^{\alpha}$	<0.001

o-Coumaric acid	163/119	0.07±0.02	0.11±0.05	0.10±0.03	0.13±0.03	0.19
Caffeic acid	179/135	5.42±0.34$^{\alpha}$	5.84±0.47$^{\alpha,\beta}$	7.05±0.55$^{\beta}$	4.83±0.45$^{\alpha}$	<0.001
Ferulic acid	193/134	11.80±0.98$^{\alpha}$	15.7±1.79$^{\alpha,\beta}$	15.25±0.94$^{\beta}$	11.16±0.83$^{\alpha}$	0.002
Sinapic acid	223/164	0.99±0.18	1.25±0.19	1.43±0.2	1.18±0.26	0.091
Hydroxyphenylpropionic acids						
3-(3-Hydroxyphenyl) propionic acid	165/121	6.22±1.09$^{\alpha}$	7.13±1.26$^{\alpha,\beta}$	10.07±2.05$^{\beta}$	4.70±0.87α	<0.001
3-(4-Hydroxyphenyl)propionic acid	165/121	287.44±27.16	371.63±45.16	389.2±39.36	313.3±36.76	0.06
Dihydrocaffeic acid	181/137	14.09±1.39$^{\alpha,\beta}$	16.22±1.75$^{\alpha,\beta}$	17.29±1.50$^{\beta}$	12.87±1.51$^{\alpha}$	0.018
Flavan-3-ols [e)]						
∑(Epi)catechin glucuronides[d)]	465/289	9.42±1.58$^{\alpha}$	24.15±3.20$^{\beta}$	26.61±5.39$^{\beta}$	6.72±1.87$^{\alpha}$	<0.001
∑ (Epi)catechin sulfates[d)]	369/289	3.04±0.49$^{\alpha}$	10.17±1.44$^{\beta}$	10.69±1.66$^{\beta}$	2.50±0.44$^{\alpha}$	<0.001
∑ Methyl(epi)catechin glucuronides[d)]	479/303	3.76±0.93$^{\alpha}$	15.95±2.41$^{\beta}$	13.84±2.74$^{\beta}$	3.32±0.84$^{\alpha}$	<0.001
∑ Methyl(epi)catechin sulfates[d)]	383/303	10.87±1.94$^{\alpha}$	25.27±2.92$^{\beta}$	25.64±3.39$^{\beta}$	7.33±1.9$^{\alpha}$	<0.001
Glycinates						
Vanilloylglycine	224/180	0.80±0.09$^{\alpha}$	1.41±0.26$^{\beta}$	1.31±0.16$^{\alpha,\beta}$	0.80±0.13$^{\alpha}$	0.001
Feruloylglycine	250/100	9.23±1.05	11.27±1.38	11.24±1.31	8.88±1.35	0.14
Hydroxyphenylvalerolactones [e]						
DHPV 1	207/163	6.73±1.21$^{\alpha}$	13.80±2.78$^{\beta}$	13.61±2.68$^{\beta}$	3.67±0.77$^{\alpha}$	<0.001
DHPV 2	207/163	18.50±3.67$^{\alpha}$	34.20±5.59$^{\beta}$	37.04±4.31$^{\beta}$	7.80±1.97$^{\gamma}$	<0.001
∑ DHPV glucuronides [d)]	383/207	70.62±14.54$^{\alpha}$	157.76±27.06$^{\beta}$	177.49±24.62$^{\beta}$	36.52±8.84$^{\alpha}$	<0.001
∑ DHPV sulfates[d)]	287/207	527.13±55.87$^{\alpha}$	876.9±101.81$^{\beta}$	913.43±114.12$^{\beta}$	418.39±71.73$^{\alpha}$	<0.001
MHPV	221/162	ND	ND	ND	ND	
MHPV glucuronide [d)]	397/221	23.81±4.43$^{\alpha,\gamma}$	37.36±6.57$^{\alpha,\beta}$	38.43±7.08$^{\beta}$	20.24±4.19$^{\gamma}$	<0.001
∑MHPV sulfates [d)]	301/221	32.29±5.52$^{\alpha,\beta}$	38.42±6.12$^{\alpha,\beta}$	43.13±6.52$^{\beta}$	24.9±4.26$^{\alpha}$	0.006
Stilbenes [d,f)]						
Resveratrol Biomarker [g)]	-	692.21±208.33$^{\alpha}$	5352.45±661.99$^{\beta}$	5824.25±722.19$^{\beta}$	238.00±84.61$^{\alpha}$	<0.001
∑Resveratrol Microbial Metabolites	-	506.39±107.97$^{\alpha}$	4208.95±430.76$^{\beta}$	5230.62±508.44$^{\beta}$	283.86±76.23$^{\alpha}$	<0.001
∑Total resveratrol metabolites	-	811.54±211.27$^{\alpha}$	6282.25±770.39$^{\beta}$	7090.29±822.66$^{\beta}$	306.08±90.44$^{\alpha}$	<0.001
Other polyphenols						
Enterolactone	297/254	8.73±1.10$^{\alpha}$	11.4±2.19$^{\alpha,\beta}$	14.81±3.40$^{\beta}$	7.82±0.94$^{\alpha}$	0.001
Pyrogallol	125/69	1.96±0.43$^{\alpha}$	8.00±1.19$^{\beta}$	8.08±1.78$^{\beta}$	2.99±0.58$^{\alpha}$	<0.001

[a)]BAS, baseline; DRW, dealcoholized red wine; DHPV, dihydroxyphenyl-c-valerolactone; MHPV, methoxyhydroxyphenyl-c-valerolactone; MRM, Multiple Reaction Monitoring; RW, red wine.
[b)]Results are expressed as mean ± SEM.
[c)]Changes in variables were determined by using the ANOVA analysis for repeated measures. Means in a row with different superscript Greek letters are significantly different, $P < 0.05$ (Bonferroni post hoc test).
[d)]Metabolites determined in non-hydrolyzed samples.
[e)]Identification of metabolites described previously by Boto-Ordoñez et al.[4]
[f)] Data obtained from a previous study by Rotches-Ribalta et al.[11]
[g)] Resveratrol Biomarker described by Zamora-Ros et al.[9].

Supporting Information Table S5. Fasting plasma concentrations of metabolites in 33 subjects at baseline and after the three intervention periods.[a)]

Metabolites	MRM	Plasma samples (µmol/L)[b)]				
		BAS	RW	DRW	GIN	*P*[c)]
Hydroxybenzoic acids						
4-Hydroxybenzoic acid	137/93	3.26±0.14	3.47±0.12	3.44±0.09	3.02±0.17	0.22
3-Hydroxybenzoic acid	137/93	ND	ND	ND	ND	
2,4-Dihydroxybenzoic acid	153/109	ND	ND	ND	ND	
2,6-Dihydroxybenzoic acid	153/109	0.08±0.01	0.10±0.01	0.10±0.01	0.08±0.01	0.38
2,5-Dihydroxybenzoic acid	153/109	0.04±0.01	0.05±0.01	0.07±0.02	0.03±0.01	0.08
3,5-Dihydroxybenzoic acid	153/109	ND	ND	ND	ND	
Protocatechuic acid	153/109	1.74±0.06	1.74±0.08	1.86±0.08	1.69±0.07	0.73
Syringic acid	197/121	ND	ND	ND	ND	
4-Hydroxyhippuric acid	194/100	0.17±0.02	0.16±0.02	0.17±0.02	0.15±0.01	0.85
3-Hydroxyhippuric acid	194/150	0.85±0.53	1.03±0.45	1.21±0.62	0.69±0.32	0.89
Gallic acid metabolites						
Gallic acid	169/125	0.02±0.001$^{\alpha}$	0.04±0.005$^{\beta}$	0.03±0.002$^{\alpha,\beta}$	0.02±0.002$^{\alpha}$	<0.001
Methylgallic acid [e)]	167/108	0.04±0.004$^{\alpha}$	0.07±0.02$^{\beta}$	0.06±0.01$^{\alpha,\beta}$	0.04±0.01$^{\alpha}$	0.037
Methylgallic sulfate [d),e)]	263/183	0.002±0.0004$^{\alpha}$	0.02±0.004$^{\beta}$	0.01±0.002$^{\beta}$	0.001±0.0003$^{\alpha}$	<0.001
Ethylgallate metabolites						
Ethylgallate	197/169	0.03±0.01	0.04±0.01	0.09±0.06	0.03±0.02	0.93
Ethylgallate sulfate [d),e)]	277/197	ND	ND	ND	ND	
Ethylgallate glucuronide 1 [d),e)]	373/197	0.10±0.018.9	0.36±0.21	0.12±0.03	0.17±0.07	0.16
Ethylgallate glucuronide 2 [d),e)]	373/197	0.09±0.011.63	0.11±0.02	0.10±0.01	0.09±0.02	0.45
Hydroxyphenylacetic acids						
Phenylacetic acid	135/91	0.37±0.03	0.38±0.03	0.38±0.03	0.36±0.04	0.76
3-Hydroxyphenylacetic acid	151/107	0.22±0.03$^{\alpha}$	0.38±0.06$^{\beta,\gamma}$	0.40±0.04$^{\beta}$	0.24±0.04$^{\alpha,\gamma}$	0.002
2-Hydroxyphenylacetic acid	151/107	0.08±0.01	0.10±0.01	0.10±0.01	0.08±0.01	0.33
3,4-Dihydroxyphenylacetic acid	167/123	0.03±0.01	0.03±0.01	0.03±0.01	0.02±0.01	0.80
Homovanillic acid	181/137	ND	ND	ND	ND	
Hydroxycinnamic acids						
p-Coumaric acid	163/119	0.005±0.002$^{\alpha}$	0.02±0.003$^{\beta}$	0.02±0.003$^{\beta}$	0.02±0.003$^{\beta}$	<0.001

m-Coumaric acid	163/119	ND	ND	ND	ND	
o-Coumaric acid	163/119	ND	ND	ND	ND	
Caffeic acid	179/135	0.09±0.01	0.09±0.01	0.11±0.01	0.08±0.01	0.19
Ferulic acid	193/134	1.63±0.06	1.65±0.06	1.69±0.06	1.55±0.04	0.56
Sinapic cid	223/164	ND	ND	ND	ND	
Hydroxyphenylpropanoic acids						
3-(4-Hydroxyphenyl)propionic acid	165/121	3.34±0.17	3.44±0.23	3.54±0.17	3.05±0.24	0.30
3-(3-Hydroxyphenyl)propionic acid	165/121	0.21±0.05	0.21±0.05	0.29±0.06	0.15±0.04	0.14
Dihydrocaffeic acid	181/137	ND	ND	ND	ND	
Flavan-3-ols [e)]						
(Epi)catechin glucuronide[4]	465/289	0.02±0.01$^{\alpha}$	0.05±0.01$^{\beta}$	0.04±0.01$^{\beta}$	0.02±0.01$^{\alpha}$	<0.001
Methyl(epi)catechin glucuronide[4]	479/303	0.01±0.001$^{\alpha,\gamma}$	0.03±0.01$^{\beta}$	0.02±0.01$^{\alpha,\beta}$	0.01±0.001$^{\gamma}$	0.004
Glycynates						
Vanilloylglycine	224/180	0.03±0.001	0.03±0.001	0.03±0.001	0.03±0.001	0.24
Feruloylglycine	250/100	0.08±0.002	0.09±0.003	0.09±0.003	0.08±0.003	0.44
Hydroxyphenylvalerolactones [e)]						
DHPV 1	207/163	0.07±0.02$^{\alpha,\gamma}$	0.16±0.03$^{\beta}$	0.10±0.02$^{\alpha,\beta}$	0.04±0.02$^{\gamma}$	<0.001
DHPV 2	207/163	0.17±0.04$^{\alpha,\gamma}$	0.45±0.1$^{\beta}$	0.29±0.06$^{\alpha,\beta}$	0.10±0.03$^{\gamma}$	<0.001
∑ DHPV glucuronides[d)]	383/207	0.18±0.05$^{\alpha,\beta}$	0.46±0.13$^{\beta}$	0.29±0.06$^{\alpha,\beta}$	0.14±0.08$^{\alpha}$	<0.001
∑ DHPV sulfates [d)]	287/207	ND	ND	ND	ND	
MPHV	221/162	ND	ND	ND	ND	
MHPV glucuronide [d)]	397/221	ND	ND	ND	ND	
∑ MPHV sulfates [d)]	301/221	ND	ND	ND	ND	
Other polyphenols						
Enterolactone	297/254	0.01±0.002	0.01±0.002	0.02±0.01	0.01±0.002	0.18
Pyrogallol	125/69	ND	ND	ND	ND	

[a)]BAS, baseline; DRW, dealcoholized red wine; DHPV, dihydroxyphenyl-c-valerolactone; MHPV, Methoxy-hydroxyphenyl-c–valerolactone; MRM, Multiple Reaction Monitoring; ND, no detected; RW, red wine.

[b)]Results are expressed as mean ± SEM.

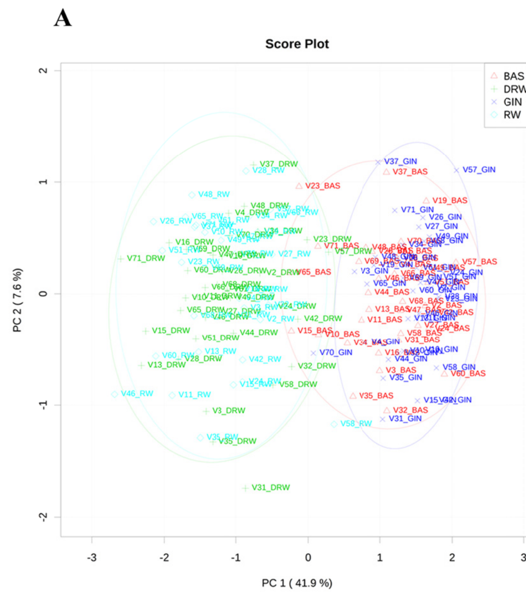
[c)]Changes in variables were determined by using the ANOVA analysis for repeated measures. Means in a row with different superscript Greek letters are significantly different, $P < 0.05$ (Bonferroni post hoc test).

[d)]Metabolites determined in non-hydrolyzed samples.

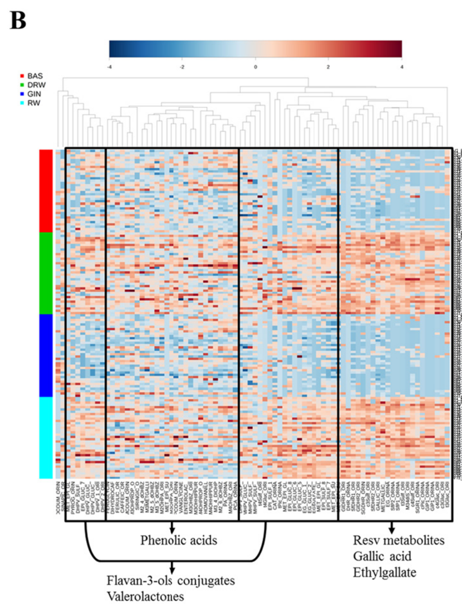
[e)]Identification of metabolites described previously by Boto-Ordoñez et al.[4]

Supporting Information Figure S1. A) Principal component analysis (PCA) using range scaled data for unsupervised segregation of the samples (red: BAS; green: DRW; dark blue: GIN; light blue: RW). **B**) Heat map visualization constructed based on the metabolites concentration in urine at baseline and after consumption of RW, DRW and GIN. The rows represent volunteers by treatment (red: BAS, green: DRW; dark blue: GIN; light blue: RW) and the columns are metabolites. The color of the heat map indicates: lowest concentration (blue) and highest concentration (red). Baseline (BAS); dealcoholized red wine (DRW); red wine (RW). Resv, Resveratrol Metbaolites.

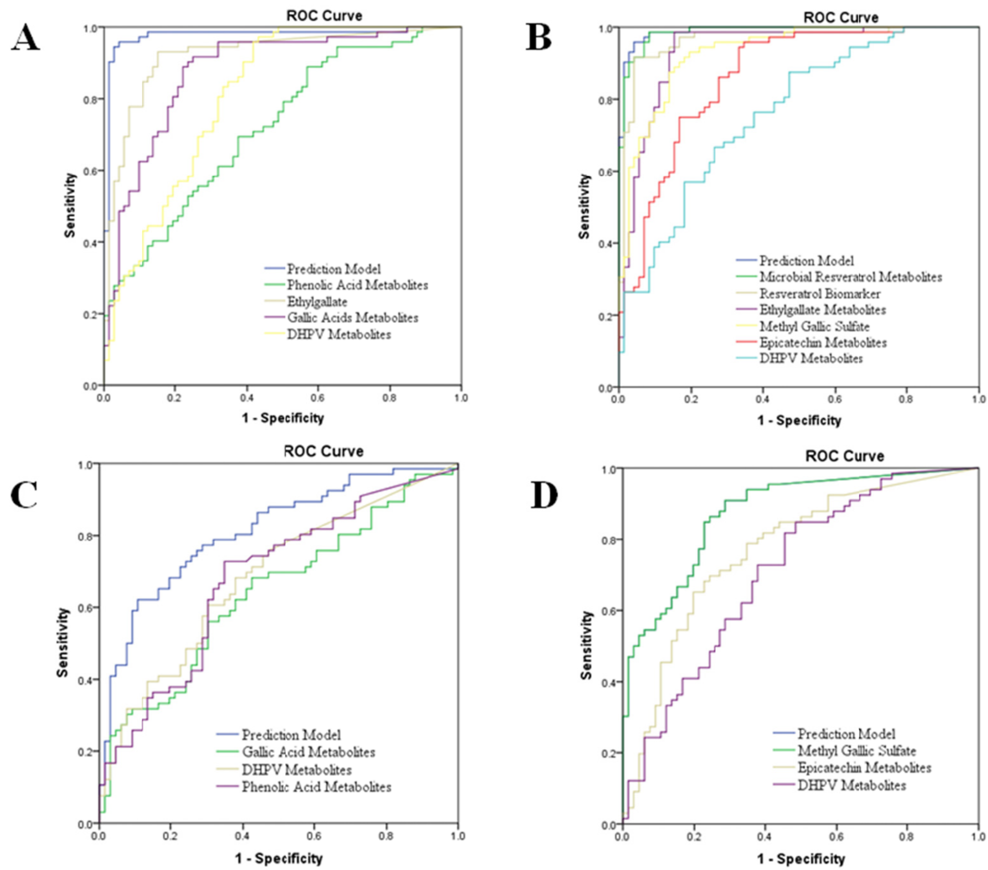
A



B



Supporting Information Figure S2. ROC curves of the phenolic metabolites groups and the prediction model: **A**) hydrolyzed urine samples; **B**) non-hydrolyzed urine samples; **C**) hydrolyzed fasting plasma samples; **D**) non-hydrolyzed fasting plasma samples.



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4.5 EFECTO DE LOS POLIFENOLES DEL VINO EN LA MODULACIÓN DE LA MICROBIOTA HUMANA Y LOS MARCADORES BIOQUÍMICOS

En el marco de una colaboración con el grupo del Dr. Francisco Tinahones, del Hospital Universitario Virgen de la Victoria de Málaga (CIBER-OBN), y mediante la concesión de una estancia predoctoral del Ministerio de Educación, Cultura y Deportes (Septiembre-Diciembre 2010), se ha estudiado la relación entre el consumo de vino tino, vino tinto sin alcohol y ginebra y la composición de la microbiota colónica, así como la relación con otros marcadores bioquímicos.

Este trabajo ha sido publicado en una de las revistas considerada por el *Science Citation Index*, con un índice de impacto de 6.504 y situada en el primer cuartil de la categoría *Nutrition & Dietetics* (3 de 76).

Publicación 5: María Isabel Queipo-Ortuño, María Boto-Ordóñez, Mora Murri, Juan Miguel Gomez-Zumaquero, Mercedes Clemente-Postigo, Ramon Estruch, Fernando Cardona Diaz, Cristina Andrés-Lacueva, Francisco J. Tinahones. Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. *Am J Clin Nutr,* 2012; 95: 1323–34

Resumen:

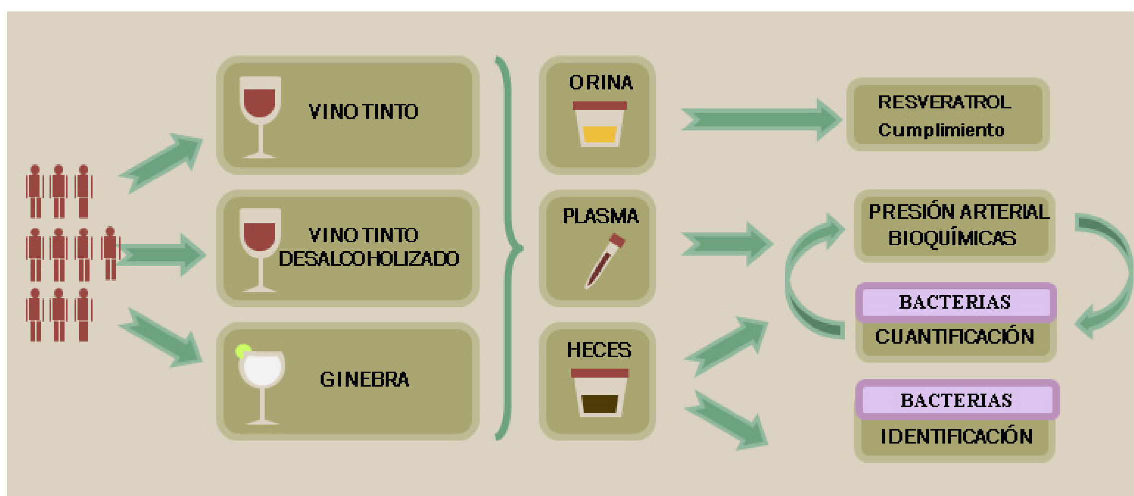
Pocos estudios han investigado el efecto de los polifenoles de la dieta sobre la compleja microbiota intestinal humana, centrándose principalmente en moléculas individuales de polifenoles y poblaciones bacterianas concretas. En este estudio, el objetivo fue evaluar el efecto de una ingesta moderada de polifenoles del vino tinto en determinados grupos microbianos intestinales y la implicación en los beneficios para la salud del individuo. Para ello, 10 voluntarios fueron sometidos a un estudio de intervención controlado, aleatorizado y cruzado, donde tras un período de lavado, todos los sujetos recibieron vino tinto, la cantidad equivalente de vino tinto desalcoholizado, o ginebra durante 20 días cada uno. Los cambios en la microbiota fecal se midieron mediante el análisis del ADN fecal total tras su amplificación por PCR,

seguido de una electroforesis en gel por gradiente de desnaturalización y finalmente cuantificación por qPCR. Conjuntamente se midieron varios marcadores bioquímicos.

Como resultado se obtuvo que la composición bacteriana dominante no permaneció constante durante los diferentes periodos de intervención. En comparación con el momento inicial, el consumo diario de polifenoles de vino tinto aumentó significativamente el número de *Enterococcus, Bifidobacterium, Eggerthella lenta*, después de los dos vinos y *Bacteroides uniformis, Prevotella, Bacteroides, Blautia coccoides-Eubacterium rectale* tras el vino alcoholizado ($P< 0.05$). En el caso de los filos, todos aumentaron significativamente después de consumo de vino tinto con alcohol respecto al basal, excepto las *Actinobacterias*. En paralelo, la presión arterial sistólica y diastólica y las concentraciones plasmáticas de triglicéridos, colesterol total, colesterol HDL, y de proteína C-reactiva disminuyeron significativamente ($P<0.05$) tras la intervención. Por otra parte, cambios en el colesterol y la proteína C reactiva fueron ligados a los cambios en el número de bifidobacterias., así como la reducción de la presión arterial, los triglicéridos y el HDL a los cambios en el número de *Bacteroides*.

Este estudio demostró que el consumo de vino tinto puede modular significativamente el crecimiento de microbiota intestinal seleccionada en los seres humanos, lo que sugiere posibles efectos beneficiosos asociados a un papel prebiótico de los polifenoles del vino.

Figura Resultados R5: Gráfico resumen del papel de los polifenoles del vino sobre la microbiota humana y marcadores bioquímicos.



The American Journal of Clinical Nutrition

Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers[1–4]

María Isabel Queipo-Ortuño, María Boto-Ordóñez, Mora Murri, Juan Miguel Gomez-Zumaquero, Mercedes Clemente-Postigo, Ramon Estruch, Fernando Cardona Diaz, Cristina Andrés-Lacueva, and Francisco J Tinahones

ABSTRACT

Background: Few studies have investigated the effect of dietary polyphenols on the complex human gut microbiota, and they focused mainly on single polyphenol molecules and select bacterial populations.

Objective: The objective was to evaluate the effect of a moderate intake of red wine polyphenols on select gut microbial groups implicated in host health benefits.

Design: Ten healthy male volunteers underwent a randomized, crossover, controlled intervention study. After a washout period, all of the subjects received red wine, the equivalent amount of de-alcoholized red wine, or gin for 20 d each. Total fecal DNA was submitted to polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis and real-time quantitative PCR to monitor and quantify changes in fecal microbiota. Several biochemical markers were measured.

Results: The dominant bacterial composition did not remain constant over the different intake periods. Compared with baseline, the daily consumption of red wine polyphenol for 4 wk significantly increased the number of *Enterococcus, Prevotella, Bacteroides, Bifidobacterium, Bacteroides uniformis, Eggerthella lenta, and Blautia coccoides–Eubacterium rectale* groups ($P < 0.05$). In parallel, systolic and diastolic blood pressures and triglyceride, total cholesterol, HDL cholesterol, and C-reactive protein concentrations decreased significantly ($P < 0.05$). Moreover, changes in cholesterol and C-reactive protein concentrations were linked to changes in the bifidobacteria number.

Conclusion: This study showed that red wine consumption can significantly modulate the growth of select gut microbiota in humans, which suggests possible prebiotic benefits associated with the inclusion of red wine polyphenols in the diet. This trial was registered at controlled-trials.com as ISRCTN88720134. *Am J Clin Nutr* 2012;95:1323–34.

INTRODUCTION

The human large intestine is an extremely active fermentation site and is inhabited by different bacterial species, reaching their highest concentrations in the colon (up to 10^{12} cells per gram of feces) (1). Not only does the composition of this bacterial ecosystem vary substantially among individuals, it is also dynamic and susceptible to change driven by dietary factors and diverse disease conditions. A balanced gut microbiota composition confers benefits to the host, whereas microbial imbalances are associated with metabolically mediated disorders (2). The intake of phytochemicals and their derived products exerts significant effects on the intestinal environment, modulating the gut microbiota composition and probably their functional effects in mammalian tissues (3). Polyphenols are phytochemicals abundantly present in our diet in diverse products, including tea, coffee, wine, fruit, vegetables, and chocolate (4). Polyphenol bioavailability and absorption may be influenced by its chemical structure, food matrix, and enterohepatic circulation, and a high percentage is not absorbed in the small intestine, arriving intact at the colon, where polyphenols may exert their regulatory function (5, 6). In an in vitro study using a gastrointestinal model, dietary polyphenol absorption was tested, and it was estimated that ~42% of dietary polyphenols become bioaccessible in the large intestine, where they may interact with microflora, and ~10% remained in the food matrix and were inaccessible after the whole digestion process (7). During wine consumption, polyphenolic oligomers such as procyanidins, conjugated polyphenols, esters, and phase II metabolites arrive in the colon (6, 8). Resveratrol metabolism has been extensively studied, suggesting a low bio-

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availability after intestinal and liver metabolism and a fast excretion. At least 70% of the resveratrol ingested is absorbed and readily metabolized to resveratrol glucuronides and sulfates and found later in urine and plasma, where it is available for enterohepatic circulation (9–11). The polyphenols in red wine may modify the gut microbial composition by their antimicrobial properties and this, in turn, may affect their functional relations with the host (12, 13). Indeed, consumption of polyphenols has been suggested to have diverse benefits, such as improved gut health and a reduced risk of coronary artery disease (14).

Although a few intervention studies have investigated the effects of dietary polyphenols on the modulation of the human intestinal microbiota, the current study is the first to be conducted after wine consumption. Most studies addressing this topic have focused mainly on single-polyphenol molecules and selected bacterial groups (14, 15). The purpose of the current study was to evaluate the possible prebiotic effect of red wine polyphenols on the human gut microbiota by designing a randomized, crossover, controlled intervention. This aim was based on the hypothesis that the moderate intake of red wine polyphenols may modulate the gut microbiota, influencing the growth of specific bacterial groups capable of producing host health benefits.

SUBJECTS AND METHODS

Study subjects and design

The study involved 10 healthy adult men aged 48 ± 2 y (range: 45–50 y). The participants were not receiving treatment for diabetes, hypertension, or dyslipidemia, nor did they have any acute or chronic inflammatory diseases, infectious diseases, viral infections, cancer, or a previous cardiovascular event at study entry. They had not received any antibiotic therapy, prebiotics, probiotics, symbiotics, or vitamin supplements or any other medical treatment influencing intestinal microbiota during the 3 mo before the start of the study or during the study (including the washout period). A randomized, crossover, controlled intervention study was performed. The study was divided into 4 consecutive periods: an initial washout period of 15 d (baseline) during which the participants didnot consume any alcohol or red wine, followed by 3 consecutive periods of 20 d during which the participants drank only de-alcoholized red wine (272 mL/d), red wine (272 mL/d), or gin (100 mL/d). Each participant provided 4 different fecal samples: a first baseline sample after the washout period and a sample at the end of each 20-d period. Fasting blood samples and 24-h urine were also collected at baseline and after each period. The participants were asked not to change their dietary pattern and lifestyle habits during the study. The subjects were asked to avoid alcoholic beverages during the study. At baseline and after each intervention period, a medical examination and a nutrient intake and physical activity–structured questionnaires were made. This information was converted into dietary data by using the Professional Diet Balancer software (Cardinal Health Systems Inc). The Ethics Committee of the Virgen de la Victoria Hospital approved the clinical protocol. All the participants gave written informed consent.

Anthropometric measures

Body weight, height, and waist and hip circumferences were measured according to standardized procedures (16).

Laboratory measurements

Blood samples were collected after an overnight fast. The serum was separated in aliquots that were immediately frozen at −80°C. Serum biochemical measures were measured in duplicate. Serum albumin, glucose, cholesterol, HDL cholesterol, triglycerides (Randox Laboratories Ltd), bilirubin (Dimension Vista System), uric acid, C-reactive protein (Dimension autoanalyzer; Dade Behring Inc), γ-glutamyl transpeptidase, glutamate-oxaloacetate transaminase, and glutamic pyruvic transaminase (Wako Bioproducts) were all measured by using standard enzymatic methods. LDL cholesterol was calculated by using the Friedewald formula. Insulin was analyzed by using an immunoradiometric assay (BioSource International), showing a 0.3% cross-reaction with proinsulin. The intra- and interassay CVs were 1.9% and 6.3%, respectively.

In the 24-h urine samples, resveratrol metabolites were measured as a biomarker of consumption of de-alcoholized red wine and red wine intervention compliance (17, 18), jointly with dihydroresveratrol metabolites (9, 11), by using the technique described by Urpi-Sarda et al (19). The resveratrol metabolites were quantified by using the commercial and available standards; *trans*- and *cis*-resveratrol-3-*O*-glucuronide (98% purity each), *cis*-resveratrol-4′-*O*-glucuronide (96% purity), and *trans*-resveratrol-3-*O*-sulfate (98% purity) were purchased from Toronto Research Chemicals Inc. The *trans*-resveratrol-3-*O*-sulfate calibration curve was used to quantify *trans*-resveratrol-4′-*O*-sulfate, *cis*-resveratrol-4′-*O*-sulfate, and *cis*-resveratrol-3-*O*-sulfate. Dihydroresveratrol was provided by Biopharmalab SL. The concentrations of dihydroresveratrol metabolites were quantified by using the dihydroresveratrol calibration curve (11, 20).

Similarly, ethylglucuronide was measured in 24-h urine samples, as a biomarker of alcohol intake, by liquid chromatography (LC Agilent series 1200 coupled with a hybrid quadrupole time-of-flight QSTAR Elite; Applied Biosystems/MDS Sciex).

DNA extraction from fecal samples

Fecal samples were collected and immediately stored at −80°C until analyzed. DNA was extracted from 200 mg stool by using the QIAamp DNA stool Mini kit (Qiagen) following the manufacturer's instructions. The DNA concentration was determined by absorbance at 260 nm, and the purity was estimated by determining the A260/A280 ratio with a Nanodrop spectrophotometer (Nanodrop Technologies).

Analysis of fecal microbiota by polymerase chain reaction–denaturing gradient gel electrophoresis

Fecal samples from each subject were examined by determining polymerase chain reaction (PCR)[5]–denaturing gradient gel electrophoresis (DGGE) profiles. The V2-V3 region of the 16S rRNA genes (positions 339–539 in the *Escherichia coli* gene) of bacteria in the fecal samples was amplified with the

[5]Abbreviations used: ATCC, American Type Culture Collection; BP, blood pressure; CECT, Spanish Type Culture Collection; CRP, C-reactive protein; Cs, similarity coefficient; DBP, diastolic blood pressure; DGGE, denaturing gradient gel electrophoresis; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; SBP, systolic blood pressure.

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primers HDA1-GC (5′-**CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G**CC TAC GGG AGG CAG CAG T-3′ (the GC clamp is in boldface) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′) generating a 200-bp product. Aliquots (2 μL) of DNA were amplified by real-time quantitative PCR (qPCR) (20 μL final volume) in a 7500 Fast RT-PCR Systems instrument by using Fast SYBR Green Master Mix and 200 nmol/L of each of the universal primers HDA1-GC/HDA2 with the following amplification program: initial denaturation at 95° for 20 s, amplification by using 45 cycles including denaturation at 95°C for 3 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min.

After real-time qPCR, 15 μL of the products was mixed with 6 μL loading dye before loading. Electrophoresis was performed with a DCode Universal Mutation Detection System instrument (Bio-Rad); 6% polyacrylamide gels were prepared and electrophoresed with 1 × TAE buffer prepared from 50 × TAE buffer (2 mol Tris base/L, 1 mol glacial acetic acid/L, and 50 mmol EDTA/L). The denaturing gradient was formed by using two 6% acrylamide (acrylamide/bisacrylamide ratio, 37.5:1) stock solutions (Bio-Rad). The gels contained a 20–80% gradient of urea and formamide, increasing in the direction of electrophoresis. Electrophoretic runs were in a Tris-acetate-EDTA buffer (TAE 1×) (40 mmol Tris/L, 20 mmol acetic acid/L, and 1 mmol EDTA /L; pH 7.4) at 130 V and 60°C for 4.5 h. Electrophoresis was stopped when a xylene cyanol dye marker reached the bottom of the gel. Gels were stained with ethidium bromide (0.5 mg/L) for 5 min, rinsed with deionized water, viewed by ultraviolet transillumination, and photographed with Gelcapture image acquisition software (DNR Bio-Imaging Systems Ltd). Samples were obtained from each subject after each period and analyzed on the same DGGE gel to avoid the possible influence of variations in electrophoretic conditions between different gels. Similarities between banding patterns in the DGGE profile were calculated based on the presence and absence of bands and expressed as a Cs (similarity coefficient). Gels were analyzed by using BioNumerics software (Applied Maths). Normalized banding patterns were used for cluster analysis. The Dice similarity coefficient was used to calculate pairwise comparisons of the DGGE fingerprint profiles obtained. A Cs value of 100% indicates that the DGGE profiles are identical, whereas completely different profiles result in a Cs value of 0%. The unweighted pair group method with arithmetic mean algorithm was used for construction of dendrograms.

Sequencing of selected bands from DGGE gels

Bands of specific interest were excised from DGGE gels with a sterile razor, placed in 40 μL sterile water, and incubated at 4°C for diffusion of DNA into the water. DNA was used in a second PCR with HDA1/2 primers without a gas chromatograph clamp (initial denaturation 95° for 20 s, followed by 45 cycles including denaturation at 95°C for 3 s, annealing at 55°C for 15 s, and extension at 72°C for 10 s). PCR products were diluted until 20 ng/μL, purified with ExoSAP-IT (USB Corporation), and sequenced in an ABI 3130 (Applied Biosystems) by using the BigDie-Kit-Standard. The nucleotide sequence data obtained were analyzed by using MicroSeqID v2.1.1 software (Applied Biosystems).

Microbial quantification by real-time qPCR

Specific primers targeting different bacterial genera were used to characterize the fecal microbiota by real-time qPCR (21–29) (**Table 1**). Briefly, quantitative PCR experiments were performed with a LightCycler 2.0 PCR sequence detection system by using the FastStart DNA Master SYBR Green kit (Roche Diagnostics). All PCR tests were carried out in duplicate with a final volume of 20 μL, containing 100 ng of each fecal DNA preparation and 200 nmol/L of each primer (Table 1). The thermal cycling conditions used were as follows: an initial DNA denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, primer annealing at optimal temperature (Table 1) for 20 s, and extension at 72°C for 15 s. Finally, melt curve analysis was performed by slowly cooling the PCRs from 95°C to 60°C (0.05°C per cycle) with simultaneous measurement of the SYBR Green I signal intensity. Melting-point-determination analysis allowed the confirmation of the specificity of the amplification products.

The bacterial concentration from each sample was calculated by comparing the Ct values obtained from the standard curves with the LightCycler 4.0 software. Standard curves were created by using a serial 10-fold dilution of DNA from pure cultures, corresponding to 10^1–10^{10} copies/g feces. The different strains used were obtained from the Spanish Type Culture Collection (CECT) (*Bacteroides vulgatus* National Collection of Type Cultures 11154, *Fusobacterium varium* National Collection of Type Cultures 10560, *Enterococcus faecalis* CECT 184, *Enterobacter cloacae* CECT 194, and *Clostridium perfringens* CECT 376) and the American Type Culture Collection (ATCC) (*Bifidobacterium bifidum* ATCC 15696, *Lactobacillus casei* ATCC 334D-5, *Prevotella intermedia* ATCC 25611D-5, *Clostridium histolyticum* ATCC 19401, *Eggerthella lenta* ATCC 25559, *Bacteroides uniformis* ATCC 8492, and *Ruminococus productus* ATCC 27340D-5). The data presented are the mean values of duplicate real-time qPCR analyses.

De-alcoholized red wine, red wine, and gin composition

De-alcoholized red wine and red wine used in this study were produced with the Merlot grape variety, from the Penedès appellation. The de-alcoholized red wine had the same composition and polyphenolic compounds as the red wine, except for ethanol (only 0.42%). The phenolic profile of de-alcoholized red wine and red wine was determined by HPLC with diode-array detection as described previously (30), and the resveratrol and piceid contents were determined by HPLC with diode-array detection as described by Romero-Pérez et al (31). The total phenolic content of several distilled alcoholic beverages was determined with the Folin-Ciocalteu method (32), and the alcoholic beverage selected was gin (38% alcohol) because the amount of phenols was not detectable. A description of the daily phenolic and alcoholic doses during treatment periods from both the wines and gin is included in **Table 2**. No significant differences in the phenolic content were identified between de-alcoholized red wine and regular red wine.

Statistical analysis

The results are expressed as means ± SDs. The statistical analysis was performed with SPSS 15.0 software (SPSS Inc).

TABLE 1
Primers used for real-time polymerase chain reaction

Target group	Oligonucleotide sequence (5′-3′)	Reference	Amplicon size
			bp
Bacteroidetes	CATGTGGTTTAATTCGATGAT AGCTGACGACAACCATGCAG	Guo et al, 2008 (21)	126
Bacteroides	GAGAGGAAGGTCCCCCAC CGCTACTTGGCTGGTTCAG	Guo et al, 2008 (21)	106
Lactobacillus	GAGGCAGCAGTAGGGAATCTTC GGCCAGTTACTACCTCTATCCTTCTTC	Delroisse et al, 2008 (22)	126
Fusobacterium	CCCTTCAGTGCCGCAGT GTCGCAGGATGTCAAGAC	Friswell et al, 2010 (23)	273
Firmicutes	ATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	Guo et al, 2008 (21)	126
Actinobacteria	CGCGGCCTATCAGCTTGTTG CCGTACTCCCCAGGCGGGG	Stach et al, 2003 (24)	600
Bifidobacterium	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	Matsuki et al, 2002 (25)	550
Prevotella	GGTTCTGAGAGGAAGGTCCCC TCCTGCACGCTACTTGGCTG	Bekele et al, 2010 (26)	121
Enterococcus	CCCTTATTGTTAGTTGCCATCATT ACTCGTTCTTCCCATGT	Rinttilä et al 2004 (27)	144
Proteobacteria	CATGACGTTACCCGCAGAAGAAG CTCTACGAGACTCAAGCTTGC	Friswell et al, 2010 (23)	195
Clostridium cluster IV	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	Matsuki et al, 2004 (25)	239
Eggerthella lenta	TGGCGAACGGGTGAGTAA AGGCCCGGGAA CGTATTCAC	Lau et al, 2004 (28)	1221
Blautia coccoides–Eubacterium rectale group	CGGTACCTGACTAAGAAGC AGTTTCATTCTTGCGAACG	Rinttilä et al, 2004 (27)	429
Clostridium histolyticum group	ATGCAAGTCGAGCGA(G/T)G TATGCGGTATTAATCT(C/T)CCTTT	Rinttilä et al, 2004 (27)	120
Bacteroides uniformis	TCCGTTTTCCACTTATAAGA GGGTTBCCCCATTCGG	Liu et al, 2003 (29)	350

The bacterial copy numbers were converted into logarithm values before the statistical analysis. The Friedman test was used to check changes in bacterial number, biochemical variables, and compliance between the intervention treatment. Wilcoxon's signed-rank tests with a Bonferroni post hoc test was used to compare the treatments. One-factor ANOVA for repeated measures with the Bonferroni post hoc test was used to compare changes in the dietary analysis in response to the intervention treatments. Within and between the intervention periods, the differences are expressed as means and 95% CIs. Univariate correlations were calculated by using Pearson's *r*. A multivariate regression analysis was performed to identify individual bacteria as independent predictors for blood pressure (BP), lipid markers, and C-reactive protein (CRP). Statistical significance was set at a *P* value <0.05. All data are presented as means ± SDs.

RESULTS

Diet and intervention compliance

The drinks were well tolerated by all of the volunteers, all of whom completed the study, and no intolerance or adverse events were reported. Throughout the study, the participants maintained their usual diet and physical activity, taking care that both remained as stable as possible over the 4 periods. No significant differences in dietary intake data were observed during the study (**Table 3**).

Alcoholic intake was monitored after the 3 treatment periods by urinary ethylglucuronide output. After the red wine and gin intakes, urinary ethylglucuronide concentrations were significantly increased compared with baseline [358% (95% CI: 146, 570%) and 342% (95% CI: 159, 525%), respectively ($P < 0.05$)] and the de-alcoholized red wine period [606% (95% CI: 328, 885%) and 625% (95% CI: 319, 931), respectively ($P < 0.05$)]. No significant differences were observed between the de-alcoholized red wine and washout periods [36% (95% CI: 25, 47%; $P = 0.638$)] or between the red wine and gin periods [24% (95% CI: 2, 46%; $P = 1.000$)]. Compliance with the 3 interventions was ensured by counting the empty bottles returned and by analyzing the participants' reports. No carryover effect was observed in the study.

Resveratrol absorption and metabolism

Resveratrol metabolites were analyzed in 24-h urine samples as biomarkers of red wine intake (17–19). Resveratrol metabolites derived from phase II metabolism were significantly increased in urine after both red wines as compared with baseline and after gin intake. Dihydroresveratrol, produced by intestinal microbiota, also had a significantly higher concentration (9, 12).

The resveratrol metabolite concentrations were significantly higher after the de-alcoholized red wine and the red wine periods

TABLE 2
Daily polyphenol and alcohol consumption from 272 mL red wine, 272 mL de-alcoholized red wine, and 100 mL gin used in this study[1]

	De-alcoholized red wine	Red wine	Gin	P^2
Total phenols (mEq GA)	733.02 ± 23.61[3]	797.86 ± 102.63	ND	0.426
Phenolic compounds (mg/dose)				
Anthocyanins				
Delphinidin-3-glucoside	4.00 ± 0.44	4.15 ± 0.24	ND	0.589
Petunidin-3-glucoside	3.27 ± 0.31	3.34 ± 0.29	ND	0.755
Peonidin-3-glucoside	1.82 ± 0.16	1.84 ± 0.17	ND	0.797
Malvidin-3-glucoside	13.56 ± 1.16	13.28 ± 1.21	ND	0.787
Malvidin-(6-acetyl)-3-glucoside	2.83 ± 0.33	2.98 ± 0.26	ND	0.563
Malvidin-(6-coumaroyl)-3-glucoside	0.96 ± 0.09	1.13 ± 0.07	ND	0.066
Flavonols				
Quercetin-3-glucuronide	3.06 ± 0.39	3.23 ± 0.38	ND	0.770
Quercetin	6.48 ± 0.64	7.25 ± 0.21	ND	0.161
Isorhamnetin	0.80 ± 0.04	0.91 ± 0.07	ND	0.114
Stilbenes				
trans-Resveratrol	0.74 ± 0.06	0.79 ± 0.10	ND	0.352
cis-Resveratrol	0.75 ± 0.04	0.76 ± 0.04	ND	0.761
trans-Piceid	2.86 ± 0.26	2.56 ± 0.31	ND	0.160
cis-Piceid	1.93 ± 0.24	2.10 ± 0.09	ND	0.226
Flavan-3-ols				
Catechin	34.39 ± 3.63	33.60 ± 3.07	ND	0.786
Epicatechin	19.20 ± 2.24	18.46 ± 2.11	ND	0.699
Procyanidin B1	17.50 ± 2.10	17.52 ± 1.52	ND	0.712
Procyanidin B2	12.92 ± 1.44	12.41 ± 0.74	ND	0.502
Procyanidin B3	7.48 ± 0.08	6.85 ± 0.08	ND	0.526
Procyanidin B4	13.19 ± 1.35	13.33 ± 1.54	ND	0.934
Hydroxybenzoic acids				
GA acid	19.90 ± 1.91	18.63 ± 1.74	ND	0.306
Protocatechuic acid	1.59 ± 0.14	1.42 ± 0.17	ND	0.246
Hydroxycinnamic acids				
2-*S*-Glutathionylcaftaric	2.93 ± 0.34	2.80 ± 0.27	ND	0.956
trans-Caftaric	5.23 ± 0.44	5.06 ± 0.39	ND	0.595
trans-Caffeic	3.31 ± 0.25	3.13 ± 0.22	ND	0.246
trans-Coutaric	1.53 ± 0.14	1.42 ± 0.12	ND	0.182
Tyrosols				
Tyrosol	13.01 ± 1.06	11.86 ± 1.29	ND	0.298
Alcoholic content (g)	<1	30	30	

[1] GA, gallic acid; ND, not detected.
[2] Reflects the comparison between red wine and de-alcoholized red wine polyphenols (Student's *t* test for independent samples).
[3] Mean ± SD (all such values; $n = 2$).

[5.03 μmol (95% CI: 2.25, 7.80) and 4.49 μmol (95% CI: 1.36, 7.63), respectively] than at baseline [0.21 μmol (95% CI: 0.08, 0.33)] and after gin consumption [0.17 μmol (95% CI: −0.01, 0.35)] ($P < 0.001$). The resveratrol metabolite concentrations did not change significantly after the gin period compared with baseline ($P > 0.05$). Total dihydroresveratrol concentrations after de-alcoholized red wine and red wine were 3.70 μmol (95% CI: 1.21, 6.20) and 4.64 μmol (95% CI: 0.38, 8.91), respectively, which were significantly increased compared with baseline (0.13 μmol; 95% CI: 0.05, 0.20) and gin (0.17 μmol; 95% CI: −0.02, 0.35) ($P < 0.001$). The results are expressed in **Figure 1**.

Anthropometric and biochemical measurements

The anthropometric and biochemical variables of the participants during the study are shown in **Table 4**. A significant decrease was observed in diastolic BP (DBP; mm Hg) after the red wine period with respect to the washout period, whereas the systolic BP (SBP; mm Hg) decreased significantly after both the red wine and the de-alcoholized red wine periods. The highest decrease in DBP and SBP was nevertheless observed after the red wine period (97.40 ± 15.21 compared with 86.50 ± 11.60 and 145.40 ± 23.86 compared with 129.50 ± 17.60; $P < 0.05$). The effect of the 3 interventions on BMI and plasma concentrations of glucose, glutamic pyruvic transaminase, and LDL cholesterol did not differ significantly. However, a significant decrease was seen in the concentrations of glutamate-oxaloacetate transaminase, γ-glutamyl transpeptidase, triglycerides, HDL cholesterol, and CRP after both the de-alcoholized red wine and the red wine periods compared with the washout period. Finally, the uric acid and total cholesterol concentrations decreased significantly from baseline only after the red wine period.

PCR-DGGE and bacterial band identification

The generated host-specific fingerprints showed interindividual variation in the composition of the fecal microbiota, showing

TABLE 3
Result of energy and dietary intakes in the 10 subjects studied at baseline and after the 3 treatments[1]

	Baseline (washout period)	De-alcoholized red wine intervention	Red wine intervention	Gin intervention	P
Energy (kcal/d)	1721.3 (1421.0, 2294.0)	1813.7 (1515.6, 2165.6)	1792.3 (1535.0, 2111.8)	1849.1 (1602.6, 2173.4)	0.052
Total protein (g/d)	75.7 (64.8, 94.2)	85.7 (70.8, 103.6)	83.5 (69.8, 100.0)	91.9 (77.2, 111.8)	0.228
Carbohydrates (g/d)	167.0 (136.0,227.0)	151.2 (124.5, 182.7)	151.8 (126.1, 180.7)	155.8 (129.1, 187.1)	0.892
Dietary fiber (g/d)	16 (14.1, 17.9)	17.1 (14.6, 19.7)	16.9 (14.5, 19.5)	16.9 (13.8, 19.9)	0.834
Sugars (g/d)	69.5 (56.4, 82.7)	66.1 (30.7, 101.5)	70.1 (37.9, 102.3)	78.4 (37.9, 118.9)	0.527
Total lipids (g)	79.8 (63.6, 108)	91.7 (72.7, 111.1)	90.6 (78.8, 103.6)	91.5 (76.6, 105.7)	0.084
SFA (g/d)	24.0 (19.4, 32.1)	25.8 (20.4, 31.65)	26.0 (22.7, 29.2)	29.6 (23.5, 35.6)	0.164
MUFA (g/d)	40.8 (30.9, 57.0)	47.2 (36.5, 58.0)	46.9 (39.3, 54.7)	43.9 (35.3, 53.1)	0.176
PUFA (g/d)	11.6 (10.1, 13.0)	12.2 (9.8, 14.7)	11.8 (9.8, 13.9)	12.9 (8.4, 17.5)	0.598
Cholesterol (mg/d)	329.6 (307.4, 351.9)	351.0 (275.9, 426.1)	313.8 (261.9, 365.7)	350.3 (280.1, 420.5)	0.567
Vitamin C (mg/d)	82.3 (67.1, 97.5)	70.9 (49.7, 92.3)	72.2 (32.9, 111.4)	67.5 (50.6, 84.4)	0.564
Vitamin A (μg RE/d)	690.3 (574.3, 806.3)	798.6 (456.8, 1140.4)	679.3 (550.9, 807.6)	805.1 (449.5, 1160.7)	0.661
Vitamin E (mg/d)	5.5 (4.6, 6.6)	5.5 (3.9, 7.4)	6.2 (4.9, 7.7)	5.6 (3.9, 7.5)	0.816
Folic acid (μg/d)	237.9 (204.7, 271.3)	229.1 (181.1, 277.1)	209.3 (175.0, 243.6)	222.3 (159.3, 285.3)	0.633
Total polyphenols (mg/d)	425.0 (250.0, 651.0)	372.6 (189.4, 592.0)	369.5 (203.1, 584.2)	379.6 (225.1, 538.2)	0.942

[1] All values are means; 95% CIs in parentheses. $n = 10$ subjects. Energy, nutrient, and total polyphenol contributions from interventions were excluded. No changes were observed between baseline and interventions determined, $P > 0.05$ (repeated-measures 1-factor ANOVA with post hoc Bonferroni test). RE, retinol equivalent.

a high heterogeneity in electrophoretic patterns between the different subjects and between the different intake periods. Variations were found in the presence or absence (qualitative) and intensity (quantitative) of the bands.

DGGE band profiles (mean of bands: 16.8) were stable for each subject at baseline. Nevertheless, differences in band richness were found between the de-alcoholized red wine, the red wine, and the gin periods (de-alcoholized red wine, mean of bands: 17.4; red wine, mean of bands: 18.2; gin, mean of bands: 14.6). On the other hand, some bands were seen in fingerprints from all the participants, indicating that specific species of the predominant microbiota were common to the participants.

The Dice similarity coefficient was used to calculate the similarity coefficient between DGGE band profiles in the 4 different sampling periods for each participant. The median similarity coefficient values were 34.68%, 26.42%, and 28.33% for de-alcoholized red wine, red wine, and gin, respectively. These data showed that the dominant bacterial composition did not remain constant over the different intervention periods with respect to baseline. Cluster analysis of DGGE profiling confirmed that banding patterns were not closely related for the participants, revealing grouping according to the intake period. The DGGE gel and the results of the cluster analysis are shown in **Figure 2**.

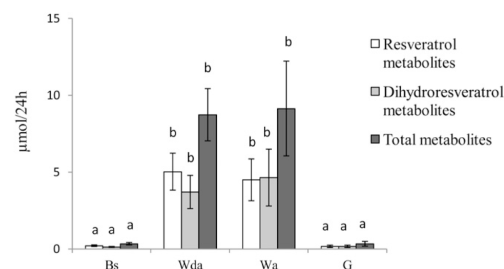

FIGURE 1. Mean (±SEM) resveratrol metabolites, dihydroresveratrol metabolites, and total metabolites (resveratrol and dihydroresveratrol) quantified in 24-h urine samples from 10 volunteers at Bs, Wda, Wa, and G. The changes between the intervention treatments were analyzed by using the Friedman test. Wilcoxon's signed-rank test was used to compare the treatments with one another. Values in a box of the same shade of gray with different lowercase letters are significantly different, $P < 0.05$ (Bonferroni post hoc test). Bs, baseline sample; G, sample after gin consumption; Wa, sample after red wine consumption; Wda, sample after de-alcoholized red wine consumption.

Selected bands from all volunteer profiles at baseline and in the different intake periods were sequenced and the sequence homology matches for bands were analyzed by MicroSeqID v2.1.1 software. Bacterial identification showed that most of the bacteria represented in our fingerprints corresponded to 5 phyla. Most of the sequences belonged to *Firmicutes* and *Bacteroidetes*, with the rest distributed among *Proteobacteria, Actinobacteria*, and *Fusobacteria* (**Table 5**). Nevertheless, we also observed important differences between baseline and the different intake periods and between polyphenol intake periods (de-alcoholized red wine and red wine) and the gin period in the distribution ratio of different genera within the *Bacteroidetes* and *Firmicutes* phyla. At baseline, the *Bacteroides* and *Prevotellaceae* frequencies were lower than those after polyphenol intake periods, whereas the *Clostridium* frequency was similar. After the de-alcoholized red wine and the red wine periods, the *Bacteroides, Clostridium*, and *Prevotellaceae* frequencies were similar; however, an increase in the *Bacteroides* and *Clostridium* frequencies and a disappearance of *Prevotellaceae* after the gin period were observed (Table 5).

Effect of wine polyphenol intake and ethanol on human fecal microbiota

Changes in the bacterial population content were assessed in the fecal samples of all volunteers at baseline and after each intervention period. The results obtained in the real-time qPCR experiments with the different primers are shown in **Tables 6** and **7.** Relevant differences were found in the bacteria number of 4 phyla after the different intake periods. *Proteobacteria, Fusobacteria, Firmicutes*, and *Bacteroidetes* changed significantly over the study. Only the *Actinobacteria* phyla did not change

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TABLE 4
Anthropometric and biochemical variables during the study[1]

	Baseline (washout period)	De-alcoholized red wine period	Red wine period	Gin period	P^2
Weight (kg)	97.8 ± 21.3	97.8 ± 19.4	96.4 ± 20.6	97.2 ± 19.6	0.306
Waist (cm)	106.7 ± 14.3	106.5 ± 14.4	105.1 ± 14.5	105.7 ± 13.5	0.392
Hip (cm)	111.0 ± 10.4	109.0 ± 12.8	110.2 ± 11.1	110.8 ± 10.3	0.908
DBP (mm Hg)	97.4 ± 15.2[a]	91.0 ± 12.9[a]	86.5 ± 11.6[b]	98.4 ± 14.3[a]	0.026
SBP (mm Hg)	145.4 ± 23.9[a]	135.1 ± 24.6[b]	129.5 ± 17.6[b]	142.7 ± 22.3[a]	0.026
BMI (kg/m^2)	27.6 ± 3.2	27.6 ± 3.1	27.5 ± 2.9	27.6 ± 2.8	0.241
Glucose (mg/dL)	111.3 ± 23.1	104.5 ± 24.2	108.5 ± 16.4	108.8 ± 17.2	0.772
Uric acid (mg/dL)	5.7 ± 1.1[a]	5.3 ± 1.0[a]	5.0 ± 0.8[b]	5.4 ± 1.5[a]	0.018
GOT (mg/dL)	22.0 ± 7.3[a]	14.3 ± 4.0[b]	17.6 ± 13.4[b]	19.1 ± 8.0[a]	0.021
GPT (mg/dL)	46.4 ± 12.6	41.2 ± 7.7	42.0 ± 9.3	43.1 ± 6.9	0.888
GGT (mg/dL)	36.9 ± 25.6[a]	30.1 ± 13.5[b]	36.1 ± 16.3[b]	38.0 ± 27.7[a]	0.012
Triglycerides (mg/dL)	245.4 ± 231.7[a]	171.7 ± 206.7[b]	179.4 ± 177.1[b]	190.1 ± 222.5[b]	0.001
Cholesterol (mg/dL)	257.5 ± 88.6[a]	241.2 ± 94.9[a]	188.6 ± 61.6[b]	235.3 ± 91.4[a]	0.008
LDL cholesterol (mg/dL)	129.6 ± 41.9	123.5 ± 28.1	125.7 ± 30.3	130.6 ± 22.0	0.266
HDL cholesterol (mg/dL)	58.5 ± 16.7[a]	48.8 ± 17.1[b]	49.7 ± 14.3[b]	52.3 ± 16.5[a]	0.001
CRP (mg/L)	6.9 ± 2.6[a]	4.3 ± 2.3[b]	4.6 ± 2.5[b]	6.8 ± 3.7[a]	0.001

[1] All values are means ± SDs; n = 10 subjects. Means in a row with different superscript letters are significantly different, $P < 0.05$ (Wilcoxon's signed-rank test with post hoc Bonferroni test). CRP, C-reactive protein; DBP, diastolic blood pressure, GGT, γ-glutamyl transferase; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; SBP, systolic blood pressure.

[2] Derived by using the Friedman test.

significantly after any of the periods. After the red wine period, the bacterial concentrations of *Proteobacteria, Fusobacteria, Firmicutes* and *Bacteroidetes* were significantly increased compared with the washout period. On the other hand, the de-alcoholized red wine significantly increased the *Fusobacteria* concentration with respect to baseline. The concentrations of *Bacteroidetes* and *Firmicutes* decreased significantly after the de-alcoholized red wine period compared with the red wine period (Table 6).

Moreover, a significant change in the number of *Enterococcus*, *Clostridium*, *Clostridium histolyticum*, *Blautia coccoides–Eubacterium rectale*, *Bacteroides*, *Bacteroides uniformis*, *Prevotella*, Bifidobacterium, and *Eggerthella lenta* were found during the study. Within Firmicutes, the genus *Enterococcus* and the *Blautia coccoides–Eubacterium rectale* group increased significantly after consumption of de-alcoholized red wine and red wine compared with baseline. *Lactobacillus* concentrations remained unchanged throughout the study ($P > 0.05$), whereas *Clostridium* and the *Clostridium histolyticum* group increased significantly after gin consumption. Within Bacteroidetes, the genus *Bacteroides* and the *B. uniformis* species increased significantly after red wine intake, whereas the number of *Prevotella* decreased significantly after gin intake and increased significantly after red wine intake, both with respect to baseline. Finally, within the Actinobacteria phyla, we found that interventions with red wine and de-alcoholized red wine led to a significant increase in the number of Bifidobacterium and *Eggerthella lenta* compared with baseline (Table 7).

Bacterial changes predicted modifications in lipid markers, CRP, and BP

In addition to the reduction after polyphenol interventions (de-alcoholized red wine and red wine) in BP and concentrations of triglycerides, cholesterol, HDL cholesterol, and CRP with respect to baseline (Table 4), we also found after the same periods a significant univariate correlation between changes in the amount of specific bacteria and DBP (*Bacteroides*: $r = -0.406$, $P = 0.026$), SBP (*Enterococcus*: $r = -0.362$, $P = 0.049$; *Bacteroides*: $r = -0.362$, $P = 0.049$), plasma triglycerides (*Bacteroides*: $r = -0.364$, $P = 0.048$), cholesterol (*Bifidobacterium*: $r = -0.401$, $P = 0.028$; *Bacteroides*: $r = -0.363$, $P = 0.049$), HDL cholesterol (*Lactobacillus:* $r = -0.447$, $P = 0.013$; *Bacteroides*: $r = -0.469$, $P = 0.009$), and CRP (*Bifidobacteria:* $r = -0.430$, $P = 0.018$; *Lactobacillus*: $r = -0.405$, $P = 0.027$). With the use of a multivariate regression analysis that included all the bacterial groups analyzed, only the increment of *Bacteroides* predicted the DBP ($P = 0.48$, $R^2 = 0.364$) and SBP ($P = 0.03$, $R^2 = 0.369$) reductions and triglyceride ($P = 0.048$, $R^2 = 0.364$) and HDL cholesterol ($P = 0.001$, $R^2 = 0.732$) reductions. On the other hand, *Bifidobacterium* growth predicted the decrease in cholesterol ($P = 0.012$, $R^2 = 0.583$) and CRP ($P = 0.018$, $R^2 = 0.430$). This suggested that these bacterial groups could be partly implicated in the observed reduction in BP, plasma lipid markers, and CRP.

DISCUSSION

In this study we observed that red wine, de-alcoholized red wine, and gin produced significant changes in the fecal microbiota of all the participants. The DGGE analysis of the predominant fecal microbiota generated complex and relatively stable and unique profiles for each study subject. DGGE analysis showed low similarity index values between the different intake periods, with clustering of banding patterns characteristic for each intake period. These results indicate that changes in the gut microbiota composition of healthy subjects occur after a dietary intervention, which disagrees with previous studies regarding the subject specificity of the predominant fecal communities and their stability over time and resistance to perturbations (33, 34). Because DGGE is considered a semiquantitative tool for monitoring the dynamics

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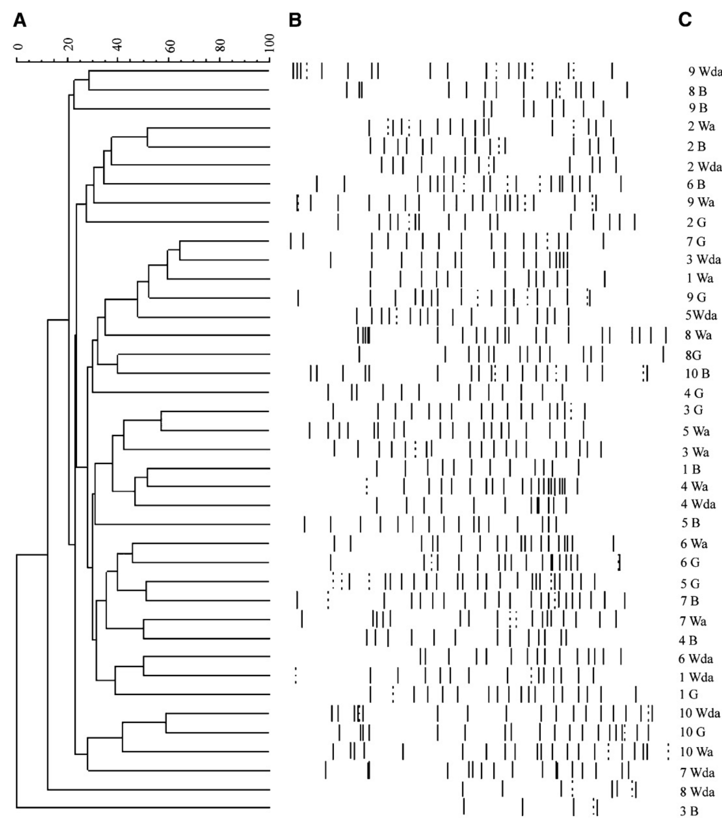

FIGURE 2. Dendrograms of electrophoretic band patterns obtained in the denaturing gel-gradient electrophoresis experiment with universal primers in the 4 different fecal samples collected from the 10 healthy subjects. A: cluster analysis; B: DGGE profiles of fecal samples; C: line graph. B, baseline sample; G, sample after gin consumption; Wa, sample after red wine consumption; Wda, sample after de-alcoholized red wine consumption.

of the predominant bacterial species of fecal microbiota, additional analysis with real-time qPCR was performed to obtain a quantitative estimation of the effect of the ethanol and polyphenol intakes on fecal bacteria populations. We found significant changes in the relative amounts of this microbiota, depending on the kind of beverage consumed. Sequencing results also showed differences in the microbiota composition during the study. After ethanol intake (gin period), there was an increase in the *Bacteroides* and *Clostridium* frequencies and a disappearance of *Prevotellaceae* as compared with polyphenol (de-alcoholized red wine period) or polyphenol plus ethanol intake (red wine period) or baseline (no polyphenol, no alcohol). These results suggest that the dominant genera in the intestinal microbiota of the volunteers was different at baseline and after polyphenol intake than after only ethanol intake. Moreover, the diversity of the fecal microbiota, according to the number of bands in DGGE profiles, was higher after the red wine period. We also found that phenolic compounds altered fecal microbiota, and consequently the *Bacteroides/Firmicutes* balance. After the red wine period, which was rich in polyphenols, there was a significant increase in the concentration of *Firmicutes* and *Bacteroidetes* in stool samples. However, no significant differences were detected in the number of *Lactobacillus* species after any intake period, which indicates that polyphenol or ethanol consumption had no effect on the growth of these bacteria. Similar results were reported by Tzounis et al (35), who found that after catechin intake the growth of *Lactobacillus* spp. remained relatively unaffected in an in vitro study, and by Yamakoshi et al (36), who observed no change in that genera after ingestion of procyanidin-rich extracts in healthy adults. Nevertheless, Tzounis et al recently found a significant increase in the growth of *Lactobacillus* spp. after a regular intake of a high-cocoa flavonol drink in both in vivo and in vitro studies (37). Although the *Actinobacteria* phyla did not change after any intake period, we found a significant increase in the *Proteobacteria, Firmicutes*, and *Bacteroidetes* phyla after the red wine period but not after the gin or de-alcoholized red wine periods, as compared with the initial washout period. These findings indicate that small ethanol doses plus polyphenol intake for a short time can generate an important

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TABLE 5
Bacterial identification after the sequencing of the bands from the denaturing gradient gel electrophoresis analysis of fecal samples at baseline and in the 3 intake periods

Type bacteria genus (sequencing results of the bands)	Baseline (washout period)[1] (*n* = 35)	De-alcoholized red wine periods[1] (*n* = 38)	Red wine period[1] (*n* = 41)	Gin period[1] (*n* = 37)	Sequence similarity
	n (%)	*n (%)*	*n (%)*	*n (%)*	%
Phylum *Bacteroidetes*					
Genus *Bacteroides*	10 (28.57)	15 (39.47)	14 (34.14)	18 (48.64)	99.86
Unclassified *Prevotellaceae*	7 (20)	11 (28.94)	11 (26.82)	0	94.71
Phylum *Firmicutes*					
Genus *Clostridium*	5 (25.71)	4 (10.52)	5 (12.19)	13 (35.13)	98.73
Genus *Veillonella*	3 (8.57)	3 (7.89)	3 (7.31)	1 (2.70)	97.49
Phylum *Actinobacteria*					
Unclassified *Actinobacteria*	1 (2.85)	2 (5.26)	2 (4.87)	1 (2.70)	94.52
Phylum *Proteobacteria*					
Genus *Pseudomona*	1 (2.85)	1 (2.63)	1 (2.43)	1 (2.70)	97.70
Genus *Acinetobacter*	1 (2.85)	1 (2.63)	1 (2.43)	1 (2.70)	97.37
Unclassified *Enterobacteriales*	1 (2.85)	2 (5.26)	1 (2.43)	1 (2.70)	93.39
Unclassified *Campylobacterales*	1 (2.85)	1 (2.63)	2 (4.87)	0	94.27
Phylum *Fusobacterium*					
Unclassified *Fusobacteriaceae*	1 (2.85)	1 (2.63)	1 (2.43)	1 (2.70)	94.28

[1] Refers to the frequency (and percentage) of each unique bacteria genus in the baseline or de-alcoholized red wine or red wine or gin intake periods; *n* is the number of bands sequenced and identified in each intake period.

change in the gut microbiota, which may have influenced the host metabolism. Moreover, after the red wine period, there was a significant increase in the number of *Enterococcus, Bacteroides*, and *Prevotella* genera and an important decrease in the *Clostridium* genera and *Clostridium histolyticum* group. Similar results were found by others in rat models. Smith et al (38) found that the *Bacteroides* group increased significantly when a tannin-rich diet was given to rats, and Dolara et al (39) reported that when rats were treated with red-wine polyphenols they had significantly lower levels of *Clostridium* spp., and no change was observed in *Actynomices* genera within *Actinobacterias*, as in the current study. Predominant red wine polyphenols, such as flavan-3-ol monomers and procyanidins, are associated with antimicrobial activity, exerting their inhibitory effect on certain bacterial groups by binding to bacterial membranes (14, 40). Differences in cell surface structures could explain why Gram-positive clostridial-type bacteria are more sensitive to the bactericidal effects of these compounds than are Gram-negative *Prevotella* and *Bacteroides* species (41). The significant decrease found by us in the *Clostridium histolyticum group* suggests that red wine polyphenols have an inhibitory effect on the growth of these bacteria, which includes *Clostridium perfringens*—an important pathogen closely related with the progression of colonic cancer and the onset of inflammatory bowel disease (42). The same result was reported by Tzounis et al (37) in an intervention study using cocoa-derived flavonols. On the other hand, this study showed that polyphenol and polyphenol plus ethanol intake positively affected the growth of the *Blautia coccoides–Eubacterium rectale* group, *Bifidobacterium, Eggerthella lenta*, and *Bacteroides uniformis.* The last 2 microorganisms are able to degrade resveratrol into dihydroresveratrol, which has antiproliferative effects in human prostate cancer cells and has less antioxidant activity and less ability to inhibit DNA synthesis than resveratrol (13, 20, 43). We found that the significant increase in dihydroresveratrol found in urine in our study after polyphenol intake was related with the significant increase in numbers of *Eggerthella lenta* and *Bacteroides uniformis*.

A study by Barcenilla et al (44) showed that most of the butyrate-producing isolates from human fecal samples are related to the *Blautia coccoides–Eubacterium rectale* group. The presence of butyrate in the colon may be important for the prevention of colon cancer (45) or ulcerative colitis (46). In addition, the increased growth of *Bifidobacteria* has been associated with positive effects in the large intestine (47). This bacterial group has the capacity to produce beneficial organic acids (lactate and acetate) and the ability to inhibit the growth of pathogenic bacteria (48). Other previous in vitro studies using human feces inoculated with (+)-catechin have reported an increase in the growth of *Blautia coccoides–Eubacterium rectale* group and *Bifidobacterium* spp., which suggests a moderate prebiotic effect of monomeric flavan-3-ol on the intestinal microbiota (35). Vendrame et al (49) found a significant increase in the amount of *Bifidobacterium* after 6 wk of consumption of a wild blueberry drink, which suggests an important role of the polyphenol present in wild blueberries on the intestinal microbiota composition modulation. The similar data found in our study could suggest a possible prebiotic effect of the red wine polyphenol on the microbiota.

Bacterial population shifts caused by wine polyphenols may have an effect on host health. Our results clearly showed that dealcoholized red wine and red wine significantly decrease SBP and transaminase concentrations. These results agree with those of other studies, which have reported that polyphenols suppress serum transaminase elevations and reduce elevated BP (50, 51). Uric acid concentrations decreased significantly during the red wine period. This can be explained by the significant increase of *Proteobacteria* observed in this stage, which has previously been reported to degrade uric acid (52). During the study we found that the regular intake of red wine polyphenols generated significant decreases in the plasma concentrations of triglycerides,

TABLE 6
Real-time polymerase chain reaction quantification of microbiota phyla during the study[1]

	Baseline (washout period)	De-alcoholized red wine period	Red wine period	Gin period	P^2
	log_{10} *copies/g feces*	log_{10} *copies/g feces*	log_{10} *copies/g feces*	log_{10} *copies/g feces*	
Proteobacteria	7.21 ± 1.50^{a}	7.37 ± 1.92^{a,b}	8.68 ± 2.43^{b}	6.74 ± 2.19^{a}	0.017
Actinobacteria	8.32 ± 2.78	8.53 ± 2.56	9.05 ± 2.48	7.66 ± 2.94	0.084
Fusobacteria	5.97 ± 1.71^{a}	7.04 ± 1.09^{b,c}	7.83 ± 0.99^{b}	5.81 ± 1.72^{a,c}	0.015
Firmicutes	8.62 ± 0.50^{a}	8.54 ± 0.52^{a}	9.41 ± 0.68^{b}	7.88 ± 0.86^{a}	0.006
Bacteroidetes	8.96 ± 0.87^{a}	9.44 ± 0.82^{a}	9.98 ± 0.64^{b}	8.46 ± 1.38^{a}	0.001

[1] All values are means ± SDs; n = 10 subjects. Wilcoxon's signed-rank test was used to compare the treatments with each other. Means in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test).
[2] Derived by using the Friedman test.

total cholesterol, and HDL cholesterol. A previous study using a rat model showed that red wine polyphenols reduced circulating triglycerides and total cholesterol (53). Additionally, gut microbiota increase energy metabolism and have systemic effects on host lipid metabolism, especially increasing triglyceride clearance (54). We found that these significant reductions observed in BP, triglycerides, and HDL cholesterol may have been due in part to the polyphenol-induced increase in the growth of *Bacteroides* genera. The decrease observed in the cholesterol concentration could be related to the significant increase in *Bifidobacterium*—a bacteria genera previously associated with the reduction of plasma cholesterol concentrations (55). Finally, we noted a significant reduction in the concentration of CRP after de-alcholized red wine and red wine treatment. This could have been due to the increase seen in *Bifidobacterium* number. CRP is a blood marker of inflammation, and its concentration is a specific predictor of cardiovascular event risk in healthy subjects. Its reduction in our study links polyphenol intake to cardiovascular benefits in the host (56, 57).

One of the main limitations of our study was the lack of washout periods between interventions. The inclusion of washout periods between interventions would extend the study a further 6 wk, which would make it difficult to ensure compliance, and the subjects would be more inclined to withdraw from the study. Nonetheless, no carryover effect was observed in the study, and the absence of a washout period was therefore unlikely to affect the results obtained.

Finally, a limitation of the 16S rRNA gene–based method is that the function of the identified bacteria is unknown. Future studies using a microbial metagenomic sequencing analysis will be done to obtain information about the functional diversity of the bacterial community analyzed here and their effects on polyphenol metabolism and health.

In conclusion, this was the first in vivo study to show that regular moderate consumption of red wine could have a noteworthy effect on the growth of select gut microbiota. We found that red wine polyphenols can inhibit nonbeneficial bacteria from the human microbiota and potentiate the growth of probiotic bacteria such as bifidobacteria, which could be implicated in the reduction of CRP and cholesterol observed in our study, promoting health benefits in the host. Although further research is required, the results of this study suggest the possible prebiotic benefits associated with the inclusion of red wine polyphenols in the diet.

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TABLE 7
Genera, species, and groups within the phyla *Bacteroidetes, Firmicutes*, and *Actinobacteria* amplified by real-time polymerase chain reaction[1]

	Baseline (washout period)	De-alcoholized red wine period	Red wine period	Gin period	P^2
	log_{10} *copies/g feces*	log_{10} *copies/g feces*	log_{10} *copies/g feces*	log_{10} *copies/g feces*	
Enterococcus	5.18 ± 1.2^{a}	6.94 ± 1.5^{b}	7.10 ± 1.1^{b}	4.84 ± 1.75^{a}	0.001
Lactobacillus	5.24 ± 1.81	5.66 ± 1.71	5.59 ± 2.02	4.91 ± 2.12	0.204
Clostridium	4.70 ± 2.17^{a}	3.98 ± 2.08^{a}	3.34 ± 1.78^{a}	6.48 ± 1.64^{b}	0.013
Clostridium histolyticum group	3.62 ± 2.09^{a}	3.75 ± 2.12^{a}	3.19 ± 2.19^{a}	6.62 ± 1.52^{b}	0.001
Blautia coccoides–Eubacterium rectale group	4.95 ± 1.33^{a}	6.64 ± 1.26^{b,c}	6.86 ± 1.43^{b}	5.13 ± 1.17^{a,c}	0.001
Bacteroides	7.61 ± 1.71^{a}	8.37 ± 0.95^{a,b,c}	9.42 ± 0.85^{b}	6.46 ± 1.81^{a,c}	0.046
Bacteroides uniformis	8.52 ± 0.97^{a}	9.37 ± 1.12^{a,b}	9.98 ± 1.02^{b}	7.96 ± 1.53^{a}	0.001
Prevotella	6.87 ± 0.67^{a}	7.19 ± 0.64^{a,b}	7.85 ± 0.81^{b}	4.88 ± 0.57^{c}	0.013
Bifidobacterium	7.12 ± 2.28^{a}	9.93 ± 1.85^{b}	9.88 ± 1.78^{b}	6.87 ± 2.74^{a}	0.031
Eggerthella lenta	7.68 ± 1.41^{a}	9.84 ± 1.65^{b}	9.97 ± 1.77^{b}	7.26 ± 1.87^{a}	0.001

[1] Values are means ± SD; n = 10 subjects. Wilcoxon's signed-rank test was used to compare the treatments with each other. Means in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test).
[2] Derived by using the Friedman test.

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The authors' responsibilities were as follows—MIQ-O, CA-L, and FJT: conceived and designed the research; MIQ-O, MM, MB-O, JMG-Z, MC-P, and FCD: conducted the research; MIQ-O, MM, and FCD: performed the statistical analysis and interpreted the data; MIQ-O, MB-O, MM, FCD, and FJT: wrote the manuscript; and MIQ-O, CA-L, FJT, and RE: critically revised the manuscript. All authors have read and approved the final manuscript. None of the authors had any financial or other contractual agreements that might have caused conflicts of interest or that could be perceived as causing conflicts of interest.

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4.6 EFECTO PREBIÓTICO DE LOS POLIFENOLES DEL VINO TINTO SOBRE LAS PRINCIPALES BACTERIAS COLÓNICAS.

La asociación de los cambios observados para los grupos bacterianos aumentados después del consumo de vino tinto con los cambios en el perfil metabólico urinario queda reflejada en el artículo presentado a continuación que se encuentra actualmente en proceso de publicación.

Publicación 6: María Boto-Ordóñez, Mireia Urpi-Sarda, María Isabel Queipo-Ortuño, Sara Tulipani, Francisco J. Tinahones, Cristina Andres-Lacueva. High levels of *Bifidobacteria* are associated with increased levels of anthocyanin metabolites: A randomized clinical trial. *En proceso de publicación.*

Resumen:

Los beneficios sobre la salud asociados a los alimentos y bebidas que contienen polifenoles se han estudiado a fondo, sin embargo, los mecanismos de acción siguen siendo mayoritariamente desconocidos. Uno de los mecanismos propuestos es a través de la interacción con la microbiota. La interacción con la microbiota intestinal no sólo genera metabolitos fenólicos, si no que también puede modular la composición bacteriana, actuando como un compuesto prebiótico. La mayoría de los estudios se centran sólo en uno de estos aspectos, pero se necesita una visión global para comprender el verdadero papel en el cuerpo humano. En el presente estudio la relación entre los cambios en el contenido de microbiota fecal y los cambios en los metabolitos fenólicos presentes en orina se analizó después de una intervención con vino tinto. Nueve participantes siguieron un ensayo de intervención, aleatorizado, controlado y cruzado con vino tinto, vino tinto desalcoholizado y ginebra. Los ácidos fenólicos en la orina (n>60) fueron identificados y cuantificados por UPLC-MS/MS y la composición microbiana de muestras fecales obtenidas mediante PCR cuantitativa a tiempo real. Después de la intervención del vino, sólo dos géneros *Bifidobacterium* y *Enterococcus* y la especie *Eggerthella lenta* se modificaron significativamente después de ambos períodos con vino tinto, pero no en la intervención con ginebra. Cuando los

participantes se dividieron en tertiles de cambios de bacterias fecales, los del tertil más alto de bifidobacterias tenían mayores cambios de concentraciones urinarias de siríngico, *p*-cumárico, ácido 4-hidroxibenzoico y homovanílico, todos ellos metabolitos de antocianinas, en comparación con los del tertil 1 ($P<0.05$, todos). Además, los participantes en el tertil más alto de *Eggerthella lenta*, tenían menores cambios en la concentración urinaria de ácidos hidroxicinámicos ($P<0.05$, todos). Tras un análisis de regresión lineal por pasos, sólo los cambios de ácidos siríngico y 4-hidroxibenzoico podían predecir 68,5% cambios en bifidobacterias. Este estudio confirma la importancia del papel de los polifenoles como sustrato bacteriano y su capacidad moduladora, un campo importante para el desarrollo en la industria alimentaria de nuevos productos con características prebióticas y probióticas.

Figura R6: Gráfico resumen del papel de los polifenoles del vino sobre la microbiota humana y su posible asociación.

High levels of *Bifidobacteria* are associated with increased levels of anthocyanin microbial metabolites: A randomized clinical trial

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Keywords: Wine, Microbiota, Bifidobacteria, Anthocyanins, *in vivo*, prebiotic.

ABSTRACT

The health benefits associated with the consumption of polyphenol-rich foods have been studied in depth, however, the full mechanism of action remains unknown. One of the proposed mechanisms is through microbiota interaction. In the present study, we aimed to explore the relationship between changes in fecal microbiota and changes in urinary phenolic metabolites after wine interventions. Nine participants followed a randomized, crossover, controlled interventional trial with a 20 days consumption of red wine, dealcoholized red wine and gin. Polyphenol metabolites ($n>60$) in urine were identified and quantified by UPLC-MS/MS and the microbial content of fecal samples was quantified by from real-time quantitative PCR. Interventions with both red wine and dealcoholized red wine increased the fecal concentration of *Bifidobacterium*, *Enterococcus* and *Eggerthella lenta*, compared to gin intervention and baseline. When participants were categorized in tertiles of changes in fecal bacteria, those in the highest tertile of *Bifidobacteria* had higher urinary concentration changes in syringic, *p*-coumaric, 4-hydroxybenzoic and homovanillic acid, all anthocyanin metabolites, than those in tertile 1 ($P<0.05$, all). In addition, changes of *Bifidobacteria* correlated positively with changes of these metabolites ($r=0.5-0.7$, $P<0.05$, all). Moreover, those participants in the highest tertile of *Eggerthella lenta* had lower urinary concentration changes of hydrocinnamic acids ($P<0.05$, all). Finally, the 68.5% changes in *Bifidobacteria* can be predicted by syringic acid and 4-hydroxybenzoic acid changes. This study confirms the important role of polyphenols as bacterial substrates and their modulatory capacity as an important field in the research of new products with prebiotic and probiotic characteristics for the food industry.

Introduction

In the nutrition research field the link between food consumption and human health has been explored and discussed in depth. From macronutrients[1] to micronutrients[2] and phytochemicals,[3] the knowledge of their true role in the prevention of diseases has been limited to the high individual variability and understanding of the human metabolism. In this process, microbiota have been positioned as a key point due to their relation with some diseases such as obesity[4] and metabolic syndrome[5] or in host defense.[6] The connection between diet and microbiota is described in two ways: i) food may have a prebiotic effect modulating bacterial composition;[7, 8] ii) and behaving as a metabolic entity, producing metabolites derived from food consumption.[9] One of the diet components that have been associated with health benefits through microbiota interaction is polyphenols.[10] Most dietary polyphenols arrive intact at the colon and may interact with microbiota to be metabolized before being absorbed.[11] This interaction could involve hydrolysis, demethylation, reduction, decarboxylation, dehydroxylation and isomerization of these compounds into more simple components[12] modulating absorption and biological activity.[13] Concurrently, polyphenols and metabolites formed in the intestine have the ability to promote and inhibit the growth of bacterial groups.[9] In the case of red wine (RW), it has been described that the interaction of polyphenols with microbiota releases a wide range of microbial metabolites found in biofluids[14, 15] and also increases the number of *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium*, *Bacteroides uniformis*, *Eggerthella lenta*, and *Blautia coccoides-Eubacterium rectale group* along with the phyla *Proteobacteria*, *Fusobacteria*, *Firmicutes* and *Bacteroidetes.*[7] However, the link between bacterial groups and polyphenolic metabolites after food consumption has just been proved in *in vitro* studies, where microbiota incubated with polyphenols release phenolic metabolites whose presence may modulate their growth.[16, 17]

For that reason, we embarked on a study to evaluate the associations between changes in bacterial number produced at intestinal level and urinary changes in microbial phenolic acids in a randomized, controlled intervention study with 20 days consumption of RW, dealcoholized red wine (DRW) and gin.

Materials and methods

Study subjects and design

The study was an open, randomized, crossover, controlled intervention trial[7] that involved 9 adult men aged between 45 and 50. The study design was divided into 3 consecutive periods of 20 days each with an initial washout period (baseline) during which the participants did not consume any alcohol or red wine. This period was followed in a random order by 3 consecutive

periods during which the participants drank DRW (272 mL/d), or RW (272 mL/d, containing 30 g ethanol), or gin (100 mL/d, containing 30 g ethanol).

At baseline, and after each intervention period, participants provided fecal and 24 h urine samples, which were stored at -80 ºC until analysis. They were asked to maintain their dietary pattern and lifestyle and to avoid alcoholic beverages during the whole study. Participants had not received treatment for diabetes, hypertension, or dyslipidemia, any antibiotic therapy, prebiotics, probiotics, symbiotics, or vitamin supplements or any other medical treatment influencing intestinal microbiota during the 3 months before the start of the study or during the study (including the washout period). They did not have any acute or chronic inflammatory diseases, infectious diseases, viral infections, or cancer, and had not had a previous cardiovascular event at study entry. The Ethics Committee of the Virgen de la Victoria Hospital approved the clinical protocol. All the participants gave written informed consent. This trial was registered at controlled-trials.com as ISRCTN88720134.

Red wine, dealcoholized red wine and gin

The RW and DRW used in this study were elaborated with the Merlot grape variety, from the Penedès appellation (Catalonia). No differences in phenolic composition were found in the RW and DRW.[7]

Chemical and reagents

Available phenolic acids and flavanols and *β*-glucuronidase/sulfatase (from *Helix pomatia*) were purchased from Sigma-Aldrich (St. Louis, MO, USA), PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany), and Extrasynthèse (Genay, France) as previously described.[14] HPLC grade solvents were purchased from Scharlau Chemie, S.A. (Sentmenat, Spain) and Panreac Química, S.A.U. (Castellar del Vallès, Spain). Ultrapure water (Milli-Q) was obtained from Millipore System (Bedford, MA, USA).

Sample extraction

Microbial-derived and conjugated metabolites present in urine were analyzed using solid-phase extraction (SPE) with an Oasis® MCX and HLB 96-well plates (Waters, Milford, Massachusetts), respectively, as previously described.[14, 18, 19] Briefly, urine samples (1mL) were loaded onto the conditioned cartridge plate, washed and eluted with methanol or acidified methanol, respectively, and evaporated to dryness. Reconstitution of the residues was carried out with 100 µL of taxifolin in mobile phase.

UPLC-MS/MS Analysis

Metabolites in urine were analyzed by UPLC-MS/MS equipped with a binary solvent manager and a refrigerated autosampler plate (Waters Acquity UPLC system, Milford, MA, USA), coupled to an AB Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray, ionizing in negative mode (PE Sciex). An Acquity UPLC BEH C18 column (Milford, MA, USA) (1.7 μm, 2.1 mm × 5 mm), using a prefilter, working at 40 ◦C and with an injection volume of 5 μL, was used as described before.[14] Mobile phases used were: A (0.1% formic acid) and B (0.1% formic acid in acetonitrile) at a flow rate of 500 μL/min with the following proportions (v/v) of phase A [t(min),%A]: (0,92); (2.5,50); (2.6,0); (3,0); (3.1,92); (3.5,92). The MS/MS parameters used were as previously described.[14] Phase II and microbial metabolites were quantified using the Multiple Reaction Monitoring (MRM) mode with a dwell time of 10 ms. Calibration curves were constructed with available standards in synthetic urine and subjected to the same procedure as the samples. If standard was not available, metabolites were quantified using the most similar compound standard curve and results were expressed as their equivalents.[14] The metabolites analyzed for this study are shown in the supplementary data. Quality parameters of the methodology accomplish with accuracy, precision and recovery <15%.[19]

DNA extraction from fecal samples and analysis of fecal microbiota by polymerase chain reaction (PCR)

Extraction of DNA was from 200 mg stools by using a QIAamp DNA Stool Mini Kit (Qiagen) and concentration and purity were estimated with a NanoDrop spectrophotometer (NanoDrop Technologies). For bacterial quantification to characterize the fecal microbiota, specific primers targeting different bacterial genera were used by PCR as previously described.[7] Briefly, the LightCycler 2.0 PCR sequence detection system, by using the FastStart DNA Master SYBR Green Kit (Roche Diagnostics), was used for quantitative PCR experiments. Comparison among Ct values obtained from the standard curves with the LightCycler 4.0 software was carried out to calculate bacterial concentration. Standard curves were created by using a serial 10-fold dilution of DNA from pure cultures, corresponding to 10^1–10^{10} copies/g feces. The data presented were the mean values of duplicate real-time qPCR analyses.

Statistical analysis

Before the statistical analysis, a cube root transformation and a range scaling of the data for phenolic data through the MetaboAnalyst Web-based platform was performed for normalization[14, 20] and the bacterial copy numbers were converted into logarithm values.[7] We only considered bacteria with significant changes after both wines compared to gin and baseline.

These changes of bacteria were for two bacterial genera (*Bifidobacterium*, *Enterococcus*) and one species *(Eggerthella lenta*). Changes of bacteria and phenolic acids after wine intervention were assessed checking the difference compared to baseline, and changes of bacteria were divided into tertiles. The IBM SPSS Statistics software program for Windows version 20 (Chicago, IL) was used to compare differences of urinary metabolites through bacteria tertiles by a one-way analysis of variance (ANOVA). If changes of metabolites and bacteria presented a significant Spearman correlation, lineal regression stepwise analysis was performed in order to establish which of these metabolites were predictors of bacterial changes. Statistical significance was considered to be $P<0.05$.

Results

Tertiles of bacterial group changes after wine consumption

In this study, we considered the bacteria that showed significant modifications after both wine interventions and gin intervention compared to baseline.[7] No differences in number of bacteria (means±SD, $\log_{10}$ copies/g feces) were observed after DRW and RW: *Bifidobacterium* (9.93±1.85 and 9.88±1.78, respectively), *Eggerthella lenta* (9.84±1.65 and 9.97±1.77, respectively) and *Enterococcus* (6.94±1.5 and 7.10±1.1, respectively).[7] Tertiles of differences were calculated between bacterial number after wine interventions and baseline. The tertile distribution is presented in Fig. 1. The third tertile showed higher mean increases of *Bifidobacterium* (5.52±0.88 $\log_{10}$ copies/g feces), *Enterococcus* (2.83±0.51 $\log_{10}$ copies/g feces) and *Eggerthella lenta* (3.47±1.03 $l\log_{10}$ copies/g feces) while the first tertile showed lower mean increases of *Bifidobacterium* (0.38±0.57 $\log_{10}$ copies/g feces), *Enterococcus* (0.50±0.81 $\log_{10}$ copies/g feces) and *Eggerthella lenta* (1.10±0.51 $\log_{10}$ copies/g feces). The increases through tertiles of bacteria were significant ($P<0.001$).

Relationship among changes in bacterial population and urinary phenolic metabolites

The tertiles of bacterial modifications were used to analyze phenolic metabolite changes after wine interventions (Table 1). From the lowest to the highest changes in *Bifidobacteria* tertiles, participants had a higher excretion of four phenolic metabolites related to anthocyanin metabolism (Figure 2): syringic, *p*-coumaric, 4-hydroxybenzoic and homovanillic acids. On the other hand, higher increases of *Eggerthella lenta* corresponded to lower excretion of hydroxycinnamates and syringic acid. In addition, higher increases of *Enterococcus* corresponded to lower excretion of 3,4-dihydroxyphenylacetic acid.

Additionally, correlation analysis indicated that only differences in *Bifidobacteria* were significantly correlated with differences in syringic ($r=0.537$, $P<0.022$), *p*-coumaric ($r=0.621$, $P<0.006$), 4-hydroxybenzoic ($r=0.677$, $P<0.002$) and homovanillic acids ($r=0.507$, $P<0.032$).

Linear regression stepwise analysis evaluating *Bifidobacteria* changes included only two metabolites in the model (R^2=0.685; P<0.001), syringic acid and 4-hydroxybenzoic acid. This model explained 68.5% of the *Bifidobacterium* changes.

Discussion

The increased knowledge about the role of microbiota in human health and the possible modulation through food consumption is an interesting field for developing new products in the food industry such as probiotics and prebiotics.[21] Food has demonstrated the capacity to modulate the growth of intestinal bacteria in several clinical trials[8, 22] and produce bioactive metabolites.[23]

One of the main studied bacteria to be affected by food intake is *Bifidobacteria*. *Bifidobacterium* is one of the predominant genera in the human intestine, and it is considered health-promoting constituent of the microbiota.[24] In this study, the unique metabolites correlated to *Bifidobacterium* were those derived from anthocyanin degradation: 4-hydroxybenzoic, syringic, p-coumaric and homovanillic acid. The concentration of anthocyanins in wine is high but lower than flavanols, which are the main wine polyphenols.[15, 25] Nevertheless, their dietetic distribution in Mediterranean diets is more limited than flavan-3-ols and their metabolites have been proposed as excellent markers of wine consumption.[14] Anthocyanins were first supposed to have low bioavailability,[26] but in the last few years, studies with isotopically labeled anthocyanins have demonstrated that anthocyanins reach the colon where they are transformed, releasing new metabolites that differ from the original compound.[27]

Microbial metabolism of anthocyanins at colonic level involves reactions of breakage in the C-ring, resulting in hydroxylated aromatic compounds derived from the A-ring, and release of the B-ring in numerous phenolic acids, different depending on their hydroxylated pattern,[12] as well as deglycosylation.[28] Furthermore, *Bifidobacterium* enzymatic activity for polyphenols has not only been associated with ring fission,[28] but also hydrolysis[29] and glycosidase activity.[28] Figure 2 shows the principal origin of microbial metabolites derived from anthocyanin structure associated with *Bifidobacteria* increase. One of these phenolic acids is 4-hydroxybenzoic acid, which has been proposed as a pelargonidin metabolite,[30] and comes from microbial degradation of *p*-coumaric[31] or could come from syringic acid demethylation, a reaction associated with certain intestinal bacteria.[11] Moreover, some studies have shown that the concentration of 4-hydroxybenzoic acid increased in plasma and urine after strawberry consumption by healthy volunteers[32] and in the urine of rats fed with wine powder.[33] Syringic acid may come from malvidin degradation described from *Lactobacillus* and *Bifidobacterium.*[28, 34] These two

metabolites were the ones that entered the stepwise logistic regression, indicating that they were the strongest contributors to *Bifidobacteria* change after wine consumption. In the same study, *p*-coumaric was also formed when delphinidin and malvidin were incubated with these bacteria[28] via hydrolysis of *p*-coumaroyl-acylated anthocyanins, which are abundant in red wine (Fig. 2). Homovanillic acid has also been described as coming from malvidin glycoside degradation via demethoxylation and was one of the main urinary metabolites after berry purée consumption by humans.[35]

It is difficult to establish whether these compounds are primary anthocyanin metabolites or are derived from other sources. Homovanillic acid could also be formed from ferulic acid,[35] additionally *p*-coumaric acid could come from dehydroxylation of caffeic acid, and syringic from gallic acid.[36] Moreover, some of these metabolites, such as gallic acid, are also present in original wine composition.[15, 25]

Previous studies have already shown the role of anthocyanins in the bifidogenic effect as Guglielmetti *et al*. found after consumption of a wild blueberry drink by humans.[37] Biological effects associated with these changes have already been described. *Bifidobacterium* has been associated with antiobesity effects[38] and cholesterol regulation.[39] Metabolite 4-hydroxybenzoic could be responsible for the antioxidant properties of polyphenol consumption, inhibiting tyrosine nitration through the formation of 4-hydroxy-3-nitrobenzoic acid, which is less reactive than nitrotyrosine.[40] Syringic acid has been proved to increase nitric oxide production[41] and *p*-coumaric acid has inhibitory activity over angiotensin-converting enzymes.[42] The biological activities attributed to the increase in metabolites could be responsible for benefits observed in blood pressure and improving plasma lipid profile or inflammation in this study.[22, 43]

The other bacteria species modified after red wine consumption was *Eggerthella lenta*, which is significantly abundant in intestinal microbiota.[24] Significant inverse associations were found between changes in *Eggerthella lenta* tertiles and changes in hydroxycinnamic acid concentrations and between changes in *Enterococcus* tertiles and changes in 3,4-dihydroxyphenylacetic acid concentration. This was probably due to the fact that both bacterial groups could be inhibited by phenolic compounds including gallic acid and resveratrol metabolites in *in vitro* studies.[44-46] In addition, *Enterococcus* genus bacteria have been inhibited by cloudberry intake.[47]

Conclusion

Bacteria changes after red wine consumption, with or without alcohol content have been associated with the excretion of phenolic metabolites. Specifically, *Bifidobacteria* increase, correlates with increases in microbial metabolites derived from wine anthocyanins. This study contributes with new data to understanding the role of phenolic compounds in the maintenance of intestinal health and opens the way to considering anthocyanins not only as new prebiotics, but also as being responsible for health benefits associated with the consumption of anthocyanin-rich food.

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Table 1 Changes in urinary phenolic metabolites after wine interventions (mean value ± standard desviation) according to changes in bacterial tertiles.

	Changes in bacterial population tertiles			*P*-trend
	Changes in Bifidobacterium tertiles [a]			
	1 (n=6) (<1.18)	2 (n=6) (1.18-4.47)	3 (n=6) (>4.47)	
4-Hydroxybenzoic acid[b]	-20.34±15.6	-2.87±22.07	18.04±34.2	0.013
Syringic acid	-0.91±1.75	-0.5±1.23	1.37±1.28	0.024
P-Coumaric acid	0.82±1.04	1.16±1.32	2.05±1.29	0.038
Homovanillic acid	-81.83±116.86	-29.65±89.66	20.28±93.29	0.043
	Changes in Enterococcus tertiles			
	1 (n=6) (<1.36)	2 (n=6) (1.36-2.07)	3 (n=6) (>2.07)	
3,4-(dihydroxyphenyl)acetic acid	-0.22±12.06	-1.88±6.94	-13.09±9.04	0.039
	Changes in Eggerthella lenta tertiles			
	1 (n=6) (<1.88)	2 (n=6) (1.88-2.29)	3 (n=6) (>2.29)	
Caffeic acid	3.36±2.69	0.69±2.12	-1.76±3.42	0.018
Ferulic acid	11.3±12.41	-3.58±3.12	-2.95±3.6	0.009
Syringic acid	1.16±1.21	-0.6±2.16	-0.6±1.07	0.037
Feruloylglycine	14.67±18.45	4.73±7.56	-5.46±7.41	0.018

[a] $\log_{10}$ copies/g feces
[b] µmol/24 h.

Table 2 Stepwise linear regression model showing the best metabolite predictors of *Bifidobacterium* change.

	B(SD)	β	*P*
Bifidobacterium			
Intercept	2.75 (0.37)		<0.001
4-Hydroxybenzoic acid	3.60 (1.27)	0.513	0.013
Syringic acid	3.63(1.51)	0.402	0.042

Fig. 1 Tertiles of bacterial differences of two genera (*Bifidobacterium, Enterococcus*) and one species (*Eggerthella lenta*) between wine interventions and baseline (mean±SD).

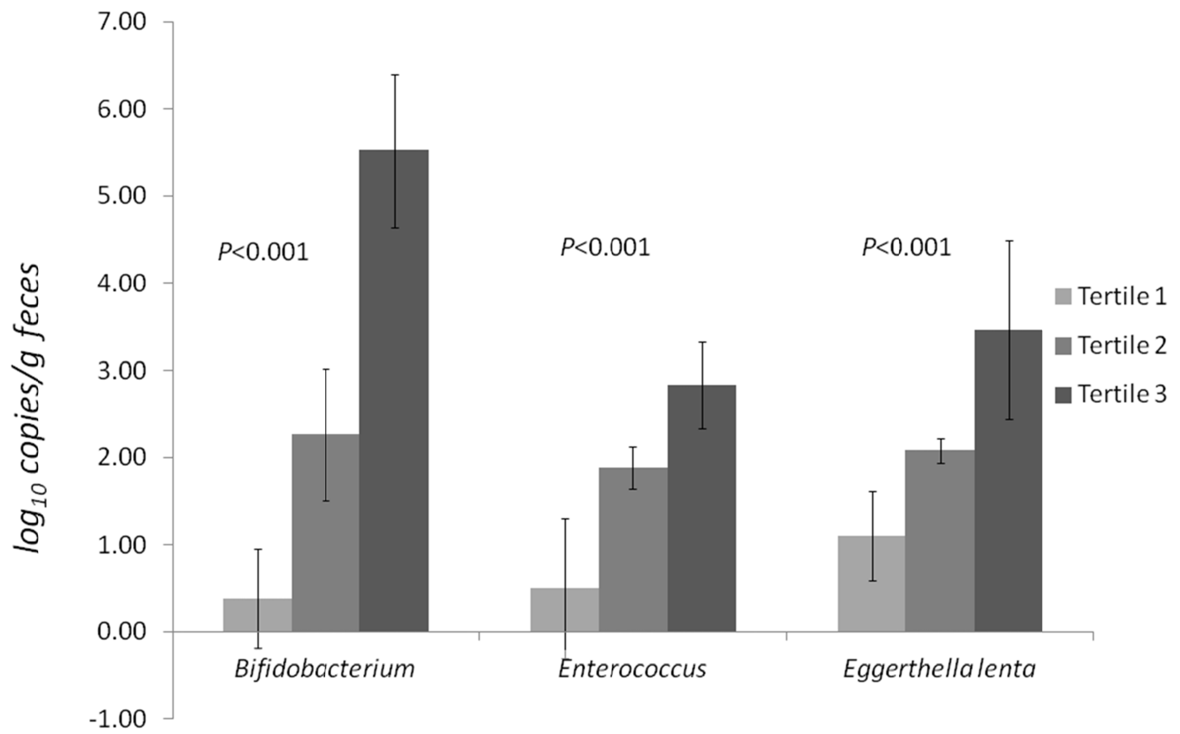
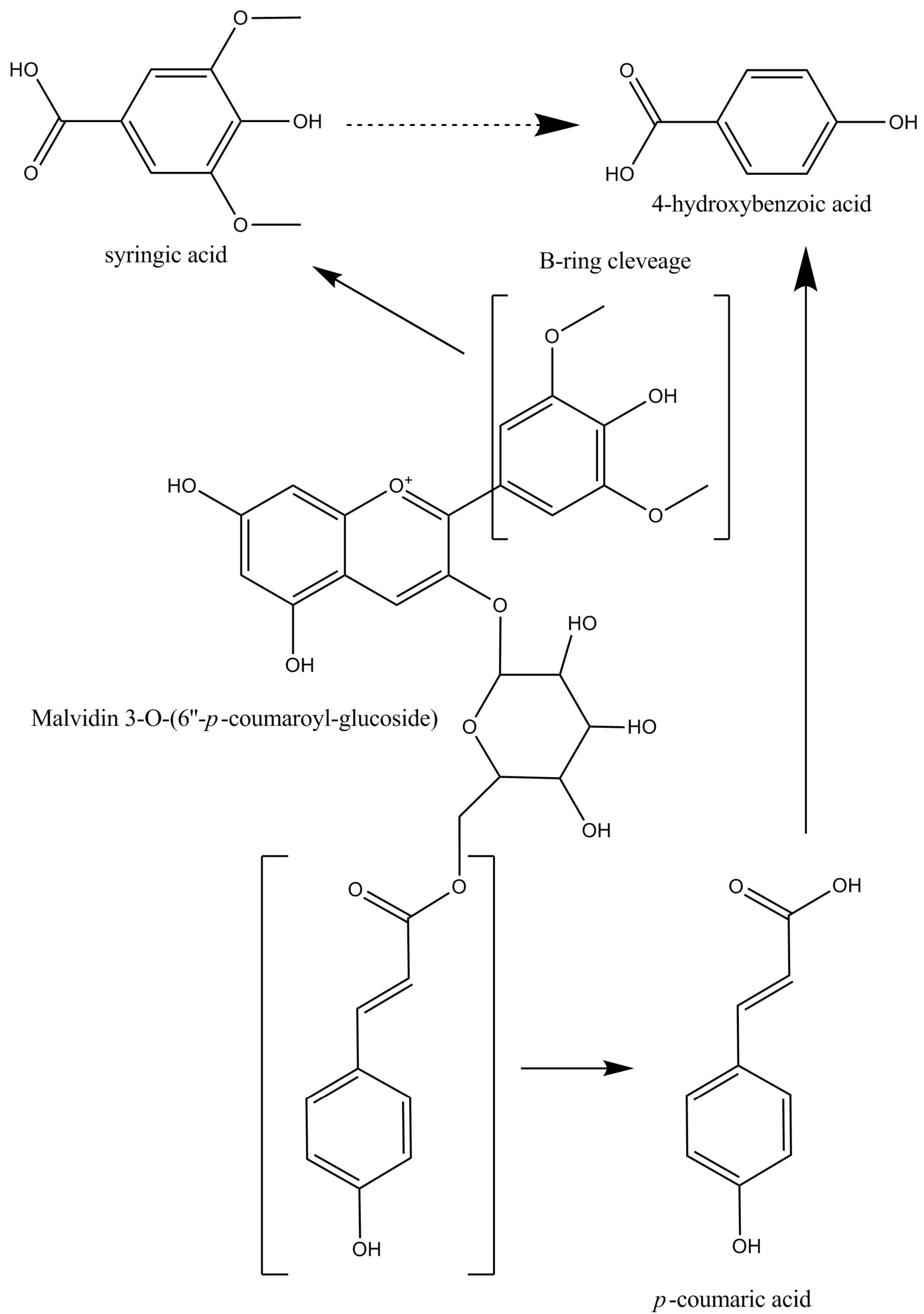


Fig. 2 Proposed metabolic route of anthocyanin degradation by *Bifidobacteria*.



Supplemental Data: Multiple Reaction Monitoring (MRM) Transitions, Declustering Potential (DP) and Collision Energy (CE) for each Microbial and Conjugate Metabolite Identified in this Study.

Analyte	MRM transitions	Identified by	DP	CE	Quantified as
Hydroxybenzoic acids					
2,4-dihydroxybenzoic acid	153/109	STD[a]	-50	-20	STD
2,6-dihydroxybenzoic acid	153/109	STD	-50	-20	STD
2,5-dihydroxybenzoic acid	153/109	STD	-50	-20	STD
3,5-dihydroxybenzoic acid	153/109	STD	-50	-20	STD
Protocatechuic acid	153/109	STD	-50	-20	STD
Vanillic acid	167/152	STD	-50	-20	STD
Syringic acid	197/121	STD	-50	-25	STD
4-hydroxybenzoic acid	137/93	STD	-50	-16	STD
3-hydroxybenzoic acid	137/93	STD	-50	-16	STD
4-hydroxyhippuric acid	194/100	STD	-50	-20	STD
3-hydroxyhippuric acid	194/150	PIS[b]	-50	-20	4-hydroxyhippuric acid
Gallic acid	169/125	STD	-40	-20	STD
4-O-methyl gallic acid	167/108	STD	-50	-26	STD
Methyl gallic acid	167/108	PIS	-50	-26	4-O-methyl gallic acid
Methyl gallic sulfate	263/183	PIS	-50	-25	Gallic acid
Ethylgallate	197/169	STD	-50	-25	Gallic acid
Ethylgallate sulfate	277/197	PIS	-50	-25	Gallic acid
Ethylgallate glucuronide 1,2	373/197	PIS	-50	-25	Epicatechin-5-*O*-glucuronide
Hydroxyphenylacetic acids					
Phenylacetic acid	135/91	STD	-30	-12	STD
3-hydroxyphenylacetic acid	151/107	STD	-50	-12	STD
2-hydroxyphenylacetic acid	151/107	STD	-50	-12	STD
3,4-dihydroxyphenylacetic acid	167/123	STD	-50	-12	STD
Homovanillic acid	181/137	STD	-40	-10	Vanillic acid
Hydroxycinnamic acids					
m-coumaric acid	163/119	STD	-50	-30	STD
o-coumaric acid	163/119	STD	-50	-30	STD
p-coumaric acid	163/119	STD	-50	-30	STD
Caffeic acid	179/135	STD	-50	-21	STD
Ferulic acid	193/134	STD	-50	-25	STD
Sinapic acid	223/164	STD	-50	-25	STD
Hydroxyphenylpropionic acids					
3-(4-hydroxyphenyl)propionic acid	165/121	STD	-30	-16	STD
3-(3-hydroxyphenyl)propionic acid	165/121	STD	-30	-16	STD
Dihydrocaffeic acid	181/137	STD	-40	-10	STD
Flavan-3-ols					
(Epi)catechin glucuronide 1,2,3,4	465/289	PIS	-50	-25	Epicatechin-5-*O*-glucuronide
(Epi)catechin sulfate 1,2,3	369/289	PIS	-50	-25	(Epi)Catechin

Methyl (epi)catechin glucuronide 1,2,3	479/303	PIS	-50	-30	Epicatechin-5-*O*-glucuronide
Methyl (epi)catechin sulfate 1,2,3	383/303	PIS	-50	-25	(Epi)Catechin
Glycinates					
Vanilloylglycine	224/180	PIS	-50	-25	4-hydroxyhippuric acid
Feruloylglycine	250/100	PIS	-50	-25	4-hydroxyhippuric acid
Hydroxyphenylvalerolactones					
DHPV 1	207/163	PIS	-50	-25	(Epi)Catechin
DHPV 2	207/163	PIS	-50	-25	(Epi)Catechin
DHPV glucuronide 1,2	383/207	PIS	-50	-25	Epicatechin-5-*O*-glucuronide
DHPV sulfate 1,2	287/207	PIS	-50	-25	(Epi)Catechin
MHPV 1	221/162	PIS	-50	-25	(Epi)Catechin
MHPV glucuronide 1	397/221	PIS	-50	-25	Epicatechin-5-*O*-glucuronide
MHPV sulfate 1,2	301/221	PIS	-50	-25	(Epi)Catechin
Other polyphenols					
Enterolactone	297/253	STD	-50	-25	STD
Pyrogallol	125/69	STD	-50	-25	STD

[a]STD, Standard available; [b]PIS, Product Ion Scan.

5.DISCUSIÓN GENERAL

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La relación entre dieta y salud está ampliamente estudiada, sin embargo, los mecanismos por lo que ello ocurre, están solo parcialmente elucidados. Para poder establecer una relación de causalidad entre la ingesta y los parámetros de salud, lo primero que se necesita conocer es la exposición exacta de los componentes de la dieta al individuo. En este punto juegan un papel clave los biomarcadores nutricionales. Así mismo, resultan también un factor limitante para el desarrollo de nuevos alimentos funcionales, cuyos estudios en humanos deben ir acompañados de marcadores válidos (van Loveren *et al.*, 2012).

La primera aproximación en la identificación de biomarcadores nutricionales requiere un conocimiento exhaustivo del compuesto a estudiar y su metabolismo, y así poder determinar la ingesta a través de los marcadores nutricionales y asociarlos con los parámetros de salud en estudios clínicos y epidemiológicos. Con ello, el desarrollo de bases de datos con información cualitativa y cuantitativa sobre metabolismo es esencial e imprescindible para facilitar el acceso rápido y efectivo al gran volumen de información repartido en la bibliografía (Scalbert *et al.*, 2011). Hasta este momento, las bases de datos con información sobre metabolismo y farmacocinética, se han limitado a aquellas procedentes del metabolismo de fármacos (Reimschuessel *et al.*, 2005; Knox *et al.*, 2011), sin embargo, en el campo de la alimentación, y más concretamente en el de los polifenoles, el acceso al conocimiento del metabolismo fenólico era a través de búsqueda bibliográfica manual. A esto hay que añadir también el hecho de que en el campo de los polifenoles la variabilidad entre los estudios de metabolismo es muy amplia, y pocos son los metabolitos con una composición definida por falta de estándares comerciales o por la utilización de métodos con hidrólisis enzimática de la muestra biológica. Otros factores como la dosis, la frecuencia de la intervención (puntual o repetida), el tipo de tratamiento (alimento o compuesto puro), el método analítico (LC-MS o GC-MS entre otros), la especie (humanos, animales) y la muestra biológica (orina, plasma, tejido), hace que su comparabilidad sea muy complicada. Por esta razón se planteó el desarrollo del módulo de metabolismo de Phenol-Explorer (módulo 2.0) (resultado 1) gracias a una colaboración del grupo de investigación con los Drs Augustin Scalbert y Claudine Manach de la Unidad de Nutrición Humana del

INRA (Instituto Nacional de Investigaciones Agronómicas, Clermont-Ferrand, Francia) y la Universidad de Alberta (Canadá). En este módulo se presenta lo que es hasta el momento la mayor base de datos en el estudio del metabolismo de los polifenoles (Rothwell *et al.*, 2012), combinándolo con la ya existente en composición fenólica de alimentos (Neveu *et al.*, 2010).

El módulo de metabolismo de Phenol-Explorer permite la visualización de todos los metabolitos identificados en el plasma y en la orina tras el consumo de polifenoles o alimentos ricos en polifenoles y que, junto con sus concentraciones urinarias y sus parámetros farmacocinéticos, permite comparar un mismo metabolito en diferentes estudios o diferentes metabolitos de un mismo estudio. Del mismo modo, los investigadores interesados en metabolitos de polifenoles con una cierta actividad biológica pueden conocer a través de Phenol-Explorer de qué alimentos procede este determinado metabolito. Este avance supone un adelanto para la comunidad científica tanto a la hora de planear una investigación como para valorar los resultados de los estudios en este campo.

La utilidad de este trabajo queda reflejada en su aplicación para el estudio del metabolismo del vino, que se presenta en la presente Tesis Doctoral como el resultado 2 (Boto-Ordonez *et al.*, 2013b). En este trabajo la unión del conocimiento de los metabolitos derivados del consumo de vino y productos de la uva, con el metabolismo de aquellos polifenoles presentes en el vino y que se han consumido de manera aislada, ha permitido ampliar el rango de posibles metabolitos presentes *in vivo* (n = 97). Sólo Forester y Waterhouse (Forester y Waterhouse, 2009) habían revisado los metabolitos obtenidos tras el consumo de vino por grupos fenólicos, recogiendo también aquellos metabolitos formados *in vitro* y con una posición de conjugación indefinida (ej. Resveratrol glucurónido). Aquellos metabolitos que cumplían con las condiciones de inclusión del Phenol-Explorer 2.0 coincidieron con los descritos en esta revisión, demostrando que a través de la base de datos se puede reproducir de manera eficiente los resultados de las búsquedas bibliográficas exhaustivas, pero con un menor número final. Esto es debido a la capacidad de Phenol-Explorer para combinar alimentos y compuestos puros. Los compuestos puros se administran a dosis más altas que las presentes en los alimentos y por consiguiente, las concentraciones

en fluidos también son mayores. Este hecho facilita su detección y ha permitido valorar otros polifenoles del vino en la huella metabólica derivada de su consumo diferentes a las (epi)catequinas (Donovan *et al.*, 1999; Bell *et al.*, 2000) o resveratrol (Urpi-Sarda *et al.*, 2005; Vitaglione *et al.*, 2005).

La complejidad de su composición y metabolismo queda reflejada en la ruta metabólica presentada en el resultado 2. Hasta ahora, no existen rutas metabólicas sobre el metabolismo de polifenoles después del consumo de alimentos en bases de datos disponibles, y este es el primer intento de desarrollarlas. En ella se recogen metabolitos generados por fenómenos de metilación, glucuronidación, sulfatación, oxidación, etc. (Manach *et al.*, 2004), y abre la posibilidad de aplicarlo en el estudio de otros alimentos. En dicha ruta metabólica, basada en el conocimiento previo del metabolismo de los polifenoles, los distintos grupos de polifenoles presentes en el vino convergen en metabolitos comunes, principalmente ácidos fenólicos. Algunos de los metabolitos descritos aunque están presentes en la composición original del vino, pueden ser el resultado del metabolismo de la microbiota (Boto-Ordonez *et al.*, 2013b), sugiriendo así que los ácidos fenólicos son metabolitos comunes de los principales sustratos (Williamson y Clifford, 2010). La importancia del estudio de dichos metabolitos se debe a sus posibles actividades biológicas, incluyendo su efecto sobre la flora intestinal, la modulación de la biodisponibilidad y por tanto sus posibles efectos en el organismo, y finalmente su papel como biomarcadores de ingesta.

A la hora de buscar biomarcadores nutricionales, se requieren estudios de intervención para la validación analítica de los compuestos propuestos. Con este fin, el conocimiento generado en el resultado 2 fue aplicado a un estudio clínico de intervención con vino tinto desalcoholizado (Boto-Ordonez *et al.*, 2013c) (resultado 3), enfatizando aquellos metabolitos provenientes del metabolismo de la microbiota. El resultado fue el perfil metabólico más extenso después del consumo de vino (n=61), identificando nuevos metabolitos, y valorando y proponiendo las rutas metabólicas de grupos de polifenoles del vino hasta ahora inexplorados para este alimento *in vivo*. Los 49 metabolitos aumentados tras el consumo de vino desalcoholizado representan la proporción de los compuestos fenólicos ingerida a través de este alimento, ya que la ingesta dietética fue mantenida durante el estudio, y la transformación microbiana de

diferentes clases de polifenoles del vino, especialmente antocianos y flavanoles. Dichos metabolitos, tales como ácidos hidroxifenilpropiónicos, hidroxibenzoicos o catequinas, han demostrado tener la capacidad *in vitro* de inhibir el crecimiento de bacterias patógenas y no patógenas (Puupponen-Pimia *et al.*, 2001; Alakomi *et al.*, 2007) proponiendo estos cambios como una parte responsable de los beneficios para la salud de los compuestos fenólicos en el organismo.

El efecto prebiótico de los componentes y metabolitos del vino se observó en el resultado 5 (Queipo-Ortuno *et al.*, 2012), donde en el estudio clínico de intervención con similares características al anterior, se tomaron muestras de heces para valorar los cambios microbianos tras el consumo de vino tinto, vino tinto desalcoholizado y ginebra y su posible relación con las mejoras bioquímicas en parámetros de salud. En este estudio, 4 filos y 9 géneros y especies sufrieron modificaciones tras las intervenciones. No es la primera vez que se indica que la ingesta de polifenoles puede generar un importante cambio en la microbiota intestinal. Yamakoshi *et al*. demostraron que la ingesta repetida de extracto de procianidinas tenía la capacidad para aumentar significativamente las *Bifidobacterias* y disminuir las *Enterobacterias* (Yamakoshi *et al.*, 2011) así como, la ingesta de cacao durante 4 semanas podía aumentar las *Bifidobacterias* y disminuir el *Clostridium hystolyticum group* en un estudio en humanos (Tzounis *et al.*, 2011). En el presente estudio las *Bifidobacterias* aumentaron, como se había visto previamente al administrar una bebida de arándanos (Vendrame *et al.*, 2011), conjuntamente con la *Eggerthella lenta* y *Bacteroides uniformis*, bacterias capaces de metabolizar resveratrol *in vitro* (Jung *et al.*, 2009). En sentido contrario, los clostridiales, asociados a enfermedades intestinales (Kelly y LaMont, 2008), aumentaron tras el consumo de ginebra, pero no después de los dos vinos, que se mantuvieron estables.

De manera adicional a las modificaciones bacterianas, se observó que el consumo regular de polifenoles del vino tinto también afectó a los parámetros bioquímicos, con una disminución significativa de la presión arterial, los niveles plasmáticos de triglicéridos, la proteína-C-reactiva, el colesterol total y el colesterol HDL. Estos beneficios del consumo de vino sobre estos parámetros ya se conocían (Chiva-Blanch *et al.*, 2012a; Chiva-Blanch *et al.*, 2013), pero no ha sido hasta este momento cuando

se han podido relacionar con la composición bacteriana intestinal. Las reducciones significativas de la presión arterial, de los triglicéridos y del colesterol-HDL pueden ser debidos en parte al incremento de *Bacteroides* tras el consumo de polifenoles, cuya correlación resultó ser significativa. Así mismo el aumento de Bifidobacterias correlacionó con la disminución del colesterol total y la PCR, todos ellos parámetros vinculados a una mejora en el estado del individuo (Hage y Szalai, 2007).

Los cambios en el número de bacterias tras la intervención con vino, independientemente de su contenido alcohólico, se pudieron asociar con cambios en los metabolitos urinarios (resultado 6)(Boto-Ordonez *et al.*, 2013d). Uno de los géneros predominantes de la microbiota intestinal, la *Bifidobacteria* (Arumugam *et al.*, 2011), fue asociada a los cambios de metabolitos de antocianos como el homovanílico, el *p*-cumárico, el 4-hidroxibenzoico y el siríngico, pudiendo la combinación de estos dos últimos llegar a predecir el 68.5% de los cambios en el número de la misma. El poder catabólico de la bifidobacterias sobre los antocianos ya se había demostrado en estudios *in vitro* (Ávila *et al.*, 2009; Hidalgo *et al.*, 2012), así como las actividades biológicas de dichos metabolitos sobre parámetros de salud como la presión arterial o el metabolismo lipídico, cambios observados tras el consumo de vino tinto (Queipo-Ortuno *et al.*, 2012; Clemente-Postigo *et al.*, 2013). El género *Enterococcus* y la bacteria *Eggerthella lenta* tuvieron una asociación inversa con la excreción de metabolitos fenólicos, probablemente por la inhibición de las mismos con compuestos presentes en el vino (Özkan *et al.*, 2004; Lee *et al.*, 2006b; Jung *et al.*, 2009).

Como se ha demostrado, la microbiota juega también un papel muy importante en la generación de metabolitos, y por tanto el número de posibles biomarcadores de ingesta con una mayor vida media en matrices orgánicas. Este aumento en el número de metabolitos requiere enfoques metabólicos globales con el fin de evaluar el papel de metabolitos individuales o grupos de metabolitos, en la discriminación de consumo de un alimento seleccionado, y es aquí donde nuevas herramienta estadísticas son necesarias (Scalbert *et al.*, 2013). Con este objetivo, en el resultado 4, se estableció una estrategia de búsqueda de biomarcadores mediante una regresión logística binaria en pasos para calcular una ecuación que valorase la importancia de cada metabolito como biomarcador, y su posible transposición entre orina de 24 horas y plasma en

ayunas en el ensayo clínico de intervención con vino tinto, vino desalcoholizado y ginebra (Boto-Ordonez *et al.*, 2013a). Los resultados del modelo se evaluaron y compararon con los grupos de metabolitos que provenían de una misma clase de polifenol del vino y con los metabolitos individuales a través de curvas COR dando sus valores de sensibilidad, selectividad y AUC.

Este tipo de estudios tradicionalmente aplicados en la determinación temprana de enfermedades y en el campo de la epidemiología (Moons *et al.*, 2012), se aplica por primera vez en la identificación de biomarcadores de alimentos. El análisis de regresión logística por pasos propuso un modelo donde entraron metabolitos derivados principalmente del ácido gálico y etilgalato, todos ellos metabolitos descritos después del consumos de alimentos ricos en polifenoles como el té, cacao o las bayas (Zhang y Zuo, 2004; Neveu *et al.*, 2010). Tras evaluar y comparar los resultados del modelo con los grupos de metabolitos de un polifenoles o con metabolitos individuales, el modelo resultó ser tan buen discriminante del consumo de vino como el biomarcador del resveratrol y sus metabolitos microbianos en orina de 24 horas (AUC >96%), mejorando también los resultados en plasma en ayunas (AUC >80%).

El tipo de muestra es un factor determinante en la evaluación de los biomarcadores. La orina de 24 horas se ha descrito como la mejor muestra para determinar biomarcadores derivados de los polifenoles ya que proporciona una mejor medida del total y mejor índice de consumo (Spencer *et al.*, 2008). Sin embargo, por razones prácticas, la orina de 24 horas no es una muestra fácil de obtener en los estudios epidemiológicos a gran escala. En consecuencia, se evaluaron las correlaciones que existen entre esta muestra y el plasma en ayunas para poder ser considerado en la determinación de biomarcadores. La alta correlación entre los modelos de predicción entre plasma y orina, nos indicó que aquellos voluntarios que fueron mejor clasificados como los consumidores de vino en orina, lo eran también en el plasma en muestras con hidrólisis y sin hidrólisis (r=0.565 y r=0.599, respectivamente, $P<0.001$). Además, 18 metabolitos individuales y grupos de metabolitos como el ácido gálico, valerolactonas, flavan-3-oles) mostraron correlaciones positivas (r=0.3-0.6). Estas correlaciones aunque significativas, se consideraron clínicamente moderadas al tener

valores de correlación inferiores a 0.75 (Aubertin-Leheudre *et al.*, 2008), pero similares a los estudios previos con alquilresorcinol en plasma y orina (Aubertin-Leheudre *et al.*, 2008). Los presentes resultados podrían abrir la posibilidad de encontrar los metabolitos en el plasma y establecerlos como biomarcadores de consumo y efecto, pero se necesitan estudios más amplios en población sin intervención para confirmar y generalizar esta afirmación.

6. CONCLUSIONES

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Los resultados obtenidos en los diferentes trabajos que constituyen la presente Tesis Doctoral derivan en las siguientes conclusiones:

1. Se ha desarrollado el módulo de metabolismo de la base de datos Phenol-Explorer. Con esta nueva plataforma, Phenol-Explorer pasa a ser la mayor base de datos de metabolismo de los polifenoles, que en conjunto, con el conocimiento de la composición de polifenoles de los alimentos y sus modificaciones durante el procesado, facilitará el diseño e interpretación de estudios relacionados con estos compuestos y su papel sobre la salud humana.

2. Se ha diseñado la ruta metabólica de polifenoles más completa tras el consumo de vino, productos derivados y polifenoles puros presentes en su composición original, a través del uso de Phenol-Explorer con 97 metabolitos descritos en plasma y/o orina.

3. Se ha descrito el más amplio perfil metabólico de metabolitos fenólicos, mayoritarimente aquellos originados por la microbiota intestinal (n=61), mediante UPLC-MS/MS en orina después del consumo de vino tinto desalcoholizado en un estudio clínico de intervención. Esta descripción permite destacar el importante papel de las bacterias intestinales en la degradación de los compuestos fenólicos de los alimentos, modulando su biodisponibilidad y los posibles efectos asociados en el organismo.

4. Tras el consumo de vino desalcoholizado, 49 biomarcadores cuantificados en orina derivados de los polifenoles del vino han aumentado significativamente comparado con el momento inicial, siendo los de mayor relevancia e incremento aquellos derivados de los antocianos seguidos por los metabolitos de los flavan-3-oles.

5. La combinación de diversos biomarcadores en orina de 24 horas, procedentes del consumo de vino mejora su capacidad discriminatoria y predictiva respecto a la consideración de biomarcadores individuales. Por ejemplo, tras un análisis de regresión logística observamos que la combinación de aquellos metabolitos

derivados del ácido gálico y etilgalato, o los metabolitos microbianos del resveratrol, presentan una capacidad discriminatoria y predictiva comparable e incluso superior, a los metabolitos clásicos de resveratrol (glucurónidos y sulfatos).

6. Se ha establecido la correlación existente entre el perfil metabólico en plasma/ayunas y orina de 24 horas en estudios de intervención. Se ha observado que la muestra de orina de 24 horas proporciona un mayor poder discriminatorio entre consumidores o no consumidores de vino respecto a la de plasma. Este nuevo enfoque es una herramienta prometedora que tiene un gran potencial para la identificación de posibles marcadores biológicos para evaluar el cumplimiento en los estudios clínicos, la identificación de los patrones de alimentación e incluso establecer asociaciones entre el consumo de polifenoles y sus potenciales beneficios para la salud.

7. Se ha establecido el efecto prebiótico de los polifenoles del vino sobre la microbiota intestinal y se ha observado un aumento significativo de *Enterococcus*, *Bifidobacterium* y *Eggerthella lenta*, tras la ingesta de los polifenoles independientemente de la presencia conjunta de alcohol. Sin embargo se observa un aumento de *Bacteroides uniformis, Prevotella, Bacteroides, Blautia coccoides-Eubacterium rectale* y los filos *Bacteroidetes*, *Fusobacterias, Firmicutes* y *Proteobacterias* sólo tras el consumo de los polifenoles del vino en presencia de alcohol. Estas modificaciones se encuentran asociadas a la mejora de parámetros bioquímicos como el colesterol o la proteína-C reactiva.

8. Los aumentos de las bacterias debidos a los polifenoles del vino, se han asociado con aumentos de la excreción de metabolitos fenólicos, y más concretamente los aumentos de las bifidobacterias se correlacionaban con aumentos de metabolitos microbianos derivados de los antocianos del vino.

7.BIBLIOGRAFÍA

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8.ANEXO

8. ANEXOS

8.1 OTRAS PUBLICACIONES EN REVISTAS CIENTÍFICAS

ESTUDIO DE LA ENDOTOXEMIA POSTPRANDIAL

Publicación I: Clemente-Postigo M, Queipo-Ortuño MI, Boto-Ordóñez M, Coin-Aragüez L, Roca-Rodriguez MM, Delgado-Lista J, Cardona F, Andres-Lacueva C, Tinahones FJ. Effect of acute and chronic red wine consumption on lipopolysaccharide concentrations. *Am J Clin Nutr*, 2013; 97: 1053-61 y erratum *Am J Clin Nutr,* 2013; 98: 512.

Se analizó el efecto sobre las concentraciones de LPS de consumo vino tinto tras una ingesta crónica y aguda en relación con el alto consumo de grasa. Este trabajo pertenece a la estancia realizada en el grupo de investigación del Doctor Francisco J. Tinahones (Hospital Universitario Virgen de la Victoria, Málaga), dentro del miniproyecto CONSOLIDER "*Microbiota humana y polifenoles del vino*", donde se colaboró en el estudio de intervención y análisis de la LPS en el estudio agudo.

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Effect of acute and chronic red wine consumption on lipopolysaccharide concentrations[1–3]

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ABSTRACT

Background: Chronic red wine (RW) consumption has been associated with decreased cardiovascular disease risk, mainly attributed to an improvement in lipid profile. RW intake is also able to change the composition of gut microbiota. High fat intake has recently been reported to increase metabolic endotoxemia. The gut microbiota has been proposed as the main resource of plasma lipopolysaccharides (LPSs) in metabolic endotoxemia.

Objective: We analyzed the effect on LPS concentrations of chronic RW consumption and acute RW intake in relation to high fat intake in middle-aged men.

Design: For the chronic study, 10 middle-aged male volunteers were randomly assigned in a crossover trial, and after a washout period, all subjects received RW, dealcoholized red wine (DRW), or gin for 20 d. Serum endotoxin and LPS-binding protein (LBP) concentrations were determined after the washout period and after each of the treatments, and changes in fecal microbiota were quantified. For the acute study, 5 adult men underwent a fat overload or a fat overload together with the consumption of RW, DRW, or gin. Baseline and postprandial serum LPS and LBP concentrations and postprandial chylomicron LPS concentrations were measured.

Results: There were no significant differences in the change in LPS or LBP concentrations between chronic RW, DRW, and gin consumption. *Bifidobacterium* and *Prevotella* amounts were significantly increased by RW and correlated negatively with LPS concentrations. There were no differences in postprandial serum LPS, LBP, or chylomicron LPS concentrations between acute RW, DRW, or gin intake together with a fatty meal.

Conclusion: Chronic RW consumption increases *Bifidobacterium* and *Prevotella* amounts, which may have beneficial effects by leading to lower LPS concentrations. This trial was registered at controlled-trials.com as ISRCTN88720134. *Am J Clin Nutr* 2013;97:1053–61.

INTRODUCTION

Epidemiologic studies have reported that moderate red wine (RW)[4] consumption is associated with decreased risk of cardiovascular disease (CVD) (1–3). This reduction in CVD risk has been associated with an improvement in the lipid profile (ie, an increase in HDL cholesterol and decrease in LDL cholesterol as well as a reduction in lipid oxidation) (4–6). These beneficial effects have been attributed mainly to antiinflammatory and antioxidant properties exerted by polyphenols of RW (eg, resveratrol, flavonoids, catechin, epicatechin, and phenolic acids) (7). However, the exact mechanisms whereby phenolics exert their beneficial effects are not clear.

Although chronic RW consumption seems to improve the lipid profile, the acute consumption of RW with a high-fat or mixed meal appears to increase postprandial triglycerides and delay the apolipoprotein B-48 response to a greater extent than does water or nonalcohol consumption (8–10), possibly because of an impaired

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[4] Abbreviations used: ATCC, American Type Culture Collection; CECT, Spanish Type Culture Collection; CRP, C-reactive protein; CVD, cardiovascular disease; DRW, dealcoholized red wine; DRW+FO, dealcoholized red wine together with the fat overload; EU, endotoxin units; FO, fat overload; Gin+FO, gin together with the fat overload; LAL, limulus amebocyte lysate; LBP, LPS-binding protein; PCR, polymerase chain reaction; RW, red wine; RW+FO, red wine together with the fat overload.

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hydrolysis and clearance of triglyceride-rich lipoproteins (ie, chylomicrons and VLDL) (11); these increases seem to be common to other alcoholic beverages (12). However, others authors have found no differences in the postprandial lipid response between consuming and not consuming RW (13). Despite an enhanced postprandial lipid response to a meal induced by RW, a moderate dose of RW prevents the increased nuclear factor κB activation induced by a high-fat meal (9).

Recently, RW has been shown to be able to modify the gut-microbiota composition. In addition, these changes in gut microbiota, together with RW consumption, have been negatively correlated with plasma C-reactive protein (CRP), triglyceride, total cholesterol, and HDL-cholesterol concentrations. These findings suggest that, at least in part, beneficial effects of RW can be mediated by its prebiotic effects in the gut microbiota (14). In fact, *ob/ob* mice treated with prebiotics, which specifically modify gut microbiota, displayed lower LPS concentrations induced by a high-fat diet than did their untreated littermates (15). Human studies have also shown that LPS concentrations are higher in subjects who consume a high-fat diet (16), and after a high-fat meal, postprandial LPS concentrations rise significantly (17, 18), with an association between metabolic endotoxemia and energy intake (16).

Thus, in view of the previous results of our group that showed that the chronic consumption of RW was able to change the gut microbiota, we investigated whether these prebiotic effects also imply a change in LPS concentrations and which bacterium groups are related to endotoxemia. In addition, we also studied whether the acute intake of RW changes the postprandial LPS increase induced by a fatty meal.

SUBJECTS AND METHODS

Study subjects and design

Study and population characteristics for the chronic study have been previously published (14). Briefly, the chronic study was a randomized, crossover, controlled intervention study. Ten adult men aged 48 ± 2 y (age range: 45–50 y) were recruited in the outpatient clinic of the Endocrinology Department of our institution from June 2010 to December 2010. Data were determined for all subjects for BMI (in kg/m^2) (27.6 ± 3.2), waist (106.7 ± 14.3 cm) and hip (111.0 ± 10.4 cm) circumferences, and the HOMA-IR (3.66 ± 2.29) calculated as described in Laboratory measurements. Concentrations of glucose (111.3 ± 23.1 mg/dL), triglycerides (245.4 ± 231.7 mg/dL), cholesterol (257.5 ± 88.6 mg/dL), HDL cholesterol (58.5 ± 16.7 mg/dL), and LDL cholesterol (129.6 ± 41.9 mg/dL) were also measured. Transaminase concentrations of participants were normal. More detailed information about the characteristics of the chronic study population are shown elsewhere (14). The study was divided into 4 consecutive periods as follows: an initial washout period of 15 d (baseline) during which participants did not consume any alcohol or RW, followed by 3 consecutive periods of 20 d each during which participants drank only RW (272 mL/d), dealcoholized red wine (DRW) (272 mL/d), or gin (100 mL/d), respectively. After the washout period, participants were individually randomly assigned by the dietitian in a crossover design to 3 treatment sequences in which test beverages were provided. Random assignment was based on a computer-generated random-number table, which resulted in 6 possible diet sequences. Participants were not blinded to the type of drink they ingested. Each participant provided 4 different fecal samples as follows: a first baseline sample after the washout period and a sample at the end of each 20-d period. Fasting blood samples and 24-h urine were also collected at baseline and after each period. At baseline and after each intervention period, a medical examination was given and structured nutrient intake and physical activity questionnaires were completed (14).

For the acute study, a total of 5 adult men aged 41.8 ± 15.01 y (range: 30–54 y) were recruited. BMI (28.37 ± 6.09) and waist (107.80 ± 16.80 cm) and hip (111.40 ± 8.44 cm) circumferences were determined. Biochemical variables of participants are described in **Table 1**. The study, which had a 4-way crossover

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TABLE 1
Biochemical variables of participants in the acute study[1]

	FO	RW+FO	DRW+FO	Gin+FO	*P*
Glucose (mg/dL)	97.80 ± 14.53	96.40 ± 9.24	98.20 ± 12.36	102.00 ± 8.86	NS
HOMA-IR	3.46 ± 1.68	4.59 ± 3.89	4.22 ± 1.95	5.39 ± 5.19	NS
TG at 0 h (mg/dL)	174.60 ± 42.22*	179.00 ± 92.72*	238.60 ± 132.94	187.40 ± 74.35	NS
TG at 3 h (mg/dL)	292.40 ± 50.91	285.40 ± 65.62	318.00 ± 124.79	280.75 ± 79.40	NS
ΔTG (mg/dL)[2]	117.80 ± 48.09	106.40 ± 78.40	79.40 ± 117.18	75.00 ± 57.11	NS
Cholesterol (mg/dL)	210.20 ± 23.78	205.00 ± 44.96	212.00 ± 69.73	202.60 ± 53.20	NS
HDL cholesterol (mg/dL)	54.00 ± 17.39	50.00 ± 15.13	53.20 ± 21.90	52.20 ± 16.30	NS
LDL cholesterol (mg/dL)	126.60 ± 41.48	127.40 ± 48.05	121.80 ± 53.18	116.80 ± 49.99	NS
Apo A1 (mg/dL)	160.80 ± 48.63	158.25 ± 33.77	164.00 ± 46.09	161.40 ± 46.32	NS
Apo B (mg/dL)	100.04 ± 28.12	95.12 ± 42.77	98.35 ± 45.09	97.28 ± 42.03	NS
GPT (units/L)	53.40 ± 15.73	52.60 ± 16.30	56.60 ± 16.20	57.60 ± 14.22	NS
GGT (units/L)	63.60 ± 53.39	61.20 ± 53.80	64.80 ± 52.06	63.00 ± 52.88	NS
CRP (mg/dL)	6.46 ± 5.43	6.27 ± 5.17	10.37 ± 13.24	4.36 ± 1.40	NS

[1] All values are means ± SDs. $n = 5$. *P* values were determined by using Friedman's test. *$P < 0.05$ between 0 and 3 h according to Wilcoxon's signed-rank test. Apo A1, apolipoprotein 1; Apo B, apolipoprotein B; CRP, C-reactive protein; DRW+FO, dealcoholized red wine together with the fat overload; FO, fat overload; GGT, γ-glutamyltransferase; Gin+FO, gin together with the fat overload; GPT, glutamic pyruvate transaminase; RW+FO, red wine together with the fat overload; TG, triglycerides; ΔTG, change in triglyceride concentrations.

[2] Calculated as the difference between postprandial and baseline values.

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design, included 4 separate intervention days, each of which was preceded by a 2-d washout period. Participants were randomly assigned to the 4 different treatments as follows: a 50-g fat overload (FO) that consisted of a preparation (patent P201030776), 272 mL red wine together with the fat overload (RW+FO), 272 mL dealcoholized red wine together with the fat overload (DRW+FO), and 100 mL gin together with the fat overload (Gin +FO). At baseline and 3 h after intake, blood samples were obtained. Only water was permitted during the process, and no physical exercise was undertaken. The high-fat preparation of 100 mL contained 50 g fat, of which 10 g were saturated, 29.46 g were monounsaturated, and 10.625 g were polyunsaturated. Each 100 mL contained <1 g lauric acid, <1 g myristic acid, 4.8 g palmitic acid, 1.4 g stearic acid, 27.7 g oleic acid, 9.6 g linoleic acid, 1.4 g behenic acid, and 0.5 g lignoceric acid. The fat was supplied as emulsified triglycerides, and it was an exclusively fat preparation without carbohydrates or proteins. This test has been previously validated in another study by our group (18). All participants consumed the same diet on the day before the FO.

Participants in both the chronic and the acute studies had no history of diabetes, hypertension, or dyslipidemia; they had no acute or chronic inflammatory diseases, infectious diseases, viral infections, cancer, or a previous cardiovascular event; nor were they receiving treatment at study entry. Participants had not received any antibiotic therapy, prebiotics, probiotics, synbiotics, vitamin supplements, or any other medical treatment that influenced intestinal microbiota during the 3 mo before the start of the study, nor did they receive any of these during the study (including the initial washout period). Participants did not smoke and were moderate alcohol consumers (≤25 g alcohol/d). Participants were asked not to change their dietary patterns and lifestyle habits during the study, except that they were asked to avoid alcoholic beverages during the study. The Ethics Committee of the Virgen de la Victoria Hospital approved the clinical protocol. All participants gave written informed consent.

Laboratory measurements

Blood samples were obtained from the antecubital vein and placed in evacuated tubes (BD Vacutainer). The serum was separated by centrifugation for 10 min at 4000 rpm and frozen at −80°C until analysis. Serum glucose, uric acid, cholesterol, triglycerides, HDL cholesterol, CRP, and transaminases were measured in a Dimension autoanalyzer (Dade Behring Inc) by using enzymatic methods (Randox Laboratories Ltd and Wako Bioproducts). LDL cholesterol was calculated by using Friedewald's formula. Serum LPS-binding protein (LBP) was measured by using an immunoassay kit (HyCult Biotechnology) according to the manufacturer's protocol. Insulin concentrations were quantified by radioimmunoassay (BioSource SA). The insulin resistance was calculated from the HOMA-IR with the following formula (19):

$$\text{Insulin resistance} = [\text{fasting serum insulin } (\mu\text{U/mL}) \times \text{fasting blood glucose (mmol/L)}] \div 22.5 \qquad (1)$$

Chylomicrons were separated from serum by ultracentrifugation at 30,000 rpm for 30 min at room temperature (Beckman Coulter, TLA 100.3). The top layer was carefully isolated and resuspended in endotoxin-free saline solution to the initial volume. In the 24-h urine samples, resveratrol metabolites were measured as a biomarker of consumption of DRW- and RW-intervention compliance, jointly with dihydroresveratrol metabolites as previously described (14).

Limulus amebocyte lysate assays

Baseline and postprandial serum concentrations of LPS as well as chylomicron concentrations of LPS at 3 h were measured by endotoxin assay, based on a Limulus amebocyte extract with a chromogenic limulus amebocyte lysate (LAL) assay (QCL-1000; Lonza Group Ltd). Samples were diluted in pyrogen-free water and heated to 70°C for 10 min to inactivate endotoxin-neutralizing agents that inhibit the activity of endotoxin in the LAL assay. Pyrosperse reagent (Lonza Group Ltd), which is a metallo-modified polyanionic dispersant, was added to test samples at a ratio of 1:200 (vol:vol) before LAL testing to minimize interference in the reaction. All samples were tested in duplicate, and results were accepted when the intraassay CV was <10%. The endotoxin content was expressed as endotoxin units (EU) per milliliter. Exhaustive care was taken to avoid environmental endotoxin contamination, and all material used for both sample preparation and the test was pyrogen-free.

DNA extraction from fecal samples

Fecal samples were collected and immediately stored at −80°C until analysis. DNA extraction from 200 mg of stools was done by using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. The DNA concentration was determined by absorbance at 260 nm, and the purity was estimated by determining the A260:A280 ratio with a Nanodrop spectrophotometer (Nanodrop Technologies).

Microbial quantification by quantitative real-time polymerase chain reaction

Specific primers that targeted different bacterial phyla (*Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria*) and genera (*Bacteroides*, *Prevotella*, *Lactobacillus*, *Clostridium* cluster IV, *Clostridium histolyticum*, *Blautia coccoides–Eubacterium rectale* group, *Enterococcus*, and *Bifidobacteria*) described by Queipo-Ortuño et al (14) were used to characterize the fecal microbiota by quantitative real-time polymerase chain reaction (PCR). In addition, specific primers for *Parabacteroides distasonis* (20) and the PrimerDesign genesig Kit (all strains) (e.coli-sp) genomes for *Escherichia coli* (PrimerDesign Ltd) were also used. Briefly, quantitative PCR experiments were performed with a LightCycler 2.0 PCR sequence-detection system (Roche Applied Science) by using the FastStart DNA Master SYBR Green kit (Roche Diagnostics). All PCR tests were carried out in duplicate with a final volume of 20 μL that contained 100 ng of each fecal DNA preparation and 200 nmol/L of each primer. Thermal cycling conditions used were as follows: an initial DNA denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, primer annealing at the optimal temperature for each primer for 20 s (14), and extension at 72°C for 15 s. Finally, a melt-curve analysis was performed by slowly cooling the PCRs from 95°C to 60°C (0.05°C per cycle) with simultaneous measurement of the SYBR Green I

signal intensity. Melting-point–determination analysis allowed the confirmation of the specificity of amplification products.

The bacterial concentration from each sample was calculated by comparing the threshold cycle (Ct) values obtained from standard curves with LightCycler 4.0 software (Roche Applied Science). Standard curves were created by using serial 10-fold dilution of DNA from pure cultures corresponding to 10^1–10^{10} copies/g feces. The different strains used were obtained from the Spanish Collection of Type Cultures (CECT) (*Enterococcus faecalis* CECT 184, *Enterobacter cloacae* CECT 194, *Clostridium perfringens* CECT 376), the National Collection of Type Cultures (NCTC) (*Bacteroides vulgatus* NCTC 11154) and the American Type Culture Collection (ATCC) [*Bifidobacterium bifidum* ATCC 15696, *Lactobacillus casei* ATCC 334D-5, *Prevotella intermedia* ATCC 25611D-5, *C. histolyticum*, ATCC 19401, *Bacteroides uniformis* ATCC 8492, *Ruminococus productus*, ATCC 27340D-5, *P. distasonis* (*B. distasonis*) ATCC 8503]. Data presented are mean values of duplicate real-time quantitative PCR analyses.

DRW, RW, and gin compositions

The DRW and RW used in this study were produced with the Merlot grape variety from the Penedès appellation. The DRW had the same composition and polyphenolic compounds as the RW except for ethanol (only 0.42%). Phenolic profiles of DRW and RW were determined by using HPLC with diode-array detection, and resveratrol and piceid contents were determined by using HPLC with diode-array detection as described by Queipo-Ortuño et al (14). The total phenolic content of several distilled alcoholic beverages was determined with the Folin-Ciocalteu method, and the alcoholic beverage selected was gin (38% alcohol) because the amount of phenols was not detectable. No significant differences were identified in the phenolic content between DRW and regular RW (14).

Statistical analysis

The sample size was determined with the ENE 3.0 statistical program (GlaxoSmithKline) with assumption of a maximum loss of 10% of participants. To detect mean differences for LPS concentrations of 0.04 with a conservative SD of 0.03, 7 subjects were needed to complete the study (α risk = 0.05; power = 0.8). However, the sample size was 10 subjects. LPS concentrations were used to set the sample size, but changes in all endpoints were of equal interest.

Results are expressed as means ± SDs. Bacterium data represent the bacterial copy number per gram of feces converted into logarithmic values. Friedman's test was used to check changes in bacterial numbers, LPS concentrations, and biochemical variables between intervention treatments. Pearson's correlation analyses were done to study associations between variables of the chronic study, and Spearman's correlation coefficient test was used to analyze the association between variables of the acute study. Statistical significance was set at $P < 0.05$. Analyses were performed with SPSS software (version 15.0 for Windows; SPSS Iberica).

RESULTS

Chronic RW, DRW, and gin intake

Resveratrol metabolite concentrations as well as total dihydroresveratrol concentrations were significantly higher after DRW and RW periods ($P < 0.001$), and these concentrations did not change significantly after the gin period ($P > 0.05$). These results have been previously reported by our group (14).

There were significant differences between the 4 measurements according to Friedman's test in LBP and LPS concentrations (**Figure 1**). However, no significant differences were shown between the magnitude of the change in LPS or LBP concentrations induced by each drink, although a tendency toward a higher decrease after chronic RW consumption was found [changes in LPS concentrations: −0.066 ± 0.045 for RW, −0.047 ± 0.087 for DRW, and −0.016 ± 0.109 for gin (P = 0.097); changes in LBP concentrations: −2.52 ± 2.75 for RW, −1.83 ± 1.09 for DRW, and 0.21 ± 2.17 for gin (P = 0.080)].

The change in LPS concentration after each intervention period correlated positively with the HOMA-IR, significantly so after the DRW intervention (DRW: r = 0.782, P = 0.008). No significant correlation was seen between changes in LPS concentrations after each intervention and other biochemical variables.

As has been previously reported by our group, higher bacteria amounts were observed from *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* phyla after RW consumption, and there were significant differences in the number of *Enterococcus*, *Clostridium*, *Bacteroides*, *Prevotella*, *Bifidobacterium*, *C. histolyticum*, and *B. coccoides–E. rectale* groups during the study, although there were no differences in the *Lactobacillus* group (14).

No significant differences were seen between treatments in *P. distasonis* (baseline: 8.23 ± 2.00; DRW: 8.51 ± 1.54; RW: 8.78 ± 1.81; gin: 8.17 ± 1.70; P = 0.159) or *E. coli* amounts (baseline: 8.20 ± 2.19; DRW: 8.11 ± 1.85; RW: 8.33 ± 2.82; gin: 8.73 ± 1.80; P = 0.706).

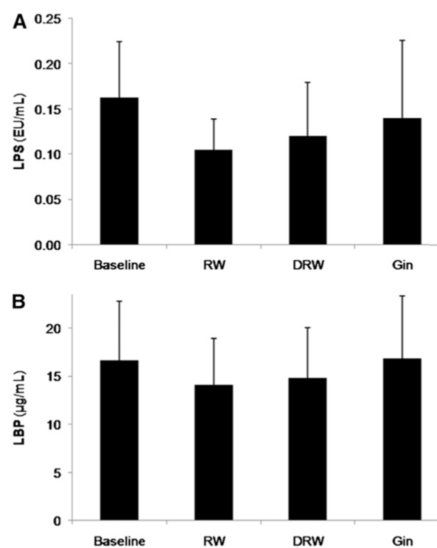

FIGURE 1. Mean (±SD) serum LPS (A) and LBP (B) concentrations in the 4 groups of the chronic study (n = 10). There were significant differences between treatments according to Friedman's test in LPS and LBP concentrations ($P < 0.05$). DRW, dealcoholized red wine; EU, endotoxin units; LBP, LPS-binding protein; RW, red wine.

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LPS concentrations correlated negatively with *Prevotella* and with the *Bifidobacterium* group (**Figure 2**). Although great changes have been reported in some of the bacterium groups analyzed, no significant correlation was seen between other bacterium groups (*Enterococcus*, *Clostridium*, *Bacteroides*, *C. histolyticum*, *Lactobacillus*, and *B. coccoides–E. rectale* group) and LPS concentrations (data not shown).

The difference between bacterium amounts before and after each treatment was calculated to determine whether differences existed in the magnitude of the change caused by each type of drink (**Table 2**). The major bacterium phyla, those groups significantly associated with LPS concentrations, and bacterium species that were not analyzed previously are shown in Table 2. At the phylum level, the changes in *Firmicutes* and *Bacteroidetes* differed significantly between the 3 treatments (Table 2). Changes in *Actinobacteria* and *Proteobacteria* tended to be higher after RW treatment than after gin and DRW treatments ($P = 0.061$ and $P = 0.067$, respectively). At the genera level, gin tended to decrease bacterium amounts, except for *E. coli*. These changes differed significantly between intervention periods in *Prevotella* and *Bifidobacterium*; RW consumption significantly raised *Prevotella* amounts in comparison with DRW and gin consumption. The amount of *Bifidobacterium* was also higher with RW and DRW compared with gin (Table 2).

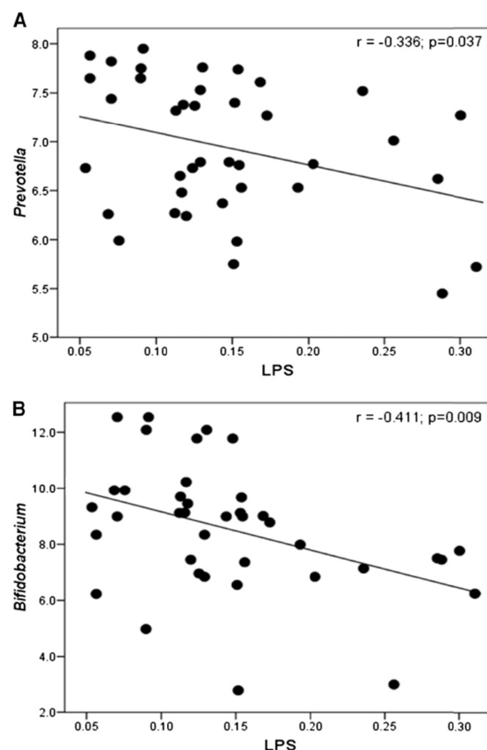

FIGURE 2. Correlations between serum LPS concentrations and bacterium amounts ($n = 10$). The linear relation was determined by Pearson's correlation test between serum LPS concentrations and *Prevotella* amounts (A) and serum LPS concentrations and *Bifidobacterium* amounts (B).

Acute RW, DRW, and gin intake plus a fat overload

Biochemical variables of participants in the acute study are summarized in Table 1. Changes in triglyceride concentrations (calculated as the difference between postprandial and baseline values) were not significantly different between treatments (Table 1).

Serum LPS concentrations tended to rise at 3 h after the intake of FO, RW+FO, and Gin+FO, although differences were not significant. No significant differences were seen in serum LPS concentrations between the 4 treatments at 0 or 3 h (**Figure 3**A). There were no significant differences for the change in serum LPS (calculated as the difference between postprandial and baseline concentrations) between treatments (FO: 0.081 ± 0.186 EU/mL; RW+FO: 0.102 ± 0.127 EU/mL; DRW+FO: 0.039 ± 0.133 EU/mL; and Gin+FO, 0.150 ± 0.069 EU/mL; $P = 0.960$). Neither baseline nor postprandial serum LPS concentrations correlated significantly with the biochemical variables analyzed.

There were no significant differences in baseline or postprandial LBP concentrations between treatments (Figure 3B), and there were no significant differences in the change in serum LBP (FO: −0.999 ± 1.764 μg/mL; RW+FO: 0.353 ± 2.675 μg/mL; DRW+FO: 0.613 ± 2.273 μg/mL; and Gin+FO: −1.362 ± 4.37 μg/mL; $P = 0.753$).

Chylomicron LPS concentrations at 3 h after intake did not differ between treatments (**Figure 4**). Postprandial chylomicron LPS concentrations showed a positive correlation with the change in triglycerides calculated as the difference between postprandial and baseline triglyceride concentrations ($r = 0.517$, $P = 0.028$).

DISCUSSION

The results of this study showed that the chronic consumption of alcoholized RW leads to lower serum LPS concentrations in middle-aged men. However, we did not find that the acute intake of alcoholized RW, DRW, or gin modified the LPS increase induced by a fat overload. This is the first study to examine the possible acute and chronic effects of RW, DRW, and gin on LPS concentrations in middle-aged men.

Animal studies have shown that acute (21, 22) or chronic (21, 23) ethanol consumption leads to high plasma LPS concentrations, and this has been associated with the development of alcoholic liver disease. The gut microbiota has been proposed as the LPS resource in these cases because it has been reported that alcohol administration also promotes leaky gut and, thus, enhances LPS translocation from the gut to the systemic circulation (24, 25). However, there is little evidence in humans that supports the hypothesis that the LPS translocation promoted by alcohol consumption is related to gut microbiota (26). Our study did not find an increase in serum LPS concentrations after gin consumption, probably because the study was carried out in healthy subjects or because only a moderate dose of alcohol was administrated, and longer or higher doses of alcohol might be necessary to detect a significant effect on endotoxemia. This is in concordance with a previous study that showed that a subgroup of alcoholic subjects displayed dysbiosis and higher endotoxin concentrations than those of a group of nonalcoholic subjects who consumed no more than a moderate amount of alcohol (26).

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TABLE 2
Changes in gut bacterial phyla after DRW, RW, and gin periods compared with at baseline[1]

	RW	DRW	Gin	*P*
	log_{10} copies/g feces	*log_{10} copies/g feces*	*log_{10} copies/g feces*	
Firmicutes	0.590 ± 0.693[a]	0.025 ± 0.295[b]	−0.739 ± 1.652[c]	0.004
Bacteroidetes	0.350 ± 0.558[a]	−0.023 ± 0.403[b]	−1.005 ± 2.057[c]	0.001
Actinobacteria	0.732 ± 0.677	0.211 ± 0.709	−0.656 ± 1.879	0.061
Proteobacteria	1.476 ± 1.197	0.168 ± 1.466	−0.462 ± 1.577	0.067
Parabacteroides distasonis	0.541 ± 1.315	0.274 ± 1.339	−0.060 ± 0.835	0.061
Prevotella	0.318 ± 0.444[a]	−0.021 ± 0.290[b]	−0.004 ± 0.446[b]	0.006
Bifidobacterium	2.829 ± 2.327[a]	2.715 ± 2.179[a]	0.131 ± 0.642[b]	0.002
Escherichia coli	−0.145 ± 1.817	−0.299 ± 2.602	0.391 ± 1.428	0.368

[1] All values are means ± SDs. $n = 10$. The change was calculated as the difference between the respective interventional period and baseline real-time polymerase chain reaction data of each bacteria group. *P* values were based on Friedman's test. Wilcoxon's signed-rank test was used to compare treatments with each other ($P < 0.05$). Different superscript letters indicate significant differences between intervention periods. DRW, dealcoholized red wine; RW, red wine.

However, we did find a reduction in LPS concentrations with chronic RW consumption. The gut microbiota has been proposed as the major source of LPS in metabolic endotoxemia (27). Chronic RW consumption is able to modify the gut microbiota by the increase in different bacterial phyla such as *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*, and this effect is less noticeable or disappears with DRW consumption (14). In addition, previous studies have described how polyphenolic extract compounds can also modify the amount of these phyla, increasing *Bacteroidetes* and *Actinobacteria* but lowering *Firmicutes* (28–31). In our study, chronic RW consumption reduced LPS concentrations, but the effect of chronic DRW consumption on LPS concentrations was not so evident, which suggests that in vivo effects of polyphenol on the gut microbiota can differ when RW polyphenols are consumed together in their natural source in the presence of alcohol. In addition, compared with RW and DRW, gin produced the opposite effect on the gut microbiota considering that its consumption tended to reduce all bacterial phyla (14). These findings highlight the opposing effects between the intake of an exclusively alcoholic beverage and consumption of a beverage with both alcohol and polyphenols.

Our results indicate that the effect of chronic RW consumption on LPS concentrations could be mediated by the modulation of the gut microflora considering that a negative correlation was observed between *Bifidobacterium* and *Prevotella* genera. *Bifidobacterium* belongs to the *Actinobacteria* phyla, and its abundance has been related to a healthy and well-functioning type of gut bacteria (30). Tzounis et al (31) showed that a high–cocoa-flavonol drink was able to modulate human gut microbiota by increasing fecal bifidobacteria and lactobacilli with a concomitant reduction in plasma triglycerides and CRP. Moreover, when *Bifidobacterium* sp. was increased by means of prebiotic carbohydrates in mice, gut permeability was decreased and, consequently, circulating LPS concentrations diminished after the treatment (32). These findings could constitute a plausible explanation of how chronic RW consumption led to lower LPS concentrations in our study because, in addition, we have previously shown that chronic RW intake increases *Bifidobacterium* sp. (14). However, additional human studies will be necessary to confirm this hypothesis. Swanson et al (33) have recently reported that RW consumption for 7 d increased gut permeability in subjects with inactive inflammatory bowel disease but not in healthy subjects. These results would indicate that the previous health status of the individual could modify the response to RW consumption. In contrast to Swanson et al (33), our data suggest that RW consumption for periods of time longer than 1 wk might be able to diminish gut permeability in healthy subjects.

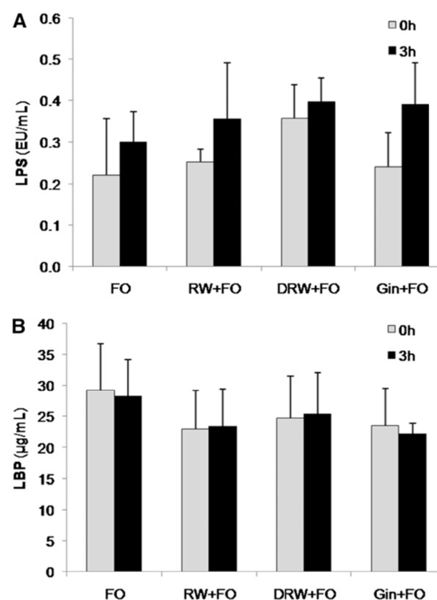

FIGURE 3. Mean (±SD) baseline and postprandial serum LPS (A) and LBP (B) concentrations in the 4 groups of the acute study ($n = 5$). There were no significant differences according to Friedman's test when different treatments were compared. DRW+FO, dealcoholized red wine together with the fat overload; EU, endotoxin units; FO, fat overload; Gin+FO, gin together with the fat overload; LBP, LPS-binding protein; RW+FO, Red wine together with the fat overload.

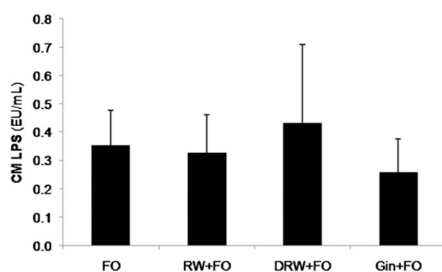

FIGURE 4. Mean (±SD) postprandial chylomicron LPS concentrations in the 4 groups of the acute study (n = 5). There were no significant differences according to Friedman's test. CM, chylomicron; DRW+FO, dealcoholized red wine together with the fat overload; EU, endotoxin units; FO, fat overload; Gin+FO, gin together with the fat overload; RW+FO, red wine together with the fat overload.

Prevotella is a Gram-negative bacterium belonging to the *Bacteroidetes* phyla. The prevalence of this bacterial phylum is related to healthy conditions, and it is diminished in some pathologic conditions such as obesity and alcoholic liver disease (26, 34). Interestingly, endotoxemia is also elevated in these situations (23, 25, 26, 35), and studies in high-fat diet–fed mice that were treated with antibiotics reported an increase in *Bacteroides-Prevotella* spp. and a decrease in endotoxemia (36). Our results are in agreement with these findings because the same tendency was found with a negative correlation between *Prevotella* amounts and endotoxemia. Nevertheless, the reasons why these Gram-negative bacteria are related to a reduction in LPS concentrations are not yet understood.

Moderate alcohol consumption has also been associated with lower risk of diabetes (37). RW consumption improves insulin resistance in both diabetic patients (38) and insulin-resistant nondiabetic patients (39). In addition, endotoxemia has been related to increased risk of diabetes (35), and differences in gut microbiota have been reported between diabetic and nondiabetic patients (40). This direct relation between LPS concentrations and insulin resistance was seen in the current study, in which we showed positive correlations between the change in LPS concentrations after each intervention and the reduction in the degree of insulin resistance, with highly significant correlations given by the DRW intervention.

In agreement with the LPS results, LBP concentrations were also diminished after 20 d of RW consumption. Previous studies have reported that alcoholic patients displayed higher LBP concentrations than those in moderate consumers of alcohol (41). However, to our knowledge, the effect of long-term RW consumption on LBP concentrations has not been previously analyzed. LPS is able to stimulate LBP synthesis (42); thus, the reduction in LBP concentrations after RW consumption may be due to lower LPS concentrations. Nevertheless, additional experimental studies will be necessary to confirm this hypothesis.

The postprandial response to an FO leads to an increase in inflammation and oxidative stress markers, which have been attributed to the rise in plasma lipid concentrations (43–45). Previous studies have explored the capacity of RW to modulate the postprandial response to high-fat or mixed meals and have shown that RW is not able to attenuate the postprandial lipid response (8–10). However, other studies reported a decrease in inflammation and lipid-oxidation products when RW was administered with the high-fat meal (9, 46). In addition, small amounts of LPS are absorbed from the gut (47), and there is evidence that chylomicron formation promotes LPS absorption (18, 48), which would explain why a high-fat meal raises postprandial LPS concentrations (17, 18). Thus, when we designed the study, we also considered whether the reduction after RW intake of the inflammation induced by a high-fat meal might be related to a decrease in postprandial LPS concentrations by means of a lower translocation of LPS from the gut. Nevertheless, we showed that neither RW, DRW, nor gin intake significantly modified the LPS increase induced by a high-fat meal. We did not observe a significant effect of the FO or any of the beverages tested on LBP concentrations either. However, previous studies have shown that a high-fat, high-carbohydrate meal was able to raise LBP concentrations (49, 50) and that a resveratrol and polyphenol preparation was able to attenuate this postprandial increase (49). It is noteworthy that the maximum peak of postprandial LBP concentrations was different in these 2 studies, and thus, it seems that the time of the postprandial LBP response may be variable, and it could be difficult to detect without an analysis of postprandial concentrations of LBP at various times.

It must be taken into consideration that the relatively small sample size of the postprandial study might have limited the finding of significant effects of RW, DRW, or gin. Nevertheless, in view of our results, an acute effect of great magnitude of RW on LPS and LBP concentrations can be ruled out.

Thus, our results support the hypothesis that chronic RW consumption is able to lower LPS concentrations by means of the modulation of the composition of the gut microbiota while acute RW intake does not modify the postprandial LPS response, probably because changing gut microbiota requires a longer stimulus than a meal. However, additional studies will be necessary to elucidate the exact mechanisms that explain how the changes in the gut microbiota induced by RW modulate the degree of endotoxemia in humans. These findings could be of clinical relevance because high LPS concentrations have been associated with higher risk of CVD in epidemiologic studies (51, 52), and it has also been shown that LPSs induce the release of proinflammatory cytokines from macrophages and endothelial cells, which promote plaque formation and rupture, oxidation of LDLs, and thrombogenesis (27).

One of the limitations of our study was the lack of washout periods between interventions. The inclusion of washout periods between interventions would have extended the study an additional 6 wk, which would have made it difficult to ensure compliance, and subjects would have been more inclined to withdraw from the study. Nonetheless, no carryover effect was observed in this study considering that resveratrol or dihydroresveratrol metabolites were specifically increased by RW and DRW, and their concentrations were similar between baseline and the gin-consumption period (14). Thus, the absence of a washout period was unlikely to have affected the results obtained.

In conclusion, this is the first in vivo human study to explore the relation of both chronic and acute RW, DRW, as well as gin consumption with endotoxemia, showing that chronic RW consumption increases *Bifidobacterium* and *Prevotella* amounts, which may have beneficial effects by leading to lower LPS concentrations.

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The authors' responsibilities were as follows—FJT and CA-L: conceived and designed the research; MC-P, MIQ-O, LC-A, and MB-O: conducted the research; MC-P, FC, MdMR-R, and FJT: performed the statistical analysis and interpreted data; MC-P and FJT: wrote the manuscript; MC-P, JD-L, FC, CA-L, and FJT: critically revised the manuscript; and all authors: read and approved the final manuscript. None of the authors had a conflict of interest.

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Erratum

Clemente-Postigo M, Queipo-Ortuño MI, Boto-Ordoñez M, Coin-Aragüez L, Roca-Rodriguez MdM, Delgado-Lista J, Cardona F, Andres-Lacueva C, Tinahones FJ. Effect of acute and chronic red wine consumption on lipopolysaccharide concentrations. Am J Clin Nutr 2013;97:1053–61.

On page 1053, footnote 2 should include the following additional funding information: "The study was also supported by CP07/00095 from the ISCIII, and MdMR-R was a recipient of a fellowship from ISCIII (Rio Hortega CM11/00030), Spanish Ministry of Economy and Competitiveness, Madrid, Spain."

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Erratum

Schernhammer ES, Bertrand KA, Birmann BM, Sampson L, Willett WC, Feskanich D. Consumption of artificial sweetener– and sugar-containing soda and risk of lymphoma and leukemia in men and women. Am J Clin Nutr 2012;96:1419–28.

The supplemental data for this article were inadvertently missed during production and were therefore not posted online. The supplemental data file (Table 1) is now available online.

doi: 10.3945/ajcn.113.066365.

Erratum

Wilkinson SB, Tarnopolsky MA, MacDonald MJ, MacDonald JR, Armstrong D, Phillips SM. Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. Am J Clin Nutr 2007;85:1031–40.

On page 1039, an error appears in the legend to Figure 5. The solid circle line should represent skim milk, and the open circle line should represent the soy-protein beverage. The first sentence of the figure legend should read as follows: "Mean (±SEM) total amino acid (TAA) chemical net balance (NB) after consumption of a nonfat milk-protein beverage (●) or an isonitrogenous, isoenergetic, macronutrient-matched (750 kJ, 18.2 g protein, 1.5 g fat, and 23 g carbohydrate) soy-protein beverage (○)."

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El estudio de la LPS después de sobrecarga grasa en pacientes con obesidad mórbida y su relación con la hipertrigliceridemia postprandial pertenece a la estancia realizada en el grupo de investigación del Doctor Francisco J. Tinahones (Hospital Universitario Virgen de la Victoria, Málaga), donde se colaboró en el análisis de las muestras.

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Endotoxin increase after fat overload is related to postprandial hypertriglyceridemia in morbidly obese patients

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Abstract The low-grade inflammation observed in obesity has been associated with a high-fat diet, though this relation is not fully understood. Bacterial endotoxin, produced by gut microbiota, may be the linking factor. However, this has not been confirmed in obese patients. To study the relationship between a high-fat diet and bacterial endotoxin, we analyzed postprandial endotoxemia in morbidly obese patients after a fat overload. The endotoxin levels were determined in serum and the chylomicron fraction at baseline and 3 h after a fat overload in 40 morbidly obese patients and their levels related with the degree of insulin resistance and postprandial hypertriglyceridemia. The morbidly obese patients with the highest postprandial hypertriglyceridemia showed a significant increase in lipopolysaccharide (LPS) levels in serum and the chylomicron fraction after the fat overload. Postprandial chylomicron LPS levels correlated positively with the difference between postprandial triglycerides and baseline triglycerides. There were no significant correlations between C-reactive protein (CRP) and LPS levels. The main variables contributing to serum LPS levels after fat overload were baseline and postprandial triglyceride levels but not glucose or insulin resistance. Additionally, superoxide dismutase activity decreased significantly after the fat overload. Postprandial LPS increase after a fat overload is related to postprandial hypertriglyceridemia but not to degree of insulin resistance in morbidly obese patients.—Clemente-Postigo, M., M. I. Queipo-Ortuño, M. Murri, M. Boto-Ordoñez, P. Perez-Martinez, C. Andres-Lacueva, F. Cardona, and F. J. Tinahones. **Endotoxin increase after fat overload is related to postprandial hypertriglyceridemia in morbidly obese patients.** ***J. Lipid Res.*** **2012.** 53: **973–978.**

Supplementary key words chylomicrons • insulin resistance • nutrition • triglycerides • metabolic endotoxemia • obesity

Obesity is an increasingly prevalent health problem, very often accompanied by other diseases, the most common being insulin resistance, type 2 diabetes mellitus, and cardiovascular complications (1, 2). Furthermore, an association has been reported between obesity and both oxidative stress and increased inflammation (3, 4). Obesity facilitates the development of a low-grade inflammatory state, characterized by increased plasma levels of proinflammatory cytokines (4). However, the factors that trigger this low-grade inflammation in obesity are unclear. Postprandial lipidemia has recently emerged as a potential candidate because the ingestion of a high-fat meal leads to the systemic increase of a wide range of inflammatory mediators (5–7) and an increase in oxidative stress markers (8). However, the cause of these postprandial events that occur in association with the postprandial triglyceride response remains poorly understood. A possible link is bacterial endotoxin [lipopolysaccharide (LPS)], a component of the Gram-negative bacteria cell wall that is present in large quantities in the human gut (9). Endotoxins circulate in the plasma

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Abbreviations: BMI, body mass index; CM, chylomicron; CRP, C-reactive protein; GGT, gamma-glutamyl transferase; HOMA-IR, homeostasis model assessment of insulin resistance; IL, interleukin; LAL, Limulus amebocyte lysate; LPS, lipopolysaccharide; SOD, superoxide dismutase; TNFα, tumor necrosis factor alpha; ΔTG; Delta triglycerides.

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of healthy human subjects at low concentrations (known as metabolic endotoxemia), and an elevated concentration of circulating LPS has been associated with a higher risk for atherosclerosis (10). There is evidence that metabolic plasma LPS levels are modulated by food content: the higher the fat content, the higher the concentration of plasma LPS (11). Small amounts of LPS are absorbed from the gut in healthy animals (12), and there is evidence that chylomicrons likely also transport significant amounts of absorbed gut LPS (13–15). In concordance with these data, some studies have shown that a high-fat meal leads to an increase in postprandial endotoxemia (7, 14).

Obesity tends to be accompanied by the consumption of a high-fat diet, and interestingly, the proportion of Gram-negative bacteria in microflora is higher in obese subjects than in lean subjects (16, 17). Thus, these conditions would enhance the translocation of endogenous LPS from the gut during fat absorption, which would lead to the low-grade inflammation observed in these patients (7, 18). However, no studies have yet examined metabolic endotoxemia in obese patients. This is of particular interest, as metabolic endotoxemia could be involved in the development of obesity-related comorbidities, as it has been associated with the development of insulin resistance in mice (18).

Thus, the aim of this study was to analyze postprandial endotoxemia in morbidly obese patients after a fat overload and to determine its relationship with postprandial hypertriglyceridemia and insulin resistance.

MATERIAL AND METHODS

Subjects and study design

A total of 40 morbidly obese patients [body mass index (BMI) >40 kg/m^2] were selected from our database according to their degree of insulin resistance (HOMA-IR): 20 morbidly obese patients with HOMA-IR < 5 and 20 morbidly obese patients with HOMA-IR > 8. Those patients with intermediate values of HOMA-IR (HOMA-IR > 5 or < 8) were excluded. The cut-off point for the HOMA-IR was taken from previous studies carried out in a healthy population with no carbohydrate metabolism disorders (2). Patients were excluded if they had cardiovascular disease, arthritis, acute inflammatory disease, infectious disease, renal disease, were receiving treatment for hyperlipidemia or diabetes or were taking medications that could influence gastric emptying or the absorption time. All the patients were recruited by endocrinologists and gave informed consent to the study, which was approved by the Ethics Committee of Virgen de la Victoria Clinical University Hospital, Malaga, Spain.

After an overnight fast, all 40 participants underwent a 50 g fat overload with a preparation (patent No. P201030776). Only water was permitted during the process and no physical exercise was undertaken. The preparation of 100 ml contained 50 g fat, of which 10 g were saturated, 29.46 g were monounsaturated, and 10.625 g were polyunsaturated. Each 100 ml contained <1 g lauric acid, <1 g myristic acid, 4.8 g palmitic acid, 1.4 g stearic acid, 27.7 g oleic acid, 9.6 g linoleic acid, 1.4 g behenic acid and 0.5 g lignoceric acid. This test was previously validated in another study by our group (19). All the participants followed the same diet on the day prior to fat overload.

The 40 patients were classified according to their degree of insulin resistance (HOMA-IR, calculated as described below) and the Delta triglycerides (ΔTG), measured as the difference

TABLE 1. Anthropometric and biochemical variables of the study groups

	Group 1 (n = 10)	Group 2 (n = 10)	Group 3 (n = 10)	Group 4 (n = 10)	All (n = 40)
Age (years)	40.00 ± 11.58a	38.71 ± 8.63a	43.14 ± 6.568a	42.00 ± 6.00a	41.41 ± 9.36
Weight (Kg)	129.77 ± 27.50a, b	152.64 ± 23.90b, c	118.96 ± 11.93a	156.95 ± 11.29c	139.38 ± 25.48
BMI (kg/m^2)	51.69 ± 9.16a, b	59.85 ± 8.86a	46,66 ± 6.54b	55,72 ± 9.56a, b	53.70 ± 9.49
Waist (cm)	130.05 ± 10.63a	147.29 ± 14.64b	123.00 ± 5.66a	152.00 ± 7.80b	137.80 ± 15.24
Hip (cm)	147.40 ± 12.62a	155.14 ± 10.24a	145.20 ± 11.28a	149.17 ± 16.52a	149.32 ± 12.61
Waist/Hip ratio	0.88 ± 0.08a, b	0.95 ± 0.04b, c	0.85 ± 0.05a	1.03 ± 0.10c	0.92 ± 0.09
Glucose (mg/dl)	93.00 ± 16.32a	110.43 ± 26.25a	96.20 ± 6.94a	103.33 ± 11.22a	100.14 ± 17.99
Insulin (μUI/ml)	15.86 ± 5.54a	39.77 ± 9.74b	12.38 ± 2.33a	42.52 ± 8.21b	26.93 ± 15.03
HOMA-IR	3.59 ± 1.15a	10.54 ± 2.01b	2.94 ± 0.64a	10.79 ± 2.19b	6.76 ± 4.00
Uric acid (mg/dl)	5.85 ± 1.60a, b	6.33 ± 1.37a	5.27 ± 1.07a, b	7.20 ± 1.98b	6.16 ± 1.61
Chol (mg/dl)	211.40 ± 31.18a	187.57 ± 44.02a	183.00 ± 37.50a	183.50 ± 27.85a	194.39 ± 35.73
FFA 0 h (mmol/L)	0.52 ± 0.20a	0.55 ± 0.21a	0.42 ± 0.16a	0.49 ± 0.17a	0.51 ± 0.19
FFA 3 h (mmol/L)	0.74 ± 0.27a	0.74 ± 0.20a	0.77 ± 0.26a	0.83 ± 0.25a	0.76 ± 0.25
TG 0 h (mg/dl)	133.97 ± 77.15a, b	215.29 ± 105.69a	98.93 ± 46.93b	110.40 ± 19.80a	142.99 ± 82.74
TG 3 h (mg/dl)	166.75 ± 70.17a	251.00 ± 106.27a	205.21 ± 43.95a	224.29 ± 29.87a	207.01 ± 75.97
ΔTG (mg/dl)	32.78 ± 22.99a	35.71 ± 22.82a	106.28 ± 29.32b	113.88 ± 31.06b	64.02 ± 45.28
HDL-C (mg/dl)	48.20 ± 5.43a	41.14 ± 6.94a, b	45.80 ± 11.08a, b	37.83 ± 5.84b	43.79 ± 7.92
LDL (mg/dl)	140.71 ± 22.43a	98.80 ± 24.92b	105.71 ± 22.31a, b	123.95 ± 19.24a, b	120.30 ± 26.16
GOT (units/L)	26.30 ± 25.95a	19.86 ± 7.47a	20.00 ± 7.87a	34.67 ± 23.50a	25.36 ± 19.52
GGT (units/L)	27.30 ± 11.73a	26.43 ± 11.34a	26.00 ± 9.14a	51.33 ± 29.66b	32.00 ± 18.86
Leptin (ng/ml)	61.17 ± 32.94a	58.05 ± 23.28a	52.63 ± 29.28a	71.47 ± 25.87a	60.71 ± 29.39
Adiponectin (ng/ml)	9.35 ± 3.25a	6.85 ± 3.33a	9.49 ± 3.12a	7.49 ± 4.01a	8.52 ± 3.49
CRP 0 h (mg/dl)	6.15 ± 6.67a, b	10.09 ± 10.70a	3.98 ± 3.94b	7.42 ± 6.03a, b	7.03 ± 7.60
CRP 3 h (mg/dl)	5.11 ± 3.93a	8.43 ± 9.67a	3.86 ± 2.94a	7.38 ± 5.64a	6.11 ± 6.13

Values are presented as means ± SD. Different letters indicate significant differences ($P < 0.05$) between the study groups (ANOVA, Bonferoni test). Group 1: Patients with HOMA-IR ⩽ 5 and ΔTG < 80 mg/dl; Group 2: patients with HOMA-IR > 8 and ΔTG < 80 mg/dl; Group 3: patients with HOMA-IR ⩽ 5 and ΔTG > 80 mg/dl; Group 4: patients with HOMA-IR > 8 and ΔTG > 80 mg/dl. BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; Chol, cholesterol; FFA, free fatty acids; TG, triglycerides; ΔTG, difference between postprandial and baseline triglyceride levels ; GOT, glutamic oxalacetic transaminase; GGT, γ-glutamyltransferase; CRP, C-reactive protein.

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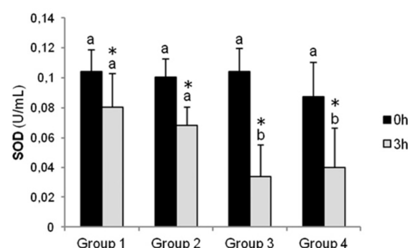

Fig. 1. Baseline and postprandial plasma superoxide dismutase activity in the four study groups. Results are presented as means ± SD. Different letters indicate significant differences between the study groups (ANOVA, Bonferroni test). *$P < 0.05$ between baseline and postprandial values. Group 1: patients with HOMA-IR ≤ 5 and ΔTG < 80 mg/dl; Group 2: patients with HOMA-IR > 8 and ΔTG < 80 mg/dl; Group 3: patients with HOMA-IR ≤ 5 and ΔTG > 80 mg/dl; Group 4: patients with HOMA-IR > 8 and ΔTG > 80 mg/dl. SOD, superoxide dismutase.

between postprandial triglycerides and baseline triglycerides, as follows: Group 1 (HOMA-IR ≤ 5 and ΔTG < 80 mg/dl), Group 2 (HOMA-IR > 8 and ΔTG < 80 mg/dl), Group 3 (HOMA-IR ≤ 5 and ΔTG > 80 mg/dl) and Group 4 (HOMA-IR > 8 and ΔTG > 80 mg/dl). The cut-off point for the triglyceride difference was determined by the median.

Biochemical analyses

At baseline and 3 h after the high-fat meal, blood samples were obtained from the antecubital vein and placed in vacutainer tubes (BD vacutainer™, London, UK). The serum was separated by centrifugation for 10 min at 4,000 rpm and frozen at −80°C until analysis. Data recorded for all subjects were age, weight, height (to calculate the BMI, calculated as the weight in kg divided by the height in square meters), and waist and hip circumferences (to calculate the waist-to-hip ratio, calculated as the waist circumference divided by the hip circumference). Serum glucose, uric acid, cholesterol, triglycerides, HDL cholesterol, free-fatty acids, C-reactive protein (CRP) and transaminases were measured in a Dimension autoanalyzer (Dade Behring Inc., Deerfield, IL) by enzymatic methods (Randox Laboratories Ltd., UK). Insulin levels were quantified by radioimmunoassay supplied by BioSource S.A. (Nivelles, Belgium). The LDL cholesterol was calculated from the Friedewald equation. The insulin resistance was calculated from the homeostasis model assessment of insulin resistance (HOMA-IR) with the formula: insulin resistance = [fasting serum insulin (μU/ml) × fasting blood glucose (mmol/L)]/22.5 (20). Leptin was analyzed by ELISA kits (DSL, Webster, TX). Adiponectin was analyzed by ELISA kits (DRG Diagnostics GmbH, Germany). Superoxide dismutase (SOD) activity was measured in plasma using commercial kits (Cayman Chemical, Ann Arbor, MI).

Chylomicrons were separated from serum by ultracentrifugation at 30,000 rpm for 30 min at room temperature (Beckman TLA 100.3). The top layer was carefully isolated and resuspended in endotoxin-free saline solution to the initial volume.

LAL assays

Serum LPS and chylomicron LPS concentrations were measured by endotoxin assay, based on a Limulus amebocyte extract with a chromogenic Limulus amebocyte lysate (LAL) assay (QCL-1000, Lonza Group Ltd.). Samples were diluted in pyrogen-free water and heated at 70°C for 10 min to inactivate endotoxin-neutralizing agents that inhibit the activity of endotoxin in the LAL assay. Then, Pyrosperse reagent (Lonza Group Ltd.), a metallo-modified polyanionic dispersant, was added to the test samples at a ratio of 1/200 (v/v) before LAL testing to minimize interference in the reaction. Internal control of recovery calculation was included in the assessment. All samples were tested in duplicate and results were accepted when the intra-assay CV was less than 10%. The endotoxin content was expressed as endotoxin units (EU) per ml. Exhaustive care was taken to avoid environmental endotoxin contamination and all material used for sample preparation and the test was pyrogen-free.

Statistical analysis

Study groups were compared by ANOVA and Bonferroni's post-hoc tests at both baseline and in the postprandial state. Analysis of the effects of the fat overload on the biological variables was done by Wilcoxon test. Spearman correlation analyses were done to study the associations between variables. Multiple linear regression analysis was performed to evaluate which variables contributed more to LPS levels. In all cases, the rejection level for a null hypothesis was an alpha = 0.05 for two tails. Calculations were performed with SPSS software (version 15.0; SPSS Iberica, Madrid, Spain).

RESULTS

The anthropometric and biochemical variables of the morbidly obese patients are summarized in **Table 1**.

Plasma CRP levels were significantly higher in group 2 than in group 3, but there was no significant difference between postprandial CRP levels (Table 1).

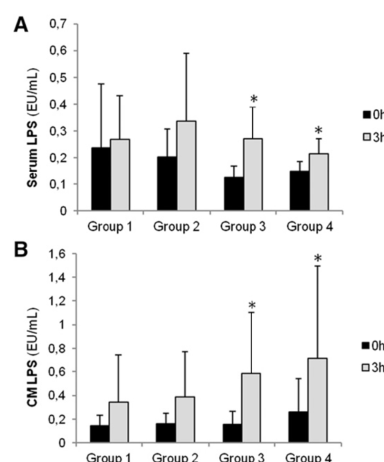

Fig. 2. Baseline and postprandial serum and chylomicron LPS levels in the four study groups. Results are presented as means ± SD. * $P < 0.05$ between baseline and postprandial values; Group 1: patients with HOMA-IR ≤ 5 and ΔTG < 80 mg/dl; Group 2: patients with HOMA-IR > 8 and ΔTG < 80 mg/dl; Group 3: patients with HOMA-IR ≤ 5 and ΔTG > 80 mg/dl; Group 4: patients with HOMA-IR > 8 and ΔTG > 80 mg/dl. LPS, lipopolysaccharide; CM, chylomicron.

TABLE 2. Correlations between baseline and postprandial LPS levels in all the study subjects

	TG 0 h	TG 3 h	ΔTG	Insulin	HOMA-IR	Glucose
LPS S 0 h	0.480[a]	0.374	−0.257	−0.060	0.008	0.283
LPS S 3 h	0.852[b]	0.837[b]	−0.180	−0.069	0.063	0.442[a]
ΔLPS S	0.416[a]	0.489[a]	0.042	−0.016	0.056	0.188
LPS CM 0 h	−0.048	0.170	0.371	0.098	0.071	−0.022
LPS CM 3 h	−0.045	0.192	0.401[a]	−0.009	−0.032	−0.008
ΔLPS QM	−0.041	0.192	0.394[a]	−0.053	−0.072	−0.001

Correlations were determined by Spearman's correlation coefficient test. LPS, lipopolysaccharide; S, serum; CM, chylomicron; TG, triglycerides; ΔTG, difference between postprandial and baseline triglyceride levels; HOMA-IR, Homeostasis model assessment of insulin resistance.
[a] $P < 0.05$.
[b] $P < 0.01$.

No differences were seen between the study groups in baseline plasma SOD activity, but after fat overload, this was significantly lower in the groups with ΔTG>80 mg/dl. After the fat overload, there was a significant drop in SOD activity in all the groups, significantly more pronounced in the groups with ΔTG>80 mg/dl (**Fig. 1**).

Although groups 3 and 4 appear to have lower serum LPS levels, no significant differences were found between groups at 0 h or 3 h in either serum or chylomicron LPS levels; only the groups with the highest ΔTG had a significant increase over baseline after fat overload in both serum and chylomicron LPS levels (**Fig. 2**).

Plasma LPS levels at 0 h correlated positively with triglyceride levels at 0 h. In addition, plasma LPS levels at 3 h correlated positively with triglyceride levels at both 0 h and 3 h, and with plasma glucose levels. Chylomicron LPS levels at 3 h as well as Delta chylomicron LPS (ΔCM LPS; measured as the difference between postprandial chylomicron LPS and baseline chylomicron LPS levels) showed a positive correlation with the ΔTG (**Table 2**). Inflammatory (CRP) and hormonal (adiponectin and leptin) variables as well as anthropometric variables (BMI, waist, and age) showed no significant correlations with LPS levels (data not shown).

We carried out a multiple regression analysis with those factors associated with serum LPS levels at 0 h and 3 h. We considered as independent variables age, sex, BMI, variables with significant differences between the study groups in univariate analysis [waist circumference, insulin, HOMA-IR, triglyceride levels, ΔTG, uric acid, HDL-C, LDL-C, gamma-glutamyl transferase (GGT), and CRP], and variables that correlated significantly with LPS levels (**Table 3**). This analysis confirmed that the baseline triglyceride level was the best variable to predict the baseline serum LPS level. In addition, triglyceride levels at 0 h and 3 h were significantly and independently associated with LPS levels after fat overload.

A multiple regression analysis was also done with chylomicron LPS levels at 0 h and 3 h as dependent variables and the same independent variables as described above. The ΔTG was the only variable significantly and independently associated with chylomicron LPS at 3 h (**Table 4**). In contrast, no significant association was found with chylomicron LPS levels at 0 h.

TABLE 3. Multiple linear regression analysis with serum LPS levels at both 0 h and 3 h after the fat overload as the dependent variables, in all the study subjects

	Serum LPS 0 h ($R = 0.480$; $R^2 = 0.230$)		
	β	P	95% CI
TG 0 h	0.480	0.013	0.000–0.001
	Serum LPS 3 h ($R = 0.885$; $R^2 = 0.783$)		
	β	P	95% CI
TG 0 h	0.483	0.015	−0.001–0.003
TG 3 h	0.407	0.033	0.000–0.002

TG, triglycerides. Independent variables: Triglycerides 0 h, Triglycerides 3 h, ΔTG (difference between postprandial and baseline triglyceride levels), HOMA-IR, insulin, glucose, body mass index, C-reactive protein, age, sex, waist circumference, uric acid, HDL-C, LDL-C and GGT.

DISCUSSION

The results of this study show that a fat overload leads to increased LPS levels related with chylomicrons. This increase was associated with postprandial hypertriglyceridemia but not with insulin resistance in morbidly obese patients.

The degree of metabolic endotoxemia is related to fat ingestion and some authors have suggested that it might be responsible, at least in part, for the low-grade inflammation observed in obese patients (7, 18). However, to date, all studies dealing with endotoxemia and fat intake have been carried out in healthy lean subjects (7, 14). Thus, ours is the first study to examine this hypothesis in morbidly obese patients and to attempt to clarify the relationship between metabolic endotoxemia, hypertriglyceridemia, and insulin resistance in obesity.

The intestinal microflora is considered a source of circulating LPS (9). In fact, small amounts of LPS are

TABLE 4. Multiple linear regression analysis with chylomicron LPS levels at 3 h after the fat overload as the dependent variable, in all the study subjects

	Chylomicron LPS 3 h ($R = 0.401$; $R^2 = 0.161$)		
	β	P	95% CI
ΔTG	0.401	0.047	0.000-0.009

TG, triglycerides. Independent variables: Triglycerides 0 h, Triglycerides 3 h, ΔTG, (difference between postprandial and baseline triglyceride levels), HOMA-IR, insulin, glucose, body mass index, C-reactive protein, age, sex, waist circumference, uric acid, HDL-C, LDL-C and GGT.

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absorbed from the gut in healthy animals (12) and bioactived LPS is detectable in low amounts in the blood of healthy human subjects, even in the apparent absence of infections (21, 22). Chylomicrons have been associated with metabolic endotoxemia. Both animal and in vitro studies have demonstrated that chylomicron formation promotes LPS absorption (13). A recent study has also shown human chylomicrons can be postprandial carriers of LPS in healthy humans (14).

Our study agrees with the idea of chylomicron LPS transport because the patients with higher increases in triglyceride levels over baseline displayed higher levels of chylomicron LPS after the fat overload. Concordantly with the idea that chylomicrons promote LPS absorption, a high-fat meal leads to increased endotoxemia in healthy humans (7, 14). In consequence, it has been hypothesized that endogenous LPS levels could be responsible for the low-grade inflammation observed in obese subjects who have a high fat intake.

Obesity is now considered to be a condition that facilitates the development of a low-grade inflammatory state, characterized by increased plasma levels of proinflammatory cytokines such as tumor necrosis factor α, interleukins (IL), and cytokine-like proteins known as adipokines (4). It has been reported that patients with morbid obesity have a greater postprandial response to fat overload, and the postprandial response is associated with a greater increase in oxidative stress and inflammation (8, 23). Bacterial endotoxin is increasingly being considered as a potential inflammatory mediator of obesity, diabetes, and atherosclerosis (10, 17, 24, 25). Laugerette et al. (14) showed that healthy subjects following a mixed meal containing lipids from different food products undergo a transient increase in endotoxemia associated with raised inflammation biomarkers such as sCD14 and an early peak of IL-6. Others have reported postprandial endotoxemia in healthy humans after a fat load (50 g of butter on toast) but failed to observe postprandial inflammation (7). We found no relationship between CRP levels and LPS levels in our patients. In fact, CRP seems to be more related to obesity or insulin resistance. This agrees with previous studies finding no relationship between CRP and endotoxemia or postprandial response (7). This is probably because CRP is a marker of long-term inflammation rather than short-term inflammation (which is the situation we studied in the postprandial state 3 h after fat load), and this may also explain the differences found in CRP levels according to the degree of insulin resistance but not to postprandial response. Clarifying the relationship between obesity and metabolic endotoxemia will require further studies exploring this association in obese patients. However, we did find that patients with high triglyceride increases after fat overload showed a decrease in antioxidant defenses because they had lower postprandial SOD activity than the patient groups with lower triglyceride increases after fat overload. Thus, these results show for the first time a possible link between oxidative stress and metabolic endotoxemia.

Studies in mice showed a link between postprandial endotoxemia and postprandial hypertriglyceridemia when comparing the groups of mice fed with different diets (11, 18). Our study shows that the postprandial triglyceride level was more influential than the degree of insulin resistance in postprandial LPS levels and that serum LPS rose significantly after fat overload only in morbidly obese patients displaying the highest triglyceride increase after a fatty meal. It is worth noting, though, that despite the lack of a significant difference between groups in fasting LPS levels, the serum LPS increase might be driven by lower baseline LPS levels in groups 3 and 4. Interestingly, we found that the baseline triglyceride level was the variable that best predicted the baseline LPS level in serum. This shows a clear relationship between triglyceride metabolism and endotoxemia, though further studies will be necessary to elucidate what other mechanisms related to triglyceride metabolism, apart from chylomicrons, could be determining fasting endotoxemia.

The specific mechanisms leading to insulin resistance have been partially characterized and have revealed an incomplete picture of a complex cross-talk integrating metabolic, nutritional, and inflammatory signaling pathways, eventually leading to the development of obesity-induced insulin resistance (26). Among the various nutritional factors involved in the development of insulin resistance, postprandial hypertriglyceridemia-associated increased endotoxemia could be responsible for a higher oxidative stress increase and the degree of inflammation that influences the insulin signaling pathways. Cani et al. (18) showed that LPS-infused mice had higher glucose and insulin levels than control mice, suggesting that LPS could initiate insulin resistance and the development of diabetes. However, here we show that in morbidly obese patients, LPS levels are associated with triglyceride levels but not the degree of insulin resistance.

A noteworthy finding is that chylomicron LPS values were slightly higher than serum values, a result previously reported by other authors (13, 14). It would be expected that chylomicron LPS levels were lower than (or at most the same as) serum LPS levels. It has been previously reported that certain proteins in serum could interfere with LAL enzymatic reaction (27) in spite of using heating to eliminate serum complement factor or dispersant agents. Thus, a plausible explanation for these discrepancies between serum and chylomicron LPS levels could be that LAL reaction-interfering serum molecules were not isolated in the chylomicron fraction during ultracentrifugation and they stayed in the remaining fraction.

In conclusion, LPS levels rose after a fat overload in morbidly obese persons and those patients with a high postprandial triglyceride increase showed a higher increase in chylomicron LPS levels after the fat overload as well as higher oxidative stress.

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ANÁLISIS DE METABOLITOS DEL VINO EN DIFERENTES MATRICES

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El análisis de la ácidos fenólicos fecales tras el consumo de vino tinto, vino tinto desalcoholizado y ginebra, permitió una mejor aproximación al estudio del papel de la microbiota intestinal, identificando metabolitos microbianos que se encontraron presentes en orina de esos mismos individuos, resultados presentes en esta Tesis Doctoral. Este trabajo pertenece a la estancia realizada en el grupo de investigación del Doctor Francisco J. Tinahones (Hospital Universitario Virgen de la Victoria, Málaga), dentro del miniproyecto CONSOLIDER “*Microbiota humana y polifenoles del vino*”.

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Comparative Study of Microbial-Derived Phenolic Metabolites in Human Feces after Intake of Gin, Red Wine, and Dealcoholized Red Wine

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S *Supporting Information*

ABSTRACT: The analysis of microbial phenolic metabolites in fecal samples from in vivo studies is crucial to understanding the potential modulatory effects derived from polyphenol consumption and its overall health effects, particularly at the gut level. In this study, the composition of microbial phenolic metabolites in human feces collected after regular consumption of either red wine, dealcoholized red wine, or gin was analyzed by UPLC-ESI-MS/MS. Red wine interventions produce a change in the content of eight phenolic acids, which are probably derived from the catabolism of flavan-3-ols and anthocyanins, the main flavonoids in red wine. Moreover, alcohol seemed not to influence the formation of phenolic metabolites by the gut microbiota. A principal component analysis revealed large interindividual differences in the formation of microbial metabolites after each red wine polyphenol intervention, but not after the gin intervention, indicating differences in the gut microbial composition among subjects.

KEYWORDS: *red wine polyphenols, gut microbiota, microbial metabolites, feces, UPLC-ESI-MS/MS*

INTRODUCTION

Recent studies seem to indicate that the biological activity and health effects derived from the consumption of polyphenol-rich foods, such as wine, are mainly due to the phenolic metabolites formed in the gastrointestinal tract rather than the original forms present in foods.[1−5] The contribution of either phase II metabolism or colonic catabolism to the overall bioavailability of polyphenols is directly influenced by their chemical structure. Wine polyphenols comprise a wide range of nonflavonoid and flavonoid compounds. Flavan-3-ols and anthocyanins are among the most abundant and bioactive flavonoids in red wine. Flavan-3-ols occur as monomeric, oligomeric, or polymeric forms; the latter two are also known as proanthocyanidins or condensed tannins. Main flavanol monomeric units include (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-*O*-gallate. Anthocyanins identified in red wine include the 3-*O*-monoglucosides and the 3-*O*-acylated monoglucosides of five main anthocyanidins: delphinidin, cyanidin, petunidin, peonidin, and malvidin. Such compounds can be acylated in the glucose molecule through esterification with acetic, *p*-coumaric, and caffeic acids.

Among the different structures, monomeric flavan-3-ols and anthocyanin glucosides are mainly absorbed in the small intestine and then reach the colon through heterohepatic recirculation in conjugated form, whereas proanthocyanidins with a degree of polymerization ≥3 directly reach the colon in their native form.[4] Although the colonic microbial catabolic pathways of these compounds are still under consideration and hence, microbial phenolic metabolites derived from wine consumption are not fully elucidated, in vitro fermentations carried with human feces in the presence of grape seed, wine extracts or individual compounds, have allowed the identification of a wide range of metabolites including phenyl-valerolactones and phenolic acids of different side chain length and hydroxylation pattern.[3]

Recent in vitro studies suggest that the polyphenol food source influences the amount and profile of microbial metabolite that may be produced by the colonic microbiota.[6−9] In fact, in vivo studies have revealed the differential impact of black tea or red wine/grape juice polyphenolic extracts on the urinary human metabolome.[10,11] These changes in the metabolome may have profound health effect implications at the gut level and systemically, because for some polyphenols microbial catabolism constitutes a way of their conversion into more bioactive forms.[3] Although the known bioactive properties of microbial metabolites are still limited, some metabolites have revealed antioxidant, antithrombotic, anti-inflammatory, and antiproliferative activ-

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ities, as well as inhibition of pathogenic bacteria and modulation of lipid metabolism.[3]

Moreover, considering that the colon is the site where the bacteria−polyphenol interaction occurs, modulation of the microbiota growth and metabolism is excepted to occur at the colon level.[12] Recent studies indicate that flavan-3-ol-rich sources such as chocolate, green tea, black currant or grape seed extracts may modulate the intestinal microbiota in vivo, producing changes in beneficial bacteria such as *Lactobacillus*, but inhibiting other groups such as *Clostridium* spp.[13−17] In the case of red wine polyphenols, evidence is still scarce.[18] Despite these modulatory effects found in vivo, very few studies have attempted to study the metabolome of the corresponding fecal samples,[19,20] limiting the analysis to urine samples. On the other hand, most of the knowledge accumulated on the profile of microbial metabolites in feces comes from culture samples collected in in vitro fermentation studies.[3] The integration of both microbiome and metabolome analyses in fecal samples from in vivo polyphenol feeding trials studies is crucial not only to link bacteria to the production of certain metabolites but also to understand how the modulatory effects of these compounds result in benefits to the bacteria−host mutualism, in favor of disease prevention or health improvement.

In a recent study carried out by our group and comprising three different interventions (red wine, dealcholized red wine, and gin), it was found that the daily consumption of red wine polyphenol for 4 weeks by human subjects significantly increased the number of *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium*, *Bacteroides uniformis*, *Eggerthella lenta*, and *Blautia coccoides−Eubacterium rectale* groups in fecal samples compared to baseline.[21] In addition, it was found that positive changes in cholesterol and C-reactive protein concentration were linked to changes in the *Bifidobacterium* number.

Because of the impact of regular wine consumption on the modulation of the microbiota composition and its correlation with some biomarkers of health effect, in the present work fecal samples were chosen to study the profile of microbial phenolic metabolites derived from red wine consumption and compared to that from samples collected after dealcoholized red wine and gin consumption. For this purpose, a powerful UPLC-ESI-MS/MS method was validated and used for the screening of more than 60 metabolites in fecal samples.

■ MATERIALS AND METHODS

Standards and Reagents. Phenolic compounds targeted in this study ($n = 60$) are listed in Table 1-OSM in the Supporting Information. Standards of mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, and cinnamic acids were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), Phytolab (Vestenbergsgreuth, Germany), or Extrasynthèse (Genay, France). Valerolactone standards (5-(3′,4′,-dihydroxyphenyl)-γ-valerolactone and 5-(4′-hydroxyphenyl)-γ-valerolactone) were previously synthesized.[22] Signals of 4-hydroxyvaleric acids (4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric, 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid, 4-hydroxy-5-(phenyl)valeric acid and 4-hydroxy-5-(3′,4,′5′-trihydroxyphenyl)-valeric acid), 5-(3′,4,′5′-trihydroxyphenyl)-γ-valerolactone and other metabolites (dihydroresveratrol, 1-(3′,4′,5′,-trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol, 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol) were also considered. Acetic acid was purchased from Scharlau (Barcelona, Spain). Acetonitrile (HPLC grade) was purchased from Labscan (POCH S.A, Gliwice, Poland). Ultrapure water was obtained using a Milli-Q system (Waters Millipore, Milford, MA, USA).

Stock solutions of phenolic standards (250 $\mu g\ mL^{-1}$) were prepared by exact weighing of the analytes and dissolution with acetonitrile/water (1:4, v/v). Diluted solutions of 5 and 50 $\mu g\ mL^{-1}$ were prepared to optimize the MS/MS parameters. According to their response, analytes were classified in five different groups[7] (from highest to lowest response), and a stock standard pool solution was prepared by weighing individual compounds to achieve the following starting concentrations: 25, 50, 100, 200, and 500 $\mu g\ mL^{-1}$. Other solutions were prepared via serial dilutions and used in the generation of the calibration curves (11 different calibration levels from 10- to 10000-fold dilutions of the initial pool solution).

Design of the Human Intervention Study. A randomized, crossover, controlled intervention study was performed to study the influence of moderate red wine intake on the gut microbiota.[21] The study involved eight healthy adults (ages ranging from 45 to 50 years). The participants were not receiving treatment for diabetes, hypertension, or dyslipidemia, nor did they have any acute or chronic inflammatory diseases, infectious diseases, viral infections, cancer, or a previous cardiovascular event at study entry. They had not received any antibiotic therapy, prebiotics, probiotics, symbiotics, or vitamin supplements or any other medical treatment influencing intestinal microbiota during the 3 months before the start of the study or during the study (including the washout period). The study was divided into four consecutive periods: an initial washout period of 2 weeks (baseline) during which the participants did not consume any alcohol or red wine, followed by three consecutive periods of 20 days during which the participants drank only dealcoholized red wine (272 mL/day), red wine (272 mL/day), or gin (100 mL/day).

Red wine and dealcoholized red wine used in this study were produced with the Merlot grape variety, from the Penedès appellation (Spain). The dealcoholized red wine had the same composition and polyphenolic compounds as the red wine, except for the ethanol (only 0.42%).[21]

Each participant provided four different fecal samples: a first baseline sample after the washout period and a sample at the end of each 20 day period. The participants were asked not to change their dietary pattern and lifestyle habits during the study. The subjects were asked to avoid alcoholic beverages during the study. All of the participants gave written informed consent.

Sample Collection and Preparation of Fecal Solutions. Fecal samples were stored at −80 °C awaiting analysis. For preparation of fecal solutions, samples were thawed at room temperature and weighed (1.0 g) in 15 mL sterile conical tubes. Ten milliliters of sterile saline solution (NaCl 0.9%, Fresenius Kabi, Spain) was added and vortexed and centrifuged (10 min, 10000 rpm, 4 °C) two times. The supernatant (fecal water) was filtered (0.22 μm) and diluted with acetonitrile (1:4, v/v, acetonitrile/fecal water). Two microliters of sample was injected into the chromatographic system.

Analysis of Phenolic Metabolites in Fecal Solutions. Phenolic metabolites were analyzed by UPLC-ESI-MS/MS.[20] The liquid chromatographic system was a Waters Aquity UPLC (Milford, MA, USA) equipped with a binary pump, an autosampler thermostated at 10 °C, and a heated column compartment (40 °C). The column employed was a BEH-C18, 2.1 × 100 mm, and 1.7 μm particle size from Waters. The mobile phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient program was as follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium time was 2.4 min, resulting in a total run time of 18 min. The flow rate was set constant at 0.5 $mL\ min^{-1}$, and the injection volume was 2 μL.

The LC effluent was pumped to an Acquity TQD tandem quadrupole mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N_2) flow rate, 750 L/h; cone gas (N_2) flow rate, 60 L/h. The ESI was operated in negative mode. The MS/MS parameters (cone voltage and collision energy) of each analyte were initially optimized by direct infusion experiments using 10 $\mu g\ mL^{-1}$ solutions at a flow rate of 5 $\mu L\ min^{-1}$, and the most sensitive transition (precursor and product ions) was selected for quantification purposes using the multiple reaction monitoring (MRM) mode. Apart from the phenolic standards, signals of valeric acids (4-

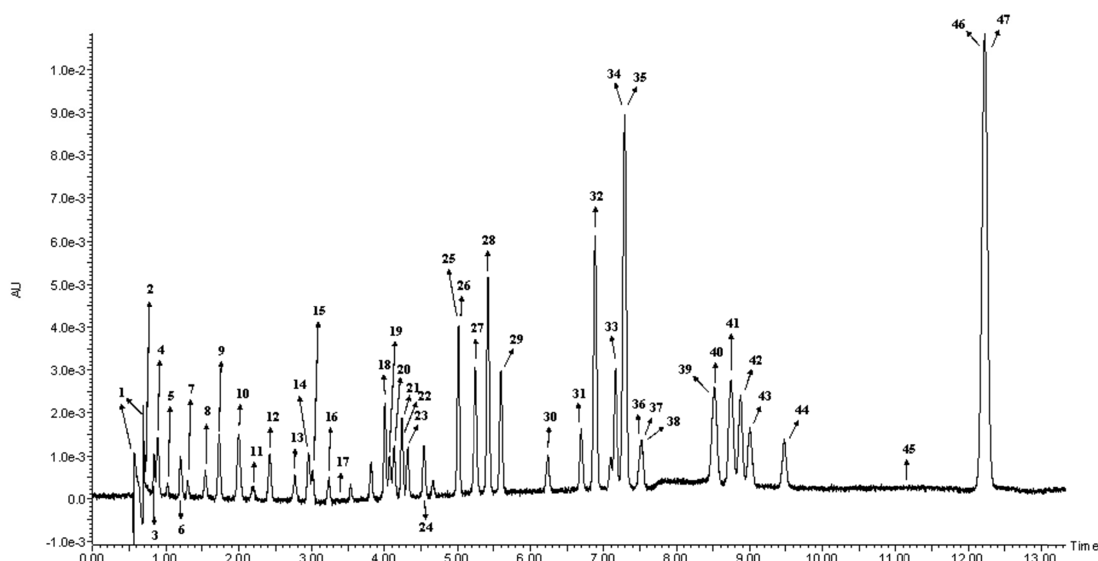

Figure 1. UPLC-DAD chromatogram of a solution of phenolic standards (n = 47). Peaks: (1) 3,4-dihydroxymandelic acid; (2) phloroglucinol; (3) 4-hydroxymandelic acid; (4) gallic acid; (5) pyrogallol; (6) 4-hydroxy-3-methoxymandelic acid; (7) 3-hydroxymandelic acid; (8) 3,5-dihydroxybenzoic acid; (9) protocatechuic acid; (10) 3-hydroxy-4-methoxymandelic acid; (11) 4-hydroxyhippuric acid; (12) catechol/pyrocatechol; (13) 3,4-dihydroxyphenylacetic acid; (14) 3-*O*-methylgallic acid; (15) 4-hydroxybenzoic acid; (16) 4-*O*-methylgallic acid; (17) mandelic acid; (18) 4-hydroxyphenylacetic acid; (19) 3-(3,4-dihydroxyphenyl)propionic acid; (20) 3-hydroxybenzoic acid; (21) hippuric acid; (22) caffeic acid; (23) vanillic acid; (24) 3-hydroxyphenylacetic acid; (25) syringic acid; (26) 4-hydroxy-3-methoxyphenylacetic acid; (27) 4-methylcatechol; (28) 3-(4-hydroxyphenyl)propionic acid; (29) *p*-coumaric acid; (30) 3-(3-hydroxyphenyl)propionic acid; (31) ferulic acid; (32) *m*-coumaric acid; (33) 3,4-dimethoxybenzoic acid; (34) benzoic acid; (35) isoferulic acid; (36) phenylacetic acid; (37) salicylic acid; (38) 3,4-dimethoxyphenylacetic acid; (39) 4-methoxyphenylacetic acid; (40) 4-methoxybenzoic acid; (41) 4-ethylcatechol; (42) 3,4,5-trimethoxybenzoic acid; (43) 3-methoxybenzoic acid; (44) 3-(3,4-dimethoxyphenyl)propionic; (45) phenylpropionic acid; (46) 3,4,5-trimethoxycinnamic acid; (47) *trans*-cinnamic acid.

hydroxy-5-(3′,4′-dihydroxyphenyl)valeric, 4-hydroxy-5-(3′-hydroxyphenyl)valeric, and 4-hydroxy-5-(phenyl)valeric acids) were optimized using samples from in vitro fermentations of flavan-3-ols.[7] These latter metabolites were quantified using the calibration curves of 3-(3,4-dihydroxyphenyl)propionic, 3-(3-hydroxyphenyl)propionic acids, and propionic acids, respectively. 5-(3′-Hydroxyphenyl)-γ-valerolactone was quantified using the calibration curve of 5-(4′-hydroxyphenyl)-γ-valerolactone. Data acquisition and processing were realized with MassLynx 4.1 software.

Statistical Analysis. The statistical methods used for the data analysis were as follows: t test for dependent samples and its corresponding nonparametric Wilcoxon matched pairs test to evaluate differences in means of the content of phenolic acid metabolites in feces between two levels of the four interventions (baseline, dealcoholized red wine, red wine, and gin); and principal component analysis (PCA), from correlation matrix, to examine the relationships between analyzed variables and samples. A value of P = 0.05 was fixed for the level of significance of the tests. The Statistica program for Windows, version 7.1 (StatSoft Inc., 1984–2006, www.statsoft.com), was used for data processing.

RESULTS AND DISCUSSION

Validation of the UPLC-ESI-MS/MS Method for Analysis of Phenolic Metabolites in Fecal Solutions. Ultrahigh-pressure LC improves chromatographic resolution, speed, and sensitivity and, when coupled to MS, facilitates rapid and high-throughput analysis. Therefore, a UPLC column was used, and the choice for the mobile phase was adopted from a previous method developed in our laboratory.[20] The chromatographic optimization allowed the separation, within 15.5 min, of the phenolic standards selected for this work, including phenols and mandelic, benzoic, hippuric, phenylacetic, phenylpropionic, valeric, and cinnamic acids (Figure 1).

Flow injection analysis of the studied compounds was performed by directly injecting individual standard solutions into the source operated in negative mode. The deprotonated molecular ion $[M - H]^-$ was selected as the precursor ion in all cases. Further identification of the most abundant product ions and selection of the optimum collision energies for each analyte were carried out in the product ion scan mode. Table 1-OMS in the Supporting Information reports the MS/MS parameters (MRM transitions, cone voltages, and collision energies) for the 60 phenolic metabolites targeted. These compounds were selected on the basis of previous studies reported in the literature, availability, analogous chemical structures, etc.

The method validation was carried out in terms of linearity, precision and accuracy, and limits of detection (LOD) and quantification (LOQ). Calibration curves were prepared according to the method of external standard at 11 different concentration levels in triplicate. According to their response, five different starting concentrations were employed (25, 50, 100, 200, and 500 μg mL^{-1}), and dilutions from 1:10 to 1:10000 were made. Weighted least-squares regression analysis was applied. As expected from the differences observed in MS responses, the linear concentration range largely varies between the different analytes, even between the ones included in the same calibration range. LOD and LOQ were determined by injection of the pool standard dilutions and following the criterion of signal-to-noise ratio (S/N) ≥3 and ≥8, respectively. The accuracy and precision study was made by using the 1:100 dilution of the initial stock

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Table 1. Mean ± Standard Deviation Values ($n = 8$) of the Microbial Phenolic Concentration (Micrograms per Gram) in Human Feces of the Studied Subjects at Baseline and after the Three Treatments

compound	baseline (washout period)	dealcoholized red wine	red wine	gin
benzoic acids				
gallic acid	ND[a]	0.364 ± 0.825	0.266 ± 0.445	0.121 ± 0.224
3,5-dihydroxybenzoic acid	0.010 ± 0.029	0.046*[b] ± 0.056	0.150* ± 0.189	0.026 ± 0.028
protocatechuic acid	0.277 ± 0.270	0.554 ± 0.408	1.080* ± 1.020	0.565 ± 0.300
3-*O*-methylgallic acid	ND	0.134* ± 0.145	0.293* ± 0.273	0.082 ± 0.165
4-hydroxybenzoic acid	0.227 ± 0.218	0.267 ± 0.197	0.447 ± 0.361	0.270 ± 0.219
3-hydroxybenzoic acid	0.061 ± 0.068	0.219 ± 0.293	0.249 ± 0.429	0.082 ± 0.121
vanillic acid	0.018 ± 0.034	0.151* ± 0.170	0.343* ± 0.363	0.063 ± 0.093
syringic acid	ND	0.495* ± 0.592	1.270* ± 1.390	0.461 ± 0.654
benzoic acid	47.344 ± 47.317	69.735 ± 79.125	93.000 ± 114.132	33.761 ± 31.376
salicylic acid	0.206 ± 0.450	0.069 ± 0.094	0.093 ± 0.165	0.026 ± 0.055
phenylacetic acids				
4-hydroxyphenylacetic acid	1.900 ± 2.510	1.480 ± 1.270	2.040 ± 1.290	1.320 ± 0.560
3-hydroxyphenylacetic acid	1.340 ± 2.910	4.920 ± 6.590	6.150 ± 6.580	2.290 ± 3.670
phenylacetic acid	35.395 ± 23.871	37.508 ± 26.739	37.600 ± 9.772	31.872 ± 20.803
phenylpropionic acids				
3-(3,4-dihydroxyphenyl)propionic acid	0.016 ± 0.046	0.149 ± 0.234	0.107 ± 0.258	0.089 ± 0.173
3-(3-hydroxyphenyl)propionic acid	4.410 ± 10.600	1.140 ± 1.580	0.899 ± 0.886	0.828 ± 0.556
phenylpropionic acid	5.950 ± 5.260	16.700* ± 13.200	11.900* ± 4.200	9.450 ± 7.920
valeric acids				
4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid	ND	0.017 ± 0.033	0.069 ± 0.198	0.003 ± 0.008
4-hydroxy-5-(3′-hydroxyphenyl)valeric acid	0.041 ± 0.116	ND	1.170 ± 3.310	0.347 ± 0.728
4-hydroxy-5-(phenyl)valeric acid	6.270 ± 9.270	29.700* ± 30.600	31.400* ± 21.100	33.200 ± 49.400
cinnamic acids				
caffeic acid	0.265 ± 0.376	0.302 ± 0.178	0.605 ± 0.713	0.346 ± 0.293
p-coumaric acid	0.049 ± 0.032	0.415* ± 0.389	0.728* ± 0.573	0.347 ± 0.393
ferulic acid	0.557 ± 0.527	1.860 ± 2.380	2.200 ± 2.490	2.490 ± 4.350

[a]ND, not detected. [b]The asterisk indicates the mean value is significantly different from the baseline concentration ($p < 0.05$).

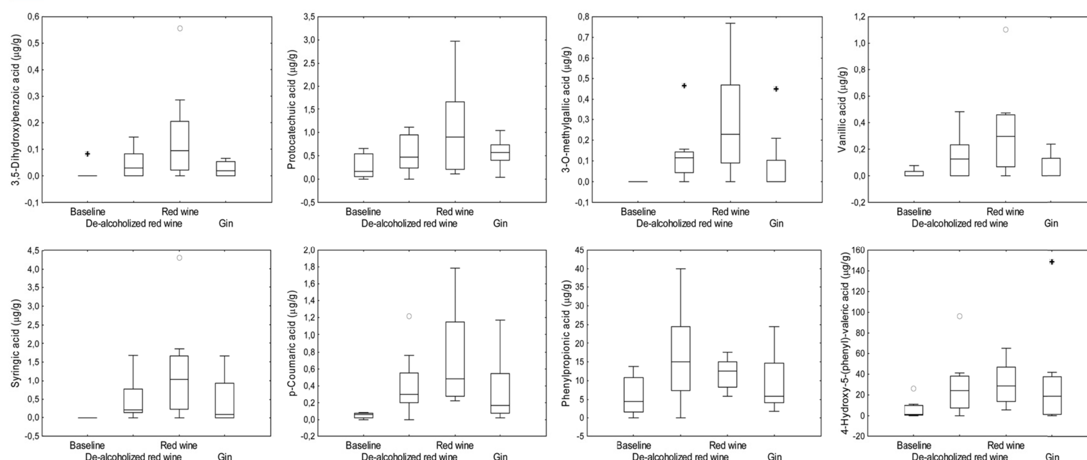

Figure 2. Box and whiskers plots (median, 25th and 75th percentiles, nonoutlier range) of the microbial metabolite content in feces (μg/g) at baseline and after dealcoholized red wine, regular red wine, and gin interventions. The outliers (o) and extremes (∗) are also included.

standard pool solution (0.25, 0.50, 1.0, 2.0, and 5.0 μg mL^{-1}), performing three different injections/day on three different days. The accuracy of the method (expressed as 100 times the mean observed concentration/added concentration) ranged from 80 to 126%. The precision (interday assay), expressed as the relative standard deviation (% RSD), was <15% in all cases. Results of the method validation (concentration range, linearity, LOD, LOQ, accuracy and precision) agreed with those from our previous publication.[20]

Analysis of Phenolic Acid Metabolites in Fecal Solutions. Fecal samples from the volunteers ($n = 8$) at baseline and after the three consecutive periods of beverage consumption (dealcoholized red wine, red wine, and gin) were analyzed with the described method. Results expressed as the

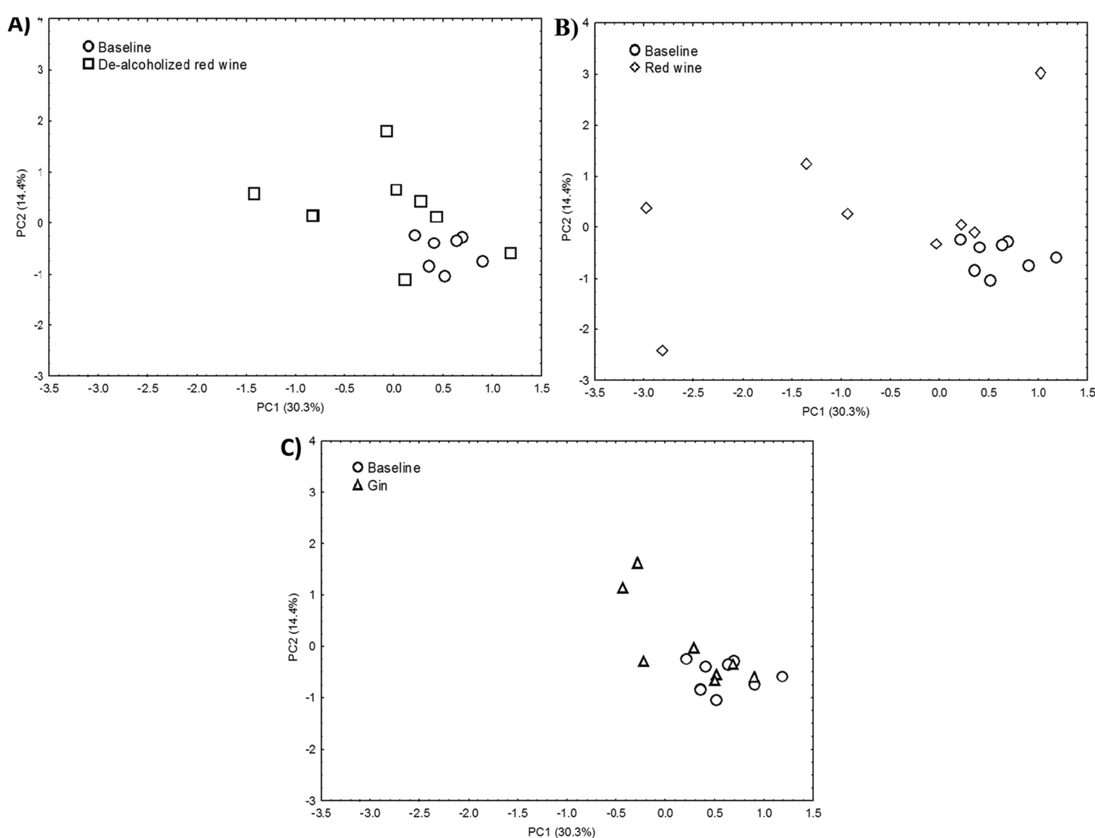

Figure 3. Representation of the samples in the plane defined by the first two principal components (PC1 and PC2) resulting from a PCA of both baseline and after dealcoholized red wine (A), red wine (B), and gin (C) interventions.

mean ± standard deviation (SD) are shown in Table 1. Among the microbial metabolites, benzoic acid, phenylacetic acid, phenylpropionic acid, 4-hydroxy-5-phenylvaleric acid, and ferulic acid were the most abundant metabolites of each group at baseline. In a study carried out by Jener et al.,[19] a concentration range of 15.6−149.5 mg/L of phenylacetic acid was found in fecal water (that is, the upper water layer after homogenization of feces in a stomacher), which is equivalent to a content of 10.1−97.2 μg/g of fresh feces considering an average fecal water content of 65%. These data are in line with our findings because the content of phenylacetic acid at baseline varied from 17.02 to 73.16 μg/g. In the same paper,[19] a concentration range of 0.85−42.8 μg/g was found for phenylpropionic acid, which is in accordance with the range of 3.16−13.75 μg/g obtained in the present work. The content of benzoic acid reported[19] is included within the range of 1.56−10.6 μg/g, considerably lower than the mean value obtained by us (47.3 μg/g). This is due to the high content of benzoic acid found for one of the volunteers of the study (142.8 μg/g), which substantially increased the mean value of this compound.

Both the *t* test for dependent samples and the Wilcoxon matched pairs test showed significant differences in phenolic content between the baseline and the red wine intervention for eight metabolites: 3,5-dihydroxybenzoic acid, 3-*O*-methylgallic acid, *p*-coumaric acid, phenylpropionic acid, protocatechuic acid, vanillic acid, syringic acid, and 4-hydroxy-5-(phenyl)valeric acid (Table 1). When comparing data at baseline and after dealcoholized red wine intervention, the *t* test and the Wilcoxon matched pairs showed significant differences for the content of 3,5-dihydroxybenzoic acid, 3-*O*-methylgallic acid, *p*-coumaric acid, phenylpropionic acid, vanillic acid, syringic acid, and 4-hydroxy-5-phenylvaleric acid (Table 1). Therefore, except for protocatechuic acid, the same metabolites were changed after the consumption of polyphenols in the form of either red wine or dealcoholized wine. Moreover, no significant differences were found for any of these metabolites when samples from the red wine and dealcoholized red wine interventions were compared (data not shown), indicating that the alcoholic matrix of wine does not seem to affect the profile of microbial metabolites and, thus, the bioavailabity and biotransformation of red wine polyphenols, as previously reported for resveratrol metabolites.[23] Finally, as expected, no significant differences were found in the content of any metabolite between samples at baseline and after gin intervention.

Figure 2 illustrates the box and whiskers plots (median, 25th and 75th percentiles, nonoutlier range) of the microbial metabolites mentioned above, which showed significant differences ($P < 0.05$) between the baseline and the red wine interventions. The outliers (*o*) and extremes (∗) values are also shown. Although, as mentioned above, differences between the

red wine and dealcoholized red wine interventions were not significant for these compounds, in all cases a slightly higher content was found after the red wine intervention compared to the dealcoholized red wine intervention, with the exceptions of phenylpropionic and 4-hydroxy-5-(phenyl)valeric acids. One of the main limitations of our study is that no washout period was carried out between interventions, so these findings could be due to a possible cumulative effect derived from the previous intervention.

On the basis of the proposed catabolic pathway of flavan-3-ols, 4-hydroxy-5-(phenyl)valeric acids are considered to arise from the first steps of the microbial degradation of flavan-3-ols as a result of the fission of the heterocyclic C-ring of the flavonoid molecule. Shortening of the side-chain length by subsequent β-oxidation reactions results in phenylpropionic, phenylacetic, and benzoic acid derivatives. Phenylpropionic acid usually results from the dehydroxylation of 3-(3,4-dihydroxyphenyl)propionic acid, which has been reported as one of the most abundant final microbial metabolites derived from flavan-3-ols, both in vivo and in vitro.[6,7,24] *O*-Methylated benzoic acids including syringic and vanillic acids could arise from the catabolism of red wine anthocyanins.[25−28] The B-ring of malvidin, the major anthocyanin in red wine, could be degraded into 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid), whereas peonidin could be degraded into 4-hydroxy-3-methoxybenzoic acid (vanillic acid). Cyanidin, a nonmethylated form, would result in 3,4-dihydroxybenzoic acid (protocatechuic acid). 4-*O*-Methylgallic acid is a potential urinary biomarker of red wine consumption, resulting from hepatic or renal metabolism of gallic acid,[29,30] which is first released from hydrolysis of grape seed galloylated flavan-3-ols.[7] Likewise, both 4-*O*- and 3-*O*-methylgallic acids have been reported as important urinary biomarkers of black tea consumption.[31] However, the presence of 3-*O*-methylgallic acid in fecal samples is most likely to arise from the catabolism of petunidin. Finally, *p*-coumaric acid could be formed from the hydrolysis of *p*-coumaroyl-acylated anthocyanins, which are also very abundant in red wine.[32]Therefore, metabolites significantly changing after the red wine interventions come from the catabolism of both flavan-3-ols and anthocyanins, the major flavonoids in red wine.

To summarize the changes in the phenolic metabolites as a consequence of moderate intake of red wine polyphenols, a PCA was applied. Figure 3 represents the plane defined by the first two principal components (PC1 and PC2) that resulted from the PCA using the data from samples at baseline and after dealcoholized red wine (A), red wine (B), and gin (C) interventions, respectively. The first principal component (PC1), explaining 30.3% of the total variance, was negatively correlated (loadings ≤ -0.7) with 3,5-dihydroxybenzoic acid, protocatechuic acid, 3-*O*-methylgallic acid, vanillic acid, syringic acid, and *p*-coumaric acid. The second principal component explained 14.4% of the total variance. Panels A and B of Figure 3 show that samples collected after the intake of either red wine or dealcoholized red wine could be easily differentiated from those at baseline. On the other hand, Figure 3C shows no differentiation between samples at baseline and after the gin intervention. Therefore, interindividual variability between subjects considerably increased as a result of the different wine interventions in comparison to baseline conditions, but not after the gin intervention, reflecting variations in the microbiota composition between subjects. These findings indicate that red wine polyphenols have a profound impact on the fecal metabolite profile. Although some connections have been made between certain bacterial clusters including *Actinobacteria* and *Clostridium* with metabolites arising from the first stages of microbial degradation of flavan-3-ols, such as phenylvalerolactones,[33] the association between changes in metabolic profile and microbial groups is still difficult to establish and would require in-depth studies at the metagenomic or transcriptomic levels.

In conclusion, the validated method allowed the analysis of 60 microbial phenolic metabolites in fecal solutions. Results obtained indicate that the microbial metabolic profile of feces is significantly modified after moderate intake of red wine polyphenols. Also important was the fact that the presence of alcohol in red wine did not seem to affect the performance of the microbial catabolism of polyphenols and, therefore, the potential bioavailability of these compounds. Metabolites derived from the catabolism of both flavan-3-ols and anthocyanins seem to contribute to explain changes in the fecal metabolic profile. Some of these metabolites may help to explain some of the numerous health benefits derived from red wine polyphenols, including the modulation of the microbial population reported in our previous study.[21] Although our results need to be further corroborated with a larger number of volunteers, our study showed that the regular consumption of red wine could affect the metabolic profile of feces and produce positive changes in the microbiota composition.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table 1-OSM. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Table 1-OSM. Retention times and MS/MS parameters for the investigated phenolic metabolites.

Compound	Rt (min)	MRM transition (m/z)	Cone voltage (V)	Collision energy (V)
Mandelic acids				
3,4-Dihydroxymandelic acid	0.57	183>137	32	14
4-Hydroxymandelic acid	0.86	167>123	30	12
4-Hydroxy-3-methoxymandelic acid	1.22	197>137	33	20
3-Hydroxymandelic acid	1.32	167>121	30	16
3-Hydroxy-4-methoxymandelic acid	2.01	197>137	30	24
Mandelic acid	3.40	151>107	27	8
Benzoic acids				
Gallic acid	0.91	169>125	33	15
3,5-Dihydroxybenzoic acid	1.57	153>109	30	12
Protocatechuic acid	1.76	153>109	30	14
3-*O*-methylgallic acid	2.98	183>168	33	12
4-Hydroxybenzoic acid	3.04	137>93	27	12
4-*O*-methylgallic acid	3.25	183>168	30	12
3-Hydroxybenzoic acid	4.16	137>93	30	18
Vanillic acid	4.34	167>152	30	12
Syringic acid	5.03	197>182	30	12
3,4-Dimethoxybenzoic acid	7.18	181>107	30	30
Benzoic acid	7.31	121>77	27	12
Salicylic acid	7.54	137>93	27	17
4-Methoxybenzoic acid	8.56	151>107	32	12
3,4,5-Trimethoxybenzoic acid	8.89	211>167	30	12
3-Methoxybenzoic acid	9.03	151>107	30	12
Phenols				
Phloroglucinol	0.77	125>83	33	12
Pyrogallol	1.04	125>79	36	16
Catechol/Pyrocatechol	2.45	109>81	44	12
4-Methylcatechol	5.27	123>108	35	14
4-Ethylcatechol	8.77	137>122	30	16
Tyrosol	3.85	137>106	35	15
Hippuric acids				
4-Hydroxyhippuric acid	2.22	194>100	27	11
Hippuric acid	4.26	178>134	30	10
Phenylacetic acids				
3,4-Dihydroxyphenylacetic acid	2.79	167>123	20	12
4-Hydroxyphenylacetic acid	4.03	151>107	24	6
3-Hydroxyphenylacetic acid	4.56	151>107	25	16
4-Hydroxy-3-methoxyphenylacetic acid	5.03	181>137	27	8
Phenylacetic acid	7.50	135>91	21	18
3,4-Dimethoxyphenylacetic acid	7.55	195>136	25	14
4-Methoxyphenylacetic acid	8.52	165>106	30	12
Phenylpropionic acids				
3-(3,4-Dihydroxyphenyl)-propionic acid	4.09	181>137	32	12
3-(4-Hydroxyphenyl)-propionic acid	5.45	165>121	33	12
3-(3-Hydroxyphenyl)-propionic acid	6.26	165>121	32	12
3-(3,4-Dimethoxyphenyl)-propionic acid	9.50	209>150	36	12
Phenylpropionic acid	11.14	149>105	33	10
Valeric acids				
4-Hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid	4.05	225>163	30	15
4-Hydroxy-5-(3'-hydroxyphenyl)-valeric acid	5.74	209 > 147	32	15
4-Hydroxy-5-(phenyl)-valeric acid	11.96	193 > 175	35	15
4-Hydroxy-5-(3',4,'5'-trihydroxyphenyl)-valeric acid	nd	241>179	30	15

Valerolactones				
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	5.48	207>163	40	18
5-(3′-hydroxyphenyl)-γ-valerolactone	7.96	191>147	35	18
5-(4′-hydroxyphenyl)-γ-valerolactone	7.05	191>147	35	18
5-(3′,4,′5′-trihydroxyphenyl)-γ-valerolactone	nd	223>179	40	18
γ-Valerolactone	2.31	101>55	25	7
Cinnamic acids				
Caffeic acid	4.26	179>135	35	16
p-Coumaric acid	5.62	163>119	30	14
Ferulic acid	6.72	193>134	30	16
m-Coumaric acid	6.91	163>119	30	18
Isoferulic acid	7.32	193>134	27	14
t-Cinnamic acid	12.24	147>103	27	12
3,4,5-Trimethoxycinnamic acid	12.26	237>103	30	18
Other metabolites				
Dihydroresveratrol	nd	227>185	40	20
1-(3′,4′,5′,-Trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol	nd	307>263	33	13
1-(3′,4′-Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol	5.35	291>247	33	13

nd- not determined

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El estudio del perfil metabólico del resveratrol tras un consumo regular y moderado de vino tinto en comparación con el mismo vino desalcoholizado junto con la optimización del método analítico, sirvieron como medida de cumplimiento de la intervención en los trabajos presentados en la presente Tesis Doctoral al tratarse del mismo ensayo clínico, donde se colaboró en el análisis de muestras.

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Gut and microbial resveratrol metabolite profiling after moderate long-term consumption of red wine *versus* dealcoholized red wine in humans by an optimized ultra-high-pressure liquid chromatography tandem mass spectrometry method

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A B S T R A C T

Resveratrol exerts a variety of biological and pharmacological activities, which are observed despite its extremely low bioavailability and rapid clearance from the circulation due to extensive sulfation and glucuronidation in the intestine and liver. In order to more accurately quantify all known resveratrol metabolites, a sensitive and optimized analytical assay was developed and validated by pure standards. Methodology improvements aimed to the chromatographic detection of disulfates and sulfoglucuronides, improving resolution of sulfates, by using a buffered solution, with recovery values of resveratrol and its metabolites, even of sulfates, of 99%. The adapted methodology was then applied to a clinical study with high cardiovascular risk subjects, after the moderate consumption of red wine (RW) or dealcoholized red wine (DRW) for 28 days. Up to 21 resveratrol metabolites, including those formed by gut and microbial metabolism, were identified in 24-h urine samples. Interestingly, after long-term consumption of RW and DRW, resveratrol metabolite concentration significantly increased in urine with no differences between the two interventions, indicating that bioavailability and biotransformation of resveratrol is not affected by the alcoholic matrix of wine. In summary, we established a sensitive analytical assay for the quantification of a wide resveratrol metabolic profile in human urine, also regarding gut microbial-derived metabolites, which may also be applied to blood and tissue samples. The resveratrol metabolic pattern might therefore act as an excellent marker for the efficacy of resveratrol in clinical and epidemiological studies for the study of the beneficial effects of grape product consumption. In this sense, having a more precise concentration value of all the resveratrol metabolites in target tissues would finally lead to a better interpretation of the obtained results.

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1. Introduction

Resveratrol (RV) is a stilbene mainly consumed through grape and grape products, such as red wine [1]. Although several studies have already demonstrated the RV ability to protect against several diseases [2], it has aroused some controversia due to its low bioavailability [3], since it is rapidly metabolized after its oral absorption [4]. Indeed, free RV found in plasma samples constituted less than 2% of the total RV consumed [5], and therefore it is mainly the metabolic forms which will reach the target tissues. Thus, more studies are necessary in order to investigate the biological mechanisms for which RV or its metabolites could exert

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a biological activity. Actually, scarce information is now available regarding the possible benefits of these metabolites [6–9], which mainly focuses on the sulfate conjugates. Prior to studying the possible beneficial effects of RV metabolites, development of highly sensitive and selective techniques is necessary for their identification and quantification in biological samples. Several liquid chromatography–mass spectrometry (LC–MS) techniques have already been validated for the analysis of RV metabolites [10–16], and although it is now widely accepted that RV is mainly metabolized to glucuronide and sulfate conjugates [3], including several isomers of mono and di-conjugates [10–12,17,18], less information is now available regarding piceid metabolism [11,19], as well as those metabolites derived from gut microbial action, represented by dihydroresveratrol (DHR) and its phase II metabolites [19–21]. Standards of these metabolites are rarely available and their quantification in biological samples is made by expressing results as aglycone equivalents [10–12], which is useful as a first approach, but this is open to error, since metabolite ionization in LC-ESI–MS techniques could be different from their aglycones. Several studies attempted to avoid this known technical drawback by measuring RV metabolites indirectly after enzymatic hydrolysis [20,22,23] or with diode-array detection (DAD) [17,24]. However, other drawbacks are shown using these techniques, such as the low efficiency of enzymatic hydrolysis, as well as losing all information of the metabolic profile, and the low sensitivity for DAD. Studies on the RV metabolic profile need more sensitive methodologies only achieved by LC-ESI–MS techniques, which are being used not only for RV metabolites [10–12] but also for other polyphenols [25,26], despite their quantification drawback. Nowadays, some purified standards of RV metabolites, along with the labeled C^{13}-resveratrol are already commercially available, allowing an accurate quantification of these compounds in biological samples; so, data from clinical and epidemiological studies could be more precise. Moreover, other drawbacks in chromatography, bearing in mind the different chemical characteristics of the high number of determined and identified metabolites, need to be minimized. It is known that while aglycone, glucuronided and glucosided metabolites of RV and other polyphenols are well resolved when reverse analytical columns are used, other metabolites, such as sulfate conjugates, show poor chromatographic behavior [21]. Although in our previous methodology [10] we solved this problem with the addition of more apolar mobile phase, such as acetone, the determination of disulfate metabolites was not evaluated. Thus, analytical solutions are required in this aspect.

Therefore, the main aim of this study is to validate a methodology for the analysis of RV metabolites in urine samples, through optimization of solid-phase extraction (SPE) and ultra-performance liquid chromatography (UPLC) analysis, using the recent available standards of glucuronide and sulfate conjugates and gut microbial metabolites. This methodology was applied to a randomized, crossover, controlled clinical trial, where volunteers with cardiovascular risk factors consumed, for one month, a daily moderate dose of RW or the same dose of dealcoholized RW (DRW) in order to investigate a possible alcoholic matrix effect on the bioavailability of a detailed RV metabolic profile, which will be accurately quantified with the corresponding standards.

2. Materials and methods

2.1. Standards and reagents

All samples and standards were handled with no exposure to light. Standards of *trans*-RV (99% purity) and *trans*-3,4′,5-trihydroxystilbene-3-β-D-glucopyranoside (*trans*-piceid) (97% purity) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and taxifolin (>90% purity) from Extrasynthese (Genay, France).

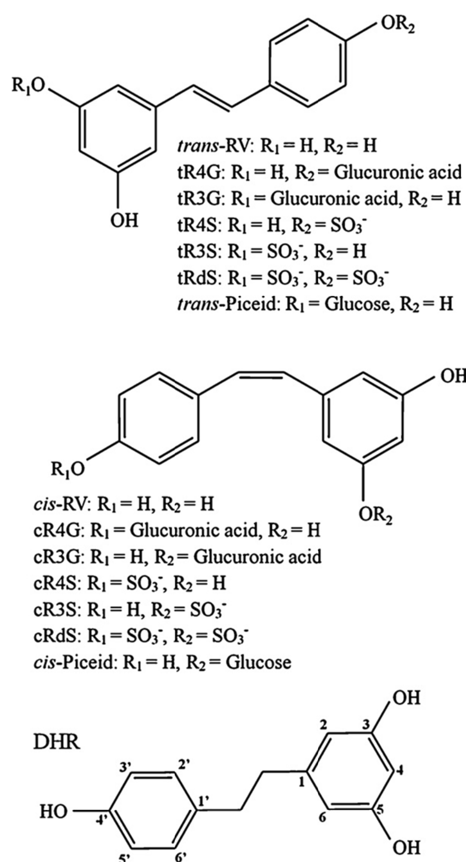

Fig. 1. Chemical structures of RV and its metabolites identified using the available commercial standards.

Standards of *cis*-RV (97% purity), *trans*- and *cis*-resveratrol-3-*O*-glucuronide (tR3G and cR3G, respectively) (98% purity each), *trans*- and *cis*-resveratrol-4′-*O*-glucuronide (tR4G and cR4G, respectively) (98% and 96% purity, respectively), *trans*-resveratrol-3-*O*-sulfate (tR3S) (98% purity) and *trans*-RV-$^{13}C_6$, to be used as internal standard, were acquired from Toronto Research Chemicals Inc. (North York, ON, Canada). *trans*-Resveratrol-4′-*O*-sulfate (tR4S) (>95% purity) and *trans*-resveratrol-3,4′-*O*-disulfate (tRdS) (>91% purity) were obtained as reported previously [27]. DHR aglycone (80% purity) was synthesized following the work by Thakkar et al. [28] (see Section 2.2), using palladium on charcoal and celite, which were purchased from Sigma–Aldrich (St. Louis, MO, USA). Standards were prepared as 80% (v/v) methanol stock solutions.

Methanol, ethyl acetate and acetonitrile (ACN) of LC grade and ammonium acetate (>99%) were purchased from Scharlau Chemie, S.A. (Sentmenat, Spain). LC grade solvents glacial acetic acid, acetone and ammonia (35%) were purchased from Panreac Quimica, S.A.U. (Castellar del Vallès, Spain) and the deuterated dimethyl sulfoxide (DMSO-d_6, 99.96% deuterated) from Euriso-top, SAS (Cedex, France). Ultrapure water (MilliQ) was obtained from Millipore (Bedford, MA, USA) and blank human urine from volunteers after one week of polyphenol-free diet.

Table 1
MRM transitions and optimized parameters of collision energy (CE) and declustering potential (DP).

Analyte	MRM transitions	Identified by	CE	DP
Mobile phase A: 0.05 mL/L acetic acid				
trans-RV	227/185	STD	−30	−55
cis-RV		STD		
tR4G	403/227	STD	−40	−50
tR3G		STD		
cR4G		STD		
cR3G		STD		
trans-Piceid	389/227	STD	−25	−50
cis-Piceid		Isomerized STD		
Pic-G	565/227	PIS		
DHR	229/123	STD	−25	−45
DHR-G	405/229	PIS		
Mobile phase A: ammonium acetate 10 mM				
tR4S	307/227	STD	−30	−50
tR3S		STD		
cR4S		Isomerized STD		
cR3S		Isomerized STD		
tR34dS	387/227	STD	−20	−35
cR34dS		Isomerized STD		
RV-SG	483/307	PIS	−20	−35
Pic-S	469/227	PIS	−25	−50
DHR-S	309/229	PIS	−25	−45
DHR-SG	485/309	PIS		
t-RV-$^{13}C_6$ (IS)	233/191	STD	−25	−55
Taxifolin (IS)	303/285	STD	−25	−50

2.2. Synthesis and structural identification of DHR

For DHR synthesis, 100 mg of *trans*-RV were dissolved in ethanol (120 mL) and hydrogenated at 40 psi in the presence of 10% palladium on charcoal for 24 h. The solution was filtered through celite to remove the catalyst and was evaporated to dryness. DHR structure was confirmed by nuclear magnetic resonance (^{1}H NMR) measurements using a Varian 400-MHz instrument VNMR System (Varian, Palo Alto, CA). Synthesized DHR was dissolved in deuterated DMSO (99.96% deuterated) using dinitrobenzene (1.6 mmol/L) as internal standard. The ^{1}H NMR spectrum was acquired with the following signals numbered according to Fig. 1: δ (ppm) 9.0 (broad band, 3H, —OH), 6.97 (doublet, 2H, J = 8.6 Hz, H-2′,6′), 6.63 (doublet, 2H, J = 8.6 Hz, H-3′,5′), 6.03 (doublet, 2H, J = 4.0 Hz, H-2,6), 6.00 (double doublet, 1H, J = 4.0 Hz, H-4), 2.68–2.56 (multiplet, 4H, —CH_2—CH_2—). Standard purity was calculated following the validated protocol by Malz and Jancke [29], obtaining 80% purity, for quantifying purposes. Only one peak was detected after a full-scan MS experiment (*m*/*z* 229) and no peaks of the parent compound RV were detected, since 97% purity related to the starting material was shown.

2.3. Intervention beverages

RW and DRW of the Merlot variety (Penedes appellation) were selected for its large amount of RV and piceid, in comparison to other red varieties [30]. The phenolic composition of both wines was analyzed throughout the study period [31–33] and no significant differences were obtained for any phenolic compound between RW and DRW (Supplementary Table S1).

2.4. Human experimental design

A total of 73 high-risk subjects with high cardiovascular risk, aged ≥55 years, were recruited for a randomized, crossover, controlled clinical trial [34], although in this work, we analyzed available urine from 59 included subjects to study the metabolic profile of RV. Subjects were firstly asked to follow a 15-day run-in period in which they consumed neither grape-derived products nor alcoholic beverages and, after that, they were requested to consume 272 mL of RW (30 g ethanol/day) or DRW every day for 4 weeks, following the same background diet. Twenty-four hour urine samples were collected on the last run-in period day and on the last day after each intervention period. Aliquots were immediately stored at −80 °C until analysis, after noting the corresponding total excreted volume.

This trial was registered in the Current Controlled Trials at the International Standard Randomized Controlled Trial Number Register, at controlled-trials.com, as ISRCTN88720134.

2.5. Sample extraction

RV metabolites were extracted from urine samples by SPE as previously described [10] with slight modifications. Briefly, 1 mL of urine with 100 μL of *trans*-RV-$^{13}C_6$ as internal standard (1.71 μmol/L) was loaded onto a preconditioned Waters Oasis® HLB 96-well SPE plate (30 mg) (Milford, MA, USA). After washing the plate, elution of RV metabolites was achieved with 0.5 mL of 1 mol/L acetic acid in methanol, 2 × 0.5 mL of 1 mol/L acetic acid in ethyl acetate and 0.5 mL of ammonia 5% (v/v) in methanol. The eluate was evaporated to dryness and then reconstituted with a solution of 100 μL of taxifolin (1.64 μmol/L) as secondary internal standard diluted in acidified water (0.5 mL/L acetic acid) with 10% organic mobile phase.

2.6. Chromatography optimization

The mobile phase has a significant influence on the ionization efficiency. Therefore, mobile phases were examined in order to optimize chromatographic conditions for RV metabolites, using the available pure standards (1 mg/L) in MRM mode. These mobile phases included: 0.5 mL/L acetic acid [11], and a buffered solution with ammonium acetate (10 mmol/L) at different pH (3, 4 and 5),

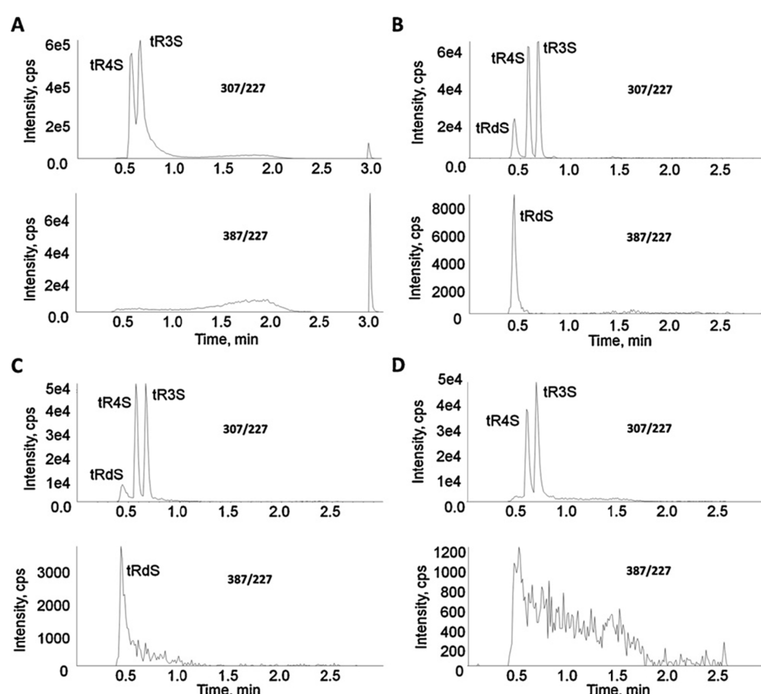

Fig. 2. Representative MRM chromatograms of tR3S (peak 5 – Fig. S1) and tR4S (peak 6 – Fig. S1) (MRM 307/227) and tRdS (peak 9 – Fig. S1) (MRM 387/227) standards, using (A) 0.5 mL/L acetic acid, (B) ammonium acetate 10 mmol/L at pH 5, (C) ammonium acetate 10 mmol/L at pH 4 and (D) ammonium acetate 10 mmol/L at pH 3.

as the aqueous solvent A and acetone:ACN (70:30, v/v) as solvent B in gradient elution (see Section 2.7). Ammonium acetate, along with formate salt, has already been used for the determination of RV-sulfates [13,27,35] as well as for other sulfate metabolites [36,37]. Acetic acid 10% (v/v) was used to adjust the mobile phase at different pH values.

2.7. UPLC–MS/MS analysis

The analysis of RV metabolites in urine samples was carried out by UPLC coupled to tandem mass spectrometry (UPLC–MS/MS) adapted from a previous validated methodology [10]. A Waters Acquity UPLC system (Milford, MA, USA), equipped with a binary solvent manager and a refrigerated autosampler plate, was used to couple an AB Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray, ionizing in negative mode. An Acquity UPLC BEH C18 (Milford, MA, USA) analytical column (1.7 μm, 2.1 mm × 5 mm), maintained at 40 °C, was used for chromatographic separation at a flow rate of 1 mL/min, split (1:1) before MS analysis. Injection volume was 5 μL. Two different aqueous mobile phases A were used: 0.5 mL/L acetic acid and then ammonium acetate 10 mmol/L at pH 5. Mobile phase B consisted of acetone:ACN (70:30). A linear gradient profile was applied with the following proportions (v/v) of phase A [t(min),%A]: (0,90); (1,70); (2,0); (2.3,0); (2.31,90); (3,90). MS and MS/MS parameters were optimized in infusion experiments for each RV metabolite standard (Table 1) in the corresponding mobile phase A/B (1:1) at a flow rate of 5 μL/min in a mass spectrometer using a model 11 syringe pump (Harvard Apparatus, Holliston, MA), with the general parameters of collision cell exit potential (−15), focusing potential (−200 V), entrance potential (−10 V) and capillary voltage (−3500 V) obtained.

The availability of pure standards of RV phase II metabolites allows their identification by comparing the retention time at the corresponding MRM transitions. Other RV and piceid phase II metabolites, along with the gut microbial ones, were identified by product ion scan (PIS) (Supplementary Figs. S1 and S2) [19]. For quantitative analysis, calibration curves in blank human urine were constructed with available standards subjected to the same SPE procedure as the samples, using the MRM transitions shown in Table 1. When the metabolite standard was not available, concentrations were estimated using the most similar compound standard curve and expressed as their equivalents (Table 3). A commercial standard of cR3G was not available when urine samples were analyzed and, thus, they were quantified using a cR4G standard curve, although afterwards cR3G was validated for this method. After quantification, values under LOQ were considered as the LOD value and values under LOD were considered zero.

2.8. Assay validation

Selectivity, linearity, detection and quantification limits, recovery, accuracy and precision of the available standards were evaluated for the optimized method, according to the Food and Drug Administration (FDA) [38]. The selectivity of the method was assessed by analyzing blank human urine in order to ensure that there were no interferences at the same retention time and MRM transition for the analytes. Five calibration curves in blank human urine of all available RV metabolite standards were performed on three different analysis days for linearity evaluation at

Table 2
Limits of detection (LOD), recovery, accuracy and precision data ($n = 8$) obtained from the UPLC–MS/MS of all available standards in blank human urine for each chromatographic method.

Analyte	LOD (μg/L)	Recovery (mean ± SD, %)	Added Conc. (μg/L)	Calculed Conc. (mean, μg/L)	Accuracy (%)	Precision (RSD, %)
Mobile phase A: 0.5 mL/L acetic acid						
trans-RV	0.48 ± 0.03	105.1 ± 11.5	50.0	54.1	108.18	6.81
			500.0	506.1	101.21	9.30
			1000.0	974.4	97.44	7.68
cis-RV	0.55 ± 0.04	101.6 ± 8.4	51.8	53.5	102.86	9.31
			518.0	482.0	93.04	8.66
			1036.0	960.5	92.71	11.54
tR4G	0.76 ± 0.10	101.2 ± 5.1	50.0	54.9	109.50	0.39
			500.0	546.5	109.30	0.39
			1000.0	958.0	95.80	1.18
tR3G	1.41 ± 0.20	98.7 ± 7.2	51.2	51.4	100.76	4.04
			512.0	473.0	92.38	7.90
			1024.0	970.3	94.76	7.94
cR4G	1.75 ± 0.20	102.0 ± 6.4	51.5	50.3	96.79	8.69
			515.0	482.6	93.72	7.84
			1030.0	988.5	95.98	10.14
cR3G	1.53 ± 0.15	96.1 ± 9.1	50.5	48.6	95.28	6.22
			505.0	497.6	98.53	12.32
			1011.0	1063.6	105.20	12.12
trans-Piceid	0.42 ± 0.05	96.5 ± 5.0	50.0	47.8	95.51	8.63
			500.0	523.6	104.72	9.21
			1000.0	1002.0	100.20	10.57
DHR	0.77 ± 0.03	96.1 ± 5.4	51.6	51.3	98.67	9.13
			516.0	531.7	103.05	5.83
			1032.0	1004.0	97.29	10.32
Mobile phase B: ammonium acetate 10 mM						
tR4S	0.53 ± 0.05	97.4 ± 10.0	50.0	54.4	108.81	8.27
			500.0	523.4	104.68	5.55
			1000.0	954.7	95.47	9.05
tR3S	0.36 ± 0.04	95.6 ± 9.9	50.0	51.5	103.04	6.04
			500.0	546.1	109.23	5.19
			1000.0	981.4	98.14	9.19
tR34dS	0.62 ± 0.09	100.5 ± 6.4	53.3	49.7	93.74	10.08
			533.0	510.1	95.70	6.11
			1065.0	1043.3	97.96	10.97
trans-Piceid	0.85 ± 0.10	–	50.0	49.8	99.53	12.09
			500.0	486.2	97.23	6.06
			1000.0	919.4	91.94	11.03
DHR	1.05 ± 0.13	–	51.6	51.3	98.68	12.42
			516.0	495.0	95.93	7.48
			1032.0	904.9	87.68	9.60

eight concentration points from 1 to 1000 μg/L. Detection limit was defined as the analyte concentration that produced a signal-to-noise ratio of at least 3. The lowest concentration with accepted precision and accuracy criteria for each standard on the calibration curve was the lower limit of quantification (LLOQ). Three different concentrations (low, medium and high) of each available standard were used to evaluate their precision and accuracy ($n = 8$). The latter was obtained as the percentage of the ratio between the mean calculated concentration and the known added concentration and precision as its relative standard deviation. The same concentration levels were used to perform the extraction recovery, for which the standards were spiked to blank human urine before extraction and compared to the standards spiked to extracted blank human urine.

Validation parameters were evaluated for *trans*- and *cis*-RV, the available four glucuronide isomers, *trans*-piceid and DHR using the chromatographic method with 0.5 mL/L acetic acid as mobile phase A, while for sulfate and disulfate conjugates the validation procedure was performed using the mobile phase A consisting of ammonium acetate 10 mmol/L. Standards of *trans*-piceid and DHR were also validated for the ammonium acetate 10 mmol/L mobile phase A, since their calibration curves were used to quantify their respective sulfate and sulfoglucuronide (SG) conjugates.

2.9. Statistical analysis

As urinary RV data were skewed (Kolmogorov and Levene tests) and the natural logarithm of the variable did not normalized the data, comparisons between interventions were performed using the nonparametric Friedman test and the paired Wilcoxon test. IBM SPSS Statistics software, version 20 (Chicago, IL), was used to perform the statistical analysis. Statistical significance was defined as $P \leq 0.05$.

3. Results and discussion

3.1. Method optimization for sulfate metabolites

The availability of purified standards of RV metabolites, and specifically tRdS, has allowed some improvements of the previously validated methodology for the analysis of the RV metabolic profile

Table 3
24-h urinary excretion (mean ± SEM) of individual metabolites at baseline (BAS) and after regular consumption of dealcoholized red wine (DRW) and red wine (RW).

Metabolites	Urinary excretion (nmol/24 h)		
	Baseline	DRW	RW
RV phase II metabolites			
trans-RV	n.d.	n.d.	n.d.
cis-RV	n.d.	n.d.	n.d.
tR4G	85.82 ± 28.36*	838.11 ± 259.78	390.58 ± 145.55
tR3G	89.58 ± 24.70*	192.88 ± 29.34	192.69 ± 29.72
cR4G	53.59 ± 15.97*	487.35 ± 84.54	449.89 ± 60.64
cR3G[a]	292.35 ± 97.22*	2410.23 ± 267.05	2304.85 ± 231.88
tR4S	80.33 ± 61.95*	140.56 ± 77.24	91.08 ± 47.40
tR3S	55.83 ± 25.32*	488.62 ± 69.93	591.88 ± 93.27
cR4S[b]	180.43 ± 62.57*	1044.86 ± 152.15	931.97 ± 143.70
cR3S[c]	83.34 ± 41.40*	891.18 ± 132.27	753.16 ± 132.13
tR34dS	47.63 ± 16.78*	414.45 ± 58.92	418.60 ± 53.37
cR34dS[d]	n.d.	n.d.	n.d.
RV-SG[d]	35.35 ± 19.36*	211.14 ± 30.73	170.17 ± 22.37
Sum of the total RV phase II metabolites	1004.26 ± 252.75*	7119.38 ± 658.16	6294.88 ± 620.00
RV glucosides			
trans-Piceid	0.00 ± 0.00*	2.94 ± 0.53	2.63 ± 0.55
cis-Piceid[e]	2.26 ± 0.92*	14.71 ± 1.78	17.84 ± 2.41
Pic-G[e]	3.80 ± 1.52*	29.64 ± 2.97	31.80 ± 4.55
Pic-S 1[e]	6.91 ± 2.44*	50.46 ± 6.11	54.68 ± 7.06
Pic-S 2[e]	4.39 ± 1.56*	44.86 ± 5.08	39.42 ± 4.01
Sum of the total RV glucosides	17.36 ± 5.87*	142.60 ± 13.23	146.38 ± 13.40
Gut microbial metabolism			
DHR	1.46 ± 1.01*	18.08 ± 2.47	20.27 ± 3.56
DHR-G 1[f]	7.94 ± 3.29*	92.18 ± 8.03	73.16 ± 8.95
DHR-G 2[f]	58.21 ± 22.06*	500.81 ± 43.85	455.99 ± 42.34
DHR-S 1[f]	515.83 ± 166.71*	3286.05 ± 279.89	2799.04 ± 254.89
DHR-S 2[f]	144.81 ± 27.51*	839.04 ± 97.16	681.17 ± 106.40
DHR-SG[f]	49.75 ± 17.43*	333.12 ± 34.09	300.42 ± 34.22
Sum of the total gut microbial metabolism of RV	778.00 ± 222.87*	5069.28 ± 383.77	4330.04 ± 362.33

Changes in excretion values in response to the intervention treatment were determined by Wilcoxon non-parametric test for 2 related samples. n.d., not detected.
[a] Expressed as cR4G equivalents.
[b] Expressed as tR4S equivalents.
[c] Expressed as tR3S equivalents.
[d] Expressed as tRdS equivalents.
[e] Expressed as *trans*-piceid equivalents.
[f] Expressed as DHR equivalents.
* $P < 0.05$, comparing baseline to DRW and RW interventions.

[10,11], including also a reduction of time analysis with UPLC. The poor chromatographic behavior previously reported for RV-sulfates [21] was improved using ammonium acetate (10 mmol/L) as mobile phase. In our previous publication we improved sulfate metabolite resolution using a ternary mobile phase B with acetone [10], but disulfate metabolites were not previously considered. In fact, the use of a buffered solution in chromatography analysis allowed the identification of tRdS, since it was not observed when 0.5 mL/L acetic acid was used (Fig. 2A). Certainly, ammonium acetate improves the separation and enhances peak shapes of sulfate conjugates, avoiding adsorption and tailing previously shown [3]. MRM analysis of metabolites (Fig. 2) supports these improvements, since monosulfate conjugates provided a signal-to-noise ratio around 7-fold higher for tR4S and tR3S, respectively, with ammonium acetate at pH 5 compared with 0.5 mL/L acetic acid (Fig. 2A and B). Regarding pH values, mobile phase at pH = 5 provided signal-to-noise ratios from 9- to 20-fold higher for tRdS and around 7-fold higher for tR4S and tR3S, compared to mobile phases at pH 4 and 3. Moreover, the higher the pH value, the better the resolution of sulfate peak shapes shown (Fig. 2). However, the signal-to-noise ratios for aglycones (*trans*- and *cis*-RV and DHR), as well as their glucoside (*trans*-piceid) and glucuronide conjugates, was up to 4-fold higher with 0.5 mL/L acetic acid mobile phase, compared to the buffered solution. Bearing in mind these differences in chromatography resolution, both aqueous mobile phases were used for sample analysis, since low chromatographic times were achieved with UPLC. Regarding the benefits of ammonia solution for sulfate detection, we added a final stage of the elution process in the SPE with 5% ammonium hydroxide in methanol, obtaining recoveries from 96% to 105% for all studied standards (Table 2).

3.2. Quality parameters of the method

Selectivity of the method was evaluated in blank human urine and no interference peaks were observed at the same retention time of the metabolites. The five calibration curves were determined by weighted ($1/x^2$) least-square regression analysis, and linearity over the concentration range was studied by obtaining correlation coefficients for all analytes higher than 0.994, with accuracy values ranging from 85 to 115%. Detection limit for each standard ranged from 0.36 to 1.75 μg/L (Table 2), and the lower limit of quantification (LLOQ) was 5 μg/L, which evinced the great sensitivity achieved by the analytical method. Results for accuracy and precision, displayed in Table 2, met the acceptance criteria of the FDA [38]. Thus, results obtained with this new adapted methodology will be repetitive and exact at low, medium and high concentrations, since accuracy values were from 88 to 111%, with mean variations in precision up to 12%. Mean recovery values for all

the compounds were 99% (Table 2), with 95% for the RV isotope (*trans*-RV-$^{13}C_6$), which was used as internal standard.

3.3. Identification of metabolite profiling of RV

Up to 15 RV and derivates were unequivocally identified using the available commercial standards, including also *cis*-isomers of RV sulfates and piceid, which were identified by isomerization of the *trans*-isomer standard solutions after 30 min in the sunlight [11] (Table 1). Although *cis*-resveratrol-3,4′-*O*-disulfate (cRdS) was also identified after isomerization of the *trans*-standard, it was not found in any sample after RW or DRW consumption. Also, neither *trans*- nor *cis*-RV were found in urine samples, as was previously reported [1,17], due to their extensive metabolism [39].

The presence of other metabolites, whose standard were not yet available, was also studied through MS/MS experiments. We identified up to 9 RV metabolites using the PIS (Supplementary Figs. S1 and S2): RV-SG, piceid-glucuronide (Pic-G), two piceid-sulfates (Pic-S), two DHR-glucuronides (DHR-G), two DHR-sulfates (DHR-S) and a DHR-SG. Other metabolites such as RV-diglucuronides (MRM 579/403), DHR-diglucuronides (MRM 581/229) and DHR-disulfates (MRM 389/229) were also sought, although any peak could be confirmed in PIS experiments.

Piceid and its phase II metabolites were also previously detected in human samples [11,19] and in rats [15,40,41], indicating that RV glucoside might be absorbed in its intact form, conjugated and finally excreted in urine. Gut microbial metabolism of RV was observed with the presence of DHR and its glucuronide and sulfate conjugates, as already published in some previous works in humans [19–21], in rats [14,15,42] and in pigs [43] after RV or RW administration. However, it should be borne in mind that this is the first work linking all the resveratrol derivates and their metabolites in a unique metabolic profile of up to 21 compounds, considering resveratrol and piceid phase II metabolites and also those obtained by microbial action.

3.4. Quantitative expression of RV metabolites

With the recent commercial availability of RV metabolites, an accurate quantification of these compounds in biological samples could be achieved. In this work, we have been able to quantify 6 RV phase II metabolites with the corresponding standard: tR4G, tR3G, cR4G, tR4S, tR3S and tRdS. phase II metabolites of RV have already been studied, with glucuronide and sulfate forms being the most reported ones [3,39], although many studies measured them indirectly as free aglycone after enzymatic hydrolysis [20–23], or directly expressing results as aglycone equivalents [10–12]. In this work, the effect of quantifying a RV metabolite with its corresponding standard or as aglycone equivalents has been evaluated in the urine samples of this study (Fig. 3). Statistical differences between both quantification methods were obtained for the evaluated metabolites, with higher excretion values using aglycone equivalents, except for tR4G (Fig. 3). However, while only 0.7–1.2-fold higher values were achieved for glucuronide conjugates, amounts of sulfate metabolites were more than 8-fold higher when expressed as aglycone equivalents. Thus, sulfate conjugates seem to be greatly overestimated compared to glucuronides, when quantified as RV equivalents.

Despite all these reported differences, a wide RV metabolic profile of up to 21 compounds was considered, with 8 of those accurately quantified as the real metabolite and 13 expressed as equivalents of the most similar compound (Table 3), since commercial standards are not yet available. It would now be very interesting to have these standards available, in order to finally achieve a more accurate concentration of the whole metabolic profile of RV and its relation to the beneficial effects of grape product consumption. To

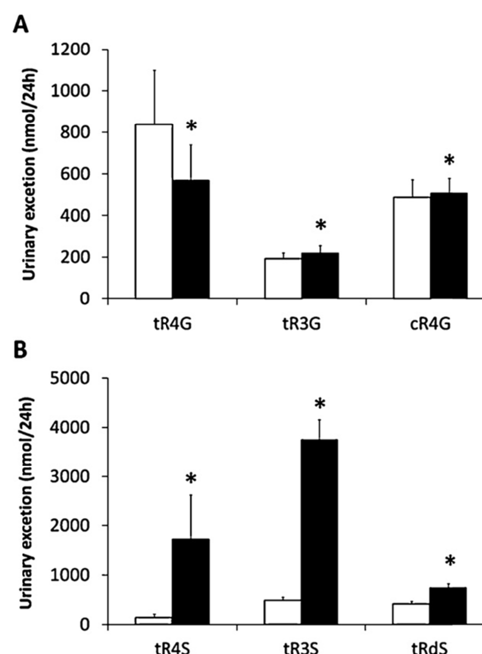

Fig. 3. Mean excreted amounts (and SEM) of RV glucuronides (A) and RV sulfates (B) accurately quantified with their corresponding standard (white boxes) or as RV equivalents (black boxes) after the consumption of DRW. *$P < 0.05$ when comparing both quantification expressions (Wilcoxon test).

our knowledge, few studies have been performed on the possible biological activity of RV metabolites [6–9] and, although the *in vitro* activity of these metabolites has been shown to be less effective than that observed for RV [6,8,9], it is thought that, *in vivo*, enzymes could release the aglycone [7], which exerts its biological activity.

3.5. Effect of alcohol on the bioavailability of RV

Statistical analysis of 24-h urinary excretion of all the RV metabolites showed significant differences between baseline period and both interventions ($P < 0.001$, Friedman test). In fact, these differences in the excreted amounts of all the metabolites studied were significantly higher after a regular consumption of RW and DRW compared with values obtained after the run-in period (Table 3) ($P < 0.05$). Increments on excreted RV metabolites after RW consumption were previously observed [10,44,45] and, indeed, Zamora-Ros et al. described urinary glucuronide and sulfate conjugates of RV as biomarkers of moderate wine intake [44,45].

Interestingly, when the 24-h excretion values of volunteers after 4 weeks of moderate RW and DRW consumption were compared, no differences were obtained for any individual metabolite or for the sum of RV phase II metabolites, RV glucosides and those of gut microbial metabolites (Table 3) ($P > 0.05$). Therefore, excretion of RV metabolites, and thus RV bioavailability, would not be influenced by the alcoholic matrix of wine. Previous reports showed that alcohol from RW could improve polyphenol absorption by increasing their solubility [46], as was reported for quercetin in *in vitro* and *in vivo* studies [22,47]. However, different conclusions are reached in the few *in vivo* human studies focused on the bioavailability of RV related to alcohol matrix effects. In one of the first RV bioavailability studies in humans [22], pure *trans*-RV was

dissolved in three different matrices considering absence/presence of alcohol – white wine, grape juice and vegetable juice – resulting in an equivalent absorption of this polyphenol either in aqueous or alcoholic matrices. These results are in line with those obtained by Ortuño et al. [20], who compared pharmacokinetics of RV after wine or grape juice administration, obtaining similar values for *trans*-RV when samples were hydrolyzed. Moreover, another study concluded that the combined intake of 2000 mg of *trans*-RV with 500 mg of quercetin and 5% of alcohol (100 mL) did not improve RV absorption [48]. The same results were obtained for other polyphenols, such as catechin, malvidin-3-glucoside, caffeic acid and production of 4-O-methylgallic acid when interventions with RW and DRW were compared [49–51]. It is important to note that the above-mentioned studies on RV bioavailability only looked for RV aglycone, as itself or after enzymatic hydrolysis, with their results being in line of those obtained in this study. Thus, the utility of this method to give a global value for the resveratrol metabolism, largely used in clinical and epidemiological studies [34,45,52], might not be discarded, since similar results were obtained not only for the individual compounds but also for the total sum. However, the importance of the study of the large metabolic profile must be highlighted, since different concentration values of RV metabolites could reach target tissues, and thus they could have different capacities to exert their possible biological activity.

4. Conclusions

Although several studies have already demonstrated the RV ability to protect against several diseases, its low bioavailability makes them quite controversial. Thus it is important to have a sensitive and accurate methodology for the resveratrol analysis in biological samples, providing a complete profiling of the metabolism, including those formed by gut and microbial metabolism, in order to elucidate their possible biological activities. In this work, the analysis of RV metabolite profiling has been optimized, specifically for disulfate and sulfate metabolites, improving their chromatographic resolution and their extraction, as well, by using ammonium solutions. The improved methodology using combined mobile phases to consider the different chemical characteristics and analytical behavior of all the compounds constituting the metabolic profile has been validated for 11 RV derivates in a wide concentration range, with the recent availability of pure standards, which also allows an accurate quantification of biological samples. Furthermore, the study of the metabolic profile of RV in humans has been extended to a total of 21 metabolites in order to get the most comprehensive metabolic profile of resveratrol described in the literature with the inclusion of tentative identification of piceid and gut microbial-derived metabolites, which have aroused a great interest in this field, due to the microbiota effect on polyphenols activity. The analysis of all these metabolites has been applied to a clinical study where RV metabolites were excreted in higher proportions after long-term consumption of RW and DRW, with no differences observed between the interventions. Thus, it seems that RV bioavailability would not be influenced by the alcoholic matrix of wine. Having a more precise concentration value of all the RV metabolites in target tissues would finally lead to a better interpretation of the obtained results from clinical and epidemiological studies for the study of the beneficial effects of grape product consumption, since it has been demonstrated that the resveratrol metabolic pattern might act as an excellent marker for the efficacy of resveratrol in such studies. However, not only a global sum of total metabolites could be provided, but also the individual excretion of all the metabolic profile and, thus, it would be achieved higher possibilities to know which compounds would possibly have higher biological activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2012.09.093.

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SUPPLEMENTARY DATA

Table S1. Phenolic composition of intervention beverages, Red Wine (RW) and dealcoholized RW (DRW), during the study period.

Phenolic compound (mg/L) [a]	**DRW**	**RW**	***P***
trans-RV [b]	2.73 ± 0.23	2.92 ± 0.36	0.352
cis-RV [b]	2.75 ± 0.15	2.79 ± 0.15	0.761
trans-Piceid [b]	10.53 ± 0.96	9.41 ± 1.12	0.160
cis-Piceid [b]	7.08 ± 0.87	7.71 ± 0.34	0.226
Gallic acid [c]	73.17 ± 7.01	68.48 ± 6.40	0.306
Protocatechuic acid [c]	5.85 ± 0.51	5.22 ± 0.62	0.246
Tyrosol [c]	47.81 ± 3.90	43.59 ± 4.73	0.298
Catechin [c]	126.45 ± 13.35	123.51 ± 11.30	0.786
Epicatechin [c]	70.57 ± 8.22	67.86 ± 7.74	0.699
trans-Caftaric [c]	19.21 ± 1.62	18.62 ± 1.44	0.595
trans-Caffeic [c]	12.18 ± 0.92	11.50 ± 0.79	0.246
trans-Coutaric [c]	5.62 ± 0.52	5.21 ± 0.45	0.182
2-S-Glutathionylcaftaric [c]	10.76 ± 1.26	10.30 ± 1.00	0.956
Quercetin-3-glucuronide [c]	11.25 ± 1.42	11.88 ± 1.38	0.770
Quercetin [c]	23.82 ± 2.37	26.66 ± 0.78	0.161
Isorhamnetin [c]	2.96 ± 0.14	3.34 ± 0.27	0.114
Delphinidin-3-glucoside [c]	14.71 ± 1.62	15.25 ± 0.89	0.589
Petunidin-3-glucoside [c]	12.04 ± 1.15	12.29 ± 1.06	0.755
Peonidin-3-glucoside [c]	6.68 ± 0.57	6.78 ± 0.62	0.797
Malvidin-3-glucoside [c]	49.86 ± 4.27	48.83 ± 4.45	0.787
Malvidin-(6-acetyl)-3-glucoside [c]	10.41 ± 1.20	10.97 ± 0.96	0.563
Malvidin-(6-coumaroyl)-3-glucoside [c]	3.54 ± 0.33	4.15 ± 0.27	0.066
Total Phenol (meqAG/L) [d]	2694.92 ± 86.79	2933.35 ± 377.31	0.426

[a] Values are expressed as Mean ± SD (n=5). [b]Analyzed following the work by Romero-Perez, et al. (Romero-Perez *et al.*, 1999). [c]Determined as previously described by Ibern-Gomez, et al. (Ibern-Gomez *et al.*, 2002). [d]Analyzed by Folin-Ciocalteu methodology (Singleton i Rossi, 1965)

Figure S1. Representative MRM trace chromatogram in urine samples after RW intake using different aqueous mobile phases: **A)** Acetic acid (0.05%): RV glucuronides (403/227): 1, tR4G, 2, tR3G, 3, cR4G, 4, cR3G; RV glucosides (389/227): 11, *trans*-piceid, 12, *cis*-piceid; 13, Pic-G (565/227); 16, DHR (229/123); 17, 18, DHR-G (405/229); and **B)** Ammonium acetate (10 mmol/L at pH 5): RV sulfates (307/227): 5, tR4S, 6, tR3S, 7, cR4S, 8, cR3S; 9, tRdS (387/227); 10, RV-SG (483/307); 14, 15, Pic-S (469/227); 19, 20, DHR-S (309/229) and 21, DHR-SG (485/309).

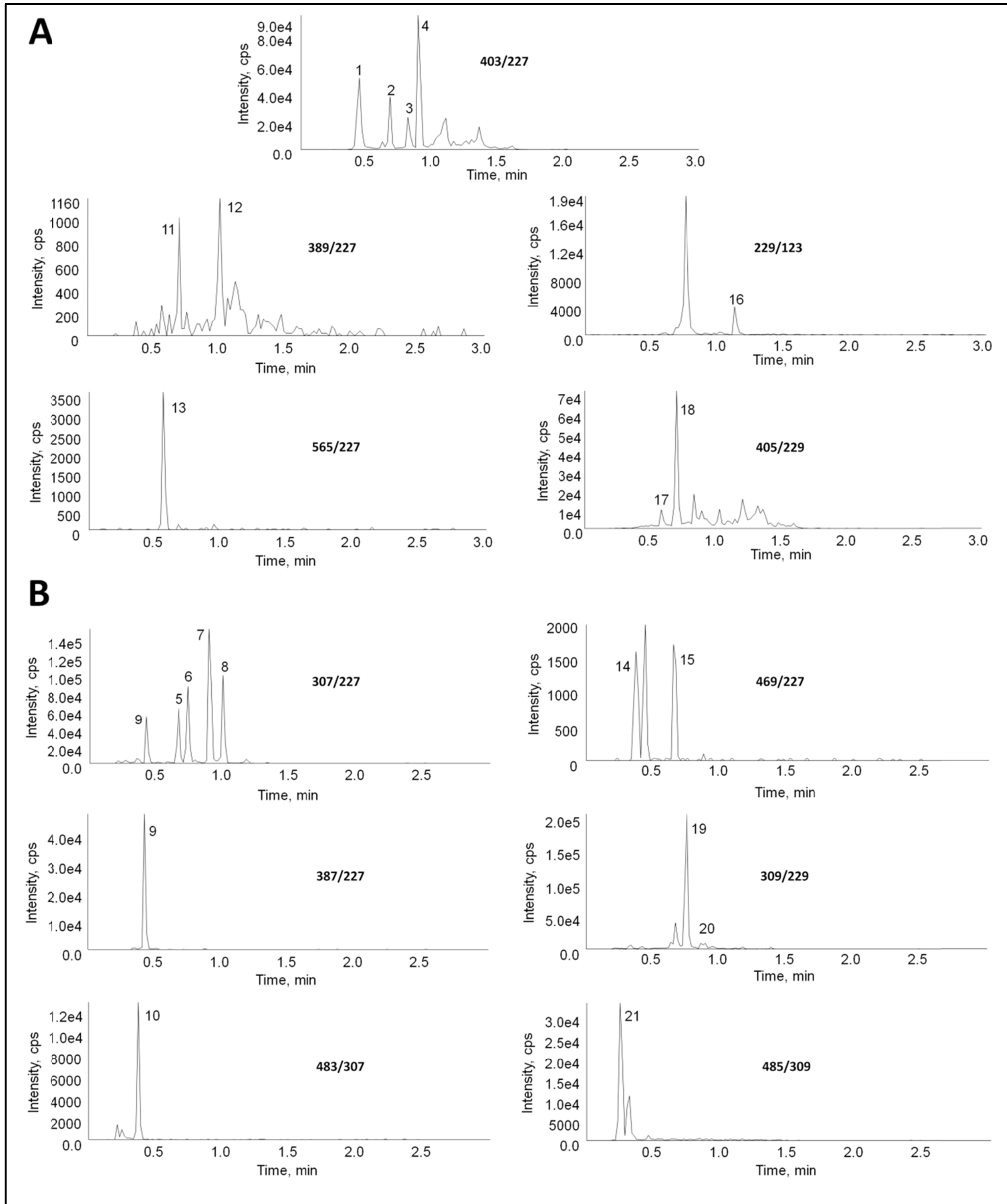


Figure S2. Product ion scan (PIS) of RV metabolites in urine samples after RW intake using the aqueous mobile phase of 0.05% acetic acid: A, Pic-G (peak 13, Figure S1, *m/z* 565) and B, DHR-G (peaks 17-18, Figure S1, *m/z* 405). Typical losses of the glucuronide moiety (-176 u) were shown, as well as *m/z* 175 and *m/z* 113, corresponding to the glucuronic acid moieties.

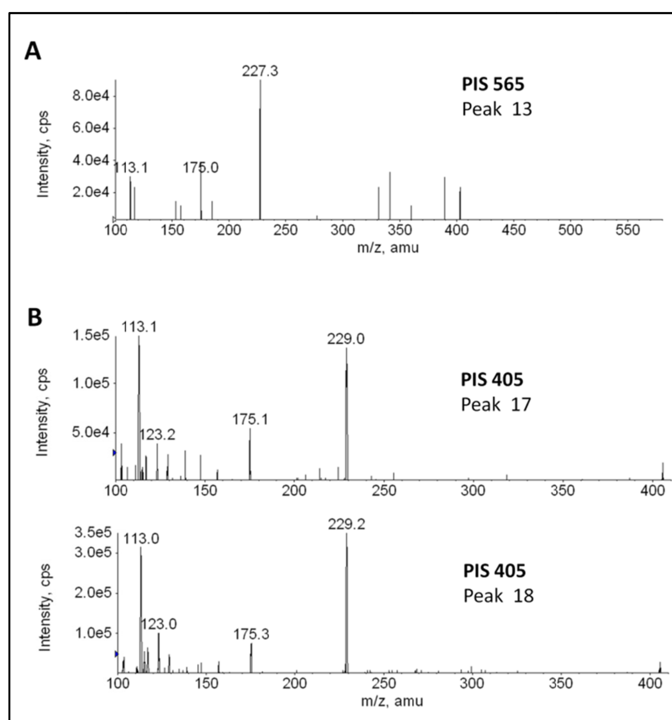
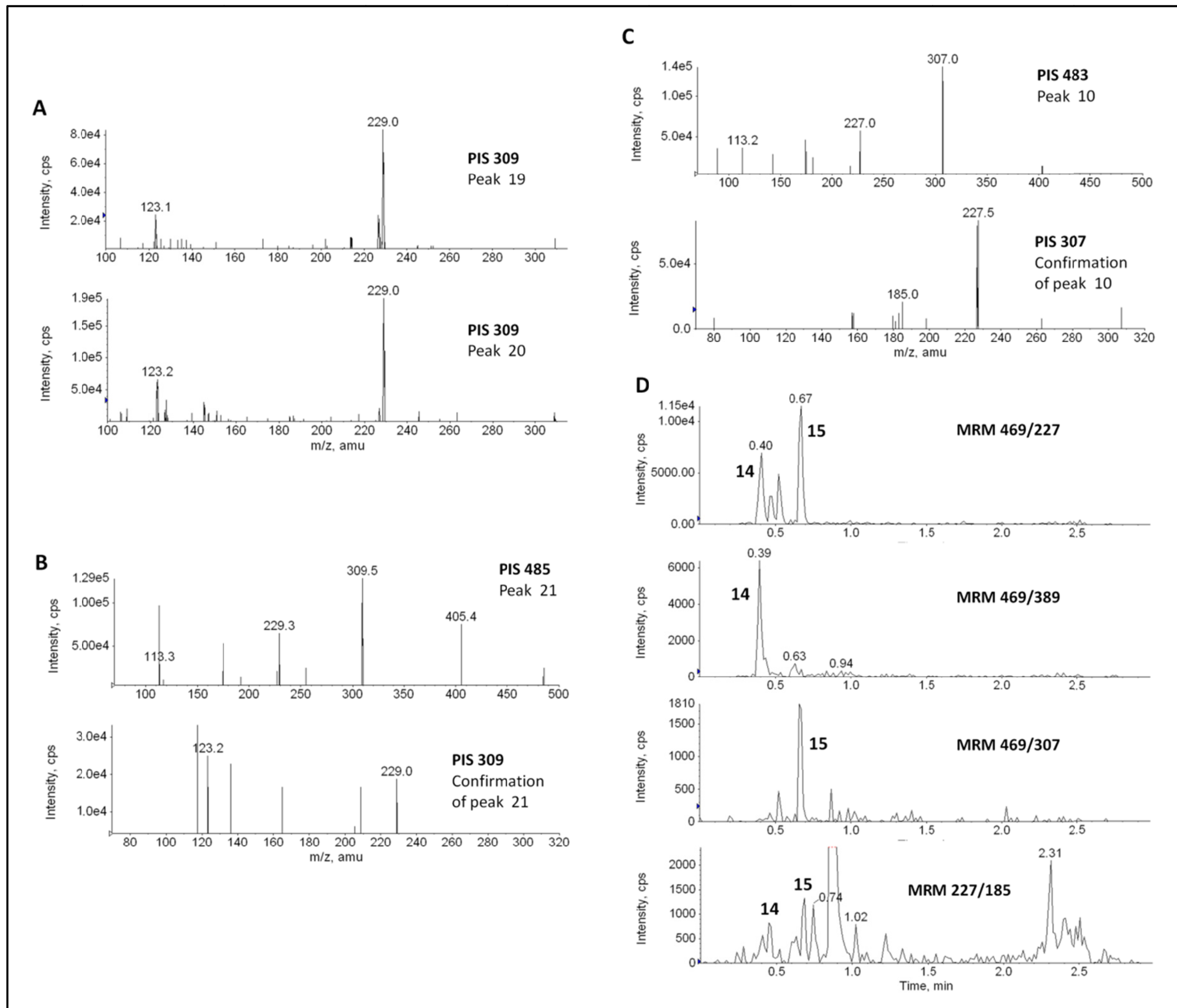


Figure S3. Product ion scan (PIS) of RV metabolites in urine samples after RW intake using the aqueous mobile phase of ammonuim acetate (pH 5), where the typical losses of the sulfate group (-80 u) could be shown: A, DHR-S (peaks 19-20, m/z 309); B, DHR-SG (peak 21, m/z 485) with its confirmation through the PIS of m/z 229 in CID-MS/MS experiment, and C, RV-SG (peak 10, m/z 483) with its confirmation through the PIS of m/z 227 in CID-MS/MS experiment. Pic-S (peaks 14-15, m/z 469) (D) were identified and confirmed by MRM experiments due to the low concentrations in urine samples.



Publicación V: Andres-Lacueva C, Macarulla MT, Rotches-Ribalta M, Boto-Ordóñez M, Urpi-Sarda M, Rodríguez VM, Portillo MP. Distribution of resveratrol metabolites in liver, adipose tissue, and skeletal muscle in rats fed different doses of this polyphenol. *J Agric Food Chem,* 2012; 60: 4833-40.

En este trabajo se estudia la distribución de los metabolitos de resveratrol en hígado, tejido adiposo y músculo esquelético de ratas alimentadas con diferentes dosis de este polifenol. El estudio del metabolismo del resveratrol fue de gran ayuda en el transcurso de la Tesis Doctoral, para mejorar el conocimiento de la metodología de extracción en fase sólida y la cromatografía acoplada a espectometría de masas.

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Distribution of Resveratrol Metabolites in Liver, Adipose Tissue, and Skeletal Muscle in Rats Fed Different Doses of This Polyphenol

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ABSTRACT: This study aimed to characterize resveratrol metabolite profiles in liver, skeletal muscle, and adipose tissue in rats treated for 6 weeks with 6, 30, or 60 mg of *trans*-resveratrol/kg body weight/d. Resveratrol metabolites were quantified by liquid chromatography−tandem mass spectrometry. The greatest number of metabolites was found in liver followed by adipose tissue. A great number of metabolites in muscle was below the limit of detection. The amounts of sulfate conjugates tended to increase when resveratrol dosage was enhanced, while the glucuronide ones increased only between 6 and 30 mg/kg/d. Microbiota metabolites were detected in higher amounts than resveratrol conjugates in liver, while the opposite occurred in adipose tissue and muscle. So, the largest amounts of resveratrol metabolites were found in liver, intermediate amounts in adipose tissue, and the lowest amounts in muscle. Sulfate conjugates, but not glucuronides, showed a dose−response pattern. Microbiota metabolites were predominant in liver.

KEYWORDS: *resveratrol, glucuronide metabolites, sulfate metabolites, dihydroresveratrol, liver, adipose tissue, skeletal muscle*

INTRODUCTION

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a phytoalexin polyphenolic compound occurring naturally in various plants, including grapes, berries, and peanuts, in response to stress or as a defense mechanism against fungal, viral, bacterial infections and damage from exposure to ultraviolet radiation.[1]

Resveratrol has been reported to have several beneficial effects on health.[2] However, the efficacy of orally administered resveratrol depends on its absorption, metabolism, and tissue distribution, since it is quickly absorbed in the intestine, via simple intestinal transepithelial diffusion at a high rate (77−80%).[3,4] Active transport might occur as well, but this is only likely for resveratrol metabolites.[5] Most resveratrol undergoes rapid and extensive metabolism in enterocytes, before entering into the bloodstream, resulting in up to a 20-fold higher concentration of conjugates, and less than 1% resveratrol. Furthermore, it undergoes rapid first-pass metabolism in the liver.[6] Consequently, resveratrol bioavailability is very low and only a small proportion reaches plasma.[7] Besides this intestinal and hepatic metabolism, it has been proposed that bacterial metabolism should also be taken into account.[4] Several approaches that may increase the availability of resveratrol are under evaluation, such as dose escalation studies,[8] as well as repeated or long-term dosing, which might result in saturation of metabolism, leading to higher plasma and tissue levels of resveratrol.[4]

In vitro studies have suggested that concentrations in the range of 5−50 μM are needed to find significant changes in cells.[9−14] Taking into account the low bioavailability of orally administered resveratrol, the doses usually administered in in vivo studies are unlikely to furnish resveratrol levels sufficiently compatible with those that modulate biological actions in vitro.[15,16] A great number of bioavailability studies have shown this.[6−8,17] Nevertheless, beneficial effects of resveratrol under in vivo conditions have been reported. This may be due to the fact that some resveratrol metabolites show biological activities[18] or due to the potential conversion of resveratrol metabolites back to resveratrol in target organs.[19]

Thus, the cause−effect relationship between resveratrol concentration in the systemic bloodstream and its reported biological effects is still a conundrum.[15] Consequently, it is essential (a) to establish whether resveratrol metabolites can conceivably contribute to, or account for, different effects of resveratrol in vivo and (b) to quantify resveratrol metabolites in vivo. Localization of tissue uptake, which determines target tissues, and the target/dose relationship are important to understand the positive health effects described for resveratrol.[20]

In this context, the present study aimed to characterize the resveratrol metabolite profile in liver, skeletal muscle, and adipose tissue in rats fed on obesogenic diets supplemented with resveratrol. Different doses of this polyphenol were used for supplementation in order to assess potential saturation of metabolic pathways responsible for transformation of resveratrol into metabolites. These tissues were chosen because they

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play a crucial role in the recently described body-fat-lowering effect of resveratrol. An interesting aspect of this study is that very few data have been published concerning metabolites found in two of these tissues, adipose tissue and skeletal muscle, as well as tissue distribution after a long-term treatment with resveratrol.

■ MATERIAL AND METHODS

Animals and Experimental Design. The experiment was conducted with 24 male Sprague−Dawley rats with an initial body weight of 180 ± 2 g purchased from Harlan Ibérica (Barcelona, Spain) and took place in accordance with the institution's guide for the care and use of laboratory animals (Reference protocol approval CUEID CEEA/30/2010). Animals from this cohort were previously used to study the potential body-fat-lowering effect of resveratrol.[21]

The animals were individually housed in polycarbonate metabolic cages (Techniplast Gazzada, Guguggiate, Italy) and placed in an air-conditioned room (22 ± 2 °C) with a 12-h day−night rhythm (light on at 21:00). After a 6-day adaptation period, the animals were fed on a commercial obesogenic diet supplied by Harlan Iberica (ref TD.06415) for 6 weeks. All animals had free access to food and water.

Before starting our animal studies with resveratrol, we analyzed the stability of this polyphenol when added into the diet in a reservoir protected from light. We found that resveratrol degraded almost completely over the feeding time.[21] Therefore, we concluded that mixing it in the diet, the most common method used to test the biological effects of a functional molecule intake, was not a suitable system. In a great number of studies performed by our group we have observed that the rats started eating the diet immediately when it was replaced every day at the beginning of the dark period. Taking advantage of this situation, we added resveratrol by using an ethanol solution (30 mg/mL) on the surface of the food reservoir. The amount of resveratrol solution added to the diet was adjusted on a daily basis according to the animal weight to reach the following doses: 6 mg resveratrol/kg body weight/d in RSV1 group, 30 mg resveratrol/kg body weight/d in RSV2 group, and 60 mg resveratrol/kg body weight/d in RSV3 group. These doses are equivalent to 68, 340, and 680 mg in a 70-kg person.[22] In order to avoid differences in the amount of ethanol received by each animal, ethanol was completed to reach 2 mL/kg body weight/d. Because a very small volume was added (0.36−0.69 mL for body weights ranging from 180 g, the initial body weight, to 345 g, the final body weight), the rats ate all the resveratrol provided in the first minutes of feeding period without any degradation. *trans*-Resveratrol (95% purity) was provided by Monteloeder (Elche, Spain) and added to the diet as explained above for 6 weeks.

Tissue Removal. At the end of the experimental period rats were fasted 12 h and sacrificed under anesthesia (chloral hydrate) by cardiac exsanguination. Subcutaneous white adipose tissue, gastrocnemious muscle, and liver were dissected, weighed, immediately frozen, and stored at −80 °C until analysis. Tissue removal was performed 24 h after resveratrol administration.

Standards and Reagents. All samples and standards were handled with no exposure to light. *trans*-Resveratrol (99% purity) and ethyl gallate, as internal standard (96% purity), were purchased from Sigma-Aldrich (St. Louis, MO). The external standard taxifolin (>90% purity) was purchased from Extrasynthese (Genay, France). Standards of *cis*-resveratrol (97% purity) and *trans*-resveratrol metabolites, i.e., *trans*-resveratrol-3-*O*-glucuronide (98% purity), *trans*-resveratrol-4′-*O*-glucuronide (98% purity) and *trans*-resveratrol-3-*O*-sulfate (98% purity), were acquired from Toronto Research Chemicals Inc. (North York, Canada). *trans*-Resveratrol-4′-*O*-sulfate and *trans*-resveratrol-3,4′-disulfate were obtained as reported previously for identification purposes.[23] Dihydroresveratrol aglycon was synthesized following the work by Thakkar et al.[24] Standards were prepared as stock solutions in 80% (v/v) methanol.

Liquid chromatography (LC) grade solvents methanol, ethyl acetate, acetonitrile, and ammonium acetate (>99%) were purchased from Scharlau Chemie, S.A. (Sentmenat, Spain). LC grade solvents glacial acetic acid, acetone, and ammonia (35%) were purchased from Panreac Quimica, SAU (Castellar del Vallès, Spain). Deuterated dimethyl sulfoxide (DMSO-d_6, 99.96% deuterated) was purchased from Euriso-top, SAS (Saint-Aubin Cedex, France). Ultrapure water (Milli-Q) was obtained from Millipore (Bedford, MA).

Structural Identification of Dihydroresveratrol. Dihydroresveratrol structure was confirmed by nuclear magnetic resonance (^{1}H NMR) measurements using a Varian 400-MHz instrument VNMR System (Varian, Palo Alto, CA). Synthesized dihydroresveratrol was dissolved in deuterated DMSO-d_6 using dinitrobenzene (1.6 mmol/L) as internal standard. ^{1}H NMR spectra were acquired with the following data: δ (ppm) 9.0 (br, 3H, −OH), 6.97 (d, 2H, J = 8.6 Hz, H-2′,6′), 6.63 (d, 2H, J = 8.6 Hz, H-3′,5′), 6.03 (d, 2H, J = 4.0 Hz, H-2,6), 6.00 (dd, 1H, J = 4.0 Hz, H-4), 2.68−2.56 (m, 4H, $-CH_2CH_2-$). Standard purity was calculated following the protocol validated by Malz and Jancke,[25] obtaining 80% purity. Only one peak was detected after a full scan MS experiment, and no peaks of the parent compound resveratrol (m/z 227) were detected.

Sample Preparation and Determination of Resveratrol Metabolites in Tissues. Analysis was carried out by LC−MS/MS after a solid-phase extraction (SPE), as described previously by Urpi-Sarda et al.[26] and Andres-Lacueva et al.[27] with some modifications. Briefly, ~100 mg of frozen samples was extracted three times with 1 mL of a solution of 1.5 M formic acid with 5% of methanol using a mixer mill (Retsch MM 400, Qiagen, Hilden, Germany) at 30 Hz for 0.5 min and centrifuged each time at 14 000 rpm. After this homogenization, the tissue structure was broken and metabolites linked to protein were released. Pooled supernatant fractions with the internal standard were loaded onto a preconditioned Waters Oasis HLB 96-well plate (30 mg). Acetic acid 2 M in water (1 mL) and in water/methanol (85/15 v/v) (1 mL) were used to wash the plate. Elution was achieved with 0.5 mL of 1 M acetic acid in methanol, 1.5 mL of 1 M acetic acid in ethyl acetate, and 0.5 mL of methanol with 5% (v/v) ammonia. The eluate was evaporated to dryness. The residue was reconstituted with taxifolin dissolved in mobile phase as an additional external standard.

Liquid chromatography analyses were performed using an ACQUITY UPLC (Waters, Milford, MA) and a triple quadrupole mass spectrometer (API 3000) from Applied Biosystems (PE Sciex, Concord, Canada), equipped with a Turbo IonSpray source operated in the negative-ion mode. An ACQUITY UPLC BEH C_{18} column, 50 × 2.1 mm i.d., 1.7 μm, was used for chromatographic separation with mobile phase A (0.05% acetic acid in water, for glucuronide metabolites, or 10 mM ammonium acetate solution for sulfate metabolites) and mobile phase B (acetone:acetonitrile, 70:30). The linear gradient for the determination of resveratrol metabolites at a flow rate of 1 mL/min was [t (min), %B] (0, 10), (1, 30), (2, 100), (2.3, 100), (2.31, 10), (3, 10)]. In each case the sample volume injected was 5 μL. MS/MS parameters used were as follows: capillary voltage, −3500 V; focusing potential, −200 V; entrance potential, −10 V; nebulizer gas, 12 (arbitrary units), curtain gas, 12 (arbitrary units), collision gas, 6 (arbitrary units), auxiliary gas temperature, 400 °C; auxiliary gas flow rate, 7000 cm^3/min.

For quantification of resveratrol metabolites in tissue samples, the multiple reaction monitoring (MRM) mode was used with the following transitions: 227/185 for resveratrol; 403/227 and 307/227 for glucuronide and sulfate conjugates of resveratrol, respectively; 579/403 for resveratrol diglucuronides; 387/227 for resveratrol disulfates; 389/227 for resveratrol glucosides; 229/123 for dihydroresveratrol; 405/229 and 309/229 for glucuronide and sulfate conjugates of dihydroresveratrol and 197/169 and 303/285 for ethylgallate and taxifolin, respectively. Control tissues were used to prepare calibration curves since no resveratrol or its metabolites were detected. Calibration curves were prepared, in the range of expected concentrations, by supplementation with known concentrations of available standards. All of them were quantified using a six-point calibration curve between the limit of quantification (LOQ) and 10 μg/g determined by weighted ($1/x^2$) linear regression. Dihydroresveratrol metabolites and *cis*-resveratrol-3-*O*-sulfate were quantified using the calibration curve of dihydroresveratrol and *trans*-resveratrol-3-*O*-

Table 1. Limits of Detection (LOD) and Limits of Quantification (LOQ) for Each Tissue and Mean Recovery Efficiency from the Three Tissues[a]

	LOD (nmol/g tissue)			LOQ (nmol/g tissue)			
analyte	liver	adipose tissue	skeletal muscle	liver	adipose tissue	skeletal muscle	recovery (%)
trans-resveratrol	0.21 ± 0.01	0.07 ± 0.02	0.27 ± 0.02	0.70 ± 0.02	0.23 ± 0.05	0.90 ± 0.05	105.1 ± 11.5
cis-resveratrol	0.22 ± 0.02	0.14 ± 0.03	0.29 ± 0.02	0.73 ± 0.05	0.46 ± 0.08	1.31 ± 0.07	101.6 ± 8.4
trans- resveratrol-4′-*O*-glucuronide	0.11 ± 0.03	0.20 ± 0.04	0.29 ± 0.03	0.35 ± 0.09	0.66 ± 0.09	0.82 ± 0.94	101.2 ± 5.1
trans- resveratrol-3-*O*-glucuronide	0.09 ± 0.02	0.15 ± 0.04	0.21 ± 0.06	0.31 ± 0.06	0.51 ± 0.07	0.89 ± 0.07	98.7 ± 7.2
trans- resveratrol-3-*O*-sulfate	0.30 ± 0.00	0.06 ± 0.00	0.18 ± 0.04	1.00 ± 0.01	0.19 ± 0.01	0.61 ± 0.08	97.4 ± 10.0
dihydroresveratrol	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.21 ± 0.00	0.15 ± 0.01	0.15 ± 0.02	96.1 ± 5.4

[a]Values are means ± SD.

Table 2. Resveratrol Metabolite Profile (nmol/g tissue) in Liver and Adipose Tissue from Rats Receiving 30 mg of Resveratrol/kg Body Weight/d for 6 Weeks

	liver			adipose tissue		
analyte	mean ± SEM[c]	range	*n*	mean ± SEM[c]	range	*n*
cis-resveratrol	nq	nd–nq	–	nd	–	–
trans-resveratrol-3-*O*-glucuronide	8.66 ± 3.24	nd–28.79	7/8	1.31 ± 0.43	nd–3.24	6/8
trans-resveratrol-4′-*O*-sulfate[a]	1.07 ± 0.27	nq–2.64	7/8	nq	nd–nq	–
trans-resveratrol-3-*O*-sulfate	nq	nd–nq	–	0.24 ± 0.06	nd–0.41	6/8
cis-resveratrol-3-*O*-sulfate[a]	nq	nd–nq	–	nq	nd–0.41	4/8
trans-resveratrol-3,4′-disulfate[a]	nq	nd–nq	–	nq	nd–nq	–
dihydroresveratrol	nq	nd–0.42	2/8	nd	–	–
dihydroresveratrol glucuronide[b]	5.50 ± 1.45	1.77–14.86	8/8	nq	nd–0.18	2/8
dihydroresveratrol sulfate[b]	16.44 ± 4.27	2.90–41.35	8/8	0.32 ± 0.08	nd–0.65	6/8

[a]Quantified as *trans*-resveratrol-3-*O*-sulfate equivalents. [b]Quantified as dihydroresveratrol equivalents. [c]Values <LOQ considered as the LOD value. *n* is the number of rats in which the metabolite was quantified/total rats measured. nd and nq indicate not detected and not quantifiable, respectively (below the limit of detection or quantification, respectively; see Table 1).

Table 3. Resveratrol Metabolite Profile (nmol/g tissue) in Liver and Adipose Tissue from Rats Receiving 60 mg of Resveratrol/kg Body Weight/d for 6 Weeks

	liver			adipose tissue		
analyte	mean ± SEM[c]	range	*n*	mean ± SEM[c]	range	*n*
cis-resveratrol	nq	nd–nq	–	nq	nd–nq	–
trans-resveratrol-3-*O*-glucuronide	10.54 ± 3.06	2.26–25.36	8/8	1.08 ± 0.29	nq–2.33	6/8
trans-resveratrol-4′-*O*-sulfate[a]	3.01 ± 1.14	nq–8.55	6/8	0.26 ± 0.06	nq–0.65	6/8
trans-resveratrol-3-*O*-sulfate	nq	nd–nq	–	0.19 ± 0.06	nd–0.39	5/8
cis-resveratrol-3-*O*-sulfate[a]	nq	nd–nq	–	0.19 ± 0.06	nd–0.32	5/8
trans-resveratrol-3,4′-disulfate[a]	nq	nd–2.39	2/8	nq	nd–nq	–
dihydroresveratrol	0.75 ± 0.20	nd–1.42	7/8	nd	–	–
dihydroresveratrol glucuronide[b]	9.55 ± 2.45	2.65–18.96	8/8	nq	nd–0.30	4/8
dihydroresveratrol sulfate[b]	74.17 ± 23.39	13.00–186.68	8/8	1.02 ± 0.37	0.34–3.65	8/8

[a]Quantified as *trans*-resveratrol-3-*O*-sulfate equivalents. [b]Quantified as dihydroresveratrol equivalents. [c]Values <LOQ considered as the LOD value. *n* is the number of rats in which the metabolite was quantified/total rats measured. nd and nq indicate not detected and not quantifiable, respectively (below the limit of detection or quantification, respectively, see Table 1).

sulfate, respectively, and expressed as their equivalents, as was done previously,[8,26] since no standards are available.

The mean recovery efficiency of the available standards from the different samples was 99.2 ± 11.5%, ranging from 95.6 ± 9.9% (in the case of *trans*-resveratrol-4′-*O*-sulfate) to 105.1 ± 11.5% (in the case of *trans*-resveratrol) (Table 1). Calibration curves were linear over the concentration range with correlation coefficients for all the analytes >0.99. The detection limit (LOD) was defined as the concentration of analyte that produced a signal-to-noise ratio of at least 3, and the limit of quantification (LOQ) was the lowest standard with a signal-to-noise ratio of at least 10. The LOD and LOQ for each standard and evaluated tissue are shown in Table 1. The method had accuracy values (mean ± SD) of 97.25 ± 3.97%, 100.18 ± 3.05%, and 97.69 ± 5.49% for liver, adipose tissue, and skeletal muscle, respectively, and precision values ranged from 3.26 to 13.65% for liver, from 2.65 to 12.48% for adipose tissue, and from 3.22 to 12.85% for skeletal muscle, which met the acceptance criteria of the FDA.[28]

Statistical Analysis. Results are presented as mean ± SEM. Statistical analysis was performed using SPSS 17.0 (SPSS, Chicago, IL). Data were analyzed by one-way ANOVA followed by Newman Keuls post hoc test. Significance was assessed at the $P < 0.05$ level.

■ RESULTS

The profile of resveratrol metabolites in liver and adipose tissues is detailed in Tables 2 and 3. These metabolites included five phase II metabolites of resveratrol, *cis*-resveratrol, and dihydroresveratrol and its glucurono- and sulfo-conjugates (as microbial metabolites).

When the lowest dose of resveratrol was administered to rats (RSV1 group), two microbial metabolites of resveratrol (dihydroresveratrol conjugates) were found in quantifiable amounts in liver of all eight rats (mean ± SEM): dihydroresveratrol glucuronide (1.48 ± 0.32 nmol/g) and dihydroresveratrol sulfate (2.23 ± 0.32 nmol/g). Otherwise, two metabolites were found in adipose tissue of three rats (range): *trans*-resveratrol-3-*O*-sulfate [not detected (nd)−0.31 nmol/g] and *cis*-resveratrol-3-*O*-sulfate (nd−0.26 nmol/g). Any metabolite has been detected in skeletal muscle.

When higher doses were administered to rats (RSV2 and RSV3 groups) (Tables 2 and 3), the number of quantifiable compounds increased up to four in liver and three in adipose tissue in the RSV2 treatment and five metabolites in liver and adipose tissue in RSV3 treatment. The metabolite mainly detected in liver for all the treatment groups was dihydroresveratrol sulfate, a microbial metabolite, followed by *trans*-resveratrol-3-*O*-glucuronide and dihydroresveratrol glucuronide. Thus, it seems that microbial metabolites predominated in liver. *trans*-Resveratrol-3-*O*-glucuronide was quantified in only one rat in skeletal muscle after RSV2 and RSV3 treatments: 1.02 and 0.76 nmol/g, respectively. Dihydroresveratrol sulfate was quantified in skeletal muscle of two rats after RSV2 treatment (range: nd−0.31 nmol/g) and in six rats after RSV3 treatment [mean ± SEM (range): 0.30 ± 0.10 nmol/g (nd−0.86 nmol/g)]. *trans*-Resveratrol, *trans*-resveratrol-4′-*O*-glucuronide, and resveratrol diglucuronide were not detected in any tissue.

The highest concentrations of resveratrol metabolites were found in liver, followed by adipose tissue and skeletal muscle (Figure 1). A dose−response pattern was found in liver when total resveratrol metabolites were quantified. By contrast, in adipose tissue this pattern was only observed when RSV1 was compared with RSV2 and RSV3 groups (Figure 1). In skeletal muscle no resveratrol metabolites were detected in the RSV1 group, and no significant differences between groups RSV2 and RSV3 were observed (Figure 1).

Total glucuronide and sulfate metabolites in the three tissues are shown in Figure 1. When the distribution between these two kinds of metabolites was analyzed, different patterns of response were also found among tissues. In liver and adipose tissue, animals from the RSV2 group showed significantly higher amounts of glucuronide metabolites than animals from RSV1. Similar amounts of these metabolites were shown in the three tissues of animals from the RSV2 and RSV3 groups, since no statistical differences were obtained between them. A dose−response pattern in all the evaluated tissues was observed for sulfate conjugates. This pattern was also observed when metabolites derived from microbiota were considered (dihydroresveratrol and its conjugates), in the three tissues. Larger amounts of these metabolites than of resveratrol conjugates were detected in liver while the opposite was true in adipose tissue and skeletal muscle (Figure 2).

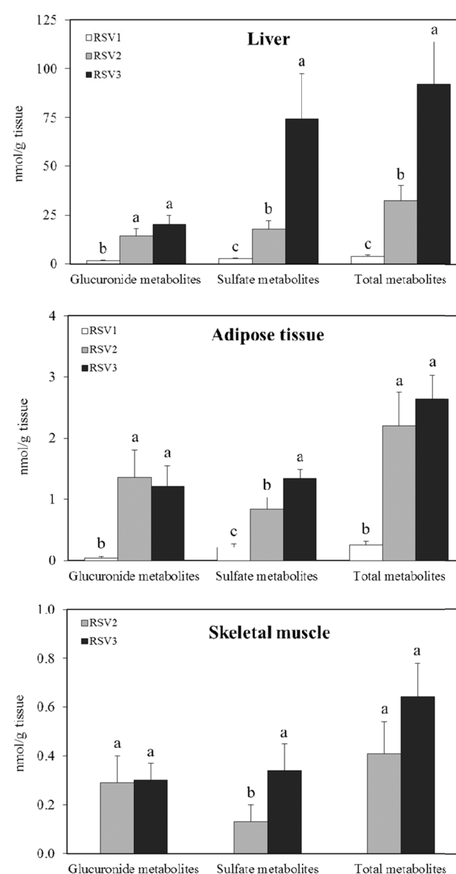

Figure 1. Glucuronide, sulfate, and total analyzed resveratrol metabolites in liver, adipose tissue, and skeletal muscle from rats receiving 6 mg/kg/d (RSV1), 30 mg/kg/d (RSV2), or 60 mg/kg/d (RSV3) of resveratrol for 6 weeks. Results are expressed as mean ± SEM. Statistics shows comparisons among the three dose groups for each metabolite type. Bars not sharing a common letter are significantly different ($P < 0.05$). No detectable amounts of resveratrol metabolites were found in the RSV1 group for the skeletal muscle. Data concerning total metabolites in adipose tissue from rats treated with 30 mg/kg/d have been previously presented.[43] (Values <LOQ considered as the LOD value.)

■ DISCUSSION

Resveratrol is a polyphenol with a very low availability. Due to its extensive metabolism, free resveratrol reaches very low concentrations in plasma and tissues.[6] Despite that, a great number of published studies have reported beneficial effects on health in animal experimental models. It can be hypothesized that resveratrol metabolites may be active per se, or that they provide a pool for local or systemic regeneration of resveratrol in vivo.[29] Due to these facts, it is essential to quantify resveratrol metabolites in tissues in in vivo studies. Nevertheless, tissue distribution of resveratrol and its metabolites has rarely been assessed compared with the number of papers that refer to the plasmatic concentrations of this polyphenol so far.

It is important to point out that our experimental design was not specifically planned to carry out a pharmacokinetic study. In fact, our first interest was to analyze the effects of resveratrol on body fat accumulation and serum parameters, and thus, rats were fed on an obesogenic diet.[21] We observed that the lowest dose (6 mg/kg/d) was without effect, the intermediate dose (30 mg/kg/d) induced a significant reduction in body fat, and the highest dose (60 mg/kg/d) did not improve the effect of 30 mg/kg/d. In the light of these results, we considered it very interesting to determine the amount of resveratrol and resveratrol metabolites found in target tissues involved in the

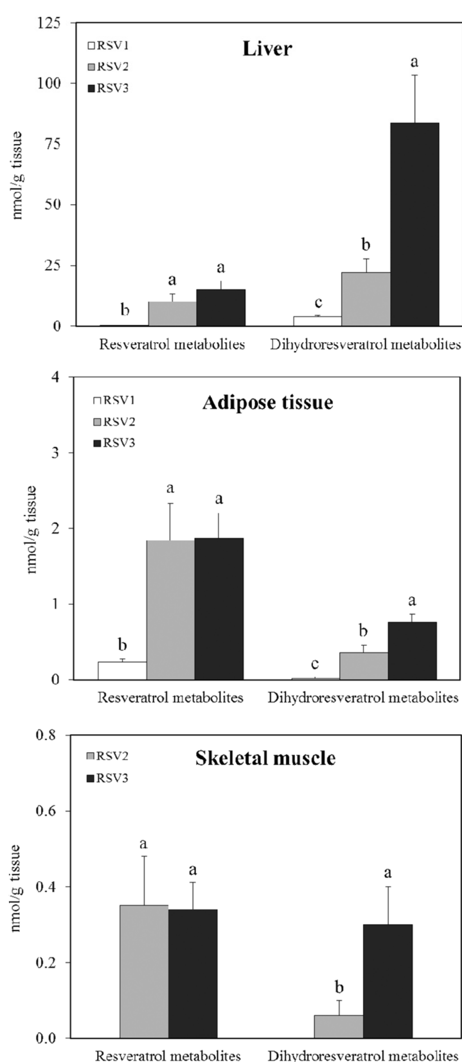

Figure 2. Resveratrol (glucuronide and sulfate conjugates) and dihydroresveratrol (free and its glucurono- and sulfoconjugates; microbial metabolism) metabolites in liver, adipose tissue, and skeletal muscle from rats receiving 6 mg/kg/d (RSV1), 30 mg/kg/d (RSV2), or 60 mg/kg/d (RSV3) of resveratrol for 6 weeks. Results are expressed as mean ± SEM. Statistics shows comparisons among the three dose groups for each metabolite type. Bars not sharing a common letter are significantly different ($P < 0.05$). No detectable amounts resveratrol metabolites were found in the RSV1 group for the skeletal muscle. (Values <LOQ considered as the LOD value.)

body-fat-lowering effect of this polyphenol, such as adipose tissue, liver, and skeletal muscle,[30−32] and we carried out the present study in the same cohort of animals, which were fed an obesogenic diet and fasted overnight before sacrifice.

These experimental conditions were not ideal for this type of study, and obviously this is a limitation of this work. Nevertheless, an interesting aspect should also be emphasized. Usually, resveratrol metabolite distribution has been analyzed after a single dose of resveratrol, or repeated doses for a short time.[6,15,33,34] By contrast, in the present study resveratrol metabolite distribution was analyzed after a long-term treatment (6 weeks) and this can provide additional data on this issue.

With regard to diet composition, feeding rats an obesogenic diet (high-fat diet) can modify resveratrol absorption. Vaz-da-Silva et al.[35] showed that a high-fat breakfast delayed the absorption of *trans*-resveratrol compared with fasted intake, but it did not influence the extent of absorption in humans after a single dose of 400 mg. La Porte et al.[36] reported decreased and delayed absorption of *trans*-resveratrol in combination with a high-fat breakfast as compared with a standard breakfast in humans treated with 2000 mg of resveratrol twice daily. Despite this fact, we believe that results concerning resveratrol metabolite distribution obtained under the experimental conditions which allow resveratrol to show body fat-lowering properties, can be helpful to explain this biological effect. The distribution of resveratrol under other experimental conditions should also be analyzed in order to gain more insight into this issue.

In the present study, resveratrol metabolites, but not their parent compound, were detected in the three tissues analyzed by the method described. Taking into account that rats were fasted overnight, the results are to be expected because other authors have shown that after its administration, free resveratrol is either absent or present only as trace amounts in plasma and tissues and that resveratrol metabolite concentrations are much higher than the concentrations of the parent compound.[4,5] It has been also reported that resveratrol is detected in plasma as soon as 15 min after administration, reaching peak concentrations at 60 min and then sharply decreasing. In contrast, some resveratrol metabolites are detectable in plasma even 3 h after administration; at this time only trace amounts of free resveratrol are found in plasma.[3,37]

Our results are in line with those published by Wenzel et al.,[6] who found resveratrol metabolites, but not free resveratrol, in kidneys after an overnight fast when rats were administered 300 mg/kg/d of this polyphenol. In liver they found a small amount of free resveratrol and higher amounts of its metabolites but they used a dose far higher than that used in the present study. Nevertheless, the fact that resveratrol was present but below the LOD cannot be discarded.

Most of the studies devoted to analyzing resveratrol metabolic profile in tissues focus on liver, kidney, lungs, brain, and testis.[6,34,38,39] However, as far as we know, only one study has evaluated resveratrol metabolites in adipose tissue and skeletal muscle.[40] In recent years the effectiveness of resveratrol as a body-fat-lowering agent has been demonstrated in rodents.[21,30−32,41] Adipose tissue and skeletal muscle, as well as liver, are targets for resveratrol; in fact different actions of this polyphenol, such as decreasing lipogenesis, increasing lipolysis, and increasing fatty acid oxidation, take place in these tissues and organs and they are mechanisms of action that explain the effect of resveratrol on the reduction of body fat accumulation.[21,30−32,41−43] Thus, describing the resveratrol metabolic profile in these tissues is an important issue.

As expected, in general terms, resveratrol metabolite amounts increased with increasing concentrations of the polyphenol in the diet. A clear dose−response pattern was found in liver when the three experimental groups were analyzed. By contrast, this pattern was observed in adipose tissue only when RSV1 and RSV2 groups were compared. In skeletal muscle, resveratrol

metabolites were not detected in the RSV1 group, and very similar amounts were detected in the RSV2 and RSV3 groups. This could suggest that these two tissues have a maximal capacity for resveratrol metabolite incorporation, which cannot be exceeded by increasing resveratrol administration. In the present study, the highest amounts of total resveratrol metabolites for the three analyzed doses were detected in liver. Indeed, other authors have reported that this organ has been found to be subjected to an important accumulation of these compounds in rodents.[6,34,38,39] Resveratrol metabolite amounts in adipose tissue were much higher than those in skeletal muscle. It has been reported that resveratrol is mainly distributed in abundant blood-supplied tissues, such as liver, lung, and kidney.[34,40] This is logical, but other factors also may influence this process because although skeletal muscle has a more active blood flow than adipose tissue, lower amounts of resveratrol metabolites were detected in this tissue. Thus, the potential contribution of differences among tissues in terms of transporters that excreted xenobiotics from different organs (members of the family of ATP-binding cassette) cannot be discarded.[34]

In this work, the resveratrol metabolic profile evaluated in the three tissues under fasting conditions and during long-term treatment includes glucuronide and sulfate conjugates of resveratrol together with those derived from the microbiota. Although glucuronide and sulfate conjugates of resveratrol have blood and tissue peaks about 3−6 h postadministration, it could be hypothesized that resveratrol is accumulated in tissues and released slowly over a longer period of time, as previously published for resveratrol[44] or other polyphenols.[45]

Only a few studies have considered the determination of microbial metabolites after resveratrol administration: Azorín-Ortuño et al. in a great number of tissues[40] and Wang et al.[46] in urine samples. Dihydroresveratrol conjugates appear later in the organism after the colonic microbial degradation of resveratrol and further absorption and phase II metabolism. Thus, in our study, dihydroresveratrol as free or as glucuronide and sulfate conjugates was assessed. These were the main metabolites in liver, but not in adipose tissue and skeletal muscle.

When the distribution between glucuronide and sulfate metabolites was analyzed, it was noteworthy that while sulfates increased when resveratrol dosage was enhanced, glucuronides increased between 6 and 30 mg/kg/d, but remained unchanged when resveratrol dosage reached 60 mg/kg/d. These results may suggest that glucuronidation, but not sulfation, is a saturable metabolic pathway, at least in the range of doses used in the present study. Nevertheless, the potential degradation of glucuronide metabolites, as well as a more rapid elimination, cannot be discarded. Consequently, further studies are needed to better analyze this issue. In the literature there are other studies that provide data that lend support to this theory. Thus, Kapetanovic et al.[47] observed a shift from glucuronidation to sulfation of resveratrol possibly due to the saturation of the glucuronide pathway. Maier-Salamon et al.[48] have already described a possible saturation of the enzymes responsible for resveratrol glucuronidation (UDP-glucuronosyltransferases UGT1A7 and UGT1A10), when increasing administered resveratrol concentrations, while noncompetitive substrate inhibition was shown for resveratrol sulfation.

These data can provide a clue to explain the results that we obtained, by using this cohort of animals, when the effects of resveratrol on body fat accumulation were assessed.[21] As we have explained before in this section, while resveratrol reduced body fat at a dose of 30 mg/kg/d, it did not at 6 mg/kg/d. Surprisingly, rats treated with 60 mg/kg/d resveratrol did not show further reduction. This means that a plateau was reached when the dose of resveratrol increased. Moreover, in a previous experiment that we performed in 3T-L1 adipocytes, glucuronide, but not sulfate metabolites, were shown to be active in reducing triacylglycerol accumulation (data submitted). Thus, it can be hypothesized that the lack of increase in glucuronides, metabolites that could be responsible in part for the body-fat-lowering effect of resveratrol, when the dose of this polyphenol increases from 30 to 60 mg/kg/d could help to explain the above-mentioned plateau effect. Nevertheless, the levels of these metabolites detected in adipose tissue and skeletal muscle are very low and thus it is difficult to understand how they are contributing to the effects observed. Consequently, more studies are needed to explain the in vivo effects induced by resveratrol after a long-term treatment.

In conclusion, this study describes that the largest amounts of resveratrol metabolites were found in liver, intermediate amounts were observed in adipose tissue, and the lowest amounts were seen in skeletal muscle. In general terms, a dose−response pattern was found in sulfate conjugates but not glucuronides. Metabolites derived from microbiota were detected in greater amounts in liver than resveratrol conjugates were, whereas the opposite was the case in adipose tissue and skeletal muscle.

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Notes

The authors declare no competing financial interest.

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8.2 OTROS CAPÍTULOS DE LIBRO

Capítulo de libro I: Trespalacios MP, Tulipani S, Garcia-Aloy M, Zamora-Ros R, Rotchés-Ribalta M, Rabassa M, Urpi-Sarda M, Boto M, Vázquez R, Llorach R, Andrés-Lacueva C. "Nutritional biomarkers: applications and challenges", dentro del libro "Food intake: regulation, assessing and controlling" Nova Science Publishers, Inc, 2013, 6, 117-56. ISBN:978-1-61324-183-87.

NUTRITIONAL BIOMARKERS: APPLICATIONS AND CHALLENGES

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ABSTRACT

Diet is implicated in a myriad of diseases, ranging from events in the first weeks of life to chronic and age-related illnesses, for example, cancer and cardiovascular disease. A primary goal of nutrition research is to optimize health via dietary interventions, by prevention, delay, or reduction in the severity of disease.

To date, several methods for dietary assessment have been used, such as food frequency questionnaires, food diaries, food records, or 24-h dietary recalls. However, they are associated with different measurement errors between diet observed and true intake, which may obscure some disease risk associations. Thus, nutrition epidemiologists started using different biomarkers in biological samples that are more objective measures and are independent of errors related to dietary assessment methods. A nutritional biomarker can be any biological compound that is an indicator of nutritional status with respect to the intake or metabolism of dietary constituents. An ideal nutritional biomarker would accurately reflect its dietary intake level and it would be specific, sensitive and applicable to many populations. It should also be reproducible, validated and reliable.

The discovery and assessment of nutritional biomarkers highly depend on appropriate analytical tools. An ideal technique for biomarkers discovery and monitoring, unfortunately, is not currently available, the critical limiting factor being the lack of standardized technologies and methodologies in biomarker discovery and validation process.

Nevertheless, recent technological advances have improved the detection capabilities of the instrumental techniques commonly used in nutrition research. The current tendency is to make use of multiple analytical platforms to maximize the range of metabolic profile studied in a sample. Of the emerging multiple platforms for biomarker discovery, perhaps none has garnered more recent attention than metabolomics. The main analytical and statistical strategies used in nutritional metabolomics are discussed, and some applications to biomarkers discovery presented. Also the main factors affecting the utility of a biomarker in reflecting properly dietary exposures are discussed. Notwithstanding the current main challenges for metabolomics, several examples of successful applications to nutritional research exist. Concluding, the development of new robust nutritional biomarkers may help to improve disease risk stratification by better characterizing the metabolic phenotype at the individual level. Consequently, new nutritional biomarkers are needed to objectively evaluate the effect of diet on health and to check adherence to dietary recommendations and healthy eating patterns.

Capítulo de libro II: Rabassa M, Zamora-Ros R, Tulipani S, Khymenets O, Urpi-Sarda M, Garcia-Aloy M, Boto M, Vázquez R, Rotches-Ribalta M, Chiva G, Trespalacios MP, Llorach R, Andres-Lacueva C. "Flavonoids from food and its implication in human health", dentro del libro "Applications of natural products in food" Nova Science Publishers, Inc, 2012, 12, 427-42. ISBN:978-1-61942-049-6.

FLAVONOIDS FROM FOOD AND ITS IMPLICATION IN HUMAN HEALTH

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ABSTRACT

Flavonoids comprise a large group of phenolic compounds synthesized by plants as secondary metabolites, with a common diphenylpropane skeleton (C6-C3-C6). Over 9,000 structurally distinct flavonoids have been identified in nature, although less than 200 were found in a sample French population. Flavonoids can be classified according to their chemical structure into eight subclasses: flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols or flavanols, anthocyanidins, isoflavones and proanthocyanidins.

Flavonoids occur ubiquitously in plants and plant products. They are generally found in glycosylated forms, mainly with glucose or rhamnose moieties, but other sugars may also be involved. Flavonoids in foods contribute to their flavor, providing color, astringency and bitterness. Flavonols are the most frequent flavonoids in foods, with onions, apples and tea being the main food sources. Flavones are widely distributed in plants, but in lower amounts than flavonols. The richest sources are herbs and spices. Dihydroflavonols have been identified in a few foods, such as wines and oregano. Flavanones are characteristic compounds of citrus fruits and their derived juices. Anthocyanidins are responsible for the colours of fruits and vegetables, like pink, red, blue or purple. They are abundant in berries and their derived products, such as juices and wines. Flavan-3-ols are found as aglycones in foods. Catechin and epicatechin are the main flavan-3-ol contributors, with tea, apples, red wine and chocolate being their most common food sources. Proanthocyanidins are oligomeric and polymeric flavan-3-ols, also known as condensed tannins, and are responsible for the astringency of various fruits or beverages and for

the bitterness of chocolate. Theaflavins and thearubigins are derived flavanol compounds that are only found in tea. Isoflavones are found almost exclusively in leguminous plants, particularly in soybeans and their processed products.

The bioavailability of flavonoids varies greatly among and within their subclasses, in terms of their chemical structure and sugar moiety type. Flavonoids are usually absorbed by both the small intestine and colon as aglycones after being hydrolyzed by either enzymes or the microbiota with a high intra- and inter-individuality. After absorption, they are quickly metabolized in the intestinal cells or in the liver. Therefore, the biological activity of flavonoids may be due to their conjugated forms or the aglycones that may be set free in target tissues. Finally, they are eliminated by renal and/or liver excretion. Most flavonoids are known to exert similar biological effects – such as antioxidant, anticarcinogenic, anti-inflammatory, antiobesity, antidiabetic, antiallergic, hepatoprotective, and gastroprotective activities – *in vitro*. But each individual compound has different effective concentrations, may use different mechanism of actions and even exert different effects.

Epidemiological studies have suggested that flavonoids may provide protection against several chronic diseases, such as cardiovascular and neurodegenerative diseases, diabetes, obesity and some cancers. However, scientific evidence on the role of flavonoid intake in disease prevention is promising but not conclusive, and further clinical and epidemiological studies are needed.

Capítulo de libro III: Rotches-Ribalta M, Urpi-Sarda M, Tulipani S, Trespalacios P, Khymenets O, Rabassa-Bonet M, Boto-Ordóñez M, Vazquez-Fresno R, Garcia-Aloy M, Llorach R and Andres-Lacueva C. "Sources and food matrix on the bioavailability of resveratrol", dentro del libro "Resveratrol: Sources, Production and Health Benefits" Nova Science Publishers, Inc, 2013, 20, 441-464. ISBN:978-1-62081-997-5.

SOURCES AND FOOD MATRIX ON THE BIOAVAILABILITY OF RESVERATROL

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ABSTRACT

Resveratrol consumption has been positively associated with the prevention of a number of diseases, mainly due to its reported antioxidant activity and anti-inflammatory and antitumor effects, and has, therefore, the potential to be evaluated for therapeutic use. However, scarce studies have been carried out in humans and the results yielded are still controversial. Before making nutritional recommendations regarding this promising molecule, other aspects such as dietary intake and bioavailability should be considered.

Resveratrol and other stilbenes, such as piceid, have been identified in a large number of plants, although they have been found in a few dietary components, with red wine and grape products as the major sources. Therefore, urinary resveratrol metabolites have been described as nutritional biomarkers of wine consumption, providing an additional tool for clinical and epidemiological studies, since they reflect a more objective assessment of nutrient intake than self-reported dietary data. Its estimated intake or exposure is essential in order to relate resveratrol consumption with its possible health effects *in vivo*. However, resveratrol is rapidly metabolized, and therefore the bioactivity of this compound will be related to its metabolites. Identification and measurement of metabolic forms are key prerequisites for understanding the role of these compounds, since they will be the ones that reach the peripheral circulation and tissues where they could exert their biological effect. Phase II metabolism of resveratrol has been widely studied, with the glucuronide and sulfate forms being the main resveratrol conjugates. However, few studies have focused on the microbial metabolism of resveratrol, which leads to dihydroresveratrol formation, although this could give a better idea about effects of long-term resveratrol consumption. Furthermore, the matrix in which resveratrol is delivered could play an important role in its bioavailability.

This chapter, therefore, provides an overview of the metabolism of resveratrol after short- and long-term consumption and the microbiota and food matrix effects on its bioavailability. This needs to be considered in order to gain a better understanding of the links between resveratrol intake and health.

Capítulo de libro IV: Vázquez-Fresno R, Tulipani S, Khymenets O, Urpi-Sarda M, Garcia-Aloy M, Rabassa M, Boto-Ordóñez M, Rotches-Ribalta M, Llorach R and Andres-Lacueva C. "Emerging Applications of Metabolomics to Polyphenols and CVD Biomarker Discovery" dentro del libro "Polyphenols in human health and disease" Academic Press Inc, 2013, 79, 1023-40. ISBN:978-0-12-398456-2.

EMERGING APPLICATIONS OF METABOLOMICS TO POLYPHENOLS AND CVD BIOMARKER DISCOVERY

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Over the last decade, a growing body of interventional and epidemiological studies has furnished new evidence on the beneficial properties of dietary polyphenols in the reduction of the risk of cardiovascular disease (CVD). However, further research is required on the discovery and validation of new polyphenol intake biomarkers and their effect on the promotion of cardiovascular health. To successfully face the challenge of biomarker discovery, the application of emerging "omics" technologies and hypothesis-generating approaches are very encouraging. Metabolomics is a particularly promising strategy for obtaining a comprehensive overview of the low-molecular-weight metabolites present in a biological system at a given moment, and to explore unknown metabolic pathways impacted on diet. Despite being the youngest of the "omics" technologies, it already counts for a series of successful applications in the identification of potential biomarkers related to the prediction, early diagnosis and prognosis of CVD.

This chapter will review the state of the art in the application of the metabolomics approach to polyphenol and CVD biomarker discovery, and their implications in cardiovascular risk assessment.

8.3 COMUNICACIONES EN CONGRESOS

Durante el periodo predoctoral han presentado hasta 22 comunicaciones en congresos, 4 de las cuales presentan algunos de los resultados recogidos en la presente Tesis Doctoral. Estas 4 comunicaciones se han presentado como póster en tres congresos internacionales: la V y VI edición de la "*International Conference on Polyphenols and Health*", celebradas en Sitges y Buenos Aires respectivamente, y el Metabolomics Society Conference, Glasgow. Más adelante, se exponen el resto de comunicaciones presentadas en diferentes congresos.

Comunicación de póster: María Boto-Ordóñez, Maria Isabel Queipo-Ortuño, Mireia Urpi-Sarda, Sara Tulipani, Rafael Llorach, Maria Rotches-Ribalta, Ramon Estruch, Francisco Tinahones, Cristina Andrés-Lacueva. Plasma and urine modifications in phenolic acids microbial metabolites after a long-term consumption of red wine compared with dealcoholized red wine and gin are related with changes in microflora. 5th International Conference on Polyphenols and Health. Sitges-Barcelona (España), Octubre 2011. *Ganador del premio al mejor poster de la Sección de Microbiota.*

PLASMA AND URINE MODIFICATIONS IN PHENOLIC ACIDS MICROBIAL METABOLITES AFTER A LONG-TERM CONSUMPTION OF RED WINE COMPARED WITH DEALCOHOLIZED RED WINE AND GIN ARE RELATED WITH CHANGES IN MICROFLORA.

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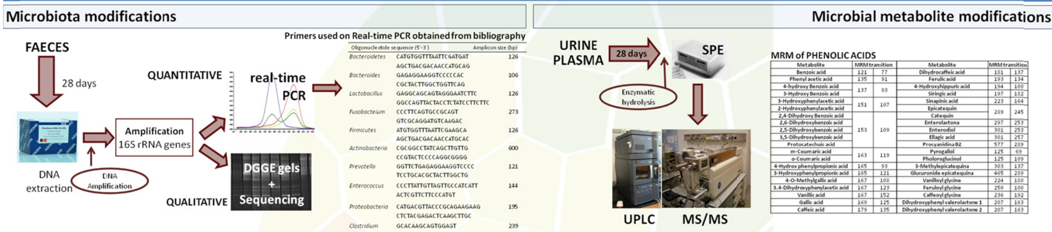
INTRODUCTION

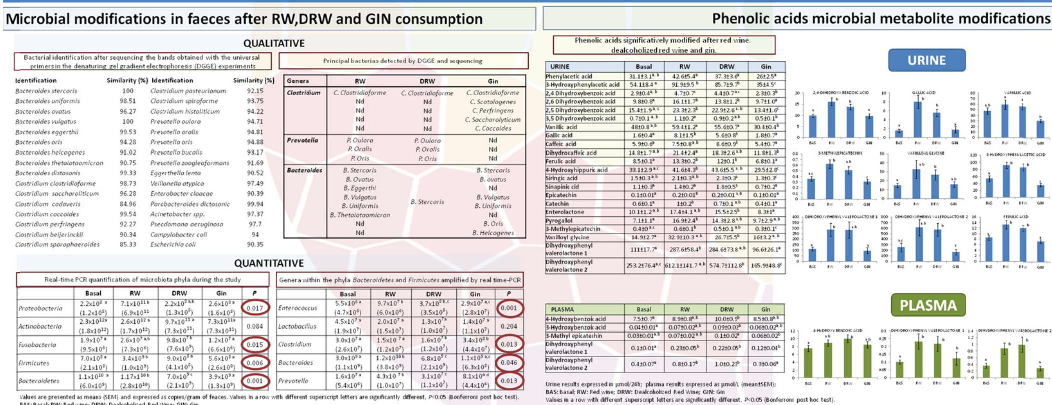
Last few years it has been observed that changes on the diet are related with changes in human microbiota. It is also known that some species are predominant in some situations over others, and thanks to the new techniques those species are being identified. Polyphenol rich food and drinks interaction with microbiota is largely know because microbial metabolites derived from polyphenol degradation in gut, mainly phenolic acids, had been described in biofluids after consumption. However, the role of polyphenols and metabolites in gut bacterias is still unknown, and promoter or inhibitor activities in relation with that food are poorly studied. In this work we present a parallel approach: (1) modifications in microbial metabolites after red wine, dealcoholized red wine, and gin consumption, and (2) modifications of bacterias present in human faeces after the same treatments in order to establish a relation of the phenolic content of those drinks and phenomenon occurred in gut that may affect host health.

STUDY DESIGN

MATERIAL AND METHODS



RESULTS



DISCUSSION

Decrease of *Clostridium spp* has been reported in *in vitro* studies using flavonols and in animal studies using wine poliphenols (4,5). *Bacteroides spp* is decreased in animal studies after consumption of red wine polyphenols (4, 7). Bacteria *Eggerthella lenta* and *E.Coli* indentified in feaces has been described to metabolize flavanols and catechins (5,8). *Enterococcus spp* capacity to produce phenolic acids metabolites from wine in *in vitro* studies (6).

Gallic acid, described as biomarker of grape products intake, was found in a concentration significantly higher after red wine and dealcoholized red wine than baseline and gin (P<0.05) (9). Metabolites derived from procyanidins microbial catabolism, hydroxyphenylvalerolactones, 3-hydroxyphenylacetic acid, ferulic acid, vanillic acid and 4-hydroxyhippuric acid, were found in plasma and urine in a higher amount after moderate red wine and dealcoholized red wine consumption compared with baseline and gin period ($P<0.05$)(1,10)

CONCLUSIONS

- Moderate ethanol and red wine polyphenol consumption can generate an important change in the gut microbiota, modifying the number of some dominant phyla, accompanied by changes in metabolites present in host biofluids.
- *Clostridum spp, Enterococcus spp* and *Bacteroides spp* that were determined and modified by red wine, dealcoholized red wine and gin intakes have already been described to have an important role on polyphenol microbial metabolism and inhibiting and promoting colonic bacterias. Other bacterias as *Egerthella lenta* and *Escherichia Coli*, identified in volunteers faeces, had also been related with polyphenol metabolism.
- Phenolic acids metabolites derived from microbial catabolism of wine polyphenols were present in a higher amount after red wine and dealcoholized red wine intakes in comparison with baseline and gin periods, and may play a key role in the prevention of diseases.

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Póster: <u>María Boto-Ordóñez</u>, Joseph Rothwell, Cristina Andres-Lacueva, Vanessa Neveu, Claudine Manach, Rafael Llorach, Augustin Scalbert, Mireia Urpi-Sarda. Prediction of metabolic profile of polyphenols after wine and wine products intake using the Phenol-Explorer database**.** 9th Metabolomics Society Conference, Glasgow (Reino Unido), Julio 2013 y 6th International Conference on Polyphenols and Health. Buenos Aires (Argentina), Octubre 2013.

Prediction of metabolic profile of polyphenols after wine and wine products intake using the Phenol-Explorer database

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INTRODUCTION

BACKGROUND

A moderate intake of red wine has been associated with a reduction in mortality rate and a lower incidence of cardiovascular diseases and other diseases [1-3], but the molecular mechanisms involved are not fully understood. Considering the important role of polyphenols abundant in wine, knowledge of the metabolic profile of polyphenols derived from the consumption of red wine could be key to understand its health benefits and associated mechanisms. However , the metabolism of wine phenolic compounds has scarcely studied and limited to a few *in vivo* studies focusing on a single component such as resveratrol or catechin.

AIM

For that reason, by using Phenol-Explorer 2.0/3.0, we aim to predict the profile of wine polyphenol metabolic space in biofluids as accurately as possible, determine those metabolites described in intervention studies with grape products, mainly red wine, and those metabolites derived from the consumption of compounds present in wine.

WINE INTAKE
Clinical Studies
METABOLISM BIOAVAILABILITY
WINE COMPONENTS
WINE POLYPHENOLS
WINE METABOLITES
HEALTH BENEFITS
Epidemiological Studies

SEARCH STRATEGY

METABOLITES FROM FOOD

To retrieve the list of metabolites from wine-derived food sources and extracts, "Metabolism" would be selected from the site's main toolbar followed by "**Administered Food & Food Extracts**".

METABOLITES FROM PURE COMPOUNDS

Similarly, the database can be used to retrieve those metabolites reported after consumption of **some pure polyphenol**s found in red wine. There are two possible routes to these data: 1) by selecting an individual polyphenol listed as a constituent of red wine and then selecting "Show Metabolites", or 2) through the "Metabolism" tab followed by "Administered Pure Compounds" and then "Metabolites".

RESULTS

METABOLITES DISTRIBUTION

Venn diagrams showing the overlap between metabolites from A) phenolic wine constituents, wine products (WP) and pure compounds metabolites; B) plasma and urine; and C) human and animal metabolites distribution

A total number of 97 metabolites may be expected after wine consumption. A total of 37 metabolites derived from consumption of WP. A total of 90 metabolites reported after consumption of pure compounds, of which 30 were common to both groups. When these metabolites were compared with red wine constituents, 24 compounds were common to both groups while 10 polyphenols were common to WP and pure compound metabolites and wine composition. In relation to biofluid, 90 metabolites have been described in urine and 59 in plasma, 52 being common to both. Twenty nine metabolites were found only in human studies, 37 only in animal studies and 31 were common to both groups.

A: RED WINE POLYPHENOL COMPOSITION 51; 14; 0; 10; PURE COMPOUND METABOLITES 46; 20; 7 WP METABOLITES

B: PLASMA 7; 52; URINE 38

C: HUMANS 29; 31; ANIMALS 37

Classes of polyphenol metabolites identified after intake of red wine, red wine products and pure wine polyphenols

After determination of 97 metabolites derived from wine intake, These results corresponded with the fact that more than half of total metabolites were aglycones (57%) followed by glucuronidated (18%), methylated (16 %), sulfated (6%), glycosylated (5%) and glycinated (3%) conjugates.

	TOTAL	Aglycone	Glycoside	Glucuronide	Sulfate	Methylated	Glycinated
Flavonoids							
Flavanols	11	4	0	4	0	5	0
Anthocyanins	5	0	5	0	0	3	0
Flavanones	6	4	0	2	0	0	0
Flavonols	2	1	0	0	0	1	0
Phenolic acids							
Hydroxybenzoic acids	17	14	0	0	0	2	1
Hydroxycinnamic acids	15	7	0	7	0	0	1
Hydroxyphenylacetic acids	10	9	0	0	0	1	0
Hydroxyphenylpropanoic acids	8	5	0	0	0	2	1
Hydroxyphenylpentanoic acids /lactones	7	6	0	0	0	1	0
Stilbenes							
Stilbenes	12	2	0	4	6	0	0
Other polyphenols							
Alkylphenols	1	1	0	0	0	0	0
Tyrosols	1	1	0	0	0	0	0
Others (1,3,5-trimethoxybenzene, 4-ethylbenzoic acid)	2	1	0	0	0	1	0
TOTAL		**55**	**5**	**17**	**6**	**16**	**3**

METABOLIC PATHWAY

Proposed metabolic pathway for the metabolism of wine polyphenols.

The identities of metabolites of WP and pure polyphenols have allowed the proposal of a tentative global pathway of wine polyphenol metabolism Nearly all the compounds from wine, with the exception of stilbenes, lead to common metabolites, mainly phenolic acids from microbial metabolism.

TYROSOLS
SINAPIC ACID
ANTHOCYANINS
FLAVANONES
GALLIC ACID
GALLATES
FLAVONOL
PROCYANIDINS
CATECHINS
STILBENES

Note: Metabolites reported to be of microbial origin are indicated in italics.

CONCLUSIONS

In conclusion, the Phenol-Explorer database has allowed to build up a comprehensive pathway for wine polyphenol metabolites. Such an in-silico metabolite prediction will greatly facilitate the analysis of metabolic profile from samples collected from wine consumers in both intervention and observational studies. It should help identifying metabolites most promising as biomarkers of wine consumption. Analyzing the whole spectrum of metabolites derived from wine intake should also be key to understand the health effects of wine.

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A Novel Urinary Targeted Metabolomic Approach For Discriminatory Analysis of Food Intake Biomarkers.

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INTRODUCTION

Biomarkers in the food research field have been discussed in depth with regard to their ability to solve classical problems regarding estimating an index of quantitative exposure to individual food *(1-3)*, and recently, identifying dietary patterns that establish possible relations with health benefits. In particular, the consumption of grape-derived products such as red wine (RW) and dealcoholized RW (DRW) has been associated with a protective effect on cardiovascular diseases *(4, 5)*. These associations were first linked to phytochemicals found in foods, however, in recent years there has been an increased attention on the role of metabolites formed in the organism, especially those formed by microbiota.

In the case of RW, resveratrol metabolites have been described as being good biomarkers of wine intake *(6)*, and also gallic acid has been suggested as a marker, due to its increased excretion after consumption *(7)*. Both compounds were determined in 24-h urine which has been suggested as being better for biomarker determination than others, but since it is difficult to obtain in large epidemiological studies, other samples such as fasting plasma need to be assessed to identify biomarkers.

Here, we describe for the first time a new screening strategy for the evaluation and discrimination of biomarkers of intake, specifically RW, through a targeted metabolomic approach in a clinical intervention trial where several biological samples such as fasting plasma and 24-h urine samples were considered.

STUDY DESIGN

n = 36 male volunteers

Crossover /Randomized/Controlled

METHODS

Host and microbial wine metabolites (>60) including resveratrol, catechins, valerolactones and phenolic acids were analyzed by targeted UPLC-MS/MS using hydrolyzed and non-hydrolyzed samples.

STATISTICS

-PCA, clustering for population distribution
-Logistic regression analysis using the stepwise variable selection was used for built the prediction model
-ROC curves were used for biomarker evaluation

RESULTS

BIOMARKER DISCOVERY AND EVALUATION

FIGURE 1. Population distribution

Wine Consumers — Non Wine Consumers

STEPWISE LOGISTIC REGRESSION — With significant metabolites after Wine Intervention

TABLE 1 : Stepwise logistic regression model for discriminating wine consumers obtained from the training set

	Coefficient	Standard error	*p* value	Coefficient CI 95%
URINE				
Hydrolyzed samples				
2,4-Dihydroxybenzoic Acid	0.60	0.23	0.007	0.16, 1.05
Gallic Acid	0.84	0.24	<0.001	0.38, 1.31
Ethylgallate	0.48	0.18	0.009	0.12, 0.83
Constant	-4.47	0.94	<0.001	-6.31, -2.63
Non-Hydrolyzed samples				
Methylgallic Acid Sulfate	0.17	0.06	0.005	0.05, 0.29
Ethylgallate Sulfate	0.41	0.10	<0.001	0.21, 0.62
Constant	-4.19	0.91	<0.001	-5.98, -2.41
PLASMA				
Hydrolyzed samples				
3-Hydroxyphenylacetic Acid	2.38	1.02	0.020	0.39, 4.38
Gallic acid	62.21	22.41	0.006	18.29, 106.12
P-Coumaric acid	40.91	15.43	0.008	10.67, 71.14
Constant	-3.09	0.79	<0.001	-4.64, -1.54
Non-Hydrolyzed samples				
Methylgallic Acid Sulfate	525.00	118.68	<0.001	292.39, 757.61
Constant	-1.63	0.35	<0.001	-2.32, -0.94

PHENOLIC GROUPS VS PREDICTION MODELS

TABLE 2: Threshold, sensitivity, specificity and AUC for phenolic groups and prediction model obtained from stepwise logistic regression in hydrolyzed and non-hydrolyzed urine and plasma samples

	Threshold	Sensitivity (%)	Specificity (%)	AUC (%)
URINE				
Hydrolyzed samples				
Gallic acid Metabolites	4.89	88.89	77.80	87.75
DHPV Metabolites	18.17	83.33	66.70	81.15
Phenolic acid Metabolites	207.2	69.44	62.50	71.95
Ethylgallate	0.69	93.06	84.72	92.35
Prediction Model		**91.66**	**91.66**	**96.14**
Non-hydrolyzed samples				
(Epi)catechin Metabolites	39.84	75.00	83.33	86.32
DHPV Metabolites	695.53	66.70	73.60	76.33
Ethylgallate Metabolites	152.31	98.60	84.70	93.67
Methylgallic Sulfate	5.49	87.50	86.11	93.23
Resveratrol Biomarker	1966.05	91.67	95.83	96.45
Microbial Resveratrol Metabolites	1424.19	95.83	93.06	98.77
Prediction Model		**95.83**	**94.44**	**98.40**
PLASMA				
Hydrolyzed samples				
Gallic acid Metabolites	0.06	68.18	57.58	64.10
DHPV Metabolites	0.14	68.18	62.12	68.37
Phenolic acid Metabolites	0.24	72.73	65.15	68.02
Prediction Model		**75.76**	**74.24**	**81.18**
Non-hydrolyzed samples				
(Epi)catechin Metabolites	0.03	69.70	75.76	76.92
DHPV Metabolites	0.06	72.73	62.12	71.12
Methylgallic Sulfate	0.002	84.85	77.27	87.50
Prediction Model		**84.85**	**77.27**	**87.50**

ROC CURVES

FIGURE 2. ROC curves of the phenolic metabolites groups and the prediction model: A) hydrolyzed urine samples; B) non-hydrolyzed urine samples; C) hydrolyzed fasting plasma samples; D) non-hydrolyzed fasting plasma samples

MATRIX CORRELATION

Spearman's correlations between fasting plasma and 24-h urine samples for individual, phenolic metabolite groups and the prediction model

PLASMA — PLASMA/URINE CORRELATIONS — URINE

- The best correlations were observed among model scores from hydrolyzed and non-hydrolyzed samples, indicating that those volunteers that were better classified as wine consumers in urine, they were also in plasma (r=0.565 and r=0.599, respectively P<0.001).
- Gallic acid metabolites in plasma were also significantly correlated with their presence in hydrolyzed and non-hydrolyzed urine samples (r=0.451 and r=0.587, respectively, P<0.001), as well as valerolactone metabolites (r=0.348 and r=0.356, respectively, P<0.001).

SUMMARY RESULTS

- After a logistic regression analysis, prediction models for 24h urine in both hydrolyzed and non-hydrolyzed samples obtained AUC over 96%.
- Resveratrol, ethylgallate and gallic acid metabolites in non-hydrolyzed urine samples resulted also to be good predictors of wine consumption (AUC>93%).
- Microbial Resveratrol (AUC 98%) resulted to be similar to phase II metabolites (AUC 96%) in non-hydrolyzed samples.
- Lower values of AUC were obtained in fasting plasma samples for prediction models in hydrolyzed and non-hydrolyzed samples (81-87%) and phenolic groups (64-87%) in hydrolyzed and non-hydrolyzed samples.
- The highest correlations between fasting plasma and 24h urine were obtained for the prediction models score (r=0.6, P<0.001) followed by gallic acid metabolites (r=0.5-0.6, P<0.001).

CONCLUSIONS

In conclusion, this study proposes the use of a strategy that combines targeted metabolomic analysis and logistic regression in interventional studies to identify, evaluate, compare and validate biomarkers of food intake, more precisely biomarkers of wine intake. Correlations between fasting plasma and urine provide the opportunity to discriminate metabolites that could be good urinary biomarkers of consumption in urine, and that could also be found in plasma. This new approach is a promising tool that has great potential for identifying possible biomarkers to evaluate compliance in clinical studies, identifying eating patterns and making associations between polyphenol consumption and health benefits.

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