UNIVERSIDAD DE CÓRDOBA

TESIS DOCTORAL

Programa de Biomedicina

DIFERENCIAS ENTRE LA MICROBIOTA INTESTINAL DE HOMBRES Y MUJERES Y SU RELACIÓN CON LA PREVALENCIA DE DESARROLLO DE ENFERMEDADES METABÓLICAS

DIFFERENCES BETWEEN THE GUT MICROBIOTA OF MEN AND WOMEN AND THEIR RELATIONSHIP TO THE PREVALENCE OF DEVELOPING METABOLIC DISEASES

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Entregada en el IDEP en julio de 2022

TITULO: *DIFERENCIAS ENTRE LA MICROBIOTA INTESTINAL DE HOMBRES Y MUJERES Y SU RELACIÓN CON LA PREVALENCIA DE DESARROLLO DE ENFERMEDADES METABÓLICAS*

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Memoria de Tesis Doctoral presentada por José Antonio Santos Marcos, Licenciado en Biología por la Universidad de Córdoba, para optar al grado de Doctor en Biomedicina por la Universidad de Córdoba.

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TÍTULO DE LA TESIS

DIFERENCIAS ENTRE LA MICROBIOTA INTESTINAL DE HOMBRES Y MUJERES Y SU RELACIÓN CON LA PREVALENCIA DE DESARROLLO DE ENFERMEDADES METABÓLICAS

DOCTORANDO

JOSÉ ANTONIO SANTOS MARCOS

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

El trabajo de Tesis Doctoral titulado "Diferencias entre la microbiota intestinal de hombres y mujeres y su relación con la prevalencia de desarrollo de enfermedades metabólicas" ha sido realizado satisfactoriamente por el doctorando José Antonio Santos Marcos, Licenciado en Biología, bajo nuestra dirección en el Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC) entre los años 2015 y 2022. Este trabajo se ha basado en evaluar las diferencias entre la microbiota intestinal de hombres y mujeres y su relación con la prevalencia de desarrollo de enfermedades metabólicas y cardiovasculares, así como en evaluar la influencia que ejercen en el desarrollo de estas enfermedades las hormonas sexuales, el factor dietético, y la comunicación entre la microbiota y su huésped vía microARN. Con los resultados obtenidos se han cumplido los objetivos planteados, habiendo participado el doctorando en su difusión en dos congresos internacionales, el XXXIX Congreso de la Sociedad Española de Bioquímica y Biología Molecular (Salamanca, 2016), y el 5th Congress on Targeting Microbiota (Berlín, 2017), así como en el V Congreso de Investigadores en Formación de la Universidad de Córdoba (Córdoba, 2016). La calidad y relevancia científica de los resultados han permitido difundirlos en cuatro publicaciones originales de revistas internacionales de primer decil y cuartil, y segundo cuartil, que se citan a continuación:

1. **Santos-Marcos JA**, Rangel-Zuñiga OA, Jimenez-Lucena R, Quintana-Navarro GM, Garcia-Carpintero S, Malagon MM, Landa BB, Tena-Sempere M, Perez-Martinez P, Lopez-Miranda J, Perez-Jimenez F, Camargo A. Influence of gender and menopausal status on gut microbiota. Maturitas. 2018 Oct;116:43-53. doi: 10.1016/j.maturitas.2018.07.008. Epub 2018 Jul 19. PMID: 30244778. **Factor de impacto: 3,65 (Q1)**
2. **Santos-Marcos JA**, Haro C, Vega-Rojas A, Alcala-Diaz JF, Molina-Abril H, Leon-Acuña A, Lopez-Moreno J, Landa BB, Tena-Sempere M, Perez-Martinez P, Lopez-Miranda J, Perez-Jimenez F, Camargo A. Sex Differences in the Gut Microbiota as Potential

Determinants of Gender Predisposition to Disease. Mol Nutr Food Res. 2019 Apr;63(7):e1800870. doi: 10.1002/mnfr.201800870. Epub 2019 Feb 13. PMID: 30636111. **Factor de impacto: 5,31 (D1)**

3. **Santos-Marcos JA***, Barroso A*, Rangel-Zuñiga OA, Perdices-Lopez C, Haro C, Sanchez-Garrido MA, Molina-Abril H, Ohlsson C, Perez-Martinez P, Poutanen M, Lopez-Miranda J, Perez-Jimenez F, Tena-Sempere M, Camargo A. Interplay between gonadal hormones and postnatal overfeeding in defining sex-dependent differences in gut microbiota architecture. Aging (Albany NY). 2020 Oct 27;12(20):19979-20000. doi: 10.18632/aging.104140. Epub 2020 Oct 27. PMID: 33107844; PMCID: PMC7655199. ***Igual contribución. Factor de impacto: 5,68 (Q1)**
4. Barroso A*, **Santos-Marcos JA***, Perdices-Lopez C, Vega-Rojas A, Sanchez-Garrido MA, Krylova Y, Molina-Abril H, Ohlsson C, Perez-Martinez P, Poutanen M, Lopez-Miranda J, Tena-Sempere M, Camargo A. Neonatal exposure to androgens dynamically alters gut microbiota architecture. J Endocrinol. 2020 Oct;247(1):69-85. doi: 10.1530/JOE-20-0277. PMID: 32755996. ***Igual contribución. Factor de impacto: 4,29 (Q2)**

Además, el doctorando ha participado como primer autor en la elaboración de una publicación de revisión bibliográfica, sobre el papel de la dieta y la microbiota intestinal en el desarrollo del síndrome metabólico (citada a continuación), y actualmente está ultimando otra revisión bibliográfica, en este caso sobre la relación de las hormonas sexuales y la microbiota con el dimorfismo sexual de las enfermedades metabólicas.

Santos-Marcos JA, Perez-Jimenez F, Camargo A. The role of diet and intestinal microbiota in the development of metabolic syndrome. J Nutr Biochem. 2019 Aug;70:1-27. doi: 10.1016/j.jnutbio.2019.03.017. Epub 2019 Apr 8. PMID: 31082615. **Factor de impacto: 4,49 (Q1)**

Basándonos en lo expuesto anteriormente, estimamos que el trabajo realizado por el doctorando José Antonio Santos Marcos reúne los méritos suficientes para ser defendido ante el tribunal correspondiente y poder optar al grado de Doctor por la Universidad de Córdoba, por lo que se autoriza la presentación de esta tesis doctoral.

Córdoba, 8 de julio de 2022

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AGRADECIMIENTOS

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Tras el largo camino recorrido desde el comienzo de esta tesis, llega el momento de reflexionar y tomo conciencia de que, lejos de ser únicamente el fruto de mi trabajo, lo es también de un conjunto de personas que, en mayor o en menor medida, directa o indirectamente, han contribuido en su realización. A todas ellas, quiero expresarles mi más profundo agradecimiento.

Comienzo mi agradecimiento con quienes han contribuido en la parte técnica. A la Dra. Carmen Haro, un ejemplo de bondad cuya ayuda comenzó en el máster y continuó en la tesis con su participación en las publicaciones. A Andrés Morales, técnico de laboratorio, por su colaboración en la realización de la parte experimental, por su forma de ser y su cercanía en el trato personal. Al Dr. Oriol Rangel, por su cordialidad en el trato y su participación en el análisis de datos. A la Dra. Alexia Barroso y a su director de tesis, el Dr. Manuel Tena, por su participación en dos de las publicaciones y por su amabilidad y disponibilidad en todo momento.

Continúo mi agradecimiento con mi director de tesis, el Dr. Francisco Pérez Jiménez, una eminencia en su campo y un referente en el Hospital Reina Sofía de Córdoba en toda una vida dedicada a la Medicina. Gracias por haberme dado la oportunidad de realizar esta tesis dedicándome parte de su tiempo al final de su vida profesional. En este punto quiero tener una mención especial para mi también director de tesis y paisano, el Dr. Antonio Camargo García, por haber visto en mí la capacidad de abordar esta tesis durante la dirección de mi Trabajo de Fin de Máster y haberme brindado esta oportunidad. Sin duda, has sido la pieza clave para poder llevar a cabo este trabajo, y contigo, la tesis ha trascendido hasta la complicidad y la amistad. Gracias por todas las horas que me has dedicado por las tardes en el IMIBIC y los fines de semana en mi casa, durante tus visitas a Castro, y por su prolongación con la cerveza de mediodía en "Las Cabrerizas". Son, sin duda, recuerdos que siempre estarán presentes en mi memoria.

No puedo olvidarme de mis amigos de siempre, que han contribuido a ser lo que soy, por su amistad y por su interés en el desarrollo de esta tesis. A David Hidalgo, compañero desde la infancia y amigo desde siempre. Gracias por tu participación en la publicación de revisión bibliográfica, por tu amistad y por contagiarme tu positivismo a la hora de entender la vida. A Jesús Alba, por tu amistad y por tu ejemplo de honradez y de buen hacer en el trabajo. A Cristóbal Priego, compañero de piso, junto con Jesús, en la carrera, y amigo desde entonces. A Juan Barrera, compañero de viajes y otro ejemplo de honradez y de trabajador incansable. A Javier Pérez, por los kilómetros y las charlas compartidas con la bicicleta. A Rafael Blanco, por contagiarme tu pasión por la investigación. A Antonio Jiménez, amigo y confidente, gracias por las veladas de conversación amena, por tu sincera amistad. Gracias a todos por vuestra amistad y por saber que estáis ahí siempre que os necesito.

Quiero finalizar mi agradecimiento con mi familia, con la que siempre puedo contar. Con mis hermanos, y en especial con mis padres, que incluso antes de mi nacimiento ya habían decidido sacrificarse en un país lejano por el bien de sus hijos, y en los que siempre he visto un ejemplo de trabajo, de sacrificio, y de buen hacer. A mi madre, por alegarse de mis éxitos, por su incondicional apoyo, por su positivismo y su ejemplo de bondad hacia los demás. A mi padre, que ya no está, pero que permanece en mi recuerdo constantemente y permanecerá hasta el fin de mis días. Por haberme inculcado desde siempre hasta contagiarme su "apego a la tierra", por haberme animado siempre al estudio como forma de prosperar en la vida, por su ejemplo de trabajo constante e incansable. A él le debo gran parte de lo que soy y sé que se hubiese enorgullecido al ver culminada esta etapa de mi vida. A su memoria dedico esta tesis.

Mi eterna gratitud a todos.

INFORME SOBRE LA CALIDAD DE LAS PUBLICACIONES DE LA TESIS

1. **Santos-Marcos JA,** Rangel-Zuñiga OA, Jimenez-Lucena R, Quintana-Navarro GM, Garcia-Carpintero S, Malagon MM, Landa BB, Tena-Sempere M, Perez-Martinez P, Lopez-Miranda J, Perez-Jimenez F, Camargo A. Influence of gender and menopausal status on gut microbiota. Maturitas. 2018 Oct;116:43-53. doi: 10.1016/j.maturitas.2018.07.008. Epub 2018 Jul 19. PMID: 30244778.

   Factor de impacto de la revista (JCR): **3,65**
   Categoría: OBSTETRICS & GYNECOLOGY
   Puesto: 9/83; **Q1**

2. **Santos-Marcos JA,** Haro C, Vega-Rojas A, Alcala-Diaz JF, Molina-Abril H, Leon-Acuña A, Lopez-Moreno J, Landa BB, Tena-Sempere M, Perez-Martinez P, Lopez-Miranda J, Perez-Jimenez F, Camargo A. Sex Differences in the Gut Microbiota as Potential Determinants of Gender Predisposition to Disease. Mol Nutr Food Res. 2019 Apr;63(7):e1800870. doi: 10.1002/mnfr.201800870. Epub 2019 Feb 13. PMID: 30636111.

   Factor de impacto de la revista (JCR): **5,31**
   Categoría: FOOD SCIENCE & TECHNOLOGY
   Puesto: 9/135; **D1**

3. **Santos-Marcos JA***, Barroso A*, Rangel-Zuñiga OA, Perdices-Lopez C, Haro C, Sanchez-Garrido MA, Molina-Abril H, Ohlsson C, Perez-Martinez P, Poutanen M, Lopez-Miranda J, Perez-Jimenez F, Tena-Sempere M, Camargo A. Interplay between gonadal hormones and postnatal overfeeding in defining sex-dependent differences in gut microbiota architecture. Aging (Albany NY). 2020 Oct 27;12(20):19979-20000. doi: 10.18632/aging.104140. Epub 2020 Oct 27. PMID: 33107844; PMCID: PMC7655199. ***Igual contribución**

   Factor de impacto de la revista (JCR): **5,68**
   Categoría: GERIATRICS & GERONTOLOGY
   Puesto: 10/53; **Q1**

4. Barroso A*, **Santos-Marcos JA*,** Perdices-Lopez C, Vega-Rojas A, Sanchez-Garrido MA, Krylova Y, Molina-Abril H, Ohlsson C, Perez-Martinez P, Poutanen M, Lopez-Miranda J, Tena-Sempere M, Camargo A. Neonatal exposure to androgens dynamically alters gut microbiota architecture. J Endocrinol. 2020 Oct;247(1):69-85. doi: 10.1530/JOE-20-0277. PMID: 32755996. ***Igual contribución**

   Factor de impacto de la revista (JCR): **4,29**
   Categoría: ENDOCRINOLOGY & METABOLISM
   Puesto: 61/145; **Q2**

NOTA: Los índices de impacto y la posición en la categoría corresponden al año de paginación del artículo.

ÍNDICE

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ABREVIATURAS

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ACS, Acil-CoA sintetasa

AGA, Alteración de la glucemia en ayunas

AGCC, Ácidos grasos de cadena corta

AgRP, Proteína relacionada con agutí (del inglés "*Agouti*-Related *Protein"*)

AMPK, Proteína quinasa activada por AMP (del inglés *"AMP-activated protein kinase"*)

ARNlnc, ARN largo no codificante

ATG, Alteración de la tolerancia a la glucosa

AUC, Área bajo la curva (del inglés "Area under the curve")

BMI, Body mass index

CCK, Colecistoquinina

CN-DC, Camada normal y dieta control

CP-DAG, Camada pequeña y dieta alta en grasa

CYP7A1, Colesterol 7α-hidroxilasa (Citocromo P450 Familia 7 Subfamilia A Miembro 1)

DHT, Dihidrotestosterona

DPN, Día posnatal

DT2, Diabetes de tipo 2

F/B, *Firmicutes/Bacteroidetes*

FCF, Factor de crecimiento de fibroblastos

FT, Factores de transcripción

FXR, Receptor farnesoide X (del inglés "F*arnesoid X receptor")*

GFHS, Globulina fijadora de hormonas sexuales

GH, Hormona del crecimiento (del inglés *"Growth hormone"*)

GiP, Péptido inhibidor gástrico (del inglés "Gastric inhibitory peptide")

GLP-1, Péptido similar al glucagón-1 (del inglés *"Glucagon-like peptide-1"*)

GnRH, Hormona liberadora de gonadotropina (del inglés *"Gonadotropin-releasing hormone"*)

GTT, Glucose tolerance test

HDL, Lipoproteínas de alta densidad (del inglés *"High-density lipoproteins"*)

IAL, Índice de andrógenos libres

IMC, Índice de masa corporal

ITT, Insulin tolerance test

JAK2, Janus quinasa 2

LDL, Lipoproteínas de baja densidad (del inglés *"Low-density lipoproteins"*)

LEfSe, Análisis lineal discriminante del efecto del tamaño (del inglés *"Linear discriminant analysis (LDA) effect size"*)

LG, Libres de gérmenes

LPL, Lipoproteína lipasa

LPS, Lipopolisacárido

MCP-1, Proteína quimiotáctica de monocitos-1 (del inglés *"Monocyte chemoattractant protein-1")*

MetS, Metabolic syndrome

miARN, MicroARN

NF-κB, Factor nuclear kappa B (del inglés *"Nuclear factor κB"*)

NL-CD, Normal litter and control diet

NpY, Neuropéptido Y

O-DMA, O-desmetilangolensina

PCOS, Polycystic ovary syndrome

PCR, Proteína C reactiva

PND, postnatal day

POMC, Proopiomelanocortina

PYY, Péptido YY

RA, Receptor de andrógenos

REα, Receptor de estrógenos alfa

REβ, Receptor de estrógenos beta

SL-HFD, Small litter and high fat diet

SM, Síndrome metabólico

SOP, Síndrome del ovario poliquístico

STAT, Transductor de señales y activador de la transcripción (del inglés *"Signal transducer and activator of transcription"*)

T2D, Type 2 diabetes

Th17, Células colaboradoras T17 (Del inglés *"Helper"*)

TLR, Receptor tipo Toll (del inglés *"Toll-like receptors"*)

TMF, trasplante de microbiota fecal

TNF-α, Factor de necrosis tumoral alfa (del inglés *"Tumour necrosis factor"*)

Treg, Células T reguladoras

TTG, Test de tolerancia a la glucosa

TTI, Test de tolerancia a la insulina

I. RESUMEN

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1.1. Introducción

La creciente incidencia de las enfermedades metabólicas en la población mundial, particularmente de la obesidad, el síndrome metabólico (SM), y la diabetes de tipo 2 (DT2), ha hecho de ellas un problema sanitario, social, y económico de primer orden. El hecho de que estas patologías muestren un marcado dimorfismo sexual en su desarrollo y prevalencia hace suponer la implicación en ellas de las hormonas sexuales.

La obesidad, además de constituir una patología per se, constituye un factor de riesgo para el SM, que a su vez influye en la DT2. Dentro de este marco, se han descrito dos patrones de distribución de la grasa, un patrón periférico, típico de mujeres premenopáusicas, y un patrón central, típico de mujeres posmenopáusicas y hombres. Ambos patrones, sujetos a una base genética regulada por las hormonas sexuales, están relacionados con el desarrollo de las enfermedades metabólicas, mostrando la grasa central (abdominal visceral) un perfil patológico frente a un perfil protector de la grasa periférica (subcutánea).

La influencia de las hormonas sexuales en las enfermedades metabólicas está avalada por situaciones en las que sus niveles están alterados. Hombres y mujeres transgénero muestran una redistribución de la grasa corporal tras el tratamiento con esteroides sexuales, al igual que ocurre tras los cambios hormonales de la menopausia. La disminución del nivel de estrógeno, tras la menopausia y tras una ooforectomía, eleva el riesgo de sufrir DT2, mientras que la terapia hormonal con estrógenos en mujeres posmenopáusicas reduce su incidencia. Las mujeres con hiperandrogenismo debido al síndrome del ovario poliquístico (SOP) muestran una mayor adiposidad central y un mayor riesgo de sufrir SM. En los hombres, la terapia de privación de andrógenos aumenta la masa grasa y la prevalencia del SM y la DT2, mientras que el tratamiento con testosterona disminuye la grasa visceral, el SM, y la DT2.

En los últimos años, durante el desarrollo de esta tesis, nuestro grupo de investigación, junto con otros grupos de investigación, ha aportado evidencias que apoyan la idea de la existencia de un dimorfismo sexual en la composición de la microbiota intestinal, en el que las hormonas sexuales parecen desempeñar un papel destacado.

La alteración o la protección de la mucosa intestinal por parte de la microbiota intestinal es un factor clave en el mantenimiento de la llamada barrera intestinal, que limita el acceso de los microorganismos al torrente sanguíneo y, por tanto, influye en el estado inflamatorio descrito en procesos como la obesidad y el SM. La acción de la microbiota intestinal se extiende al sistema nervioso central a través del eje intestino-cerebro para influir en la ingesta de alimentos, e incluso al hígado a través del eje intestino-hígado para regular el metabolismo de los nutrientes. La interacción entre la microbiota intestinal y su huésped parece influir así en el desarrollo de las enfermedades metabólicas, en donde los cambios en la microbiota podrían conformar, al menos en

parte, los mecanismos patogénicos de la obesidad, la resistencia a insulina, y el desarrollo del SM. Además, en ratones se ha constatado la influencia de la microbiota intestinal en el nivel de las hormonas sexuales, pues la colonización por microbios comensales eleva la testosterona en los ratones macho, mientras que la transferencia de la microbiota intestinal de los machos adultos a las hembras inmaduras altera su microbiota y eleva la testosterona.

La relación entre la microbiota intestinal, las hormonas sexuales, y el desarrollo de ciertas enfermedades ofrece un nuevo campo de investigación en la prevención de estas enfermedades mediante la manipulación de la microbiota intestinal. En este sentido, los trasplantes fecales han cobrado gran interés como terapia alternativa en el tratamiento de enfermedades como el SM, en donde la transferencia de la microbiota fecal de donantes sanos a pacientes con esta patología mejora la sensibilidad a la insulina. En la misma línea, han surgido terapias basadas en la modificación de la microbiota mediante intervención dietética y el uso de prebióticos y probióticos.

1.2. Hipótesis

Hipótesis: Existen diferencias entre la microbiota intestinal de hombres y mujeres, lo que podría a su vez ser un determinante en la prevalencia en el desarrollo de enfermedades metabólicas y cardiovasculares. Hipótesis nula: No existen diferencias entre la microbiota intestinal de hombres y mujeres.

1.3. Objetivos

OBJETIVO PRINCIPAL:

1. Evaluar la existencia de diferencias en la microbiota intestinal asociadas al estado hormonal entre mujeres pre y posmenopáusicas (disminución de estrógenos), así como explorar las diferencias con la microbiota de hombres de similar edad, índice de masa corporal y hábitos nutricionales, mediante secuenciación masiva del ADN bacteriano.

OBJETIVOS SECUNDARIOS:

2. Evaluar las diferencias en la microbiota intestinal de pacientes con síndrome metabólico en función del sexo y determinar si dos dietas saludables, la dieta mediterránea y una dieta baja en grasa, pueden modular la disbiosis microbiana de forma diferencial en función del sexo.
3. Determinar la contribución de las hormonas sexuales y la obesidad a las diferencias en la estructura y composición de la microbiota intestinal entre sexos mediante la caracterización de las diferencias en la microbiota intestinal ante la reducción de las hormonas gonadales en ratas hembra sometidas a ovariectomía y ratas macho a orquiectomía, así como analizar el efecto de la obesidad inducida por la dieta en la microbiota intestinal en estos modelos. Además, se evaluará la potencial implicación

de los miARN intestinales en la interacción entre la microbiota intestinal y su huésped.

4. Explorar la influencia de los esteroides sexuales y de una dieta obesogénica (sobrealimentación posnatal) desde las primeras fases del desarrollo mediante un modelo de ratas androgenizadas, en la modificación persistente de la estructura de la microbiota intestinal, así como la posible interacción entre la microbiota intestinal y el huésped a través de la regulación microbiana de la expresión de miARN en el intestino delgado y grueso.

1.4. Características del estudio

Publicación 1: Este estudio se realizó con 76 individuos incluidos en el grupo control de personas sanas del estudio ONCOVER, un estudio centrado en el desarrollo de un sistema de detección de compuestos volátiles para el diagnóstico precoz del cáncer de pulmón, colon, mama, y próstata. Los 76 individuos conformaban cuatro grupos: 17 mujeres premenopáusicas, 20 mujeres posmenopáusicas, y 19 y 20 hombres como grupo de control para las mujeres pre y posmenopáusicas respectivamente. Para la confección de los grupos se tuvieron en cuenta aspectos como la edad, el índice de masa corporal (IMC), y los hábitos nutricionales. Se analizaron las diferencias asociadas al sexo y al estado hormonal de la microbiota intestinal, la endotoxemia, las incretinas intestinales, las citoquinas proinflamatorias, y los niveles plasmáticos de las hormonas reguladoras de la homeostasis energética entre las mujeres pre y posmenopáusicas y entre ellas y sus respectivos grupos control de hombres.

Publicación 2: Este estudio se realizó en el marco del estudio CORDIOPREV, un ensayo prospectivo, aleatorio y abierto en 1002 pacientes con cardiopatía coronaria. Se analizó la microbiota intestinal al inicio y después de 3 años de intervención dietética en 123 mujeres y 123 hombres, pareados por las principales variables metabólicas y categorizados según la presencia o no de SM en función de los criterios del Panel de Tratamiento de Adultos III (1). Además del tratamiento convencional para la cardiopatía, los pacientes siguieron dos modelos de dieta, la dieta mediterránea, y una dieta baja en grasa. Los cuatro grupos experimentales fueron: 79 mujeres y 79 hombres con SM (ambos grupos con las mismas combinaciones de criterios del SM), y 44 mujeres y 44 hombres sin SM. El efecto del consumo de ambas dietas en la microbiota intestinal de los pacientes con SM se estudió en 99 de los 158 pacientes con SM (por motivo de disponibilidad de muestras fecales a los 3 años de seguimiento).

Publicación 3: El estudio se realizó con un modelo animal, concretamente con ratas Wistar, machos y hembras, criadas en el vivario de la Universidad de Córdoba y alimentadas con una dieta control (10%, 20% y 70% de calorías procedentes de grasas, proteínas y carbohidratos, respectivamente) o una dieta alta en grasa (45%, 20% y 35% de calorías procedentes de grasas, proteínas y carbohidratos, respectivamente). En el día posnatal (DPN)-1, las crías se separaron en dos tamaños de camada: camadas

pequeñas (4 crías por camada), como modelo de sobrealimentación posnatal, y camadas normales (12 crías por camada), como modelo de alimentación normal. Tras su destete, en el DPN-23 se hicieron subgrupos de hembras y machos de camadas normales y pequeñas, que fueron alimentados ad libitum con la dieta control o la dieta alta en grasa, respectivamente, para generar dos grupos experimentales, camada normal y dieta control (CN-DC), y camada pequeña y dieta alta en grasa (CP-DAG), representativos del fenotipo magro y obeso respectivamente. Subconjuntos de animales de cada grupo fueron sometidos en el DPN-90 a una gonadectomía como modelo de cese de las secreciones gonadales. En el DPN-120, los animales fueron sometidos a un test de tolerancia a la glucosa (TTG) y, una semana más tarde, a un test de tolerancia a la insulina (TTI) para evaluar el desarrollo de la resistencia a la insulina. Los experimentos finalizaron en el DPN-150. En esta última fase se controlaron los índices fenotípicos y los parámetros bioquímicos/hormonales del suero, y se diseccionó el intestino delgado y grueso para obtener muestras fecales con las que analizar la microbiota y el ARN.

Publicación 4: El estudio se llevó a cabo con un modelo animal, concretamente con ratas Wistar, machos y hembras, criadas en el vivario de la Universidad de Córdoba y alimentadas con una dieta control (10%, 20% y 70% de calorías procedentes de grasas, proteínas y carbohidratos, respectivamente) o con una dieta alta en grasa (45%, 20% y 35% de calorías procedentes de grasas, proteínas y carbohidratos, respectivamente). En el DPN-1, las crías se separaron en dos tamaños de camada: pequeña (4 crías por camada), como modelo de sobrealimentación posnatal, y normal (12 crías por camada), como modelo de alimentación normal. A continuación, subconjuntos de hembras de ambos tamaños de camada fueron androgenizados con propionato de testosterona, mientras que otras hembras fueron inyectadas con vehículo (aceite de oliva). Tras su destete, en el DPN-23 se hicieron subgrupos de hembras androgenizadas o tratadas con vehículo y machos de camada normal y camada pequeña, que fueron alimentados ad libitum con la dieta control o la dieta alta en grasa, respectivamente, para generar dos grupos experimentales, CN-DC y CP-DAG, representativos del fenotipo magro y obeso respectivamente. El impacto a corto y largo plazo de la androgenización neonatal y la dieta obesogénica en analizó en el DPN-50 y el DPN-150, representativos de ratas adultas jóvenes (DPN-50) y adultas (DPN-150). Las ratas de todos los grupos se pesaron y fueron sometidas a un TTG y a un TTI. Se midieron los niveles de glucosa y su variación se estimó como área bajo la curva (AUC). Tras el sacrificio de las ratas se tomaron muestras de sangre, heces, y tejidos, para analizar los índices fenotípicos, los parámetros bioquímicos/hormonales, la microbiota, y el ARN.

1.5. Resultados

Publicación 1: En cuanto a la microbiota intestinal, las mayores diferencias se observaron entre las mujeres posmenopáusicas y premenopáusicas, y entre estas últimas y su grupo control de hombres. Con respecto a las mujeres premenopáusicas, las posmenopáusicas tenían mayor abundancia del filo *Firmicutes* y menor abundancia

del filo *Actinobacteria*, mayor abundancia de los géneros *Lachnospira* y *Roseburia* y menor abundancia de los géneros *Ruminococcus (Lachnospiraceae), Parabacteroides, Prevotella*, y *Bilophila,* si bien este último género no alcanzó la significación estadística. Respecto a sus grupos control de hombres, las mujeres posmenopáusicas presentaban una mayor la relación *Firmicutes/Bacteroidetes (F/B)*, mientras que las premenopáusicas presentaban una mayor abundancia de los géneros *Ruminococcus (Lachnospiraceae)*, *Bilophila*, *Prevotella,* y *Oscillospira*, pero en este último género sin alcanzar la significación estadística. En lo referente a los marcadores inflamatorios, las mujeres premenopáusicas tenían un menor nivel de IL-6 y proteína quimiotáctica de monocitos-1 (MCP-1) que las posmenopáusicas. Con respecto a las hormonas reguladoras implicadas en la homeostasis energética y a las incretinas, el nivel del péptido similar al glucagón-1 (GLP-1) fue menor en las mujeres posmenopáusicas que en las premenopáusicas, el del péptido inhibidor gástrico (GiP) y de leptina mayor en el conjunto de las mujeres que en el de los hombres, y el de adiponectina mayor en las mujeres premenopáusicas que en su grupo control de hombres. Finalmente, el análisis PICRUST de la microbiota intestinal evidenció una tendencia estadística hacia un mayor metabolismo del propanoato y del butanoato en las mujeres premenopáusicas en comparación con los otros grupos.

Publicación 2: El análisis lineal discriminante del efecto del tamaño (LEfSe) reveló que las diferencias en la microbiota intestinal eran más acusadas en las mujeres y hombres sin SM que en las mujeres y hombres con SM. El análisis univariante (ANOVA) mostró diferencias específicas de sexo en los grupos con SM ausentes en los grupos sin SM; en este caso una mayor abundancia de los géneros *Phascolarctobacterium, Collinsella*, *Alistipes*, y *Anaerotruncus* en mujeres con SM que en hombres con SM, al contrario que con los géneros *Faecalibacterium* y *Prevotella*. Además, hubo una mayor abundancia de los géneros *Ruminococcus* (*Lachnospiraceae*) y *Bilophila* en las mujeres con y sin SM que en sus grupos masculinos, así como una mayor abundancia de los géneros *Clostridium* y *SMB53* en hombres que en mujeres, tanto con SM como sin SM.

En cuanto al efecto diferencial de la dieta en la composición de la microbiota intestinal, se observó una mayor proporción de los géneros *Desulfovibrio*, *Roseburia,* y *Holdemania* en hombres con SM que en mujeres con SM tras el consumo de la dieta baja en grasa, así como una mayor proporción de un género desconocido de la familia *Rikenellaceae* en hombres con SM que en mujeres con SM tras el consumo de la dieta mediterránea.

Publicación 3: La gonadectomía no modificó la mayoría de las diferencias observadas a nivel de filo encontradas entre machos y hembras sin gonadectomía, tanto con alimentación normal como con sobrealimentación. No obstante, en cuanto a los géneros relacionados con las patologías metabólicas, la gonadectomía redujo la abundancia de *Roseburia* y *Butyricimonas* en las hembras sometidas a sobrealimentación. También se detectó una relación *F/B* más alta en hembras con gonadectomía que en machos ante la sobrealimentación.

Nuestros resultados mostraron una relación entre la microbiota intestinal y la expresión intestinal de varios microARN (miARN) asociados a vías relacionadas con los esteroides sexuales. Las funciones de KEGG, en las que se asignaron los miARN seleccionados, incluían el metabolismo de los lípidos, los aminoácidos, los cofactores y las vitaminas, la transducción de señales y los sistemas endocrinos. En concreto, estaban implicadas las vías de señalización de la insulina, la hormona liberadora de gonadotropina (GnRH), el estrógeno y la prolactina, y la maduración de ovocitos mediada por la progesterona.

Publicación 4: Con respecto a las hembras intactas, la androgenización neonatal aumentó el peso en el DPN-50 y el DPN-150, tanto con alimentación normal como con sobrealimentación, al tiempo que produjo varios efectos hormonales, entre ellos una reducción de la progesterona en el DPN-50 y el DPN-150, un aumento de estradiol y de estrona en el DPN-50 en el DPN-50, todo ello en ambos tipos de alimentación (niveles altos frente a no detectados), así como un aumento de estrona en el DPN-150 bajo sobrealimentación.

La androgenización perjudicó el equilibrio energético y la homeostasis de la glucosa, mostrando las ratas androgenizadas en el DPN-50 una mayor AUC de glucosa que las hembras control, que alcanzó la significación estadística ante la sobrealimentación, pero no en la alimentación normal. El TTG mostró niveles de glucosa más altos (menor tolerancia a la glucosa) en las hembras androgenizadas (y en los machos) en el DPN-50 que en las hembras control. Además, el TTI mostró una mayor AUC de glucosa (menor sensibilidad a la insulina) en las hembras androgenizadas que en las hembras control sometidas a sobrealimentación en el DPN-50 y el DPN-150. El TTI también mostró a los 0 minutos niveles de glucosa más altos en las hembras androgenizadas que en las hembras control en el DPN-50 bajo alimentación normal y sobrealimentación, así como niveles más altos en las hembras androgenizadas (y en los machos) que en las hembras intactas en el DPN-150 bajo sobrealimentación.

La androgenización modificó la microbiota intestinal con respecto a las hembras control. A nivel de filo, en el DPN-50 bajo alimentación normal aumentó *Bacteroidetes* y disminuyeron *Firmicutes* y *Euryarchaeota*, mientras que con la sobrealimentación se redujeron *Euryarchaeota*, *Verrucomicrobia* y *Cyanobacteria*. Por su parte, en el DPN-150 bajo alimentación normal aumentó el filo *Cyanobacteria* y disminuyeron *Euryarchaeota* y *Actinobacteria*, mientras que con la sobrealimentación aumentó *Bacteroidetes*.

Nuestros resultados mostraron una relación entre la microbiota intestinal y la expresión intestinal de varios miARN asociados a vías relacionadas con los esteroides sexuales. En este contexto, se observaron asociaciones mediadas por miARN entre la microbiota intestinal y: 1) Procesos relacionados con las hormonas sexuales: biosíntesis de esteroides, meiosis de ovocitos, y maduración de ovocitos mediada por progesterona; 2) Metabolismo: vía de señalización de la insulina, biosíntesis de ácidos grasos, y metabolismo de ácidos grasos; 3) Integridad de la barrera intestinal: adhesión focal, unión adherente, y biosíntesis de mucina tipo O-glicano.

1.6. Conclusiones

1. Nuestros resultados reflejan que hombres y mujeres difieren en la composición de su microbiota intestinal y que estas diferencias dependen del estado hormonal de la mujer (premenopausia vs posmenopausia), lo que podría estar relacionado con el dimorfismo sexual observado en la incidencia de las enfermedades metabólicas y cardiovasculares, dada la relación de estas enfermedades con la microbiota intestinal.
2. Nuestros resultados sugieren que las alteraciones de la microbiota intestinal asociadas al síndrome metabólico parecen ser diferentes entre hombres y mujeres. Estos resultados sugieren a su vez la implicación de la microbiota intestinal en la diferencia en la incidencia de enfermedades metabólicas entre sexos. Además, los diferentes cambios inducidos por la dieta mediterránea o una dieta baja en grasa en la microbiota intestinal según el sexo sugieren que las mujeres y los hombres podrían beneficiarse de forma diferente de una dieta específica en función de su sexo.
3. Nuestros resultados muestran la contribución de las hormonas gonadales a la definición de las diferencias dependientes del sexo en la microbiota intestinal, así como su potencial papel en la formación de esta microbiota como consecuencia de la interacción entre el sexo y la nutrición. De hecho, la eliminación de las hormonas sexuales modifica la microbiota intestinal hacia un perfil más deletéreo, especialmente en las hembras tras una sobrealimentación posnatal, lo que confirma la implicación de la dieta en estos procesos. Por otro lado, nuestros resultados sugieren que la implicación de la microbiota intestinal en las enfermedades metabólicas podría estar mediada por la interacción entre la microbiota y su huésped a través de los miARN intestinales.
4. Nuestros resultados sugieren que la alteración nutricional y hormonal en los primeros períodos del desarrollo alteran la programación metabólica y la estructura de la microbiota intestinal, cuyas consecuencias se prolongan en el tiempo. La relación observada entre los cambios en la microbiota intestinal y la expresión de miARN en el intestino delgado y grueso sugiere un posible mecanismo de comunicación cruzada entre la microbiota intestinal y el huésped que puede contribuir a amplificar el desajuste metabólico causado por la obesidad.

I. ABSTRACT

1.1. Introduction

The increasing incidence of metabolic diseases in the world population, particularly obesity, metabolic syndrome (MetS), and type 2 diabetes (T2D), has made them a major health, social and economic problem. The fact that these pathologies show a marked sexual dimorphism in their development and prevalence suggests that sex hormones are involved.

Obesity, in addition to being a pathology per se, is a risk factor for MetS, which in turn influences T2D. Within this framework, two patterns of fat distribution have been described, a central pattern, typical of postmenopausal women and men, and a peripheral pattern, typical of premenopausal women. Both patterns, subject to a genetic basis regulated by sex hormones, are related to the development of metabolic diseases, with central (visceral abdominal) fat showing a pathological profile versus a protective profile of peripheral (subcutaneous) fat.

The influence of sex hormones on metabolic diseases is supported by situations in which their levels are altered. Transgender men and women show a redistribution of body fat after sex steroid treatment, as occurs after the hormonal changes of menopause. Decreased estrogen levels after menopause and after oophorectomy increase the risk of T2D, whereas estrogen hormone therapy in postmenopausal women reduces its incidence. Women with hyperandrogenism due to polycystic ovary syndrome (PCOS) show increased central adiposity and increased risk of MetS. In men, androgen deprivation therapy increases fat mass and the prevalence of MetS and T2D, while testosterone treatment decreases visceral fat, T2D and MetS

In recent years, during the development of this thesis, our research group, together with other research groups, has provided evidence supporting the idea of the existence of a sexual dimorphism in the composition of the gut microbiota, in which sex hormones seem to play a prominent role.

The alteration or protection of the gut mucosa by the gut microbiota is a key factor in the maintenance of the so-called gut barrier, which limits the access of microorganisms to the bloodstream and thus influences the inflammatory state described in processes such as obesity and MetS. The action of the gut microbiota extends to the central nervous system via the gut-brain axis to influence food intake, and even to the liver via the gut-liver axis to regulate nutrient metabolism. The interaction between the gut microbiota and its host thus appears to influence the development of metabolic diseases, where changes in the microbiota could shape, at least in part, the pathogenic mechanisms of obesity, insulin resistance, and the development of MetS. In addition, the influence of gut microbiota on sex hormone levels has been demonstrated in mice, where colonisation by commensal microbes raises testosterone in male mice, while

transfer of gut microbiota from adult males to immature females alters their microbiota and raises testosterone.

The relationship between gut microbiota, sex hormones, and disease development offers a new field of research in disease prevention through manipulation of the gut microbiota. In this regard, faecal transplants have become of great interest as an alternative therapy in the treatment of diseases such as MetS, where the transfer of faecal microbiota from healthy donors to patients with MetS improves insulin sensitivity. In the same vein, therapies based on modification of the microbiota through dietary intervention, as well as the use of prebiotics and probiotics, have emerged.

1.2. Hypothesis

Hypothesis: There are differences between the gut microbiota of men and women, which could in turn be a determinant in the prevalence in the development of metabolic and cardiovascular diseases. Null hypothesis: There are no differences between the gut microbiota of men and women.

1.3. Objectives

MAIN OBJECTIVE:

1. To evaluate the existence of differences in gut microbiota associated with hormonal status between pre- and postmenopausal women (estrogen depletion), as well as to explore the differences with the microbiota of men of similar age, body mass index and nutritional habits, by means of massive sequencing of bacterial DNA.

SECONDARY OBJECTIVES:

2. To evaluate the differences in the gut microbiota of patients with metabolic syndrome according to sex and to determine whether two healthy diets, the Mediterranean diet and a low-fat diet, can modulate microbial dysbiosis differentially according to sex.
3. To determine the contribution of sex hormones and obesity to sex differences in gut microbiota structure and composition by characterising differences in gut microbiota upon gonadal hormone depletion in ovariectomised female rats and orchiectomised male rats, and to analyse the effect of diet-induced obesity on gut microbiota in these models. In addition, the potential involvement of gut miRNAs in the interaction between the gut microbiota and its host will be evaluated.
4. To explore the influence of sex steroids and an obesogenic diet (postnatal overfeeding) from the early stages of development using an androgenised rat model on the persistent modification of gut microbiota structure, as well as the possible interaction between gut microbiota and host through microbial regulation of miRNA expression in the small and large intestine.

1.4. Characteristics of the study

Publication 1: This study was conducted with 76 individuals included in the healthy control group of the ONCOVER study, a study focused on the development of a screening system for volatile compounds for the early diagnosis of lung, colon, breast, and prostate cancer. The 76 individuals comprised four groups: 17 premenopausal women, 20 postmenopausal women, and 19 and 20 age-matched men as a control group for the premenopausal and postmenopausal women, respectively. Age, body mass index (BMI), and nutritional habits were taken into account for grouping. Sex- and hormonal status-associated differences in gut microbiota, endotoxaemia, gut incretins, proinflammatory cytokines and plasma levels of hormones regulating energy homeostasis were analysed among the pre- and postmenopausal women and among their respective control groups of men.

Publication 2: This study was conducted as part of the CORDIOPREV study, a prospective, randomised, open-label trial in 1002 patients with coronary heart disease. Gut microbiota was analysed at baseline and after 3 years of dietary intervention in 123 women and 123 men, matched for major metabolic variables and categorised according to the presence or absence of MetS according to the criteria of the Adult Treatment Panel III (Expert Panel 2001) (1). In addition to conventional treatment for heart disease, patients followed two dietary patterns, the Mediterranean diet and a low-fat diet. The four experimental groups were: 79 women and 79 men with MetS (both groups shared the same combinations of MetS criteria), and 44 women and 44 men without MetS. The effect of consumption of both diets on the gut microbiota of patients with MetS was performed in a smaller population (99 of 158 patients with MetS), due to the availability of faecal samples at 3 years follow-up.

Publication 3: The study was conducted with an animal model, specifically with male and female Wistar rats, bred in the vivarium of the University of Cordoba and fed a control diet (10%, 20% and 70% of calories from fat, protein and carbohydrate, respectively) or a high-fat diet (45%, 20% and 35% of calories from fat, protein and carbohydrate, respectively). On postnatal day (PND)-1, pups were separated into two litter sizes: small litters, with 4 pups per litter (as a postnatal overfeeding model), and normal litters, with 12 pups per litter (as a normal feeding model). After weaning, subgroups of females and males from normal and small litters were subgrouped in PND-23 and fed ad libitum with control diet or high fat diet, respectively, to generate two experimental groups, normal litter and control diet (NL-CD), and small litter and high fat diet (SL-HFD), representative of the lean and obese phenotype, respectively. Subsets of animals from each group were subjected in PND-90 to gonadectomy as a model for cessation of gonadal secretions. In PND-120, animals were subjected to a glucose tolerance test (GTT) and, one week later, to an insulin tolerance test (ITT) to assess the development of insulin resistance. The experiments ended at PND-150. In this last phase, phenotypic indices and serum

biochemical/hormonal parameters were monitored, the small and large intestine was dissected, and faecal samples were obtained for microbiota and RNA analysis.

Publication 4: The study was carried out with an animal model, namely male and female Wistar rats, bred in the vivarium of the University of Cordoba and fed a control diet (10%, 20% and 70% of calories from fat, protein and carbohydrate, respectively) or a high-fat diet (45%, 20% and 35% of calories from fat, protein and carbohydrate, respectively). In PND-1, pups were separated into two litter sizes: small, with 4 pups per litter (as a postnatal overfeeding model), and normal, with 12 pups per litter (as a normal feeding model). Sub-sets of females of both litter sizes were then androgenised with testosterone propionate, while other females were injected with vehicle (olive oil). After weaning, subgroups of androgenised or vehicle-treated females and males of normal and small litter size were fed ad libitum with control diet or high fat diet, respectively, to generate two experimental groups, NL-CD and SL-HFD, representative of the lean and obese phenotype, respectively, in PND-23. The short- and long-term impact of neonatal androgenisation and obesogenic diet was analysed in PND-50 and PND-150, representative of young adult (PND-50) and adult (PND-150) rats. Rats in all groups were weighed and subjected to GTT and ITT. Glucose levels were measured and their variation was estimated as area under the curve (AUC). After sacrifice of the rats, blood, faeces, and tissue samples were taken for analysis of phenotypic indices, biochemical/hormonal parameters, microbiota, and RNA.

1.5. Results

Publication 1: In terms of gut microbiota, the greatest differences were observed between postmenopausal and premenopausal women, and between the latter and their male control group. Compared to premenopausal women, postmenopausal women had higher abundance of the phylum *Firmicutes* and lower abundance of the phylum *Actinobacteria*, higher abundance of the genera *Lachnospira* and *Roseburia* and lower abundance of the genera *Prevotella*, *Parabacteroides*, *Bilophila*, and *Ruminococcus (Lachnospiraceae)*, although the latter genus did not reach statistical significance. Compared to their male control groups, postmenopausal women had a higher *Firmicutes/Bacteroidetes (F/B)* ratio, while premenopausal women had a higher abundance of the genera *Ruminococcus (Lachnospiraceae)*, *Bilophila*, *Prevotella*, and *Oscillospira*, but the latter genus did not reach statistical significance. Regarding inflammatory markers, premenopausal women had lower levels of IL-6 and monocyte chemotactic protein-1 (MCP-1) than postmenopausal women. With regard to regulatory hormones involved in energy homeostasis and incretins, the level of glucagon-like peptide-1 (GLP-1) was lower in postmenopausal women than in premenopausal women, the level of gastric inhibitory peptide (GiP) and leptin was higher in women as a whole than in men, and the level of adiponectin was higher in premenopausal women than in their male control group. Finally, PICRUST analysis of the gut microbiota showed a

statistical trend towards higher propionate and butanoate metabolism in the premenopausal women compared to the other groups.

Publication 2: Linear discriminant analysis (LDA) effect size (LEfSe) revealed that differences in gut microbiota were more pronounced for women and men without MetS than for women and men with MetS. Univariate analysis (ANOVA) showed sex-specific differences in the MetS groups absent in the non-MetS groups; in this case a higher abundance of the genera *Phascolarctobacterium*, *Anaerotruncus, Collinsella*, and *Alistipes*, in MetS women than in MetS men, as opposed to the genera *Faecalibacterium* and *Prevotella*. In addition, there was a higher abundance of the genera *Ruminococcus (Lachnospiraceae)* and *Bilophila* in women with and without MetS than in their male counterparts, as well as a higher abundance of the genera *Clostridium* and *SMB53* in men than in women, both with and without MetS.

Regarding the differential effect of diet on gut microbiota composition, a higher proportion of the genera *Desulfovibrio*, *Roseburia*, and *Holdemania* was observed in men with MetS than in women with MetS after consumption of the low-fat diet, as well as a higher proportion of an unknown genus of the family *Rikenellaceae* in men with MetS than in women with MetS after consumption of the Mediterranean diet.

Publication 3: Gonadectomy did not modify most of the observed phylum-level differences found between males and females without gonadectomy, both with normal feeding and with overfeeding. However, for genera related to metabolic pathologies, gonadectomy reduced the abundance of *Roseburia* and *Butyricimonas* in overfed females. A higher *F/B* ratio was also detected in gonadectomised females than in overfed males.

Our results showed a relationship between gut microbiota and gut expression of several miRNAs associated with sex steroid-related pathways. The functions of KEGG, in which the selected miRNAs were mapped, included lipid, amino acid, cofactor and vitamin metabolism, signal transduction and endocrine systems. In particular, insulin, gonadotropin-releasing hormone (GnRH), estrogen and prolactin signalling pathways were involved, as well as progesterone-mediated oocyte maturation.

Publication 4: Relative to intact females, neonatal androgenisation increased weight in PND-50 and PND-150, both under normal feeding and overfeeding, while producing several hormonal effects, including reduced progesterone in PND-50 and PND-150, and increased estradiol and estrone in PND-50, all under both types of feeding (high vs. undetected levels), as well as increased estrone in PND-150 under overfeeding.

Androgenisation impaired energy balance and glucose homeostasis, with androgenised rats in PND-50 showing a higher glucose AUC than control females, which reached statistical significance under overfeeding, but not in normal feeding. GTT showed higher glucose levels (lower glucose tolerance) in androgenised females (and males) in PND-50 than in control females. In addition, ITT showed a higher glucose AUC (lower insulin

sensitivity) in androgenised females than in control females subjected to overfeeding in PND-50 and PND-150. ITT also showed higher glucose levels at 0 minutes in androgenised females than in control females in PND-50 under normal feeding and overfeeding, as well as higher levels in androgenised females (and males) than in intact females in PND-150 under overfeeding.

Androgenisation modified the gut microbiota with respect to control females. At the phylum level, in PND-50 under normal feeding *Bacteroidetes* increased and *Firmicutes* and *Euryarchaeota* decreased, while with overfeeding *Euryarchaeota*, *Verrucomicrobia* and *Cyanobacteria* decreased. In PND-150 under normal feeding, the phylum *Cyanobacteria* increased and *Euryarchaeota* and *Actinobacteria* decreased, while overfeeding increased *Bacteroidetes*.

Our results showed a relationship between gut microbiota and gut expression of several miRNAs associated with sex steroid-related pathways. In this context, miRNA-mediated associations were observed between gut microbiota and: 1) Sex hormone-related processes: steroid biosynthesis, oocyte meiosis, and progesterone-mediated oocyte maturation; 2) Metabolism: insulin signalling pathway, fatty acid biosynthesis, and fatty acid metabolism; 3) Intestinal barrier integrity: focal adhesion, adherens junction, and O-glycan-like mucin biosynthesis; 4) Intestinal barrier integrity: focal adhesion, adherens junction, and O-glycan-like mucin biosynthesis.

1.6. Conclusions

1. Our results show that men and women differ in the composition of their gut microbiota and that these differences depend on the hormonal status of women (premenopausal vs. postmenopausal), which could be related to the sexual dimorphism observed in the incidence of metabolic and cardiovascular diseases, given the relationship of these diseases with the gut microbiota.
2. Our results suggest that alterations in gut microbiota associated with metabolic syndrome appear to be different between men and women. These results in turn suggest the involvement of the gut microbiota in the difference in the incidence of metabolic diseases between sexes. Furthermore, the different changes induced by the Mediterranean diet or a low-fat diet in the gut microbiota according to sex suggest that women and men may benefit differently from a specific diet, depending on their sex.
3. Our results show the contribution of gonadal hormones to the definition of sex-dependent differences in gut microbiota, as well as their potential role in the formation of this microbiota as a consequence of the interaction between sex and nutrition. Indeed, the elimination of sex hormones modifies the gut microbiota towards a more deleterious profile, especially in females after postnatal overfeeding, confirming the involvement of diet in these processes. On the other hand, our results suggest that the involvement of the gut microbiota in metabolic

diseases could be mediated by the interaction between the microbiota and its host via gut miRNAs.

4. Our results suggest that nutritional and hormonal disruption in early development alters the metabolic programming and structure of the gut microbiota, the consequences of which are prolonged over time. The observed relationship between changes in gut microbiota and miRNA expression in the small and large intestine suggests a possible mechanism of cross-communication between gut microbiota and host that may contribute to amplify the metabolic mismatch caused by obesity.

II. INTRODUCCIÓN

II. INTRODUCCIÓN

Las diferencias entre los sexos, o dimorfismo sexual, que abarca diferencias tan dispares como las referentes a los gametos, las gónadas, el comportamiento, la morfología, o la fisiología, dependen de la expresión génica diferencial de genes ubicados en distintos cromosomas; pero de algún modo han de estar influenciadas por los cromosomas sexuales, ya que en ellos reside la diferencia genética entre ambos sexos (2). De hecho, el dimorfismo sexual comienza a manifestarse a nivel genético, donde el patrón de secreción de la hormona del crecimiento (GH), dependiente a su vez de las hormonas sexuales (3), condiciona una expresión génica sexualmente dimórfica (4).

Las enfermedades metabólicas, y en particular la obesidad, el SM, y la DT2, han derivado en un grave problema sanitario, social, y económico, a nivel mundial dada su creciente incidencia (5-7). Estas patologías muestran un marcado dimorfismo sexual en su desarrollo y prevalencia, influenciadas por las hormonas sexuales (8) (Figura 1). Dicha influencia queda patente en aquellas condiciones en las que el nivel normal de estas hormonas se ve alterado. Así, los hombres y las mujeres transgénero muestran una redistribución de la grasa tras el tratamiento con esteroides sexuales (andrógenos y estrógenos) (9). Los cambios hormonales de la menopausia también son responsables de una redistribución de la grasa (10) y de un mayor riesgo de DT2 (11), mientras que la terapia hormonal con estrógenos y progestágenos en mujeres posmenopáusicas reduce su incidencia (12). La terapia de privación de andrógenos en hombres con cáncer de próstata provoca un aumento de la masa grasa (13), una mayor prevalencia del SM (14), y un mayor riesgo de DT2 (15). Por el contrario, el tratamiento con testosterona disminuye la grasa visceral en hombres envejecidos no obesos con síntomas de deficiencia de andrógenos (16), así como la DT2 y el SM en pacientes con hipogonadismo. Por otra parte, las mujeres con hiperandrogenismo, debido al SOP, muestran un aumento de la adiposidad central (17) y un mayor riesgo de SM (18), mientras que la disminución de estrógenos inducida por la ooforectomía en mujeres posmenopáusicas aumenta el riesgo de DT2 (19).

En línea con lo anterior, hay que destacar que se han descrito dos patrones de distribución de la grasa corporal, un patrón abdominal (visceral), típico de hombres y mujeres posmenopáusicas, y un patrón periférico (subcutáneo), típico de mujeres premenopáusicas (20, 21). Ambos patrones, regulados por las hormonas sexuales (22), están relacionados con el desarrollo de enfermedades metabólicas, mostrando la distribución de la grasa central un perfil patológico (23) frente a un perfil protector de la grasa periférica (24).

La microbiota intestinal es una comunidad simbiótica, formada en su mayoría por bacterias (dominios Archaea y Bacteria), que participa activamente en la fisiología del huésped al intervenir en procesos como la absorción de nutrientes (energía), la respuesta inmunitaria, la permeabilidad intestinal, y la producción de hormonas y vitaminas (25-27), al tiempo que enriquece el metabolismo de los glicanos, los

aminoácidos, los xenobióticos, la metanogénesis, y la biosíntesis de vitaminas e isoprenoides (28). Por ello, la microbiota intestinal parece desempeñar un papel clave en la salud humana (29), mientras que la alteración de su composición o diversidad, conocida como disbiosis, así como una interacción alterada con su huésped, pueden dar lugar a diversas patologías (30, 31).

En los últimos años, durante el desarrollo de esta tesis, nuestro grupo de investigación ha aportado evidencias que apoyan la idea de la existencia de un dimorfismo sexual en la composición de la microbiota intestinal, en consonancia con otros estudios (32) en los que las hormonas sexuales parecen tener un papel destacado (33). Además, la interacción entre la microbiota intestinal y su huésped parecer ser clave para el desarrollo de las enfermedades metabólicas (26). En este sentido, la microbiota intestinal interviene en el mantenimiento de la llamada barrera intestinal (34), que limita el acceso de los microorganismos al torrente sanguíneo e influye, por ello, en el estado inflamatorio descrito en procesos como la obesidad y el SM (35). La acción de la microbiota intestinal también se extiende al sistema nervioso central para influir en la ingesta de alimentos a través del eje intestino-cerebro (36), e incluso al hígado para regular el metabolismo de los nutrientes a través del eje intestino-hígado (37).

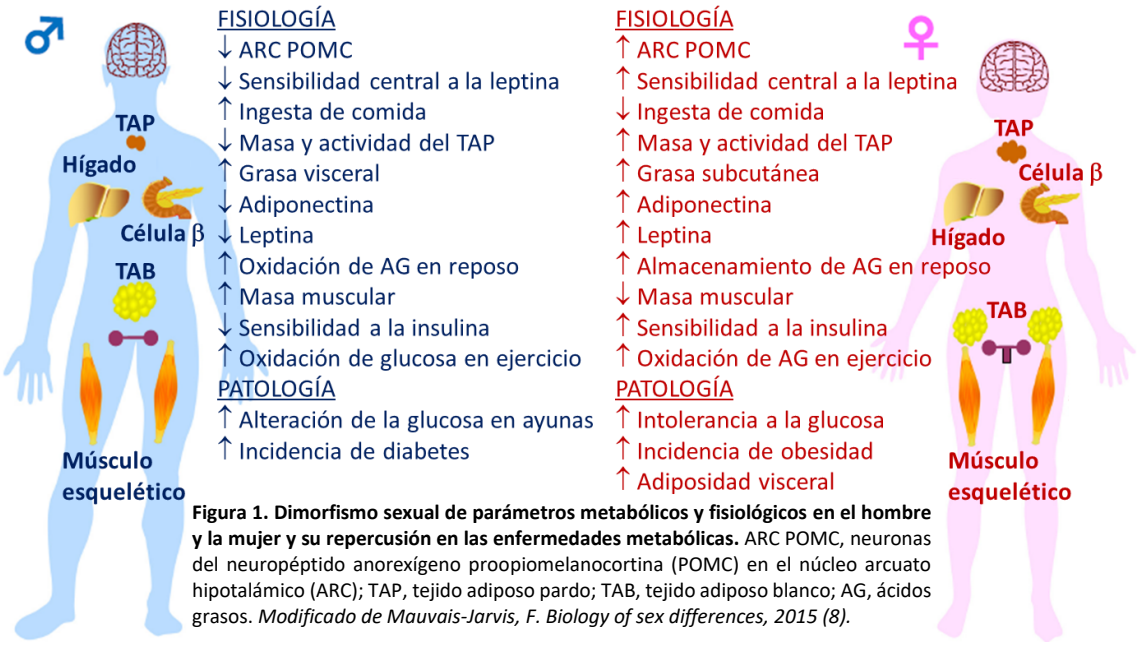

Figura 1. Dimorfismo sexual de parámetros metabólicos y fisiológicos en el hombre y la mujer y su repercusión en las enfermedades metabólicas. ARC POMC, neuronas del neuropéptido anorexígeno proopiomelanocortina (POMC) en el núcleo arcuato hipotalámico (ARC); TAP, tejido adiposo pardo; TAB, tejido adiposo blanco; AG, ácidos grasos. *Modificado de Mauvais-Jarvis, F. Biology of sex differences, 2015 (8).*

2.1. Nivel genético del dimorfismo sexual: El hígado como modelo de expresión génica sexualmente dimórfica

La expresión génica dependiente del sexo, que repercute finamente en patologías sexualmente dimórficas (38-40), ha sido ampliamente estudiada en el hígado, un órgano clave en el metabolismo general del cuerpo, como lo demuestran las complicaciones metabólicas derivadas de un trasplante hepático (41). Los mecanismos responsables del dimorfismo sexual dependen de la GH, que lleva a cabo su acción principalmente a través de la proteína janus quinasa 2 (JAK2) y del transductor de señales y activador de

la transcripción (STAT), implicando también a varios factores de transcripción (FT), al grado de condensación del ADN, e incluso a diferentes tipos de ARN. (Figura 2)

2.1.1. Patrón de secreción de la GH: Clave del dimorfismo sexual de la expresión génica

La GH juega un papel clave en la regulación de la expresión de numerosos genes (42) y, en este sentido, la regulación sexualmente dimórfica de algunos genes depende de las diferencias de sexo en la secreción hipofisaria de la GH, que es pulsátil (intermitente) en los hombres frente a casi continua en las mujeres (43, 44). Esto se ha corroborado en ratones, en donde la hipofisectomía anula la especificidad del sexo de aproximadamente el 90% de los genes dependientes del sexo en el hígado, de los que unos responden a la GH positivamente (clase I), y otros negativamente (clase II) (45, 46). Por otra parte, el dimorfismo en la secreción de GH a nivel central (hipotálamo y pituitaria) y su acción a nivel periférico están regulados por los esteroides sexuales (testosterona y estrógenos) (3, 47, 48), por lo que podría considerarse a la GH como una intermediaria en el papel de las hormonas sexuales en la expresión génica dependiente del sexo.

2.1.2. JAK2 y STAT: Mecanismo de acción de la GH

Tras unirse a su receptor y provocar la rotación de sus subunidades, la GH induce la activación de la JAK2 intracelular (49, 50), la cual inicia una fosforilación de proteínas que culmina en la inducción de varias redes de señalización intracelular (51, 52).

Entre las moléculas activadas por la GH a través de esta vía hay varios FTs, como STAT, un factor clave en la regulación de la expresión de muchos genes, incluyendo algunos sexualmente dimórficos expresados en el hígado (53, 54). En realidad, STAT comprende una familia de proteínas, entre las que destaca STAT5 (STAT5a y STAT5b) por su papel clave en la regulación de la transcripción de muchos genes hepáticos de forma dependiente del sexo. En este sentido, la deficiencia de STAT5b en ratones knockout provoca la pérdida de las respuestas sexualmente dimórficas asociadas al patrón de secreción de la GH dependiente del sexo, con respuestas específicas de los machos knockout disminuidas hasta los niveles de las hembras de tipo salvaje y respuestas de las hembras knockout aumentadas hasta un nivel intermedio a las de los machos y hembras de tipo salvaje (55, 56). Estudios posteriores han confirmado que STAT5b permite y reprime en el hígado la expresión de genes específicos de machos y hembras respectivamente (53, 57). Por otro lado, STAT5a parece jugar un papel clave en la regulación de los genes hepáticos específicos del sexo en el hígado femenino (58). Otro estudio basado en la deleción del locus Stat5a-Stat5b en el hígado de ratón demostró que la regulación positiva y negativa de los genes específicos de machos y hembras, respectivamente, requerían la acción de STAT5ab, y que STAT5b era el mayor responsable del dimorfismo sexual hepático dependiente de STAT5ab (59).

En cuanto a su mecanismo de acción, tras su fosforilación, las proteínas STAT se desplazan del citosol al núcleo como dímeros para unirse a sitios específicos del ADN (60, 61). En este caso, la pauta masculina de GH intermitente activa la fosforilación de

STAT5 y su translocación del citosol al núcleo, mientras que la pauta femenina de GH continua desensibiliza su fosforilación y ocasiona un bajo nivel de STAT5 activo (62-64).

2.1.3. Condensación de la cromatina: Factor epigenético

En el dimorfismo sexual de la expresión génica también influye la organización tridimensional del genoma (65). Este dimorfismo parece estar relacionado con el grado de metilación de la cromatina, que en última instancia influye en su grado de condensación y, por tanto, en su accesibilidad; habiéndose identificado a este sentido sitios de control transcripcional específicos del sexo (66, 67). A este respecto, la testosterona induce una desmetilación del ADN específica del sexo en el hígado de ratón mediante la activación del receptor de andrógenos (RA) (68), que una vez establecida, persiste incluso en ausencia de testosterona y suele actuar principalmente sobre secuencias reguladoras de genes expresados diferencialmente en el hígado masculino.

2.1.4. ARN largo no codificante (ARNlnc) y microARN (miARN)

Varios tipos de ARN, como los miARN y los ARN largos no codificantes (ARNlnc), también participan en la regulación de la expresión génica dependiente del sexo.

Un ejemplo es la expresión sexo-específica del citocromo P450 2b9 (Cyp2b9) en el hígado de ratones hembra, que implica a varios miARNs a través de una regulación postranscripcional (69). En la misma línea, el miR-1948, con sesgo masculino, y el miR-802, con sesgo femenino, se localizan en regiones de la cromatina cuya accesibilidad depende del sexo y están regulados por STAT5, dependiente a su vez de la secreción de la GH (70). Preferentemente, el miR-1948-5p reprime e induce los ARNm de sesgo femenino y masculino, respectivamente, en el hígado masculino, mientras que el miR-802-5p lo hace al contrario en el hígado femenino.

En cuanto a los ARNlnc, la expresión de algunos de ellos, implicados en el dimorfismo sexual del metabolismo y la patología hepática, está regulada en el hígado de ratón por el perfil de secreción de la GH (71). En este sentido, se han identificado en ratones hipofisectomizados ARNlnc específicos del sexo con una fuerte regulación negativa de los genes sexualmente dimórficos, lo que sugiere que pueden contribuir al control de la expresión génica hepática dependiente del sexo (72). Más recientemente, se han descrito transcritos antisentido de ARNlnc nucleares implicados en la expresión dependiente del sexo de los genes Cyp2a4 y Cyp2a5 en el hígado de ratón (73).

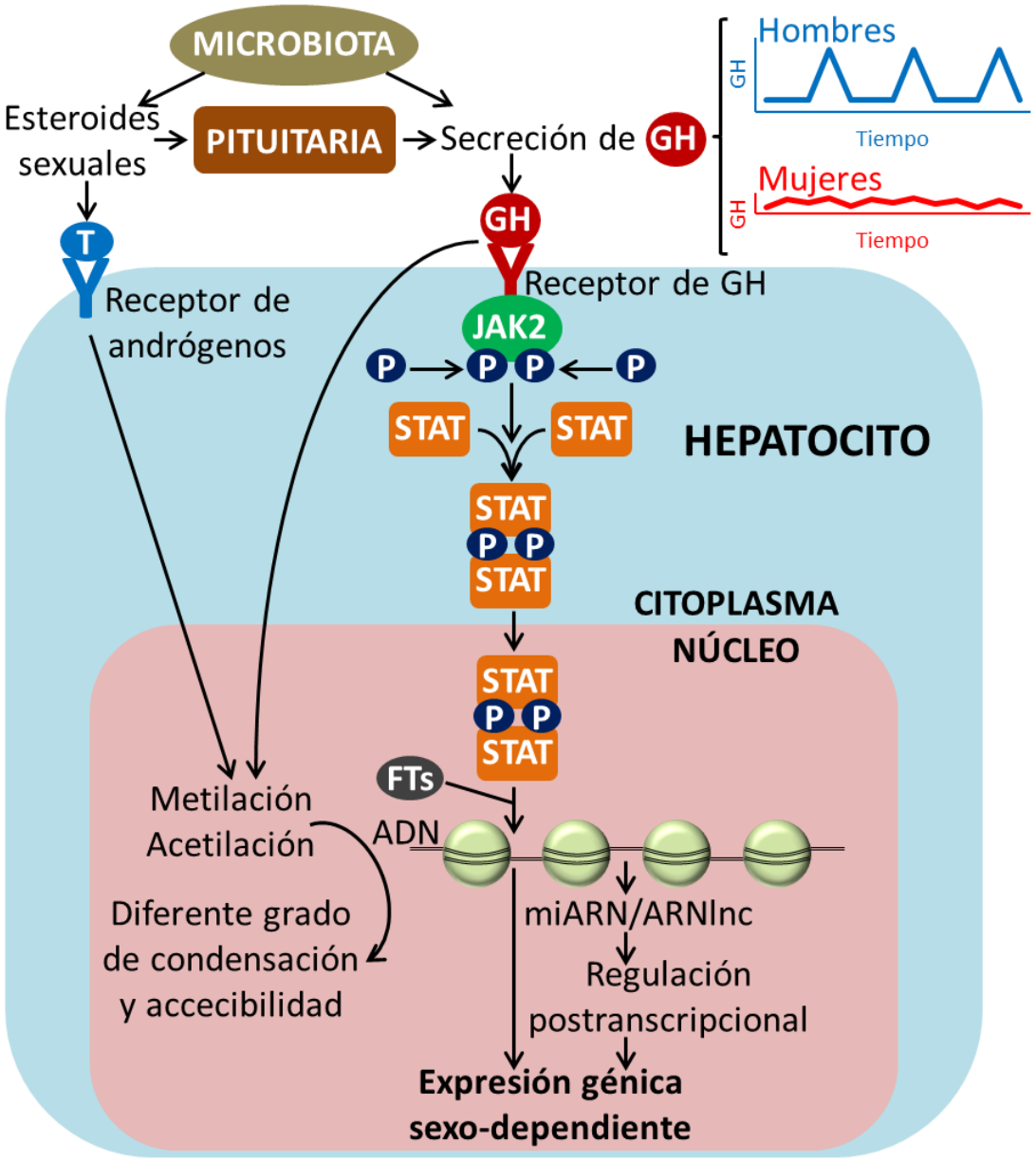

Figura 2. Mecanismo general de la expresión génica sexo-dependiente en el hígado. La microbiota influye en los niveles de esteroides sexuales y en el patrón de secreción de la hormona del crecimiento (GH), el cual se manifiesta de forma intermitente en los hombres y casi continua en las mujeres. La unión de la GH a su receptor activa la vía de la Janus quinasa 2 (JAK2), que fosforila al transductor de señales y activador de la transcripción (STAT). Al fosforilarse, STAT accede al núcleo como dímero y, con otros factores de transcripción (FTs), se une a secuencias reguladoras del ADN para inducir la expresión génica dependiente del sexo y obtener diferentes tipos de ARN (microARN (miARN) y ARN largo no codificante (ARNlnc)); estos últimos responsables de la regulación postranscripcional sexo-dependiente. El grado de condensación del ADN dependiente de los esteroides sexuales y de la GH determina la accesibilidad de los factores de transcripción a las secuencias reguladoras. T, testosterona.

2.2. Enfermedades metabólicas sexualmente dimórficas (Tabla 1)

La prevalencia de las enfermedades metabólicas responde a un dimorfismo sexual (8). La prevalencia global de la obesidad es mayor en la mujer, pues su propensión a ganar grasa abdominal aumenta con la edad. De hecho, en la actualidad, la prevalencia de la obesidad visceral asociada al SM es mucho mayor en las mujeres en muchas regiones del mundo. Por otra parte, la prevalencia de la DT2 se invierte según la etapa de la vida, pues hay más hombres diabéticos antes de la pubertad y más mujeres diabéticas tras la menopausia.

2.2.1. Obesidad

La prevalencia de la obesidad, establecida a partir de un IMC de 30 kg/m^2, ha aumentado en el mundo desarrollado tanto en adultos como en niños. Esta patología, resultante de complejas relaciones genéticas, socioeconómicas y culturales, implica graves problemas sanitarios, económicos y sociales (5). La obesidad está relacionada con la inflamación de bajo grado que conduce a un estado de resistencia a la insulina, implicada a su vez en el desarrollo de trastornos metabólicos como el SM y la DT2 (74). (Figura 3)

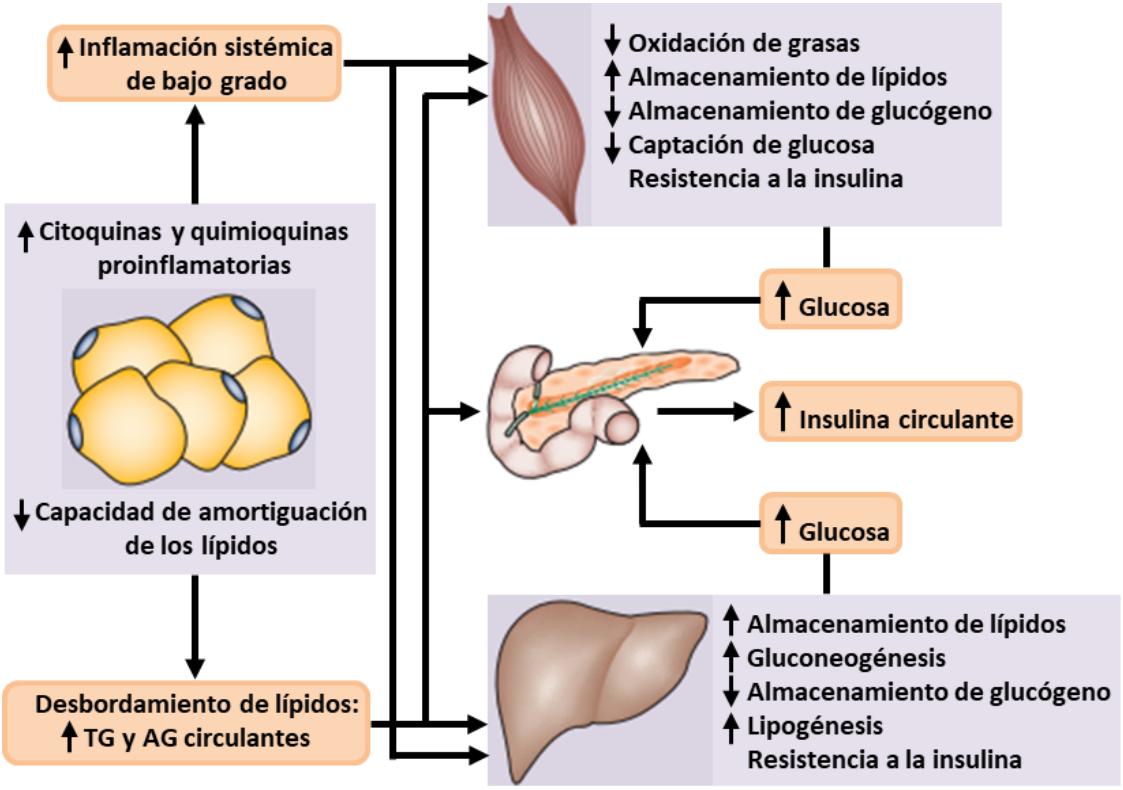

Figura 3. Resistencia a la insulina inducida por la obesidad. Ante un balance energético positivo (ingesta de energía mayor a su gasto), el tejido adiposo supera su capacidad de amortiguación para almacenar el exceso de energía en forma de triglicéridos (TG), originando un desbordamiento de lípidos en la circulación y una acumulación ectópica de grasa en tejidos no adiposos (hígado, músculo esquelético y páncreas), lo que origina resistencia a la insulina. Junto con la reducción de la capacidad de amortiguación de los lípidos, el tejido adiposo se inflama y aumenta la secreción de citoquinas proinflamatorias y adipoquinas, que también podría contribuir a la resistencia a la insulina y a una alteración de la homeostasis de la glucosa. AG, ácidos grasos. *Modificado de Canfora, EE et al. Nature reviews. Endocrinology, 2015 (298).*

En realidad, los trastornos metabólicos están relacionados con la distribución de la grasa corporal, que presenta un dimorfismo sexual al acumularse preferentemente alrededor del tronco y el abdomen en los hombres (distribución androide) y alrededor de las caderas y los muslos en las mujeres (distribución ginoide) (21) (Figura 4). La adiposidad abdominal se subdivide en subcutánea y visceral, siendo esta última la que se ha asociado a un aumento de las complicaciones metabólicas tanto en hombres como en mujeres (23, 75, 76) al provocar un aumento de la glucosa y los triglicéridos en sangre, una disminución de las lipoproteínas de alta densidad (HDL), y un aumento de las lipoproteínas de baja densidad (LDL), así como un incremento de los marcadores inflamatorios (77). Por el contrario, la grasa glúteo-femoral se ha asociado a un perfil lipídico y glucémico protector y a una disminución del riesgo metabólico, pareciendo

ejercer su efecto protector a través del almacenamiento de ácidos grasos a largo plazo y de un perfil de adipoquinas beneficioso (asociación positiva con los niveles de leptina y adiponectina y asociación negativa con el nivel de citoquinas inflamatorias) (24).

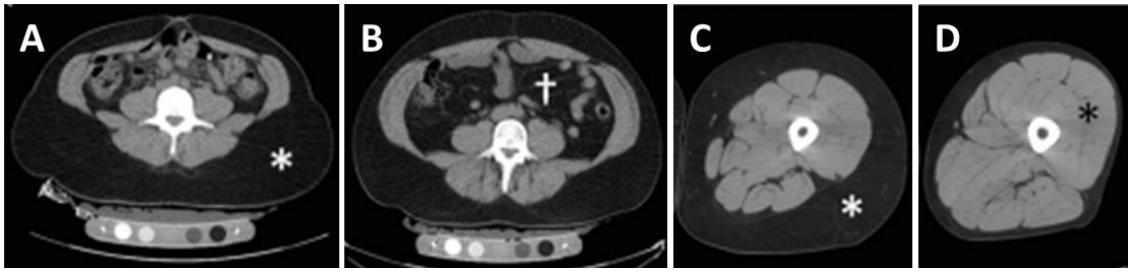

Figura 4. Composición corporal del abdomen (A) y el muslo (C) en una mujer de 39 años y un hombre de 37 años (B, D) con obesidad (IMC, 33 kg/m^2) mediante tomografía computarizada. La mujer tiene más tejido adiposo subcutáneo en abdomen y muslo (asteriscos blancos), mientras que el hombre tiene más tejido adiposo visceral (cruz) y masa muscular (asterisco negro). La mujer tenía un mejor perfil metabólico en comparación con el hombre (colesterol LDL sérico, 57 mg/dL frente a 147 mg/dL; colesterol-HDL, 68 mg/dL frente a 32 mg/dL; triglicéridos, 45 mg/dL frente a 159 mg/dL; insulina, 4,9 µU/mL frente a 7,0 µU/mL; HOMA-IR, 0,98 frente a 7,00). *Modificado de Bredella MA. Advances in Experimental Medicine and Biology, 2017 (21).*

2.2.1.1. Implicación de las hormonas sexuales en la obesidad

Los esteroides sexuales modulan la distribución de la grasa corporal. En este sentido, los cambios hormonales de la pubertad se han asociado con una diferente ganancia de peso corporal entre los sexos, debido al aumento de la masa magra y de la masa grasa en los niños y niñas respectivamente, así como con la distribución de la grasa androide y ginoide (20), pues las hormonas sexuales parecen regular ciertos genes implicados en la distribución sexualmente dimórfica de la grasa corporal (22, 78, 79).

En los hombres, la testosterona inhibe la captación de triglicéridos en la región intraabdominal y parece promover su acumulación en la región subcutánea (80), a la vez que reduce la lipólisis estimulada por catecolaminas en la grasa subcutánea pero no en la visceral (81). Estos procesos parecen estar influenciados por el gen del RA, ya que en los modelos de ratón knockout RA, la deleción de este gen aumenta la adiposidad al disminuir la lipólisis (82-84) (Figura 5). Además, parece que la proteína caveolina-1 (CAV1) desempeña un papel importante en la acumulación de grasa y que está regulada de forma diferente por los estrógenos (estradiol) y los andrógenos (dihidrotestosterona (DHT)) (85).

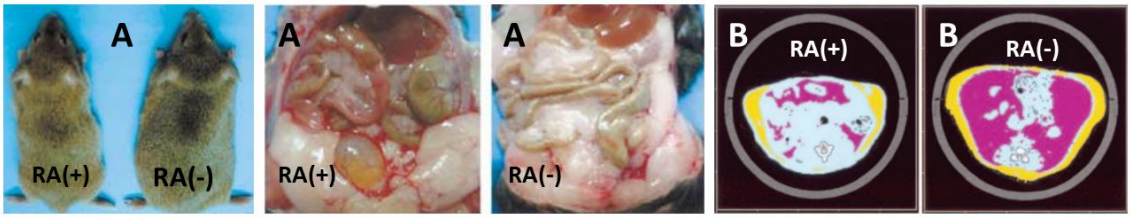

Figura 5. Aspecto externo e intraabdominal (A) y distribución de la grasa obtenida por tomografía computarizada (B) en ratones macho deficientes del receptor de andrógenos (RA(-)) y salvajes (RA(+)). La edad de los ratones era de 30 semanas en A y de 40 semanas en B. Las áreas rosa y amarilla en B representan la grasa visceral y subcutánea, respectivamente.
A: Modificado de Sato, T et al. Biochemical and Biophysical Research Communications, 2003 (82).
B: Modificado de Fan, W et al. Diabetes, 2005 (83).

A nivel celular, se han observado diferencias en el efecto de los esteroides sexuales sobre la función de los adipocitos en el tejido adiposo blanco en aspectos como la

diferenciación de los adipocitos, la lipólisis/lipogénesis, la sensibilidad a la insulina, y la producción/secreción de adipoquinas (86). En este sentido, la testosterona y la DHT regulan la diferenciación de las células mesenquimales pluripotentes, promoviendo e inhibiendo su diferenciación en miocitos y adipocitos, respectivamente, de forma dependiente del RA (87). De forma similar, la DHT inhibe la diferenciación adipogénica de las células madre mesenquimales y de los preadipocitos de forma dependiente del RA, aumenta la lipólisis y reduce la acumulación de lípidos (88). Un estudio reciente ha mostrado que, en ratones castrados (modelo de hipogonadismo masculino), la masa grasa aumenta merced a la hipertrofia de adipocitos y la adipogénesis (89), mientras que al someter a estos ratones a una terapia de sustitución hormonal, la testosterona impedía la expansión de la masa grasa visceral y subcutánea. Además, la adipogénesis obesogénica también se elevaba al inhibir la actividad del RA. Por otra parte, este estudio también mostró una regulación diferencial de la distribución de la grasa, ya que el estradiol derivado de la testosterona y la DHT bloquean el aumento de la grasa visceral y subcutánea respectivamente.

A nivel enzimático, la acción de la lipoproteína lipasa (LPL), una enzima clave en la captación y almacenamiento de lípidos por parte de los adipocitos (90), parece estar suprimida por el estradiol en el tejido adiposo de las mujeres obesas (91) y por la testosterona en el tejido adiposo de los hombres obesos (92). Esta supresión es mayor en el muslo comparado con el abdomen en el caso de los hombres, por lo que podría ser un elemento clave en su acumulación central de grasa. Además, la deficiencia de testosterona en los hombres aumenta la actividad de la LPL y de la acil-CoA sintetasa (ACS) e induce la acumulación de ácidos grasos en el tejido adiposo femoral (93, 94), mientras que la administración de testosterona reduce la actividad de la LPL abdominal y la captación de triglicéridos en esta zona (95). En la mujer, la deficiencia de esteroides sexuales después de la menopausia influye en la actividad de la ACS y de la diacilglicerol aciltransferasa y promueve un mayor almacenamiento de ácidos grasos en el tejido adiposo subcutáneo 96). Por otra parte, en las mujeres premenopáusicas, los factores adipogénicos femorales responden a la supresión aguda de hormonas sexuales en mayor medida que los abdominales (97).

2.2.1.2. Obesidad en el hombre

La concentración de testosterona se correlaciona negativamente con la obesidad central (98, 99), y el tratamiento con esta hormona disminuye la grasa visceral en hombres con síntomas de deficiencia de andrógenos y niveles séricos de testosterona bajos y normales (16). En este contexto, la oxandrolona, un esteroide artificial similar a la testosterona, reduce principalmente la grasa abdominal en hombres de edad avanzada, y dentro de ella, reduce en mayor medida la grasa visceral que la subcutánea (100). Además, la terapia de reemplazo de testosterona mejora el control glucémico, la resistencia a la insulina y la dislipidemia en pacientes con hipogonadismo, en parte reduciendo la obesidad central (101-103). Por otro lado, la terapia de privación de

andrógenos en hombres con cáncer de próstata conduce a un aumento de la masa grasa (13, 104, 105). No obstante, y en contra de lo que podría suponerse, el aumento de la grasa abdominal en este último caso parece deberse a la acumulación de grasa subcutánea y no de grasa intraabdominal (106, 107). Además, la disminución de la testosterona propia del envejecimiento se acompaña de un aumento de la adiposidad, con una acumulación preferente de grasa abdominal de tipo visceral (108). Otro hecho destacado es la redistribución de la grasa observada en los hombres transexuales tras el tratamiento con esteroides sexuales (9, 109, 110).

2.2.1.3. Obesidad en la mujer

En la mujer, la obesidad central se ha correlacionado con un aumento de la testosterona y una disminución del estradiol (98). Los cambios hormonales de la menopausia conducen a una redistribución de la grasa, independientemente de la grasa total y de la edad, hacia un fenotipo más central y androide (10, 111, 112); si bien algunos estudios sugieren que la distribución de la grasa hacia la parte superior del cuerpo tras la menopausia puede deberse al envejecimiento más que a la menopausia per se (113, 114). No obstante, el cambio hacia la distribución de la grasa central y androide en las mujeres perimenopáusicas y posmenopáusicas puede ser contrarrestado con una terapia de sustitución hormonal (115). Otros hechos a destacar son la mayor adiposidad central observada en mujeres con hiperandrogenismo debido al SOP (17, 116, 117) y la redistribución de la grasa en las mujeres transexuales tras el tratamiento con esteroides sexuales (9, 109, 110).

2.2.2. Síndrome metabólico (SM)

El SM es una condición patológica caracterizada por la presencia de obesidad abdominal, resistencia a la insulina, hipertensión e hiperlipidemia, que se ha extendido por todo el mundo y contribuye a la propagación de enfermedades como la DT2, la enfermedad coronaria, y el accidente cerebrovascular (7). Además de reducir la calidad de vida, el SM tiene un importante impacto económico en la sanidad pública debido a las elevadas tasas de morbilidad que genera, ya que el mayor riesgo de desarrollar DT2 aumenta en más de un 30% la probabilidad de sufrir una enfermedad cardiovascular, que constituye actualmente la principal causa de mortalidad a nivel mundial (118-120).

2.2.2.1. Implicación de las hormonas sexuales en el síndrome metabólico

Distintos estudios confirman el papel de las hormonas sexuales en el desarrollo del SM. Se ha observado una asociación inversa entre los niveles séricos de la globulina fijadora de hormonas sexuales (GFHS) y la prevalencia del SM en escolares de 12 a 16 años, siendo la GFHS un marcador más sensible de SM en los niños, pero no en las niñas, lo que indica un dimorfismo de sexo ya a una edad temprana (121). A edades más avanzadas se sigue observando una asociación entre los niveles más bajos de GFHS y el SM tanto en hombres como en mujeres, mientras que los niveles de testosterona total y libre son más bajos en los hombres y más altos en las mujeres con SM (122-124). Sin

embargo, se ha sugerido que un nivel bajo de GFHS estaría asociado a una mayor prevalencia de SM en hombres y mujeres premenopáusicas, pero no en mujeres posmenopáusicas, por lo que el nivel de GFHS en plasma podría ser un predictor significativo de SM solamente en hombres y mujeres premenopáusicas (125).

2.2.2.2. Síndrome metabólico en el hombre

En los hombres, el SM parece estar relacionado con la testosterona, pero no con el estradiol (126, 127). En este sentido, los niveles de testosterona se han asociado negativamente con el riesgo del SM (128), y recientemente se ha constatado que esta asociación es aplicable a todos los parámetros de esta patología (129); mientras que la terapia de reemplazo de testosterona parece mejorar la mayoría de estos parámetros (glucosa, triglicéridos, circunferencia de la cintura y colesterol-HDL) (130).

Otros estudios especifican que el SM se asocia inversamente tanto con la testosterona total como con la GFHS (131-133), y que tanto la testosterona como la GFHS muestran una asociación inversa con las concentraciones de insulina, glucosa y triglicéridos, así como una asociación positiva con el colesterol-HDL (134-136). Por su parte, otros estudios apuntan a la GFHS como la más influyente en el desarrollo del SM (137, 138) y como un factor de riesgo independiente y dominante (139-141) y un buen marcador temprano del SM (132, 133).

En cuanto a la testosterona libre, aunque también se ha descrito su asociación inversa con el SM, la mayoría de los estudios indican que esta asociación es menor que en el caso de la testosterona total y la GFHS (142-144), e incluso se ha descrito que esta relación no existe (140) o que puede ser positiva (137).

Por otra parte, los hombres con hipogonadismo (deficiencia de testosterona), resultante de la terapia de privación de andrógenos contra el cáncer de próstata, muestran niveles más bajos de testosterona total y libre, así como una mayor prevalencia del SM (14). Dentro de los parámetros del SM, estos hombres tenían una mayor prevalencia de obesidad abdominal e hiperglucemia, así como niveles elevados de triglicéridos en comparación con los controles. En línea con lo anterior, el tratamiento con testosterona en hombres con hipogonadismo restablece los niveles fisiológicos de testosterona y mejora los componentes del SM, aumentando el colesterol-HDL y reduciendo el colesterol total, el colesterol-LDL, los triglicéridos, y la glucosa (102, 103, 145).

2.2.2.3. Síndrome metabólico en la mujer

El nivel de estrógenos también parece influir en la prevalencia del SM. La depleción de estrógenos inducida por la ooforectomía en ratas empeora varios de los componentes del SM (lípidos, glucosa, HDL y LDL) (146, 147), mientras que en las mujeres menores de 50 años aumenta su prevalencia (148, 149). Además, las mujeres sometidas a histerectomía (a menudo acompañada de ooforectomía bilateral para prevenir el cáncer de ovario) experimentan un aumento del nivel de glucosa en sangre (150) y de la hipertensión (151).

La menopausia provoca una disminución del nivel de GFHS, al menos en parte debido a la disminución de estrógenos, mientras que el nivel de testosterona no se altera durante los años de la menopausia (152). En este sentido, la menopausia puede considerarse un predictor (factor de riesgo) del SM y de todos sus componentes individuales, independientemente de la edad (153, 154). Además, se ha descrito una asociación inversa entre la GFHS y el SM, especialmente entre las mujeres posmenopáusicas (155).

En cuanto a la testosterona, su exceso (hiperandrogenismo) en las mujeres con SOP es un potente predictor de los trastornos metabólicos característicos del SM, siendo esta patología más prevalente en las pacientes con SOP que en las mujeres sanas (156, 18). Sin embargo, aunque al hiperandrogenismo se le ha otorgado un papel destacado en las alteraciones metabólicas asociadas al SOP (157), un reciente estudio de revisión y metaanálisis ha indicado que la mayor prevalencia del SM en las mujeres con SOP estaría asociada a la obesidad y a las características metabólicas, pero no al índice de hiperandrogenismo (158).

2.2.3. Diabetes de tipo 2 (DT2)

El término diabetes engloba un grupo de enfermedades, diferenciadas por sus mecanismos de desarrollo, que reducen la capacidad de regular el nivel de glucosa en sangre y conducen a una hiperglucemia prolongada. Hay dos formas principales de diabetes, la insulinodependiente (diabetes de tipo 1, T1D) y la no insulinodependiente (diabetes de tipo 2, DT2), debidas, respectivamente, a un proceso autoinmune y metabólico. La DT2 se caracteriza por una producción insuficiente de insulina por parte de las células beta del páncreas y una alteración del metabolismo hepático de la glucosa, así como por la resistencia a la insulina, que conduce a una menor capacidad de respuesta de los tejidos a la misma (159, 160). Esta patología, que ha derivado en una pandemia, afectando aproximadamente al 9% de la población mundial (6), está condicionada por varios factores, como la genética, el sedentarismo, la inactividad física, el tabaquismo, el consumo de alcohol, el estrés oxidativo, y la dieta (161). Sin embargo, la obesidad se considera el principal factor de riesgo de la DT2, condicionando tanto el desarrollo de la resistencia a la insulina como el desarrollo de la enfermedad (162).

2.2.3.1. Implicación de las hormonas sexuales en la diabetes

La alteración de la glucemia en ayunas (AGA) y la alteración de la tolerancia a la glucosa (ATG), que se producen como paso previo a la DT2, muestran un dimorfismo sexual, siendo la ATG más frecuente en las mujeres y la AGA en los hombres (163-165). Se ha sugerido que las hormonas sexuales pueden ser responsables de este dimorfismo, pues la terapia hormonal con estrógenos en mujeres menopáusicas con DT2 modifica ambos parámetros (165). Además, la incidencia de esta patología es mayor en el hombre que en la mujer (166, 167). Por otra parte, la menopausia implica un mayor riesgo de DT2, mientras que la terapia hormonal con estrógenos para la menopausia puede retrasar su aparición (11).

2.2.3.2. La diabetes en el hombre

Los hombres con DT2 tienen niveles más bajos de testosterona total y testosterona libre (168-170). En relación con esto, se ha sugerido que los niveles bajos de testosterona y GFHS están relacionados con el desarrollo de la resistencia a la insulina y la posterior DT2 en los hombres (23, 128). Además, la combinación de niveles elevados de GFHS y niveles bajos de testosterona se ha asociado con un aumento de la mortalidad en hombres con DT2 (171, 172). Otros estudios han demostrado que en los hombres con DT2, los niveles bajos de testosterona per se están asociados con un aumento de la mortalidad, mientras que el reemplazo de testosterona puede mejorar su supervivencia (173, 174). Del mismo modo, la proporción de hombres con DT2 se reduce después del tratamiento con testosterona (103, 175), mientras que la terapia de privación de andrógenos en el cáncer de próstata conlleva un mayor riesgo de sufrir esta patología (15, 176, 177).

En consonancia con lo anterior, los hombres con DT2 tienden a tener niveles bajos de testosterona, y la mayoría de ellos tienen hipogonadismo (178, 179). De hecho, se ha confirmado que los pacientes obesos y diabéticos con hipogonadismo y niveles bajos de testosterona muestran una mejora de la resistencia a la insulina y del control glucémico tras someterse a una terapia de sustitución de testosterona (103, 145, 180).

Con respecto a las hormonas femeninas, los hombres con niveles elevados de estradiol tienen un mayor riesgo de sufrir DT2, y esta concentración elevada de estradiol, junto con una concentración baja de SHBH, conlleva un efecto perjudicial aditivo sobre el riesgo de la DT2 (23, 181).

2.2.3.3. La diabetes en la mujer

A diferencia de los hombres, un nivel elevado de testosterona en la mujer está relacionado con la resistencia a la insulina y la DT2 (128, 182, 183). Sin embargo, un estudio en mujeres chinas ha demostrado que, aunque los valores elevados de GFHS se asocian con una menor probabilidad de DT2, los niveles de estradiol y testosterona no muestran ninguna asociación con esta patología (181). Estos resultados contradictorios respecto a la relación entre la testosterona y la incidencia de DT2 pueden deberse a la medición de la testosterona, ya que algunos autores utilizan la testosterona total y otros la testosterona libre, y según un estudio reciente, el método de análisis puede diferir entre los estudios (184). Además, el índice de andrógenos libres (IAL) usado en algunos estudios no es un indicador fiable de la testosterona libre cuando la concentración de GFHS es inferior a 30 nmol/L, lo que llevaría a posibles errores de investigación en mujeres con niveles bajos de GFHS (185). En consecuencia, se ha informado que en las mujeres no hay asociación entre la testosterona total y la DT2, aunque un mayor nivel de testosterona libre se asocia con un mayor riesgo de DT2 (186).

Al igual que en el hombre, el nivel de GFHS también se ha asociado inversamente con el riesgo de DT2 en la mujer (128, 155, 170, 187). De hecho, en la mujer, la asociación entre

la GFHS baja y la DT2 parece ser más fuerte que en el hombre (182, 183). Aunque esta asociación inversa entre la GFHS y la DT2 es persistente en diferentes grupos étnicos (188), según un estudio en mujeres hispanas posmenopáusicas con y sin DT2, los niveles medios de GFHS no fueron significativamente diferentes en ambos grupos (189). Estos resultados contradictorios pueden deberse a que los niveles de hormonas sexuales y GFHS pueden variar en mujeres posmenopáusicas según las diferencias raciales/étnicas (190, 191).

Con respecto al estradiol, las mujeres posmenopáusicas con DT2 tienen niveles de estradiol más altos que las mujeres sanas (182, 187, 189). Sin embargo, los datos de un conjunto de pruebas basadas en una menarquia o menopausia más temprana y en la práctica de la histerectomía y la ooforectomía sugieren que los niveles de estradiol no fisiológicos (superiores o inferiores a los valores normales) pueden ser responsables de una mayor incidencia de DT2. En este sentido, el inicio temprano de la menarquia parece aumentar el riesgo de DT2 (192-194). No obstante, algunos estudios sugieren que parte del riesgo de DT2 debido a la menarquia temprana puede deberse al aumento de la adiposidad (19, 195, 196), ya que la menarquia temprana se asocia con un aumento del IMC en la edad adulta (197, 198). Por otro lado, la menopausia precoz o la insuficiencia ovárica prematura conllevan un mayor riesgo de desarrollar DT2 (199-201). Resultados similares se han observado en mujeres posmenopáusicas con ooforectomía bilateral (19, 202). Además, la terapia hormonal con estrógenos y progestágenos en mujeres posmenopáusicas (tanto con el útero intacto como con histerectomía) reduce la incidencia de la DT2 (12, 203, 204).

	HOMBRE	MUJER
↑T	➢ Disminución de la obesidad central ➢ Disminución de la grasa visceral	➢ Aumento de la obesidad central ➢ Aumento del SM ➢ Aumento de la DT2
↓T	➢ Aumento de la masa grasa (acumulación de grasa subcutánea, no acumulación de grasa intraabdominal) ➢ Aumento de la adiposidad (acumulación preferente de grasa abdominal de tipo visceral) (envejecimiento) ➢ Aumento del SM ➢ Aumento de la DT2	
↑E		➢ Aumento de la DT2 (valor no fisiológico)*
↓E		➢ Aumento de la obesidad central ➢ Aumento del SM ➢ Aumento de la DT2 (valor no fisiológico)*

Tabla 1. Resumen de la influencia de los valeres elevados (↑) y disminuidos (↓) de las hormonas sexuales, testosterona (T) y estradiol (E), en la obesidad, el síndrome metabólico (SM), y la diabetes de tipo 2 (DT2). (*): Un valor no fisiológico de estradiol (aumentado o disminuido) sería responsable de un mismo efecto, el aumento del riesgo de desarrollar la DT2.

2.3. Interacción entre la microbiota intestinal y las hormonas sexuales

2.3.1. Pruebas de la interacción entre la microbiota intestinal y las hormonas sexuales

La composición de la microbiota intestinal depende del sexo (32) y puede, a su vez, influir en los niveles de las hormonas sexuales, como por ejemplo, en el nivel de estrógenos no ováricos en los hombres y en las mujeres posmenopáusicas a través de la circulación enterohepática (205).

2.3.1.1. Estudios en roedores

Los estudios en ratones muestran un cambio en los niveles de estradiol y testosterona tras la colonización microbiana (206, 207). En lo referente a los cambios hormonales asociados a la pubertad, la α-diversidad microbiana no parece mostrar diferencias dependientes del sexo en ratones prepúberes, cosa que sí ocurre en ratones pospúberes, en los que, tras la reducción de los niveles de andrógenos mediante castración, la microbiota de los machos castrados muestra más similitudes con la microbiota de las hembras que con la de los machos no castrados (208). En la misma línea, el tratamiento con testosterona evita los cambios en la composición de la microbiota intestinal en ratones macho castrados (209).

La microbiota también parece estar involucrada en los ritmos diurnos específicos del sexo de la expresión génica y el metabolismo de los esteroides, pues en ratones libres de gérmenes (LG) se ha observado una secreción alterada de la GH, con niveles bajos tanto en machos como en hembras y un patrón de secreción de las hembras similar al de los machos (210). Esto podría explicar el dimorfismo sexual atenuado del hígado observado en estos ratones, ya que el tratamiento continuado con GH restauraba en parte el dimorfismo sexual en los hepatocitos de las hembras.

2.3.1.2. Estudios en humanos

Los hombres y mujeres con niveles séricos elevados de testosterona y estradiol, respectivamente, albergan una microbiota intestinal más diversa, con bacterias correlacionadas con los niveles de testosterona (*Acinetobacter*, *Dorea*, *Ruminococcus* y *Megamonas*) y estradiol (*Slackia* y *Butyricimonas*) (33). Además, la microbiota intestinal está influenciada por los cambios de los niveles de estrógenos y andrógenos debidos a factores como el embarazo, la pubertad, la menopausia, o el SOP. En este sentido, la microbiota cambia del primer al tercer trimestre del embarazo, con una riqueza reducida y un aumento general de *Proteobacteria* y *Actinobacteria* (211), mientras que la de las mujeres con SOP (hiperandrogénicas) está marcadamente alterada (212-214). En cuanto a la pubertad, la microbiota intestinal de las niñas se vuelve más similar a la de los adultos con la progresión de la pubertad, lo que sugiere que la microbiota podría afectar al momento de la pubertad, posiblemente regulando los niveles de las hormonas sexuales (215-217). (Figura 6)

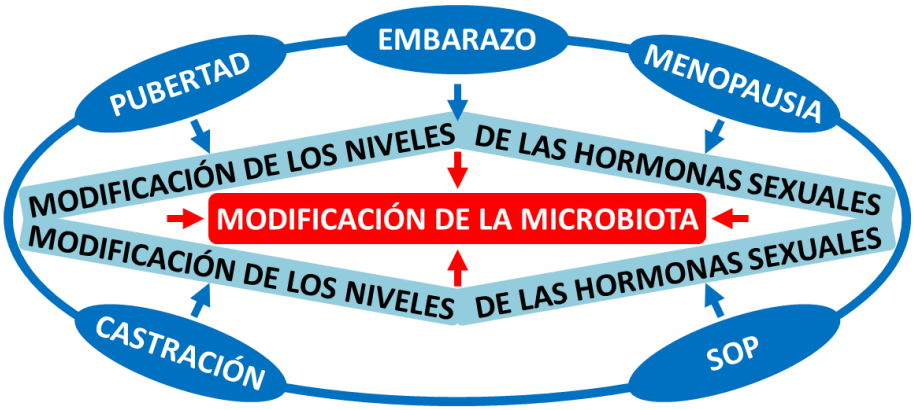

Figura 6. Interacción entre la microbiota intestinal y las hormonas sexuales. Diversas circunstancias, como la pubertad, el embarazo, la menopausia, el síndrome del ovario poliquístico (SOP), y la castración, se traducen en una modificación de los niveles de las hormonas sexuales (testosterona y estradiol), lo que a su vez deriva en una modificación de la composición de la microbiota intestinal.

Los niveles de estrógenos en la orina de hombres y mujeres posmenopáusicas están fuertemente asociados con la riqueza y la α-diversidad de la microbiota intestinal, mientras que en las mujeres premenopáusicas, en las que estos niveles son muy variables durante los ciclos menstruales, no se observa esta asociación (205, 218). Coincidiendo con nuestros resultados, un reciente estudio también ha constatado que la microbiota intestinal de las mujeres posmenopáusicas es más similar a la de los hombres que a la de las mujeres premenopáusicas, sin que se observen realmente diferencias significativas entre las mujeres posmenopáusicas y los hombres (219). Este estudio también mostró una asociación entre los esteroides gonadales y las diferencias en la microbiota, con vías de biosíntesis y degradación de esteroides enriquecidas en las mujeres premenopáusicas y asociadas significativamente con el nivel de testosterona en plasma. Además, la microbiota permitió predecir el nivel de testosterona circulante tanto en humanos como en ratones macho (tratados con antibióticos) tras la transferencia de materia fecal humana.

2.3.2. Mecanismo de acción entre la microbiota intestinal y las hormonas sexuales

2.3.2.1. Ácidos biliares

Parte del sesgo sexual de la microbiota intestinal podría depender de los ácidos biliares, ya que su reserva es mayor en los hombres que en las mujeres (220, 221) y, tras ser sintetizados en el hígado a partir del colesterol, son metabolizados por la microbiota intestinal en ácidos biliares secundarios, que a su vez pueden modificar la estructura de la microbiota y provocar diversas patologías 222-224) (Figura 7). En este sentido, la microbiota intestinal regula el metabolismo secundario de los ácidos biliares e inhibe su síntesis en el hígado mediante la regulación de la expresión del factor de crecimiento de fibroblastos (FCF) 15 en el íleon y de la colesterol 7α-hidroxilasa (CYP7A1) en el hígado a través de mecanismos dependientes del receptor farnesoide X (FXR) (también conocido como NR1H4, del inglés "Nuclear receptor subfamily 1, group H, member 4") (225, 226), un receptor nuclear para los ácidos biliares. En línea con esto, el FCF15 reprime la expresión de CYP7A1 en el hígado (227), la enzima que cataliza y regula el

primer paso de la síntesis de ácidos biliares (228). Además, se ha observado que una reducción de los ácidos biliares conduce a la proliferación bacteriana y que el FXR inhibe el sobrecrecimiento bacteriano (229).

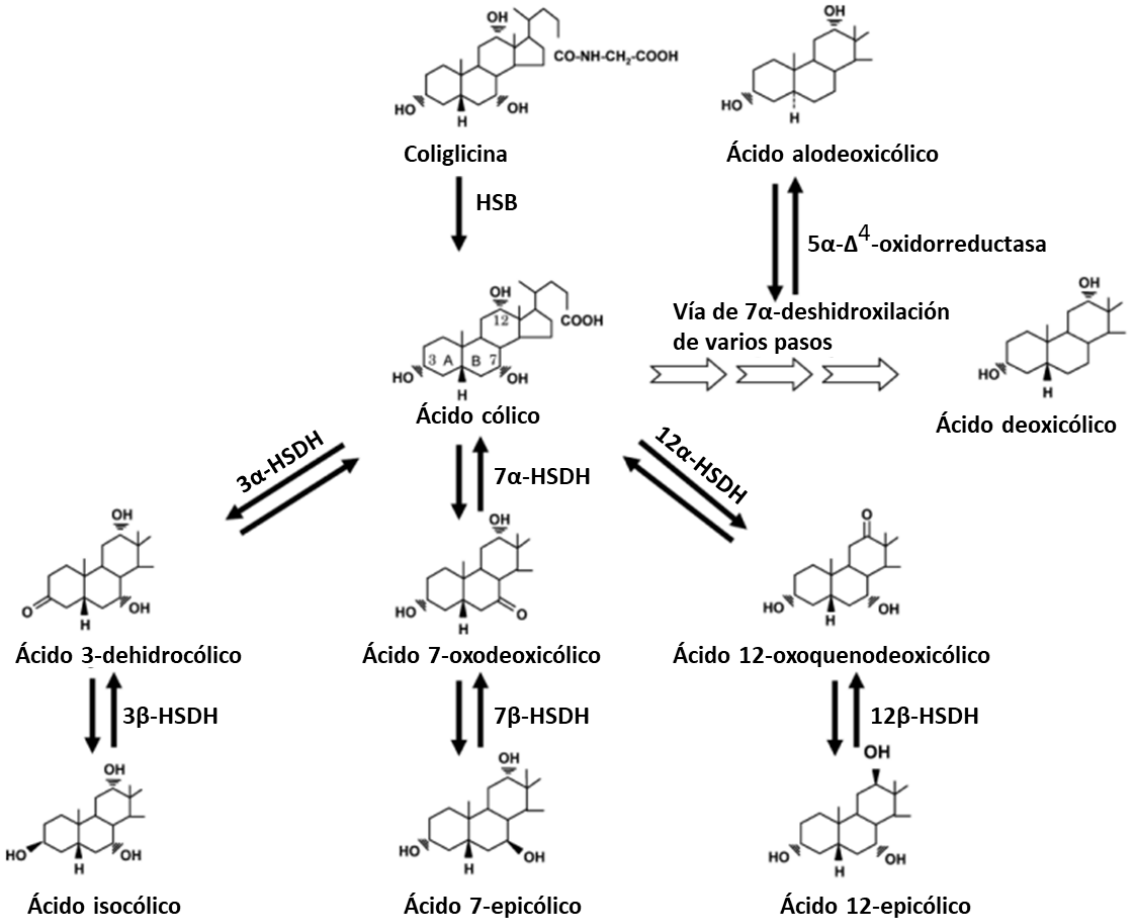

Figura 7. Reacciones bacterianas de biotransformación de sales biliares en el tracto intestinal humano. HSB, hidrolasa de sales biliares; HSDH, hidroxiesteroide deshidrogenasa. *Modificado de Ridlon, JM et al. Journal of lipid research, 2006 (222).*

Varios estudios han confirmado la relación entre los ácidos biliares, las hormonas sexuales, y la composición de la microbiota intestinal. En ratas, la administración de ácido cólico induce cambios en la microbiota similares a los inducidos por las dietas altas en grasa, aumentando los niveles de *Firmicutes* a expensas de *Bacteroidetes* (230). Además, el trasplante de microbiota fecal (TMF) de un donante delgado modifica la microbiota intestinal y los perfiles de ácidos biliares y los asemeja a los del donante delgado (231), mientras que la gonadectomía en ratones altera el patrón de ácidos biliares (209), al igual que en ratas LG y tratadas con antibióticos (232). Dado que la testosterona se sintetiza a partir de los ácidos biliares (233), y como se ha descrito anteriormente, los niveles de ácidos biliares se ven alterados por la microbiota, es plausible que la microbiota pudiera influir indirectamente en el nivel de testosterona.

2.3.2.2. Acción enzimática

La comunidad microbiana comensal puede modificar los niveles de las hormonas sexuales a través de la actividad de sus enzimas, habiéndose definido en este sentido el

"estroboloma" como el conjunto de genes de la microbiota intestinal capaces de activar los estrógenos a partir de sus glucurónidos inactivos, y especialmente gracias a las enzimas β-glucuronidasas, que desconjugan los estrógenos en sus formas activas (234-236) (Figura 8). Estos estrógenos activos pasan al torrente sanguíneo y actúan sobre los receptores de estrógenos alfa (REα) y beta (REβ) (237). Igualmente, la microbiota intestinal participa en el metabolismo y la desglucuronización intestinal de la DHT y la testosterona, originando niveles extremadamente altos del andrógeno más potente, la DHT (238).

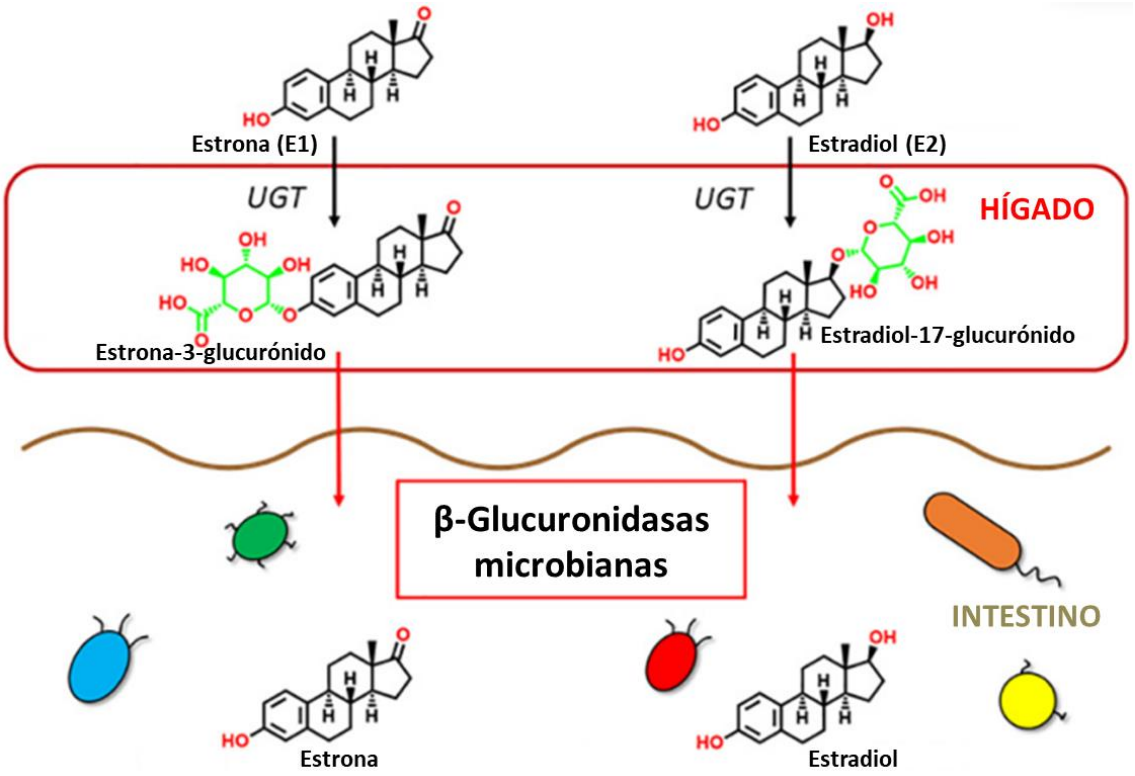

Figura 8. β-glucuronidasas microbianas. Las enzimas β-glucuronidasas de la microbiota intestinal desconjugan en el intestino la estrona-3- y el estradiol-17-glucurónidos a las agliconas estrona y estradiol, respectivamente. Esta reactivación permite que los estrógenos no ligados vuelvan a circular por el torrente sanguíneo. UGT, UDP-glucuronosiltransferasa. *Modificado de Ervin, SM et al. The Journal of biological chemistry, 2019 (236).*

Otro mecanismo de acción de la microbiota intestinal se debería a sus enzimas hidroxiesteroides deshidrogenasas, que intervienen en el metabolismo de las hormonas esteroideas y controlan la unión de los esteroides a sus receptores nucleares, haciéndolos actuar como activadores o inhibidores (239, 240). (Figura 7)

2.3.2.3. Fitoestrógenos

Además de las tres formas principales de estrógenos (hormonas esteroides derivadas del colesterol), el estradiol (E2, predominante en las mujeres no embarazadas antes de la menopausia), la estrona (E1, predominante después de la menopausia) y el estriol (E3, predominante durante el embarazo), existen unos compuestos vegetales, llamados fitoestrógenos, que muestran similitudes estructurales y funcionales con los estrógenos (241) (Figura 9). Entre los fitoestrógenos están las isoflavonas, como la genisteína y la daidzeína, que abundan principalmente en la soja y se activan tras ser metabolizadas

por la microbiota intestinal, pudiendo entonces provocar distintos efectos fisiológicos (242). En este sentido, la microbiota intestinal permite obtener O-desmetilangolensina (O-DMA) y equol a partir de la daidzeína, ambos con actividad estrogénica (243-246).

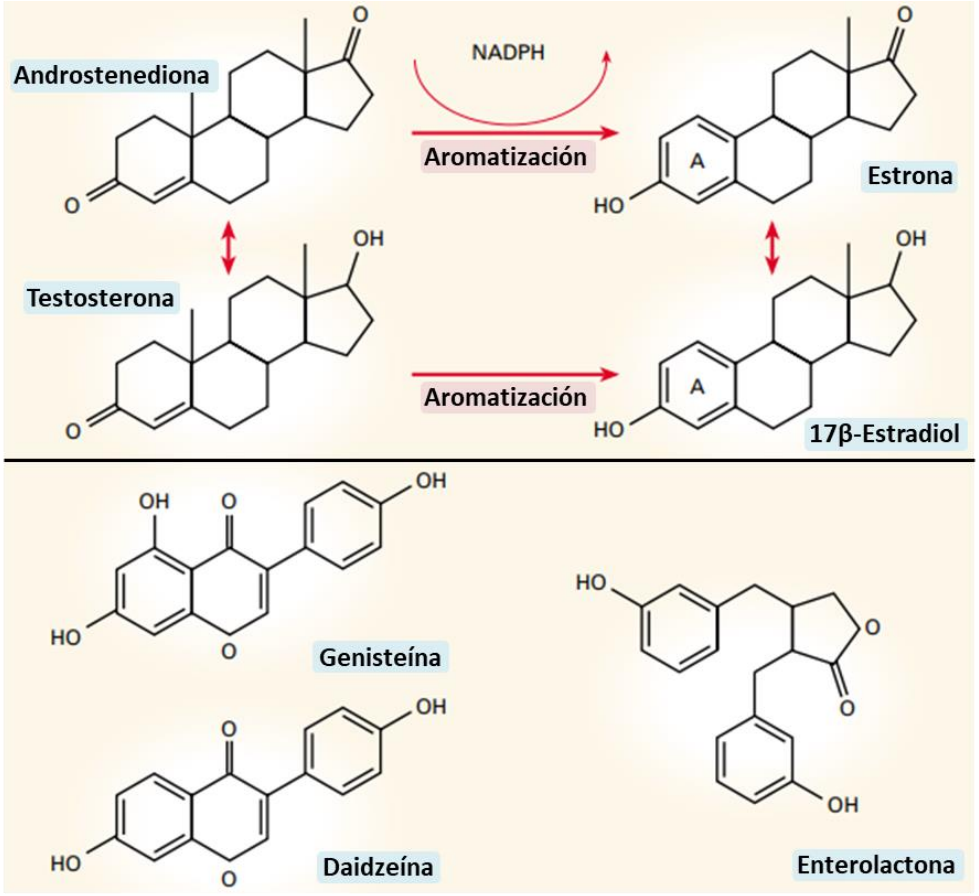

Figura 9. Estructura y producción de estrógenos endógenos y estructura de los fitoestrógenos. La androstenediona y la testosterona son los precursores obligatorios de los estrógenos (panel superior). La genisteína y la daidzeína son isoflavonoides, mientras que la enterolactona es un lignano (panel inferior). En los isoflavonoides, el número y la posición de los sustituyentes hidroxilos determinan el grado de homología estérica con el 17b-estradiol y, por tanto, su afinidad de unión al receptor de estrógenos. Los grupos fenilos de los isoflavonoides y lignanos median la capacidad antioxidante de estos compuestos. *Modificado de Gruber, CJ et al. The New England journal of medicine, 2002 (241).*

Al igual que los estrógenos, los fitoestrógenos causan efectos fisiológicos al afectar a la señalización celular, ya que pueden inducir o inhibir la acción de los estrógenos activando o inhibiendo los REα o REβ, al tiempo que pueden causar efectos epigenéticos y desencadenar cascadas de señalización intracelular (247-249). En relación con esto, se ha sugerido que los fitoestrógenos pueden mejorar varias patologías mediante la modulación del sistema endocrino, incluyendo los síntomas de la menopausia (242) (Figura 10), y que pueden revertir los síntomas de la endotoxemia metabólica (250). En este sentido, el metabolito fitoestrógeno equol se ha asociado a un menor riesgo de enfermedades femeninas relacionadas con las hormonas al favorecer la excreción urinaria de estrógenos y modificar sus niveles en sangre en la mujer (251, 252), mientras que la no producción de O-DMA se ha asociado con la obesidad (243, 245).

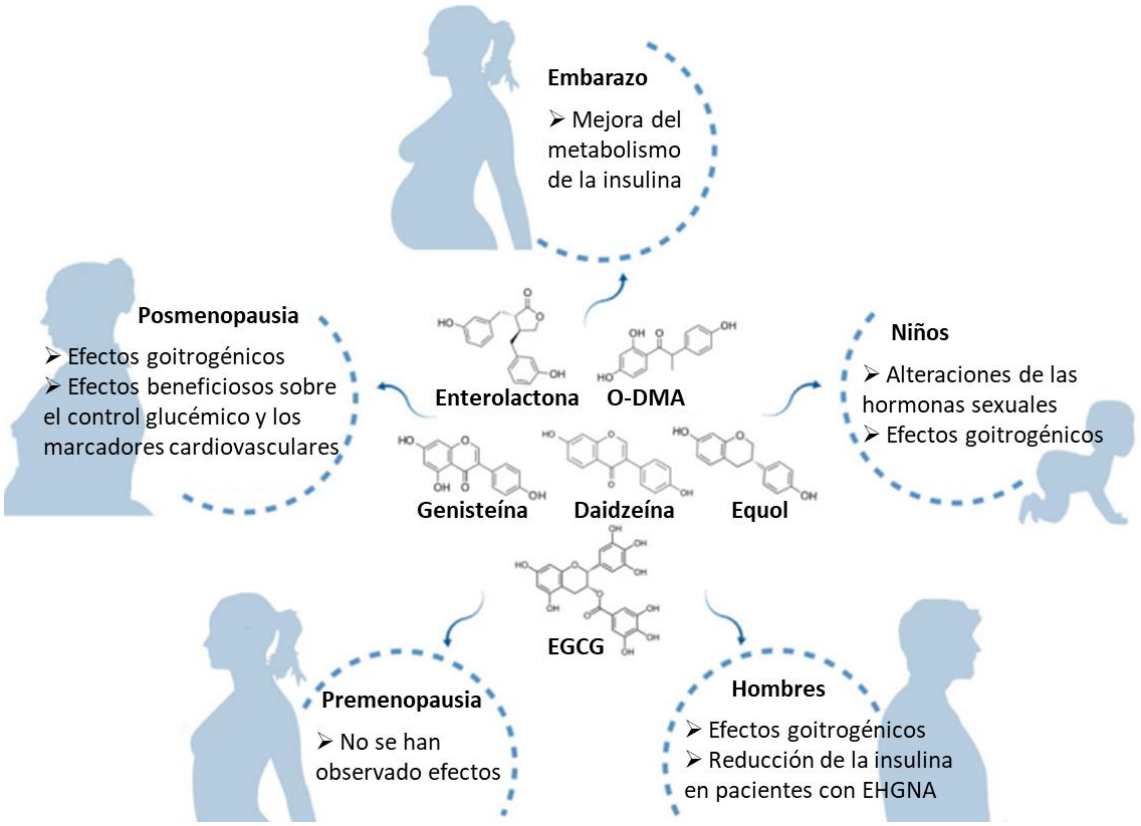

Figura 10. Resumen de los efectos de los fitoestrógenos de la dieta en diferentes etapas de la vida. EGCG, epigalocatequina-3-galato; EHGNA, enfermedad del hígado graso no alcohólico; O-DMA, O-desmetilangolensina. *Modificado de Domínguez-López, I et al. Nutrients, 2020 (242).*

2.4. Aspectos clave de la acción de la microbiota intestinal en las enfermedades metabólicas (Figura 15)

2.4.1. Pruebas de la implicación de la microbiota intestinal en las enfermedades metabólicas

Desde que en 2005 se descubrió un aumento de la relación *F/B* en ratones obesos en comparación con ratones delgados (253), muchos estudios han abordado el papel de la microbiota intestinal en la obesidad y en patologías asociadas, como la DT2 (254). En humanos, la proporción relativa de *Bacteroidetes* disminuye en personas obesas en comparación con las delgadas y aumenta con la pérdida de peso, lo que otorga a la obesidad un componente microbiano (255). Además, la "microbiota obesa" posee una mayor capacidad para extraer energía de la dieta, siendo este rasgo transmisible, pues la colonización de ratones LG con una "microbiota obesa" provoca un mayor aumento de la grasa corporal que con una "microbiota magra" (256). La mayor diversidad metabólica encontrada en *Firmicutes* frente a *Bacteroidetes* (348 vías metabólicas frente a 76, respectivamente) (257), apoya la idea de que el filo *Firmicutes* podría extraer más energía de la dieta y apoya aun más la idea de que la microbiota intestinal desempeña un papel importante en el desarrollo de la obesidad.

No obstante, si bien la implicación de la relación *F/B* en la obesidad está bien establecida en estudios en animales, en humanos los resultados son contradictorios. En este

sentido, aunque algunos estudios han constatado que esta relación es mayor en los individuos obesos (como en ratones) (255, 258), otros estudios no confirman estas observaciones (259-261) o incluso muestran una menor abundancia de *Firmicutes* en individuos obesos (262). Además, en un estudio basado en dietas de adelgazamiento no se encontró ninguna relación entre los individuos obesos y no obesos y la relación *F/B* (258). Sin embargo, otro estudio ha mostrado una marcada disbiosis caracterizada por un aumento de la relación *F/B* en personas obesas con SM en comparación con personas obesas sin SM y con personas no obesas (263), lo que sugiere que la relación *F/B* puede estar relacionada con la presencia o ausencia de rasgos metabólicos en humanos más que con la obesidad en sí.

Siguiendo con lo anterior, se ha comprobado que la microbiota intestinal está implicada en el SM (264, 265), pudiendo conducir a una inflamación de "bajo grado" que, a su vez, puede promover el desarrollo de esta patología (266). Ratones genéticamente deficientes en el receptor tipo Toll (TLR) 5 presentan rasgos del SM, así como una microbiota intestinal alterada que confiere muchas características de este síndrome al ser transferida a ratones LG (267). Esta implicación de la microbiota intestinal en el SM se ha confirmado al erradicarse su desarrollo en ratas ante un tratamiento con antibióticos o ante la transferencia fecal de ratas sin esta patología (268).

Estudios en ratones (269, 270) y en humanos (271-273) también han mostrado diferencias en la microbiota de individuos con DT2. Pacientes con DT2 presentan una microbiota intestinal con una menor abundancia de bacterias productoras de butirato, un aumento de varios patógenos oportunistas, y un enriquecimiento de las funciones microbianas que permiten la reducción de sulfatos y la resistencia al estrés oxidativo (274, 275). También se ha comprobado que las intervenciones dietéticas mejoran la DT2 al modular la disbiosis de la microbiota, recuperando una comunidad equilibrada de productores de ácidos grasos de cadena corta (AGCC) (276), aumentando la relación *F/B* y la abundancia del género *Lactobacillus* (277), y suprimiendo el estado inflamatorio (278, 279). Por otra parte, el uso de probióticos mejora esta patología en ratones al mejorar la función de la barrera intestinal y aumentar la proporción de bacterias productoras de AGCC (280). En línea con lo anterior, un estudio reciente sugiere que la microbiota intestinal puede contribuir a la inflamación sistémica crónica y a la DT2 a través de la translocación bacteriana, ya que los pacientes diabéticos tenían una microbiota con menos géneros productores de AGCC y más bacterias gramnegativas productoras de endotoxinas (281). El uso de metformina en humanos, un fármaco usado contra la DT2, y el senósido A en ratones, el principal ingrediente activo del *Rhizoma Rhei* (ruibarbo), modifican la microbiota intestinal y mejoran la DT2 (282, 283). Concretamente, se ha destacado que la metformina aumenta la proporción de especies beneficiosas, como *Akkermansia muciniphila*. Últimamente también se ha observado que el TMF de humanos con tolerancia normal a la glucosa a ratones diabéticos mejora varios parámetros metabólicos implicados en la DT2 y modifica la microbiota al

disminuir los niveles de *Desulfovibrio* y *Clostridium coccoides* y aumentar los de *Akkermansia muciniphila* (284). En otro estudio, el TMF en un modelo de ratón con DT2 disminuyó la respuesta inflamatoria, mejoró la resistencia a la insulina, e inhibió la apoptosis de las células beta pancreáticas (285).

2.4.2. Inflamación

La microbiota intestinal se ha relacionado con enfermedades caracterizadas por una inflamación crónica de bajo grado, como la obesidad y la DT2. En concreto, el estado inflamatorio estaría influenciado principalmente por el lipopolisacárido (LPS), la barrera intestinal y varios metabolitos derivados del metabolismo bacteriano.

2.4.2.1. Lipopolisacárido (LPS)

El LPS, una endotoxina de la membrana externa de las bacterias gramnegativas, participa en la inflamación crónica al activar los TLR e inducir la secreción de citoquinas proinflamatorias potencialmente diabetogénicas, como la IL-6 y el factor de necrosis tumoral alfa (TNF-α), así como de componentes clave de la respuesta inmunitaria en el tejido adiposo. También se ha correlacionado con los niveles de insulina, al tiempo que los pacientes con DT2 presentan una mayor cantidad de LPS circulante (286). Además, la endotoxemia metabólica, definida como una alta concentración de LPS en el torrente sanguíneo, se ha relacionado con la resistencia a la insulina, la hiperplasia de adipocitos, y la reducción de la función de las células beta pancreáticas (287).

La relación entre los niveles elevados de LPS circulante y las enfermedades metabólicas se ha comprobado mediante la infusión crónica de LPS en ratones, lo que da lugar a un aumento de la glucemia en ayunas, de la insulinemia, y de la resistencia a la insulina, así como a un aumento de la infiltración de macrófagos en el tejido adiposo (288). Además, el anterior estudio mostró que la ablación del correceptor CD14 del LPS revertía las enfermedades metabólicas inducidas por este compuesto.

Se han propuesto dos mecanismos de absorción de LPS, no excluyentes, desde el intestino al sistema circulatorio (289): (1) transporte facilitado por quilomicrones (lipoproteínas que transportan los lípidos de la dieta a los tejidos periféricos), apoyado por el hecho de que el nivel de LPS aumenta cuando las células son estimuladas con ácidos grasos que promueven la formación de quilomicrones, al tiempo que la inhibición de la formación de quilomicrones bloquea la absorción de LPS; (2) transporte extracelular a través de las uniones estrechas del epitelio, apoyado por el hecho de que la reducción de la permeabilidad intestinal y la mejora de la integridad de las uniones estrechas reducen los niveles plasmáticos de LPS, las concentraciones de citoquinas inflamatorias circulantes, y la inflamación hepática. (Figura 11)

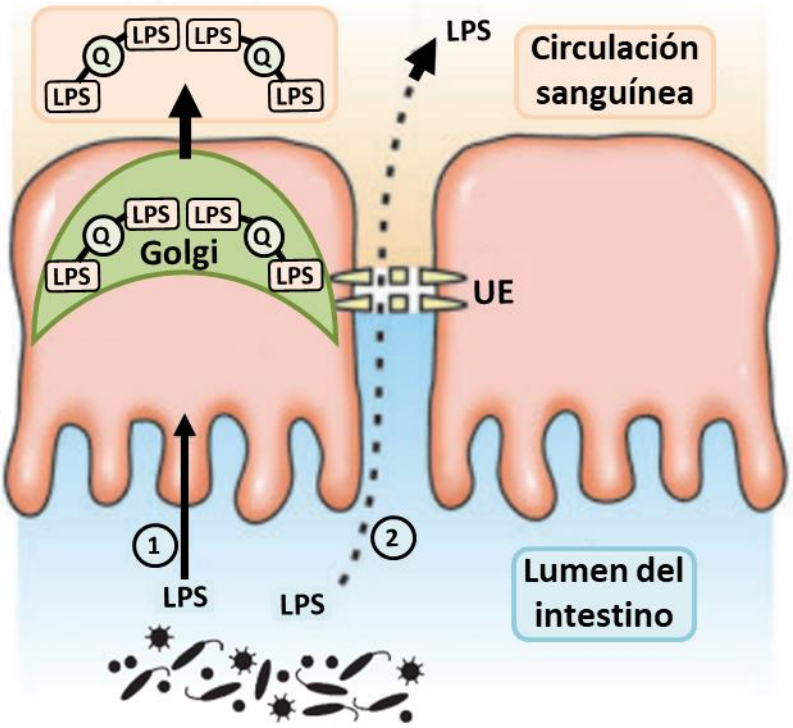

Figura 11. Mecanismos de absorción intestinal del lipopolisacárido (LPS). (1) Absorción intracelular facilitada por los quilomicrones (Q), vía aparato de Golgi. (2) Absorción extracelular a través de las uniones estrechas (UE) del epitelio. *Modificado de Caesar, R et al. Journal of internal medicine, 2010 (289).*

2.4.2.2. Integridad de la barrera intestinal

Mientras que el epitelio intestinal del intestino delgado tiene una capa de mucosa no adherida, el del colon presenta dos, la interna, adherida, y la externa, menos densa y no adherida (290). Esta mucosa, compuesta por glicanos o mucinas (proteínas altamente glicosiladas secretadas por las células caliciformes, entre las que destaca la proteína MUC2), y que forma la llamada barrera intestinal, representa una barrera para las bacterias intestinales y proporciona así protección contra la inflamación (35) implicada en la patogénesis de la resistencia a la insulina, que a su vez está relacionada con la obesidad y la DT2 (291). En este sentido, la microbiota intestinal influye en la integridad y permeabilidad de la barrera intestinal y, por tanto, en el estado inflamatorio, al interaccionar con los O-glicanos de tipo mucina (26, 34), lo que a su vez puede derivar en enfermedades metabólicas como la resistencia a la insulina. En relación con esto, el aumento de la permeabilidad intestinal se ha asociado con el riesgo de DT2 (292), y la inflamación de bajo grado y la resistencia a la insulina que caracterizan tanto a la obesidad como a la DT2 están mediadas por el LPS bacteriano (endotoxemia metabólica) (26, 288). De hecho, en ratones LG, el mantenimiento de la estructura de la mucosa intestinal requiere la presencia de microbiota intestinal (293, 294) y puede ser modificada por el TMF (295). (Figura 12)

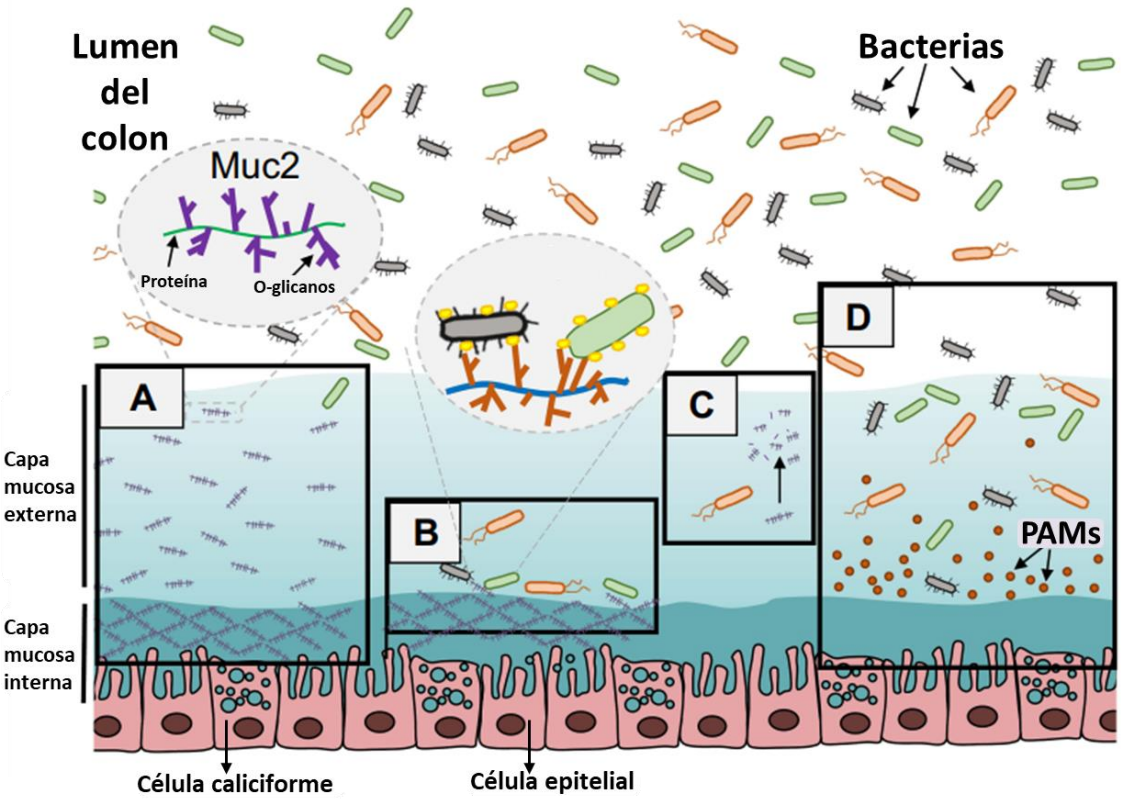

Figura 12. Interacciones entre los O-glicanos de tipo mucina y la microbiota intestinal. (A) La distribución de los glicanos de la mucosa externa es débil, y los glicanos de la mucosa interna se entrelazan en una red para actuar como barrera de la mucosa; (B) Interacción de las bacterias con los glicanos; (C) Descomposición bacteriana de los glicanos en oligosacáridos; (D) los péptidos antimicrobianos (PAMs) inhiben los patógenos bacterianos en la capa mucosa. *Modificado de Zhang, Y et al. Journal of inflammation research, 2021 (34).*

2.4.2.3. Metabolitos derivados de la microbiota intestinal

Los AGCC (principalmente el acético, propiónico, y butírico) procedentes de la fermentación bacteriana de la fibra alimentaria se han relacionado con una disminución de la inflamación (296, 297), así como con una mejora de la homeostasis de la glucosa y de la sensibilidad a la insulina (298). Estos compuestos mejoran la función de la barrera intestinal y el estado inflamatorio a través de varios mecanismos: 1) la regulación al alza de las proteínas de unión estrecha (299-301); 2) la regulación del ensamblaje de la unión estrecha por un mecanismo dependiente de la activación de la proteína quinasa activada por AMP (AMPK) (302-304); 3) el aumento de las células T reguladoras (Treg) (300, 305); 4) el aumento de las citoquinas antiinflamatorias y la disminución de las citoquinas inflamatorias (305, 306).

El estado inflamatorio parece depender en gran medida del equilibrio entre las células Treg, que producen la citoquina antiinflamatoria IL-10, y las células colaboradoras T17 (Th17), que producen la citoquina inflamatoria IL-17, de modo que un aumento de la proporción Treg/Th17 reduce el estado inflamatorio. En este sentido, el tratamiento de la enfermedad inflamatoria intestinal con partenólido (una lactona sesquiterpénica extraída originalmente de la planta *Tanacetum balsamita*) reduce la inflamación de forma dependiente de la microbiota intestinal, ya que mejora el equilibrio Treg/Th17 en la mucosa intestinal al aumentar la producción de AGCC (307). El butirato es un actor

clave en esta regulación del equilibrio Treg/Th17 al inducir la diferenciación intestinal de las células Treg por un mecanismo dependiente de la acetilación de las histonas en las regiones promotoras de ciertos genes a través de la inhibición de la histona desacetilasa (308, 309). Este aumento de las células Treg se traduce en un aumento de los niveles de citoquinas anti-Th17 (IL-10 e IL-12) y en una reducción de los niveles de IL-17 (310). Además, los AGCC también parecen estar implicados en la reducción de otros factores proinflamatorios, como el TNF-α, la IL-1β, la IL-6, y el NO (311, 312), así como en la inhibición de la actividad del factor nuclear kappa B (NF-κB) (313, 314), que se ha relacionado con los procesos inflamatorios (315).

Por otra parte, metabolitos bacterianos distintos a los AGCC, como el ácido cafeico, el ácido 4-hidroxifenilpropiónico, y el ácido 4-hidroxifenilacético, pueden mediar en la inflamación, posiblemente a través del receptor de hidrocarburos de arilo y la modulación de la relación Treg/Th17 (316). En relación con esto, los ácidos biliares secundarios resultantes de la desconjugación bacteriana de los ácidos biliares también parecen aumentar la diferenciación de las células Treg en el intestino (317, 318).

2.4.3. Eje intestino-cerebro

La influencia de la microbiota en el desarrollo de la obesidad y las patologías relacionadas con ella podría deberse, en parte, a la alteración de los niveles de las hormonas intestinales que intervienen en el eje intestino-cerebro, por lo que el sistema nervioso central regularía la ingesta de alimentos a través de los productos de la actividad de la microbiota intestinal, incluidos los AGCC (36). La ausencia de microbiota intestinal puede inducir el consumo de nutrientes obesogénicos, como las grasas y los azúcares, debido al aumento de la expresión de sus receptores y transportadores, junto con los niveles más bajos de leptina y grelina circulantes y la disminución de la expresión de los péptidos de saciedad intestinal (colecistoquinina (CCK), péptido YY (PYY), y GLP-1) (319, 320). En conjunto, estos últimos compuestos parecen mediar el control de la función motora gastrointestinal y la ingesta de alimentos (321, 322). Por otra parte, el eje intestino-cerebro, activado por el GLP-1 para el control de la secreción de insulina y el vaciado gástrico, se ve afectado por bacterias del íleon (323). Más concretamente, los AGCC derivados de los microbios pueden inducir un aumento de los niveles de GLP-1 (324-326). (Figuras 13 y 14)

Tanto el PYY como la CCK, producidos por las células L intestinales, son hormonas anorexigénicas que inhiben la ingesta de alimentos y reducen el aumento de peso (327, 328).

El GLP-1 es una hormona incretina que media en la liberación de insulina en las células beta pancreáticas para mantener la normoglucemia (37, 329) y reduce la entrada de nutrientes en la circulación aumentando la saciedad y reduciendo la velocidad del vaciado gástrico (330, 331). Más concretamente, este neuropéptido modula los mecanismos centrales de la ingesta de alimentos en el hipotálamo estimulando la

actividad de las neuronas anorexigénicas de la proopiomelanocortina (POMC) e inhibiendo la actividad de las neuronas orexigénicas de la proteína relacionada con agutí (AgRP)/neuropéptido Y (NpY) (332).

Tanto la hormona orexigénica grelina como la hormona anorexigénica leptina juegan un papel clave a través del eje intestino-cerebro en la regulación metabólica y la homeostasis energética y, por tanto, en el desarrollo de la obesidad (321, 333, 334). La grelina está relacionada con la adiposidad y el aumento de peso excesivo al inducir un aumento de la tasa de vaciado gástrico y una disminución del gasto energético (335-337), al tiempo que aumenta la ingesta de alimentos al estimular las neuronas orexigénicas AgRP/NpY e inhibir las neuronas anorexigénicas POMC en el hipotálamo (338). La grelina también está implicada en la secreción de la GH (339, 340), que desempeña un papel clave en la expresión génica sexualmente dimórfica (véase el punto 2.1.1 "Patrón de secreción de la GH: Clave del dimorfismo sexual de la expresión génica"). En este sentido, el dimorfismo sexual observado en las enfermedades metabólicas podría deberse, al menos en parte, a la influencia de la microbiota en los niveles de grelina y, por tanto, en la liberación de la GH. En cuanto a la leptina, reduce la ingesta de alimentos, el peso corporal y la insulina circulante, eleva la concentración circulante de grelina, y promueve la liberación de GH (341-343).

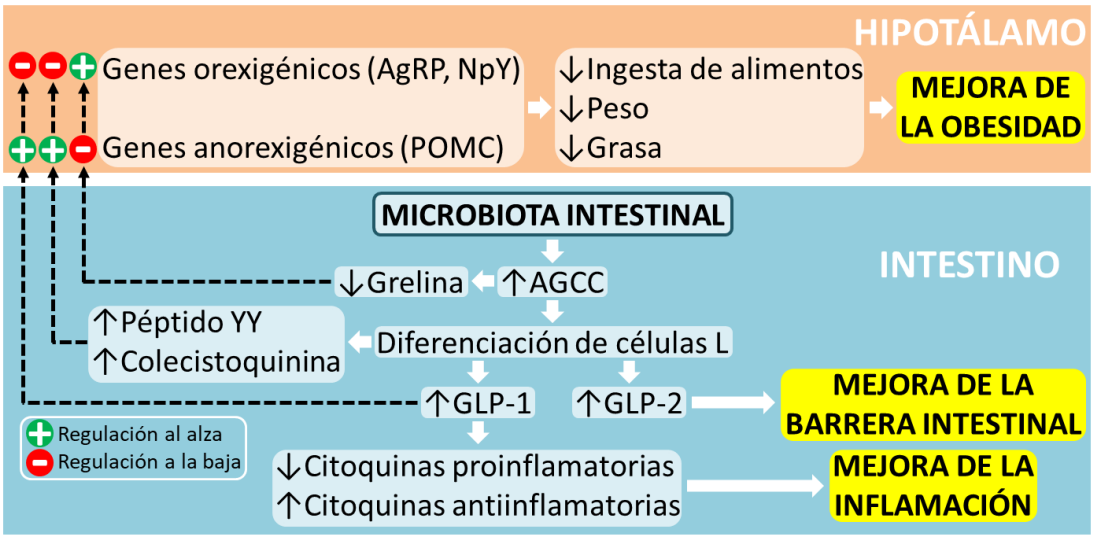

Figura 13. Eje intestino-cerebro. Los AGCC derivados de la fermentación de los polisacáridos disminuyen la liberación de grelina y favorecen la diferenciación de las células L, que inducen un aumento del péptido YY (PYY), colecistoquinina, y péptido similar al glucagón-1 (GLP-1). Estas hormonas mejoran la obesidad reduciendo la ingesta de alimentos, el aumento de peso, y la acumulación de grasa a través de su acción en el hipotálamo. El GLP-1 también reduce las citoquinas proinflamatorias y aumenta las citoquinas antiinflamatorias, lo que mejora del estado inflamatorio. Las células L también producen el péptido similar al glucagón-2 (GLP-2), que mejora la función de la barrera intestinal. *Modificado de Santos-Marcos, JA et al. The Journal of nutritional biochemistry, 2019 (434).*

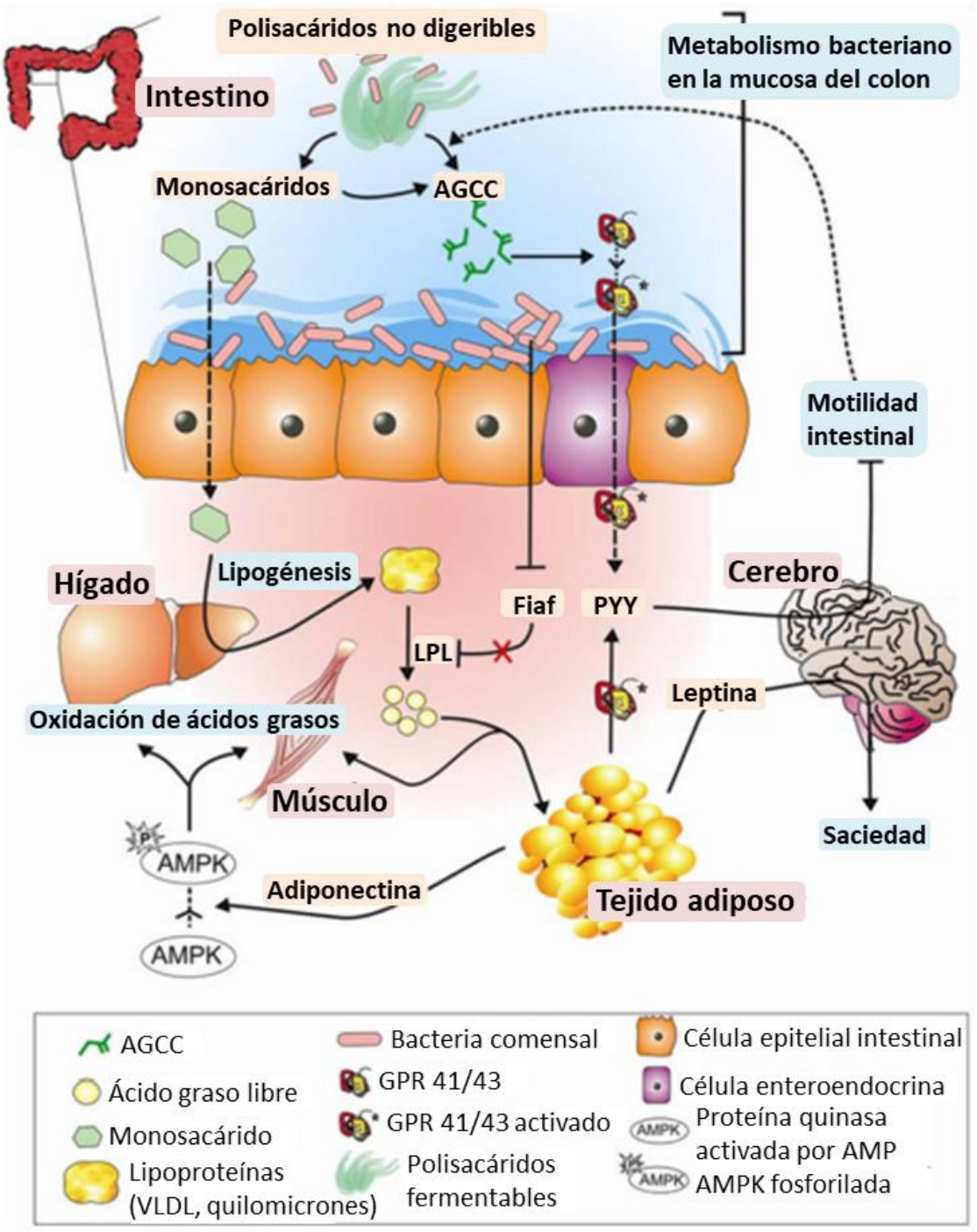

Figura 14. Regulación del metabolismo energético del huésped por la microbiota intestinal. Al descomponer los polisacáridos no digeribles, la microbiota intestinal produce monosacáridos y ácidos grasos de cadena corta (AGCC). Los AGCC se unen a los receptores GPR 41/43 y estimulan la producción del péptido YY (PYY), que inhibe la motilidad intestinal y permite a la microbiota digerir más polisacáridos. La microbiota intestinal también regula el metabolismo energético reduciendo la expresión del factor adiposo inducido por el ayuno (Fiaf) de las células epiteliales del intestino. La supresión de la liberación de Fiaf provoca la degradación de las lipoproteínas y el depósito de ácidos grasos libres en el tejido adiposo. La adiposidad en el hígado y el músculo esquelético también está regulada por la microbiota a través de los cambios en los niveles de la proteína quinasa activada por monofosfato de adenosina fosforilada (AMPK). LPL, lipoproteína lipasa; VLDL, lipoproteína de muy baja densidad. *Modificado de Krajmalnik-Brown, R et al. Nutrition in clinical practice, 2012 (287).*

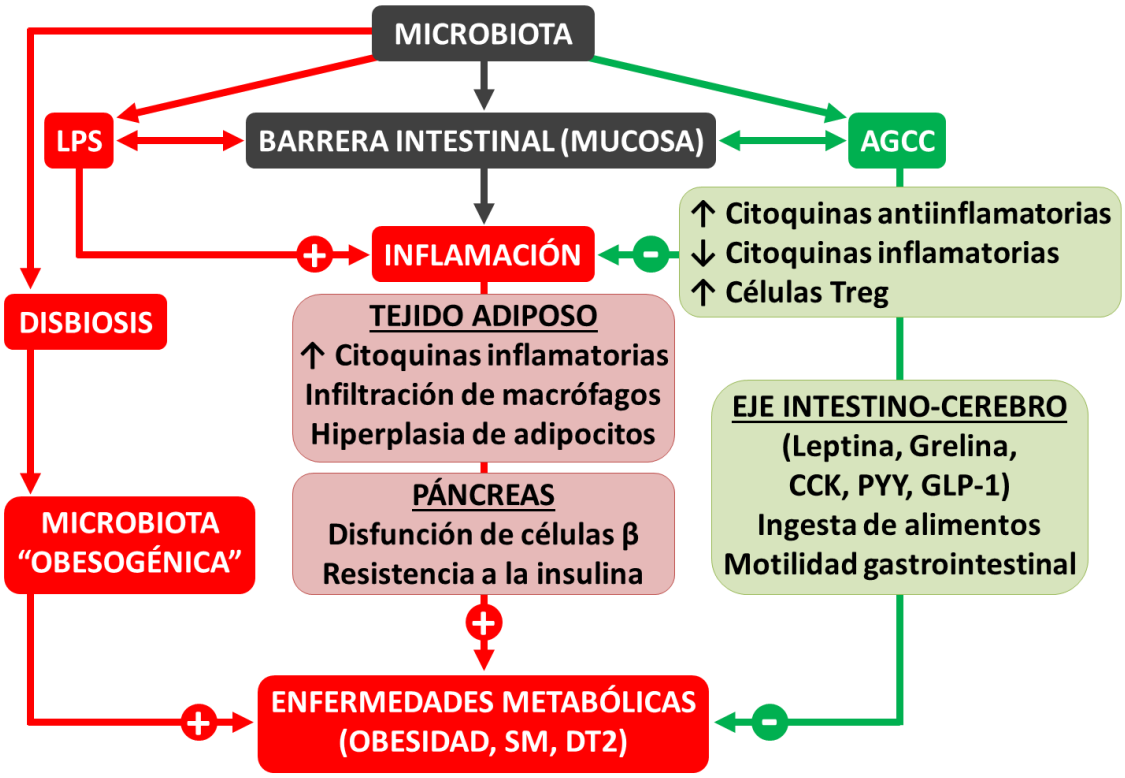

Figura 15. Implicación de la microbiota intestinal en las enfermedades metabólicas. Una microbiota "obesogénica" (mayor relación *Firmicutes/Bacteroidetes*), con mayor capacidad de extraer energía de la dieta, puede contribuir al estado de obesidad. Las enfermedades metabólicas están asociadas a una inflamación crónica de bajo grado y a los desajustes que este estado provoca en el tejido adiposo y en el páncreas. La microbiota puede influir en el estado inflamatorio mediante el lipopolisacárido (LPS), la barrera intestinal y varios de sus metabolitos (especialmente los ácidos grasos de cadena corta (AGCC)). El LPS potencia la inflamación induciendo la infiltración de macrófagos y de citoquinas proinflamatorias en el tejido adiposo. La estructura y permeabilidad de la barrera intestinal (mucosa), que protege contra la inflamación impidiendo la translocación bacteriana, se ve afectada, positiva o negativamente, por la presencia o ausencia de diferentes tipos de bacterias. Los AGCC mejoran la barrera intestinal reforzando las uniones estrechas, reducen la inflamación aumentando las células T reguladoras (células Treg) y las citoquinas antiinflamatorias y disminuyendo las citoquinas inflamatorias, y mejoran la homeostasis de la glucosa y la sensibilidad a la insulina. Los AGCC también intervienen en el eje intestino-cerebro regulando los niveles de hormonas que intervienen en el control de la función motora gastrointestinal y la ingesta de alimentos, como la leptina, la grelina, el péptido YY (PYY), la colecistoquinina (CCK), y el péptido similar al glucagón-1 (GLP-1).

2.4.4. Representantes de la microbiota intestinal relacionados con las enfermedades metabólicas

- Taxones bacterianos productores de AGCC. Los AGCC derivados de la microbiota intestinal, y especialmente el butirato, reducen la respuesta inflamatoria en el intestino al disminuir la expresión de citoquinas proinflamatorias a través de la inhibición de la activación del NF-κB y de la degradación de la proteína inhibidora del NF-κB (IκBα) (344), al tiempo que regulan el metabolismo energético (345) y el estrés oxidativo (346) y mejoran la sensibilidad a la insulina (347). La disbiosis de la microbiota intestinal de los pacientes con SM muestra una reducción de especies bacterianas con una importante actividad sacarolítica (348-350), lo que puede reducir la producción de propionato y acetato (351, 352) y reducir así la abundancia de bacterias que consumen acetato y producen butirato (353, 354). Una reducción de los taxones bacterianos productores de AGCC y, por tanto, una menor disponibilidad de estas moléculas, influye en el eje intestino-cerebro, ya que estos compuestos inducen la liberación de moléculas como el GLP-1 o la grelina, que actúan a nivel

cerebral y modulan la acción de la insulina y el apetito, lo que a su vez influye en el desarrollo de la obesidad y el SM (328, 335, 355). (Figuras 13, 14, y 15)

- *Akkermansia muciniphila*. Esta especie, que degrada la mucina y reside en la capa mucosa (356), participa en la integridad de la barrera intestinal restaurando el grosor de la capa mucosa mediante el aumento de las células caliciformes productoras de mucina (357, 358) e induciendo la expresión de ocludina (proteína de unión intercelular) (359, 360). También actúa contra los trastornos metabólicos inducidos por una dieta alta en grasa (peso corporal, adiposidad, endotoxemia metabólica, marcador de inflamación del tejido adiposo CD11c, hiperglucemia en ayunas, y resistencia a la insulina). Por otra parte, induce un aumento de los niveles intestinales de endocannabinoides (acilgliceroles) que controlan la inflamación, la barrera intestinal y la secreción de péptidos intestinales (357). Además, la proteína Amuc_1100, específica de su membrana externa, mejora la barrera intestinal y diversos procesos de la fisiología intestinal al interactuar con los receptores TLR2 y TLR4 (361-363), al tiempo que induce la producción de la citoquina antiinflamatoria IL-10. También contribuye a la disminución de la inflamación del tejido adiposo reduciendo la infiltración de macrófagos, restableciendo las células Treg, reduciendo las citoquinas proinflamatorias (como la IL 6 y la IL-1β), y aumentando los factores antiinflamatorios (como el α-tocoferol y el β-sitosterol) (358, 359, 364).

- *Faecalibacterium prausnitzii*. Considerada como una de las bacterias productoras de butirato más abundantes del intestino (365, 366), su escasez se ha relacionado con enfermedades asociadas a la inflamación, como la DT2 (276). De hecho, a esta especie se le han descrito efectos antiinflamatorios al bloquear la producción de IL-8 y la activación del NF-κB (ambos relacionados con procesos inflamatorios), así como al inducir un aumento de la secreción de la IL-10 antiinflamatoria y una disminución de la secreción de las citoquinas proinflamatorias IL-12 y TNF-α (367). Asimismo, en sujetos obesos sometidos a una cirugía bariátrica se ha observado una correlación negativa entre esta especie y la inflamación de bajo grado, y concretamente con las concentraciones séricas de marcadores inflamatorios circulantes, como la proteína C reactiva (PCR) y la IL-6 (368).

- Género *Lactobacillus*. Especies de este género se han asociado con un aumento de peso, mientras que otras se han asociado a una "protección" del peso (369). Las asociadas al aumento de peso carecen de las enzimas del catabolismo de la fructosa, de la defensa contra el estrés oxidativo, y de la síntesis de dextrina, L-ramnosa y acetato, al tiempo que codifican las tiolasas implicadas en el metabolismo de los lípidos. Además, las especies asociadas al aumento de peso codifican más bacteriocinas que las asociadas a la protección del peso. También se ha citado que algunas especies mejoran el daño que otras bacterias causan en la barrera intestinal (370). En este sentido, *L. plantarum* ha sido citada por mejorar las uniones estrechas epiteliales (371-373). En cuanto a la inflamación, este género inhibe la respuesta

inflamatoria reduciendo la expresión de citoquinas proinflamatorias a través de la modulación del TLR, del NF-κB, y de las vías de señalización de la proteína quinasa activada por mitógenos (MAPK) (374, 375), además de inducir la secreción de la β-defensina 2 humana, implicada en la defensa del huésped (376). Efectos similares a los anteriores se han observado con *L. fructosus*, *L. acidophilus*, *L. fermentum*, *L. casei* y *L. rhamnosus* (326, 377-382). Especialmente, el probiótico *L. rhamnosus GG* puede reducir la señalización inflamatoria en los epitelios intestinales inmaduros de ratón (383), a la vez que mejora la sensibilidad a la insulina y reduce la adiposidad mediante la secreción de adiponectina y la activación de la AMPK (384).

- Género *Bifidobacterium*. Especies de este género se han asociado a un estado delgado y saludable (262, 385, 386). Se ha observado que mejora la barrera intestinal aumentando las proteínas de unión estrecha (387) y modulando la función de las células caliciformes mediante la secreción de metabolitos, aumentando así la producción de la proteínas MUC2 (388). También aumenta las proteínas Reg I intestinales (389), que participan en la estructura vellosa intestinal (390). Además, reduce la inflamación por varios mecanismos: 1) disminuyendo las citoquinas proinflamatorias (IL-6 e IL-17) y aumentando las citoquinas antiinflamatorias (IL-4 e IL-10) (387, 391); 2) disminuyendo la translocación bacteriana (392-394); 3) previniendo la captación de LPS (387); 4) mejorando la función de los macrófagos y de las células dendríticas en relación con la fagocitosis, la producción de citoquinas y la inducción de la proliferación de los linfocitos T (391). Por otra parte, se ha sugerido que el acetato producido por este género mejora la defensa intestinal mediada por las células epiteliales al inducir efectos antiinflamatorios y/o antiapoptóticos, protegiendo así al huésped contra la infección (395). Además, *Bifidobacterium* es uno de los principales géneros que producen péptidos antibacterianos, concretamente bacteriocinas, contra microorganismos patógenos (396, 397).
- Género *Roseburia*. Este género es menos abundante en pacientes con DT2 (276), y se ha constatado su producción de butirato (398, 399). Además, su abundancia se correlaciona negativamente con las alteraciones inducidas por el consumo de grasas (aumento de peso, adiposidad, hiperglucemia en ayunas, acumulación de colesterol y triglicéridos en suero y en hígado, e intolerancia a la glucosa) (400, 401).
- Género *Prevotella*. Este género participa en la integridad de la barrera intestinal al degradar las mucinas (402) de la capa mucosa (403). Además, produce propionato, succinato, y acetato, utilizando gran variedad de polisacáridos (404, 405); habiéndose descrito como uno de los géneros más abundantes en dietas con alto contenido en polisacáridos (406-408). Por el contrario, algunos estudios han descrito un efecto perjudicial de este género. Concretamente, se ha demostrado que *P. intermedia* induce la producción de TNF-α por un mecanismo inducido por el LPS (409), y que produce lípidos dihidroceramidos fosforilados, que a su vez inducen la secreción de la citoquina proinflamatoria IL-6 (410).

2.5. Modulación de la microbiota intestinal mediante la dieta como terapia para el tratamiento de las enfermedades metabólicas

La dieta es uno de los principales factores que determinan la composición de la microbiota intestinal, cuya primera evidencia provino del estudio de Cani et al. (288) al demostrar que una dieta rica en grasa aumentaba la proporción de LPS en el intestino y modificaba la composición de la microbiota intestinal; lo cual se relacionaba con un aumento de la obesidad y del estado inflamatorio de "bajo grado". Otros estudios han descrito el efecto en la composición de la microbiota intestinal de dietas tan distintas como las dietas vegetarianas y omnívoras, o las dietas de zonas geográficas tan distantes como África y Europa (406, 411).

2.5.1. Estudios en modelos animales

El estudio pionero de Turnbaugh et al. (412) demostró que la obesidad inducida por una dieta occidental en ratones modificaba la composición de la microbiota intestinal. Este grupo de investigación también ha demostrado que la estructura inicial de la microbiota intestinal se altera rápidamente con la dieta, incluso en un día (413). Además, en ratones colonizados con una microbiota de un donante alimentado con una dieta occidental obesogénica aumentaba la adiposidad, lo cual demuestra que este rasgo es transmisible mediante el trasplante de microbiota.

Diferentes estudios constatan que una dieta rica en grasa aumenta la permeabilidad intestinal y, en consecuencia, incrementa el LPS y la translocación de bacterias a través de la mucosa intestinal, lo que conlleva un aumento de peso, una morfología alterada del tejido adiposo, resistencia a la insulina e inflamación, así como una alteración de la microbiota intestinal al aumentar el filo *Firmicutes* y disminuir el filo *Bacteroidetes* (288, 394, 414, 415). Por otra parte, una dieta baja en grasa y alta en fibra beneficia a las bacterias beneficiosas y la producción de AGCC, y especialmente del butirato; mientras que una dieta alta en grasa/baja en fibra beneficia a las bacterias perjudiciales (416). En la misma línea, también se ha comprobado que una dieta baja en grasa y alta en fibra aumenta la abundancia del género beneficioso *Bifidobacterium* (417).

2.5.2. Estudios en humanos

La composición de la microbiota intestinal en los seres humanos puede modificarse rápidamente, en cuestión de días, mediante cambios en los hábitos alimentarios (259, 418). En este sentido, una dieta basada en productos animales incrementa los microorganismos tolerantes a la bilis (*Alistipes*, *Bilophila* y *Bacteroides*) y disminuye los niveles de *Firmicutes* que metabolizan los polisacáridos vegetales (*Eubacterium rectale, Ruminococcus bromii,* y *Roseburia*,) (418, 419). (Figura 16)

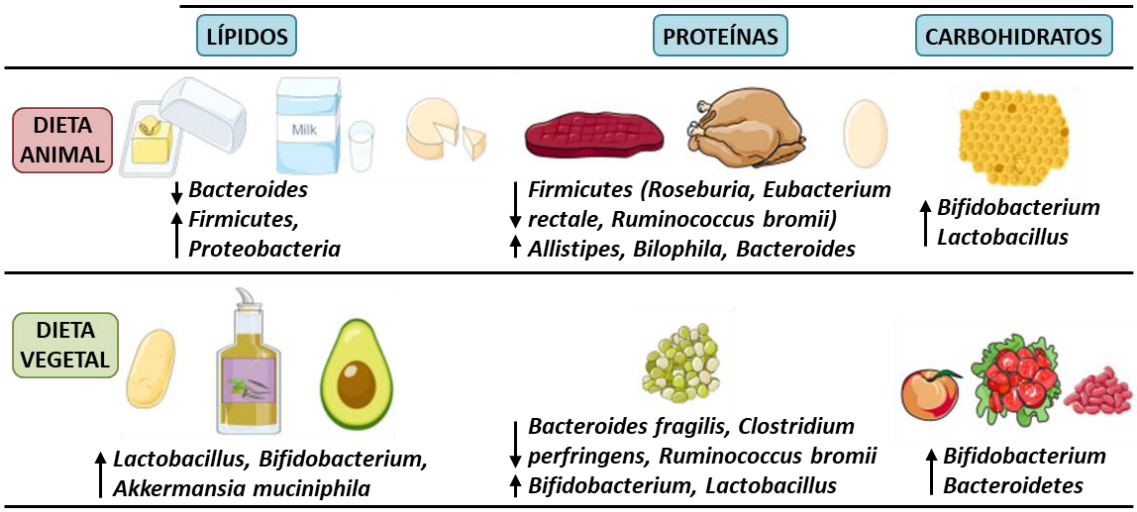

Figura 16. Cambios en la microbiota intestinal provocados por los macronutrientes (lípidos, proteínas y carbohidratos) de origen animal o vegetal. *Modificado de Puértolas-Balint, F and Schroeder, BO. Frontiers in immunology, 2020 (419).*

El contenido de carbohidratos y de fibra en la dieta parecen influir en la composición de la microbiota intestinal. Así, al comparar la microbiota intestinal de niños europeos, con una dieta occidental moderna (rica en grasa y azúcares), y de niños africanos, con una dieta rural (rica en polisacáridos), la microbiota "africana" presenta más *Bacteroidetes* y menos *Firmicutes*, mientras que la familia *Enterobacteriaceae* (*Shigella* y *Escherichia*) era más abundante en la microbiota "europea" (406). Continuando con el anterior estudio, a nivel de géneros, *Prevotella* y *Xylanibacter*, que hidrolizan la celulosa y el xilano (420-422), fueron los géneros principales en los niños africanos, pero estuvieron ausentes en los europeos, mientras que los géneros principales en los niños europeos fueron *Faecalibacterium* y *Bacteroides*, estando *Bacteroides* ausente en los africanos. Además, los AGCC eran más abundantes en los niños africanos. Estos datos llevaron a los autores de este estudio a suponer que la microbiota intestinal de la dieta africana rica en polisacáridos permitía maximizar la energía procedente de la fibra y protegía de la inflamación y las enfermedades colónicas no infecciosas.

Las dietas ricas en carbohidratos han demostrado ser beneficiosas para la salud humana al modificar la microbiota intestinal, observándose este efecto en un estudio de Duncan et al. (353) en el que se administraron tres dietas a sujetos obesos sanos: mantenimiento (13% de proteínas, 52% de carbohidratos y 35% de grasas), alta proteína/medio carbohidrato (30% de proteínas, 35% de carbohidratos, 35% de grasas) y alta proteína/bajo carbohidrato (30% de proteínas, 4% de carbohidratos, 66% de grasas). El resultado fue una reducción de los AGCC fecales (sucesivamente: 114 mM, 74 mM, y 56 mM) y del butirato fecal (sucesivamente: 18 mM, 9 mM, y 4 mM) con la disminución de la ingesta de carbohidratos, demostrando así estos resultados la relación entre la producción de butirato y la ingesta de carbohidratos fermentables en la dieta. En cuanto al contenido bacteriano, la abundancia de los géneros *Roseburia* y *Bifidobacterium*, así como de *Eubacterium rectale*, se redujo con la disminución de la ingesta de carbohidratos. También se observó una correlación entre la abundancia de bacterias productoras de butirato y la disminución del butirato fecal.

En consonancia con los estudios que muestran el efecto beneficioso de las dietas ricas en carbohidratos, varios estudios muestran que las comunidades microbianas se agrupan en dos "enterotipos" básicos, *Bacteroides* y *Prevotella*, los cuales están estrechamente asociados con las dietas basadas en carbohidratos y en proteínas y grasas animales, respectivamente (259, 407, 408).

2.5.3. La dieta como herramienta terapéutica en enfermedades metabólicas

Los estudios sobre intervenciones dietéticas han demostrado que el consumo de dietas saludables puede restaurar, al menos parcialmente, la microbiota intestinal en condiciones de disfunción metabólica hacia un perfil beneficioso para la salud humana.

Las dietas ricas en fibra aumentan la abundancia de especies bacterianas sacarolíticas (423, 424). En comparación con una dieta alta en proteínas y baja en carbohidratos (29% de proteínas, 5% de carbohidratos, 66% de grasas), una dieta alta en proteínas y moderada en carbohidratos (28% de proteínas, 35% de carbohidratos, 37% de grasas) resulta más beneficiosa para los sujetos obesos (425). Ambas dietas aumentaron los ácidos grasos de cadena ramificada, el ácido fenilacético y los compuestos N-nitrosos, pero la dieta baja en carbohidratos redujo el butirato fecal, lo que fue concomitante con una reducción del grupo *Roseburia*/*Eubacterium* y una reducción de los ácidos fenólicos antioxidantes derivados de la fibra. En la misma línea, una dieta muy baja en carbohidratos y alta en grasa también resulta perjudicial, pues reduce el butirato y el total de AGCC y *Bifidobacterium* en comparación con una dieta alta en carbohidratos, alta en fibra, y baja en grasa (426).

Tanto la dieta mediterránea (15% de proteínas, <50% de hidratos de carbono, >35% de grasas (22% monoinsaturadas)), como una dieta baja en grasa (15% de proteínas, >55% de hidratos de carbono, <3% de grasas (12%-14% monoinsaturadas)), mejoran el desarrollo de la DT2, mejorando la sensibilidad a la insulina al modular de diferente modo la microbiota intestinal (427). Así, la dieta baja en grasa aumentó el género *Prevotella* y *Faecalibacterium prausnitzii* y disminuyó el género *Roseburia*, mientras que la dieta mediterránea disminuyó el género *Prevotella* y aumentó *Parabacteroides distasonis* y los géneros *Roseburia* y *Oscillospira*. Este efecto antidiabético también se ha observado en pacientes diabéticos tras consumir la dieta macrobiótica Ma-Pi 2, rica en fibra y carente de grasas y proteínas de origen animal y azúcares añadidos, y una dieta control recomendada para el tratamiento de la DT2 (10%-20% de proteínas, 40%-60% de carbohidratos, 30% de grasas, ≥20 g de fibra) (276), en donde ambas dietas modulaban la disbiosis de la microbiota intestinal aumentando su diversidad y restableciendo una comunidad equilibrada de productores de AGCC beneficiosos para la salud (*Faecalibacterium*, *Roseburia*, *Lachnospira*, *Bacteroides*, y *Akkermansia*). Sin embargo, la dieta rica en fibra fue la única que evitó el aumento de bacterias proinflamatorias, como *Collinsella* y *Streptococcus*.

La intervención dietética también ha demostrado ser beneficiosa en la lucha contra el SM, como muestra un estudio en sujetos con riesgo de SM (con un mínimo de dos de sus características) ante distintos tipos dieta (428). Al final de la intervención, las dietas con alto contenido en grasas monoinsaturadas redujeron las bacterias totales, el colesterol total, y el colesterol-LDL. Las dietas altas en carbohidratos incrementaron el género *Bifidobacterium* y redujeron la glucosa y el colesterol en ayunas. La dieta alta en carbohidratos y de alto índice glucémico incrementó el género *Bacteroides*, mientras que la dieta alta en carbohidratos y de bajo índice glucémico y la dieta alta en grasas saturadas incrementaron *Faecalibacterium prausnitzii*. Por último, la dieta alta en grasas saturadas aumentó el nivel de AGCC fecales. Estos datos indican que las dietas altas en carbohidratos pueden modular las bacterias sacarolíticas fecales, que las dietas altas en grasas monoinsaturadas reducen el número de bacterias, y que las dietas altas en grasas saturadas aumentan la excreción de AGCC.

Otros estudios indican que la dieta mediterránea (15% de proteínas, <50% de carbohidratos, >35% de grasas (22% monoinsaturadas)) y dietas bajas en grasa (15% de proteínas, >55% de carbohidratos, <3% de grasas (12%-14% monoinsaturadas)) también pueden modular la composición de la microbiota intestinal en sujetos con SM, pues si bien la microbiota de sujetos sanos y con SM mostraba diferencias en un principio, tales diferencias desaparecían tras la intervención dietética (262, 264). Uno de estos estudios también mostró que la dieta mediterránea restablecía parcialmente la población de varias especies bacterianas con importante actividad sacarolítica en pacientes con SM, como *Parabacteroides distasonis*, *Bacteroides thetaiotaomicron*, *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis* y *Bifidobacterium longum* (264). Otros estudios indican que además del aumento de la abundancia de *Bacteroides* y *Prevotella*, que forman parte del filo *Bacteroidetes* y reducen la relación *F/B*, el consumo de la dieta mediterránea o de dietas bajas en grasa aumenta la abundancia de otros géneros con actividad sacarolítica, como *Faecalibacterium* (348-350, 367). Sin embargo, el consumo de la dieta mediterránea, rica en compuestos fenólicos antioxidantes procedentes de alimentos como la fruta fresca, las verduras, el vino tinto, y el aceite de oliva, también aumentó la abundancia de *Roseburia* y *Ruminococcus* (que también participa en la actividad sacarolítica), lo que implica un mayor potencial de la dieta mediterránea para restaurar la funcionalidad de la microbiota intestinal que la dieta baja en grasa, cuya riqueza en compuestos fenólicos era menor. No obstante, el consumo de estas dietas restablecía parcialmente la disbiosis de la microbiota intestinal de los pacientes con SM pero sin que desapareciera esta patología, lo que sugiere que podría ser necesaria una intervención dietética más prolongada.

III. HIPÓTESIS

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El punto de partida de esta tesis se basa en la hipótesis de que “existen diferencias entre la microbiota intestinal de hombres y mujeres, lo que podría a su vez ser un determinante en la prevalencia en el desarrollo de enfermedades metabólicas y cardiovasculares”; siendo la hipótesis nula que “no existen diferencias entre la microbiota intestinal de hombres y mujeres”.

IV. OBJETIVOS

IV. OBJETIVOS

Los objetivos de esta tesis se agrupan en un objetivo principal y tres objetivos secundarios:

OBJETIVO PRINCIPAL:

1. Evaluar la existencia de diferencias en la microbiota intestinal asociadas al estado hormonal entre mujeres pre y posmenopáusicas (disminución de estrógenos), así como explorar las diferencias con la microbiota de hombres de similar edad, índice de masa corporal y hábitos nutricionales, mediante secuenciación masiva del ADN bacteriano.

OBJETIVOS SECUNDARIOS:

2. Evaluar las diferencias en la microbiota intestinal de pacientes con síndrome metabólico en función del sexo y determinar si dos dietas saludables, la dieta mediterránea y una dieta baja en grasa, pueden modular la disbiosis microbiana de forma diferencial en función del sexo.
3. Determinar la contribución de las hormonas sexuales y la obesidad a las diferencias en la estructura y composición de la microbiota intestinal entre sexos mediante la caracterización de las diferencias en la microbiota intestinal ante la reducción de las hormonas gonadales en ratas hembra sometidas a ovariectomía y ratas macho a orquiectomía, así como analizar el efecto de la obesidad inducida por la dieta en la microbiota intestinal en estos modelos. Además, se evaluará la potencial implicación de los miARN intestinales en la interacción entre la microbiota intestinal y su huésped.
4. Explorar la influencia de los esteroides sexuales y de una dieta obesogénica (sobrealimentación posnatal) desde las primeras fases del desarrollo mediante un modelo de ratas androgenizadas, en la modificación persistente de la estructura de la microbiota intestinal, así como la posible interacción entre la microbiota intestinal y el huésped a través de la regulación microbiana de la expresión de miARN en el intestino delgado y grueso.

V. PUBLICACIONES

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5.1. Publicación 1

Citación: Santos-Marcos JA, Rangel-Zuñiga OA, Jimenez-Lucena R, Quintana-Navarro GM, Garcia-Carpintero S, Malagon MM, Landa BB, Tena-Sempere M, Perez-Martinez P, Lopez-Miranda J, Perez-Jimenez F, Camargo A. Influence of gender and menopausal status on gut microbiota. Maturitas. 2018 Oct;116:43-53. doi: 10.1016/j.maturitas.2018.07.008. Epub 2018 Jul 19. PMID: 30244778.

Maturitas 116 (2018) 43–53

Contents lists available at ScienceDirect

Maturitas

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

Influence of gender and menopausal status on gut microbiota

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ARTICLE INFO

Keywords:
Gut microbiota
Sexual dimorphisms
Sex hormones
Metabolic diseases
Obesity

ABSTRACT

Objectives: We explore the differences in the gut microbiota associated with gender and hormonal status.
Study design: We included 76 individuals in this study: 17 pre-menopausal women, 19 men matched by age, as a control group for the pre-menopausal women, 20 post-menopausal women and 20 men matched by age as a control group for the post-menopausal women; all 4 groups were also matched by body mass index (BMI) and nutritional background.
Main measurements: We analyzed the differences in the gut microbiota, endotoxemia, intestinal incretins, pro-inflammatory cytokines, and plasma levels of energy homeostasis regulatory hormones between pre- and post-menopausal women and compared them with their respective male control groups.
Results: We found a higher *Firmicutes/Bacteroidetes* ratio, a higher relative abundance of *Lachnospira* and *Roseburia*, and higher GLP-1 plasma levels in pre-menopausal women than in post-menopausal women, who had similar levels to men. In contrast, we observed a lower relative abundance of the *Prevotella*, *Parabacteroides* and *Bilophila* genera, and IL-6 and MCP-1 plasma levels in pre-menopausal women than in post-menopausal women, who had similar levels to the men. We also found higher GiP and leptin plasma levels in women than in men, irrespective of the menopausal status of the women. In addition, adiponectin levels were higher in pre-menopausal women than in their corresponding age-matched male control group.
Conclusions: Our results suggest that the differences in the composition of gut microbiota between genders and between women of different hormonal status may be related to the sexual dimorphism observed in the incidence of metabolic diseases and their co-morbidities.

1. Introduction

The incidence of metabolic diseases and their co-morbidities is sexually dimorphic [1]. Sexual asymmetry in glucose homeostasis has also been described. The prevalence of pre-diabetic syndromes, such as impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), differs by sex, with IFG more prevalent in men, while IGT is more prevalent in women [2]. Moreover, the prevalence of type 2 diabetes mellitus (T2DM) is also marked by sexual dimorphism: there are more diabetic men before puberty and more diabetic women after menopause [2]. Furthermore, it has been suggested that females have stronger immune responses, on the basis of the higher frequency and severity of infectious diseases in males than in females, which in turn makes females more likely to develop autoimmune diseases [3].

The gut microbiota is a symbiotic community that acts as an organ which is fully integrated in the host's metabolism [4]. Interestingly, studies performed over the last few years have shown that the gut microbiota seems to be related with development of metabolic diseases [5], in which sexual dimorphism has been described [2]. Moreover, it has been suggested that gut microbiota composition can be regulated by

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https://doi.org/10.1016/j.maturitas.2018.07.008
Received 17 April 2018; Received in revised form 5 July 2018; Accepted 18 July 2018
0378-5122/

estrogen levels [6–8]. We therefore hypothesized that differences in the gut microbiota, between gender and hormonal status, may be a determinant of the sexual dimorphism in metabolic diseases.

Several studies have suggested that differences in the gut microbiota may trigger the pathogenic mechanisms that involve the development of obesity and insulin resistance [5]. In fact, gender differences in fat distribution have been reported, and these are related with differences in sex hormone levels [9]. In line with this, the sexual dimorphism in total body fat content seen in rodents has been to shown to be absent in germ-free animals, which suggests that the gut microbiota plays a role in host adiposity [10]. In addition, microbiota has also been shown to act by regulating innate and adaptive immunity [4], which may be involved in the higher prevalence of autoimmune diseases in women than in men.

Microbial exposure and sex hormones seem to exert potent effects on autoimmune diseases, such as type 1 diabetes mellitus [11] and autoimmune encephalomyelitis [12], whereas other studies have also related gut microbiota with phospholipid metabolism and cardiovascular risk [13], in which sex hormones are thought to play an important role [14]. On the basis of the potential role of gut microbiota in the sexual dimorphism of metabolic diseases, we aimed to explore the differences in the gut microbiota associated to hormonal status between pre- and post-menopausal women (estrogen depletion), and to compare these with their respective male control groups with a similar age, body mass index and nutritional background.

2. Material and methods

2.1. Study participants

The current work was conducted on a subgroup of patients from the healthy control group (without oncological diseases) included in the ONCOVER study (http://www.proyecto-oncover.es/), which is focused in the development of a volatile compounds detection system for the early diagnosis of lung, colon, breast and prostate cancer. Recruitment was carried out among the free-living population without oncological diseases or disabling diseases or whose severity implied a life expectancy of less than three years. These inclusion and exclusion criteria were assessed according to their medical history, biochemical measurements and physical examination by clinicians. The use of antibiotics in the 3 months prior to sampling was included as an exclusion criteria for the current study. We analyzed 76 patients (39 men and 37 women): 17 pre-menopausal women (estradiol = 109.40 ± 41.46 pg/mL), 19 men matched by age as a control group for the pre-menopausal women, 20 post-menopausal women (years after last menstrual cycle: 6.38 ± 1.14; Spearman correlation between estradiol levels and years after menopause: R = –0.575 P = 0.025; estradiol = 11.49 ± 4.47 pg/mL) and 20 men matched by age as a control group for the post-menopausal women; all 4 groups were also matched by BMI. All the patients gave their written informed consent to participate in the study. The protocol was approved by the Human Investigation Review Committee of Reina Sofia University Hospital (2012/000069), following the Helsinki declaration and good clinical practice. Diet assessment and Clinical plasma parameters were determined as previously [15]. The metabolic characteristics of the subjects in the study are shown in Table 1.

2.2. Measurement of pro-inflammatory cytokines, energy homeostasis regulatory hormones and intestinal incretins in plasma

The patients had fasted (food/drugs) for 12 h and were asked to refrain from smoking during the fasting period and from alcohol intake during the preceding 7 days. They were also asked to avoid strenuous physical activity the day before the test and pre-menopausal women were asked not to attend during the days of menstruation. A fasting blood sample was taken in tubes containing EDTA to give a final concentration of 0.1% EDTA. The plasma was separated from the red cells by centrifugation at 1500 × *g* for 15 min at 4 °C, and then frozen until the biomolecular measurements were taken. The plasma levels of MCP-1, IL-6 and TNF-alpha levels were measured by an IL-1 beta/IL-1F2 Quantikine HS ELISA Kit, IL-6 Quantikine HS ELISA Kit and TNF-alpha Quantikine ELISA Kit, from R&D Systems, Inc.™ (MN, USA), following the manufacturer's instructions. The level of estradiol was determined using the commercial kit: Estradiol EIA kit (Cayman Chemical; Cat. No. 582251), according to the manufacturer's instructions. The plasma levels of adiponectin, leptin and resistin levels were measured by Total Adiponectin/Acrp30 Quantikine ELISA Kit, Leptin Quantikine ELISA Kit, and Resistin Quantikine ELISA Kit, from R&D Systems, Inc.™ (MN, USA), following the manufacturer's instructions. The plasma levels of GLP-1 and GiP were measured using the GLP-1 (7–36)-Amide EIA kit and GiP EIA kit (Phoenix Europe GmbH, Germany), according to the manufacturer's instructions. The endotoxin lipopolysaccharide (LPS) was measured using the limulus amebocyte lysate test (QCL-1000 Chromogenic LAL (Lonza Iberica S.A., Spain), as previously described [16]. LPS Binding Protein (LBP) measurements were made using the human LBP ELISA kit (Hycult biotech, Netherlands), according to the manufacturer's instructions.

2.3. Sequencing the V1-V2 microbial 16S rRNA gene on the Illumina MiSeq

DNA extraction from fecal samples was performed by the QIAamp DNA kit Stool Mini Kit Handbook (Qiagen, Hilden, Germany), following the manufacturer's instructions. The 76 samples were amplified in triplicate by polymerase chain reaction (PCR) to generate an amplification library. The bacterial 16S rRNA gene V1-V2 hypervariable regions were amplified in polymerase chain reactions (PCR) using universal bacterial primers 8F (AGAGTTTGATCMTGGCTCAG) and 357R (CTGCTGCCTYCCGTA) complemented with 8 nt index and Illumina adapter sequences. PCR was performed with Smart-Taq Hot Red 2X PCR Mix (Naxo, Estonia), 1 μl of extracted DNA (10 ng/μl), 0.2 μM of each primer, using the following cycling parameters: 15 min denaturation at 95 °C followed by 3 cycles (30 s at 95 °C, 30 s at 50 °C, 60 s at 72 °C), 28 cycles (30 s at 95 °C, 30 s at 65 °C, 60 s at 72 °C) and a final extension at 72 °C for 7 min. Oligonucleotides were removed from pooled PCR product library using the QIAquick® PCR Purification Kit (Qiagen, Inc.). Single end sequencing of V2 hypervariable region was performed on Illumina MiSeq next generation sequencing platform using the v3 kit (a service provided by The Estonian Genome Center Core Facility).

2.4. Upstream analysis of the 16S rRNA gene sequences

The 16S rRNA gene sequences obtained were analyzed using QIIME 1.9.1 with default parameters unless indicated otherwise [17]. Raw sequencing data was de-multiplexed and low quality and short (< 150 nt) readings were discarded. The readings were clustered using a closed-reference OTU picking protocol that assigned readings to reference sequences. Briefly, the processing involved the following steps: (1) demultiplexing and filtering of short (< 150 nt) and low quality readings; (2) de novo clustering of the sequences into operational taxonomic units (OTUs) with the USEARCH61 program using a 97% similarity threshold [18] and the Greengenes v13-8 database [19]; (3) taxonomical assignment of each OTU by running the RDP Classifier [20] at 85% bootstrap confidence on a selected representative sequence from each OTU; (4) alignment of representative sequences using PyNAST [21] with the Greengenes core-set alignment template.

The differences between the bacterial communities were calculated in QIIME using rarefaction curves of alpha-diversity indexes including estimates of community richness (such as the Chao1 estimator and the observed number of OTUs present in each sample and Phylogenetic diversity (PD) or the length of the phylogenetic branch observed in each sample). The upper limit of rarefaction depths was 16,998 sequences per sample. Beta diversity was estimated using weighted and

Table 1
Metabolic characteristics of the participants in the study. Values correspond to the mean ± SEM. The statistical differences between groups were evaluated by One-way ANOVA. BMI, body mass index; TG, triacylglycerides; HDL-c, high density lipoprotein-cholesterol; LDL-c, low density lipoprotein-cholesterol; HbA1c, glycated hemoglobin; BP, blood pressure. In each row, values with different letters in superscript differ statistically in the Bonferroni's corrected post hoc multiple comparison test ($P < 0.05$).

	Pre-menopausal women (N = 17)	Men control group pre (N = 19)	Post-menopausal women (N = 20)	Men control group post (N = 20)	P-value
Age (years)	46.12 ± 0.82[a]	46.58 ± 0.61[a]	55.85 ± 0.66[b]	56.20 ± 0.72[b]	< 0.001
Last menstrual cycle (years)	–	–	50.13 ± 0.76	–	n.a.
BMI (kg/m^2)	26.31 ± 1.52	27.10 ± 0.91	28.94 ± 1.25	28.53 ± 1.13	0.393
Waist circumference (cm)	88.94 ± 3.15	95.68 ± 2.24	92.65 ± 3.00	99.30 ± 2.88	0.079
HDL-c (mg/dL)	57.00 ± 3.37[a]	41.79 ± 2.14[b]	54.50 ± 2.82[a,c]	45.40 ± 1.92[b,c]	< 0.001
LDL-c (mg/dL)	118.53 ± 6.97	132.42 ± 5.97	137.40 ± 7.13	145.65 ± 7.21	0.057
TG (mg/dL)	88.88 ± 9.97	115.95 ± 14.92	102.95 ± 12.76	107.95 ± 13.24	0.545
Glucose (mg/dL)	87.00 ± 2.75[a]	98.21 ± 5.48[a,b]	92.05 ± 2.38[a]	108.40 ± 4.29[b]	0.002
Insulin (mU/L)	5.39 ± 0.79	6.37 ± 0.90	6.87 ± 0.92	8.08 ± 0.99	0.233
HbA1c (%)	5.11 ± 0.11	5.32 ± 0.17	5.54 ± 0.08	5.55 ± 0.13	0.063
Sistolic BP (mm Hg)	127.96 ± 6.30	129.53 ± 3.85	132.83 ± 3.66	136.18 ± 2.86	0.518
Diastolic BP (mm Hg)	77.89 ± 2.43	81.62 ± 2.54	77.35 ± 2.39	85.50 ± 1.56	0.041

unweighted UniFrac distance [22]. Beta-diversity distance matrices were built after sub-sampling all the samples to an even depth of 16,998 sequences per sample, which is the same as the depth used with script alpha_rarefaction.py. Relative taxonomic abundance was measured as the proportion of readings over the total in each sample assigned to a given taxonomy.

2.5. Statistical analysis

All the data shown in this study have been expressed as mean ± SEM. Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and the calculation of Variables Importance Project (VIP) score from the PLS-DA model was performed with Metaboanalyst 3.0 [23]. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was performed against the GreenGenes database with default settings (type of functional predictions: KEGG Orthologs) to predict the functional profiling of the microbial communities based on the 16S rRNA gene sequences (http://picrust.github.io/picrust/) [24]. PASW statistical software, version 20.0 (IBM Inc., Chicago, IL, USA) and R software, version 3.0.2 (R Foundation for Statistical Computing, http://www.R-project.org/) were used for univariate statistical analyses of data. We used the Kolmogorov-Smirnov test to evaluate the normality of the variables and the One-way ANOVA to test the differences in the relative abundance of bacterial taxa between the groups. Post hoc statistical analysis was completed by using Bonferroni's comparison test. Multiple comparisons in the large-scale analyses were assessed by False Discovery Rate using the Benjamini and Hochberg method. *p-values* and *q-values* < 0.05 were considered statistically significant in all the statistical analyses.

3. Results

3.1. Baseline characteristics of the study participants

No statistically significant differences were found in BMI, waist circumference, LDL-c, triacylglycerides (TG), insulin, glycated hemoglobin (HbA1c), and systolic blood pressure between groups, although the women had higher HDL-c and lower glucose levels ($P < 0.001$ and $P = 0.002$, respectively). The pre-menopausal women group and their corresponding age-matched men (Male-pre group) were younger than the post-menopausal women and their age-matched male counterparts ($P < 0.001$) (Table 1). In addition, no differences were found in the dietary habits between groups (Supplemental Table S1).

3.2. Diversity and relative abundance of the gut microbiota

The PLS-DA analysis showed that the biggest differences in gut microbiota were in pre- versus post-menopausal women (Q2 = 0.866; R2 = 0.541) and pre-menopausal women versus the 'male-pre' group (Q2 = 0.844; R2 = 0.654) (Fig. 1; Supplemental Table S2). However, PCA did not yield relevant results. Moreover, no significant differences were found in bacterial richness or diversity between any of the study groups (Supplementary Fig. S1).

We also performed a Variables Importance Projection analysis (VIP), which identified *Roseburia* as the bacterial taxon with highest relevance to discriminate between pre- and post-menopausal women groups, and *Prevotella* as the bacterial taxon with highest relevance to discriminate between pre-menopausal women and the 'male-pre' group (Fig. 2).

3.3. Gut microbiota differences between pre- and post-menopausal women

In order to assess the differences in gut microbiota composition according to the hormonal status in women, we compared the gut microbiota composition of pre- and post-menopausal women at the phylum and genus level.

We found a higher *Firmicutes* proportion in post-menopausal women than in pre-menopausal women. In fact, we also found a higher *Firmicutes/Bacteroidetes* ratio in post-menopausal women ($P = 0.017$) than their corresponding male (age-matched) control group. We also observed lower presence of the *Actinobacteria* phylum in post-menopausal women ($P = 0.001$) than in pre-menopausal women (Fig. 3).

Moreover, we found that the relative abundance of the *Lachnospira* ($P = 0.047$) and *Roseburia* ($P = 0.003$) genera was greater in post-menopausal women than in pre-menopausal women. By contrast, the relative abundance of *Parabacteroides* ($P = 0.002$), *Prevotella* ($P < 0.001$) and *Bilophila* was lower in post-menopausal women than in pre-menopausal women at the genera level, while *Ruminococcus* (*Lachnospiraceae*) ($P = 0.067$) showed a statistical trend. In addition, the relative abundance of *Ruminococcus* (*Lachnospiraceae*) ($P = 0.003$), *Bilophila* ($P < 0.001$), and *Prevotella* ($P < 0001$) was higher in pre-menopausal women than in their corresponding male (age-matched) control group, while *Oscillospira* ($P = 0.051$) showed a statistical trend (Fig. 4; Supplemental Table S3).

We also analyzed the relationship between estradiol levels and relative abundance of the bacterial taxa. Here, we found two bacterial taxa, the *Gammaproteobacteria* class and an unknown family from *Mixococcales*, that positively correlate ($R = 0.575$, $P = 0.013$ and $R = 0.521$, $P = 0.039$, respectively), and the bacterial family *Prevotellaceae* that negatively correlates ($R = –0.523$ $P = 0.018$) with

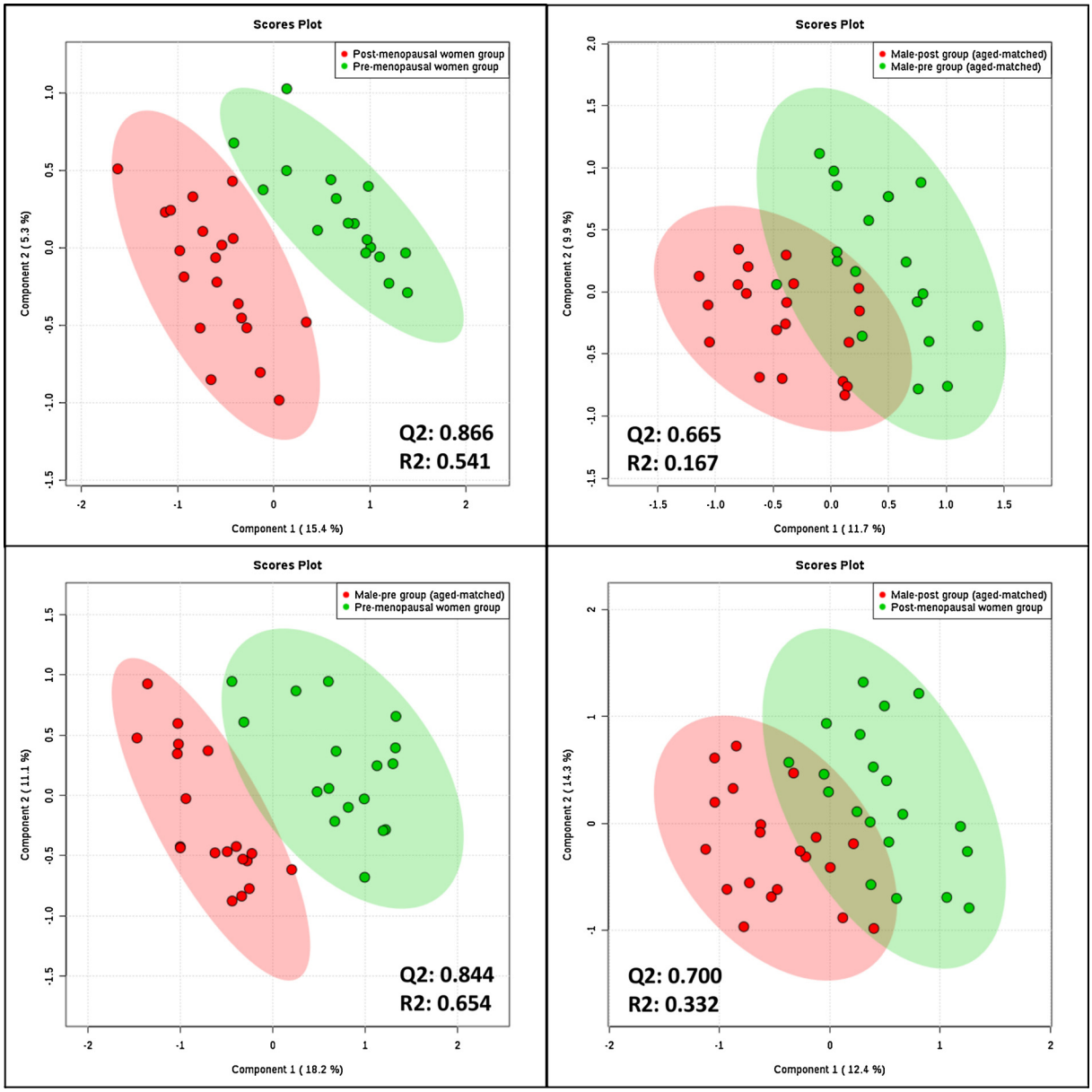

Fig. 1. Partial least squares discriminant analysis.

estradiol levels.

3.4. Gender differences in the gut microbiota composition

When we also evaluated the specific differences in gut microbiota composition by gender independently of the menopause (without differences between post- and pre-menopausal women), we found that the relative abundance of *Actinobacteria* phylum was greater (P = 0.001 Q = 0.004) and the *Sutterella* genus (P = 0.001 Q = 0.005) was lower in women (post- and pre-menopausal women groups together) than in men (both male control groups together). In addition, we observed that the relative abundance of the *Paraprevotellaceae* family (P = 0.007 Q = 0.052) tended to be lower in women (post- and pre-menopausal women groups together) than in men (both male control groups together), although it did not reach statistical significance.

3.5. Ageing-dependent menopause-independent differences in gut microbiota composition

We also assessed the differences in gut microbiota by age and we found a higher relative abundance of the *Coprococcus* genus in pre-menopausal women and their age-matched male control groups together (46.36 ± 0.50 years as the mean age), than in the post-menopausal women and their corresponding age-matched male control groups together (56.03 ± 0.49 years as the mean age) (P = 0.002 Q = 0.011).

3.6. Functional profiles of microbial communities between gender and between pre- and post-menopausal women

To study the potential function of gut microbiota, a PICRUST analysis was performed to predicate and identify differentially enriched pathways between groups. In fact, 19 from 328 pathways were differentially represented (Fig. 5). Moreover, taking into account the importance of SCFA in metabolic diseases [25], we also focused the

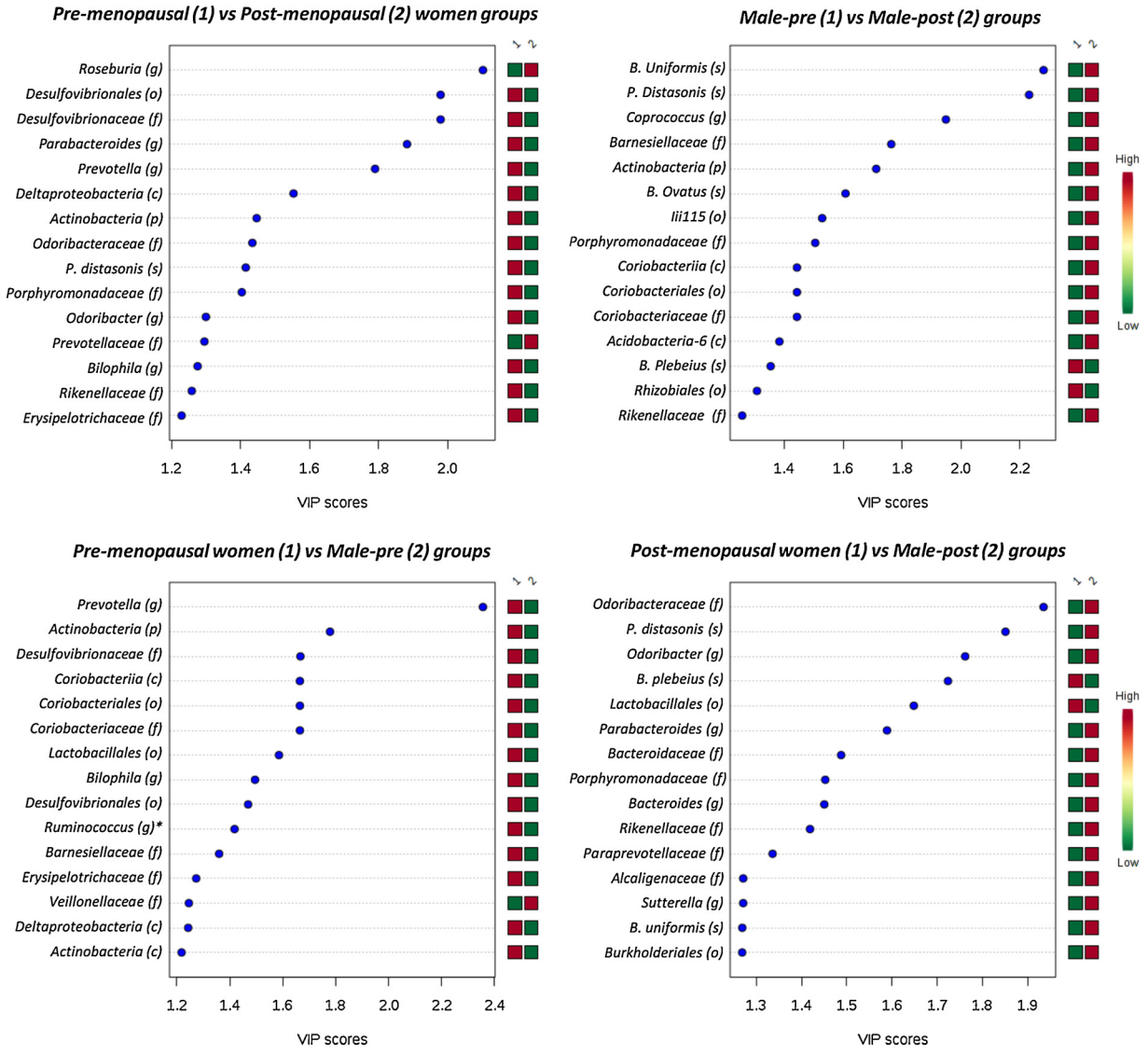

Fig. 2. Variables Importance Projection analysis. Bacterial taxa: (p) phylum; (c) class; (o) order, (f) family; (g) genus; (s) bacterial specie. * bacterial species of the *Ruminococcus* genus catalogued in *Lachnospiraceae* family.

functional analysis on the higher propanoate and butanoate metabolism. We observed a statistical trend for higher propanoate and butanoate metabolism in pre-menopausal women compared with the other groups (Supplemental Fig. S2).

3.7. Differences in endotoxemia and intestinal incretin plasma levels between pre- and post-menopausal women

The gut microbiota composition may affect the integrity of the intestinal barrier, which in turn influences the intestinal absorption of bacterial components such as the endotoxin lipopolysaccharides (LPS). These compounds are known to activate the toll-like receptors, inducing inflammation, which may promote insulin resistance (IR) [26,27]. In addition, differences in gut microbiota composition, mainly in terms of saccharolitic activity, may also modulate the release of incretins by L-cells in the intestine through short-chain fatty acid (SCFA) signaling [28,29], and this, in turn, increases the action and release of insulin. Whereas we did not find any differences in LPS and LBP plasma levels between groups, we observed higher GLP-1 plasma levels in pre-menopausal women than in post-menopausal women (P = 0.030). In addition, we observed higher GiP levels in women than in men (P = 0.018) (Fig. 6).

3.8. Differences in pro-inflammatory cytokine plasma levels between pre- and post-menopausal women

Taking into account the fact that metabolic diseases such as metabolic syndrome (MetS) and T2DM are characterized by a pro-inflammatory state [30,31], we tested whether the hormonal status in women may affect the inflammatory status. Moreover, we observed lower IL-6 and MCP-1 plasma levels in pre-menopausal women than in postmenopausal women (P = 0.036 and P = 0.045, respectively), who had similar levels to those found in men (Fig. 7).

3.9. Differences in energy homeostasis regulatory hormone plasma levels between pre- and post-menopausal women

The gut microbiota is involved in maintaining the energy balance [4]. Here, we measured adiponectin, a hormone with insulin sensitivity and anti-inflammatory functions [32], leptin, a mediator of long-term regulation of the energy balance which suppresses food intake [33], and resistin, which is considered an important link between obesity, insulin resistance and T2DM [34]. Thus, differences in the gut microbiota composition may somehow affect the plasma levels of adiponectin, resistin, and leptin.

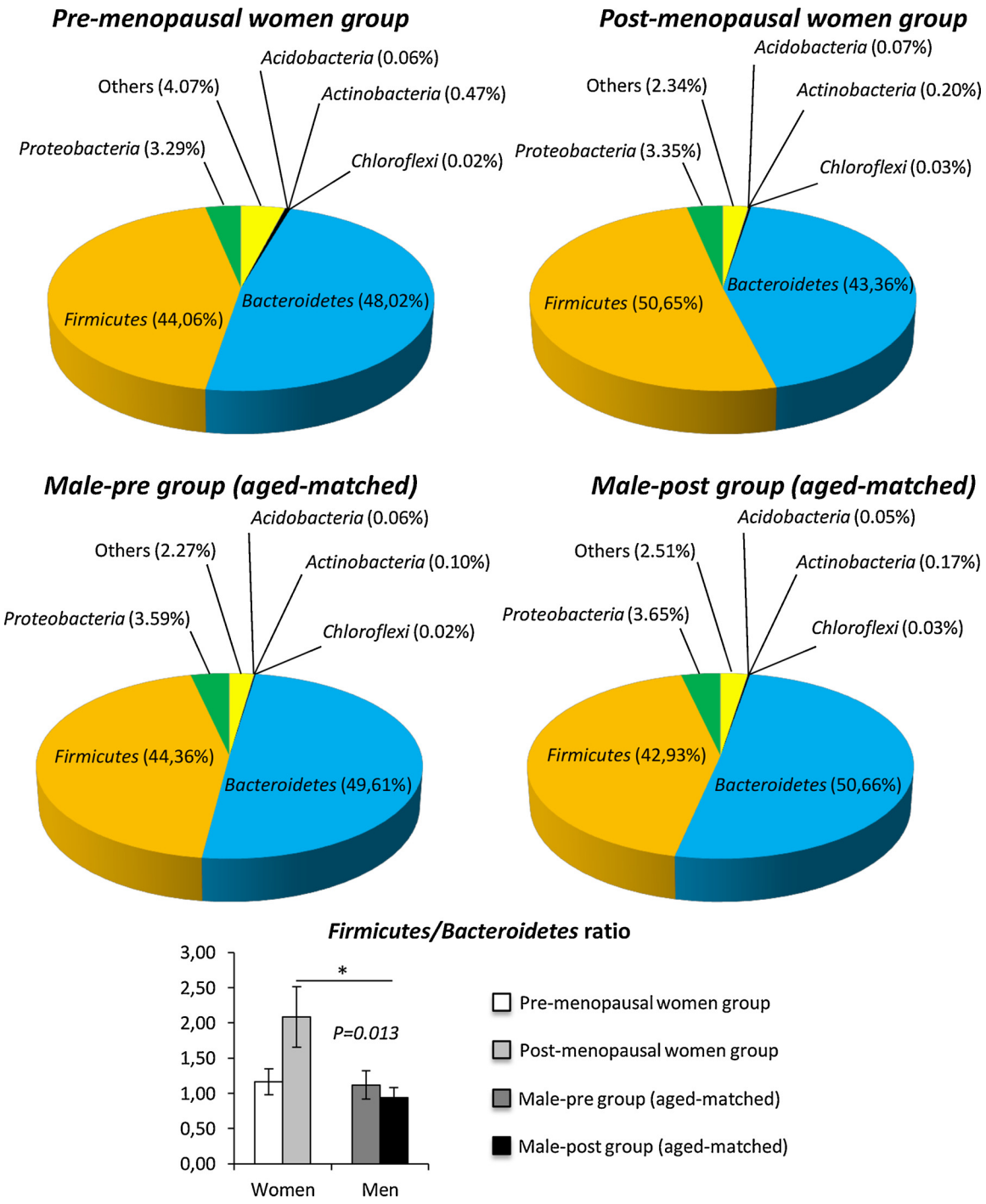

Fig. 3. Gut microbiota composition between groups at phylum level. Values represent relative abundance of the phylum in each group. Male-pre group: group of men age-matched with the pre-menopausal women. Male-post group: group of men age-matched with the post-menopausal women. All four groups are matched by BMI. **Bar Graphic**: values represent mean ± SEM of the *Firmicutes/Bacteroidetes* ratio. One-way ANOVA statistical analysis P-value. *P < 0.05 in the post hoc Bonferroni's multiple comparison tests.

We observed higher leptin levels in women than in men (P < 0.001), whereas no differences were found between women with different hormonal status (pre- and post-menopause). Moreover, we observed higher adiponectin levels in pre-menopausal women than in their corresponding age-matched male control group (P = 0.016), whereas no differences were found between post-menopausal women and their corresponding age-matched male control group (Fig. 7).

4. Discussion

Studies in animal models have identified a direct interaction between gut microbiota, sex hormones and the development of disease [11], suggesting that the potential differences in the composition of the gut microbiota between genders may be related with the dimorphism observed in the incidence of metabolic diseases, and the increase in their incidence after menopause in women [14]. In line with this, our study showed that the gut microbiota of pre-menopausal women is different from that of post-menopausal women and the 'male-pre' group

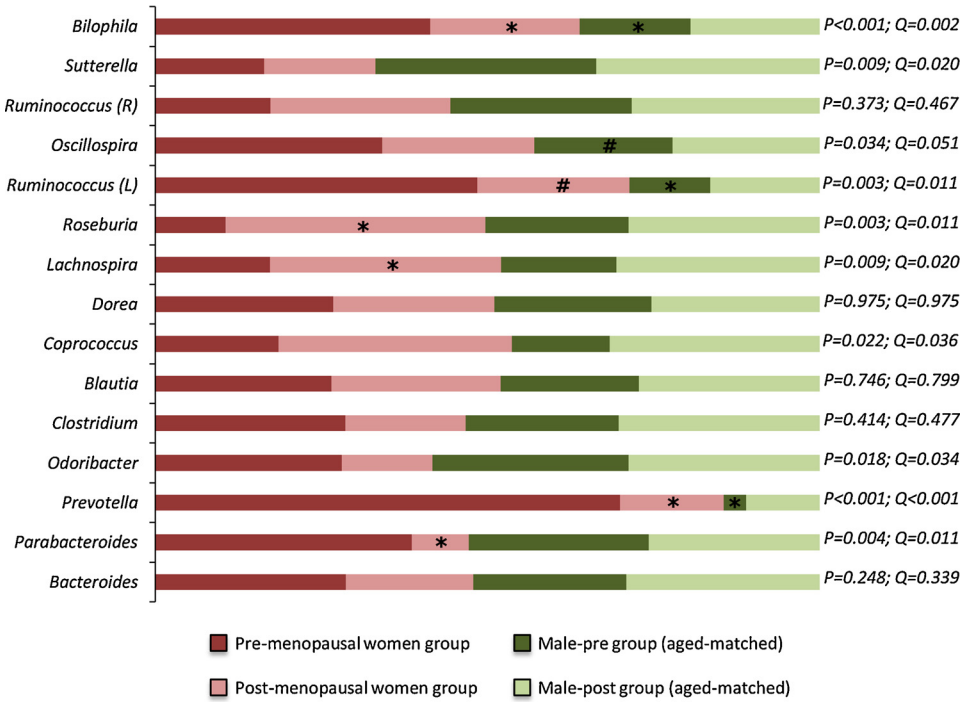

Fig. 4. Gut microbiota composition between groups at genera level. Values represent mean of the genus, proportional in the four experimental groups (mean ± SEM of the relative abundance are shown in Supplemental Table S3). Statistical analysis was performed with the relative abundance of each genus in the groups. One-way ANOVA statistical analysis P-value. Q-value: False Discovery Rate (FDR) using the Benjamini and Hochberg method. *P < 0.05 in the post hoc Bonferroni's multiple comparison tests versus pre-menopausal women. #P < 0.1 in the post hoc Bonferroni's multiple comparison tests versus pre-menopausal women. Male-pre group: group of men age-matched with the pre-menopausal women. Male-post group: group of men age-matched with the post-menopausal women. *Ruminococcus* (L): bacterial species of the *Ruminococcus* genus catalogued in *Lachnospiraceae* family. *Ruminococcus* (R): bacterial species of the *Ruminococcus* genus catalogued in *Ruminococcaceae* family. All four groups are matched by BMI.

in terms of global taxa relative abundance on the basis of the OPLS-DA. Moreover, these differences in the bacterial composition of the microbiota were also associated to different predicted gut microbial functions, as assessed by pathways enrichment analysis (PICRUSt). This is of great importance, since both gut microbial composition and function may be potentially related with the dimorphism observed in

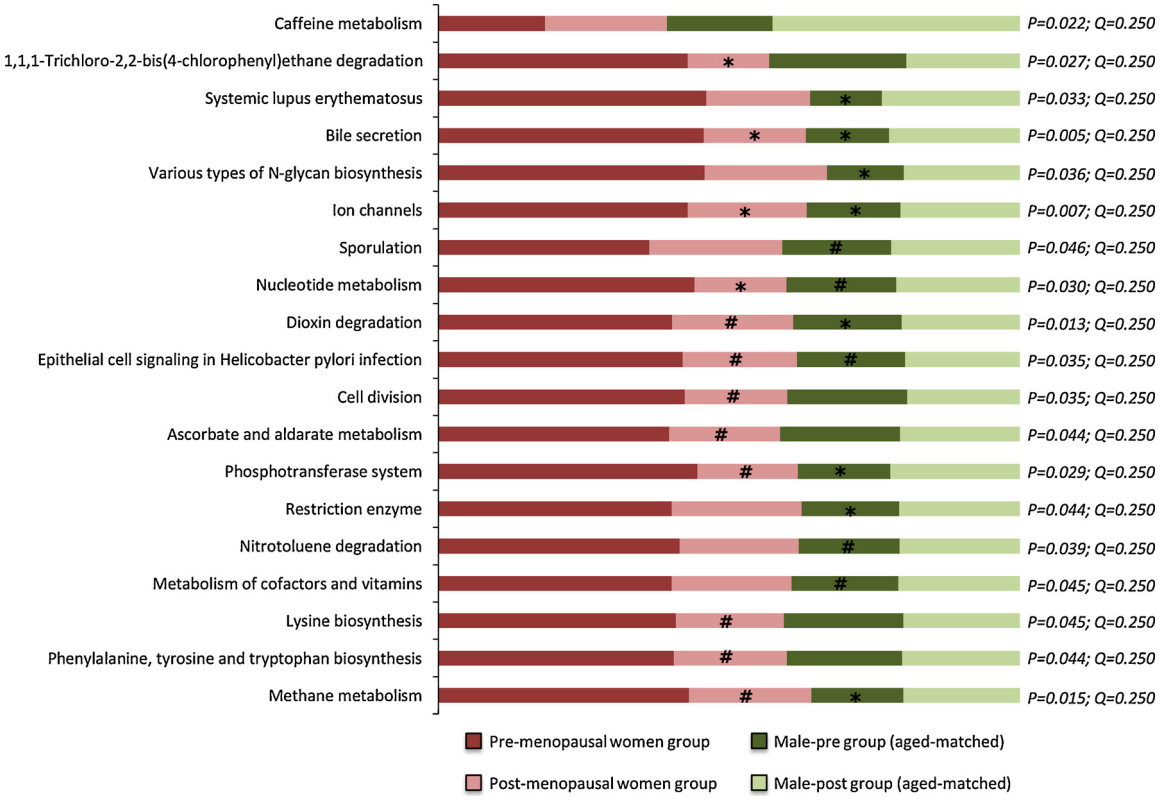

Fig. 5. Inferred gut microbiome functions by PICRUSt from 16S rRNA gene sequences between groups. Functional profiles of microbial communities were predicted using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (http://picrust.github.io/picrust/). Values represent the mean, proportional in the four experimental groups (mean ± SEM of the sequences are shown in Supplemental Table S4). One-way ANOVA statistical analysis P-value. Q-value: False Discovery Rate using the Benjamini and Hochberg method. *P < 0.05 in the post hoc Bonferroni's multiple comparison tests versus pre-menopausal women. #P < 0.1 in the post hoc Bonferroni's multiple comparison tests versus pre-menopausal women. Male-pre group: group of men age-matched with the pre-menopausal women. Male-post group: group of men age-matched with the post-menopausal women. All four groups are matched by BMI.

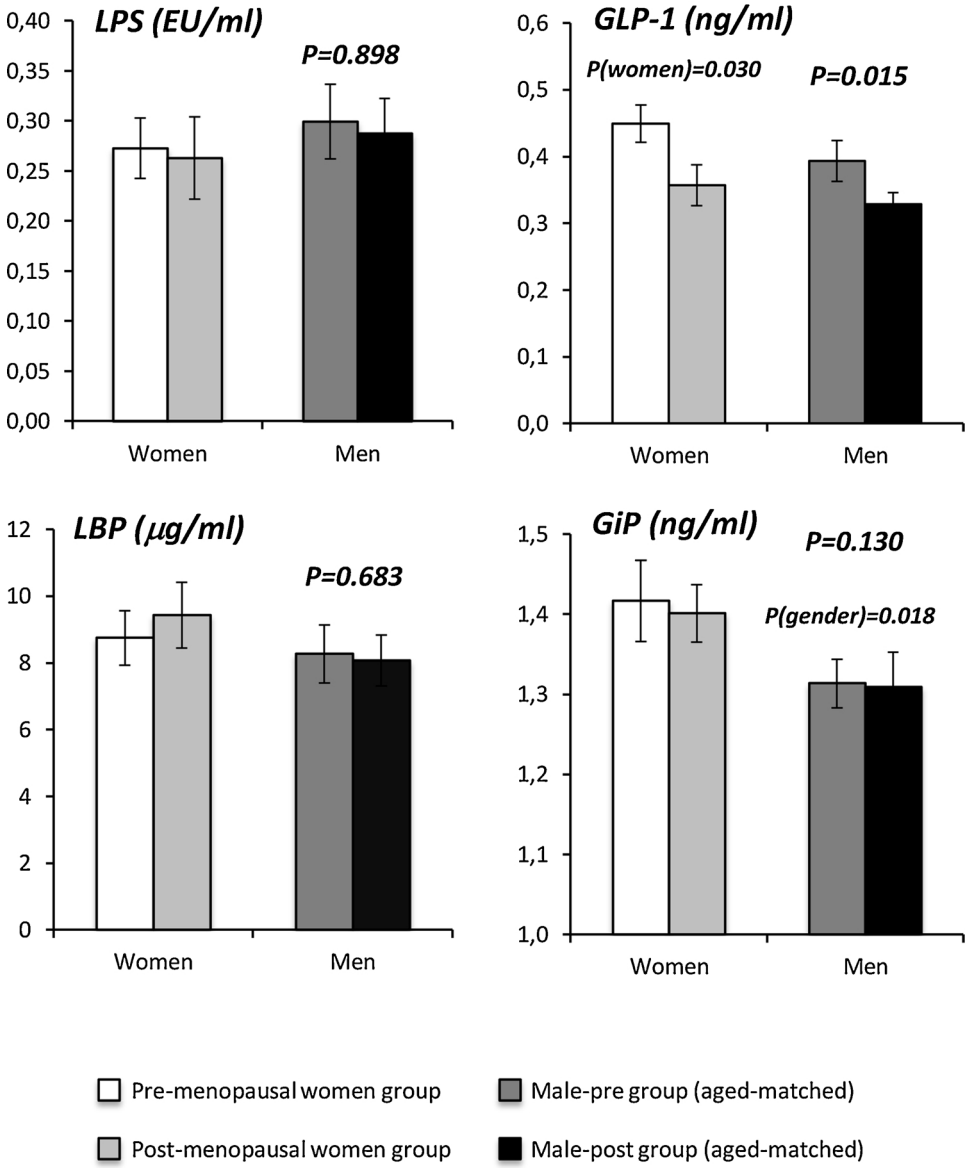

Fig. 6. Endotoxemia and intestinal incretins levels in the study groups. Values represent mean ± SEM of the relative expression of the indicated biomolecule. One-way ANOVA statistical analysis P-value. P-value (women): One-way ANOVA statistical analysis between pre- and postmenopausal groups. P-value (gender): One-way ANOVA statistical analysis between women (pre- and post-menopausal women together) and men (both male groups together) groups. Experimental groups: pre-menopausal women; male-pre: age-matched men as a control group for the pre-menopausal women; post-menopausal women; male-post: age-matched men as a control group for the post-menopausal women. All four groups are matched by BMI.

the incidence of metabolic diseases.

In fact, our study showed that the relative abundance of three short-chain fatty acid (SCFA)-producing genera *Prevotella*, *Ruminoccocus*, and *Roseburia* [35–37] was dependent on gender and hormonal status. Although there are other SCFA-producing bacteria, taking into account the differences in the relative abundance of these three bacterial genera together, our results suggest a decrease in the relative abundance of SCFA-producing bacteria in post-menopausal women and in both control groups of men compared with pre-menopausal women. Moreover, the relative abundance of *Parabacteroides*, a genus associated to sulphate assimilation but also a producer of SCFA [38], was lower in post- than in pre-menopausal women. Altogether, these observations might explain, at least partially, the dimorphism in the incidence of metabolic diseases such as T2DM (which is higher in men), while more women suffer from diabetes after menopause [2]. This may be due to a gut microbiota with a higher saccharolytic activity in pre-menopausal women, which may exert certain protection against metabolic diseases. This idea is supported by the PICRUSt analysis, which showed a potential higher propanoate and butanoate metabolism in pre-menopausal women compared with the other groups, suggesting higher SCFA production. In addition, the SCFA derived from the anaerobic breakdown of dietary fiber by the saccharolytic flora play an important role in maintaining intestinal health and exert anti-inflammatory properties [39]. This is particularly important, as inflammation has been shown to promote insulin resistance [26], which plays a major role in the pathogenesis of T2DM [40]. This idea is supported in pre-menopausal women by the trend for lower plasma levels of inflammatory markers observed in our study.

In addition, our study showed that as well as *Parabacteroides*, another sulphate metabolism-related genus *Bilophila*, a sulphite-reducing bacterial genus, was more abundant in women than in men. In this context, taking into account that these genera have been associated with inflammatory bowel disease (IBD) [41], it is tempting to hypothesize that the differences in gut microbiota composition reported here might contribute towards determining gender differences in the prevalence of IBD.

Gender differences in fat distribution and their relationship with the differences in sex hormone levels have been reported [9], although we still know very little about the cellular and molecular mechanisms underlying this phenomenon. However, experimentation in animal models has demonstrated that the gut microbiota may determine how excess energy is stored depending on gender, as the sexual dimorphism in total body fat content seen in rodents (males exhibiting higher fat content than females) is not evident in germ-free animals [10]. This

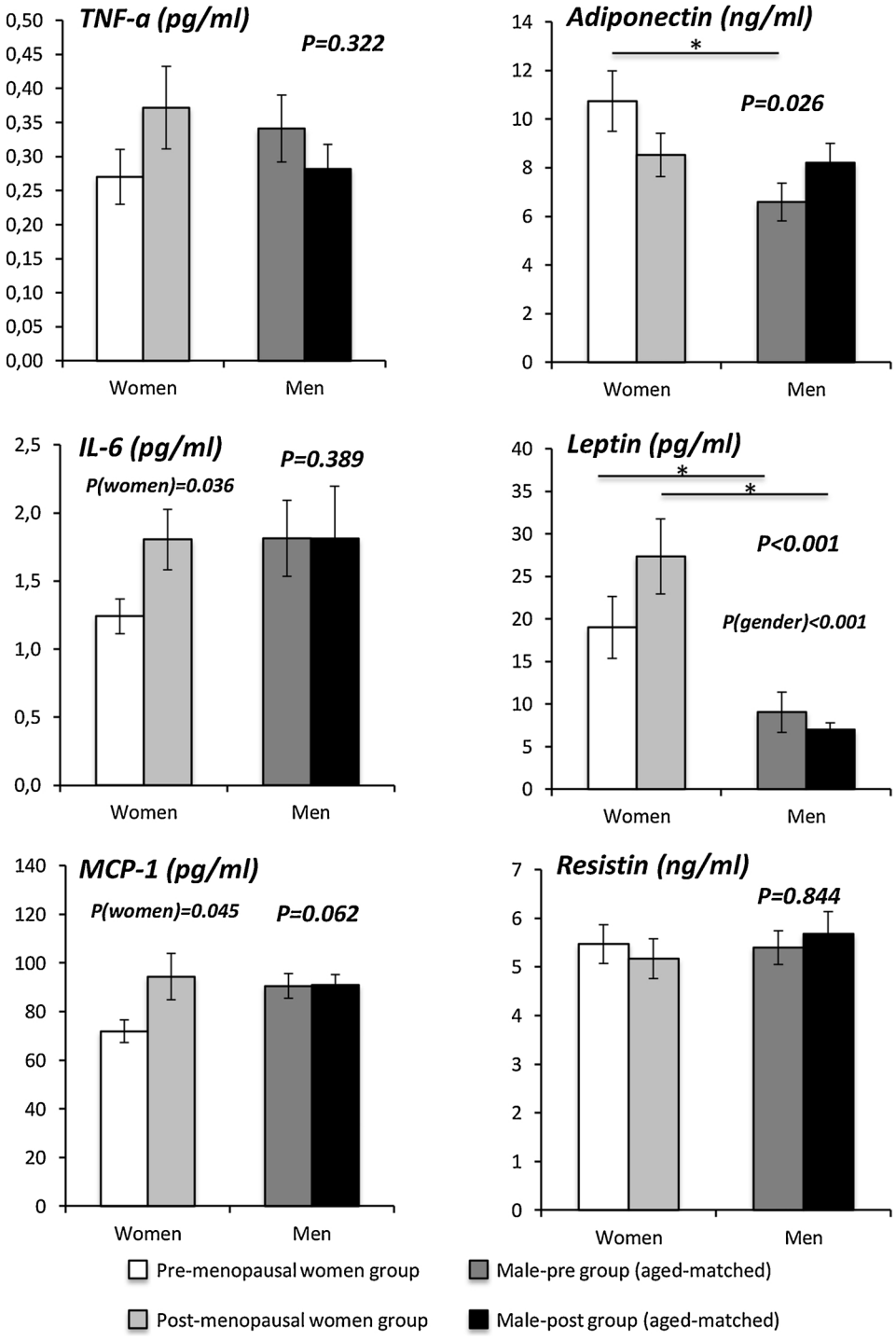

Fig. 7. Pro-inflammatory citokines and energy homeostasis regulatory hormones plasma levels. Values represent mean ± SEM of the relative expression of the indicated biomolecule. P-value: One-way ANOVA statistical analysis between the 4 groups. *P < 0.05 in the post hoc Bonferroni's multiple comparison tests. P-value (women): One-way ANOVA statistical analysis between pre- and post-menopausal groups. P-value (gender): One-way ANOVA statistical analysis between women (pre- and post-menopausal women together) and men (both male groups together). Experimental groups: pre-menopausal women; male-pre: age-matched men as a control group for the pre-menopausal women; post-menopausal women; male-post: age-matched men as a control group for the post-menopausal women. All four groups are matched by BMI.

latter point is especially important, as the part of the body where the fat is stored is an important factor in many obesity-related metabolic diseases. Moreover, it has been described that the decline in estradiol production during menopause in women accelerates the accumulation of abdominal fat, thus increasing the risk of developing obesity, T2DM and MetS [42].

It is therefore plausible that the gender-related differences found in our study regarding bacterial composition, such as an increase in the *Firmicutes/Bacteroidetes* ratio after menopause, may have an impact on how men and women differentially store excess energy. In fact, the plasma levels of the regulatory hormones involved in energy homeostasis seem to display sexual dimorphism. Thus, the two major adipokines, leptin and adiponectin, are characterized by higher circulating levels in women than men [43], a finding which is confirmed by the current work. However, we also observed that adiponectin plasma levels were higher only in pre-menopausal women, which may be related with the differences in fat distribution between men and women, as well as by the differences in the hormonal status between pre- and post-menopausal women. This may be related, in turn, to the modifications in the *Firmicutes/Bacteroidetes* ratio produced by the differences in hormonal status between pre- and post-menopausal women. Moreover, we also observed a sexual dimorphism in the plasma levels of intestinal incretins, which are hormones released from enteroendocrine cells in the gut which act to potentiate glucose clearance in response to the ingestion of food [44], which acts in parallel with the greater effect of incretin in pre-menopausal women (GLP-1) or women independently of

the hormonal status (GiP). These differences may reflect the differences in the gut microbiota between men and women, at least in the case of GLP-1, as it has been described that SCFA derived from the anaerobic breakdown of dietary fiber by the saccharolytic flora, which is higher in pre-menopausal women than in men and post-menopausal women, induces the release of GLP-1 by L-cells in the intestine [28].

Importantly, to the best of our knowledge, our study represents the first approach to exploring the differences in the gut microbiota associated to hormonal status between pre- and post-menopausal women (estrogen depletion). However, it is important to mention one limitation of the current study concerning the sample size, which was large enough to discern differences between groups after adjustment by the False Discovery Rate, but was not able to detect small differences.

5. Conclusions

Our results suggest that the differences in the composition of the gut microbiota between genders are influenced by the hormonal status in women, which might play a role in the dimorphism observed in the incidence of metabolic diseases and their co-morbidities. Our findings may be a relevant factor in developing strategies based on transferring gut microbiota from pre-menopausal women to post-menopausal women or to men, as a protection therapy against metabolic diseases, such as metabolic syndrome or type 2 diabetes mellitus.

Contributors

Jose A. Santos-Marcos collected data and performed the experiments, and helped in the data analysis and results interpretation, contributed to the writing of the manuscript and revised it critically for important intellectual content.

Oriol A. Rangel-Zuñiga drafted the manuscript, collected data and performed the experiments, and helped in the data analysis and results interpretation.

Rosa Jimenez-Lucena collected data and performed the experiments, and helped in the data analysis and results interpretation, contributed to the writing of the manuscript and revised it critically for important intellectual content.

Gracia Quintana-Navarro collected data and performed the experiments, and helped in the data analysis and results interpretation.

Sonia Garcia-Carpintero collected data and performed the experiments, and helped in the data analysis and results interpretation.

Maria M. Malagon contributed to the writing of the manuscript and revised it critically for important intellectual content.

Blanca B. Landa contributed to the writing of the manuscript and revised it critically for important intellectual content.

Manuel Tena-Sempere contributed to the writing of the manuscript and revised it critically for important intellectual content.

Pablo Perez-Martinez contributed to the writing of the manuscript and revised it critically for important intellectual content.

Jose Lopez-Miranda conceived and designed the experiments, and contributed to the writing of the manuscript and revised it critically for important intellectual content.

Francisco Perez-Jimenez conceived and designed the experiments, and contributed to the writing of the manuscript and revised it critically for important intellectual content.

Antonio Camargo conceived and designed the experiments, and contributed to the writing of the manuscript and revised it critically for important intellectual content.

Francisco Perez-Jimenez and Antonio Camargo have responsibility for the contents of the article.

Conflict of interest

None of the authors has any conflict of interest that could affect the performance of the work or the interpretation of the data.

Funding

The ONCOVER study is supported by the University of Córdoba, the Maimonides Biomedical Research Institute of Córdoba, the Ministerio de Economía y Competitividad and the Fondo Europeo de Desarrollo Regional (FEDER). The CORDIOPREV study is supported by the Fundación Patrimonio Comunal Olivarero, Junta de Andalucía (Consejería de Salud, Consejería de Agricultura y Pesca, Consejería de Innovación, Ciencia y Empresa), Diputaciones de Jaén y Córdoba, Centro de Excelencia en Investigación sobre Aceite de Oliva y Salud and Ministerio de Medio Ambiente, Medio Rural y Marino, Gobierno de España; Instituto de Salud Carlos III (CP14/00114 to A C). Ministerio de Economía y Competitividad (AGL2012/39615, PIE14/00005, and PIE 14/00031 to J L-M; AGL2015-67896-P to J L-M and A C; CP14/00114 to A C; PI13/00619 to F P-J and P P-M; PI16/01777 to F P-J and P P-M BFU2016-76711-R to MMM); Consejería de Innovación, Ciencia y Empresa, Proyectos de Investigación de Excelencia, Junta de Andalucía (CVI-7450 to J L-M); and by the Fondo Europeo de Desarrollo Regional (FEDER). Antonio Camargo is supported by an ISCIII research contract (Programa Miguel-Servet CP14/00114).

Ethical approval

All the patients gave their written informed consent to participate in the study. The protocol was approved by the Human Investigation Review Committee of Reina Sofia University Hospital (2012/000069), following the Helsinki declaration and good clinical practice.

Provenance and peer review

This article has undergone peer review.

Research data

The datasets generated for this study can be found in the NCBI's BioProject database (http://www.ncbi.nlm.nih.gov/bioproject/436982).

Acknowledgments

The CIBEROBN is an initiative of the Instituto de Salud Carlos III, Madrid, Spain. We would like to thank the Córdoba branch of the Biobank of the Sistema Sanitario Público de Andalucía (Andalusia, Spain) for providing the biological human samples. Our thanks also go to José Andrés Morales Martínez for technical assistance. We would also like to thank the EASP (Escuela Andaluza de Salud Pública), Granada, Spain.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.maturitas.2018.07.008.

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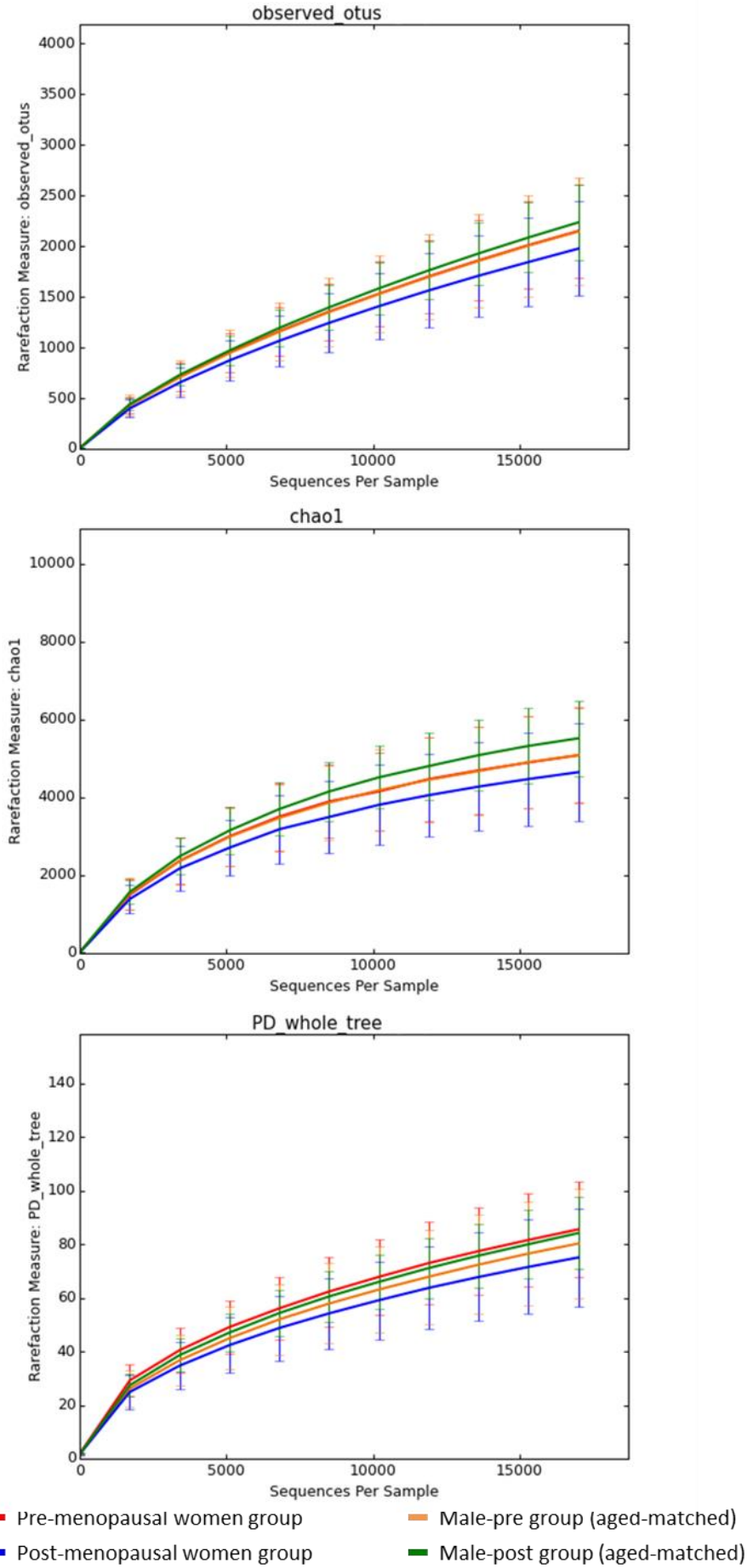
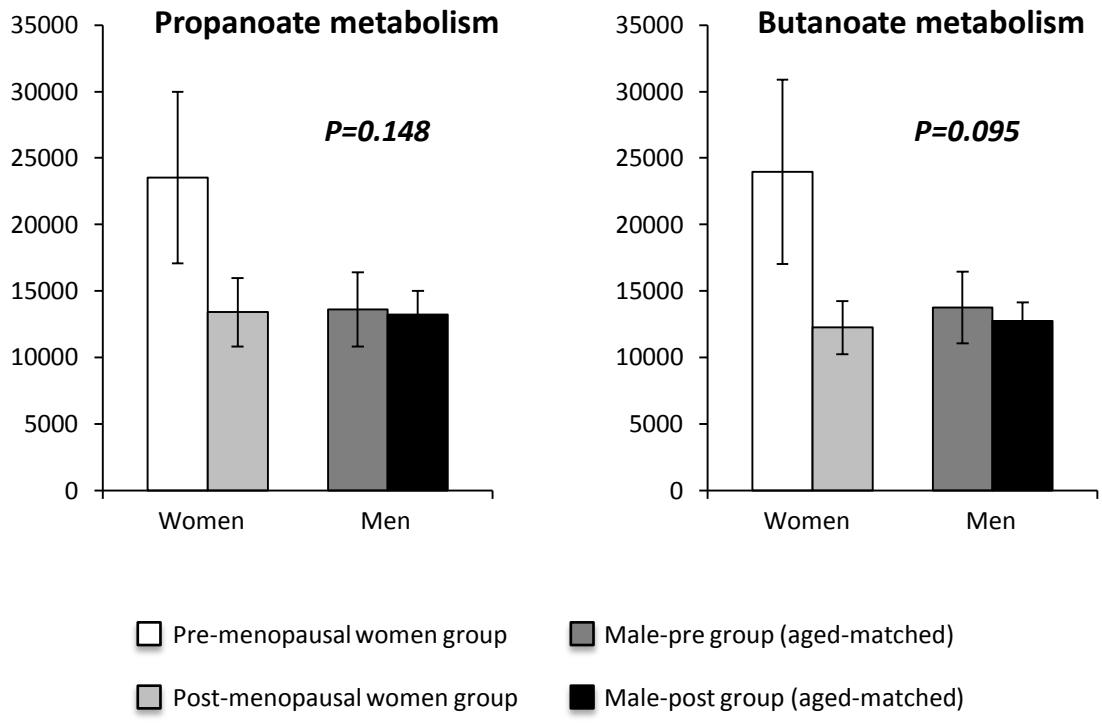


Supplemental Figure 1. Evaluation of microbial diversity using a variety of alpha diversity metrics.



Supplemental Figure 2. Functional analysis of the propanoate and butanoate metabolism in gut microbiome by PICRUSt from 16S rRNA gene sequences between groups.

	Mediterranean diet score
Pre-menopausal women group	8.67±0.46
Men control group pre	8.89±0.53
Post-menopausal women group	9.18±0.58
Men control group post	9.30±0.48
ANOVA P-value	0.817
ANOVA P-value (age)	0.366
ANOVA P-value (gender)	0.712

Supplemental Table 1. Dietary assessment of the participant in the study.

Values correspond to the mean±SEM of a 14-item questionnaire to assess adherence to the Mediterranean Diet. Men control group pre: control group for pre-menopausal women matched by age and BMI. Men control group post: control group for post-menopausal women matched by age and BMI. The statistical differences between groups were evaluated by One-way ANOVA. ANOVA p-value: analysis between the 4 groups. ANOVA p-value (age): analysis between PRE groups (women and men) combined versus POST groups (women and men) combined. ANOVA p-value (gender): analysis between women groups combined versus men groups combined.

Pre-menopausal women group versus post-menopausal women group					
Measure	*1 comps*	*2 comps*	*3 comps*	*4 comps*	*5 comps*
Accuracy	0.804	0.826	0.826	0.826	0.870
R2	0.663	0.866	**0.915**	0.955	0.961
Q2	0.504	0.541	**0.595**	0.560	0.558
Male-pre group versus male-post group					
Measure	*1 comps*	*2 comps*	*3 comps*	*4 comps*	*5 comps*
Accuracy	0.640	0.660	0.640	0.660	0.600
R2	0.486	**0.666**	0.776	0.860	0.897
Q2	0.133	**0.167**	0.040	-0.050	-0.143
Pre-menopausal women group versus male-pre group					
Measure	*1 comps*	*2 comps*	*3 comps*	*4 comps*	*5 comps*
Accuracy	0.896	0.917	0.917	0.958	0.958
R2	0.693	0.844	0.934	0.967	**0.976**
Q2	0.582	0.654	0.717	0.756	**0.767**
Post-menopausal women group versus male-post group					
Measure	*1 comps*	*2 comps*	*3 comps*	*4 comps*	*5 comps*
Accuracy	0.717	0.739	0.783	0.870	0.804
R2	0.557	0.700	0.844	**0.907**	0.938
Q2	0.300	0.332	0.376	**0.439**	0.430

Supplemental Table 2. Partial least squares discriminant analysis details.

	Pre-menopausal women (N=17)	*Men control group pre (N=19)*	*Post-menopausal women (N=20)*	*Men control group post (N=20)*	*P-value*	*Q-value*
Bacteroides	0.19263±0.02717	0.15495±0.03006	0.12834±0.02119	0.19517±0.02988	0.248	0.339
Parabacteroides	0.00759±0.00136	0.00533±0.00140	0.00168±0.00038	0.00505±0.00105	0.004	0.011
Prevotella	0.05395±0.01240	0.00263±0.00085	0.01199±0.00396	0.00853±0.00367	<0.001	<0.001
Odoribacter	0.00382±0.00056	0.00401±0.00082	0.00186±0.00026	0.00392±0.00046	0.018	0.034
Clostridium	0.00283±0.00079	0.00227±0.00035	0.00179±0.00029	0.00299±0.00071	0.414	0.477
Blautia	0.00260±0.00047	0.00204±0.00036	0.00250±0.00047	0.00267±0.00045	0.746	0.799
Coprococcus	0.00426±0.00069	0.00338±0.00082	0.00809±0.00176	0.00728±0.00121	0.022	0.036
Dorea	0.00133±0.00027	0.00117±0.00027	0.00121±0.00024	0.00126±0.00021	0.975	0.975
Lachnospira	0.00760±0.00107	0.00765±0.00131	0.01534±0.00253	0.01349±0.00226	0.009	0.020
Roseburia	0.00236±0.00058	0.00482±0.00100	0.00877±0.00145	0.00645±0.00106	0.003	0.011
Ruminococcus (L)	0.00280±0.00086	0.00070±0.00019	0.00132±0.00029	0.00096±0.00018	0.003	0.011
Oscillospira	0.01095±0.00153	0.00667±0.00076	0.00733±0.00111	0.00710±0.00093	0.034	0.051
Ruminococcus (R)	0.00452±0.00061	0.00711±0.00128	0.00706±0.00150	0.00737±0.00128	0.373	0.467
Sutterella	0.00854±0.00176	0.01729±0.00353	0.00869±0.00138	0.01751±0.00266	0.009	0.020
Bilophila	0.00329±0.00055	0.00133±0.00022	0.00179±0.00026	0.00154±0.00021	<0.001	0.002

Supplemental Table 3. Relative abundance of the genera in the study groups. Values represent mean±SEM of the relative expression of the indicated gene. One-way ANOVA statistical analysis P-value. Q-value: False Discovery Rate (FDR) using Benjamini and Hochberg method. Experimental groups: pre-menopausal women; male-pre: age-matched men as a control group for the pre-menopausal women; post menopausal women; male-post: age-matched men as a control group for the post-menopausal women. Ruminococcus (L): bacterial species of the Ruminococcus genus belonging to Lachnospiraceae family. Ruminococcus (R): bacterial species of the Ruminococcus genus belonging to Ruminococcaceae family. All the four groups are matched by BMI.

5.2. Publicación 2

Citación: Santos-Marcos JA, Haro C, Vega-Rojas A, Alcala-Diaz JF, Molina-Abril H, Leon-Acuña A, Lopez-Moreno J, Landa BB, Tena-Sempere M, Perez-Martinez P, Lopez-Miranda J, Perez-Jimenez F, Camargo A. Sex Differences in the Gut Microbiota as Potential Determinants of Gender Predisposition to Disease. Mol Nutr Food Res. 2019 Apr;63(7):e1800870. doi: 10.1002/mnfr.201800870. Epub 2019 Feb 13. PMID: 30636111.

Molecular Nutrition & Food Research
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Sex Differences in the Gut Microbiota as Potential Determinants of Gender Predisposition to Disease

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Scope: Dysbiosis of gut microbiota is involved in metabolic syndrome (MetS) development, which has a different incidence between men (M) and women (W). The differences in gut microbiota in MetS patients are explored according to gender, and whether consuming two healthy diets, Mediterranean (MED) and low-fat (LF), may, over time, differentially shape the gut microbiota dysbiosis according to gender is evaluated.

Materials and Methods: All the women from the CORDIOPREV study whose feces samples were available and a similar number of men, matched by the main metabolic variables ($N = 246$, 123 women and 123 men), and categorized according to the presence or not of MetS are included. Gut microbiota is analyzed at baseline and after 3 years of dietary intervention.

Results: Higher abundance of *Collinsella*, *Alistipes*, *Anaerotruncus*, and *Phascolarctobacterium* genera is observed in MetS-W than in MetS-M, whereas the abundance of *Faecalibacterium* and *Prevotella* genera is higher in MetS-M than in MetS-W. Moreover, higher levels of *Desulfovibrio*, *Roseburia*, and *Holdemania* are observed in men than in women after the consumption of the LF diet.

Conclusion: The results suggest the potential involvement of differences in gut microbiota in the unequal incidence of metabolic diseases between genders, and a sex-dependent effect on shaping the gut microbiota according to diet.

1. Introduction

The incidence of metabolic diseases and their comorbidities are sexually dimorphic.[1] For example, the prevalence of MetS, a cluster of characteristics associated with an increased risk of type 2 diabetes mellitus (T2DM) and cardiovascular diseases,[2] differs by age, ethnicity, gender, diet, and levels of physical activity.[3] This syndrome is conventionally diagnosed when a threshold of three of five criteria is reached; yet, ten different combinations of such criteria are in fact possible, each with a different pathophysiology. Moreover, the prevalence of each metabolic syndrome risk factor also differs by gender and country.[4,5]

Certain fundamental aspects of metabolic homeostasis are regulated differently in males and females.[6] For example, women have a propensity to store fat in subcutaneous adipose tissue compared to the preferential visceral fat deposition in men.[7] However, females seem to be more predisposed to obesity and metabolic syndrome than men,[8]

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DOI: 10.1002/mnfr.201800870

despite the fact that visceral fat has a stronger association with cardiovascular and metabolic disease.[9] It has also been described that glucose homeostasis is subject to sexual asymmetry. In fact, the prevalence of prediabetic syndromes differs by genders. Whereas impaired fasting glucose (IFG) is more prevalent in men, impaired glucose tolerance (IGT) is more prevalent in women.[6] In addition, there are more diabetic males before puberty and more diabetic females after menopause.[10]

Gut microbiota is now recognized as an organ integrated in the metabolism of the host.[11] It has been proposed that alteration of the gut microbiota structure may trigger the development of metabolic diseases such as obesity, MetS, and T2DM.[12–14] Taking into account that previous data indicate that the composition of the gut microbiota is different between genders,[15–18] we hypothesized that the microbiota gender difference might determine the differences in the predisposition to develop MetS between women and men.

Dietary strategies are of great interest in preventing and treating metabolic diseases[19] and it has been suggested that they act by modifying the microbiome.[20] Although it has been suggested that the composition of the gut microbiota in adulthood is very stable over time,[21] the consumption of different diets can shape the gut microbiota and could potentially be used to restore an imbalanced microbiome to a healthy, balanced microbiome.[20] However, the effectiveness of diets may be dependent on microbiota composition of the recipient, as has been shown in the case of fecal transplant response,[22] suggesting that a different gut microbiota composition in men and women before dietary intervention may be a determinant in the effect of diet in modifying the microbiome between genders.

Based on this previous evidence, we explored the gut microbiota composition in men and women with or without MetS and also evaluated if the consumption of Mediterranean (MED) and low-fat (LF) diets for 3 years may differentially shape the gut microbiota composition according to gender.

2. Experimental Section

2.1. Study Participants

The current work was conducted in the framework of the CORDIOPREV study (Clinical Trials.gov.Identifier: NCT00924937), an ongoing prospective, randomized, open, controlled trial in 1002 patients with coronary heart disease (CHD) who had their last coronary event over 6 months before enrolling. The patients, in addition to conventional treatment for CHD, followed MED and LF diets over a period of 7 years. The inclusion and exclusion criteria have been previously described,[23] and are summarized thus: patients were eligible if they were over 20 years of age but below 75, had established CHD without clinical events in the last 6 months, were thought to be capable of following a long-term dietary intervention, and did not have severe diseases or an estimated life expectancy of below 7 years. For the current study, the use of antibiotics was added as one of the exclusion criteria. All the patients gave their informed consent in writing to participate in the study. The trial protocol and all the amendments were approved by the local ethics committees, following the Helsinki Declaration and good clinical practice.

The criteria of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) were followed to assess the presence of MetS.[24] All the women from the CORDIOPREV study whose feces samples were available, and a similar number of men (N = 246, 123 women and 123 men), were included in this study and categorized according to the presence or not of MetS as follows: MetS-W, 79 women with MetS; MetS-M, 79 men with MetS; NonMetS-W, 44 women without MetS; NonMetS-M, 44 men without MetS. Each woman was individually matched to one man according to the presence of MetS, T2DM (presence, treatment, and if treatment was with metformine), fasting glucose criteria for MetS, HbA1c levels (according to American Diabetes Association criteria for normoglucemia, prediabetes, and T2DM), triacylglycerides (TAG), HDL-c, hypertension criteria for MetS and age (ranges: <40, 40–49, 50–59, 60–69, and >70). This matching was performed in order to avoid differences between genders in variables that may affect gut microbiota composition different to the MetS. In fact, the women and men shared the same combinations of MetS criteria (Table S1, Supporting Information). In addition, we checked the nutritional background between genders and groups of patients according the presence of MetS (Table S2, Supporting Information), which may also affect gut microbiota composition. Moreover, there were no differences on physical activity between groups.

The baseline metabolic characteristics of the patients in the study are shown in **Table 1**. The effect of the consumption of MED or LF diets for 3 years in shaping the gut microbiota of the MetS patients was performed in a smaller population (99 of the 158 MetS patients), due to the availability of fecal samples at 3 years of follow-up. The baseline metabolic characteristics and the presence of MetS criteria of the patients included in the diet effect analysis are shown in Table S5*, Supporting Information.

(*): S4

2.2. Diet Assessment

The adherence to the MED diet was assessed by a validated 14-item questionnaire[25] and the adherence to the LF diet by a 9-point score. This was performed once before the start of the dietary intervention and then yearly. The Spanish food composition tables and a validated food frequency questionnaire[26] were used to calculate the intake of fiber.

2.3. Clinical Plasma Parameters

Tubes containing 0.1% EDTA were used to collect the blood, which were then centrifuged at 1500 × *g* for 15 min at 4 °C to separate the plasma and red blood cells. The analytes were determined, blinded to the team members, from frozen samples at the Lipid and Atherosclerosis Unit at Reina Sofia University Hospital by members of the laboratory research team, as previously described.[17]

2.4. DNA Extraction from Fecal Samples

The patients were given a box with carbonic ice and a sterile plastic bottle with a screw cap to collect the fecal samples. The

Table 1. Baseline characteristic of the participants in the study. Values correspond to the mean ± SEM.

	MetS-W ($N = 79$)	MetS-M ($N = 79$)	NonMetS-W ($N = 44$)	NonMetS-M ($N = 44$)	p-Value
Age (years)	62.95 ± 1.00	61.63 ± 0.99	61.48 ± 1.27	60.61 ± 1.34	0.526
BMI (kg m^{-2})	32.18 ± 0.46^{a}	31.83 ± 0.51$^{a, c}$	29.41 ± 0.88^{b}	29.88 ± 0.61$^{b, c}$	0.002
Waist circumference (cm)	102.37 ± 1.19^{a}	110.83 ± 1.45^{b}	93.29 ± 2.13^{c}	103.61 ± 1.58^{a}	<0.001
HDL-c (mg dL^{-1})	42.56 ± 1.21^{a}	37.35 ± 1.01^{b}	57.30 ± 1.63^{c}	45.91 ± 1.56^{a}	<0.001
LDL-c (mg dL^{-1})	88.48 ± 3.34^{a,b}	82.71 ± 2.58^{a}	97.73 ± 4.03^{b}	94.30 ± 4.33^{a,b}	0.014
Total cholesterol (mg dL^{-1})	164.46 ± 4.53^{a,b}	156.84 ± 2.94^{a}	175.77 ± 4.54^{b}	160.55 ± 5.25^{a,b}	0.026
TAG (mg dL^{-1})	159.36 ± 9.39^{a}	168.40 ± 8.58^{a}	96.27 ± 5.31^{b}	99.93 ± 5.38^{b}	<0.001
Glucose (mg dL^{-1})	130.13 ± 6.50^{a}	126.49 ± 4.68^{a}	90.07 ± 1.90^{b}	93.27 ± 2.43^{b}	<0.001
Insulin (mU L^{-1})	12.58 ± 1.62^{a,b}	14.48 ± 1.66^{a}	7.01 ± 0.62^{b}	8.58 ± 1.02^{a,b}	0.003
HbA1c (%)	7.11 ± 0.17^{a}	6.99 ± 0.15^{a}	6.09 ± 0.08^{b}	6.27 ± 0.11^{b}	<0.001
Sistolic BP (mm Hg)	145.06 ± 2.22^{a}	146.60 ± 2.23^{a}	133.70 ± 3.19^{b}	127.84 ± 2.58^{b}	<0.001
Diastolic BP (mm Hg)	75.28 ± 1.22^{a}	80.10 ± 1.31^{b}	72.86 ± 1.36^{a}	70.07 ± 1.75^{a}	<0.001

HDL-c, HDL-cholesterol; LDL-c, LDL-cholesterol; TAG, triacylglycerides; and BP, blood pressure. The statistical differences between groups were evaluated by one-way ANOVA. In each row, values with different letters in superscript differ statistically in the Bonferroni's post hoc test ($p < 0.05$).

samples were kept frozen after delivery to the laboratory staff and stored at –80 °C. DNA was extracted using the QIAamp DNA kit Stool Mini Kit Handbook (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA samples were stored at –20 °C, after quantification with the Nanodrop ND-1000 v3.5.2 spectrophotometer (Nanodrop Technology, Cambridge, UK).

2.5. Sequencing and Bioinformatics

For each DNA (fecal) sample, the hypervariable regions V3 and V4 of the 16S rRNA gene were amplified by polymerase chain reaction using the primer pair 5′-TCGTCGGCAGCGTCAGATG TGTATAAGAGACAG-3′ and 5′-GTCTCGTGGGCTCGGAGATG TGTATAAGAGACAG-3′,[27] which was further sequenced on a MiSeq Illumina platform (Illumina, San Diego, CA). Briefly, PCR was performed using a KAPA HiFi HotStart ReadyMix (KAPABIOSYSTEMS), 1.25 µL of extracted DNA (5 ng μL^{-1} in 10 mM Tris pH8.5) and 0.2 µM of each primer, using the following cycle parameters: 3 min denaturation at 95 °C followed by 25 cycles (30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C) and a final extension at 72 °C for 5 min. The 16S V3 and V4 amplicon purification was performed using Agentcourt AMPure XP beads (Beckman Coulter). A second PCR reaction attaches dual indices and Illumina sequencing adapters using the Nextera XT Index Kit. This PCR was performed with a KAPA HiFi HotStart ReadyMix (KAPABIOSYSTEMS), 5 µL of the previous amplicon, 5 µL of each Nextera XT Index Primer 1(N7xx), and 5 µL of each Nextera XT Index Primer 2(S5xx), using the following cycle parameters: 3 min denaturation at 95 °C followed by 8 cycles (30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C), and a final extension at 72 °C for 5 min. The PCR product purification was performed using Agentcourt AMPure XP beads (Beckman Coulter). Sequence outputs were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) program, version 1.9.1,[28] using QIIME default parameters. The 16S paired reads were assembled using the script multiple_join_paired_ends.py, which joins forward and reverse demultiplexed reads. The output file was processed for quality filtering by split_libraries_fastq.py. High quality sequences were grouped into Operational Taxonomic Units (OTUs) with a sequence identity threshold of 97%, and taxonomy was assigned by interrogating the high quality sequences with the Greengenes database (13_5).[29] Bacterial richness and diversity across the samples were calculated using the Chao1, Simpson, and Shannon indexes.[30] Principal component analysis of community structure (beta-diversity) was done using the unweighted and weighted UniFrac distance metrics[31] and analyzed by permutational multivariate analysis of variance (PERMANOVA) using the script compare_categories.py. Linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.harvard.edu/galaxy/) was used to compare groups at baseline and visualize the results using taxonomic bar charts and cladograms.[32]

2.6. Statistical Analysis

R software, version 3.0.2 (R Foundation for Statistical Computing, http://www.R-project.org/) was used for men and women matching with the MatchIt 3.0.2. PASW statistical software package, version 20.0 (IBM Inc., Chicago, IL, USA), which was used for univariate statistical analyses of the data. The statistical differences in the abundance of bacterial groups at baseline were tested by one-way analysis of variance (ANOVA). This test was adjusted by the false discovery rate (FDR) using the Benjamini and Hochberg method. Gut microbiota changes according to the dietary intervention were assessed by ANOVA for repeated measures, with time as intrasubject factor, and diet and gender as the intersubject factors. Post hoc statistical analysis was completed using Bonferroni's comparison test. To evaluate the specific differences between bacterial taxa, the abundance of taxa present in at least 75% of the human fecal DNA samples in each of the study groups was compared. Moreover, the frequency of occurrence of the bacterial taxa identified in at least 50% of the samples was analyzed in each of the groups in the study using the χ^2 test. p-Values < 0.05 and q-values < 0.1 were considered statistically significant in all the statistical analyses. All the data shown in this study have been expressed as mean ± SEM.

3. Results

3.1. Baseline Characteristics of the Study Participants

The differences in the main anthropometric and metabolic variables between groups are shown in Table 1. Overall, both MetS groups of women and men had higher waist circumference, glucose, TAG, and systolic blood pressure, and lower HDL-c plasma levels than NonMetS groups (women and men). The MetS criteria were similar between genders (Table S1, Supporting Information). In addition, the nutritional background was similar between genders, and between MetS and NonMetS groups (Table S2, Supporting Information).

3.2. Relationship between Anthropometric and Metabolic Variables with Gut Microbiota Composition

We studied the relationship between the main anthropometric and metabolic variables at baseline and the gut microbiota composition using Pearson's correlation test. We found a positive relationship between waist circumference and the *Acidaminococcus* genus ($R = 0.592$, $p < 0.001$) in men with MetS, and a negative relationship between c-HDL plasma concentration and the *Desulfovibrio* genus ($R = –0.568$, $p < 0.001$) in men without MetS. Moreover, we also observed positive relationships between HbA1C (%) and an unknown genus from the *S24-4* family ($R = 0.514$, $p = 0.001$), the plasma concentration of TAG and an unknown genus from *Ruminococcaceae* family ($R = 0.523$, $p < 0.001$), and insulin levels and the *Acidaminococcus* genus ($R = 0.539$, $p < 0.001$) in women without MetS.

3.3. Diversity of the Gut Microbiota according to Gender in MetS Patients

No significant differences in the richness and alpha-diversity parameters (Chao1, Simpson, and Shannon) were observed (Table S3, Supporting Information). Regarding beta-diversity, we detected significant differences between groups in the principal component analysis, based on unweighted (qualitative) and weighted (quantitative) UniFrac distance metrics (Figure S1, Supporting Information) analyzed by PERMANOVA ($p = 0.004$ and $p = 0.006$, respectively). Moreover, we found significant differences in unweighted and weighted UniFrac distance metrics between NonMetS women and men (Figure S2, Supporting Information; PERMANOVA; $p = 0.030$ and $p = 0.001$, respectively). However, we found significant differences in unweighted UniFrac distance metrics between women and men with MetS (Figure S3, Supporting Information), but no statistically significant differences were found in weighted UniFrac distance metrics (PERMANOVA; $p = 0.036$ and $p = 0.437$, respectively).

3.4. Differences in the Gut Microbiota between Genders in MetS: LEfSe Analysis

In order to evaluate whether the dysbiosis of the gut microbiota in MetS differs by gender, we assessed the global differences of the gut microbiota between women and men with MetS. Moreover, to discern whether these gender differences were specific in MetS, we compared these differences with those found between genders in the NonMetS groups.

We used LEfSe to compare the estimated phylotypes of the women and men in the MetS and NonMetS groups (**Figure 1**). The NonMetS men's gut microbiota was characterized by a preponderance of *Firmicutes* phylum and several genera from the *Clostridiales* order, such as *Clostridium*, *Coprococcus*, *Dorea*, *Lachnospira*, *Roseburia*, and *Veillonella*. By contrast, the MetS men's gut microbiota was characterized by a preponderance of genera such as *Clostridium*, *SMB53*, *Coprococcus*, *Roseburia*, and *Faecalibacterium*, but by a lower abundance of the *Ruminococcus*, *Anaerotruncus*, and *Phascolarctobacterium* genera.

On the other hand, the NonMetS women's gut microbiota was characterized by a preponderance of families and genera from the *Bacteroidales* order, such as *Bacteroides*, *Barneciellaceae*, *Butyricimonas*, *Parabacteroides*, and *Rikenellaceae*, whereas the MetS women's gut microbiota was characterized by a preponderance of the *Cyanobacteria* phylum and *Parabacteroides* genus, but by lower *Prevotella* genus.

3.5. Differences in the Abundance of the Microbial Taxa between Genders in MetS: Univariate Analysis

Further, we explored the MetS-specific differences in the abundance of bacterial taxa at genus level between women and men by one-way ANOVA, adjusting the FDR by the Benjamini and Hochberg method. Moreover, we also analyzed the differences between women and men in NonMetS control groups to discern whether the gender differences found were dependent or independent of MetS (**Figure 2**).

In fact, we found specific gender differences in MetS groups not present in the NonMetS control groups. We observed a higher abundance of the *Collinsella*, *Alistipes*, *Anaerotruncus*, and *Phascolarctobacterium* genera in MetS-W than in MetS-M ($p = 0.003$, $q = 0.021$; $p = 0.014$, $q = 0.088$; $p < 0.001$, $q = 0.001$; and $p = 0.016$, $q = 0.090$, respectively), whereas the abundance of *Faecalibacterium* and *Prevotella* genera was higher in MetS-M than in MetS-W ($p = 0.002$, $q = 0.015$ and $p < 0.001$, $q = 0.005$, respectively).

Moreover, we observed gender differences in MetS groups also present in NonMetS control groups—in other words, gender differences that were independent of MetS. We found higher levels of *Ruminococcus* (*Lachnospiraceae* family) and *Bilophila* genera in both MetS-W ($p < 0.001$, $q < 0.001$ and $p = 0.002$, $q = 0.017$, respectively) and non-MetS-W ($p = 0.009$, $q = 0.032$ and $p < 0.001$, $q = 0.005$, respectively) than in their corresponding male groups. By contrast, we observed higher levels of *Clostridium* and *SMB53* genera in MetS-M than in MetS-W ($p < 0.001$, $q = 0.002$ and $p < 0.001$, $q = 0.001$, respectively), and in non-MetS-M than in NonMetS-W (both, $p = 0.002$, $q = 0.011$).

3.6. Bacterial Prevalence Differentially Associated to Genders in MetS

We also analyzed the MetS-associated differences in bacterial genera prevalence between the genders using the χ^2 test.

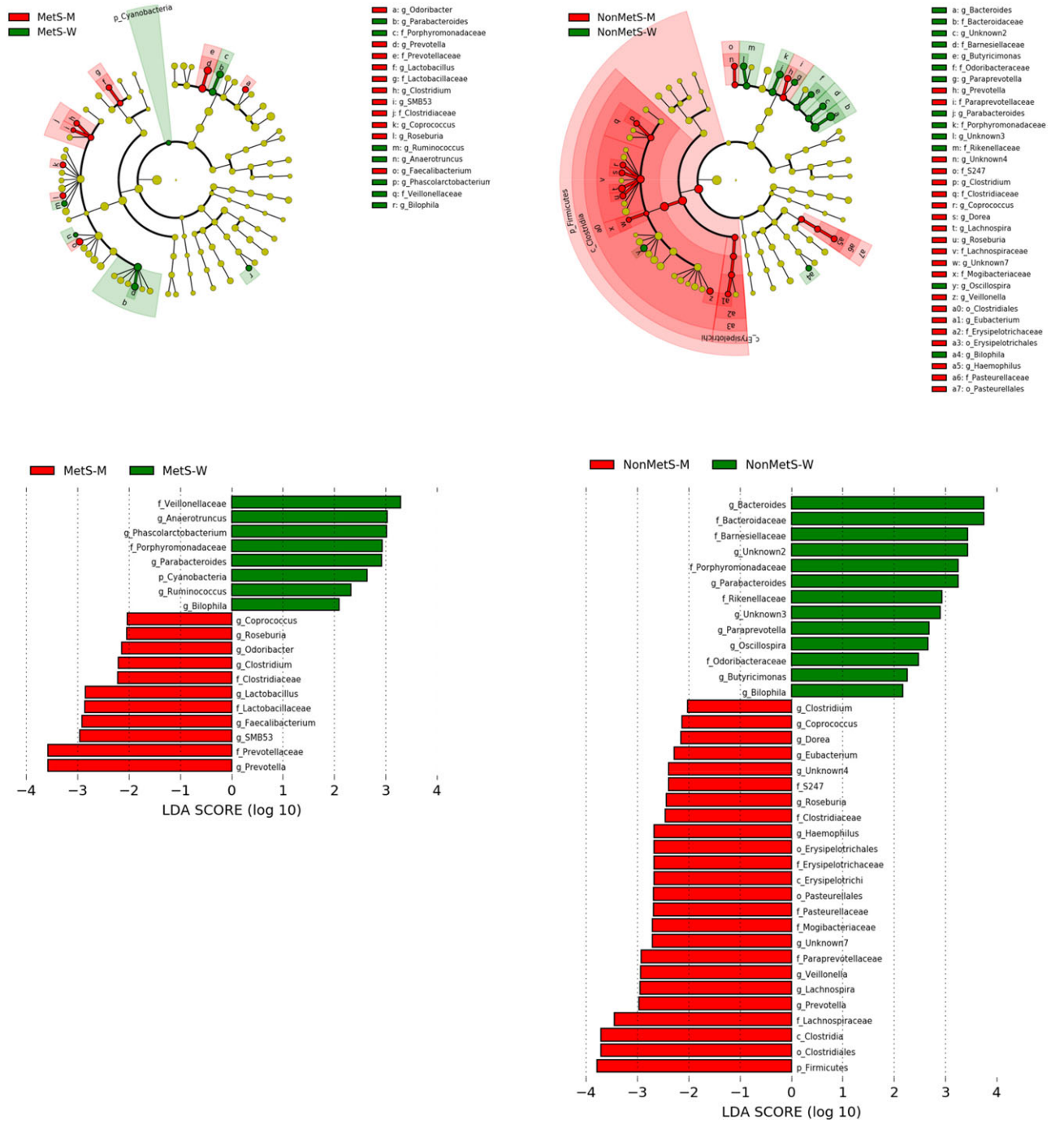

Figure 1. Linear discriminant analysis effect size. Cladogram representing the taxonomic hierarchical structure of the identified differences between genders using Linear discriminant analysis effect size. Each filled circle represents one phylotype. Red, bacterial taxa statistically overrepresented in men; green, bacterial taxa overrepresented in women. Phylum and class are indicated in their names on the cladogram and the order, family, or genus are given in the key.

Moreover, we analyzed the differences between women and men in the NonMetS groups to discern whether the gender differences found were dependent or independent of *MetS* (**Table 2**). A higher presence of *Eggerthella* (67.1% vs 39.2%; χ^2 test $p < 0.001$) and *Acidaminococcus* (91.1% vs 79.8%; χ^2 test $p = 0.042$) genera was observed in fecal samples from MetS-W compared to MetS-M. Likewise, a higher presence of *Slackia* (79.8% vs 59.5%; χ^2 test $p = 0.006$), *Odoribacter* (100% vs 93.7%; χ^2 test $p = 0.023$) genera, and an unknown genus from *RF39* order (59.5% vs 39.2%; χ^2 test $p = 0.011$) was observed in fecal samples from MetS-M compared to MetS-W.

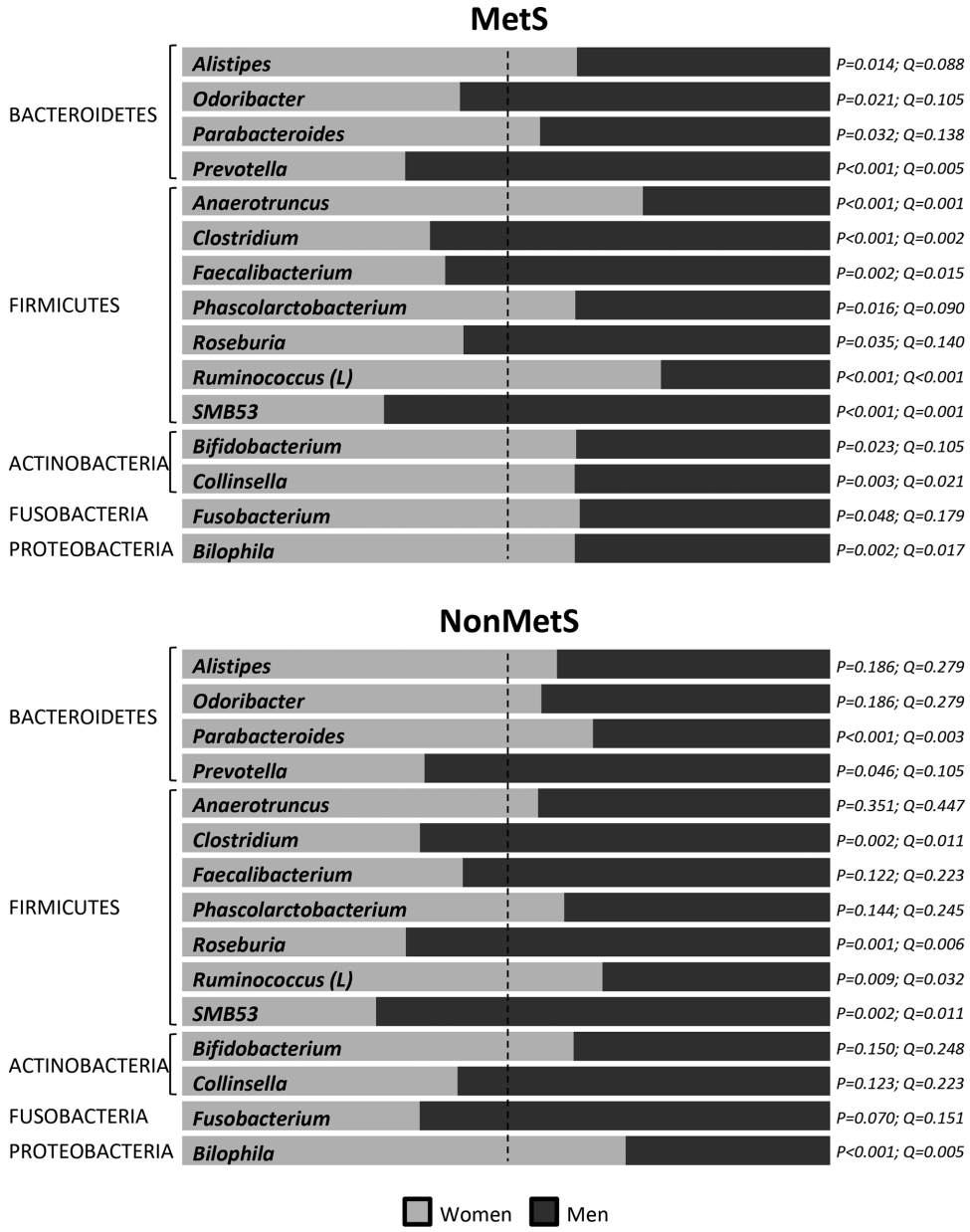

Figure 2. Differences in the gut microbiota composition according to gender in metabolic syndrome. Values represent mean of the genus, proportional in the four experimental groups (mean ± SEM of the abundance are shown in Table S6*, Supporting Information). *Ruminococcus* (L): *Ruminococcus* genus belonging to the *Lachnospiraceae* family. Statistical analysis was performed to find statistically significant differences in the abundance of each genus in the groups. *p*-Values: One-way ANOVA statistical analysis. *q*-Values: ANOVA *p*-values adjusted by the FDR using the Benjamini and Hochberg method. (*): S5

3.7. Differential Diet Effect on Gut Microbiota Composition

In the next step, we explored whether gender determines a differential shaping of the gut microbiota from MetS patients by the consumption for 3 years of an MED or an LF diet. We observed higher levels of *Desulfovibrio*, *Roseburia*, and *Holdemania* genera in MetS-M than in MetS-W after the consumption for 3 years of the LF diet ($p = 0.001$, $p = 0.001$, and $p = 0.023$), whereas no differences in the abundance of these bacterial genera between genders were observed after the consumption of the MED diet. By contrast, we found higher levels on an unknown genus from the *Rikenellaceae* family in MetS-M than in MetS-W after the consumption for 3 years of the MED diet ($p = 0.018$), whereas no differences were observed after the consumption of the LF diet (**Figure 3**).

Moreover, we also observed several gender-specific differences in the gut microbiota from MetS patients, regardless of the diet consumed. We observed a higher abundance of *Suterella* genus, and an unknown genera from *Erysipelotrichaceae* family and *Enterobacteriaceae* family in MetS-W than MetS-M after 3 years of dietary intervention ($p = 0.001$, $p = 0.001$, and $p = 0.031$, respectively). By contrast, we observed a higher abundance of

Table 2. Analysis of the bacterial genera presence according to metabolic syndrome between women and men. Values correspond to the number of patients in which the bacterial genus was detected; percentage is shown between brackets. The χ^2 test was applied to establish differences in bacterial prevalence between the groups studied. Values: number of patients in which the genus was detected; (% of patients in which the genus was detected); [Relative abundance of this genus in the group].

	MetS-W ($N = 79$)	MetS-M ($N = 79$)	χ^2	NonMetS-W ($N = 44$)	NonMetS-M ($N = 44$)	χ^2
Eggerthella	53 (67.09)	31 (39.24)	<0.001	24 (54.55)	20 (45.45)	0.394
	[0.00027]	[0.00014]		[0.00052]	[0.00005]	
Slackia	47 (59.49)	63 (79.75)	0.006	28 (63.64)	36 (81.82)	0.056
	[0.00072]	[0.00110]		[0.00127]	[0.00213]	
Odoribacter	74 (93.67)	79 (100)	0.023	43 (97.73)	44 (100)	0.315
	[0.00318]	[0.00424]		[0.00352]	[0.00283]	
Acidaminococcus	72 (91.14)	63 (79.75)	0.042	39 (88.64)	42 (95.45)	0.237
	[0.00137]	[0.00270]		[0.00149]	[0.00028]	
Unknown genus[a)]	31 (39.24)	47 (59.49)	0.011	21 (47.73)	27 (61.36)	0.199
	[0.00018]	[0.00016]		[0.00018]	[0.00026]	
Anaerotruncus	67 (84.81)	60 (75.95)	0.161	38 (86.36)	30 (68.18)	0.042
	[0.00013]	[0.00005]		[0.00008]	[0.00007]	
Catenibacterium	43 (54.43)	50 (63.29)	0.258	23 (52.27)	33 (75.00)	0.027
	[0.00196]	[0.00234]		[0.00144]	[0.00340]	

[a)] Unknown genus (Unknown family; RF39 order).

Phascolarctobacterium genus and an unknown genera from the *Clostridiales* order and *Coriobacteriaceae* and *S24-7* families in MetS-M than MetS-W after 3 years of dietary intervention ($p = 0.044$, $p = 0.005$, $p = 0.021$, and $p<0.001$, respectively; **Figure 4**).

4. Discussion

This work provides evidence of a different gut microbiota composition in MetS, according to gender. Moreover, our study showed a differential shaping of the gut microbiota, according to the gender, in MetS patients, after the consumption for 3 years of an MED or an LF diet.

Previous data has indicated that the variability of the human population, related with factors such as gender, seems to affect gut microbiota composition.[16–18] However, to the best of our knowledge, the gender differences in gut microbiota in the presence of metabolic disease have not been studied in humans. Nevertheless, studies in animal models have identified a direct interaction between gender differences in gut microbiota and the development of disease,[33] suggesting that microbiota dysbiosis may be related with the dimorphism observed in the incidence of metabolic diseases.[34–36] To explore gender differences in MetS, we compared women and men with the same pattern of MetS criteria, to avoid a factor of confusion since different combinations of MetS criteria may be associated with different gut dysbiosis and therefore may hinder the identification of gender differences. Moreover, we also took into account the fact that the baseline nutritional background was similar in all the participants, as this parameter can also modify the microbial composition of the intestine.[20]

The present work has shown that the microbiota of women with MetS is different to the microbiota of men with MetS. However, this difference is less pronounced in MetS than in NonMetS groups, on the basis of the linear discriminant analysis effect size. A lower bacterial diversity associated to disease, which has been previously described,[37] may be responsible for this observation. In fact, we observed significant differences in quantitative and qualitative metrics (weighted and unweighted UniFrac distances) between genders in NonMetS groups, whereas only qualitative metrics (unweighted UniFrac distance) showed differences between genders in MetS groups. This also suggests that low-abundance taxa may be important in determining the differences in gut microbiota composition between genders in disease, on the basis that weighted UniFrac incorporates abundances to calculate distance, and the impact of low-abundance features is diminished, whereas unweighted UniFrac is more sensitive to differences in low-abundance features.[31] However, the impact of differences in low-abundance features between genders requires further research.

Taking into account the potential role of gut microbiota in the development of MetS, we explored the specific gender differences between MetS groups that did not exist between NonMetS women and men groups. Thus, we observed differences in the abundance of bacterial genera previously related with the presence of T2DM, such as *Collinsella*, *Faecalibacterium*, and *Prevotella*,[38] with insulin resistance, such as *Phascolarctobacterium*,[39] and directly with MetS, such as *Anaerotruncus*.[40] Considering the fact that the SCFA-producers *Faecalibacterium* and *Prevotella*[38] are more abundant in men than in women, our results reinforce the idea that the loss of certain functions or features of the gut microbiota, such as the loss of the capacity to degrade carbohydrates to SCFA, is present in MetS, as previously described,[41] but the current work suggests that this might occur due to the differential alteration of the gut microbiota in women and in men. Moreover, these alterations may also differentially affect the extent to which SCFA production might potentially be reduced in the gut of women and men.

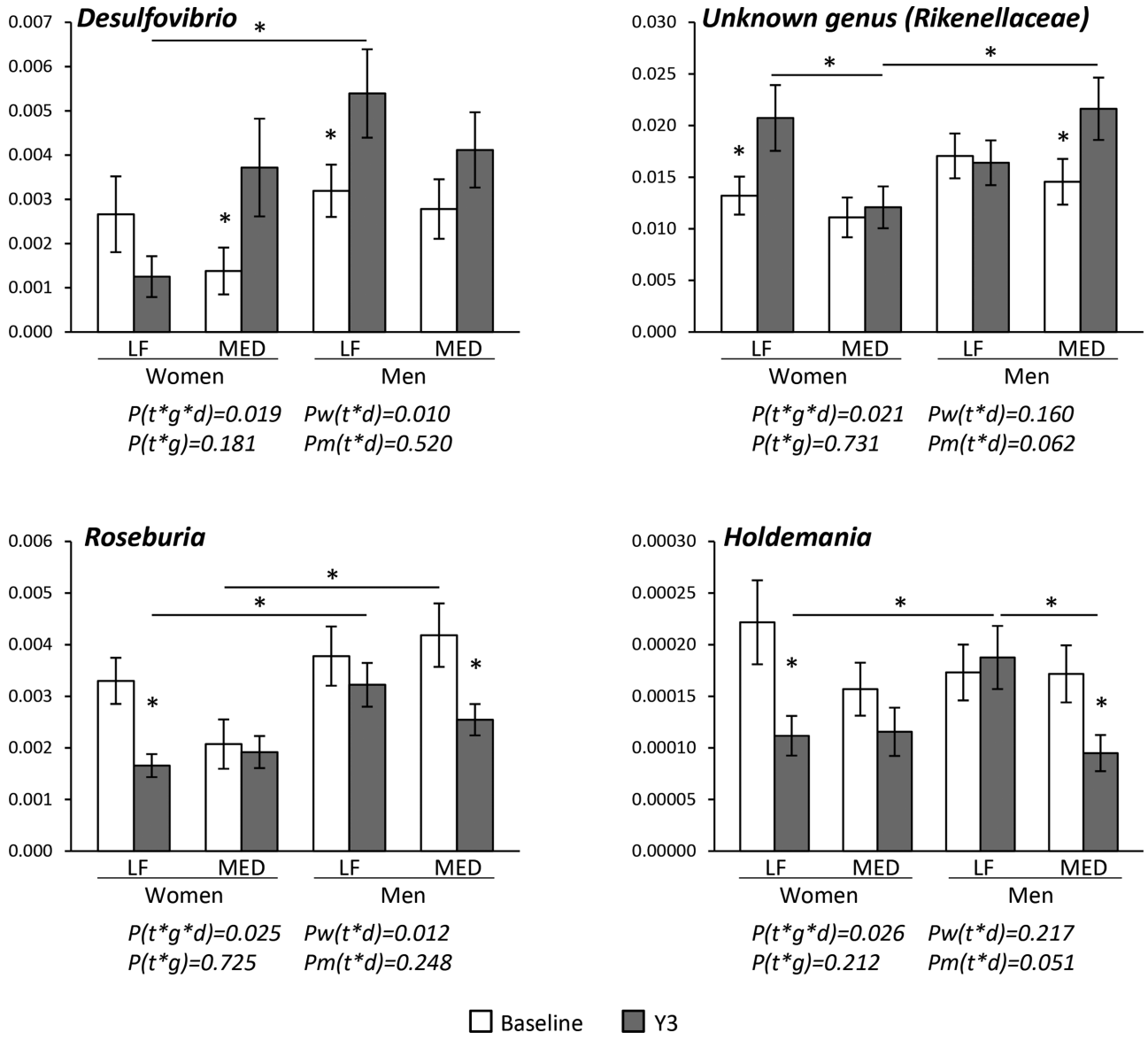

Figure 3. Gut microbiota composition changes according to gender and diet. Values represent mean of the bacterial taxa abundance ± SEM. LF, low-fat diet; MED, Mediterranean diet. The statistically significant differences between baseline and follow-up were tested by ANOVA for repeated measures, with time (t) as intrasubject factor, and diet (d) and gender (g) as the intersubject factors. $*p < 0.05$ in the post hoc Bonferroni's multiple comparison test.

The fact that the same combinations of MetS criteria in women and men were associated to different alterations of the gut microbiota might help to explain the differences in the prevalence of MetS between genders,[3] and the differences in the prevalence of each metabolic syndrome risk factor, which also differs by gender and country.[4,5] Thus, it is plausible to think that the different dysbiosis or microbial imbalances in gut microbiota composition between men and women could be differentially favored or attained, which may therefore affect the incidence of the associated metabolic diseases between genders. In addition, given that each MetS combination may be associated to a different microbial dysbiosis, we identified a gender-dependent dysbiosis between women and men with the same combinations of MetS criteria.

Dietary strategies, which presumably act by modifying the microbiome,[20] are of great interest in preventing and treating metabolic disorders.[19] In fact, we have previously shown that the long-term consumption of both MED or LF diets partially restores the gut microbiota dysbiosis in obese people with the full characteristics of MetS (five criteria), whereas no significant modifications of gut microbiota were observed in NonMetS patients after the dietary intervention.[42] Based on this, in the current work, we analyzed whether the long-term consumption of these diets induced different microbiota changes in men than in women with MetS. In fact, we found two different effects: a) bacterial taxa whose abundance changed differentially between genders as consequence of the dietary intervention and regardless of diet, and b) bacterial taxa whose abundance was differentially

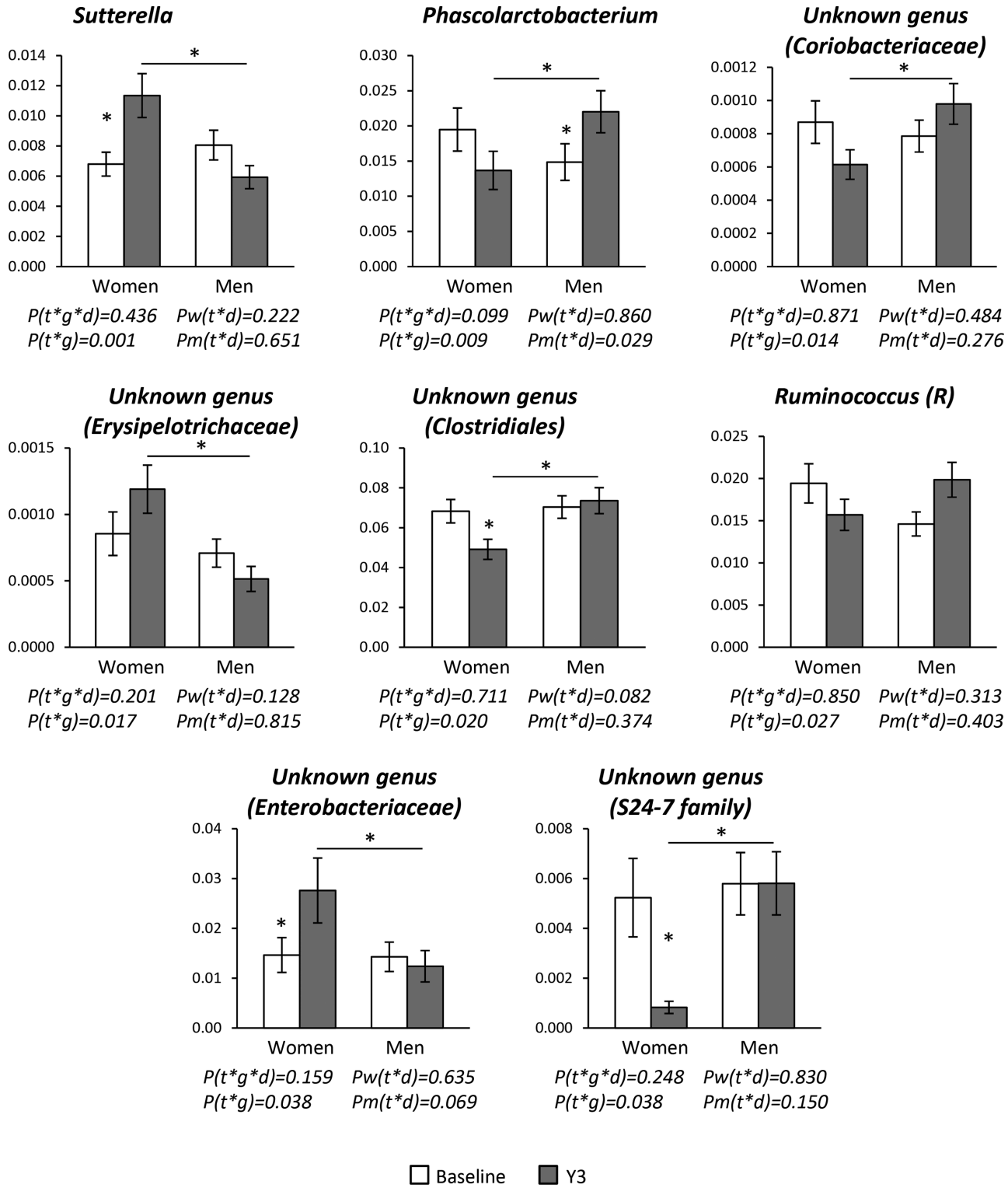

Figure 4. Gut microbiota composition changes according to gender. Values represent mean of the bacterial taxa abundance ± SEM. LF, low-fat diet; MED, Mediterranean diet. The statistically significant differences between baseline and follow-up were tested by ANOVA for repeated measures, with time (t) as intrasubject factor, and diet (d) and gender (g) as the intersubject factors. *$p < 0.05$ in the post hoc Bonferroni's multiple comparison test. (R): *Ruminococcus* from *Ruminococcaceae* family.

modified by the consumption of each diet, with a different effect in women than in men.

This latter is especially important, as our results suggest a new hypothesis based on the fact that women and men could differentially benefit from the consumption of the MED and LF diets. In fact, we observed a higher abundance of *Roseburia*, an SCFA-producer genus[43] and *Holdemania*, a genus whose abundance is linked to impaired lipid and glucose metabolism,[44] in men than in women after 3 years of the consumption of the LF diet. Moreover, we observed a higher abundance of *Desulfovibrio* in men than in women after 3 years of the consumption of this diet. The fact that this is a hydrogen sulfide-producing bacterial genus related with gastrointestinal disorders such as ulcerative colitis, Crohn's disease, and irritable bowel syndrome,[45] suggests that the consumption of an LF diet has a detrimental effect in men with this pathology, whereas its consumption in women would be advisable. By contrast, the abundance of an unknown genus from the *Rikenellaceae* family, which has been shown to negatively correlate with MetS components,[46] was higher in men than in women after 3 years of consumption of the MED diet. In addition, this bacterial genus was also higher in women who consumed the LF diet than in women who consumed the MED diet.

Our study has limitations. One limitation lies in the fact that although low-abundance taxa may be important in determining the differences in gut microbiota composition between genders in MetS, on the basis of beta-diversity metrics, the potential impact that differences in low-abundance features between genders may have on host physiology remains to be explored. Another limitation is that the 16S rRNA sequencing is suitable for microbiota analysis from phylum to genus levels, but is limited in its ability to identify bacterial species. Therefore, the analysis of the MetS-associated differences in bacterial taxa prevalence between the genders was performed at genus levels, and different bacterial species may be contributing to the prevalence of the genera.

In conclusion, our results suggest the potential involvement of the gut microbiota in the different incidence of metabolic diseases between genders. According to our results, which show the relationship between gut microbiota and metabolic disease, MetS-associated dysbiosis seems to be gender-dependent, and the alteration of the intestinal microbiota from normobiosis to dysbiosis may be different in women and men. Moreover, different diet-induced changes of gut microbiota by gender suggest that women and men could differentially benefit from the consumption of a specific diet, depending on their gender and disease.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

J.A.S.-M. and C.H. contributed equally to this work. J.A.S.-M., C.H., A.V.-R., J.F.A.-D., H.M.-A., A.L.-A., and J.L.-M. conducted the research; J.A.S.-M., C.H., A.V.-R., J.F.A.-D., H.M.-A., B.B.L., M.T.-S., and P.P.-M. analyzed the data or performed statistical analyses; J.A.S.-M., C.H., M.T.-S., F.P.J., and A.C. wrote the paper; J.L.-M., F.P.-J., and A.C. designed the research; J.L.-M., F.P.J., and A.C. had primary responsibility for the final content. The CIBEROBN is an initiative of the Instituto de Salud Carlos III, Madrid, Spain. The CORDIOPREV study is supported by the Fundación Patrimonio Comunal Olivarero, Junta de Andalucía (Consejería de Salud, Consejería de Agricultura y Pesca, Consejería de Innovación, Ciencia y Empresa), Diputaciones de Jaén y Córdoba, Centro de Excelencia en Investigación sobre Aceite de Oliva y Salud and Ministerio de Medio Ambiente, Medio Rural y Marino, Gobierno de España; Instituto de Salud Carlos III (CP14/00114 to A.C.). Ministerio de Economía y Competitividad (AGL2012/39615, PIE14/00005, and PIE 14/00031 to J.L.-M.; AGL2015-67896-P to J.L.-M. and A.C.; CP14/00114 to A C; PI13/00619 to F.P.-J.; PI16/01777 to F.P.-J. and P.P.-M.); Consejería de Innovación, Ciencia y Empresa, Proyectos de Investigación de Excelencia, Junta de Andalucía (CVI-7450 to J.L.-M.); and by the Fondo Europeo de Desarrollo Regional (FEDER). Antonio Camargo is supported by an ISCIII research contract (Programa Miguel-Servet CP14/00114). The authors would like to thank the Córdoba branch of the Biobank of the Sistema Sanitario Público de Andalucía (Andalusia, Spain) for providing the biological human samples. The authors also thank José Andrés Morales Martínez for technical assistance. The authors would also like to thank the EASP (Escuela Andaluza de Salud Pública), Granada, Spain.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

dysbiosis, gut microbiota,metabolic diseases, obesity, sexual dimorphism

Received: August 21, 2018
Revised: December 17, 2018
Published online:

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Number of MetS criteria	*MetS-W (N=79)*	*MetS-M (N=79)*	*NonMetS-W (N=44)*	*NonMetS-M (N=44)*
5	16/79 (20.25%)	14/79 (17.72%)	0/44 (0.00%)	0/44 (0.00%)
4	28/79 (35.44%)	40/79 (50.63%)	0/44 (0.00%)	0/44 (0.00%)
3	35/79 (44.30%)	25/79 (31.65%)	0/44 (0.00%)	0/44 (0.00%)
2	0/79 (0.00%)	0/79 (0.00%)	20/44 (45.45%)	22/44 (50.00%)
1	0/79 (0.00%)	0/79 (0.00%)	22/44 (50.00%)	19/44 (43.18%)
0	0/79 (0.00%)	0/79 (0.00%)	2/44 (4.55%)	3/44 (6.82%)
Criteria of MetS				
Waist circumference (cm)	71/79 (89.87%)	73/79 (92.41%)	24/44 (54.55%)	24/44 (54.55%)
HDL-c (mg/dL)	67/79 (84.81%)	64/79 (81.01%)	9/44 (20.45%)	11/44 (25.00%)
TAG (mg/dL)	37/79 (46.84%)	40/79 (50.63%)	3/44 (6.82%)	2/44 (4.55%)
Glucose (mg/dL)	57/79 (72.15%)	57/79 (72.15%)	7/44 (15.91%)	7/44 (15.91%)
Blood pressure (mm Hg)	65/79 (82.28%)	71/79 (89.87%)	19/44 (43.18%)	19/44 (43.18%)

Supplemental Table 1. Metabolic syndrome criteria in the participants in the study. MetS, metabolic syndrome; HDL-c, high density lipoprotein-cholesterol; TAG, triacylglycerides.

	MED diet score	*Fiber intake (g/1000 kcal)*
MetS-W	8.14±0.19	12.69±0.36
MetS-M	8.48±0.25	11.61±0.38
P-value	*0.268*	*0.041*
NonMetS-W	8.30±0.31	12.73±0.54
NonMetS-M	8.95±0.33	10.95±0.39
P-value	0.147	0.009

Supplemental Table 2. Dietary assessment of the participant in the study. Values correspond to the mean±SEM of a 14-item questionnaire to assess adherence to the Mediterranean diet and a 9-point score to assess adherence to low-fat diet. Fiber intake was calculated using the Spanish food composition tables. The statistical differences between groups were evaluated by One-way ANOVA.

	MetS-W (N=79)	*MetS-M (N=79)*	*NonMetS-W (N=44)*	*NonMetS-M (N=44)*	*P-value*
Chao-1	182.29±3.71	186.67±3.27	186.11±4.52	193.84±3.79	0.241
Simpson (1-D)	0.94±0.00	0.94±0.00	0.94±0.00	0.94±0.00	0.970
Shannon (H)	3.19±0.02	3.18±0.02	3.20±0.03	3.21±0.02	0.900

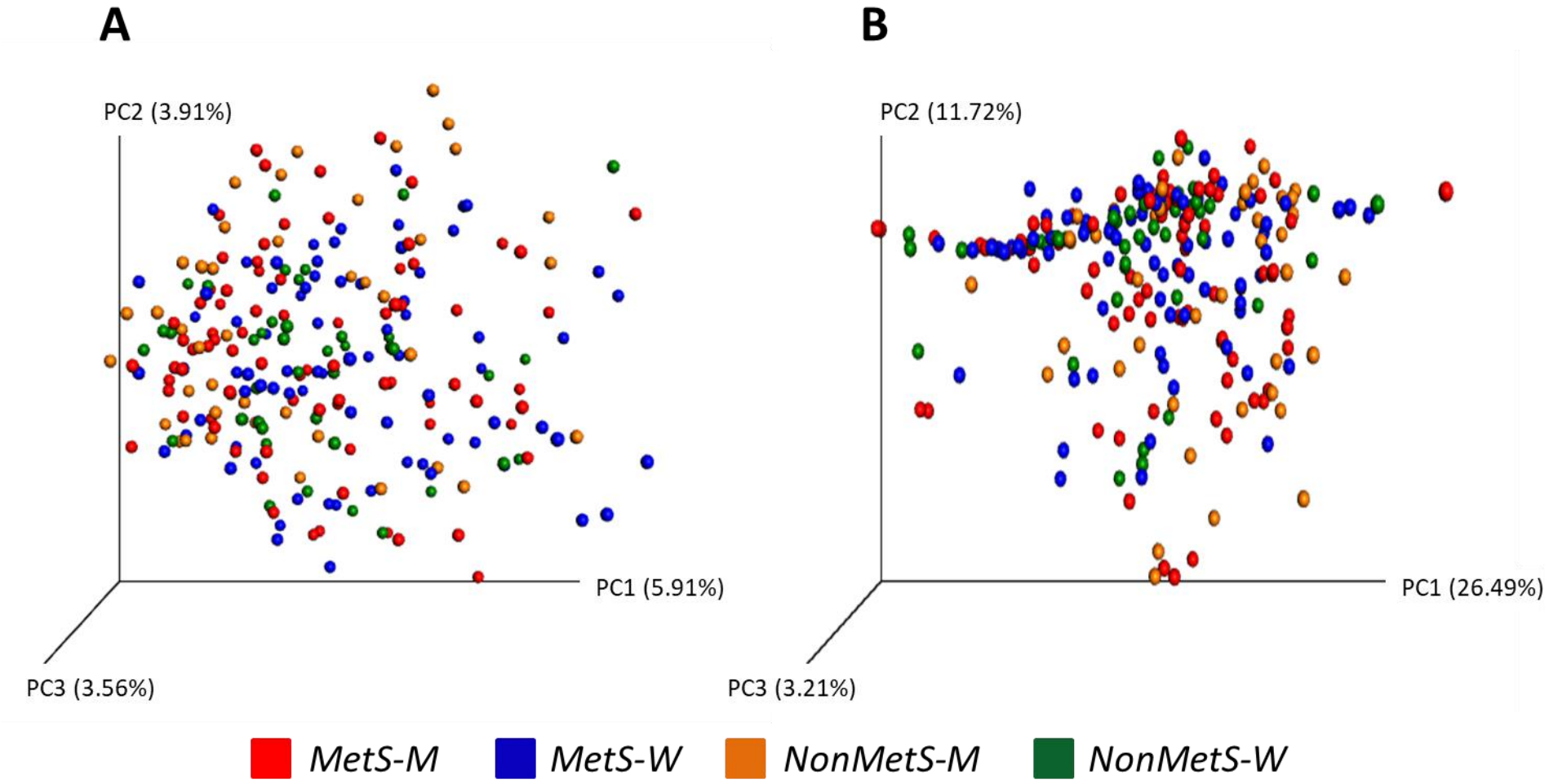
Supplemental Table 3. Diversity of the gut microbiota according to gender. Values correspond to the mean±SEM. The statistical differences between groups were evaluated by One-way ANOVA.

	MetS-W		*MetS-M*		
	LF diet (N=25)	*MED diet (N=21)*	*LF diet (N=29)*	*MED diet (N=24)*	*P-value*
Age (years)	63.60±1.64	59.52±1.80	62.52±1.55	61.50±1.73	0.401
BMI (kg/m^2)	31.51±0.83	33.77±0.88	32.55±1.00	31.25±0.71	0.213
Waist circumference (cm)	101.52±2.03^{a,c}	104.40±2.22^{a,c}	112.97±2.30^{b}	106.86±1.32^{a,b}	0.001
HDL-c (mg/dL)	39.84±1.66^{a}	43.38±1.78^{a,b}	39.93±2.33^{a}	35.38±1.23^{a,c}	0.043
LDL-c(mg/dL)	77.22±6.15	96.90±6.91	83.43±3.26	80.46±4.57	0.064
Total cholesterol (mg/dL)	151.88±7.63^{a,b}	178.62±9.61^{a,c}	156.03±4.48^{a}	153.42±5.39^{a}	0.031
TAG (mg/dL)	172.32±18.69	160.25±14.18	150.07±13.30	185.88±15.03	0.381
Glucose (mg/dL)	118.21±8.72	134.14±15.69	122.79±8.27	124.46±7.41	0.750
Insulin (mU/L)	17.80±4.39	10.88±1.88	11.70±1.75	16.46±4.13	0.374
HbA1c (%)	7.01±0.32	7.10±0.32	6.83±0.26	6.83±0.25	0.884
Systolic BP (mm Hg)	148.28±4.25	137.71±3.27	144.62±3.33	145.83±4.44	0.304
Diastolic BP (mm Hg)	75.64±2.67	75.29±1.87	79.50±1.98	80.58±2.97	0.317
Number of MetS criteria					
5	5/25 (20.00%)	3/21 (14.29%)	4/29 (13.79%)	3/24 (16.67%)	
4	10/25 (40.00%)	9/21 (42.86%)	14/29 (48.28%)	12/24 (50.00%)	
3	10/25 (40.00%)	9/21 (42.86%)	11/29 (37.93%)	8/24 (33.33%)	
Criteria of MetS per group					
Waist circumference (cm)	22/25 (88.00%)	21/21 (100%)	27/29 (93.10%)	21/24 (87.50%)	
HDL-c (mg/dL)	23/25 (92.00%)	17/21 (80.95%)	22/29 (75.86%)	21/24 (87.50%)	
TAG (mg/dL)	13/25 (52.00%)	11/21 (52.38%)	14/29 (48.28%)	13/24 (54.17%)	
Glucose (mg/dL)	17/25 (68.00%)	13/21 (61.90%)	20/29 (68.97%)	17/24 (70.83%)	
Blood pressure (mm Hg)	20/25 (80.00%)	16/21 (76.19%)	26/29 (89.66%)	20/24 (83.33%)	

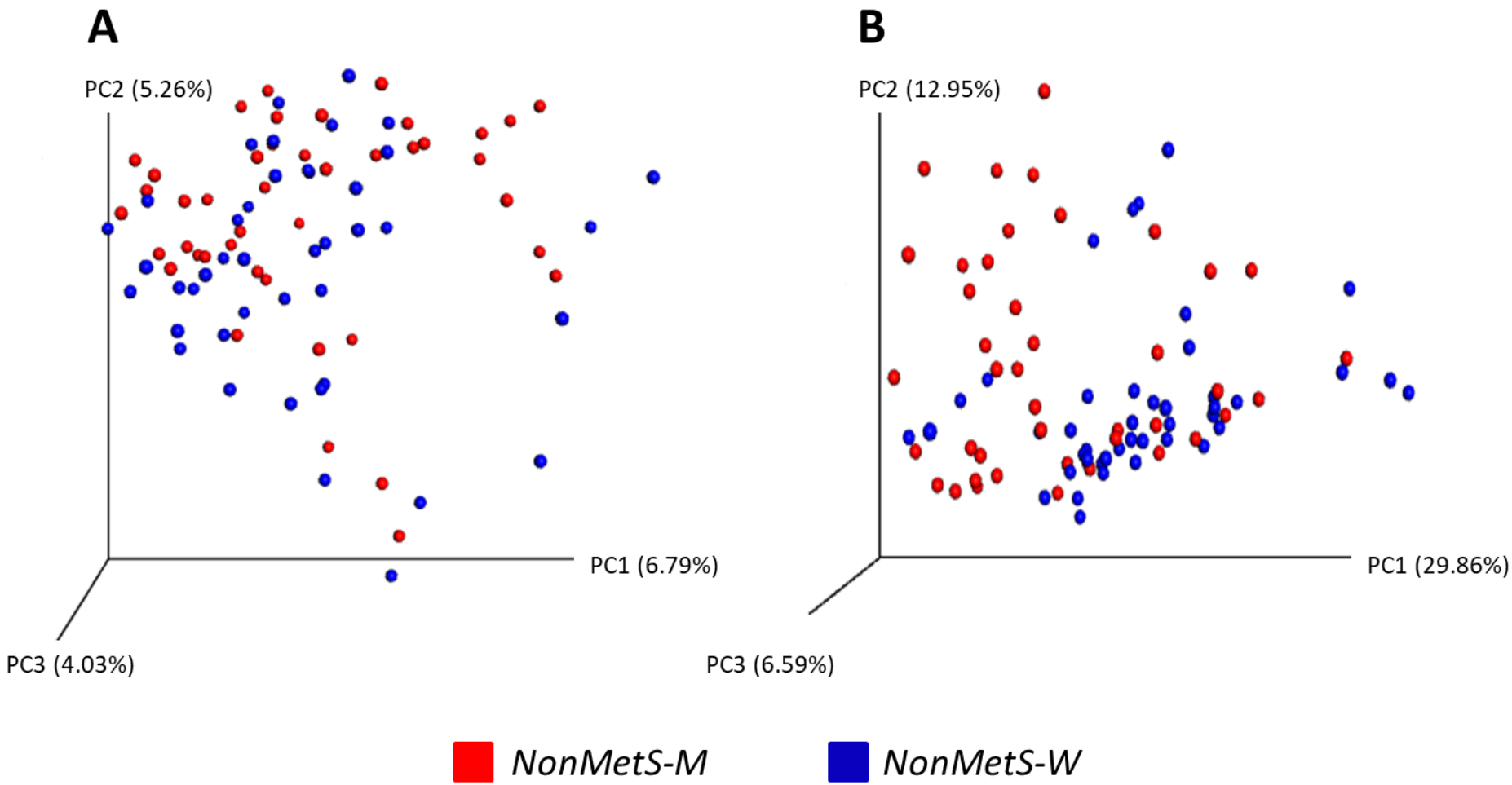
Supplemental Table 4. Metabolic characteristics of the participants in the dietary intervention study. Values correspond to the mean±SEM. The statistical differences between groups were evaluated by One-way ANOVA. BMI, body mass index; HDL-c, high density lipoprotein-cholesterol; LDL-c, low density lipoprotein-cholesterol; TAG, triacylglycerides; HbA1c, glycated hemoglobin; BP, blood pressure. In each row, values with different letters in superscript differ statistically in the Bonferroni's corrected post hoc multiple comparison test ($P<0.05$).

	MetS-W	*MetS-M*	*P-value*	*Q-value*	*NonMetS-W*	*NonMetS-M*	*P-value*	*Q-value*
Alistipes	0.00098±0.00012	0.00063±0.00008	0.014	0.088	0.00100±0.00016	0.00073±0.00013	0.186	0.279
Odoribacter	0.00318±0.00029	0.00424±0.00035	0.021	0.105	0.00352±0.00037	0.00283±0.00036	0.186	0.279
Parabacteroides	0.03119±0.00210	0.02528±0.00175	0.032	0.138	0.03448±0.00285	0.01991±0.00229	>0.001	0.003
Prevotella	0.03667±0.00505	0.06986±0.00779	<0.001	0.005	0.04342±0.00777	0.07263±0.01219	0.046	0.105
Anaerotruncus	0.00013±0.00002	0.00005±0.00001	<0.001	0.001	0.00008±0.00001	0.00007±0.00001	0.351	0.447
Clostridium	0.00116±0.00011	0.00187±0.00015	<0.001	0.002	0.00132±0.00018	0.00227±0.00025	0.002	0.011
Faecalibacterium	0.01566±0.00140	0.02290±0.00177	0.002	0.015	0.02407±0.00282	0.03150±0.00383	0.122	0.223
Phascolarctobacterium	0.02562±0.00299	0.01661±0.00219	0.016	0.090	0.02193±0.00368	0.01527±0.00263	0.144	0.245
Roseburia	0.00302±0.00028	0.00393±0.00033	0.035	0.140	0.00274±0.00034	0.00520±0.00061	0.001	0.006
Ruminococcus	0.00207±0.00025	0.00073±0.00008	<0.001	<0.001	0.00159±0.00023	0.00086±0.00014	0.009	0.032
SMB53	0.00010±0.00001	0.00023±0.00003	<0.001	0.001	0.00029±0.00006	0.00068±0.00011	0.002	0.011
Bifidobacterium	0.00374±0.00050	0.00241±0.00029	0.023	0.105	0.00514±0.00105	0.00337±0.00062	0.150	0.248
Collinsella	0.00228±0.00024	0.00148±0.00012	0.003	0.021	0.00192±0.00026	0.00259±0.00035	0.123	0.223
Fusobacterium	0.00017±0.00003	0.00010±0.00002	0.048	0.179	0.00010±0.00002	0.00017±0.00003	0.070	0.151
Bilophila	0.00245±0.00023	0.00159±0.00015	0.002	0.017	0.00250±0.00030	0.00115±0.00020	<0.001	0.005

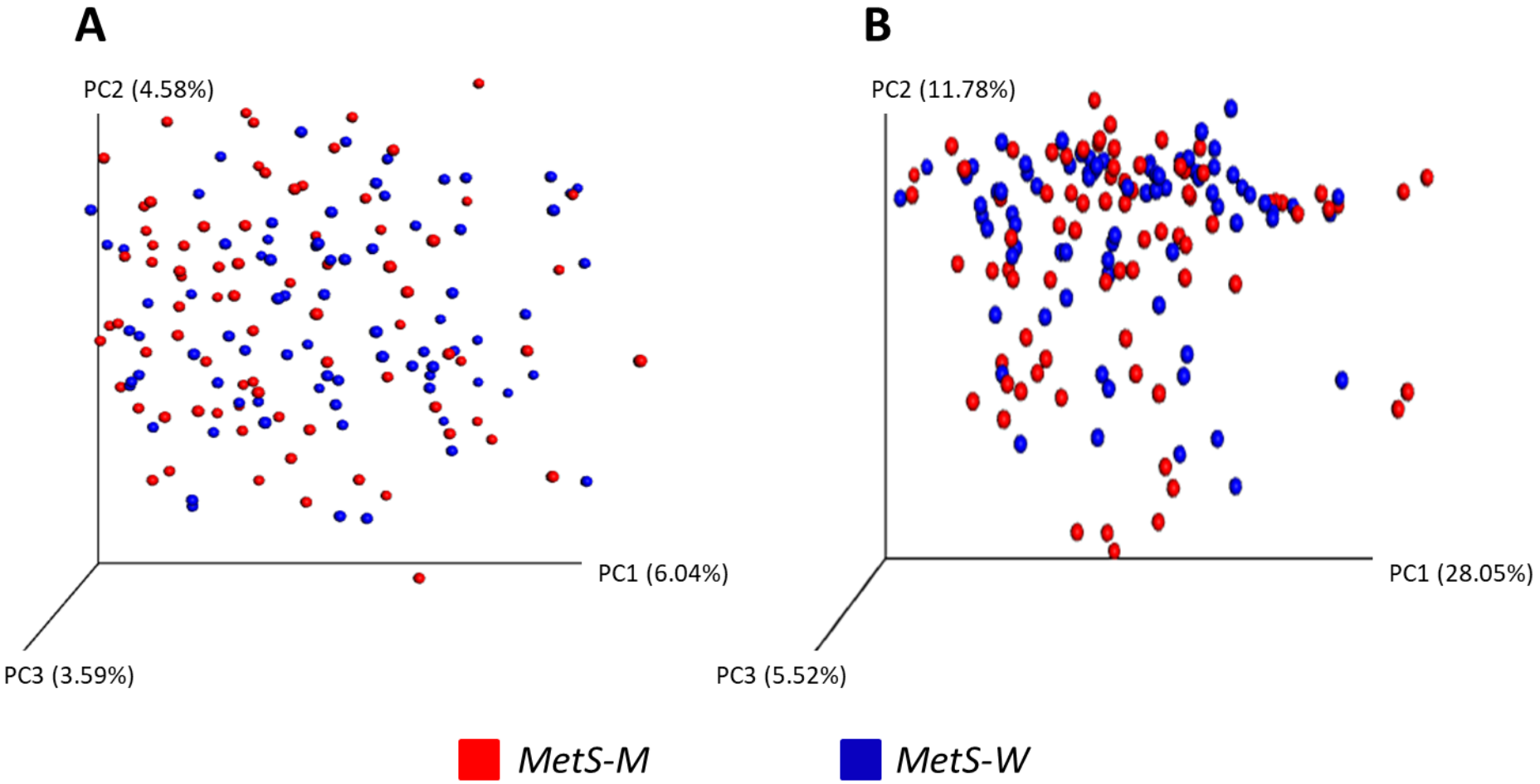
Supplemental Table 5. Relative abundance of bacterial taxa according to metabolic disease and gender. Values represent mean ±SEM of the abundance of genera shown in **Figure 2**). Statistical analysis was performed with the relative abundance of each bacterial taxa. P-value: One-way ANOVA statistical analysis. Q-value: False Discovery Rate (FDR) using the Benjamini and Hochberg method.



Supplemental Figure 1. Principal Component Analysis plots of unweighted (A) and weighted (B) Unifrac distance metrics between groups.



Supplemental Figure 2. Principal Component Analysis plots of unweighted (A) and weighted (B) Unifrac distance metrics between non-Metabolic Syndrome women and men.



Supplemental Figure 3. Principal Component Analysis plots of unweighted (A) and weighted (B) Unifrac distance metrics between women and men with Metabolic Syndrome.

5.3. Publicación 3

Citación: Santos-Marcos JA*, Barroso A*, Rangel-Zuñiga OA, Perdices-Lopez C, Haro C, Sanchez-Garrido MA, Molina-Abril H, Ohlsson C, Perez-Martinez P, Poutanen M, Lopez-Miranda J, Perez-Jimenez F, Tena-Sempere M, Camargo A. Interplay between gonadal hormones and postnatal overfeeding in defining sex-dependent differences in gut microbiota architecture. Aging (Albany NY). 2020 Oct 27;12(20):19979-20000. doi: 10.18632/aging.104140. Epub 2020 Oct 27. PMID: 33107844; PMCID: PMC7655199. *Igual contribución

Research Paper

Interplay between gonadal hormones and postnatal overfeeding in defining sex-dependent differences in gut microbiota architecture

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Keywords: gut microbiota, sex steroids, gender, metabolism, miRNAs
Received: June 18, 2020 **Accepted:** September 20, 2020 **Published:** October 27, 2020

ABSTRACT

Aging is associated with a decline in sex hormones, variable between sexes, that has an impact on many different body systems and might contribute to age-related disease progression. We aimed to characterize the sex differences in gut microbiota, and to explore the impact of depletion of gonadal hormones, alone or combined with postnatal overfeeding, in rats. Many of the differences in the gut microbiota between sexes persisted after gonadectomy, but removal of gonadal hormones shaped several gut microbiota features towards a more deleterious profile, the effect being greater in females than in males, mainly when animals were concurrently overfed. Moreover, we identified several intestinal miRNAs as potential mediators of the impact of changes in gut microbiota on host organism physiology. Our study points out that gonadal hormones contribute to defining sex-dependent differences of gut microbiota, and discloses a potential role of gonadal hormones in shaping gut microbiota, as consequence of the interaction between sex and nutrition. Our data suggest that the changes in gut microbiota, observed in conditions of sex hormone decline, as those caused by ageing in men and menopause in women, might exert different effects on the host organism, which are putatively mediated by gut microbiota-intestinal miRNA cross-talk.

INTRODUCTION

Aging is the largest risk factor for cardiovascular diseases (CVD) [1]. However, coronary heart disease usually starts in women 10 years later than in men, a difference that increases to 20 years for cardiac events such as myocardial infarction [2, 3]. It has been shown that sex steroid hormones play a key role in CVD

susceptibility, but the differences in sex steroid profiles between elder men and women are smaller when compared to earlier in life [4]; for instance, sex steroid cardio-protection in women disappears after menopause [5]. Likewise, the decline in testosterone (T) seen in aging men is associated with a greater likelihood of CVD [6]. The mechanisms involved in the sex difference in CVD are not yet fully understood, but it is crucial to develop strategies and therapies aimed at reducing the incidence of CVD.

The gut microbiota has been shown to be involved in the development of CVD [7], suggesting a potential role in the dimorphism of their incidence, as gender, in addition to other factors, such as age, genetic make-up and nutritional habits, impacts on gut microbiota architecture [8–10]. In fact, in recent years there has been accumulating evidence suggesting that the differences in the intestinal microbiota according to gender may be associated with the sex differences observed in the development of autoimmune, metabolic and CV diseases [11, 12]. Moreover, diet and nutrition influence the host and the microbial metabolites [13], which might be associated with the onset of human pathologies [14]. In fact, the composition of the intestinal microbiota depends on the interactions between diet and the host's gender, and the therapies used to restore the dysbiosis of the gut microbiota associated to disease should be gender-specific.

We have previously shown that the intestinal microbiota from post-menopausal women presents a higher *Firmicutes/Bacteroidetes* (*F/B*) ratio than men, and a lesser abundance of short chain fatty acids (SCFA)-producing bacteria compared with the intestinal microbiota from pre-menopausal women, highlighting the influence of estrogens on gut microbiota architecture [15]. Moreover, we have also shown the differences in the intestinal microbiota architecture between post-menopausal women and age-matched men, which may stem from the actual differences in sex hormone levels in elder men and women and/or may reflect the residual influence of the dramatic differences in sex steroid profiles in early life between the sexes, and which may have a persistent effect on gut microbiota over time [9]. Moreover, intestinal microbiota transplant experiments in germ-free mice have recently demonstrated that the sex of the recipient animal shapes the composition of the intestinal microbiota [10]. In addition, it has been shown that males have a less diverse gut microbiota than their female littermates, a difference which is minimized with the castration of males, showing the influence of androgens on gut microbiome composition [16]. In fact, it has been shown that sex steroid manipulation during periods of early development alters gut microbiota [17].

However, the gender contribution to the sex differences in the gut microbiota, independently of sex steroid hormones, is not well understood, and may contribute to explaining the differences between genders in the incidence of cardiometabolic diseases. This set of interrelated conditions includes CVD, such as coronary heart disease, as well as metabolic diseases, such as type 2 diabetes and obesity. In order to shed light on the sex differences in the gut microbiota and the contribution of gonadal hormones and obesity to such differences, we explored here the sex-specific architecture of gut microbiota in gonadal-intact and gonadectomized rats of both sexes, alone or in combination with postnatal overnutrition.

RESULTS

Sex differences in gut microbiota according to nutritional status

We first explored differences between gonadal-intact male and female rats. In these studies, we found a higher α-diversity of the bacterial community in gonadal-intact females than in males, as assessed by both Shannon and Observed OTUs indexes under normal feeding (NL-CD) or postnatal overfeeding (SL-HFD) conditions (Supplementary Figure 1A).

In terms of bacterial composition, NL-CD males were characterized by higher *Elusimicrobia*, *Cyanobacteria*, and *Verrucomicrobia* phyla, whereas females were characterized by higher *Euryarchaeota* and *TM7* phyla. In postnatal overfed rats (SL-HFD), differences in *Cyanobacteria*, *Euryarchaeota* and *TM7* remained between sexes, in addition to higher *Bacteroidetes* and *Spirochaetes* phyla in males and higher *Firmicutes* phyla in females (Figure 1A; Supplementary Figure 2A). Moreover, whereas no differences in the *F/B* ratio were observed between sexes in animals under normal feeding, we observed a higher ratio in females than in males subjected to postnatal overfeeding (Figure 2).

Gut microbiota differences between sexes in gonadectomized animals

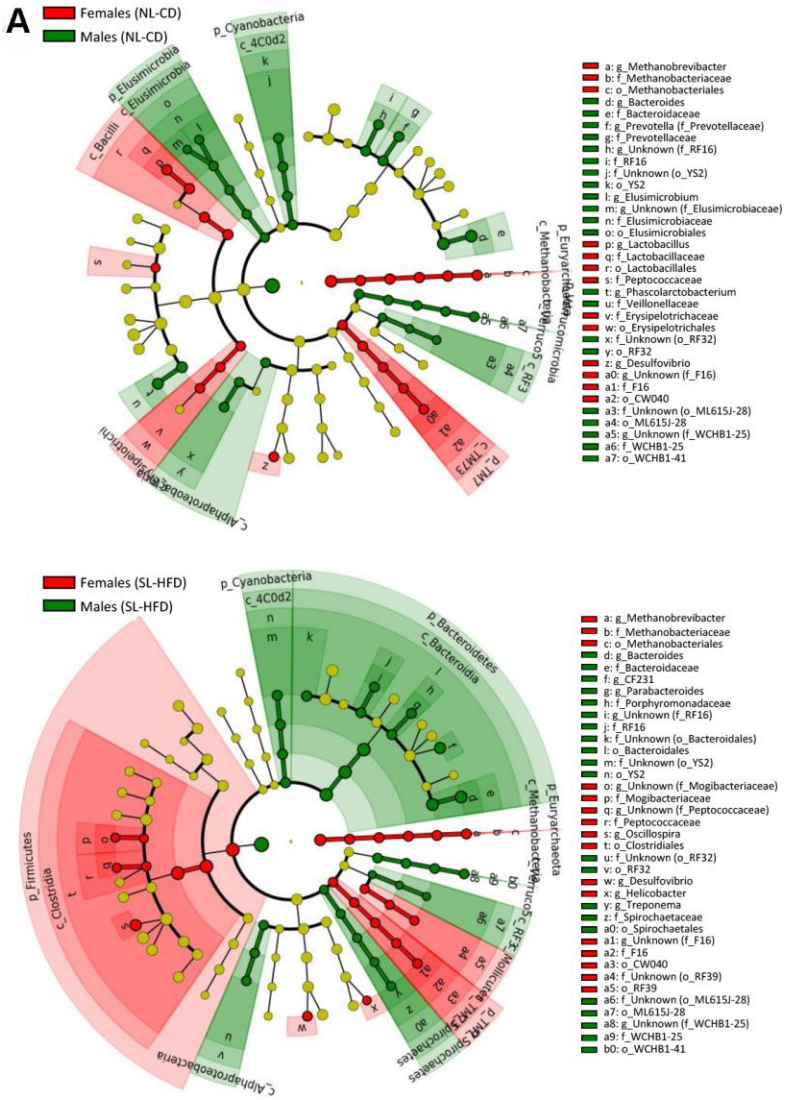
We next evaluated the differences between gonadectomized (GNX) males and females, under normal feeding or overfeeding conditions.

We found no differences in the α-diversity of the bacterial community between GNX males and females, regardless of the feeding condition (Supplementary Figure 1A). In terms of bacterial composition, most of the differences at phylum level found between male and female intact rats, both under normal feeding and postnatal overfeeding conditions, were also presented in

GNX animals. In fact, differences in *TM7*, *Cyanobacteria*, and *Euryarchaeota* phyla under normal feeding remained, whereas differences in *Elusimicrobia* and *Verrucomicrobia* phyla were absent. In addition, GNX males had higher *Proteobacteria* than GNX females. In postnatal overfeeding condition, differences in *Cyanobacteria*, *Euryarchaeota*, *Firmicutes* and *TM7* remained between GNX males and females, whereas differences in *Bacteroidetes* and *Spirochaetes* disappeared. Additionally, GNX males had higher *Elusimicrobia* than GNX females (Figure 1B; Supplementary Figure 2B). Moreover, whereas no differences in the *F/B* ratio were observed between sexes in GNX animals under normal feeding, we detected a higher *F/B* ratio in GNX females than in GNX males following postnatal overnutrition (Figure 2). In addition, from 55 bacterial genera included in LEfSe analysis, the abundance of 11 of these was different between sexes under normal feeding conditions, and the difference in the abundance of 6 of these disappeared after gonadectomy. Moreover, 8 additional bacterial genera were differentially represented between sexes only after gonadectomy in conditions of normal feeding. By contrast, the abundance of 13 genera was different between sexes under postnatal overfeeding condition, and the difference in the abundance of 5 of these disappeared after gonadectomy. In addition, 9 additional bacterial genera were differentially represented between sexes only after gonadectomy in overfed animals (Supplementary Table 1).

Impact of postnatal overfeeding in sex steroid hormones levels

Next, we evaluated the relationship between the obesogenic insult (postnatal overfeeding) and circulating sex steroids by measuring their plasma



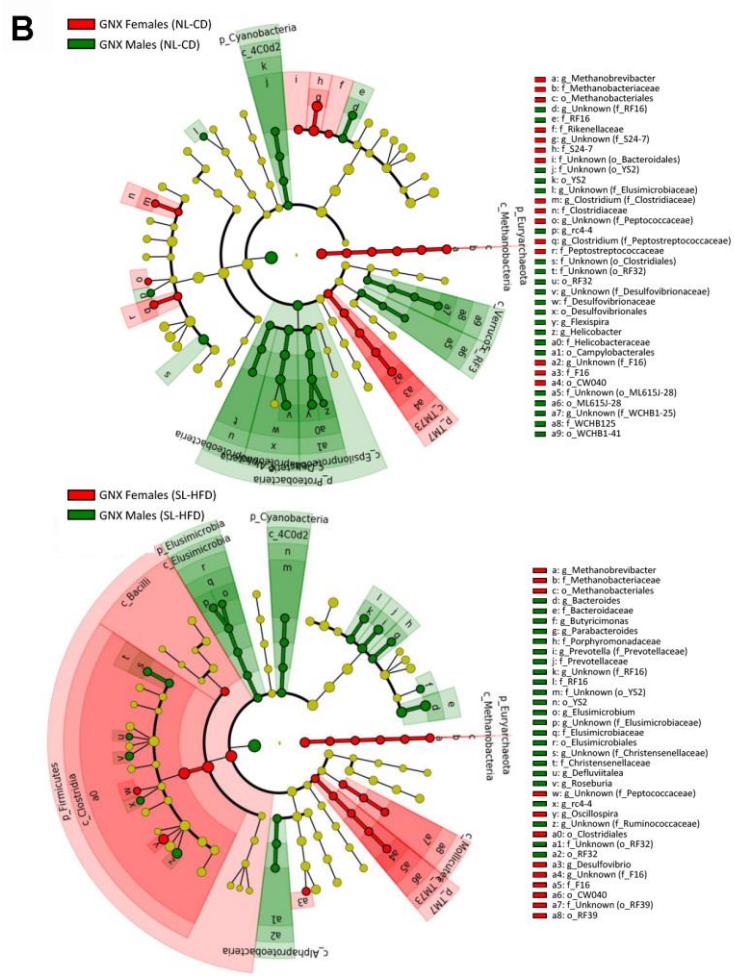


Figure 1. LEfSe analysis between sexes under normal feeding and overfeeding conditions in intact (**A**) and gonadectomized (**B**) animals. Cladogram representing the taxonomic hierarchical structure of the identified differences between genders using Linear discriminant analysis effect size (LEfSe). Each filled circle represents one phylotype. Red denotes bacterial taxa statistically overrepresented in females; green denotes bacterial taxa overrepresented in males. Phylum and class are indicated by their names on the cladogram and the order, family, or genus are given in the key.

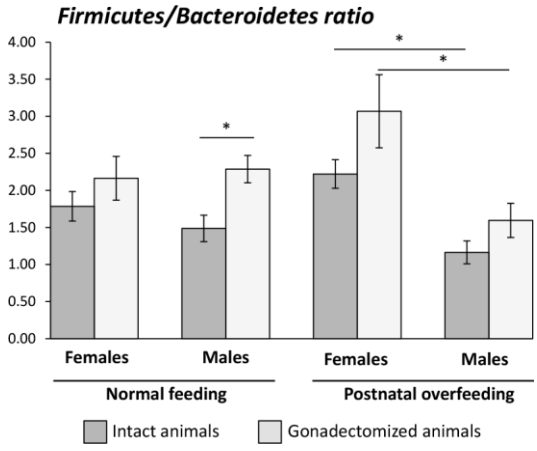


Figure 2. ***Firmicutes/Bacteroidetes*** **ratio in intact and gonadectomized animals under normal feeding and overfeeding conditions.** *P<0.05 in One-way ANOVA statistical analysis.

levels using the sensitive gas chromatography-tandem mass spectrometry method.

We found lower T, dihydrotestosterone, and androstenedione serum levels in males subjected to postnatal overfeeding that in those with normal feeding. No differences in the serum concentrations of these hormones, whose levels were much lower than in males, were found in females, regardless of their nutritional status. However, we found lower serum levels of estradiol (E_2) in females under normal feeding than in those subjected to postnatal overfeeding, whereas no differences in progesterone or estrone (E_1) levels (the latter was only detected in females) were found (Table 1). Because of the surgical removal of the gonads, sex steroid levels were not assessed in GNX male or female rats.

Sex-dependent metabolic disruption after gonadectomy

Further, we studied the sex-dependent metabolic alterations, alone or in combination with postnatal overnutrition, caused by GNX in males and females.

No differences in body weight (BW) were found between gonadal-intact and GNX males. By contrast, the BW of GNX females was higher than in intact females. We also observed in females an increase in plasma leptin levels in parallel with changes in BW after gonadectomy (Table 2).

Glucose tolerance, as measured by the area under the curve (AUC) of glucose during glucose tolerance test (GTT), was significantly worse in males subjected to postnatal overfeeding (SL-HFD), in both gonadal-intact and GNX conditions. However, GNX per se did not alter AUC GTT values in any of the two nutritional conditions. Nonetheless, we observed a higher ΔAUC GTT (as a net increment of the AUC over basal levels) of glucose in GNX males vs. intact males that were raised under postnatal overfeeding conditions. In turn, in gonadal-intact female rats, AUC GTT was not altered by SL-HFD, while in GNX females, the same obesogenic diet tended to increase glucose intolerance, although this change did not reach statistical significance (P=0.086). Regarding insulin sensitivity, obese (SL-HFD) males displayed insulin resistance, defined by significantly higher AUC during the insulin tolerance test (ITT) values than in lean (NL-CD) males, but GNX did not worsen insulin sensitivity neither in NL-CD or SL-HFD conditions. In contrast, higher insulin resistance was detected in SL-HFD females only when they were previously ovariectomized (Table 2).

Gonadal hormone contribution to gut microbiota structure

Next, we evaluated the hormonal contribution to gut microbiota structure by comparing the gut microbiota of intact versus GNX males and intact versus GNX females.

We found a higher α-diversity of the bacterial community in GNX males than in intact males as assessed by both Shannon and Observed OTUs indexes under normal feeding conditions, while no differences were found in overfed males (a trend for higher α-diversity in GNX males was observed). However, ovariectomy in females did not change these diversity indexes (Supplementary Figure 1B).

In terms of bacterial composition under normal feeding conditions, we observed that the gut microbiota of GNX males was characterized by higher *Firmicutes*, *Deferribacteres* and *TM7* phyla, and lower *Bacteroidetes* phylum, compared with intact males. By contrast, in animals subjected to postnatal overfeeding, the gut microbiota from GNX and intact males differed in the minority phylum *Elusimicrobia*, which was more abundant in GNX males. On the other hand, gonadectomy slightly impacted on the gut microbiota from females under normal feeding, with lower *Proteobacteria* phylum in GNX females, whereas in conditions of postnatal overfeeding, the gut microbiota from GNX females was characterized by higher *Elusimicrobia* and *Spirochaetes* phyla and lower *Actinobacteria* phylum (Figure 3; Supplementary Figure 3).

Microbiota putatively modulates host metabolism via miRNAs

Finally, we evaluated the potential role of miRNAs on the dialogue (cross-talk) between gut microbiota and host organism in response to changes in sex hormones and nutritional status.

To this end, we analyzed the relationship between the bacterial taxa identified by LEfSe analysis according to gender, sex hormones and obesity, and the expression levels of the miRNAs in the small and large intestine, determined by expression microarray analysis. Of note, we did not include in the analysis all the bacterial taxa but only those identified by LEfSe analysis in order to reduce random associations (Supplementary Tables 2–5; Figures 4, 5). From 758 miRNAs tested, the expression of 99 and 101 miRNAs was detectable in the large and small intestine, respectively, in at least 7 of the 8 experimental groups. From these, 54 miRNAs were detectable in both the large and small intestine. From

Table 1. Sex steroid plasma levels in intact animals under normal feeding and postnatal overfeeding conditions.

	Males			*Females*		
Sex steroid (pg/ml)	***NL-CD***	***SL-HFD***	***p-value***	***NL-CD***	***SL-HFD***	***p-value***
Testosterone	9347.12±2850.11	2070.24±421.49	0.027	80.49±19.95	122.16±36.97	0.323
Dihydrotestosterone	87.23±22.91	22.63±5.31	0.016	3.74±1.05	8.15±2.33	0.097
Androstenedione	669.91±169.78	133.64±20.40	0.007	47.47±2.94	73.38±14.72	0.089
Estradiol	0.32±0.32	n.d.	n.a.	3.19±0.13	8.46±2.33	0.032
Progesterone	909.23±125.72	765.00±317.08	0.679	18380.32±2037.92	16474.30±3361.90	0.626
Estrone	n.d.	n.d.	n.a.	1.00±0.27	2.79±1.68	0.278

NL-CD: normal litter, control diet. SL-HFD: small litter, high fat diet. Plasma was collected at PND-150 for determination of sex steroids by mass spectrometry. p-value: One-way ANOVA statistical analysis. n.d.: not detectable; n.a.: not available.

Table 2. Metabolic parameters in intact and gonadectomized animals under normal feeding and under postnatal overfeeding.

		Males			*Females*		
		non-GNX	***GNX***	***p-value***	***non-GNX***	***GNX***	***p-value***
Body weight (g)	***NL-CD***	356.96±12.47	328.78±7.71	**0.079**	223.53±5.62	256.31±5.45	**0.006**
	SL-HFD	478.61±7.65	481.31±15.16	**0.826**	260.30±8.65	319.73±8.92	**<0.001**
	p-value	**<0.001**	**<0.001**		**0.011**	**<0.001**	
Leptin (ng/ml)	***NL-CD***	10.26±2.90	8.69±2.44	**0.691**	3.77±0.51	7.43±0.95	**0.014**
	SL-HFD	34.03±5.02	32.45±3.79	**0.813**	10.19±1.81	22.62±4.09	**0.010**
	p-value	**0.005**	**0.001**		**0.014**	**0.005**	
AUC GTT	***NL-CD***	18230.00±818.40	17381.43±1473.46	**0.404**	21231.25±1434.01	20641.25±1452.55	**0.601**
	SL-HFD	23947.50±1215.50	24797.50±1255.93	**0.558**	22875.00±1000.87	24111.25±1329.62	**0.514**
	p-value	**0.001**	**0.005**		**0.330**	**0.086**	
Δ AUC GTT	***NL-CD***	4835.00±531.22	3855.71±758.97	**0.118**	6126.25±769.10	7021.25±625.65	**0.348**
	SL-HFD	3798.75±1084.42	8687.50±1328.90	**0.023**	8340.00±1237.97	6066.25±542.50	**0.089**
	p-value	**0.394**	**0.003**		**0.078**	**0.230**	
AUC ITT	***NL-CD***	5986.25±240.34	7126.25±585.95	**0.130**	6593.75±215.38	5890.00±264.53	**0.111**
	SL-HFD	7705.00±268.30	7623.75±327.63	**0.841**	6275.00±138.07	7401.25±553.07	**0.132**
	p-value	**0.005**	**0.561**		**0.327**	**0.030**	

NL-CD: normal litter, control diet. SL-HFD: small litter, high fat diet. GNX: gonadectomized animals. Non-GNX: intact animals. Glucose tolerance test (GTT) was performed at PND-120. Insulin tolerance test (ITT) was performed one week later than GTT. Body weight corresponds to PND-150 animals. p-value: One-way ANOVA statistical analysis.

the correlation analysis, we selected 27 miRNAs in the small intestine and 25 in the large intestine (1 miRNA were shared by both the large and small intestine), in which Pearson's correlation coefficient was > 0.9 or < -0.9 and P<0.01. Further, we performed a supplemental analysis with the 51 selected miRNAs using the DIANAtools V.3. DIANA-miRPath is a web-server which provides accurate statistics and can accommodate advanced pipelines. miRPath can utilize predicted miRNA targets (in CDS or 3'-UTR regions) provided by the DIANA-microT-CDS algorithm or even experimentally validated miRNA interactions derived from DIANA-TarBase [18]. Thus, in addition to several KEGG pathways related with metabolism, our approach detected miRNA-mediated associations between the gut microbiota and sex steroid-related pathways. The functions of KEGG, in which selected miRNAs were assigned, included the metabolism of lipids, amino acids, cofactors and vitamins, signal transduction, and endocrine systems. Specifically, insulin, GnRH, estrogen, and prolactin signaling pathways, as well as progesterone-mediated oocyte maturation, were involved (Supplementary Tables 6, 7).

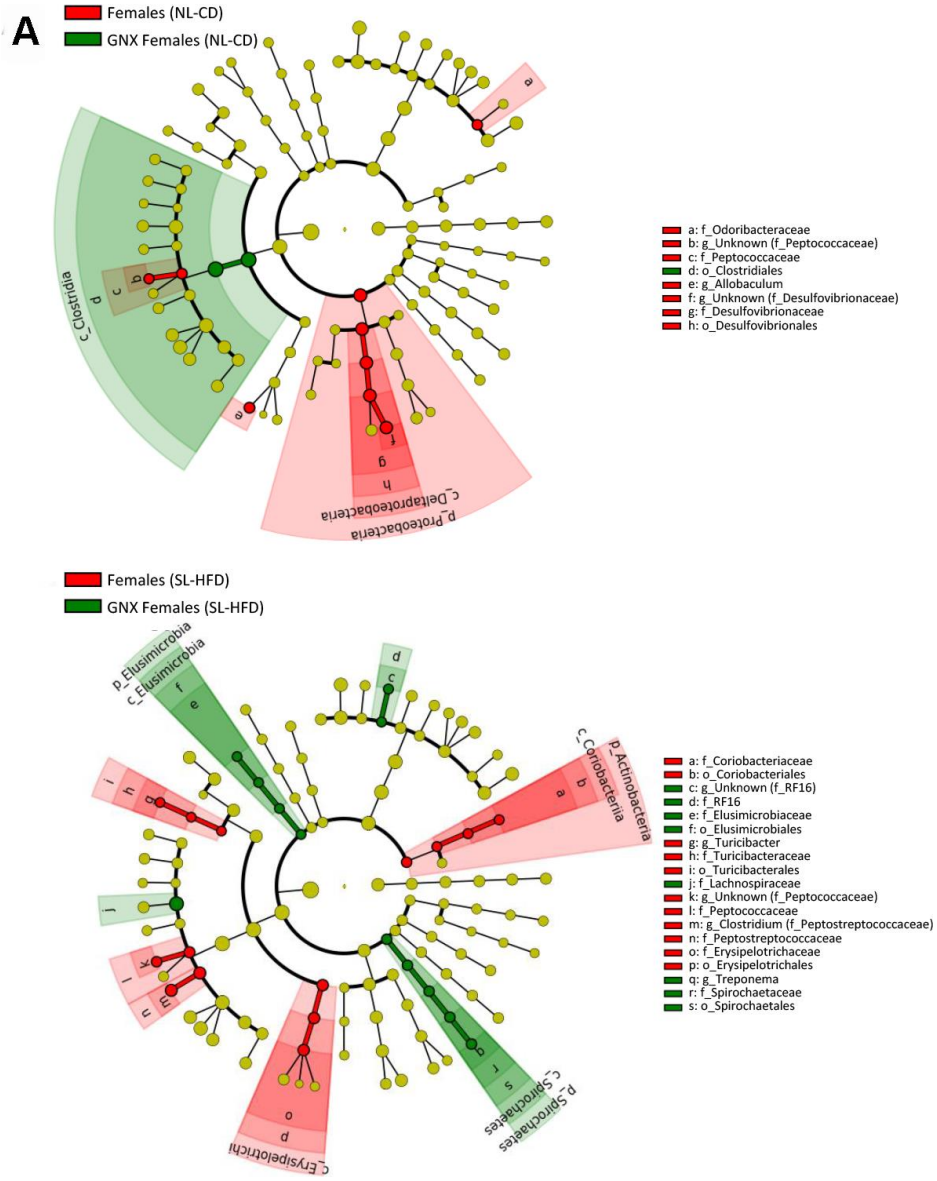
DISCUSSION

Our study documents that many of the differences in the gut microbiota found between males and females, both under normal and overfeeding conditions, persisted after gonadectomy. However, removing the sex hormones shaped several gut microbiota features towards a more deleterious profile, especially in

females, mainly when animals were subjected to postnatal overfeeding. In addition, our study also shows that overnutrition in females significantly increased *F/B* ratio as compared with males.

Previous observations in humans showed that the *F/B* ratio, which is of major importance in the development of obesity as it increases in this condition [19], is higher in women than in men under obesity conditions [9], and increases in women after menopause [15]. Consistent with this, our study showed that the *F/B* ratio was higher in females than in males subjected to postnatal overfeeding, both in intact and GNX animals, even taking into account that the gonadectomy of males, as previously shown in mice [20], increased the *F/B* ratio. In line with this, our study showed that this increase was proportional to the prevailing T levels, being higher in normal fed animals than in postnatal overfed males, which showed a decline of endogenous T levels due to obesity [21].

By contrast to males, in which no changes in BW were observed after gonadectomy, in females, ovariectomy caused an increase in BW in parallel with the rise in leptin levels. This observation may be explained on the basis of the anti-obesity effect of estrogens through decreasing food intake and increasing energy expenditure [22]. In fact, animal studies have shown that while females are relatively resistant to diet-induced obesity, ovariectomy reverses this protective effect [23], whereas estrogens protect ovariectomized females from obesity [24].



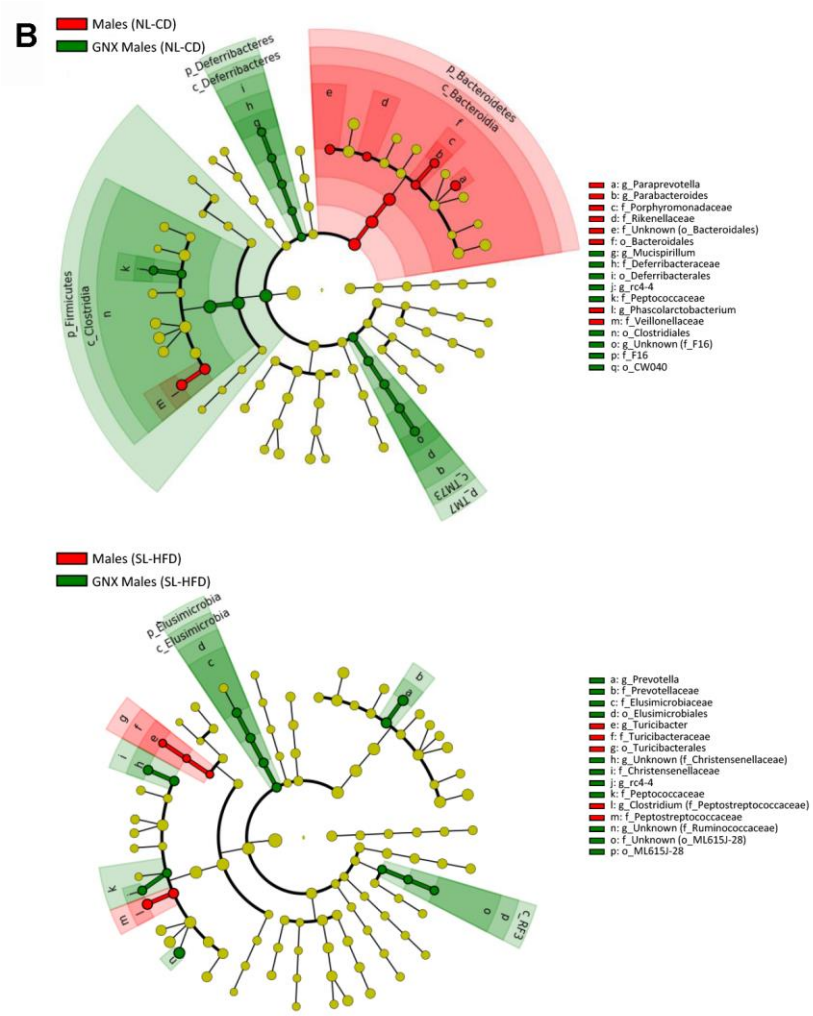


Figure 3. LEfSe analysis between intact and gonadectomized animals under normal feeding and overfeeding conditions in females (**A**) and males (**B**). Cladogram representing the taxonomic hierarchical structure of the identified differences between genders using Linear discriminant analysis effect size (LEfSe). Each filled circle represents one phylotype. Red denotes bacterial taxa statistically overrepresented in intact animals; green denotes bacterial taxa overrepresented in gonadectomized animals. Phylum and class are indicated by their names on the cladogram and the order, family, or genus are given in the key.

It has been proposed that the gender differences in the incidence during adulthood of cardiometabolic diseases - a set of interrelated cardiovascular and metabolic diseases - may be explained, at least partially, by sex-specific effects of dietary factors during early stages of life, in addition to maternal conditions in the uterus [25]. Herein, we show that the postnatal overfeeding (continued with an obesogenic diet after weaning) of females had a discernible impact on the *F/B* ratio, a phenomenon that was not observed in males. This contrasts with previous observations from studies in animal models, mostly performed only in males, that showed an obese microbiota pattern characterized by a high *F/B* ratio [19]. Thus, our study suggests that persistent overnutrition since lactation may have a durable influence on the sensitivity of gut microbiota to diet-induced changes in the adulthood. This idea is supported by the fact that obesity in childhood, which is associated with a higher risk of obesity in adulthood [26], is linked to alterations in gut microbiota at an early age [27]. Moreover, the influence of postnatal overfeeding in shaping gut microbiota in females, but not in males, may also help to explain the inconsistent results surrounding changes in *F/B* ratio in several studies in humans, as the period of life in which overfeeding triggered obesity seems to be important for determining gut microbiota dysbiosis. In fact, while several studies have shown an increased *F/B* ratio in obesity [28, 29], others did not confirm these observations [30], or even showed that this ratio was reduced in obese subjects [31].

Moreover, postnatal overfeeding and gonadectomy also impacted differentially on several bacterial taxa at lower hierarchical levels. In relation to metabolic disease, our study showed that the lower abundance of *Bacteroides* genus and *Prevotellaceae* family in females as compared with males, which has been associated to metabolic syndrome in humans [32], disappeared after gonadectomy under normal feeding conditions, but not under postnatal overfeeding conditions. In addition, we also observed a higher abundance of *Clostridiaceae* family in females after gonadectomy under normal feeding conditions; this family is also related with metabolic syndrome in humans [33].

All together, these alterations in gut microbiota suggest a higher impact of GNX in females when animals were postnatally overfed, a phenomenon which is consistent with previous observations in humans, in which the differences in the gut microbiota between men and postmenopausal women are influenced by the grade of obesity [9, 15]. In addition, the combination of both overfeeding and sex steroid removal by gonadectomy seems to have a more deleterious effect in females than in males, as suggested by the abundance of two SCFA-producing bacterial genera, *Butyricimonas* and *Roseburia*. In fact, the lower abundance of these bacterial genera in GNX females under postnatal overfeeding supports the idea that the microbiota in males, but presumably not in females, is able to adapt itself when it is exposed to high caloric supply early on life, and is able to maintain a higher SCFA production than in females. This, therefore, may impact differentially on disease predisposition between genders, and might also affect disease incidence. In fact, it has been described that metabolic diseases increase after menopause in women in parallel with estrogen depletion [5], which is also related with gender differences in fat distribution [34].

We also explored whether the dialogue, or cross-talk, between gut microbiota and host organism in response to changes in sex hormones and nutritional status can

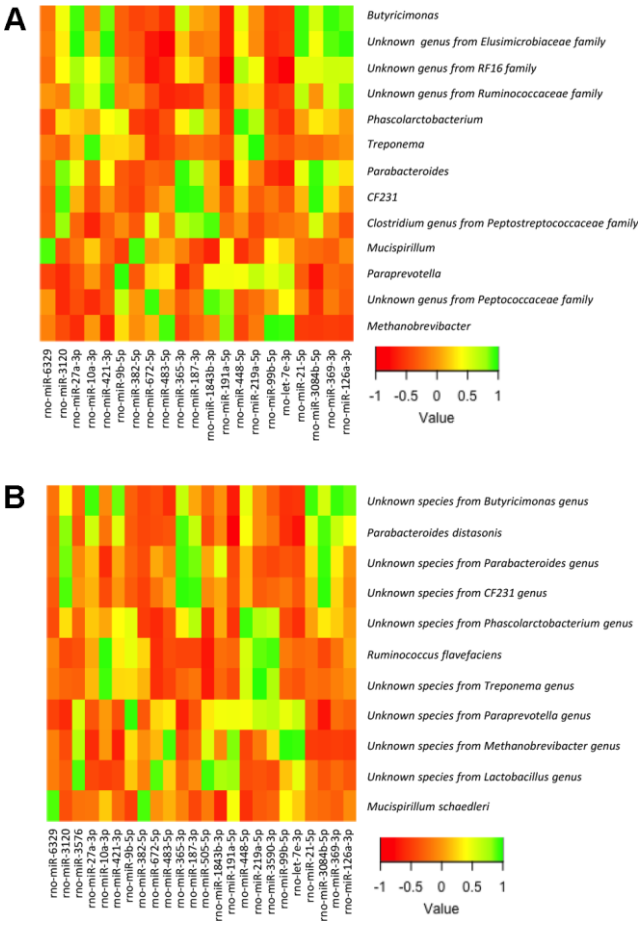


Figure 4. Heatmap from the Pearson's correlation coefficient between the bacterial genera (**A**) and species (**B**) identified by LEfSe analysis and the expression levels of the miRNAs in the large intestine.

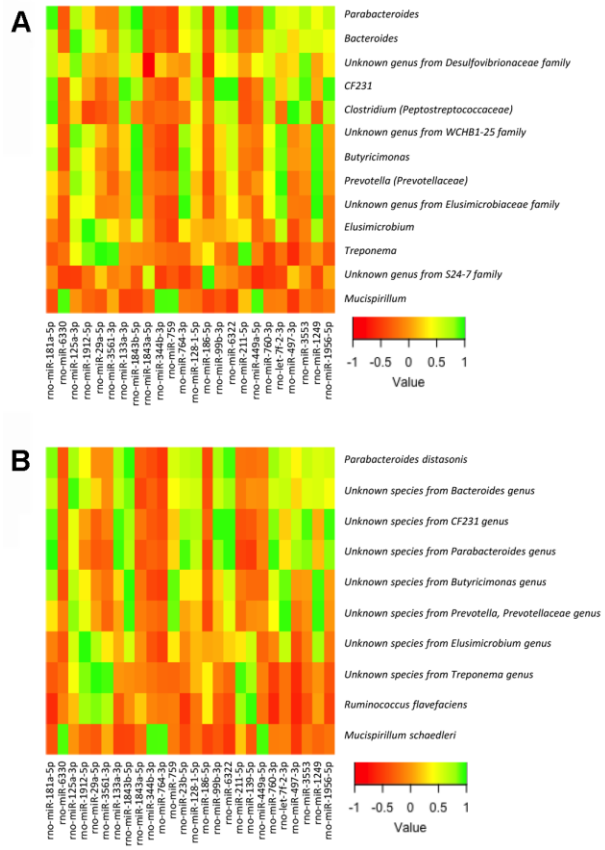


Figure 5. Heatmap from the Pearson's correlation coefficient between the bacterial genera (**A**) and species (**B**) identified by LEfSe analysis and the expression levels of the miRNAs in the small intestine.

take place through regulation of miRNA expression in the small and large intestines, which is increasingly recognized as transmitters or decoders of dysbiosis into cardiometabolic diseases [35, 36]. Based on KEGG pathways, our study identified miR-23b-5p and miR-186-5p, expressed in the small intestine, as potential modulators of steroid biosynthesis, in response to gut microbiota changes. In fact, we found a relationship in terms of abundance-expression of these miRNAs with an unknown bacterial species from the *Parabacteroides* genus (in the case of miR-23b-5p) and with an unknown genus from the *S24-7* family (miR-186-5p). These findings point out that these bacterial taxa might be related in modulating steroid biosynthesis. In addition, the expression in small intestine of another two miRNAs, miR-181a-5p and miR-139-5p, both involved in the estrogen signaling pathway, was related with the intestinal abundance of *Parabacteroides* and *Clostridium* (from *Peptostreptococcaceae* family) in the first case, and with *Ruminococcus flavefaciens* in the second.

We also identified platelet activation as one of the pathways that may be modulated by gut microbiota-miRNAs cross-talk in response to sex steroid-related alterations. In fact, it was recently shown that T reduces platelet activation in elderly people [37]. Taking into account the decline in T seen in aging [6], a potential role of the gut microbiota through miRNA actions

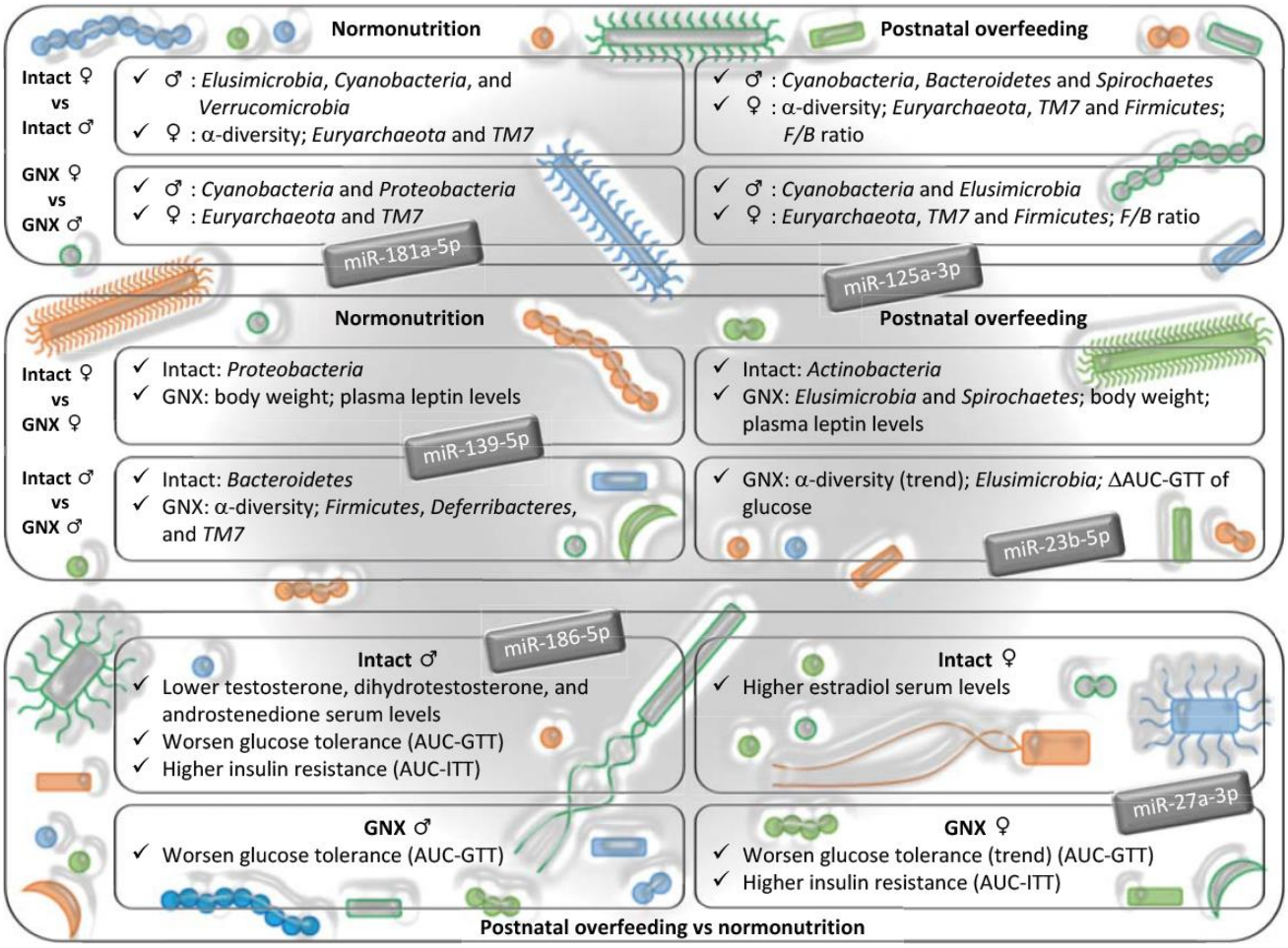


Figure 6. Impact of gonadal hormone depletion, alone or combined with postnatal overfeeding, on the sex-differences in gut microbiota, subsequent metabolic alterations and potential miRNAs involved. Upper panel: gender differences in intact and gonadectomized animals. GNX, gonadectomized animals. The bacterial taxa indicated are more abundant in the gender shown by the symbol. Intermediate panel: impact of depletion of gonadal hormones. The variables indicated are more abundant in the animal model shown (intact or GNX animals). ΔAUC, delta area under the curve. GTT, glucose tolerance test. Lower panel: effect of postnatal overfeeding on intact and gonadectomized animals (in this panel, text refers to effect found in postnatal overfeeding as compared with normonutrition). ITT, insulin tolerance test. miRNAs shown are putatively involved in the dialogue between gut microbiota and host organism in response to changes in sex hormones and nutritional status, and related with the insulin signaling pathway, steroid biosynthesis, the estrogen signaling pathway, adherens junctions and progesterone-mediated oocyte maturation.

inducing changes in blood platelets might be suggested. This idea is also supported by the previously described aging-induced changes in the gut microbiota [38].

Moreover, our study showed that gut microbiota-miRNAs cross-talk may also influence the intestinal barrier integrity through modulation of adherens junctions, which, together with the tight junction, provide important adhesive contacts between epithelial cells, and are involved in intestinal barrier permeability [39]. However, this potential mechanism would be complementary to the direct effect through bacterial species involved in the stability of the mucosal layer [40]. In addition, diet may also exert its effect through the cross-talk between gut microbiota and the intestinal expression of miRNAs, as evidenced by the relationship between miR-125a-3p, involved in adherens junctions, and the abundance of *Bacteroides* in the small intestine, associated to a meat-rich diet, as most of the species are bile acid resistant [41].

Intestinal absorption of bacterial components, such as endotoxin lipopolysaccharide, induces inflammation through toll-like receptor activation, which may promote insulin resistance [42]. In line with this, our study also showed the relationship between the intestinal expression of the insulin signaling-related miR-27a-3p, and the abundance of *Butyricimonas* in the large intestine. Moreover, this bacterial genus is a butyrate-producer [43], which may also be involved in insulin signaling, as SCFA increases the action and release of insulin through intestinal incretins [44, 45]. Furthermore, SCFA are also involved in energy metabolism and appetite regulation [46], which may be partially responsible of the weight gain in females after GNX, a condition in which the abundance of this genus is higher in males than in females (GNX, SL-HFD). Additionally, miR-27a-3p is also involved in mediating sex-steroid actions in other tissues, such as progesterone-mediated oocyte maturation, therefore supporting the view that the cross-talk between gut microbiota and the host via specific miRNAs may also involve gonadal steroid mediated events. This idea is also supported by the relationship found between miR-181a-5p, related with the estrogen signaling pathway, and the abundance of *Parabacteroides* in the small intestine, a genus associated to sulphate assimilation but also a producer of SCFA [47]. Overall, our results support the idea that gut microbiota-miRNA cross-talk may serve as decoder of changes in the gut microbiota composition into the host metabolism, in line with previous data [35, 36].

In conclusion, our study documents the contribution of gonadal hormones to defining sex-dependent differences on gut microbiota, and discloses a potential role of gonadal hormones in shaping gut microbiota, as consequence of the interaction between sex and nutrition (Figure 6). Thus, the development of therapies aimed at restoring gut microbiota alterations in elderly people, in order to reduce the risk of diseases such as CVD, should be gender-specific. Our data suggest that the changes in gut microbiota, observed in conditions of sex hormone decline, such as those caused by ageing in men and menopause in women, may exert different effects on the host organism, which are putatively mediated by gut microbiota-miRNA cross-talk.

MATERIALS AND METHODS

Animals and diets

Wistar male and female rats bred in the vivarium of the University of Cordoba were used. The animals were maintained at 22 ± 1°C under constant conditions of light (14 hours) with free access to water. The experimental animals were fed a control diet (CD), D12450B (10%, 20%, and 70% calories from fat, protein, and carbohydrate, respectively), or a high-fat diet (HFD), D12451 (45%, 20%, and 35% calories from fat, protein and carbohydrate, respectively; Research Diets Inc., New Brunswick, NJ, USA). All the experimental protocols were approved by Cordoba University Ethical Committee for animal experimentation and conducted in accordance with the European Union guidelines for use of experimental animals.

Experimental design

On postnatal day (PND)-1, pups were cross-fostered and reared in two different litter sizes: small litters (SLs) (4 pups per litter; as a model of postnatal overnutrition) or normal litters (NLs) (12 pups per litter), as extensively described previously [48–50]. The animals were weaned at PND-23 and housed in groups of four or five rats per cage. From weaning onwards, subgroups of NL and SL females and males were fed CD or HFD *ad libitum*, respectively; thus, two experimental groups (NL-CD and SL-HFD) were generated, representative of the lean and obese phenotype, respectively. On PND-90, subsets of animals from each group were subjected to gonadectomy, via bilateral abdominal approach in the case of females, or via scrotal route in case of males, as a model of cessation of gonadal secretions. At PND-120, the animals were subjected to a GTT, and one week later to an ITT to assess the development of insulin resistance in the different experimental models.

Experiments were terminated at PND-150, both in gonadal-intact and GNX animals; the latter, 60 days after surgical removal of the gonads. At this stage,

phenotypic indices and serum biochemical/hormonal parameters were monitored; sampling in the groups of intact females was carried out at the same stage of the ovarian cycle, namely, diestrus-1. Rats were euthanized by decapitation and trunk blood was collected for analyses. Additionally, sections of small and large intestine were dissected and fecal samples were obtained from the different study groups directly from stool expulsion stimulated by manual handling. Samples were frozen in liquid nitrogen and stored at -80 °C until analysis.

Phenotypic indices and hormonal measurements

Terminal BW was monitored on PND-150 intact and GNX rats. Glucose concentrations were measured in blood samples, taken from the experimental animals at PND-120 after overnight fasting. In PND-150, serum levels of leptin were assayed by double-antibody RIA, using the kit provided by EMD MILLIPORE (St. Charles, MO, USA). The sensitivity limit of the assay was 0.8 ng/mL, and the intra- and inter-assay coefficients of variation were less than 4% and 9%, respectively. In addition, in intact animals of both experimental groups (NL-CD and SL-HFD), sex steroid plasma levels were determined using a thoroughly validated, sensitive gas chromatography-tandem mass spectrometry method, in keeping with previous references [51, 52]. Next, the serum levels of T, dihydrotestosterone, androstenedione, progesterone, E_1 and E_2 were measured. The lowest levels of quantification in the assay were: 8 pg/mL for T, 2.5 pg/mL for dihydrotestosterone, 12 pg/mL for androstenedione, 74 pg/mL for progesterone, and 0.5 pg/mL for E_1 and E_2, in line with previous references [51, 52].

Glucose tolerance tests and insulin tolerance tests

To assess glucose handling in all the experimental groups, the rats were subjected to GTT on approximately PND-120. The rats were fasted overnight and subsequently received an intraperitoneal (ip) bolus of glucose (1 g/kg BW). Blood glucose levels were determined before (0) and at 20, 60, and 120 minutes post administration. After complete recovery, one week later, insulin sensitivity was assessed using ITT. For this, the rats were fasted overnight, followed by an ip injection of 1UI insulin (Sigma-Aldrich, St. Louis, MO) per kg BW. Blood glucose levels were measured before (0) and at 20, 60, and 120 minutes after insulin administration. Integral glucose changes and net increases in integral glucose levels were estimated as area under the curve (AUC) and delta area under the curve (ΔAUC), respectively, during the 120 min period after the glucose or insulin administration, as calculated by the trapezoidal method. All glucose concentrations were measured using a handheld glucometer (ACCU-CHECK Aviva; Roche Diagnostics).

Intestinal microbiota analysis

DNA extraction from feces was performed using the QIAamp DNAStool Mini Kit Handbook (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The microbiota composition analysis of the fecal samples was performed on a MiSeq Illumina platform (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Briefly, polymerase chain reaction (PCR) was performed using 0.2 μM of the primer 5'-TCGTCGGCAGCGTCAGATGTG TATAAGAGACAG-3' and 5'-GTCTCGTGGGCTC GGAGATGTGTATAAGAGACAG-3' [53] to generate amplicons containing the hypervariable region V3 of the 16s rRNA gene. KAPA HiFi HotStart ReadyMix (KAPABIOSYSTEMS) and 1.25 μl of extracted DNA (5 ng/μl in 10 mM Tris pH8.5) were used with the following PCR parameters: 3 minutes denaturation at 95°C, followed by 25 cycles (30 s at 95°C, 30 s at 60°C, 30 s at 72°C) and a final extension at 72°C for 5 min. The amplicon purification was performed using Agentcourt AMPure XP beads (Beckman Coulter). A second PCR reaction attaches dual indices and Illumina sequencing adapters. For this, the Nextera XT Index Kit was used. This PCR was performed with a KAPA HiFi HotStart ReadyMix (KAPABIOSYSTEMS), 5 μl of the previous amplicon, 5 μl of each Nextera XT Index Primer 1(N7xx) and 5 uL of each Nextera XT Index Primer 2(S5xx), with the following cycle parameters: 3 minutes denaturation at 95°C, followed by 8 cycles (30 s at 95°C, 30 s at 55°C, 30 s at 72°C), and a final extension at 72°C for 5 min. The PCR product purification was performed using Agentcourt AMPure XP beads (Beckman Coulter). Sequencing data were analyzed and visualised using QIIME 2 v. 2019.7 [54]. Demultiplexed single-end reads containing V3 hypervariable region were truncated at 212 bp (Quality score median >30), and denoised using the DADA2 method [55]. After filtering, the high-quality reads of the 64 samples (n = 8 for each group) ranging from 224,029 to 18,682 sequence counts were taken, with the rarefaction depth established at 18,500 sequence counts. Bacterial α-diversity across the samples was calculated using the observed OTUs and Shannon indexes [56]. Principal component analysis of community structure (beta-diversity) was performed using the unweighted and weighted UniFrac distance metrics [57] and analyzed by permutational multivariate analysis of variance (PERMANOVA). Taxonomy was assigned to the high-quality reads using q2-feature-classifier [58] with a sequence identity threshold of 99%, interrogating the sequences with the Greengenes database (13_8) [59]. To be consistent with the taxonomic data obtained from 16S

rRNA, only taxa in the bacteria domain were included in the statistical analysis. The relative taxonomic abundance was measured as the proportion of reads over the total in each sample assigned to a given taxonomy. To exclude bacterial taxa that were not present in the majority of samples, a cut-off for exclusion was fixed; only bacterial taxa containing sequence reads in at least 75% of total samples were considered. Linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.harvard.edu/galaxy/) was used to compare groups at baseline and visualize the results using taxonomic bar charts and cladograms [60].

RNA isolation from the small and large intestine

Frozen tissue was ground to a fine powder in liquid nitrogen, using a mortar and pestle. RNA was isolated with the commercial kit Direct-zol™ RNA MiniPrep Plus (Zymo Research Corp., CA, USA, and quantified using the v3.5.2 Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Cambridge, UK).

miRNA expression analysis

miRNA expression profiles were generated using the SurePrint Rat miRNA Microarrays (Rat miRNA 8x15K Microarray, Release 21.0, Agilent Technologies Inc., Santa Clara, CA, USA). RNA samples of each experimental group were pooled and labeled using the miRNA Labeling and Hyb Kit (Agilent Technologies Inc.), according to the manufacturer's instructions. Hybridization was performed using this latter kit, also according to the manufacturer's instructions. Microarray images of each slide were obtained with a Gene Pix 4000B scanner (Axon Instruments, Union City, CA, USA). Image quantization was performed using Agilent Feature Extraction Software (Agilent Technologies Inc.). Raw microarray data were analyzed using the limma R package [61]. Spots with foreground mean and median differing by more than 50 were filtered out and data quality was checked using limma tools. Background correction was performed using saddle-point approximation in the normal-exponential convolution method Normexp [62]. Next, within arrays Print-tip loess [63] and between arrays quantile were used for normalization. Finally, replicate spots in the array data were averaged.

Software for miRNA analysis

To identify the role of selected miRNAs in the cellular processes, we performed an analysis using the DIANAtools V.3. DIANA-miRPath is a web-server (http://diana.imis.athena-innovation.gr/DianaTools/index.php), which provides accurate statistics and can accommodate advanced pipelines. DIANA-miRPath can utilize predicted miRNA targets (in CDS or 3'-UTR regions) provided by the DIANA-microT-CDS algorithm or even experimentally validated miRNA interactions derived from DIANA-TarBase [18].

Statistical analysis

The PASW statistical software package, version 20.0 (IBM Inc., Chicago, IL, USA), was used for statistical analysis of the data. We used One-way ANOVA to test the differences in the plasma metabolites between groups of animals. Pearson's correlation test was used to evaluate the relationship between miRNA intestinal expression and bacterial taxa abundance. Data are presented as mean ± standard error of the mean. P-values <0.05 were considered statistically significant in all the statistical analyses.

Abbreviations

CVD: cardiovascular diseases; T: testosterone; F/B: *Firmicutes/Bacteroidetes*; SCFA: short chain fatty acids; NL-CD: normal feeding condition; SL-HFD: postnatal overfeeding; GNX: gonadectomized; E_2: estradiol; E_1: estrone; AUC: area under the curve; ΔAUC: delta area under the curve; GTT: glucose tolerance test; ITT: insulin tolerance test; LEfSe: Linear discriminant analysis (LDA) effect size; CD: control diet; HFD: high-fat diet; PND: postnatal day; SL: small litter; NL: normal litter; BW: body weight.

AUTHOR CONTRIBUTIONS

JAS-M, AB, OAR-Z, and CP-L conducted the experiments. JAS-M and AB wrote the original draft. CH, MAS-G and HM-A performed the statistical analysis. CO, PP-M, MP JL-M and FP-J supervised the statistical analysis, and reviewed and edited the manuscript. MT-S and AC designed and supervised the study, and reviewed and edited the manuscript.

ACKNOWLEDGMENTS

The CIBEROBN is an initiative of the Instituto de Salud Carlos III, Madrid, Spain.

CONFLICTS OF INTEREST

None of the authors has any conflict of interest that could affect the performance of the work or the interpretation of the data.

FUNDING

This work was supported by research grants from Ministerio de Economia y Competitividad (CP14/00114

to A.C.; PIE14/00005 to JLM and MTS; AGL2015/-67896-P to JL-M and AC); Ministerio de Ciencia e Innovacion (FIS PI19/00299 to AC; DTS19/00007 to AC; PI16/01777 to PPM); Fondo Europeo de Desarrollo Regional (FEDER). A.C. is supported by an ISCIII research contract, Instituto de Salud Carlos III (Programa Miguel-Servet CP14/00114 and CPII19/00007).

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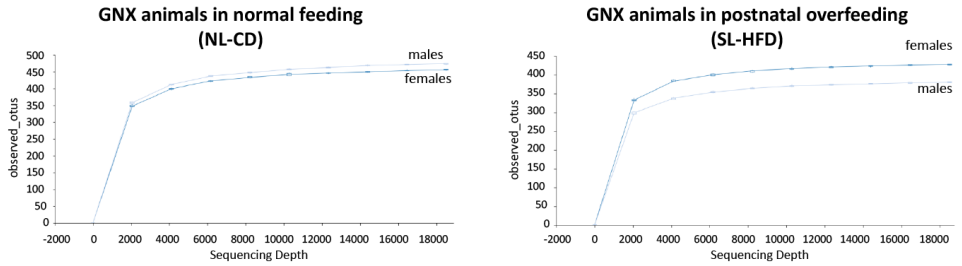
SUPPLEMENTARY MATERIALS

Supplementary Figures

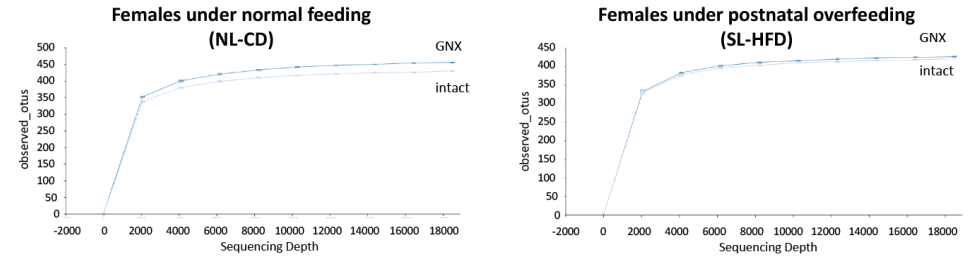
A **Sex effect on alpha diversity.**



	Observed_otus	Shannon
GNX animals in normal feeding	0.495	0.834
GNX animals in postnatal overfeeding	0.294	0.753
Intact animals in normal feeding	0.027	0.016
Intact animals in postnatal overfeeding	0.021	0.027

Kruskal-Wallis statistical analysis p-values.

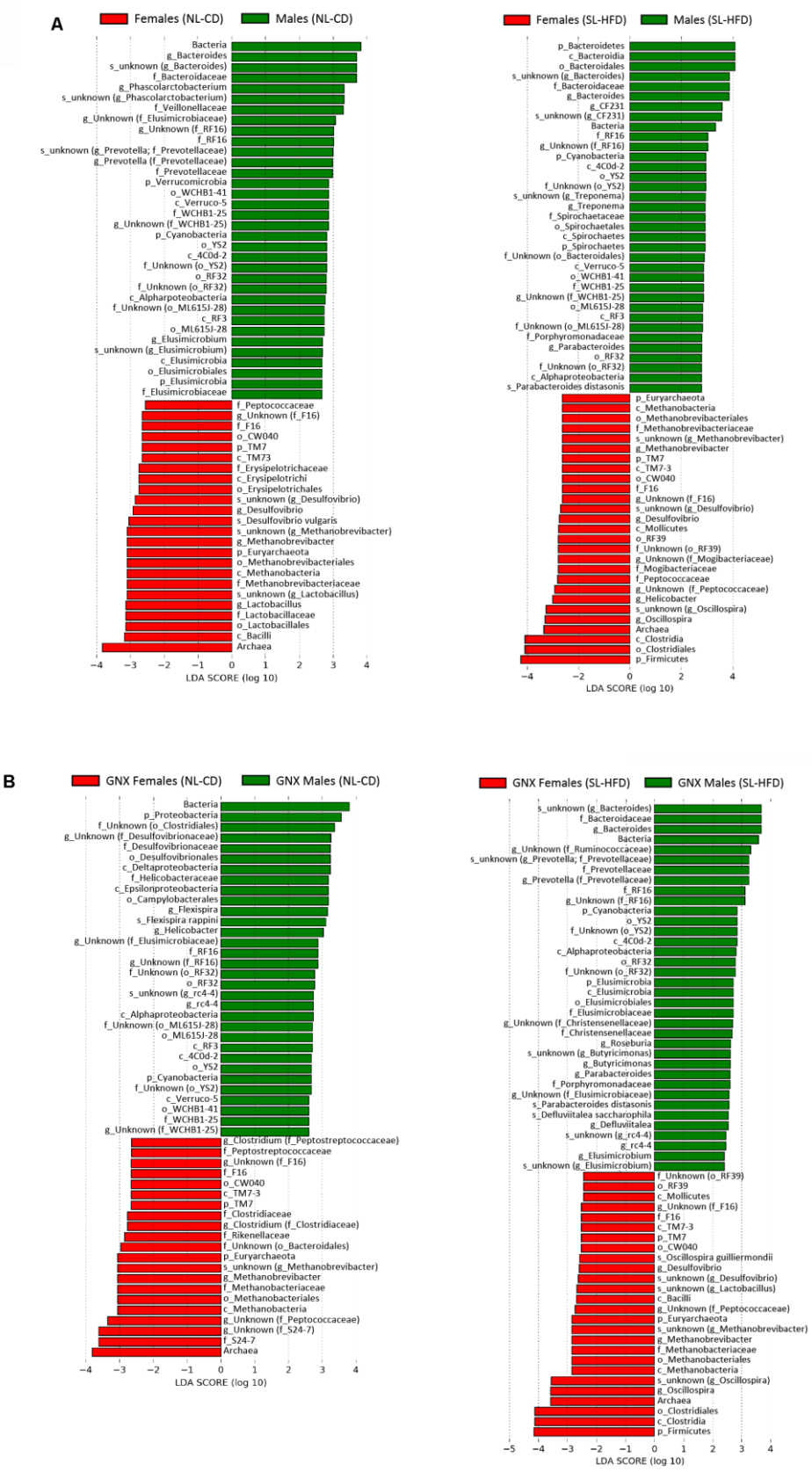
B **Gonadectomy effect on alpha diversity.**



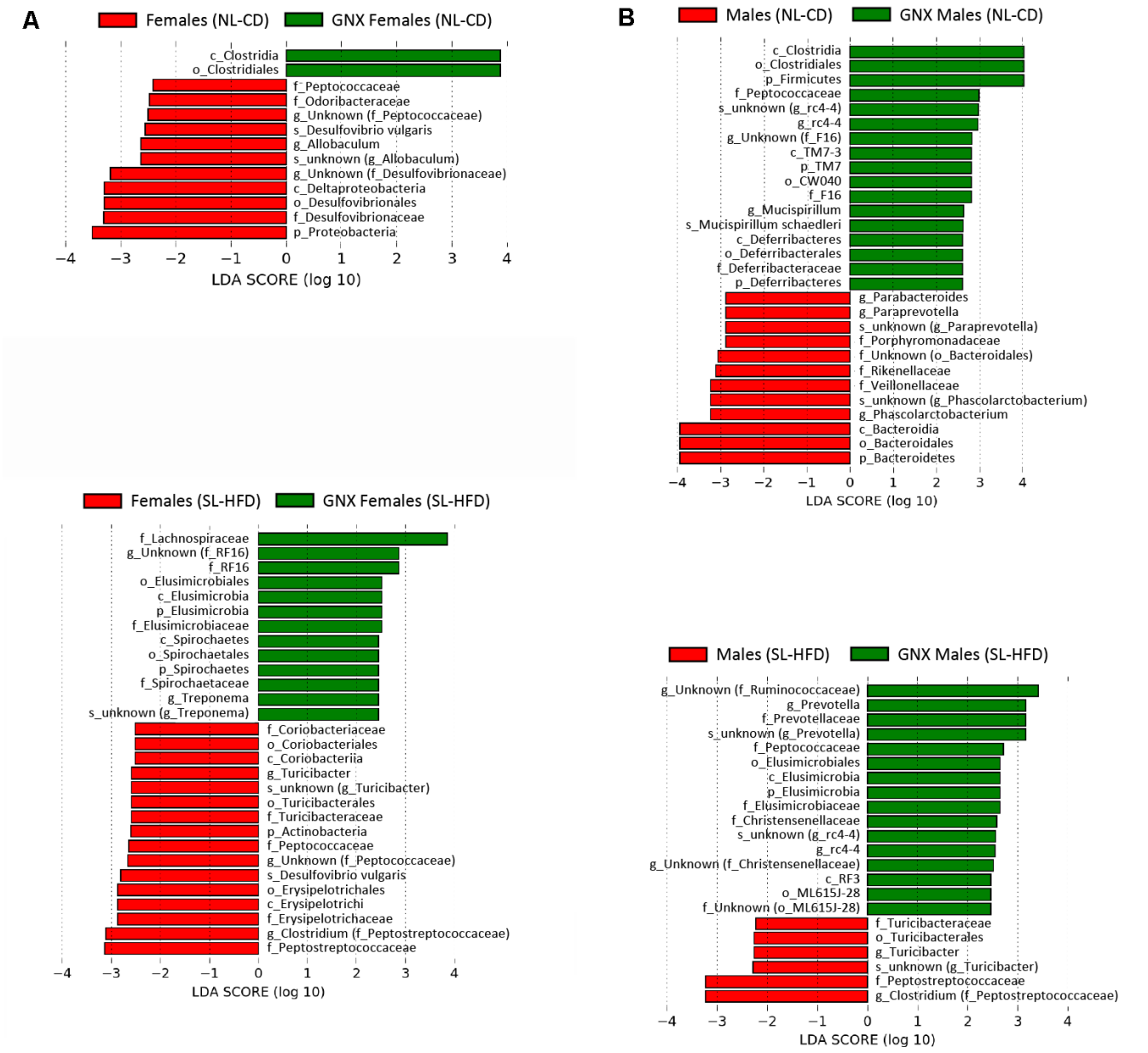
	Observed_otus	Shannon
Females under normal feeding	0.600	0.916
Females under postnatal overfeeding	1.000	0.208
Males under normal feeding	0.046	0.027
Males under postnatal overfeeding	0.059	0.074

Kruskal-Wallis statistical analysis p-values.

Supplementary Figure 1. Diversity indexes according to sex, gonadectomy and feeding conditions. (**A**) Sex effect on alpha diversity; (**B**) Gonadectomy effect on alpha diversity.



Supplementary Figure 2. Linear discriminant analysis (LDA) scores between sexes under normal feeding and overfeeding conditions, in intact (**A**) and gonadectomized (**B**) animals.



Supplementary Figure 3. Linear discriminant analysis (LDA) scores between intact and gonadectomized animals, under normal feeding and overfeeding conditions in females (**A**) and males (**B**).

Supplementary Tables

Please browse Full Text version to see the data of Supplementary Tables 2 to 7.

Supplementary Table 1. Bacterial genera with different abundance between sexes in intact and gonadectomized animals.

	Normal feeding		*Postnatal overfeeding*	
	non-GNX	***GNX***	***non-GNX***	***GNX***
Methanobrevibacter	F	F	F	F
Bacteroides	M		M	M
Parabacteroides			M	M
Prevotella (Prevotellaceae)	M			M
Unknown (f_RF16)	M	M	M	M
Unknown (f_S24-7)		F		
Butyricimonas				M
CF231			M	
Unknown (Elusimicrobiaceae)	M	M		M
Elusimicrobium	M			M
Lactobacillus	F			
Unknown (Christensenellaceae)				M
Clostridium (Clostridiaceae)		F		
Roseburia				M
Unknown (Peptococcaceae)		F	F	F
rc4-4		M		M
Clostridium (Peptostreptococcaceae)		F		
Unknown (Ruminococcaceae)				M
Oscillospira			F	F
Phascolarctobacterium	M			
Unknown (Mogibacteriaceae)			F	
Unknown (Desulfovibrionaceae)		M		
Desulfovibrio	F		F	F
Flexispira		M		
Helicobacter		M	F	
Treponema			M	
Unknown (f_F16)	F	F	F	F
Unknown (f_WCHB1-25)	M	M	M	
Defluviitalea				M

GNX: gonadectomized animals. Non-GNX: intact animals. M: higher abundance in males. F: higher abundance in females.

Supplementary Table 2. Relationship between the bacterial genera identified by LEfSe analysis and the expression levels of the miRNAs in large intestine.

Supplementary Table 3. Relationship between the bacterial species identified by LEfSe analysis and the expression levels of the miRNAs in large intestine.

Supplementary Table 4. Relationship between the bacterial genera identified by LEfSe analysis and the expression levels of the miRNAs in small intestine.

Supplementary Table 5. Relationship between the bacterial species identified by LEfSe analysis and the expression levels of the miRNAs in small intestine.

Supplementary Table 6. KEGG pathways related with miRNAs expression in the intestine.

Supplementary Table 7. KEGG pathways associated with microbiome-related miRNAs.

Supplementary Table 2. Relationship between the bacterial genera identified by LEfSe analysis and the expression levels of the miRNAs in large intestine. Pearson's correlation analysis coefficient (Corr.) and P-value.

		rno-miR-6329	rno-miR-3120	rno-miR-27a-3p	rno-miR-10a-3p	rno-miR-421-3p	rno-miR-9b-5p	rno-miR-382-5p	rno-miR-672-5p	rno-miR-483-5p	rno-miR-365-3p	rno-miR-187-3p	rno-miR-1843b-3p	rno-miR-191a-5p	rno-miR-448-5p	rno-miR-219a-5p	rno-miR-99b-5p	rno-let-7e-3p	rno-miR-21-5p	rno-miR-3084b-5p	rno-miR-369-3p	rno-miR-126a-3p
Methanobrevibacter	Corr.	-0.129	-0.408	-0.587	-0.006	-0.658	0.268	-0.043	0.127	**0.912**	-0.432	-0.245	-0.089	0.838	-0.451	-0.158	**0.956**	**0.931**	-0.495	-0.520	-0.488	-0.519
	p-value	0.782	0.363	0.126	0.990	0.108	0.560	0.927	0.786	**0.004**	0.332	0.597	0.850	0.019	0.309	0.735	**0.001**	**0.002**	0.212	0.232	0.220	0.232
Parabacteroides	Corr.	-0.250	0.845	0.486	-0.323	0.402	-0.330	-0.434	-0.246	-0.313	**0.929**	0.711	0.078	-0.688	0.337	-0.134	-0.561	-0.668	0.481	**0.932**	0.568	0.288
	p-value	0.588	0.017	0.222	0.479	0.371	0.470	0.331	0.596	0.495	**0.002**	0.073	0.867	0.088	0.461	0.775	0.190	0.101	0.228	**0.002**	0.142	0.532
Unknown (f_RF16)	Corr.	-0.230	0.398	0.750	0.381	0.836	-0.085	-0.299	-0.721	-0.615	0.305	0.170	-0.250	**-0.917**	0.714	0.451	-0.666	-0.801	0.519	0.515	0.600	0.586
	p-value	0.619	0.376	0.032	0.399	0.019	0.856	0.515	0.068	0.142	0.507	0.716	0.588	**0.004**	0.072	0.310	0.102	0.031	0.187	0.237	0.116	0.167
Butyricimonas	Corr.	-0.200	0.456	**0.921**	-0.075	0.863	-0.300	-0.448	-0.384	-0.584	0.634	0.058	-0.039	-0.681	0.496	-0.041	-0.565	-0.543	**0.925**	0.576	**0.935**	0.867
	p-value	0.667	0.304	**0.001**	0.874	0.012	0.513	0.313	0.395	0.168	0.126	0.901	0.934	0.092	0.257	0.930	0.186	0.208	**0.001**	0.176	**0.001**	0.011
CF231	Corr.	-0.339	**0.904**	0.106	-0.414	-0.016	-0.314	-0.471	-0.175	0.034	**0.953**	**0.922**	0.059	-0.387	0.130	-0.311	-0.170	-0.351	0.125	**0.950**	0.241	-0.086
	p-value	0.458	**0.005**	0.803	0.356	0.973	0.493	0.286	0.707	0.943	**0.001**	**0.003**	0.899	0.391	0.782	0.497	0.716	0.440	0.768	**0.001**	0.565	0.854
Paraprevotella	Corr.	-0.464	-0.605	-0.361	0.007	-0.386	**0.946**	-0.342	0.245	0.310	-0.644	-0.361	0.448	0.449	0.434	0.640	0.488	0.621	-0.320	-0.721	-0.225	-0.301
	p-value	0.294	0.150	0.380	0.988	0.392	**0.001**	0.453	0.597	0.498	0.119	0.426	0.313	0.312	0.331	0.121	0.267	0.137	0.439	0.068	0.592	0.511
Mucispirillum	Corr.	**0.903**	-0.388	-0.173	0.201	-0.236	-0.477	**0.909**	-0.035	0.102	-0.115	-0.440	-0.639	0.437	-0.561	-0.330	0.374	0.216	-0.184	-0.241	-0.308	-0.064
	p-value	**0.005**	0.389	0.681	0.665	0.611	0.279	**0.005**	0.941	0.827	0.806	0.323	0.123	0.327	0.190	0.469	0.408	0.642	0.662	0.603	0.458	0.892
Unknown (Elusimicrobiaceae)	Corr.	-0.161	0.332	**0.982**	0.117	**0.968**	-0.303	-0.357	-0.692	-0.773	0.188	-0.153	-0.105	-0.685	0.365	-0.066	-0.588	-0.518	**0.922**	0.440	0.888	**0.952**
	p-value	0.761	0.467	**0.000**	0.825	**0.001**	0.560	0.487	0.128	0.072	0.722	0.773	0.842	0.090	0.477	0.902	0.219	0.292	**0.003**	0.383	0.008	**0.003**
Unknown (Peptococcaceae)	Corr.	-0.018	-0.600	-0.395	-0.588	-0.356	0.638	-0.006	**0.928**	0.036	-0.343	-0.153	0.896	0.817	-0.340	-0.204	0.023	0.393	-0.091	-0.580	-0.149	-0.325
	p-value	0.972	0.208	0.381	0.165	0.434	0.173	0.991	**0.003**	0.946	0.506	0.743	0.016	0.047	0.510	0.698	0.965	0.440	0.845	0.227	0.750	0.529
Clostridium (Peptostreptococcaceae)	Corr.	-0.318	0.812	-0.271	-0.644	-0.281	-0.070	-0.341	0.522	-0.120	0.671	0.812	**0.901**	-0.232	-0.031	-0.443	-0.245	-0.180	-0.107	0.569	-0.079	-0.381
	p-value	0.486	0.026	0.516	0.118	0.542	0.881	0.454	0.230	0.798	0.099	0.026	**0.006**	0.616	0.948	0.320	0.596	0.700	0.800	0.183	0.853	0.399

Unknown (Ruminococcaceae)	Corr.	-0.015	-0.105	0.814	0.513	**0.919**	-0.120	-0.054	-0.395	-0.644	-0.578	-0.529	-0.142	-0.596	0.199	0.250	-0.545	-0.462	0.669	-0.023	0.588	0.850
	p-value	0.974	0.823	0.014	0.239	**0.003**	0.797	0.908	0.381	0.118	0.174	0.222	0.761	0.158	0.668	0.589	0.205	0.297	0.070	0.962	0.125	0.015
Phascolarctobacterium	Corr.	-0.405	0.279	0.191	0.063	0.397	0.498	-0.499	-0.610	-0.385	0.340	0.690	0.079	-0.552	**0.926**	0.713	-0.397	-0.573	0.039	0.314	0.208	0.019
	p-value	0.368	0.544	0.651	0.892	0.378	0.256	0.254	0.146	0.394	0.455	0.086	0.866	0.199	**0.003**	0.072	0.378	0.179	0.926	0.492	0.622	0.967
Treponema	Corr.	-0.144	-0.270	0.131	**0.922**	0.252	0.258	0.119	-0.569	-0.458	-0.256	-0.179	-0.260	-0.408	0.578	**0.979**	-0.294	-0.374	-0.211	-0.239	-0.124	-0.023
	p-value	0.757	0.559	0.757	**0.003**	0.586	0.576	0.800	0.183	0.301	0.580	0.700	0.573	0.364	0.174	**0.000**	0.522	0.409	0.616	0.606	0.769	0.961

Supplementary Table 3. Relationship between the bacterial species identified by LEfSe analysis and the expression levels of the miRNAs in large intestine. Pearson's correlation analysis coefficient (Corr.) and P-value.

		rno-miR-6329	rno-miR-3120	rno-miR-3576	rno-miR-27a-3p	rno-miR-10a-3p	rno-miR-421-3p	rno-miR-9b-5p	rno-miR-382-5p	rno-miR-672-5p	rno-miR-483-5p	rno-miR-365-3p	rno-miR-187-3p	rno-miR-505-5p	rno-miR-1843b-3p	rno-miR-191a-5p	rno-miR-448-5p	rno-miR-219a-5p	rno-miR-3590-3p	rno-miR-99b-5p	rno-let-7e-3p	rno-miR-21-5p	rno-miR-3084b-5p	rno-miR-369-3p	rno-miR-126a-3p
Unknown (Methanobrevibacter)	Corr.	-0.129	-0.408	0.689	-0.587	-0.006	-0.658	0.268	-0.043	0.127	**0.912**	-0.433	-0.245	0.530	-0.089	0.838	-0.452	-0.158	0.259	**0.956**	**0.931**	-0.495	-0.520	-0.488	-0.519
	p-value	0.783	0.363	0.087	0.126	0.990	0.108	0.561	0.928	0.786	**0.004**	0.332	0.597	0.221	0.850	0.019	0.309	0.735	0.574	**0.001**	**0.002**	0.212	0.232	0.220	0.232
Unknown (Parabacteroides)	Corr.	-0.250	0.855	-0.087	0.148	-0.592	0.058	-0.279	-0.408	0.069	-0.115	**0.916**	0.853	0.063	0.504	-0.421	0.127	-0.368	-0.437	-0.357	-0.421	0.254	**0.932**	0.330	-0.025
	p-value	0.589	0.014	0.854	0.727	0.162	0.902	0.545	0.364	0.883	0.807	**0.004**	0.015	0.893	0.248	0.347	0.787	0.417	0.327	0.432	0.346	0.544	**0.002**	0.425	0.957
Parabacteroides distasonis	Corr.	-0.267	0.829	-0.352	0.587	-0.185	0.521	-0.323	-0.439	-0.400	-0.358	0.899	0.635	-0.410	-0.076	-0.768	0.420	-0.047	-0.188	-0.578	-0.724	0.531	**0.907**	0.62	0.394
	p-value	0.563	0.021	0.439	0.126	0.692	0.230	0.480	0.324	0.374	0.430	0.006	0.126	0.361	0.872	0.044	0.349	0.921	0.686	0.174	0.066	0.176	**0.005**	0.101	0.382
Unknown (Butyricimonas)	Corr.	-0.200	0.456	-0.273	**0.921**	-0.074	0.863	-0.300	-0.448	-0.384	-0.585	0.634	0.058	-0.292	-0.039	-0.681	0.496	-0.041	-0.310	-0.565	-0.543	**0.925**	0.575	**0.935**	0.867
	p-value	0.667	0.304	0.553	**0.001**	0.874	0.012	0.513	0.313	0.395	0.168	0.126	0.901	0.525	0.934	0.092	0.257	0.930	0.499	0.186	0.208	**0.001**	0.177	**0.001**	0.011
Unknown (CF231)	Corr.	-0.339	**0.904**	-0.017	0.107	-0.414	-0.015	-0.314	-0.471	-0.175	0.034	**0.953**	**0.922**	-0.080	0.060	-0.388	0.130	-0.311	-0.246	-0.170	-0.351	0.126	**0.951**	0.242	-0.086
	p-value	0.458	**0.005**	0.971	0.802	0.356	0.974	0.493	0.286	0.707	0.943	**0.001**	**0.003**	0.865	0.899	0.390	0.782	0.497	0.594	0.715	0.440	0.767	**0.001**	0.564	0.855
Unknown (Paraprevotella)	Corr.	-0.464	-0.605	0.568	-0.361	0.007	-0.386	**0.946**	-0.342	0.245	0.310	-0.644	-0.362	0.306	0.448	0.449	0.433	0.640	0.682	0.488	0.621	-0.320	-0.721	-0.225	-0.301
	p-value	0.294	0.150	0.184	0.380	0.988	0.392	**0.001**	0.453	0.596	0.498	0.118	0.426	0.504	0.313	0.312	0.332	0.122	0.091	0.267	0.136	0.439	0.068	0.592	0.511
Mucispirillum schaedleri	Corr.	**0.904**	-0.389	-0.200	-0.172	0.201	-0.235	-0.478	**0.909**	-0.033	0.101	-0.116	-0.441	-0.157	-0.638	0.435	-0.560	-0.329	-0.281	0.370	0.213	-0.183	-0.241	-0.307	-0.063
	p-value	**0.005**	0.389	0.667	0.683	0.665	0.612	0.278	**0.005**	0.943	0.830	0.805	0.322	0.737	0.123	0.329	0.191	0.471	0.542	0.414	0.646	0.664	0.602	0.460	0.893
Unknown (Lactobacillus)	Corr.	-0.133	-0.311	**0.902**	-0.431	-0.492	-0.443	0.179	-0.128	0.891	0.208	-0.276	-0.065	**0.917**	0.739	0.789	-0.399	-0.365	-0.424	0.172	0.493	-0.168	-0.371	-0.238	-0.346

	p-value	0.776	0.496	**0.005**	0.286	0.262	0.319	0.702	0.785	0.007	0.655	0.548	0.891	**0.004**	0.057	0.035	0.375	0.420	0.343	0.713	0.261	0.690	0.412	0.571	0.447
	Corr.	-0.100	-0.427	-0.383	0.068	**0.953**	0.330	0.481	0.236	-0.585	-0.394	-0.454	-0.446	-0.704	-0.222	-0.253	0.561	0.877	**0.911**	-0.142	-0.249	-0.219	-0.428	-0.186	0.070
Ruminococcus flavefaciens	p-value	0.832	0.339	0.396	0.874	**0.001**	0.469	0.275	0.611	0.168	0.382	0.306	0.316	0.077	0.632	0.583	0.190	0.009	**0.004**	0.762	0.590	0.602	0.338	0.660	0.882
	Corr.	-0.405	0.279	-0.280	0.191	0.065	0.398	0.498	-0.499	-0.610	-0.386	0.340	0.689	-0.607	0.079	-0.553	**0.926**	0.714	0.638	-0.398	-0.573	0.040	0.314	0.208	0.020
Unknown (Phascolarctobacterium)	p-value	0.368	0.545	0.543	0.650	0.890	0.376	0.256	0.254	0.145	0.392	0.456	0.087	0.148	0.866	0.198	**0.003**	0.072	0.123	0.377	0.179	0.925	0.493	0.621	0.966
	Corr.	-0.144	-0.270	-0.300	0.131	**0.922**	0.252	0.258	0.119	-0.569	-0.458	-0.256	-0.180	-0.690	-0.260	-0.408	0.578	**0.979**	0.724	-0.294	-0.373	-0.211	-0.239	-0.124	-0.023
Unknown (Treponema)	p-value	0.758	0.558	0.513	0.757	**0.003**	0.585	0.576	0.800	0.183	0.301	0.580	0.700	0.086	0.573	0.364	0.174	**<0.001**	0.066	0.522	0.409	0.616	0.605	0.769	0.962

Supplementary Table 4. Relationship between the bacterial genera identified by LEfSe analysis and the expression levels of the miRNAs in small intestine. Pearson's correlation analysis coefficient (Corr.) and P-value.

		rno-miR-181a-5p	rno-miR-6330	rno-miR-125a-3p	rno-miR-1912-5p	rno-miR-29a-5p	rno-miR-3561-3p	rno-miR-133a-3p	rno-miR-1843b-5p	rno-miR-1843a-5p	rno-miR-344b-3p	rno-miR-764-3p	rno-miR-759	rno-miR-128-1-5p	rno-miR-186-5p	rno-miR-99b-3p	rno-miR-6322	rno-miR-211-5p	rno-miR-449a-5p	rno-miR-760-3p	rno-let-7f-2-3p	rno-miR-497-3p	rno-miR-3553	rno-miR-1249	rno-miR-1956-5p
Bacteroides	Corr.	0.690	-0.277	**0.917**	0.592	0.247	-0.047	0.656	0.851	-0.446	-0.287	-0.449	0.409	0.59	-0.393	0.610	0.809	-0.159	-0.044	0.584	0.623	0.250	0.530	0.548	0.437
	p-value	0.086	0.547	**0.004**	0.122	0.593	0.920	0.109	0.015	0.268	0.533	0.265	0.363	0.123	0.383	0.108	0.015	0.734	0.925	0.169	0.135	0.589	0.221	0.203	0.279
Parabacteroides	Corr.	**0.926**	-0.334	0.705	0.261	-0.121	-0.130	0.813	**0.980**	-0.385	-0.360	-0.483	0.479	0.718	-0.460	0.783	**0.956**	-0.263	-0.133	0.851	0.523	0.489	0.713	0.452	0.659
	p-value	**0.003**	0.465	0.077	0.533	0.797	0.781	0.026	**<0.001**	0.347	0.428	0.225	0.277	0.045	0.299	0.022	**<0.001**	0.569	0.777	0.015	0.228	0.266	0.072	0.309	0.075
Prevotella	Corr.	0.291	-0.107	0.818	0.481	0.035	-0.273	0.056	**0.929**	-0.133	-0.211	-0.267	0.862	0.166	-0.438	0.151	0.485	-0.221	-0.017	0.391	**0.943**	-0.183	-0.043	**0.940**	-0.006
	p-value	0.526	0.820	0.025	0.227	0.941	0.554	0.905	**0.002**	0.753	0.649	0.522	0.013	0.695	0.325	0.721	0.223	0.635	0.971	0.386	**0.001**	0.694	0.927	**0.002**	0.989
Unknown (f_S24-7)	Corr.	-0.090	-0.504	-0.486	-0.199	0.201	-0.122	-0.032	-0.351	0.570	-0.494	-0.334	-0.513	-0.300	**0.930**	0.008	-0.079	-0.259	-0.593	-0.496	-0.438	0.205	-0.030	-0.446	0.064
	p-value	0.848	0.249	0.268	0.636	0.665	0.795	0.946	0.440	0.140	0.260	0.418	0.239	0.470	**0.002**	0.985	0.853	0.574	0.161	0.258	0.326	0.659	0.949	0.316	0.881
Butyricimonas	Corr.	0.797	-0.389	0.670	0.349	-0.070	-0.199	0.234	**0.966**	-0.177	-0.436	-0.494	0.874	0.330	-0.349	0.241	0.590	-0.155	-0.240	0.535	0.867	0.000	0.020	**0.919**	0.032
	p-value	0.032	0.389	0.099	0.397	0.882	0.669	0.614	**<0.001**	0.674	0.328	0.213	0.010	0.424	0.443	0.565	0.124	0.739	0.604	0.216	0.011	1.000	0.966	**0.003**	0.940
CF231	Corr.	0.878	-0.274	0.488	0.031	-0.298	-0.129	**0.912**	0.761	-0.181	-0.336	-0.398	0.196	0.727	-0.281	**0.948**	**0.948**	-0.367	-0.137	0.833	0.217	0.631	**0.904**	0.086	**0.924**
	p-value	0.009	0.552	0.266	0.942	0.517	0.782	**0.004**	0.047	0.668	0.462	0.329	0.674	0.041	0.541	**<0.001**	**<0.001**	0.418	0.770	0.020	0.641	0.129	**0.005**	0.855	**0.001**
Mucispirillum	Corr.	-0.384	**0.901**	-0.03	-0.266	-0.340	0.081	-0.458	-0.447	-0.211	**0.905**	**0.904**	-0.158	-0.185	-0.457	-0.351	-0.540	0.177	**0.909**	-0.135	-0.131	-0.545	-0.152	-0.255	-0.181
	p-value	0.395	**0.006**	0.949	0.524	0.456	0.862	0.302	0.314	0.616	**0.005**	**0.002**	0.735	0.661	0.302	0.395	0.167	0.705	**0.005**	0.773	0.779	0.206	0.744	0.580	0.667
Unknown	Corr.	0.227	-0.352	0.466	0.426	-0.102	-0.101	0.045	**0.911**	-0.070	-0.394	-0.437	**0.937**	0.226	-0.339	0.066	0.384	0.010	-0.274	0.435	0.851	-0.153	-0.170	**0.958**	-0.154

(Elusimicrobiaceae)	p-value	0.665	0.493	0.352	0.341	0.847	0.849	0.933	**0.011**	0.881	0.439	0.327	**0.006**	0.626	0.511	0.888	0.395	0.985	0.600	0.388	0.032	0.773	0.747	**0.003**	0.742
Elusimicrobium	Corr.	-0.131	-0.348	0.569	**0.966**	0.713	0.322	0.096	0.572	-0.031	-0.373	-0.484	0.328	0.132	0.064	0.086	0.228	0.309	-0.258	-0.096	0.634	-0.407	-0.114	0.756	-0.167
	p-value	0.780	0.444	0.182	**<0.001**	0.072	0.481	0.839	0.180	0.942	0.410	0.224	0.472	0.756	0.891	0.840	0.588	0.499	0.576	0.837	0.126	0.364	0.807	0.049	0.692
Clostridium (Peptostreptococcaceae)	Corr.	**0.911**	-0.253	0.178	-0.452	-0.361	-0.229	0.832	-0.055	-0.301	-0.214	-0.261	-0.149	0.487	-0.226	0.641	0.676	-0.362	-0.212	0.716	-0.187	**0.970**	0.732	-0.270	0.721
	p-value	**0.004**	0.585	0.703	0.261	0.427	0.622	0.020	0.906	0.469	0.645	0.532	0.750	0.221	0.627	0.086	0.066	0.425	0.647	0.070	0.688	**<0.001**	0.061	0.559	0.044
Unknown (Desulfovibrionaceae)	Corr.	0.712	0.271	0.844	0.123	0.035	0.079	0.639	0.245	**-0.918**	0.217	0.035	-0.027	0.625	-0.595	0.401	0.485	0.027	0.437	0.582	0.193	0.271	0.866	0.054	0.336
	p-value	0.073	0.557	0.017	0.772	0.941	0.866	0.122	0.597	**0.001**	0.640	0.934	0.954	0.098	0.159	0.325	0.223	0.954	0.327	0.170	0.678	0.557	0.012	0.908	0.416
Treponema	Corr.	-0.338	-0.212	0.438	0.837	**0.966**	**0.914**	-0.015	-0.045	-0.095	-0.174	-0.249	-0.259	0.125	0.353	-0.129	-0.261	**0.910**	-0.128	-0.501	-0.201	-0.603	-0.216	-0.022	-0.364
	p-value	0.458	0.649	0.325	0.010	**<0.001**	**0.004**	0.974	0.923	0.823	0.710	0.552	0.575	0.768	0.437	0.762	0.532	**0.004**	0.784	0.252	0.666	0.152	0.642	0.962	0.376
Unknown (f_WCHB1-25)	Corr.	0.499	-0.338	0.860	0.578	0.147	-0.131	0.246	**0.956**	-0.205	-0.391	-0.483	0.790	0.327	-0.334	0.263	0.586	-0.111	-0.180	0.438	0.881	-0.080	0.043	**0.914**	0.037
	p-value	0.254	0.458	0.013	0.133	0.752	0.779	0.595	**0.001**	0.625	0.386	0.226	0.034	0.429	0.465	0.530	0.127	0.813	0.700	0.326	0.009	0.865	0.927	**0.004**	0.931

Supplementary Table 5. Relationship between the bacterial species identified by LEfSe analysis and the expression levels of the miRNAs in small intestine. Pearson's correlation analysis coefficient (Corr.) and P-value.

		rno-miR-181a-5p	rno-miR-6330	rno-miR-125a-3p	rno-miR-1912-5p	rno-miR-29a-5p	rno-miR-3561-3p	rno-miR-133a-3p	rno-miR-1843b-5p	rno-miR-1843a-5p	rno-miR-344b-3p	rno-miR-764-3p	rno-miR-759	rno-miR-23b-5p	rno-miR-128-1-5p	rno-miR-186-5p	rno-miR-99b-3p	rno-miR-6322	rno-miR-211-5p	rno-miR-139-5p	rno-miR-449a-5p	rno-miR-760-3p	rno-let-7f-2-3p	rno-miR-497-3p	rno-miR-3553	rno-miR-1249	rno-miR-1956-5p
Unknown (Bacteroides)	Corr.	0.684	-0.273	**0.919**	0.596	0.249	-0.048	0.651	0.849	-0.442	-0.285	-0.446	0.410	0.559	0.587	-0.392	0.608	0.807	-0.160	-0.040	-0.041	0.579	0.626	0.241	0.527	0.550	0.435
	p-value	0.090	0.553	**0.003**	0.119	0.591	0.919	0.113	0.016	0.273	0.536	0.268	0.361	0.192	0.126	0.385	0.110	0.016	0.732	0.932	0.931	0.173	0.132	0.602	0.224	0.200	0.281
Unknown (Parabacteroides)	Corr.	**0.970**	-0.233	0.483	-0.106	-0.328	-0.291	**0.927**	0.728	-0.361	-0.273	-0.343	0.252	**0.906**	0.650	-0.452	0.824	**0.948**	-0.457	-0.498	-0.097	**0.931**	0.290	0.754	0.832	0.156	0.824
	p-value	**<0.001**	0.616	0.272	0.802	0.473	0.527	**0.003**	0.064	0.379	0.553	0.406	0.585	**0.005**	0.081	0.309	0.012	**<0.001**	0.303	0.256	0.836	**0.002**	0.528	0.050	0.020	0.739	0.012
Parabacteroides distasonis	Corr.	0.838	-0.357	0.745	0.409	-0.050	-0.059	0.735	**0.962**	-0.311	-0.396	-0.526	0.557	0.656	0.704	-0.428	0.752	**0.920**	-0.182	-0.206	-0.167	0.786	0.586	0.364	0.628	0.547	0.590
	p-value	0.019	0.432	0.055	0.315	0.915	0.899	0.060	**0.001**	0.453	0.379	0.180	0.194	0.109	0.051	0.338	0.031	**0.001**	0.696	0.657	0.720	0.036	0.167	0.422	0.131	0.204	0.124
Unknown (Prevotella, Prevotellaceae)	Corr.	0.301	-0.115	0.823	0.487	0.039	-0.268	0.064	**0.931**	-0.134	-0.219	-0.276	0.861	0.057	0.172	-0.434	0.158	0.490	-0.218	-0.055	-0.023	0.393	**0.942**	-0.181	-0.038	**0.940**	-0.002
	p-value	0.512	0.806	0.023	0.221	0.933	0.560	0.892	**0.002**	0.752	0.637	0.508	0.013	0.904	0.684	0.331	0.709	0.217	0.638	0.907	0.961	0.383	**0.002**	0.698	0.935	**0.002**	0.996
Unknown (Butyricimonas)	Corr.	0.796	-0.389	0.670	0.349	-0.070	-0.199	0.234	**0.966**	-0.177	-0.436	-0.494	0.874	0.326	0.330	-0.349	0.241	0.590	-0.155	-0.234	-0.240	0.534	0.867	-0.001	0.020	**0.919**	0.032
	p-value	0.032	0.389	0.099	0.397	0.882	0.669	0.614	**<0.001**	0.674	0.328	0.214	0.010	0.475	0.424	0.442	0.566	0.124	0.740	0.614	0.605	0.217	0.011	0.999	0.967	**0.003**	0.941
Unknown (CF231)	Corr.	0.878	-0.274	0.488	0.031	-0.298	-0.130	**0.912**	0.761	-0.181	-0.336	-0.398	0.197	0.790	0.727	-0.281	**0.948**	**0.948**	-0.367	-0.333	-0.137	0.833	0.217	0.631	**0.904**	0.086	**0.924**
	p-value	0.009	0.552	0.267	0.942	0.516	0.782	**0.004**	0.047	0.669	0.462	0.329	0.672	0.034	0.041	0.541	**<0.001**	**<0.001**	0.418	0.465	0.769	0.020	0.64	0.129	**0.005**	0.854	**0.001**
Mucispirillum schaedleri	Corr.	-0.383	**0.900**	-0.028	-0.266	-0.339	0.082	-0.458	-0.446	-0.214	**0.905**	**0.904**	-0.158	-0.422	-0.185	-0.458	-0.352	-0.541	0.178	-0.336	**0.910**	-0.135	-0.131	-0.545	-0.153	-0.255	-0.183
	p-value	0.396	**0.006**	0.952	0.525	0.457	0.861	0.302	0.316	0.611	**0.005**	**0.002**	0.735	0.346	0.662	0.301	0.393	0.167	0.703	0.462	**0.004**	0.773	0.780	0.206	0.743	0.581	0.665
Unknown (Elusimicrobium)	Corr.	-0.131	-0.349	0.569	**0.966**	0.713	0.322	0.096	0.572	-0.031	-0.373	-0.484	0.328	-0.078	0.132	0.064	0.086	0.228	0.310	0.606	-0.258	-0.096	0.634	-0.408	-0.114	0.756	-0.167

	p-value	0.780	0.444	0.182	**<0.001**	0.072	0.481	0.838	0.180	0.941	0.410	0.224	0.472	0.869	0.755	0.891	0.840	0.588	0.499	0.149	0.576	0.838	0.126	0.364	0.807	0.049	0.692
	Corr.	-0.593	-0.120	0.155	0.780	0.839	0.720	-0.316	-0.051	0.193	-0.095	-0.152	-0.140	-0.554	-0.176	0.339	-0.294	-0.400	0.791	**0.940**	-0.195	-0.622	-0.201	-0.638	-0.383	0.031	-0.456
Ruminococcus flavefaciens	p-value	0.160	0.797	0.741	0.022	0.018	0.068	0.490	0.914	0.647	0.839	0.720	0.765	0.197	0.677	0.457	0.480	0.326	0.034	**0.002**	0.675	0.136	0.666	0.123	0.396	0.948	0.256
	Corr.	-0.338	-0.212	0.438	0.837	**0.966**	**0.913**	-0.015	-0.045	-0.095	-0.173	-0.249	-0.259	-0.179	0.125	0.353	-0.129	-0.262	**0.910**	0.761	-0.128	-0.501	-0.200	-0.603	-0.216	-0.022	-0.364
Unknown (Treponema)	p-value	0.458	0.649	0.325	0.010	**<0.001**	**0.004**	0.974	0.923	0.823	0.710	0.553	0.575	0.701	0.768	0.437	0.761	0.531	**0.004**	0.047	0.785	0.252	0.666	0.152	0.642	0.962	0.376

Supplementary Table 6. KEGG pathways related with miRNAs expression in the intestine.

KEGG pathway	P-value
1. Metabolism	
1.0 Global and overview maps	
2-Oxocarboxylic acid metabolism	0.011
Fatty acid metabolism	0.009
1.3 Lipid metabolism	
Fatty acid biosynthesis	0.001
Fatty acid elongation	0.028
1.5 Amino acid metabolism	
Lysine degradation	<0.001
1.7 Glycan biosynthesis and metabolism	
Other types of O-glycan biosynthesis	0.003
1.8 Metabolism of cofactors and vitamins	
Vitamin B6 metabolism	0.011
2. Genetic Information Processing	
2.1 Transcription	
Spliceosome	0.002
2.2 Translation	
mRNA surveillance pathway	0.006
2.3 Folding, sorting and degradation	
Protein processing in endoplasmic reticulum	<0.001
Ubiquitin mediated proteolysis	0.025
RNA degradation	0.012
3. Environmental Information Processing	
3.2 Signal transduction	
Rap1 signaling pathway	0.011
MAPK signaling pathway	<0.001
ErbB signaling pathway	0.018
TGF-beta signaling pathway	<0.001
Hippo signaling pathway	<0.001
VEGF signaling pathway	0.029
TNF signaling pathway	0.002
HIF-1 signaling pathway	0.006
FoxO signaling pathway	<0.001
Sphingolipid signaling pathway	<0.001
AMPK signaling pathway	0.002
mTOR signaling pathway	0.005
3.3 Signaling molecules and interaction	
ECM-receptor interaction	0.005
4. Cellular Processes	
4.1 Transport and catabolism	
Endocytosis	0.001
4.2 Cell growth and death	
Cell cycle	<0.001
Oocyte meiosis	<0.001
p53 signaling pathway	0.001
4.3 Cellular community - eukaryotes	
Focal adhesion	<0.001
Adherens junction	<0.001
Signaling pathways regulating pluripotency of stem cells	<0.001
5. Organismal Systems	
5.1 Immune system	
Platelet activation	0.030
Fc gamma R-mediated phagocytosis	0.005
5.2 Endocrine system	
Insulin signaling pathway	0.001

GnRH signaling pathway	0.012
Estrogen signaling pathway	0.001
Progesterone-mediated oocyte maturation	0.001
Prolactin signaling pathway	0.030
Thyroid hormone signaling pathway	<0.001
5.6 Nervous system	
Neurotrophin signaling pathway	<0.001
5.8 Development	
Axon guidance	<0.001
5.10 Environmental adaptation	
Circadian rhythm	0.013
6. Human Diseases	
6.1 Cancers: Overview	
Pathways in cancer	<0.001
Central carbon metabolism in cancer	0.005
Choline metabolism in cancer	0.006
Transcriptional misregulation in cancer	<0.001
Proteoglycans in cancer	<0.001
Viral carcinogenesis	<0.001
6.2 Cancers: Specific types	
Colorectal cancer	<0.001
Pancreatic cancer	<0.001
Glioma	<0.001
Thyroid cancer	<0.001
Acute myeloid leukemia	0.007
Chronic myeloid leukemia	<0.001
Melanoma	0.007
Renal cell carcinoma	<0.001
Bladder cancer	<0.001
Prostate cancer	<0.001
Endometrial cancer	<0.001
Small cell lung cancer	0.012
Non-small cell lung cancer	<0.001
6.4 Neurodegenerative diseases	
Prion diseases	<0.001
6.8 Infectious diseases: Bacterial	
Epithelial cell signaling in Helicobacter pylori infection	0.047
Salmonella infection	0.005
Shigellosis	<0.001
Bacterial invasion of epithelial cells	<0.001
6.9 Infectious diseases: Viral	
Human T-cell leukemia virus 1 infection	0.032
Hepatitis B	<0.001
Hepatitis C	0.032
Epstein-Barr virus infection	0.001
6.10 Infectious diseases: Parasitic	
Toxoplasmosis	0.033
Chagas disease (American trypanosomiasis)	0.005

Supplementary Table 7. KEGG pathways associated with microbiome-related miRNAs.

KEGG pathway	Human miRNA
2-Oxocarboxylic acid metabolism (hsa01210)	hsa-miR-505-5p-L
Acute myeloid leukemia (hsa05221)	hsa-miR-181a-5p-S
Adherens junction (hsa04520)	hsa-miR-27a-3p-L; hsa-miR-10a-3p-L; hsa-miR-99b-5p-L; hsa-miR-181a-5p-S;hsa-miR-125a-3p-S;hsa-miR-186-5p-S; hsa-miR-99b-3p-S; hsa-miR-139-5p-S
Adrenergic signaling in cardiomyocytes (hsa04261)	hsa-miR-181a-5p-S
Allograft rejection (hsa05330)	hsa-miR-382-5p-L
Alzheimer's disease (hsa05010)	hsa-let-7e-3p-L
Amoebiasis (hsa05146)	hsa-miR-382-5p-L
AMPK signaling pathway (hsa04152)	hsa-miR-27a-3p-L; hsa-miR-186-5p-S
Antigen processing and presentation (hsa04612)	hsa-miR-382-5p-L; hsa-miR-211-5p-S
Arrhythmogenic right ventricular cardiomyopathy (ARVC) (hsa05412)	hsa-miR-505-5p-L
Axon guidance (hsa04360)	hsa-miR-27a-3p-L; hsa-miR-505-5p-L; hsa-miR-99b-5p-L
Bacterial invasion of epithelial cells (hsa05100)	hsa-miR-27a-3p-L; hsa-miR-21-5p-L; hsa-miR-181a-5p-S; hsa-miR-99b-3p-S
Biosynthesis of unsaturated fatty acids (hsa01040)	hsa-miR-21-5p-L
Biotin metabolism (hsa00780)	hsa-miR-181a-5p-S
Bladder cancer (hsa05219)	hsa-miR-27a-3p-L; hsa-miR-181a-5p-S
Cell cycle (hsa04110)	hsa-miR-27a-3p-L; hsa-miR-21-5p-L; hsa-miR-186-5p-S; hsa-miR-99b-3p-S
Central carbon metabolism in cancer (hsa05230)	hsa-miR-27a-3p-L; hsa-miR-382-5p-L; hsa-miR-181a-5p-S; hsa-miR-125a-3p-S
Chronic myeloid leukemia (hsa05220)	hsa-miR-27a-3p-L; hsa-miR-181a-5p-S; hsa-miR-186-5p-S
Circadian entrainment (hsa04713)	hsa-miR-369-3p-L
Colorectal cancer (hsa05210)	hsa-miR-27a-3p-L; hsa-miR-10a-3p-L; hsa-miR-21-5p-L; hsa-miR-181a-5p-S; hsa-miR-125a-3p-S; hsa-miR-186-5p-S
Cytokine-cytokine receptor interaction (hsa04060)	hsa-miR-211-5p-S
Dorso-ventral axis formation (hsa04320)	hsa-miR-181a-5p-S
ECM-receptor interaction (hsa04512)	hsa-miR-27a-3p-L; hsa-miR-382-5p-L; hsa-miR-125a-3p-S; hsa-miR-23b-5p-S; hsa-miR-211-5p-S
Endometrial cancer (hsa05213)	hsa-miR-27a-3p-L; hsa-miR-21-5p-L; hsa-miR-181a-5p-S; hsa-miR-139-5p-S
Epithelial cell signaling in Helicobacter pylori infection (hsa05120)	hsa-miR-10a-3p-L
Epstein-Barr virus infection (hsa05169)	hsa-miR-186-5p-S

ErbB signaling pathway (hsa04012)	hsa-miR-27a-3p-L; hsa-miR-139-5p-S
Estrogen signaling pathway (hsa04915)	hsa-miR-181a-5p-S; hsa-miR-139-5p-S
Fatty acid biosynthesis (hsa00061)	hsa-miR-27a-3p-L
Fatty acid degradation (hsa00071)	hsa-miR-21-5p-L; hsa-miR-29a-5p-S
Fatty acid elongation (hsa00062)	hsa-miR-21-5p-L; hsa-miR-29a-5p-S
Fatty acid metabolism (hsa01212)	hsa-miR-27a-3p-L; hsa-miR-21-5p-L
Focal adhesion (hsa04510)	hsa-miR-27a-3p-L; hsa-miR-125a-3p-S; hsa-miR-186-5p-S
FoxO signaling pathway (hsa04068)	hsa-miR-27a-3p-L; hsa-miR-10a-3p-L; hsa-miR-21-5p-L; hsa-miR-181a-5p-S; hsa-miR-125a-3p-S
Glioma (hsa05214)	hsa-miR-27a-3p-L; hsa-miR-181a-5p-S; hsa-miR-139-5p-S
Glycerolipid metabolism (hsa00561)	hsa-let-7e-3p-L
Glycosphingolipid biosynthesis - ganglio series (hsa00604)	hsa-miR-187-3p-L
Glycosphingolipid biosynthesis - lacto and neolacto series (hsa00601)	hsa-miR-505-5p-L
Hepatitis B (hsa05161)	hsa-miR-27a-3p-L; hsa-miR-10a-3p-L; hsa-miR-21-5p-L; hsa-miR-181a-5p-S
HIF-1 signaling pathway (hsa04066)	hsa-miR-181a-5p-S
Hippo signaling pathway (hsa04390)	hsa-miR-27a-3p-L; hsa-miR-10a-3p-L; hsa-miR-21-5p-L; hsa-miR-181a-5p-S; hsa-miR-29a-5p-S; hsa-miR-99b-3p-S; hsa-miR-139-5p-S
Insulin signaling pathway (hsa04910)	hsa-miR-27a-3p-L
Leukocyte transendothelial migration (hsa04670)	hsa-miR-99b-5p-L
Long-term depression (hsa04730)	hsa-miR-99b-5p-L
Lysine degradation (hsa00310)	hsa-miR-27a-3p-L; hsa-miR-505-5p-L; hsa-miR-21-5p-L; hsa-miR-181a-5p-S; hsa-miR-125a-3p-S; hsa-miR-186-5p-S
Melanoma (hsa05218)	hsa-miR-27a-3p-L; hsa-miR-181a-5p-S
Metabolism of xenobiotics by cytochrome P450 (hsa00980)	hsa-miR-497-3p-S
Morphine addiction (hsa05032)	hsa-miR-369-3p-L
mRNA surveillance pathway (hsa03015)	hsa-miR-29a-5p-S
mTOR signaling pathway (hsa04150)	hsa-miR-27a-3p-L; hsa-miR-382-5p-L
Mucin type O-Glycan biosynthesis (hsa00512)	hsa-miR-27a-3p-L; hsa-miR-99b-5p-L; hsa-miR-29a-5p-S
Neurotrophin signaling pathway (hsa04722)	hsa-miR-27a-3p-L; hsa-miR-181a-5p-S; hsa-miR-139-5p-S
NF-kappa B signaling pathway (hsa04064)	hsa-miR-139-5p-S
Non-homologous end-joining (hsa03450)	hsa-miR-99b-3p-S

Non-small cell lung cancer (hsa05223)	hsa-miR-27a-3p-L; hsa-miR-181a-5p-S
One carbon pool by folate (hsa00670)	hsa-miR-125a-3p-S
Oocyte meiosis (hsa04114)	hsa-miR-27a-3p-L; hsa-miR-29a-5p-S
Other types of O-glycan biosynthesis (hsa00514)	hsa-miR-505-5p-L
p53 signaling pathway (hsa04115)	hsa-miR-27a-3p-L; hsa-miR-21-5p-L; hsa-miR-181a-5p-S; hsa-miR-186-5p-S; hsa-miR-497-3p-S
Pancreatic cancer (hsa05212)	hsa-miR-27a-3p-L; hsa-miR-181a-5p-S
Pantothenate and CoA biosynthesis (hsa00770)	hsa-miR-181a-5p-S
Parkinson's disease (hsa05012)	hsa-miR-23b-5p-S; hsa-miR-99b-3p-S
Pathways in cancer (hsa05200)	hsa-miR-27a-3p-L; hsa-miR-10a-3p-L; hsa-miR-21-5p-L; hsa-miR-181a-5p-S; hsa-miR-186-5p-S
Phosphatidylinositol signaling system (hsa04070)	hsa-miR-483-5p-L
PI3K-Akt signaling pathway (hsa04151)	hsa-miR-382-5p-L; hsa-miR-181a-5p-S
PPAR signaling pathway (hsa03320)	hsa-let-7e-3p-L
Prion diseases (hsa05020)	hsa-miR-27a-3p-L; hsa-miR-382-5p-L; hsa-miR-186-5p-S
Progesterone-mediated oocyte maturation (hsa04914)	hsa-miR-27a-3p-L; hsa-miR-186-5p-S
Prolactin signaling pathway (hsa04917)	hsa-miR-21-5p-L; hsa-miR-181a-5p-S
Prostate cancer (hsa05215)	hsa-miR-27a-3p-L; hsa-miR-181a-5p-S; hsa-miR-186-5p-S; hsa-miR-139-5p-S
Protein processing in endoplasmic reticulum (hsa04141)	hsa-miR-27a-3p-L; hsa-miR-483-5p-L; hsa-miR-181a-5p-S; hsa-miR-186-5p-S; hsa-miR-211-5p-S
Proteoglycans in cancer (hsa05205)	hsa-miR-27a-3p-L; hsa-miR-10a-3p-L; hsa-miR-21-5p-L; hsa-miR-181a-5p-S; hsa-miR-125a-3p-S; hsa-miR-29a-5p-S; hsa-miR-186-5p-S
Regulation of actin cytoskeleton (hsa04810)	hsa-miR-181a-5p-S; hsa-miR-186-5p-S
Renal cell carcinoma (hsa05211)	hsa-miR-27a-3p-L; hsa-miR-181a-5p-S
RNA degradation (hsa03018)	hsa-miR-187-3p-L; hsa-miR-181a-5p-S; hsa-miR-497-3p-S
Salmonella infection (hsa05132)	hsa-miR-505-5p-L
Serotonergic synapse (hsa04726)	hsa-miR-483-5p-L
Shigellosis (hsa05131)	hsa-miR-27a-3p-L; hsa-miR-10a-3p-L
Signaling pathways regulating pluripotency of stem cells (hsa04550)	hsa-miR-27a-3p-L; hsa-let-7e-3p-L; hsa-miR-181a-5p-S
Small cell lung cancer (hsa05222)	hsa-miR-181a-5p-S
Sphingolipid metabolism (hsa00600)	hsa-miR-21-5p-L; hsa-miR-186-5p-S
Sphingolipid signaling pathway (hsa04071)	hsa-miR-27a-3p-L; hsa-miR-181a-5p-S

Spliceosome (hsa03040)	hsa-miR-99b-5p-L
Steroid biosynthesis (hsa00100)	hsa-miR-23b-5p-S; hsa-miR-186-5p-S
Sulfur metabolism (hsa00920)	hsa-miR-505-5p-L
Synaptic vesicle cycle (hsa04721)	hsa-miR-187-3p-L
Terpenoid backbone biosynthesis (hsa00900)	hsa-miR-181a-5p-S
TGF-beta signaling pathway (hsa04350)	hsa-miR-27a-3p-L; hsa-miR-10a-3p-L; hsa-miR-125a-3p-S; hsa-miR-29a-5p-S; hsa-miR-186-5p-S
Thyroid cancer (hsa05216)	hsa-miR-27a-3p-L; hsa-miR-21-5p-L; hsa-miR-181a-5p-S; hsa-miR-125a-3p-S
Thyroid hormone signaling pathway (hsa04919)	hsa-miR-27a-3p-L; hsa-miR-21-5p-L
Transcriptional misregulation in cancer (hsa05202)	hsa-miR-27a-3p-L; hsa-miR-21-5p-L; hsa-miR-181a-5p-S; hsa-miR-186-5p-S
Tyrosine metabolism (hsa00350)	hsa-miR-369-3p-L
Ubiquitin mediated proteolysis (hsa04120)	hsa-miR-27a-3p-L
Valine, leucine and isoleucine biosynthesis (hsa00290)	hsa-miR-505-5p-L
Viral carcinogenesis (hsa05203)	hsa-miR-27a-3p-L; hsa-miR-181a-5p-S; hsa-miR-125a-3p-S; hsa-miR-186-5p-S
Viral myocarditis (hsa05416)	hsa-miR-382-5p-L
Vitamin B6 metabolism (hsa00750)	hsa-miR-186-5p-S

hsa: *Homo sapiens*; -L: large intestine; -S: small intestine

5.4. Publicación 4

Citación: Barroso A*, Santos-Marcos JA*, Perdices-Lopez C, Vega-Rojas A, Sanchez-Garrido MA, Krylova Y, Molina-Abril H, Ohlsson C, Perez-Martinez P, Poutanen M, Lopez-Miranda J, Tena-Sempere M, Camargo A. Neonatal exposure to androgens dynamically alters gut microbiota architecture. J Endocrinol. 2020 Oct;247(1):69-85. doi: 10.1530/JOE-20-0277. PMID: 32755996. *Igual contribución

RESEARCH

Neonatal exposure to androgens dynamically alters gut microbiota architecture

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Abstract

Gonadal steroids strongly contribute to the metabolic programming that shapes the susceptibility to the manifestation of diseases later in life, and the effect is often sexually dimorphic. Microbiome signatures, together with metabolic traits and sex steroid levels, were analyzed at adulthood in neonatally androgenized female rats, and compared with those of control male and female rats. Exposure of female rats to high doses of androgens on early postnatal life resulted in persistent alterations of the sex steroid profile later on life, namely lower progesterone and higher estradiol and estrone levels, with no effect on endogenous androgens. Neonatally androgenized females were heavier (10% at early adulthood and 26% at adulthood) than controls and had impaired glucose homeostasis observed by higher AUC of glucose in GTT and ITT when subjected to obesogenic manipulations. Androgenized female displayed overt alterations in gut microbiota, indicated especially by higher *Bacteroidetes* and lower *Firmicutes* abundance at early adulthood, which disappeared when animals were concurrently overfed at adulthood. Notably, these changes in gut microbiota were related with the intestinal expression of several miRNAs, such as miR-27a-3p, miR-29a-5p, and miR-100-3p. Our results suggest that nutritional and hormonal disruption at early developmental periods not only alters the metabolic programming of the individual later in life but also perturbs the architecture of gut microbiota, which may interact with the host by a cross-talk mediated by intestinal miRNAs; phenomena that may contribute to amplify the metabolic derangement caused by obesity, as seen in neonatally androgenized female rats.

Key Words

- gut microbiota
- hormones
- sex steroids
- metabolic diseases
- obesity

Journal of Endocrinology (2020) **247**, 69–85

https://joe.bioscientifica.com
https://doi.org/10.1530/JOE-20-0277

Introduction

Sex steroids are key metabolic regulators in different tissues, with proven roles in the control of food intake and energy homeostasis, whose deregulation is frequently linked to the manifestation of diseases (Mauvais-Jarvis *et al.* 2013). In addition, the incidence of metabolic diseases and their co-morbidities is sexually dimorphic, and varies depending on the gonadal status; for example, increases after menopause (Mauvais-Jarvis 2015).

However, the influence of gonadal factors is rather complex. For instance, male hypogonadism is considered a risk factor for the development of cardiovascular complications associated to type 2 diabetes, suggesting that low testosterone in adult men is detrimental for metabolic and cardiovascular homeostasis (Rao *et al.* 2013). By contrast, androgen excess in women, a characteristic of polycystic ovary syndrome (PCOS), is associated to insulin resistance, which also contributes to the excessive ovarian androgen production (Witchel *et al.* 2019).

Early metabolic programming by sex steroids also contributes to define differences in susceptibility to later development of the metabolic disease. Inappropriate exposures to sex steroids during early maturational periods (e.g. excessive androgenization in utero) have been linked to the development of insulin resistance and PCOS (Witchel & Tena-Sempere 2013). Hence, gonadal hormones are likely to influence metabolic homeostasis via multiple, frequently redundant, regulatory systems, and can be considered as genuine modifiers of metabolic homeostasis, which may contribute to generate differences in susceptibility for the developing of cardiometabolic disease (Faulkner & Belin de Chantemele 2019).

Gut microbiota is now recognized as an organ integrated in the metabolism of the host (Tremaroli & Backhed 2012), and it has been proposed that alteration of the gut microbiota structure may trigger the development of cardiometabolic diseases (Jie *et al.* 2017). Notably, sex hormones appear to be one of the factors driving gut microbiota differences between males and females, which may also contribute to the sexual dimorphism in disease susceptibility (Cross *et al.* 2018). In addition, it has been shown that the gut microbiota structure is influenced by the combined effects of sex, gonadal hormones, and obesity, with specific abnormalities in women with PCOS (Insenser *et al.* 2018).

In this study, we aimed to explore, using suitable preclinical (rat) models, the putative role of sex steroid milieu at early developmental periods, alone or in combination with obesogenic insults later in life, as persistent modifiers of gut microbiota architecture, in the context of development of metabolic alterations. In order to shed light on the putative mechanisms for the integration between gut microbiota and host, we also explored the cross-talk between gut microbiota and the host through regulation of the expression of miRNAs in small and large intestine, as intestinal miRNAs are increasingly recognized as potential decoders of the impact of dysbiosis into cardiometabolic diseases (Liu *et al.* 2016, Serino 2016).

Materials and methods

Animals and diets

Wistar male and female rats bred in the vivarium of the University of Cordoba were used. The animals were maintained at 22 ± 1°C under constant conditions of light (14 h) with free access to water. The experimental animals were fed a control diet (CD), D12450B (10, 20, and 70% calories from fat, protein, and carbohydrate, respectively), or a high fat diet (HFD), D12451 (45%, 20%, and 35% calories from fat, protein and carbohydrate, respectively; Research Diets Inc., New Brunswick, NJ, USA).

Experimental design

On postnatal day (PND)-1, males and female pups were cross-fostered and reared in two different litter sizes: small litters (SLs: 4 pups per litter; as a model of postnatal overnutrition) or normal litters (NLs: 12 pups per litter), as extensively described previously (Castellano *et al.* 2011, Sanchez-Garrido *et al.* 2013, 2014). Immediately afterwards, subsets of female pups of the two litter sizes were androgenized by a single s.c. injection of a bolus of 1.25 mg of testosterone propionate dissolved in olive oil (100 µL), whereas the remaining female pups were injected with vehicle (olive oil), following previously validated protocols (Pinilla *et al.* 2002). After weaning on PND-23, groups of androgenized or vehicle-treated females, as well as males, were randomly pooled within each category (androgenization or vehicle; NL or SL) to avoid the bias of differences in body weight (BW) between the different subgroups, and were housed in a number of four-five rats per cage. From weaning onwards, the groups of SL rats (either males, vehicle-treated or androgenized females) were fed with an HFD, while the NL groups received a CD; all animals had access to food ad libitum.

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https://doi.org/10.1530/JOE-20-0277

Analyses were applied at two age points, on PND-50 and PND-150, representative of young adult (PND-50) or adult rats (PND-150), in order to check the short- and long-term the impact of the different stressors (neonatal androgenization, and obesogenic dietary patterns). Rats of both age-groups were killed by decapitation, and trunk blood and fecal and tissue samples were collected for analyses. These analyses included phenotypic indices and biochemical/hormonal parameters in serum, as well as fecal and intestinal samples. In intact females, sampling was carried out at the same stage of the ovarian cycle, namely diestrus-1, and was conducted between 9:00 h and noon to avoid the potential interference of circadian variations (Castellano *et al.* 2011). Small and large intestine sections were dissected and fecal samples were obtained from the different study groups directly from stool expulsion stimulated by manual handling. The samples obtained were frozen in liquid nitrogen and stored at −80°C until analysis. All the experimental protocols were approved by Cordoba University Ethical Committee of animal experimentation and conducted in accordance with the European Union guidelines for the use of experimental animals.

Phenotypic indices and hormonal measurements

Body weight (BW) and basal blood glucose levels were recorded in all the experimental groups, at the two age-points (PND-50 and -150); glucose levels were determined after overnight fasting. In addition, serum level of leptin was assayed at the same ages by a double-antibody RIA, using the kit provided by EMD MILLIPORE. The limit of sensitivity of the assay was 0.801 ng/mL, and the intra- and inter-assay coefficients of variation were less than 4 and 9%, respectively. At the two ages, the serum levels of ghrelin were also measured using a Bio-Plex Rat Diabetes Assay, provided by Bio-Rad Laboratories. The limit of sensitivity of the assay was 0.3 pg/mL, and the intra- and inter-assay CVs were 4 and 4%, respectively. In all experimental groups, sex steroid plasma levels were determined at PND-50 and -150, using a sensitive gas chromatography-tandem mass spectrometry method, thoroughly validated as described in previous references (Nilsson *et al.* 2015, Velasco *et al.* 2019).

Glucose tolerance tests and insulin tolerance tests

Rats of all experimental groups were subjected to glucose tolerance tests (GTTs) at the two age groups indicated previously. Rats were fasted overnight and subsequently received an intraperitoneal (ip) bolus of glucose (1 g/kg BW). Glucose levels were determined in blood before (0) and at 20, 60, and 120 min post-administration. After complete recovery 1 week later, insulin sensitivity was assessed by an insulin tolerance tests (ITT). Rats were fasted overnight, following an ip injection of 1UI insulin (Sigma-Aldrich) per kg body weight. Blood glucose levels were measured before (0) and at 20, 60, and 120 min after insulin administration. Integral glucose changes levels were estimated as area under the curve (AUC), during the 120 min period after glucose or insulin administration, as calculated by the trapezoidal method. All glucose concentrations were measured using a handheld glucometer (ACCU-CHECK Aviva; Roche Diagnostics).

Intestinal microbiota analysis

DNA extraction from feces was performed using the QIAamp DNAStool Mini Kit Handbook (QIAGEN), following the manufacturer's instructions.

The microbiota composition analysis of the fecal samples was performed on a MiSeq Illumina platform (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Briefly, PCR (PCR) was performed using 0.2 µM of each one of the primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′ and 5′-GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAG-3′ (Klindworth *et al.* 2013) to generate amplicons containing the hypervariable region V3 of the 16s rRNA gene, KAPA HiFi HotStart ReadyMix (KAPABIOSYSTEMS), and 1.25 µL of extracted DNA (5 ng/µL in 10 mM Tris pH 8.5), using the following cycle parameters: 3 min denaturation at 95°C followed by 25 cycles (30 s at 95°C, 30 s at 60°C, 30 s at 72°C) and a final extension at 72°C for 5 min. The amplicon purification was performed using Agentcourt AMPure XP beads (Beckman Coulter).

A second PCR reaction attaches dual indices and Illumina sequencing adapters using the Nextera XT Index Kit. This PCR was performed with a KAPA HiFi HotStart ReadyMix (KAPABIOSYSTEMS), 5 µL of the previous amplicon, 5 µL of each Nextera XT Index Primer 1 (N7xx) and 5 µL of each Nextera XT Index Primer 2 (S5xx), using the following cycle parameters: 3 min denaturation at 95°C followed by eight cycles (30 s at 95°C, 30 s at 55°C, 30 s at 72°C), and a final extension at 72°C for 5 min. The PCR product purification was performed using Agentcourt AMPure XP beads (Beckman Coulter). Raw data are available at NCBI Sequence Read Archive (SRA) under the accession number PRJNA631334. Sequencing

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data were analyzed and visualized using QIIME 2 v. 2019.7 (Bolyen *et al.* 2019). Demultiplexed single-end reads containing V3 hypervariable region were truncated at 212 bp (Quality score median >30), and denoised using DADA2 method (Callahan *et al.* 2016).

After filtering, the high-quality reads of the 64 samples ($n=8$ for each group) ranging from 170,919 to 18,682 sequence counts, and rarefaction depth was established at 18,500 sequence counts. Bacterial richness and diversity across the samples were calculated using the observed OTUs and Shannon indexes (Hammer *et al.* 2001). Principal component analysis of community structure (beta-diversity) was done using the unweighted and weighted UniFrac distance metrics (Lozupone & Knight 2005) and analyzed by permutational multivariate ANOVA (PERMANOVA). Taxonomy was assigned to the high-quality reads using q2-feature-classifier (Bokulich *et al.* 2018) with a sequence identity threshold of 99% interrogating the sequences with the Greengenes database (13_8) (McDonald *et al.* 2012). To be consistent with the 16S rRNA obtained taxonomic data, only taxa in the bacteria domain were included in the statistical analysis.

The relative taxonomic abundance was measured as the proportion of reads over the total in each sample assigned to a given taxonomy. To exclude bacterial taxa that were not present in the majority of samples, a cut-off for exclusion was fixed; only bacterial taxa containing sequence reads in at least 75% of total samples were considered. Linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.harvard.edu/galaxy/) was used to compare groups at baseline and visualize the results using taxonomic bar charts and cladograms (Segata *et al.* 2011).

RNA isolation from small and large intestine

Frozen tissue was ground to a fine powder in liquid nitrogen, using a mortar and pestle. RNA was isolated with the commercial kit Direct-zol™ RNA MiniPrep Plus (Zymo Research Corp., CA, USA), and quantified using the spectrophotometer v3.5.2 Nanodrop ND-1000 (Nanodrop Technologies, Cambridge, UK).

miRNA expression analysis

miRNA expression profiles were generated using the SurePrint Rat miRNA Microarrays, Rat miRNA 8x15K Microarray (Release 21.0) (Agilent Technologies Inc.). RNA samples of each experimental group were pooled and labeled using the miRNA Labeling and Hyb Kit (Agilent Technologies Inc.), according to the manufacturer's instructions. Hybridization was performed using this latter kit, also according to the manufacturer's instructions. Microarray images of each slide were obtained with a Gene Pix 4000B scanner (Axon Instruments, Union City, CA, USA). Image quantization was performed using Agilent Feature Extraction Software (Agilent Technologies Inc.). Raw microarray data were analyzed using the limma R package (Smyth 2005). Spots with foreground mean and median differing by more than 50 were filtered out and data quality was checked using limma tools. Background correction was performed using saddle-point approximation in the normal-exponential convolution method Normexp (Ritchie *et al.* 2007). Then within arrays Print-tip loess (Yang *et al.* 2001) and between arrays quantile were used for normalization. Finally, replicate spots in the array data were averaged.

Software for miRNA analysis

To identify the role of selected miRNAs in the cellular processes, we performed an analysis using the DIANA tools V.3. DIANA-miRPath is a web-server (http://diana.imis.athena-innovation.gr/DianaTools/index.php), which provides accurate statistics and can accommodate advanced pipelines. miRPath can utilize predicted miRNA targets (in CDS or 3′-UTR regions) provided by the DIANA-microT-CDS algorithm or even experimentally validated miRNA interactions derived from DIANA-TarBase (Vlachos *et al.* 2015).

Statistical analysis

PASW statistical software package, version 20.0 (IBM Inc.), was used for statistical analyses of the data. We used One-way ANOVA to test the differences between animal's groups. We used ANOVA for repeated measures for the time-course glucose levels analysis in the GTT and ITT with time as intra-individual factor and animal's group as inter-individual factor. *Post hoc* was performed by Bonferroni multiple comparisons test. Pearson's correlation test was used to evaluate the relationship between miRNAs intestinal expression and bacterial taxa abundance. Data are presented as mean ±S.E.M. *P*-values <0.05 were considered statistically significant in all the statistical analyses.

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Results

Effect of androgenization on sex steroid profile according to the nutritional status

In order to evaluate the early metabolic programming by sex steroids on the susceptibility to later development of metabolic disease, female rats were subjected to neonatal androgenization by single injection of a bolus of testosterone propionate (1.25 mg, s.c.) on PND-1. By measuring testosterone, dihydrotestosterone and androstenedione later on life, we did not observe differences in androgen levels between androgenized and not androgenized females, with the exception of 49.7% lower androstenedione levels in androgenized females ($P=0.014$) under NL-CD conditions at PND-150. As expected, in none of the female study groups the androgen levels reached that of the males, with the exception of testosterone and androstenedione for which the difference between the males and androgenized females under NL-CD condition at PND-50 did not reach the statistical significance. Of importance, neonatal androgenization reduced progesterone levels in the females at PND-50 and -150 in both NL-CD (0.22 and 0.15-fold change) and SL-HFD (0.19 and 0.28-fold change) conditions, compared to control females (all, P-values < 0.05). Thus, the progesterone levels in androgenized females turned toward that measured in males. Interestingly, estradiol (E2) levels were markedly (16.61 and 8.91-fold change, $P < 0.001$ and $P=0.018$, respectively) increased in females at PND-50 as a consequence of neonatal androgenization, in both NL-CD and SL-HFD conditions, whereas no statistically significant differences were found between the groups at PND-150. Moreover, estrone (E1) level was markedly higher in androgenized females at PND-50, in both NL-CD (26.28-fold change, $P=0.009$) and SL-HFD (high levels vs not detected), and at PND-150 in SL-HFD (31.89-fold change, $P=0.011$) conditions compared to intact females. As expected, only very low or non-detectable level of E2 and E1 was measured in the males (Fig. 1).

Early manipulation of the sex steroid milieu impairs energy balance and glucose homeostasis

Neonatal androgenization increased BW of the females measured at 50 and 150 days, in both NL-CD and SL-HFD conditions compared to intact females. We found a grossly similar profile in leptin levels in both intact and androgenized females, but increased leptin levels were observed in androgenized females compared to intact rats under SL-HFD at PND-150. In these rats, leptin levels were close to the ones observed in males. By contrast, ghrelin levels were lower in androgenized females than males (NL-CD and SL-HFD) and intact females (NL-CD) at PND-50, with no differences between groups at PND-150 (Supplementary Fig. 1, see section on supplementary materials given at the end of this article).

In addition, GTT and ITT were applied to the experimental groups to assess the potential alterations in glucose homeostasis. We found that androgenized female rats at PND-50 displayed an AUC of glucose higher than intact females, which reached the statistical

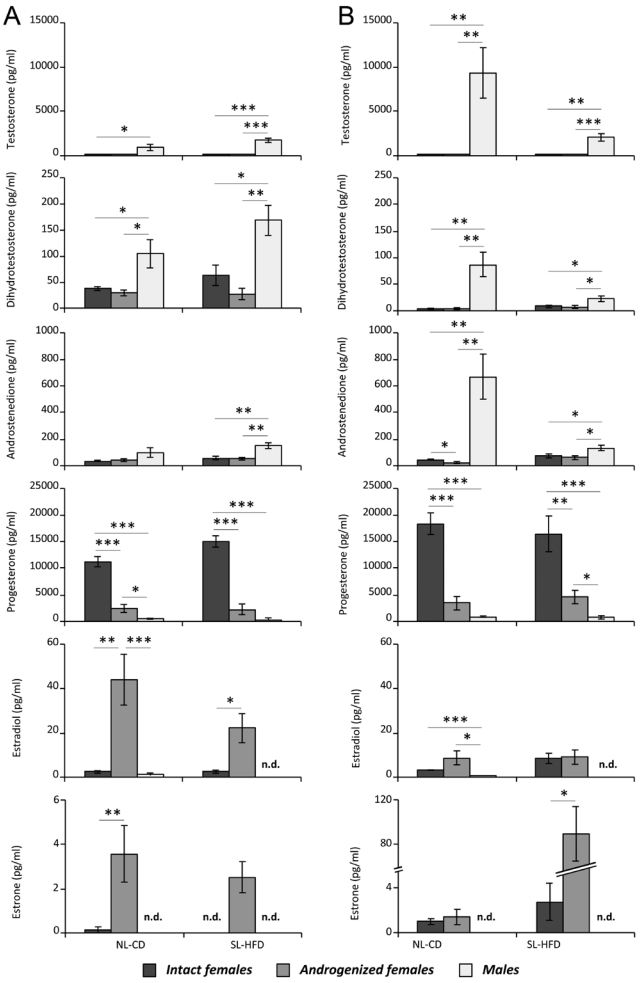

Figure 1
Serum sex steroid levels in intact (non-androgenized) animals and androgenized females, under normal feeding (NL-CD) and postnatal overfeeding (SL-HFD) at PND-50 (A) and PND-150 (B). NL-CD, normal litter, control diet; SL-HFD, small litter, high fat diet; PND, postnatal day. *P-value < 0.05, **P-value < 0.01, and ***P-value < 0.001 in the one-way ANOVA statistical analysis. n.d., not detectable.

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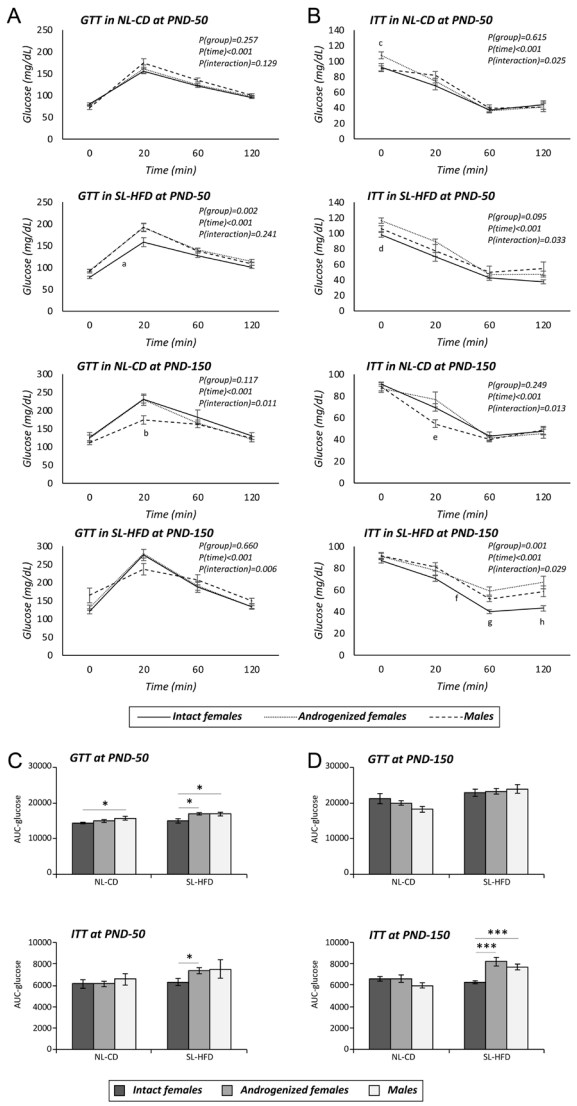

Figure 2

Metabolic parameters in intact animals and androgenized females. Time-course profiles and area under curve of serum glucose in glucose and insulin tolerance tests, under normal feeding (NL-CD) and postnatal overfeeding (SL-HFD). NL-CD, normal litter, control diet; SL-HFD, small litter, high fat diet; PND, postnatal day. Glucose tolerance test (GTT) was performed in young adult (<PND-50) and adult (between PND-120 and PND-150) animals. After complete recovery 1 week later, insulin tolerance tests (ITTs) was assessed. Time-course profiles (A and B) ANOVA for repeated measures statistical analysis. (a) Intact females lower than androgenized females ($P = 0.004$) and males ($P = 0.010$). (b) Males lower than intact females ($P = 0.014$) and androgenized females ($P = 0.017$) at time point of 20 min. (c) Androgenized females higher than males ($P = 0.031$) and a trend with intact females ($P = 0.064$) at time point of 0 min. (d) Androgenized females higher than intact females ($P = 0.004$) at time point of 0 min. (e) Androgenized females higher than males ($P = 0.012$) at time point of 20 min. (f) Intact females lower than androgenized females ($P = 0.001$) and males ($P = 0.009$). (g) Intact females lower than androgenized females ($P < 0.001$) and males ($P = 0.022$) at time point of 60 min. (h) Intact females lower than androgenized females ($P = 0.005$) and a trend with males ($P = 0.076$) at time point of 120 min. Area under curve (AUC) (C and D) One-way ANOVA statistical analysis. *P-value < 0.05 and ***P-value < 0.001.

significance under SL-HFD, but not under NL-CD (Fig. 2). In fact, the analysis of the time-course glucose levels on the GTT showed higher glucose levels (indicative of lower glucose tolerance) in androgenized females (and males) at PND-50 than in intact females (Fig. 2). In addition, we observed in the ITT a higher AUC of glucose (indicative of lower insulin sensitivity) in androgenized females than in intact females under SL-HFD, but not under NL-CD, at both PND-50 and PND-150 (Fig. 2). Moreover, time-course glucose levels at 0 min on the ITT showed higher glucose levels in androgenized females than intact females at PND-50 under both NL-CD and SL-HFD conditions. In addition, glucose levels on the ITT showed higher glucose levels of androgenized females (and males) than intact females at PND-150 under SL-HFD condition, whereas no differences were shown under NL-CD (Fig. 2).

Impact of early androgenization on gut microbiota diversity

We evaluated whether the disruption on the early metabolic programming by neonatal androgenization impacts on the gut microbiota architecture. These studies showed that there were no significant differences in the α-diversity measured by the Shannon and observed OTUs indexes, and by the phylogenetic diversity (Faith) between androgenized and intact females at any age (PND-50 nor PND-150) and nutritional condition (NL-CD and SL-HFD). However, there was a higher α-diversity at PND-50 of the bacterial community in androgenized females than in males under NL-CD condition, but these differences were not found when animals were raised under SL-HFD condition. By contrast, lack of differences or only trends were found between androgenized females and males, at PND-150 under both NL-CD and SL-HFD conditions, with the exception of Observed OTUs index (Supplementary Table 1).

In term of bacterial β-diversity, we found significant unweighted UniFrac distances among androgenized, intact female and male groups (all P and Q values < 0.05), but no differences at PND-150 in the SL-HFD groups between androgenized and intact females. In contrast, we found significant differences in weighted UniFrac distances among the main groups (androgenized, intact females and males) under NL-CD, and between androgenized females and intact females under SL-HFD condition. However, no differences were found between androgenized females and males under SL-HFD condition (Supplementary Table 1).

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Impact of early androgenization on gut microbiota composition at PND-50

Further, we analyzed the differences in the microbiota structure by LEfSe analysis (Fig. 3 and Supplementary Figs 2, 3). The data showed that in NL-CD animals at PND-50 the gut microbiota from androgenized females was characterized by higher abundance of *Bacteroidetes* phylum and by a reduced abundance of *Firmicutes* and *Euryarchaeota* phyla compared to intact females. At genus level, we observed that androgenization increased the abundance of *Bacteroides*, *Helicobacter* and *CF231* genera in addition to one unknown genus from *S24-7* family. The androgenization, furthermore, reduced the abundance of *Methanobrevibacter*, *Desulfovibrio* and *p-75-a5* genera, in addition to an unknown genus from *Desulfovibrionaceae* family. In contrast, when animals were raised under SL-HFD conditions, no differences in the majority phyla, *Firmicutes* and *Bacteroidetes*, were found between androgenized and intact females, but we found that androgenization reduced the *Euryarchaeota*, *Verrucomicrobia*, and *Cyanobacteria* phyla. We observed a lower *Firmicutes*/*Bacteroidetes* (F/B) ratio in androgenized females than intact females ($P=0.022$) at PND-50 under NL-CD condition, whereas no differences were observed under SL-HFD condition (Supplementary Table 2).

At genus level, we found that androgenization increased *Clostridium* (*Clostridiaceae*), *Clostridium* (*Lachnospiraceae*), and *Lactobacillus*. We also observed that androgenization reduced *Ruminococcus* (*Ruminococcaceae*), *Oscillospira*, *Clostridium* (*Peptostreptococcaceae*), *Methanobrevibacter*, *Phascolarctobacterium*, *Flexispira*, *Dehalobacterium*, *Alistipes*,

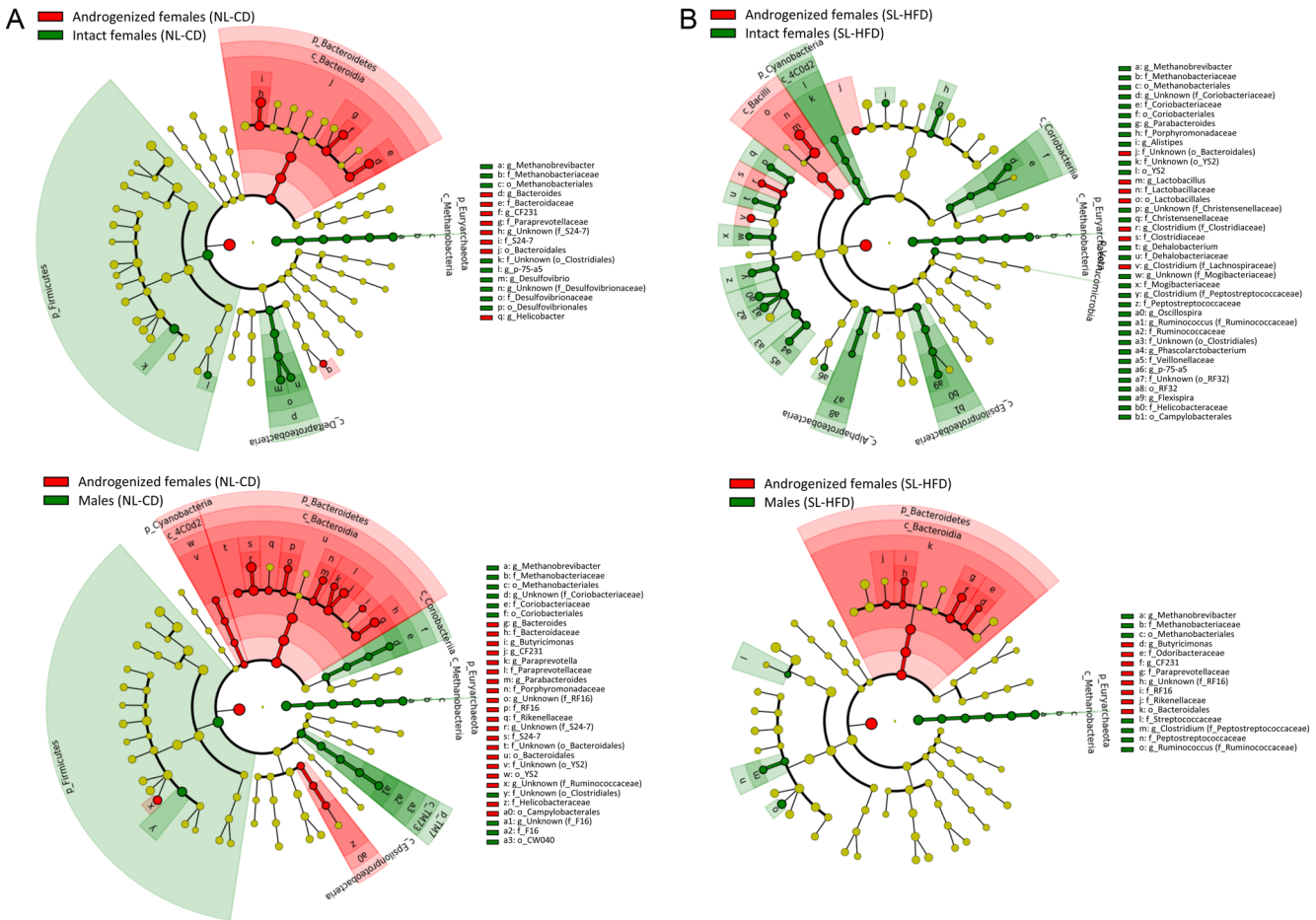

Figure 3
LEfSe analysis between androgenized females and intact females (and males) under normal feeding (A) and postnatal overfeeding (B) conditions at PND-50. Cladogram representing the taxonomic hierarchical structure of the identified differences between groups using Linear discriminant analysis effect size (LEfSe). Each filled circle represents one phylotype. Red, bacterial taxa statistically overrepresented in androgenized females; green, bacterial taxa overrepresented in females (or males). Phylum and class are indicated in their names on the cladogram and the order, family, or genus are given in the key. A full colour version of this figure is available at https://doi.org/10.1530/JOE-20-0277.

p-75-a5, and *Parabacteroides* genera, in addition to 3 unknown genera from unknown from *Christensenellaceae*, *Coriobacteriaceae*, and *Mogibacteriaceae* families.

Impact of early androgenization on gut microbiota composition at PND-150

Analyses of the differences in community structure by LEfSe analysis at PND-150 (Fig. 4 and Supplementary Figs 4, 5) revealed that, in NL-CD animals, androgenization increased *Cyanobacteria* phylum, and reduced *Euryarchaeota* and *Actinobacteria* phyla, but no differences in the majority phyla, *Firmicutes* and *Bacteroidetes*, were found as compared with intact females. At genus level, we found that androgenization increased *CF231* genus in addition to three unknown genera from the *WCHB1-25*, *Elusimicrobiaceae*, and *RF16* families, whereas it reduced *Methanobrevibacter* and *Turicibacter*, in addition to two unknown genera from *S24-7*, and *Coriobacteriaceae* families. In turn, in SL-HFD animals, we found that androgenization increased *Bacteroidetes* phylum, in addition to discrete changes at genus level, as an increase in *Alistipes* and *Odoribacter* genera, and differences in two unknown additional genera from *RF16* (increase), and *Coriobacteriaceae* (decrease) families. Moreover, we observed no differences in F/B ratio between androgenized and intact females at PND-150 under both NL-CD and SL-HFD conditions (Supplementary Table 2).

Microbiota putatively modulates host metabolism via changes in miRNAs

In order to evaluate the cross-talk between gut microbiota and the changes in sex hormones and obesity of the host,

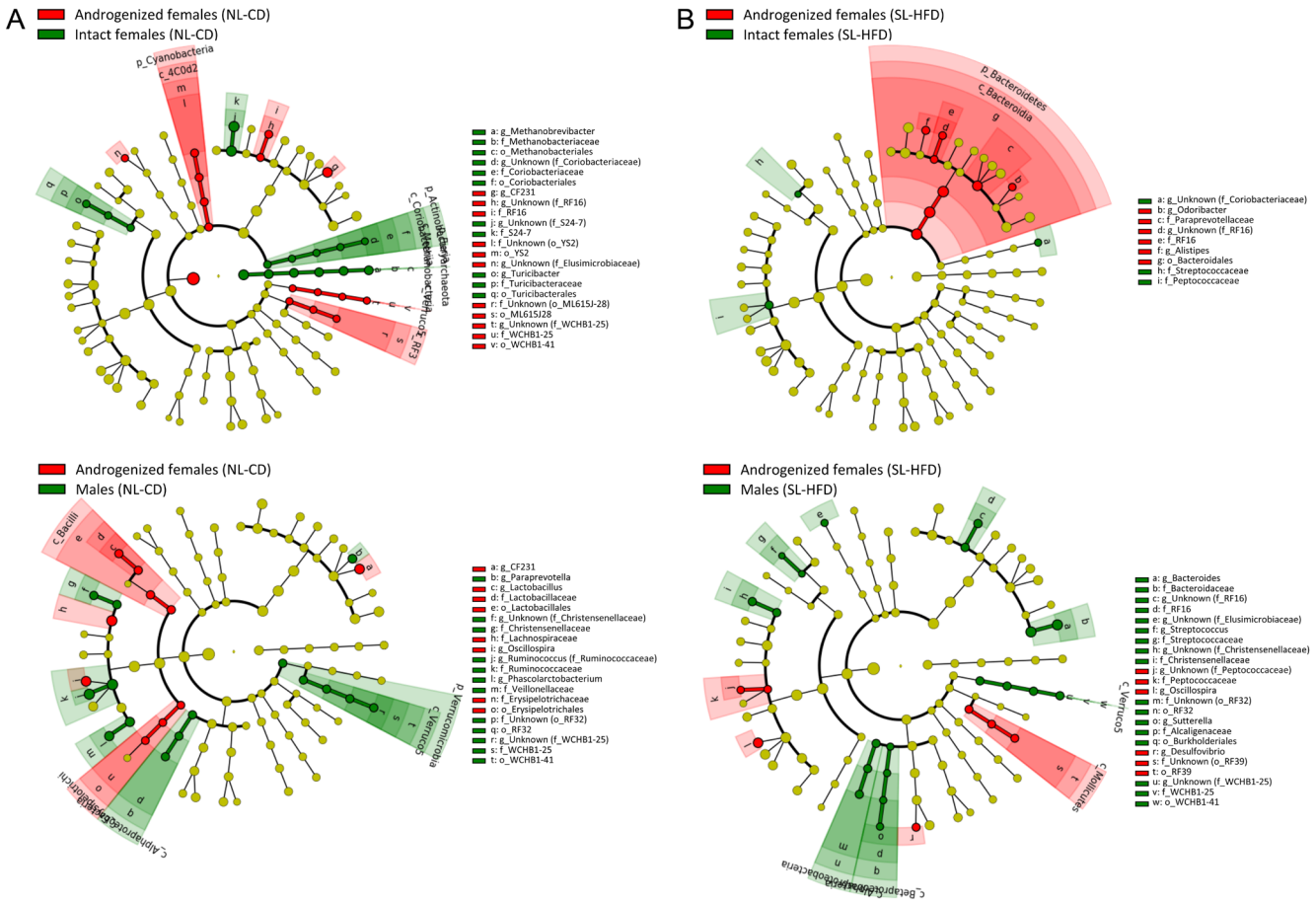

Figure 4
LEfSe analysis between androgenized females and intact females (and males) under normal feeding (A) and postnatal overfeeding (B) conditions at PND-150. Cladogram representing the taxonomic hierarchical structure of the identified differences between groups using Linear discriminant analysis effect size (LEfSe). Each filled circle represents one phylotype. Red, bacterial taxa statistically overrepresented in androgenized females; green, bacterial taxa overrepresented in females (or males). Phylum and class are indicated in their names on the cladogram and the order, family, or genus are given in the key. A full colour version of this figure is available at https://doi.org/10.1530/JOE-20-0277.

we analyzed the relationship between the bacterial taxa identified by LEfSe analysis according to sex hormones and obesity, and the expression levels of the miRNAs in small and large intestine. First of all, microarray analysis testing 758 miRNAs detected the expression of 60 miRNAs in large intestine and 103 miRNAs in small intestine of the animals in our experimental conditions. Of note, the expression of 25 of these miRNAs was detected in both large and small intestine. We performed a correlation analysis between the bacterial taxa identified by LEfSe analysis and the intestinal expression of miRNAs. From this correlation analysis, we selected 30 miRNAs in small intestine and 23 miRNAs in large intestine (4 miRNAs expressed in both large and small intestine showed a relationship with any bacterial taxa identified by LEfSe analysis) in which Pearson correlation coefficient was >0.9 or <–0.9, and a *P*-value < 0.01 (Figs 5, 6 and

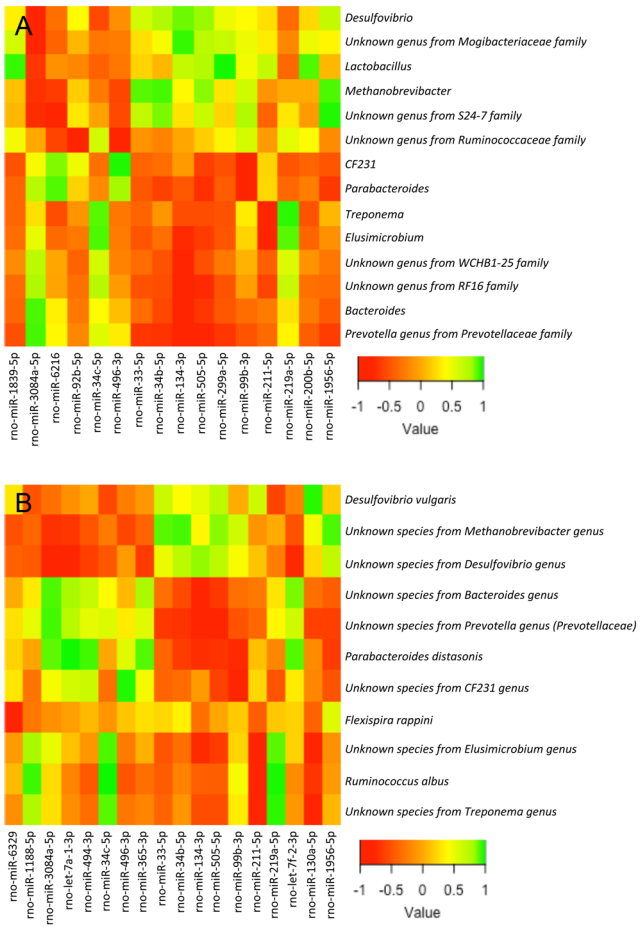

Figure 5
Heatmap from the Pearson's correlation coefficient between the bacterial genera (A) and species (B) identified by LEfSe analyses and the expression levels of the miRNAs in large intestine. A full colour version of this figure is available at https://doi.org/10.1530/JOE-20-0277.

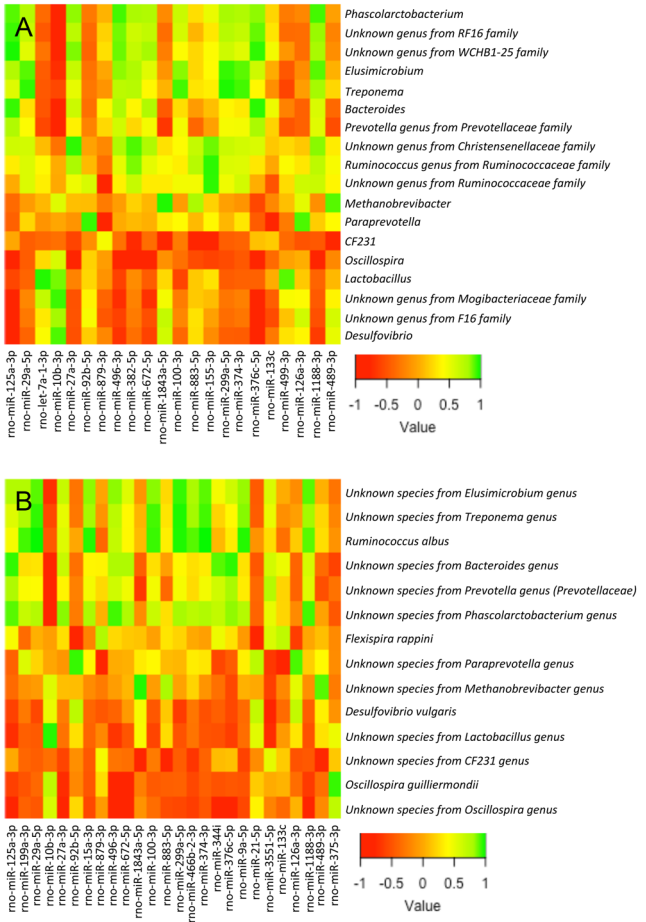

Figure 6
Heatmap from the Pearson's correlation coefficient between the bacterial genera (A) and species (B) identified by LEfSe analyses and the expression levels of the miRNAs in small intestine. A full colour version of this figure is available at https://doi.org/10.1530/JOE-20-0277.

Supplementary Tables 3, 4, 5, 6). Further, we performed a complementary analysis with the selected miRNAs in large and small intestine separately using the DIANAtools V.3. KEGG pathways in which selected miRNAs were assigned are shown in Tables 1 and 2, and their associated miRNAs are shown in Table 3. Of note, we found that several of the selected miRNAs were associated to (1) sex hormone-related processes: steroid biosynthesis (miR-15a-3p, miR-100-3p, miR-199a-3p), oocyte meiosis (miR-27a-3p, miR-29a-5p), progesterone-mediated oocyte maturation (miR-21-5p, miR-27a-3p, hsa-miR-34a-5p); (2) metabolism: insulin signaling pathway (miR-15a-3p, miR-27a-3p), fatty acid biosynthesis (miR-15a-3p, miR-27a-3p, miR-34a-5p, miR-34c-5p, miR-199a-3p) fatty acid metabolism (miR-15a-3p, miR-21-5p, miR-27a-3p, miR-34a-5p, miR-34c-5p, miR-92b-5p, miR-199a-3p); and (3) intestinal barrier integrity: focal adhesion (miR-27a-3p, miR-125a-3p), adherens junction

Table 1 KEGG pathways related with miRNAs expression in the small intestine.

KEGG pathway	*P*-value
1. Metabolism	
1.0 Global and overview maps	
Fatty acid metabolism	0.002
1.1 Carbohydrate metabolism	
Inositol phosphate metabolism	0.023
1.3 Lipid metabolism	
Fatty acid biosynthesis	<0.001
1.5 Amino acid metabolism	
Lysine degradation	<0.001
1.7 Glycan biosynthesis and metabolism	
Mucin type O-glycan biosynthesis	0.038
Other types of O-glycan biosynthesis	0.013
Glycosaminoglycan biosynthesis – keratan sulfate	0.011
2. Genetic information processing	
2.1 Transcription	
Spliceosome	0.047
2.2 Translation	
mRNA surveillance pathway	0.022
2.3 Folding. sorting and degradation	
Protein processing in endoplasmic reticulum	<0.001
RNA degradation	0.041
2.4 Replication and repair	
3. Environmental information processing	
3.2 Signal transduction	
MAPK signaling pathway	0.009
TGF-beta signaling pathway	<0.001
Hippo signaling pathway	<0.001
TNF signaling pathway	0.016
HIF-1 signaling pathway	0.007
FoxO signaling pathway	<0.001
Sphingolipid signaling pathway	0.002
PI3K-Akt signaling pathway	0.047
AMPK signaling pathway	<0.001
mTOR signaling pathway	<0.001
3.3 Signaling molecules and interaction	
ECM-receptor interaction	<0.001
4. Cellularprocesses	
4.1 Transport and catabolism	
Endocytosis	0.009
4.2 Cell growth and death	
Cell cycle	<0.001
Oocyte meiosis	0.001
p53 signaling pathway	0.003
4.3 Cellular community – eukaryotes	
Focal adhesion	<0.001
Adherens junction	<0.001
Signaling pathways regulating pluripotency of stem cells	<0.001
4.5 Cell motility	
5. Organismal systems	
5.1 Immune system	
Leukocyte transendothelial migration	0.034
5.2 Endocrine system	
Insulin signaling pathway	<0.001
Thyroid hormone signaling pathway	<0.001
5.3 Circulatory system	
Adrenergic signaling in cardiomyocytes	0.016

(Continued)

Table 1 Continued.

KEGG pathway	*P*-value
5.6 Nervous system	
Neurotrophin signaling pathway	<0.001
5.10 Environmental adaptation	
Circadian rhythm	0.005
6. Human diseases	
6.1 Cancers: overview	
Pathways in cancer	<0.001
Transcriptional misregulation in cancer	0.016
Proteoglycans in cancer	<0.001
Viral carcinogenesis	<0.001
6.2 Cancers: specific types	
Colorectal cancer	<0.001
Pancreatic cancer	0.004
Glioma	0.001
Thyroid cancer	<0.001
Chronic myeloid leukemia	<0.001
Renal cell carcinoma	<0.001
Prostate cancer	0.019
Endometrial cancer	0.001
Non-small cell lung cancer	0.030
6.4 Neurodegenerative diseases	
Prion diseases	<0.001
6.6 Cardiovascular diseases	
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.047
6.8 Infectious diseases: bacterial	
Shigellosis	0.009
Bacterial invasion of epithelial cells	<0.001
6.9 Infectious diseases: viral	
Hepatitis B	<0.001
Epstein-Barr virus infection	0.004
6.10 Infectious diseases: parasitic	
Chagas disease (American trypanosomiasis)	0.002

(miR-27a-3p, miR-34a-5p, miR-99b-3p, miR-125a-3p, miR-130a-5p, miR-200b-5p), mucin type O-Glycan biosynthesis (miR-27a-3p, miR-29a-5p, miR-100-3p).

Discussion

Our study shows a disruption of the metabolic programming by an early (neonatal) exposure to high doses of androgen, namely testosterone propionate, in female rats. Androgenized females were heavier than intact females and displayed impaired glucose homeostasis, when concurrently exposed to an obesogenic insult (SL-HFD condition), and these phenotypic alterations were paralleled by alterations in the gut microbiota. Moreover, changes in gut microbiota were related with perturbations of miRNA expression profiles in small and large intestine, which might reflect a potential mechanism of the cross-talk between gut microbiota and host. Admittedly, our model of neonatal androgenization does not fully recapitulate the phenotype of a particular human disease. However, it putatively shares features with human conditions linked to inappropriate exposures to androgens during early developmental periods, which include not only PCOS, for which the neonatally androgenized rat has been used previously as model (Walters *et al.* 2012, Romero-Ruiz *et al.* 2019), but also other pathologies and conditions, ranging from congenital adrenal hyperplasia to inappropriate exposures to environmental compounds with androgenic (or anti-androgenic) activity. This reinforces the translational value of our current dataset.

Sex steroids are important determinants of metabolic programming, and an inappropriate exposure to sex steroids during early maturational periods has been shown to influence metabolic homeostasis (Witchel & Tena-Sempere 2013). In fact, it has been shown that sex steroid manipulations, and particularly postnatal androgenization, alters gut microbiota composition (Moreno-Indias *et al.* 2016). However, this latter study did not describe whether postnatal androgenization alters sex steroid plasma levels later on life, neither it used dynamic test to evaluate the impact of postnatal androgenization

Table 2 KEGG pathways related with miRNAs expression in the large intestine.

KEGG pathway	*P*-value
1. Metabolism	
1.0 Global and overview maps	
2-Oxocarboxylic acid metabolism	0.022
Fatty acid metabolism	<0.001
1.2 Energy metabolism	
Sulfur metabolism	0.002
1.3 Lipid metabolism	
Fatty acid biosynthesis	<0.001
1.5 Amino acid metabolism	
Valine. leucine and isoleucine biosynthesis	0.001
1.7 Glycan biosynthesis and metabolism	
Other types of O-glycan biosynthesis	0.002
Glycosphingolipid biosynthesis – lacto and neolacto series	0.002
1.8 Metabolism of cofactors and vitamins	
Pantothenate and CoA biosynthesis	0.027
1.12 Chemical structure transformation maps	
2. Genetic information processing	
2.4 Replication and repair	
3. Environmental information processing	
3.2 Signal transduction	
Hippo signaling pathway	0.022
TNF signaling pathway	0.022
3.3 Signaling molecules and interaction	
4. Cellular processes	
4.3 Cellular community – eukaryotes	
Focal adhesion	0.042
Adherens junction	<0.001
4.5 Cell motility	
Regulation of actin cytoskeleton	0.022
6. Human diseases	
6.1 Cancers:overview	
Pathways in cancer	0.022
6.2 Cancers: specific types	
Glioma	0.020
6.8 Infectious diseases: bacterial	
Pathogenic *Escherichia coli* infection	0.007
Salmonella infection	0.016
Shigellosis	0.005
Bacterial invasion of epithelial cells	0.003

on glucose homeostasis. More importantly, this previous study did not include analyses in males or at two different age-points; the latter have allowed us to highlight important temporal changes in microbiota composition between early and late-adulthood. Finally, the previous study did not analyze the effect of postnatal androgenization on gut microbiota under lean (NL-CD) and obese (SL-HFD) condition separately, as in that former study the groups of androgenized females under NL-CD and SL-HFD conditions were merged for microbiota analyses.

Notably, our study showed that whereas androgenization had no effect on endogenous androgens levels later in life (except for a slight reduction on the androstenedione levels), it caused an overt alteration of serum E2 and E1 levels, in addition to reduced levels of progesterone. Albeit the positive role of estrogen receptor activation on insulin sensitivity has been described in different tissues (Yan *et al.* 2019), it has been pointed out also that the constant maintenance of high estrogen levels could lead to insulin resistance, as a consequence of increasing insulin secretion (Polderman *et al.* 1994, Alonso-Magdalena *et al.* 2006). This suggests that the increased serum E2 and E1 levels might contribute to the lower insulin sensitivity observed in androgenized females compared to the intact adult females, an effect exacerbated under SL-HFD condition.

Despite the proven anti-obesity effects of estrogens, through decreasing food intake and increasing energy expenditure in females (Eckel 2011, Xu *et al.* 2019),

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T a b l e KEGG pathways associated with microbiome-related miRNAs.

KEGG pathway	Human miRNA
2-Oxocarboxylic acid metabolism (hsa01210)	hsa-miR-505-5p-L
Adherens junction (hsa04520)	hsa-miR-99b-3p-SL; hsa-miR-200b-5p-L; hsa-miR-130a-5p-L; hsa-miR-125a-3p-S; hsa-miR-27a-3p-S
Allograft rejection (hsa05330)	hsa-miR-10b-3p-S; hsa-miR-92b-5p-SL; hsa-miR-382-5p-S
alpha-Linolenic acid metabolism (hsa00592)	hsa-miR-92b-5p-SL
Amoebiasis (hsa05146)	hsa-miR-382-5p-S
AMPK signaling pathway (hsa04152)	hsa-miR-27a-3p-S; hsa-miR-15a-3p-S
Antigen processing and presentation (hsa04612)	hsa-miR-10b-3p-S; hsa-miR-92b-5p-SL; hsa-miR-382-5p-S; hsa-miR-211-5p-L
Apoptosis (hsa04210)	hsa-miR-200b-5p-L
Arrhythmogenic right ventricular cardiomyopathy (ARVC) (hsa05412)	hsa-miR-100-3p-S; hsa-miR-505-5p-L
Axon guidance (hsa04360)	hsa-miR-505-5p-L; hsa-miR-27a-3p-S
Bacterial invasion of epithelial cells (hsa05100)	hsa-miR-99b-3p-SL; hsa-miR-27a-3p-S; hsa-miR-21-5p-S
Biosynthesis of unsaturated fatty acids (hsa01040)	hsa-miR-21-5p-S
Bladder cancer (hsa05219)	hsa-miR-27a-3p-S
Cell cycle (hsa04110)	hsa-miR-99b-3p-SL; hsa-miR-27a-3p-S; hsa-miR-21-5p-S
Central carbon metabolism in cancer (hsa05230)	hsa-miR-34c-5p-L; hsa-miR-382-5p-S; hsa-miR-125a-3p-S; hsa-miR-27a-3p-S
Chronic myeloid leukemia (hsa05220)	hsa-miR-27a-3p-S; hsa-miR-15a-3p-S
Circadian entrainment (hsa04713)	hsa-miR-10b-3p-S; hsa-miR-200b-5p-L
Colorectal cancer (hsa05210)	hsa-miR-125a-3p-S; hsa-miR-27a-3p-S; hsa-miR-15a-3p-S; hsa-miR-21-5p-S
Cytokine-cytokine receptor interaction (hsa04060)	hsa-miR-211-5p-L
D-Glutamine and D-glutamate metabolism (hsa00471)	hsa-miR-200b-5p-L
ECM-receptor interaction (hsa04512)	hsa-miR-382-5p-S; hsa-miR-100-3p-S; hsa-miR-211-5p-L; hsa-miR-130a-5p-L; hsa-miR-125a-3p-S; hsa-miR-27a-3p-S
Endometrial cancer (hsa05213)	hsa-miR-27a-3p-S; hsa-miR-21-5p-S
ErbB signaling pathway (hsa04012)	hsa-miR-27a-3p-S
Ether lipid metabolism (hsa00565)	hsa-miR-100-3p-S
Fatty acid biosynthesis (hsa00061)	hsa-miR-34c-5p-L; hsa-miR-199a-3p-S; hsa-miR-27a-3p-S; hsa-miR-15a-3p-S
Fatty acid degradation (hsa00071)	hsa-miR-92b-5p-SL; hsa-miR-29a-5p-S; hsa-miR-21-5p-S
Fatty acid elongation (hsa00062)	hsa-miR-29a-5p-S; hsa-miR-21-5p-S
Fatty acid metabolism (hsa01212)	hsa-miR-92b-5p-SL; hsa-miR-34c-5p-L; hsa-miR-199a-3p-S; hsa-miR-27a-3p-S; hsa-miR-15a-3p-S; hsa-miR-21-5p-S
Focal adhesion (hsa04510)	hsa-miR-125a-3p-S; hsa-miR-27a-3p-S
FoxO signaling pathway (hsa04068)	hsa-miR-125a-3p-S; hsa-miR-27a-3p-S; hsa-miR-21-5p-S
Glioma (hsa05214)	hsa-miR-34c-5p-L; hsa-miR-27a-3p-S; hsa-miR-15a-3p-S
Glycosphingolipid biosynthesis – lacto and neolacto series (hsa00601)	hsa-miR-505-5p-L; hsa-miR-130a-5p-L
Graft-vs-host disease (hsa05332)	hsa-miR-10b-3p-S
Hepatitis B (hsa05161)	hsa-miR-27a-3p-S; hsa-miR-15a-3p-S; hsa-miR-21-5p-S
Hippo signaling pathway (hsa04390)	hsa-miR-99b-3p-SL; hsa-miR-29a-5p-S; hsa-miR-27a-3p-S; hsa-miR-21-5p-S
HTLV-I infection (hsa05166)	hsa-miR-15a-3p-S
Insulin signaling pathway (hsa04910)	hsa-miR-27a-3p-S; hsa-miR-15a-3p-S
Leukocyte transendothelial migration (hsa04670)	hsa-miR-200b-5p-L
Lysine degradation (hsa00310)	hsa-miR-505-5p-L; hsa-miR-125a-3p-S; hsa-miR-27a-3p-S; hsa-miR-21-5p-S
Melanoma (hsa05218)	hsa-miR-27a-3p-S
mRNA surveillance pathway (hsa03015)	hsa-miR-29a-5p-S
mTOR signaling pathway (hsa04150)	hsa-miR-382-5p-S; hsa-miR-27a-3p-S
Mucin type O-Glycan biosynthesis (hsa00512)	hsa-miR-100-3p-S; hsa-miR-29a-5p-S; hsa-miR-27a-3p-S
Neurotrophin signaling pathway (hsa04722)	hsa-miR-27a-3p-S
NF-kappa B signaling pathway (hsa04064)	hsa-miR-200b-5p-L
Non-homologous end-joining (hsa03450)	hsa-miR-99b-3p-SL
Non-small cell lung cancer (hsa05223)	hsa-miR-27a-3p-S
One carbon pool by folate (hsa00670)	hsa-miR-125a-3p-S
Oocyte meiosis (hsa04114)	hsa-miR-29a-5p-S; hsa-miR-27a-3p-S
Other types of O-glycan biosynthesis (hsa00514)	hsa-miR-505-5p-L
p53 signaling pathway (hsa04115)	hsa-miR-27a-3p-S; hsa-miR-21-5p-S
Pancreatic cancer (hsa05212)	hsa-miR-27a-3p-S

(Continued)

Table 3 Continued.

KEGG pathway	Human miRNA
Pantothenate and CoA biosynthesis (hsa00770)	hsa-miR-34c-5p-L; hsa-miR-100-3p-S
Parkinson's disease (hsa05012)	hsa-miR-99b-3p-SL
Pathways in cancer (hsa05200)	hsa-miR-27a-3p-S; hsa-miR-21-5p-S
PI3K-Akt signaling pathway (hsa04151)	hsa-miR-382-5p-S
Prion diseases (hsa05020)	hsa-miR-382-5p-S; hsa-miR-27a-3p-S
Progesterone-mediated oocyte maturation (hsa04914)	hsa-miR-27a-3p-S
Prolactin signaling pathway (hsa04917)	hsa-miR-21-5p-S
Prostate cancer (hsa05215)	hsa-miR-27a-3p-S
Protein processing in endoplasmic reticulum (hsa04141)	hsa-miR-211-5p-L; hsa-miR-27a-3p-S
Proteoglycans in cancer (hsa05205)	hsa-miR-125a-3p-S; hsa-miR-199a-3p-S; hsa-miR-29a-5p-S; hsa-miR-27a-3p-S; hsa-miR-15a-3p-S; hsa-miR-21-5p-S
Renal cell carcinoma (hsa05211)	hsa-miR-100-3p-S; hsa-miR-27a-3p-S
Salmonella infection (hsa05132)	hsa-miR-505-5p-L
Shigellosis (hsa05131)	hsa-miR-27a-3p-S
Signaling pathways regulating pluripotency of stem cells (hsa04550)	hsa-miR-27a-3p-S
Sphingolipid metabolism (hsa00600)	hsa-miR-21-5p-S
Sphingolipid signaling pathway (hsa04071)	hsa-miR-27a-3p-S
Steroid biosynthesis (hsa00100)	hsa-miR-100-3p-S; hsa-miR-199a-3p-S; hsa-miR-15a-3p-S
Sulfur metabolism (hsa00920)	hsa-miR-505-5p-L
Taste transduction (hsa04742)	hsa-miR-100-3p-S
TGF-beta signaling pathway (hsa04350)	hsa-miR-125a-3p-S; hsa-miR-29a-5p-S; hsa-miR-27a-3p-S
Thyroid cancer (hsa05216)	hsa-miR-125a-3p-S; hsa-miR-27a-3p-S; hsa-miR-21-5p-S
Thyroid hormone signaling pathway (hsa04919)	hsa-miR-27a-3p-S; hsa-miR-21-5p-S
Transcriptional misregulation in cancer (hsa05202)	hsa-miR-27a-3p-S; hsa-miR-21-5p-S
Ubiquitin mediated proteolysis (hsa04120)	hsa-miR-27a-3p-S
Valine, leucine and isoleucine biosynthesis (hsa00290)	hsa-miR-505-5p-L; hsa-miR-130a-5p-L
Viral carcinogenesis (hsa05203)	hsa-miR-125a-3p-S; hsa-miR-27a-3p-S
Viral myocarditis (hsa05416)	hsa-miR-382-5p-S

hsa, *Homo sapiens*; L, large intestine; S, small intestine.

our study shows that androgenized females, which presented higher estrogen levels, were heavier than intact females, suggesting the contribution of another factors or mediators for such an obese phenotype. These might include changes in gut microbiota, as it has been previously shown that the structure of gut microbiota is altered in parallel to changes on sex hormones levels (Markle *et al.* 2013, Insenser *et al.* 2018, Santos-Marcos *et al.* 2018). However, the causality between these two phenomena has not been described so far. Our study conclusively documents that early androgenization of females alters the gut microbiota profile, evidenced by the fact that the gut microbiota under NL-CD condition from androgenized females at PND-50 was characterized by higher *Bacteroidetes* and lower *Firmicutes* than the intact females. However, differences in these major phyla disappeared in older rats (PND-150), while in those androgenization reduced the abundance of *Actinobacteria* and *Euryarchaeota* and increased *Cyanobacteria*.

Different hormonal changes take place through the lifespan (Horstman *et al.* 2012). For example, major hormonal changes occur during puberty (Koolschijn *et al.* 2014), which may also influence gut microbiota composition (Kim *et al.* 2019). Our study showed that the differences between androgenized females and intact females were more exacerbated at early adulthood (PND-50) than later in life (late adulthood at PND-150). In fact, the differences observed between androgenized and intact females under NL-CD at PND-50 in the major phyla, *Bacteroidetes* and *Firmicutes*, were not observed at PND-150. These differences also disappeared at early adulthood (PND-50) when animals were overfed, suggesting an additional effect of obesity, reducing the differences in gut microbiota between androgenized and intact females, as observed later in life. However, postnatal overfeeding increased the F/B ratio in both androgenized and intact females as expected taking into account that the F/B ratio, which has great importance in the development of obesity, is commonly increased in this condition (Turnbaugh *et al.* 2006). Moreover, postnatal overfeeding reduced the alterations caused by androgenization in gut microbiota in the late adulthood

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(PND-150), with only four genera differing between groups, but androgenized females were characterized by higher *Bacteroidetes* than intact females. Overall, our study supports the idea that the interaction of the hormonal status during the lifespan with the nutritional conditions might modulate gut microbiota.

As intestinal miRNAs are increasingly recognized as potential decoders of dysbiosis into cardiometabolic diseases, we also explored the putative cross-talk between gut microbiota and host through regulation of miRNA expression in small and large intestine (Liu *et al.* 2016, Serino 2016). Of note, we did not include in the analysis all the bacterial taxa but only those identified by LEfSe analysis in order to reduce the random association. This approach allowed us to detect those miRNAs that were affected by neonatal androgenization, and changed in parallel with changes in bacterial taxa, identified by LEfSe analysis. Among those, we could identify the miRNAs that potentially mediate the interaction between sex hormones, gut microbiota and host metabolism.

In addition to several KEGG pathways related with metabolism, such as fatty acid metabolism or sphingolipid signaling pathway, which in turn are related with the development of type 2 diabetes mellitus (Imamura *et al.* 2017) and cardiovascular disease (Razquin *et al.* 2018), our approach detected also potential miRNA-mediated relationships between the gut microbiota, metabolism and sex steroid-related pathways. For example, the expression levels of miR-27a-3p were positively related with *Oscillospira*, a short chain fatty acids (SCFA)-producer bacterial genus (Gophna *et al.* 2017), which in turn may be related with glucose homeostasis, as SCFA increases the action and release of insulin through intestinal incretins (Freeland & Wolever 2010, Tarini & Wolever 2010). Moreover, SCFAs are also involved in energy metabolism and appetite regulation through the hormones leptin and ghrelin (Shen *et al.* 2013, Byrne *et al.* 2015, Lv *et al.* 2018), whose plasma levels were altered in our study and may be at least partially responsible of the weight gain in androgenized females. In addition, miR-27a-3p is involved in fatty acid metabolism, insulin signaling, oocyte maturation, in addition to contribute to the intestinal barrier integrity by processes such as adherens junction, focal adhesion and mucin type O-glycan biosynthesis. In addition, other miRNAs showed by this study, such as miR-29a-5p, and miR-100-3p, may also link metabolism and sex hormone actions on the basis of their previously reported involvement in mediating sex-steroid actions in other tissues, such as progesterone-mediated oocyte maturation (Navakanitworakul *et al.* 2016, Tesfaye *et al.* 2018, Tu *et al.* 2019). These evidences suggest that the cross-talk between gut microbiota and the host via specific miRNAs may involve also gonadal steroid mediated events. In addition, the expression of these miRNAs was related with the intestinal abundance of *Treponema*, a bacterial genus that enhances the ability to extract calories from resistant starch and oligosaccharides, as well as carbohydrates that escape digestion in the small intestine and are fermented in the gut (Flint *et al.* 2008).

Taken together, our results support the idea that changes in the gut microbiome composition, in response to alterations in sex hormones, may trigger a further gene expression response in the host for selective gut microbial colonization, via miRNAs as a mediator of this process. Thus, the potential contribution of intestinal bacteria to the expression levels of miRNAs as a gut microbiota-intestine cross-talk system, in line with previous studies (Liu *et al.* 2016, Serino 2016), would link changes in the gut microbiota composition to processes such as metabolism and sex hormone metabolic programming.

Our study suggests that nutritional and hormonal disruption in early periods of the development alters metabolic programming with durable consequences later in life. Moreover, this disruption dynamically affects the structure of gut microbiota, currently considered as an integrated organ, which may interact with the host via a cross-talk mediated by intestinal miRNAs, which may serve as an additional link between early mal-programming and impaired metabolic health later in life.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/JOE-20-0277.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by research grants from Ministerio de Economia y Competitividad (CP14/00114 to A C; PIE14/00005 to J L-M and M T-S; AGL2015-67896-P to J L-M and A C); Ministerio de Ciencia e Innovacion (FIS PI19/00299 to A C; DTS19/00007 to A C; PI16/01777 to P P M); Fondo Europeo de Desarrollo Regional (FEDER). A C is supported by an ISCIII research contract, Instituto de Salud Carlos III (Programa Miguel Servet CP14/00114 and CPII19/00007).

Authors contribution statement

A Barroso and Jose A Santos-Marcos contributed to the animal experiments, acquisition, analysis and interpretation of data, and drafting of the

manuscript. C Perdices-Lopez, A Vega-Rojas, M A Sanchez-Garrido, and Y Krylova contributed to the animal experiments, analysis and interpretation of data. H Molina-Abril contributed to analysis and interpretation of data. C Ohlsson, P Perez Martinez, M Poutanen, J Lopez-Miranda contributed to interpretation of data and edited the manuscript. M Tena-Sempere and A Camargo designed and conducted the experiments, approval of the manuscript and are the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Acknowledgements

The CIBEROBN is an initiative of the Instituto de Salud Carlos III, Madrid, Spain.

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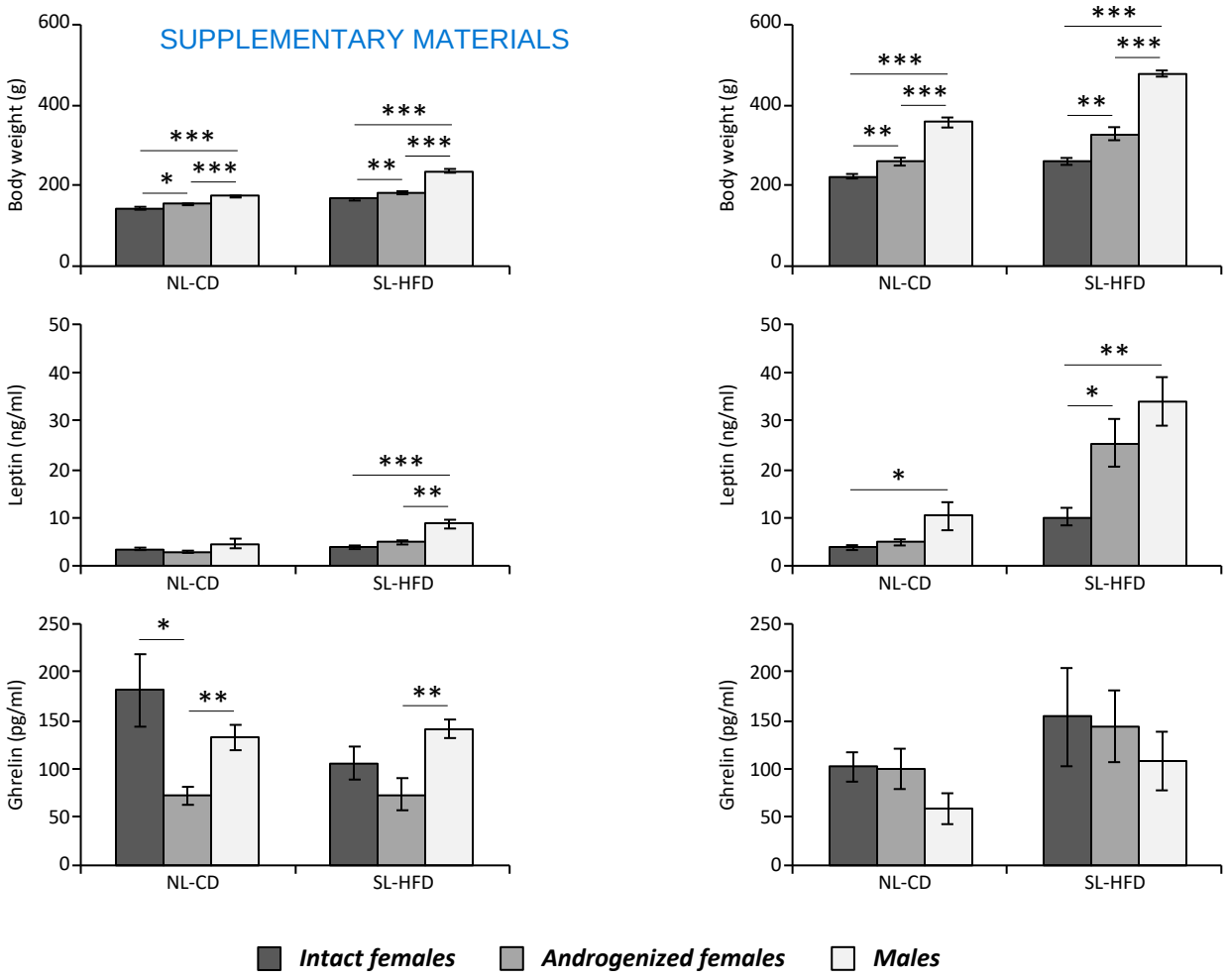
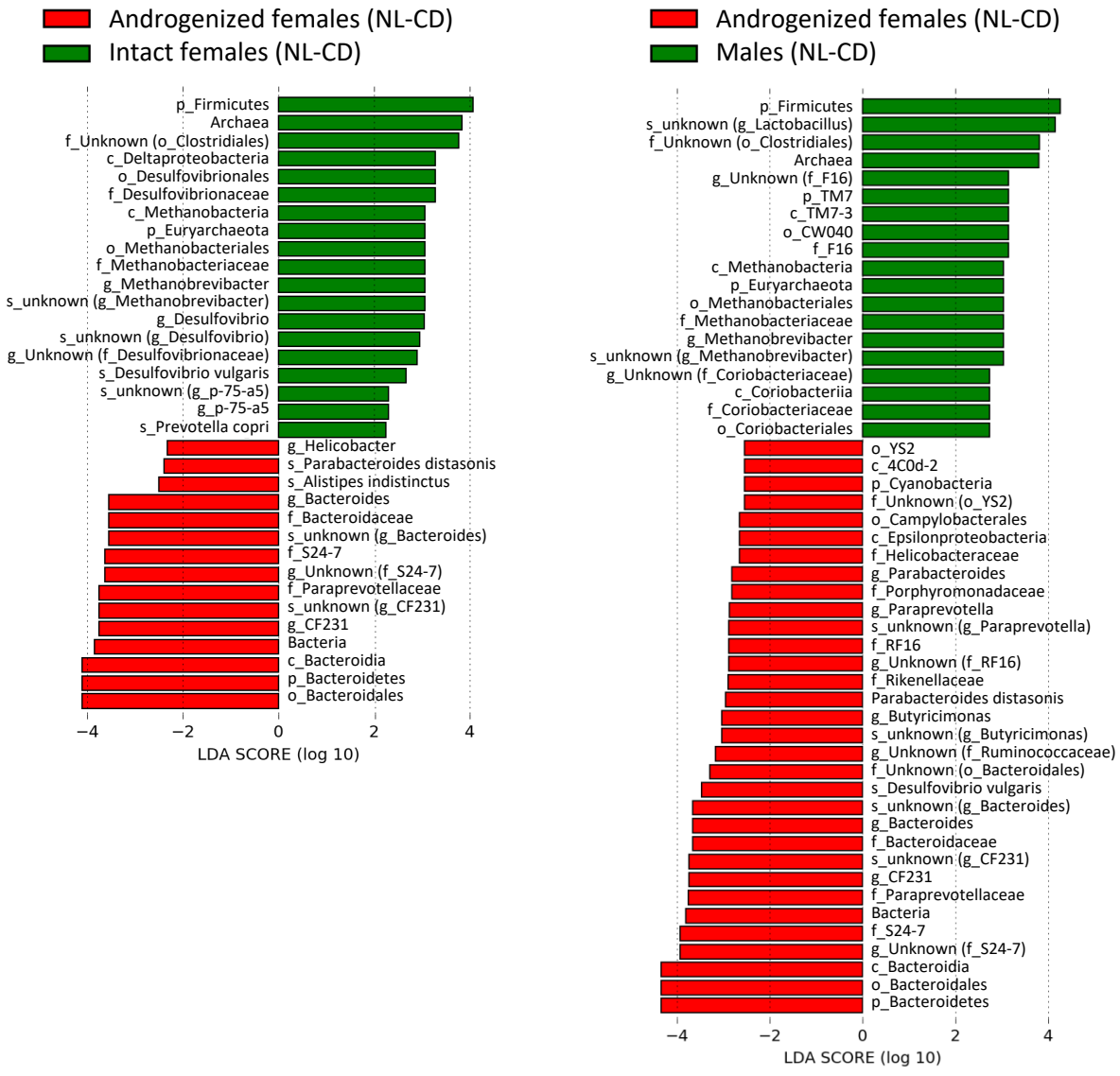
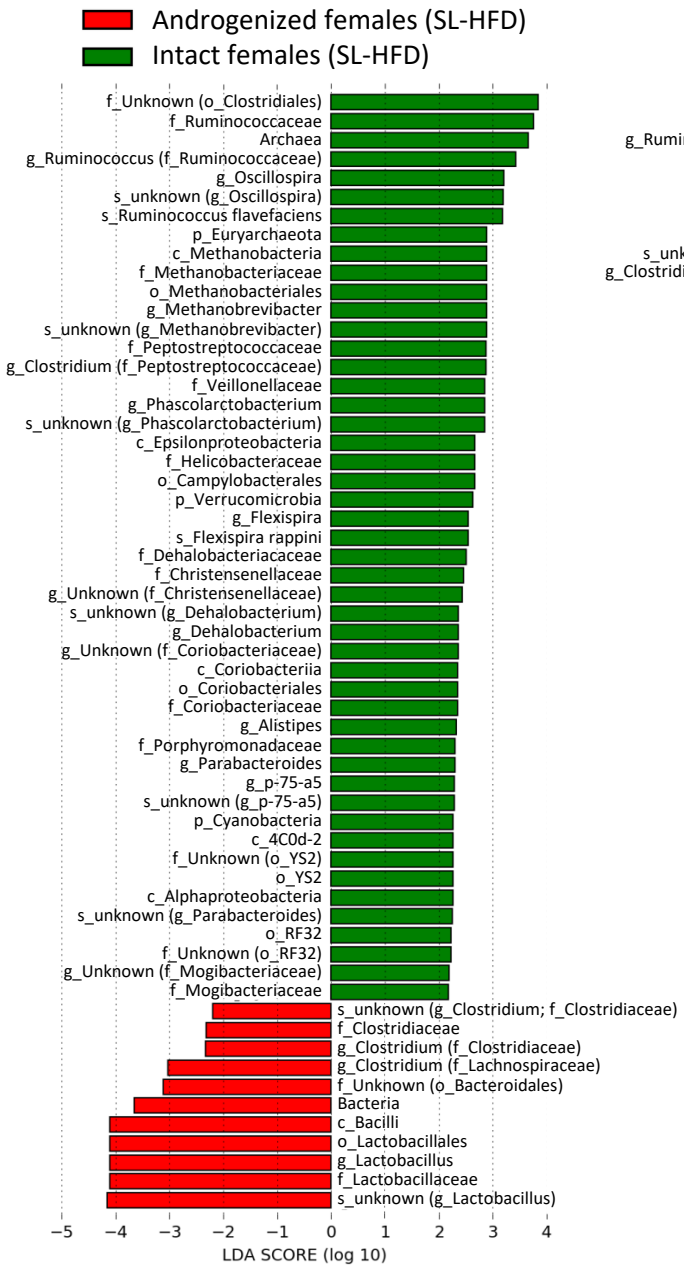
Received in final form 28 July 2020
Accepted 4 August 2020
Accepted Manuscript published online 4 August 2020



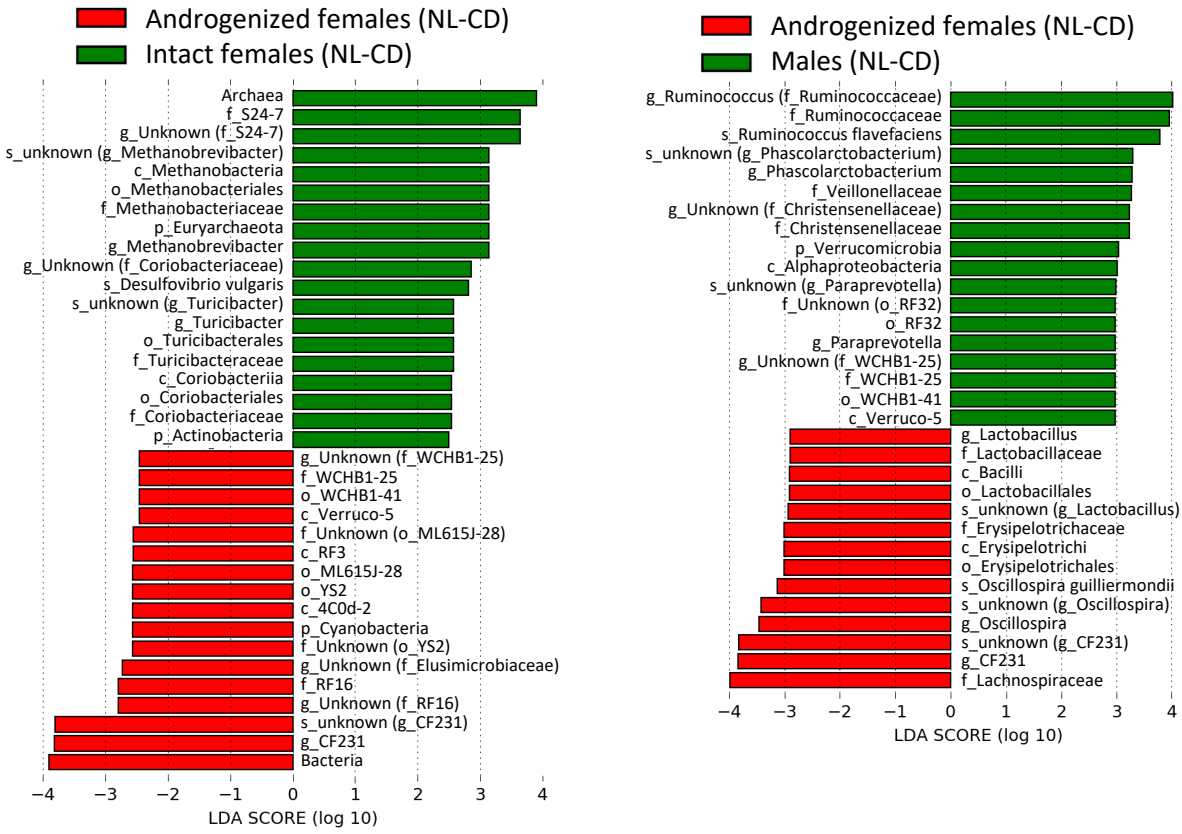
Supplementary Figure 1. Body mass and energy balance-related hormones in intact (non-androgenized) animals and androgenized females, under normal feeding (NL-CD) and postnatal overfeeding (SL-HFD).



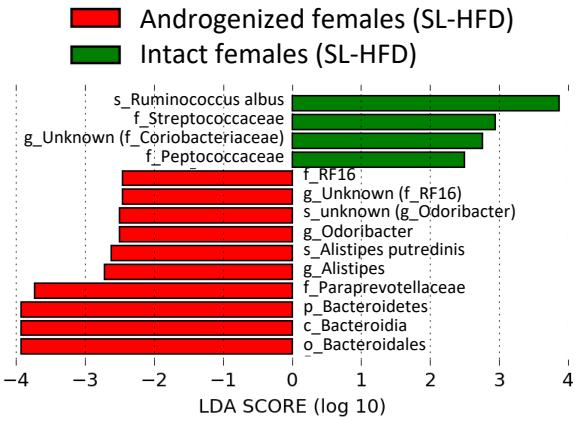
Supplementary Figure 2. Linear discriminant analysis (LDA) scores between androgenized females and intact females (and males) under normal feeding condition at PND-50.

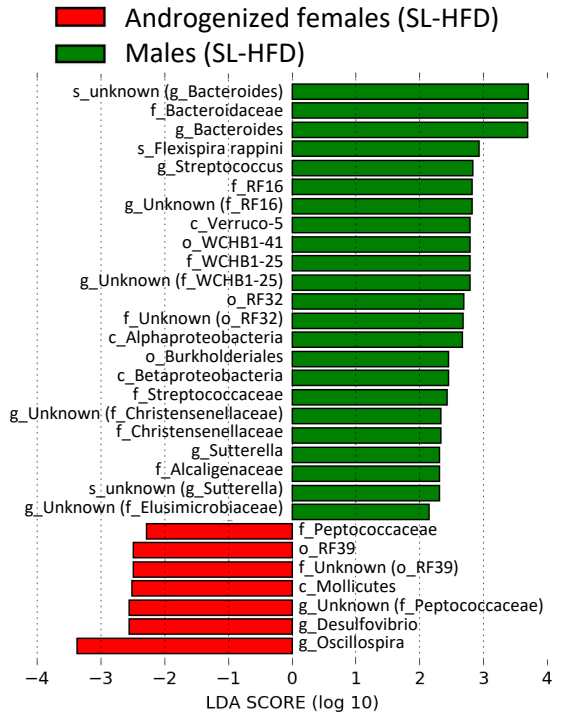


Supplementary Figure 3. Linear discriminant analysis (LDA) scores between androgenized females and intact females (and males) under postnatal overfeeding condition at PND-50.



Supplementary Figure 4. Linear discriminant analysis (LDA) scores between androgenized females and intact females (and males) under normal feeding condition at PND-150.





Supplementary Figure 5. Linear discriminant analysis (LDA) scores between androgenized females and intact females (and males) postnatal overfeeding condition at PND-150.

α-diversity indexes		*NL-CD at PND-50*			*NL-CD at PND-150*		
Faith_pd index		*H*	*P-value*	*Q-value*	*H*	*P-value*	*Q-value*
Androgenized females	Intact females	0.27	0.599	0.599	0.10	0.753	0.753
	Males	4.41	0.036	0.053	1.86	0.172	0.258
Shannon index		*H*	*P-value*	*Q-value*	*H*	*P-value*	*Q-value*
Androgenized females	Intact females	0.89	0.344	0.344	2.82	0.093	0.139
	Males	6.89	0.009	0.026	1.86	0.172	0.172
Observed OTUs		*H*	*P-value*	*Q-value*	*H*	*P-value*	*Q-value*
Androgenized females	Intact females	<0.01	0.958	0.958	0.18	0.674	0.674
	Males	5.84	0.016	0.047	3.98	0.046	0.069
β-diversity distances		*NL-CD at PND-50*			*NL-CD at PND-150*		
Unweighted unifrac		*pseudo-F*	*P-value*	*Q-value*	*pseudo-F*	*P-value*	*Q-value*
Androgenized females	Intact females	1.77	0.003	0.003	1.99	0.002	0.003
	Males	2.75	0.001	0.002	2.12	0.025	0.025
Weighted unifrac		*pseudo-F*	*P-value*	*Q-value*	*pseudo-F*	*P-value*	*Q-value*
Androgenized females	Intact females	3.86	0.012	0.018	2.86	0.027	0.027
	Males	9.54	0.002	0.006	3.44	0.004	0.006
α-diversity indexes		*SL-HFD at PND-50*			*SL-HFD at PND-150*		
Faith_pd index		*H*	*P-value*	*Q-value*	*H*	*P-value*	*Q-value*
Androgenized females	Intact females	1.86	0.172	0.172	0.40	0.529	0.529
	Males	2.16	0.141	0.172	3.19	0.074	0.223
Shannon index		*H*	*P-value*	*Q-value*	*H*	*P-value*	*Q-value*
Androgenized females	Intact females	4.42	0.036	0.107	0.04	0.834	0.833
	Males	1.59	0.208	0.208	3.57	0.059	0.088
Observed OTUs		*H*	*P-value*	*Q-value*	*H*	*P-value*	*Q-value*
Androgenized females	Intact females	0.80	0.372	0.372	0.01	0.916	0.916
	Males	1.87	0.172	0.258	5.34	0.021	0.031
β-diversity distances		*SL-HFD at PND-50*			*SL-HFD at PND-150*		
Unweighted unifrac		*pseudo-F*	*P-value*	*Q-value*	*pseudo-F*	*P-value*	*Q-value*
Androgenized females	Intact females	1.90	0.012	0.017	1.32	0.112	0.112
	Males	1.93	0.017	0.017	2.66	0.006	0.017
Weighted unifrac		*pseudo-F*	*P-value*	*Q-value*	*pseudo-F*	*P-value*	*Q-value*
Androgenized females	Intact females	5.78	0.001	0.003	2.56	0.017	0.026
	Males	2.13	0.107	0.107	2.02	0.062	0.062

Supplementary Table 1. Impact of androgenization on gut microbiota diversity. NL-CD: normal litter, control diet. SL-HFD: small litter, high fat diet. PND: postnatal day. α-diversity indexes: P-value. β-diversity distances: PERMANOVA P-value. Q-value: False Discovery Rate.

	Firmicutes/Bacteroidetes ratio		
	NL-CD PND-50	***SL-HFD PND-50***	*ANOVA P-value*
androgenized females	1.07±0.13	2.94±0.38	<0.001
intact females	2.11±0.038	2.78±0.44	0.275
ANOVA P-value	0.022	0.781	
androgenized females	1.07±0.13	2.94±0.38	<0.001
males	3.26±0.45	6.48±1.52	0.062
ANOVA P-value	<0.001	0.041	
	Firmicutes/Bacteroidetes ratio		
	NL-CD PND-150	***SL-HFD PND-150***	*ANOVA P-value*
androgenized females	1.52±0.20	1.64±0.31	0.738
intact females	1.79±0.20	2.22±0.19	0.139
ANOVA P-value	0.356	0.133	
androgenized females	1.52±0.20	1.64±0.31	0.738
males	1.49±0.18	1.16±0.15	0.193
ANOVA P-value	0.356	0.188	

Supplementary Table 2. Firmicutes/Bacteroidetes ratio between androgenized females and intact females (and males). NL-CD: normal litter, control diet. SL-HFD: small litter, high fat diet. PND: postnatal day.

		rno-miR-1839-5p	rno-miR-3084a-5p	rno-miR-6216	rno-miR-92b-5p	rno-miR-34c-5p	rno-miR-496-3p	rno-miR-33-5p	rno-miR-34b-5p	rno-miR-134-3p	rno-miR-505-5p	rno-miR-299a-5p	rno-miR-99b-3p	rno-miR-211-5p	rno-miR-219a-5p	rno-miR-200b-5p	rno-miR-1956-5p
Methanobrevibacter	Corr.	0.119	-0.705	-0.639	0.177	-0.272	-0.582	**0.902**	**0.919**	0.385	0.843	0.284	0.631	-0.120	0.011	0.021	**0.915**
	p-value	0.822	0.118	0.172	0.738	0.602	0.225	**0.014**	**0.010**	0.451	0.035	0.586	0.179	0.820	0.983	0.968	**0.010**
Bacteroides	Corr.	-0.391	**0.917**	0.339	-0.278	0.399	0.106	-0.349	-0.568	**-0.969**	-0.673	-0.476	-0.344	-0.266	0.277	-0.268	-0.457
	p-value	0.444	**0.010**	0.510	0.593	0.434	0.841	0.498	0.240	**0.001**	0.143	0.340	0.505	0.611	0.595	0.608	0.362
Parabacteroides	Corr.	-0.404	0.742	**0.907**	0.200	-0.176	0.812	-0.475	-0.610	-0.472	-0.711	-0.453	-0.893	0.219	-0.377	-0.247	-0.658
	p-value	0.427	0.091	**0.012**	0.704	0.739	0.050	0.341	0.199	0.344	0.113	0.367	0.017	0.677	0.462	0.637	0.156
Prevotella (Prevotellaceae)	Corr.	-0.526	**0.923**	0.373	-0.198	0.558	0.324	-0.676	-0.694	-0.824	**-0.945**	-0.669	-0.503	-0.373	0.365	-0.440	-0.634
	p-value	0.284	**0.009**	0.466	0.707	0.249	0.531	0.141	0.126	0.044	**0.004**	0.146	0.310	0.467	0.477	0.383	0.177
Unknown (f_RF16)	Corr.	-0.359	0.772	-0.021	-0.324	0.747	-0.087	-0.512	-0.539	**-0.931**	-0.738	-0.496	-0.053	-0.589	0.667	-0.325	-0.359
	p-value	0.484	0.072	0.968	0.530	0.088	0.870	0.299	0.270	**0.007**	0.094	0.317	0.921	0.218	0.148	0.529	0.485
Unknown (f_S24-7)	Corr.	0.081	-0.729	-0.784	0.296	-0.040	-0.564	0.691	0.863	0.274	0.662	0.203	0.761	-0.409	0.291	-0.073	**0.962**
	p-value	0.878	0.100	0.065	0.569	0.941	0.244	0.128	0.027	0.599	0.152	0.700	0.079	0.421	0.575	0.890	**0.002**
CF231	Corr.	-0.506	0.439	0.855	0.477	-0.324	**0.967**	-0.402	-0.348	-0.074	-0.617	-0.522	**-0.951**	0.215	-0.494	-0.403	-0.491
	p-value	0.306	0.384	0.030	0.339	0.532	**0.002**	0.430	0.500	0.889	0.192	0.288	**0.004**	0.682	0.319	0.428	0.323
Elusimicrobium	Corr.	-0.347	0.509	-0.364	-0.281	**0.915**	-0.262	-0.488	-0.336	-0.744	-0.646	-0.490	0.209	-0.817	**0.902**	-0.399	-0.141
	p-value	0.501	0.303	0.479	0.589	**0.010**	0.617	0.327	0.515	0.090	0.166	0.324	0.691	0.047	**0.014**	0.434	0.790
Lactobacillus	Corr.	**0.940**	-0.672	-0.141	-0.197	-0.420	-0.292	0.202	0.072	0.687	0.750	**0.979**	0.456	0.674	-0.382	**0.911**	0.080
	p-value	**0.005**	0.144	0.790	0.708	0.407	0.575	0.701	0.893	0.132	0.086	**0.001**	0.363	0.142	0.455	**0.011**	0.880
Unknown (Ruminococcaceae)	Corr.	0.456	0.006	-0.587	**-0.908**	0.615	-0.819	-0.124	-0.214	-0.017	0.171	0.354	0.596	-0.078	0.532	0.416	-0.168
	p-value	0.363	0.991	0.221	**0.012**	0.194	0.046	0.815	0.685	0.975	0.746	0.492	0.212	0.883	0.277	0.412	0.750
Unknown (Mogibacteriaceae)	Corr.	0.611	**-0.915**	-0.411	0.076	-0.301	-0.258	0.265	0.429	**0.941**	0.717	0.666	0.492	0.326	-0.199	0.488	0.366
	p-value	0.198	**0.010**	0.419	0.887	0.562	0.622	0.612	0.396	**0.005**	0.109	0.149	0.322	0.529	0.705	0.326	0.476
Desulfovibrio	Corr.	0.338	**-0.949**	-0.341	0.404	-0.563	-0.151	0.624	0.764	0.876	0.827	0.484	0.387	0.258	-0.359	0.231	0.678

	p-value	0.512	**0.004**	0.508	0.427	0.245	0.775	0.186	0.077	0.022	0.042	0.331	0.448	0.622	0.485	0.660	0.139
Treponema	Corr.	-0.389	0.273	-0.536	-0.125	**0.903**	-0.289	-0.396	-0.110	-0.543	-0.542	-0.510	0.317	**-0.929**	**0.951**	-0.497	0.067
	p-value	0.446	0.601	0.273	0.813	**0.014**	0.579	0.437	0.835	0.266	0.266	0.301	0.541	**0.007**	**0.003**	0.316	0.899
Unknown (f_WCHB1-25)	Corr.	-0.171	0.726	-0.018	-0.410	0.612	-0.216	-0.344	-0.508	**-0.946**	-0.531	-0.278	0.055	-0.428	0.561	-0.112	-0.302
	p-value	0.746	0.103	0.972	0.419	0.196	0.681	0.504	0.304	**0.004**	0.278	0.594	0.917	0.397	0.247	0.832	0.561

Supplementary Table 3. Relationship between the bacterial genera identified by LEfSe analysis and the expression levels of the miRNAs in large intestine. Pearson's correlation analysis coefficient (Corr.) and P-value.

		rno-miR-6329	rno-miR-1188-5p	rno-miR-3084a-5p	rno-let-7a-1-3p	rno-miR-494-3p	rno-miR-34c-5p	rno-miR-496-3p	rno-miR-365-3p	rno-miR-33-5p	rno-miR-34b-5p	rno-miR-134-3p	rno-miR-505-5p	rno-miR-99b-3p	rno-miR-211-5p	rno-miR-219a-5p	rno-let-7f-2-3p	rno-miR-130a-5p	rno-miR-1956-5p
Unknown (Methanobrevibacter)	Corr.	-0.532	-0.384	-0.704	-0.675	-0.494	-0.271	-0.584	-0.394	**0.902**	**0.918**	0.384	0.843	0.632	-0.120	0.012	-0.511	0.460	**0.915**
	p-value	0.277	0.452	0.118	0.142	0.319	0.603	0.224	0.440	**0.014**	**0.010**	0.452	0.035	0.178	0.821	0.982	0.300	0.358	**0.011**
Unknown (Bacteroides)	Corr.	0.018	0.308	**0.912**	0.805	0.671	0.402	0.086	0.788	-0.331	-0.554	**-0.973**	-0.659	-0.328	-0.273	0.286	0.862	-0.326	-0.441
	p-value	0.973	0.553	**0.011**	0.054	0.145	0.429	0.871	0.063	0.522	0.254	**0.001**	0.154	0.526	0.600	0.583	0.027	0.529	0.381
Parabacteroides distasonis	Corr.	0.196	-0.028	0.896	**0.983**	**0.924**	-0.004	0.547	**0.907**	-0.399	-0.644	-0.723	-0.692	-0.744	0.118	-0.198	**0.907**	-0.164	-0.643
	p-value	0.710	0.958	0.016	**0.000**	**0.009**	0.994	0.262	**0.013**	0.434	0.167	0.105	0.127	0.090	0.824	0.707	**0.013**	0.756	0.168
Unknown (Prevotella, Prevotellaceae)	Corr.	0.270	0.506	**0.926**	0.730	0.532	0.561	0.314	0.551	-0.666	-0.689	-0.833	**-0.940**	-0.495	-0.378	0.370	0.629	-0.608	-0.626
	p-value	0.605	0.306	**0.008**	0.099	0.278	0.247	0.545	0.257	0.149	0.130	0.040	**0.005**	0.318	0.460	0.470	0.181	0.200	0.184
Unknown (CF231)	Corr.	0.297	-0.306	0.446	0.616	0.653	-0.326	**0.965**	0.453	-0.398	-0.350	-0.079	-0.616	**-0.955**	0.221	-0.498	0.320	-0.200	-0.495
	p-value	0.568	0.555	0.375	0.193	0.160	0.529	**0.002**	0.367	0.434	0.497	0.882	0.193	**0.003**	0.674	0.314	0.536	0.705	0.318
Unknown (Elusimicrobium)	Corr.	-0.083	0.804	0.509	0.120	-0.109	**0.916**	-0.265	0.049	-0.486	-0.336	-0.745	-0.644	0.210	-0.817	**0.902**	0.200	-0.789	-0.140
	p-value	0.876	0.054	0.302	0.821	0.838	**0.010**	0.612	0.927	0.329	0.515	0.089	0.167	0.689	0.047	**0.014**	0.703	0.062	0.791
Ruminococcus albus	Corr.	0.086	**0.934**	0.243	-0.212	-0.468	**0.989**	-0.493	-0.343	-0.450	-0.231	-0.433	-0.435	0.442	-0.775	**0.975**	-0.162	-0.679	-0.074
	p-value	0.872	**0.006**	0.642	0.687	0.349	**0.000**	0.320	0.505	0.370	0.660	0.391	0.389	0.380	0.070	**0.001**	0.759	0.138	0.889
Unknown (Desulfovibrio)	Corr.	-0.423	-0.460	**-0.977**	-0.820	-0.621	-0.479	-0.081	-0.654	0.487	0.717	0.834	0.720	0.428	0.131	-0.248	-0.753	0.233	0.691
	p-value	0.403	0.359	**0.001**	0.045	0.189	0.336	0.879	0.159	0.327	0.108	0.039	0.107	0.397	0.805	0.635	0.084	0.656	0.129
Desulfovibrio vulgaris	Corr.	0.292	-0.501	-0.327	-0.136	0.000	-0.571	-0.247	-0.099	0.652	0.388	0.536	0.716	0.019	0.634	-0.586	-0.215	**0.957**	0.173
	p-value	0.575	0.312	0.527	0.797	0.999	0.236	0.637	0.852	0.160	0.447	0.273	0.109	0.971	0.176	0.222	0.683	**0.003**	0.744
Flexispira rappini	Corr.	**-0.939**	-0.289	-0.132	-0.051	0.085	-0.107	0.139	0.259	0.225	0.343	-0.326	-0.012	0.164	-0.442	0.152	0.198	-0.405	0.546
	p-value	**0.006**	0.578	0.803	0.923	0.873	0.840	0.793	0.620	0.668	0.506	0.528	0.982	0.756	0.381	0.774	0.706	0.426	0.263
Unknown (Treponema)	Corr.	-0.160	0.775	0.273	-0.146	-0.346	**0.904**	-0.292	-0.207	-0.394	-0.110	-0.544	-0.541	0.319	**-0.929**	**0.952**	-0.093	-0.837	0.068
	p-value	0.762	0.070	0.601	0.783	0.502	**0.013**	0.575	0.694	0.439	0.836	0.265	0.268	0.538	**0.007**	**0.003**	0.861	0.038	0.898

Supplementary Table 4. Relationship between the bacterial species identified by LEfSe analysis and the expression levels of the miRNAs in large intestine. Pearson's correlation analysis coefficient (Corr.) and P-value.

		rno-miR-125a-3p	rno-miR-29a-5p	rno-let-7a-1-3p	rno-miR-10b-3p	rno-miR-27a-3p	rno-miR-92b-5p	rno-miR-879-3p	rno-miR-496-3p	rno-miR-382-5p	rno-miR-672-5p	rno-miR-1843a-5p	rno-miR-100-3p	rno-miR-883-5p	rno-miR-155-3p	rno-miR-299a-5p	rno-miR-374-3p	rno-miR-376c-5p	rno-miR-133c	rno-miR-499-3p	rno-miR-126a-3p	rno-miR-1188-3p	rno-miR-489-3p
Methanobrevibacter	Corr.	-0.367	-0.071	0.080	0.556	0.109	0.104	-0.269	-0.062	0.198	0.070	**0.964**	-0.062	0.785	0.194	-0.041	-0.113	-0.572	-0.184	0.108	0.534	-0.151	**0.903**
	p-value	0.474	0.893	0.881	0.252	0.838	0.845	0.607	0.907	0.707	0.895	**0.002**	0.908	0.064	0.712	0.939	0.832	0.236	0.728	0.839	0.276	0.775	**0.014**
Bacteroides	Corr.	**0.939**	0.278	-0.491	-0.896	0.648	-0.334	0.364	0.770	0.366	0.763	-0.461	0.252	-0.079	0.112	0.369	0.312	**0.952**	0.485	-0.232	-0.416	0.584	-0.380
	p-value	**0.005**	0.593	0.322	0.016	0.164	0.518	0.479	0.073	0.475	0.078	0.358	0.630	0.882	0.833	0.471	0.548	**0.003**	0.329	0.658	0.413	0.224	0.457
Prevotella (Prevotellaceae)	Corr.	0.768	0.412	-0.567	**-0.943**	0.345	-0.231	0.230	0.618	0.223	0.456	-0.674	0.455	-0.392	-0.041	0.442	0.454	0.799	0.199	-0.487	-0.416	0.552	-0.542
	p-value	0.075	0.417	0.240	**0.005**	0.504	0.659	0.661	0.191	0.671	0.363	0.142	0.365	0.442	0.938	0.380	0.366	0.057	0.705	0.327	0.412	0.256	0.267
Unknown (f_RF16)	Corr.	**0.932**	0.669	-0.470	-0.876	0.720	-0.370	0.196	0.870	0.638	0.694	-0.347	0.606	0.064	0.301	0.737	0.683	0.883	0.288	-0.359	-0.349	0.828	-0.214
	p-value	**0.007**	0.146	0.347	0.022	0.107	0.470	0.710	0.024	0.173	0.126	0.500	0.202	0.904	0.563	0.094	0.135	0.020	0.580	0.484	0.497	0.042	0.685
CF231	Corr.	-0.001	-0.440	-0.376	-0.334	-0.593	-0.155	0.445	-0.314	-0.744	-0.344	-0.775	-0.260	**-0.910**	-0.836	-0.441	-0.411	0.115	0.167	-0.369	-0.504	-0.467	-0.764
	p-value	0.998	0.382	0.463	0.517	0.215	0.770	0.377	0.544	0.090	0.504	0.070	0.618	**0.012**	0.038	0.381	0.418	0.828	0.752	0.471	0.307	0.350	0.077
Paraprevotella	Corr.	-0.419	0.218	-0.116	-0.014	-0.116	**0.938**	**-0.950**	0.017	0.087	0.034	0.317	0.387	0.088	0.377	0.066	0.293	-0.452	**-0.919**	-0.265	**0.914**	0.177	0.388
	p-value	0.408	0.679	0.827	0.979	0.827	**0.006**	**0.004**	0.975	0.870	0.949	0.541	0.448	0.869	0.462	0.901	0.573	0.368	**0.009**	0.612	**0.011**	0.738	0.448
Elusimicrobium	Corr.	0.757	**0.901**	-0.451	-0.727	0.666	-0.314	-0.019	0.838	0.774	0.552	-0.118	0.844	0.224	0.399	**0.940**	0.897	0.635	0.025	-0.480	-0.170	0.900	0.045
	p-value	0.081	**0.014**	0.369	0.102	0.149	0.544	0.971	0.037	0.071	0.256	0.824	0.035	0.670	0.433	**0.005**	0.015	0.176	0.963	0.336	0.747	0.015	0.932
Lactobacillus	Corr.	-0.577	-0.395	**0.961**	0.867	-0.217	0.224	-0.125	-0.616	-0.170	-0.448	0.229	-0.592	0.157	0.279	-0.446	-0.421	-0.421	0.022	**0.900**	0.159	-0.396	0.077
	p-value	0.231	0.439	**0.002**	0.025	0.679	0.669	0.814	0.193	0.747	0.373	0.663	0.215	0.767	0.593	0.376	0.405	0.406	0.967	**0.014**	0.763	0.437	0.885
Unknown (Christensenellaceae)	Corr.	0.629	0.612	0.091	-0.316	**0.948**	-0.038	-0.187	0.773	**0.910**	0.776	0.273	0.411	0.675	0.876	0.656	0.615	0.577	0.151	0.231	0.152	0.846	0.321
	p-value	0.181	0.196	0.864	0.542	**0.004**	0.944	0.723	0.071	**0.012**	0.069	0.601	0.418	0.141	0.022	0.158	0.194	0.230	0.775	0.660	0.774	0.034	0.535
Unknown (Ruminococcaceae)	Corr.	0.012	0.576	0.337	0.004	0.479	0.591	-0.768	0.345	0.667	0.317	0.315	0.457	0.460	**0.950**	0.477	0.613	0.029	-0.504	0.243	0.638	0.654	0.369
	p-value	0.981	0.231	0.514	0.994	0.336	0.216	0.074	0.503	0.148	0.540	0.543	0.362	0.358	**0.004**	0.339	0.195	0.956	0.307	0.642	0.173	0.159	0.471
Oscillospira	Corr.	-0.847	-0.452	0.235	0.585	**-0.975**	0.198	-0.068	-0.897	-0.774	**-0.956**	-0.132	-0.327	-0.548	-0.604	-0.547	-0.465	-0.774	-0.368	-0.037	0.030	-0.776	-0.185
	p-value	0.033	0.368	0.654	0.223	**0.001**	0.706	0.898	0.015	0.071	**0.003**	0.803	0.527	0.261	0.204	0.262	0.353	0.071	0.473	0.945	0.955	0.070	0.725
Ruminococcus (Ruminococcaceae)	Corr.	0.325	0.570	0.376	0.025	0.787	0.084	-0.336	0.523	0.860	0.522	0.422	0.332	0.739	**0.953**	0.576	0.559	0.291	-0.001	0.417	0.291	0.712	0.439
	p-value	0.529	0.237	0.462	0.963	0.063	0.874	0.514	0.287	0.028	0.288	0.404	0.520	0.094	**0.003**	0.231	0.249	0.575	0.998	0.410	0.576	0.112	0.384

Phascolarctobacterium	Corr.	**0.903**	0.751	-0.373	-0.795	0.836	-0.275	0.053	**0.924**	0.787	0.767	-0.176	0.654	0.254	0.503	0.810	0.763	0.838	0.213	-0.265	-0.193	**0.924**	-0.046
	p-value	**0.014**	0.085	0.466	0.059	0.038	0.597	0.921	**0.009**	0.063	0.075	0.739	0.159	0.627	0.309	0.051	0.077	0.037	0.685	0.612	0.713	**0.008**	0.932
Unknown (Mogibacteriaceae)	Corr.	-0.881	-0.199	0.684	**0.933**	-0.510	0.354	-0.402	-0.721	-0.225	-0.707	0.450	-0.253	0.157	0.103	-0.297	-0.233	-0.853	-0.442	0.429	0.416	-0.458	0.360
	p-value	0.021	0.705	0.134	**0.007**	0.301	0.491	0.429	0.106	0.668	0.116	0.371	0.628	0.767	0.847	0.568	0.656	0.031	0.381	0.397	0.412	0.361	0.484
Desulfovibrio	Corr.	-0.836	-0.401	0.417	**0.913**	-0.516	0.174	-0.194	-0.689	-0.340	-0.598	0.621	-0.381	0.263	-0.163	-0.435	-0.448	-0.898	-0.274	0.276	0.362	-0.639	0.511
	p-value	0.038	0.431	0.410	**0.011**	0.294	0.741	0.713	0.130	0.510	0.210	0.188	0.456	0.614	0.758	0.388	0.373	0.015	0.600	0.597	0.480	0.172	0.300
Treponema	Corr.	0.556	**0.945**	-0.467	-0.567	0.517	-0.289	-0.120	0.719	0.741	0.377	0.050	**0.923**	0.284	0.334	**0.967**	**0.925**	0.377	-0.148	-0.588	-0.062	0.812	0.220
	p-value	0.252	**0.005**	0.350	0.240	0.293	0.579	0.821	0.107	0.092	0.461	0.925	**0.009**	0.585	0.518	**0.002**	**0.008**	0.461	0.780	0.219	0.908	0.050	0.676
Unknown (f_F16)	Corr.	-0.886	-0.273	0.309	0.813	-0.505	0.437	-0.475	-0.607	-0.271	-0.531	0.679	-0.194	0.277	-0.043	-0.350	-0.294	**-0.966**	-0.548	0.127	0.617	-0.516	0.607
	p-value	0.019	0.600	0.551	0.049	0.306	0.386	0.341	0.201	0.604	0.279	0.138	0.712	0.595	0.936	0.496	0.572	**0.002**	0.260	0.810	0.192	0.295	0.202
Unknown (f_WCHB1-25)	Corr.	**0.972**	0.540	-0.311	-0.771	0.857	-0.395	0.264	0.880	0.682	0.811	-0.249	0.414	0.218	0.413	0.634	0.549	**0.946**	0.459	-0.105	-0.350	0.801	-0.160
	p-value	**0.001**	0.268	0.549	0.073	0.029	0.439	0.613	0.021	0.136	0.050	0.635	0.414	0.678	0.415	0.177	0.259	**0.004**	0.360	0.843	0.497	0.055	0.761

Supplementary Table 5. Relationship between the bacterial genera identified by LEfSe analysis and the expression levels of the miRNAs in small intestine. Pearson's correlation analysis coefficient (Corr.) and P-value.

		rno-miR-125a-3p	rno-miR-199a-3p	rno-miR-29a-5p	rno-miR-10b-3p	rno-miR-27a-3p	rno-miR-92b-5p	rno-miR-15a-3p	rno-miR-879-3p	rno-miR-496-3p	rno-miR-672-5p	rno-miR-1843a-5p	rno-miR-100-3p	rno-miR-883-5p	rno-miR-299a-5p	rno-miR-466b-2-3p	rno-miR-374-3p	rno-miR-344i	rno-miR-376c-5p	rno-miR-9a-5p	rno-miR-21-5p	rno-miR-3551-5p	rno-miR-133c	rno-miR-126a-3p	rno-miR-1188-3p	rno-miR-489-3p	rno-miR-375-3p
Unknown (Methanobrevibacter)	Corr.	-0.366	-0.130	-0.071	0.556	0.110	0.105	-0.162	-0.269	-0.061	0.072	**0.964**	-0.061	0.786	-0.040	0.207	-0.112	-0.328	-0.571	0.047	0.006	-0.626	-0.184	0.534	-0.150	**0.903**	-0.264
	p-value	0.475	0.806	0.894	0.252	0.836	0.844	0.759	0.606	0.908	0.893	**0.002**	0.908	0.064	0.940	0.694	0.833	0.525	0.237	0.930	0.992	0.184	0.728	0.275	0.776	**0.014**	0.614
Unknown (Bacteroides)	Corr.	**0.943**	0.261	0.285	-0.894	0.664	-0.330	0.365	0.355	0.782	0.778	-0.440	0.258	-0.056	0.377	0.264	0.318	**0.902**	**0.950**	0.276	-0.475	0.616	0.483	-0.402	0.594	-0.359	-0.604
	p-value	**0.005**	0.617	0.584	0.016	0.151	0.523	0.476	0.490	0.066	0.068	0.383	0.622	0.916	0.461	0.613	0.539	**0.014**	**0.004**	0.597	0.341	0.193	0.332	0.430	0.214	0.485	0.204
Unknown (Prevotella, Prevotellaceae)	Corr.	0.777	0.419	0.416	**-0.947**	0.358	-0.234	0.455	0.231	0.629	0.470	-0.665	0.457	-0.379	0.448	0.234	0.457	0.627	0.805	0.321	-0.376	0.748	0.204	-0.413	0.560	-0.532	-0.320
	p-value	0.069	0.409	0.412	**0.004**	0.486	0.655	0.365	0.660	0.181	0.347	0.150	0.362	0.459	0.373	0.656	0.362	0.183	0.053	0.535	0.462	0.087	0.698	0.415	0.248	0.277	0.536
Unknown (CF231)	Corr.	0.001	-0.386	-0.444	-0.339	-0.589	-0.147	-0.474	0.441	-0.310	-0.336	-0.775	-0.263	**-0.909**	-0.445	-0.680	-0.413	0.091	0.119	-0.597	-0.166	0.523	0.166	-0.499	-0.465	-0.764	0.153
	p-value	0.998	0.450	0.378	0.511	0.219	0.781	0.342	0.382	0.550	0.515	0.070	0.615	**0.012**	0.376	0.137	0.415	0.864	0.823	0.211	0.754	0.287	0.753	0.314	0.352	0.077	0.772
Unknown (Paraprevotella)	Corr.	-0.415	0.567	0.217	-0.014	-0.108	**0.939**	0.370	**-0.951**	0.021	0.041	0.321	0.385	0.095	0.066	0.322	0.293	-0.579	-0.449	0.342	0.724	-0.665	**-0.916**	**0.917**	0.180	0.392	-0.166
	p-value	0.413	0.240	0.679	0.979	0.838	**0.006**	0.470	**0.004**	0.968	0.938	0.535	0.451	0.858	0.901	0.534	0.573	0.229	0.372	0.507	0.104	0.149	**0.010**	**0.010**	0.733	0.443	0.754
Unknown (Elusimicrobium)	Corr.	0.758	0.758	**0.901**	-0.727	0.669	-0.312	0.840	-0.021	0.840	0.555	-0.116	0.844	0.227	**0.940**	0.786	0.897	0.452	0.635	0.829	-0.462	0.582	0.024	-0.168	**0.901**	0.047	-0.310
	p-value	0.081	0.081	**0.014**	0.102	0.146	0.547	0.036	0.968	0.036	0.253	0.827	0.034	0.666	**0.005**	0.064	0.015	0.368	0.175	0.041	0.356	0.225	0.963	0.750	**0.014**	0.929	0.550
Unknown (Lactobacillus)	Corr.	-0.721	-0.431	-0.464	**0.958**	-0.318	0.268	-0.412	-0.185	-0.679	-0.491	0.431	-0.593	0.244	-0.512	-0.307	-0.495	-0.524	-0.633	-0.350	0.537	-0.732	-0.087	0.296	-0.522	0.275	0.486
	p-value	0.106	0.393	0.354	**0.003**	0.539	0.608	0.416	0.725	0.138	0.322	0.393	0.215	0.641	0.300	0.555	0.318	0.286	0.177	0.496	0.272	0.098	0.870	0.569	0.288	0.597	0.329
Unknown (Oscillospira)	Corr.	-0.859	-0.417	-0.479	0.585	**-0.981**	0.228	-0.567	-0.083	**-0.903**	**-0.945**	-0.124	-0.346	-0.549	-0.575	-0.632	-0.488	-0.792	-0.783	-0.574	0.359	-0.213	-0.378	0.054	-0.790	-0.181	0.697
	p-value	0.029	0.411	0.337	0.222	**0.001**	0.664	0.240	0.876	**0.014**	**0.004**	0.814	0.502	0.259	0.233	0.178	0.326	0.061	0.065	0.233	0.485	0.686	0.461	0.919	0.061	0.732	0.123
Oscillospira guilliermondii	Corr.	-0.618	-0.503	-0.379	0.698	-0.730	-0.182	-0.487	0.253	-0.850	**-0.933**	-0.243	-0.459	-0.434	-0.423	-0.571	-0.432	-0.509	-0.481	-0.498	0.167	0.016	0.056	-0.347	-0.652	-0.338	**0.939**
	p-value	0.191	0.310	0.459	0.123	0.099	0.730	0.327	0.628	0.032	**0.007**	0.642	0.360	0.389	0.404	0.236	0.392	0.303	0.334	0.315	0.751	0.975	0.916	0.501	0.160	0.513	**0.005**
Ruminococcus albus	Corr.	0.441	**0.925**	**0.995**	-0.472	0.553	-0.002	**0.960**	-0.390	0.683	0.373	0.089	**0.935**	0.330	**0.966**	**0.904**	**0.996**	0.078	0.318	**0.961**	-0.125	0.253	-0.333	0.164	0.900	0.253	-0.124
	p-value	0.381	**0.008**	**0.000**	0.345	0.255	0.997	**0.002**	0.445	0.135	0.466	0.867	**0.006**	0.523	**0.002**	**0.013**	**0.000**	0.884	0.538	**0.002**	0.813	0.629	0.519	0.756	0.014	0.629	0.814
Unknown (Phascolarctobacterium)	Corr.	**0.901**	0.666	0.749	-0.792	0.844	-0.265	0.788	0.043	**0.927**	0.775	-0.165	0.652	0.265	0.808	0.721	0.763	0.692	0.836	0.745	-0.399	0.507	0.210	-0.181	**0.927**	-0.036	-0.481
	p-value	**0.014**	0.148	0.086	0.061	0.035	0.612	0.062	0.935	**0.008**	0.070	0.755	0.161	0.612	0.052	0.106	0.078	0.128	0.038	0.089	0.433	0.305	0.690	0.731	**0.008**	0.946	0.334
Desulfovibrio vulgaris	Corr.	-0.616	-0.273	-0.568	0.502	-0.257	0.717	-0.393	-0.476	-0.413	-0.084	0.444	-0.482	0.161	-0.639	-0.297	-0.523	-0.426	-0.567	-0.372	0.681	**-0.901**	-0.356	0.687	-0.450	0.339	-0.131

	p-value	0.193	0.601	0.240	0.310	0.623	0.109	0.441	0.340	0.416	0.874	0.378	0.333	0.760	0.172	0.568	0.287	0.400	0.240	0.468	0.137	**0.014**	0.488	0.132	0.370	0.511	0.804
Flexispira rappini	Corr.	0.436	-0.361	0.034	-0.056	0.261	**-0.934**	-0.221	0.761	0.229	0.158	0.130	-0.045	0.286	0.206	0.009	-0.053	0.482	0.278	-0.082	**-0.926**	0.580	0.698	-0.636	-0.018	0.112	-0.172
	p-value	0.388	0.482	0.949	0.916	0.618	**0.006**	0.673	0.079	0.663	0.765	0.806	0.933	0.583	0.695	0.987	0.920	0.333	0.594	0.877	**0.008**	0.227	0.123	0.174	0.973	0.833	0.744
Unknown (Treponema)	Corr.	0.557	0.771	**0.945**	-0.567	0.520	-0.287	0.813	-0.122	0.721	0.380	0.052	**0.923**	0.287	**0.968**	0.815	**0.926**	0.206	0.378	0.849	-0.454	0.501	-0.149	-0.059	0.814	0.222	-0.209
	p-value	0.251	0.072	**0.004**	0.240	0.291	0.581	0.049	0.818	0.106	0.458	0.922	**0.009**	0.582	**0.002**	0.048	**0.008**	0.695	0.461	0.032	0.365	0.311	0.779	0.911	0.049	0.673	0.692

Supplementary Table 6. Relationship between the bacterial species identified by LEfSe analysis and the expression levels of the miRNAs in small intestine. Pearson's correlation analysis coefficient (Corr.) and p-value.

VI. DISCUSIÓN GLOBAL

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La creciente incidencia de las enfermedades metabólicas en la población mundial, y en particular de la obesidad, el SM, y la DT2, ha hecho de ellas un problema sanitario, social, y económico de primer orden (5-7). Esta incidencia es sexualmente dimórfica, con una influencia de las hormonas sexuales avalada por situaciones en las que sus niveles están alterados: redistribución de la grasa corporal tras la menopausia (10) y tras el tratamiento con esteroides sexuales en hombres y mujeres transgénero (9); incremento de la incidencia de la DT2 tras la menopausia (11) y tras una ooforectomía (19), y reducción de su incidencia ante una terapia hormonal con estrógeno tras la menopausia (12); mayor adiposidad central (17) y mayor riesgo de SM (18) en mujeres con hiperandrogenismo debido al SOP; aumento de la masa grasa (13) y de la prevalencia del SM (14) y la DT2 (15) en hombres tras una terapia de privación de andrógenos; y disminución de la grasa visceral (16), de la DT2 y del SM (103) en hombres tras un tratamiento con testosterona.

Coincidiendo con nuestros resultados, durante el desarrollo de esta tesis, otros estudios han constatado que en la composición de la microbiota intestinal, una comunidad simbiótica que actúa como un órgano integrado en el metabolismo del huésped (429), existe un dimorfismo sexual (32) en el que las hormonas sexuales parecen estar implicadas (33). En modelos animales se ha constatado la influencia de la microbiota intestinal en el nivel de las hormonas sexuales, pues la colonización por microbios comensales eleva la testosterona en los ratones macho, mientras que la transferencia de microbiota intestinal de machos adultos a hembras inmaduras altera su microbiota y eleva la testosterona (206). Los cambios en la microbiota intestinal, y la interacción con su huésped parecen influir en los mecanismos patogénicos de la obesidad, la resistencia a la insulina, y el SM (26, 254, 430). Por otra parte, en los últimos años se ha constatado que, en respuesta a los cambios en las hormonas sexuales y el estado nutricional, la microbiota intestinal puede establecer una comunicación cruzada con su huésped regulando la expresión de miARN en el intestino delgado y grueso, lo que situaría a estas moléculas como transmisores o decodificadores de la disbiosis en las enfermedades metabólicas (431, 432).

La relación entre la microbiota intestinal, las hormonas sexuales, y el desarrollo de la enfermedad ofrece un nuevo campo de investigación en la prevención y tratamiento de la enfermedad mediante la manipulación de la microbiota intestinal. En este sentido, los trasplantes fecales han cobrado gran interés como terapia alternativa en el tratamiento de enfermedades como el SM, en donde la transferencia de la microbiota fecal de donantes sanos a pacientes con SM mejora la sensibilidad a la insulina (433). En la misma línea, han surgido terapias basadas en la modificación de la microbiota intestinal mediante intervención dietética, así como en el uso de prebióticos y probióticos (434).

En una primera fase, que incluye nuestras dos primeras publicaciones, abordamos la modificación de la microbiota intestinal en humanos ante los cambios en el nivel de las

hormonas sexuales, sirviéndonos para ello un modelo natural que conlleva cambios hormonales drásticos, la mujer premenopáusica y posmenopáusica. En esta fase también analizamos, en función del sexo, tanto los cambios en la microbiota intestinal en pacientes con SM como la influencia de la dieta en la modulación de la disbiosis microbiana.

En estos estudios hemos comprobado que la composición de la microbiota intestinal se modifica ante cambios en los niveles de las hormonas sexuales y de un modo diferente según el sexo en pacientes con SM. Dentro de este contexto, es interesante resaltar los cambios producidos en géneros bacterianos productores de AGCC, los cuales permiten a la microbiota intestinal extender su acción al sistema nervioso central a través del eje intestino-cerebro para influir en la ingesta de alimentos (36). En este sentido, las diferencias en la abundancia relativa de géneros productores de AGCC (*Prevotella*, *Ruminoccocus*, *Roseburia*, y *Parabacteroides*) (349, 399, 435, 436) sugieren una menor producción de AGCC en mujeres posmenopáusicas y hombres en comparación con las mujeres premenopáusicas. Esto podría otorgar una mayor protección a las mujeres premenopáusicas frente a las enfermedades metabólicas gracias a una microbiota intestinal con mayor actividad sacarolítica, y podría explicar, al menos parcialmente, el dimorfismo en la incidencia de enfermedades metabólicas como la DT2 (mayor en mujeres posmenopáusicas y hombres) (8). Esta idea estaría respaldada por el mayor metabolismo potencial de propanoato y butanoato que refleja el análisis PICRUSt en las mujeres premenopáusicas, lo que sugiere una mayor producción de AGCC. Estos AGCC reducen la inflamación (437) que favorece la resistencia a la insulina (289) implicada en la patogénesis de la DT2 (438), lo que estaría en consonancia con el menor nivel de los marcadores inflamatorios IL-6 y MCP-1 observado en las mujeres premenopáusicas. En los pacientes con SM hemos encontrado diferencias en la abundancia de géneros bacterianos previamente relacionados con la DT2, como *Collinsella*, *Faecalibacterium* y *Prevotella* (276), con la resistencia a la insulina, como *Phascolarctobacterium* (399), y directamente con el SM, como *Anaerotruncus* (265). Al ser *Faecalibacterium* y *Prevotella* géneros productores de AGCC (276) y más abundantes en hombres con SM que en mujeres con SM, estos resultados refuerzan la idea de que la pérdida de ciertas funciones de la microbiota intestinal, como la de degradar los hidratos de carbono a AGCC, está implicada en el SM, como se ha descrito anteriormente (264); si bien los resultados de nuestro trabajo sugieren que esto podría ocurrir por la alteración diferencial de la microbiota intestinal en hombres y mujeres.

El LEfSe ha revelado que, si bien la microbiota es diferente en mujeres y hombres con SM, esta diferencia es menor que en mujeres y hombres sin SM. Una menor diversidad bacteriana asociada a la enfermedad, descrita anteriormente (439), puede ser la responsable de este resultado. De hecho, nuestros resultados mostraron diferencias en las métricas cuantitativas y cualitativas (distancias UniFrac ponderadas y no ponderadas, respectivamente) en los grupos sin SM pero solamente en las métricas cualitativas en

los grupos con SM. Esto sugiere la importancia de los taxones con una baja abundancia en la determinación de la enfermedad, ya que el UniFrac ponderado incorpora las abundancias para calcular la distancia, y el impacto de los rasgos de baja abundancia disminuye, mientras que el UniFrac no ponderado es más sensible a las diferencias en los rasgos de baja abundancia (440).

La obesidad, además de constituir una patología per se, constituye un factor de riesgo para el SM (441), que a su vez influye en la DT2 (442). En este contexto, se han descrito dos patrones de distribución de la grasa, un patrón central, en hombres y mujeres posmenopáusicas, y un patrón periférico, en mujeres premenopáusicas (20, 21), ambos regulados por las hormonas sexuales (22) y relacionados con las enfermedades metabólicas, en donde la grasa central (abdominal visceral) muestra un perfil patológico (23) frente a un perfil protector de la grasa periférica (subcutánea) (24). En este sentido, los mayores niveles plasmáticos de leptina y adiponectina (hormonas implicadas en la homeostasis energética) que hemos encontrado en las mujeres con respecto a los hombres apoyan el dimorfismo sexual en el metabolismo energético y la diferente distribución de la grasa corporal entre hombres y mujeres, a lo que también podría contribuir la mayor proporción *F/B* de las mujeres posmenopáusicas con respecto a los hombres. En cuanto a los niveles plasmáticos de las incretinas, hormonas liberadas por las células enteroendocrinas que potencian el aclaramiento de la glucosa en respuesta a la ingestión de alimentos (443), también hemos observado algunas diferencias, con un mayor nivel de GLP-1 en las mujeres premenopáusicas que en las posmenopáusicas y de GiP en las mujeres que en los hombres. Estas diferencias podrían estar asociadas a las diferencias de la microbiota intestinal, al menos en el caso del GLP-1, ya que la producción de AGCC por la microbiota sacarolítica, mayor en mujeres premenopáusicas al igual que el GLP-1, inducen la liberación de GLP-1 por parte de las células L del intestino (444).

Dado el presumible papel de las intervenciones dietéticas en la modificación de la microbiota intestinal (445), y por ello su interés en la prevención y el tratamiento de los trastornos metabólicos (446), y sumado a que en nuestro grupo de investigación se ha demostrado anteriormente que el consumo a largo plazo de la dieta mediterránea o una dieta baja en grasa restablece parcialmente la disbiosis de la microbiota intestinal en personas obesas con SM sin modificar significativamente la microbiota intestinal en individuos sin SM (263), analizamos si el consumo de estas dietas modificaba diferencialmente la microbiota en hombres y mujeres con SM y observamos una serie de taxones bacterianos cuya abundancia se modificó de forma diferencial según el tipo de dieta y con un efecto diferente según el sexo. Esto último sugiere que mujeres y hombres podrían beneficiarse de forma diferente ante el consumo de la dieta mediterránea y una dieta baja en grasa. De hecho, tras el consumo de la dieta baja en grasa, los resultados reflejan una mayor abundancia en los hombres que en las mujeres de los géneros *Roseburia*, productor de AGCC (435), *Holdemania*, relacionado con el

deterioro del metabolismo de los lípidos y la glucosa (447), y *Desulfovibrio*, productor de sulfuro de hidrógeno y relacionado con trastornos gastrointestinales como la colitis ulcerosa, la enfermedad de Crohn, y el síndrome del intestino irritable (448). Esto último sugiere que el consumo de una dieta baja en grasa sería recomendable en mujeres, pero no así en hombres con esta patología.

En una segunda fase, profundizamos nuestro estudio en las diferencias entre la microbiota intestinal de hombres y mujeres ante la reducción de las hormonas gonadales mediante un modelo animal de rata hembra sometida a una ovariectomía y de rata macho sometida a una orquiectomía, en comparación con ratas macho y hembra normales, al tiempo que analizamos en estos modelos el efecto en la microbiota intestinal de una dieta obesogénica. También hemos explorado, mediante ratas androgenizadas, la influencia de los esteroides sexuales y de una dieta obesogénica (sobrealimentación posnatal), durante las primeras fases del desarrollo, en la modificación persistente de la estructura de la microbiota intestinal. Estos estudios también nos han permitido analizar la posible interacción entre la microbiota intestinal y el huésped a través de la regulación de la expresión microbiana de miARN en el intestino delgado y grueso.

Nuestros resultados en esta fase indican que muchas de las diferencias iniciales en la microbiota intestinal entre machos y hembras persisten tras la gonadectomía, tanto en condiciones normales como obesogénicas. No obstante, la gonadectomía modificó la microbiota intestinal hacia un perfil más deletéreo, y especialmente en las hembras tras una sobrealimentación posnatal, en las que aumentó significativamente la relación *F/B* en comparación con los machos. Esto concuerda con observaciones previas en humanos, donde se ha descrito un aumento de esta relación en el desarrollo de la obesidad (256), siendo mayor en mujeres que en hombres (449), y aun más tras la menopausia como hemos comprobado nosotros previamente (450). Por otra parte, el mayor efecto deletéreo en las hembras, bajo el efecto combinado de una condición obesogénica y la eliminación de esteroides sexuales por gonadectomía, también se confirma por la menor abundancia en ellas de *Butyricimonas* y *Roseburia*, dos géneros productores de AGCC, lo que apoya la idea de que la microbiota en los machos se adapta mejor que en las hembras ante un alto aporte calórico en las primeras etapas de la vida y mantiene una mayor producción de AGCC. Esto podría tener un impacto diferencial entre los sexos en la predisposición e incidencia ante las enfermedades metabólicas, como apunta el hecho de que estas enfermedades aumenten tras la menopausia en paralelo a la reducción de los estrógenos (451), lo cual está también relacionado con las diferencias de sexo en la distribución de la grasa (452).

A pesar del efecto antiobesidad de los estrógenos, gracias a la disminución de la ingesta de alimentos y al aumento del gasto energético (453, 454), hemos observado un aumento del peso en las hembras androgenizadas en paralelo a un aumento de los estrógenos, lo que sugiere la contribución de otros factores en el fenotipo obeso. Estos

factores podrían incluir cambios en la microbiota intestinal, pues se ha demostrado que su estructura se altera ante cambios en el nivel de las hormonas sexuales (206, 212, 450). Sin embargo, hasta ahora no se había descrito la causalidad entre estos dos fenómenos, y nuestro estudio documenta de forma concluyente que la androgenización temprana altera el perfil de la microbiota intestinal, evidenciado por un aumento de *Bacteroidetes* y una disminución de *Firmicutes* en las hembras androgenizadas con respecto a las hembras control bajo alimentación normal en el DPN-50. Sin embargo, las diferencias en estos filos principales desaparecieron en las ratas de mayor edad (DPN-150), mientras que en ellas la androgenización redujo la abundancia de *Actinobacteria* y *Euryarchaeota* y aumentó la abundancia de *Cyanobacteria*. Estas diferencias también desaparecieron a principios de la edad adulta (DPN-50) cuando los animales fueron sobrealimentados, lo que sugiere un efecto adicional de la obesidad.

La exposición temprana (neonatal) a altas dosis de andrógenos alteró la programación metabólica de las ratas hembra. Su homeostasis de la glucosa hembras se deterioró al ser expuestas a un patrón obesogénico, dándose un paralelismo entre estas alteraciones fenotípicas y las alteraciones de la microbiota intestinal. Nuestros datos han demostrado que la androgenización aumenta los niveles séricos de estrona y estradiol y reduce los de progesterona. Aunque se ha descrito el papel positivo de la activación del receptor de estrógenos sobre la sensibilidad a la insulina (455), también se ha señalado que los niveles elevados de estrógenos podrían conducir a una resistencia a la insulina ante el aumento de la secreción de esta última (456, 457). Esto sugiere que el aumento de los niveles séricos de estrona y estradiol podría contribuir a la menor sensibilidad a la insulina observada en las hembras androgenizadas en comparación con las hembras control, un efecto exacerbado bajo la condición obesogénica.

La influencia del sexo en la diferente incidencia de las enfermedades metabólicas durante la edad adulta parecen explicarse, al menos parcialmente, por la influencia de la dieta durante las primeras etapas de la vida, además de por las condiciones maternas en el útero (458). Ante esto, nuestros resultados sugieren que la influencia persistente de la condición obesogénica desde la lactancia sobre la microbiota intestinal podría perdurar en la edad adulta. Esta idea estaría respaldada por el hecho de que la obesidad en la infancia, que se asocia a un mayor riesgo de obesidad en la edad adulta (459), está relacionada con alteraciones en la microbiota intestinal a una edad temprana (460).

Ante la relación descrita entre los miARN y las enfermedades metabólicas (431, 432), abordamos la posible implicación de estas moléculas en la comunicación entre la microbiota intestinal y su huésped en respuesta a los cambios en las hormonas sexuales y el estado nutricional, comprobando en ambos estudios que los cambios en la microbiota intestinal se relacionaron con alteraciones en la expresión de miARN en el intestino delgado y grueso.

En este sentido, con respecto a las vías KEGG, hemos observado una relación entre la expresión del miR-27a-3p, relacionado con la señalización de la insulina, y la abundancia

del género *Butyricimonas* en el estudio de gonadectomía y del género *Oscillospira* en el estudio de androgenización, siendo ambos géneros productores de AGCC (461, 462), por lo que este miARN podría estar implicado en la homeostasis de la glucosa a través de la señalización de la insulina, ya que los AGCC aumentan la liberación de insulina a través de las incretinas intestinales (444, 463). Además, la implicación de los AGCC en el metabolismo energético y en la regulación del apetito (464-466) podría ser parcialmente responsable del aumento de peso en las hembras tras la gonadectomía (CP-DAG) y en las hembras androgenizadas. Por otra parte, este miARN también está implicado en el metabolismo de los ácidos grasos, la señalización de la insulina, y la maduración de los ovocitos, al tiempo que contribuye a la integridad de la barrera intestinal mediante procesos como la unión adherente, la adhesión focal, y la biosíntesis de la mucina tipo O-glicano.

En relación con los AGCC, el estudio de androgenización mostró que el miR-181a-5p, relacionado con la vía de señalización de estrógenos, también lo estaba con la abundancia de *Parabacteroides*, un género productor de AGCC y asociado a la asimilación de sulfatos (399). En este mismo estudio, además de varias vías KEGG relacionadas con el metabolismo, como el metabolismo de los ácidos grasos o la vía de señalización de los esfingolípidos, a su vez relacionadas con el desarrollo de la DT2 (467) y las enfermedades cardiovasculares (468), otros miARNs, como el miR-29a-5p y el miR-100-3p, también podrían enlazar el metabolismo y las acciones de las hormonas sexuales en otros tejidos, como la maduración de ovocitos mediada por la progesterona (469-471). Además, la expresión de estos miARNs se relacionó con la abundancia intestinal de *Treponema*, un género bacteriano que potencia la capacidad de extraer calorías de los glúcidos complejos, así como de los carbohidratos que son fermentados tras escapar a la digestión intestinal (472). En su conjunto, estas evidencias sugieren que la comunicación cruzada entre la microbiota intestinal y el huésped a través de miARNs específicos puede implicar también eventos mediados por los esteroides gonadales.

El estudio basado en la gonadectomía ha mostrado que los miARNs miR-23b-5p y miR 186-5p podrían actuar como moduladores de la biosíntesis de esteroides, mientras que los miARNs miR-181a-5p y miR-139-5p podrían estar implicados en la vía de señalización del estrógeno. La dieta también podría ejercer su efecto a través de la interacción microbiota-miARN, como demuestra la relación entre el miR-125a-3p, implicado en las uniones adherentes, y la abundancia del género *Bacteroides*, asociada a una dieta rica en carne (259).

Como conclusión, los resultados derivados de esta tesis doctoral reflejan que hombres y mujeres difieren en la composición de su microbiota intestinal y que estas diferencias dependen de los niveles de las hormonas sexuales, así como del estatus nutricional. Así mismo, nuestros resultados sugieren que estas diferencias podrían estar relacionadas con el dimorfismo sexual observado en la incidencia de las enfermedades metabólicas y cardiovasculares, dada la relación de estas enfermedades con la microbiota intestinal.

Además, nuestros resultados mostraron la contribución de las hormonas gonadales a la definición de las diferencias dependientes del sexo en la microbiota intestinal, y revela un papel potencial de las hormonas gonadales en la formación de la microbiota intestinal, como consecuencia de la interacción entre el sexo y la nutrición, mediante un mecanismo de comunicación cruzada entre la microbiota intestinal y el huésped mediado por miARN del intestino delgado y grueso.

VII. CONCLUSIONES

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1. Nuestros resultados reflejan que hombres y mujeres difieren en la composición de su microbiota intestinal y que estas diferencias dependen del estado hormonal de la mujer (premenopausia vs posmenopausia), lo que podría estar relacionado con el dimorfismo sexual observado en la incidencia de las enfermedades metabólicas y cardiovasculares, dada la relación de estas enfermedades con la microbiota intestinal.
2. Nuestros resultados sugieren que las alteraciones de la microbiota intestinal asociadas al síndrome metabólico parecen ser diferentes entre hombres y mujeres. Estos resultados sugieren a su vez la implicación de la microbiota intestinal en la diferencia en la incidencia de enfermedades metabólicas entre sexos. Además, los diferentes cambios inducidos por la dieta mediterránea o una dieta baja en grasa en la microbiota intestinal según el sexo sugieren que las mujeres y los hombres podrían beneficiarse de forma diferente de una dieta específica en función de su sexo.
3. Nuestros resultados muestran la contribución de las hormonas gonadales a la definición de las diferencias dependientes del sexo en la microbiota intestinal, así como su potencial papel en la formación de esta microbiota como consecuencia de la interacción entre el sexo y la nutrición. De hecho, la eliminación de las hormonas sexuales modifica la microbiota intestinal hacia un perfil más deletéreo, especialmente en las hembras tras una sobrealimentación posnatal, lo que confirma la implicación de la dieta en estos procesos. Por otro lado, nuestros resultados sugieren que la implicación de la microbiota intestinal en las enfermedades metabólicas podría estar mediada por la interacción entre la microbiota y su huésped a través de los miARN intestinales.
4. Nuestros resultados sugieren que la alteración nutricional y hormonal en los primeros períodos del desarrollo alteran la programación metabólica y la estructura de la microbiota intestinal, cuyas consecuencias se prolongan en el tiempo. La relación observada entre los cambios en la microbiota intestinal y la expresión de miARN en el intestino delgado y grueso sugiere un posible mecanismo de comunicación cruzada entre la microbiota intestinal y el huésped que puede contribuir a amplificar el desajuste metabólico causado por la obesidad.

VIII. REFERENCIAS

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IX. MATERIAL SUPLEMENTARIO

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9.1. Artículo de revisión bibliográfica

Citación: Santos-Marcos JA, Perez-Jimenez F, Camargo A. The role of diet and intestinal microbiota in the development of metabolic syndrome. J Nutr Biochem. 2019 Aug;70:1-27. doi: 10.1016/j.jnutbio.2019.03.017. Epub 2019 Apr 8. PMID: 31082615.

Available online at www.sciencedirect.com

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Journal of Nutritional Biochemistry 70 (2019) 1–27

Journal of Nutritional Biochemistry

The role of diet and intestinal microbiota in the development of metabolic syndrome

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Received 20 September 2018; received in revised form 28 January 2019; accepted 25 March 2019

Abstract

Metabolic syndrome (MetS) is a cluster of metabolic factors that increase the risk of cardiovascular disease and type 2 diabetes mellitus (T2DM), which is in itself a major cardiovascular disease risk factor. The aim of this review is to summarize the data related to the influence of the gut microbiota on the development of obesity and the MetS, highlighting the role of diet in controlling the MetS by modifying the gut microbiota. The main alterations in the gut microbiota of individuals with MetS consist of an increased *Firmicutes*/*Bacteriodetes* ratio and a reduced capacity to degrade carbohydrates to short-chain fatty acids, which in turn is related with the metabolic dysfunction of the host organism rather than with obesity itself. In addition to a low-fat, high-carbohydrate diet, with its high fiber intake, a diet with 30% fat content but with a high content in fruit and vegetables, such as the Mediterranean diet, is beneficial and partially restores the dysbiosis found in individuals with MetS. Overall, the shaping of the gut microbiota through the administration of prebiotics or probiotics increases the short-chain fatty acid production and is therefore a valid alternative in MetS treatment.

Keywords: Gut microbiota; Dysbiosis; Obesity; Metabolic syndrome; Inflammation; Diet

Contents

Abbreviations: AgRP, agouti-related protein; AMPK, AMP-activated protein kinase; CRP, C-reactive protein; CVD, cardiovascular disease; F/B, Firmicutes to Bacteroidetes; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; GPR43, G-protein-coupled receptor 43, also named "free fatty acid receptor 2" or FFAR2; IFN-γ, interferon-gamma; IKK-β, inhibitor of nuclear factor kappa-β kinase; LDLR, LDL (low-density lipoprotein) receptor; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MetS, metabolic syndrome; NF-κB, nuclear factor kappa B; NKT cells, natural killer T cells; NpY, neuropeptide Y; POMC, proopiomelanocortin; PYY, peptide YY; SCFA, short-chain fatty acids; SREBP2, sterol regulatory element-binding protein 2; T2DM, type 2 diabetes mellitus; Th17 cells, helper T17 cells; TLR, Toll-like receptor; TNF, tumor necrosis factor; Treg cells, regulatory T cells.

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https://doi.org/10.1016/j.jnutbio.2019.03.017
0955-2863/

1. Introduction

Metabolic syndrome (MetS) is a cluster of metabolic factors that increase the risk of cardiovascular disease (CVD) and T2DM, which is in itself a CVD risk factor [1,2]. In addition to reducing the patients' quality of life, the MetS has a significant economic impact on public health expenditure due to the high morbidity rates generated since the greater risk of developing T2DM increases by over 30% the probability of CVD, which is currently the leading cause of mortality worldwide [3–5].

Gut microbiota is a symbiotic community made up of thousands of microbial species (belonging to the *Archaea, Bacteria* and *Eukarya* domains, as well as viruses) that actively participates in the host's physiology by intervening in processes such as energy/nutrient absorption, immune response, intestinal permeability and hormone and vitamin production [6–8]. In fact, the gut microbiota significantly enriches the metabolism of glycans, amino acids and xenobiotics, methanogenesis, and the biosynthesis of vitamins and isoprenoids [9]. In line with this, the gut microbiota seems to play a key role in human health (for a general review, see [10]). However, alterations in the gut microbiota composition or diversity, known as dysbiosis, as well as an altered interaction between the gut microbiota and the host can lead to several diseases, including MetS [11,12]. In addition, the composition of the gut microbiota is influenced by several factors, including geography, age, sex, hormonal status, host genetics and diet [13–23].

Since 2005, when Ley et al. [24] found a reduction in the abundance of *Bacteroidetes* in obese mice compared with lean mice and a proportional increase in *Firmicutes*, many studies have focused on the role of the gut microbiota in obesity, the main hallmark of the MetS [25]. Furthermore, it has been shown that the relative proportion of *Bacteroidetes* decreased in obese people in comparison with lean people and that this proportion increased with weight loss in two types of low-calorie diet, thus demonstrating that obesity has a microbial component [26]. Moreover, Turnbaugh et al. [27] showed that the "obese microbiota" had an increased capacity to harvest energy from the diet and that this trait was transmissible because the colonization of germ-free mice with an "obese microbiota" caused a greater increase in body fat than colonization with a "lean microbiota," which shows that the gut microbiota plays a major role in the development of obesity. Over the following years, different studies based on co-housing [28], antibiotics [7,29] or fecal microbiota transplantation [27,30] have supported the relationship between the intestinal microbiota and the development of obesity.

The aim of this review is to summarize the data related to the influence of the gut microbiota on the development of obesity and the MetS, highlighting the role of diet in controlling the MetS by modifying the gut microbiota.

2. Methods

PubMed databases were used to search for reviews and research studies published in English using the search terms: microbiota and metabolic syndrome, microbiota and diet, microbiota and dysbiosis, microbiota and obesity, microbiota and inflammation, *Firmicutes* and *Bacteroidetes*, microbiota and short chain fatty acids, microbiota and intestinal barrier integrity, *F. prausnitzii*, *Lactobacillus*, *Bifidobacterium*, *Roseburia*, *A. muciniphila*, *Prevotella*, microbiota and prebiotics, microbiota and probiotics, microbiota and synbiotics. Following this search, an initial selection of articles was made according to their titles and abstracts. Subsequently, a second selection was made based on a critical reading of the articles.

3. Gut microbiota in metabolic syndrome

The involvement of gut microbiota in the MetS was shown by Vijay-Kumar et al., who demonstrated that mice who were genetically deficient in Toll-like receptor 5 (TLR5) exhibited MetS features and an altered gut microbiota, which conferred many features of this syndrome when it was transferred to wild-type germ-free mice [31]. This potential role of the gut microbiota in the MetS was confirmed by Di Luccia et al., who showed that the development of MetS in high-fructose-diet-fed rats was eradicated by antibiotic treatment or fecal transfer from control rats [32].

In line with this, several features of the gut microbiota composition have been shown to play a prominent role in intestinal barrier integrity, inflammation and obesity, all of which are factors that lead to the development of MetS.

3.1 *Firmicutes* to *Bacteroidetes* (F/B) ratio: The first studies dealing with the alteration in gut microbiota in obesity showed an increased F/B ratio [24]. The fact that the colonization of germ-free mice with an "obese microbiota," in which the relative abundance of *Firmicutes* was greater than in the "lean microbiota," caused a bigger increase in body fat than colonization with a "lean microbiota" [27] showed that this obesity-associated gut microbiota, with an increased ability to extract energy from the diet, is transmissible. The greater metabolic diversity found in *Firmicutes* with respect to *Bacteroidetes* (348 metabolic pathways versus 76, respectively) [33], supports the idea that the *Firmicutes* phylum could extract more energy from the diet.

Besides, both *in vivo* and *in vitro* studies [34,35] have shown that *Bacteroidetes* were enriched in the luminal content, while *Firmicutes* tended to colonize the mucin layer. Thus, most of the butyrate-producing bacteria are located in the mucin layer rather than the lumen, which may enhance butyrate bioavailability. This spatial separation of both groups of bacteria, added to the fact that mucosal bacteria may interact more closely with the host physiology, could be a basis for understanding the differences in the F/B, especially if we take into account the alteration undergone by the mucosa in the processes related to low-grade inflammation and obesity. In fact, the mucous layer represents a barrier that separates the gut bacteria from the intestinal epithelium and protects against inflammation [36].

Although the involvement of the F/B ratio in obesity is strongly supported by animal studies, studies in humans have in fact yielded contradictory results. Just as with mice, several studies in humans found that this ratio is greater in obese individuals [26,37]. However, others did not confirm these observations [20,38,39] or even showed that the relative abundance of *Firmicutes* was less in obese subjects [40]. In addition, in a study based on weight-loss diets, Duncan et al. [37] did not find any link between obese and non-obese individuals and the F/B ratio. However, a recent study showed a marked dysbiosis characterized by an increased F/B ratio in obese people with MetS compared with obese people without MetS and non-obese people [41], which suggests that the F/B ratio may be related to the presence or absence of metabolic traits in humans rather than to obesity itself.

Overall, more studies are needed which take into account co-founding factors, including geography, age, sex, hormonal status, host genetics and diet [13–22], which may potentially affect the composition of the gut microbiota in order to assess the alteration of the F/B ratio in obesity and MetS.

3.2 Short-chain fatty acids (SCFA)-producing bacterial taxa: Several studies have shown evidence that alterations in the gut microbiota may lead to obesity and MetS, directly or as a consequence

of the disturbances in the gut microbiota that cause "low-grade" inflammation, which may in turn promote the development of MetS [42–44]. In fact, metabolites derived from the gut microbiota, such as SCFA, have been shown to take part in this "low-grade" inflammation, in addition to other functions, such as appetite and the regulation of energy intake [45–48].

It has been reported that the dysbiosis present in the gut microbiota of MetS patients [43] is characterized by a reduction in the abundance of several bacterial species within the *Bacteroides* and *Ruminococcus* genera with important saccharolytic activity, such as *B. fragilis* group, *P. distasonis*, *B. thetaiotaomicron* and the *R. flavefaciens* subgroup [49–51]. This suggests a reduction in the carbohydrate degradation capacity in MetS patients, which may also cause a reduction in propionate and acetate production [52,53]. The latter point is particularly relevant in this context, as a reduction of acetate levels in the gut may also reduce the abundance of beneficial bacteria such as *F. prausnitzii* and *E. rectale*. These bacteria consume acetate and produce butyrate [54,55], in addition to the decrease in *E. rectale*, *F. nucleatum* and *F. prausnitzii*, which directly degrade carbohydrate to produce butyrate. In addition, a further study in obese patients with MetS compared with obese people without MetS and non-obese people [41] showed a similar reduction of the genera with saccharolytic activity, especially *Bacteroides* and *Prevotella* (the main two genera belonging to the *Bacteroidetes* phylum) as well as *Roseburia*, *Ruminococcus* and *Faecalibacterium* [49–51,56].

Taken together, these studies suggest that the loss of certain functions or features of the gut microbiota, such as the loss of the capacity to degrade carbohydrates to SCFA, may be related with the metabolic dysfunction of the host organism [14,15]. Taking into account that amino acids can also serve as precursors for the synthesis of SCFA by bacteria [57], the loss of the capacity to degrade carbohydrates to SCFA may unbalance the interplay between the gut microbiota and amino acid and SCFA homeostasis, thus shifting the bacterial activity towards SCFA production from amino acids. This is consistent with the reduced insulin sensitivity observed in MetS patients and may be related with the high branched-chain amino acid concentration found in insulin resistance states [58] and a dysregulation in the production of incretins. These processes are involved in the regulation of the feeding and energy balance, presumably through SCFA as signal molecules [59,60], which may in turn contribute to the development of T2DM and MetS [61,62].

A reduction in the SCFA-producing bacterial taxa and therefore a lower availability of these molecules seem to influence the gut–brain axis. In fact, SCFA have been shown to induce the release of molecules such as GLP-1 or ghrelin that act at brain level and modulate insulin action and appetite, which in turn influence the development of obesity and MetS [63–67].

3.3 *Faecalibacterium prausnitzii* has been reported as one of the most abundant butyrate-producing bacteria in the gut [68–70], and its shortage has been linked to several inflammatory-related diseases in humans. For example, a lower abundance of this genus has been reported in patients with rheumatoid arthritis [71] and in type 2 diabetic patients [72], in addition to obesity and MetS [43]. Regarding its role in the inflammatory state, in a study in human cells and mice by Sokol et al. [56], in *F. prausnitzii,* although no *in vitro* antibacterial effects were shown, anti-inflammatory effects were evident in Caco-2 cells in part due to secreted metabolites with the ability to block nuclear factor kappa B (NF-κB) activation and IL-8 production (both related to inflammatory processes). Additionally, in the same study, this bacterium induced an increased secretion of anti-inflammatory IL-10 and a decreased secretion of proinflammatory tumor necrosis factor (TNF)-α and IL-12 cytokines in mice with induced colitis.

Furthermore, in a study with lean controls and obese patients undergoing bariatric-surgery-induced weight loss, Furet et al. [73] showed a consistent correlation between *F. prausnitzii* and low-grade inflammation, which was negatively correlated with serum concentrations of inflammatory circulating markers, such as C-reactive protein (CRP) and IL-6, regardless of the calorie intake.

3.4. Bacterial species belonging to *Lactobacillus* genus have been associated with obesity, and their relationship with weight gain and loss is dependent on the metabolic features of the different bacterial species. In fact, certain species of *Lactobacillus* are present in normal-weight individuals, while other species are present in obese individuals [74]. Moreover, it has been described that although species associated with weight gain lack the enzymes of fructose catabolism, which provides a defense against oxidative stress and the synthesis of dextrin, L-rhamnose and acetate, they encode the thiolases involved in the metabolism of lipids, unlike weight protection-associated species. In addition, weight-gain-associated species encoded more bacteriocins than weight-protection-associated species [75].

In terms of inflammation, Paolillo et al. [76] demonstrated that *L. plantarum* significantly induces the secretion of human β-defensin 2, an inducible peptide that is involved in host defense and represents a link between the innate and adaptive immune responses. Moreover, two studies in humans have shown the potential role in the integrity of the intestinal barrier of two different strains of this bacterial species, MB452 and WCFS1 [77,78], by inducing changes in the epithelial tight junctions.

There are also many studies in rodents that show other mechanisms of action for this genus. For example, Lin et al. [79] reported for the first time that the probiotic *L. rhamnosus GG* can reduce inflammatory signaling in the immature intestinal epithelia of mice. Besides, in a study performed by Kim et al., [80] *L. rhamnosus GG* improved insulin sensitivity and reduced adiposity in mice by adiponectin secretion and the subsequent activation of AMP-activated protein kinase (AMPK).

3.5. Other bacterial species belonging to the *Bifidobacterium* genus have been associated with a lean, healthy status [40,81,82]. Mechanistically, studies in animal models have shown that *Bifidobacterium* supplementation reduces bacterial translocation, improves mucosal damage [83,84] and normalizes the endotoxemia level and inflammatory status, as well as other metabolic parameters [85,86]. On the other hand, it has been suggested that acetate produced by *Bifidobacterium* improves intestinal defense mediated by epithelial cells by inducing anti-inflammatory and/or antiapoptotic effects, thus protecting the host against infection [87]. Besides, *Bifidobacterium* is one of the main genera that produce antibacterial peptides, namely, bacteriocins, against pathogenic microorganisms (for a general review, see [88,89]). Finally, *Bifidobacterium* produce conjugated linoleic acid, which can also provide numerous health benefits (for general review, see [90]).

3.6. The *Roseburia* genus has been reported to be less abundant in patients with colorectal cancer [91], ulcerative colitis [92] and T2DM [72]. In fact, *Roseburia* has been reported as a butyrate-producing genus [93,94], and its abundance decreases in parallel with the carbohydrate intake [54]. In addition, the abundance of *Roseburia* negatively correlates with high-fat-induced alterations (weight gain, adiposity, fasting hyperglycemia, serum and hepatic cholesterol and triglyceride accumulation, and glucose intolerance [95,96].

3.7. *Akkermansia muciniphila*, a mucin-degrading bacterium that resides in the mucus layer [97], has been associated with inflammatory processes, one of the hallmarks of the MetS. In fact, *A. muciniphila* restores the thickness of the inner mucus layer and works against high-fat-diet-induced metabolic disorders (namely, body weight, adiposity, metabolic endotoxemia, adipose tissue inflammation marker CD11c, fasting hyperglycemia and insulin resistance), as well as increasing the gut levels of endocannabinoids (acylglycerols) that control inflammation, the gut barrier and gut peptide secretion [98]. In line with this, it has been reported that the abundance of *A. muciniphila* shows a positive correlation with the lipid metabolism, and an inverse correlation with lipid synthesis, inflammatory markers (i.e., insulin, leptin, glucose and triglycerides) and several serum markers of insulin resistance, cardiovascular risk and adiposity [99], presumably by

attenuating adipose tissue inflammation by restoring regulatory T (Treg) cells and the reduction of the expression of proinflammatory IL-6 and IL-1β cytokines in the visceral adipose tissue [100].

Taken as a whole, all these results support the idea that *A. muciniphila* affects the integrity of the intestinal barrier, metabolic inflammation and fat storage and that this relationship plays a key role in the development of the MetS. In addition, results similar to those from animal studies have been reported in humans. For instance, Karlsson et al. showed that the abundance of *A. muciniphila* was negatively correlated with body weight in preschool children [101], while Lim et al. found that this bacterium was enriched in healthy individuals [12].

3.8. *Prevotella* is another genus involved in the intestinal barrier integrity, related with its property for the degradation of the mucins [102] which constitute the mucosal layer surrounding the wall of the digestive tract [103]. Besides, *Prevotella* is one of the most important genera involved in the conversion of plant lignans to health beneficial antioxidants in the rumen of cows [104]. Related to this, *Prevotella* is able to produce propionate, succinate and acetate [105] by using a wide variety of polysaccharides [106]. In fact, *Prevotella* has been described as one of the most abundant genera in high-polysaccharide diets [15,107,108]. By contrast, a few studies have described a detrimental effect of this genus. For example, *Prevotella* (*P. intermedia*) has been shown to induce TNF-α production in a dose-dependent way by a lipopolysaccharide (LPS)-induced mechanism [109] and to produce phosphorylated dihydroceramide lipids, which in turn lead to proinflammatory IL-6 cytokine secretion [110].

4. Modulation of gut microbiota by diet as a therapy for metabolic syndrome treatment

Diet is one of the main factors that determine the gut microbiota composition. The first evidence came from the study by Cani et al. [111], who demonstrated that a high-fat diet increased the proportion of bacterial LPS in the gut by modifying the composition of the gut microbiota, which was related with an increase in obesity and the "low-grade" inflammatory state. Further studies have described the effect on the gut microbiota composition of dietary habits as different as vegetarian versus omnivore diets, as well as in people from distant geographical areas, such as Africa and Europe [15,112]. Nowadays, the link between diet, gut microbiota and certain diseases, including the MetS, is recognized. In fact, the gut microbiota may be considered as a target for the MetS by dietary intervention as a therapeutic treatment which could potentially modify the gut microbiota composition.

4.1. Evidence from studies in animal models

A pioneer study by Turnbaugh et al. [113] analyzed the interrelationship between diet, gut microbial ecology and energy balance in mice fed on a Western diet. Diet-induced obesity resulted in an increase in the *Mollicutes* class from the *Firmicutes* phylum. Experiments performed by transplanting human fecal microbial communities into germ-free mice have shown that the initial structure of the gut microbiota is rapidly altered by diet [114]. In fact, when a low-fat, plant-based, high-polysaccharide diet was changed to a high-fat/high-sugar Western diet, the structure, the metabolic pathways and gene expression of the gut microbiota were transformed in a single day. Moreover, mice colonized with a microbiota from humanized donors and fed an obese Western diet showed increased adiposity, which showed that this trait was transmissible through microbiota transplantation. In terms of the gut microbiota structure, when compared to mice fed the low-fat, plant-based, high-polysaccharide diet, the Western diet induced in mice an increase in the *Erysipelotrichi* and *Bacilli* classes, both belonging to the *Firmicutes* phylum, and a significant decrease in members of the *Bacteroidetes* phylum members.

Different studies have shown that a high-fat diet is responsible for an increase in gut permeability and, consequently, an increase in LPS and bacteria translocation through the intestinal mucosa, which leads to weight gain, altered morphology in the adipose tissue, insulin resistance and inflammation, as well as altering the gut microbiota by increasing *Firmicutes* and decreasing *Bacteroidetes* [111,115–117].

By contrast, a study in pigs by Heintriz et al. [118] showed that beneficial bacteria and SCFA production, especially butyrate, was stimulated with a low-fat/high-fiber diet, while bacterial groups associated with unhealthy conditions were stimulated by a high-fat/low-fiber diet. A low-fat/high-fiber diet also modified the gut microbiota by increasing *Bifidobacterium* spp., a well-known health-promoting genus whose growth is stimulated by dietary fibers [119].

4.2. Diet-induced changes in gut microbiota in humans

Several studies have shown that the composition of the gut microbiota in humans can be rapidly shaped by changes in dietary habits (Table 1). In fact, a short-term controlled-feeding experiment designed in a study including 10 subjects showed that both high-fat, low-fiber and low-fat, high-fiber diets can alter the microbiome composition in a single day [20], as previously reported by Turnbaugh et al. [114] in mice. This was also confirmed by David et al. [14], who showed that the short-term consumption (over 3 days) of diets composed entirely of animal or plant products alters the microbial community structure and overrides any interindividual differences in microbial gene expression. In fact, the animal-based diet increased the abundance of bile-tolerant microorganisms (*Alistipes*, *Bilophila*, and *Bacteroides*) and decreased the levels of *Firmicutes*, which metabolize dietary plant polysaccharides (*Roseburia*, *E. rectale* and *R. bromii*). This study also showed that the microbial activity mirrored differences between herbivorous and carnivorous mammals, reflecting trade-offs between carbohydrate and protein fermentation [14].

Overall, the content of carbohydrates in the diet and the intake of fiber seem to significantly influence gut microbiota composition. De Filippo et al. [15] compared the gut microbiota of European children, characterized by a modern Western diet (rich in fat and sugar), and African children, characterized by a rural diet (rich in polysaccharide), and found significant differences between the two groups. The gut microbiota of African children presented a greater abundance of *Bacteroidetes* and less *Firmicutes*, while *Enterobacteriaceae* (*Shigella* and *Escherichia*) were significantly more abundant in European children. At the genus level, *Prevotella* and *Xylanibacter*, known to be involved in cellulose and xylan hydrolysis [120–122], were the major genera in African children but were absent in European children, while *Faecalibacterium* and *Bacteroides* became the major genera in European children, with *Bacteroides* absent in African children. In addition, SCFA were significantly higher in African than in European children. Based on these data, these authors hypothesized that the gut microbiota related to the high-polysaccharide diet of African individuals allowed them to maximize the intake of energy from fibers and protected them from inflammation and non-infectious colonic diseases.

The role of high-carbohydrate diets has been widely investigated in studies of dietary intervention, which have showed that these diets are beneficial for human health by modifying the gut microbiota. In fact, the potential effect of carbohydrate content can be observed in a study by Duncan et al. [54], who gave three different diets in succession to healthy obese subjects: maintenance (13% protein, 52% carbohydrate and 35% fat as calories), high protein/medium carbohydrate (30% protein, 35% carbohydrate, 35% fat) and high protein/low carbohydrate (30% protein, 4% carbohydrate, 66% fat as calories). This resulted in a significant reduction in total fecal short-chain fatty acids (in succession: 114 mM, 74 mM and 56 mM) and a disproportionate and significant reduction in fecal butyrate (in succession: 18 mM, 9 mM and 4 mM) with decreasing carbohydrate intake. Therefore, these results demonstrated the relationship between the production of butyrate and the intake of fermentable carbohydrates in the diet.

Table 1
Diet-induced changes in gut microbiota in humans

Diet	Time	*N*	Results	Interpretation	Ref.
Entirely animal or plant diets.	5 days	6 male and 4 female	-Gut microbiome can rapidly respond to altered diet. -Animal diet increased bile-tolerant microorganisms (*Alistipes*, *Bilophila* and *Bacteroides*) and decreased *Firmicutes* that metabolize plant polysaccharides (*Roseburia*, *E. rectale*, *R. bromii*). -The plant-based diet did not change the weight of the subjects, while the animal-based diet decreased it significantly by day 3.	-Microbiota changes on the animal-based diet could be linked to altered fecal bile acid profiles and the potential for human enteric disease. -Gut microbiome can rapidly respond to altered diet, potentially facilitating the diversity of human dietary lifestyles.	[14]
Rural diet (rich in fiber) versus modern Western diet (rich in fat and sugar).	Long-term	15 healthy children (9 male and 6 female) living in Burkina Faso and Italy	-Rural diet: more *Bacteroidetes* and less *Firmicutes*, and *Prevotella* and *Xylanibacter* as major genera. -Western diet: more *Enterobacteriaceae* (*Shigella* and *Escherichia*), and *Faecalibacterium* and *Bacteroides* as major genera. -More SCFA associated with the rural than with the western diet.	This study shows the importance of preserving the treasure of microbial diversity from ancient rural communities worldwide. The results make it possible to hypothesize that gut microbiota coevolved with the polysaccharide-rich diet of rural individuals, allowing them to maximize energy intake from fibers by producing high levels of SCFA that supply the host with an additional amount of energy, while also protecting them from inflammations and non-infectious colonic diseases.	[15]
High-fat/low-fiber or low-fat/high-fiber diet.	Long-term	98 healthy subjects	Enterotypes strongly associated with long-term diets: protein and animal fat with *Bacteroides* versus carbohydrates with *Prevotella*.	Long-term diet is particularly strongly associated with enterotype partitioning. Therefore, if an enterotype is causally related to a disease, long-term dietary interventions may allow modulation of an individual's enterotype to improve health.	[20]
	Days 1 and 10	10 healthy subjects	-Microbiome changed within 24 h of initiating the diets. -Enterotypes remained stable during the 10-day study.		
Usual diets of each area.	Long-term	-Bacterial analysis: 531 -Gene analysis: 110 -Individuals (wide range of age) from the Amazonas (Venezuela), rural Malawian communities and USA metropolitan areas	-Pronounced differences in bacterial species and functional genes between individuals of USA compared to the other two countries. -Obvious differences in early childhood and adulthood. -The similarity of fecal microbiomes among family members extends across cultures. -No strong evidence for discrete clustering, but rather for variation driven in adults by a trade-off between *Prevotella* and *Bacteroides*. -Age-associated changes in the microbial genes involved in vitamin biosynthesis and metabolism.	It is necessary to consider the microbiome when evaluating human development, nutritional needs, physiological variations and the impact of Westernization.	[21]
Mediterranean diet versus low-fat, high-carbohydrate diet.	2 years	106 subjects: -33 obese with MetS -32 obese without MetS -41 non-obese	-Marked dysbiosis in obese with MetS compared obese without MetS and non-obese. -Dysbiosis was reversed by consumption of both diets and no significant microbiota changes were observed in obese without MetS and non-obese. -Both diets decreased the triacylglycerides levels in the MetS-obese group after 2 years.	These results suggest that the chronic intake of two healthy dietary patterns partially restores the gut microbiome dysbiosis in obese patients with coronary heart disease, depending on the degree of metabolic dysfunction.	[41]
Mediterranean diet versus low-fat, high-carbohydrate diet.	2 years	239 patients: -138 MetS patients -101 subjects without MetS	Mediterranean diet partially restores the population of *P. distasonis, B. thetaiotaomicron, F. prausnitzii, B. adolescentis and B. longum* in MetS patients.	The Mediterranean diet could be a useful tool to restore potentially beneficial members of the gut microbiota, although the stability of these changes over time still remains to be assessed.	[43]
Successive diets: -Maintenance (*) (399 g carbohydrate/day). -High protein/ medium (164 g/day) carbohydrate (**). -High protein/low (24 g/day)	3 days (*) 4 weeks (**)	19 healthy, obese subjects	-Reduction in fecal total SCFA and butyrate with decreasing carbohydrate. -Fecal ammonia also declined with decreased carbohydrate intake. -Reduction in *Roseburia* spp. and *E. rectale* subgroup of cluster XIVa and bifidobacteria with decreasing carbohydrate. -Correlation between the abundance of butyrate-producing	Because this study was of limited duration and it is unknown whether the relatively short period of reduced butyrate and SCFA supply to the colonic mucosa would have long-term consequences for gut health, it may become important if low-carbohydrate diets are	[54]

(continued on next page)

Table 1 (*continued*)

Diet	Time	*N*	Results	Interpretation	Ref.
carbohydrate (**).			bacteria related to *Roseburia* spp. and *E. rectale* with fecal butyrate decline.	consumed for longer periods without ensuring that adequate forms of appropriate fermentable substrates comprise part of the diet.	
Macrobiotic Ma-Pi 2 diet (rich in fiber and lacking fat and protein from animal source and added sugars) versus control diet (energy from 40%–60% carbohydrate, 10%–20% protein, 30% fat and ≥20 g/1000 kcal fiber).	21 days	56 overweight or obese T2DM patiens (28 in each diet)	-Both diets modulated gut microbiome dysbiosis by increasing the gut microbiota diversity and restoring a balanced community of health-promoting SCFA producers (*Faecalibacterium*, *Roseburia*, *Lachnospira*, *Bacteroides* and *Akkermansia*). -Ma-Pi 2 diet: prevented the increase of proinflammatory bacteria, such as *Collinsella* and *Streptococcus*. -Reduction of fasting blood glucose and postprandial blood glucose levels in both diet groups (higher in the Ma-Pi 2 diet). -Higher reduction in HOMA-IR, total cholesterol, LDL-cholesterol and LDL:HDL in the Ma-Pi 2 diet. -Reduction of the plasma TNF-α levels in both diets. -Reduction of the plasma levels of CRP and IL-6 in Ma-Pi 2 diet.	The Ma-Pi 2 diet was effective in counteracting the increase of possible proinflammatory groups, such as *Collinsella* and *Streptococcus*, showing the potential to reverse proinflammatory dysbiosis in T2DM and possibly explaining the greater efficacy in improving the metabolic control.	[72]
Subjects were divided into vegetarians and non-vegetarians by a dendrogram of the PCR-DGGE.		13 healthy Thai	*Prevotella* and *Bacteroides* as the most abundant genera in the vegetarians and non-vegetarians, respectively.	These findings on the microbiota of healthy Thais consuming different diets could provide helpful data for predicting the health of South East Asians with similar diets.	[107]
-Vegetarians/non-vegetarians. -Vegetarian group: ovolacto vegetarians, lactovegetarians, an ovovegetarian, and vegans.	Vegetarians for at least 3 years before the study	36 healthy vegetarian and 36 healthy non-vegetarian Thais	-*P. copri* as the key species of vegetarians. -*Bacteroides vulgatus*, *Escherichia coli* and *Escherichia hermanii* as the key species of non-vegetarians. -Pathogenic bacteria: *B. wadsworthia*, *E. coli*, and *E. hermannii* in non-vegetarians and only *K. pneumoniae* in vegetarians.	The microbiota of vegetarians with high abundance of *P. copri* and low potential pathogen variety would be a way to maintain good health in Thais.	[108]
Successive diets: -Control diet. -High in resistant starch. -Non-starch polysaccharides. -Reduced carbohydrate weight loss.	10 weeks	14 overweight men	-The gut microbiota rapidly changed when each diet was applied. -Resistant starch diet: increased *R. bromii*, *E. rectale* and *Oscillibacter* group. -Reduced carbohydrate weight loss: increased *Oscillibacter* group and decreased *E. rectale* and *C. aerofaciens*.	Dietary non-digestible carbohydrate can produce marked changes in the gut microbiota, but these depend on the initial composition of an individual's gut microbiota.	[124]
Diets supplemented with resistant starch or non-starch polysaccharides and a weight-loss diet.	10 weeks	14 obese males	-The diet explained around 10% of the total variance in microbiota composition. -Resistant starch diet: decreased the diversity of the microbiota and increased *Ruminococcaceae* phylotypes. -Non-starch polysaccharides diet: increased mostly *Lachnospiraceae* phylotypes. -Weight-loss diet: decreased bifidobacteria. -Lower fecal concentrations of the main SCFA on the resistant starch and weight-loss diets compared with the weight maintenance and non-starch polysaccharides diets. -Slightly stronger influence of diet on insulin sensitivity than on the microbiota or SCFA. -Positive correlation between fecal bifidobacteria and plasma insulin.	The dietary responsiveness of the individual's microbiota varied substantially and associated inversely with its diversity, suggesting that individuals can be stratified into responders and non-responders based on the features of their intestinal microbiota.	[125]
Successive diets: -Weight-maintenance diet (*). -High-protein and moderate-carbohydrate (**) diet. -High-protein and low-carbohydrate (**) diet.	7 days (*) 4 weeks (**)	17 obese men	-High-protein diets: increased branched-chain fatty acids, phenylacetic acid and N-nitroso compounds. -High-protein and low-carbohydrate diet: reduced fecal butyrate and *Roseburia*/*Eubacterium* group reduced fiber-derived antioxidant phenolic acids.	Because weight-loss diets that were high in protein but reduced in total carbohydrates and fiber resulted in a significant decrease in fecal cancer-protective metabolites and increased concentrations of hazardous metabolites, long-term adherence to such diets may increase risk of colonic disease.	[126]

Very low-carbohydrate, high-fat diet versus high-carbohydrate, high-fiber, low-fat diet.	8 weeks	91 overweight and obese subjects	-Very low-carbohydrate, high-fat diet reduced butyrate and total SCFA and bifidobacteria compared to high-carbohydrate, high-fiber, low-fat diet. -Detrimental effects of very low-carbohydrate, high-fat diet on fecal SCFA compared with a high-carbohydrate, high-fiber, low-fat diet.	Long-term consumption of a very low-carbohydrate, high-fat diet, may increase the risk of development of gastrointestinal disorders.	[127]
Mediterranean diet versus low-fat, high-complex carbohydrate diet.	1 year	20 obese patients (men)	-Both diets exert a protective effect on the development of type 2 diabetes by changing the gut microbiota. -Low-fat, high-complex carbohydrate diet: increased the *Prevotella* genus and *F. prausnitzii* and decreased the *Roseburia* genus. -Mediterranean diet: decreased the *Prevotella* and increased *P. distasonis* and the *Roseburia* and *Oscillospira* genera. -Increase in the insulin sensitivity index for both diets. -Changes in amino acid, peptide and sphingolipid metabolism were the main diet-induced effects on the fecal metabolome.	Long-term consumption of the Mediterranean and low-fat, high-complex carbohydrate diets could exert a protective effect on the development of type 2 diabetes by different specific changes in the gut microbiota, increasing the abundance of the *Roseburia* genus and *F. prausnitzii*, respectively.	[128]
-High-saturated-fat diet (baseline) (*). -High-saturated-fat diet (**). -High-monounsaturated fat with high- or low-glycemic-index diets (**). -High-carbohydrate with high- or low-glycemic index diets (**).	4 weeks (*) 24 weeks (**)	88 subjects at MetS risk	-High-monounsaturated-fat diets: reduced total bacteria and plasma total and LDL-cholesterol. -High-carbohydrate diets: increased fecal *Bifidobacterium* and reduced fasting glucose and cholesterol. -High-carbohydrate and high-glycemic-index diets: increased fecal *Bacteroides*. -High-carbohydrate and low-glycemic-index diet and high-saturated-fat diet increased *F. prausnitzii*. -High-saturated-fat diet: increased fecal SCFA levels. -Decrease in body fat percentage with high carbohydrate/high glycemic index compared to baseline also accompanied by a slight decrease in body weight. -Increase in waist circumference compared to baseline in the high-monounsaturated-fat/low-glycemic-index group. -Decrease in NEFA concentration with high-carbohydrate/low-glycemic-index diet compared to the control high-saturated fat diet and to high-carbohydrate/high-glycemic-index diet. -Increase of NEFA with high-carbohydrate/high-glycemic-index diet, while it decreased after intervention with high-carbohydrate/low-glycemic-index diet. -Plasma total and LDL cholesterol decreased in all intervention groups, while HDL cholesterol was decreased after high-carbohydrate/high-glycemic-index diet. -Lower fasting plasma glucose concentrations with both high-carbohydrate diets, and high-carbohydrate/high-glycemic-index diet also decreased plasma insulin concentrations. -Soluble ICAM-1 was higher after high-monounsaturated-fat with high-glycemic-index diet. -Increase of the fecal concentrations of acetate, propionate and n-butyrate after the high-saturated-fat, control diet.	-High-carbohydrate diets, irrespective of glycemic index, can modulate human fecal saccharolytic bacteria, -High-fat diets reduced bacterial numbers and, in the high-saturated-fat diet, increased excretion of SCFA, which may suggest a compensatory mechanism to eliminate excess dietary energy.	[129]

Regarding the bacterial content, the amount of *Roseburia* spp., *E. rectale* and *Bifidobacterium* spp. significantly decreased with decreasing carbohydrate intake. A correlation was also noted between the abundance of butyrate-producing bacteria related to *Roseburia* spp. and *E. rectale* and the decrease in fecal butyrate.

Individuals can be clustered according to their gut microbiota structure, which also is also related with the diet consumed. Arumugam et al. [123] analyzed the fecal metagenomes of individuals from four countries and identified three robust clusters, or "enterotypes," which were not nation- or continent-specific and which would imply, according to these authors, the existence of well-balanced, diet-dependent microbial states. These enterotypes were established according to the abundance of one of three genera: *Bacteroides*, *Prevotella* and *Ruminococcus*. However, Yatsunenko et al. [21] analyzed the fecal samples of healthy individuals from Venezuela, Malawi and the USA, over a wide age range, and did not observe any evidence for discrete clustering in enterotypes but rather for variation in adults through compensation between *Prevotella* and *Bacteroides*. In line with the studies showing the beneficial effect for human health of high-carbohydrate diets, Wu et al. [20] verified the link between long-term dietary patterns and the gut microbiota. This study showed that fecal communities clustered into *Bacteroides* and *Prevotella* enterotypes, with *Prevotella* and *Bacteroides* closely associated with carbohydrate-based diets and protein and animal fat-based diets, respectively. Similar results were reported by Ruengsomwon et al. [107,108] in two studies with vegetarian and non-vegetarian Thai subjects, with *Prevotella* and *Bacteroides* as the most abundant genera in the vegetarian and non-vegetarian groups, respectively.

4.3. Diet as therapeutic tool in human metabolic disease

Dietary interventions studies have shown that the composition of the gut microbiota can be altered, potentially towards a beneficial profile for human health. In fact, several studies have shown that the intestinal microbiota, under conditions of metabolic dysfunction, can be restored, at least partially, by the consumption of healthy diets.

First of all, high-fiber diets have been shown to increase the abundance of saccharolytic bacterial species. In a dietary intervention performed by Walker et al. [124] in overweight men who received diets high in resistant starch or non-starch polysaccharides, a resistant starch diet promoted an increase in the *R. bromii*, *E. rectale* and *Oscillibacter* groups, whereas a reduced-carbohydrate weight-loss diet promoted an increase in the *Oscillibacter* group and a decrease in *E. rectale* and *C. aerofaciens*. The authors of this study concluded that marked changes in the gut microbiota can be produced by non-digestible carbohydrate, although these changes depend on the initial composition of the each individual's gut microbiota [124]. An increase in the abundance of saccharolytic bacterial species was also observed in a study in obese males with diets supplemented with resistant starch, or non-starch polysaccharides, and a weight-loss diet, in which diet accounted for around 10% of the variance in gut microbiota composition [125]. In this study, each diet induced distinct changes in the gut microbiota: the resistant starch diet significantly decreased the diversity of the microbiota and increased multiple *R. phylotypes*, whereas mostly *Lachnospiraceae* phylotypes increased in the non-starch polysaccharides diet and *Bifidobacterium* decreased significantly in the weight-loss diet [125].

Moreover, when compared with a high-protein, low-carbohydrate diet (29% protein, 5% carbohydrate, 66% fat), a high-protein, moderate-carbohydrate diet (28% protein, 35% carbohydrate, 37% fat) has been shown to be more beneficial for obese men [126]. Both high-protein diets increased branched-chain fatty acids, phenylacetic acid and N-nitroso compounds, but high-protein and low-carbohydrate diets reduced fecal butyrate, which was concomitant with a reduction in the *Roseburia*/*Eubacterium* group and reduced fiber-derived antioxidant phenolic acids. According to these data, high-protein diets may increase the risk of colonic disease.

In addition, a very low-carbohydrate, high-fat diet is detrimental. In fact, in a dietary intervention study, the effects of a very low-carbohydrate, high-fat diet (35% protein, 4% carbohydrate, 61% fat) was compared with a high-carbohydrate, high-fiber, low-fat diet (24% protein, 46% carbohydrate, 30% fat) in overweight or obese men and women with abdominal obesity and at least one other metabolic risk factor [127]. In this parallel study, the participants were assigned to either an energy-restricted (about 6–7 MJ, 30% deficit) planned isoenergetic very low-carbohydrate, high-fat diet or a high-carbohydrate, high-fiber, low-fat diet for 8 weeks. The very low-carbohydrate, high-fat diet reduced butyrate and total SCFA and *Bifidobacterium* compared to the high-carbohydrate, high-fiber, low-fat diet. Under energy-restricted conditions, a short-term very low-carbohydrate, high-fat diet had detrimental effects on fecal SCFA compared with a high-carbohydrate, high-fiber, low-fat diet. According to the authors, these data suggest that the long-term consumption of a very low-carbohydrate, high-fat diet may increase the risk of development of gastrointestinal disorders.

A further study also showed in an obese human population that the long-term consumption of the Mediterranean [15% protein, <50% carbohydrate, >35% fat (22% monounsaturated)] and a low-fat diets [15% protein, >55% carbohydrate, <3% fat (12%–14% monounsaturated)] exerts a protective effect on the development of T2DM, improving insulin sensitivity by different specific changes in the gut microbiota [128]. In fact, the consumption of a low-fat diet increased the *Prevotella* genus and *F. prausnitzii* genera and decreased the *Roseburia* genus, whereas the Mediterranean diet decreased the *Prevotella* genus and increased the *P. distasonis*, and *Roseburia* and *Oscillospira* genera.

In line with this, the antidiabetic effect observed which shapes the gut microbiota by dietary intervention was also shown by Candela et al. [72], who compared two diets, the macrobiotic Ma-Pi 2 diet, rich in fiber and lacking fat and protein from animal sources and added sugars, and a recommended control diet for T2DM treatment (10%–20% protein, 40%–60% carbohydrate, 30% fat, ≥20 g of fiber). This study, carried out with T2DM patients, demonstrated that both diets modulated gut microbiome dysbiosis by increasing the gut microbiota diversity and restoring a balanced community of health-promoting SCFA producers (*Faecalibacterium*, *Roseburia*, *Lachnospira*, *Bacteroides* and *Akkermansia*). However, only the high-fiber diet prevented the increase of proinflammatory bacteria, such as *Collinsella* and *Streptococcus*.

Specifically in MetS disease, the Mediterranean diet, in addition to low-fat diets, has been shown to be beneficial. In a dietary intervention, subjects at MetS risk (with a minimum of two MetS features) followed a high-saturated-fat diet for 4 weeks (baseline) and then randomized onto one of five diets for 24 weeks [129]: high-saturated-fat diet [17% protein, 45% carbohydrate, 38% fat (12% monounsaturated), glycemic index (GI) 64%], high-monounsaturated-fat [17% protein, 45% carbohydrate, 38% fat (20% monounsaturated)] with high- (64%) or low- (53%) GI diets, and high-carbohydrate [27% protein, 55% carbohydrate, 28% fat (11% monounsaturated)] with high- (64%) or low- (51%) GI diets. At the end of the intervention, the high-monounsaturated-fat diets reduced the total bacteria, the serum total and LDL-cholesterol. High-carbohydrate diets increased fecal *Bifidobacterium* and reduced fasting glucose and cholesterol. The high-carbohydrate and high-glycemic-index diet also increased fecal *Bacteroides*, whereas high-carbohydrate and low-glycemic-index diet and high-saturated-fat diet increased *F. prausnitzii*. Finally, the high-saturated-fat diet increased the fecal SCFA level. In summary, high-carbohydrate diets can modulate fecal saccharolytic bacteria and high-monounsaturated fat diets reduced bacterial numbers, whereas the high-saturated fat diet increased excretion of SCFA, which may reflect, according to these authors, a compensatory mechanism to eliminate excess dietary energy [129].

Several studies have shown that the consumption of Mediterranean [15% protein, <50% carbohydrate, >35% fat (22% monounsaturated)] and low-fat [15% protein, >55% carbohydrate, <3% fat (12%–14% monounsaturated)] diets influenced the gut microbiota composition in the MetS

[41,43]. In fact, these studies showed that the gut microbiota of MetS patients altered the composition to the pattern found in metabolically healthy people, whereas no significant microbiota changes were observed in non-MetS individuals after the dietary intervention. This was presumed by the dysbiosis observed in MetS patients compared with non-MetS individuals, suggesting that the consumption of Mediterranean and low-fat diets may help in maintaining the the gut microbiota homeostasis, which is particularly important in conditions of an alteration of microbiota such as obesity and MetS.

One of the studies, performed by quantitative PCR in a handful of bacterial genera, showed that the consumption of a Mediterranean diet rather than a low-fat diet partially restores the population of several bacterial species with important saccharolytic activity, such as *P. distasonis*, *B. thetaiotaomicron*, *F. prausnitzii*, *B. adolescentis* and *B. longum* in MetS patients [43]. A latter study included MetS patients who met all the five defining criteria for this syndrome and whose dysbiotic pattern was reversed by the consumption of both Mediterranean or low-fat diets, altering its microbiota composition to the pattern found in metabolically healthy people [41]. In addition to the increase in the abundance of *Bacteroides* and *Prevotella*, which make up the *Bacteriodetes* phylum and in turn reduce the F/B ratio, the consumption of Mediterranean or low-fat diets increased the abundance of other genera with saccharolytic activity, such as *Faecalibacterium* [49–51,56]. This in turn may restore, at least partially, the fiber-derived SCFA synthesis by gut microbiota. However, the consumption of the Mediterranean diet, rich in antioxidant phenolic compounds from foods such as fresh fruit, vegetables, red wine and olive oil, also increased the abundance of *Roseburia* and *Ruminococcus* (which is also involved in saccharolytic activity [49–51,56]), which implies a greater potential of the Mediterranean diet for restoring gut microbiota functionality than the low-fat diet, which was more abundant in whole grains, significantly lower in sources of phenolic compounds and lower in fiber than the former. However, these studies showed that the consumption of Mediterranean or low-fat diets partially restored the alteration in the gut microbiota composition observed in MetS patients, without the disappearance of the syndrome, which suggests that longer periods of consumption may be required.

Although dietary intervention studies in humans have pointed to nutritional intervention as a potential therapeutic tool to restore, at least partially, the dysbiosis found in individuals with MetS and other metabolic diseases, most of these studies do not focus on molecular mechanisms beyond gut microbiota changes. However, these studies mainly linked changes in gut microbiota with intestinal barrier integrity and SCFA production.

5. Modulation of gut microbiota by prebiotics and probiotics as a therapy for metabolic syndrome treatment

Over the last few years, the use of prebiotics and probiotics to promote human health by modulating the host's microbiota composition has seen a remarkable increase. Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [130], while prebiotics were defined in 1995 as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health" [131]. However, this concept was modified in 2004 by establishing three criteria: a) resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; b) fermentation by intestinal microflora; c) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing [132] (Fig. 1).

5.1. Prebiotics in animal models

Although there is a wide variety of prebiotics, most of the studies have focused on the use of inulin and fructo-oligosaccharides (for a general review, see [132]) (Table 2). However, the results obtained are highly dependent on the specific nature of the prebiotic [133] and/or the particular

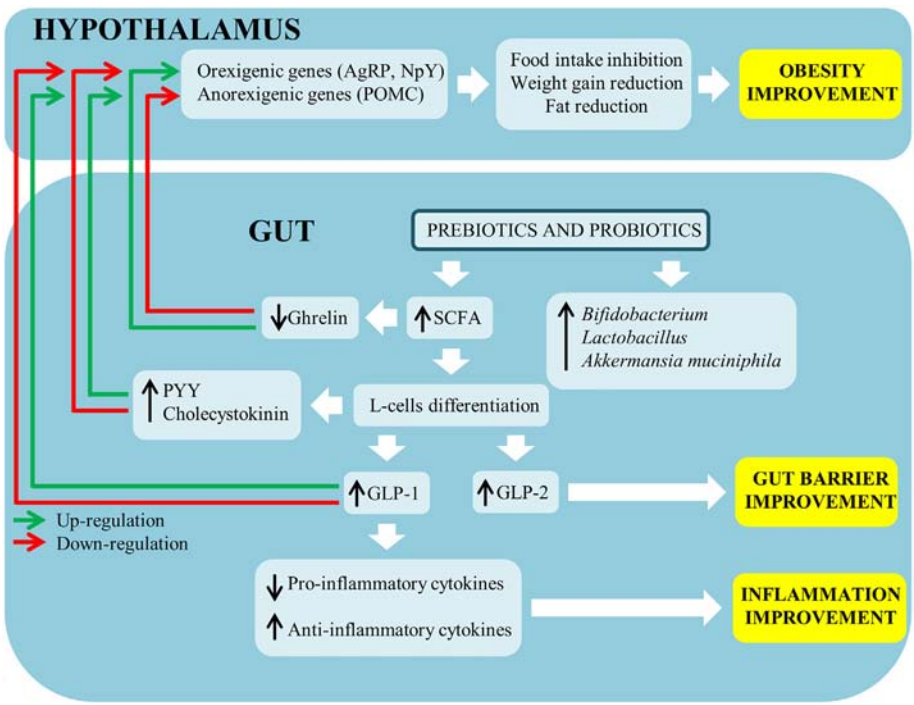

Fig. 1. Overview of how prebiotics and probiotics act in the gut and regulate food intake. The SCFA derived from the fermentation of nondigestible polysaccharides prebiotics or the involvement of probiotics lead to a decrease in ghrelin release and differentiation of L-cells, which induce an increase in PYY, cholecystokinin and GLP-1. Altogether, these hormones are able to improve obesity status by reducing food intake, weight gain and fat accumulation through their action in the hypothalamus. GLP-1 also reduces proinflammatory cytokines and increases anti-inflammatory cytokines, leading to an improvement in the inflammatory status. In addition, L-cells also produce GLP-2, which is responsible for an improvement in the intestinal barrier function. Both, prebiotics and probiotics restore the normobiosis of the gut microbiota, for example, by increasing *Lactobacillus*, *Bifidobacterium* and *A. muciniphyla*.

Table 2
Prebiotics in animal models

Prebiotic	Time	Animal	Results	Interpretation	Ref.
Fructans with different degrees of polymerization.	3 weeks	Standard-diet fed male Wistar rats.	-The consumption of fructans led to a lower dietary energy intake and a decrease in epididymal fat mass. -Fructans modulates GLP-1 and ghrelin production (higher GLP-1 and lower ghrelin with consumption of fructans).	This study demonstrates the potential modulation of GLP-1 and ghrelin by fermentable fibers such as fructans.	[64]
Oligofructose.	14 weeks	HF-diet-fed mice.	-Oligofructose restored bifidobacteria and other physiologically positive bacteria levels, normalized endotoxemia and improved body weight gain and energy intake, and reduced fat mass development by increasing colonic GLP-1. -*Bifidobacterium* spp. positively correlated with improved glucose tolerance, glucose-induced insulin secretion and normalized inflammatory tone.	The gut microbiota could contribute towards the pathophysiological regulation of endotoxemia and set the tone of inflammation for occurrence of diabetes and/or obesity.	[85]
Xylo-oligosaccharides.	Diferent fermentation time: (0 h, 24 h and 48 h).	*In vitro* study with different strains of *Bifidobacteria*, *Lactobacilli* and *Pediococci* spp.	Acetate was found to be the major SCFA produced as the end product of fermentation.	The fermentation pattern of xylo-oligosaccharides depends on the nature of xylo-oligosaccharides, the bacterial strain tested and the time of action.	[133]
-Standard diet with o without short-chain fructo-oligosaccharides (*). -Two distinct high-fat diets with or without short- chain fructo-oligosaccharides (**).	3 weeks (*). 4 weeks (**).	Mice with induced metabolic disorders associated to obesity.	Short-chain fructo-oligosaccharides improved the harmful effects of obesity (decrease in energy intake, body weight gain, glycemia, and epididymal fat mass) when consumed as supplement of a high-fat, carbohydrate-free diet, in which short-chain fructo-oligosaccharides promoted colonic proglucagon expression and insulin secretion, whereas no effects were observed when consumed in combination with a high-fat diet with relatively high carbohydrates content.	These results support an association between the increase in proglucagon expression in the proximal colon and short-chain fructo-oligosaccharides effects on glycemia, fat mass development and/or body weight gain.	[134]
Chito-oligosaccharides and resistant starch.	6 weeks	High-fat-fed rats.	-The combined use of both prebiotics led to the highest level of fecal fat excretion and enhanced the excretion of bile acids in the feces. -The combined use of both prebiotics developed an increased richness and diversity in the gut bacterial community and increased the abundance of both *Lactobacillus* and *Bifidobacterium*. -The combined use of both prebiotics led to a highest abundance of *Allobaculum* and *Blautia*, which is highly related to the highest production of short-chain fatty acids. -The combined use of both prebiotics was associated with a greater degree of thickness of the mucosa layer.	The resulting shift in the gut microbiome, increased metabolite (SCFA) production and increased thickness of the mucosal layer may provide profound influences of chito-oligosaccharides and resistant starch consumption on gut protection.	[135]
Oligofructose (*). Restriction diet (**).	6 weeks (*). 4 weeks (**).	Diabetic male Wistar rats.	-Oligofructose improved diabetes hallmarks by regulating GLP-1 production. -Oligofructose improved glucose tolerance and reduced food intake.	The fermentation of oligofructose in the colon promotes the expression and secretion of colonic peptides, namely GLP-1(7–36) amide, with beneficial consequences on glycemia, insulin secretion and hyperphagia in diabetic rats.	[136]
-Standard diet with or without oligofructose (*). -High-fat diet with or without oligofructose (**).	35 days (*). 15 days (**).	Standard-diet-fed male Wistar rats.	-Oligofructose leads to an increase in GLP-1 and GLP-2 contents in the proximal colon, with an increase on the portal concentration of GLP-1. -Oligofructose leads to a lower ghrelin level, energy consumption, body weight and epididymal adipose tissue weight. -Oligofructose decreased serum triglyceride accumulation.	Oligofructose modulates the production of intestinal peptides involved in the regulation of appetite and body weight (GLP-1 and ghrelin) and could be proposed as interesting nutrients to consider in the treatment of fat intake and associated metabolic disorders.	[137]
-Mix of a fermentable dietary fiber (oligofructose). -Mix of a non-fermentable. dietary fiber (microcrystalline cellulose).	Different times for different experiments.	ob/ob Mice.	-Prebiotics changed the gut microbiota of the caecum, with a higher total bacteria count, *Lactobacillus* spp., *Bifidobacterium* spp., and the *C. coccoides–E. rectale* cluster. -Prebiotics increased GLP-1 and GLP-2 and decreased the glucose-dependent insulinotropic polypeptides (GIPs) production.	A selective gut microbiota change controls and increases endogenous GLP-2 production, and consequently improves gut barrier functions by a GLP-2-dependent mechanism, contributing to the improvement of gut barrier functions during obesity and diabetes.	[138]

			-Prebiotics also modulated two pancreatic peptides: increased amylin and decreased pancreatic polypeptide (PP). -Prebiotics lead to: decreased food intake, lower plasma lipopolysaccharide (LPS) and cytokines, decreased hepatic expression of inflammatory and oxidative stress markers, lower intestinal permeability and improved tight-junction integrity. -Prebiotics lead to lower visceral, epididymal and subcutaneous adipose depots and a higher muscle mass.		
Maltodextrin or fructo-oligosaccharides.	Different times for different experiments.	Obesogenic-diet fed rats.	Prebiotic consumption led to: lower glycemic response to oral glucose, higher GLP-1 responses to oral glucose, reduced energy intake and adipose tissue weight gain, enhanced cecal GLP-1 production, and increase of GLP-1 contents and luminal propionate concentrations in the large intestine.	Prebiotic promotes GLP-1 production, which would help to prevent excess energy intake and fat accumulation.	[139]
Diets varying in inulin concentrations.	3 weeks.	High-fat-fed male rats.	-Inulin dose-dependently modulated gut microbiota (increased *Bacteroidetes* and *Bifidobacterium* spp., and decreased *Clostridium* clusters I and IV). -Inulin dose-dependently decreased caloric intake, respiratory quotient and body weight and body fat, improved glucose tolerance, increased butyryl-CoA: acetate CoA-transferase in cecum, modulated the abundance of key enzymes that regulate butyrate synthesis, up-regulated peptide YY, cholecystokinin and proglucagon transcripts in the cecum and colon, and increased plasma peptide YY and glucagon-like peptide-1 concentrations.	Inulin dose-dependently decreases caloric intake, modulates gut microbiota and up-regulates satiety hormones, with metabolic effects largely independent of caloric restriction.	[140]
Inulin.	12 weeks.	High-fat-diet diabetic rats.	-Inulin treatment reduced the relative abundance of *Firmicutes*, increased the abundance of *Bacteroidetes* and normalized the composition of the gut microbiota (increased *Lactobacillus* and SCFA-producing bacteria *Lachnospiraceae*, *Phascolarctobacterium* and *Bacteroides* and decreased *Desulfovibrio*, which produce lipopolysaccharide LPS). -Inulin reduced fasting blood glucose levels and alleviated glucose intolerance (reduced serum insulin levels and HOMA-IR) and blood lipid panels (reduced total cholesterol and triglycerides). -Inulin increased the serum glucagon-like peptide-1 level and reduced serum IL-6 level, Il6 expression in epididymal adipose tissue, and Pepck, G6pc expression in liver. -Inulin increased body weight in diabetic rats compared with the control diabetic rats.	Inulin treatment can alleviate gut microbiota dysbiosis and enhance serum GLP-1 level to suppress IL-6 secretion and production and hepatic gluconeogenesis, leading to moderation of insulin tolerance.	[141]
Oligofructose.	4 weeks.	Male Wistar rats.	-Oligofructose promoted L-cell differentiation in the proximal colon by promoting the expression of two differentiation factors: neurogenin 3 and NeuroD, thus contributing to a higher GLP-1 production. -Oligofructose reduced food intake, energy intake and body weight gain, which was associated with lower epidydimal, inguinal and visceral adipose tissue.	Dietary fibers may be a tool to decrease food intake and fat mass development.	[142]
Oligofructose.	4 weeks.	High-fat-fed diabetic mice.	-Oligofructose improved glucose tolerance, fasting blood glucose, glucose-stimulated insulin secretion and insulin-sensitive hepatic glucose production and reduced body weight gain. -Oligofructose reduced the levels of two intracellular inflammatory effectors: nuclear factor-KB and inhibitor of KB kinase β.	Antidiabetic actions of oligofructose treatment require a functional GLP-1 receptor and highlight the therapeutic potential of enhancing endogenous GLP-1 secretion for the treatment of type 2 diabetes.	[146]

(continued on next page)

Table 2 (*continued*)

Prebiotic	Time	Animal	Results	Interpretation	Ref.
-Bovine milk oligosaccharides. -Inulin.	1, 3 or 6 weeks.	High-fat-fed mice.	-Bovine milk oligosaccharides and inulin increased abundance of beneficial microbes *Bifidobacterium* and *Lactobacillus*. -Bovine milk oligosaccharides attenuated weight gain, decreased adiposity and decreased caloric intake. -Bovine milk oligosaccharides and inulin abolished the increase in paracellular and transcellular intestinal permeability. -Inulin altered phylogenetic diversity and decreased species richness.	Bovine milk oligosaccharides prevent increased intestinal permeability and microbial dysbiosis and was partially effective to prevent weight gain diet-induced obesity.	[148]
Inulin oligofructose.	8 weeks.	Diet-induced metabolic syndrome male Wistar rats.	-Inulin oligofructose reduced body weight gain, plasma concentrations of free fatty acids and triglycerides. -Inulin oligofructose increased fecal output, fecal lipid excretion, and weight of caecum and colon. -Inulin oligofructose improved ileal morphology by reducing inflammation and improving the density of crypt cells. -Inulin oligofructose attenuated abdominal fat pads, fasting blood glucose concentrations, systolic blood pressure, left ventricular diastolic stiffness, plasma alanine transaminase, increases in inflammatory cell infiltration in the heart and liver, lipid droplets in the liver and plasma lipids as well as impaired glucose and insulin tolerance.	Increasing soluble fiber intake with inulin oligofructose improves the metabolic syndrome hallmarks by decreasing gastrointestinal carbohydrate and lipid uptake.	[149]
Fructooligosaccharides.	12 weeks.	Fructose-fed obese male Wistar rats.	Fructooligosaccharides decreased body weight, cholesterol, triglycerides, and reduced IL-6, IFN-γ, MCP-1, IL-1β and VEGF levels.	*P. decompositum* has anti-inflammatory and hypolipidemic properties that might be used as an alternative treatment for the control of obesity.	[152]
Polydextrose.	60 days.	Partially gastrectomized male Wistar rats.	-Polydextrose decreased the pH of the cecum, increased the cecal wall and marked production of SCFA, especially acetic and propionic acids. -Polydextrose increased hemoglobin values and hematocrit in the gastrectomized group and decreased the hepatic ferroportin expression. -Hepatic iron levels were lower in gastrectomized animals.	Considering that SCFAs play a central role in the increasing nutrients uptake, this mechanism may be involved in altering the hematology profile observed in these animals but not enough to reverse iron deficiency anemia in post-gastrectomy rats.	[153]
Galacto-oligosaccharides.	16 weeks.	Colorectal induced male Wister rats.	-Galacto-oligosaccharides increased beneficial bacteria (bifidobacteria and lactobacilli) and decreased harmful bacteria. -Galacto-oligosaccharides reduced aberrant crypt foci formation, had protective effects against induced body weight loss and showed higher level of cecal and fecal SCFA (acetate, propionate and butyrate).	Novel galacto-oligosaccharides exhibit protective activity against aberrant crypt foci formation *in vivo*, which could be useful in the fight against colorectal cancer.	[154]
Inulin and polyphenol-rich pomegranate extract alone or in combination.	4 weeks.	Diet induced obesity mice.	-Inulin and polyphenol-rich pomegranate extract alone or in combination changed the gut microbiota in a different way. -Inulin and polyphenol-rich pomegranate extract increased *Bifidobacterium*. -Inulin increased serum lipopolysaccharide and monocyte chemoattractant protein 1, which was reversed with inulin + pomegranate extract. Inulin + pomegranate extract increased Bifidobacteriaceae and Rikenellaceae, which may have contributed to the reduction of endotoxemia markers. -Inulin supplementation showed lower species richness of gut microbiota, and the reduction was reversed by the addition of pomegranate extract.	The gut microbiota and their biological pathways are differentially effected by dietary pomegranate extract and inulin fed combined or alone. It is therefore very important to consider the interaction among bioactive components of food when evaluating potential prebiotic effects.	[155]

nutritional background. For example, the consumption of short-chain fructo-oligosaccharides improved the harmful effects of obesity when consumed as a supplement to a high-fat, -carbohydrate-free diet (78% fat, 28% protein, <1% carbohydrates), whereas no effects were observed when they were consumed in combination with a high-fat diet with a relatively high carbohydrate content (58% fat, 16% protein, 26% carbohydrates) [134]. Moreover, the effects of the combined use of different prebiotics may exert additional beneficial properties, as shown by Shang et al. [135]. In fact, this study shows that the combination of chito-oligosaccharides and resistant starch induces a greater thickness of the mucosa layer and increases the production of SCFA than when consumed separately.

Several studies have shown that the gut microbiota influences obesity and the metabolic syndrome through the gut–brain axis so that food intake is regulated in the central nervous system by the products of gut microbiota activity. Here, many studies have reported that prebiotics reduce food intake, body weight and adiposity, and improve most of the obesity-related disorders through the modulation of the production and secretion of intestinal hormones [64,85,136–141]. In fact, it has been shown that prebiotic fermentation promotes L-cell differentiation in the gut, which may contribute towards increasing glucagon-like peptide-1 (GLP-1) production [142]. In addition, SCFA derived from the anaerobic breakdown of dietary fiber by the saccharolytic flora induce the release of GLP-1 by L-cells in the intestine [143–145]. Moreover, Cani et al. demonstrated that the antidiabetic actions of prebiotic treatment (improvement of glucose tolerance, fasting blood glucose, glucose-stimulated insulin secretion and insulin-sensitive hepatic glucose production) require a functional GLP-1 receptor [146]. In addition, prebiotics up-regulate peptide YY (PYY) and cholecystokinin in the gut [140], both also produced by intestinal L-cells and considered as anorexigenic hormones which inhibit food intake and reduce weight gain [63,65]. In the serum, prebiotics kept a low level of ghrelin [64], which is synthesized mainly in the stomach, enhances appetite and stimulates food intake and weight gain, leading to greater adiposity [66,67]. In this case, it has been reported that ghrelin stimulates orexigenic agouti-related protein (AgRP)/neuropeptide Y (NpY) neurons and inhibits anorexigenic proopiomelanocortin (POMC) neurons in the hypothalamus [147].

Another mechanism by which prebiotics seem to exert their beneficial effect is by increasing the thickness of the mucosal layer, improving tight-junction integrity and reducing the intestinal permeability in the gut compared to the increased intestinal permeability observed in obesity [135,138,148], as well as improving ileal morphology by reducing inflammation and improving the density of crypt cells [149]. Similarly, prebiotics also increase the intestinotrophic growth hormone glucagon-like peptide-2 (GLP-2) level in the gut [137,138], which has been related to an improvement in gut barrier functions [138,150,151]. Thus, an improvement in intestinal permeability by consuming prebiotics has been associated with a lower level of inflammation by decreasing the levels of proinflammatory cytokines, such as IL-1α, IL-1β, IL-6, monocyte chemotactic protein-1 (MCP-1) and interferon-gamma (IFN-γ) in serum [85,141,152].

Overall, most of the beneficial effects described above are likely to be caused by SCFA, whose increased production induced by prebiotics has been established [135,153,154]. Moreover, the consumption of prebiotics has been shown to induce changes in the gut microbiota composition and frequently increases the health-promoting genera *Bifidobacterium* and/or *Lactobacillus* [85,138,140,141,148,154,155].

5.2. Prebiotics in humans

Human studies have also shown that the effect of prebiotics is highly dependent on the nutritional background (Table 3). For example, a study by Healey et al. showed different results in two different low-fiber (20.6% protein, 40.6% carbohydrate, 34.4% fat, 2.2% fiber) and higher-fiber (19.1% protein, 39.3% carbohydrate, 35.9% fat, 3.1% fiber) diets when added to an inulin-type fructan prebiotic (16g/d) [156]. Prebiotic intervention led to an increase in *Bifidobacterium* in the low-fiber group, and an increase in *Bifidobacterium* and *Faecalibacterium* and a decrease in *Coprococcus*, *Dorea* and *Ruminococcus* (*Lachnospiraceae* family) in the high-fiber group.

In the case of human studies, the regulation of food intake through the gut–brain axis has also been highlighted. In this case, human consumption of prebiotics has been linked with a reduction in food intake, body weight and adiposity, and an improvement in most disorders linked to obesity, mainly through the increase in GLP-1 and PYY levels [157,158], as well as a decrease in ghrelin [158]. Moreover, the consumption of prebiotics by humans has also been associated with an improvement of the inflammatory status, both at the endoscopic and at the histological level [159]. Linked to this, the consumption of prebiotics increases the anti-inflammatory cytokine IL-4 and decreases proinflammatory cytokines IL-6, IL-12, and IFN-γ [160,161].

However, the influence of prebiotics in the production of SCFA is not entirely clear in humans. In this area, some studies have shown an increase in SCFA after the consumption of prebiotics [159,162–165], while others have not detected any such increase [166–168]. Moreover, a number of other discrepancies have been noted in the different studies on the consumption of prebiotics in humans. For example, the study by Clarke et al. [163], cited in SCFA production, shows contradictory results as the prebiotics increased and the anti-inflammatory cytokines IL-4 and IL-10 decreased, respectively; moreover, it also increased LPS levels in serum. In addition, other studies have reported no effects after prebiotic consumption. For example, the consumption of galacto-oligosaccharides seems to have no effect on gut-derived hormones, incretins, lipopolysaccharide-binding protein or other inflammation markers despite increasing bacterial species such as *Bifidobacterium* [166]. In line with this, a study by Cani et al. showed no effect on fasting glucose or insulin plasma levels after the prebiotic treatment, although a lower AUC of glucose after a test meal was observed in the group of individuals who consumed the prebiotic [157].

Moreover, the study by Rahat-Rozenbloom et al. [164] showed that an increase in SCFA reduces ghrelin levels, although this was not accompanied by the stimulation of GLP-1 and PYY, as found in animal models. In addition, a study by Boll et al. [162] did not show any link between the increase of SCFA and GLP-1 (and GLP-2) serum levels.

In particular, it has been reported that prebiotics increase *F. prausnitzii* [169] and decrease *Bacteroides* spp. [159,161]. Just as in studies in animal models, most of the studies in humans show that prebiotic consumption increases *Bifidobacterium* and/or *Lactobacillus* genera [163,166–168]. At the metabolic level, it has been shown that the ingestion of prebiotics improves fat oxidation in overweight to obese men [165]. Prebiotics can even improve the healthy effects of a high-carbohydrate, low-fat diet (15% protein, 55% carbohydrate, 30% fat) by lowering hepatic lipogenesis and serum triacylglycerol concentration [170]. Moreover, the application of a mixture of galacto-oligosaccharides in overweight adults with three or more risk factors associated with MetS produced positive effects on the gut microbiota composition, the immune response, insulin, total cholesterol and triglyceride concentrations [171].

5.3. Probiotics in animal models

Most of the research with probiotics has mainly tested the effect of the *Bifidobacterium* and *Lactobacillus* species and is made up of comparative studies between "normal" mice and high-fat-diet-induced obese mice (Table 4). The main mechanism by which probiotics were found to improve metabolic disorders is through modulating the gut microbiota composition and its metabolic products, mainly SCFA, which in turn lead to decreased obesity and improved insulin resistance and inflammation.

Regarding the regulation of food intake through the gut–brain axis, probiotics have been shown to increase SCFA production, which in turn stimulates the release of GLP-1 from intestinal L-cells [143–145].

Table 3
Prebiotics in humans

Prebiotic	Time	*N*	Results	Interpretation	Ref.
Resistant starch or non-starch polysaccharides.	10 weeks. Randomized crossover design.	14 obese males	-The diet explained around 10% of the total variance in microbiota composition. -Resistant starch increased multiple *Ruminococcaceae* phylotypes. -Non-starch polysaccharides increased mostly *Lachnospiraceae* phylotypes and decreased the diversity of the microbiota significantly. -Bifidobacteria decreased significantly on the weight-loss diet. -Positive correlation between fecal bifidobacteria and plasma insulin. -Lower fecal concentrations of the main SCFA acetate, propionate and butyrate, as well as succinate on the resistant starch and weight-loss diets, whereas most of the minor fermentation acids were increased. -The weight-loss diet had a strikingly strong impact on SCFA, as well as on insulin sensitivity.	The dietary responsiveness of the individual's microbiota varied substantially and associated inversely with its diversity, suggesting that individuals can be stratified into responders and non-responders based on the features of their intestinal microbiota.	[125]
Low dietary fiber versus high dietary fiber plus inulin-type fructan.	9 weeks.	34 healthy participants.	-Prebiotics led to an increase in *Bifidobacterium* in low-fiber group, and an increase in *Bifidobacterium* and *Faecalibacterium* and decreases in *Coprococcus*, *Dorea* and *Ruminococcus* (*Lachnospiraceae* family) in higher-fiber group. -The prebiotic intervention led to a significant reduction in satisfaction before lunch and in hunger after dinner, and a significant increase in fullness and satisfaction after lunch.	High dietary fiber intake have a greater gut microbiota response and is therefore more likely to benefit from an inulin-type fructan prebiotic than low dietary fiber intake. Future studies aiming to modulate the gut microbiota and improve host health, using an inulin-type fructan prebiotic, should take habitual dietary fiber intake into account.	[156]
Mixture of glucosyl-$(fructosyl)_n$-fructose and $(fructosyl)_m$-fructose extracted from chicory roots.	2 weeks.	10 healthy adults (5 men and 5 women).	Prebiotics increased plasma GLP-1 and PYY, whereas postprandial plasma glucose responses decreased after the standardized meal. -Prebiotic treatment significantly lowered hunger at time 180 min, whereas this variable remained unchanged after dextrin maltose treatment. -The area under the curve for plasma glucose concentration was significantly lower after the prebiotic treatment.	Prebiotic supplementation is associated with an increase in plasma gut peptide concentrations (glucagon-like peptide 1 and peptide YY), which may contribute in part to changes in appetite sensation and glucose excursion responses after a meal in healthy subjects.	[157]
Oligofructose.	12 weeks.	48 healthy overweight and obese adults.	Prebiotics reduced caloric intake, body weight, glucose, insulin and ghrelin, and increased PYY.	Independent of other lifestyle changes, oligofructose supplementation has the potential to promote weight loss and improve glucose regulation in overweight adults. Suppressed ghrelin and enhanced PYY may contribute in part to the reduction in energy intake.	[158]
Inulin.	3 weeks.	20 patients with an ileal pouch-anal anastomosis.	-Prebiotics increased butyrate concentrations, lowered pH, decreased *B. fragilis*, and diminished secondary bile acids in feces. -Prebiotics reduced the intestinal mucosa inflammation both at the endoscopic and histological level.	Enteral inulin supplementation leads to a decrease of inflammation-associated factors and to a reduction of inflammation of pouch mucosa.	[159]
Oligofructose-enriched inulin.	2 months.	46 diabetic females.	-Prebiotics improved the glycemic status and lipid profile. -Prebiotics increased the anti-inflammatory cytokine IL-4 and decreased pro-inflammatory cytokines IL-12 and IFN-γ. -Prebiotics significantly reduced body mass index, waist circumference, hip circumference, diastolic blood pressure, fasting serum glucose, HbA1c and serum lipids.	This study shows several beneficial effects of oligofructose-enriched inulin on the improvement of the glycemic status, lipid profile, and immune markers in patients with T2DM. Further studies are needed to confirming our findings and to better clarify the underlying mechanisms.	[160]

Oligofructose-enriched inulin.	16 weeks.	42 overweight or obesity, but otherwise healthy.	-Prebiotics decreased body weight, percent body fat, percent trunk fat, serum triglycerides and proinflammatory cytokine IL-6. -Prebiotics increased *Bifidobacterium* and decreased *B. vulgatus*. -Primary bile acids increased in fecal samples in the placebo group but not in the prebiotic group over the 16-week study period.	Oligofructose-enriched inulin treatment selectively altered the intestinal microbiota and significantly reduced body weight z-score, percent body fat, percent trunk fat and serum level of IL6 in children with overweight or obesity.	[161]
Arabinoxylan oligosaccharides and/or resistant starch.	3 h	19 healthy subjects (9 men and 10 women).	-Arabinoxylan oligosaccharides treatment led to a decrease in glucose response and fasting insulin, as well as an increase in acetate, butyrate and total SCFA.	Arabinoxylan oligosaccharides have the potential of improving glucose tolerance by improving insulin sensitivity and increasing gut fermentation.	[162]
β2-1 Fructans.	Two 28-day treatments separated by a 14-day washout period.	30 adults (13 men and 17 women).	Prebiotics increased serum lipopolysaccharide, fecal SCFA, the anti-inflammatory cytokine IL-4, circulating percentages of CD282+/TLR2+ myeloid dendritic cells, and indigestion; but decreased the anti-inflammatory cytokine IL-10. -Prebiotics increased fecal bifidobacteria, but this change was not directly related to any of the determined host parameters.	No differences in host well-being were associated with either treatment, although the self-reported incidence of gastrointestinal symptoms and headaches increased during the β2-1 fructan phase.	[163]
Inulin or resistant starch	Intervals over 6 h	13 healthy overweight/obese and 12 lean subjects.	-Inulin increased SCFA (acetate, propionate and butyrate). -Although neither inulin nor resistant starch reduced ghrelin compared to glucose, ghrelin at 6 h after inulin was significantly lower than that after glucose.	Acute increases in colonic SCFA do not affect GLP-1 or PYY responses in lean or overweight/obese subjects but may reduce ghrelin. The results do not support the hypothesis that SCFA acutely stimulate PYY and GLP-1 secretion; however, a longer adaptation to increased colonic fermentation or a larger sample size may yield different results.	[164]
Inulin.	2 days with at least 5 days of washout period in between.	14 healthy, overweight to obese men.	-Fat oxidation was higher in the early postprandial phase after inulin treatment, while carbohydrate oxidation was lower. -Inulin intake led to higher plasma acetate concentrations in the late postprandial phase. -Plasma free fatty acids were higher in the early and lower in the late postprandial period after inulin ingestion. -The inulin treatment resulted in a lower glucose response in the early postprandial phase and in a lower plasma insulin concentration.	Ingestion of the prebiotic inulin improves fat oxidation and promotes SCFA production in overweight to obese men. Overall, replacing digestible carbohydrates with the fermentable inulin may favor human substrate metabolism.	[165]
Galacto-oligosaccharides.	12 weeks. Double-blind, placebo-controlled, randomized, parallel trial.	44 overweight or obese prediabetic men and women.	-Prebiotics increased fecal *Bifidobacterium*. -Prebiotics did not affect the SCFA concentrations, gut-derived hormones, inflammation markers, insulin sensitivity, body composition, and energy and substrate metabolism.	Twelve-week supplementation of galacto-oligosaccharides selectively increased fecal *Bifidobacterium* spp. abundance, but this did not produce significant changes in insulin sensitivity or related substrate and energy metabolism in overweight or obese prediabetic men and women.	[166]
Partially hydrolyzed guar gum dietary fiber.	2 weeks.	10 healthy volunteers.	-Prebiotics increased *Bifidobacterium*, the *C. coccoides* group, the *Roseburia*/*E. rectale* group, *E. hallii* and butyrate-producing bacterium strain SS2/1. -Prebiotics did not affect the fecal SCFAs (acetate, propionate and butyrate) concentrations.	Partially hydrolyzed guar gum may benefit health by stimulating *Bifidobacterium* and butyrate-producing bacteria in the human large intestine.	[167]
Agave fructans	3 weeks followed by a 2-week washout period.	38 healthy volunteers.	-Prebiotics increased fecal bifidobacteria and lactobacilli. -Prebiotics did not affect the SCFA, secretory IgA, and	Agave fructans are well tolerated in healthy human subjects and increased bifidobacteria	[168]

(continued on next page)

Table 3 (*continued*)

Prebiotic	Time	*N*	Results	Interpretation	Ref.
	Placebo-controlled, randomized, double-blind, crossover study.		PGE2 concentrations.	and lactobacilli numbers *in vitro* and *in vivo* but did not influence other products of fermentation.	
Inulin.	16 days. Randomized balanced crossover study design.	12 healthy adults.	-Prebiotics increased *F. prausnitzii*. -*Bifidobacterium* spp. showed the strongest stimulation upon inulin ingestion. -Inulin did not affect the fecal SCFA concentrations, while lactate was slightly increased.	*B. adolescentis* plays a major role in the response to inulin *in vivo*. Furthermore, a significant stimulation was found for *F. prausnitzii*, confirming that ingestion of prebiotics is likely to lead to microbiota changes beyond the lactic acid bacteria. Since interindividual variation may have a major influence, studies involving larger numbers of volunteers together with more detailed analysis of the microbiota will be necessary to further define those bacteria that respond to inulin.	[169]
Inulin.	3 weeks. Double-blind, randomized, placebo-controlled crossover study.	8 healthy subjects.	Prebiotics decreased hepatic lipogenesis and plasma triacylglycerol concentrations.	The addition of high-performance inulin to a moderately high-carbohydrate, low-fat diet has a beneficial effect on plasma lipids by decreasing hepatic lipogenesis and plasma triacylglycerol concentrations. These results support the use of non-digestible carbohydrate for reducing risk factors for atherosclerosis.	[170]
Galactooligosaccharides.	12 weeks, with a 4-week washout period between interventions. Double-blind, randomized, placebo (maltodextrin)-controlled, crossover study.	45 overweight adults with metabolic syndrome.	-Galacto-oligosaccharides increased the number of fecal bifidobacteria at the expense of less desirable groups of bacteria. -Galacto-oligosaccharides increased fecal secretory IgA and decreased fecal calprotectin, plasma C-reactive protein, insulin, total cholesterol (TC), triglycerides and the TC:HDL cholesterol ratio.	Administration of galacto-oligosaccharides to overweight adults resulted in positive effects on the composition of the gut microbiota, the immune response, and insulin, total cholesterol and triglycerides concentrations. Therefore, galacto-oligosaccharides may be a useful candidate for the enhancement of gastrointestinal health, immune function and the reduction of metabolic syndrome risk factors in overweight adults.	[171]

Table 4
Probiotics in animal models

Prebiotic	Time	*N*	Results	Interpretation	Ref.
L. rhamnosus GG.	13 weeks.	High-fat-fed mice.	-*L. rhamnosus* GG increased and decreased fatty acid oxidative genes and the gluconeogenic genes expression in the liver, respectively. -*L. rhamnosus* GG attenuated weight gain and enhanced insulin sensitivity in high-fat-diet group. -GLUT4 mRNA expression in skeletal muscle and adiponectin production in adipose tissue were increased. Activation of AMPK increased in both tissues.	*L. rhamnosus* GG treatment improves insulin sensitivity and reduces fat accumulation by stimulating adiponectin secretion and consequent activation of AMPK in skeletal muscle and adipose tissue.	[80]
B. animalis subsp. *lactis* 420.	6 weeks.	Myd88 knockout and ob/ob mice.	-Probiotic reduced bacterial translocation and proinflammatory cytokines TNF-α, IL-1b, PAI-1 and IL-6. -Glucose intolerance was moderately blunted by the probiotic treatment, although fasting glycemia remained unaffected. -Insulin sensitivity and fasting hyperinsulinemia were completely normalized by the probiotic treatment.	The early onset of high-fat-diet-induced hyperglycemia is characterized by an increased bacterial translocation from intestine towards tissues, fueling a continuous metabolic bacteremia, which could represent new therapeutic targets.	[115]
-*B. animalis* ssp. *lactis* GCL2505. -*B. longum* ssp. *longum* JCM1217T (BloJ).	7 weeks.	High-fat-fed mice.	-*B. animalis* ssp. *lactis* GCL2505 increased *Bifidobacterium* and altered the overall structure of gut microbiota. -*B. animalis* ssp. *lactis* GCL2505 reduced visceral and subcutaneous fat and improved glucose tolerance, SCFA (especially acetate) and gut and plasma GLP-1.	*Bifidobacterium animalis* ssp. *lactis* GCL2505, a highly viable and proliferative probiotic, improves metabolic disorders by modulating gut microbiota, which results in the elevation of SCFA, especially acetate.	[143]
L. casei CCFM419.	12 weeks	High-fat and streptozotocin-induced diabetes mice.	-Probiotic increased *Bacteroidetes* and decreased *Firmicutes*, and enriched *Bifidobacterium*, *Lactobacillus*, and SCFA-producing bacteria, including *Allobaculum* and *Bacteroides*. -Probiotic attenuated several symptoms of diabetes, including fasting blood glucose, postprandial blood glucose, glucose intolerance, and insulin resistance. -Probiotic decreased the levels of the inflammatory markers TNF-α and IL-6 levels, and increased intestinal GLP-1 level, which is associated with the SCFA production (probiotic led to an increased level of acetic acid and butyrate). -Probiotic reduced LDL-cholesterol levels.	*L. casei* CCFM419 modified the gut flora-SCFA-inflammation/GLP-1 mechanism to ameliorate type 2 diabetes.	[144]
VSL#3.	21 weeks.	Male mice fed a low-/high-fat diet.	-Probiotic treatment reduced *Firmicutes* and increased *Bacteriodetes*, bifidobacteria and the total abundance of butyrate-producing bacteria. -Probiotic treatment reduced the hunger-inducing hormone ghrelin and increased butyrate level, which stimulated the release of GLP-1 from intestinal L-cells, resulting in reduced food intake, reduced body weight gain and insulin resistance by modulating the gut microbiota. -Probiotic treatment reduced fasting and fed blood glucose levels, improved glucose tolerance and insulin tolerance, and suppressed hyperinsulinemia. -Probiotic treatment decreased serum triglyceride and free fatty acid levels and reduced fat accumulation in the liver (hepatic steatosis). -Probiotic treatment decreased resistin and increased adiponectin levels. -Probiotic treatment decreased the circulating levels of inflammatory cytokines, i.e. IL-6, MCP-1, and TNF-α.	These results suggest that probiotics reduce the inflammatory state that is often associated with obesity and insulin resistance. Probiotics could modulate the gut microbiota–SCFA–hormone axis and thus are of potential therapeutic utility to counter obesity and diabetes.	[145]
-Monostrain *L. salivarius* Ls33. -Multi-strain *L. rhamnosus* LMG S-28148 and *B. animalis*	16 weeks.	High-fat-induced obese mice.	-Multi-strain mixture changed the gut microbiota composition by decreasing *Lactobacillaceae* and increasing *A. muciniphila* and *Rikenellaceae*.	These results provide crucial clues for the design and use of more efficient probiotic preparations in obesity management and may bring new insights	[173]

(continued on next page)

Table 4 (*continued*)

Prebiotic	Time	*N*	Results	Interpretation	Ref.
subsp. *lactis* LMG P-28149.			-Multi-strain mixture improved adiposity, insulin resistance and dyslipidemia through adipose tissue immune cell remodeling, mainly affecting macrophages. -Multi-strain mixture reduced body weight gain, white adipose tissue mass, fasting glucose, insulin levels, and blood leptin levels, while adiponectin levels were higher. -Multi-strain mixture reduced total cholesterol, HDL and LDL cholesterol. -Multi-strain mixture modified the fatty acids uptake and restored of the SCFA receptor GPR43 level. -Multi-strain mixture decreased monocyte/macrophage markers and increased Treg cells. -Multi-strain mixture increased IL-10 and reduced IL-17 and TNF-α. -An *in vitro* gut model showed that the probiotic mixture favors the production of butyrate and propionate. -The protective effect of the probiotic multistrain mixture is mainly supported by *B. animalis* subsp. *lactis*.	into the mechanisms by which host–microbe interactions govern such protective effects.	
B. pseudocatenulatum CECT 7765.	7 weeks.	High-fat-fed mice.	-Probiotic increased bifidobacteria and reduced enterobacteria and the inflammatory properties of the gut content. -Probiotic reduced caloric intake and body weight gain. -Probiotic reduced serum cholesterol, triglyceride and glucose levels and decreased insulin resistance and improved glucose tolerance. -Probiotic reduced leptin, IL-6, IL-10 and MCP-1, and increased IL-4. -Probiotic reduced hepatic steatosis, adipocyte size and enterocyte fat absorption. -Probiotic improved the function of macrophages and dendritic cells, cytokine production and induction of T-lymphocyte proliferation.	*B. pseudocatenulatum* CECT 7765 was shown to ameliorate both metabolic and immunological dysfunctions related to obesity in high-fat-diet-fed mice.	[177]
L. curvatus HY7601 and *L. plantarum* KY1032.	10 weeks.	High-fat-diet-fed male mice.	-The diversity of the gut microbiota and its composition were significantly altered in the diet-induced obese mice and after probiotic treatment. -Probiotics reduced body weight gain, fat accumulation, plasma insulin, leptin, total-cholesterol and liver toxicity biomarkers. -Probiotics reduced TNF-α, IL-6, IL-1b and MCP-1 in adipose tissue. -Probiotics up-regulated fatty acid oxidation-related genes in the liver.	The gut microbiota of diet-induced obese mice appears to be modulated in mice receiving probiotic treatment. Probiotic treatment might reduce diet-induced obesity and modulate genes associated with metabolism and inflammation in the liver and adipose tissue.	[178]
B. adolescentis IM38.	6 weeks.	High-fat-induced obese mice.	-Probiotic lowered the *Proteobacteria*/*Bacteroidetes* ratio. -Probiotic reduced whole-body and epididymal fat weight gain, LPS, TNF and IL-17 and increased IL-10. -Probiotic inhibited differentiation into Th17 cells and NF-KB activation expression in the colon. -Probiotic increased tight junction proteins.	These results suggest that IM38 can inhibit HFD-induced LPS production in gut microbiota through the regulation of *Proteobacteria*/*Bacteroidetes* ratio and NF-KB activation in the colon, which ultimately attenuates colitis. Thus, IM38 may be a suitable ingredient of functional foods designed for treating or preventing colitis.	[179]
VSL#3.	4 weeks.	High-fat-fed male mice.	Probiotics reduced weight and improved hepatic steatosis, insulin resistance, and NKT cell depletion, decreasing TNF-α and IKK-β, and increasing IL-4.	Probiotics improve high-fat-diet-induced steatosis and insulin resistance. These effects of probiotic are likely due to increased hepatic NKT cell numbers and reduced inflammatory signaling.	[180]
B. longum.	12 weeks.	High-fat metabolic-syndrome-induced rats.	-Probiotics increased the gut bifidobacterial content. -Probiotics improved high-fat-diet-induced metabolic disorders (increase in body weight, fat deposits, systolic blood pressure, fasting glucose, fasting triglycerides and reduced insulin sensitivity), normalized LPS, IL-1b plasma levels, and intestinal myeloperoxidase, and increased intestinal Reg I proteins.	Increasing *Bifidobacterium* in the gut improved high-fat-fed-induced metabolic syndrome by reducing metabolic endotoxin concentrations and intestinal inflammation, as well as upgrading the expression of intestinal Reg I as a regulator of growth factor. Thus, specific strategies for modifying gut microbiota in favor of *Bifidobacterium* could be useful tools for reducing the impact of high-fat feeding on the occurrence of metabolic syndrome.	[186]

-*L. paracasei* CNCM I-4270. -*L. rhamnosus* I-3690. -*B. animalis* subsp. *lactis* I-2494.	12 weeks.	High-fat-fed mice.	-The probiotic strains shifted the overall structure of the high-fat diet disrupted gut microbiota toward that of lean mice fed a normal (chow) diet. -Probiotics attenuated weight gain and macrophage infiltration into epididymal adipose tissue and improved glucose–insulin homeostasis and hepatic steatosis. -Probiotics restored gut microbiota composition. -*L. paracasei* CNCM I-4270 and *L. rhamnosus* I-3690 increased cecal acetate but did not affect circulating lipopolysaccharide-binding protein. -*B. animalis* subsp. *lactis* I-2494 decreased adipose and hepatic TNF-α, and attenuated endotoxin load and systemic inflammation.	These results suggest that *Lactobacillus* and *Bifidobacterium* differentially attenuate obesity comorbidities in part through strain-specific impacts on metabolic syndrome associated phylotypes of gut microbiota in mice.	[189]
B. breve B-3.	8 weeks.	High-fat-induced obese mice.	-Probiotics increased bifidobacteria. -Probiotics reduced body weight and epididymal fat, improved total-cholesterol, fasting glucose and insulin levels, and stimulate adiponectin secretion. -Probiotics up-regulated the expression of genes related to fat metabolism and insulin sensitivity in the gut and epididymal fat tissue.	These results suggest that the use of *B. breve* B-3 would be effective in reducing the risk of obesity.	[191]
L. acidophilus IMV B-7279, *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKL and *B. animalis* VKB separately and in various compositions.	4 weeks.	High-calorie-induced obese female mice.	-The number of *Lactobacillus* spp., *Bifidobacterium* spp. and coliform bacteria increased, the number of staphylococci and streptococci decreased, and the number of microscopic fungi significantly decreased in the gut of obese mice after treatment with *L. casei* IMV B-7280, *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* (separately) or their compositions. -*L. casei* IMV B-7280 (separately) and a composition of *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 decreased the obese mice weight, decreased cholesterol, restored the liver morphology and beneficially modulated the gut microbiota. -The size of the liver slightly decreased after treatment with *L. delbrueckii* subsp. *bulgaricus* IMV B-7281, *B. animalis* VKB or probiotic compositions; and a reduction of the mesenteric fat size after injection of all the probiotic bacteria (separately) and probiotic compositions was observed.	*L. casei* IMV B-7280 (separately) and a composition of *B. animalis* VKL/*B. animalis* VKB/*L. casei* IMV B-7280 are effective at decreasing the weight of obese mice, decreasing cholesterol level, restoring the liver morphology and beneficially modulating the gut microbiome in high-calorie induced obesity.	[193]
L. acidophilus NS1.	10 weeks.	High-fat-fed male mice	-Probiotics reduced body weight gain and decreased triglycerides, total cholesterol and LDL cholesterol levels. -Probiotics increased SREBP2 and LDL receptor expression in the liver.	These results suggest that the oral administration of *L. acidophilus* NS1 to mice fed an HFD increased the expression of SREBP2 and LDLR in the liver, which was inhibited by high fat intake, thus leading to a decrease in plasma cholesterol levels. *Lactobacillus acidophilus* NS1 could be a useful probiotic microorganism for cholesterol-lowering dairy products and the improvement of hyperlipidemia and hepatic lipid metabolism.	[194]

In addition, Yadav et al. reported that probiotics could reduce the expression of the orexigenic (appetite-increasing) genes, such as AgRP and NpY, and increase the expression of anorexigenic (appetite-suppressing) POMC in the hypothalamus. Based on these data, these authors have suggested that probiotics might modulate central food intake mechanisms in the hypothalamus [145]. Further, this idea was confirmed by Secher et al., who demonstrated that GLP-1 directly stimulated POMC neurons and indirectly inhibited the activity of AgRP and NpY neurons [172]. In line with this, probiotics have also been shown to restore the expression of the SCFA receptor G-protein-coupled receptor 43 (GPR43) in the gut [173], whose stimulation suppresses insulin-dependent fat accumulation in adipose tissue [174] and promotes GLP-1 secretion in L-cells [175,176].

Many studies have reported that probiotics reduce inflammatory status by decreasing proinflammatory cytokines IL-17, IL-6, IL-1α and IL-1β [115,144,173,177–179] and increasing anti-inflammatory cytokines IL-4 and IL-10 [177,179,180]. In addition, probiotics promote a decrease in proinflammatory cytokine TNF-α [115,144,173,179,180], which is known to mediate the insulin resistance associated with obesity in animal models [181,182]. In line with this, it has been reported that GLP-1 attenuates macrophage infiltration and improves inflammatory macrophage-derived insulin resistance by inhibiting the NF-κB pathway and the secretion of inflammatory cytokines in macrophages, including TNF-β, IL-6 and IL-1β [183]. Moreover, GLP-1 also improves glucose tolerance by stimulating insulin production and inhibiting glucagon production in the pancreas [184].

In the gut, probiotics increase tight junction proteins [179], reduce bacterial translocation [115] and restore the abundance of *A. muciniphila* [173]. They also inhibit differentiation into helper T17 (Th17) cells, reduce the proinflammatory cytokine IL-17 [179] and inhibit NF-κB activation expression in the colon, as well as decreasing LPS levels in both blood and colonic fluid [179]. In fact, either LPS or free fatty acids stimulate NF-κB signaling and the production of inflammatory cytokines in adipocytes, such as TNF-α and IL-6, which in turn leads to insulin resistance [185]. Probiotics increase intestinal Reg I proteins [186], which act as a regulator of cell growth needed to generate and maintain the villous structure of the small intestine [187]. As regards Treg cells, SCFA from bacterial fermentation regulate their size and function in the colon in a GPR43-dependent way [188].

In the adipose tissue, probiotic treatment decreases and increases the number of macrophages and anti-inflammatory Treg cells, respectively [173,189]. Linked to this, probiotics reduce adipose tissue and MCP-1 serum levels [177,178], whose expression contributes to the macrophage infiltration into this tissue and insulin resistance [190]. Moreover, probiotics also stimulate adiponectin secretion and the subsequent activation of AMPK in the adipose tissue and skeletal muscle, which induces glucose uptake and fatty acid oxidation [80,191].

In the liver, probiotics act to improve insulin resistance by increasing natural killer T (NKT) cells, decreasing TNF-α and the inhibitor of nuclear factor kappa-β kinase (IKK-β), and increasing IL-4 [180]. Here, it has been shown that IKK-β and NF-κB activation in the liver leads to hepatic inflammation, which develops insulin resistance both locally in the liver and systemically [192]. In this organ, probiotics act at the metabolic level by up-regulating fatty acid oxidation genes and down-regulating gluconeogenic genes [80,178]. Finally, several studies have reported that probiotics reduce the serum level of cholesterol and LDL [191,193,194]. In particular, one of these studies has shown that probiotics increase the expression of sterol regulatory element-binding protein 2 (SREBP2) and LDL (low-density lipoprotein) receptor (LDLR), which in turn leads to a decrease in serum cholesterol levels [194]. SREBP2 is a key transcription factor for LDLR gene expression [195], while LDLR is necessary for the cellular uptake of circulating cholesterol [196].

5.4. Probiotics in humans

In general, studies in humans are based on random double-blind placebo procedures and have reported that the consumption of probiotics also leads to weight loss and, therefore, to a decrease in the grade of obesity by modifying the gut microbiota [197–199]. However, these studies are less conclusive with respect to the changes in the characteristic markers of metabolic disorders compared with experimentation in animal models (Table 5).

In addition, one study showed that weight loss was not significant in all the participants, although it was significant in women when analyzing both sexes separately [200], and that it was associated with a significant reduction in circulating leptin level, mainly in women. In terms of inflammatory status, the use of probiotics in humans has been shown to prevent the absorption of LPS into the bloodstream, which reduces endotoxemia and improves metabolic disorders [201], as evidenced by the decrease in gamma-glutamyl transferase, an important predictor for developing MetS [202,203], and CRP, which impairs the insulin signaling pathway that promotes glucose transport, which in turn leads to insulin resistance [204].

Although we have highlighted the positive effects of probiotics, there are studies that show either no effects or negative effects of the consumption of probiotics. For example, several clinical trials testing the use of the *Lactobacillus* species have not demonstrated any effects on metabolic markers. In the study by Lewis et al., although the ingestion of *L. acidophilus* seems to reduce cholesterol levels, no changes were observed in serum lipids and anthropomorphic measurements [205]. Moreover, other studies in obese or MetS patients show no effects on metabolic markers after the ingestion of *L. casei* Shirota and *L. paracasei* F19, respectively [206,207].

In summary, both prebiotics and probiotics appear to exert healthy effects through SCFA production, mainly via three mechanisms (Fig. 1):

1) Improvement in obesity through the gut–brain axis; reducing food intake, body weight and adiposity; and improving most of the obesity-related disorders by modulating the production and secretion of intestinal hormones (up-regulating GLP-1, PYY and cholecystokinin and down-regulating ghrelin), which regulate orexigenic genes (AgRP and NpY) and anorexigenic genes (POMC) in the hypothalamus. Moreover, prebiotics and probiotics act by increasing fat oxidation, decreasing hepatic lipogenesis and serum triacylglycerol concentration and suppressing fat accumulation in adipose tissue. In addition, antidiabetic mechanisms have also been shown, due to GLP-1 action, which lead to an improvement in glucose tolerance (stimulating insulin production and inhibiting glucagon production in the pancreas), fasting blood glucose, glucose-stimulated insulin secretion and insulin-sensitive hepatic glucose production.
2) Improvement in gut barrier integrity by increasing the intestinotrophic GLP-2 level in the gut, and generating and maintaining the villous structure of the small intestine by increasing intestinal Reg I proteins.
3) Improvement in inflammation and a reduction in bacterial translocation, as a consequence of improved intestinal permeability, leading to a reduced inflammation level by decreasing the levels of proinflammatory cytokines, thus attenuating macrophage infiltration through GLP-1.

5.5. Studies showing synbiotic treatments

Growing evidence supports the idea that symbiotic treatments (prebiotics plus probiotics) allow us to obtain better results for human health. For this reason, numerous studies have emerged in recent years based on the combined use of prebiotics and probiotics. For example, Stewart et al. determined *in vitro* whether *L. reuteri* supplementation could improve the fermentation of dextrin, inulin

Table 5
Probiotics in humans

Prebiotic	Time	*N*	Results	Interpretation	Ref.
B. breve B-3.	12 weeks.	52 adults with a tendency for obesity.	Probiotics lowered fat mass and improved some blood parameters related to liver functions and inflammation, such as γ-glutamyltranspeptidase and high-sensitivity C-reactive protein.	These results suggest the beneficial potential of B. breve B-3 in improving metabolic disorders.	[197]
Lactobacillus.	6 months.	41 patients undergoing Roux-en-Y gastric bypass.	Probiotics improved bacterial overgrowth, vitamin B12 availability, and weight loss after Roux-en-Y gastric bypass.	Probiotic administration improves bacterial overgrowth, vitamin B12 availability, and weight loss after Roux-en-Y gastric bypass. These data may provide further evidence that altering the gastrointestinal microbiota can influence weight loss.	[198]
Yogurt, enriched by *L. acidophilus* La5, *Bifidobacterium* BB12, and *L. casei* DN001 108.	8 weeks.	75 healthy overweight and obese subjects.	-Low-calorie diet and probiotic yogurt had synergistic effects on T-cells subset specific gene expression in peripheral blood mononuclear cells, fat and body weight among overweight and obese subjects. -Low-calorie diet with probiotic yogurt led to higher reduction in body mass index, fat percentage and leptin level. -Probiotic yogurt without a low-calorie diet led to a more evident reduction in serum levels of hs-CRP.	These results suggest that the weight-loss diet and probiotic yogurt had synergistic effects on T-cells subset specific gene expression in PBMCs, fat percentage and body weight among overweight and obese individuals.	[199]
L. rhamnosus CGMCC1.3724.	24 weeks.	125 healthy overweight men and women.	-Probiotics reduced the *Subdoligranulum* genus in females. -Probiotics induced weight loss in women associated not only with reductions in fat mass and circulating leptin but also with the relative abundance of *Lachnospiraceae*. -Probiotic decreased fasting leptin concentrations in the population, including males and females. This effect was mainly driven by an important effect in females.	The present study shows that the *Lactobacillus rhamnosus* CGMCC1.3724 formulation helps obese women to achieve sustainable weight loss.	[200]
S. thermophilus (KCTC 11870BP), *L. plantarum* (KCTC 10782BP), *L. acidophilus* (KCTC 11906BP), *L. rhamnosus* (KCTC 12202BP), *B. lactis* (KCTC 11904BP), *B. longum* (KCTC 12200BP), and *B. breve* (KCTC 12201BP).	8 weeks.	50 female subjects.	Modulation of gut microbiota by probiotic administration might increase the level of gram negative bacteria of obese subjects and prevent LPS absorption into blood circulation, which reduces endotoxemia and improves metabolic disorders.	Correlations between gut microbiota and change in body composition indicate that probiotics may influence energy metabolism in obesity. Correlation between endotoxin level and weight reduction indicates that probiotics may play an important role in prevention of endotoxin production, which can lead to gut microbiota dysbiosis associated with obesity.	[201]

and wheat psyllium and concluded that it was possible to improve inulin fermentation but not that of wheat dextrin or psyllium [208]. In a study in rats, metabolic endotoxemia, gut inflammation, F/B ratio and *Enterobaceriaceae* content were equally reduced by the consumption of the prebiotic xylooligosaccharides, probiotic *L. paracasei* HII01 and synbiotics, while an increase in the *Bifidobacterium* level alone was found with prebiotic treatment [209]. A study in humans which tested prebiotic galactooligosaccharides and the probiotic strains *B. adolescentis* IVS-1 and *B. lactis* BB-12 on their own or as synbiotic combinations observed improvements in colonic permeability but no synergistic effects [210]. Based on these cases, it is logical to think that the synergistic effect of synbiotics depends in each case on the type of prebiotic and probiotic used. Therefore, rather than using synbiotics systematically, each individual case should be analyzed.

6. Conclusions

In conclusion, the main alterations in the gut microbiota of individuals with MetS are an increased *Firmicutes*/*Bacteriodetes* ratio and a reduced capacity to degrade carbohydrates to short-chain fatty

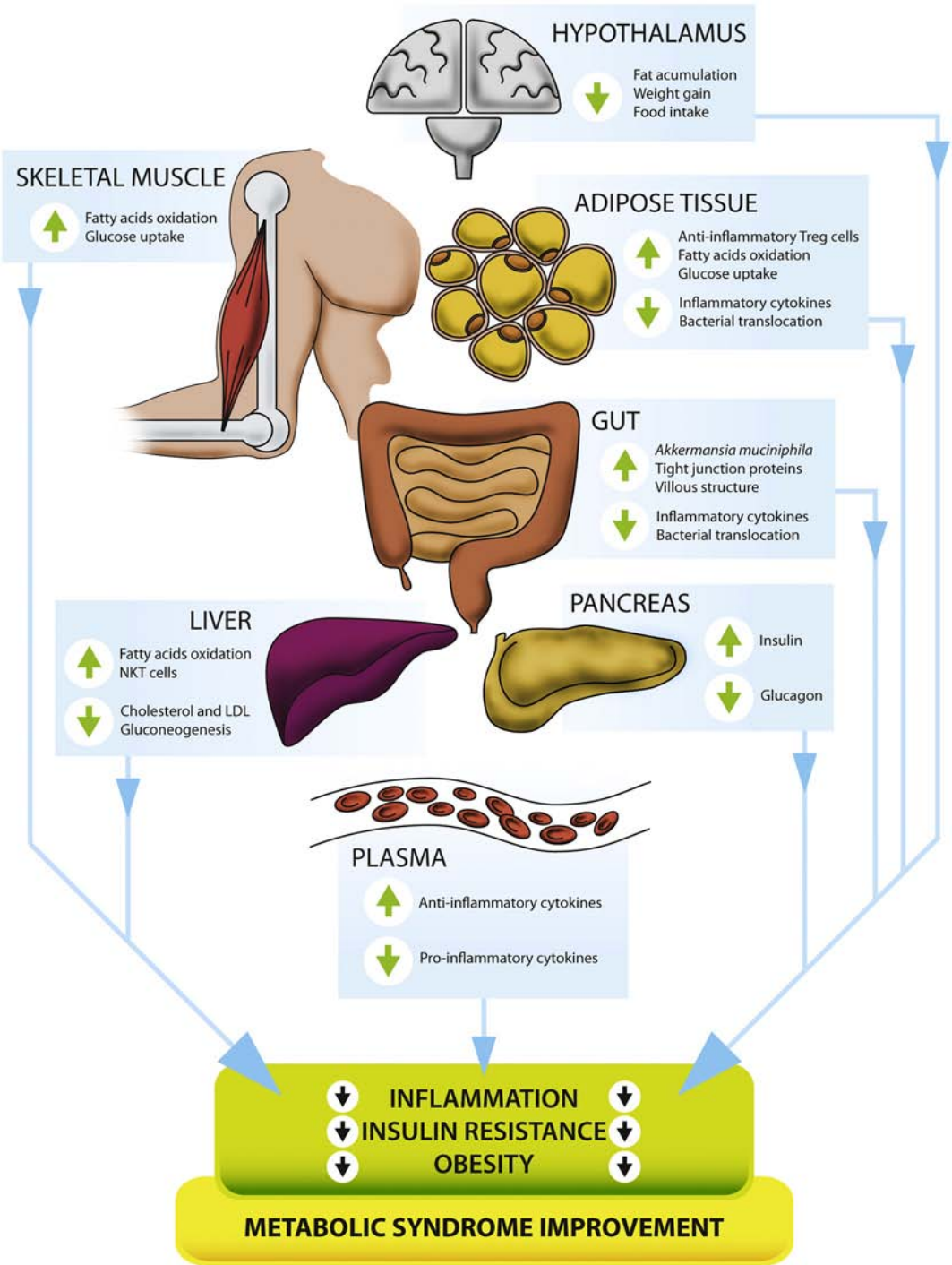

Fig. 2. Overview of the microbiome-related effects of nutritional strategies at systemic level. At present, the nutritional strategies act in different tissues and organs and have been shown to be able to decrease obesity, inflammation and insulin resistance, leading to an improvement of the metabolic syndrome.

acids, which are related with the grade of metabolic dysfunction of the host organism rather than with obesity itself. In addition to a low-fat, high-carbohydrate diet and a high fiber intake, a diet with higher fat content such as the Mediterranean diet, which has a high content in fruit and vegetables, is beneficial and partially restores the dysbiosis found in individuals with MetS. Overall, prebiotics and probiotics increase short-chain fatty acid production and therefore constitute a valid alternative for the MetS treatment by shaping the gut microbiota (Fig. 2).

However, the findings about the role of diet and intestinal microbiota in the development of metabolic syndrome may not be generalizable because of differences between populations, such as genetic variability and dietary background, as well as different methodologies, such as different macro- and micronutrients in diets and different doses of prebiotics and probiotics, among other factors. Therefore, further research is needed to extend this knowledge that may pave the way for novel diagnostics and therapeutics based on microbiota.

Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

Acknowledgments

The CIBEROBN is an initiative of the Instituto de Salud Carlos III (ISCIII), Madrid, Spain. Antonio Camargo is supported by an ISCIII research contract (Programa Miguel-Servet CP14/00114). We would like to thank David Hidalgo Cruz for providing technical support on imaging preparation.

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