Universidad de Oviedo
Programa de Doctorado en Biotecnología Alimentaria

Isoflavonas de soja y poblaciones microbianas intestinales: identificación y caracterización de microorganismos involucrados en su metabolismo

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La Comisión Académica del Programa de Doctorado Ingeniería Química, Ambiental y Bioalimentaria en su reunión de fecha 02/10/2017, acordó la presentación de la tesis doctoral a la Comisión de Doctorado, previa comprobación de la acreditación en el *Documento de Actividades* del autor de la Tesis doctoral de los requisitos formativos previstos en la memoria de verificación del Programa de Doctorado, y de que la tesis presentada y la documentación que la acompaña cumplen con la normativa vigente, según lo establecido en el Art.30.7 del Reglamento de los Estudios de Doctorado, aprobado por el Consejo de Gobierno, en su sesión del día 17 de junio de 2013 (BOPA del 25 de junio de 2013)

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La memoria de la Tesis Doctoral presentada recoge el trabajo realizado por la doctoranda Lucía Guadamuro García en el contexto del Programa de Doctorado de Ingeniería Química, Ambiental y Bioalimentaria. Una vez comprobado que la tesis presentada y la documentación que la acompaña cumplen la normativa vigente, la Comisión Académica del Programa de Doctorado autoriza su presentación y depósito.

Oviedo, 2 de Octubre 2017

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AGCC: Ácidos grasos de cadena corta

BAL: Bacterias ácido-lácticas

CCR: Cáncer colorectal

DGGE: "Denaturing gradient gel electrophoresis" (Electroforesis en gel con gradiente desnaturalizante)

DHD: Dihidrodaidzeína

EFSA: "European Food Safety Authority" (Autoridad Europea de Seguridad Alimentaria)

EMA: "Ethidium monoazide" (Monoazida de etidio)

EII: Enfermedad inflamatoria intestinal

ERs: Receptores estrogénicos

FOS: Fructo-oligosacáridos

GOS: Galacto-oligosacáridos

LDL: "Low-density lipoproteins" (Lipoproteínas de baja densidad)

PDA: "Photodiode array detector" (Detector de fotodiodos en serie)

***O*-DMA:** *O*-desmetilangolensina

ORF: "Open reading frame" (Marco de lectura abierto)

qPCR: PCR cuantitativa

QPS: "Qualified presumption of safety" (Presunción cualificada de seguridad)

SHBG: "Sex hormone binding globulin" (Globulina fijadora de hormonas sexuales)

SPRR2A: "Small proline rich protein 2A" (Proteína pequeña 2A rica en prolina)

TGI: Tracto gastrointestinal

THS: Terapia hormonal sustitutoria

UHPLC: "Ultra high performance liquid chromatography" (Cromatrografía líquida de ultra-alto rendimiento)

UPLC-MS: "Ultra performance liquid chromatography-mass spectrometry" (Cromatrografía líquida de ultra rendimiento acoplada a espectrometría de masas)

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Resumen

Los efectos beneficiosos de la soja en la salud se atribuyen a su alto contenido en proteínas de gran valor biológico y a su contenido en isoflavonas. Las isoflavonas son polifenoles con similitud química con el 17-β-estradiol que poseen actividad estrogénica. En las plantas las isoflavonas se encuentran conjugadas con azúcares en forma de glucósidos. La desglicosilación de los glucósidos de la soja libera las correspondientes agliconas, con mayor biodisponibilidad y actividad que los glucósidos. Las agliconas se metabolizan en el intestino dando lugar a compuestos con mayor actividad biológica (como el equol a partir de la daidzeína) o inactivos (como el *O*-DMA). Poco se conoce de las rutas y los microorganismos involucrados en este metabolismo. Al igual que otros polifenoles, las isoflavonas (o sus metabolitos) muestran cierta actividad antimicrobiana. Algunos metabolitos podrían también promover el desarrollo de determinados biotipos. La modulación de las poblaciones intestinales podría ser uno de los mecanismos de los efectos beneficiosos de las isoflavonas en la salud.

En este contexto, la Tesis Doctoral que aquí se presenta ha perseguido dos objetivos principales: (i) estudiar el efecto de la ingesta de isoflavonas sobre las poblaciones microbianas intestinales y su metabolismo y (ii) identificar y seleccionar microorganismos que activen las isoflavonas y que puedan emplearse como cultivos iniciadores funcionales. Ante la falta de metodología, fue necesario también mejorar o desarrollar métodos de extracción, detección y cuantificación de las isoflavonas y sus metabolitos a partir de muestras biológicas humanas.

Entre los resultados más importantes, podemos mencionar el desarrollo de un método de extracción en fase sólida y separación por cromatografía líquida de ultra-alto rendimiento (UHPLC) para el análisis simultáneo de agliconas y equol. Se desarrolló también un método de UPLC acoplado a espectrometría de masas de ionización por electroespray (UPLC-ESI-MS/MS) para detectar en heces compuestos de naturaleza fenólica.

Los efectos de las isoflavonas sobre las poblaciones microbianas intestinales se estudiaron en muestras de heces de un grupo de mujeres menopáusicas en tratamiento con isoflavonas. Observamos grandes variaciones en las comunidades intestinales de partida entre muestras de distintas voluntarias. Sin embargo, durante

el tratamiento se comprobó que se incrementaban algunos biotipos asociados con el metabolismo de fitoestrógenos. En una mujer productora de equol se comprobó que durante el tratamiento aumentaban las secuencias de la familia *Coriobacteriaceae* a la que pertenecen géneros relacionados con el metabolismo de las isoflavonas. El incremento de estos biotipos se observó también en fermentaciones fecales con isoflavonas y coincidió con un aumento de los ácidos grasos de cadena corta (AGCC), metabolitos microbianos relacionados con salud intestinal.

En cuanto al segundo objetivo -de una orientación más aplicada y tecnológica-, en primer lugar, se llevó a cabo una caracterización de microorganismos por su actividad β-glucosidasa (involucrada en la activación de isoflavonas), seleccionando ocho cepas de lactobacilos y dos de bifidobacterias. Estas cepas se emplearon para fermentar dos bebidas de soja comerciales en las que se evaluó el desarrollo de las cepas, los parámetros físico-químicos y microbiológicos básicos y las características sensoriales de los productos fermentados. Con el objetivo de profundizar en el conocimiento de las β-glucosidasas implicadas en la liberación de agliconas, se secuenció el genoma de una de las cepas con mayor actividad: *Bifidobacterium pseudocatenulatum* IPLA 36007. Cuatro genes que codificaban β-glucosidasas se sintetizaron *in vitro*, se clonaron en un sistema de sobreexpresión y las enzimas recombinantes se purificaron y se caracterizaron. Todas ellas actuaban sobre la daidzina y la genistina. Esta y otras cepas estudiadas son firmes candidatos para su utilización como cultivos iniciadores en la elaboración de productos fermentados a base de soja.

Summary

The beneficial health effects of soy are attributed to its content in proteins of high biological value and that of isoflavones. Isoflavones are polyphenols with chemical similarity to 17-β-estradiol possessing oestrogenic activity. In plants, isoflavones are conjugated to sugars in the form of glycosides. The deglycosylation of these compounds releases the corresponding aglycones, with greater bioavailability and activity than the glycosides. Aglycones are metabolized in the gut giving rise to compounds with more biological activity than their precursors (such as equol from daidzein) or inactive molecules (such as *O*-DMA). Little is known of the pathways and microorganisms involved in this metabolism. Like other polyphenols, isoflavones (or their metabolites) have shown some antimicrobial activity. Isoflavone metabolites could also promote the development of certain microbial biotypes. Modulation of intestinal populations could be one of the mechanisms for the beneficial effects of soy isoflavones in health.

In this context, the PhD report presented here had two main objectives: (i) to study the effect of isoflavone intake on the intestinal microbial populations and their metabolism and (ii) to identify and select microorganisms activating isoflavones that could be used as functional starter cultures. In the absence of appropriate methodology, it was also necessary to improve or develop methods for extracting, detecting and quantifying isoflavones and their metabolites from human biological samples.

Among the most important results, we can highlight the development of a solid phase extraction method followed by separation by ultra-high performance liquid chromatography (UHPLC) for the simultaneous detection of aglycones and equol. A UPLC method coupled to electrospray ionization mass spectrometry (UPLC-ESI-MS / MS) was also developed to detect compounds of phenolic nature in faeces.

The effects of isoflavones on the intestinal microbial populations were studied in faecal samples from a group of menopausal women under isoflavone treatment. Large variations in the intestinal communities between faecal samples of different volunteers were observed. However, along the treatment, biotypes associated with the metabolism of phytoestrogens were shown to increase. In an

equol-producing woman, sequences of the family *Coriobacteriaceae*, to which various genera related to the metabolism of isoflavones belong, were shown to increase. The increase of these biotypes even under *in vitro* culture conditions agrees well with an enhancement in the production of short chain fatty acids, microbial metabolites related to intestinal health.

As for the second objective –of a more applied and technological interest-, microorganisms with high β-glucosidase activity (involved in the activation of isoflavones) were characterized. Eight strains of lactobacilli and two of bifidobacteria were used to ferment two commercial soy beverages. In the fermented soy products, growth of the strains, basic physical-chemical and microbiological parameters and sensorial characteristics were evaluated. In order to increase our knowledge on the β-glucosidases involved in the release of aglycones from isoflavone glycosides, the genome of one of the strains with the strongest β-glucosidase activity (*Bifidobacterium pseudocatenulatum* IPLA 36007) was sequenced. From its genome, four genes encoding β-glucosidases were synthesized *in vitro*, cloned into an overexpression system and the recombinant enzymes were purified and characterized. All β-glucosidases acted on daidzin and genistin. This and other strains studied are strong candidates for use as starter cultures in the production of fermented soy products.

INTRODUCCIÓN

INTRODUCTION

INTRODUCCIÓN

1. ISOFLAVONAS

1.1. Polifenoles

Los compuestos polifenólicos son un grupo de sustancias de origen vegetal que se caracterizan por tener al menos un anillo aromático unido a uno o más grupos hidroxilo. En las plantas los polifenoles se encuentran como glicósidos con diferentes unidades de azúcares y grupos acilados en distintas posiciones del esqueleto fenólico central. Es el grupo de compuestos más numeroso y de mayor distribución en los vegetales, donde se han descrito unas 8000 estructuras fenólicas diferentes, de las cuales muchas forman parte de la dieta humana (Crozier y cols., 2009). Frutas, verduras, granos enteros y otros tipos de alimentos y bebidas como el té, el chocolate, el vino y la soja y sus productos derivados, son fuentes ricas en polifenoles. Los polifenoles son potentes antioxidantes que complementan la acción de las vitaminas y enzimas en la respuesta al estrés oxidativo generado por la presencia de un exceso de radicales libres en el organismo (Pandey y Rizvi, 2009). Por ese motivo, se piensa que los polifenoles podrían tener un papel relevante en la prevención de enfermedades neurodegenerativas, enfermedades cardiovasculares o de ciertos tipos de cáncer (Tsao, 2010).

La diversidad y amplia distribución de los polifenoles han dado lugar a diferentes formas de clasificar estos compuestos, principalmente en función de su origen, su función biológica y su estructura química (del Rio y cols., 2013). En cuanto a su estructura química, los polifenoles pueden clasificarse en dos grandes grupos: flavonoides y no-flavonoides. Los no-flavonoides son un grupo de compuesto fenólicos heterogéneo que abarca desde moléculas simples, como el ácido benzoico y los hidroxicinamatos, hasta moléculas más complejas, como los estilbenos, lignanos y taninos hidrosolubles (Crozier y cols., 2009; del Rio y cols., 2013). Los flavonoides son compuestos polifenólicos con 15 átomos de carbono organizados en dos anillos aromáticos conectados por un puente de tres carbonos. Las subclases principales de los flavonoides son las isoflavonas, las chalconas, los neoflavonoides, las flavonas, los flavonoles, las flavanonas, los flavanonoles, los flavanoles, las proantocianidinas y las antocianidinas (Tsao, 2010) (Figura 1).

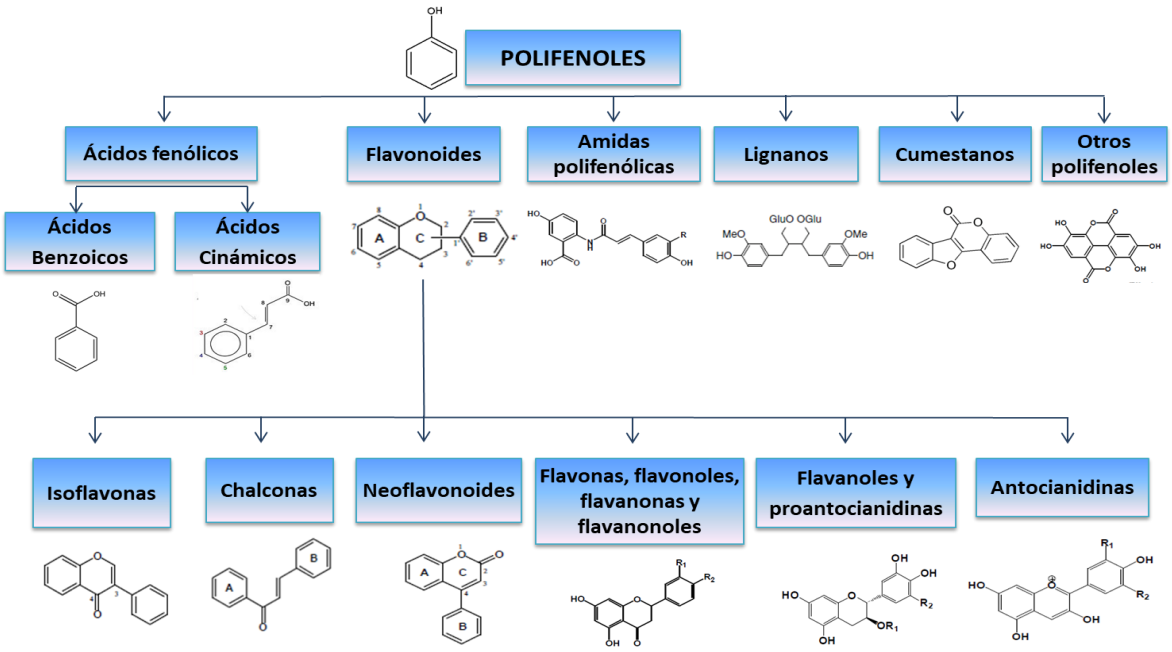


Figura 1. Clasificación de los polifenoles en función de su estructura química. Adaptado de Tsao (2010.

1.2. Fitoestrógenos

Los fitoestrógenos son un grupo heterogéneo de polifenoles con estructuras similares a la del 17-β-estradiol, lo que les permite interaccionar con los receptores estrogénicos (ERs) de las hormonas esteroideas y mostrar una cierta actividad hormonal (Pilšáková y cols., 2010). Se conocen alrededor de unos 100 tipos de fitoestrógenos diferentes que se dividen según su estructura química en flavonoides, entre los que destacan las isoflavonas (ej., daidzeína, genisteína, gliciteína); lignanos (ej., enterodiol, enterolactona); cumestanos (ej., cumestanol) y estilbenos (ej., resveratrol) (Crozier y cols., 2009). Por su actividad y relevancia, las isoflavonas y el estilbeno resveratrol han sido los fitoestrógenos más estudiados (Bilal y cols., 2014). Como polifenoles que son, los fitoestrógenos de los alimentos se encuentran en forma de complejos, frecuentemente como conjugados glicosilados biológicamente inactivos y poco absorbibles (del Rio y cols., 2013). Su biodisponibilidad (la proporción absorbida de una molécula o de sus metabolitos) depende de la liberación de los azúcares por enzimas tisulares y sobre todo por enzimas producidas por la microbiota gastrointestinal (Landete y cols., 2015).

Diversos estudios epidemiológicos sugieren que una dieta rica en alimentos que contienen fitoestrógenos reduce el riesgo de padecer algunas enfermedades crónicas, entre las que cabe destacar las enfermedades cardiovasculares, neurodegenerativas y

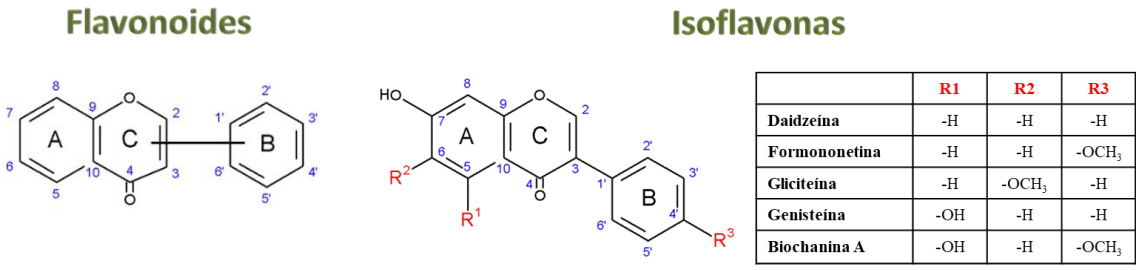
algunos tipos tipo de cáncer (Peeters y cols., 2007; Pilšáková y cols., 2010; Wada y cols., 2015; Bilal y cols., 2014).

1.3. Flavonoides e isoflavonas

Los flavonoides son moléculas que poseen en común una estructura química C6-C3-C6: dos anillos de seis carbonos de naturaleza fenólica unidos por un anillo pirano (Figura 2). Las variaciones en el anillo C y el patrón de hidroxilaciones dan lugar a los diferentes flavonoides (Tsao, 2010). Las isoflavonas son los compuestos más destacados dentro del grupo de los flavonoides. Su incorporación en la dieta parece ser clave para el buen funcionamiento de nuestro organismo (Messina, 2016).

1.4. Estructura química y propiedades de las isoflavonas

Las isoflavonas poseen la estructura general de los flavonoides con el anillo fenólico (B) unido por el carbono 3 al anillo cromano (C) (Figura 2). Los distintos tipos de isoflavonas se caracterizan por presentar sustituciones con diversos radicales en distintas posiciones. Así, en el caso de las isoflavonas de la soja, la diferencia entre los tipos principales (genisteína, daidzeína y gliciteína) radica en los sustituyentes que se encuentran en las posiciones 5 (radical 1) y 6 (radical 2) del anillo A y en la posición 4' del anillo B (radical 3).



	R1	R2	R3
Daidzeína	-H	-H	-H
Formononetina	-H	-H	$-OCH_3$
Gliciteína	-H	$-OCH_3$	-H
Genisteína	-OH	-H	-H
Biochanina A	-OH	-H	$-OCH_3$

Figura 2. Estructura del esqueleto central de los flavonoides y de las isoflavonas mostrando la numeración de los carbonos y las posiciones de sustitución que dan lugar a las diferentes agliconas.

Como en otros polifenoles, las formas conjugadas son la forma característica en las que las isoflavonas se encuentran en los vegetales. La conjugación más frecuente consiste en una β-glucosilación a partir del grupo hidroxilo de la posición 7 del anillo A (del Rio y cols., 2013). También es frecuente que las isoflavonas se encuentren en

forma de acetil, malonil o succinil glucósidos. Las formas conjugadas poseen mayores pesos moleculares y son más hidrofílicas que las agliconas, por lo que son más solubles, pero apenas son absorbidos en el intestino delgado (Izumi y cols., 2000). Por esta razón es necesaria la liberación de las agliconas, formas más hidrofóbicas que se absorben más rápidamente y en mayor cantidad en el intestino (Barnes y cols., 2011).

La primera vía de actuación de las isoflavonas en el organismo viene dada por la semejanza estructural que presentan con el 17-β-estradiol, motivo por el interaccionan con los ERs (Barnes y cols., 2000). Los ERs se localizan principalmente en la membrana del núcleo de las células de los tejidos en que se expresan (Pilšáková y cols., 2010). En el organismo hay dos tipos de ERs, los ER-α y los ER-β, que se expresan de forma diferencial en diferentes tejidos. La interacción de los estrógenos con los ERs desencadena una cascada de señalización celular que termina por afectar al proceso de transcripción (Belcher y Zsarnovszky, 2001), así como a la actividad enzimática de las proteínas G, la enzima adenilato ciclasa, las fosfolipasas y las proteín quinasas (Pilšáková y cols., 2010). La afinidad de la genisteína por los ER-β es comparable a la afinidad que posee el 17-β-estradiol; esta afinidad es unas 20 a 30 veces superior a la que presenta por los ERα (Kuiper y cols., 1998). La afinidad del resto de isoflavonas por los ERs es del orden de 100 a 500 veces inferior a la de la genisteína. Esta diferente afinidad explica que los efectos de las isoflavonas sean menores o inapreciables en los tejidos con receptores ER-α (tejido mamario y endometrio), respecto a los que se inducen en los tejidos donde predominan células con ER-β (hueso, pared vascular, tracto urogenital y sistema nervioso central). Las isoflavonas tienen una acción estrogénica o antiestrogénica en función del tipo y número de receptores que estén disponibles, lo que depende a su vez de la concentración relativa de isoflavonas y hormonas endógenas (Hwang y cols., 2006). De esta manera, cuando los niveles endógenos de 17-β-estradiol son elevados (por ejemplo durante la fase folicular del ciclo menstrual de la mujer), las isoflavonas son incapaces de unirse a los ERs. En cambio, cuando los niveles endógenos son bajos (por ejemplo durante la menopausia o después de una ovariectomía), la acción de las isoflavonas es mayor (Pilšáková y cols., 2010). Tras la unión de las isoflavonas al receptor, el complejo que se forma y las rutas de señalización intracelular desencadenadas son similares, aunque de menor magnitud, a los que se inducen por el 17-β-estradiol.

Otra vía de actuación de las isoflavonas es a través de su interacción con el metabolismo de las hormonas sexuales esteroideas (Pilšáková y cols., 2010). Las

isoflavonas pueden inhibir la actividad de la enzima 5α-reductasa (que cataliza la conversión de la testosterona en 5α-dihidrotestosterona). En altas concentraciones pueden activar la aromatasa P450 (que cataliza la conversión de testosterona a 17-β-estradiol) o inhibirla cuando se encuentran en baja concentración (Almstrup y cols., 2002). Así mismo, se ha descrito que las isoflavonas pueden unirse a la globulina fijadora de hormonas sexuales (SHBG) y estimular su síntesis, lo que puede afectar a la concentración de hormonas circulantes (Adlercreutz y cols., 1987).

1.5. Las isoflavonas de la soja

Las isoflavonas se encuentran en más de 300 plantas, con una mayor concentración en raíces y semillas (Aguiar y cols., 2007). De todas ellas la soja es una de las principales fuentes de isoflavonas de la dieta (Kaufman y cols., 1997). La concentración (entre 1,2 y 4,2 mg por g de peso seco) depende de las características del suelo en el que se cultiva, del clima, de la madurez de la planta en el momento de la recolección de la semilla o del procesado a que se somete con posterioridad (Caldwell y cols., 2005; Thompson y cols., 2006; Zhang y cols., 2014). Al igual que otros compuestos fenólicos, las isoflavonas de la soja se encuentran en la mayor parte como conjugados glicosilados (>80%). Como ya se adelantaba para los polifenoles, los glicósidos mayoritarios de las isoflavonas (genistina, daidzina y glicitina) apenas son absorbidos en el intestino y presentan una baja actividad estrogénica (Nielsen y Williamson, 2007); solo tras la hidrólisis adquieren su máxima biodisponibilidad y actividad biológica (Crozier y cols., 2009; de Cremoux y cols., 2010). La desglicosilación de los glicósidos de isoflavonas de soja da como resultado las agliconas genisteína, daidzeína y gliciteína, respectivamente.

1.6. Extractos de isoflavonas

Además de las presentes en los alimentos de la dieta, las isoflavonas se ingieren también como extractos concentrados en estos compuestos (de venta en farmacias y parafarmacias). Estos concentrados se utilizan para tratar los síntomas de la menopausia que resultan del descenso de estrógenos en el organismo (sofocos, sequedad vaginal, insomnio, fatiga, etc.) (Figura 3). Actualmente se encuentran en el mercado preparados de distintas casas comerciales: 12st Century®, Fisiogen®, SoyLife^{TM}, AllNatural®, LifeExtension®, etc. Estos concentrados, con cantidades variables de las distintas isoflavonas y distintas proporciones de formas glicosiladas y agliconas, se obtienen de

diversas plantas y se elaboran mediante varios métodos de extracción y concentración. Las tres principales fuentes de concentrados de isoflavonas son las semillas de soja, el trébol rojo y la raíz de "kudzu" (nombre común en chino de la planta *Pueraria lobata* que se utiliza en la medicina tradicional china) (Autoridad Europea de Seguridad Alimentaria (EFSA), 2015). Las semillas de soja contienen una mezcla de genisteína y daidzeína (>80% glicosiladas), mientras que los extractos de trébol rojo contienen formononetina y biochanina A (también glicosiladas mayoritariamente) y el kudzu contiene esencialmente glicósidos de daidzeína y genisteína (en una relación de dos tercios a un tercio) y pueranina (Delmonte y cols., 2006).

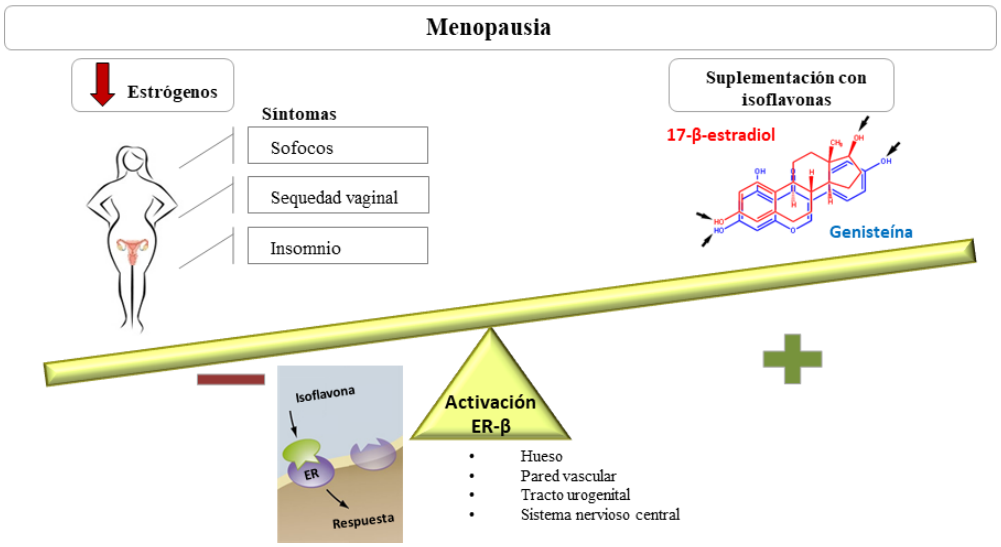


Figura 3. Esquema mostrando los síntomas típicos de la menopausia y las posibles vías de actuación de las isoflavonas.

De forma muy reciente, la EFSA evaluó los aspectos de seguridad que supone el consumo de suplementos de isoflavonas en mujeres peri- y post-menopaúsicas sobre los órganos endocrinos (glándula mamaria, útero y tiroides) (EFSA, 2015). Tras revisar de forma sistemática la evidencia científica en la literatura, los expertos concluyeron que la ingesta de estos suplementos es segura en mujeres que presentan una función tiroidea normal y que no padecen cáncer de pecho o de útero dependientes de estrógenos (EFSA, 2015).

1.7. Metabolismo de las isoflavonas

El metabolismo de las isoflavonas comienza, como ya se ha comentado, con su desglicosilación en el intestino delgado mediante enzimas de los enterocitos y enzimas microbianas, liberando las agliconas. En los enterocitos del intestino delgado, las agliconas sufren transformaciones metabólicas para convertirse en metabolitos sulfatados, glucoronidados y metilados que pasan al torrente sanguíneo. Desde allí, estos metabolitos alcanzan el hígado, donde son metabolizados o secretados con la bilis, lo que los devuelve al intestino. Las isoflavonas que no son absorbidas y las que se secretan al intestino son desconjugadas por las enzimas microbianas, pudiendo ser reabsorbidas de nuevo, metabolizadas o excretadas (Kemperman y cols., 2010) (Figura 4).

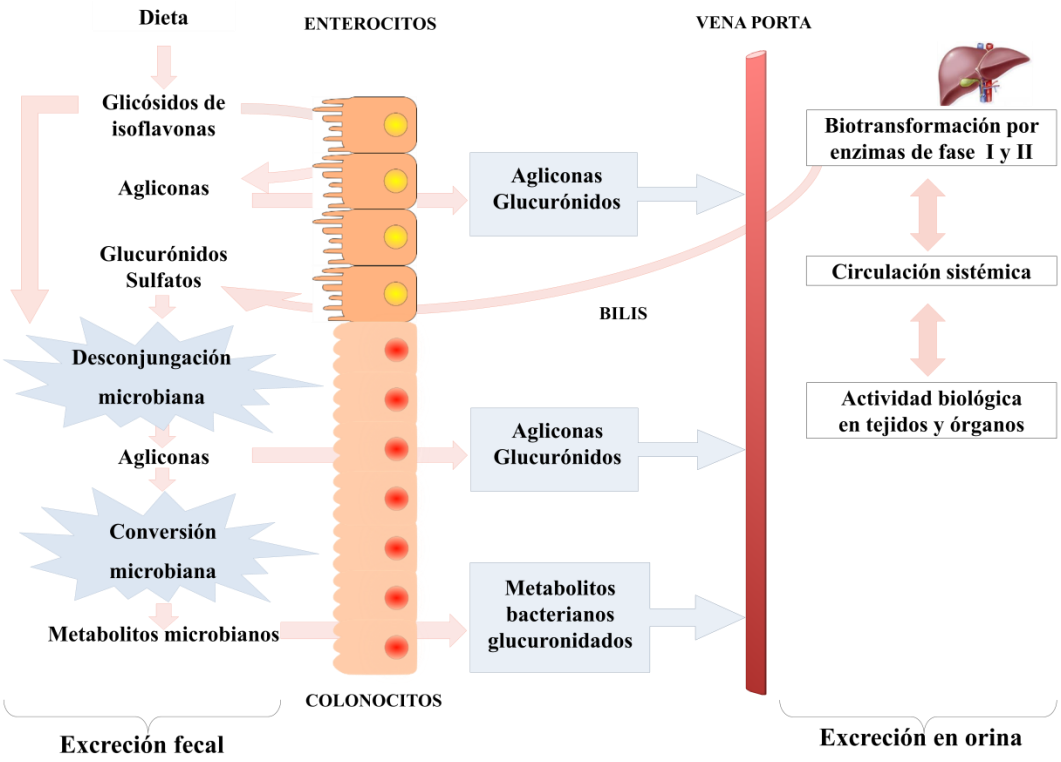


Figura 4. Diagrama general del metabolismo de las isoflavonas en el organismo. Adaptado de Kemperman y cols. (2010).

1.8. Propiedades beneficiosas de las isoflavonas

La ingesta de soja se ha asociado repetidamente con diversos efectos beneficiosos en la salud. Los efectos de las isoflavonas parecen estar mediados por las actividades hormonales (Yuan y cols., 2007), antioxidantes (Arora y cols., 1998) e inhibidoras de enzimas que actúan sobre el ADN (Crozier y cols., 2009). Al contrario que las correlaciones epidemiológicas, los estudios intervencionales no son tan concluyentes, tal y como ha puesto de manifiesto la EFSA en una revisión sistemática reciente (EFSA, 2012). En general, existe un consenso entre la comunidad científica sobre la falta de estudios a largo plazo, así como un escaso conocimiento de los cambios que se producen en los marcadores metabólicos de salud durante las intervenciones nutricionales con soja o con isoflavonas (Decroos y cols., 2006; Charles y cols., 2009).

1.8.1. Isoflavonas y menopausia

Las mujeres asiáticas sufren menos sofocos, sudoraciones nocturnas y otros síntomas de la menopausia (Figura 3) en comparación a las mujeres caucásicas, lo que se ha relacionado con una mayor ingesta de alimentos a base de soja (Messina, 2000). Además de estas evidencias epidemiológicas, las isoflavonas se comenzaron a utilizar masivamente como tratamiento alternativo de la Terapia Hormonal Sustitutoria (THS). Su utilización se disparó tras la publicación de los resultados de la Women's Health Initiative en el año 2002 (http://www.nhlbi.nih.gov/whi/), donde se sugería que había una mayor incidencia de cáncer de pecho, útero y enfermedades cardiovasculares en mujeres menopaúsicas tratadas con THS (Borrelli y Ernst, 2010; Molla y cols., 2011). En la actualidad, las conclusiones de la WHI están siendo sometidas a una revisión crítica en la que parece que la relación de la incidencia de las enfermedades con el tratamiento no es tan clara como se pensaba en un principio (Lobo, 2016; Langer, 2017). En cuanto a la eficacia de las isoflavonas la mayoría de las revisiones y meta-análisis recientes muestran resultados no concluyentes (Jacobs y cols., 2009; Messina, 2010; Taku y cols., 2010). En una de las últimas revisiones sistemáticas, los autores concluyen tan solo que los "extractos que contienen altos niveles de genisteína parecen reducir el número de sofocos diarios y requieren ser investigados en mayor profundidad" (Lethaby y cols., 2013). Las diferencias en la respuesta a las isoflavonas pudieran estar mediadas por la formación de diferentes metabolitos finales en distintos individuos (Peeters y cols., 2007; Franke y cols., 2014). Estas diferencias pudieran ser debidas a las grandes diferencias interindividuales en la composición y/o actividad de la

microbiota intestinal (Qin y cols., 2010). Esto mismo parece sugerirse también de gran parte de los trabajos más recientes sobre isoflavonas y menopausia (Aso y cols., 2012; Jenks y cols., 2012; Crawford y cols., 2013; Newton y cols., 2015).

1.8.2. Efectos beneficiosos de las isoflavonas en otras patologías

1.8.2.1. Las isoflavonas y el sistema cardiovascular

En los países asiáticos la incidencia de enfermedades cardiovasculares es aproximadamente ocho veces menor que en los países occidentales (Rosell y cols., 2004; Yamori, 2006). Además de los factores genéticos, se piensa que esa gran diferencia tiene una base nutricional. Las revisiones sistemáticas y los meta-análisis sugieren que el consumo de soja reduce poco, pero de manera significativa, el colesterol total y el colesterol unido a lipoproteínas de baja densidad (LDL) (Reynolds y cols., 2006; Harland y Haffner, 2008). Resultados parecidos se han demostrado también con la ingesta de isoflavonas purificadas (Taku y cols., 2008). Diversos datos sugieren que las isoflavonas regulan la actividad de los vasos sanguíneos (Hermenegildo y cols., 2005), el metabolismo lipídico y el transporte de colesterol (Pilšákeová y cols., 2010; González-Granillo y cols., 2012). Todos estos factores pueden tener una influencia sobre el sistema cardiovascular.

1.8.2.2. Isoflavonas y hueso

Bajos niveles de 17-β-estradiol en suero se han asociado con una biodisponibilidad reducida de calcio y la activación de citoquinas que aceleran la reabsorción del hueso, lo que conduce finalmente a la osteoporosis (Wei y cols., 2012). Existen trabajos que sugieren que las isoflavonas podrían prevenir esta patología (Cassidy y cols., 2006; Wei y cols., 2012). Los últimos meta-análisis que analizan los resultados de ensayos controlados y aleatorizados concluyen que la intervención con isoflavonas atenúa significativamente la pérdida de hueso espinal en mujeres menopáusicas, inhibe la reabsorción ósea y estimula la formación de hueso nuevo (Taku y cols., 2010; Taku y cols., 2011; Wei y cols., 2012). Otros autores sin embargo, no encuentran relaciones estadísticas significativas entre consumo de isoflavonas y calcificación (Tai y cols., 2012). Muchos factores endógenos (genética, microbiota intestinal) y exógenos (tipo de isoflavona y concentración, dieta y estilo de vida) pudieran influir también en la respuesta fisiológica de los pacientes (Ho y cols., 2008).

1.8.2.3. Isoflavonas y cánceres dependientes de estrógenos

La incidencia del cáncer de próstata, colon y algunos cánceres de mama es mucho menor en los países orientales que en los occidentales (Perabo y cols., 2008; Fritz y cols., 2013; He y Chen, 2013; Sugiyama y cols., 2013; Mahmoud y cols., 2014). Sin embargo, parece que los individuos asiáticos que emigran a países occidentales y cambian sus hábitos dietéticos terminan padeciendo estos tipos de cáncer con una frecuencia similar a la de la población de acogida (He y Chen, 2013), lo que se ha relacionado con el abandono del consumo de soja. En intervenciones controladas, se han detectado daidzeína, genisteína y sus metabolitos en tejido de próstata (Hong y cols., 2002) y mama (Maubach y cols., 2003) tras la ingestión de isoflavonas. Estos metabolitos podrían interferir con el metabolismo de los estrógenos y con otros procesos no hormonales (procesos epigenéticos, unión a receptores activados por proliferadores de peroxisomas, inducción de la apoptosis, estimulación de la autofagia, etc.) que pudieran finalmente incidir en estas patologías (Pilšáková y cols., 2010; He y Chen, 2013).

1.8.2.4. Isoflavonas y sistema nervioso central

Las isoflavonas, al igual que el 17-β-estradiol, son capaces de atravesar la barrera hemato-encefálica. Sin embargo, poco se conoce sobre la influencia de estos fitoestrógenos en el sistema nervioso central. Los estudios epidemiológicos revelan de nuevo que las tasas de demencia son inferiores en las poblaciones asiáticas (Liu y cols., 2003). Los estudios con animales han demostrado que las isoflavonas tienen efectos potencialmente beneficiosos sobre algunos problemas relacionados con la edad, como la reducción de la capacidad de aprendizaje, la memoria y la ansiedad (Lephart y cols., 2002; Sarkaki y cols., 2008). Por su parte, las intervenciones en el hombre centradas en las funciones cognitivas reportan en general efectos beneficiosos de las isoflavonas (Fournier y cols., 2007). En este sentido, el consumo de isoflavonas a largo plazo se ha asociado en mujeres menopáusicas con una mejora del aprendizaje, el pensamiento lógico y la habilidad planificadora (Duffy y cols., 2003; Karvaj y cols., 2007; Kreijkamp-Kaspers y cols., 2007).

1.8.2.5. Isoflavonas y otros efectos sobre la salud

Algunos autores han sugerido también que las isoflavonas presentan un efecto anti-edad sobre la piel (proporcionando protección frente al daño ocasionado por la luz

ultravioleta) modulan la obesidad y la diabetes de tipo 2 y podrían mejorar la enfermedad renal crónica (Yuan y cols., 2007; Charles y cols., 2009; Jackson y cols., 2011; Jing y Wei-Jie, 2016).

Todas estas observaciones, sin embargo, han de ser sustanciadas con cuidadosas investigaciones científicas para separar los efectos reales de las meras asociaciones estadísticas. Solo de esta forma nos podremos beneficiar en el futuro del consumo de soja y sus productos derivados, tanto en el estado de salud como en algunas situaciones de enfermedad.

2. MICROBIOTA INTESTINAL

2.1. Composición y variabilidad

El término microbiota intestinal hace referencia al conjunto de microorganismos que habitan en el tracto gastrointestinal (TGI) y que conviven e interactúan con el hospedador. Recientemente se ha estimado que en el cuerpo humano hay aproximadamente la misma cantidad de microorganismos que células eucariotas ($\sim 10^{13}$) o incluso un número mayor si no se computan las células anucleadas (los glóbulos rojos) (Sender y cols., 2016). El estómago y el intestino delgado sólo permiten el establecimiento de un número relativamente bajo de microorganismos, capaces de tolerar las condiciones de pH, oxígeno y el rápido tránsito de alimento y secreciones que tiene lugar en estas posiciones. En contraste, las condiciones del intestino grueso favorecen el establecimiento de una comunidad microbiana mucho más densa y compleja (Figura 5), dominada por bacterias anaerobias estrictas (Sekirov y cols., 2010).

La microbiota intestinal constituye un sistema microbiano diverso en el que dominan bacterias de los filos *Firmicutes* y *Bacteroidetes,* seguidas por otras de los filos *Proteobacteria*, *Actinobacteria*, *Fusobacteria* y *Verrucomicrobia* (Rajilić- Stojanović y cols., 2007). La mayoría de *Firmicutes* presentes en el TGI pertenecen a la clase *Clostridia*, principalmente a los grupos XIVa y IV (Rajilić- Stojanović y cols., 2007). A nivel de género son abundantes *Faecalibacterium, Roseburia, Blautia, Ruminococcus, Eubacterium,* así como los que componen la familia *Lachnospiraceae*. Dentro del filo *Firmicutes* también se encuentran bacterias ácido-lácticas (BAL) pertenecientes a los géneros *Enterococcus, Streptococcus* y *Lactobacillus,* entre otros (Figura 5). Hongos y arqueas componen una parte minoritaria; entre el 0,05% y el 1%, respectivamente, de los componentes de la microbiota intestinal (Tannock, 2007). La microbiota se completa

con el conjunto de virus intestinales, formado en su mayoría por bacteriófagos y cuyo número se estima que sobrepasa unas 100 veces el número de bacterias (Virgin, 2014).

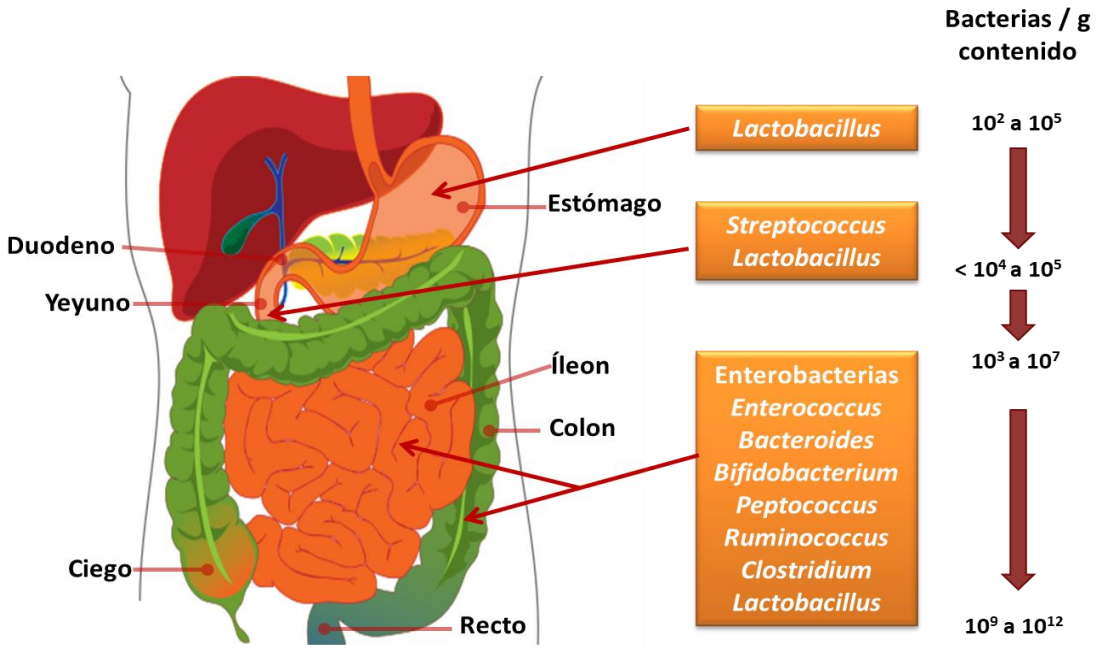


Figura 5. Variaciones longitudinales de la microbiota a lo largo del tracto gastrointestinal. Adaptado de Konturek y cols. (2015).

Aunque la microbiota intestinal está constituida por miembros de unos pocos filos, a nivel de especie presenta una enorme diversidad. Así, se ha estimado que en el TGI pueden encontrarse entre 1000 y 1500 especies bacterianas diferentes (Qin y cols., 2010). El mantenimiento de la diversidad microbiana parece ser importante para mantener el estado de salud, ya que muchas patologías intestinales cursan con una pérdida de diversidad (Mosca y cols., 2016). Otra característica importante de la microbiota es su extremada variabilidad interindividual (Qin y cols., 2010). En estudios con gemelos monocigóticos se ha comprobado que, en algunos casos, estos comparten menos del 50% de las especies (Turnbaugh y cols., 2010). Algunos autores han planteado la existencia de un núcleo común de microorganismos dominantes y prevalentes en la microbiota fecal de la mayoría de adultos sanos. Este núcleo bacteriano de la microbiota intestinal (denominado "core") constaría de algo más de 50 taxones que se encuentran en más del 80% de los sujetos analizados y estaría formado por especies de los géneros *Faecalibacterium, Ruminococcus, Eubacterium, Dorea, Bacteroides, Alistipes* y *Bifidobacterium* según Qin y colaboradores (2010).

A pesar de esa gran diversidad y variabilidad, la funcionalidad de la microbiota parece ser relativamente estable entre individuos como se desprende de los estudios del contenido génico (Lozupone y cols, 2012). En el metagenoma de la microbiota intestinal, entendiendo por tal el conjunto de genomas de los miembros de esta microbiota (Marchesi y Ravel, 2015), están codificados más de 3 millones de genes, lo que equivale a 150 veces más genes que los presentes en el genoma humano (Qin y cols, 2010). El metagenoma microbiano intestinal codifica funciones importantes para el correcto funcionamiento del organismo, incluyendo la fermentación de compuestos de la dieta y sustratos endógenos lo que da lugar a la producción, entre otros, de metabolitos tan importantes como los ácidos grasos de cadena corta (AGCC).

2.2. Funciones de la microbiota

La microbiota intestinal desempeña un papel clave en el correcto funcionamiento de nuestro organismo a través de diversas funciones, tal y como se relaciona a continuación.

2.2.1. Metabolismo de nutrientes

El metagenoma intestinal codifica enzimas de rutas bioquímicas que el hospedador no posee. Este hecho dota de una función metabólica importante a la comunidad microbiana intestinal. Diversos sistemas enzimáticos permiten el aprovechamiento de energía de los alimentos mediante la utilización de sustratos de la dieta no digeribles por nuestro organismo. En algunos casos, los metabolitos que se generan son esenciales como los aminoácidos ramificados, ciertos AGCC como el butirato, y otros compuestos como la vitamina K y algunas vitaminas hidrosolubles (cianocobalamina, biotina, ácido fólico, ácido pantoténico) (Jandhyala y cols., 2015).

2.2.2. Protección frente a patógenos

Mediante la adhesión al epitelio intestinal los componentes de la microbiota limitan el contacto físico de los microorganismos patógenos con el hospedador, ejerciendo una barrera protectora ante posibles infecciones (Jandhyala y cols., 2015). A este efecto barrera también se añade el hecho de que una gran parte de las bacterias intestinales producen sustancias antimicrobianas como bacteriocinas y AGCC. Estos compuestos inhiben el crecimiento de otros microorganismos, siendo un mecanismo de antibiosis más allá de la competencia por los nutrientes del medio. Por otro lado, la

microbiota intestinal, a través de metabolitos y componentes estructurales es capaz de inducir la síntesis de proteínas antimicrobianas en las células eucariotas, así como de incrementar la producción local de inmunoglobulinas (Jandhyala y cols., 2015).

2.2.3. Mantenimiento de la integridad estructural de la barrera intestinal

La microbiota intestinal contribuye al mantenimiento de la barrera del epitelio intestinal preservando las uniones estrechas intercelulares y la estimulando la reparación epitelial tras una lesión. Por ejemplo, se ha reportado que la especie *Bacteroides thetaiotaomicron* induce la expresión de la proteína pequeña 2A rica en prolina (SPRR2A), necesaria para el mantenimiento de los desmosomas en las vellosidades epiteliales (Sekirov y cols., 2010).

2.2.4. Desarrollo del sistema inmunológico e inmunomodulación

La microbiota intestinal contribuye al normal desarrollo del sistema inmunológico del hospedador a través de la interacción constante con las células de la mucosa del TGI. Esta interacción induce una especie de "educación" del sistema inmunológico, en el que se favorece la tolerancia hacia antígenos de la microbiota y de la dieta, al mismo tiempo que se desarrolla una respuesta rápida frente a microorganismos patógenos. Todo ello conduce al desarrollo de un sistema inmunológico funcional y a la denominada "homeostasis inmunológica" (Weng y Walker, 2013).

2.3. Papel de la microbiota en la salud y en la enfermedad

En condiciones normales la microbiota del TGI está en equilibrio, sin embargo diversos factores pueden afectar a la dinámica de las poblaciones microbianas y conducir a desequilibrios de las mismas, que se conocen con el término de disbiosis. Una microbiota descompensada puede alterar la homeostasis y llevar a una disfunción o estado de enfermedad. En los últimos años se han relacionado diversas disbiosis de la microbiota del TGI con algunas enfermedades infecciosas y no infecciosas (no solo en el TGI). Sin embargo, en muchos de los casos aún no se sabe con certeza si las disbiosis son causa o consecuencia de las patologías.

2.3.1. Enfermedades infecciosas

Uno de los desórdenes intestinales más comunes y mejor documentados es la diarrea asociada al tratamiento con antibióticos. La incidencia de esta diarrea se sitúa entre un 5 y un 25% de los casos de terapia antibiótica, siendo mucho más frecuente cuando se utilizan antimicrobianos de amplio espectro. El tratamiento con antibióticos perturba el balance de la microbiota intestinal normal permitiendo la proliferación de patógenos que se encuentran de manera habitual en pequeños números, como *Clostridium difficile*, causante de la colitis pseudomembranosa (Bergogne-Bérézin, 2000).

2.3.2. Enfermedades inflamatorias

La enfermedad inflamatoria intestinal (EII) es una patología crónica que afecta al colon y/o al intestino delgado. Dentro de este término se engloban dos enfermedades comunes que afectan al 0,1% de la población en las sociedades occidentales y que presentan una incidencia en aumento: la enfermedad de Crohn y la colitis ulcerosa. La etiología de estas enfermedades es todavía poco clara, aunque en ambos casos parece demostrado que el sistema inmunológico reacciona de manera anormal contra componentes de la microbiota intestinal. De forma reciente se ha observado que existe una pérdida de la diversidad y estabilidad de la microbiota en pacientes con enfermedad de Crohn (Vindigni y cols., 2016).

2.3.3. Enfermedades metabólicas

La microbiota intestinal se ha señalado en los últimos años como uno de los factores implicados en la regulación del peso corporal (Vrieze y cols., 2012). De esta forma, se han asociado alteraciones en la microbiota con ciertas enfermedades y trastornos metabólicos como la obesidad, la diabetes de tipo II y el síndrome metabólico (Cani y Delzenne, 2009). Aunque aún existe cierta controversia, se han descrito diferencias en la microbiota entre individuos delgados y obesos, con un incremento de la proporción relativa del filo *Firmicutes* en detrimento de la proporción de *Bacteroidetes* en individuos obesos (Turnbaugh y cols, 2009).

2.3.4. Enfermedades de base inmunológica

En las sociedades desarrolladas la incidencia de alergias y de enfermedades con componente autoinmune ha crecido de modo exponencial durante la segunda mitad del

siglo XX. La hipótesis de la higiene excesiva sugiere que la falta de una exposición adecuada a microorganismos en edades tempranas de la vida podría ser la casusa de la creciente aparición de disbiosis microbianas en diversas patologías de base inmunológica (Hua y cols., 2016). En este sentido, cabe mencionar que estudios recientes en modelos animales con esclerosis múltiple y artritis reumatoide sugieren una fuerte influencia de la microbiota intestinal en el desarrollo de estas enfermedades (Lee y Mazmanian, 2010).

2.3.5. Cáncer colorectal

En el desarrollo de cáncer de colon (CCR), además de factores hereditarios, parecen estar implicados factores de interacción entre la microbiota colónica, la dieta y el epitelio del colon (Zackular y cols., 2013). En el hombre, diversos estudios apuntan a la existencia de disbiosis microbiana intestinal en pacientes con CCR, además se ha asociado la presencia de microorganismos concretos a su patogénesis como *Fusobacterium nucleatum* (Coleman y Nunes, 2016). La disbiosis intestinal, a través de la producción de metabolitos bacterianos tóxicos, la reducción de metabolitos microbianos beneficiosos y la ruptura de la barrera intestinal puede conducir a una activación inmunológica anormal, lo que a su vez produce la inflamación que contribuye al desarrollo y progreso del CCR (Sun y Kato, 2016).

2.4. Microbiota intestinal y dieta

La dieta es un factor relevante cuando se habla de la microbiota intestinal, ya que el consumo de ciertos alimentos puede afectar el establecimiento, desarrollo y competencia de los microorganismos que del TGI. Así lo sugieren diversos estudios que comparan la microbiota intestinal de poblaciones occidentales, cuya dieta habitualmente contiene una cantidad reducida de fibra y alta cantidad de grasas y carbohidratos refinados, con la de poblaciones de áreas rurales de África y Sudamérica (Graf y cols., 2015). De forma reciente se ha comprobado que la microbiota intestinal responde rápidamente a cambios en la dieta, aunque aún no se conoce con exactitud en qué medida los hábitos dietarios determinan la composición microbiana a largo plazo (David y cols., 2014). Algunos autores apuntan a que las intervenciones dietarias probablemente producen un impacto mayor sobre la funcionalidad de la microbiota que sobre su composición *per se* (Sonnenburg y Bäckhed, 2016). Sin embargo, debido a las grandes diferencias en la microbiota intestinal entre individuos, los cambios en la dieta

pueden tener efectos muy variables en diferentes personas. Así, en estudios intervencionales se ha descrito que en unos individuos se producen incrementos de ciertas especies microbianas como respuesta a un cambio en la dieta, mientras que en otros individuos las mismas poblaciones pueden disminuir (Cotillard y cols., 2013). De forma análoga, sujetos con una baja diversidad microbiana pueden responder con un aumento de esta diversidad, mientras que los sujetos que parten de una alta diversidad microbiana no responden de igual forma frente al mismo cambio dietario (Walker y cols., 2011).

Entre los componentes de la dieta se sabe que las grasas y la fibra presentan efectos acusados y diferentes sobre microbiota intestinal (Martínez y cols., 2010; Jumpertz y cols., 2011). Las grasas saturadas se asocian con una mayor prevalencia de *Bacteroides*, mientras que *Prevotella* parece disminuir con el aumento de la ingesta de grasa (Wu y cols., 2011). En lo que respecta a la fibra, los estudios intervencionales indican que estimula el crecimiento de bacterias productoras de butirato como *Roseburia, Eubacterium rectale* y *Faecalibacterium prausnitzii*, produciendo también un incremento de bifidobacterias y lactobacilos así como un cambio en la prevalencia de *Parabacteroides* en detrimento de *Bacteroides* (Graf y cols., 2015).

El impacto de muchos componentes y microcomponentes de la dieta, incluyendo compuestos fenólicos, no es tan conocido. En el caso de los polifenoles, varios estudios clínicos han reportado un efecto modulador de la microbiota con la ingesta de alimentos ricos en estas sustancias como cacao, té, uvas, vino, bayas, granadas y nueces (Dueñas y cols., 2015). En realidad, existe una interacción recíproca entre polifenoles y dieta: los polifenoles modulan la microbiota intestinal y los microorganismos modifican la actividad de los polifenoles, lo que a su vez afecta a su biodisponibilidad y actividad. De donde se puede concluir que el perfil de metabolitos resultantes de la ingesta de polifenoles y su efectividad depende en gran medida de la microbiota intestinal de cada individuo (Tomás-Barberán y cols., 2016).

2.5. Alimentación funcional

Hasta hace poco tiempo, la meta en la alimentación humana era asegurar un aporte adecuado de energía y de los macro y micronutrientes esenciales. A finales del siglo pasado apareció un nuevo concepto de nutrición que procura, además de los objetivos nutricionales, la promoción del bienestar y la prevención de las enfermedades. El conocimiento de la importancia de la dieta sobre la salud ha aumentado

considerablemente en los últimos años y se han identificado alimentos o componentes de los mismos que juegan un papel clave en la promoción de la salud (Alkerwi, 2014). Aquellos alimentos con un valor añadido por encima de los meros requerimientos nutricionales se conocen como alimentos funcionales. Estos alimentos contribuirían al mantenimiento y la prolongación del estado de salud (Katan y de Roos, 2004). El concepto de alimento funcional, que surgió en Japón en los años 80, requiere que su ingesta se efectúe como componente habitual de la dieta y que ejerza sus efectos en las cantidades que se ingieren con una dieta equilibrada. En el contexto de la alimentación funcional tienen un papel destacado, y en general bien reconocido, los probióticos y los prebióticos.

2.5.1. Probióticos

La Organización Mundial de la Salud (OMS) y la Asociación Científica Internacional para los Probióticos y Prebióticos (ISAPP) definen los probióticos como "*microorganismos vivos que, cuando son administrados en cantidades adecuadas, confieren un beneficio sobre la salud del hospedador*" (Hill y cols., 2014). Los probióticos se comercializan como suplementos alimenticios o como alimentos funcionales (siendo el principal vehículo de administración los productos lácteos fermentados). Los microorganismos probióticos más utilizados en la actualidad pertenecen a especies de los géneros *Lactobacillus* y *Bifidobacterium*. El tratamiento con probióticos va dirigido a mejorar la resistencia a infecciones y a tratar trastornos intestinales como el síndrome del intestino irritable, otros desórdenes intestinales crónicos o intolerancia a la lactosa, pero también existen probióticos de aplicación extraintestinal para tratar afecciones como la candidiasis vaginal recurrente o diversos problemas de piel (Sanders y cols., 2007).

2.5.2. Prebióticos

Los prebióticos constituyen otra estrategia de modulación de la microbiota intestinal. Se trata de compuestos que inducen cambios beneficiosos al fortalecer los niveles de ciertas bacterias autóctonas de la microbiota o al promover la producción de metabolitos microbianos beneficiosos. Recientemente, la ISAPP ha definido prebiótico como "*un sustrato que es utilizado selectivamente por microorganismos del hospedador confiriendo un efecto beneficioso sobre la salud*" (Gibson y cols., 2017). Estos compuestos, debido a su estructura química, no son absorbidos en la parte superior del

TGI ni son hidrolizados por las enzimas celulares. Los prebióticos más aceptados y estudiados son oligosacáridos no digeribles como los galacto-oligosacáridos (GOS) y los fructo-oligosacáridos (FOS). La ingesta de estos compuestos en una dieta normal es en general baja, de forma que para alcanzar las dosis efectivas han de añadirse a los alimentos o consumirse como suplementos alimenticios.

Las poblaciones bacterianas a quienes van dirigidos los prebióticos actuales son fundamentalmente las de lactobacilos y bifidobacterias. Sin embargo, de forma reciente, diversos microorganismos comensales del TGI considerados beneficiosos como *Faecalibacterium, Roseburia* y *Eubacterium* (productores todos ellos de butirato) se consideran dianas alternativas para los prebióticos del futuro (Gibson, 2017).

3. MICROBIOTA E ISOFLAVONAS

Como polifenoles que son, las isoflavonas no absorbidas y las excretadas de nuevo al TGI son metabolizadas por componentes de la microbiota. Al mismo tiempo, es posible que pudieran modular también la composición y/o la actividad de las poblaciones intestinales. En este apartado se explican en mayor detalle las interacciones de la microbiota intestinal con las isoflavonas y su metabolismo.

3.1. Desglicosilación de isoflavonas

Como ya se ha comentado, los glicósidos de las isoflavonas incrementan su absorción y funcionalidad cuando pierden sus residuos azucarados. Este paso lo realizan enzimas tisulares y enzimas de la microbiota intestinal (de Cremoux y cols., 2010). Enzimas capaces de llevar a cabo la desglicosilación son corrientes en bacterias de géneros abundantes como *Escherichia, Bacteroides* y *Clostridium* entre otros, lo que sugiere que la desglicosilación en el intestino no es un paso limitante en el metabolismo de las isoflavonas (Aura, 2008; Landete y cols., 2015). Las BAL y las bifidobacterias también están involucradas en la liberación de agliconas de los glicósidos de isoflavonas (Tsangalis y cols., 2002; Otieno y Shah, 2007; Champagne y cols., 2010; Rekha y Vijayalakshmi, 2010). De hecho, la secuenciación del genoma de diversas cepas de estos grupos ha revelado una impresionante cantidad de genes que codifican glicosilasas, incluyendo β-glucosidasas, enzimas que se consideran responsables de la liberación de las agliconas (Schell y cols., 2002).

3.2. Metabolismo de isoflavonas y producción de compuestos bioactivos

Las agliconas de las isoflavonas pueden continuar siendo metabolizadas en el intestino mediante reacciones de deshidroxilación, reducción, rotura de los anillos o desmetilación (Figura 6). Por ejemplo, la genisteína puede ser convertida en dihidrogenisteína, 6'-hidroxi-*O*-desmetilangolensina, *p*-etil fenol o 5′ -hidroxi-equol (Heinonen y cols., 2003; Steer y cols., 2003; Hosoda y cols., 2011). La gliciteína por su parte se puede convertir en dihidrogliciteína, dihidro-6,7,4′ -trihidroxiisoflavona, 5′ -*O*-metil-*O*-desmetilangolensina o 6-*O*-metil-equol (Simons y cols., 2005). Los principales metabolitos microbianos de la daidzeína, por su parte, son la hidroxidaidzeína (DHD) y el equol (Atkinson y cols., 2005; Rüfer y cols., 2008). La daidzeína también puede ser degradada hacia compuestos biológicamente inactivos como la *O*-desmetilangolensina (*O*-DMA) (Gardana y cols., 2014) (Figura 6). A pesar de conocer muchos metabolitos, no se conocen con exactitud las poblaciones microbianas que los producen. Tampoco se conocen las interacciones de las isoflavonas con la mayoría de las poblaciones microbianas del TGI. La identificación de microorganismos involucrados en la conversión de isoflavonas en compuestos más activos permitiría diseñar estrategias para conseguir incrementar las poblaciones microbianas deseables, favoreciendo la transformación adecuada de las isoflavonas y minimizando la formación de compuestos inactivos. Estas estrategias podrían basarse en la modulación de las poblaciones microbianas intestinales a través del uso de prebióticos (Steer y cols., 2003) y/o probióticos (Ding y Shah, 2010).

3.2.1. Equol

El equol [$C_{15}H_{12}O(OH)_2$] se identificó por primera vez en 1932 a partir de la orina de yeguas preñadas (Marrian y Haslewood, 1932). Más tarde, en 1982 fue el primer flavonoide detectado en orina y sangre humanas (revisión de Setchell y cols., 2001). El equol es un metabolito de la daidzeína y con una actividad estrogénica y una acción antioxidante mayor que cualquier otra aglicona o metabolito de las isoflavonas (Setchell y cols., 2002; Sánchez-Calvo y cols., 2013). Químicamente el equol es más estable que su molécula precursora y más fácilmente absorbible en el colon (Setchell y cols., 2002; Yuan y cols., 2007). La estructura química no planar del equol podría ser responsable, al menos en parte, de su fuerte actividad estrogénica (Crozier y cols., 2009). Las propiedades antiandrogénicas del equol son únicas, ya que se une específicamente a los receptores de la 5α-dihidrotestosterona, pero no a los receptores

de la testosterona (Lund y cols., 2004). Todas las especies animales estudiadas hasta ahora (ratón, rata, oveja, pollo y otras aves, cabra, vaca) producen equol en respuesta al consumo de soja (Setchell y cols., 2002). Sin embargo, en el hombre solamente alrededor de un 20-35% de los individuos occidentales lo produce, frente a un 50-55% de los individuos asiáticos (Setchell y Cole, 2006; Bolca y cols., 2007; Hall y cols., 2007; Peeters y cols., 2007). En cambio, entre un 80 a 90% de los sujetos producen *O*-DMA a partir de daidzeína (Atkinson y cols., 2005). Dada su mayor actividad, los individuos productores de equol podrían ser los que más se benefician del consumo de isoflavonas.

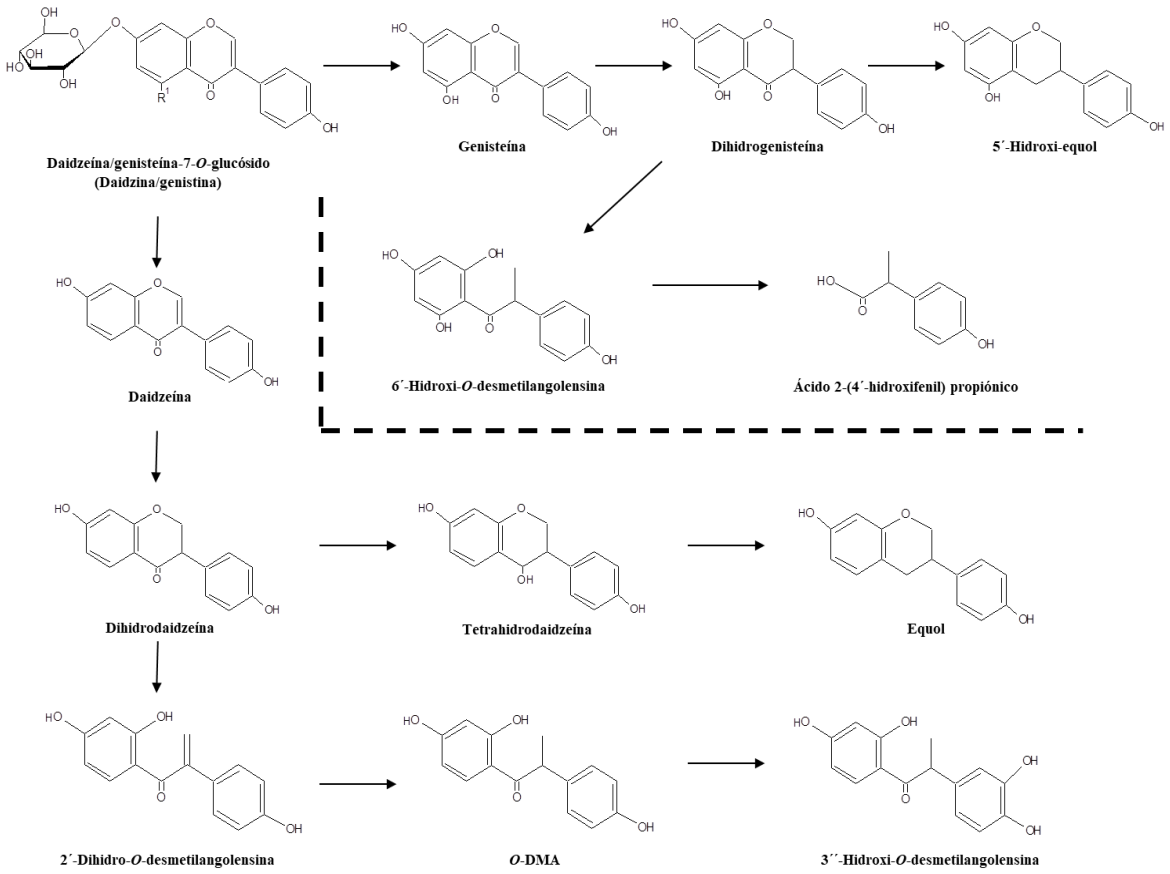


Figura 6. Metabolismo de los glúcosidos de isoflavonas genistina y daidzina.

3.2.1.1. Microorganismos productores de equol

El equol es una molécula ópticamente activa con átomos de carbono asimétricos que dan lugar a diferentes enantiómeros (isómeros ópticos). Sin embargo, la síntesis biológica da lugar únicamente a la forma S (S-equol) (Setchell y cols., 2005; Jackson y cols., 2011; Schwen y cols., 2012). La biosíntesis bacteriana de equol parece proceder a través de los intermediarios dihidrodaidzeína y tetrahidrodaidzeína (Figura 6). Nuestro conocimiento sobre los microorganismos que producen equol, aunque limitado, ha ido en aumento en los últimos años (Setchell y cols., 2002; Atkinson y cols., 2005; Yuan y cols., 2007; Setchell y Clerici, 2010). El equol no se detecta en la orina o plasma de la mayoría de los niños menores de 12 años (Cao y cols., 2009; Brown y cols., 2014), lo que sugiere que las bacterias productoras se instalan en el TGI en una etapa tardía. Inicialmente se describieron combinaciones de bacterias intestinales capaces de producir este compuesto (Decroos y cols., 2005; Wang y cols., 2007). Sin embargo, en las últimas décadas, se han identificado cepas únicas del intestino humano y de otros animales capaces de producir equol (Wang y cols., 2005; Uchiyama y cols., 2007; Maruo y cols., 2008; Yokoyama y Suzuki, 2008; Yu y cols., 2008; Tsuji y cols., 2010).

Aunque no se conoce con seguridad si la producción de equol es un carácter específico de familia, especie o cepa bacteriana (Clavel y Mapesa, 2013), casi todos los microorganismos productores aislados hasta el momento pertenecen a la familia *Coriobacteriaceae* (Clavel y cols., 2014). Además de metabolizar las isoflavonas, los miembros de esta familia también metabolizan hormonas esteroideas y ácidos biliares (Ridlon y cols., 2006), lo que apunta a una especialización funcional de estos tipos bacterianos en el TGI. La familia *Coriobacteriaceae* incluye los géneros *Adlercreutzia*, *Assacharobacter*, *Eggerthella*, *Enterorhabdus*, *Paraeggerthela* y *Slackia* (Clavel y cols., 2014). Las cepas productoras de equol se han identificado como *Assacharobacter celatus* (Minamida y cols., 2008), *Enterorhabdus mucosicola* (Clavel y cols., 2009), *Slackia isoflavoniconvertens* (Matthies y cols., 2009) y *Slackia equolifaciens* (Jin y cols., 2010). Algunos aislados solo han sido identificados a nivel de género, como las cepas *Eggerthella* sp. YY7918 (Yokoyama y cols., 2011), *Paraeggerthela* sp. 380 SNR40-432 (Abiru y cols., 2013) o *Slackia* sp. NATTS (Sugiyama y cols., 2014), pudiendo representar especies nuevas respecto a las ya descritas.

Fuera de las coriobacterias solo hay identificada una cepa productora de equol de origen intestinal que pertenece a otro grupo bacteriano, *Lactococcus garvieae* 20-92 (Uchiyama y cols., 2007). Los genes de *L. garvieae* involucrados en la producción de

equol han sido clonados y caracterizados (Shimada y cols., 2010; Shimada y cols., 2011; Shimada y cols., 2012), y se ha visto que presentan una secuencia y organización similares los de las coriobacterias (Schröder y cols., 2013), *Eggerthella spp*. (Yokoyama y cols., 2011) y *Slackia sp*. NATTS (Shimada y cols., 2010; Shimada y cols., 2011; Shimada y cols., 2012; Tsuji y cols., 2012). Esta similitud hace sospechar que la maquinaria genética se ha transferido horizontalmente desde algún miembro de esta familia a *L. garvieae*.

Algunas bacterias productoras de equol también actúan sobre la genisteína, generando 5-hidroxi equol (Matthies y cols., 2009; Jin y cols., 2010 Matthies y cols., 2012; Abiru y cols., 2013), compuesto que bien pudiera tener propiedades similares al equol. Cepas productoras de equol y/o 5-hidroxi equol bien caracterizadas podrían usarse para la producción biotecnológica de equol a gran escala. De hecho, el escaso número de estudios intervencionales en los que se utiliza equol se atribuye frecuentemente al alto coste de su producción (Selvaraj, 2004). La maquinaria genética pudiera también transferirse mediante técnicas de ingeniería genética a otros grupos microbianos más fáciles de cultivar, incluyendo bacterias de grado alimentario (Matthies y cols., 2012). Estos microorganismos o el equol purificado bien pudieran servir para el diseño de nuevos alimentos funcionales.

3.2.1.2. Fenotipo productor de equol

Algunos estudios sugieren que el estatus productor de equol es estable (Setchell y cols., 2003; Wiseman y cols., 2004), mientras que otros demuestran conversiones entre productores y no productores (Akaza y cols., 2004; Frankenfeld y cols., 2005; Franke y cols., 2012). Se debate también si la dieta influye en la capacidad de producir equol y en la cantidad total producida (Frankenfeld, 2011a; Franke y cols., 2012). Algunos estudios han demostrado que la frecuencia de productores de equol entre vegetarianos es significativamente mayor que en el resto de la población (59% frente al 25%) (Setchell y Cole, 2006). Decroos y colaboradores demostraron que la producción de equol se incrementa en presencia de propionato y butirato, por lo que una dieta rica en carbohidratos podría estimular su producción (Decroos y cols., 2005). También el consumo de leche y productos lácteos (Frankenfeld, 2011a) y de almidón resistente (Tousen y cols., 2011) se han relacionado con una mayor producción de equol. De manera similar en animales, el consumo combinado de daidzeína y lactulosa parece promover la producción de equol (Zheng y cols., 2014). Todas estas observaciones

sugieren que la formación de equol puede ser incrementada mediante la dieta, la cual podría estar modulando las poblaciones microbianas intestinales productoras de equol.

3.3. Isoflavonas y alimentos funcionales

La combinación de soja (o isoflavonas) con un microorganismo activador de las isoflavonas resultará en una mejora en la biodisponibilidad de las agliconas (Wei y cols., 2007; Champagne y cols., 2010). Del mismo modo, la utilización combinada de isoflavonas y bacterias productoras de equol posibilitaría la formación de este compuesto (en el alimento y/o en el intestino) con independencia de los tipos microbianos presentes en el TGI. Por tanto, la utilización combinada de isoflavonas y microorganismos puede favorecer el desarrollo de alimentos funcionales para aumentar y extender a la población general los beneficios sobre la salud de las isoflavonas y sus metabolitos más activos.

OBJETIVOS

OBJECTIVES

OBJETIVOS DE LA TESIS

Las isoflavonas se han relacionado con una variedad de efectos beneficiosos sobre la salud, entre los que destaca su utilidad para tratar los síntomas de la menopausia. Sin embargo, distintos trabajos reportan resultados de eficacia contradictorios debido, muy probablemente, a la versatilidad y gran variabilidad interindividual en la composición y actividad de la microbiota intestinal. Ésta podría influir en el perfil de metabolitos derivados de las isoflavonas, lo que podría relacionarse, en último término, con sus efectos fisiológicos. En particular, solo unas pocas personas son capaces de producir equol, el metabolito de las isoflavonas con mayor actividad estrogénica y antioxidante. Las personas con un fenotipo productor de equol (y quizá de otros compuestos) bien pudieran ser las únicas que, debido a las actividades de su microbiota intrínseca característica, se aprovechen en todo su potencial de los beneficios del consumo de soja o de isoflavonas.

En este contexto, esta Tesis Doctoral persigue dos objetivos principales:

Por un lado, nos hemos propuesto estudiar las relaciones de las poblaciones microbianas intestinales con la ingesta y metabolismo de las isoflavonas de soja. Los estudios sobre los mecanismos de los efectos beneficiosos de las isoflavonas sobre la salud son todavía escasos. La hipótesis de este trabajo es que las isoflavonas podrían modular las comunidades microbianas intestinales, incrementando las poblaciones beneficiosas y/o inhibiendo otras perjudiciales, lo que resultaría en todo caso en un efecto beneficioso neto sobre la salud. Los efectos beneficiosos pudieran también ser el resultado de la interacción de las isoflavonas con los microorganismos intestinales y estar mediados por metabolitos microbianos que resultan de una transformación directa de las isoflavonas (equol) o de la formación de otros compuestos beneficiosos (AGCC). El trabajo se realizó con la participación de un grupo de 16 mujeres voluntarias que iniciaban un tratamiento con concentrados de isoflavonas de soja para paliar los síntomas de la menopausia. El estudio de los cambios en la composición microbiana en muestras de heces y de los metabolitos en heces y orina se llevó a cabo a lo largo de un periodo de seis meses de tratamiento, durante el cual las mujeres tomaban un suplemento dietético diario con un contenido en isoflavonas de 80 mg de los que entre un 50 y 70% eran genistina (Fisiogen, Zambon).

El segundo objetivo, con una orientación más tecnológica, se ha dirigido a identificar y caracterizar microorganismos intestinales que actúen sobre las isoflavonas, y cuya utilización como cultivos iniciadores en la fermentación de leche de soja o sus productos derivados o su empleo como probióticos pudiera resultar en promover o incrementar los efectos beneficiosos de las isoflavonas. En este sentido, la búsqueda y selección de cepas se ha centrado en microorganismos que llevan a cabo el primer paso de activación de las isoflavonas: la desglicosilación de los glicósidos y la consiguiente liberación de las agliconas. Esta hidrólisis resulta esencial para incrementar la biodisponibilidad de las isoflavonas y su absorción, así como, probablemente, para una correcta transformación metabólica posterior. Cepas con estas características pudieran ser útiles para el desarrollo de alimentos funcionales a base de soja.

Para alcanzar los objetivos principales generales se establecieron unos objetivos parciales más concretos, tal y como se relaciona de manera esquemática a continuación:

Objetivo 1.- Estudiar las relaciones de las poblaciones microbianas intestinales con la ingesta y metabolismo de las isoflavonas de soja

Subobjetivo 1.1.: Analizar en muestras de heces y orina las principales isoflavonas de soja y sus principales metabolitos.

Subobjetivo 1.2.: Estudiar los cambios en la composición microbiana de muestras de heces mediante cultivo y técnicas independientes de cultivo durante el tratamiento con isoflavonas de soja.

Subobjetivo 1.3.: Relacionar los tipos microbianos intestinales influenciados por las isoflavonas y los metabolitos derivados producidos.

Objetivo 2.- Identificar y caracterizar cepas bacterianas activadoras de isoflavonas y sus actividades

Subobjetivo 2.1.: Caracterizar microorganismos que lleven a cabo la hidrólisis y transformación de las isoflavonas para su utilización en la elaboración de alimentos funcionales a base de soja.

Subobjetivo 2.2.: Estudiar los mecanismos microbianos involucrados en la desglicosilación de los glicósidos de isoflavonas.

TRABAJO EXPERIMENTAL

EXPERIMENTAL WORK

CAPÍTULO 1

Desarrollo de métodos de análisis de isoflavonas y sus metabolitos excretados en heces y orina

El abordaje del estudio de los efectos de las isoflavonas de soja sobre los microorganismos intestinales comenzó con el desarrollo de metodologías para el análisis en muestras de orina y heces humanas de las principales isoflavonas de la soja y sus metabolitos derivados más comunes.

En este capítulo se describe el desarrollo de un método simple, basado en la separación mediante extracción en fase sólida y cromatografía líquida de ultra-alto rendimiento (UHPLC en sus siglas en inglés), para el análisis simultáneo de las agliconas mayoritarias de las isoflavonas de la soja (daidzeína y genisteína) y el metabolito microbiano con mayor actividad biológica (equol) en muestras de orina humana. Las isoflavonas se identificaron mediante un detector de fotodiodos en serie (PDA), mientras que el equol se identificó mediante un detector de fluorescencia, ya que este compuesto posee una absorción de luz ultravioleta muy baja y apenas se detecta mediante PDA. En un segundo trabajo, se optimizó un método basado en UPLC acoplado a espectrometría de masas de ionización por electroespray (UPLC-ESI-MS/MS) para detectar además de los compuestos mencionados anteriormente dihidrodaidzeína (DHD) y *O*-desmetilangolensina (*O*-DMA), junto con otros 40 compuestos de naturaleza fenólica en heces.

Estos métodos se utilizaron para monitorizar la excreción de metabolitos microbianos de naturaleza fenólica derivados de la ingesta de isoflavonas en muestras biológicas (orina y heces) de las mujeres durante el tratamiento con isoflavonas. En los experimentos llevados a cabo con el método de UHPLC se observó que solo cuatro de las 16 mujeres del estudio (25%) fueron capaces de metabolizar la daidzeína hacia equol, en consonancia con los porcentajes descritos previamente para las poblaciones occidentales. Por su parte, de los experimentos realizados con el sistema de UPLC-ESI-MS/MS aplicados al análisis de muestras de heces se observó, tras la intervención con isoflavonas, un incremento de diversos derivados fenólicos como el ácido 3-hidroxifenilacético y el ácido fenilpropiónico, que provienen, seguramente, del

metabolismo de las isoflavonas suplementadas. Ambos compuestos resultan también del metabolismo de otros polifenoles de la dieta.

El Capítulo 1 recoge el trabajo correspondiente al Subobjetivo 1.1.: Analizar en muestras de heces y orina las principales isoflavonas de soja y sus principales metabolitos, y engloba los dos artículos que se relacionan a continuación:

- **Artículo 1: Guadamuro, L.**, Jiménez-Girón, A. M., Delgado, S., Flórez, A. B., Suárez, A., Martín-Álvarez, P. J., Bartolomé, B., Moreno-Arribas, V., & Mayo, B. (2015). Profiling of phenolic metabolites in feces from menopausal women after long-term isoflavone supplementation. *Journal of Agricultural and Food Chemistry*, 64, 210-216.
- **Artículo 2:** Redruello, B., **Guadamuro, L.**, Cuesta, I., Álvarez-Buylla, J.R., Mayo, B., & Delgado, S. (2015). A novel UHPLC method for the rapid and simultaneous determination of daidzein, genistein and equol in human urine. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* 1005, 1-8.

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Profiling of Phenolic Metabolites in Feces from Menopausal Women after Long-Term Isoflavone Supplementation

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Supporting Information

ABSTRACT: Phenolic compounds were screened by UPLC-ESI-MS/MS in the feces of 15 menopausal women before and after long-term isoflavone treatment. In total, 44 compounds were detected. Large intertreatment, interindividual, and intersample variations were observed in terms of the number of compounds and their concentration. Four compounds, the aglycones daidzein and genistein and the daidzein derivatives dihydrodaidzein and *O*-desmethylangolensin, were associated with isoflavone metabolism; these were identified only after the isoflavone treatment. In addition, 4-ethylcatechol, 3-hydroxyphenylacetic acid, and 3-phenylpropionic acid differed significantly in pre- and postintervention samples, whereas the concentration of 4-hydroxy-5-phenylvaleric acid showed a trend toward increasing over the treatment. The phenolic profiles of equol-producing and -non-producing groups were similar, with the exceptions of 3-hydroxyphenylacetic acid and 3-phenylpropionic acid, which showed higher concentrations in equol-non-producing women. These findings may help to trace isoflavone-derived metabolites in feces during isoflavone interventions and to design new studies to address their biological effects.

KEYWORDS: *isoflavones, equol, menopause, gut microbiota, fecal phenolic metabolites*

INTRODUCTION

The role of dietary polyphenols in human health is a topic of growing research interest. These compounds have been reported to possess antioxidant, anticarcinogenic, neuroprotective, antimicrobial, cardioprotective, and antidiabetic properties in humans.[1] Before having any potential beneficial effect, however, polyphenols need to be metabolized by the intestinal microbiota; this increases their bioavailability and/or leads to the production of key active metabolites.[2,3] Large interindividual differences in the composition and activity of the gut microbiota have, however, been reported,[4,5] potentially leading to the formation of unwanted, inactive, or even toxic metabolites.[6−8] It is therefore conceivable that only those people carrying the appropriate microorganisms fully benefit from the intake of (at least some) dietary polyphenols.[9] Studies that identify the metabolites and microorganisms that provide beneficial effects are therefore crucial.[10] Knowledge gained on the metabolic pathways involved might then be of help in designing strategies and recommendations aimed at increasing the production of desirable compounds and at inhibiting their degradation, via the modulation of the intestinal microbiota.[11]

Some polyphenols, such as isoflavones and lignans, are phytoestrogens that mimic mammalian estrogens both structurally and functionally,[12] of which isoflavones are among the best studied. Isoflavones are present at relatively high concentrations in soy and soy-derived foods and have received interest for their effectiveness in reducing menopausal symptoms in women.[13,14] They have also been implicated in the prevention of hormone-dependent and age-related diseases, such as osteoporosis,[15] cardiovascular diseases,[16] and certain types of cancer.[17,18] In soy, isoflavones are found mostly as glycosides (>80%) (i.e., daidzin, genistin, and glycitin), which show low bioavailability and bioactivity.[7,19] For full activity, isoflavone glycosides need to be deglycosylated (i.e., to daidzein, genistein, and glycitein) and/or transformed into more active compounds (such as equol from daidzein). These processes are mainly performed by microbial enzymes of constituents of the gut microbiota.[20] Some studies have investigated the metabolism of isoflavones in humans by means of the urinary profile after isoflavone supplementation[21−23] and of incubations with fecal microbiota.[24] However, studies of isoflavone-derived metabolites in feces are scarce, although these biological samples are a route of excretion and indeed a good approach to study phenolic metabolism and its interaction with the human gut microbiota.[25]

Therefore, the aim of the present work was to study the phenolic profiles of feces from menopausal women before and during a period of isoflavone supplementation. To this end, a previously developed UPLC-ESI-MS/MS analytical method[26] was adapted to screen for phenolic compounds including known phenolic metabolites (mandelic acids, benzoic acids,

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Table 1. Optimized MS/MS Conditions and Validation Parameters of the UPLC-ESI-MS/MS Method for the Detection and Quantification of Microbial Phenolics

compound	MRM transitions	cone voltage (V)	collision energy (V)	t_R (min)	LOD[a] (ng/mL)	LOQ[a] (ng/mL)	linear concentration range (μg/mL)	r^2	accuracy (%)	precision (% RSD)[b]
enterodiol	301/253	20	25	3.23	6.2	16.4	0.04−5.23	0.999	117.41	5.40
DHD[a]	255/149	25	15	11.46	4.4	11.6	0.037−4.78	0.999	117.39	7.24
daidzein	255/199	20	25	12.00	5.6	15.1	0.07−0.58	0.999	94.63	11.10
genistein	269/133	40	32	14.64	4.1	10.9	0.03−2.26	0.997	113.45	12.29
equol	241/121	40	15	14.66	24.2	64.7	0.32−20.4	0.997	101.40	11.19
enterolactone	297/253	30	25	14.67	0.4	1.3	0.035−1.138	0.997	94.73	4.68
O-DMA[a]	257/108	20	25	14.69	0.4	1.2	0.03−0.56	0.998	112.00	8.99
skatole	132/117	30	20	14.73	17.4	46.5	0.071−4.73	0.999	113.02	11.42

[a]Abbreviations: LOD, limit of detection; LOQ, limit of quantification; DHD, dihydrodaidzein; *O*-DMA, *O*-desmethylangolensin. [b]Percent of the relative standard deviation (RSD) (n = 3).

phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-hydroxyvaleric acids, and valerolactones) and also isoflavones and isoflavone-derived metabolites. In total, 44 phenolic compounds were identified in feces, and their presence and concentrations were checked for any association with isoflavone supplementation. Correlations were also sought with equol production status, as quantified previously in urine from the same individuals,[27] in an attempt to identify those compounds linked to the synthesis of this active, microbial-derived metabolite.

MATERIALS AND METHODS

Chemical Reagents. Acetic acid was purchased from Scharlau (Barcelona, Spain). Acetonitrile and methanol (HPLC grade) were procured from Labscan (Poch, Gliwice, Poland). Ultrapure water was obtained using a Milli-Q system (Waters Millipore, Milford, MA, USA). The phenolic standards used in this study (for the mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, and cinnamic acids) were purchased from Sigma-Aldrich (St. Louis, MO, USA), Phytolab (Vestenbergsgreuth, Germany), or Extrasynthèse (Genay, France). The isoflavones daidzein, genistein, and equol were obtained from LC Laboratories (Woburn, MA, USA) and *O*-desmethylangolensin (*O*-DMA) and dihydrodaidzein (DHD) from Apin Chemicals (Oxford, UK). Enterolactone, enterodiol, and skatole were purchased from Sigma-Aldrich. The 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(4′-hydroxyphenyl)-γ-valerolactone standards used were those synthesized in a previous study.[28] The compound 4-hydroxybenzoic-2,3,5,6-d_4 acid, used as an internal standard, was purchased from Sigma-Aldrich.

Human Intervention Study. This study was approved by the Research Ethics Committee of the *Principado de Asturias*, Spain. Menopausal women were recruited at the Obstetrics and Gynaecology Units of the *Hospital de Cabueñes* (Gijón, Spain). The selection of donors and later sampling was performed following the hospital's standardized protocols. All volunteers gave their written informed consent prior to participation. Exclusion criteria included recent antibiotic treatment, gastrointestinal disorders and related diseases, and a previous record of consumption of soy and soy foods. Participants (n = 15; age range, 48−61 years, mean 53.4 years; body weight range, 52−73 kg, average 67.6) consumed for 6 months one tablet a day containing 80 mg of isoflavones containing genistin/genistein in the range of 55−72% (Fisiogen; Zambon, Bresso, Italy); HPLC analysis of the tablets indicated that >95% of the isoflavones are in the glycosidated form (data not shown). Fecal samples were collected at four time points: before the start of the intervention (t = 0) and at 1 (t = 1), 3 (t = 3), and 6 (t = 6) months of treatment. Freshly voided stools were collected in sterile plastic containers and transported to the laboratory by a courier service. Stool samples were kept frozen at −80 °C until analysis. During the intervention study, volunteers consumed their usual diet.

Preparation of Fecal Solutions. For the preparation of fecal solutions, frozen feces were thawed at room temperature and weighed (0.20−1.0 g, according to the amount available) in 15 mL sterile conical tubes. Ten-fold dilutions were prepared in Milli-Q water spiked with the required internal standard (IS), vortexed, and centrifuged twice (10 min, 10000 rpm, 4 °C). Supernatants (fecal solutions) were passed through a 0.22 μm pore diameter filter and diluted with acetonitrile (1:4, v/v, acetonitrile/fecal solution). Aliquots of 2.0 μL of samples with a 2.5 μg/mL final concentration of IS were injected into the chromatography system. All analyses were performed in duplicate.

Analysis of Phenolic Compounds, Including Isoflavone-Derived Metabolites in Feces. Phenolic compounds were detected using a UPLC-ESI-MS/MS system involving a modification of the method described by Sánchez-Patán et al.[26] The liquid chromatography system was a Waters Acquity UPLC (Milford, MA, USA) equipped with a binary pump, an autosampler set thermostatically at 10 °C, and a heated (40 °C) column compartment. The column employed was a Waters BEH-C18 model (2.1 × 100 mm, particle size 1.7 μm). Mobile phases A and B were 2% acetic acid in water and 2% acetic acid in acetonitrile, respectively. 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. The equilibrium time was 2.4 min, and the flow rate was set at a constant 0.5 mL/min, resulting in a total run time of 18 min. 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 both negative and positive ion modes. For quantification, data were collected in multiple reactions monitoring (MRM) mode, tracking the transition of parent and product ions specific to each compound. The MS/MS parameters (cone voltage, collision energy, and MRM transition) for 38 of the compounds screened (mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-hydroxyvaleric acids, and valerolactones) were those previously reported.[29] The MS/MS parameters for the isoflavones, lignans, and skatole were optimized by direct infusion in the mass spectrometer (Table 1).

Dilutions from the stocks were prepared with Milli-Q water and used in the generation of calibration curves. For this, 11 concentrations ranging from 36.5 to 0.04 μg/mL (2−1000-fold dilution) were injected into the system (performed in triplicate). All metabolites were quantified using the calibration curves for their corresponding standards, except for those for which standards were not available. In the latter cases, metabolites were relatively quantified using calibration curves of chemically related compounds. In this way, 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric, 4-hydroxy-5-(3′-hydroxyphenyl)valeric, and 4-hydroxy-5-phenylvaleric acids were quantified using the calibration curves for 3-(3,4-dihydroxyphenyl)-propionic, 3-(3-hydroxyphenyl)propionic, and propionic acids, respectively. Similarly, 5-(3′-hydroxyphenyl)-γ-valerolactone was

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Table 2. Identification and Quantification of Phenolic and Indolic Metabolites in Feces from Menopausal Women before ($t = 0$) and during ($t = 1$ through $t = 6$) a 6 Month Intervention with Soy Isoflavone Concentrates

compound	metabolite concentration[a] (μg/g feces)			
	$t = 0$	$t = 1$	$t = 3$	$t = 6$
skatole	3.91 ± 1.36 ($n = 9$)[b]	4.21 ± 1.44 ($n = 8$)	3.7 ± 2.34 ($n = 10$)	4.15 ± 2.34 ($n = 7$)
isoflavones				
daidzein	nd[c]	1.19 ± 1.26 ($n = 9$)	1.74 ± 2.15 ($n = 11$)	1.66 ± 2.51 ($n = 12$)
dihydrodaidzein	nd	2.18 ± 1.67 ($n = 7$)	1.54 ± 1.76 ($n = 9$)	1.96 ± 2.8 ($n = 10$)
O-desmethylangolensin	nd	0.16 ± 0.15 ($n = 10$)	0.37 ± 0.25 ($n = 7$)	0.11 ± 0.09 ($n = 7$)
genistein	nd	0.48 ($n = 1$)	nd	1.39 ± 1.3 ($n = 3$)
lignans				
enterolactone	0.36 ± 0.23 ($n = 14$)	0.38 ± 0.19 ($n = 15$)	0.31 ± 0.15 ($n = 15$)	0.54 ± 0.57 ($n = 15$)
enterodiol	0.54 ± 0.15 ($n = 3$)	0.58 ± 0.11 ($n = 2$)	0.74 ± 0.06 ($n = 3$)	0.72 ± 0.12 ($n = 3$)
mandelic acids				
3-hydroxymandelic acid	1.75 ± 0.01 ($n = 7$)	1.77 ± 0.04 ($n = 8$)	1.76 ± 0.03 ($n = 9$)	1.75 ± 0.02 ($n = 8$)
benzoic acids				
gallic acid	1.17 ± 1.2 ($n = 3$)	1.56 ± 0.94 ($n = 2$)	8.86 ± 15.75 ($n = 5$)	2.47 ± 3.77 ($n = 6$)
3,5-dihydrobenzoic acid	0.53 ± 0.29 ($n = 8$)	0.63 ± 0.34 ($n = 8$)	0.68 ± 0.79 ($n = 6$)	0.42 ± 0.52 ($n = 8$)
protocatechuic acid	0.83 ± 0.89 ($n = 14$)	0.78 ± 1.09 ($n = 14$)	0.57 ± 0.52 ($n = 15$)	0.69 ± 0.46 ($n = 14$)
3-*O*-methylgallic acid	0.37 ± 0.06 ($n = 3$)	0.25 ± 0.05 ($n = 4$)	0.27 ± 0.06 ($n = 4$)	0.75 ± 0.94 ($n = 5$)
4-hydroxybenzoic acid	1.47 ± 2.07 ($n = 13$)	0.69 ± 0.98 ($n = 12$)	0.98 ± 1.14 ($n = 10$)	1.39 ± 1.9 ($n = 12$)
4-*O*-methylgallic acid	1.13 ($n = 1$)	nd	1.15 ± 0.05 ($n = 2$)	1.24 ± 0.14 ($n = 2$)
3-hydroxybenzoic acid	1.17 ± 1.29 ($n = 9$)	0.71 ± 0.23 ($n = 10$)	1.01 ± 0.53 ($n = 12$)	0.88 ± 0.58 ($n = 11$)
vanillic acid	1.26 ± 0.83 ($n = 3$)	0.43 ($n = 1$)	1.3 ± 0.25 ($n = 2$)	0.44 ± 0.09 ($n = 3$)
syringic acid	0.88 ± 0.14 ($n = 3$)	0.4 ± 0.09 ($n = 3$)	0.2 ($n = 1$)	1.45 ± 0.97 ($n = 3$)
benzoic acid	16.41 ± 26.1 ($n = 7$)	2.43 ± 2.63 ($n = 6$)	14.62 ± 31.94 ($n = 7$)	2.25 ± 1.52 ($n = 7$)
salicylic acid	0.18 ± 0.02 ($n = 3$)	1.47 ± 1.78 ($n = 3$)	0.34 ± 0.09 ($n = 3$)	0.37 ± 0.28 ($n = 3$)
phenols				
catechol/pyrocatechol	1.17 ± 0.49 ($n = 5$)	2.14 ± 2.42 ($n = 6$)	2.52 ± 2.37 ($n = 8$)	1.46 ± 1.34 ($n = 6$)
4-methylcatechol	1.2 ± 0.25 ($n = 2$)	1.52 ± 0.88 ($n = 2$)	1.17 ± 0.44 ($n = 4$)	0.81 ± 0.04 ($n = 2$)
4-ethylcatechol	0.21 ± 0.04 ($n = 5$)	0.28 ± 0.09 ($n = 10$)	0.25 ± 0.06 ($n = 11$)	0.20 ± 0.02 ($n = 10$)
hippuric acids				
4-hydroxyhippuric acid	18.05 ($n = 1$)	3.65 ($n = 1$)	8.12 ± 5.22 ($n = 3$)	9.43 ± 5.15 ($n = 3$)
phenylacetic acids				
phenylacetic acid	98.52 ± 48.8 ($n = 15$)	120.48 ± 46 ($n = 14$)	104.5 ± 51.71 ($n = 14$)	101 ± 46.37 ($n = 14$)
3,4-dihydroxyphenylacetic acid	2.3 ± 4.08 ($n = 10$)	0.94 ± 0.36 ($n = 8$)	3.17 ± 3.34 ($n = 10$)	3.4 ± 5.66 ($n = 8$)
4-hydroxyphenylacetic acid	7.88 ± 10.83 ($n = 15$)	4.13 ± 2.83 ($n = 15$)	8.09 ± 9.56 ($n = 15$)	8.15 ± 14.1 ($n = 14$)
3-hydroxyphenylacetic acid	8.27 ± 9.36 ($n = 11$)	9.92 ± 11.11 ($n = 13$)	10.8 ± 16.17 ($n = 14$)	6.09 ± 5.32 ($n = 12$)
4-hydroxy-3-methoxyphenylacetic acid	1.84 ± 0.86 ($n = 3$)	2.35 ± 0.19 ($n = 2$)	1.5 ($n = 1$)	2.59 ± 1.5 ($n = 2$)
phenylpropionic acids				
3-(3′,4′-dihydroxyphenyl)propionic acid	10.89 ± 16.92 ($n = 7$)	4 ± 1.35 ($n = 9$)	4.8 ± 2.5 ($n = 9$)	21.22 ± 48.96 ($n = 9$)
3-(2′,4′-dihydroxyphenyl)propionic acid	38.61 ± 43.42 ($n = 10$)	51.09 ± 76.33 ($n = 8$)	61.85 ± 74.98 ($n = 8$)	53.35 ± 80.44 ($n = 6$)
3-(3′-hydroxyphenyl)propionic acid	66.37 ± 111.29 ($n = 15$)	62.61 ± 151.72 ($n = 15$)	64.51 ± 147.35 ($n = 15$)	59.17 ± 110.54 ($n = 15$)
3-phenylpropionic acid	34.39 ± 26.06 ($n = 12$)	45.13 ± 40.25 ($n = 14$)	52.77 ± 26.11 ($n = 14$)	56 ± 35.32 ($n = 12$)
dihydroxyphenylpropan-2-ol	2.36 ($n = 1$)	1.98 ($n = 1$)	2.88 ($n = 1$)	5.41 ($n = 1$)
valeric acids				
4-hydroxy-5-(3′,4′-dyhidroxyphenyl)valeric acid	4.85 ± 3.1 ($n = 3$)	10.07 ± 6.46 ($n = 2$)	5.29 ± 3.18 ($n = 5$)	4.36 ± 3.6 ($n = 3$)
4-hydroxy-5-(3′-hidroxyphenyl)valeric acid	0.79 ± 0.68 ($n = 2$)	0.99 ± 0.6 ($n = 3$)	0.26 ± 0.12 ($n = 4$)	0.3 ± 0.21 ($n = 2$)
4-hydroxy-5-phenylvaleric acid	54.03 ± 53.59 ($n = 12$)	82.64 ± 85.09 ($n = 14$)	69.28 ± 78.74 ($n = 14$)	83.15 ± 116.09 ($n = 13$)
valerolactones				
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	6.4 ± 5.86 ($n = 4$)	6.36 ± 8.73 ($n = 4$)	5.56 ± 7.11 ($n = 8$)	4.56 ± 8.52 ($n = 6$)
5-(3′-hydroxyphenyl)-γ-valerolactone	nd	39.44 ($n = 1$)	14.71 ± 12.83 ($n = 3$)	35.27 ($n = 1$)
5-(4′-hydroxyphenyl)-γ-valerolactone	3.85 ± 1.6 ($n = 2$)	3.72 ± 2.35 ($n = 2$)	12.04 ± 5.67 ($n = 3$)	8.77 ± 4.98 ($n = 3$)
cinnamic acids				
caffeic acid	0.73 ± 0.41 ($n = 13$)	0.64 ± 0.45 ($n = 13$)	0.76 ± 0.53 ($n = 14$)	1.06 ± 1.15 ($n = 14$)
p-coumaric acid	1.58 ± 0.62 ($n = 14$)	1.56 ± 0.84 ($n = 13$)	1.66 ± 0.69 ($n = 11$)	1.49 ± 0.37 ($n = 14$)
m-coumaric acid	0.41 ± 0.12 ($n = 2$)	0.29 ($n = 1$)	0.3 ± 0.03 ($n = 2$)	0.38 ± 0.11 ($n = 2$)
ferulic acid	1.61 ± 0.28 ($n = 7$)	1.57 ± 0.31 ($n = 10$)	1.62 ± 0.46 ($n = 10$)	2.35 ± 1.16 ($n = 9$)
isoferulic acid	4.06 ($n = 1$)	nd	3.67 ± 0.23 ($n = 2$)	5.24 ± 0.75 ($n = 2$)

[a]Means and standard deviations were calculated excluding cases with values below the limit of quantification. [b]In parentheses, number of cases considered for each of the compounds. [c]nd, not detected.

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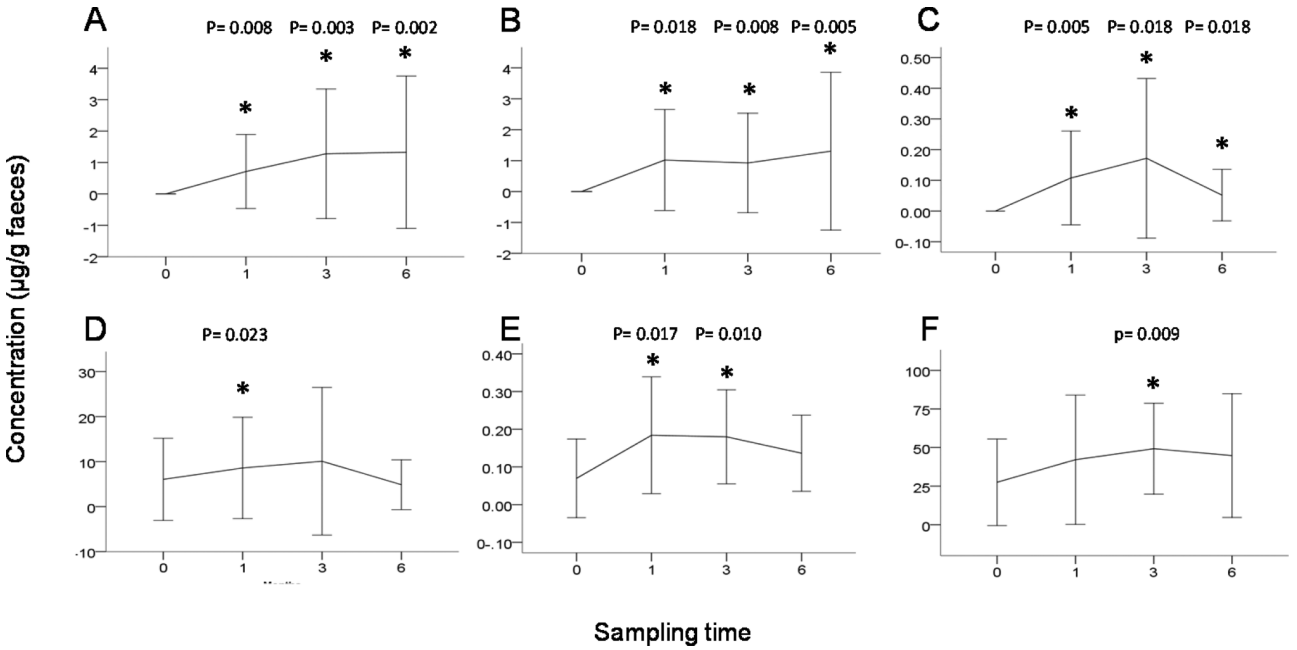

Figure 1. Progress of the concentration of phenolics in feces of the menopausal women (n = 15) of this study along with isoflavone treatment: mean (±SD) concentrations of daidzein (A), dihydrodaidzein (B), *O*-desmethylangolensin (C), 3-hydroxyphenylacetic acid (D), 4-ethylcatechol (E), and 3-phenylpropionic acid (F). Asterisks indicate statistical significance of the data by using the Wilcoxon nonparametric test as compared to those at basal time (t = 0) before isoflavone treatment.

quantified using the calibration curve for 5-(4′-hydroxyphenyl)-γ-valerolactone. Dihydroxyphenylpropan-2-ol was quantified using the calibration curve for 3-(3′,4′-dyhydroxyphenyl)propionic acid. Data acquisition and processing were performed using MassLynx 4.1 software.

Statistical Analysis. The Shapiro−Wilk test was used to verify the normal distribution of the data. The nonparametric Mann−Whitney test was used to compare the differences in the content of phenolic compounds in the feces of equol-producing and -non-producing women. The nonparametric Wilcoxon signed-rank test was used to examine the differences in the content of the identified compounds over the course of the isoflavone treatment. Significance was set at P = 0.05. Calculations were performed using SPSS v. 22.00 (IBM, Armonk, NY, USA) and Statistica v. 7.1 software for Windows (StatSoft, Tulsa, OK, USA).

RESULTS

The validation of the UPLC-ESI-MS/MS method for analysis of phenolic metabolites in fecal solutions, including isoflavone-derived compounds, in terms of limits of detection (LOD) and quantification (LOQ), calibration range, linearity, precision, and accuracy was undertaken following official methods as reported elsewhere.[26,29] The optimized MS/MS conditions and the validation parameters of the UPLC-ESI-MS/MS method used for the detection and quantification of isoflavone-derived phenolics are related in Table 1. The chromatographic optimization implemented in this study allowed the separation within 22 min of the phenolic standards selected for this work, which included isoflavones and their metabolites, lignans and skatole.

Fifteen women due to begin a treatment regimen with soy isoflavone concentrates designed to mitigate menopausal symptoms were recruited for the present study. Four fecal samples from each woman were analyzed by the described method, one at t = 0 and during t = 1, t = 3 and t = 6 months. In total, 44 phenolic compounds were identified and quantified (Table 2). Large interindividual, intertreatment, and even intersample variations were observed for both the number of compounds detected and their absolute or relative concentration (data not shown). The 3-phenylpropionic acid derivative 3-(3′-hydroxyphenyl)propionic acid was the only compound present in all fecal samples from all women. A few other compounds belonging to different chemical families, notably enterolactone, protocatechuic acid, phenylacetic acid, 3- and 4-hydroxyphenylacetic acid, 4-hydroxy-5-phenylvaleric acid, caffeic acid, and *p*-coumaric acid, were present in most samples, although at different concentrations. The metabolite 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol (also called dihydroxyphenylpropan-2-ol), in contrast, was found in all fecal samples of only one women, whereas enterodiol, 4-*O*-methylgallic acid, vanillic acid, syringic acid, salicylic acid, 4-hydroxyhippuric acid, 4-hydroxy-3-methoxyphenylacetic acid, 5-(4′-hydroxyphenyl)-γ-valerolactone, *m*-coumaric acid, and isoferulic acid were identified in the samples of always fewer than four women (Table 2).

The concentrations of some of the compounds, such as gallic acid, benzoic acid, and 3-(3′,4′-dihydroxyphenyl)propionic acid, varied between the samples of some women, apparently at random, whereas those of 3-(2′,4′-dihydroxyphenyl)-propionic acid, 4-hydroxy-5-phenylvaleric acid, 5-(3′-hydroxyphenyl)-γ-valerolactone, and 5-(4′-hydroxyphenyl)-γ-valerolactone (this latter compound except at t = 1) increased consistently over the treatment period as compared to the basal concentration. Finally, catechol/pyrocatechol and benzoic acid showed the widest variations between samples at different times (Table 2).

Four compounds were directly associated with isoflavone metabolites: the aglycones daidzein and genistein and the daidzein derivatives dihydrodaidzein and *O*-desmethylangolensin (*O*-DMA). Surprisingly, equol was not detected in any of the fecal samples. As expected, neither isoflavones nor their

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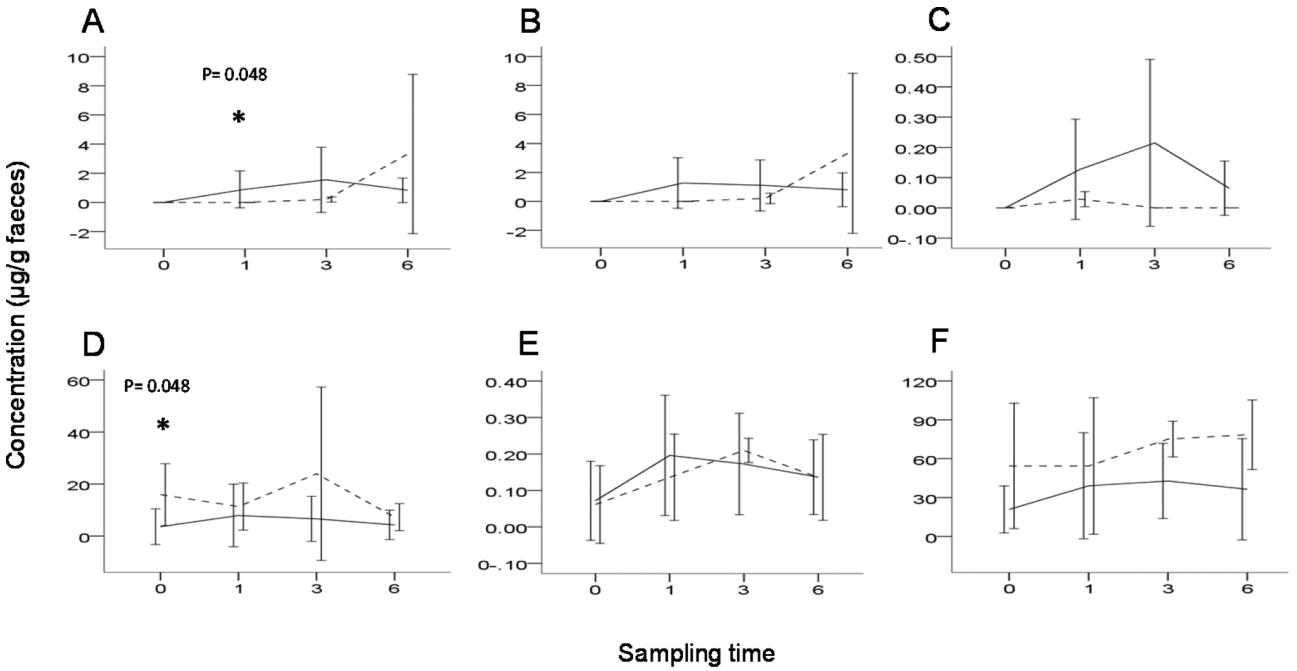

Figure 2. Changes of the concentration of phenolics in feces along with isoflavone treatment in equol-non-producing (n = 12) (continuous line) and -producing (n = 3) (broken line) menopausal women: mean (±SD) concentrations of daidzein (A), dihydrodaidzein (B), *O*-desmethylangolensin (C), 3-hydroxyphenylacetic acid (D), 4-ethylcatechol (E), and 3-phenylpropionic acid (F). Asterisks indicate statistical significance between groups at each sampling point by the nonparametric Mann−Whitney test.

derived metabolites dihydrodaidzein and *O*-DMA were detected in the samples of any of the women before the start of the intervention (Table 2). These compounds were usually found in most of the fecal samples of the women during treatment, except for genistein, which was observed only occasionally. The Wilcoxon nonparametric test showed the concentrations of daidzein, dihydrodaidzein, and *O*-DMA to be significantly different among samples collected before and over the treatment periods (Figure 1). In addition, notable differences were seen in the concentration of 4-ethylcatechol, 3-hydroxyphenylacetic acid, and 3-phenylpropionic acid between the samples collected before and after treatment (Figure 1). Finally, 4-hydroxy-5-phenylvaleric acid showed a trend toward an increase in its concentration over the supplementation period (data not shown).

The equol production status of the women had been assessed in previous works by determining the concentration of this compound in urine.[23,27] According to the definition by Rowland et al.,[30] 3 of the 15 women of the study had been classified as equol producers: isoflavone treatment increased their urine equol concentration to >1000 nM after beginning isoflavone intake. No significant levels of isoflavones were detected in the feces of the equol-producing women at t = 1 or t = 3, suggesting they were completely transformed or degraded into other compounds. When the fecal phenolic profiles of the equol-producing and -non-producing women wre compared, similar results were found, except with respect to the compounds 3-hydroxyphenylacetic acid and 3-phenylpropionic acid, which were always found at lower concentration in the feces of the equol-non-producing women (Supplementary Tables 1 and 2). The evolution during the treatment in equol-non-producing and producing menopausal women of these and some other isoflavone-derived compounds is depicted in Figure 2. Because data did not follow a normal distribution (Shapiro−Wilk test), the nonparametric Mann−Whitney test was applied for searching statistical significance of the differences in concentration between the two groups. Differences were observed, but most of them without statistical significance, except for phenylacetic acid, the concentration of which in feces differed significantly between equol-producing and -non-producing women at all sampling points (Figure 3). The concentration of this compound was shown to be higher during the isoflavone intervention in the equol-non-production group.

DISCUSSION

A total of 44 bacterial-derived, phenolic compounds were detected and quantified in feces from menopausal women before and after a long-term daily administration of isoflavones.

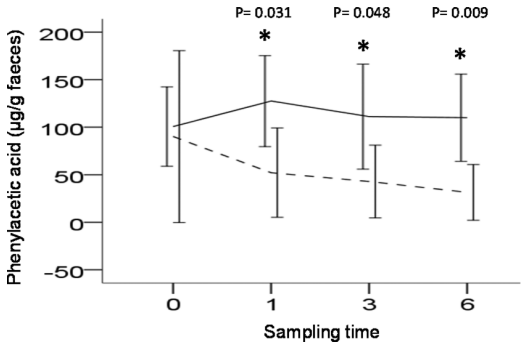

Figure 3. Changes of phenylacetic acid concentration (mean ± SD) in feces along with isoflavone treatment in equol-non-producing (n = 12) (continuous line) and -producing (n = 3) (broken line) menopausal women. Asterisks indicate statistical significance between samples at each sampling point by the nonparametric Mann−Whitney test.

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As reported by other authors for different intervention[29,31] and in vitro[32,33] studies, each subject returned an individual phenolic profile at each sampling point, although overall their profiles were quite similar. Wide intersubject, intertreatment, and intraindividual variations were encountered in both the number of compounds observed and their concentrations. In general, both the number of phenolics and their concentration agree well with those reported for human feces elsewhere after the intake of polyphenols from other sources.[29,32]

Four of the identified compounds were directly associated with isoflavone metabolism: the aglycones daidzein and genistein and the daidzein derivatives dihydrodaidzein and *O*-DMA.[34] Low consumption of soy and soy products has been reported in Spain.[35] Therefore, although women were not refrained from any food, isoflavone supplements were the only measurable source of isoflavones in this work, which agrees well with the absence of isoflavone derivatives in the fecal samples from all women at $t = 0$. During the treatment, the fact that isoflavone-derived compounds were not detected in all samples suggests that further degradation of isoflavones and their metabolite derivatives must occur in feces. Although three women were equol producers, as it had been determined by urine analysis,[27] equol was not found in any fecal sample. This confirms reports suggesting that urine is the main equol excretion route in humans.[36−38] Excretion via the feces is known, however, in animals that produce large amounts of this compound;[39] whether further degradation of equol in human feces occurs has yet to be investigated.

Phenylacetic and phenylpropionic acids are well-known isoflavone degradation products.[2,24,34] However, these compounds, along with the valeric acids and valerolactones, are intermediate degradation products of many other phenolics, including quercetins, anthocyanins, flavanols, and procyanidins.[2] The fact that compounds of these families increased consistently over the treatment in this study strongly suggests that in this study they appeared from the catabolism of isoflavones. The number of women whose fecal samples contained 4-ethylcatechol doubled between $t = 0$ and $t = 6$, although the final concentration was always quite similar. This compound is a well-known metabolic end product of caffeic acid, which is metabolized via hydroxycinnamate decarboxylase and vinylphenol reductase;[40] whether it is also produced via the degradation of isoflavones is not yet known.

The small differences in the baseline profiles of the equol-producing and equol-non-producing women might be related to dietary habits (which were not controlled in this study) and/or personal metabolic background with regard to phenolic compounds.[2,8,34] However, the differences between these groups with respect to the isoflavone metabolite contents of their feces during treatment almost certainly reflect variations in isoflavone catabolism. The differences in the degradation pathways followed could be due to variation in the subjects' genetic background or to diversity in the composition and/or activity of the microbiota they carried. Unfortunately, the small number of equol-producing women in this study, together with the wide interindividual microbial diversity in the feces of members of the general population,[4,5] hinders strong conclusions from the comparison of the phenolic compound profiles of equol-producing and -non-producing subjects.

In conclusion, this study reports on a complete inventory of the phenolic compounds present in feces of menopausal women after isoflavone treatment. To the best of our knowledge, this is the first study tracking phenolic metabolites in feces after isoflavone supplementation in humans. The data provided valuable information on baseline levels of the phenolic metabolites in the feces of these women. It also shows that the intake of isoflavones can significantly alter the presence and/or concentration of different phenolics, which could be indicative of an individual-dependent metabolism of these compounds. Although wide interindividual variation was seen, these data may help to trace isoflavone-derived metabolites in feces during isoflavone interventions and in the design of in vitro and in vivo (cell cultures, animal models) studies aimed to examine the biological effects of isoflavone supplementation at the gut level.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b05102.

Phenolics identified and quantified in the equol-producing ($n = 3$) and equol-non-producing women ($n = 12$) (Supplementary Tables 1 and 2, respectively) (PDF)

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Notes

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A novel UHPLC method for the rapid and simultaneous determination of daidzein, genistein and equol in human urine

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ABSTRACT

This work reports on a novel method involving reverse-phased ultra-high performance liquid chromatography (UHPLC) plus a spectrophotometric photodiode array/fluorescence (FLR) detection system for determining the concentration of equol and major soy isoflavones (daidzein and genistein) in human urine. The proposed method was validated in terms of its linearity, sensitivity, accuracy (recovery) and precision (intra- and inter-day repeatability). The isoflavone profiles of urine samples from a group of menopausal women following oral soy isoflavone supplementation were determined and compared. Screening for equol-producer status was accomplished with high sensitivity (detection limit of the FLR detector 2.93 nM). The method involves a short chromatographic run time compared to conventional HPLC methods while allowing for the simultaneous and reliable quantification of daidzein, genistein and equol in human urine. It also allows for the rapid screening of multiple urine samples when testing for equol production status and checking patient adherence to isoflavone treatment regimens.

1. Introduction

Isoflavones (plant secondary metabolites of the family flavonoids) are present in high concentrations in soy and soy products. The scientific and medical interest surrounding these compounds is linked to their beneficial health effects, particularly in age-related and hormone-dependent diseases, a consequence of their estrogenic activity [1]. For dietary isoflavones to act, a previous activation and/or conversion into derived (more active) metabolites must occur. Conversions of isoflavones are mostly brought about by enzymes from the human intestinal microbiota [2]. However, a marked inter-individual diversity within the intestinal microbial communities has been described. This has an influence on isoflavone metabolism, which could explain at least in part the wide variations in physiological effects after isoflavone treatments [3]. In the plant, soy isoflavones mainly occur in a glycosylated form, of which daidzin and genistin are a majority. Such glycosides are readily hydrolysed in the intestine, and the aglycones released (daidzein and genistein) – more absorbable and active that their dietary precursors – may undergo further metabolic modifications [4]. One of these microbial metabolites is equol, which is formed from daidzein and has the highest estrogenic activity [5]. The prevalence of equol producers among Western individuals is 20–35% compared to 50–55% among Asian adults, who routinely include soy-based foods in their diet [4]. The effects of consuming isoflavones on human health seem to be stronger in equol producer individuals [6]. For both gynecologists and Western women seeking medical care for their menopause symptoms, it is important to follow and potentially anticipate outputs of isoflavone treatments. In this respect, a quick determination of excretion of isoflavones and equol can bring key information about the personal metabolism of these compounds.

In biological fluids (serum, plasma, urine, breast milk and amniotic and prostatic fluid), isoflavones are largely found as glucuronide and sulphate conjugates, along with small amounts of aglycones [7]. The urine is the main route for the elimination of these compounds; indeed, urinary excretion is used as an indicator of the intake and metabolism of isoflavones.

The isolation of isoflavones (and other flavonoids) and their derivatives from physiological fluids usually relies on liquid–liquid

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extraction or solid phase extraction (SPE) (normally involving reversed phase C-18 silica gel cartridges) [8]. Different methods have been developed for their later identification and quantification, such as gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC), capillary electrophoresis, and immunoassays [9–11]. HPLC (involving different detection technologies) is one of the most frequently used methods since it is highly efficient, the results are reproducible, the equipment required is widely available, and the system has been extensively studied. The great majority of HPLC methods for the separation of isoflavones involve the use of reversed phase columns with either methanol or acetonitrile, plus water and a small amount of acid, as the mobile phase. Current commercially available ultra-high performance liquid chromatography (UHPLC) equipments are capable of the rapid chromatographic separation of many different compounds, including flavonoids and isoflavones [12–15]. Since all isoflavones show maximum UV absorption in the 240–270 nm range, UV and diode array detectors are widely employed to distinguish them. However, equol cannot be detected with any degree of sensitivity by such systems given the molecule's poor UV absorption characteristics [5]. Although equol's fluorescent emission has been reported [16], no chromatographic methods based on this feature are available for reliable quantification of equol, the isoflavone-derived microbial metabolite with the highest biological activity [5].

The present work describes a simple method involving SPE, UHPLC separation and photodiode array (PDA) and fluorescence (FLR) detection, for the simultaneous analysis of the main isoflavone aglycones (daidzein and genistein) and equol in human urine. The method was used to monitor the urine content of these compounds in a group of menopausal women taking daily, oral, soy isoflavone supplements to combat menopause-related symptoms.

2. Materials and methods

2.1. Chemicals, standards and urine samples

HPLC-grade methanol and ortho-phosphoric acid were obtained from Scharlau Chemie S.A. (Barcelona, Spain). Dimethyl sulphoxide (DMSO), ethyl acetate, sodium acetate and β-glucuronidase-sulphatase from *Helix pomatia* (extract type H-1) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Ultra pure water was obtained using a Milli-Q system (Bedford, MA, USA).

Genistein, daidzein, equol and the internal standard (IS) 4-hydroxy-benzophenone (4-HBPH) were obtained from Sigma–Aldrich. All purchased analytes were ≥98% pure.

Urine samples were obtained from 16 menopausal women taking 80 mg Fisiogen capsules (Zambon S.A., Barcelona, Spain) (a dietary supplement rich in soy isoflavones) daily for 6 months. The participants were recruited at the Obstetrics and Gynaecology Service of the Hospital de Cabueñes (Gijón, Spain). The study was approved by the Ethical Research Committee of the Principado de Asturias region, Spain. Blank urine samples were provided from volunteer women not consuming isoflavones, these served as controls during the validation of the method. All urine samples were stored at −20 °C until analysis.

2.2. Preparation of standard solutions

Standard stock solutions (2 mM) of daidzein, genistein and equol were prepared in methanol. An IS stock solution (450 μM) was also prepared in the same solvent. All solutions were kept at 4 °C in opaque vials until further use. The three standard stock solutions were then mixed to produce a combined standard solution containing 400 μM of each analyte (prepared in triplicate). Serial dilutions were then prepared with methanol to obtain a series of working solutions at concentrations from 0.005–363.60 μM each one contained the IS at 45 μM. The same working solutions were used to construct the calibration curves and in the validation study (see below).

2.3. Solid phase extraction of analytes from urine

Analytes extraction was performed using a vacuum manifold system (Vac Elut-12 Manifold Processing Station, Agilent Technologies, Santa Clara, CA, USA) with a 12 positions rack and Bond Elut-C18 200 mg SPE cartridges (Agilent Technologies). Extraction was performed according to the generic protocol recommended by the supplier of the cartridges. With some modifications, the extraction procedure was based on that reported previously for the analytical measurement of daizein, genistein and equol in urine by HPLC [17]. Prior to extraction, the cartridge's sorbent was preconditioned with 3 mL of methanol and equilibrated with 3 mL of sodium acetate buffer (0.1 M, pH 4.5). Urine samples (2.99 mL) were mixed with 10 μL of the IS stock solution (450 μM) and 3 mL of sodium acetate buffer (0.1 M, pH 4.5) before being loaded onto the SPE cartridges. Cartridge's washing was then performed with 3 mL of sodium acetate buffer. Before the elution step, 200 μL of ethyl acetate were passed through the cartridge to completely remove any aqueous traces from the eluate. This volume of ethyl acetate was calculated based on the bead volume (120 μL per 100 mg sorbent), being less than the minimum elution volume (500 μL) recommended for this type of cartridge. Analytes were finally eluted with 1 mL of ethyl acetate. The eluent was filtered through a 0.2 μm PTFE filter (VWR, Barcelona, Spain), the solvent evaporated to dryness under vacuum at room temperature, and the urine residue re-dissolved in 100 μL of methanol. Samples were either injected immediately into the chromatographic system or kept at −20 °C in opaque vials with screw caps until use.

Control urine samples from volunteer women not consuming isoflavones were used to validate the method. These were prepared in triplicate by spiking 2.90 mL blank urine samples with 0.1 mL of one of four concentrations of the standard working solutions (11.36 μM, 45.45 μM, 90.91 μM and 181.81 μM) plus IS. SPE was then performed as above.

When quantifying compounds in urine from the Fisiogen-treated women, their samples were first treated with 10 μl of a β-glucuronidase-sulphatase from *H. pomatia* (100 unit/μL) for 20 h at 37 °C prior to the addition of IS and the SPE step. This enzymatic hydrolysis was performed to ensure the release of conjugated isoflavone forms.

2.4. Equipment and chromatographic conditions

The UHPLC system used (an H-Class Acquity UPLC™) (Waters, Milford, MA, USA) consisted of a quaternary pump, a flow-through-needle auto-sampler, and a coupled PDA and FLR modules. Chromatographic separation was performed in a reversed phase Acquity UPLC™ BEH C18 1.7 μm column (2.1 mm × 100 mm) (Waters) in an oven at 40 °C. Samples were kept at 16 °C in the auto-sampler until injection. The mobile phase consisted of 0.05% ortho-phosphoric acid in water (eluent A) and 100% methanol (eluent B). Samples (1 μL) were applied to the column and eluted at a flow rate of 0.45 mL/min according to the following linear gradient: 0 min – 10% B; 1 min – 12% B; 3.5 min – 25% B; 6.5 min – 25% B; 10 min – 50% B; 12 min – 90% B; 16 min – 90% B. The column was then returned to the initial conditions within 1 min and equilibrated for 5 min (equivalent to 5.6 column volumes) before the next sample injection.

Daidzein, genistein and equol were identified by their retention times and spectral characteristics at 260 nm. Equol was additionally

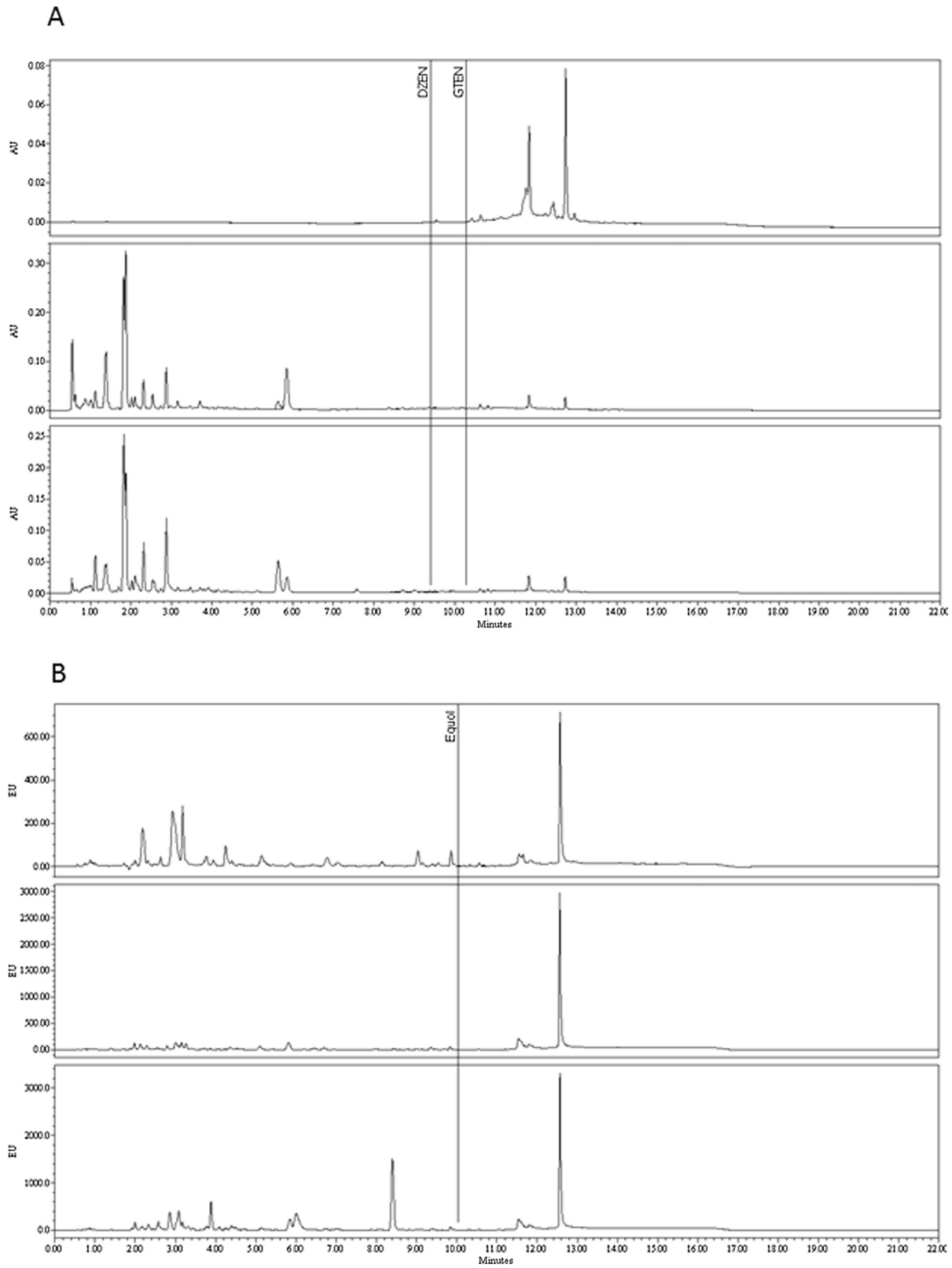

Fig. 1. Chromatograms from the blank urine samples used as controls to develop the UHPLC method. Three representative urine samples from women not consuming isoflavones were subjected to UHPLC separation plus PDA (A) and FLR (B) detection. Vertical lines denote the reference retention times for the indicated analytes. No interfering peaks were detected. (DZEN: daidzein, GTEN: genistein).

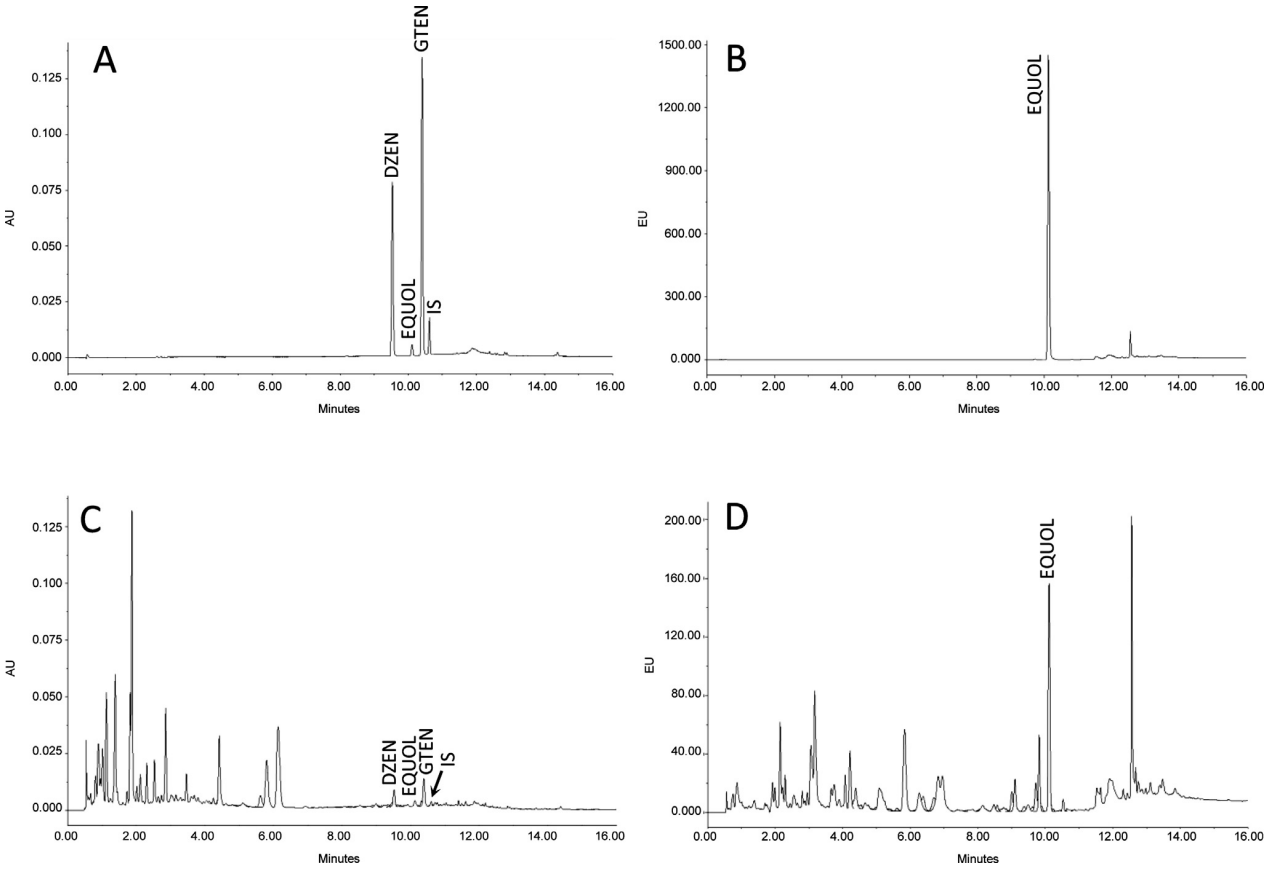

Fig. 2. Chromatographic profile of daidzein, genistein and equol. The elution profile of a daidzein, genistein and equol standard mixture at 90 μM under PDA (A) and FLR (B) detection is shown. The elution profile of blank urine spiked with the same standard mixture is also shown with PDA (C) and FLR (D) detectors. (DZEN: daidzein, GTEN: genistein, IS: internal standard).

Table 1
Comparison of the efficiency (in%) of daidzein, genistein and equol determination in urine samples with or without calibration by the internal standard (IS).

Level (μM)	Without IS			With IS		
	Daidzein	Genistein	Equol	Daidzein	Genistein	Equol
11.36	38.09 (16.67)[a]	36.56 (22.54)	26.92 (18.31)	149.21 (9.78)	140.43 (12.12)	110.14 (3.03)
45.45	43.42 (38.78)	41.55 (44.89)	31.24 (47.13)	111.10 (8.43)	126.56 (14.78)	102.43 (18.23)
90.91	16.04 (8.08)	15.33 (8.31)	12.23 (8.67)	77.52 (3.51)	73.64 (4.81)	58.56 (3.67)
181.81	19.89 (24.56)	20.31 (28.62)	17.78 (29.50)	80.12 (5.34)	80.81 (2.67)	74.60 (6.14)

[a] In brackets, relative standard deviation (RSD) among triplicates.

identified by fluorescence (280 nm excitation, 310 nm emission). Data were acquired and analysed using Empower v.2.0 software (Waters).

2.5. Method validation

The method was validated according to Taverniers et al. [18]. This included the determination of its selectivity, linearity, sensitivity (limit of detection [LOD] and limit of quantification [LOQ]), precision (intra- and inter-day repeatability), and accuracy (recovery).

The selectivity of the method was investigated by analysing blank urine from different volunteers after ensuring they had not consumed soy-based foods or any other product rich in isoflavones. The resulting chromatograms were examined to determine the presence of any endogenous constituents that might interfere with the analytes of interest. When any interfering peak was found, the linear gradient of elution was optimised until a clean chromatogram was obtained.

The calibration curves for daidzein, genistein and equol were constructed by plotting each of the peak area/IS area ratios against the known concentrations. The linearity of the method was verified by analysing the variance of the regression (triplicate curves). The LOD and LOQ were calculated using the following equations:

$$\text{LOD} = (\text{SD} \times 3.3)/\text{slope of the calibration curve}$$

$$\text{LOQ} = (\text{SD} \times 10)/\text{slope of the calibration curve}$$

where SD is the standard deviation of the blank measurements.

Six replicates of a standard solution were tested over the same day to examine the intra-day precision of the UHPLC system in terms of retention time and peak area. Inter-day precision was assessed by testing the same standard solution over four consecutive days.

The precision of the UHPLC method was examined using blank control urine spiked with 0.10 mL of the 11.36 μM, 45.45 μM, 90.91 μM or 181.81 μM standard working solutions. Three

Table 2
Linearity and sensitivity of the developed UHPLC-PDA-FLR method.

Compound	Linearity		LOQ[b]	LOD[c]
	R^2	Linear range[a] (μM)	(nM)	(nM)
Daidzein	0.9981	0.01–363.61	14.39	4.75
Genistein	0.9978	0.04–363.61	45.96	15.17
Equol	0.9976	0.01–181.81	8.89	2.93

[a] Concentration range between the limit of quantification and the upper linear limit.
[b] Limit of quantification. Signal/noise ratio = 10.
[c] Limit of detection. Signal/noise ratio = 3.

Table 3
Precision and recovery of daidzein, genistein and equol in human urine.

Compound	Intra-day (n = 3)				Inter-day (n = 4)			
	Added (μM)	Found (μM)	RSD[a] (%)	Recovery[b] (%)	Added (μM)	Found (μM)	RSD[a] (%)	Recovery[b] (%)
Daidzein	0.00	nd	–	–	0.00	nd	–	–
	11.36	12.48	17.68	102.94	11.36	12.68	2.85	112.50
	45.45	42.82	4.17	94.22	45.45	50.04	3.98	107.12
	90.91	68.31	5.54	75.14	90.91	71.47	6.26	77.13
	181.81	130.17	0.66	70.11	181.81	178.75	6.89	97.57
Genistein	0.00	nd	–	–	0.00	nd	–	–
	11.36	7.46	13.90	74.59	11.36	15.82	5.62	114.83
	45.45	35.84	4.23	78.85	45.45	46.73	7.41	102.81
	90.91	69.88	12.42	76.87	90.91	66.39	4.93	76.86
	181.81	131.42	2.51	72.28	181.81	178.54	8.50	97.83
Equol	0.00	nd	–	–	0.00	nd	–	–
	11.36	10.96	2.19	96.08	11.36	10.33	4.40	91.27
	45.45	47.85	7.84	104.54	45.45	41.06	5.10	90.13
	90.91	97.46	1.04	107.15	90.91	74.41	4.25	81.74
	181.81	190.13	12.78	104.40	181.81	150.60	8.11	82.77

nd: not detected.
–: not applicable.
[a] RSD, relative standard deviation of the concentrations found.
[b] Percentage of the means difference between the content after addition and the initial urine content.

replicates of the same control urine were used to test the intra-day precision. Two control urine samples (prepared in triplicate), each tested twice over two consecutive days, were used to examine the inter-day precision. Non-spiked control urine was used as a blank to determine the initial isoflavone and equol contents.

The accuracy of the system was tested by recovery analysis. The percentage recovery at each concentration was calculated as: [(amount found in the spiked sample) − (amount found in the blank)/(amount added)] × 100.

2.6. Statistical analysis

Regression analyses were performed using Empower v.2.0 (Waters) and Excel 2010 software (Microsoft, Redmond, WA, USA). Means and standard deviations were calculated with the same Excel software.

3. Results and discussion

3.1. Optimisation of the UHPLC separation step and of daidzein, genistein and equol detection

Urine samples from several volunteer women not consuming isoflavones were used to provide control chromatographic profiles. These samples were used to monitor for the presence of any contaminant peak that might interfere with the detection of the analytes. Fig. 1 shows the chromatograms of three blank urine samples.

Since the proposed isoflavone determination method is based on the official AOAC HPLC method for food samples [19], a geometric scaling factor (from the ratio of the cross-sectional areas of the AOAC-cited column to that of the present column) was used to adapt the mobile phase flow and the injection volume to the UHPLC system. A pre-injection isocratic volume (213 μL) was added before the elution gradient to compensate for the smaller dwell volume of the UHPLC system.

Acetonitrile-based gradients allow faster elution schemes than methanol ones [13]; consequently, initial attempts were made with acetonitrile as the organic solvent. However, importunate co-elution of daidzein, genistein and/or equol with unkown, urine-vehicled compounds present in the control samples was systematically found. Therefore, a change to a methanol-based gradient was made. It allowed a better separation of all the urine compounds and the appearance of plain areas within the chromatogram where the analytes of interest eluted. A major drawback of methanol-based gradients is the high pressure that the column supports. To decrease the pressure of the UHPLC system we reduced the flow rate (from 0.65 mL/min in the original method [19] to 0.45 mL/min), which allowed maintaining the column original temperature (40 °C). The presence of a peak shoulder accompanying genistein and the incomplete resolution of equol made necessary a fine tuning of the proportion of the methanol/aqueous mixture within the second half of the elution gradient until the profile described in Section 2.4 was reached. With all these settings, an accurate baseline separation of a daidzein, genistein and equol mixture was achieved within 12 min of using the linear gradient (Fig. 2, panels A and B). The same good resolution was obtained when urine samples were spiked with the mixture of standards (Fig. 2, panels C and D). No interfering peaks appeared in any of the chromatographic profiles, indicating the good specificity of the method.

As phenolic compounds, all phytoestrogens have at least one aromatic ring with a maximum ultraviolet absorption in the

Table 4
Daidzein, genistein and equol (ng/mL) in urine samples of the treated menopausal women in this study.

Sample	Months	Daidzein	Genistein	Equol
WA	0	nd	nd	41.19
	1	nd	nd	7,754.26
	3	nd	nd	99.33
	6	nd	nd	431.24
WB	0	nd	nd	11.31
	1	10,987.82	10,821.30	19.38
	3	6,834.54	10,603.33	19.38
	6	4,086.32	4,921.96	9.69
WC	0	nd	nd	2.42
	1	1,331.33	1,147.63	227.73
	3	5,506.62	4,160.80	384.41
	6	7,940.44	5,800.24	293.95
WD	0	nd	nd	0.80[a]
	1	397.44	170.25	nd
	3	4,238.85	918.82	2.42
	6	541.51	292.75	nd
WE	0	nd	40.54	nd
	1	3,066.01	2,426.76	nd
	3	2,518.58	1,610.63	nd
	6	2,051.64	1,861.95	nd
WF	0	nd	nd	nd
	1	nd	227.89	nd
	3	nd	nd	nd
	6	nd	434.19	nd
WG	0	36.43	10.81[a]	13.74
	1	13,380.96	4,662.53	9,681.91
	3	11,728.47	6,352.45	6,984.64
	6	10,836.12	4,592.27	4,540.14
WH	0	nd	nd	4.85
	1	3,986.33	3,223.96	5.64
	3	2,503.33	2,033.10	4.85
	6	3,360.92	1,898.90	5.64
WI	0	nd	nd	4.87
	1	32,981.82	2,538.93	16.98
	3	–	–	–
	6	28,813.44	7,000.03	9.04
WJ	0	nd	nd	nd
	1	605.91	457.60	3.08
	3	519.47	197.28	41.50
	6	555.06	286.45	12.99
WK	0	nd	nd	nd
	1	504.21	163.04	0.80[a]
	3	1,398.27	622.44	0.80[a]
	6	138.12	57.64	nd
WL	0	nd	nd	nd
	1	18,126.60	10,550.17	nd
	3	2,671.12	657.57	nd
	6	3,972.78	1,382.74	0.80[a]
WM	0	nd	nd	nd
	1	9,326.86	5,028.27	13.74
	3	1,333.00	408.95	2.42
	6	1,910.11	1,134.12	0.80[a]
WN	0	nd	nd	126.78
	1	nd	nd	0.80[a]
	3	nd	nd	2.42
	6	nd	nd	2.42
WO	0	nd	nd	nd
	1	955.07	849.45	1.62[a]
	3	60.18	53.16	4.85
	6	1,565.22	1,413.36	1.62[a]
WP	0	nd	nd	2.42
	1	6,464.23	4,714.80	384.41
	3	4,510.04	3,258.20	383.59
	6	3,194.83	2,177.24	312.53

nd: not detected.
–: urine sample not provided.
[a] Between LOD and LOQ.

230–280 nm range. Equol is also a fluorescent compound [16]. Two different wavelengths, 260 nm and 280 nm, were evaluated until reliable simultaneous results were achieved at 260 nm using the PDA detector. For instance, the PDA signal retrieved at 260 nm for a daidzein 50 μM standard increased by 74% when compared with its signal at 280 nm. An equivalent standard of genistein produced a signal at 260 nm 16% higher than that at 280 nm. The equol signal, although detected at 260 nm, improved dramatically (between 100 and 600 folds, depending on the concentration) when the FLR detector was employed. To the best of our knowledge, this is the first report on the simultaneous determination of daidzein, genistein and equol by UHPLC-PDA-FLR in urine samples. Daidzein and genistein are the main aglycones derived from soy supplements (which are rich in their precursors daidzin and genistin, and to a minor extent in glycitin), meanwhile equol is the microbial metabolite derived from daidzein with the highest estrogenic activity and antioxidant capacity reported [5]. These three compounds are considered the most relevant and useful to bring information about metabolism and excretion of soy isoflavones.

3.2. Improvement of SPE by calibration with an internal standard

The efficiency of the extraction procedure was calculated by comparing the peak areas for the analytes of interest with those obtained after spiking urine with the standard solutions, SPE and concentration. Triplicate samples with four different concentrations of the analytes were used. The percentage mean difference between the non-extracted standard solutions and the spiked/extracted urine was deemed to represent the extraction performance. The percentage recovery for the different compounds varied between 12% and 43% – a poor extraction performance (Table 1). Further, the relative standard deviation (RSD) among triplicate samples was in most cases >15%, far above normally accepted values [18]. This was understood to be a sign of a clear matrix effect, either of the urine, the SPE cartridge, or both. In the present study, the extraction procedure for isoflavones followed those previously described and consisted on an acid treatment of the native urine in acetate buffer followed by an elution with a highly efficient organic solvent (ethyl acetate) [17,20,21]. Although this, we cannot ruled out losses of analytes during sample preparation and recovering through the reversed phase C-18 silical gel cartridges. The problem was solved by spiking the urine samples with an IS. A suitable IS candidate for the analysis of isoflavones should be similar in structure to isoflavones but not present in the biological sample. Additionally, it should be completely separable from the rest of compounds analysed under the same chromatographic conditions. Apigenin (the flavone analog to the isoflavone genistein, used in the official AOAC HPLC method for determination of isoflavone in soy foods [19]), was our first candidate. However, a peak eluting at the retention time of apigenin was observed in some of the blank urine samples used. Therefore, 4-hydroxy-benzophenone (4-HBPH) was finally chosen as IS, since it has been previously utilized with good results for the determination of isoflavones in biological samples, including urine [17,20]. Then, calibration curves and validation studies for the determination of daidzein, genistein and equol were performed by relative quantitation against the peak area of 4-HBPH. Table 1 shows the results obtained.

3.3. Method validation

The precision of the UHPLC equipment was evaluated taking into account both the peak area and the retention time of a calibration standard mixture at 45.45 μM. The intra-day precision test was based on six runs of the mixture within one day; the RSD was always <0.85% for peak area and <0.05% for retention times. The inter-day precision test was performed by injecting the standard mixture on

four independent days and were always <5%, i.e., within normally acceptable limits [18]. Together, these results indicate satisfactory precision.

Table 2 shows the results of the linearity and sensitivity tests. Least-squares analysis returned correlation coefficients of $R^2 > 0.997$ for all the standard curves, indicating excellent linearity. A wide linear concentration range was obtained for all calibration curves, covering between four and five orders of magnitude from the lowest to the highest concentration.

Sensitivity was calculated on the basis of calibration curves, and is expressed in nano-molar (Table 2). The LOD was 15.17 nM and 4.75 for genistein and daidzein, respectively and 2.93 nM for equol. The LOQ for equol was 8.89 nM – two and five times lower than that for daidzein and genistein respectively. When the LOQs were converted to ng/mL, results of 4 ng/mL, 12 ng/mL and 2 ng/mL were returned for daidzein, genistein and equol respectively. The sensitivity of the present method is between three and 10 times higher than that reported for previous UHPLC-UV [12–15] and HPLC-UV [22] procedures for determining daidzein and genistein in human urine, and 2–100 times more sensitive than HPLC-based methods for the detection of equol [10,16,20,23]. The increase in the equol detection limit achieved is important for determining how much is really excreted via the urine, and for differentiating between equol producers and non-producers [24].

The precision and accuracy of the method were determined in urine samples spiked with mixtures of standards at various concentrations (Table 3). The percentage of variation among triplicates (precision) or RSD was <15% in intra-day assays, and <7.5% in inter-day tests. The recovery (accuracy) of the analytes was within the 70–114% range. These values all indicate the method to show good precision and accuracy [18].

Together the results of the validation analyses reveal the proposed method can be used to reliably identify and quantify the target compounds in real urine samples with high sensitivity.

3.4. Use of the method in an experimental setting

The method was used to determine daidzein, genistein and equol in 64 samples of urine from 16 menopausal women taking a daily isoflavone supplement over a 6 month period. Urine samples were taken before the start of the treatment (time 0), and after one (t1), three (t3) and six (t6) months of supplementation. To determine the total daidzein and genistein, a pre-extraction step with β-glucuronidase/sulphatase was performed to hydrolyse all conjugated forms. After isoflavone supplementation the concentrations detected in urine for daidzein ranged from 36.43 ng/mL to 32,981.82 ng/mL (143.30 nM to 129,732.20 nM, respectively), while genistein varied from 10.81 ng/mL to 10,821.30 ng/mL (40.00 nM to 40,043.30 nM, respectively) and equol between 0.80 ng/mL and 9681.91 ng/mL (3.30 nM and 39,963.30 nM, respectively) (Table 4). These results revealed large qualitative and quantitative differences among individuals under an identical isoflavone treatment and confirm the versatility and suitability of the method for quantifying wide ranges of daidzein, genistein and equol concentrations in urine. The greatest variation in inter-individual urinary excretion was recorded for equol, which is consistent with previous reports [24–26]. In the present work, strong equol producers (25%; women WA, WC, WG and WP; >1000 nM (242.27 ng/mL) equol in urine according to the threshold of Rowland et al. [24]), were easily distinguishable from poor equol producers (women WB, WH. WI, WJ, WM and WO; between 2.42 and 24.22 ng/mL corresponding to 10–100 nM) and non-producers (women WD, WE, WF, WK, WL and WN; with less than 2.42 ng/mL excretion, <10 nM). Isoflavones bioconversion and bioavailability greatly depend on their transformation by tissue enzymes and those from the gastrointestinal microbiota. However, our current knowledge on the intestinal microbial metabolism and transformation of isoflavones is still very limited [4]. Intestinal degradation of isoflavones into compounds considered to be biologically inactive, such as conversion of daidzein into *O*-desmethylangolensin (*O*-DMA), can also occur. Equol and *O*-DMA are likely produced by different bacteria, and the bacteria involved may further differ between individuals [2,6].

As expected, at t0 the target compounds were either not detected or found at very low concentrations in the urine of most of the women. Although genistin/genistein is specified to be in higher proportions (55–72%) than other soy isoflavones (28–45%) in the Fisiogen capsules, urine daidzein concentrations in the menopausal women after this isoflavone treatment were always similar to or higher than those recorded for genistein. We cannot completely discard concomitant ingestion of other soy-derived foods or isoflavone rich products by some of the menopausal women enrolled in this study. On the other hand, and in contraposition with previous reports [27,28], we observed higher excretion of daidzein in strong equol producers than in non-producers, at least at month three and six of the isoflavone challenge. Additionally, no daidzein or genistein was detected in the urine of three of the women (WA, WF and WN). All together, these results suggest a possible poor adherence to the supplement regimen of some of these patients.

UHPLC methods have previously been used in the analysis of isoflavones in soy products [29,30] and in some biological fluids: the blood of animals following soy-based nutritional supplementation [31,32] and human urine in studies of male infertility [21] and treated patients with cardiovascular disease [12–15]. Apart from this, literature contains nothing on the simultaneous determination of the main soy isoflavones and equol by UHPLC in urine of isoflavone-treated menopausal women. The present manuscript reports valuable data about the content of the main isoflavone-derived compounds in the urine of menopausal women receiving long-term treatment with a soy-isoflavone concentrate. The great inter-individual differences observed in the levels of daidzein, genistein and equol's excretion could be mainly explained by personal variations in the metabolism of isoflavones already described [3,6]. To establish significant urinary average concentrations of these metabolites, which could ultimately be associated with physiological responses, larger numbers of menopausal women enrolled in isoflavone medications need to be studied.

4. Conclusions

The proposed method allows the simultaneous and reliable quantification of daidzein, genistein and equol in human urine with high sensitivity. It also allows for the rapid screening of multiple urine samples when testing for equol production status and checking patient adherence and response to isoflavone treatment regimens.

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CAPÍTULO 2

Estudio de la composición y evolución de la microbiota fecal de mujeres menopáusicas durante el tratamiento con isoflavonas

En este capítulo se incluyen los resultados de dos trabajos sobre el efecto del tratamiento con isoflavonas sobre las comunidades bacterianas fecales en un grupo de mujeres menopáusicas. En los trabajos se utilizaron de forma combinada métodos de cultivo convencionales y métodos independientes de cultivo como la PCR cuantitativa (qPCR), la electroforesis en gel con gradiente desnaturalizante (DGGE) y la pirosecuenciación de amplicones para determinar la composición y los cambios de las principales poblaciones microbianas intestinales con el objetivo de relacionar la ingesta de isoflavonas con la evolución de las comunidades microbianas y la producción de equol.

Como resultados principales se observaron grandes variaciones en las comunidades microbianas de las mujeres menopaúsicas, tanto antes de iniciar el tratamiento como durante la intervención con isoflavonas. La técnica de DGGE reveló en algunas mujeres incrementos de microorganismos tras la ingesta de isoflavonas que se asocian en la literatura con el metabolismo de fitoestrógenos, como *Lactonifactor longoviformis*, *Faecalibacterium prausnitzii*, *Bifidobacterium* spp. o *Ruminococcus* spp. En muestras procedentes de una mujer productora de equol se utilizó la tecnología de pirosecuenciación para estudiar con más detalle la composición y evolución de las comunidades microbianas. Esta técnica mostró un incremento de secuencias de miembros de la familia *Coriobacteriaceae* al que pertenecen géneros relacionados con el metabolismo de las isoflavonas como *Eggerthella*, *Collinsella, Slakia* o *Coribacterium*. Se observó también que durante el tratamiento con isoflavonas se producía un incremento de secuencias de las familias *Ruminococcaceae* y *Lachnospiraceae*, ambas relacionadas con la producción de AGCC.

Los resultados que se presentan en este Capítulo corresponden al Subobjetivo 1.2.: Estudiar los cambios en la composición microbiana de muestras de heces mediante cultivo y técnicas independientes de cultivo durante el tratamiento con isoflavonas de soja, y se describen en los siguientes artículos:

- **Artículo 3: Guadamuro, L.**, Delgado, S., Redruello, B., Flórez, A. B., Suárez, A., Martínez-Camblor, P., & Mayo, B. (2015). Equol status and changes in fecal microbiota in menopausal women receiving long-term treatment for menopause symptoms with a soy-isoflavone concentrate. *Frontiers in Microbiology*, *6*, 777.
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Equol status and changes in fecal microbiota in menopausal women receiving long-term treatment for menopause symptoms with a soy-isoflavone concentrate

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The knowledge regarding the intestinal microbial types involved in isoflavone bioavailability and metabolism is still limited. The present work reports the influence of a treatment with isoflavones for 6 months on the fecal bacterial communities of 16 menopausal women, as determined by culturing and culture-independent microbial techniques. Changes in fecal communities were analyzed with respect to the women's equol-producing phenotype. Compared to baseline, at 1 and 3 months the counts for all microbial populations in the feces of equol-producing women had increased strongly. In contrast, among the non-producers, the counts for all microbial populations at 1 month were similar to those at baseline, and decreased significantly by 3 and 6 months. Following isoflavone intake, major bands in the denaturing gradient gel electrophoresis (DGGE) profiles appeared and disappeared, suggesting important changes in majority populations. In some women, increases were seen in the intensity of specific DGGE bands corresponding to microorganisms known to be involved in the metabolism of dietary phytoestrogens (*Lactonifactor longoviformis*, *Faecalibacterium prausnitzii*, *Bifidobacterium* sp., *Ruminococcus* sp.). Real-Time quantitative PCR revealed that the *Clostridium leptum* and *C. coccoides* populations increased in equol producers, while those of bifidobacteria and enterobacteria decreased, and *vice versa* in the non-producers. Finally, the *Atopobium* population increased in both groups, but especially in the non-producers at three months. As the main findings of this study, (i) variations in the microbial communities over the 6-month period of isoflavone supplementation were large; (ii) no changes in the fecal microbial populations that were convincingly treatment-specific were seen; and (iii) the production of equol did not appear to be associated with the presence of, or increase in the population of, any of the majority bacterial types analyzed.

Keywords: soy isoflavone, equol, intestinal microbiology, fecal microbiota, menopause, probiotics

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Introduction

Compared to Caucasian women, fewer Asian women suffer discomfort during menopause. They also have better intestinal health, and enjoy lower rates of cardiovascular disease (Bhupathy et al., 2010) and cancer (Virk-Baker et al., 2010). The better health of these women has been associated with a higher intake of soy foods (Messina, 2000). Soy contains many biologically active compounds (Kang et al., 2010), but its beneficial health effects have been attributed to its isoflavone content (Sánchez-Calvo et al., 2013). At the molecular level, the health benefits of dietary isoflavones appear to be mediated through their hormonal (Yuan et al., 2007), antioxidant (Wang et al., 2008), and enzyme-inhibitory (Crozier et al., 2009) activities.

In nature, isoflavones (daidzin, genistin, glycitin) mostly (>80%) appear conjugated with sugars as isoflavone-glycosides, the bioavailability and bioactivity of which are low (Crozier et al., 2009; de Cremoux et al., 2010). For their full activity to be realized, aglycones (daidzein, genistein, glycitein) need to be released from these isoflavone-glycosides and, occasionally, metabolized (Sánchez-Calvo et al., 2013). The transformations necessary are mostly performed by the enzymes of the gut microbiota. However, though improving, our knowledge of the gut microbes, their enzymes, and the pathways involved in the metabolism of isoflavones, is still limited (Atkinson et al., 2005; Yuan et al., 2007; Kemperman et al., 2010; Clavel and Mapesa, 2013). The metabolism of isoflavones is thought to occur in sequential steps involving several enzymes produced by a number of microbial types (Clavel and Mapesa, 2013; Sánchez-Calvo et al., 2013). The release of aglycones from conjugated isoflavones starts via the action of the widely distributed glycosyl hydrolases (members of the β-glucosidase family; Cantarel et al., 2009). The aglycones formed then undergo dehydroxylation, reduction, the breakage of the pyrone ring, and demethylation, etc., giving rise to compounds either of greater biological activity (such as equol and 5-hydroxy equol) or inactive molecules [such as *o*-demethylangolensin (*O*-DMA); Clavel and Mapesa, 2013]. Due to the inter-individual diversity in microbiota composition (Frankenfeld, 2011), only around 30–50% of Western women are capable of producing equol, while around 80–90% produce *O*-DMA. It may be that equol-producing women benefit more fully from the intake of isoflavones (Sánchez-Calvo et al., 2013).

Like other polyphenols, isoflavones have antimicrobial activity, which can modulate the diversity and composition of the gut microbiota after their consumption (Kemperman et al., 2010). The inhibition of pathogens or an increase in the size of beneficial populations might then contribute toward health benefits. However, studies on how isoflavones influence the composition and activity of the gut microbial community, and its effect on human health, are scarce (Clavel et al., 2005; Bolca et al., 2007; Nakatsu et al., 2014). Further, the results of the studies that are available are hard to compare, a consequence of differences in treatment regimen, target group, and the techniques employed. However, understanding how microorganisms and metabolites interact and elicit a physiological response (or lack thereof) is crucial if the results of observational and interventional studies are to be properly interpreted. Investigations that assess *in vivo* the response of gut populations to isoflavone consumption are much needed.

The main aim of the present study was to determine the effect of long-term dietary supplementation with an isoflavone concentrate on the fecal microbial communities of menopausal women, via both culturing and culture-independent techniques. Women were entering a treatment of menopause symptoms with an isoflavone concentrate, which made unnecessary the design of an intervention study. The microbial results were correlated with equol production status in an attempt to identify those microbial populations and/or numbers linked to the production of this active, microbial-derived compound.

Materials and Methods

Human Volunteers and Urine and Stool Samples

This study was approved by the Research Ethics Committee of the Principado de Asturias, Spain. The selection of donors and later sampling was performed following standardized protocols recommended by the above committee. Sixteen menopausal women (age range 48–61; average 53.4) were recruited at the Obstetrics and Gynaecology Service of the Hospital de Cabueñes (Gijón, Spain). No participants suffered from any disease or intestinal disorder. Additionally, they had received no treatment with antibiotics or any other medication for at least 6 months prior to the start of the study. All participants consumed one tablet containing 80 mg of an isoflavone concentrate (Fisiogen; Zambon, Bresso, Italy) per day for 6 months. Urine and fecal samples were collected at four time points: before the start of the treatment ($t = 0$), and at one ($t = 1$), three ($t = 3$), and six ($t = 6$) months. Morning urine samples and freshly voided stools were collected by the volunteers themselves, the latter in sterile plastic containers, in which they were maintained under anaerobic conditions via the use of Anaerocult A (Merck, Darmstadt, Germany). All samples were a transported to the laboratory by courier. Stool samples were subjected to microbial analyses within 2 h of arrival; dilutions for culture-independent techniques were kept frozen at −80°C until use, as were urine samples for later equol and creatinine analysis.

Determination of Equol and Creatinine in Urine Samples

Three milliliters of thawed urine samples were diluted with 3 mL of 0.1 M acetate buffer (pH 4.5) and treated for 20 h at 37°C with 10 μL of extract type H-1 crude enzyme solution from *Helix pomatia* (Sigma–Aldrich, St. Louis, MO, USA). This has β-glucuronidase (100 units/μL) and sulphatase (7.5 units/μL) activities. Equol was extracted using Bond Elut-C18 200 mg solid-phase cartridges (Agilent Technologies, Santa Clara, CA, USA), pre-conditioned with 3 mL of methanol and 3 mL of acetate buffer 0.1 M pH 4.5. Treated urine was passed through the cartridges, which were then washed with 3 mL acetate buffer 0.1 M (pH 4.5). To remove any residual water, 200 μL of ethyl acetate were eluted through the cartridges and rejected; this

roughly corresponded to the column dead volume. Equol was then eluted with 1 mL of ethyl acetate, filtered through a 0.2 μm PTFE membrane, and then evaporated to dryness under vacuum at room temperature. Prior to analysis, extracts were dissolved in 100 μL methanol [high-performance liquid chromatography (HPLC) grade) and kept at 4°C in opaque vials with screw caps. A 2 mM stock solution of equol (Sigma–Aldrich) in methanol was used to prepare a calibration curve covering six concentrations from 0.005 to 363.60 μM.

Equol in urine was determined using an H-Class Acquity UPLC™ ultra-high-performance liquid chromatography (UHPLC) system (Waters) equipped with a BEH reversed-phase C18 chromatographic column (1.7 μm, 2.1 mm × 100 mm; Waters, Palo Alto, CA, USA; Redruello et al., unpublished). The temperature of the column was set at 40°C. The mobile phase consisted of H_2O supplemented with 0.05% phosphoric acid (solvent A) and 100% methanol (solvent B). Samples were applied onto the column and eluted at a flow rate of 0.45 mL/min according to the following linear gradient: 0 min 90% A/10% B; 1 min 88% A/12% B; 3.5 min 75% A/25% B; 6.5 min 75% A/25% B; 10 min 50% A/50% B; 12 min 10% A/90% B; and 16 min 10% A/90% B. This was followed by washing with 10%A/90% B for 4 min and then 5 min with 90% A/10% B to re-equilibrate the column. Equol was measured using a fluorescence detector (excitation 280 nm, emission 310 nm).

As a single urine sample was analyzed, creatinine was determined to normalize equol excretion values. Equol was measured using a kinetic-photometric method based on the Jaffe reaction (Heinegård and Tiderström, 1973). For this, urine was treated with an alkaline picrate solution resulting in a bright orange–red complex. The formation rate of the complex over a prefixed interval of time (measured as an increase in absorbance) is proportional to the concentration of creatinine in the sample. Reactions were performed in 96-well microplates and measurements made using a Benchmark Plus microplate spectrophotometer (Bio-Rad, Richmond, CA, USA). Two hundred microliters of 10-fold diluted urine were added to a 2 mL solution of 25 mM picric acid prepared in 300 mM phosphate buffer (pH 12.1) containing 2 g/L SDS (to avoid protein precipitation). The reaction was allowed to proceed at 37°C and mixed every 5 s. The absorbance at 510 nm was measured over 6 min and the reaction rate determined as the tangent in the linear part of the kinetic curve between 0.08 and 5 min. Each sample was assayed in triplicate.

Fecal Microbiota Analyses

Microbial Counts

All fecal samples were processed in a Mac500 anaerobic chamber (Down Whitley Scientific, West Yorkshire, UK) containing a 10% H_2, 10% CO_2, 80% N_2 atmosphere. For microbial analyses, 1 g of feces was homogenized in 9 mL of sterile Maximum Recovery Diluent (Scharlab, Barcelona, Spain). The homogenates were then serially diluted and plated in duplicate onto general and selective agar media. Total and indicator bacterial populations were counted using the media as follows: for lactobacilli, de Man-Rogosa-Sharpe agar (Scharlab) supplemented with 0.25% cysteine (VWR International, Radnor, PA, USA; MRSC); for clostridia, agarified Reinforced Clostridial Medium (RCM; Merck, Darmstadt, Germany); for *Bifidobacterium* sp., modified Columbia agar (BCCM™/LMG, Medium M144; BIF; Masco et al., 2003); for Enterobacteriaceae, Eosin Methylene Blue agar (EMB; Oxoid, Basingstoke Hampshire, UK); for *Veillonella* sp., *Veillonella* agar (VA; Merck); for *Bacteroides* and *Prevotella* (BP), specialized *Bacteroides* and *Prevotella* agar (Ly et al., 2007); and for total microorganisms, Medium for Colon Bacteria (MCB; Van der Meulen et al., 2006). Counting was performed after anaerobic incubation of the plates at 37°C for 72 h, with the exception of the EMB plates, for which enumeration analysis was performed after aerobic incubation for 24 h. Data were recorded as colony forming units (cfu)/g of feces, were transformed to logarithmic units before statistical analysis.

DNA Extraction from fecal Samples

Extraction of total bacterial DNA was based on the method of Zoetendal et al. (2006) using the QIAamp DNA Stool Minikit (Qiagen, Hilden, Germany) with some modifications of the manufacturer's protocol. Briefly, 0.2 g of thawed fecal samples were suspended in 1.8 mL of phosphate buffer saline solution (PBS; pH 7.4). The fecal suspension was homogenized by vortexing and centrifuged at 800 rpm at 4°C for 10 min to eliminate insoluble materials. Supernatants were transferred to new tubes and centrifuged at 14,000 rpm at 4°C for 5 min. Pellets were suspended in 200 μL of lysis solution (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 1.20% Triton X-100, and 20 mg/mL lysozyme). Twenty units of mutanolysin (Merck, Darmstadt, Germany) were added to the mixture, which was then incubated at 37°C for 40 min. Immediately after, 1.2 mL of the lysis buffer from the DNA-isolation kit were added, the mixture placed in a screw-cap tube containing 0.3 and 0.1 g of 0.1 and 0.5 mm zirconia/silica beads, respectively, (BioSpec Products, Bartlesville, OK, USA) and subjected to mechanical breakage in a FastPrep FP120 Cell Disrupter (Qbiogene, Carlsbad, CA, USA; three cycles at 5.5 m s^{-1} for 30 s, cooling the samples on ice between cycles). Cell extracts were loaded onto the kit's column following the manufacturer's recommendations. Finally, the DNA was eluted with 150 μL sterile molecular biology grade water (Sigma–Aldrich) and stored at –20°C until required.

Denaturing Gradient Gel Electrophoresis (DGGE) Amplification

The variable V3 region of the 16S rRNA gene was amplified by PCR using the universal primers F357 (5′-TACGGGAGGCAGCAG-3′), to which a 39 bp GC sequence was linked to give rise to GC-F357 and R518 (5′-ATTACCGCGGCTGCTGG-3′), (Muyzer et al., 1993). The V2 and V4 regions were amplified with *Bifidobacterium*-specific primers F-Bif164 (5′-GGGTGGTAATGCCGGATG-3′) and R-Bif662 (5′-CCACCGTTACACCGGGAA-3′); a 40 bp GC sequence was linked to the latter to give rise to R-Bif662-GC (Satokari et al., 2001). Each reaction mixture consisted of 0.2 mM of each deoxynucleoside triphosphate (dNTPs), 0.24 μM of each forward and reverse primer, 2 U of 5 Prime Taq polymerase (VWR International), and between 100 and 150 ng of purified DNA. The PCR amplification programs were as follows: for

primers F357-GC and R518 – an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 40 s, plus a final extension step at 72°C for 10 min; for primers F-Bif164 and R-Bif662-G – after an identical denaturation step, 35 cycles of 95°C for 30 s, 62°C for 40 s, and 72°C for 1 min, plus a final extension step at 72°C for 5 min.

DGGE Analysis

Denaturing gradient gel electrophoresis was undertaken in a DCode apparatus (Bio-Rad) at 60°C. Electrophoresis was performed at 200 V for 10 min in an 8% polyacrylamide stacking gel, followed by 75 V for 16 h in a denaturing gel in 0.5X Tris-acetate-EDTA (TAE) buffer. The urea-formamide denaturing ranges were 40–55% for amplicons obtained with primers F357-GC/R518 and 45–55% for amplicons obtained with primers F-Bif164/R-Bif662-GC. After staining in an ethidium bromide solution, the gels were visualized under UV light using a GBox system (Syngene, Cambridge, UK) equipped with GeneSys image acquisition software (Syngene). Selected bands were excised from the gels, suspended in sterile molecular grade water, and kept overnight at 4°C. Subsequently, the eluted DNA was used as a template in new amplification reactions involving the same primers without the GC-clamp. Finally, amplicons were purified using GenElute PCR Clean-Up columns (Sigma–Aldrich) and sequenced using an ABI Prism gene sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were compared using the Blast program[1] and the Classifier tool provided by the Ribosomal Database Project[2]. Sequences with a percentage nucleotide match of 97% or higher to those in databases were assigned to the corresponding species.

The gels were digitalized and analyzed using Gene Tools v.4.03 software. For each DGGE profile, the Shannon–Weaver diversity index (H index) was estimated on the basis of the number of bands (assuming them to be equivalent to the number of species) and their relative intensity. Additionally, the similarity between the DGGE profiles (presence or absence of bands and their intensities) was determined by calculating the Dice's coefficient. Clustering was performed using the unweighted pair group method with arithmetic averages (UPGMA), employing the DendroUPGMA computer program[3].

Real-Time quantitative PCR (qPCR)

Quantification of the different bacterial populations in feces was performed by qPCR using group-specific primers targeting the 16S rRNA gene (**Table 1**). Amplification reactions were performed in 96-well optical plates (Applied Biosystems) in a 7500 Fast Real-Time PCR System (Applied Biosystems). All amplifications were performed in triplicate in a final volume of 25 μL containing 2x SYBR Green PCR Master Mix (Applied Biosystems), 0.2 μM of each primer and 1 μL of template DNA (5–10 ng). The thermal cycling protocol followed consisted of an initial cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 1 min at the appropriate primer-pair annealing temperature (**Table 1**). To check for specificity, melting curve analysis was performed, increasing the temperature from 60 to 95°C at a rate of 0.2°C per second with the continuous monitoring of fluorescence. Primer efficiency was calculated from the slope of the standard curve for each primer set ($E = 10^{-1/slope}$). The different bacterial groups were expressed as relative quantities (percentage of the total bacterial 16S rDNA in the sample) according to Vigsnæs et al. (2011).

Statistical Analysis

Statistical analysis of the culturing and qPCR data was performed using free R software[4]. The Shapiro–Wilk test was used to check for the normal distribution of the data. As several variables did

[1]http://www.ncbi.nlm.nih.gov/BLAST/

[2]http://rdp.cme.msu.edu/index.jsp

[3]http://genomes.urv.es/UPGMA/

[4]http://www.r-project.org

TABLE 1 | Bacterial target groups and characteristics of primers used for quantitative PCR (qPCR) in this study.

Microbial target	Primer	Sequence 5′–3′	Annealing (°C)	Efficiency[a]	Reference
Bifidobacterium sp.	F-bifido R-bifido	CGCGTCYGGTGTGAAAG CCCCACATCCAGCATCCA	60	1.90	Delroisse et al. (2008)
Lactobacillus sp.	Lacto-F Lacto-R	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	60	1.96	Heilig et al. (2002)
Clostridium coccoides group	g-Ccoc-F g-Ccoc-R	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	60	1.93	Matsuki et al. (2004)
Clostridium leptum group	g-Clept-F g-Clept-R	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	60	1.89	Matsuki et al. (2004)
Bacteroidetes phylum	Bact934F Bact1060R	GGARCATGTGGTTTAATTCGATGAT AGCTGACGACAACCATGCAG	60	1.92	Guo et al. (2008)
Atopobium cluster	c-Atopo-F c-Atopo-R	GGGTTGAGAGACCGACC CGGRGCTTCTTCTGCAGG	60	1.90	Matsuki et al. (2002)
Enterobacteriaceae	En-lsu3F En-lsu3-R	TGCCGTAACTTCGGGAGAAGGCA TCAAGGCTCAATGTTCAGTGTC	55	1.97	Matsuda et al. (2007)
Total bacteria[b]	TBA-F TBA-R	CGGCAACGAGCGCAACCC CCATTGTAGCACGTGTGTAGCC	60	1.93	Dennan and McSweeney (2006)

[a]*Primer efficiency was calculated from the slope of the standard curve for each primer set as* $E = 10^{-1/\text{slope}}$.

[b]*TBA primers were used for quantification of total bacterial DNA. This value was used to normalize differences in DNA concentration between individual samples.*

not follow a normal distribution, comparisons were performed by using non-parametric tests. The Mann–Whitney test for independent samples was performed to examine differences between equol producers and non-producers in terms of the microbial groups studied at every sampling point. The Wilcoxon signed-rank test for related samples was used to examine differences within microbial groups at all sampling points for both equol producers and non-producers. Two-tailed probability values of $P < 0.05$ were considered significant. Multivariate statistics was performed by principal coordinates analysis (PCoA) to search for associations between equol production status and the microbial groups determined by culturing and qPCR.

Results

Urine Equol after Isoflavone Supplementation

To test whether changes in the fecal microbial communities were associated with equol production phenotype, the equol status of the women was assessed by determining the concentration of this compound in their urine by UHPLC. Urine creatinine was also determined to normalize the equol values. Equol-producing women were defined as those responding to the soy challenge by showing an increase in urine equol to over 1000 nM (the cut-off defined by Rowland et al., 2000), and having an equol/creatinine ratio of >5.

Similar (and low) equol concentrations and equol/creatinine ratios were observed in the samples from all 16 women at $t = 0$ (**Table 2**). However, at $t = 1$, 100-fold increments in the ratios were obtained in the urine of four (25%) of the women (WA, WC, WG, and WP; Supplementary Table S1). In these subjects, the urinary excretion of equol at all post $t = 0$ sampling points (with the exception of $t = 3$ in woman WA) reached values above the stated cut-off. They were therefore considered equol producers. However, large variations in absolute equol concentrations were detected among their samples, as well as differences between samples for the same individual subject at different times (Supplementary Figure S1). In general, maximum equol production was observed at $t = 1$. After this point production was either maintained (in WC and WP) or fell by $t = 3$ and $t = 6$ (in WA and WG). Low equol levels, sometimes close to the limit of detection and/or of the limit of quantification, were measured in most urine samples from the non-producer women (**Table 2**).

TABLE 2 | Average equol production during isoflavone treatment in urine samples among equol producer and non-producer women of this study.

Equol status	Sample (month)	Parameter	
		Equol[a]	Equol/Creatinine
Producers ($n = 4$)	0	59.50	0.58
	1	18716.75	77.62
	3	8114.00	38.13
	6	5702.50	28.63
Non-producers ($n = 12$)	0	39.00	0.73
	1	19.42	0.12
	3	28.08	0.23
	6	15.58	0.10

[a]*Concentration of equol in nM.*

Microbial Counts

Wide inter-individual and inter-sample variations in counts for the different microbial populations were recorded over the supplementation period (**Table 3**). Although the response seemed erratic, some general trends were appreciated for equol producers and non-producers. At $t = 0$, counts for total and indicator microbial populations were generally slightly higher in the feces of the non-producers (**Table 3**). However, counts for all microbial populations increased strongly at $t = 1$ and $t = 3$ in the feces of the equol producers. In addition, in the equol producers, most cultivable populations decreased by $t = 6$, showing a trend toward the numbers recorded at $t = 0$. In contrast, in the non-producers, the counts for all microbial populations at $t = 1$ were similar to those at $t = 0$, but decreased significantly by $t = 3$ and $t = 6$ (**Table 3**). However, at the personal level, most microbial groups changed unpredictably in each of the women (Supplementary Table S2).

Community Profiling by DGGE

The community profiles showed 12–22 distinct DGGE bands of different intensity (**Figure 1**). Profiles contained between 3 (WP samples) through 8 (WF, WH samples) bands of a high intensity, being the rest of low or very low intensity. The appearance and disappearance of intense bands in the samples of individual subjects at consecutive sampling points (see **Figure 1**, WD-1, WF-3, and WH-1) indicates major changes in the majority bacterial populations. Since DGGE is a semi-quantitative technique, increases and reductions in the intensity of bands suggests corresponding changes in the fortune of the associated species. In general, isoflavone supplementation led to a reduction in the Shannon–Weaver diversity index (H index) compared to $t = 0$. The UPGMA comparison of Dice's coefficients showed a clear clustering of the profiles by subject (with only two exceptions, WC-0 and WD-1; **Figure 1**).

Eighty-two DGGE bands, the intensity of which varied over the supplementation period compared to $t = 0$, were assigned to bacterial types after DNA isolation, re-amplification, sequencing, and sequence comparison. The most common microbial types that responded to the presence of isoflavones were *Bifidobacterium adolescentis, Faecalibacterium prausnitzii, Lactonifactor longoviformis, Flavonifractor plautii, Coprococcus* sp., *Blautia* sp., *Oribacterium* sp., *Ruminococcus* sp., and members of the family Lachnospiraceae. However, the DGGE profiles from equol-producer women did not cluster together, and an apparent association between equol production and presence of specific bands was not observed.

Since the universal prokaryotic primers showed all bifidobacterial populations to increase over the supplementation period, DGGE analyses were performed using bifidobacteria-specific primers. Compared to the complex DGGE profiles for total bacteria, the bifidobacterial profiles were rather

TABLE 3 | Viable counts of total and indicator fecal microbial populations in menopausal women treated with a soy isoflavone supplement over a 6-month period.

Equol	Month	Microbial counts[a]						
		MCB	MRSC	BIF	RCM	EMB	BP	VA
Producers ($n = 4$)	0	9.51 ± 1.12	9.29 ± 1.39	9.44 ± 1.11	9.21 ± 1.31	7.69 ± 1.19	9.87 ± 1.27	9.65 ± 1.35
	1	10.46 ± 0.68	10.55 ± 0.82	10.28 ± 0.75	9.79 ± 1.92	8.91 ± 1.53	11.16 ± 0.63	10.65 ± 0.29
	3	10.73 ± 0.75	10.37 ± 0.83	10.49 ± 0.95	10.27 ± 0.52	7.35 ± 1.01	10.87 ± 0.87	10.42 ± 0.60
	6	9.79 ± 0.25	8.96 ± 0.87	9.53 ± 0.19	9.32 ± 0.40	7.17 ± 0.57	10.08 ± 0.13	9.36 ± 0.22
Non-producers ($n = 12$)	0	10.32 ± 0.61	10.04 ± 0.81	10.21 ± 0.67	10.23 ± 0.70	8.01 ± 0.98	10.45 ± 0.60	10.16 ± 0.73
	1	10.42 ± 0.81	10.06 ± 1.00	10.30 ± 0.82	10.38 ± 0.87	7.82 ± 0.70	10.80 ± 0.71	10.64 ± 0.75
	3	9.75 ± 0.76	9.30 ± 1.15	9.65 ± 0.69	9.49 ± 0.92	7.32 ± 1.07*	10.13 ± 0.62	9.23 ± 0.85
	6	9.54 ± 0.54**	9.10 ± 0.72**	9.47 ± 0.49**	9.19 ± 0.68**	7.31 ± 1.03*	9.74 ± 0.57**	8.86 ± 0.75***

[a] *Log_{10} cfu g^{-1} of feces (mean ± SD).*

Key of media (target population): MCB, Medium for Colon Bacteria (total cultivable bacteria); MRSC; de Man, Rogosa, and Sharpe (lactobacilli); BIF, BCCMTM/LMG-Medium M144 (bifidobacteria); RCM, Reinforced Clostridium Medium (clostridia); EMB, Eosin Methylene Blue (enterobacterias); BP, Bacteroides and Prevotella medium (Bacteroides and Prevotella sp.); VA, Veillonella Agar (Veillonella sp.).

*Key of statistical significance respect to basal time ($t = 0$): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.*

simple, involving just 2–5 bands per subject (partial results are provided in Supplementary Figure S2). In contrast to the general profiles, the bifidobacterial profiles for most of the women proved stable throughout the supplementation period. In total, 18 bands were isolated from the DGGE gels and identified as before. *B. bifidum, B. longum, B. catenulatum/B. pseudocatenulatum*, and *B. adolescentis* were the most common bifidobacterial species identified. Other species, such as *B. saeculare* and *B. ruminantium*, were occasionally identified. In two of the women (WB and WG), the bands corresponding to *B. adolescentis* increased markedly over the supplementation period.

qPCR Analysis

qPCR detected wide variations in bacterial population sizes among subjects, and between samples from the same subject at different sampling points (Supplementary Table S3). The majority populations were formed by *Bacteroides* and *Clostridium*. Bifidobacteria and lactobacilli made up <10 and <0.5%, respectively, of total bacterial numbers. Over the supplementation period, opposite trends were seen between the equol producers and non-producers in terms of the change in some other bacterial populations. For example, bifidobacterial populations decreased in size in the equol producers, but increased in the non-producers, while the population of *Bacteroides* increase in equol producers and decrease in the non-producers (**Table 4**). The population of enterobacterias was shown to be significantly lower in equol producers than in non-producers. In this last group of women, increased numbers of enterobacteria were seen at $t = 3$ and $t = 6$ (**Table 4**). The two *Clostridium* clusters (leptum and coccoides) targeted by specific primers were seen to increase in the equol producers, especially at $t = 3$ and $t = 6$, while their numbers remained similar in samples from the non-producers. Finally, the *Atopobium* population increased over the supplementation period in both groups of women, but more so in the non-producers (a significant increase was detected at $t = 3$).

Discussion

The metabolism of soy isoflavones, and therefore their bioavailability and activity in humans, strongly depends on the activity of the intestinal microbiota. The underlying interactions between microbial populations and isoflavone metabolites, however, remain poorly understood (Frankenfeld, 2011; Clavel and Mapesa, 2013).

Though several methods have been proposed to determine human equol production phenotype (Rowland et al., 2000; Setchell and Cole, 2006), the cut-off for the assignment of producer or non-producer status remains rather arbitrary. In the present work, producers and non-producers were identified based on a cut-off of 1000 nM equol in urine, as defined by Rowland et al. (2000). The same number of equol producers and non-producers were also obtained when considering an equol/creatinine ratio >5 as the cut-off (Rowland et al., 2000). The frequency of equol producers differ widely among human communities (Setchell et al., 2002). That reported in the present work agrees well with values reported by other authors for Western women (Atkinson et al., 2005; Setchell and Cole, 2006; Possemiers et al., 2007; Frankenfeld, 2011; Nakatsu et al., 2014). In contrast to that reported by Franke et al. (2012), equol production phenotype proved to be stable over the study period; no conversion from equol producer to non-producer or *vice versa* was observed.

The effects of isoflavones on the gut microbiota have been little examined (Clavel et al., 2005; Bolca et al., 2007; Nakatsu et al., 2014), and with the exception of the study by Clavel et al. (2005), which lasted two months, changes in bacterial populations have only ever been monitored over short periods. Further, apart from the work of Nakatsu et al. (2014), which involved phylogenetic/metagenomic analyses, the number of populations targeted has been very limited. In the present work, the effects of isoflavones on the fecal microbiota of healthy menopausal women were analyzed at 1, 3, and 6 months of supplementation, and the results compared to those at baseline. This design, however, may have overlooked significant changes

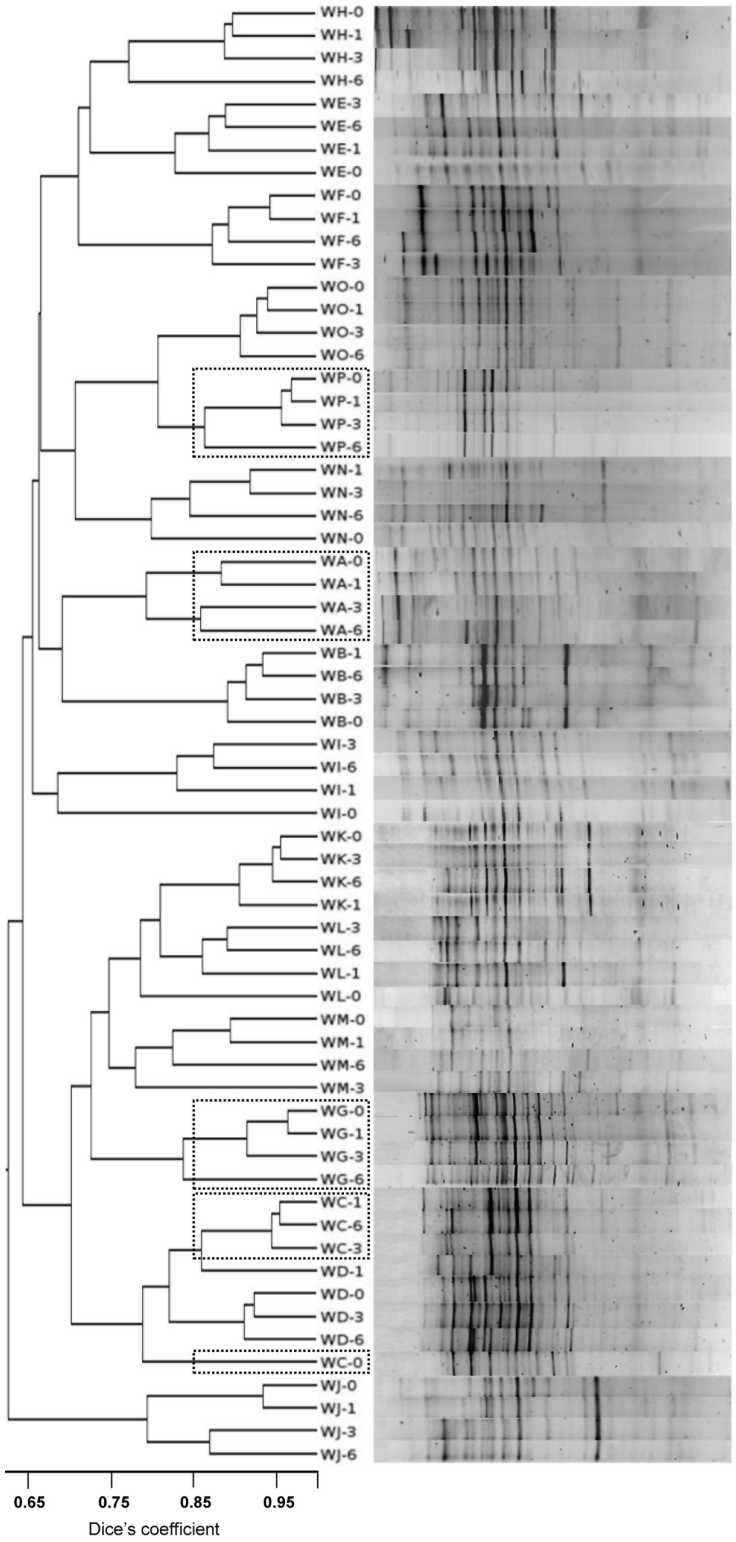

FIGURE 1 | Denaturing gradient gel electrophoresis (DGGE) profiles of the total microbial populations from fecal samples of menopausal women at different times during isoflavone treatment using universal prokaryotic primers amplifying the V2-V4 hypervariable regions of the 16S rRNA gene. Similarity of the profiles was calculated by the Dice's coefficient and clustered by the UPGMA method. Position of the profiles from equol producer women is indicated by a doted box.

TABLE 4 | Relative quantities of fecal microbial populations in the different equol status groups of the menopausal women treated with soy isoflavones of this study as determined by qPCR using universal and group-specific primers.

Equol status	Month	Microbial population[a]						
		Bifidobacteria	**Lactobacilli**	***Clostridium leptum***	***Clostridium coccoides***	***Bacteroides***	**Enterobacteria**	***Atopobium***
Producers ($n = 4$)	0	3.47 ± 5.74[a]	0.51 ± 0.91	28.70 ± 7.67	23.36 ± 14.31	73.6 ± 126.20	0.008 ± 0.001	4.87 ± 3.35
	1	1.00 ± 0.80	< 0.01 ± 0.01	19.64 ± 8.25	24.19 ± 18.58	104.9 ± 181.7	0.003 ± 0.004	5.64 ± 2.15
	3	2.05 ± 1.37	0.13 ± 0.20	35.69 ± 13.52	40.34 ± 24.18	45.31 ± 59.72	< 0.001 ± 0.001	5.96 ± 1.00
	6	1.43 ± 1.04	0.04 ± 0.03	33.14 ± 13.25	32.43 ± 24.08	113.3 ± 210.1	0.004 ± 0.006	4.70 ± 1.72
Non-producers ($n = 12$)	0	4.96 ± 6.19	0.31 ± 0.81	23.89 ± 8.75	25.33 ± 16.51	29.08 ± 63.03	0.20 ± 0.52	3.77 ± 1.97
	1	7.63 ± 7.87	0.31 ± 0.89	26.17 ± 17.11	26.97 ± 18.59	20.57 ± 31.07	0.14 ± 0.27	5.61 ± 4.25
	3	6.84 ± 9.17	0.05 ± 0.07	24.72 ± 10.89	25.15 ± 16.60	15.53 ± 14.42	0.83 ± 0.22	6.24 ± 4.90*
	6	9.27 ± 13.11	0.35 ± 0.68	19.98 ± 10.13	24.26 ± 16.80	12.55 ± 9.46	0.49 ± 0.13	4.85 ± 2.88

[a]*Percentage of the total bacterial 16S rDNA (mean ± SD), as determined using the universal prokaryotic primers TBA-F and TBA-R (**Table 1**).*
*Key of statistical significance: *$p < 0.05$ versus basal sample ($t = 0$).*

occurring soon after the start of isoflavone supplementation (Bolca et al., 2007; Nakatsu et al., 2014).

In general, the community structure and composition of the fecal bacterial populations changed significantly with the isoflavone supplementation. However, wide inter- and intra-individual (at different times) variations were detected by the different techniques employed, making it very difficult to correlate isoflavone intake with any changes in the bacterial community structure/population size, or changes in the latter with equol production. Thus, not surprisingly, PCoA of culturing and qPCR results showed no association between microbial communities and equol production status (Supplementary Figure S3). It is conceivable that isoflavones directly or indirectly affect members of the dominant microbiota (Clavel and Mapesa, 2013). In addition to the selective pressure they may exert on isoflavone-utilizing microorganisms, isoflavones or their metabolites (aglycones, equol, *O*-DMA, etc.) might modify conditions in the intestinal tract with an ensuing effect on susceptible bacterial communities. A bifidogenic effect of isoflavones has been reported in some studies (Clavel et al., 2005; Nakatsu et al., 2014), and a modest effect of this kind for most of the samples at $t = 1$ was confirmed by culturing. However, it did not persist through to $t = 3$ and $t = 6$. In equol producers, increases in population size within *Clostridium* clusters have been observed before (Clavel et al., 2005; Possemiers et al., 2007). However, such stimulatory effects may depend on the baseline sizes of these populations, which can vary widely between subjects. In contrast, a reduction was seen in the number of enterobacterias in the samples from most women (Supplementary Table S2). Since several members of the Enterobacteriaceae, including *Escherichia coli* phylotypes, harbor a ∼54 kb polyketide synthase (*pks*) pathogenicity island that encodes multi-enzymatic machinery for synthesizing a genotoxin that promotes tumorigenesis (Arthur et al., 2012), a fall in their numbers might be beneficial. However, no changes in the fecal microbial populations that were convincingly supplementation-specific were seen, suggesting that (at least some) changes might be due to normal variations caused by diet or other uncontrolled environmental factors. Together, the present enumeration results suggest that the consumption of isoflavones induces changes in the majority of microbial populations in both equol producers and non-producers, although these might be opposite.

The inadequacy of conventional culturing techniques for reflecting the microbial diversity of the intestinal ecosystem (Leser et al., 2002; Kemperman et al., 2010; Li et al., 2014) prompted the use of two culture-independent methods: DGGE for profiling the majority microbial species, and qPCR for targeting specific microbial populations. In some woman, specific bands corresponding to microorganisms previously associated with the metabolism of isoflavones and other dietary phytoestrogens (*L. longoviformis*, *F. prausnitzii*, *Bifidobacterium* sp., *Ruminococcus* sp.; Bolca et al., 2007; Clavel et al., 2007; Nakatsu et al., 2014) were enhanced in intensity. As suggested by Nakatsu et al. (2014), such increases may argue for isoflavones providing a chemical environment that selects for a subset of the initial bacterial community (Clavel and Mapesa, 2013). Alternatively, isoflavones might have antimicrobial effects on certain bacterial populations, as reported for flavonoid compounds (Orhan et al., 2010). In either case, the UPGMA analysis grouped the DGGE profiles by individual rather than by treatment (isoflavones, non-isoflavones), suggesting the changes detected by this technique depend on the initial microbial profile (personal microbiota). Furthermore, DGGE only detects changes occurring within the dominant bacterial populations (Muyzer et al., 1993); subtle changes in subdominant or minority species also occur but go unseen, and equol production might result from interactions between dominant and subdominant (or even minority) intestinal populations. In contrast, qPCR involving universal and group-specific primers produces a general microbial picture similar to that produced by state-of-the-art metagenomic techniques (Qin et al., 2010; Li et al., 2014).

Discrepancies between culturing and culture-independent methods may result from differences in lysis efficiency during DNA isolation, preferential PCR amplification (which may be

different for each primer pair), or interspecies differences in 16S rRNA operon copy number (Kemperman et al., 2010). The detection of non-cultivatable and dead cells by DNA-based techniques may account for further differences. In this sense, low recoveries of Bacteroidetes from frozen fecal samples have been reported (Bahl et al., 2012). However, in the present work *Bacteroides* sp. proved to be majority population in both equol producers and non-producers.

Conclusion

Both the culturing and the culture-independent methods used in this work detected wide microbial diversity in the studied menopausal women at baseline. Variations in the microbial communities over the six-month period of isoflavone supplementation were also large. No general patters of change due to isoflavone ingestion, or associated with equol production, were observed. The production of equol could not be correlated to the presence of, or increase in, any of the bacterial populations analyzed. This suggests isoflavone metabolism may differ in different people depending on their personal gut microbiota. Metagenomics, metabolomics, and metatranscriptomics analyses will be required to uncover the relationships between the structure and composition of the intestinal microbial communities in response to soy isoflavones and their metabolic compounds. Further research in this area could ultimately lead to the modulation of numbers of equol-producing bacteria, and thus equol producer-status, through the use of specific prebiotics and/or probiotics.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00777

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Changes in the faecal microbiota of an equol-producing menopausal woman over six months of dietary supplementation with isoflavone

Abstract

This work describes the impact of long term consumption of an isoflavone-rich dietary daily supplement on the composition and diversity of the faecal microbiota of a menopausal, equol-producing woman. Sequencing of 16S rDNA amplicons was performed on faecal samples taken at 0, 1, 3 and 6 months of treatment. Members of two genera of the family *Coriobacteriaceae* (*Eggerthella* and *Collinsella*) were found in significantly greater proportions at all sampling points during isoflavone supplementation. Different genera of the family *Ruminococcaceae* (e.g., *Ruminococcus* and *Faecalibacterium*), as well as members of the family *Lachnospiraceae*, also experienced significant expansion. Currently bacterial strains known to be involved in isoflavone metabolism and equol production have been assigned to these taxa. Our study contributes to the identification of microorganisms producing isoflavone-desirable metabolites (such as equol), which could ultimately be used as probiotics by people who cannot naturally benefit from isoflavones.

Introduction

Isoflavones are plant-derived phytoestrogens, present in especially large amounts in soy and soy-derived products. Isoflavones bind to oestrogen receptors, triggering physiological responses that might influence human health (Xiao, 2008); certainly, they have been reported beneficial in counteracting the hot flushes and vasomotor reactions experienced by menopausal women (Bolaños el al., 2010; NAMS, 2011). In soy, isoflavones are mostly conjugated with sugars, forming isoflavone glycosides, which are low availability and low bioactivity compounds (Crozier et al, 2009; de Cremoux et al., 2010). To be fully active, isoflavone glycosides need to be transformed into isoflavone aglycones via enzymes produced by the intestinal tissue and gut microorganisms (Selma et al, 2009; Sánchez-Calvo et al, 2013). Some intestinal bacteria metabolize isoflavone aglycones into other active compounds, such as equol (Atkinson et al., 2005; Sánchez-Calvo et al, 2013). Formed from the aglycone daidzein, equol is the isoflavone-derived metabolite with the strongest oestrogenic and antioxidant activities (Messina, 2016). However, only 25-30% of Western individuals have been reported to harbour the microbiota required for the conversion of daidzein into equol (Setchell and Clerici, 2010).

Although the full range of intestinal bacteria involved in equol formation remains unknown (Crozier et al., 2009; Kempermanet al., 2010), most of those recognised as equol-producers to date are members of the family *Coriobacteriaceae* (Clavel and Mapesa, 2013). Changes in the faecal community after one week of isoflavone ingestion in postmenopausal women have been previously examined (Nakatsu et al. 2014). However, the microbial changes that occur during long-term isoflavone intake in menopausal women have not been determined by high throughput sequencing methods. The few studies that reported on intestinal microbiota changes induced by long term isoflavone consumption used culture-independent identification techniques including PCR-TGGE, qPCR or FISH (Clavel et al., 2005; Bolca et al., 2007; Guadamuro et al., 2015), which are unable to determine low abundance microorganisms like those involved in equol production.

This study aimed to determine changes in the intestinal microbiota communities induced by a 6-month period of isoflavone consumption by a menopausal, equol-producing woman. Additionally, we intended to identify living

members of bacterial communities, which may ultimately be cultivated and isolated. To achieve this, we treated samples with ethidium monoazide (EMA) that enters cells with damaged membranes and binds covalently to their DNA, preventing any subsequent amplification (Nocker et al., 2006).

Material and Methods

Ethical statement, the sample donor, and supplementation regimen

Ethical approval for this study was obtained from the Bioethics Subcommittee of the Spanish Research Council (C*onsejo Superior de Investigaciones Científicas* or CSIC) and the Regional Ethics Committee for Clinical Research of the Health Service of Asturias (*Servicio de Salud del Principado de Asturias*), in compliance with the Declaration of Helsinki. Faecal samples were provided, with written consent, by a woman volunteer recruited during a previous study (Guadamuro et al., 2015) at the Gynaecology and Obstetrics Unit (in collaboration with the Gastroenterology Department). She declared having no intestinal disorders or underlying diseases, although she reported suffering menopause-related symptoms. She had undergone no medical treatment -including the taking of antibiotics- in the six months prior to beginning isoflavone supplementation, nor were any such treatments followed during the supplementation period. Supplementation consisted of the daily oral intake, for six months, of a commercial dietary supplement (Fisiogen; Zambon, Bresso, Italy) rich in soy isoflavones (80 mg/day).

Sample collection and processing

The volunteer provided samples of faeces (for molecular analysis) and urine (for equol determination) before treatment (baseline, T0) and after one (T1), three (T3) and sixth (T6) months of isoflavone supplementation. Fresh stools were collected in sterile plastic containers and kept under anaerobic conditions in jars containing Anaerocult A (Merck, Darmstadt, Germany) for transport to the laboratory under 2 h. Urine equol was determined by UHPLC/fluorescence detection as previously described (Redruello et al., 2015).

Total bacterial DNA extraction

Faecal samples (0.2 g) were suspended in 1.8 mL of phosphate buffered saline (PBS) (pH 7.4) with 6.7% sucrose. These suspensions were homogenized and centrifuged at 800 rpm for 5 min at 4ºC to eliminate pieces of insoluble material and intestinal cells, and the supernatants transferred to new tubes. These were then centrifuged again, at 14000 rpm for 5 min at 4ºC. Pelleted cells were suspended in 1 mL of cold PBS and divided into two fractions: one (0.5 mL) was kept on ice until use, and the other (0.5 mL) incubated with 100 μM EMA (Biotium, Hayward, CA., USA) in the dark for 5 min with occasional mixing. The latter samples were then exposed to a 650 W halogen light source for 5 min to induce DNA crosslinking; for this, the tubes were laid horizontally on ice on a shaker platform at about 20 cm from the light source. These cells were then washed twice with cold PBS prior to DNA extraction.

Both the EMA-treated and non-treated cells were then lysed in an enzyme solution containing 20 mM TRIS-HCl pH 8.0, 2 mM EDTA, 1.20 % Triton X-100, 20 mg mL^{-1} lysozyme (Merck) and 20 U mutanolysin (Sigma-Aldrich, Saint Louis, MO., USA). Total bacterial DNA was extracted following the protocol described by Zoetendal et al. (2006), and purified using the QIAamp DNA Stool Minikit (Qiagen, Hilden, Germany). Finally, the DNA was eluted in 100 μL sterile molecular grade water (Sigma-Aldrich), and its concentration and quality determined using an Epoch microvolume spectrophotometer (BioTek Instruments, Winooski, VT., USA).

Library construction and pyrosequencing

A segment of the 16S rRNA genes from the purified total bacterial DNA (both from the untreated and EMA-treated samples) were PCR-amplified using the universal primers Y1 (5'-TGGCTCAGGACGAACGCTGGCGGC-3') (position 20-43 on the 16S rRNA gene of *Escherichia coli*) and Y2 (5'-CCTACTGCTGCCTCCCGTAGGAGT-3')

(positions 361-338) (Young et al., 1991). These primers amplify a 348 bp stretch of the prokaryotic rDNA embracing the V1 and V2 hypervariable regions. 454-adaptors were included in both the forward (5'-CGTATCGCCTCCCTCGCGCCATCAG-3') and reverse (5'-CTATGCGCCTTGCCAGCCCGCTCAG-3') primers, followed by a 10 bp sample-specific barcode. Amplifications were performed using the NEBNext High-Fidelity 2x PCR Master Mix Kit (New England Biolabs., Ipswich, MA, USA) as follows: 95°C for 5 min, 25 cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 30 s, and a final extension step at 72°C for 5 min. The amplicons produced were purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich), and their concentration measured as above.

An amplicon library was prepared for pyrosequencing by mixing equal amounts of amplicons from the eight different samples (i.e., T0-T6 for both treatments). Pooled amplicons were then sequenced using a 1/8 picotitre plate in a 454 Titanium Genome Sequencer (Roche, Indianapolis, IN, USA).

Sequence and data analysis

Raw sequences were denoised and filtered out of the original dataset. Filtering and trimming were performed using the Galaxy Web Server (Goecks et al., 2010), employing the sliding window method. Only reads longer than 150 bp were used in further analysis. Chimeras were eliminated using the USEARCH v.6.0.307 clustering algorithm routine in *de novo* mode (Edgar et al., 2011). After demultiplexing, high quality rDNA sequences were classified taxonomically using the Ribosomal Database Project (RDP) Bayesian Classifier (Wang et al., 2007) with an 80% confidence threshold to obtain the taxonomic assignment and relative abundance of the different bacterial groups. 'Genus' was the lowest taxonomic level contemplated. The RDP Library Compare tool was used to estimate (by Northern analysis) the probability of observing differences in abundance in a given phylogenetic taxon (Wang et al., 2007). Rarefaction analysis was performed to obtain the number of operational taxonomic units (OTUs) in each sample. This allowed different diversity indices (ChaoI, Jacknife and Shannon) to be calculated and comparisons made between samples (Moreno and Halffter, 2001). Sequences at least 97% sequence similarity -the consensus species boundary threshold (Yarza et al., 2008)- were clustered into OTUs using the CD-Hit clustering method (Li and Godzik, 2006) and employed in the generation of rarefaction curves using aRarefactWin freeware (produced by S. Holland; http://strata.uga.edu/software/index.html).

Results

Urinary excretion of equol after isoflavone ingestion

The urine equol concentration increased from 9 nM at baseline to 1143 nM at T1. Similar equol concentrations were detected at T3 and T6 (1727 nM and 1382 nM respectively). According to the criterion of Rowland et al. (2000), the woman providing the study samples clearly showed an equol-producing phenotype (equol excretion in urine >1000 nM after isoflavone challenge).

Change in faecal microbiota over isoflavone supplementation

After denoising, performing chimera checks, and trimming the reads by length (150-400 bp), a mean of 6030 (±3151) high quality sequences were obtained. Taxonomic analysis grouped the sequences mainly into five phyla: Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria and Verrucomicrobia. Fifty two genera were identified, as well as five groups of clostridia (*Clostridium* cluster IV, cluster XI, cluster XIVa, cluster XVIII and *Clostridium sensu stricto*), and two taxa with family-associated *incertae sedis* (*inc. sed.*) members (*Erysipelotrichaceae inc. sed.* and *Lachnospiraceae inc. sed.*). Taxonomic groups present at an abundance of <0.1% were designed as "others". A mean of 738 sequences per sample remained unclassified.

Considerable differences were observed between the bacterial communities at T0 and

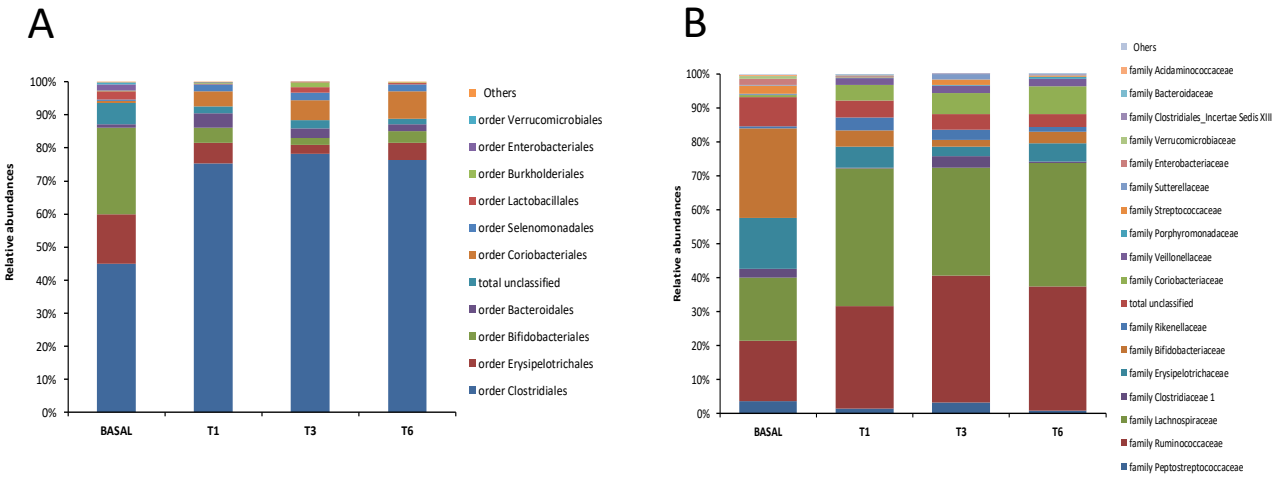

Fig. 1. Microbial composition, at the order (A) and family (B) levels, of faecal samples at T0 (basal) and at T1, T3 and T6 months after isoflavone treatment.

T1-T6. Differences were noted at the order and family levels from T1 onwards (Fig. 1). A significant (p<0.05) reduction in sequences was noted for 13 genera (including *Bifidobacterium,* members of *Erysipelotrichaceae inc. sed.*, *Streptococcus, Escherichia/Shigella, Turicibacter, Dorea*, *Akkermansia*, *Clostridium* groups XI and XVIII, *Cellulosilyticum*, *Coprobacillus*, *Flavonifractor* and *Phascolarctobacterium*) over the study period, while nine others (*Coprococcus*, *Dialister*, *Eggerthella*, *Collinsella*, *Blautia*, *Ruminococcus*, *Alistipes*, *Faecalibacterium,* and *Lachnospiraceae inc. sed.*) increased significantly (p<0.05) (Fig. 2). Isoflavone supplementation also resulted in an overall reduction of diversity and richness, as measured by the ChaoI and Jacknife indices (Fig. 3).

Differences in community composition after EMA treatment

In the faecal samples treated with EMA before DNA extraction, the mean number of sequences recovered was higher (8354 ± 2932 compared to 3705 ± 408 for the non-treated samples). However, the number of OTUs identified at 97% similarity was significanlty smaller, ranging from 105-244 per sample compared to 463-594 for the non-treated samples (Fig. 4). The most significant and consistent reductions in reads after EMA exposition were obtained for the order *Clostridiales*, particularly for those of the genera of the family *Lachnospiraceae* (*Faecalibacterium, Blautia*, *Roseburia, Dorea, Coprococcus*) and those of the phylum Actinobacteria (*Collinsella, Coriobacterium*) (Table 1).

Discussion

There is strong evidence that diet modulates the composition of the intestinal microbiota (Graf et al., 2015). Most studies, however, have concentrated on the effect of fat and fibre (Singh et al., 2017; David et al., 2014), while other dietary microcomponents, such as polyphenols, have received less attention (van Duynhoven et al., 2011). Certainly, little is known about the influence of isoflavones on the microbial populations of the gut (Clavel et al., 2005; Bolca et al., 2007; Nakatsu et al., 2014). The present work reports the impact of long term consumption of dietary isoflavones on faecal microbial composition and diversity in a menopausal woman with an equol-producing phenotype. Although several strains of the human gut -mostly members of the family *Coriobacteriaceae* (Clavel et al., 2014)- have been found to produce equol, the list of taxa able to do so is probably far from complete. In the present work, the abundance of two genera of this family -*Eggerthella* and *Collinsella*- significantly increased over the study period. Other bacteria mostly belonging to the families *Ruminococcaceae* (*Ruminococcus*, *Faecalibacterium*) and *Lachnospiraceae*

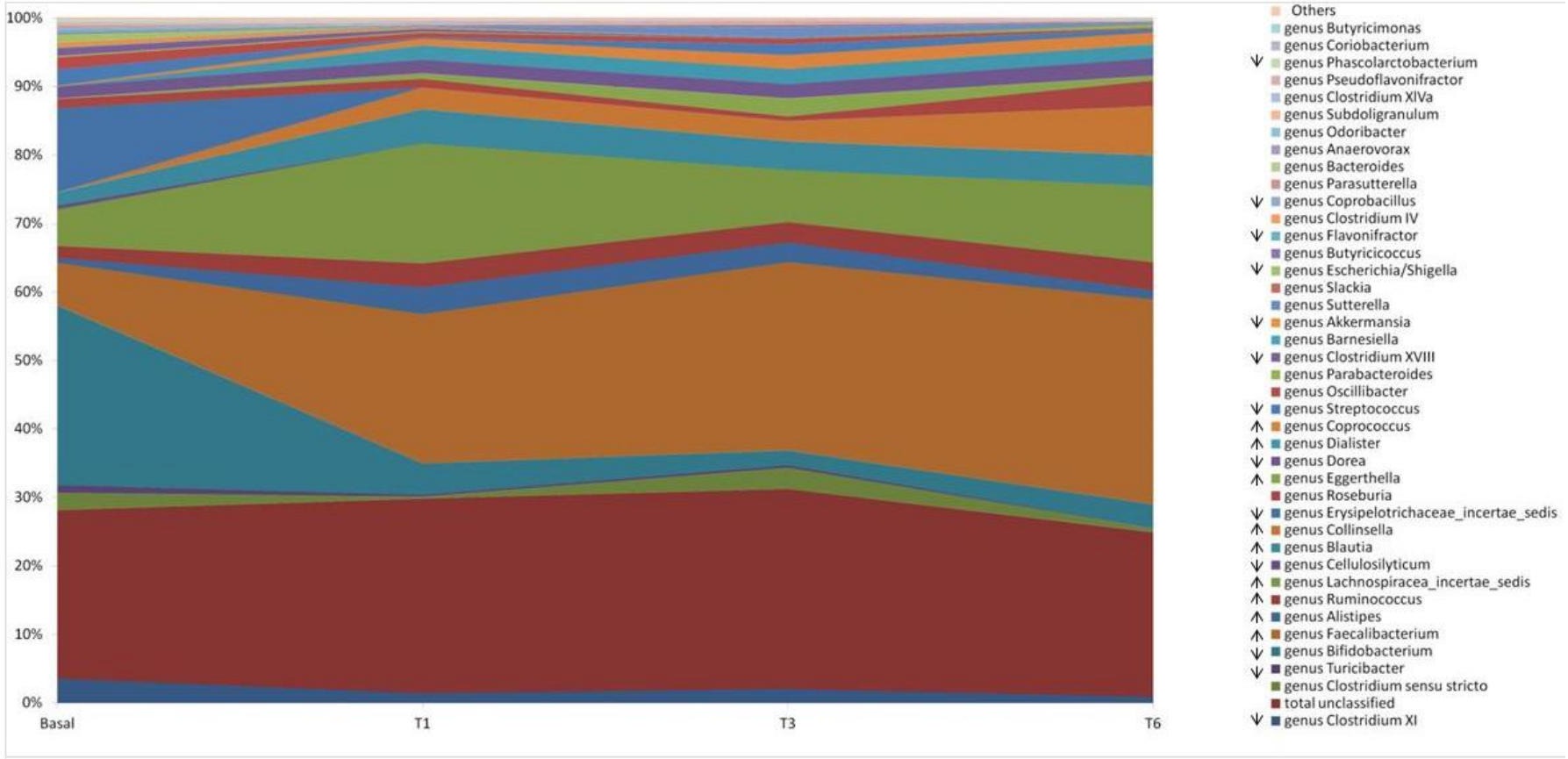

Fig. 2. Evolution of the faecal microbial composition at the genus level at different time points during isoflavone treatment (T0, T1, T3 and T6). Genera with consistent statistical significant changes during the intervention are marked in the legend with ascending and descending arrows.

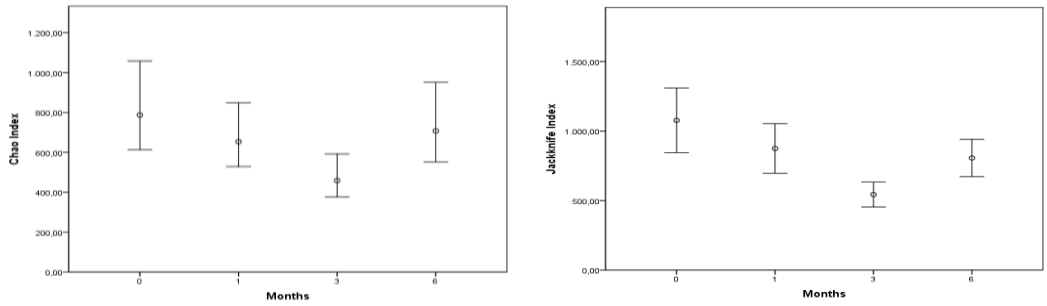

Fig. 3. Alpha diversity indices for the 16S rRNA gene sequences at 3% divergence distance of four pyrosequenced faecal samples at T0, T1, T3 and T6 treatment.

(*Coprococcus*, *Blautia and inc.sed*), plus others belonging to *Erysipelotrichaceae inc. sed.*, were also increased after isoflavone supplementation. The family *Lachnospiraceae* has a very large presence in the human gut and has been linked to the production of butyric acid (Meehan and Beiko, 2014), a compound with several beneficial effects on the gastrointestinal ecosystem (Ríos-Covián et al. 2016). The relative abundance of *Dialister*, a member of the family *Veillonellaceae* recently associated with isoflavone metabolism (Nakatsu et al., 2014) and equol production (Decroos et al., 2005), also increased significantly over the study period.

The present results reveal an increase in Gram-positive bacteria of the phylum Actinobacteria involved in isoflavone aglycones metabolism (*Collinsella, Eggerthella, Slackia, Coriobacterium*) (Clavel et al., 2014), but a reduction in other members of Actinobacteria, such as *Bifidobacterium*. Apart from the notable reduction in the abundance of reads of this genus, sequences related to *Clostridium* clusters XI and XVIII, and those of *Escherichia/Shigella*, were also less common after beginning isoflavone supplementation. Isoflavone intake may favour the development of certain bacterial types in the gut by providing an energy source (Kim and Han, 2016). The competitive advantage might indirectly lead to the inhibition of other bacteria. Conversely, like other polyphenols isoflavones and their metabolites have been shown to possess antimicrobial activity against specific bacterial groups (Kim and Lee, 2009; Sklenickova et al., 2010), such as members of the families *Clostridiaceae* and *Enteroboacteriaceae* (Vázquez et al., 2017). Different mechanisms of action have been proposed, such as the binding of these compounds to bacterial cell membranes disturbing its functioning, and via the formation of polyphenol-metal ion complexes, which could lead to iron deficiency (Kemperman et al., 2010).

A major advantage of culture-based identification is that it allows the isolation of the bacteria involved in isoflavone metabolism. The use of high throughput sequencing techniques, however, can help to detect low-abundance equol bacteria producers that have yet to be cultured. As previously suggested (Vázquez et al., 2017; Clavel et al., 2014), different bacteria may contribute towards equol production, but these might be present in the gut in low abundance, making difficult their detection by culturing.

To assess the ratio of dead/live bacteria during the isoflavone intervention, DNA from faeces was extracted with and without prior EMA treatment. This dye has been used in the

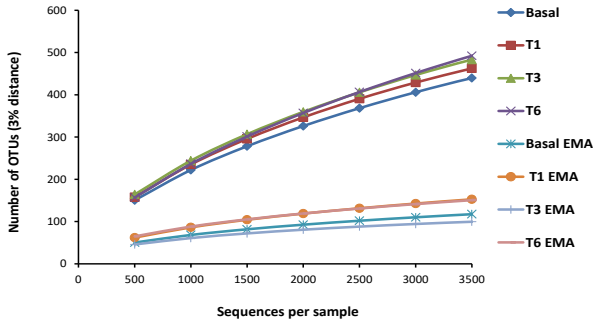

Fig. 4. Rarefaction curves of partial sequences of the bacterial 16S rRNA genes amplified from faecal samples of a menopausal woman following an isoflavone intervention at 97% similarity level using DNA extracted without (Basal, T1, T3 and T6) and with EMA ($Basal_{EMA}$, $T1_{EMA}$, $T3_{EMA}$, $T6_{EMA}$).

Table 1.- Genera showing statistical differences in relative abundance (% sequences) when comparing DNA extracted directly from faeces or after exposure to EMA.

Genera	Sample/treatment							
	Basal	Basal/EMA	T1	T1/EMA	T3	T3/EMA	T6	T6/EMA
Faecalibacterium	**6.16**	0.06***	**21.74**	1.35***	**27.46**	0.45***	**29.91**	0.80***
Lachnospiraceae incertae sedis	**5.33**	0.05***	**17.23**	0.35***	**7.60**	0.52***	**10.67**	0.93***
Streptococcus	2.37	0.00***	0.15	0.01**	1.56	0.02***	0.53	0.04***
Blautia	**2.26**	0.01***	7.06	0.29***	**5.24**	0.20***	**5.50**	0.89***
Oscillibacter	1.81	0.00***	0.76	0.10***	0.82	0.02***	0.14	0.15
Roseburia	1.37	0.04***	1.26	0.17***	0.65	0.34*	3.71	0.36***
Clostridium cluster XVIII	1.14	0.22***	0.38	0.00***	0.08	0.00	0.07	0.00*
Dorea[a]	1.09	0.00***	0.32	0.00***	0.23	0.00**	0.35	0.00***
Clostridium cluster XIVa[a]	0.33	0.00***	0.09	0.00**	0.11	0.00*	0.16	0.00***
Coprococcus	0.11	0.01*	0.61	0.09***	1.79	0.04***	1.30	0.16***
Collinsella	**0.00**	0.00	**3.16**	0.13***	**2.93**	0.13***	**7.22**	0.30***
Sutterella	0.00	0.00	0.09	0.00**	1.45	0.00***	0.14	0.00**
Coriobacterium[a]	0.00	0.00	0.12	0.00**	0.03	0.00	0.05	0.00

*p-value <0.05; **p-value <0.01; ***p-value <0.001.

[a]Genera not detected by treating feacal samples with EMA before DNA extraction.

In bold, genera enriched after isoflavone ingestion, as compared to the basal sample.

study of microbial communities found in complex environments, such as an anaerobic digestion plants (Wagner et al., 2008) and water (Nocker et al., 2010). EMA treatment considerably altered the community sequence profile of the faecal microbiota, particularly that of the dominant taxa. This finding may help in revealing the conditions needed to ensure the preservation of faecal bacteria of interest, especially in the recovery and propagation of those that produce equol.

In conclusion, the methodology followed allowed the changes in faecal microbial communities caused by isoflavone supplementation to be monitored. Isoflavone consumption was associated with significant increases in members of the families *Coriobacteriaceae*, *Ruminococcaceae* and *Lachnospiraceae*, to which strains that metabolise isoflavone and produce equol are known to belong. These taxa might be of interest in the search for isoflavone-metabolizing microorganisms, including equol-producing bacteria, which, after proper testing and safety assessment could be used as probiotics by people who cannot naturally benefit from isoflavones.

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CAPÍTULO 3

Efecto de las isoflavonas de soja sobre las comunidades microbianas fecales en cultivos *in vitro*

En este tercer capítulo se presentan los resultados de los efectos *in vitro* de las isoflavonas sobre las poblaciones microbianas de heces y sus metabolitos. El objetivo del trabajo fue determinar si la evolución de las poblaciones intestinales tenía en este contexto un comportamiento similar al que habíamos observado *in vivo* en las muestras de heces. Para este estudio se puso a punto un modelo de cultivos fecales en anaerobiosis utilizando un medio rico no selectivo, suplementado o no con isoflavonas de soja. Con este modelo se estudiaron, mediante secuenciación masiva de amplicones del ADNr 16S con la plataforma Illumina, los cambios en las comunidades bacterianas fecales y, por técnicas cromatográficas, los metabolitos bacterianos producidos durante las fermentaciones fecales.

Entre los resultados más destacados, observamos que la suplementación con isoflavonas produjo incrementos significativos de secuencias de microorganismos relacionados con la producción de AGCC como *Roseburia* spp., *Eubacterium hallii*, *Blautia obeum* y *Faecalibacterium prausnitzii*; se observó también un incremento en los cultivos de microorganismos relacionados con el metabolismo de compuestos fenólicos y/o la producción de equol (cuando este compuesto se producía) que pertenecían a los géneros *Dorea*, *Finegoldia* y *Collinsella*. Además, la concentración de AGCC fue superior en los cultivos fecales con isoflavonas inoculados con heces de mujeres con un fenotipo productor del equol.

El trabajo de este Capítulo se corresponde con el Subobjetivo 1.3.: Relacionar los tipos microbianos intestinales influenciados por las isoflavonas y los metabolitos derivados producidos, y los resultados se muestran en el siguiente artículo:

- **Artículo 5: Guadamuro, L.**, Dohrmann, A. B., Tebbe, C. C., Mayo, B., & Delgado, S. (2017). Bacterial communities and metabolic activity of faecal cultures from equol producer and non-producer menopausal women under treatment with soy isoflavones. *BMC Microbiology*, *17*, 93.

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RESEARCH ARTICLE Open Access

Bacterial communities and metabolic activity of faecal cultures from equol producer and non-producer menopausal women under treatment with soy isoflavones

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Abstract

Background: Isoflavones are polyphenols with estrogenic activity found mainly in soy and soy-derived products that need to be metabolised in the intestine by the gut bacteria to be fully active. There is little knowledge about isoflavone bioconversion and equol production in the human intestine. In this work, we developed an in vitro anaerobic culture model based on faecal slurries to assess the impact of isoflavone supplementation on the overall intestinal bacterial composition changes and associated metabolic transformations.

Results: In the faecal anaerobic batch cultures of this study bioconversion of isoflavones into equol was possible, suggesting the presence of viable equol-producing bacterial taxa within the faeces of menopausal women with an equol producer phenotype. The application of high-throughput DNA sequencing of 16S rRNA gene amplicons revealed the composition of the faecal cultures to be modified by the addition of isoflavones, with enrichment of some bacterial gut members associated with the metabolism of phenolics and/or equol production, such as *Collinsella*, *Faecalibacterium* and members of the *Clostridium* clusters IV and XIVa. In addition, the concentration of short-chain fatty acids (SCFAs) detected in the isoflavone-containing faecal cultures was higher in those inoculated with faecal slurries from equol-producing women.

Conclusions: This study constitutes the first step in the development of a faecal culturing system with isoflavones that would further allow the selection and isolation of intestinal bacterial types able to metabolize these compounds and produce equol in vitro. Although limited by the low number of faecal cultures analysed and the inter-individual bacterial diversity, the in vitro results obtained in this work tend to indicate that soy isoflavones might provide an alternative energy source for the increase of equol-producing taxa and enhancement of SCFAs production. SCFAs and equol are both considered pivotal bacterial metabolites in the triggering of intestinal health-related beneficial effects.

Keywords: Faecal slurry cultures, Faecal fermentation, Isoflavone metabolism, Equol, Intestinal microbiota, High-throughput sequencing

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Background

Isoflavones are polyphenols with estrogenic activity found at relatively high concentration in soy and soy-derived products. Epidemiological studies suggest high intakes of isoflavones to be associated with fewer and less intense menopause symptoms, and a reduced incidence of hormone-dependent and aging-associated diseases such as osteoporosis, cardiovascular diseases and cancer [1]. Although there is a growing body of scientific evidence showing beneficial effects in counteracting symptoms such as hot flushes and vasomotor reactions in menopausal women [2], the European Food Safety Authority (EFSA) has recently refuted health claims about the role of isoflavones in body functions [3]. This may be so because the functionality of isoflavones depends on their bioavailability and their conversion into (more) active metabolites within the intestinal tract. In nature, isoflavones are mostly (>80%) conjugated with sugars as isoflavone-glycosides (daidzin, genistin, glycitin) with low availability and bioactivity [4]. For full activity, aglycones (daidzein, genistein, glycitein) need to be released from their glycosides and, occasionally, metabolized to more potent metabolites, such as equol (produced from daidzein). Equol is the isoflavone-derived compound with the strongest estrogenic activity and antioxidant capacity, mechanisms by which isoflavones seem to provide the purported beneficial health effects [5]. The conversion of isoflavones into equol appears to be mainly carried out by intestinal microorganisms, especially bacteria inhabiting the distal portion of the gut [6]. Both, traditional and molecular methods have revealed marked individual diversity in intestinal bacterial communities. Such differences in the bacterial community composition may, in fact, influence the fate of isoflavone metabolic transformations [7, 8], which could ultimately contribute to differences in the physiological response to isoflavone treatment.

In that sense, only 30–50% of Western individuals produce equol [5], and only these might fully benefit from the positive health effects of isoflavone consumption. The metabolism of isoflavones involves several steps mediated by enzymes provided by different bacterial taxa [7, 8]. Even though scientific information is accumulating on the microorganisms producing equol, their enzymes and the metabolic routes involved, our current knowledge is still limited [6, 9, 10]. Most of the equol-producing bacteria characterized so far belong to the family *Coriobacteriaceae* [7]. However, it is not yet clear whether this family is the only intestinal group acting on isoflavones and producing equol.

Previously, the metabolism of daidzein by faecal bacterial consortia has been microbiologically characterized by conventional culturing methods [11–13]. The availability of high-throughput DNA sequencing techniques opens new potentials to tracking changes in the bacterial communities during isoflavone supplementation in both in vitro and in vivo systems. A better knowledge about the identity and individual variability of the intestinal bacteria that metabolize isoflavones and convert them into equol, would provide an important step for developing strategies to increase bioavailability and concentration of active compounds, e.g. by supplying suitable equol-producing probiotic bacteria.

In this study, anaerobic batch cultures of faecal samples from equol producer and non-producer menopausal women (as determined by urine equol excretion of >1000 nM) under treatment with soy isoflavones were performed, using media with and without isoflavones, to examine which bacteria take benefit from soy isoflavones and could be involved in equol production. PCR- denaturing gradient gel electrophoresis (DGGE) and high-throughput DNA sequencing were used to determine whether faecal cultures grown in the presence of isoflavones showed any change in their bacterial community composition and/or structure. These molecular analyses, based on PCR-amplified partial 16S rRNA gene sequences, were complemented by metabolic profiling of the culture supernatants using ultra-high-performance liquid chromatography (UHPLC) and gas chromatography (GC). This approach allowed the suggestion of links between structural responses in bacterial community compositions to metabolic activities.

Methods

Stool samples from isoflavone-treated menopausal women

Menopausal women who had been receiving treatment for 6 months with 80 mg/day of an isoflavone concentrate (Fisiogen; Zambon, Bresso, Italy) were recruited. Stool samples were obtained from four women whose faecal microbiota had been characterized and whose equol producer status during isoflavone treatment had been determined in a previous study [14]. Three of the women (WC, WG and WP) were equol producers (urine equol >1000 nM as defined by Rowland et al. [15]), while the fourth (WE) was a non-producer (<10 nM in urine). Faeces were collected and transported to the laboratory as previously described [14].

Faecal batch cultures

Ten-fold faecal dilutions were prepared by homogenizing 1 g of faeces in 9 ml of a pre-reduced phosphate buffer saline solution (PBS) under strict anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) in a Whitley DG500 Workstation anaerobic chamber. A 10% (*v*/*v*) aliquot of the resulting faecal slurry was used to inoculate the medium for colonic bacteria (MCB) described by van der Meulen et al. [16], modified to have a lower glucose content (2 g/l) (hereafter referred to as mMCB). This medium was prepared without and with a concentration of 160 mg/l commercial

isoflavone supplement (Fisiogen) by dissolving two capsules per liter of medium. One capsule of Fisiogen contains, as stated in the package, 80 mg of isoflavone of which 55–72% is genistin/genistein and 28–45% other soy isoflavones. It also includes as excipients E341, E460, E468, E551, E472, E464, E904, E171, and E124. The isoflavone-containing medium is hereafter referred to as $mMCB_{ISO}$. These faecal primary cultures thus prepared were incubated at 37 °C in fermentation flasks with mild stirring (250 rpm) for 24 h under anaerobic conditions. To find out whether production of equol in vitro was maintained in successive subculturings, primary cultures in $mMCB_{ISO}$ were used as inocula (10% *v*/*v*) for a second round of cultivation in fresh $mMCB_{ISO}$. These new cultures were incubated as above and referred to as secondary cultures.

Positive controls for isoflavone conversion and equol production were obtained by inoculating (at 10%) $mMCB_{ISO}$ with the strains *Slackia isoflavoniconvertens* DSM 22006 or *Slackia equolifaciens* DSM 24851. Both these strains were grown under anoxic conditions in Gifu Anaerobic Medium (GAM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.5% arginine (Merck, Darmstadt, Germany). After overnight culturing, cell of the two strains were washed twice in pre-reduced PBS before inoculating the media (mMCB and $mMCB_{ISO}$) or the faecal cultures and incubated as above.

Detection and quantification of equol and its isoflavone precursors

Equol and its isoflavone precursors [daidzin (daidzein-7-O-glucoside) and daidzein] were measured using a UHPLC procedure based on a method for equol determination in urine [17]. Briefly, after 24 h incubation, faecal cultures were centrifuged at 800 rpm for 10 min, duplicate samples of 3 ml of each supernatant collected, and their isoflavone content extracted using Bond Elute-C18 solid-phase cartridges (Agilent Technologies, Santa Clara, CA, USA). After drying, organic extracts were dissolved in 100 μl of HPLC grade methanol, and 1 μl of each sample injected into the UHPLC apparatus (Waters, Palo Alto, CA, USA). Samples were analysed in duplicate. Equol was quantified using a fluorescence detector (excitation 280 nm, emission 310 nm), while daidzin and daidzein were identified with a photodiode array (PDA) detector by comparison with their retention times and spectral characteristics at 260 nm. Quantification was performed against calibration curves prepared using commercial standards (all from Sigma-Aldrich, St. Louis, MO, USA).

Short chain fatty acids determination

Short chain fatty acids (SCFAs) in the primary and secondary cultures were determined by GC. Culture supernatants were centrifuged for 10 min at 800 rpm, and then for 10 min further at 13,200 rpm, before filtering through 0.45 μm PTFE filters. The filtered samples were then mixed 10:1 (*v*/*v*) with 1 mg/ml of 2-ethyl butyric acid (Sigma-Aldrich) dissolved in methanol as an internal standard. A chromatographic system composed of 6890 N GC apparatus (Agilent Technologies) connected to a flame ionization detector was used to identify and quantify the SCFAs as described elsewhere [18]. All samples were analysed in triplicate. The Shapiro–Wilk test was initially used to check for the normal distribution of the data. Since the data were not normally distributed, we used the non-parametric Wilcoxon signed-rank test to examine the differences in the content of SCFAs between the faecal cultures with ($mMCB_{ISO}$) and without isoflavones (mMCB). This statistic test is used to assess differences between related groups of samples and in our case the same faecal cultures were analysed under two different conditions (mMCB versus $mMCB_{ISO}$). All these calculations were performed using the SPSS software v.22.0.

Microbial community analysis

DNA extraction

Total bacterial DNA was extracted from the faecal primary and secondary cultures using the phenol-chloroform-based protocol of Zoetendal et al. [19] with modifications as described elsewhere [20]. The main modification consisted in the addition of an enzymatic lysis step before mechanical disruption in a FastPrep FP120 apparatus (Qbiogene, Carlsbad, CA, USA). After precipitation and drying, DNA samples were suspended in 100 μl of sterile molecular biology grade water (Sigma-Aldrich).

PCR-denaturing gradient gel electrophoresis (DGGE) analysis

Total DNA from the faecal cultures with and without isoflavones was used as a template for amplification of the variable region V3 of the 16S rRNA gene by PCR using the universal prokaryotic primers F357-GC and R518 [21]. DGGE electrophoresis was performed as described elsewhere [14]. DNA from *S. equolifaciens* and *S. isoflavoniconvertens* was used to provide DGGE markers. Genomic DNA from these strains was obtained from overnight cultures in GAM + 0.5% arginine using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) and subsequently employed in PCR amplifications. Amplicons were purified using GenElute PCR Clean-Up columns (Sigma-Aldrich) and mixed in equal amounts to obtain a DGGE marker.

High-throughput DNA sequencing and analysis of 16S rRNA gene amplicons

DNA samples obtained from the primary and secondary cultures were subjected to PCR amplification of the variable region V4-V5 of the 16S rRNA gene using the universal prokaryotic primers 515F and 909R [22]; these were

designed to include Illumina adapters. Double indexed amplicons were generated by the protocol of Caporaso et al. [23] with minor modifications. In brief, DNA from the samples was extracted in duplicate and amplified in triplicate; in total, 72 amplification reactions were obtained. These were subsequently paired-end sequenced in an Illumina Miseq System (Illumina, San Diego, CA, USA) by the StarSeq Company (Mainz, Germany), and treated as independent replicates.

Bioinformatic analysis was performed using the Mothur software package (v.1.34.1) following the MiSeq Standard Operating Procedure (SOP) [24]. Briefly, sequences longer than 380 bp in length, or shorter than 370 bp, and those containing ambiguous base pairs or homopolymers regions of >8 bp were removed. All other sequences were aligned using the SINA alignment service of the SILVA 16S rRNA sequence database. Chimera removal was performed using the UCHIME algorithm [25]. A random subset of 35,000 sequences per sample was used to balance numbers of reads among samples. Sequences were then clustered into operational taxonomic units (OTUs) using a 0.03 dissimilarity cut-off. Sequences were taxonomically classified using the Ribosomal Database Project (RDP) database. The Bayesian classifier with an 80% confidence threshold was used in the taxonomic assignment with the genus level as the lowest taxonomic unit considered. The MOTHUR program was also used to perform weighted UniFrac analysis, which was employed to assess the similarity of the microbial communities between samples. Construction of a heatmap was performed using the R statistical software. Clustering was accomplished using the complete linkage method with Euclidean distance measure. Multivariable statistical analysis was performed by principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) with two dimensions. Differences in the microbial composition of faecal cultures with and without isoflavones were sought by analysis of molecular variance (AMOVA) and analysis of similarities (ANOSIM). The identification of differentially abundant taxa was assessed using the Metastats software [26]. Multiple hypothesis tests were adjusted using the false discovery rate (FDR) correction [27]; an FDR q-value threshold of 0.25 was used to identify significant differences. In an attempt to assign the differential OTUs at the species level, manual sequence comparisons were performed against the Greengenes 16S rRNA gene database.

Results

Isoflavone conversion and equol production

The uninoculated medium with isoflavones, $mMCB_{ISO,}$ was shown by UHPLC to contain three times more conjugated precursor daidzin that its corresponding aglycone daidzein (Table 1). As expected, equol was never detected in the absence of isoflavones in the cultures. In the medium inoculated with *S. equolifaciens* DSM 24851, used as a positive control for equol production, daidzin levels were maintained while daidzein was transformed and converted to equol, reaching 2 μg/ml after 24 h incubation. In the primary faecal cultures from the three equol producers (WC, WG and WP) comparable amounts of equol to that produced by the positive control were recorded (1.40–3.07 μg/ml). Congruently, daidzin levels were drastically reduced in these cultures, indicating glycosidase activity from faecal bacteria towards this isoflavone glycoside. In the primary culture from the non-producing woman (WE), daidzin was also shown to decrease while daidzein accumulated, but no equol was found (limit of quantification 0.002 μg/ml). As expected, in the secondary culture from this woman equol was not detected either. In contrast, equol production was maintained to some extent in the secondary cultures from two of the three equol producers (WG and WP).

SCFA production

Comparing the results obtained in the mMCB medium, total SCFA production was greater in the faecal cultures containing isoflavones ($mMCB_{ISO}$) (Fig. 1). The concentrations of the major SCFAs (acetic, propionic and butyric acids) were particularly enhanced in the primary faecal cultures whose inocula came from the equol-producing women (Table 2); significant increases were seen for propionic acid and isobutyric and isovaleric acids (the latter two are minority, branched chain fatty acids [BCFAs]) ($p < 0.05$). In contrast, in the cultures from the equol non-producing woman (WE), no differences between the corresponding $mMCB_{ISO}$ and mMCB were seen for major or minor SCFAs in the primary cultures. Consequently, the acetic/propionic ratio for this faecal culture was five times higher than that recorded for the equol producers; indeed, the concentration of propionic and butyric acids for this culture were much lower than those recorded in the primary cultures of the equol-producing women. SCFAs production in the secondary cultures from these women was maintained to some extent, but with a major reduction in the concentration of isovaleric acid.

Bacterial community analysis by PCR-DGGE

The structural diversity of the bacterial communities in the primary faecal cultures was initially investigated by PCR-DGGE. In samples from the $mMCB_{ISO}$ medium inoculated with *S. equolifaciens* or *S. isoflavoniconvertens*, faint bands corresponding to the expected size for these species were appreciated (Additional file 1). Clear differences were seen in the bacterial DGGE patterns of the cultures for the different women. However, no changes were observed in the profiles for the mMCB and $mMCB_{ISO}$ faecal cultures from the same woman. This suggested to us

Table 1 Isoflavone levels (μg/ml) in faecal cultures from equol-producing and non-producing women in $mMCB_{ISO}$ medium

Sample	Isoflavone	Primary cultures	Secondary cultures
WC[a]	Daidzin	0.24 ± 0.09	24.69 ± 5.10
	Daidzein	1.74 ± 0.07	under LOQ[c]
	Equol	2.62 ± 0.04	under LOQ
WG[a]	Daidzin	0.05 ± 0.02	0.12 ± 0.06
	Daidzein	under LOQ	under LOQ
	Equol	3.07 ± 0.24	7.67 ± 0.64
WP[a]	Daidzin	0.09 ± 0.04	0.18 ± 0.07
	Daidzein	0.02 ± 0.01	under LOQ
	Equol	1.40 ± 0.06	4.02 ± 0.35
WE[b]	Daidzin	under LOQ	under LOQ
	Daidzein	24.10 ± 3.32	45.57 ± 5.46
	Equol	under LOQ	under LOQ
Uninoculated culture media	Daidzin	16.83 ± 2.89	N.A.
	Daidzein	5.57 ± 1.19	N.A.
	Equol	under LOQ	N.A.
Slackia equolifaciens	Daidzin	16.53 ± 3.68	N.A.
	Daidzein	0.06 ± 0.05	N.A.
	Equol	2.01 ± 0.10	N.A.

N.A. not applicable, *$mMCB_{ISO}$* modified medium for colonic bacteria supplemented with isoflavones
[a]equol-producing woman
[b]equol non-producing woman
[c]*LOQ* Limit of quantification (0.002 μg/ml for equol; 0.004 μg/ml for daidzein and 0.014 for daidzin)

that the presence of isoflavones did not affect under the experimental culture conditions employed the dominant bacterial populations. Therefore, the bacterial communities of the secondary cultures were not further analysed using this technique.

Bacterial community analysis by high-throughput DNA sequencing

The application of the Illumina technology allowed obtaining an average of 111,542 high quality PCR-amplicon sequences for each replicate. The number of high quality sequences among all the replicates ranged between 71,138–150,292, and estimated sample coverage was considered to be good for all libraries. The mean number of quality sequences retrieved per faecal culture, together with the numbers of OTUs, as defined by ≥97% sequence identity, and α-diversity indexes calculated for each sample are summarized in Additional file 2. To allow further comparative analyses between samples, each 16S rRNA gene amplicon library was rarefied to 35,000 sequences per replicate. The rarefaction curves for the normalized sequences showed that the secondary culture for subject WC (which did not render equol in vitro) was the least diverse (Fig. 2). Further, the numbers of OTUs obtained in all cultures (primary culture in mMCB, primary culture in $mMCB_{ISO}$, and secondary culture in $mMCB_{ISO}$) from the equol non-producer subject WE were smaller (2000–2300 OTUs) than for all other cultures (3000–3500 OTUs) (Additional file 2). Compared to the faecal cultures in mMCB, the presence of isoflavones had no significant influence on the richness index Sobs (number of observed species) (Fig. 3). However, a significant reduction of the bacterial diversity was recorded for the secondary cultures ($p < 0.01$).

Sequences were then taxonomically assigned using the RDP classifier. A majority of sequences in all samples belonged to the phylum Firmicutes (mean 55%), while sequences from Bacteroidetes (27%), Actinobacteria (9%) and Proteobacteria (7%) phyla were less abundant. At this taxonomic range, only 0.1% of the sequences remained unclassified/unassigned. At the genus-level, between 52 and 75 different genera were identified within each faecal culture.

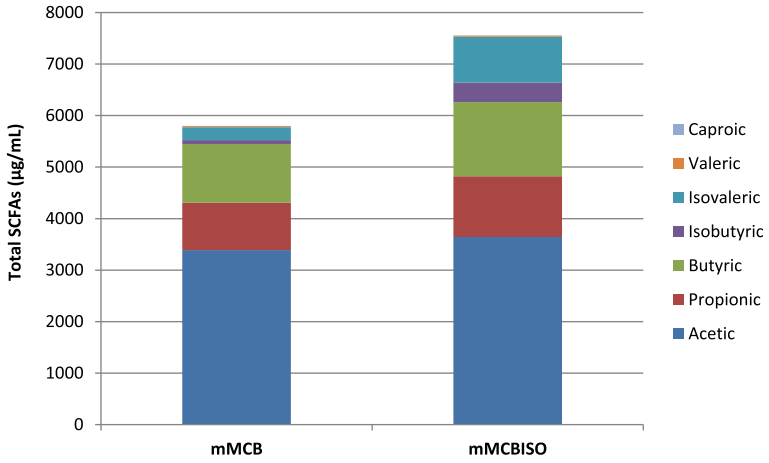

Fig. 1 Production of short chain fatty acids (SCFAs). Comparison of total SCFAs production by the primary faecal cultures in media without (mMCB) and with ($mMCB_{ISO}$) isoflavones

Table 2 Short chain fatty acid (SCFA) production (mean ± standard deviation) in faecal cultures

Equol status	Sample		SCFA (μg/ml)							
			Acetic	Propionic	Butyric	Acetic/Propionic	Isobutyric	Isovaleric	Valeric	Caproic
Producers (n = 3)	Primary cultures	mMCB	3630.78 ± 364.76	1204.29 ± 392.01	1420.04 ± 749.28	3.25 ± 0.88	97.18 ± 81.83	312.51 ± 208.9	24.52 ± 18.66	5.71 ± 5.29
		$mMCB_{ISO}$	4063.17 ± 322.93	1559.54 ± 325.14*	1859.97 ± 570.4	2.74 ± 0.76*	471.72 ± 5.28*	1114.00 ± 17.79*	27.11 ± 7.9	3.82 ± 1.37
	Secondary cultures	$mMCB_{ISO}$	3334.11 ± 655.9	1545.72 ± 632.46	1649.15 ± 189	2.34 ± 0.53	249.3 ± 223.82	613.10 ± 539.00	30.29 ± 3.97	2.67 ± 0.15
Non-producer (n = 1)	Primary cultures	mMCB	2743.95 ± 36.98	155.29 ± 2.89	404.61 ± 3.02	17.67 ± 0.13	6.66 ± 0.23	5.90 ± 0.19	8.74 ± 0.09	4.87 ± 0.02
		$mMCB_{ISO}$	2523.79 ± 25.44	167.85 ± 2.99	312.38 ± 4.2	15.04 ± 0.16	6.61 ± 0.07	5.61 ± 0.01	8.71 ± 0.26	4.71 ± 0.06
	Secondary cultures	$mMCB_{ISO}$	3537.98 ± 33.75	1246.96 ± 14.86	687.74 ± 11.19	2.84 ± 0.02	73.89 ± 1.31	407.66 ± 10.76	7.16 ± 0.12	2.26 ± 0.05
	Control media	mMCB	1000.31 ± 5.09	36.90 ± 0.15	19.52 ± 0.80	27.11 ± 0.25	4.30 ± 0.10	0.23 ± 0.00	0.00 ± 0.00	2.29 ± 0.04
		$mMCB_{ISO}$	1017.13 ± 22.67	36.63 ± 0.30	19.05 ± 0.39	27.77 ± 0.56	4.50 ± 0.10	0.32 ± 0.09	0.00 ± 0.00	2.41 ± 0.01

mMCB modified medium for colonic bacteria, $mMCB_{ISO}$ modified medium for colonic bacteria supplemented with isoflavones
Key of statistical significance: mMCB compared to $mMCB_{ISO}$: *$p < 0.05$

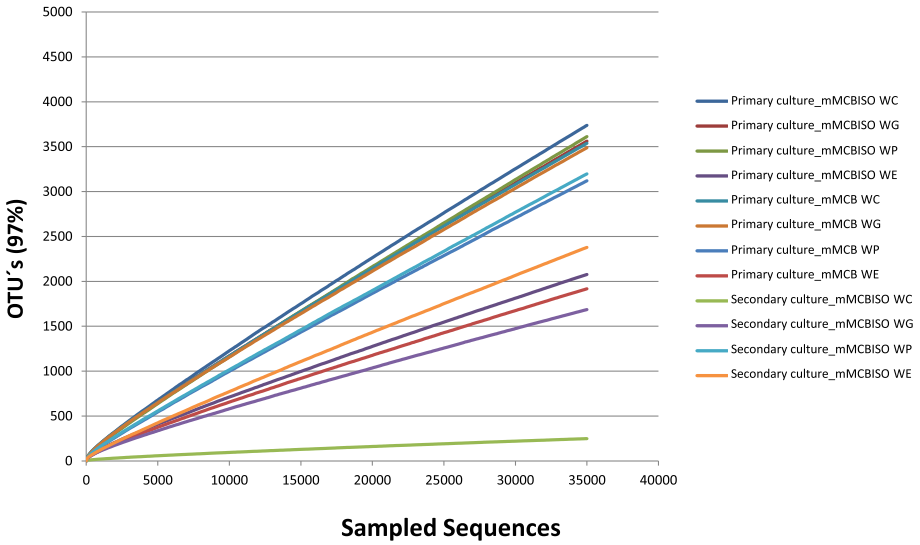

Fig. 2 Rarefaction curves calculated for each sample of the study. Rarefaction curves at a 97% similarity level of partial sequences of the bacterial 16S rRNA gene from primary and secondary faecal cultures in media with ($mMCB_{ISO}$) and without (mMCB) isoflavones

Among the dominant genera *Bacteroides* presented the highest percentage with a relative abundance between 10 and 20% of assigned reads; *Faecalibacterium* ranged between 5 and 10%; and sequences related to the *Clostridium* "sensu stricto" group also reached these percentages (5–10%), but only in the primary cultures from the equol non-producing woman (Fig. 4). Sequences assigned to the genus *Collinsella* also ranged within these abundances in the primary cultures from the equol producers.

Subdominant genera, varying in percentage from 1 to 5%, were represented by *Parabacteroides*, *Ruminococcus* and *Dorea*; the latter only in the primary cultures from equol producers. In agreement with the DGGE results, the bacterial composition profile obtained by 16S rDNA sequencing was quite similar for equivalent faecal cultures when grown with and without isoflavones (Fig. 4). Nonetheless, changes in subdominant, minor and assumingly rare genera were revealed by the high throughput DNA sequencing technique.

A heatmap depicting relative abundances across samples (including primary and secondary cultures) is shown in Fig. 5. In general, primary faecal cultures in mMCB and $mMCB_{ISO}$ from the same woman clustered together suggesting similar microbial profiles, as denoted previously by DGGE. On the other hand, the secondary cultures, with major changes in the bacterial communities' patterns, plotted in a separate branche.

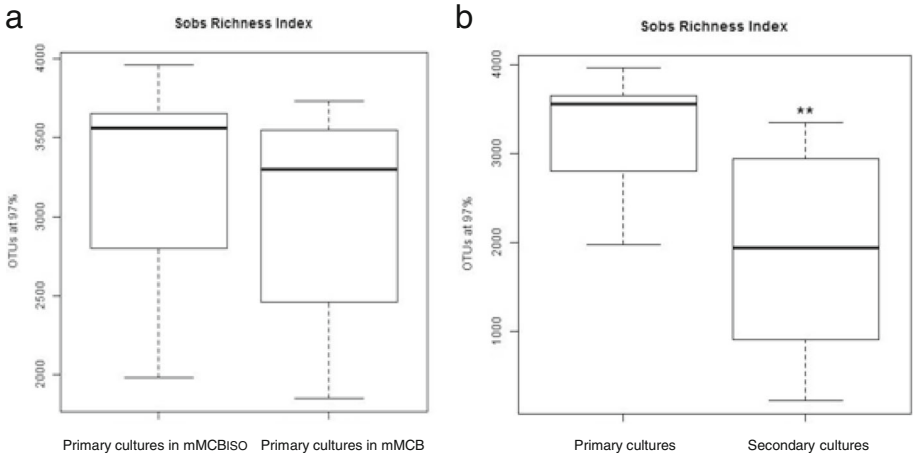

Fig. 3 Differences in richness indices between faecal cultures. Comparison of Sobs, number of observed species, for the 16S rRNA gene sequences at 3% of divergence distance of: **a**) primary faecal cultures in media with ($mMCB_{ISO}$) and without (mMCB) isoflavones, and **b**) primary and secondary cultures in media with isoflavones. *Asterisks* denoted statistically significant differences (Welch's test, p value < 0.01)

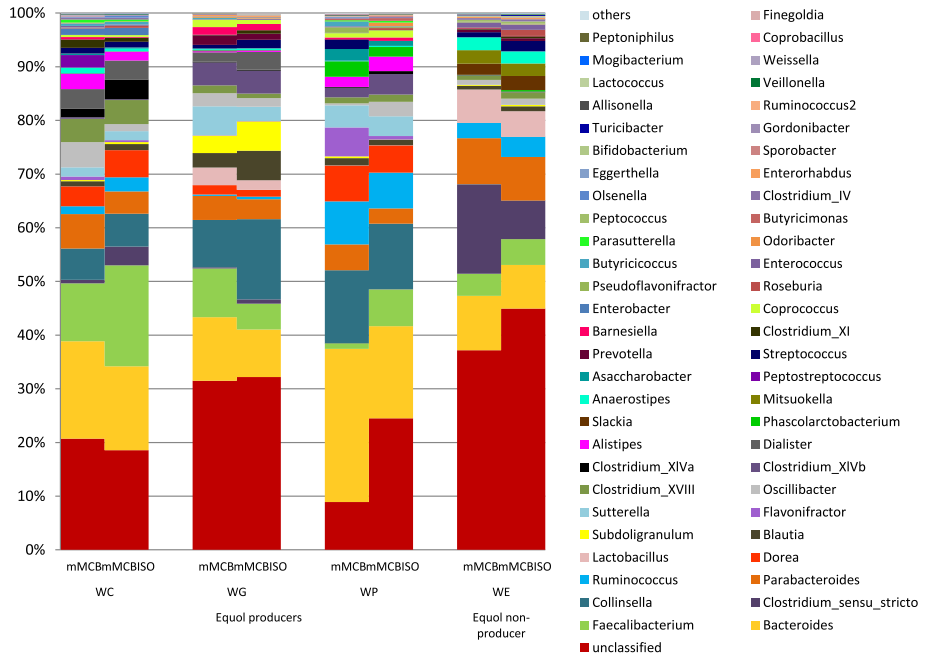

Fig. 4 Bacterial composition at the genus level of primary faecal cultures. WC, WG and WP are equol producers meanwhile WE is an equol non-producer. Composition is represented as the mean relative abundance of the six replicates sequenced. Only genera contributing with a percentage > 0.05% of the total abundance in a least one sample are shown

The application of the Metastats method (FDR correction 0.25) revealed that the presence of isoflavones in the culture medium altered the abundance at several taxonomic hierarchies. For example, the phylum Bacteroidetes was less represented in the presence of isoflavones (p = 0.001), in particular the family *Bacteroidaceae* with a decrease of reads from the genera *Bacteroides* and *Parabacteroides* (Additional file 3). On the contrary, the representation of the family *Ruminococcaceae* significantly increased with isoflavones (p = 0.011). At the genus level, increases were recorded for *Roseburia* and *Odoribacter* sequences. Reads of these two genera were still significantly more abundant when excluding from analysis the faecal culture from the equol non-producing woman (WE). If omitting samples from this woman, and by comparing faecal primary cultures with secondary cultures in the presence of isoflavones, two genera belonging to the family *Coriobacteriaceae* (capable of synthesizing equol) were significantly increased, *Slackia* (p = 0.019) and *Eggerthella* (p = 0.030). Comparison of the microbial communities in the $mMCB_{ISO}$ primary cultures for equol producers with that of the non-producer revealed a significant increase in the number of sequences belonging to 28 different genera (Additional file 4). The same number of genera showed an increased in relative abundance when comparing secondary cultures producing equol with those that did not (Additional file 5). Fifteen of the 28 genera (*Collinsella*, *Faecalibacterium*, *Dorea*, *Suterella*, *Clostridium* (groups XIVa and XIVb), *Alistipes*, *Oscillibacter*, *Barnesiella*, *Coprococcus*, *Finegoldia*, *Butyricicoccus*, *Asaccharobacter*, *Murdochiella*, *Allisonella* and *Odoribacter*) were found in both the primary and their derived secondary cultures, suggesting these groups to be more abundant in cultures yielding equol. Of note was the appearance of *Collinsella* (a member of the family *Coriobacteriaceae* not described as equol producer), which accounted for 6–15% of sequences in cultures that produced equol, while reads for this genus in isoflavone-containing cultures that did not yield equol was less than 0.04%. Furthermore, reads of the genus *Asaccharobacter*, a member of the same family harbouring equol-producing strains, increased significantly in all cultures producing equol.

To visualize the overall differences between the bacterial community structures, multivariate statistical analyses, i.e., NMDS and PCoA were applied. This revealed that the bacterial communities of the women's faecal primary and secondary cultures clustered together, with the exception of that of the secondary culture for woman WC (which, as stated above, showed a reduced diversity and did not produce equol) (Fig. 6). The cluster for the microbiota of this latter sample and those for the primary/secondary cultures for the equol non-producing subject (WE), lay at an appreciable distance from the cultures derived from equol producers (stress value 0.206 in NMDS). Further, the bacterial communities of the $mMCB_{ISO}$ cultures for the equol- producing women showed more similarity to one another than to those grown in mMCB. AMOVA revealed the clustering of the

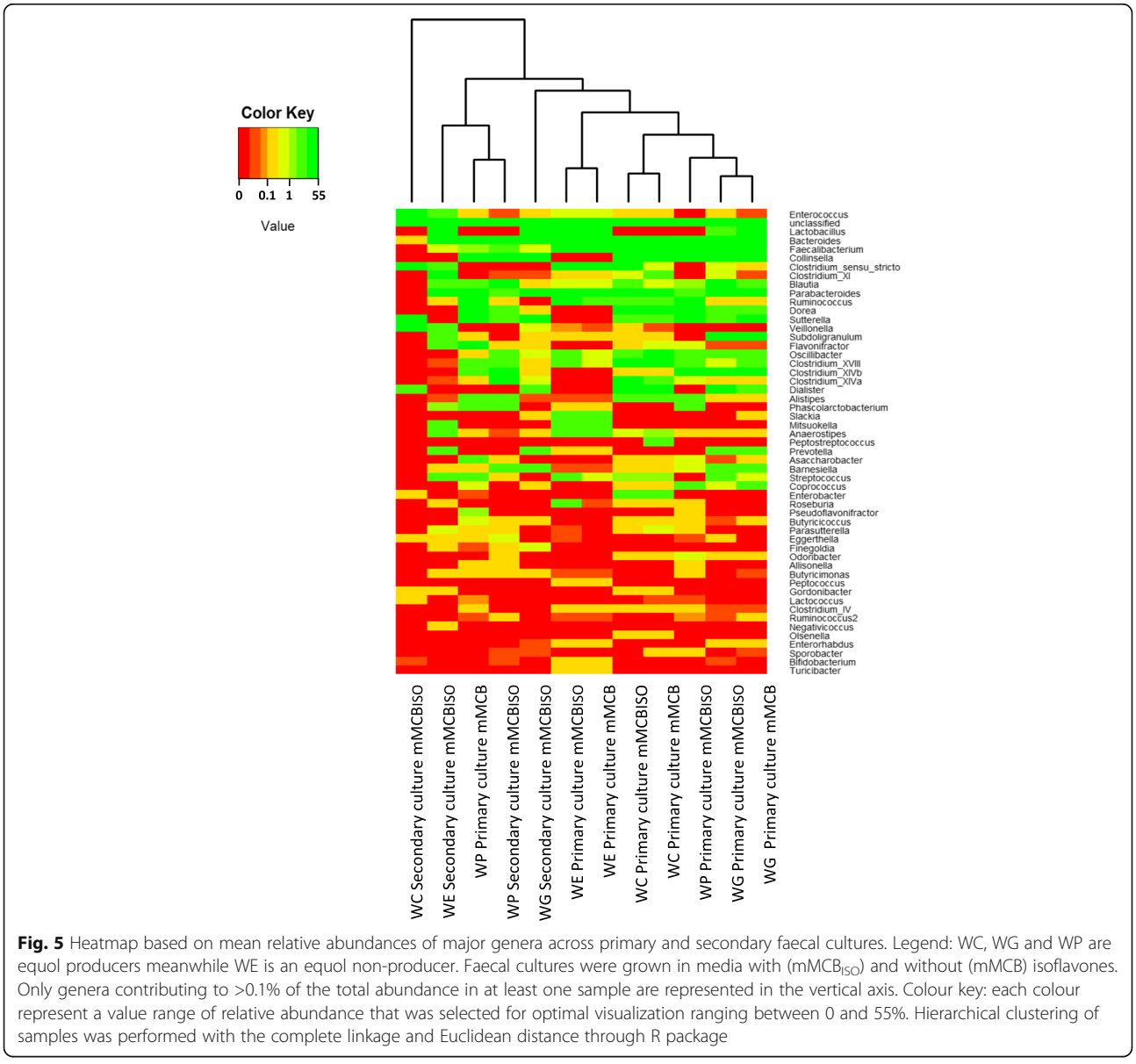

Fig. 5 Heatmap based on mean relative abundances of major genera across primary and secondary faecal cultures. Legend: WC, WG and WP are equol producers meanwhile WE is an equol non-producer. Faecal cultures were grown in media with ($mMCB_{ISO}$) and without (mMCB) isoflavones. Only genera contributing to >0.1% of the total abundance in at least one sample are represented in the vertical axis. Colour key: each colour represent a value range of relative abundance that was selected for optimal visualization ranging between 0 and 55%. Hierarchical clustering of samples was performed with the complete linkage and Euclidean distance through R package

two types of samples (in $mMCB_{ISO}$ and mMCB) to be significantly different (p = 0.036).

Finally, OTUs significantly different in abundance between primary faecal cultures grown in in $mMCB_{ISO}$ and in mMCB were manually assigned to the species level (sequence identity higher that 97%). In cultures grown with isoflavones, OTUs belonging to *Faecalibacterium prausnitzii, Eubacterium halli, Subdoligranulum variabile, Ruminococcus flavefaciens/callidus, Blautia obeum* and *Bacteroides xylanisolvens/ovatus* were increased (Additional file 6).

Discussion

In this work, the bacterial diversity and metabolic changes in cultures of faecal samples from isoflavone-treated menopausal women were investigated. For that purpose, an in vitro anaerobic batch culture system in a modified medium for colonic bacteria supplemented or not with isoflavones was developed. Production of equol was determined with a recently described UHPLC method [17] and verified by inoculating the isoflavone-containing medium with *S. equolifaciens*, an equol-producing bacterium. Similar levels of equol production to those obtained with this control strain were recorded in the primary faecal cultures for women with an equol producer phenotype (as determined previously by equol urinary excretion [14]) - but not in the faecal cultures from a non-equol producer. This strongly indicates differences among the faecal samples in types and/or numbers of intestinal bacteria with capacity to produce equol. The stability of isoflavone bioconversion into equol was further tested by making subcultures of the

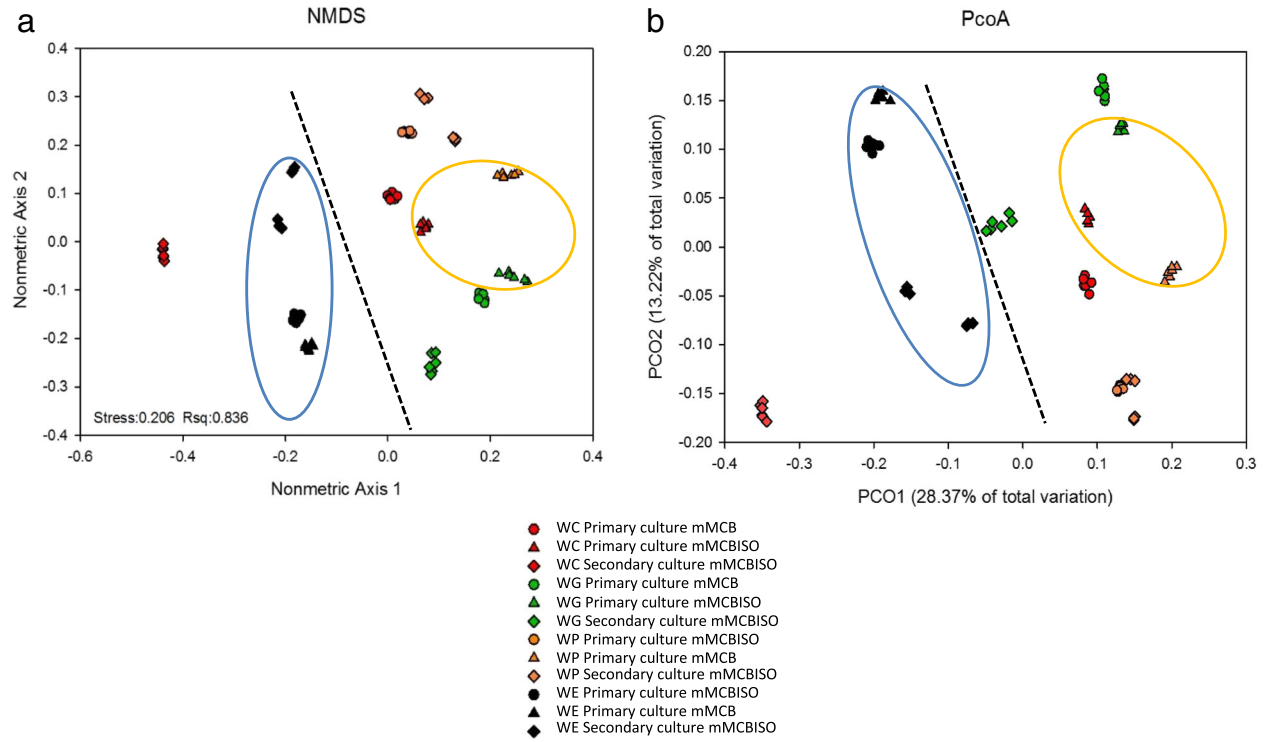

Fig. 6 Clustering of operational taxonomic units (OTUs) of primary and secondary cultures. Legend: Non-metric multidimensional scaling (NMDS) (**a**), and principal coordinates analysis (PCoA) (**b**) showing clustering of the OTUs of the faecal cultures in this study. The *blue circle* embraces samples from the equol non-producing woman; the *yellow circle* includes primary cultures in medium with isoflavones from the equol-producing women; the *dotted line* separates samples that did not render equol

faecal primary cultures in fresh media. In the presence of isoflavones, equol production was maintained in all except one of these secondary cultures, suggesting that, under the experimental conditions, bacterial types capable of metabolizing isoflavones and producing equol maintain viability and the equol-producing capability, which opens possibilities for a rational propagation and selection of these bacteria. It should be noted that with the UHPLC methodology used in this study other daidzein-derived metabolites that could be formed in the faecal cultures, such as hydroxydadidzein (DHD) and *O*-desmethylangolensin (*O*-DMA), might have passed undetected.

In our study, the bacterial composition of the faecal primary and secondary cultures was characterized by two culture-independent methods. PCR-DGGE analysis revealed no major changes in the bacterial communities between the primary cultures with and without isoflavones. This would suggest that the dominant gut populations are a priori neither involved in the metabolism of isoflavones nor influenced by the presence of these compounds. However, the 16S rDNA sequencing data revealed significant changes in different taxa in the presence of isoflavones, particularly with respect to minority groups, which cannot be tracked by the DGGE technique. Although the diversity observed in the faecal cultures was similar in the media with and without isoflavones, a larger number of OTUs was scored in samples yielding equol. This suggests a more diverse bacterial community coming from the faeces of women with an equol producer status.

Collinsella was one of the genera that increased in abundance considerably, both in the primary and secondary cultures that produced equol. This genus belongs to *Coriobacteriaceae*, a family that harbours most of the currently described equol-producing bacteria [7]. Increases in the number of *Collinsella* sequences in response to isoflavone treatment have recently been reported in vivo [28]. Although to a lesser extent, the number of reads associated with the genus *Asaccharobacter* also increased significantly in the faecal cultures producing equol. This agrees with the fact that the single species of this genus, *Asaccharobacter celatus*, has been reported to be an equol producer [29]. Other intestinal bacteria recently associated with isoflavone metabolism in humans, such as *Dorea* and *Finegoldia* [28, 30], were also significantly increased in all cultures producing equol. In agreement with the present results, isoflavones have further been described to stimulate in vivo majority microorganisms of the *Clostridium* clusters XIVa and IV, to the latter of which *F. prausnitzii* belongs [31, 14]. On the other hand, like other polyphenols, isoflavones may

have antimicrobial activity, which could modulate the diversity and composition of the faecal bacterial communities [10]. In this work, addition of isoflavones to the faecal cultures drove to a decrease of some taxa. In particular, the genera *Bacteroides* and *Parabacteroides* reduced their numbers in the presence of these compounds.

Papers reporting production of equol in slurry cultures inoculated with faeces from equol producing subjects have been published previously [12, 13, 30]. However, as far as we are aware, this is the first study that making use of a soy isoflavone extract (commercial formulation) added directly to a colonic culturing medium, describes in deep the bacterial changes and response to isoflavone enrichment by high-throughput 16S DNA sequencing. Although this, the presence in the supplement of other minor components apart from isoflavones (excipients) that might influence the faecal bacterial communities cannot be completely ruled out.

Production of SCFAs (relevant gut bacterial metabolites) was determined in the batch faecal cultures in relation with the addition of isoflavones and the production of equol. SCFAs production in the colon results from bacterial fermentation of dietary starches, fibre and sugars [32]. The SCFAs acetic (C2), propionic (C3) and butyric (C4) acids are the main fermentation end-products in the gut, and are thought to have anti-inflammatory and anti-carcinogenic activities [33]. In contrast, BCFAs, such as isobutyric and isovaleric acids, often associated with protein breakdown, have been less studied. The present data reveal changes in SCFA production in the presence of isoflavones. The increased quantities of the main SCFAs (acetic, propionic and butyric) and BCFAs in the faecal cultures with isoflavones indicates microbial activity leading to the production of these compounds. The soy isoflavone-glycosides can be initially deconjugated by endogenous intestinal bacteria with hydrolytic activity (probably β-glucosidases), as it was observed in this study with the reduction in the daidzin levels after cultivation. This activity releases isoflavone aglycones [34], but also sugars that may provide an alternative energy source to the cultures. This would agree with some studies involving prebiotic-related compounds such as beta-glucan or cellobiose, which have been shown to increase BCFAs production [35, 36]. At the genus level, *Roseburia* and *Odoribacter* sequences were both significantly increased in $mMCB_{ISO}$ as compared to the medium without isoflavones. Reduction in members of these two genera has been suggested to enhance inflammation by decreasing SCFA availability [37, 38]. Although not statistically significant, butyrate production in this work was greater in the faecal cultures that produced equol. Butyrate-producing bacteria such as *Roseburia*, *E. hallii* (both from Clostridium cluster XIVa) and *F. prausnitzii* were more abundant when faecal cultures were incubated with isoflavones, particularly in those that produce equol. When significant OTUs were assigned manually at the species level other cellulolytic butyrate producers, such as *R. flavefaciens* and *S. variabile* (both of the *Clostridium* cluster IV) [37], and propionate producers from the *Clostridium* cluster XIVa, such as *B. obeum*, were also identified.

Cross-feeding mechanisms among the more diverse faecal microbiota in equol producers might further be partly responsible for the increased production of propionic and butyric acids in isoflavone-containing cultures. Acetate is the predominant SCFA produced by bacterial fermentation in the gut, and both, *F. prausnitzii* and *E. hallii* (the sequences of which became more abundant when cultures were grown in the presence of isoflavones) are well known acetate-utilizing, butyrate-producing species [33].

Finally, isoflavone metabolism might also be affected by the presence of SCFAs; i.e., butyric acid has been shown to enhance equol production by *A. celatus* [29], and an increase production of equol by a mixed microbial culture from human faeces in the presence of propionate and butyrate has also been reported [30].

Conclusions

Faecal cultures from women with an equol producer phenotype yielded equol in vitro in faecal fermentations with isoflavones, suggesting the presence in faeces of active bacterial types able to produce this compound. The application of high-throughput DNA sequencing of 16S rRNA gene PCR amplicons revealed the composition of the faecal bacterial communities to be modified by the presence of isoflavones, including increases in equol-producing taxa. Although limited by the low number of cultures, isoflavones seem also to promote, at least in vitro, SCFAs production. This increase might be due to the growth stimulation of specific SCFA-producing bacterial types from the *Ruminococaceae* (members of the *Clostridium* cluster IV) and *Lachnospiraceae* (*Clostridium* cluster XIVa) families.

Additional files

Additional file 1: Effect of isoflavones on dominant bacterial populations determined by DGGE. PCR-DGGE profiles of the primary faecal cultures from equol-producing women grown in modified medium for colonic bacteria supplemented ($mMCB_{ISO}$) or not (mMCB) with isoflavones; A) WC samples, B) WG samples. M: DGGE marker [comprising the species *Slackia isoflavoniconvertens* (1) and *Slackia equolifaciens* (2)]. (PPTX 164 kb)

Additional file 2: Summary of sequence processing. Number of quality reads, sample coverage, richness estimators and diversity indexes of 16S rDNA libraries of primary and secondary faecal cultures. (DOCX 20 kb)

Additional file 3: Effect of isoflavones in microbial abundance. Families and genera showing significant (p value <0.05) increases (grey) and decreases in their relative abundances (% sequences) when comparing primary faecal cultures in medium with and without isoflavones. (DOCX 16 kb)

Additional file 4: Differences in microbial genera associated with equol production in primary faecal cultures. Genera showing significant increases (p value <0.05) in their relative abundances (% sequences) in medium with isoflavones when comparing primary cultures from non-producer and equol producer women. (DOCX 16 kb)

Additional file 5: Differences in microbial genera associated with equol production in secondary faecal cultures. Genera showing significant increases (p value <0.05) in their relative abundances (% sequences) in medium with isoflavones when comparing secondary cultures that rendered equol production with those that did not. (DOCX 17 kb)

Additional file 6: Effect of isoflavones in microbial species abundance. Identification of OTUs (3% distance level) showing significant increases (p value <0.05) in their relative abundances (%) in primary faecal cultures in $mMCB_{ISO}$ as compared to that in mMCB. (DOCX 17 kb)

Abbreviations
AMOVA: Analysis of molecular variance; ANOSIM: Analysis of similarities; BCFAs: Branched chain fatty acids; DGGE: Denaturing gradient gel electrophoresis; EFSA: European Food Safety Authority; FDR: False discovery rate; GC: Gas chromatography; mMCB: modified medium for colonic bacteria; $mMCB_{ISO}$: modified medium for colonic bacteria supplemented with isoflavones; NMDS: Non-metric multidimensional scaling; OTUs: Operational taxonomic units; PBS: Phosphate buffered saline; PCoA: Principal coordinates analysis; PDA: Photodiode array; RDP: Ribosomal database project; SCFAs: Short-chain fatty acids; SOP: Standard operating procedure; UHPLC: Ultra-high-performance liquid chromatography

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Availability of data and materials
All relevant data are within the paper and its additional files. The raw sequencing data are deposited in the Sequence Read Archive (SRA) of the NCBI (http://www.ncbi.nlm.nih.gov) database under accession no. SRP064029.

Authors' contributions
SD and BM contributed with the conception and design of the study. BM and CCT provided material and human resources. LG and SD were involved in all steps of the faecal cultures determinations. CCT was in charge of the experimental design for the high-throughput sequencing. ABD participated in the analysis of PCR-generated 16S rRNA gene libraries and interpretation of the data. SD drafted the manuscript. BM and CCT performed a critical revision of the manuscript. All authors approved the final version of the article.

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

Consent for publication
Not applicable.

Ethics approval and consent to participate
Human participants providing stool samples for this research signed written informed consent. Approval was obtained from the Research Ethics Committee of the Asturias Principality, Spain (approval number: 15/2011).

Publisher's Note

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CAPÍTULO 4

Estudio y caracterización de microorganismos con actividad sobre las isoflavonas para la elaboración de alimentos funcionales a base de leche de soja

En este capítulo se aborda el objetivo de "identificar cepas bacterianas con buenas propiedades tecnológicas para la elaboración de productos fermentados a base de soja". Para ello es importante que presenten actividades enzimáticas adecuadas sobre las isoflavonas y den lugar a productos con buenas propiedades sensoriales. Para llevar a cabo el estudio se partió de una colección de BAL y bifidobacterias de las que se seleccionó un conjunto de 8 cepas de lactobacilos y 2 de bifidobacterias en función de su elevada actividad β-glucosidasa. Con ellas se ensayó la fermentación de dos bebidas de soja comerciales y se evaluó el crecimiento de las cepas y su actividad en este medio. Además, se estudiaron parámetros físico-químicos básicos y algunas características sensoriales y funcionales en las bebidas de soja fermentadas. Como fruto de este trabajo, se seleccionaron finalmente cuatro cepas capaces de desglicosilar los glicósidos de isoflavonas y liberar las agliconas tras su inoculación en los preparados de soja. Tres de las cepas eran lactobacilos capaces de crecer y acidificar las bebidas de soja y la cuarta fue una cepa de *Bifidobacterium pseudocatenulatum* que, aunque no presentaba la misma tasa de crecimiento que los lactobacilos, tenía gran actividad sobre las isoflavonas y era capaz de mantenerse viable en el producto fermentado. Todas ellas presentan un gran potencial para ser utilizadas como fermentos en la elaboración de alimentos funcionales a base de soja.

A continuación, con el objetivo de profundizar en el conocimiento de los mecanismos y actividades implicados en la liberación de agliconas de los glicósidos de isoflavona, se llevó a cabo la secuenciación del genoma de la bifidobacteria seleccionada: *B. pseudocatenulatum* IPLA 36007. En el genoma de esta cepa se encontraron al menos siete marcos de lectura abiertos (ORFs) con capacidad para codificar glicosil hidrolasas anotadas como β-glucosidasas. Cuatro de ellas, se sintetizaron *in vitro*, se clonaron en un sistema de sobreexpresión de *Escherichia coli* y las proteínas recombinantes se purificaron y caracterizaron. Se comprobó que todas ellas eran verdaderas β-glucosidasas con actividad sobre daidzina y genistina, si bien

presentaban diferencias en actividad a distintos pHs y temperaturas, lo que sugiere que en condiciones fisiológicas las enzimas pueden tener actividades complementarias.

Los resultados expuestos en este Capítulo están dirigidos a la consecución del segundo objetivo general de la tesis y pretenden alcanzar los distintos subobjetivos que lo integran: Subobjetivos 2.1.: Caracterizar microorganismos que lleven a cabo la hidrólisis y transformación de las isoflavonas para su utilización en la elaboración de alimentos funcionales a base de soja y 2.2.: Estudiar los mecanismos microbianos involucrados en la desglicosilación de los glicósidos de isoflavonas. Los resultados se reportan en tres artículos, tal como se relaciona a continuación:

- **Artículo 6:** Fermentation of commercial soy beverages with lactobacilli and bifidobacteria strains featuring high β-glucosidase activity (Manuscrito en preparación)
- **Artículo 7:** Alegría, Á., Delgado, S., **Guadamuro, L.**, Flórez, A. B., Felis, G. E., Torriani, S., & Mayo, B. (2014). The genome of *Bifidobacterium pseudocatenulatum* IPLA 36007, a human intestinal strain with isoflavone-activation activity. *Gut Pathogens*, *6*, 31.
- **Artículo 8: Guadamuro, L.**, Flórez, A. B., Alegría, Á., Vázquez, L., & Mayo, B. (2017). Characterization of four β-glucosidases acting on isoflavone-glycosides from *Bifidobacterium pseudocatenulatum* IPLA 36007. *Food Research International*, *100*, 522-528

Fermentation of commercial soy beverages with lactobacilli and bifidobacteria strains featuring high β-glucosidase activity

Abstract

An increase of isoflavone aglycone content in soy-derived foods is considered to improve their biological functionality. This can be attained through fermentation of soy and soy beverages with β-glucosidase-producing, food-grade bacteria. In this study, the suitability of a set of lactobacilli (eight strains) and bifidobacteria (two strains) for the fermentation of two commercially-available soy beverages (AS and VS) with distinctive chemical composition was examined. The strains, isolated from dairy and the human gastrointestinal tract, belonged to the following species: *Bifidobacterium pseudocatenulatum* (two strains), *Lactobacillus casei* (two strains), *Lactobacillus murinus* (one strain), *Lactobacillus plantarum* (two strains), *Lactobacillus rhamnosus* (two strains), and *Lactobacillus ruminis* (one strain). In the fermented beverages, we monitored growth of the strains by plate counting, as well as measured basic physic-chemical parameters such as pH, acidity, viscosity, β-glucosidase activity of the cells, and the main volatile compounds produced. Finally, deglycosylation of the isoflavone glycosides genistin and daidzin and the appearance of the corresponding isoflavone aglycones genistein and daidzein was assessed by a high pressure liquid chromatography method. Large differences in the result of most parameters were observed for the different species and strains. Parameters also differ widely when compared values obtained for the same strains in AS and VS beverages. Most strains grew well in the beverages reaching cell counts of around $1x10^9$ cfu mL^{-1}. After 24 h of fermentation of the AS beverage, the isoflavone glycosides were fully transformed into their corresponding isoflavone aglycones by all strains except two strains, *L. ruminis* B1411 and *B. pseudocatenulatum* C63. In contrast, large amounts of undeglycosylated isoflavones were still present at this fermentation time in the VS beverage for a majority of strains. Among the strains evaluated in this study, growing (*L. casei* LP71, *L. plantarum* E112, *L. rhamnosus* E41) and non-growing (*B. pseudocatenulatum* C35) strains in soy beverages were proposed as the most suitable for evaluation of isoflavone deglycosylation under industrial conditions.

1. Introduction

The presence in soy of biologically active proteins has led to soy-derived foods being regarded as functional foods (Messina, 2016). Though a legal definition of functional foods is lacking, they are claimed to have beneficial physiological effects in the body (Shiby and Mishra, 2013). A food may be made functional by increasing or adding a potential health-promoting entity. Alternatively, concentration of adverse components may be reduced, or there may be a partial replacement of toxic by beneficial ingredients. Apart from conventional yoghurt and fermented milk products, several fermented non-dairy foods are globally gaining in interest, in particular those from cereal and soy origin (Leroy and de Vuyst, 2014).

Soy foods are allowed to make a heart beneficial health claim if they contain a mandatory 6.26 g of soy protein per serving (FDA, 1999). In addition to protein, soy contains many biologically active compounds, including high isoflavone content (Vitale et al., 2013). Soy isoflavones are a class of polyphenols of vegetal origin with structure and chemical properties similar to those of 17-β-estradiol, which have oestrogenic activity (Andres et al., 2015). Isoflavones also act independently of oestrogen receptors having other physiological effects, such as inhibition of

tyrosine kinases, activation of natural killer cells, antioxidant effects, etc. (Vitale et al., 2013). High isoflavone intake by Asian populations as compared to Caucasian populations has been associated to a better intestinal health and lower rates of cardiovascular and cancer diseases (Ko, 2014; He and Chen, 2013). These claims have contributed to a progressive increase of soy and soy-derived foods consumption in the world (Bolca et al., 2012). However, the unpleasant (or unfamiliar) odour of soy products has limited wide acceptance of soy milk and soy-derived foods by Western populations (Kaneko et al., 2011). This has favoured the development of soy-derived beverages and soy-fermented foods containing additives to counteract of soy milk strong flavours (Kaneko et al., 2014). As compared to plain soy milk products, those derived from soy beverages are consumed nowadays in much higher amounts (Nguyen et al., 2012).

In soy milk and unfermented soy foods, isoflavones mostly appear as isoflavone-glycoside conjugates (daidzin, genistin, glycitin), the bioavailability and estrogenic activity of which are low (Crozier et al., 2009; de Cremoux et al., 2010). To attain full activity, aglycones (daidzein, genistein, glycitein) need to be released from the corresponding glycosides (Islam et al., 2015). This is accomplished by cellular β-glucosidases and β-glucosidases from components of the gut microbiota (Landete et al., 2016). In addition to improving sensorial properties, fermentation of soy with β-glucosidase-producing cultures results in products with enhanced isoflavone bioavailability (Champagne et al., 2010; Wei et al., 2007). However, except in one study (Rekha and Vijayalakshmi, 2011), current starter cultures for soy milk have neither been selected for their glycosidic properties nor for any other isoflavone-related activity (Kaneko et al., 2014; Champagne et al., 2010; Raimondi et al., 2009; Chun et al., 2007; Wei et al., 2007; Chien et al., 2006; Tsangalis et al., 2002). Further, studies screening lactic acid bacteria (LAB) and bifidobacteria collections for isoflavone-degrading capability have been only recently undertaken (Raimondi et al., 2009; Gaya et al., 2016).

In this manuscript, we are reporting on the ability of a selected set of lactobacilli and bifidobacteria strains to develop in two different commercial soy beverages. These strains have been selected in a previous work for their high β-glucosidase activity and aglycone releasing-activity from isoflavone glycosides. Growth of the strains, content of isoflavones and their metabolites, and basic physic-chemical parameters of the fermented soy milks were monitored during fermentation.

2. Material and Methods

2.1. Strains and culture conditions

The strains utilized in this work were selected in a previous study and had been shown to present a high β-glucosidase activity in MRS fermentation broth with cellobiose (Delgado et al., unpublished). The strains had been isolated from dairy or from the human gastrointestinal tract, and identified as follows: *Lactobacillus casei* BA3 (faeces) and LP71 (stomach), *Lactobacillus murinus* G64 (faeces), *Lactobacillus plantarum* E112 (faeces) and LL441 (cheese), *Lactobacillus rhamnosus* E41 (faeces) and G92 (faeces), *Lactobacillus ruminis* B1411 (faeces), and *Bifidobacterium pseudocatenulatum* C35 (faeces) and C63 (intestinal content). Stock cultures of the strains were maintained at -80ºC in MRS supplemented with 20% glycerol (Merck, Germany). Strains were recovered in MRS supplemented with 0.25% cysteine (Prolabo, Belgium) (MRSC) and incubated at 37ºC for 24-72 h in an anaerobic cabinet (Don Whitley Scientific, Shipley, UK) with an anoxic atmosphere (80% N_2, 10% CO_2 and 10% H_2).

2.2. Soy beverages

Alpro-Soja (AS; CAPSA, Siero, Spain) and Vive-Soy (VS; Pascual, Aranda de Duero, Spain), two commercial soy beverages purchased from local supermarkets were inoculated and fermented with the strains above mentioned. As state on the labels, composition of the beverages is as follows, AS [water, husked soybeans (6%), brown sugar, tricalcium

phosphate, acidity corrector (monopotassium phosphate), salt, aroma, stabilizer (gellan gum), and vitamins (B2, B12, and D2)], and VS [water, soybeans (13%), fructose, stabilizer (cellulose), calcium, aromas, salt and vitamins A and D].

2.3. Fermentation of soy beverages

Strains were recovered in MRSC broth and cells were washed twice with 0.9% NaCl and suspended to an adjusted optical density at 600 nm of 1. AS and VS soy beverages were separately inoculated with cell suspensions of each strain to a final concentration of $1x10^7$-$1x10^8$ cfu mL^{-1} (mimicking the regular working concentration of dairy cultures). Lactobacilli-inoculated soy beverages were incubated for up to 72 h at 37ºC in a Hera-cell CO_2-enriched (5.0%) chamber (Thermo-Fisher, Waltham, MA USA), whereas soy beverages inoculated with bifidobacteria were incubated for up to the same time period at 37ºC in anaerobic conditions. All fermentations were done in triplicate.

2.4. Microbial analysis

The evolution of the strains during soy fermentation was determined by plating decimal dilutions of the cells in saline (0.9% Na Cl) on MRSC plates at the time of inoculation and after 24, 48 and 72 h of incubation. Counts were performed after incubation of the plates at 37ºC for 48 h under CO_2-enriched or anaerobic conditions for lactobacilli and bifidobacteria, respectively.

2.5. Analysis of isoflavones

Isoflavones were extracted from the soy beverages during fermentation at 24 and 48 h, following the official AOAC method (Collison, 2008). Briefly, the reaction mixture contained 1 mL of the fermented soy drink, 800 µL acetonitrile, 150 µL H_2O, and 50 µL of an internal standard (apigenin 2 mg mL^{-1}). The mixture was shaken vigorously for 60 min and centrifuged for 10 min at 13,500 rpm. The supernatant was filtered through a 0.45 µm PFTE membrane (Whatman; GE Healthcare Europe, Barcelona, Spain), and analysed by High Performance Liquid Chromatography (HPLC).

Soy isoflavones and their metabolites were separated and quantified in a 2795 HPLC (Waters, Palo Alto, Ca., USA) equipped with an Alltima HP C18 high carbon charge column (250 m x 4.6 nm internal diameter x 5 µm thickness) (Sigma-Aldrich). The working conditions were as follows, injection volume 20 µL, column temperature 40°C, flow 1.5 mL min^{-1} for a running time of 75 min. Elution was done in a water gradient with 0.05% (v/v) phosphoric acid (H_3PO_4) as solvent A and acetonitrile as solvent B (both from Sigma-Aldrich). The compounds were detected by a photodiode array detector (PDA 2996, Waters) at 260 nm. Quantification was performed against calibration curves prepared using commercial standards of isoflavone glycosides (daidzin and genistin) and their corresponding aglycones (all from Sigma-Aldrich, St. Louis, MO, USA).

2.6. Physic-chemical analysis of fermented soy beverages

2.6.1. pH and titratable acidity

The pH during soy fermentation was measured with a pH-meter (Crison, Barcelona, Spain) after 24, 48 and 72 hours of incubation. The titratable acidity (g of acid/100 mL) was determined in 10 mL of soy beverage with a 0.1 N Dornic NaOH solution (Panreac, Barcelona, Spain) and using 1% phenolphthalein (Panreac) as an indicator.

2.6.2. Viscosity

The viscosity in the fermented soy drinks was determined after 48 hours of incubation of the beverages with the different strains with a rotational viscometer (Fungilab, Barcelona, Spain). Measurements were taken with a spindle of 10.53 g and 1.2 cm in diameter, at 23ºC and a rotation speed of 80 rpm.

2.6.3. β-glucosidase activity

β-glucosidase activity of the cells during growth in soy was measured spectrophotometrically after 24 and 48 h of incubation by the released of *p*-nitrophenol (*p*-NP) from *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) (Sigma-Aldrich, St. Louis, Mo., USA), following the protocol by McCue and Shetty (2005) with modifications. In short, 18 mL of 0.2 M sodium acetate buffer (pH 5.5) were

added to 2 mL of soy beverage, and the mixture was centrifuged at 4ºC for 5 min at 11,000 rpm. The cell pellet was suspended in 1 mL of the sodium acetate buffer where the activity was assayed. For the reaction, 100 µL of the sample were taken and 800 µL of sodium acetate buffer was added along with 100 µL of 10 mM *p*-NPG. The reaction was incubated at 37ºC for 30 min and stopped with 1 mL 1 M sodium carbonate. Samples were centrifuged at 13,500 rpm for 10 min and the absorbance at 410 nm measured. The results were brought to a standard curve of increasing concentrations of *p*-NPG (Sigma-Aldrich). As a positive control, a sample was prepared with 100 µL (2 U mL^{-1}) of a commercial β-glucosidase enzymatic preparation from almonds (Sigma-Aldrich). A unit of β-glucosidase activity (U) was defined as the amount of enzyme which released 1 µmol of *p*-NPG per min per mL of beverage.

2.6.4. Volatile compounds

The volatile compounds in soy beverages after 48 h fermentation were analysed by head space (HS) (G1888; Agilent, Santa Clara, Ca., USA) gas chromatography (GC) (6890; Agilent) coupled to a mass spectrometer (MS) (5975B inert MSD; Agilent) in a HP-Innowax 60 m x 0.25 mm id x 0.25 µm thickness column (Agilent). Samples were equilibrated at 80ºC in the HS oven for 30 min with shaking. The CG injector was set at a temperature of 220ºC, working in "splitless" mode. A flow ramp of 1.5 mL min^{-1} was set for 1 minute and then kept constant at 1 mL min^{-1} using helium gas as a carrier. Chromatographic conditions were: 35ºC for 5 min; a first ramp of 5ºC min^{-1} to 100ºC for 10 min; a second ramp of 8ºC min^{-1} up to 220ºC; final temperature was then maintained for 5 min to ensure the column remains clean for the next injection. The transfer line of the MS was at 240ºC, and the detection was performed by electron impact (EI) in scan mode from 20 to 250 uma, at a rate of 3.12 scans s^{-1}, and with ionization energy of 70eV. Temperatures of source and quadrupole were 230 and 150ºC, respectively. Identification of the compounds was performed by comparing the EIs in the Wiley 138 spectra library (Agilent). Quantification was performed by calculating the relative abundance of the compounds as compared to an internal standard added to the soy beverages (cyclohexanone at 0.36 µg mL^{-1}; Sigma-Aldrich).

2.7. Statistical analysis.

Statistical analysis of the culturing and qPCR data was performed using free R software (www.r-project.org). Normality of the data was checked by the Shapiro-Wilk test. Data following a normal distribution were subjected to the Student's t test, while the non-parametric Mann-Whitney's U test was used otherwise.

3. Results

With small differences having sparse statistical significance, growth of the strains at a given sampling time on either AS or VS was highly similar (Fig.1 and Fig. 1cont). Under the inoculation conditions of this study, most lactobacilli strains grew in the two soy beverages between one or two logarithmic (log) units reaching final cell counts of around $1x10^9$

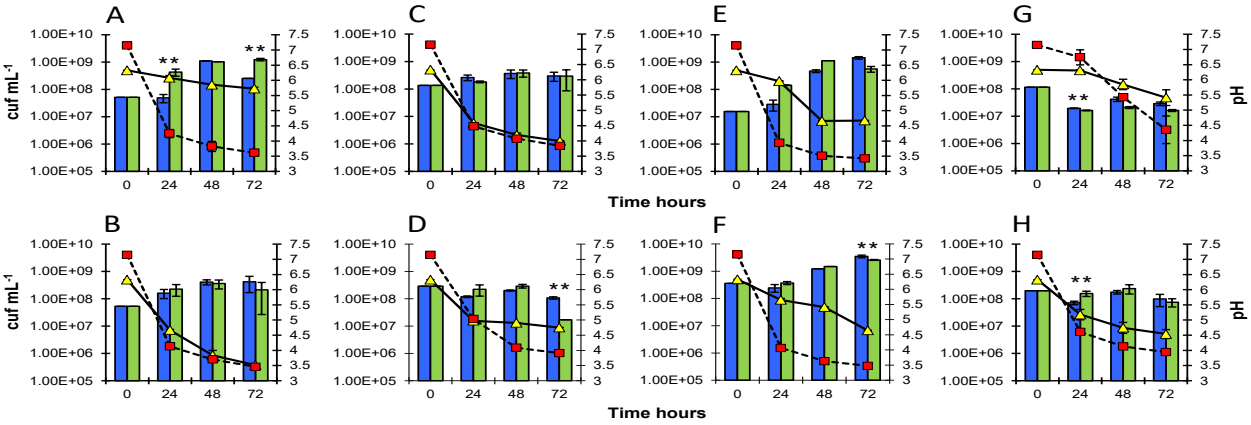

Fig. 1.- Growth and acidification curves during fermentation of two commercial soy beverages (AS and VS) with lactobacilli. Strains A to H were independently inoculated in the two soy beverages. Growth of the strains (blue boxes in AS and green boxes in VS) and pH of the fermented soy milk (yellow triangles in AS and red squares in VS) were determined during incubation at 37ºC in a CO_2-enriched chamber. Key of the strains: A, *Lactobacillus casei* BA3; B, *L. casei* LP71; C, *Lactobacillus plantarum* E112; D, *L. plantarum* L441; E, *Lactobacillus rhamnosus* E41; F, *L. rhamnosus* G92; G, *Lactobacillus murinus* G64; H, *Lactobacillus ruminis* B1411. Asterisks indicate statistical significance (P <0.05).

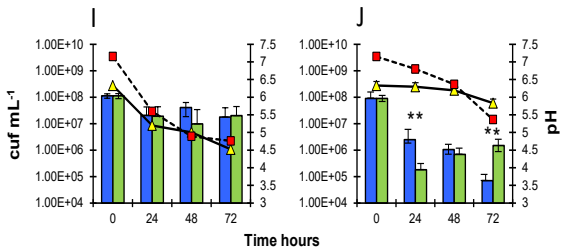

Fig. 1.cont.- Growth and acidification curves during fermentation of two commercial soy beverages (AS and VS) with bifidobacteria. Strains were independently inoculated in the two soy beverages. Growth of the strains (blue boxes in AS and green boxes in VS) and pH the fermented soy milk (yellow triangles in AS and red squares in VS) were determined during incubation at 37ºC in an anaerobic cabinet. Key of the strains: I, Bifidobacterium pseudocatenulatum C35; J, B. pseudocatenulatum C63. Asterisks indicate statistical significance (P <0.05).

cfu mL^{-1} (Fig. 1). The exceptions were *Lactobacillus plantarum* LL441, *Lactobacillus murinus* G64, and *Lactobacillus ruminis* B1411, whose inoculation numbers did not appreciably increased during incubation (Fig. 1D, 1G, and 1H, respectively). Indeed, counts of *L. ruminis* dropped near one log unit after 24 h incubation, maintaining constant counts afterwards (Fig. 1H). The same trend as for *L. ruminis* was observed for the two *Bifidobacterium pseudocatenulatum* strains assayed (Fig. 1. cont.). Counts of C35 and C63 decreased to about one and two log units at 24 h of incubation, respectively. Counts of *B. pseudocatenulatum* C35 were maintained constant afterwards (Fig. 1I), while those of the strain C63 decrease in AS and increase in VS milk (Fig. 1J).

The isoflavone compounds analysed by HPLC in this work includes the isoflavone glycosides daidzin and genistin, their derived aglycones daidzein and genistein (Supplementary Figure 1). Genistin was the main isoflavone compound in the two soy beverages (232.4±14.0 and 139.6±20.2 µmol, respectively), followed by daidzein (78.6±11.8 and 66.3±15.2 µmol, respectively). Small quantities of both genistein (3.2±2.41) and daidzein (2.3±1.3) were also detected. In the case of AS beverage, after 24 h fermentation, the isoflavone glycosides were fully transformed into their corresponding isoflavone aglycones by all strains except two strains, *L. ruminis* B1411 and *B. pseudocatenulatum* C63 (Figure 2A; Supplementary Figure 1). In contrast, large amounts of undeglosylated isoflavones were still present at this fermentation time in the VS beverage for a majority of strains (Figure 2B). Analysis of isoflavone metabolites at 48 h did not significantly increased deglycosilation; in fact, occasionally, isoflavone aglycones showed lower levels at this fermentation time (results not shown).

In contrast to counts, the pH decreases in all beverages along fermentation; although, large differences in acidification curves and final pH between the beverages fermented by distinct

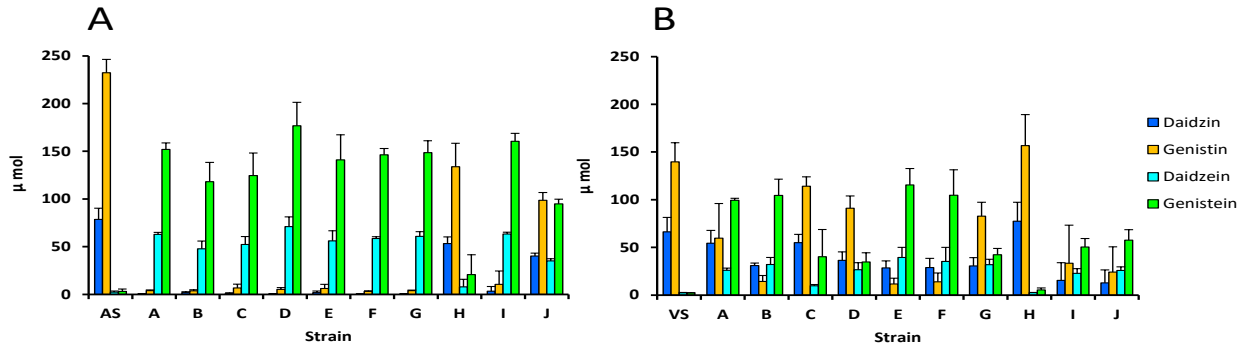

Fig. 2.- Recovery of isoflavone glycosides (daidzin and genistin) and their respective isoflavone aglycones (daidzein and genistein) after fermentation at 37ºC for 48 h of two commercial soy beverages (AS in Panel A, and VS in Panel B) with lactobacilli and bifidobacteria strains. Boxes report average results of three independent experiments. The standard deviation (SD) is indicated by vertical bars on top of the boxes. Key of the strains: A, Lactobacillus casei BA3; B, L. casei LP71; C, Lactobacillus plantarum E112; D, L. plantarum L441; E, Lactobacillus rhamnosus E41; F, L. rhamnosus G92; G, Lactobacillus murinus G64; H, Lactobacillus ruminis B1411; I, Bifidobacterium pseudocatenulatum C35; J, B. pseudocatenulatum C63. AS and VS, uninoculated soy milk AS and VS, respectively, used as controls.

Table 1.- Basic chemical properties of two commercial soy beverages after fermentation.

Strain/Soy milk		Parameter							
		Final pH at 72 h		Acidity at 72 h (% lactic acid)		Viscosity at 48 h (mPa.s)		β-glucosidase activity (mU mL^{-1})[a]	
		AS	VS	AS	VS	AS	VS	AS	VS
A	*L. casei* BA3	5.73±0.05[a]	3.61±0.11[b]	0.25[a]	1.40[b]	196.1±27.2[a]	205.5±42.9[a]	74.51	2.22
B	*L. casei* LP71	3.51±0.02[a]	3.46±0.06[a]	1.20[a]	1.45[b]	300.5±7.0[a]	248.2±22.1[b]	116.05	2.25
C	*L. plantarum* E112	4.00±0.01[a]	3.84±0.01[b]	0.78[a]	0.80[a]	424.3±30.1[a]	300.3±11.0[b]	2.88	0.11
D	*L. plantarum* L441	4.75±0.01[a]	3.91±0.01[b]	0.40[a]	0.60[a]	343.4±17.8[a]	309.8±0.6[b]	5.93	7.82
E	*L. rhamnosus* E41	4.68±.001[a]	3.43±0.01[b]	0.45[a]	1.40[b]	204.0±6.2[a]	198.3±5.4[a]	57.80	11.08
F	*L. rhamnosus* G92	4.65±0.01[a]	3.48±0.01[b]	0.30[a]	1.40[b]	181.1±1.3[a]	197.3±8.0[b]	178.52	11.09
G	*L. murinus* G64	4.54±0.15[a]	3.94±0.04[b]	0.20[a]	0.40[b]	20.7±3.9[a]	271.8±6.4[b]	0.49	0.03
H	*L. ruminis* B1411	5.41±0.26[a]	4.35±0.45[b]	0.40[a]	0.60[b]	366.9±16.2[a]	254.1±23.5[b]	13.12	6.59
I	*B. pseudocatenulatum* C35	4.52±0.21[a]	4.76±0.13[a]	0.50[a]	0.50[a]	410.3±16.6[a]	260.0±9.6[b]	4.55	13.03
J	*B. pseudocatenulatum* C63	5.83±0.01[a]	5.36±0.01[b]	0.25[a]	0.25[a]	222.1±21.5[a]	232.9±22.6[a]	1.98	7.35
AS	Uninoculated control AS	6.33±0.01	na	0.15	na	3.4±0.8	na	na	na
VS	Uninoculated control VS	na	7.15±0.01	na	0.10	na	4.15±1.05	na	na

[a]The β-glucosidase activity was measured in entire cells contained in 1 mL of fermented beverages at 24 h with *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG).
Average results of three independent experiments are reported (except for the β-glucosidase activity). Different superscripts in parameter values for a given strain indicates the results are statistically different (P <0.05).
na, not applicable.

strains were observed (Fig. 1; Table 1). Differences in the evolution of the pH in AS and VS fermented by the same strain were also noted. In general, the pH decreased sharply and reached lower levels in VS than that in AS, reaching at 72 h incubation a final pH lower than 4.0 for seven out of the ten strains (Table 1). These results almost parallels those obtained for the acidity, which was always higher in the VS beverage (Table 1). Viscosity increases from 3-4 in the soy beverages up to more than 200 mPa.s in most fermented samples (Table 1). Though no major differences were observed for each strain in the two beverages (except for *L. murinus*), a general tendency was observed in which viscosity was somewhat higher in AS, with peaks for *L. plantarum* E112 and *B. pseudocatenulatum* C35 strains.

The β-glucosidase activity of the strains during fermentation of the two beverages is summarized in Table 1. As shown in the table, large differences in enzymatic activity between strains were scored. In general, higher enzyme activities were recorded for most strains in the AS beverage, in which four strains, the two *L. casei* and the two *L. rhamnosus*, presented a notably higher β-glucosidase activity in this beverage as compared to all other strains. The enzymatic activity was shown to be lower for all strains at 48 h of incubation in both AS and VS, except for the two *L. plantarum* strains (data not shown).

The volatile compounds identified in this work in the uninoculated and corresponding fermented soy beverages are summarized in Table 2. Eight volatile compounds were detected, of which four were already present (though at different relative abundance) in the two uninoculated control soy beverages, acetaldehyde, acetic acid, ethanol and propan-2-one. Acetic acid and ethanol increased notably during fermentation by most strains in both AS and VS. High quantities of acetic acid were produced by *L. murinus* in AS and the two *B. pseudocatenulatum* strains in both AS and VS. Ethanol also increased in most fermentations, but not in those produced by the two *L. plantarum* and the *L. ruminis* strain. Moderate levels of diacetyl were produced in both AS and VS by all but three lactobacilli strains (*L. plantarum, L. murinus* and *L. ruminis*); production was particularly high by the two *B. pseudocatenulatum* strains. Noteworthy was the volatile compound profile of *L. murinus* G64, which produced larger quantities than all other strains of acetaldehyde, acetic acid, ethanol, propionaldehyde, propan-1-ol, and propan-2-one in the AS beverage. In this soy milk, G64 was also the only producer of propionaldehyde and propan-1-ol (Table 2).

Table 2.- Production of volatile compounds during fermentation of two commercial soy beverages.

Strain/Soy milk		Volatile compound[a]															
		Acetaldehyde		Acetic acid		Ethanol		Acetoin		Diacetyl		Propionaldehyde		Propan-1-ol		Propan-2-one	
		AS	VS	AS	VS	AS	VS	AS	VS	AS	VS	AS	VS	AS	VS	AS	VS
A	*L. casei* BA3	0	9.3	6.0	8.8	65.4	100.3	0	0	5.0	6.8	0	0	0	0	2.7	17.5
B	*L. casei* LP71	4.6	7.6	10.0	6.1	49.4	100.9	0.1	0	3.7	3.5	0	0	0	0	3.5	16.6
C	*L. plantarum* E112	0	4.1	11.5	18.3	32.0	68.0	0.7	1.5	6.1	14.7	0	0	0	0	3.1	9.8
D	*L. plantarum* L441	0	4.6	5.4	14.5	32.0	66.1	0	0	0	0	0	0	0	0	2.0	9.9
E	*L. rhamnosus* E41	0	9.5	5.2	5.9	79.2	98.0	0.5	0.2	6.1	7.1	0	0	0	0	2.9	15.5
F	*L. rhamnosus* G92	0	5.0	10.5	12.1	117.1	98.9	0	0.5	13.9	18.1	0	0	0	0	2.8	17.3
G	*L. murinus* G64	126.7	18.9	178.5	4.3	1325.3	113.3	0	0	0	0	20.5	0	267.3	0	17.3	9.9
H	*L. ruminis* B1411	0	5.5	4.3	8.5	31.3	62.5	0	0	0	0	0	0	0	0	2.2	8.7
I	*B. pseudocatenulatum* C35	19.2	9.7	55.5	41.6	63.3	105.3	0.2	0.3	40.8	5.4	0	0	0	0	3.8	17.3
J	*B. pseudocatenulatum* C63	10.5	8.3	33.2	21.9	57.0	102.1	0.2	0.2	44.4	3.9	0	0	0	0	3.3	17.4
AS	Uninoculated control AS	9.5	na	6.0	na	29.2	na	0	na	0	na	0	na	0	na	1.0	na
VS	Uninoculated control VS	na	7.5	na	2.6	na	66.0	na	0	na	0	na	0	na	0	na	10.3

[a]Relative abundance as compared to an internal standard (cyclohexanone at 3.6 µg mL^{-1} of sample; this was given a value of 1.0).
Average results of two independent 48-h fermentations.
na, not applicable.

4. Discussion

In this work, several lactobacilli and bifidobacteria strains of which a majority are considered by the European Food Safety Authority (EFSA) to be suitable for the

Qualified Presumption of Safety (QPS) approach to safety assessment (EFSA, 2015), have been tested for growth and isoflavone aglycone release during fermentation in two commercial soy-based beverages. In addition, some technological parameters of the fermented products were also evaluated.

Several attempts have been made to improve bioavailability of isoflavone aglycones from isoflavone glycosides in soy milk by fermentation with lactobacilli and bifidobacteria species (Tsangalis et al., 2002; Otieno et al., 2006; Raimondi et al., 2009; Rekha and Vijayalakshmi, 2010). In addition to increase absorption, fermentation might further reduce the level of antinutrients in soy milk like phytic acid and increase concentration of vitamins of the B complex (Rekha and Vijayalakshmi, 2010). However, most studies have made use of plain soy milk, while on the Western world soy is almost exclusively marketed as mixed with additives (such as those in AS and VS, see above) to mask unfamiliar soy aroma and taste. These additives can influence (positively or negatively) growth of fermenting bacteria and/or their enzyme activities (β-glucosidases), thus modifying the release of aglycones from glycosides (Chen et al., 2012). In that sense, enhanced growth of lactobacilli and bifidobacteria strains during experimental fermentation of soy and increased isoflavone aglycone release has been reported for soy milk supplemented with 2% lactose (Ding and Shah, 2010).

The growth results obtained in this work for the analysed lactobacilli and bifidobacteria strains are comparable to those reported by other authors for species and strains of these bacterial groups in either soy milk (Tsangalis et al., 2002; Chien et al., 2006; Pyo et al., 2005; Wei et al., 2007; Donkor and Shah, 2008) or soy beverages (Ding and Shah, 2010). In general, the metabolism of sugars by LAB and bifidobacteria drives to lactic and acetic acids and, as a consequence, the pH of the medium drops, leading to a decreased glycolytic flux and growth rate, compromising ultimately cell viability (Kowalczyk et al., 2014). The differences in concentration of distinct end-products (lactic and acetic acids) in AS and VS might come from the presence of different sugars in the beverages, as well as from the existence of distinct uptake, degradation and control pathways in the species and strains of this study. Further, other metabolic activities such as stress response and proteolytic activities of the strains might also influence the results, giving rise to a strain-specific chemical profile. Final pH and acidity were shown to be growth associated in most strains, but large differences between species and strains were found. A majority of the strains grew similarly in AS and VS, while the pH was usually lower and the acidity higher in the latter beverage. This suggests that AS and VS have a rather different buffering capacity, which does not affect growth but leads to a different final pH and acidity. The production of lower levels of lactic acid by bifidobacteria as compared to lactobacilli has been repeatedly reported elsewhere (Kamaly, 1997; Pyo et al., 2005).

β-glucosidases are thought to be involved in the deglycosylation of isoflavone glycosides, increasing bioavailability of isoflavones (Bowey et al., 2003). The genomes of both lactobacilli and bifidobacteria have been shown to be packed with β-glucosidase-encoding genes (Gänzle ang Follador, 2012; Milani et al., 2015). However, specificity, complementarity and regulation of the enzymes have been reporter to vary widely between species and strains (Gänzle ang Follador, 2012). Further, β-glucosidases from LAB have been reported to present all sort of localizations (intracellular, extracellular and membrane-associated) (Coulon et al., 1998; Spano et al., 2005; Michlmayr et al., 2010). In this work, the enzymatic assay was done with entire cells without cell permeabilization. Thus, the measurement might only reflect extracellular activities (secreted and/or released after cell lysis) and not the total β-glucosidase activity. In that sense, in one study involving bifidobacteria, extracellular β-glucosidase activity has been reported to be about 15% of

the total (Raimondi et al., 2009). Alternatively, enzyme production could have been higher at earlier stages of growth (for instance at the exponential phase) and thus not recorded. As compared to AS, much less β-glucosidase activity in *L. casei* and *L. rhamnosus* strains was shown in the VS (beverage that contains fructose as an ingredient). Influence of the sugar source on β-glucosidase activity in LAB strains has been previously reported (Wei et al., 2007; Iqbal and Zhu, 2009). Whatever the case, in this work, association of β-glucosidase activity with isoflavone deglycosylation was found to be only partial, as it has been reported before by Raimondi et al. (2009).

In humans, isoflavone aglycones have been reported to be absorbed faster and in higher amounts that their corresponding glycosides (Izumi et al., 2000). Therefore, increased isoflavone aglycone content in soy-derived foods improves their biological functionality. This can be accomplished by enrichment of soy foods with isoflavone aglycones prior to consumption, as for instance by fermenting soy with aglycone-releasing microorganisms (Rekha and Vijayalkashmi, 2011). Large differences in the deglycosylation ability of lactobacilli and bifidobacteria species and strains have been reported by several authors (Tsangalis et al., 2002; Otieno and Shah, 2007; Donkor and Shah, 2008; Raimondi et al., 2009; Rekha and Vijayalkashmi, 2011; Gaya et al., 2016). In this work, the use of two commercial soy beverages with different composition further allowed determining that constituents and additives of the beverages, which influence growth and/or enzyme activity, might also account for isoflavone aglycone content in the fermented product. In some studies, aglycone content during fermentation of soy milk has been reported to increased continuously for up to 72 h (Ding and Shah, 2010), while in our case, though slightly, aglycone content at 72 h incubation was usually lower than that at 48 h. A slow decrease of aglycones during fermentation and/o storage of fermented soy milks has been reported elsewhere (Iqbal and Zhu, 2009; Raimondi et al., 2009). These results might be due to differences in the size of the inoculum, although conversion of aglycones into isoflavone-derived metabolites that were not analysed in this work such as malonyl and acetyl derivatives (Ding and Shah, 2010) or degradation to other compounds (Guadamuro et al., 2016) cannot be discarded.

Evaluation of biochemical and sensory parameters will allow assessing the real technological interest of the strains for fermentation of the beverages if an acceptable fermented product can be obtained. Once again, composition of soy beverages has been shown to affect most of the parameters and compounds studied. The gradual final pH and acidity obtained by the different strains might be suitable to select appropriate strains to be used as starters for manufacturing products (more acidic, less acidic) for specific target populations. In addition to acids, volatile compounds are key components influencing both taste and aroma of the fermented soy beverages. Acetaldehyde, the most typical yoghurt volatile compound, was present in all fermented VS samples and in a relative abundance similar to that of yoghurt and other fermented milks made from cow's milk (Irigoyen et al., 2012). As in the fermented soy beverages, ethanol, acetic acid and diacetyl are also majority volatile compounds in cow's milk yoghurt (Irigoyen et al., 2012; Settachaimongkon et al., 2014). Of these, an excess of diacetyl in fermented soymilk has been related to an unpleasant odour (Kaneko et al., 2014), suggesting that the AS beverage might not be a suitable formulation to be fermented by *B. pseudocatenulatum*. However, as compared to cow's milk fermented products, in the fermented soy beverages there is a complete lack of branched alcohols and aldehydes derived from branched chain amino acids (Ile, Leu, Val) (Irigoyen et al., 2012); these might be critical for the distinct flavour between cow's- and soy-derived fermented foods. Viscosity is another important rheological parameter that affects taste properties of fermented products, thus influencing overall acceptability. In this study, the viscosity values of the fermented soy beverages were comparable to those reported in the literature for soy milk yoghurts (Rezaei et al., 2015). Under the same assay conditions,

viscosity of AS and VS fermented beverages was twice to three fold higher than that reported for stirred fermented milks made from cow's milk with lactobacilli and bifidobacteria (Salazar et al., 2009). This may be a positive or negative character depending on consumer customs and preferences.

Soy beverages and yoghurts have been reported to be good vehicles for delivering probiotic microorganisms (Farworth et al., 2007; Otieno and Sha, 2007). As some of our strains have been found to present a vast array of probiotic properties (Delgado et al., 2007; Delgado et al., 2008; Delgado et al., 2015), fermented beverages with these strains could be considered a functional food for improving health through two complementary mechanisms. Though the sensory properties of the fermented beverages were not assessed by tasting panels, growing (*L. casei* LP71, *L. plantarum* E112, *L. rhamnosus* E41) and non-growing (*B. pseudocatenulatum* C35) strains of this study showing good isoflavone deglycosylation activity were considered promising candidates for evaluation in industrial trials.

5. Conclusions

This work demonstrates that commercial soy beverages can be fermented with lactobacilli and bifidobacteria strains aimed to produce a non-dairy yoghurt-like commodity with appealing sensory properties and having high concentration of isoflavone aglycones, which provide enhanced availability of these phenolics as compared to their isoflavone glycoside precursors. However, strains to be used as starters in soy beverages should be carefully selected in order to fulfil technological and functional requirements of the pursued final product. Growing and non-growing deglycosilating strains were considered promising candidates for industrial evaluation. Beverages should incorporate suitable technological additives so as not to interfere with deglycosylation activity and, if appropriate, with bacterial development.

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The genome of *Bifidobacterium pseudocatenulatum* IPLA 36007, a human intestinal strain with isoflavone-activation activity

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Abstract

Background: *Bifidobacterium* species, including *Bifidobacterium pseudocatenulatum*, are among the dominant microbial populations of the human gastrointestinal tract. They are also major components of many commercial probiotic products. Resident and transient bifidobacteria are thought to have several beneficial health effects. However, our knowledge of how these bacteria interact and communicate with host cells remains poor. This knowledge is essential for scientific support of their purported health benefits and their rational inclusion in functional foods.

Results: This work describes the draft genome sequence of *Bifidobacterium pseudocatenulatum* IPLA 36007, a strain isolated as dominant from the feces of a healthy human. Besides several properties of probiosis, IPLA 36007 exhibited the capability of releasing aglycones from soy isoflavone glycosides. The genome contains 1,851 predicted genes, including 54 genes for tRNAs and fie copies of unique 16S, 23S and 5S rRNA genes. As key attributes of the IPLA 36007 genome we can mention the presence of a lysogenic phage, a cluster encoding type IV fimbriae, and a locus encoding a clustered, regularly interspaced, short, palindromic repeat (CRISPR)-Cas system. Four open reading frames (*orfs*) encoding β-glucosidases belonging to the glycosyl hydrolase family 3, which may act on isoflavone glycosides, were encountered. Additionally, one gene was found to code for a glycosyl hydrolase of family 1 that might also have β-glucosidase activity.

Conclusion: The availability of the *B. pseudocatenulatum* IPLA 36007 genome should allow the enzyme system involved in the release of soy isoflavone aglycones from isoflavone glycosides, and the molecular mechanisms underlying the strain's probiotic properties, to be more easily understood.

Introduction

Bifidobacterium species are majority bacteria among those inhabiting the gastrointestinal tract (GIT) of animals and humans. They play important roles in maintaining human health via the digestion of foods, production of essential vitamins, and metabolization of endogenous and exogenous compounds, as well as by preventing the colonization and/or overgrowth of pathogens in the GIT [1]. Molecular analyses have shown that members of the *Bifidobacterium catenulatum* group (which includes *B. catenulatum* and *B. pseudocatenulatum*) are abundant in fecal samples from adult humans [2,3]. *B. pseudocatenulatum* strains have a number of probiotic properties, such as the possession of antinutrient-degrading enzymes [4], the ability to bind mutagenic aromatic amines [5], and the capacity to reduce cholesterol levels [6]. However, compared to other bifidobacterial species, the genome of *B. pseudocatenulatum* has been very little explored. The Genomes Online Database (GOLD) (http://www.genomesonline.org) only contains the draft sequence of a single strain, *B. pseudocatenulatum* DSM 20438 (Gi02660), plus recently released incomplete sequences of five other strains (D2CA; TSDC19.1-1.1; TSDC19.1-1.2; TSDC19.1-1.3; and TSDC17.2-1.1). Sequence analysis of additional *B. pseudocatenulatum* strains

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would provide greater insight into the intra-specific variation of this species, and supply information on the genetics that underlay strain-specific capabilities. Recently, *B. pseudocatenulatum* has been used as a cloning host for the expression of natural [7] and synthetic [8] genes. Genomic analyses of strains of this species might allow the confident use of this bacterium in other biotechnological applications.

Bifidobacterial strains have been shown to be involved in the conversion of isoflavone glycosides into aglycones [9,10], a key step in making isoflavones bioavailable and harnessing their estrogenic activity [11]. Indeed, the genomes of sequenced bifidobacteria show an impressive array of genes coding for glycosyl hydrolases, including β-glucosidases, which are thought to be involved in the release of aglycones from dietary polyphenols such as soy isoflavones [12,13]. However, the enzyme(s) involved in the hydrolysis of soy isoflavone glycosides remain(s) mostly unknown. So far, a β-glucosidase from *Bifidobacterium animalis* subsp. *lactis* has been shown to possess aglycone-releasing activity from isoflavones by cloning and expression of its encoding gene in *Bifidobacterium bifidum* [14].

The present work provides a draft genome sequence for *B. pseudocatenulatum* IPLA 36007, an intestinal human strain able to release aglycones from the soy isoflavone glycosides daidzin and genistin. This capability endows it with properties of interest in terms of its use in functional foods.

Materials and methods

Isolation and DNA preparation

B. pseudocatenulatum IPLA 36007 was isolated among the dominant bacteria from fecal samples of a healthy human, in a study approved by The Ethic Committee of the Asturias Principality, Spain [15]. The strain was grown anaerobically at 37°C in MRS medium (Merck, Darmstadt, Germany) supplemented with 0.25% cysteine (Merck). Genomic DNA was extracted and purified from pure cultures using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, Miss., USA) following the manufacturer's instructions for extracting DNA from Gram-positive bacteria. The concentration and quality of the DNA was measured using an Epoch microvolume spectrophotometer (BioTek Instruments, Winooski, Vt., USA).

Aglycone releasing-activity from isoflavone glycosides

Strains were incubated anaerobically in a MRS basal medium without dextrose and supplemented with 2% cellobiose and 100 μM daidzin or ginistin (Sigma-Aldrich) at 37°C for 24 h. One ml cultures were centrifuged and the cells suspended in the same volume of 0.1 M sodium acetate buffer pH 4.1. Isoflavones and derivatives were then extracted with ethyl acetate (Sigma-Aldrich). The organic phase was evaporated and the dried pellet suspended in 100 μl of methanol. Five 5 μl were used for analysis by TLC in silica gel 60 F254 plates (Merck). Isoflavones were separated in a toluene:acetone (2:1) solvent system, revealed by UV light at 365 nm in a transilluminator and visualized with an ImageQuant 350 (GE Healthcare Bio-Sciences, Buckinghamshire, UK).

Genome sequencing, assembly and annotation

A genomic library of 0.5 kbp was constructed and paired-end sequenced (approximately 155-fold coverage) using a HiSeq 1000 System sequencer (Illumina, Inc., San Diego, CA, USA). Quality-filtered reads were assembled in contigs using Velvet software v.1.2.10. (https://www.ebi.ac.uk/~zerbino/velvet/). Gaps within the contigs were closed by direct sequencing of amplicons obtained by PCR with oligonucleotide primers designed to anneal in the flanking regions. The genome was annotated with the RAST annotation system (http://rast.nmpdr.org/) and the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). The KEGG Pathway (http://www.genome.jp/kegg/pathway.html), Uniprot (http://www.uniprot.org) and COG (http://www.ncbi.nlm.nih.gov/COG) databases were consulted for description of specific genes and proteins. If required, DNA and deduced protein sequences were individually subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multi-blast protein comparisons were performed with the CLC Bioinformatics Database software package (CLC bio, Aarhus, Denmark).

Nucleotide sequence accession numbers

The results of this Whole Genome Shotgun project have been deposited in the GenBank database under accession number JEOD00000000. The version described in this paper is JEOD01000000.

Results and discussion

Among a large collection of intestinal bifidobacteria strains from human origin, *B. pseudocatenulatum* IPLA 36007 showed aglycone-releasing activity from isoflavone glycosides. Figure 1 shows the conversion of daidzin into daidzein after incubation with IPLA 36007 cells. Similar activity was detected using genistin as a substrate, which was transformed into genistein (data not shown). As this strain has already shown a bunch of key properties for its use as a probiotic, including among others good survival under conditions simulating those of the GIT, absence of undesirable enzyme activities and atypical antibiotic resistances, and ability to bind human intestinal epithelial cells [16], it was selected for whole genome sequencing in order to get insights on the molecular basis of its relevant phenotypic traits.

The general features of the *B. pseudocatenulatum* IPLA 36007 genome are summarized in Table 1. The draft

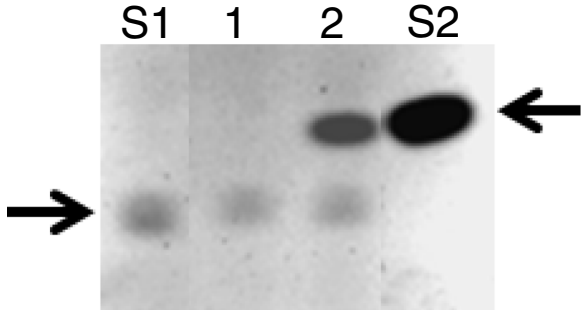


Figure 1 TLC chromatogram showing the conversion of daidzin to daidzein by *B. pseudocatenulatum* IPLA 36007. Order: line1, daidzin incubated with whole cells a negative *Bifidobacterium longum* strain; line 2, daidzin incubated with cells of *B. pseudocatenulatum* IPLA 36007; lines S1 and S2, daidzin and daidzein standards, respectively. Arrows point to the position of daidzin and daidzein.

genome sequence of IPLA 36007 included 23 contigs varying in size from 203 to 548,016 bp. According to the NCBI Prokaryotic Genome Annotation Pipeline the genome harbors 1,851 genes, of which 1,769 are coding sequences (CDS) and 22 are pseudogenes. Additionally, 60 predicted RNA genes were identified, including five copies of identical 16S, 23S and 5S rRNA genes, a non-coding RNA molecule (ncRNA), and 54 genes coding for tRNAs. The RAST server classified the CDS into 26 classes and 255 subsystems (sets of related functional roles).

This strain was demonstrated plasmid-free (data not shown), and, in agreement, no plasmid-associated genes were found. However, one integrated phage of around 43.5 kbp was recorded. The lysogenic phage region consisted of 76 CDS, and included a gene encoding a retron-type RNA-directed DNA polymerase typical of group II introns [17]. Phage-related sequences have been described in the genome of 22 strains of different bifidobacteria species, but only fragmentary information exists with regard to their functionality [18]. As in other bacteria, bifidobacterial prophages have been shown to possess classical modular genomic organization in which the DNA lysogeny module and the DNA packaging region are the most highly conserved.

A cluster of nine genes capable of encoding type IV fimbriae or pili was identified. This contained, among others, orthologous genes for *pilA, pilB, pilC, pilM,* and *pilT* (Figure 2). Operons encoding type IV fimbriae have also been found in the genome of other sequenced *B. pseudocatenulatum* strains, in other bifidobacterial species, and in some environmental actinobacteria. Such pili have been well studied in Gram-negative bacteria such as *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Moraxella bovis* and *Dichelobacter nodosus* [19]. In these pathogenic bacteria, they mediate attachment and adherence to epithelial cells, twitching motility, gliding motility, cell agglutination, and biofilm and fruiting body formation [19]. In addition, they act as receptors for bacteriophages, and are required for extracellular protein secretion and natural transformation [19]. The expression and functionality of the type IV fimbriae genes in *B. pseudocatenulatum* IPLA 36007 has yet to be demonstrated.

A locus encoding a CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins) system was identified (Figure 3). This locus contained 22 identical repeats of 33 bp (except for one repeat carrying a single C-T transition at position five) and 21 spacers ranging from 32 to 37 bp (Figure 3B). The repeats (5′-GTCGCTCTCCTCATGGAGAGCGTGGATTGAAA T-3′) were preceded by eight CRISPR-associated (*cas*) genes (Figure 3A). Spacers showed no significant homology to DNA sequences on databases. Gene content and gene order analysis showed the CRISPR-Cas system of IPLA 36007 to belong to type I-C [20]. CRISPR-Cas

Table 1 Key features of the *B. pseudocatenulatum* IPLA 36007 genome

Characteristic	Figures	Observations
Size of the genome	2,328,179 bp[a]	
G + C content	56.4%	
Contigs	23	(from 203 to 548,016 bp long)
Open reading frames (ORFs)	1851	
Coding sequences (CDS)	1769	
Pseudogenes	22	
RNA genes	60	5 rRNA operons, 54 tRNA, 1 ncRNA
Plasmidic genes	0	Plasmid free strain
Integrated phages	1	76 CDS
CRISPR-Cas system	1	22 repeats, 21 spacers, 8 CDS
Glycosyl-hydrolases and glycosyl transferases	>50	
β-glucosidases	5	5 glycosyl hydrolases family_3, 1 glycosyl hydrolase family_1

[a]bp, base pair; ncRNA, non-coding RNA.

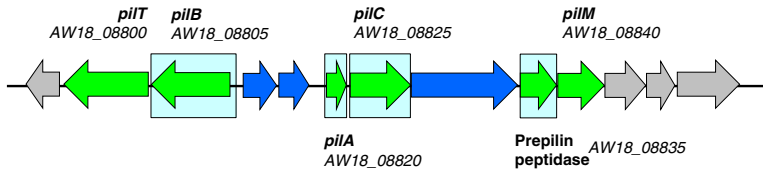
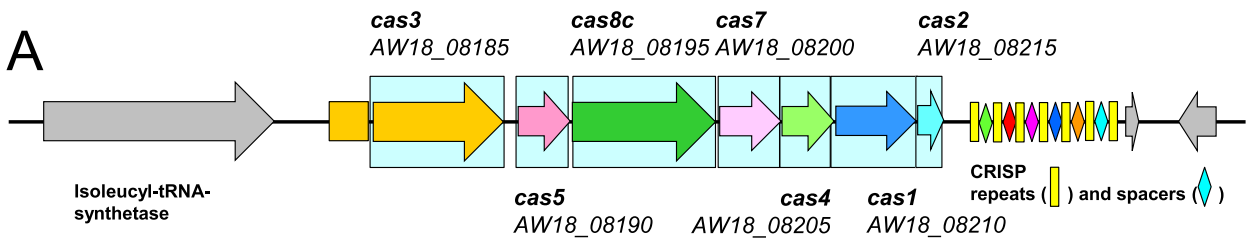


Figure 2 Locus of the type IV fimbriae in *B. pseudocatenulatum* IPLA 36007. In green, hortologous genes to those in other fimbriae/pili clusters. In blue, hypothetical genes belonging to the cluster. In grey, flanking CDS not related to the cluster. Pale blue background boxes indicate functionally coupled genes sharing conserved relative positions in the genome of at least four other species.

systems provide defenses against foreign nucleic acids derived from bacteriophages, plasmids and other sources. These systems target and digest foreign DNA in an RNA-dependent, sequence-specific manner, and are also adaptive, providing protection against previously encountered exogenous elements [19]. Physiological roles for CRISPR-*cas* systems other than in defense against foreign DNA are slowly being uncovered [21].

Bifidobacteria utilize a wide range of carbohydrates that escape digestion in the upper parts of the intestine, many of which are plant derived oligo- and polysaccharides [22]. The genome of *B. pseudocatenulatum* IPLA



B

GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	TTGGTGAGCTTGCCGGAGCCGGTGGCCGCAGCG
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	ATCATAGTGGACTATGTGACAGGCATTGCAAAAGCAA
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	TCGGCTGAATGTGTTTTGCAATGCTTGGGAACGGT
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	TTGATGACATACGCCGACTCCGCGCCGTCAATCG
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	ATCGGCAATTGCTGGGAACTGCGCAAACGAAACGAC
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	CGTATTTCGCAATCACAAGGGACGACAACAAATT
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	ATCATGCGTCTCACGGGCTTCCATGCCCTCGATA
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	CGAGATAATAGCGAGGGGTATCAGCGTTCGAGGCG
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	ATTCCGCATGCCGCTATTACGGACACGGCCAACA
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	CGTCAGAACATTGTTCTTAGTCATCAAATCATTAA
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	ATACGCAGCGCAAGGCGACTACCAGAACGCCAT
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	CTCATGCAGGACGGCGACCCGCTCAGCGCCGAC
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	GTTAGTCACCTGCGCTTTTTGCTGGATGAACTTATC
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	AGTCGCGTGACGGTCAACAATTCCGGCGGATAC
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	TGAAGGGAGCATGGTGATGACCGTGGCTGCCA
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	ATACCATCCTCGTTAAGGGTGGCGTGGTCGAACA
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	CCCGACAAGCGTAGGGGCGTGCACCGTTTTATGGT
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	ATGCGACTCGCTACGCCGTGCCCACGTCTGGAA
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	GAAGACGGGCCGTATGCCTCCGACTCCCGCAACAT
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	ACGATAGCCATGGCGCTGCCCAGGATCATCGTCG
GTCGTTCTCCTCATGGAGAGCGTGGATTGAAAT	AGCTGGGAGCAGTGCGTTCGCGCCGTACAAGGG
GTCGCTCTCCTCATGGAGAGCGTGGATTGAAAT	

Figure 3 Diagram of the CRISPR-Cas locus of *B. pseudocatenulatum* IPLA 36007. Panel A: The type I CRISPR–Cas systems seem to target DNA. Orthologous genes are color coded following protein family relationships as suggested by Makarova et al. [20]. Cas3 is a large protein with separate helicase and DNase activities. Either Cas5 or Cas7 possess RNase activity. CRISP repeats and spacers are indicated by rectangles and rhombs. In grey, bounding CDS not related to the CRISPR-Cas system. **Panel B**: Twenty two identical 33 base pair (bp) repeats (except for repeat 21, which harbors a single bp change) are separated by unrelated 32 to 37 bp spacers. Pale blue background boxes indicate functionally coupled genes sharing conserved relative positions in the genome of at least four other species.

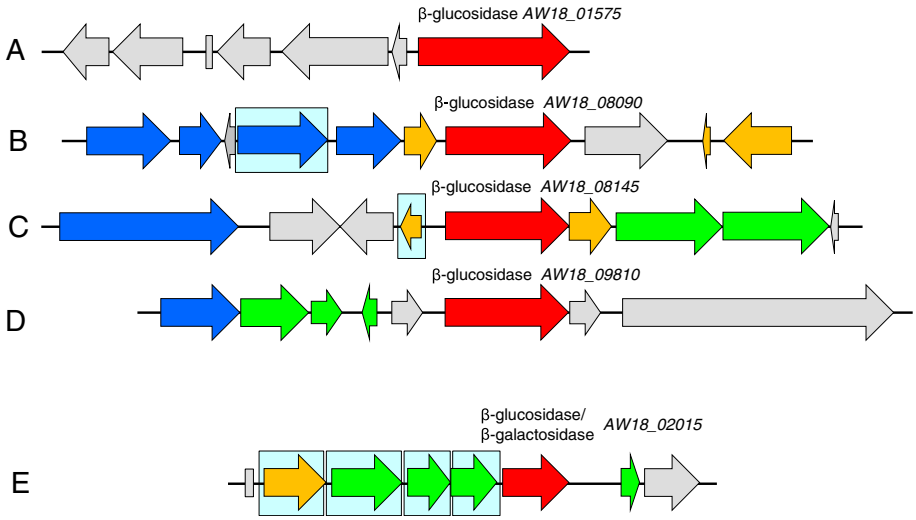


Figure 4 Genetic organization around the putative β-glucosidase-encoding genes. Five genes encoding glycosyl hydrosilases of family 3 **(A through D)** and a gene encoding a gycosyl hydrolase of family 1 **(E)** were identified in the *B. pseudocatenulatum* IPLA 36007 genome. Color code: glycosidase genes are in red; in green, genes involved in transport; in blue, genes involved in carbohydrate(s) metabolism; in brown, genes encoding regulator proteins; in gray, genes belonging to a distinct RAST subsystem; boxes represent genes encoding tRNA and 5S rRNA molecules. Pale blue background boxes indicate functionally coupled genes sharing conserved relative positions in the genome of at least four other species.

36007 contains a vast array of glycosyl transferases and glycosyl hydrolases CDS (52 genes), including genes that code for xylanases (4), pullulanases (3), amylomaltases (2) α-amylase (1) and maltodextrin glucosidase (1). It also harbors genes encoding α- (5) and β- (9) galactosidases, α- (1) and β- (7) xylosidases, α- (1) and β- (4) glucosidases, α-arabinofuranosidases (4), β- mannosidases (2), and α-rhamnosidases (1). Although belonging to the glycosyl hydrolase family 3, all four β-glucosidases (EC 3.2.1.21) shared very limited amino acid identity (26.2%) (Figure 4, A through D). Multiblast analysis of the β-glucosidases found in the IPLA 36007 genome and those present in bifidobacterial genomes suggested that homologous enzymes are present in bifidobacteria strains belonging to the *Bifidobacterium adolescentis* group (*B. adolescentis, B. dentium, B. angulatum, B. catenulatum*, etc.) (amino acid identity ranging from 80 to 98%). The homology to glycosyl hydrolases from other groups of bifidobacteria was much lower (less than 70% amino acid identity) (Additional file 1: Figure S1). Indeed, the similarity of the deduced β-glucosidases from IPLA 36007 to that of *B. animalis* subps. *lactis* SH5, which have been proved to act on soy isoflavone glycosides [14], was found to be marginal (23% amino acid identity). An additional gene encoding a putative glycoside hydrolase belonging to family 1 (EC 3.2.1.23) was detected; this might also have β-glucosidase activity (Figure 4, E). Characterization of these genes and their encoded glycosyl hydrolase enzymes would allow those acting on soy isoflavone glycosides to be identified. However, hydrolysis of isoflavone glycosides some others glycosyl hydrolases of those detected cannot be discarded.

The availability of the genome of the *B. pseudocatenulatum* IPLA 36007 strain should allow the enzymes involved in the release of soy isoflavone aglycones from isoflavone glycosides to be known. This is essential for the rational use of IPLA 36007 as a probiotic in functional foods. The ability of IPLA 36007 to colonize the GIT could be exploited to deliver the aglycone-releasing activity straightway into the human intestine. Comparison of sequences from different sequenced strains would provide greater insights into the genetic variation within this species. It will further allow the core genome and pangenome of *B. pseudocatenulatum* to be identified, while contributing towards defining the gene set required to be competitive in the human GIT.

Additional file

Additional file 1: Figure S1. Multiblast analysis of putative β-glucosidases from *Bifidobacterium pseudocatenulatum* IPLA 36007 to those on the genomes of other bifidobacteria in the databases.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BM and SD designed the study. AA, ABF and LG performed the experiments. AA, SD, BM, GEF and ST analyzed the data. BM and SD wrote the manuscript. AA, GEF and ST checked and edited the manuscript. All authors read and approved the final manuscript.

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Characterization of four β-glucosidases acting on isoflavone-glycosides from *Bifidobacterium pseudocatenulatum* IPLA 36007

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ABSTRACT

Bifidobacterium pseudocatenulatum IPLA 36007 acts on isoflavone glycosides, releasing their corresponding aglycones. This strain-specific activity might be a key step in making isoflavones bioavailable and harnessing their oestrogenic activity. To investigate the molecular mechanisms involved in this activity, four glycosyl hydrolase-encoding genes in the IPLA 36007 genome (*AW18_01575*, *AW18_09810*, *AW18_08145*, and *AW18_08090*) were selected, synthesized with heterologous promoter and terminator signals (r-β-*gluA*, r-β-*gluB*, r-β-*gluD* and r-β-*gluE*, respectively), cloned into *Escherichia coli*, overexpressed as His-tagged proteins, and the enzymes purified and characterized. All four enzymes – $GluA_{His}$, $GluB_{His}$, $GluD_{His}$ and $GluE_{His}$ – proved to have β-glucosidase activity and deglycosylated (although at different rates) the isoflavone glycosides daidzin and genistin, releasing the aglycone moieties daidzein and genistein, respectively. $GluD_{His}$ and $GluE_{His}$ were also shown to hydrolyse β-glucosyl disaccharides such as cellobiose and gentiobiose, while $GluA_{His}$ and $GluB_{His}$ did not. Differences in activity were recorded for all four β-glucosidases at different pHs and temperatures under otherwise similar assay conditions, suggesting they have complementary activities under different environmental conditions. Two of the recombinant genes, r-β-*gluA*, and r-β-*gluD*, were cloned and expressed in the model lactic acid bacterium *Lactococcus lactis*, suggesting starter and probiotic organisms could be endowed with β-glucosidase activity. *B. pseudocatenulatum* IPLA 36007 contains additional β-glucosidases to those studied in this work, indicating a high level of redundancy for this enzymatic activity. Knowledge of glycoside-degrading enzymes should facilitate the development of novel, more effective or more selective prebiotics or functional foods for the promotion of bifidobacterial numbers in the human gut. It might also be of interest in the development of novel probiotics with specific health-promoting activities.

1. Introduction

Bifidobacterium species are among the first microbes to colonize the human gastrointestinal tract, and constitute dominant bacterial populations throughout a person's life (Odamaki et al., 2016). Bifidobacteria help maintain the health of the gut in several ways, including the competitive exclusion of pathogens, modulation of the immune system, production of essential vitamins, and provision of nutrients via the degradation of non-digestible carbohydrates (O'Callaghan & van Sinderen, 2016). These perceived health-promoting activities have led to the commercial exploitation of selected bifidobacterial strains as probiotics (Picard et al., 2005). However, fundamental knowledge related to the mechanism by which bifidobacteria contribute to host health and well-being is still scarce (Sarkar & Mandal, 2016). Such knowledge is essential in order to scientifically support their purported health benefits and their rational inclusion in functional foods.

The utilization of a wide range of carbohydrates – requiring the concerted action of uptake and degrading activities – is a key metabolic trait of bifidobacteria (de Vuyst, Moens, Selak, Rivière, & Leroy, 2014). Certainly, the hydrolysis of complex dietary and host-produced carbohydrates by glycosidases would appear to be a pivotal property that allows these bacteria to colonize and survive in the gut (Pokusaeva, Fitzgerald, & van Sinderen, 2011). Genome analysis of bifidobacterial strains has revealed a vast array of genes coding for these enzymes (Lee & O'Sullivan, 2010; Pokusaeva et al., 2011). Among these, β-glucosidases (which catalyse the hydrolysis of glycosidic bonds in terminal non-reducing residues of β-glucosides and oligosaccharides with the release of glucose) may play a prominent role in the probiotic action of bifidobacteria (O'Callaghan and van Sinderen, 2016). Accordingly, the bifidobacterial genomes contain many genes coding for β-glucosidases (Pokusaeva et al., 2011).

Together with gut cell-derived enzymes (Bowey,

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Adlercreutz, & Rowland, 2003), bacterial β-glucosidases participate in the deglycosylation of dietary β-glucoside phytoestrogens such as isoflavones, lignans, stilbenes and prenylflavonoids. This results in the formation of more bioavailable compounds, which, occasionally, can be converted afterwards into more active metabolites (Ko et al., 2014; Possemiers, Bolca, Eeckhaut, Depypere, & Verstraete, 2007). In this sense, bifidobacteria have been shown involved in the conversion of soy isoflavone-glycosides into aglycones (Raimondi et al., 2008; Tsangalis, Ashton, McGill, & Shah, 2002), a key step in exploiting their health-associated benefits, directly through the oestrogenic activity of aglycones or by producing from them the actual active metabolites (e.g. equol) (Crozier, Jaganath, & Clifford, 2009). So far, however, just one β-glucosidase – from *Bifidobacterium animalis* subsp. *lactis* – with aglycone-releasing activity has been characterized at the genetic and biochemical levels (Youn, Park, & Ji, 2012).

To further our knowledge of these bifidobacterial enzymes, a selection of those produced by *Bifidobacterium pseudocatenulatum* IPLA 36007 were characterized. Four genes in the genome of IPLA 36007 coding for putative β-glucosidases (Alegría et al., 2014) were synthesized with heterologous promoter and terminator signals and cloned into *Escherichia coli*. The encoded enzymes were then overexpressed as histidine-tagged proteins and purified by affinity chromatography. Their basic biochemical properties were then determined against a model substrate and their deglycosylation activities against daidzin, genistin and other substrates tested. In addition, two of these β-glucosidase-encoding genes were cloned into *Lactococcus lactis* to determine whether this bacterium could be endowed with isoflavone deglycosylating activity.

2. Material and methods

2.1. Plasmids, bacterial strains and growth conditions

The bacterial strains and plasmid vectors used in this work are listed in Table 1. *Bifidobacterium pseudocatenulatum* IPLA 36007 was grown anaerobically at 37 °C in MRS medium (Merck, Darmstadt, Germany) supplemented with 0.25% cysteine (Merck). *Escherichia coli* was grown in either Luria Bertani (LB) or in 2xTY medium at 37 °C with shaking. *Lactococcus lactis* was grown (static) in GM17 (M17 supplemented with 0.5% w/v glucose) (Oxoid, Basingstoke, Hampshire, UK) at 32 °C. When plates were required, agar was added at 2% (w/v). For the selection of transformants and plasmid maintenance, kanamycin (40 μg mL^{-1}) or ampicillin (100 μg mL^{-1}) was added to either liquid or solid media. 5-Bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-Glu) and isopropyl-β-D-thiogalactopyranoside (IPTG) (both from Sigma-Aldrich, St. Louis, MO, USA) were used in different experiments at 0.004% (wt/vol) and 1 mM respectively.

2.2. Design of β-glucosidase gene cassettes for heterologous expression

The coding sequences of four putative β-glucosidases (*AW18_01575*, *AW18_09810*, *AW18_08145*, and *AW18_08090*) identified in the genome sequence of *B. pseudocatenulatum* IPLA 36007 (Alegría et al., 2014; GenBank accession no. JEOD01000000) were synthesized (by the GenScript company, Piscataway, NJ, USA) together with the promoter sequence of the erythromycin resistance gene in pE194 from *Staphylococcus aureus* plus the terminator sequence of the *rrnB* operon of *E. coli* (Fig. 1). As a negative control, a putative α-glucosidase-encoding gene from the genome of IPLA 36007 was selected (*AW18_01930*), synthesized, and the protein subjected to the same analysis as the β-glucosidases. These chimeric versions of the genes were denominated r-β-*gluA* (*AW18_01575*), r-β-*gluB* (*AW18_09810*), r-α-*gluC* (*AW18_01930*; α-glucosidase), r-β-*gluD* (*AW18_08145*) and r-β-*gluE* (*AW18_08090*). During synthesis, *Hin*dIII and *Bam*HI restriction enzyme sites present in the original DNA sequences were removed, while, at appropriate positions (Fig. 1), *Hin*dIII, *Bam*HI, and *Xba*I sites were inserted to allow easy cloning and subcloning. These changes were made using the third-position redundancy of the genetic code without introducing amino acid

Table 1
Bacterial strains, plasmids and primers used in this study.

Strain, plasmid, or primer	Relevant genotype, description or sequence (5′-3′)	Source or reference
Bacteria		
Bifidobacterium pseudocatenulatum IPLA 36007	Human intestinal isolate with deglycosylation activity against soy isoflavone-glycosides	Alegría et al. (2014)
Escherichia coli DH10B	Δ(*lac*) × *74*, φ80*lacZΔM15*, *araD139*, Δ(*ara,leu*)*7697*, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), Str^R, λ^-	Invitrogen
E. coli BL21(DE3)	F-, *ompT*, *gal*, *dcm*, *lon*, *hsdSB*(*rB-mB-*), λ(*DE3*[*lacI*, *lacUV5-T7 gene 1*, *ind1*, *sam7*, *nin5*])	Novagen
E. coli BL21(DE3)-pLysS	*E. coli* BL21(DE3) carrying plasmid pLysS, which harbours a lysozyme gene under the control of the T7 promoter	Novagen
Lactococcus lactis MG1363	Plasmid-free derivative of L. *lactis* SH4109	Gasson (1983)
Plasmids		
pUC57	Cloning vector, Am^r	Genscript
pUC57-r-β-*gluA*	ORF *AW18_01575* from the *B. pseudocatenulatum* IPLA 36007 genome cloned in pUC57 under promoter and terminator signals of the erythromycin resistance gene of pE194 (*Staphylococcus aureus*) and the *rrnB* operon (*E. coli*), respectively	This work
pUC57-r-β-*gluB*	The same for ORF *AW18_09810*	This work
pUC57-r-α-*gluC*	The same for ORF *AW18_01930*	This work
pUC57-r-β-*gluD*	The same for ORF *AW18_08145*	This work
pUC57-r-β-*gluE*	The same for ORF *AW18_08090*	This work
pET28a(+)	Expression vector under the T7 expression region carrying N- and C-His-Tag sequences, Km^r, 5369 bp	Novagen
pET28a(+)-r-β-*gluA*	ORF *AW18_01575* cloned in pET28a(+)	This work
pET28a(+)-r-β-*gluB*	ORF *AW18_09810* cloned in pET28a(+)	This work
pET28a(+)-r-α-*gluC*	ORF *AW18_01930* cloned in pET28a(+)	This work
pET28a(+)-r-β-*gluD*	ORF *AW18_08145* cloned in pET28a(+)	This work
pET28a(+)-r-β-*gluE*	ORF *AW18_08090* cloned in pET28a(+)	This work
p21/22	An *E. coli-L. lactis* shuttle vector derived from the pBM02 plasmid of L. *lactis* subsp. *cremoris* P8-2-47	Sánchez and Mayo (2004)
p21/22-r-β-*gluA*	ORF *AW18_01575* and promoter and terminator sequences in pUC57 cloned in p21/22	This work
p21/22-r-β-*gluD*	ORF *AW18_08145* and promoter and terminator sequences in pUC57 cloned in p21/22	This work
Oligonucleotide primers		
P21/22-F	GTAAAACGACGGCCAGTGAAGCTTCGAAATGATACACCAATCAG	This work
P21/22-R	GATCGGGTACCGAGCTCGTCTAGAGGATCCCAAACAACAGATAAAACG	This work

Am^r, Kmr, and Em^r, resistance to ampicillin, kanamycin and erythromycin, respectively.

Fig. 1. General strategy of designing the synthetic β-glucosidase-encoding genes for cloning and expression in *Escherichia coli* DH10B. P is the promoter of the erythromycin resistance gene of pE194 from *Staphylococcus aureus* and T is the terminator of the *rrnB* operon from *E. coli*.

changes.

Protein sequences of representative β-glucosidase encoded on the genome of several bifidobacteria species were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/protein/) and compared to those from *B. pseudocatenulatum* IPLA 36007 studied in this work using the Clustal X 2.0 software (Larkin et al., 2007). A phylogenetic tree was constructed with the DNAman program (Lynnon, San Ramon, CA, USA) using the neighbour-joining algorithm.

2.3. Cloning and expression of recombinant glucosidase genes in E. coli

The synthesized glucosidase genes were independently cloned into pUC57 by directional cloning, either using the corresponding restriction enzyme sites or employing the In-Fusion kit (Clontech, Mountain View, CA, USA) following the manufacturer's instructions. Electrocompetent *E. coli* DH10B cells were prepared as reported elsewhere (Sambrook & Russell, 2001) and transformed with the ligation mixtures in a Gene Pulser apparatus (Bio-Rad, Richmond, CA, USA). Transformants with β-glucosidase activity were revealed by transferring colonies to 2xTY agar plates containing ampicillin, IPTG, and the chromogenic substrate X-Glu. Plasmid DNA of positive colonies showing blue colour on the plates was purified by using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA). Constructs were verified by restriction endonuclease digestion and sequencing.

2.4. Glucosidase activity in E. coli cell-free extracts

The β-glucosidase activity of the constructs was measured in cell-free extracts using *p*-nitrophenyl(*p*NP)-β-D-glucopyranoside (*p*NP-β-Glu) (Sigma-Aldrich) as a substrate. The reaction mixture was composed of 800 µL of 40 mM sodium acetate buffer (pH 5.5), 100 µL of 10 mM of *p*NP-β-Glu or *p*NP-α-Glu, and 100 µL of each of the cell-free extracts. After 30 min at 37 °C the reaction was stopped by adding 1 mL of cold 1 M sodium carbonate, and the solution clarified by centrifugation at 19,000 × *g* for 10 min at 4 °C. The *p*NP released was determined spectrophotometrically, measuring the increase in absorbance at 410 nm against a calibration curve of *p*NP (Sigma-Aldrich). α-Glucosidase activity was measured in a similar manner by using as a substrate *p*NP-α-D-glucopyranoside (Sigma-Aldrich).

2.5. Daidzin deglycosylation activity of E. coli cell-free extracts

Overnight cultures were pelleted, suspended in 0.1 M phosphate buffer (pH 6.8) containing 100 µM daidzin (LC Labs, Woburn, MA, USA) and incubated at 37 °C for 4 h. The isoflavone aglycones were then extracted following the procedure of Guadamuro et al. (2015) with modifications. Briefly, samples were centrifuged at 800 rpm for 10 min; 3 mL of each supernatant were then collected and their isoflavone contents extracted using Bond Elut-C18 solid-phase cartridges (Agilent, Santa Clara, CA, USA). After drying, the organic extracts were dissolved in 100 µL of HPLC grade methanol. A 10 µL aliquot of the sample was then loaded onto 60 F_{254} thin layer chromatography (TLC) silica gel plates (Merck), developed in a vertical developing chamber with a 2:1 toluene:acetone (both from Merck) solvent system, and revealed under UV light (256 nm).

2.6. Cloning of the constructs in an overexpression system

To overexpress the β-glucosidases as His-tagged proteins from the chimeric glucosidase genes, plasmid DNA of the constructs in pUC57 was purified using the High Pure Plasmid Isolation kit (Roche, Basel, Switzerland), digested with the restriction enzymes *Nhe*I and *Bam*HI, and ligated with T_4 DNA ligase (Roche) to double digested pET28a(+) (Novagen, San Diego, CA, USA). The ligation mixtures were then electrotransformed into *E. coli* DH10B. Constructs in pET28a(+) were verified by digestion with restriction enzymes and sequencing. Finally, DNA of the constructs in pET28a(+) was purified as above and transformed into the overexpression host *E. coli* BL21(DE3) (Novagen).

2.7. Purification of recombinant proteins

Selected transformants of *E. coli* BL21(DE3) containing each of the pET28a(+) constructs (Table 1) were incubated overnight with agitation at 37 °C in LB broth supplemented with 25 µg mL^{-1} kanamycin. The cultures were then re-inoculated into 300 mL of fresh medium, grown until mid-exponential phase (0.5 absorbance at 600 nm), and induced for r-*glu* overexpression by the addition of 1 mM IPTG. The cells were then incubated at 37 °C for 20 h, harvested by centrifugation, suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole), and disrupted at 2.3 bar in a FastPrep cell disruptor (Constant Systems, Daventry, UK). Recombinant glucosidases were purified at 4 °C using Ni-nitrilotriacetic acid affinity chromatography as

recommended by the supplier of the latter compound (Qiagen). Purified proteins were electrophoresed under denaturing conditions in 6% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) in a mini-protean apparatus (Bio-Rad) and visualized with Coomassie Brilliant Blue R-250 (Bio-Rad).

The β-glucosidase activity of the purified proteins was measured using *p*NP-β-Glu as a substrate as described above for the cell-free extracts. Protein concentration was determined by the Bradford method using the BCA Protein Assay kit (Pierce, Rockford, IL, USA), employing bovine serum albumin as a standard. One unit of activity was defined as the amount that releases 1 μmol of *p*NP per min.

2.8. Biochemical characterization of the purified enzymes

To examine dependence on pH, the activity of the purified enzymes was measured against the substrate *p*NP-β-Glu between pH 3.0 and 8.0 (achieved using citrate or phosphate buffers) and recording the *p*NP produced. Temperature dependency was measured using the same substrate over the range 10–90 °C. The kinetics of hydrolysis was also determined using *p*NP-β-Glu (0.1–8.0 mM), after verification of the reaction rate linearity, by stopping reactions at 30 min and measuring the *p*NP released as above. Additionally, substrate specificity was evaluated using the following *p*NP-derivatives: *p*NP-β-D-galactopyranoside (*p*NP-β-Gal), *p*NP-β-D-arabinopyranoside (*p*NP-β-Ara) and *p*NP-α-D-glucopyranoside (*p*NP-α-Glu) (all from Sigma-Aldrich). Purified enzymes were also assayed against maltose, gentiobiose, melibiose, cellobiose and isomaltose (all from Sigma-Aldrich, except maltose from Merck) (2 μg purified enzyme, 10 mM of each substrate sugar in 40 mM sodium acetate buffer [pH 5.5]) overnight at 37 °C. Sugar hydrolysis was also investigated by TLC. One microliter of each reaction was loaded onto TLC plates and developed in a vertical chamber using a 2:1:1, ethyl acetate, acetic acid and water solvent system. Spots were revealed by heating at 120 °C after spraying with 5% sulphuric acid in ethanol.

The deglycosylation of daidzin and genistin was performed in 50 mM phosphate buffer (pH 6.0) with 9 μg of purified r-Glu and 750 μM daidzin- or genistin-7-O-β-D-glucoside (both from Sigma-Aldrich) in a final volume of 65 μL. After overnight incubation at 37 °C, the mixture was heated at 95 °C for 5 min and cooled on ice. Reactions were visualized by TLC. For this, 10 μL of each reaction was loaded onto the TLC plates, and developed and revealed as above.

2.9. Cloning and expression of r-β-glu genes in Lactococcus lactis MG1363

Construction of a recombinant *Lactococcus lactis* MG1363 strain expressing r-β-*glu* genes was achieved using the In-Fusion system as above. Briefly, r-β-*glu* genes were PCR amplified from the constructs in pUC57 using primers P21/22-F and P21/22-R (Table 1) in order to add to the amplicons 20 bp sequences that were identical to the extremes of *Eco*RI linearized vector p21/22 (Sánchez & Mayo, 2004). After the In-Fusion reaction, *L. lactis* MG1363 was electrotransformed with the mixture and transformants selected onto GM17 agar plates containing erythromycin (5 μg mL^{-1}) and X-Glu. Plasmid DNA was isolated from randomly selected blue colonies by a modification of the procedure of O'Sullivan and Klaenhammer (1993), involving the addition of 4 μL of mutanolysin (5 U mL^{-1}) and 20 μL of proteinase K (20 mg mL^{-1}) to the lysis buffer before phenol/chloroform extraction. Constructs were verified by digestion with restriction enzymes.

3. Results

3.1. Cloning, expression and overexpression of glycosidase genes in E. coli

Five ORFs from the genome of *B. pseudocatenulatum* IPLA 36007, encoding putative β-glucosidases (*AW18_01575*, *AW18_09810*, *AW18_08145*, and *AW18_08090*) or α-glucosidases (*AW18_01930*), were synthesized (r-β-*gluA*, r-β-*gluB*, r-α-*gluC*, r-β-*gluD* and r-β-*gluE*, respectively) under the control of synthetic heterologous promoter and terminator sequences, following the general outline shown in Fig. 1.

The synthetic genes were cloned in pUC57, and glucosidase-active clones in *E. coli* retrieved from agar plates containing chromogenic substrates. Positive transformants were obtained for all genes. The r-α-*gluC* clone and the α-glucosidase it encoded were used as β-glucosidase negative controls in the majority of subsequent experiments. TLC analysis of the reactions between isoflavone-glycosides and *E. coli* cell-free extracts revealed all clones (except, as expected r-α-*gluC*) to produce daidzein from daidzin (Supplementary Fig. 1).

For protein overexpression, DNA of all pUC-derived clones was digested with the restriction enzymes *Nhe*I and *Bam*hI and cloned into double digested pET28a(+). The new constructs were obtained and verified in *E. coli* DH10B. After the transfer of the clones to *E. coli* BL21(DE3), cultures carrying pET28a(+)-derivatives were induced with IPTG and the overexpressed proteins purified to SDS-PAGE homogeneity by Ni-nitrilotriacetic acid affinity chromatography. By way of example, Supplementary Fig. 2 shows an SDS-PAGE gel of the purification steps for $GluB_{His}$ β-glucosidase. The molecular mass of the purified enzymes agree well with the calculated molecular mass of their respective gene product. The purification yields for $GluA_{His}$, $GluB_{His}$, $GluC_{His}$, $GluD_{His}$ and $GluE_{His}$ were 0.80, 3.29, 6.39, 3.54, and 4.32 mg L^{-1} of culture respectively. The low purification yield of $GluA_{His}$ might result from toxicity of the overexpression of this protein in *E. coli*, as confirmed by the selective counting of colonies before and after IPTG induction (data not shown). Introducing the pET28a(+)-r-β-*gluA* construct into *E. coli* BL21(DE3)-pLysS in an attempt to improve the purification of $GluA_{His}$ by avoiding the leaky expression of this β-glucosidase was unsuccessful.

3.2. Characterization of purified β-glucosidases

As expected, the purified $GluA_{His}$, $GluD_{His}$ and $GluE_{His}$ showed activity against the chromogenic substrate *p*NP-β-Glu with a specific activity of 1.74, 390.18 and 219.95 μM/mg/min respectively. The low activity of $GluA_{His}$ against *p*NP-β-Glu might be explained by the low yield, as reported above, and the concomitant presence in the eluate of several protein bands without β-glucosidase activity (which might be degradation products of this toxic enzyme; data not shown). Surprisingly, the $GluB_{His}$ enzyme showed no activity against any of the *p*NP derivatives analysed. As anticipated, $GluC_{His}$ showed activity with *p*NP-α-Glu but not towards *p*NP-β-Glu.

As revealed by TLC, all four β-glucosidases were active against the isoflavone glycosides daidzin and genistin, releasing the aglycones daidzein and genistein respectively (Fig. 2). Though the products of the reactions were not quantified, $GluA_{His}$ and $GluD_{His}$ seemed to produce more aglycones than $GluB_{His}$ and $GluE_{His}$ under the same assay conditions. The TLC technique was also used to examine the activity of the purified enzymes against several α- and β-glucoside sugars. $GluD_{His}$ and $GluE_{His}$ hydrolysed β-glucosides such as cellobiose (O-β-D-glucosyl-[1 → 4]-D-glucose) and gentiobiose (O-β-D-glucosyl-[1 → 6]-D-glucose), but, as expected for a β-glucosidase, not against α-glucosides such as maltose (O-α-D-glucosyl-[1 → 4]-D-glucose), isomaltose (O-α-D-glucosyl-[1 → 6]-α-D-glucose) or melibiose (O-α-D-galactosyl-[1 → 6]-D-glucose). In contrast, $GluA_{His}$, $GluB_{His}$ degraded none of these disaccharides (Supplementary Fig. 3).

The maximum relative activity against *p*NP-β-Glu of $GluA_{His}$, $GluD_{His}$ and $GluE_{His}$ laid between pH 5.0 and 6.5. $GluA_{His}$ had an optimum pH of around 6.5; that of $GluD_{His}$ and $GluE_{His}$ was pH 5.5. $GluA_{His}$ showed an optimum temperature of 30–35 °C, with relatively strong activity between 20 and 50 °C. $GluD_{His}$ and $GluE_{His}$ showed peaks of activity at 40 and 45 °C respectively, declining sharply at lower and higher temperatures (Fig. 3). The V_{max} for $GluD_{His}$ and $GluE_{His}$ was 75.26 and 10.10 μM min^{-1} respectively; the K_m was 0.69 and 10.78 mM respectively. For technical reasons, the kinetics of $GluA_{His}$

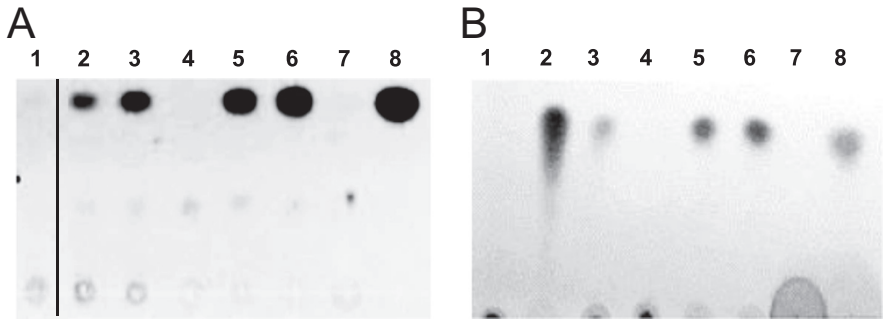

Fig. 2. TLC showing deglycosylation activity of purified β-glucosidases from *B. pseudocatenulatum* IPLA 36007 on daidzin (A) and genistin (B). Lanes 1, 2, 3, 4, 5 and 6, daidzin or genistin, after overnight incubation at 37 °C without enzyme, and with the crude extract of $GluA_{His}$ and the purified enzymes of $GluB_{His}$, $GluC_{His}$, $GluD_{His}$, and $GluE_{His}$, respectively. Lanes 7, daidzin and genistin markers, respectively. Lanes 8, daidzein and genistein markers, respectively.

(low protein concentration) and $GluB_{His}$ (lack of activity against *p*NP-β-Glu) could not be determined.

3.3. Heterologous expression in Lactococcus lactis

To provide proof of concept that the functional expression of isoflavone-deglycosylation activity can be transferred to a lactic acid bacterium (LAB), r-β-*gluA* and r-β-*gluD* were selected for cloning and expression in *Lactococcus lactis*. Plasmids of the corresponding constructs in pUC57 were digested with *Hin*dIII and *Xba*I and cloned into the *E. coli*-LAB shuttle vector p21/22. The new constructs (Table 1) were obtained in *E. coli* DH10B and then transformed into L. *lactis* MG1363 by electroporation. The β-glucosidase activity of L. *lactis* cells harbouring p21/22-r-β-*gluA* and p21/22-r-β-*gluD* on *p*NP-β-Glu was respectively 5 and 76 times greater than that of the controls. TLC analysis showed that both transformants could convert daidzin into daidzein (Supplementary Fig. 4).

4. Discussion

In this work, five genes from the genome of *B. pseudocatenulatum* IPLA 36007 thought to encode α- and β-glucosidases were cloned into *E. coli*, overexpressed, and their proteins purified and characterized. Though not well studied, evidence is accumulating for direct (Trindale, Abratt, & Reid, 2003) and reverse (Parche et al., 2006) regulatory carbon catabolite repression (CCR) mechanisms in bifidobacteria. In fact, open reading frames encoding *Lac*I-like regulatory proteins have been seen in the vicinity of most glycosidase-encoding genes (Alegría et al., 2014). To avoid regulatory carbon catabolite repression (CCR) interference during cloning and expression, the native promoter and terminator sequences of the selected genes were replaced during synthesis by heterologous signals. In this work, the promoter of the erythromycin resistance gene of pE194 from *S. aureus* and the ρ-independent terminator of the *rrnB* operon from *E. coli* were used as signals for heterologous expression of the glucosidase genes. These signals have already been shown to be recognized by Gram-positive and Gram-negative bacteria (Horinouchi & Weisblum, 1982; Schwendener & Perreten, 2015).

Compared to α-glucosidases (encoded by the so called *agl* genes in the literature) (Kelly et al., 2016; Pokusaeva et al., 2011; van den Broek, Hinz, Beldman, Vincken, & Voragen, 2008), the β-glucosidases of bifidobacteria have been poorly studied. β-Glucosidases cleave β-D-glycosidic linkages, liberating glucose moieties from phytoestrogen-glycosides (del Rio et al., 2013). As such, β-glucosidase activity in bifidobacteria has been associated with the conversion of sennosides from senna and rhubarb to sennidins (Yang, Akao, Kobashi, & Hattori, 1996), the activation of the phytotoxin cycasin into a mutagenic compound (Choi, Kim, & Ji, 1996), and the hydrolysis of isoflavone-glycosides into aglycones (Donkor & Shah, 2008; Raimondi et al., 2008; Rekha and Vijayalakshmi, 2011). The latter activity is thought to be pivotal in the absorption and subsequent metabolism of soy isoflavones, the consumption and adequate metabolism of which are considered beneficial to human health (Mayo, Guadamuro, Flórez, & Delgado, 2016).

The β-glucosidases of *B. pseudocatenulatum* IPLA 36007 characterized here are part of a vast array of genes within its genome that code for carbohydrate-modifying enzymes. Of these, 52 genes might encode different glycosyl hydrolases, and between seven and nine have already been considered that may code for β-glucosidases (Alegría et al., 2014). All four β-glucosidases characterized in this work were shown to deglycosylate the isoflavone-glycosides daidzin and genistin, releasing the aglycone moieties daidzein and genistein respectively (Fig. 2). However, although the enzymatic redundancy for deglycosylation in IPLA 36007 is here shown to be large, additional β-glucosidases from this strain may further act on soy isoflavones. All four enzymes showed differences in activity at different pHs and temperatures under otherwise similar conditions (as shown for two of them in Fig. 3), which suggests they might have complementary activities under distinct environmental conditions. However, the differential efficiency in transforming the isoflavone-glycosides into aglycones by the different enzymes could not be quantified. The K_m and V_{max} of some enzymes might be measurable using *p*NP-β-Glu, but the poor solubility of isoflavone-glycosides in water and the obligate need of using organic solvents that can otherwise be detrimental for enzyme activity, hindered the collection of results for some others.

The isoflavone-glycoside deglycosylation activity of bifidobacterial whole cells (i.e., not pure enzymes) varies widely between species and

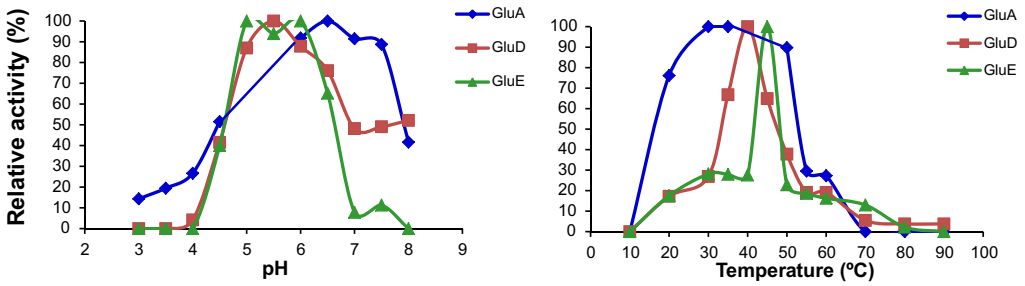

Fig. 3. Effects of pH and temperature on the activity of the purified β-glucosidases $GluA_{His}$, $GluD_{His}$ and Glu_{His} from *B. pseudocatenulatum* IPLA 36007. Experiments were performed in triplicate. Activity of each enzyme at the optimum pH and temperature was defined as 100%.

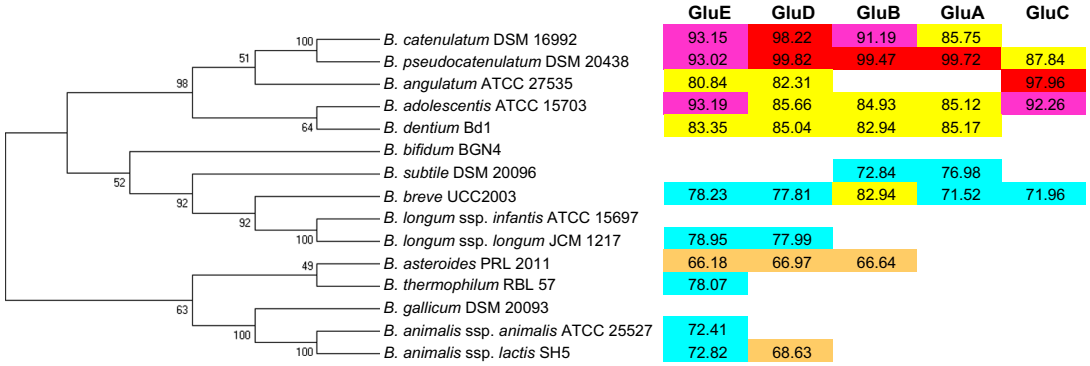

Fig. 4. Phylogenetic tree of different β-glucosidases present in the genome of sequenced strains from several bifidobacterial species (left) and amino acid sequence identity to those of *B. pseudocatenulatum* IPLA 36007 (right). Similar percentages of identity between the proteins are coded with the same colour.

strains (Raimondi et al., 2008; Wei, Chen, & Chen, 2007; Tsangalis et al., 2002). Genomes of bifidobacteria are plenty of glucosidase-encoding genes (Milani et al., 2015), of which many are β-glucosidases. Indeed, genome-sequenced bifidobacterial strains contain between two (*Bifidobacterium bifidum* S17) and 17 (*Bifidobacterium dentium* Bd1) genes encoding putative β-glucosidase (for a recent review see O'Callaghan and van Sinderen, 2016), which mostly fall within the CAZy GH families 1, 3 and 5 (http://www.cazy.org/). Orthologous genes encoding β-glucosidases related to those characterized in this work were shown to be present in representative genomes of several bifidobacteria species (Fig. 4). However, as shown in the figure, the enzymes characterized in this study showed low homology to those reported in *B. animalis* subsp. *lactis* SH5, the single strain in which isoflavone deglycosylation activity has already been characterized (Youn et al., 2012). In addition to the number of genes, substrate specificity (as it happens for the different enzymes of this study) might also account for the deglycosylating differences among species and strains. None of the deduced proteins in the present study have shown a signal peptide-like sequence that might drive secretion (suggesting the enzymes to be all intracellular) (Alegría et al., 2014). Thus variations in activity might further be owed to differences in the ability to transport isoflavone-glycosides into the cells. However, the expression of two β-glucosidase genes in *L. lactis* accompanied by the acquisition of deglycosylation phenotype argues against the need for specialized transport.

In conclusion, four β-glucosidases from *B. pseudocatenulatum* were cloned in *E. coli*, overexpressed, purified and biochemically characterized. Improved knowledge of glycoside-degrading enzymes should facilitate the development of novel, more effective or more selective prebiotic compounds to specifically enhance numbers of bifidobacteria species metabolizing them as a mean to promote health in the human gut in way similar to the current use of oligosaccharides and inulin (Oliveira et al., 2012). It should also help to design and use of novel or renew probiotics, based on their, scientifically-sound, health-related activities, such as the transformation of isoflavone-glycosides into aglycones. Probiotic strains having definite health benefits with well-known underlying operating mechanisms should replace currently-in-use "all purpose" probiotics. Finally, it could further help in the transfer of enzyme-associated functionality to technologically-relevant starter and probiotic strains. The heterologous expression of β-glucosidase genes from *B. pseudocatenulatum* in *L. lactis* confirmed the possibility of using recombinant LAB strains for the production of aglycone-enriched foods.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2017.07.024.

Authors' contribution statement

BM designed the study, provided material and human resources, contributed to discussion of the results, and wrote the manuscript. ABF, LG, AA, and LV conducted the experiments, contributed to the discussion, revised the text and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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DISCUSIÓN GENERAL

GENERAL DISCUSSION

Discusión general

Las isoflavonas han ido cobrando un interés creciente en las últimas décadas en el mundo occidental (Messina, 2010). Son muchos los trabajos que asocian su ingesta con diversos efectos positivos sobre la salud, entre los que destaca su utilidad para tratar los síntomas de la menopausia (Messina, 2016). Sin embargo, aún es necesario identificar todos los factores responsables de la variabilidad en la respuesta fisiológica y la inconsistencia de los datos clínicos, derivados, muy probablemente, de diferencias interindividuales en el metabolismo de estos compuestos. Las diferencias metabólicas pudieran estar determinadas por diferencias en la composición y funcionalidad de la microbiota intestinal (Qin y cols., 2010). De esta forma, los individuos podrían clasificarse en "metabotipos" según su capacidad para producir metabolitos microbianos específicos derivados de las isoflavonas, los cuáles podrían ser los efectores de los beneficios de las isoflavonas sobre la salud (Tomás-Barberán y Selma, 2016). De hecho, algunos metabotipos son más corrientes en ciertas enfermedades o desórdenes asociados con disbiosis microbianas intestinales, como el fenotipo no productor de equol que parece ser abundante en individuos obesos (Tomás-Barberán y Selma, 2016). Por el contrario, en los últimos años se está asociando el metabotipo productor de equol con personas que responden mejor a determinados síndromes tras la ingesta de isoflavonas (Birru y cols., 2016; Takeda y cols., 2016; Igase y cols., 2017).

En este contexto el primer objetivo de la Tesis ha sido estudiar el efecto modulador de las isoflavonas sobre las comunidades microbianas intestinales, los metabolitos microbianos de las isoflavonas y la formación de otros compuestos del metabolismo intestinal (como los derivados fenólicos o los AGCC) que resultan de la interacción de las isoflavonas con los microorganismos intestinales. Este objetivo se dirige a profundizar en los efectos y mecanismos que pudieran gobiernar la respuesta fisiológica de las isoflavonas en el hombre.

El segundo objetivo se ha dirigido a la identificación y caracterización de microorganismos y sus enzimas con actividad de desglicosilación de isoflavonas para su posible utilización en la elaboración de productos fermentados funcionales a base de soja. Los productos resultantes, enriquecidos en agliconas (formas más biodisponibles y

activas que las isoflavonas precursoras), bien pudieran promover o incrementar sus efectos beneficiosos en el consumidor.

Efecto de la ingesta de isoflavonas sobre la microbiota intestinal y perfil de metabolitos resultante

Para llevar a cabo este estudio se reclutó un grupo de 16 mujeres menopáusicas que iniciaban tratamiento para paliar los síntomas de la menopausia con un concentrado de isoflavonas de soja (Fisiogen, Zambon). Las mujeres voluntarias se reclutaron en el Servicio de Ginecología del Hospital de Cabueñes (Gijón), y su salud intestinal se evaluó en el Servicio de Digestivo del mismo hospital. El estudio de los cambios en la composición microbiana en heces y de los metabolitos en heces y orina se llevó a cabo a lo largo de un periodo de seis meses de tratamiento, durante el cual las mujeres tomaban una cápsula diaria con un contenido en isoflavonas de 80 mg. A lo largo de este tiempo, se tomaron muestras de heces y orina antes del inicio del tratamiento (muestra basal), y al primer, tercer y sexto mes de tratamiento.

Perfil de isoflavonas y polifenoles excretados

En primer lugar, dada la dificultad para detectar y cuantificar estas sustancias en muestras biológicas, fue necesario desarrollar o mejorar metodologías analíticas para la determinación de compuestos fenólicos en orina y heces humanas. En un primer trabajo se puso a punto un método, basado en una extracción en fase sólida y posterior cuantificación mediante un equipo UHPLC, para la determinación simultánea de daidzeína, genisteína y el equol en muestras de orina. Si bien existían previamente otros trabajos en los que se describen metodologías para el análisis independiente de estos compuestos por cromatografía líquida (Wilkinson y cols., 2002; Wu y cols., 2004; Baranowska y Magiera, 2011), este es el primer trabajo donde se analizan de forma simultánea todos ellos. La principal novedad del método consistió en la utilización de un detector de fluorescencia para el análisis de equol. Esta molécula posee una baja absorbancia de luz ultravioleta, a diferencia del resto de isoflavonas que presentan un máximo de absorción en el rango 240-270nm, y por ello los detectores “photodiode array” (PDA) presentan una sensibilidad demasiado baja para su detección. Con el método desarrollado en esta Tesis se alcanzó una sensibilidad en la detección de daidzeína y genisteína tres veces superior a la descrita en trabajos previos usando un sistema UHPLC (Baranowska y cols., 2011) y superior a la descrita para el equol

mediante el empleo de metodologías de HPLC (Maubach y cols., 2003). El límite de cuantificación ("limit of quantification"; LOQ) del equol también se mejoró ligeramente (de 9,4 nM a 8,89 nM). Este incremento en la sensibilidad de detección y cuantificación de equol, obtenido gracias a la combinación del uso de cartuchos de extracción en fase sólida (que permiten la concentración de la muestra biológica), el empleo de un sistema UHPLC (que permite inyectar volúmenes muy pequeños) junto con el uso de un detector de fluorescencia, resulta de suma importancia para determinar la excreción de este compuesto en orina en respuesta a la ingesta de isoflavonas, la detección y cuantificación resultó esencial para diferenciar las mujeres "productoras" de equol (en general, "respondedoras" a la ingesta de isoflavonas) de las "no productoras" (y, en general también, "no respondedoras").

Con los datos obtenidos se clasificaron las mujeres del estudio como productoras o no productoras de equol de acuerdo al límite de corte establecido por Rowland y colaboradores de una concentración de equol en orina de 1000 nM (Rowland y cols., 2000). Debido a que las muestras de orina fueron tomadas puntualmente y no recogidas durante 24 horas como establecen los procedimientos más aceptados, se determinó también la creatinina en orina y se consideró el ratio equol/creatinina para normalizar el factor de dilución de las muestras (Knudsen y cols., 2000). Estableciendo como límite un ratio equol/creatinina >5, obtuvimos el mismo número de mujeres productoras que con el criterio de excreción de equol en orina: cuatro, lo que equivale al 25% de las mujeres analizadas. Este porcentaje está en consonancia con los porcentajes de producción de equol descritos en la literatura en poblaciones humanas occidentales (Atkinson y cols., 2005; Nakatsu y cols., 2014). En contraposición a lo denotado por Frankee y cols. (2012), el fenotipo productor de equol se mostró estable a lo largo del período de tratamiento de nuestro estudio para todas las mujeres positivas. Aunque en esta Tesis solo se muestra el fenotipo durante los seis meses de ensayo, el fenotipo se mantuvo tres años después de la finalización del seguimiento (Vázquez y cols., 2017a).

En un segundo trabajo, se abordó el desarrollo de un método basado en UPLC-ESI-MS/MS para la detección y cuantificación de 40 compuestos de naturaleza fenólica en heces incluyendo daidzeína, genisteína, DHD y *O*-DMA. Este último compuesto procede, al igual que el equol, del metabolismo de la daidzeína. El *O*-DMA lo produce más del 90% de la población (Gardana y cols., 2014) y, aunque algunos autores lo consideran un metabolito biológicamente inactivo, su papel y sus efectos fisiológicos

están en permanente discusión (Frankenfeld, 2011b). La daidzeína, genisteína, DHD y *O*-DMA mostraron una asociación directa con la ingesta de isoflavonas. Sin embargo, no todos los compuestos se detectaron en todas las muestras, lo que podría deberse a una degradación o transformación de los mismos en compuestos que no se detectaban bajo las condiciones analíticas del estudio. Sorprendentemente, en las muestras de una de las mujeres del estudio (mujer WN en Tabla 4, página 6 del artículo 2) no se detectaron isoflavonas o sus metabolitos ni en orina ni en heces (artículos 1 y 2 de la Tesis), lo que nos hace sospechar que, dado que los concentrados de isoflavonas son caros y no están cubiertos por la Seguridad Social, bien pudiera haber abandonado el tratamiento con isoflavonas en una fase temprana del muestreo.

En las muestras de heces no se detectó equol en ninguna de las mujeres analizadas, ni siquiera en aquellas clasificadas como "productoras" según su nivel de excreción de equol en orina. Esto nos confirma que es la orina y no las heces la principal ruta de excreción de equol en humanos tal y como han propuesto varios autores (Hall y cols., 2007; Peeters y cols., 2007). Cabe mencionar que en ninguna de las tres mujeres clasificadas como productoras de equol se observó excreción de *O*-DMA en las heces, mientras que entre las mujeres no productoras el compuesto se detectó (al menos ocasionalmente) en 10 de ellas. Esta observación podría estar relacionada con una dominancia de la ruta de producción de equol (cuando este compuesto se produce) sobre la ruta de producción de *O*-DMA. De hecho, varios autores han observado una correlación inversa entre la excreción de equol y *O*-DMA según recoge en su revisión Frankenfeld (2011b).

A lo largo de la intervención con isoflavonas, en las muestras de heces se observaron incrementos significativos respecto al tiempo basal en los ácidos 3-hidroxifenilacético y 3-fenilpropiónico. Estos compuestos, aunque pueden resultar del metabolismos celular y microbiano de diversos compuestos polifenólicos de la dieta (Coldham y cols., 2002; Barnes y cols., 2011; del Rio y cols., 2013) muy probablemente provengan de la degradación intestinal de las isoflavonas ingeridas.

Este es el primer estudio en el que se determina el patrón de cambios de los metabolitos fenólicos en heces tras una intervención con isoflavonas en humanos. Muchos de los metabolitos microbianos resultantes de las isoflavonas todavía contienen un grupo fenólico por lo que presentan un elevado poder antioxidante (del Río y cols., 2013) lo que podría favorecer la prevención del estrés oxidativo en el tracto intestinal

y/o en otros tejidos. El estudio de la composición y concentración de estos compuestos fenólicos merece mayor investigación ya que podría explicar algunos de los efectos positivos de la ingesta de isoflavonas sobre la salud. En este sentido, algunos autores han sugerido que los efectos beneficiosos de los flavonoides en general y las isoflavonas en particular pueden estar mediados por catabolitos bacterianos que, tras su reabsorción, están presentes en la circulación por períodos de tiempo mayores que sus precursores (Kay y cols., 2009).

Evolución de las poblaciones microbianas fecales

Para estudiar el efecto de las isoflavonas a largo plazo sobre la composición de la microbiota intestinal de las mujeres menopáusicas de este trabajo se utilizaron métodos de cultivo convencionales y métodos independientes de cultivo. En concreto, los métodos independientes de cultivo utilizados fueron la PCR cuantitativa (qPCR) y la electroforesis en gel con gradiente desnaturalizante (DGGE). Nuestra hipótesis de partida era que bien podría ser que alguno de los efectos positivos de la ingesta de estos compuestos pudiera estar mediado por cambios en la microbiota intestinal. Los efectos a largo plazo de las isoflavonas sobre la microbiota intestinal han sido escasamente estudiados *in vivo* (Clavel y cols., 2005; Bolca y cols., 2007; Nakatsu y cols., 2014). Exceptuando el estudio de Clavel y cols. (2005), con una intervención de dos meses de duración, el resto de trabajos se han centrado en cambios en las poblaciones microbianas durante períodos muy cortos de tiempo. Además, con la excepción del trabajo de Nakatsu y cols., (2014), en el que se llevaron a cabo análisis metagenómicos, el número y tipo de grupos microbianos analizados es en todos los casos muy limitado, centrándose en grupos microbianos "indicadores" cuya representatividad numérica y actividad biológica en el ecosistema intestinal dista mucho de ser representativa.

En general, los datos experimentales obtenidos mostraron diversos cambios en la composición microbiana tras la ingesta de las isoflavonas. Sin embargo, dadas las grandes variaciones interindividuales observadas, resultó imposible establecer correlaciones entre la ingesta de estos compuestos y los cambios en la estructura y diversidad de las poblaciones microbianas intestinales. Tampoco se pudieron establecer correlaciones entre las poblaciones microbianas y el fenotipo productor de equol. Los estudios que nos han precedido relatan problemáticas similares para detectar cambios direccionales durante la ingesta de isoflavonas o efectos consistentes y repetibles en los

distintos consumidores (Clavel y cols., 2005; Bolca y cols., 2007; Nakatsu y cols., 2014).

Los recuentos obtenidos en los distintos medios de cultivo empleados mostraron cambios en las poblaciones microbianas en los distintos individuos, pero no se pudieron establecer patrones de cambio "generales" o "direccionales" asociados al tratamiento con isoflavonas. Se observó un incremento modesto en el grupo de las bifidobacterias tras el primer mes de intervención, sin embargo este aumento no persistió en el tercer y sexto mes. Algunos autores ya habían descrito un efecto bifidogénico de las isoflavonas (Clavel y cols., 2005, Nakatsu y cols., 2014). Sin embargo, y tal y como se comentó anteriormente, estos estudios se realizaron en períodos de intervención más cortos que los realizados en este trabajo. También observamos una disminución de las enterobacterias en las muestras de heces de la mayoría de las mujeres a lo largo del tratamiento, pero no en todas. Las isoflavonas o sus metabolitos, como otros compuesto de naturaleza polifenólica para los que se ha descrito previamente un efecto antimicrobiano (Mukne y cols., 2011), podrían presentar actividades inhibidoras sobre determinadas poblaciones susceptibles. En este sentido, resultados recientes de nuestro grupo han mostrado que las isoflavonas y sus metabolitos tienen efectos diferenciales sobre el crecimiento de distintas bacterias en el intestino (Vázquez y cols., 2017b). Por el contrario, estos compuestos podrían ejercer también un efecto estimulador sobre microorganismos que pudieran utilizarlas como fuente de energía. El resultado neto en cualquier caso, sería un aumento de unas poblaciones y disminución de otras. Sin embargo, en otras intervenciones nutricionales que nada tienen que ver con las isoflavonas (Lambert y cols., 2015; Kristensen y cols., 2016; Toscano y cols., 2017), ha quedado bien demostrado que muchos de los cambios son consecuencia de la composición de la microbiota basal (inicial) de cada individuo. De modo que, dada la gran variación inter-individual y el reducido número de mujeres de nuestro estudio, tal como hemos dicho, resulta difícil establecer correlaciones y establecer asunciones generales.

Debido a las limitaciones y posible sesgo que los métodos de cultivo convencionales presentan (entre las que se pueden mencionar la existencia de formas viables no cultivables o la presencia de poblaciones microbianas con requerimientos de cultivo específicos que no proveen los medios de cultivo empleados) (Browne y cols., 2016; Lau y cols., 2016), en el estudio de comunidades microbianas complejas se hace

necesario el empleo combinado de técnicas de cultivo y métodos moleculares independientes de cultivo (Leser y cols., 2002; Kemperman y cols., 2010; Li y cols., 2014).

Tras la ingesta de isoflavonas, los resultados obtenidos con la técnica de DGGE mostraron en algunas mujeres la intensificación de ciertas bandas de amplificación que correspondían a especies microbianas que ya habían sido asociadas con anterioridad en otros estudios con el metabolismo de polifenoles de la dieta, como *Lactonifactor longoviformis*, *Faecalibacterium prausnitzii*, *Bifidobacterium* spp., *Ruminococcus* spp. (Bolca y cols., 2007; Clavel y cols., 2007; Nakatsu y cols., 2014). Estos resultados parecen indicar que, al menos en algunas de las mujeres del estudio, las isoflavonas podrían afectar directamente a determinados miembros de la microbiota intestinal (Clavel y Mapesa, 2013). Los perfiles obtenidos con DGGE mostraron una clara agrupación de las muestras en función del factor "individuo" y no se agrupaban por el factor "tratamiento". Esto también nos está apuntando de nuevo a que los cambios producidos por las isoflavonas en las poblaciones microbianas dependen en gran medida de la microbiota basal individual de partida.

Los resultados de la qPCR mostraron también amplias variaciones intraindividuales en la abundancia de las poblaciones microbianas analizadas. Durante el período de intervención se observaron tendencias opuestas entre las mujeres productoras de equol y las no productoras en algunas de estas poblaciones. Por ejemplo, la población de bifidobacterias descendió en las mujeres productoras de equol, pero se incrementó en las no productoras; mientras que la población de *Bacteroides* mostró un comportamiento inverso. La población de enterobacterias fue significativamente menor en las heces de las mujeres con un metabotipo productor de equol en comparación con las muestras de las no productoras. En este último grupo de mujeres, las enterobacterias aumentaban de nuevo en el tercer y sexto mes de intervención. Igualmente, los dos grupos de *Clostridium* analizados mediante cebadores específicos (C. *leptum* o grupo IV y *C. coccoides* o grupo XIVa), se incrementaron en las mujeres productoras de equol, mientras que permanecieron estables en las no productoras. Por último, el género *Atopobium* se incrementó durante la intervención en los dos grupos de mujeres, pero en mayor medida en las no productoras donde el cambio en el tercer mes resultó ser estadísticamente significativo.

Ante los resultados obtenidos, se consideró que quizá la influencia de las isoflavonas podría producirse sobre poblaciones microbianas subdominantes o incluso minoritarias, que pudieran estar pasando desapercibidas por las metodologías (tanto de cultivo como independiente de cultivo) utilizadas, dado que mayoritariamente solo son útiles para el análisis de las poblaciones dominantes. Por ello en un trabajo posterior se utilizó la tecnología de secuenciación masiva (mediante pirosecuenciación) de amplicones del gen que codifica el ARNr 16S (ADNr). Con esta técnica se siguió la evolución de las comunidades bacterianas en una de las mujeres del estudio con un fenotipo productor de equol a lo largo de todo el tratamiento. Mediante esta aproximación fue posible obtener una imagen mucho más profunda de la composición y cambios en la estructura de las poblaciones microbianas presentes en las muestras de heces de esta mujer. Para distinguir las poblaciones viables de las muertas, las muestras de heces se procesaron, con anterioridad a la extracción del ADN, de forma alternativa con y sin monoazida de etidio ("ethidium monoazide"; EMA). Este compuesto penetra en el interior de las células muertas, se une covalentemente al ADN tras su activación por una luz potente e impide su posterior amplificación por PCR. El ADN así obtenido se sometió después a amplificación y los amplificaciones se secuenciaron y analizaron, comparando finalmente los resultados obtenidos con los de muestras no tratadas.

Los resultados de este trabajo mostraron cómo la ingesta de isoflavonas puede moldear la diversidad microbiana. Concretamente, y en consonancia con los resultados obtenidos por qPCR en mujeres con fenotipo productor de equol, las secuencias asignadas al género *Bifidobacterium* y *Escherichia/Shigella* disminuyeron tras la ingesta de isoflavonas y a lo largo de todo el tratamiento. Por otro lado, se observó un incremento de secuencias de la familia *Coriobacteriaceae* a la que pertenecen géneros relacionados con el metabolismo de isoflavonas (como *Eggerthella*, *Collinsella*, *Slakia* o *Coribacterium*) (Clavel y cols., 2014). En las heces de esta mujer se observó además un incremento en secuencias de los géneros *Ruminococcus* y *Faecalibacterium,* ambos géneros pertenecientes a la familia *Ruminococcaceae*, y de los géneros *Blautia* y *Coprococcus*, de la familia *Lachnospiraceae*. La familia *Lachnospiraceae* está presente en gran abundancia en el intestino humano y se ha asociado con la producción de ácido butírico (Meehan y Beiko, 2014), un AGCC con gran relevancia para la buena salud del tracto gastrointestinal (Ríos-Covián y cols., 2016). Igualmente, se observaron incrementos en secuencias del género *Dialister*, miembro de la familia *Veillonellaceae* y

recientemente asociado también con el metabolismo de isoflavonas (Nakatsu y cols., 2014). De hecho, se ha reportado el aislamiento de cepas del género *Veillonella* procedentes de heces humanas capaces de producir equol *in vitro* (Decroos y cols., 2005). Con el empleo de EMA diversos índices de diversidad bacteriana no paramétricos (ACE, Chao1) se redujeron, observándose un descenso por muestra de unas 200 unidades taxonómicas operacionales ("operational taxonomic units"; OTUs) (definidas por una similitud de secuencia ≥97%). Esto sugiere que muy posiblemente, algunos de estos tipos bacterianos se encuentran en las muestras en estados poco viables, lo que podría dificultar su propagación en cultivo y su aislamiento posterior. Sin embargo, la falta de conocimiento sobre la especificidad y eficacia de EMA en muestras biológicas complejas impide por el momento la obtención de conclusiones robustas (Wagner y cols., 2015).

Efecto de las isoflavonas sobre las poblaciones microbianas fecales *in vitro*

Para determinar si las poblaciones intestinales presentan *in vitro* un comportamiento similar al que habíamos observado en las muestras fecales *in vivo* por efecto de las isoflavonas, y para tratar de homogenizar la variabilidad debida a factores no controlados como la dieta o la genética de cada individuo, se realizó un experimento con cultivos fecales. Para ello se puso a punto un modelo de cultivos fecales en anaerobiosis utilizando un medio de cultivo rico no selectivo suplementado con isoflavonas de soja (en concreto con el mismo suplemento que las mujeres estuvieron tomando durante el periodo de intervención; Fisiogen, Zambon). El medio de cultivo se inoculó con muestras fecales de cuatro mujeres, tres de ellas clasificadas con el metabotipo productor de equol y una procedente de una mujer no productora. La diversidad y la composición bacteriana se determinaron mediante secuenciación masiva de amplicones del gen que codifica el ARNr 16S en una plataforma Illumina. Esta tecnología permite obtener una profundidad de secuenciación aún mayor que la pirosecuenciación (Mayo y cols., 2014). Además, para evaluar el impacto de la suplementación con isoflavonas en la actividad metabólica de las comunidades microbianas intestinales se cuantificó el equol y los AGCC mediante UHPLC y cromatografía de gases, respectivamente.

Con este modelo de cultivo fecal se logró la producción de equol *in vitro*, la cual se mantuvo tras sucesivos pases en dos de tres casos, lo que nos indicaba que en

nuestros ensayos los microorganismos productores de equol se mantienen viables y activos en las condiciones experimentales. Cultivos similares ya se habían descrito en la literatura para el aislamiento de bacterias productoras de equol en cultivos puros (Matthies y cols., 2009; Tsuji y cols., 2010). Existen también muchos trabajos previos donde se describe la producción de equol en cultivos inoculados con heces de sujetos productores (Atkinson y cols., 2004; Decroos y cols., 2005; Gardana y cols., 2009). Sin embargo, en ninguno de estos trabajos se utilizó un medio colónico complejo ni se había estudiado la composición y evolución de las poblaciones microbianas a lo largo de los cultivos.

La composición microbiana de los cultivos fecales se analizó en un primer momento mediante DGGE. Con esta metodología no se apreciaron cambios en las comunidades bacterianas mayoritarias tras la adición de isoflavonas al medio, lo que sugiere que las poblaciones mayoritarias quizás no se ven influidas por la presencia o no de isoflavonas en el medio de cultivo (y quizá en el intestino). Otra alternativa es que la técnica no tiene suficiente sensibilidad para detectar cambios en poblaciones subdominantes o en poblaciones minoritarias, que bien pudieran ser las responsables del metabolismo de las isoflavonas. El análisis estadístico multivariante utilizado para agrupar las muestras en función de su composición (NMDS y PCoA) mostró que los cultivos en los que se producía equol eran más similares entre sí que aquellos inoculados con heces de una mujer no productora. En contraste con los resultados obtenidos anteriormente con la técnica de la DGGE, los resultados de este trabajo sugieren la categorización de las mujeres en metabotipos ("productor", "no productor" de equol). La categorización parece ir acompañada de una composición taxonómica específica. En los cultivos que produjeron equol se observó un incremento de microorganismos relacionados con el metabolismo de las isoflavonas como *Collinsella*, *Dorea* y *Finegoldia* (Decroos y cols., 2005; Nakatsu y cols., 2014), y de forma más específica el de otros involucrados con la producción de equol como *Asaccharobacter celatus* (Minamida y cols., 2008)

La suplementación con isoflavonas en el medio de cultivo produjo también un incremento de secuencias correspondientes con microorganismos productores de ácido butírico como *Roseburia, Eubacteirum hallii* (del grupo XIVa de los clostridiales), *Faecalibacterium prausnitzii, Ruminococcus flavefaciens* y *Subdoligranulum variabile* (estas dos últimas especies pertenecientes al grupo IV de los clostridiales), como

también de productores de propiónico como *Blautia obeum*. Por otro lado, la suplementación del medio con isoflavonas produjo un descenso de las secuencias de algunos taxones como las de los géneros *Bacteroides* y *Parabacteroides*, lo que podría deberse, tal como ya se ha discutido, a una actividad antimicrobiana de las isoflavonas debida a su naturaleza polifenólica (Kemperman y cols., 2010). Cabe destacar que algunos de los géneros incrementados por la presencia de isoflavonas como *Ruminococcus* y *Faecalibacterium* de la familia *Ruminococcaceae,* y el género *Blautia*, también se vieron incrementados en las heces de esta mujer productora de equol en los resultados previos del análisis de muestras de heces mediante pirosecuenciación. Como se ha comentado con anterioridad, en el primer trabajo en el que se empleó qPCR también se habían observado incrementos en los grupos IV y XIVa de los clostridiales a los que pertenecen estos géneros. Todo ello apoya la validez de los resultados obtenidos con el modelo de cultivos fecales con isoflavonas.

La suplementación con isoflavonas en los cultivos resultó además en un incremento en la producción de los AGCC mayoritarios (acético, propiónico y butírico), como también la de los ácidos grasos de cadena corta ramificada (isobutírico e isovalérico). Los incrementos fueron estadísticamente significativos para los ácidos propiónico, isobutírico e isovalérico. La desglicosilación de las isoflavonas libera las agliconas y también azúcares. Éstos últimos podrían suponer una fuente de energía suplementaria para las bacterias intestinales capaces de desarrollarse en los cultivos. Los resultados están en consonancia con algunos estudios en los que se ha asociado la ingesta de ciertos compuestos prebióticos como beta-glucano o celobiosa con un incremento en la producción de AGCC minoritarios como el isobutírico y el isovalérico (Nilsson y cols., 2008; van Zanten y cols., 2014). Mientras que los AGCC mayoritarios en el intestino han sido extensamente estudiados (Flint y cols., 2015), el isobutírico y el isovalérico se producen por fermentación de proteínas y su metabolismo y papel en la fisiología intestinal no se conocen. Sin embargo, en pacientes con EII y con síndrome del intestino irritable se han descrito niveles reducidos de estos AGCC minoritarios (Le Gall y cols., 2011) lo que sugiere que puedan tener una gran implicación en la salud.

Este es el primer trabajo en el que se utiliza un extracto comercial de isoflavonas para suplementar el medio de cultivo y se analizan los consecuentes cambios en las poblaciones microbianas mediante secuenciación masiva de amplicones del ADNr 16S. No debe descartarse que la presencia de excipientes en los suplementos de isoflavonas

utilizados en los cultivos fecales pudiera tener alguna influencia en las comunidades microbianas. El modelo de cultivos fecales propuesto podría utilizarse en el futuro para el cultivo y aislamiento de bacterias productoras de equol. Éstas podrían utilizarse para la producción biotecnológica de equol como probióticos específicos en la población no productora.

Podemos concluir que tanto las técnicas analíticas como las distintas técnicas microbiológicas han registrado cambios a lo largo del tratamiento en las heces y orina de las mujeres de este estudio. A parte de los cambios relacionados directamente con la presencia de las isoflavonas mayoritarias (daidzeína, genisteína) y sus metabolitos derivados (dihidrodaidzeína, *O*-DMA, equol, etc.), que solo se registran tras el consumo de los suplementos, no se observaron otros cambios que pudieran relacionarse de forma directa con la ingesta de isoflavonas. Para establecer relaciones entre el consumo de isoflavonas, poblaciones microbianas intestinales y efectos beneficiosos sobre la salud, será necesario llevar a cabo estudios de intervención con mayor número de participantes y con un mejor control sobre la dieta. En esta Tesis se ha evidenciado que la complementariedad de técnicas analíticas y microbiologicas de distinto tipo permiten ahondar en la complejidad de estudio de los cambios y respuesta intestinal tras una suplementación/intervención ditetética. Numerosos y variables han sido los cambios detectados *in vivo* e *in vitro* en diversas poblaciones microbianas y/o en sus metabolitos a lo largo del tratamiento con isoflavonas. Hay que señalar que los aumentos o reducciones en los niveles de determinados microorganismos en el intestino puestos de manifiesto con las distintas tecnologías, incluyendo los de microorganismos supuestamente beneficiosos en este ecosistema como bifidobacterias, lactobacilos (Boesten y cols., 2008) y los tipos productores de AGCC (p. ej. butirato) (Ríos-Covián y cols., 2016), no son por el momento y por sí mismos marcadores válidos de salud; al menos no del mismo tipo que, por ejemplo, la reducción en el número de patógenos (Ahmed y cols., 2009). Comparando los resultados antes y durante el tratamiento, los datos microbiológicos y analíticos muestran, tras los cambios iniciales, una clara tendencia a la recuperación. Esta capacidad de autoregeneración se conoce con el nombre de "resiliencia" y tiene una gran importancia en el ecosistema intestinal (Sommer y cols., 2017). La microbiota intestinal es capaz de restaurar su equilibrio después de una perturbación externa, como puede ser la infección por un patógeno, el tratamiento con antibióticos o, como en este caso, la presencia de isoflavonas.

Cepas bacterianas con actividad desglicosiladora de isoflavonas y enzimas implicados

El Instituto de Productos Lácteos de Asturias (IPLA) del CSIC tiene como objetivos generales la creación de conocimiento a través de la investigación científica de calidad en Ciencia y Tecnología de Alimentos, a fin de favorecer y/o mejorar la salud y el bienestar de los ciudadanos, la competitividad del CSIC en el sector agroalimentario y la transmisión de conocimiento a la sociedad y el sector productivo. Para contribuir a esos objetivos generales del IPLA, en esta Tesis se planteó un subobjetivo con una orientación tecnológica para la identificación, caracterización y selección de microorganismos que tuvieran una utilidad práctica inmediata y pudieran transferirse a la industria alimentaria.

La combinación de soja con un microorganismo activador resulta de utilidad para conseguir una mejora de la biodisponibilidad de las isoflavonas que ingerimos. Las agliconas de isoflavonas se absorben más fácilmente y tienen mayor actividad biológica que sus precursores glicosilados (de Cremoux y cols., 2010). De esta forma, la utilización combinada de isoflavonas y microorganismos capaces de hidrolizarlas posibilitaría el desarrollo de alimentos funcionales a base de soja que bien pudieran aumentar y extender los beneficios sobre la salud de la soja y sus metabolitos más allá de la menopausia a la población general (Wei y cols., 2007). Los microorganismos con actividad sobre las isoflavonas podrían utilizarse para el diseño de alimentos funcionales (aportando su función durante la elaboración o el almacenamiento del alimento) (Rekha y Vijayalakshmi, 2011) o para la formulación de suplementos nutricionales (aportarían su función en el TGI tras su consumo). Para ello es importante que presenten actividades enzimáticas adecuadas sobre las isoflavonas y, pero también que, den lugar a productos con buenas propiedades sensoriales en las matrices alimentarias a las que se vayan a incorporar.

Para llevar a cabo la caracterización de microorganismos con estas propiedades se partió de una colección de BAL y bifidobacterias de las que se seleccionó un grupo de 8 cepas de lactobacilos y 2 de bifidobacterias -en su mayoría de origen intestinal- en función de su elevada actividad β-glucosidasa en extractos celulares. Estas enzimas se encargan de hidrolizar glucósidos de la dieta, incluyendo los de las isoflavonas, mediante la ruptura del enlace β-glucosídico. Con las cepas anteriores se ensayó experimentalmente la fermentación de dos bebidas de soja comerciales: Alprosoja (AS)

y ViveSoy (VS). La selección de bebidas de soja comerciales se basó en que, en la práctica, en nuestro país el consumo de leche de soja (extracto de habas de soja) sin aditivos es testimonial. En AS y VS se evaluó el crecimiento de las cepas, su actividad β-glucosidasa y su capacidad de liberar agliconas de las isoflavonas presentes en las bebidas. Además, en las bebidas de soja fermentadas se estudiaron los parámetros físico-químicos básicos y algunas características sensoriales como la viscosidad y la producción de compuestos volátiles.

La mayoría de los estudios en la literatura utilizan leche de soja pura, sin embargo los aditivos que se utilizan para mejorar el olor y sabor pueden influenciar el crecimiento de las bacterias fermentadoras y/o sus actividades enzimáticas (Chen y cols., 2012). En este trabajo se observó que los lactobacilos, con la excepción de tres cepas (*Lactobacillus plantarum* LL441, *Lactobacillus murinus* G64 y *Lactobacillus ruminis* B1411), crecieron bien en las dos bebidas de soja. Por el contrario, ninguna de las dos cepas de *Bifidobacterium pseudocatenulatum* empleadas creció en estas bebidas. Los recuentos de una de estas cepas (*B. pseudocatenulatum* C63) disminuyeron drásticamente tras la incubación, mientras que los de la otra (*B. pseudocatenulatum* C35 o IPLA 36007) se redujeron tan solo en una unidad logarítmica tras 72 h de incubación.

Tal como habíamos sospechado, la composición de cada bebida de soja influyó en la actividad desglicosiladora de las cepas. Así, en el caso de la bebida AS, se observó que todas las isoflavonas fueron desglicosiladas totalmente a las 24 horas por todas las cepas, excepto en las incubaciones con *L. ruminis* B1411 y *B. pseudocatenulatum* C6. Por el contario, en la bebida VS gran parte del contenido de isoflavonas permaneció sin ser desglicosilado por la mayoría de las cepas tras el mismo tiempo de incubación. La composición de las bebidas también influyó en la actividad β-glucosidasa de las cepas (cuantificada mediante el sustrato colorimétrico *p*-nitrofenil-β-D-glucopiranósido). En general, se observaron mayores actividades para la mayoría de cepas en la bebida AS respecto a la bebida VS, siendo especialmente mayores estas diferencias en el caso de las cepas de *L. casei* y *L. rhamnosus*. Estas diferencias en la actividad desglicosiladora pueden deberse a la influencia de los azúcares presentes en cada bebida. Estos azúcares (fructosa, sacarosa) pueden ser utilizados como fuente de carbono para el crecimiento bacteriano (Wei y cols., 2007; Iqbal y Zhu, 2009) y la activación de sus rutas de utilización puede reprimir la síntesis de las β-glucosidasas, las cuáles participan en la utilización de otros carbohidratos. Los resultados ponen de manifiesto la relevancia de

los aditivos que se añaden a los productos derivados de soja cuando se procesan posteriormente (p.ej., por fermentación).

Como fruto de este trabajo, se seleccionaron finalmente cuatro cepas capaces de desglicosilar los glicósidos de isoflavonas y liberar las agliconas tras su inoculación en los preparados de soja. Tres eran lactobacilos *(Lactobacillus casei* LP71, *Lactobacillus plantarum* E112 y *Lactobacillus rhamnosus* E41) con capacidad de crecer y acidificar las bebidas de soja, y la cuarta fue una cepa de *Bifidobacterium pseudocatenulatum* (IPLA 36007). Todas ellas presentan un gran potencial para ser utilizadas en la elaboración de alimentos funcionales a base de soja. La cepa *B. pseudocatenulatum* IPLA 36007 llevaba a cabo la activación de las isoflavonas sin crecer en las leches de soja, por lo que podría ser utilizada como cultivo adjunto en productos de soja fermentados. También podría emplearse en la elaboración de productos dulces (sin acidificar) o como probiótico participando en la biotransformación de las isoflavonas tras el consumo del microorganismo. La formación de isoflavonas activas aseguraría su presencia en el producto a consumir y/o en el intestino, aumentando la posibilidad de una correcta transformación y, al mismo tiempo, incrementando el valor añadido de productos y suplementos.

A continuación, con el objetivo de profundizar en el conocimiento de los mecanismos y actividades implicados en la liberación de agliconas de los glicósidos de isoflavonas, se llevó a cabo la secuenciación del genoma de la cepa de la bifidobacteria seleccionada: *B. pseudocatenulatum* IPLA 36007. Los genomas de las bifidobacterias contienen gran cantidad de genes que codifican glucosidasas (Milani y cols., 2015), muchos de los cuales son β-glucosidasas. Aunque en la actualidad hay descritas algunas cepas de BAL y bifidobacterias capaces de liberar agliconas de isoflavonas mediante β-glucosidasas (Tsangalis y cols., 2002; Raimondi y cols., 2008), estas enzimas están muy poco estudiadas y caracterizadas en comparación con otras glicosidasas como las β-galactosidasas (Carević y cols., 2015). Hasta ahora, solamente se ha caracterizado una β-glucosidasa con actividad desglicosilante de isoflavonas procedente de *Bifidobacterium animalis* subsp. *lactis* que ha sido clonada en *B. bifidum*.(Youn y cols., 2012). Esta enzima, sin embargo, tiene escasa relación con las β-glucosidasas de la cepa IPLA 36007.

Las especies de bifidobacterias constituyen uno de los grupos bacterianos mayoritarios que habitan el TGI humano y ayudan a mantener la salud intestinal

mediante diferentes mecanismos que incluyen la exclusión competitiva de patógenos, la modulación del sistema inmunológico, la producción de vitaminas esenciales y la aportación de nutrientes mediante la degradación de carbohidratos no digeribles de la dieta (O'Callaghan y van Sinderen, 2016). Los miembros del grupo *Bifidobacterium catenulatum* en concreto, que incluye a las especies *Bifidobacterium catenulatum* y *B. pseudocatenulatum*, son abundantes en muestras fecales de personas adultas (Junick y Blaut, 2012). Algunas cepas de *B. pseudocatenulatum* han mostrado diversas propiedades probióticas, como la posesión de enzimas degradadoras de antinutrientes (Haros y cols., 2009), la capacidad unirse a aminas aromáticas (Faridnia y cols., 2010) o de reducir los niveles de colesterol (Al-Sheraji y cols., 2012).

La cepa *B. pseudocatenulatum* IPLA 36007 había mostrado también con anterioridad una serie de propiedades de probiosis, incluyendo, entre otras, una buena supervivencia en condiciones que simulan las del TGI (elevada acidez y presencia de sales biliares), capacidad de unirse a las células epiteliales humanas, ausencia de actividades enzimáticas indeseables y la susceptibilidad a un grupo representativo de antibióticos (Delgado y cols., 2008). La capacidad de esta cepa para sobrevivir al tránsito gastrointestinal y colonizar el TGI podría explotarse para dirigir la actividad desglicosiladora de isoflavonas a posiciones distales del intestino humano. Por todo ello, decidimos explorar en mayor profundidad las características genéticas y las potencialidades bioquímicas de la cepa. En este contexto, la secuenciación del genoma completo de IPLA 36007 resulta de relevancia, no solo para el estudio del sistema enzimático implicado en la desglicosilación de las isoflavonas, sino también para la comprensión de los mecanismos moleculares subyacentes a sus propiedades probióticas. La secuenciación genómica puede ser relevante también para clarificar los aspectos de seguridad alimentaria de la cepa, dado que pertenece a una especie que por el momento no tiene la consideración de Presunción Cualificada de Seguridad (QPS) (EFSA, 2017). Este conocimiento es esencial para apoyar científicamente de forma racional su inclusión en alimentos funcionales y los supuestos beneficios que pueda proveer para la salud de los consumidores.

La secuenciación del genoma de *B. pseudocatenulatum* IPLA36007 reveló la presencia de una gran cantidad de secuencias codificantes de glicosil transferasas y glicosil hidrolasas (52 genes), incluyendo genes que codifican para xilanasas, pululanasas, amilomaltasas, α-amilasas y una maltodextrina glucosidasa. Esta gran

cantidad de genes que codifican enzimas degradadoras de carbohidratos es una característica común en las bifidobacterias (Pokusaeva y cols., 2011). Complementando las actividades fermentativas del hospedador y de otras bacterias intestinales (El Kaoutari y cols., 2013), las bifidobacterias utilizan un amplio rango de carbohidratos, muchos de los cuales son oligo- y polisacáridos derivados de plantas, que escapan a la digestión en las porciones superiores del intestino.

Entre los genes que codifican glicosil hidrolasas se encontraron cuatro genes anotados como posibles β-glucosidasas pertenecientes a la familia 3 de las glicosil hidrolasas (EC 3.2.1.21). Las cuatro β-glucosidasas compartían una limitada identidad aminoacídica (26.2 %). El análisis comparativo de las secuencias codificadoras de estas cuatro enzimas con las presentes en los genomas de otras bifidobacterias y depositadas en las bases de datos mostró que existen genes en otras cepas de bifidobacterias pertenecientes al grupo de *Bifidobacterium adolescentis* (*B. adolescentis*, *B. dentium*, *B. angulatum*, etc.) con capacidad para codificar enzimas homólogas con una identidad aminoacídica de entre el 80 y el 98%. Mientras que la homología de las β-glucosidasas de *B. pseudocatenolatum* IPLA 36007 con glicosil hidrolasas de otros grupos de bifidobacterias fue mucho menor (menos del 70%). De hecho, la similitud de las β-glucosidasas deducidas de IPLA 36007 con las β-glucosidasa de *B. animalis* subps. *lactis* SH5, la única cepa reconocida en la literatura con actividad sobre los glicósidos de isoflavonas (Youn y cols., 2012), fue muy baja (23% de identidad aminoacídica). Además de las enzimas estudiadas, cabe la posibilidad de que algunas de las otras glicosil hidrolasas de la cepa IPLA 36007 posea también actividad sobre los glicósidos de isoflavonas. La redundancia de actividades enzimáticas claves para la nutrición de los microorganismos está muy extendida entre las bacterias, tal y como se ha comprobado en otros sistemas enzimáticos como el proteolítico (Liu y cols., 2010).

Las cuatro β-glucosidasas mencionadas se sintetizaron *in vitro*, se clonaron en un sistema de sobreexpresión de *Escherichia coli* y las proteínas recombinantes se purificaron y caracterizaron. Además, dos de los genes se clonaron también en *Lactococcus lactis* para comprobar si la actividad desglicosiladora de isoflavonas podría transferirse a esta bacteria modelo, lo que posibilitaría dotar con esta actividad a otras cepas con características tecnológicas de interés en la industria de fermentación y de más fácil cultivo y manejo para su producción industrial.

Se comprobó que los cuatro genes seleccionados codificaban verdaderas β-glucosidasas activas sobre daidzina y genistina, si bien presentaron actividad diferencial a distintos pHs y temperaturas, lo que sugiere que, en condiciones fisiológicas, su actividad puede ser complementaria. Además, también mostraron diferentes especificidades de sustrato, las enzimas designadas como $GluD_{His}$ y $GluE_{His}$ mostraron actividad sobre los azúcares celobiosa (O-β-D-glucosil-[1→4]-D-glucosa) y gentiobiosa (O-β-D-glucosil-[1→6]-D-glucosa), mientras que las enzimas $GluA_{His}$ y $GluB_{His}$ no mostraron actividad sobre ninguno de los disacáridos ensayados.

Ninguna de las β-glucosidasas presentaba secuencias reconocibles de péptido señal que pudieran promover su secreción, lo que sugiere que se trata de enzimas intracelulares. Así, las variaciones de actividad detectadas entre distintas cepas podrían deberse también a diferencias en la capacidad de trasportar los glicósidos de isoflavonas al interior de las células. Sin embargo, el hecho de que la expresión de dos de las β-glucosidasas en *L. lactis* se acompañase de la adquisición del fenotipo desglicosilador parece indicar que no es necesario un transporte especializado.

La actuación de *B. pseudocatenolatum* IPLA 36007 sobre las isoflavonas de soja tal y como se encuentran en origen en la planta, junto con sus propiedades de probiosis hace de esta cepa un buen candidato para su empleo como fermento en alimentos funcionales a base de soja. Tanto esta cepa como los tres lactobacilos mencionados se han transferido de forma reciente a una empresa de cultivos iniciadores para su evaluación tecnológica y comercial.

CONCLUSIONES

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PRIMERA. Para analizar los principales metabolitos de las isoflavonas de la soja, se ha desarrollado un método de UHPLC basado en extracción en fase sólida. Este es el primer trabajo que describe un método en el que, combinando la detección mediante fotodiodo-array para las agliconas y fluorescencia para el equol, fue posible la identificación y cuantificación simultánea de daidzeína, genisteína y equol en muestras de orina humana. El método, además de más rápido y sensible que los descritos previamente, permitió clasificar a un grupo de mujeres menopáusicas en tratamiento con concentrados de isoflavonas en "productoras" y "no productoras" de equol en función de los niveles de excreción de este compuesto en orina.

SEGUNDA. Para analizar las isoflavonas en muestras fecales se ha optimizado un método de UPLC-ESI-MS/MS desarrollado previamente para el análisis de compuestos fenólicos totales en heces. El método se ha aplicado al análisis de compuestos fenólicos en muestras de heces de mujeres menopáusicas a lo largo del tratamiento con isoflavonas. El método optimizado permite detectar y cuantificar de forma simultánea 44 compuestos de naturaleza fenólica, incluyendo agliconas de isoflavonas y algunos de sus principales metabolitos.

TERCERA. La aplicación del método de UHPLC a muestras de orina permitió detectar en la población muestral a cuatro mujeres con el fenotipo "productor" de equol entre las 16 voluntarias del estudio, un porcentaje similar (25%) al descrito en diversas poblaciones occidentales. La aplicación del método de UPLC-ESI-MS/MS, por su parte, permitió determinar que el tratamiento diario a largo plazo con isoflavonas modifica el perfil fenólico de las heces, observándose un incremento significativo de los ácidos fenil-acético y fenil-propiónico. El aumento de estos compuestos tras la ingesta de isoflavonas sugiere que éstas siguen las vías habituales de degradación de otros polifenoles de la dieta.

CUARTA. La utilización de técnicas de cultivo y técnicas microbiológicas independientes de cultivo (como la qPCR y la DGGE) al estudio de los cambios en las poblaciones microbianas intestinales tras la ingesta de isoflavonas no permitió revelar asociaciones claras y uniformes entre grupos microbianos concretos o el fenotipo "productor" de equol con la ingesta de isoflavonas. La gran variación interindividual

observada en la microbiota basal de partida podría estar contribuyendo a enmascarar tendencias o patrones direccionales, aunque otros factores no controlados en el estudio (como la dieta) podrían también modular o encubrir los efectos de las isoflavonas sobre la microbiota.

QUINTA. La aplicación de técnicas microbianas moleculares más avanzadas como la secuenciación masiva de amplicones del gen que codifica el ARNr 16S a muestras de heces de una de las mujeres con fenotipo "productor" de equol permitió detectar cambios en poblaciones microbianas intestinales menos abundantes que no se habían detectado previamente con las técnicas anteriores. Mediante esta metodología se comprobó que, a largo plazo, la ingesta de isoflavonas conlleva aumentos persistentes en grupos microbianos intestinales minoritarios asociados el metabolismo de isoflavonas y/o con la producción de equol, incluyendo varios miembros de la familia *Coriobacteriaceae*. También se observaron incrementos significativos de diversos géneros de las familias *Ruminococcaceae* y *Lachnospiraceae*.

SEXTA. Se ha desarrollado un modelo de cultivos fecales en presencia de isoflavonas que ha permitido la producción *in vitro* de equol a partir de heces de mujeres con fenotipo "productor". La comparación de la composición microbiana de los cultivos fecales realizados en presencia y ausencia de isoflavonas, (determinada de nuevo mediante técnicas de secuenciación masiva), permitió revelar que diversos taxones involucrados en el metabolismo de las isoflavonas y/o la producción de equol se ven enriquecidos en los cultivos con isoflavonas. Entre ellos, y en consonancia con los resultados previos obtenidos en muestras de heces, destacaron incrementos de secuencias de los géneros *Collinsella* y *Eggerthella* de la familia *Coriobacteriaceae*, y también de *Faecalibacterium prausnitzii* y *Ruminococcus flavefaciens* (del grupo IV de los clostridiales) o *Eubacterium halii* y *Blautia obeum* (del grupo XIVa de los clostridiales), miembros de las familias *Ruminococcaceae* y *Lachnospiraceae* relacionados con la producción de ácidos grasos de cadena corta (AGCC). La concomitante detección de un aumento de la concentración de los AGCC en los cultivos fecales en presencia de isoflavonas corroboró los resultados obtenidos.

SÉPTIMA. Se caracterizó y evaluó tecnológicamente un grupo de cepas de lactobacilos y bifidobacterias seleccionadas por su actividad β-glucosidasa para su capacidad de fermentar dos bebidas comerciales de soja. Tres cepas de lactobacilos y una cepa de *Bifidobacterium pseudocatenulatum*, aisladas en su mayoría del tracto gastrointestinal,

permitieron obtener productos fermentados de soja con una alta concentración de agliconas, lo que proporciona una mayor biodisponibilidad de estos compuestos en comparación con sus precursores glicosilados, aumentando con ello el valor biológico y añadido de los productos. Por sus características bioquímicas y tecnológicas, las cuatro cepas podrían ser buenas candidatas para su ensayo y posible utilización como fermentos a escala industrial.

OCTAVA. La secuenciación y análisis del genoma de la cepa *B. pseudocatenulatum* IPLA 36007, reveló la presencia de al menos siete pautas abiertas de lectura (ORFs) con capacidad para codificar glicosil-hidrolasas de tipo β-glucosidasa. La clonación y expresión de cuatro de ellas permitió comprobar experimentalmente su actividad enzimática y la caracterización bioquímica de los enzimas purificados. Todos ellos presentaron actividad sobre la daidzina y la genistina, liberando, respectivamente, las agliconas daidzeína y genisteína. La clonación y expresión heteróloga de dos de los genes en *Lactococcus lactis* sugiere la posibilidad de transferir esta actividad β-glucosidásica a cepas de bacterias ácido-lácticas utilizadas como fermentos o probióticos, las cuáles podrían utilizarse para la elaboración de alimentos funcionales a base de soja enriquecidos en agliconas.

Conclusions

FIRST. An UHPLC method based on solid-phase extraction has been developed in order to analyse the main soy isoflavones metabolites. This is the first work describing a method that, combining a photodiode-array detector for aglycones and a fluorescence detector for equol, enabled the simultaneous identification and quantification of daidzein, genistein and equol in human urine samples. This methodology, faster and more accurate than others previously described, allowed classifying a group of menopausal woman under treatment with isoflavone concentrates as "equol producers" or "equol non-producers", according to the level of excretion of this compound in urine.

SECOND. In order to analyse isoflavones in faecal samples, an UPLC-ESI-MS/MS method previously developed for the analysis of total phenolic compounds in faeces has been optimized. The method was then applied to analyse these compounds in faecal samples from menopausal women along the treatment with isoflavones. This allowed the simultaneous detection and quantification of 44 phenolic compounds, including isoflavone aglycones and some of their main metabolites.

THIRD. The application of the UHPLC method to urine samples allowed the detection of four "equol producer" women among the 16 volunteers in our study, a similar percentage (25%) to that reported for several Western populations. The application of the UPLC-ESI-MS/MS method, on the other hand, allowed determining that a long-term daily treatment with isoflavones modifies the faecal phenolic profiles, with a significant increase of phenyl acetic and phenyl propionic acids. The increase of these compounds after ingestion of isoflavones suggests that they follow the usual degradation pathways of other polyphenolics from the diet.

FOURTH. The use of culture-dependent and culture-independent microbiological techniques (such as qPCR and DGGE) to study changes in the intestinal microbial populations during isoflavone consumption did not reveal clear and uniform associations between specific microbial groups or the "equol producer" phenotype with the isoflavone intake. The large inter-individual variation observed in the basal microbiota composition at the start of the treatment could mask trends or directional patterns. Further, other factors not controlled in the study (such as diet) could also modulate or hide away the effects of isoflavones on the microbiota.

FIFTH. The application of more advanced molecular microbial techniques such as next generation sequencing (NGS) of amplicons of the 16S rRNA gene to faecal samples from one of the women with an "equol producer" phenotype allowed to detect changes in less abundant intestinal microbial populations that had not been previously detected with other techniques. This methodology showed that, in the long term, isoflavone intake leads to persistent increases in minor intestinal microbial groups associated with isoflavone metabolism and/or equol production, such as members of the family *Coriobacteriaceae*. Significant increases of several genera of the *Ruminococcaceae* and *Lachnospiraceae* families were also observed.

SIXTH. A faecal culturing model has been developed, which allowed the production of equol *in vitro* in the presence of isoflavones when using as an inoculum faeces from women with an "equol producer" phenotypes. The comparison of the microbial composition of the faecal cultures performed in the presence and in absence of isoflavones (again determined by NGS techniques), revealed that several taxa involved in the metabolism of isoflavones and/or equol production were enriched in the faecal cultures with isoflavones. Among these, and in agreement with the previous results obtained in faeces, increases of reads from the genera *Collinsella* and *Eggerthella* of the family *Coriobacteriaceae* were found, as well as those of *Faecalibacterium prausnitzii* and *Ruminococcus flavefaciens* (*Clostridium* cluster IV) or *Eubacterium halii* and *Blautia obeum* (*Clostridium* cluster XIVa), members of the *Ruminococcaceae* and *Lachnospiraceae* families related to the production of short chain fatty acids (SCFA). The concomitant detection of an enhanced production of SCFA in the faecal cultures supplemented with isoflavones corroborated these results.

SEVENTH. A set of lactobacilli and bifidobacteria strains selected for their β-glucosidase activity were characterized and technologically evaluated for the fermentation of two commercial soy beverages. Three strains of lactobacilli and one strain of *Bifidobacterium pseudocatenulatum* (mostly isolated from the gastrointestinal tract) allowed the production of fermented soy products with a high concentration of isoflavone aglycones, providing greater bioavailability of these compounds as compared to their glycosylated precursors, thus increasing the biological and added value of the fermented products. Due to their biochemical and technological characteristics, the four strains could be good candidates to be used as starters at an industrial scale.

EIGHTH. Genome sequencing and analysis of the strain *B. pseudocatenulatum* IPLA 36007 revealed the presence of at least seven open reading frames (ORFs) capable of encoding glycosyl hydrolases of the β-glucosidase type. Cloning and expression of four of these ORFs allowed verifying experimentally their β-glucosidase activity and the biochemical characterization of the purified enzymes. All four showed activity on daidzin and genistein, releasing the corresponding aglycones daidzein and genistein. Cloning and heterologous expression of two β-glucosidase genes in *Lactococcus lactis* suggests the possibility of transferring this enzymatic activity to strains of lactic acid bacteria used as starter or probiotics for the manufacturing of functional, aglycone-enriched, soy-based foods.

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ANEXOS

ANNEXES

Informe sobre la calidad de los artículos

La información sobre la calidad de los artículos que integran esta memoria de Tesis Doctoral ha sido recogida de la Web Of Science (wos.fecyt.es/). Se han recopilado los siguientes parámetros: **el factor de impacto (FI)** de cada revista, el cual corresponde al año de publicación del artículo o en caso de los más recientes a los últimos datos publicados por Journal Citation Reports (año 2016); **el área SCI** a la que está asociada la revista y **el cuartil (Q)** de las revistas dentro de cada área, ordenadas por su factor de impacto. El número de veces que ha sido citado cada artículo **(Citas)** se ha obtenido también de la WOS en el momento de la escritura de la Tesis (Septiembre, 2017). En la tabla se incluyen los artículos de la Tesis que ya han sido publicados.

Manuscrito	Revista	Área	Q	FI	Citas
Manuscrito 1	Journal of Agricultural and Food Chemistry	Agriculture Chemistry Food Science & Technology	Q1	3,154	3
Manuscrito 2	Journal of Chromatography B	Biochemistry & Molecular Biology Chemistry	Q2	2,603	4
Manuscrito 3	Frontiers in Microbiology	Microbiology	Q1	4,076	11
Manuscrito 5	BMC Microbiology	Microbiology	Q2	2,644	0
Manuscrito 7	Gut Pathogens	Gastroenterology & Hepatology Microbiology	Q2	2,756	4
Manuscrito 8	Food Research International	Food Science & Technology	Q1	3,086	0

MATERIAL SUPLEMENTARIO ARTÍCULO 1

Supplementary Table 1: Identification and quantification of phenolic and indolic metabolites in faeces from menopausal equol-producing women (n=3) before (t=0) and during (t=1 through t=6) a six-month intervention with soy isoflavones concentrates.

Compound	Metabolite concentration[a] (µg/g feces)			
	t=0	t=1	t=3	t=6
Skatole	3.46 (n=1)[b]	1.82 (n=1)	1.99 (n=1)	nd[c]
Isoflavones				
Daidzein	nd	nd	0.29±0.01 (n=2)	4.98±4.65 (n=2)
Dihydrodaidzein	nd	nd	0.62 (n=1)	4.98±4.72 (n=2)
O-Desmethylangolensin	nd	0.04 (n=2)	nd	nd
Genistein	nd	nd	nd	3.23 (n=1)
Lignans				
Enterolactone	0.52±0.18 (n=3)	0.45±0.07 (n=3)	0.36±0.06 (n=3)	0.37±0.06 (n=3)
Enterodiol	0.75 (n=1)	nd	0.75 (n=1)	0.59 (n=1)
Mandelic acids				
3-hydroxymandelic acid	1.75±0.01 (n=2)	1.75±0.01 (n=3)	1.75±0.01 (n=2)	1.75±0.01 (n=2)
Benzoic acids				
Gallic acid	0.33 (n=1)	nd	0.57 (n=1)	nd
3,5-dihydrobenzoic acid	0.55±0.36 (n=2)	0.64±0.35 (n=2)	0.39±0.17 (n=2)	0.13±0.01 (n=2)
Protocatechuic acid	0.43±0.12 (n=2)	0.26±0.11 (n=3)	0.28±0.13 (n=3)	0.4±0.32 (n=3)
3-O-metilgallic acid	nd	nd	0.23±0.02 (n=2)	0.22 (n=1)
4-hidroxibenzoic acid	3.76±3.56 (n=2)	0.26±0.15 (n=2)	0.79±0.3 (n=2)	0.72±0.32 (n=2)
4-O-metilgallic acid	nd	nd	1.1 (n=1)	nd
3-hidroxibenzoic acid	1.24 (n=1)	0.91 (n=1)	0.69±0.38 (n=2)	0.72±0.18 (n=3)
Vanillic acid	nd	nd	1.56 (n=1)	nd
Syringic acid	nd	nd	nd	nd
Benzoic acid	3.29 (n=1)	7.95 (n=1)	3.85 (n=1)	0.94±0.33 (n=3)
Salicilyc acid	0.19 (n=1)	nd	0.23 (n=1)	0.76 (n=1)
Phenols				
Catechol/pyrocatechol	1.42 (n=1)	3.89±3.35 (n=2)	1.74±0.27 (n=2)	0.98±0.08 (n=3)
4-methylcatechol	1.45 (n=1)	nd	0.78 (n=1)	nd
4-ethylcatechol	0.18 (n=1)	0.2±0.01 (n=2)	0.21±0.03 (n=3)	0.2±0.01 (n=2)
Hippuric acids				
4-hydroxyhippuric acid	nd	nd	4.53 (n=1)	nd
Phenylacetic acids				
Phenylacetic acid	90.13±73.79 (n=3)	78.27±12.85 (n=2)	64.4±9.36 (n=2)	47.3±10.79 (n=2)
3,4-dihydroxyphenylacetic acid	3.88 (n=1)	1.48 (n=1)	4.74±2.67 (n=2)	4.86 (n=1)
4-hydroxyphenylacetic acid	4.16±1.78 (n=3)	4.01±2.01 (n=3)	9.38±8.56 (n=3)	21.88±24.47 (n=3)
3-hydroxyphenylacetic acid	15.93±9.68 (n=3)	11.34±7.38 (n=3)	23.95±27.21 (n=3)	7.27±4.23 (n=3)
4-hydroxy-3-methoxyphenylacetic acid	nd	nd	nd	nd
Phenylpropionic acids				
3-(3',4'-dihydroxyphenyl)-propionic acid	4.52±0.67 (n=2)	5.84±0.26 (n=2)	6.12±0.24 (n=2)	6.51 (n=1)
3-(2',4'-dihydroxyphenyl)-propionic acid	53.88 (n=1)	nd	24.31 (n=1)	8.85 (n=1)
3-(3'-hydroxyphenyl)-propionic acid	79.19±95.9 (n=3)	6.14±4.6 (n=3)	42.47±46.62 (n=3)	49.46±65.33 (n=3)
3-phenylpropionic acid	81.51±11.11 (n=2)	54.34±43.02 (n=3)	75.12±11.29 (n=3)	78.47±21.9 (n=3)
Dihydroxyphenylpropan-2-ol	nd	nd	nd	nd
Valeric acids				
4-hydroxy-5-(3´,4´-dyhidroxyphenyl)-valeric acid	3.84 (n=1)	nd	3.55±0.45 (n=2)	1.82±0.08 (n=2)
4-hydroxy-5-(3´-hidroxyphenyl)-valeric acid	nd	nd	0.21 (n=1)	0.1 (n=1)
4-hydroxy-5-phenylvaleric acid	82.16±47.34 (n=3)	80.78±8.93 (n=2)	29.67±11.52 (n=3)	41.57±42.5 (n=3)
Valerolactones				
5-(3´,4´-dihydroxyphenyl)-γ-valerolactone	9.64 (n=1)	nd	10.51±7.52 (n=2)	1.11±0.36 (n=2)
5-(3´-hydroxyphenyl)-γ-valerolactone	nd	nd	17.88±14.72 (n=2)	nd
5-(4´-hydroxyphenyl)-γ-valerolactone	nd	nd	18.46 (n=1)	7.25 (n=1)
Cinnamic acids				
Caffeic acid	1.17±0.2 (n=2)	1.41±0.64 (n=2)	1.85±0.06 (n=2)	1.65±1.08 (n=3)
p-coumaric acid	1.37±0.14 (n=3)	1.29±0.01 (n=2)	1.43±0.11 (n=2)	1.35±0.08 (n=3)
m-coumaric acid	0.29 (n=1)	nd	nd	nd
Ferulic acid	1.42±0.09 (n=2)	1.48±0.05 (n=2)	2.27±0.71 (n=2)	2.16±0.41 (n=2)
Isoferulic acid				

[a] Means and standard deviations were calculated excluding cases with values below the limit of quantification.
[b] In parenthesis the number of cases considered for each compound.
[c] nd, not detected.

Supplementary Table 2: Identification and quantification of phenolic and indolic metabolites in faeces from menopausal, equol non-producing women (n=12) before (t=0) and during (t=1 through t=6) a six-month intervention with soy isoflavones concentrates.

Compound	Metabolite concentration[a] (µg/g feces)			
	t=0	t=1	t=3	t=6
Skatole	3.97±1.43 (n=8)[b]	4.56±1.2 (n=7)	3.9±2.39 (n=9)	4.15±2.34 (n=7)
Isoflavones				
Daidzein	nd[c]	1.19±1.26 (n=9)	2.07±2.25 (n=9)	0.99±0.78 (n=10)
Dihydrodaidzein	nd	2.18±1.67 (n=7)	1.65±1.83 (n=8)	1.2±1.18 (n=8)
O-Desmethylangolensin	nd	0.19±0.16 (n=8)	0.37±0.25 (n=7)	0.11±0.09 (n=7)
Genistein	nd	0.48 (n=1)	nd	0.47 (n=2)
Lignans				
Enterolactone	0.32±0.23 (n=11)	0.37±0.2 (n=12)	0.3±0.16 (n=12)	0.59±0.63 (n=12)
Enterodiol	0.44 (n=2)	0.58±0.11 (n=2)	0.74±0.08 (n=2)	0.78±0.1 (n=2)
Mandelic acids				
3-hydroxymandelic acid	1.75±0.02 (n=5)	1.79±0.04 (n=5)	1.76±0.04 (n=7)	1.75±0.02 (n=6)
Benzoic acids				
Gallic acid	1.59±1.28 (n=2)	1.56±0.94 (n=2)	10.93±16.99 (n=4)	2.47±3.77 (n=6)
3,5-dihydrobenzoic acid	0.53±0.26 (n=6)	0.62±0.34 (n=6)	0.83±0.92 (n=4)	0.51±0.57 (n=6)
Protocatechuic acid	0.9±0.94 (n=12)	0.92±1.18 (n=11)	0.65±0.56 (n=12)	0.76±0.46 (n=11)
3-O-metilgallic acid	0.37±0.06 (n=3)	0.25±0.05 (n=4)	0.32±0.05 (n=2)	0.88±1 (n=4)
4-hidroxibenzoic acid	1.05±1.27 (n=11)	0.78±1.05 (n=10)	1.03±1.26 (n=8)	1.52±2.05 (n=10)
4-O-metilgallic acid	1.13 (n=1)	nd	1.2 (n=1)	1.24±0.14 (n=2)
3-hidroxibenzoic acid	1.16±1.37 (n=8)	0.69±0.23 (n=9)	1.08±0.53 (n=10)	0.94±0.66 (n=8)
Vanillic acid	1.26±0.83 (n=3)	0.43 (n=1)	1.05 (n=1)	0.44±0.09 (n=3)
Syringic acid	0.88±0.14 (n=3)	0.4±0.09 (n=3)	0.2 (n=1)	1.45±0.97 (n=3)
Benzoic acid	18.6±27.59 (n=6)	1.33±1.01 (n=5)	16.41±34.17 (n=6)	3.23±1.31 (n=4)
Salicilyc acid	0.18±0.02 (n=2)	1.47±1.78 (n=3)	0.4±0.05 (n=2)	0.18±0.06 (n=2)
Phenols				
Catechol/pyrocatechol	1.11±0.53 (n=4)	1.26±0.94 (n=4)	2.78±2.68 (n=6)	1.93±1.77 (n=3)
4-methylcatechol	0.95 (n=1)	1.52±0.88 (n=2)	1.29±0.44 (n=3)	0.81±0.04 (n=2)
4-ethylcatechol	0.22±0.04 (n=4)	0.29±0.09 (n=8)	0.26±0.06 (n=8)	0.2±0.02 (n=8)
Hippuric acids				
4-hydroxyhippuric acid	18.05 (n=1)	3.65 (n=1)	9.91±5.59 (n=2)	9.43±5.15 (n=3)
Phenylacetic acids				
Phenylacetic acid	100.62±39.92 (n=12)	127.51±45.77 (n=12)	111.18±52.85 (n=12)	109.96±43.92 (n=12)
3,4-dihydroxyphenylacetic acid	2.12±4.26 (n=9)	0.87±0.31 (n=7)	2.78±3.38 (n=8)	3.19±6.02 (n=7)
4-hydroxyphenylacetic acid	8.81±11.89 (n=12)	4.16±3 (n=12)	7.77±9.77 (n=12)	4.41±4.94 (n=11)
3-hydroxyphenylacetic acid	5.4±7.42 (n=8)	9.49±11.97 (n=10)	7.22±8.41 (n=11)	5.69±5.58 (n=9)
4-hydroxy-3-methoxyphenylacetic acid	1.84±0.86 (n=3)	2.35±0.19 (n=2)	1.5 (n=1)	2.59±1.5 (n=2)
Phenylpropionic acids				
3-(3',4'-dihydroxyphenyl)-propionic acid	13.44±19.44 (n=5)	3.48±1.04 (n=7)	4.42±2.71 (n=7)	23.06±51.63 (n=8)
3-(2',4'-dihydroxyphenyl)-propionic acid	36.91±45.45 (n=9)	51.09±76.33 (n=8)	67.21±78.71 (n=7)	62.25±85.38 (n=5)
3-(3'-hydroxyphenyl)-propionic acid	63.16±114.59 (n=12)	76.73±166.65 (n=12)	70.02±162.61 (n=12)	61.59±119.07 (n=12)
3-phenylpropionic acid	24.96±16.04 (n=10)	42.62±39.09 (n=11)	46.67±25.67 (n=11)	48.51±35.77 (n=9)
Dihydroxyphenylpropan-2-ol	2.36 (n=1)	1.98 (n=1)	2.88 (n=1)	5.41 (n=1)
Valeric acids				
4-hydroxy-5-(3´,4´-dyhidroxyphenyl)-valeric acid	5.36±3.69 (n=2)	10.07±6.46 (n=2)	6.45±3.66 (n=3)	9.45 (n=1)
4-hydroxy-5-(3´-hidroxyphenyl)-valeric acid	0.79±0.68 (n=2)	0.99±0.6 (n=3)	0.28±0.13 (n=3)	0.51 (n=1)
4-hydroxy-5-phenylvaleric acid	44.65±52.26 (n=9)	82.95±91.84 (n=12)	80.09±85.5 (n=11)	95.63±127.69 (n=10)
Valerolactones				
5-(3´,4´-dihydroxyphenyl)-γ-valerolactone	5.32±6.42 (n=3)	6.36±8.73 (n=4)	3.91±6.14 (n=6)	6.28±10 (n=4)
5-(3´-hydroxyphenyl)-γ-valerolactone	nd	39.44 (n=1)	8.36 (n=1)	35.27 (n=1)
5-(4´-hydroxyphenyl)-γ-valerolactone	3.85±1.6 (n=2)	3.72±2.35 (n=2)	8.83±4.15 (n=2)	9.53±5.96 (n=2)
Cinnamic acids				
Caffeic acid	0.65±0.39 (n=11)	0.5±0.18 (n=11)	0.58±0.31 (n=12)	0.9±1.11 (n=11)
p-coumaric acid	1.64±0.69 (n=11)	1.61±0.9 (n=11)	1.71±0.76 (n=9)	1.53±0.41 (n=11)
m-coumaric acid	0.52 (n=1)	0.29 (n=1)	0.3±0.03 (n=2)	0.38±0.11 (n=2)
Ferulic acid	1.69±0.3 (n=5)	1.59±0.34 (n=8)	1.46±0.11 (n=8)	2.4±1.29 (n=7)
Isoferulic acid	4.06 (n=1)	nd	3.45 (n=1)	5.98 (n=1)

[a] Means and standard deviations were calculated excluding cases with values below the limit of quantification.

[b] In parenthesis the number of cases considered for each compound.

[c] nd, not detected.

MATERIAL SUPLEMENTARIO ARTÍCULO 3

Supplementary Table 1.- Equol concentration during isoflavone treatment in urine samples of all 16 women in this study.

Woman	Sample	Parameter		
		Equol[a]	Creatinine[b]	Equol/Creatinine
WA	0	165	106	1.56
	1	32006	256	124.78
	3	389	166	2.34
	6	1379	179	7.70
WB	0	47	204	0.23
	1	81	302	0.27
	3	80	199	0.40
	6	41	182	0.22
WC	0	9	94	0.10
	1	1143	64	17.87
	3	1727	168	10.29
	6	1382	220	6.28
WD	0	2	155	0.01
	1	2	60	0.03
	3	9	158	0.06
	6	nd	99	nd
WE	0	nd	163	nd
	1	nd	259	nd
	3	nd	126	nd
	6	nd	146	nd
WF	0	<1.1	85	<1.1
	1	nd	120	nd
	3	<1.1	263	<1.1
	6	<1.1	230	<1.1
WG	0	58	92	0.64
	1	39965	246	162.70
	3	28829	215	133.85
	6	18741	250	75.11
WH	0	22	73	0.30
	1	24	89	0.27
	3	19	72	0.27
	6	22	108	0.20
WI	0	20	88	0.23
	1	50	316	0.16
	3	-	-	-
	6	37	149	0.25
WJ	0	nd	130	nd
	1	13	169	0.08
	3	171	115	1.49

	6	54	160	0.34
WK	0	<1.1	37	<1.1
	1	3	58	0.05
	3	4	120	0.03
	6	<1.1	75	<1.1
WL	0	<1.1	94	<1.1
	1	<1.1	117	<1.1
	3	<1.1	156	<1.1
	6	4	206	0.02
WM	0	<1.1	128	<1.1
	1	55	98	0.56
	3	9	73	0.13
	6	4	114	0.03
WN	0	377	47	7.97
	1	0	127	0.00
	3	23	81	0.29
	6	18	292	0.06
WO	0	<1.1	202	<1.1
	1	5	206	0.03
	3	22	314	0.07
	6	7	152	0.04
WP	0	6	257	0.03
	1	1753	341	5.14
	3	1511	250	6.05
	6	1308	51	25.44

Concentration: [a]nM of equol; [b]M of creatinine.
nd, not detected.

Supplementary Table 2.- Viable counts of total and indicator faecal microbial populations in postmenopausal women treated with soy isoflavones over a six-month medication.

Woman	Month	Microbial counts[a]						
		MCB	MRSC	BIF	RCM	EMB	BP	VA
WA	0	8.85E+00	8.16E+00	8.83E+00	8.08E+00	6.48E+00	9.09E+00	8.77E+00
	1	9.70E+00	9.48E+00	9.18E+00	9.97E+00	9.08E+00	1.07E+01	1.03E+01
	3	1.16E+01	1.10E+01	1.17E+01	1.08E+01	8.59E+00	1.18E+01	1.07E+01
	6	9.68E+00	7.70E+00	9.60E+00	8.78E+00	6.99E+00	9.95E+00	9.48E+00
WB	0	1.09E+01	1.10E+01	1.10E+01	1.08E+01	9.24E+00	1.09E+01	1.10E+01
	1	1.16E+01	1.14E+01	1.16E+01	1.12E+01	7.77E+00	1.13E+01	1.15E+01
	3	1.13E+01	1.13E+01	1.11E+01	1.11E+01	8.12E+00	1.12E+01	1.11E+01
	6	1.02E+01	9.99E+00	1.02E+01	1.01E+01	9.01E+00	1.03E+01	1.01E+01
WC	0	1.06E+01	1.05E+01	1.05E+01	1.05E+01	9.29E+00	1.04E+01	9.70E+00
	1	1.05E+01	1.15E+01	1.06E+01	7.02E+00	1.08E+01	1.07E+01	1.06E+01
	3	1.11E+01	1.10E+01	1.08E+01	1.06E+01	6.64E+00	1.14E+01	1.10E+01
	6	1.01E+01	9.61E+00	9.75E+00	9.67E+00	6.98E+00	1.00E+01	9.18E+00
WD	0	1.04E+01	1.06E+01	1.05E+01	1.04E+01	7.15E+00	1.05E+01	1.06E+01
	1	1.16E+01	1.12E+01	1.14E+01	1.11E+01	8.58E+00	1.14E+01	1.12E+01
	3	9.24E+00	9.73E+00	9.45E+00	9.63E+00	6.67E+00	9.97E+00	9.00E+00
	6	9.24E+00	9.18E+00	9.32E+00	9.39E+00	7.39E+00	9.32E+00	8.00E+00
WE	0	9.31E+00	9.33E+00	9.47E+00	9.27E+00	8.86E+00	9.94E+00	8.88E+00
	1	1.03E+01	9.66E+00	9.75E+00	9.72E+00	7.42E+00	1.03E+01	1.02E+01
	3	9.75E+00	9.24E+00	9.20E+00	9.56E+00	8.41E+00	9.74E+00	8.00E+00
	6	9.58E+00	9.49E+00	9.63E+00	9.73E+00	6.93E+00	1.05E+01	8.65E+00
WF	0	1.05E+01	9.02E+00	9.33E+00	9.44E+00	6.48E+00	1.03E+01	9.91E+00
	1	9.33E+00	9.87E+00	1.00E+01	1.01E+01	7.16E+00	1.03E+01	1.02E+01
	3	9.54E+00	7.70E+00	9.31E+00	8.00E+00	6.57E+00	1.02E+01	8.40E+00
	6	9.82E+00	9.04E+00	9.25E+00	8.00E+00	8.15E+00	1.01E+01	9.41E+00
WG	0	8.28E+00	8.00E+00	8.20E+00	8.08E+00	7.74E+00	8.58E+00	8.60E+00
	1	1.13E+01	1.06E+01	1.08E+01	1.11E+01	7.08E+00	1.13E+01	1.09E+01
	3	1.03E+01	9.33E+00	9.79E+00	9.84E+00	6.42E+00	1.04E+01	1.02E+01
	6	9.76E+00	9.47E+00	9.49E+00	9.57E+00	6.69E+00	1.03E+01	9.61E+00
WH	0	1.04E+01	1.02E+01	1.06E+01	1.07E+01	9.26E+00	1.07E+01	9.70E+00
	1	1.05E+01	1.05E+01	1.07E+01	1.06E+01	8.60E+00	1.18E+01	1.15E+01
	3	1.08E+01	1.07E+01	1.07E+01	1.08E+01	8.42E+00	1.15E+01	9.45E+00
	6	1.03E+01	9.44E+00	1.01E+01	9.48E+00	8.17E+00	1.04E+01	9.15E+00
WJ	0	1.00E+01	1.01E+01	9.95E+00	1.02E+01	6.84E+00	1.06E+01	1.02E+01
	1	1.06E+01	1.04E+01	1.04E+01	1.07E+01	7.24E+00	1.09E+01	1.05E+01
	3	9.95E+00	9.60E+00	9.57E+00	9.34E+00	6.84E+00	9.82E+00	8.70E+00
	6	9.87E+00	9.74E+00	9.66E+00	9.61E+00	7.46E+00	9.85E+00	9.30E+00

WI	0	9.98E+00	9.80E+00	9.70E+00	9.78E+00	7.64E+00	9.87E+00	9.82E+00
	1	1.03E+01	9.65E+00	9.72E+00	1.02E+01	7.96E+00	1.04E+01	1.04E+01
	3	1.02E+01	9.84E+00	9.96E+00	9.83E+00	6.68E+00	1.03E+01	1.04E+01
	6	9.64E+00	9.57E+00	9.57E+00	9.57E+00	5.00E+00	9.51E+00	9.33E+00
WK	0	1.06E+01	1.07E+01	1.05E+01	1.07E+01	7.86E+00	1.12E+01	1.08E+01
	1	1.03E+01	9.74E+00	9.54E+00	1.02E+01	8.23E+00	1.01E+01	1.03E+01
	3	9.40E+00	8.93E+00	9.26E+00	9.12E+00	8.16E+00	9.69E+00	9.59E+00
	6	9.71E+00	9.49E+00	9.75E+00	9.75E+00	6.70E+00	9.88E+00	9.81E+00
WL	0	1.15E+01	1.14E+01	1.15E+01	1.16E+01	9.00E+00	1.15E+01	1.15E+01
	1	9.39E+00	9.19E+00	9.53E+00	1.10E+01	7.00E+00	1.13E+01	1.13E+01
	3	9.27E+00	9.88E+00	1.01E+01	9.90E+00	8.26E+00	1.04E+01	9.15E+00
	6	9.65E+00	9.22E+00	9.76E+00	9.32E+00	8.04E+00	9.96E+00	8.00E+00
WM	0	1.02E+01	9.92E+00	1.03E+01	1.04E+01	8.02E+00	9.73E+00	9.96E+00
	1	1.10E+01	1.07E+01	1.10E+01	1.09E+01	6.71E+00	1.07E+01	1.03E+01
	3	9.47E+00	9.28E+00	9.33E+00	9.71E+00	5.48E+00	9.71E+00	9.33E+00
	6	8.40E+00	7.70E+00	8.72E+00	8.70E+00	6.60E+00	9.15E+00	7.88E+00
WN	0	9.33E+00	8.60E+00	9.46E+00	9.22E+00	8.63E+00	9.56E+00	9.34E+00
	1	9.18E+00	7.70E+00	9.07E+00	0.00E+00	8.41E+00	9.45E+00	8.95E+00
	3	8.40E+00	7.70E+00	8.74E+00	8.18E+00	8.31E+00	9.46E+00	8.74E+00
	6	8.94E+00	8.30E+00	8.74E+00	8.30E+00	7.59E+00	8.68E+00	8.40E+00
WO	0	1.07E+01	9.82E+00	1.02E+01	1.03E+01	7.15E+00	1.06E+01	1.02E+01
	1	1.10E+01	1.07E+01	1.09E+01	1.09E+01	8.75E+00	1.16E+01	1.14E+01
	3	9.64E+00	7.70E+00	9.09E+00	8.76E+00	5.93E+00	9.61E+00	8.93E+00
	6	9.08E+00	8.00E+00	8.92E+00	8.30E+00	6.64E+00	9.21E+00	8.30E+00
WP	0	1.03E+01	1.05E+01	1.02E+01	1.01E+01	7.26E+00	1.14E+01	1.15E+01
	1	1.03E+01	1.07E+01	1.05E+01	1.11E+01	8.68E+00	1.20E+01	1.09E+01
	3	9.94E+00	1.01E+01	9.67E+00	9.79E+00	7.75E+00	9.91E+00	9.67E+00
	6	9.57E+00	9.08E+00	9.29E+00	9.27E+00	8.00E+00	1.01E+01	9.18E+00

[a] Log_{10} colony forming units g^{-1} x 10^{n}.

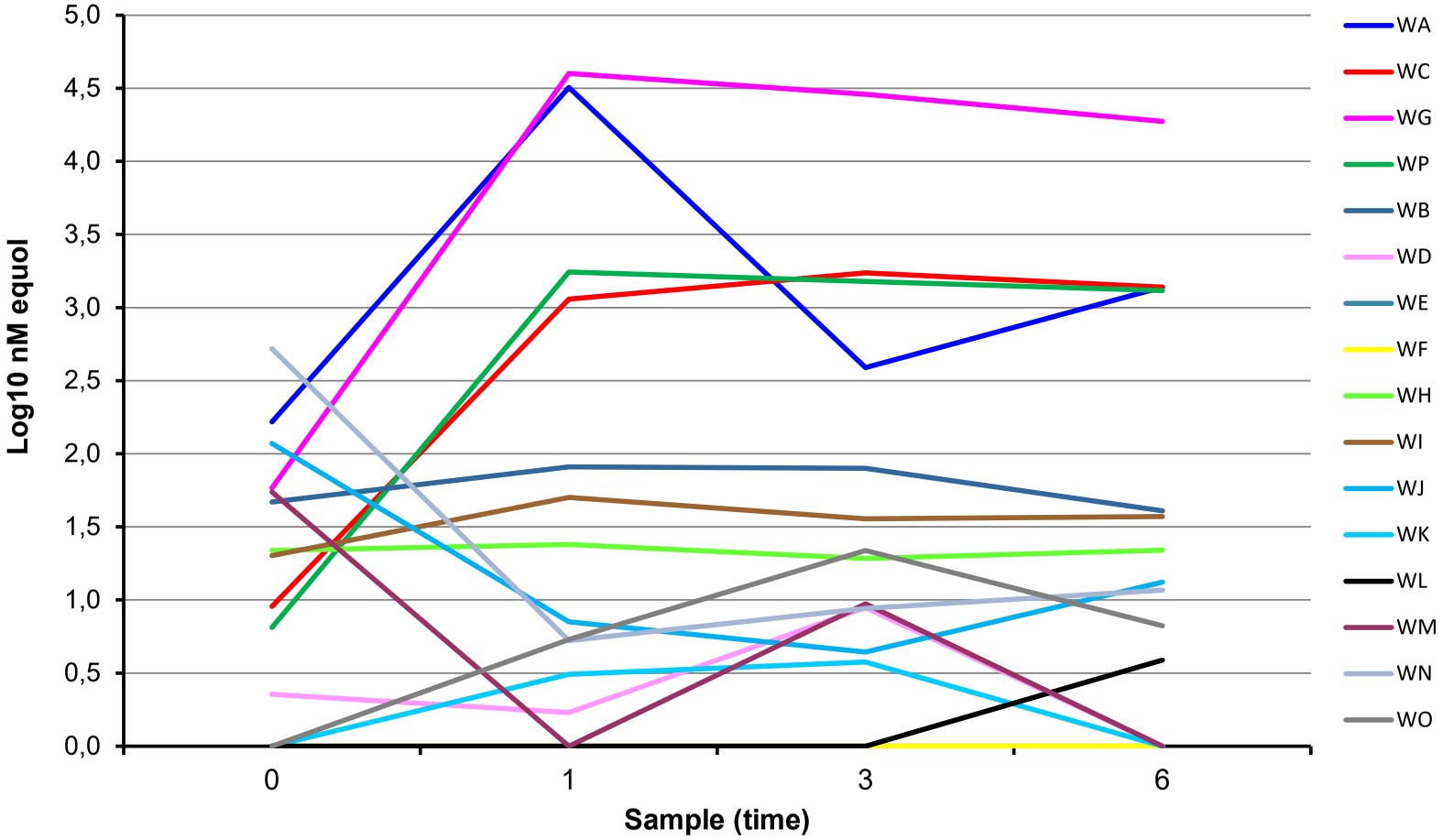
Key of the media (target population): MCB, Medium for Colon Bacteria (total cultivable bacteria); MRSC; de Man, Rogosa and Sharpe with cysteine (lactobacilli); BIF, Bifidobacteria, (*Bifidobacterium* spp.); RCM, Reinforced Clostridium Medium (clostridia); EMB, Eosin Methylene Blue (Enterobacteriaceae); BP, Bacteroides and Prevotella medium (*Bacteroides* and *Prevotella* spp.); VA, Veillonella Agar (*Veillonella* spp.).

Supplementary Table 3.- Relative quantities of fecal microbial populations in the different equol status of the postmenopausal women treated with soy isoflavones of this study as determined by qPCR using universal and group-specific primers.

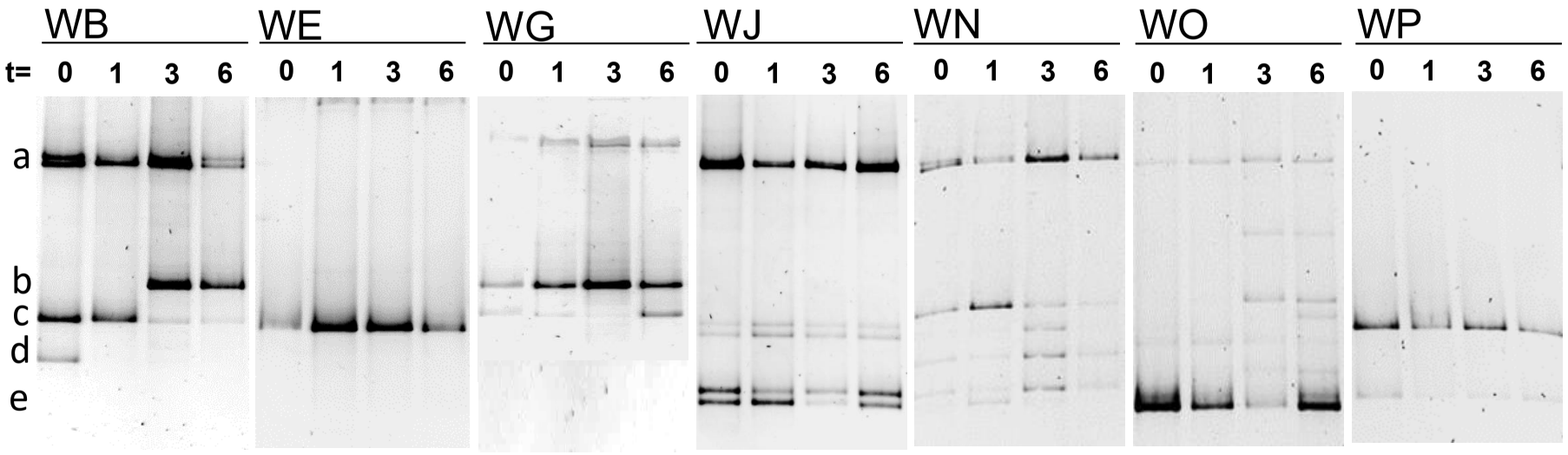
Woman	Month	Microbial population[a]						
		Bifidobacteria	Lactobacilli	*Clostridium leptum*	*Clostridium coccoides*	*Bacteroides*	Enterobacteria	*Atopobium*
WA	0	8.64E-02	9.52E-02	2.81E+01	4.11E+01	5.72E+00	0.00E+00	4.83E+00
	1	2.26E-03	2.63E-03	2.99E+01	4.66E+01	5.13E+00	0.00E+00	6.53E+00
	3	3.96E-03	4.24E-01	3.96E+01	2.89E+01	1.20E+01	0.00E+00	6.61E+00
	6	2.12E-01	2.89E-02	3.45E+01	3.46E+01	9.08E+00	0.00E+00	4.30E+00
WB	0	2.15E+01	2.85E+00	1.08E+01	7.07E+00	7.80E+00	1.48E+00	4.40E+00
	1	2.67E+01	3.81E-02	9.56E-01	1.32E+01	1.89E+01	8.11E-01	1.05E+01
	3	3.29E+01	8.97E-02	4.26E+00	5.08E+00	8.15E+00	4.68E-01	5.58E+00
	6	4.70E+01	5.37E-01	7.49E+00	7.94E+00	2.05E+01	3.77E-01	9.49E+00
WC	0	1.20E+01	1.87E+00	3.94E+01	7.37E+00	6.97E+00	1.64E-02	4.19E-01
	1	1.87E+00	4.21E-04	1.64E+01	2.72E+00	1.80E+01	6.26E-03	3.08E+00
	3	2.63E+00	6.73E-02	4.66E+01	7.38E+01	2.24E+01	1.54E-03	7.01E+00
	6	1.59E+00	2.61E-02	4.77E+01	6.56E+01	8.17E+00	6.01E-05	3.98E+00
WD	0	1.08E+01	1.31E-02	2.88E+01	5.71E+01	6.36E+00	0.00E+00	4.13E+00
	1	1.91E+00	3.12E+00	3.40E+01	4.89E+01	9.61E+00	0.00E+00	2.67E+00
	3	6.97E+00	2.61E-03	2.60E+01	4.38E+01	8.59E+00	0.00E+00	3.11E+00
	6	7.00E+00	2.18E+00	1.60E+01	5.13E+01	4.61E+00	0.00E+00	2.36E+00
WE	0	1.86E+00	2.18E-01	2.15E+01	5.54E+00	9.31E+00	2.24E-03	2.27E+00
	1	3.16E+00	3.77E-02	3.45E+01	1.12E+01	1.08E+01	4.04E-04	1.00E+00
	3	3.66E+00	2.37E-01	2.56E+01	6.16E+00	7.47E+00	4.11E-04	1.86E+00
	6	3.51E+00	6.18E-02	3.25E+01	1.39E+01	7.75E+00	2.19E-04	1.13E+00
WF	0	2.30E-01	4.30E-03	2.46E+01	4.64E+01	2.71E+01	5.08E-03	8.54E-01
	1	1.07E-03	4.04E-02	1.28E+01	7.22E+01	1.76E+01	1.33E-02	7.66E-01
	3	2.18E-01	1.35E-02	2.99E+01	5.44E+01	2.41E+01	8.38E-04	1.33E+00
	6	3.14E-01	3.46E-02	6.02E+00	6.33E+01	1.68E+01	1.29E-02	4.57E-01
WG	0	2.10E-01	4.42E-02	2.14E+01	2.71E+01	1.90E+01	2.98E-04	8.48E+00
	1	7.82E-01	2.56E-03	2.18E+01	2.97E+01	1.93E+01	8.23E-05	8.07E+00
	3	2.77E+00	1.31E-02	4.06E+01	1.79E+01	1.22E+01	1.83E-04	5.15E+00
	6	1.21E+00	8.86E-02	3.48E+01	1.63E+01	7.53E+00	9.48E-03	7.21E+00
WH	0	1.23E+00	2.91E-02	3.17E+01	5.38E+00	2.18E+02	0.00E+00	5.49E-01
	1	1.15E+01	1.99E-02	5.63E+01	2.31E+01	1.12E+02	0.00E+00	1.31E+00
	3	9.46E+00	9.99E-02	5.01E+01	2.72E+01	4.97E+01	0.00E+00	2.78E+00
	6	1.55E+01	1.20E+00	2.09E+01	1.57E+01	3.54E+01	0.00E+00	3.50E+00
WI	0	1.72E+00	9.21E-03	3.09E+01	9.52E+00	6.80E+00	0.00E+00	5.59E+00
	1	7.25E+00	2.20E-02	1.89E+01	5.81E+00	5.34E+00	0.00E+00	1.03E+01

	3	5.14E+00	5.66E-02	2.12E+01	6.14E+00	4.15E+00	0.00E+00	1.05E+01
	6	4.33E+00	1.68E-02	2.52E+01	1.05E+01	5.59E+00	0.00E+00	3.95E+00
WJ	0	9.29E+00	3.25E-01	1.29E+01	3.41E+01	4.15E+00	2.33E-03	7.31E+00
	1	5.97E+00	2.75E-01	1.41E+01	1.34E+01	2.97E+00	2.52E-03	1.37E+01
	3	5.49E+00	6.79E-03	2.01E+01	6.67E+00	2.93E+00	2.24E-04	1.72E+01
	6	9.42E+00	2.64E-02	2.12E+01	1.35E+01	3.62E+00	2.40E-03	5.95E+00
WK	0	1.30E+00	7.92E-02	3.45E+01	2.69E+01	0.00E+00	2.18E-02	4.41E+00
	1	3.44E+00	3.58E-02	3.48E+01	2.43E+01	0.00E+00	7.09E-02	4.31E+00
	3	9.08E-01	1.48E-02	3.19E+01	3.13E+01	0.00E+00	1.48E-02	7.36E+00
	6	2.45E+00	6.36E-03	4.01E+01	2.52E+01	0.00E+00	2.27E-04	5.44E+00
WL	0	1.69E+00	1.39E-02	1.76E+01	2.67E+01	1.63E+01	7.22E-02	5.70E+00
	1	1.14E-01	1.67E-02	1.93E+01	3.25E+01	2.70E+01	1.79E-01	6.25E+00
	3	6.36E-02	8.13E-03	2.09E+01	4.02E+01	2.95E+01	2.28E-02	4.47E+00
	6	1.93E+00	6.59E-02	2.10E+01	2.26E+01	1.33E+01	6.46E-04	8.12E+00
WM	0	1.32E+00	1.10E-01	3.75E+01	2.98E+01	5.13E+00	0.00E+00	3.87E+00
	1	1.65E+01	2.02E-03	5.55E+01	2.81E+01	8.63E+00	0.00E+00	8.24E+00
	3	2.19E+00	1.23E-03	2.93E+01	3.38E+01	1.96E+01	0.00E+00	1.29E+01
	6	5.11E-01	1.41E-02	2.57E+01	2.70E+01	1.15E+01	0.00E+00	6.09E+00
WN	0	5.46E+00	2.61E-02	1.93E+01	3.30E+01	6.67E+00	2.60E-02	2.83E+00
	1	1.16E+01	1.20E-02	1.61E+01	3.40E+01	3.50E+00	1.90E-02	4.71E+00
	3	1.39E+01	4.62E-02	2.17E+01	2.64E+01	2.62E+00	6.17E+00	4.84E+00
	6	2.17E+00	8.43E-03	1.38E+01	2.51E+01	4.13E+00	7.27E-04	3.41E+00
WO	0	3.26E+00	5.86E-02	1.67E+01	2.23E+01	1.22E+01	7.05E-04	3.37E+00
	1	3.40E+00	5.09E-02	1.69E+01	1.68E+01	1.02E+01	7.48E-03	3.45E+00
	3	1.24E+00	3.82E-02	1.56E+01	2.07E+01	1.41E+01	1.97E-03	2.97E+00
	6	1.72E+01	2.84E-02	9.96E+00	1.50E+01	1.48E+01	5.59E-04	8.33E+00
WP	0	1.58E+00	7.95E-03	2.59E+01	1.79E+01	2.63E+02	0.00E+00	5.75E+00
	1	1.33E+00	7.27E-03	1.05E+01	1.77E+01	3.77E+02	0.00E+00	4.88E+00
	3	2.79E+00	2.41E-02	1.59E+01	4.08E+01	1.35E+02	0.00E+00	5.06E+00
	6	2.73E+00	8.20E-03	1.56E+01	1.32E+01	4.29E+02	0.00E+00	3.32E+00

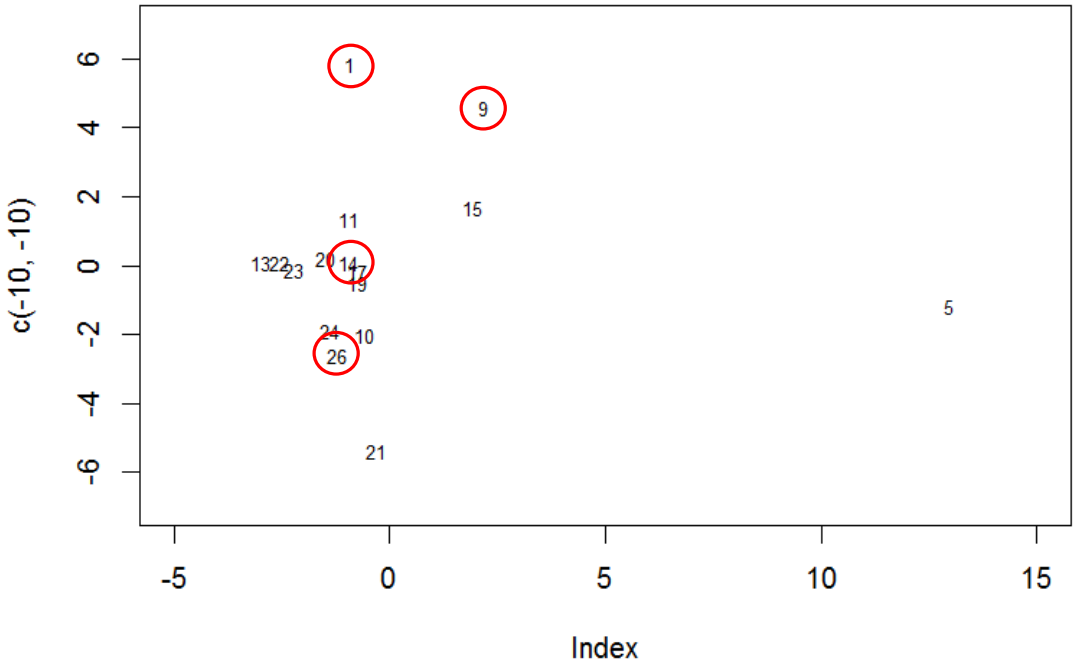
[a]% of the total bacterial 16S rDNA x 10^n, as determined using the universal prokaryotic primers TBA-F and TBA-R (Table 1).



Supplementary Figure 1.- Variation in the concentration levels of equol in urine during soy-isoflavone treatment in the 16 women of this study. Note the logarithmic scale of the equol concentration (nM).

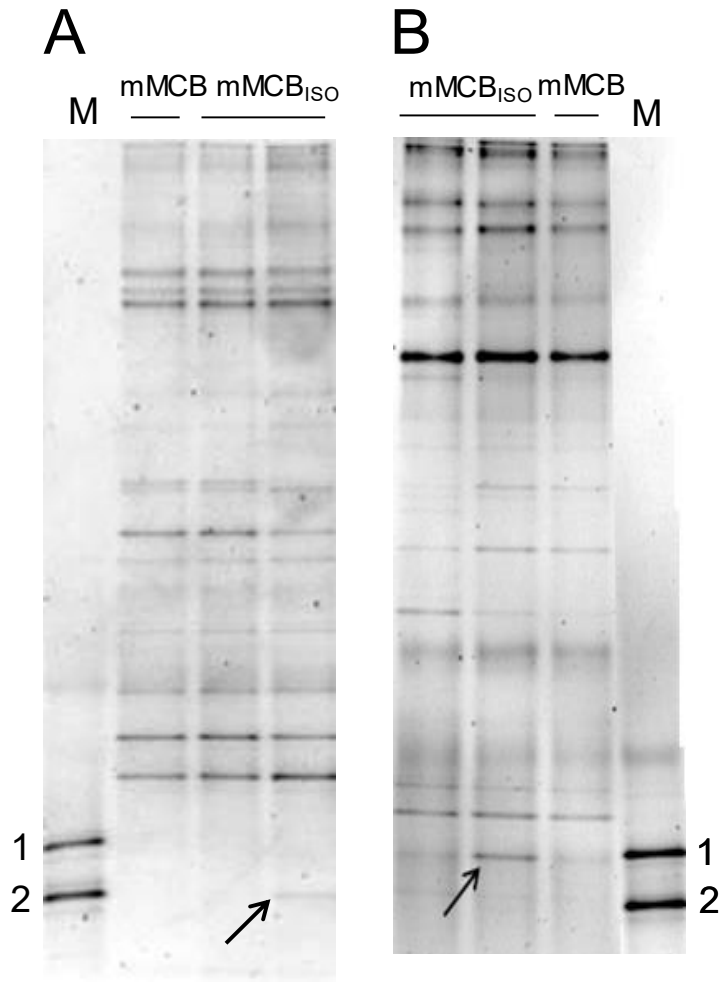


Supplementary Figure 2.- Representative DGGE profiles of bifidobacteria from faecal samples of some women of the study at different times during isoflavone treatment. Bands identified after sequencing and sequence comparison against ribosomal DNA databases: a, Bifidobacterium bifidum; b, Bifidobacterium adolescentis; c, Bifidobacterium longum; d, Bifidobacterium saeculare; e, Bifidobacterium catenulatum/pseudocatenulatum.



Supplementary Figure 3.- Principal Coordinate Analysis (PCoA) of the pooled microbial results obtained by culturing and qPCR and the equol production phenotype. Women 1, 9, 14 and 26, are equol producers and correspond to WA, WC, WG and WP in supplementary tables.

MATERIAL SUPLEMENTARIO ARTÍCULO 5



Additional file 1: Effect of isoflavones on dominant bacterial populations determined by DGGE. PCR-DGGE profiles of the primary faecal cultures from equol-producing women grown in modified medium for colonic bacteria supplemented (mMCB$_{ISO}$) or not (mMCB) with isoflavones; A) WC samples, B) WG samples. M: DGGE marker [comprising the species *Slackia isoflavoniconvertens* (1) and *Slackia equolifaciens* (2)]. (PPTX 164 kb)

Additional file 2: Summary of sequence processing. Number of quality reads, sample coverage, richness estimators and diversity indexes of 16S rDNA libraries of the primary and secondary faecal cultures.

	Library	NS	OTUs[a]	Chao1[b]	Simpson[b]	Shannon[b]	ESC[c]
WC	Primary culture mMCB	113,343	3,538	42,912.87 (36,262.24; 50,915.37)	0.03 (0.03; 0.03)	4.66 (4.64; 4.68)	0.91
	Primary culture $mMCB_{ISO}$	117,798	3,738	39,743.64 (34,111.65; 46,421.64)	0.03 (0.03; 0.03)	4.54 (4.51; 4.56)	0.90
	Secondary culture $mMCB_{ISO}$	102,101	249	1,120.57 (774.96; 1,694.67)	0.36 (0.35; 0.36)	1.47 (1.46; 1.49)	0.99
WG	Primary culture mMCB	122,186	3,489	45,965.86 (38,489.01; 55,041.67)	0.03 (0.03; 0.03)	4.47 (4.45; 4.5)	0.91
	Primary culture $mMCB_{ISO}$	93,477	3,562	49,825.82 (41,588.49; 59,851.52)	0.05 (0.05; 0.05)	4.32 (4.29; 4.35)	0.91
	Secondary culture $mMCB_{ISO}$	105,583	1,686	18,315.58 (14,525.55; 23,227.37)	0.15 (0.15; 0.15)	3.12 (3.09; 3.14)	0.96
WP	Primary culture mMCB	113,924	3,120	47,705.44 (39,083.6; 58,400.07)	0.05 (0.05; 0.05)	4.06 (4.04; 4.09)	0.92
	Primary culture $mMCB_{ISO}$	114,540	3,612	65,179.68 (53,228.06; 80,017.35)	0.05 (0.05; 0.05)	4.26 (4.24; 4.29)	0.90
	Secondary culture $mMCB_{ISO}$	101,670	3,197	62,336.79 (49,903.97; 78,089.56)	0.05 (0.05; 0.05)	4.11 (4.09; 4.14)	0.91
WE	Primary culture mMCB	115,916	1,918	18,791.06 (15,277.95; 23,230.43)	0.08 (0.07; 0.08)	3.46 (3.44; 3.49)	0.95
	Primary culture $mMCB_{ISO}$	127,122	2,077	23,154.77 (18,701.53; 28,804.76)	0.07 (0.07; 0.07)	3.66 (3.63; 3.68)	0.95
	Secondary culture $mMCB_{ISO}$	110,850	2,379	25,867.57 (21,411.11; 31,371.44)	0.05 (0.05; 0.05)	3.8 (3.78; 3.82)	0.94

Abbreviations: ESC, estimated sample coverage; NS, average number of quality sequences for each sample; OTU, operational taxonomic unit

WC, WG and WP are equol-producing women; WP is a equol non-producing woman

[a] Calculated by MOTHUR at the 3% distance level

[b] Diversity indexes calculated using MOTHUR (3% distance)

Values in brackets are 95% confidence intervals as calculated by MOTHUR

[c] ESC: $Cx = 1 - (Nx/n)$, where Nx is the number of unique sequence and n is the total number of sequences

All calculations (a, b and c) were made with a subsample of 35,000 sequences per library

Additional file 3: Effect of isoflavones in microbial abundance. Families and genera showing significant (*p* value <0.05) increases (grey) and decreases in their relative abundances (% sequences) when comparing primary faecal cultures in medium with and without isoflavones.

		Primary cultures mMCB[a]	Primary cultures $mMCB_{ISO}$[a]
Family	*p*-value[b]	% relative abundance[c]	% relative abundance[c]
Ruminococcaceae	0.011	24.214±1.484	30.167±1.667
Bacteroidaceae	0.010	17.144±1.512	12.452±0.847
Porphyromonadaceae	0.007	6.853±0.268	5.656±0.350
*Clostridiales_Incertae_Sedis*_XI	0.015	0.038±0.011	0.009±0.002
Genus			
Roseburia	0.001	0.060±0.011	0.470±0.083
Odoribacter	0.002	0.088±0.013	0.257±0.054
Bacteroides	0.016	17.144±1.512	12.452±0.847
Parabacteroides	0.018	6.088±0.341	4.721±0.433
Flavonifractor	0.019	1.543±0.468	0.281±0.055
Peptostreptococcus	0.020	0.587±0.213	0.002±0.001
Butyricicoccus	0.005	0.410±0.067	0.174±0.037
Pseudoflavonifractor	0.025	0.262±0.092	0.052±0.018
Finegoldia	0.011	0.019±0.005	0.006±0.002
Peptoniphilus	0.012	0.017±0.006	0.001±0.001

[a]mMCB: modified medium for colonic bacteria; mMCBISO: modified medium for colonic bacteria supplemented with isoflavones
[b]Significance was considered below a *p*-value of 0.05, multiple hypothesis tests correction of Benjamini and Hochberg was applied with a FDR=0.25.
[c]Mean relative abundance ± standard deviation.

Additional file 4: Differences in microbial genera associated with equol production in primary faecal cultures. Genera showing significant increases (p value <0.05) in their relative abundances (% sequences) in medium with isoflavones when comparing primary cultures from non-producer and equol producer women.

		Primary culture equol non-producer woman	Primary cultures equol producers
Genus	p-value[a]	% relative abundance[b]	% relative abundance
Collinsella	0.000	0.004±0.002	11.098±0.970
Bacteroides	0.000	8.142±0.180	13.888±0.898
Faecalibacterium	0.007	4.781±0.002	10.160±1.523
Dorea	0.000	0.013±0.002	3.808±0.446
Clostridium group XlVb	0.000	0.006±0.002	2.745±0.458
Sutterella	0.000	0.003±0.001	2.682±0.207
Dialister	0.000	0.002±0.001	2.279±0.392
Blautia	0.011	0.894±0.035	2.564±0.511
Alistipes	0.000	0.066±0.009	1.549±0.243
Clostridium group XlVa	0.004	0.019±0.006	1.483±0.384
Coprococcus	0.000	0.007±0.002	0.828±0.107
Oscillibacter	0.004	1.151±0.104	1.867±0.153
Barnesiella	0.002	0.092±0.009	0.641±0.112
Enterobacter	0.027	0.002±0.001	0.449±0.154
Asaccharobacter	0.005	0.020±0.005	0.368±0.094
Flavonifractor	0.001	0.033±0.005	0.364±0.061
Odoribacter	0.002	0.041±0.005	0.329±0.064
Butyricicoccus	0.003	0.030±0.005	0.223±0.044
Parasutterella	0.003	0.051±0.012	0.240±0.042
Olsenella	0.044	0.001±0.001	0.104±0.037
Gordonibacter	0.008	0.004±0.002	0.066±0.018
Lactococcus	0.002	0.002±0.001	0.041±0.008
Allisonella	0.034	0.000±0.000	0.038±0.014
Weissella	0.046	0.001±0.001	0.032±0.011
Coprobacillus	0.048	0.000±0.000	0.008±0.003
Finegoldia	0.021	0.001±0.001	0.008±0.002
Murdochiella	0.011	0.000±0.000	0.003±0.001
Atopobium	0.028	0.000±0.000	0.002±0.001

[a]Significance was considered below a p-value of 0.05, multiple hypothesis tests correction of Benjamini and Hochberg was applied with a FDR=0.25.

[b]Mean relative abundance ± standard deviation.

Additional file 5: Differences in microbial genera associated with equol production in secondary faecal cultures. Genera showing significant increases (p value <0.05) in their relative abundances (% sequences) in medium with isoflavones when comparing secondary cultures that rendered equol production with those that did not.

		Secondary cultures equol$^-$	Secondary cultures equol$^+$
Genus	p-value[a]	% relative abundance[b]	% relative abundance
Lactobacillus	0.044	4.802±1.528	17.616±5.420
Collinsella	0.001	0.025±0.006	12.406±1.819
Sutterella	0.001	1.669±0.507	4.665±0.613
Clostridium group XlVb	0.001	0.005±0.001	2.082±0.586
Clostridium group XlVa	0.001	0.040±0.012	1.878±0.471
Oscillibacter	0.001	0.022±0.009	1.836±0.375
Barnesiella	0.001	0.060±0.019	1.586±0.187
Alistipes	0.001	0.047±0.013	1.474±0.444
Dorea	0.001	0.003±0.001	1.229±0.354
Clostridium group XVIII	0.001	0.031±0.009	0.981±0.264
Faecalibacterium	0.013	0.473±0.147	1.346±0.301
Finegoldia	0.001	0.069±0.031	0.487±0.086
Butyricicoccus	0.001	0.023±0.009	0.313±0.051
Allisonella	0.007	0.000±0.000	0.266±0.095
Odoribacter	0.009	0.012±0.004	0.261±0.094
Ruminococcus2	0.001	0.001±0.000	0.194±0.059
Coprococcus	0.001	0.001±0.000	0.177±0.053
Slackia	0.022	0.020±0.006	0.193±0.059
Asaccharobacter	0.001	0.006±0.002	0.149±0.048
Sporobacter	0.001	0.001±0.000	0.071±0.009
Pseudoflavonifractor	0.013	0.000±0.000	0.014±0.006
Murdochiella	0.001	0.000±0.000	0.010±0.004
Anaerotruncus	0.001	0.000±0.000	0.007±0.001
Mogibacterium	0.048	0.000±0.000	0.003±0.001
Anaerovorax	0.015	0.000±0.000	0.003±0.002
Sphingopyxis	0.001	0.000±0.000	0.003±0.001
Actinomyces	0.039	0.000±0.000	0.002±0.001
Eubacterium	0.031	0.000±0.000	0.001±0.001

[a]Significance was considered below a p-value of 0.05, multiple hypothesis tests correction of Benjamini and Hochberg was applied with a FDR=0.25.

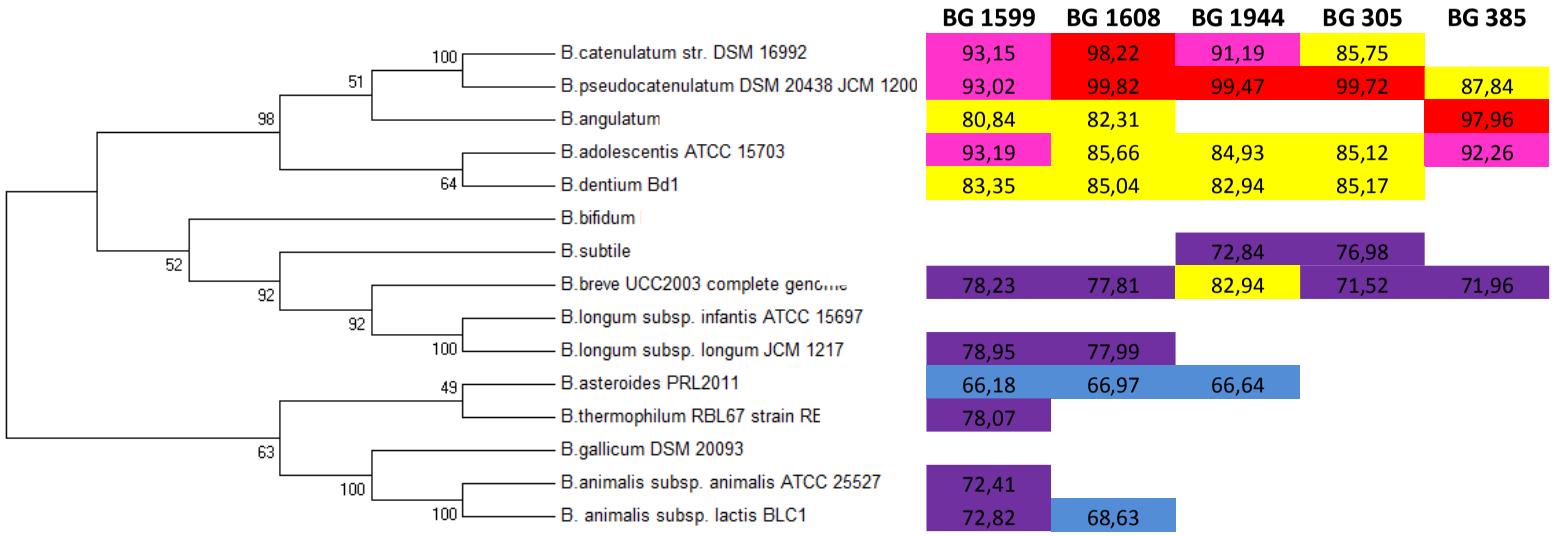
[b]Mean relative abundance ± standard deviation.

Additional file 6: Effect of isoflavones in microbial species abundance. Identification of OTUs (3% distance level) showing significant increases (p value <0.05) in their relative abundances (%) in primary faecal cultures in $mMCB_{ISO}$ as compared with mMCB.

OTU Code	Primary cultures mMCB		Primary cultures $mMCB_{ISO}$		p-value	[a]Blast match (closest relative)	Similarity (%)	Family	Cluster
	mean	standard deviation	mean	standard deviation					
Otu099029	0.00024	0.00016	0.00274	0.00087	0.00002	*Faecalibacterium prausnitzii*	98	*Ruminococcaceae*	cluster IV
Otu131074	0.00000	0.00000	0.00214	0.00122	0.00001	*Ruminococcus flavefaciens/callidus*	97	*Ruminococcaceae*	cluster IV
Otu124213	0.00000	0.00000	0.00226	0.00126	0.00000	*Subdoligranulum variabile*	98	*Ruminococcaceae*	cluster IV
Otu022359	0.00012	0.00012	0.00250	0.00101	0.00001	*Eubacterium hallii*	99	*Lachnospiraceae*	cluster XIVa
Otu067918	0.00000	0.00000	0.00226	0.00226	0.00000	*Blautia obeum*	98	*Lachnospiraceae*	cluster XIVa
Otu148782	0.00000	0.00000	0.00238	0.00238	0.00000	*Bacteroides xylanisolvens/ovatus*	99	*Bacteroidaceae*	

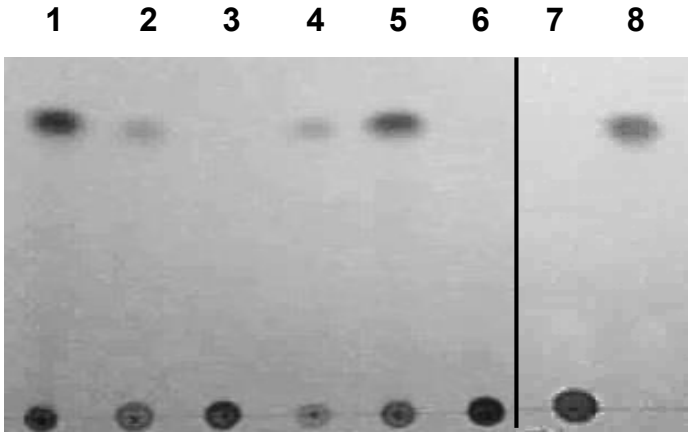
Abbreviations: OTU, operational taxonomic unit; mMCB: modified medium for colonic bacteria; mMCBISO, modified medium for colonic bacteria supplemented with isoflavones.
[a]OTUs were assigned at species level through sequence comparison against the Greengenes 16S rRNA gene database only when showing a nucleotide identity ≥97%.

MATERIAL SUPLEMENTARIO ARTÍCULO 7

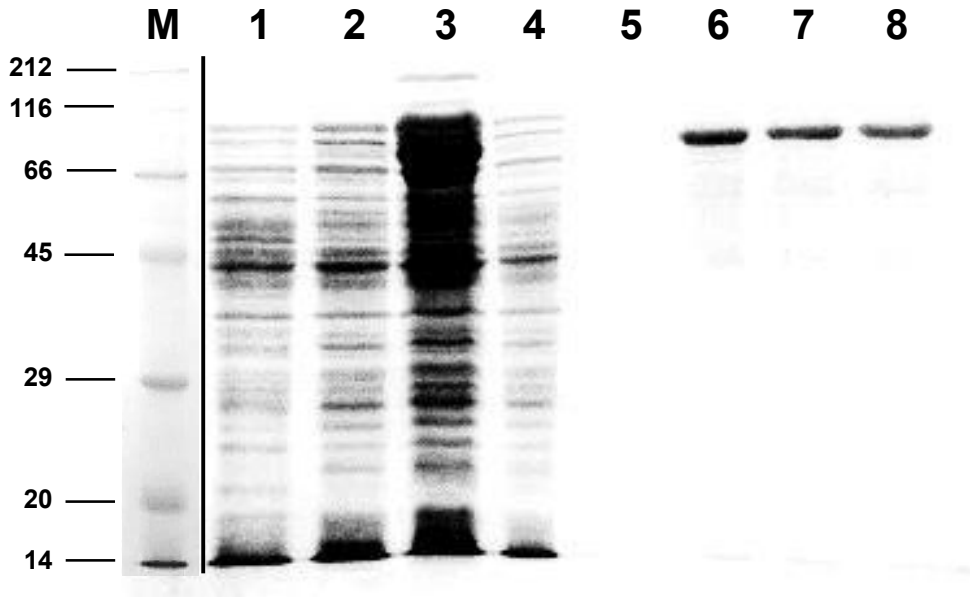


Supplementary Figure 1. Multiblast analysis of putative β-glucosidases from *Bifidobacterium pseudocatenulatum* IPLA 36007 to those on the genomes of other bifidobacteria in the databases.

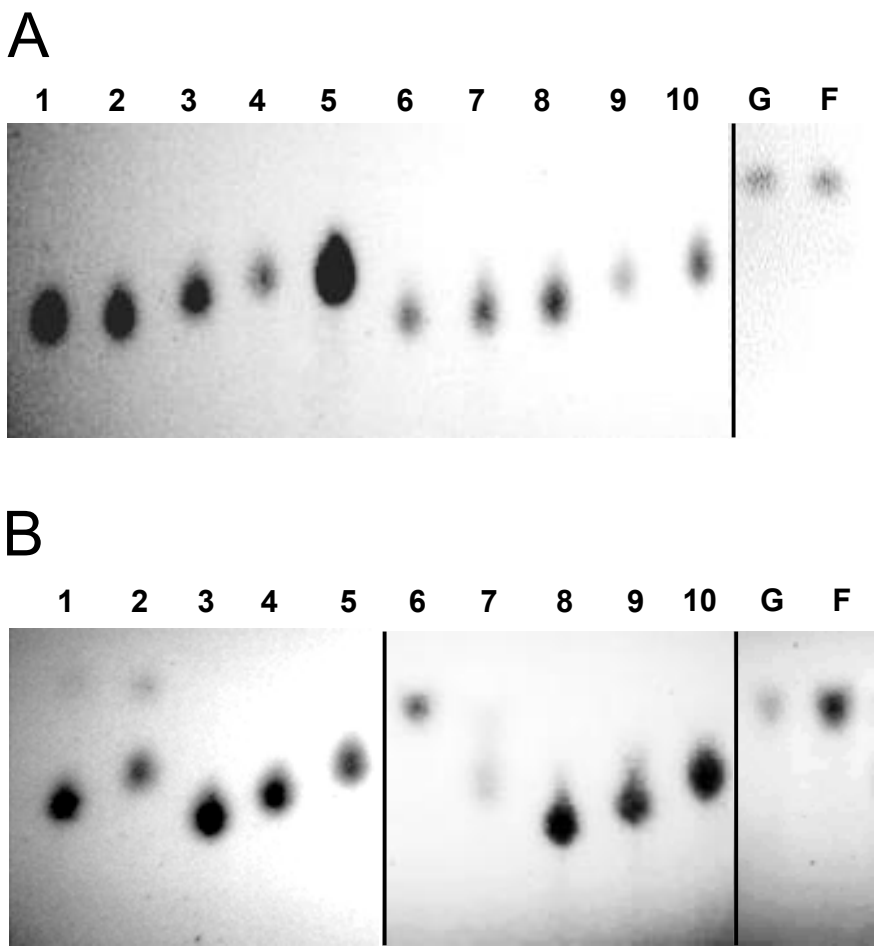
MATERIAL SUPLEMENTARIO ARTÍCULO 8



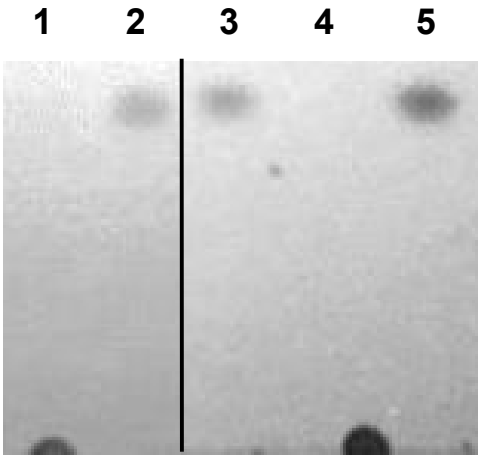
Supplementary Figure 1.- TLC showing deglycosylation of daidzin by *Escherichia coli* DH10B strain transformed with r-β-*gluA*, r-β-*gluB*, r-α-*gluC*, r-β-*gluD* and r-β-*gluE* genes from *B. pseudocatenulatum* IPLA 36007 cloned on the pUC57 vector on lanes 1, 2, 3, 4, 5, respectively. Lane 6 medium containing daidzin inoculated with *E. coli* DH10B cells harbouring pUC57 as a negative control. Lanes 7 and 8 daidzin and daidzein markers, respectively.



Supplementary Figure 2.- SDS-PAGE of purified fractions of the β-glucosidase $GluB_{His}$ during overexpression in *Escherichia coli* BL21(DE3). Order key: Lane 1, cell lysate of the expression host *E. coli* BL21(DE3); Lane 2, cell lysate of *E. coli* BL21(DE3) harbouring the cloning vector pET28a(+); Lane 3, cell lysate of *E. coli* BL21(DE3) harbouring pET28a(+)-r-β-*gluB* (overloaded); Lane 4, cell lysate of *E. coli* BL21(DE3) harbouring pET28a(+)-r-β-*gluB* after induction with 1 mM IPTG; Lane 5, washing eluate of the Ni-NTA column with 20 mM imidazole; Lanes 6, 7 and 8, consecutive elutions of GluBHis from the Ni-NTA column with 250 mM imidazole. MW, protein molecular size marker; numbers indicate the size of the markers in kDa.



Supplementary Figure 3.- Glycosidase activity of purified β-glucosidases on different sugar substrates. Order key: Panel A, Lanes 1 to 5 and lanes 6 to 10, activity of $GluA_{His}$ and $GluB_{His}$, respectively on melibiose, gentiobiose, isomaltose, cellobiose, and maltose, respectively. Panel B, lanes 1 to 5 and 6 to 10, activity of $GluD_{His}$ and $GluE_{His}$, respectively on gentiobiose, cellobiose, melibiose, isomaltose and maltose, respectively. In both panels, lanes G and F, contain glucose and fructose markers, respectively.



Supplementary Figure 4.- TLC showing deglycosylation activity on daidzin of cell-free extracts of *Lactococcus lactis* MG1363 expressing recombinant *Bifidobacterium pseudocatenulatum* IPLA 36007 β-glucosidases GluA and GluD. Order key: Lane 1, *L. lactis* MG1363 transformed with the empty p21/22 vector. Lanes 2 and 3, transformants carrying r-β-*gluA* and r-β-*gluD* genes, respectively. Lanes 4 and 5 daidzin and daidzein markers, respectively.