Universidad de Oviedo
Programa de Doctorado en Biotecnología Alimentaria

Caracterización funcional de la microbiota intestinal en algunos trastornos inmunológicos

Arancha Hevia González

Tesis Doctoral

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

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RESUMEN (en español)

La microbiota intestinal es el conjunto de microorganismos que habita el intestino humano de manera natural, estableciendo una relación estrecha con el hospedador que la alberga, al que le proporciona una serie de beneficios entre los que cabe destacar la función barrera contra bacterias potencialmente patógenas, el aprovechamiento de energía de alimentos no digeribles, y la maduración y desarrollo del sistema inmune.

Hasta el momento, tanto estudios en humanos como en animales han demostrado que cambios en las poblaciones microbianas intestinales pueden tener consecuencias para la salud, y existen numerosos trabajos científicos que relacionan a una microbiota intestinal aberrante, o disbiosis intestinal, con procesos inflamatorios, alergias, enfermedades autoinmunes e incluso cáncer.

El objetivo general de esta Tesis Doctoral es generar nuevo conocimiento sobre la microbiota intestinal en el marco de dos trastornos inmunes, el lupus eritematoso sistémico (LES) y el asma. A través del uso de técnicas independientes de cultivo, se analizaron muestras fecales y se determinó el perfil microbiano de grupos de individuos que padecían estas enfermedades, comparándolo con el de individuos sanos. De esta manera los resultados de esta Tesis Doctoral han contribuido a entender la relación entre la disbiosis intestinal que presenta la microbiota de estos individuos y su estado inmunológico.

Para el caso del LES se estudiaron 20 mujeres de entre 34 y 68 años de edad, y se compararon con un grupo control sano de 20 individuos. En alergia se analizaron muestras de 21 pacientes con asma alérgico de edades entre 24 y 57 años y se compararon con un grupo de 22 controles sanos de mismo sexo y rango de edad.

Durante la primera parte de esta tesis se optimizaron metodologías y protocolos de tratamiento de muestras fecales humanas, para separar la comunidad microbiana presente en las mismas del resto de componentes sin alterar significativamente su composición microbiológica. Posteriormente se extrajo el ADN de muestras fecales de los distintos individuos para establecer los perfiles de composición bacterianos de cada grupo (LES y alergia respiratoria) mediante la amplificación del gen del ARN ribosomal 16S bacteriano con

la plataforma de secuenciación Ion Torrent.

En el grupo de LES se obtuvieron niveles diferentes de los dos grupos bacterianos mayoritarios que colonizan el intestino, *Bacteroidetes* y *Firmicutes*, con una ratio Firmicutes/Bacteroidetes mucho menor que para los controles sanos. Por otro lado se realizó la cuantificación de metabolitos producidos por la microbiota intestinal de LES, en comparación con el grupo control, mediante metabolómica, observándose un pequeño grupo de compuestos relacionados con estrés oxidativo acumulados en la microbiota de LES. También se observaron diferencias entre individuos sanos de alto y bajo índice de masa corporal, factor que no fue determinante en el caso del LES.

Por último y mediante experimentos *in vitro*, se observó que la microbiota aislada de LES induce una respuesta Th17, y que la suplementación de la microbiota de LES con *Bifidobacterium bifidum* o con las especies comensales *Ruminococcus obeum* y *Blautia coccoides* , corrigió en cierta medida el desequilibrio inmunológico, bien reduciendo la diferenciación de células T hacia Thelper en el caso de la bifidobacteria, o bien a través de una reducción dosis-dependiente del ratio IL-17/IFNɣ en el caso de la mezcla de Firmicutes, lo cual reduce la diferenciación de células T vírgenes a células Th1.

Por otro lado, en el grupo de alérgicos no hallamos diferencias significativas en la biodiversidad microbiana en comparación con un grupo de controles sanos, ni tampoco en las abundancias relativas de ningún grupo taxonómico salvo para la especie *Bifidobacterium adolescentis*, para la que se observaron niveles menores en individuos alérgicos.

RESUMEN (en Inglés)

Gut microbiota is the global community of microorganisms that naturally inhabit the human gastrointestinal tract, establishing tight interactions with their host and bringing it a great number of benefits. It contributes to the barrier function against potential bacterial pathogens, energy harvesting from non-digestible foods, and the development and maturation of the immune system.

Nowadays, human and animal model studies have shown some changes in the gut bacterial populations that can have important consequences in health, and there is a broad range of scientific work relating an imbalanced intestinal microbiota, or gut dysbiosis, with inflammatory processes, allergy, autoimmune diseases or even cancer.

The general scope of this Doctoral Thesis is the generation of novel knowledge about gut microbiota in the frame of two immune disorders, systemic lupus erythematosus (SLE), and allergy. By means of culture-independent techniques, fecal samples from the individuals of both groups are analyzed and compared with healthy controls. In this way with this Thesis we will contribute to understand the relationship between gut disbiosis and the immunological status of the host.

In the SLE group we analysed a group of 20 women of 34-68 years old, and they were

compared with a control group of 20 healthy women. For the allergy group, the samples of 21 patients, men and women, between 24 and 57 years old were analyzed and compared with a control group of 22 healthy individuals of the same sex and age.

First of all we optimized the methodologies and protocols to analyse the fecal microbiota in SLE and allergy. We set up a protocol to separate the fecal microbiota from its complex matrix without altering its bacterial composition significantly, and we also extracted the DNA from the samples to establish the bacterial profiles of each population group, through the amplification of the 16S rRNA gene with the Ion Torrent platform.

In the SLE group we obtained different bacterial abundances in the Fila *Bacteroidetes* (B) and *Firmicutes* (F), and a decreased F/B ratio compared to the healthy controls. We also quantified metabolites produced by the microbiota of SLE and we observed higher levels of a small group of metabolites that are related to the oxidative stress different; we also identified some differences in metabolites produced by healthy controls with high and low body mass index, suggesting that in SLE, the autoimmune disease is the most relevant factor controlling gut microbiota metabolism, but in a healthy population the BMI has some effect.

We also found, in co-culture *in vitro* experiments, that the isolated microbiota of SLE induces a Th17 immune response, and that the supplementation with *Bifidobacterium bifidum* and with the commensal bacteria *Ruminococcus obeum* y *Blautia coccoides* can revert the disbiosis and this can be traduced in a partially revert the immunological status of SLE, by reducing T cell differentiation to Thelper, or inducing a dose-dependent decrease in the IL-17/IFNɣ proinflammatory ratio.

In the allergy group we found no statistically significant differences in the biodiversity of the gut microbiota compared with controls, neither in the relative abundances of any genera. However, we observed decreased levels of *Bifidobacterium adolescentis* in individuals with long-term asma.

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LISTA DE ABREVIATURAS

ADN: Ácido desoxirribonucleico

TGI: Tracto Gastrointestinal

AGCCs: Ácidos grasos de cadena corta

ARN: Ácido ribonucleico

GALT: Gut-associated lymphoid tissue (tejido linfoide asociado al intestino)

TLR: Toll-like Receptor

SNP: Single Nucleotide Polimorfism

HLA: Human leukocyte antigen (Antígenos Leucocitarios Humanos)

MHC: Major Histocompatibility Complex (Complejo Mayor de Histocompatibilidad)

NGS: Next Generation Sequencing

UPLC-MS: Ultra Performance Liquid Chromatography-Mass Spectrometry (Cromatrografía Líquida de Alto Rendimiento acoplado a detección por Espectrometría de Masas)

HMDB: Human Metabolome Database (Base de Datos del Metaboloma Humano)

PAMs: Péptidos Antimicrobianos

LP: *lamia propria*

MAMPs: Microbial-associated Molecualr Patterns (patrones moleculares asociados a microorganismos)

CD: Células Dendríticas

PRRs: Pattern recognition receptors (receptores de reconocimiento de patrones)

NLRs: NOD-like receptors (receptores tipo Nod)

CLRs: C-Lectin Receptors (lectinas de tipo C como DC-SIGN)

RLRs: RIG-I-like Receptors (receptors tipo RIG-I)

T_h: T helper cell (linfocito T helper)

T_{reg}: T regulator cell (linfocito T regulador)

SFB: bacterias filamentosas segmentadas

PSA: polisacárido capsular A

IL: interleuquina

Ig: Inmunoglobulina

EII: Enfermedad Inflamatoria Intestinal

EC: Enfermedad de Crohn

CU: Colitis Ulcerosa

SII: Síndrome del Intestino Irritable

DT1: Diabetes Mellitus Tipo 1

AR: Artritis Reumatoide

LES: Lupus Eritematoso Sistémico

CRC: Cáncer Colo-rectal

EBV: virus de Epstein–Barr

CMV: Citomegalovirus

HBV: Virus de la Hepatitis B

LPS: Lipopolisacárido

LTA: Ácido lipoteicoico

PCR: Reacción en cadena de la polimerasa

F/B: Ratio *Firmicutes/Bacteroidetes*

TNF: factor de necrosis tumoral

IFN: Interferón

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RESUMEN

SUMMARY

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INTRODUCCIÓN

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1.1. La microbiota intestinal.

El tracto gastrointestinal (TGI) de los animales alberga en su interior un gran número de microorganismos diferentes que conforman lo que se conoce como microbiota intestinal: virus, bacterias, hongos, protozoos y arqueobacterias conviven interaccionando entre sí y con las células del hospedador en el que habitan. A lo largo de la evolución humana, la microbiota intestinal ha alcanzado una configuración que ha posibilitado una relación mutualista (1). Este mismo tipo de evolución puede observarse también en otros animales, como ciertos artrópodos y nematodos en los que la bacteria *Wolbachia* sp. ha pasado de ser un parásito a un microorganismo mutualista (2). En otros organismos como las plantas, podemos encontrarnos en su rizosfera diversas especies bacterianas, como *Rhizobium* sp., importantes en la fijación del nitrógeno atmosférico (3). Durante los últimos años, el desarrollo de técnicas de secuenciación masiva de ADN ha permitido dar a conocer el amplio panorama de especies que habitan el TGI así como su metabolismo potencial, dando pie a que muchos investigadores comiencen a considerar a la microbiota intestinal como un órgano más del ser humano. Estas mismas técnicas de secuenciación han puesto de manifiesto que la microbiota participa en la fisiología intestinal normal, y que alteraciones en la composición relativa de microorganismos pueden romper este estado de equilibrio u homeostasis intestinal, teniendo repercusiones en la salud humana (4).

A la microbiota comensal se le atribuyen diversas funciones entre las que se encuentran: i) ayudar a mantener la integridad de la barrera intestinal, ii) evitar la adhesión de especies patógenas oportunistas al epitelio intestinal, iii) funciones inmunes como la modulación de la inmunidad innata y la promoción y maduración de la inmunidad adaptativa, iv) la degradación de distintos polisacáridos de la mucosa, principalmente mucina, y v) funciones

metabólicas importantes como el aprovechamiento de energía de los alimentos, fermentación y absorción de carbohidratos de la dieta no digeribles, con la consiguiente liberación de metabolitos. Algunos de estos metabolitos no solo tienen propiedades beneficiosas sino que son esenciales, como los aminoácidos ramificados, ciertos ácidos grasos de cadena corta (AGCC) como el butirato, y la vitamina K.

La microbiota tiene un papel crucial en el desarrollo del sistema inmune tal y como se dedujo de los experimentos con ratones libres de microorganismos o "*germ-free*". Este tipo de ratones nacen y crecen sin microbiota intestinal, al ser criados en condiciones de esterilidad, y entre otras anomalías desarrollan un sistema inmune inmaduro y un TGI funcionalmente anómalo. La colonización posterior con bacterias comensales de estos ratones ha evidenciado la existencia de señales concretas derivadas de los microorganismos intestinales, que son críticas para el correcto desarrollo del sistema inmune (5, 6).

El estudio y manejo de la microbiota intestinal es hoy en día un campo de actualidad para los científicos situados en la intersección de los campos de la microbiología, la inmunidad y la salud. Durante los últimos años, se han desarrollado varias iniciativas internacionales en forma de grandes consorcios de investigación, entre ellas una en Europa llamado MetaHit (http://www.metahit.eu/), y otro estadounidense denominado *Human Microbiome Project* (http://hmpdacc.org/). Ambos han contribuido enormemente al salto cualitativo sobre el conocimiento de la microbiota intestinal, principalmente a través de la creación de un catálogo genético microbiano de referencia donde se compila todo el potencial metabólico de esta comunidad microbiana (7–10).

Si bien es cierto que a día de hoy no se ha podido definir qué es una microbiota normal en términos de composición, principalmente debido a la gran variabilidad interindividual entre adultos sanos, sí que existen indicios del proceso de configuración de este ecosistema en términos de abundancias relativas de sus poblaciones, siendo las comunidades bacterianas específicas en cada parte del cuerpo humano (figura 1) (11). En el caso de la microbiota intestinal, ésta varía con la alimentación y otros factores ambientales (12–14). Se estima que cada individuo alberga más de 100 especies microbianas en su intestino, y que esta variedad llega a superar las 1000 especies diferentes si consideramos toda la población humana. Más aún, los microorganismos intestinales superan diez veces el número de células eucariotas humanas nucleadas (15–17). El conjunto de genes de la microbiota intestinal, o microbioma, está constituido por un número de genes únicos 50 veces superior al contenido en el genoma humano (9); (http://gigadb.org/dataset/view/id/100064).

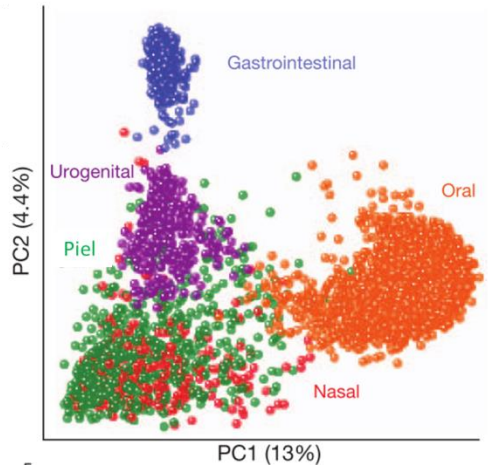

Figura 1. Perfiles de microbiota obtenidos por secuenciación masiva del gen que codifica el ARN ribosómico 16S, y que revela la diversidad bacteriana asociada a distintas localizaciones del cuerpo humano. Las distintas microbiotas se han agrupado en base a su diversidad utilizando un análisis de componentes principales. Cada color denota una localización del cuerpo humano. Adaptado de Huttenhower y cols. (18).

Se calcula que en el TGI la población bacteriana total es de 10^{13}-10^{14} células (17). El colon es el tramo más densamente poblado con una concentración de microorganismos de entre 10^{11}-10^{12} células por gramo de contenido intestinal, llegando a representar el 50-60% del peso seco de las heces (19, 20). En la microbiota intestinal las bacterias son predominantes (75%) y están representadas principalmente por especies de los filos *Firmicutes* y *Bacteroidetes,* y secundariamente por especies pertenecientes a los filos *Actinobacteria*, *Proteobacteria*, *Synergistetes*, *Fusobacteria* y *Verrucomicrobia* (21). Hongos y arqueobacterias componen un 0,05% y 1% de especies de la microbiota intestinal, respectivamente (22). El conjunto de virus intestinales o viroma intestinal está formado principalmente por bacteriofagos, y se estima que en número, èstos virus intestinales sobrepasarían en 100 veces el número de células humanas nucleadas (23).

La mayoría de *Firmicutes* presentes en el TGI pertenecen a la clase *Clostridia*, más concisamente a los grupos XIVa, IV y XVI. Dentro del filo *Firmicutes* también se encuentran especies de bacterias del ácido láctico pertenecientes a los géneros *Enterococcus* y *Lactobacillus*. A nivel de género los taxones bacterianos más abundantes son *Faecalibacterium, Roseburia*, *Blautia*, *Ruminococcus*, *Eubacterium* y especies de la familia *Lachnospiraceae*. Respecto al filo *Bacteroidetes*, sus miembros más abundantes son especies de los géneros *Bacteroides* y *Prevotella*. Del filo *Actinobacteria* podemos destacar especies de los géneros *Bifidobacterium* y *Collinsella.* Otra especie perteneciente a un filo minoratorio, pero igualmente importante en la fisiología normal del TGI, es *Akkermansia municiphila*, especializada en la degradación de mucina (filo *Verrucomicrobia*) (9, 24). En la figura 2 se

representa esquemáticamente esta distribución de grupos bacterianos más representativos a lo largo del TGI.

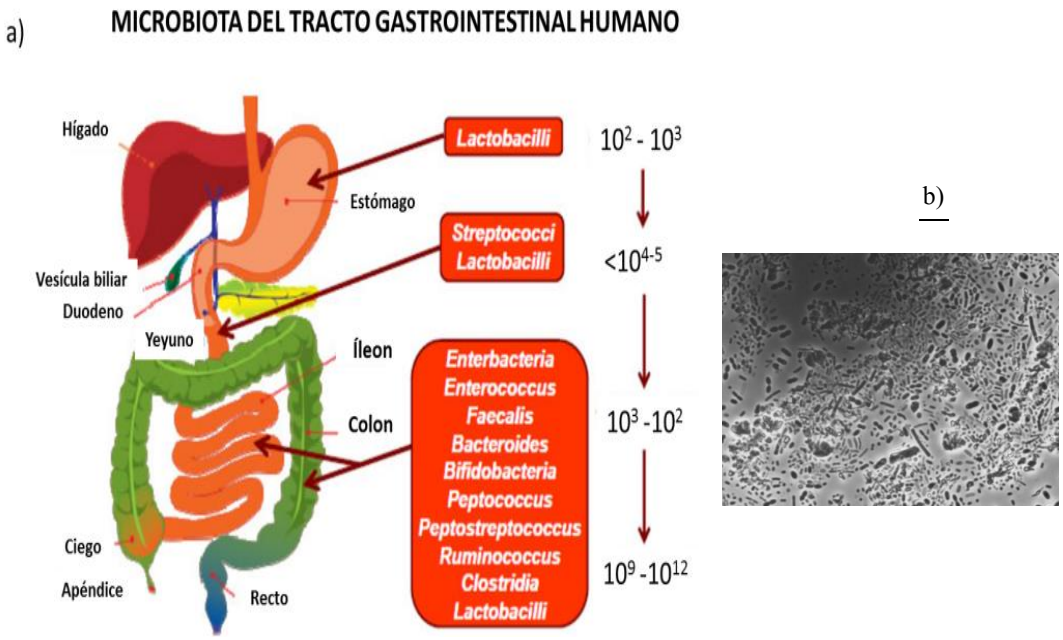

Figura 2. a) Representación esquemática de la composición microbiana a lo largo del TGI humano en el que se indican las bacterias más abundantes en cada zona y la concentración microbiana por gramo de contenido. Adaptado de Konturek y cols, *Journal of Physiology and Pharmacology*, 2015. b) Fotografía al microscopio óptico de la microbiota fecal de un individuo incluido en el estudio *Allergic Patients with Long-Term Asthma Display Low Levels of Bifidobacterium adolescentis,* PlosONE, 2016, el cual está incluido en el capítulo 3 de esta Tesis Doctoral.

1.1.1. Establecimiento de la microbiota intestinal.

El TGI es colonizado desde el mismo momento del nacimiento por una gran cantidad de microorganismos (25). La microbiota evoluciona en composición durante los primeros 2-3 años de vida para mantenerse estable durante el resto de la edad adulta en individuos sanos (26). Durante la vejez, la microbiota disminuye tanto en composición como en diversidad microbiana (27). A través de estudios en neonatos y en modelos animales con ratones "*germ-free*", se ha comprobado que la colonización del TGI a edades tempranas no solamente ayuda al desarrollo del epitelio intestinal y del sistema inmunitario asociado a mucosa intestinal (GALT), sino que previene el riesgo de padecer algunas enfermedades en la edad adulta como alergias o asma (28).

En el parto, un neonato se expone a la presencia de nuevos microorganismos que van a

colonizar gradualmente sus mucosas, entre ellas la del TGI. El establecimiento de la microbiota intestinal es un proceso natural pero complejo que comienza desde el momento mismo del nacimiento, y que se ve influenciado por diferentes factores que configuran una comunidad microbiana relativamente estable a los dos años de edad. Los primeros en establecerse son anaerobios facultativos como enterobacterias, lactobacilos y estreptococos. A los pocos días de vida se establecen microorganismos anaerobios como bifidobacterias, bacteroides, clostridios y eubacterias. Hacia los seis meses de vida, con la introducción de alimentos sólidos, la comunidad microbiana se vuelve más diversa y se incrementan rápidamente la proporción de especies de los géneros *Bacteroides*, *Clostridium* y otros anaerobios, mientras que la población relativa de bifidobacterias disminuye. En las partes superiores del TGI por el contrario, se establecen especies de *Streptococcus* que muestran tolerancia a concentraciones más altas de oxígeno, y que prácticamente se mantienen estables hasta después de los 8 años (Figura 3); (29).

Los factores más importantes que van a condicionar la colonización microbiana del TGI pueden dividirse en genéticos y ambientales. Entre los primeros podemos destacar variantes y mutaciones en los genes que codifican los receptores del sistema inmune innato, como los receptores Toll-like (TLRs), y entre los ambientales podemos citar (i) la microbiota materna, la alimentación de la madre, y su estilo de vida; (ii) la edad gestacional del niño; (iii) el tipo de nacimiento (parto natural o cesárea); (iv) el tipo de alimentación del neonato (leche materna o fórmula); (v) el estilo de vida y la localización geográfica y (vi) el uso de antibióticos, probióticos y prebióticos (30–32).

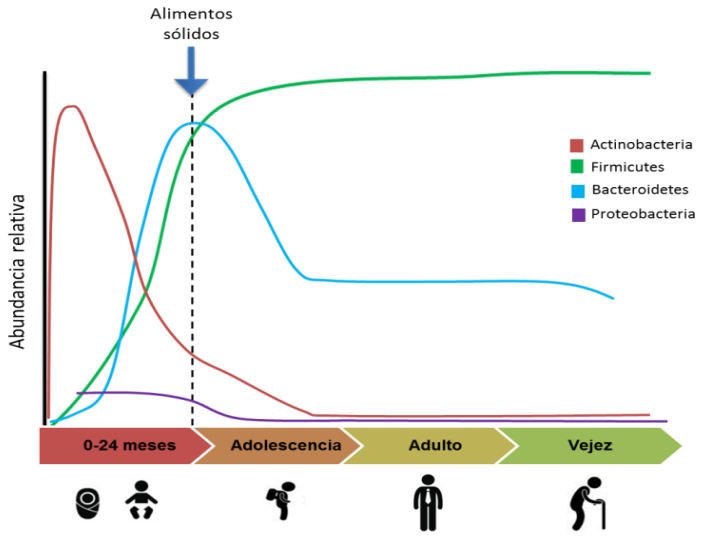

Figura 3. Establecimiento y evolución de la microbiota intestinal humana a lo largo de la vida. El gráfico muestra una visión global de la abundancia relativa de filos bacterianos clave de la composición de la microbiota.

A lo largo de la edad adulta la microbiota de cada individuo es dinámica y puede verse sometida a pequeñas fluctuaciones por factores como la dieta, la edad, y ciertos tratamientos como los antibióticos (13). A pesar de estas variaciones, cada individuo parece albergar un núcleo específico de especies asociadas o *core*, *i.e.* un conjunto de microorganismos que no varían a lo largo del tiempo, y que está formado principalmente por filotipos de microorganismos anaerobios pertenecientes a los géneros *Allistipes*, *Bifidobacterium*, *Bacteroides*, *Faecalibacterium*, *Blautia*, *Dorea* y *Ruminococcus*. (33). Se supone que este *core* de microorganismos tiene un papel fundamental para el mantenimiento de la homeostasis en el hospedador, de ahí su estabilidad.

1.1.2. Los enterotipos.

Se ha sugerido una posible clasificación de la microbiota de los seres humanos en tres grupos o enterotipos, definidos en función del grupo bacteriano más abundante y de otros grupos asociados en términos de abundancia (34). Aunque existe una significativa variabilidad inter-individual, Arumugan y colaboradores han propuesto que la mayoría de personas se podrían categorizar dentro de tres variantes de comunidades bacterianas dominadas por uno de estos tres géneros: *Bacteroides*, *Prevotella* o *Ruminococcus*. Estos grupos se han denominado enterotipos y se ha visto que la abundancia relativa de cada uno de estos tres géneros dominantes correlaciona significativamente, de forma positiva o negativa, con la de los otros dos. Una de las características de los enterotipos es que están fuertemente asociados a hábitos alimenticios característicos (34).

Se ha visto que cada uno de los tres géneros que definen los enterotipos correlaciona con otros géneros bacterianos de la microbiota, pudiendo establecerse una especie de red de coexistencia de distintos géneros para cada enterotipo. El enterotipo 1 lo define el género *Bacteroides*, asociándose también a la presencia de especies del género *Parabacteroides.* Los miembros de este enterotipo se caracterizan por ser eficientes en la obtención de energía a partir de carbohidratos no digeribles y de proteínas a través de su metabolismo fermentativo. Así, los genomas de estas bacterias contienen genes que codifican enzimas implicadas en la degradación de azúcares complejos y proteínas (galactosidasas, hexosaminidasas, proteasas, etc) (35). El enterotipo 2 se define por la presencia del género *Prevotella,* el cuál aparece asociado al género *Desulfovibrio*. Se piensa que ambos géneros pueden actuar sinérgicamente a la hora de degradar glicoproteínas como la mucina, presente en la capa de mucus intestinal. El género *Prevotella* es conocido por su capacidad de degradar glicoproteínas, mientras que *Desulfovibrio* puede aumentar el rendimiento de esta degradación al estar especializado en

uno de los pasos limitantes, la desulfatación de la mucina (36). El enterotipo 3 es el más frecuente entre la población europea, y se caracteriza por la presencia del género *Ruminococcus*, asociado a la presencia del género *Akkermansia*. Ambos son eficientes degradadores de mucina y poseen un elevado número de transportadores de azúcares, lo que sugiere eficiencia metabólica no solo en degradar sino también en captar e hidrolizar estos sustratos (37). Estas diferencias filogenéticas y funcionales entre enterotipos son el reflejo de tres combinaciones diferentes de poblaciones microbianas, que tendrían potencialmente un impacto diferente en la interacción con el hospedador.

A pesar de las potenciales utilidades de la clasificación de las poblaciones humanas en tres enterotipos microbianos, como son el desarrollo de tratamientos médicos personalizados, existe cierto debate sobre la validez de este modelo ya que, por ejemplo, los enterotipos no tienen en cuenta ni la variación temporal de la microbiota con la dieta, edad, sexo o grupo étnico. Por ejemplo Wu y colaboradores sólo encuentran dos enterotipos en su estudio, uno que combina *Ruminococcus* y *Bacteroides*, separado del enterotipo correspondiente a *Prevotella* por un continuo gradiente de abundancia de especies (38).

Otros autores dudan directamente de la existencia de los enterotipos por ser un método simplista, y sugieren que la composición de la microbiota sigue un gradiente continuo entre individuos, siendo poco posible agrupar a la microbiota en categorías teniendo como único criterio la dominancia de ciertos géneros (39). Por ejemplo, Huse y colaboradores detectaron una segregación mínima de la microbiota en 3 enterotipos, aunque sí describen 2 posibles clústeres que en todo caso no serían discretos, sino los extremos de un gradiente continuo de abundancia de especies (40). Actualmente, la evidencia científica sugiere una continua variación de las comunidades microbianas intestinales en complejas distribuciones multidimensionales, y por tanto la definición de modelos matemáticos capaces de simplificar esta complejidad en nuevos sistemas de clasificación (41).

1.1.3. Funciones de la microbiota intestinal y su papel en la salud humana.

La microbiota intestinal es una comunidad microbiana compleja que está en continua interacción con el hospedador, estableciéndose una relación muy estrecha entre ambos. Entre las principales funciones atribuidas a la microbiota intestinal se encuentran:

(i) Adhesión al epitelio intestinal limitando el contacto físico de enteropatógenos con el hospedador, ejerciendo una barrera protectora ante posibles infecciones de bacterias oportunistas presentes en el mismo nicho ecológico. A este efecto barrera también se le añade el hecho de que una gran parte de bacterias intestinales producen sustancias antimicrobianas como bacteriocinas y AGCC. Estos compuestos inhiben el crecimiento

de otros microorganismos, siendo un método de competencia directa que se une a la competencia por los nutrientes y otros recursos.

(ii) El microbioma intestinal codifica algunas enzimas e incluso vías bioquímicas no presentes en el hospedador. Este hecho dota de una función metabólica importante a la comunidad microbiana intestinal. Estas enzimas permiten el aprovechamiento de nutrientes y energía no procesados en las primeras etapas de la digestión de alimentos, con la consiguiente producción de metabolitos beneficiosos para el hospedador como AGCC (acetato, lactato, propionato y butirato mayoritariamente). También transforman químicamente algunos de ellos produciendo moléculas asimilables por el organismo, como es el caso de algunas vitaminas (K, cianocobalamina, biotina, ácido fólico, ácido pantoténico) y compuestos de baja biodisponibilidad (calcio, magnesio, hierro).

(iii) La microbiota intestinal contribuye al normal desarrollo del sistema inmune del hospedador desde el nacimiento a través de la interacción constante con las células de la mucosa del TGI. Esta interacción induce una especie de "educación" del sistema inmune, al verse favorecido el desarrollo de lo que se denomina tolerancia inmunológica hacia antígenos de la microbiota y de la dieta, mientras que se desarrolla una respuesta rápida frente a los patógenos.

(iv) De la misma forma que la microbiota intestinal tiene la capacidad de bioconversión sobre ciertos alimentos, lo mismo sucede con los fármacos. Por ejemplo, una de las moléculas prescritas en colitis ulcerosa, la sulfasalacina (ácido 5-aminosalicílico (5-ASA) unido a una molécula de sulfapiridina), no es activa hasta que llega al intestino distal. Para ser bioactiva, y ejercer su efecto antiinflamatorio, debe romperse el enlace N-N y liberarse el 5-ASA, lo cual es llevado a cabo por azoreductasas microbianas (42).

1.1.4. Factores que influyen en la microbiota intestinal.

Como se ha señalado anteriormente, existen dos tipos de factores responsables de conformar la composición y por tanto la función del microbioma humano: genéticos y ambientales (Figura 4).

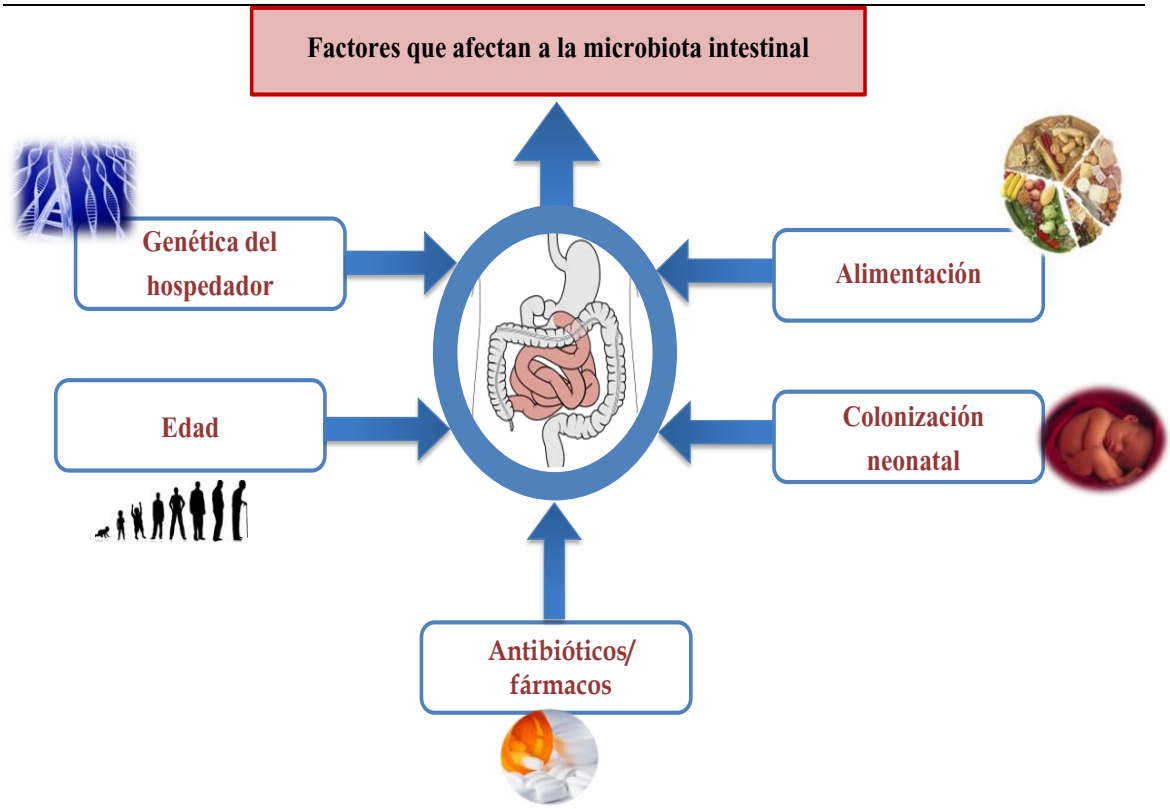

Figura 4. Factores más importantes que influyen sobre la composición y función de la microbiota intestinal. Se distinguen por un lado factores genéticos del hospedador y por otro factores ambientales. Éstos van a condicionar el diálogo directo con los microorganismos a nivel de mucosa intestinal a través, por ejemplo, de receptores moleculares presentes en las células del epitelio intestinal.

Mediante estudios de tipo "*genome-wide*" se han intentado correlacionar ciertos alelos y mutaciones en genes del hospedador con variaciones en el microbioma humano (43). Estudios realizados en gemelos homocigóticos han llegado a identificar genes individuales en los que un polimorfismo en un sólo nucleótido o SNP llega a afectar a la composición de la microbiota intestinal (44). Un ejemplo es el gen *mefV*, que codifica para la proteína pirina o marenostrina, y cuyas mutaciones están asociadas a una predisposición a la Fiebre Mediterránea Familiar. Individuos con esta mutación presentan una diversidad microbiana intestinal menor que la de individuos sin mutaciones en este gen (45).

Otros ejemplos de mutaciones que afectan la composición de la microbiota son los genes que codifican para anticuerpos secretados (principalmente el subtipo A), genes de los antígenos leucocitarios humanos (HLA) que codifican para las moléculas del complejo mayor de histocompatibilidad (MHC) y que son responsables del reconocimiento antigénico, genes

que codifican para citoquinas y defensinas y receptores del sistema inmune innato como los TLRs (44).

En un estudio reciente realizado por Goodrich y colaboradores, en el que se comparó la microbiota fecal de más de mil muestras obtenidas de pares de gemelos monocigóticos y dicigóticos, se evidenció que la microbiota intestinal era más similar entre los primeros (46). En este sentido se han identificado varios grupos taxonómicos microbianos cuyas abundancias relativas e incluso su transmisión a la descendencia se ven influenciados por la genética del hospedador (13, 47).

Por ejemplo se conoce que la familia *Christensenellaceae*, y la de otras especies microbianas como la arqueobacteria metanogénica *Methanobrevibacter smithii*, se transmiten a la descendencia siguiendo patrones parecidos a otros caracteres como el índice de masa corporal (IMC) (46). Otro estudio en el que se puso de manifiesto la importancia de los factores genéticos del hospedador sobre la microbiota intestinal se realizó en ratones a los que se eliminó de su genoma el gen *fut2*, que codifica el enzima α(1-2) fucosiltransferasa. Este enzima es responsable de la síntesis de carbohidratos que conforman el mucus que recubre al epitelio intestinal, el cual es utilizado por miembros de la microbiota como fuente de energía. Los ratones *fut2*$^{-}$*/fut2*$^{-}$ presentaron de por sí una menor α-diversidad de su microbiota medida como el número de especies únicas (índice de Chao) y comparada con la estirpe salvaje de ratones. A estos ratones *fut2*$^{-}$*/fut2*$^{-}$ se les eliminó la microbiota intestinal mediante un tratamiento con diferentes antibióticos, y posteriormente fueron colonizados con cultivos de *Bacteroides thetaiotaomicron;* se les administró además dos dietas diferentes, una con alto y otra con bajo contenido en polisacáridos. Se observó que en aquellos en dieta con bajo contenido en polisacáridos, *B. thetaiotaomicron* reprimía sus vías catabólicas, mientras sobre-expresaba un operón genético responsable de la degradación de β-galactosa. Por el contrario, no se observaron diferencias en la regulación genética de *B. thetaiotaomicron* cuando se administró la dieta con alto contenido en polisacáridos con respecto a las condiciones estándar. De aquí se concluye que no solo el fondo genético del hospedador sino la alimentación son factores clave de modificación de la función de la microbiota intestinal a través de su interacción mutua, determinando en último momento qué especies concretas se van a establecer en el TGI (48).

Entre los factores genéticos también podemos citar aquellos presentes en la propia microbiota. *Bacteroides fragilis* posee un locus genético denominado factor de colonización comensal, *fcc*, cuya delección conduce a cepas que muestran una capacidad de colonización reducida. Mientras que *B. fragilis* fcc^{+} puede penetrar en el mucus del colon y residir en las criptas, *B. fragilis* fcc^{-} es incapaz. Esto sugiere que *B. fragilis* ha evolucionado hasta desarrollar interacciones físicas con el hospedador especie-específicas que median en su

colonización, lo cual sugiere que *fcc* está implicado en la relación mutualista con el hospedador (49). Otros ejemplos de factores genéticos que condicionan la capacidad de colonización son los pili, apéndices proteicos presentes en la superficie de bifidobacterias y lactobacilos (50, 51).

1.1.4.1. Edad.

La microbiota intestinal sufre cambios desde el momento del nacimiento y se mantiene relativamente estable hasta la edad adulta, viéndose alterada de nuevo en la vejez (27). Muchos estudios han evidenciado los mismos cambios asociados a la senescencia, como es la presencia de altos niveles de *E. coli* y Bacteroidetes y una disminución del ratio Firmicutes/Bacteroidetes (52). El estudio del consorcio ELDERMET (http://eldermet.ucc.ie/) dio a conocer cómo se comporta la microbiota intestinal en individuos de avanzada edad, evidenciándose una reducción general en la biodiversidad microbiana con respecto a la edad adulta, encontrándose particularmente afectadas las proporciones relativas de las especies pertenecientes a los géneros *Bacteroides* y *Clostridium* (53).

1.1.4.2. Alimentación

Como acabamos de ver uno de los factores con más influencia sobre la microbiota intestinal son los hábitos alimenticios del hospedador. Los patrones de alimentación se asocian con especies que predominan dentro de la composición microbiana, como son las pertenecientes a los géneros *Bacteroides*, *Prevotella*, y *Ruminococcus*, y que a su vez delimitan los enterotipos. Como se explicó anteriormente estos enterotipos están asociados con dietas diferentes: el enterotipo *Bacteroides* se asocia con una dieta con alto aporte de grasas y proteínas animales y baja en fibra, y con obtención de energía principalmente de carbohidratos y proteínas a través de fermentación y proteólisis; el enterotipo *Prevotella* se correlaciona con una alimentación rica en carbohidratos y en su microbiota están presentes especies capaces de romper grupos fosfato y degradar glicoproteínas de la capa de mucina que recubre el intestino; por último el enterotipo *Ruminococcus* está dominado por especies eficientes en metabolizar y degradar mucinas. Se ha visto que en adultos que cambian su alimentación de una dieta rica en grasa y deficiente en fibra, a una baja en grasas y rica en fibra, se producen cambios en su microbiota a las 24 horas del cambio (54).

Un estudio reciente sobre el impacto de diferentes tipos de dieta en la microbiota sugiere clasificar a los individuos en dos grupos de forma similar a lo que sucede en estudios de farmacología: i) los respondedores, que presentan una menor diversidad microbiana en su TGI y cuya microbiota cambia rápidamente ante cambios en su alimentación, y ii) los no respondedores, cuya microbiota es más estable a cambios en la dieta y se caracteriza por

mayor diversidad (55). De todas formas, los mismos autores recuerdan que la alta variabilidad inter-individual prevalece a la influencia de la dieta sobre la microbiota intestinal (55).

El efecto directo de la alimentación sobre la microbiota se ha estudiado exhaustivamente en ratones gnotobióticos expuestos a dietas bien definidas; estos ratones son aquellos en los que se conoce exactamente la composición de especies que conforman su microbiota intestinal. Por ejemplo, se ha visto que dietas ricas en polisacáridos vegetales estimulan el crecimiento de especies del filo *Bacteroidetes*, que como se ha mencionado anteriormente se caracterizan por tener genomas en los que abundan genes que codifican enzimas para la degradación de polisacáridos (48). En presencia de Bacteroidetes degradadores de polisacáridos, las bacterias de los filos *Firmicutes* y *Actinobacteria* activan genes que codifican transportadores de aminoácidos y azúcares, llevando a cabo procesos fermentativos intracelulares que conducen a la liberación de AGCC, como el ácido acético (56). Otras especies del filo *Firmicutes* utilizan este ácido acético para obtener energía y producir butirato, un AGCC muy importante para la fisiología del TGI, notablemente a nivel de colonocitos (57). El ácido fórmico, producido por *Bacteroides thetaiotaomicron* en dietas ricas en fructanos, es utilizado por la arqueobacteria *Methanobrevibacter smithii* con una liberación consiguiente de metano (58).

1.1.4.3. Demográficos.

La localización geográfica en la que habita un individuo, así como su raza, son factores clave que afectan tanto a la diversidad como a la composición general de la microbiota intestinal. En estudios comparativos entre poblaciones de zonas rurales de Japón y de zonas urbanas canadienses (59), o entre individuos de áreas rurales de Sudáfrica y británicos (60), se ha visto que hay diferencias significativas en sub-comunidades de la microbiota intestinal. Estudios recientes han evidenciado que la microbiota intestinal es diferente entre individuos europeos y de ciertas regiones rurales de África (61) y también entre norteamericanos y asiáticos (62). Sin embargo, queda por esclarecerse qué parte de las diferencias en microbiota son debidas a la localización geográfica, y que parte es debida a los hábitos alimenticios y alimentarios. Estos hábitos podrían ser la causa de la mayor tasa de incidencia de cáncer colorrectal en individuos afroamericanos con respecto a individuos africanos (63).

1.1.4.4. Antibióticos y otros fármacos.

Las terapias con antibióticos dirigidas contra patógenos intestinales, afectan no solo a estos sino a toda la microbiota intestinal (y de otras partes del cuerpo) (64). Se ha visto que la microbiota no se restablece en cortos periodos de tiempo tras un tratamiento con antibióticos,

y que la dosis administrada influye en la magnitud del impacto que se genera sobre la microbiota (65). Un efecto de la administración de antibióticos es la proliferación de patógenos oportunistas como *Clostridium difficile* o *Candida albicans* (66). A esto hay que añadir que su uso favorece la aparición de resistencias a antibióticos, lo cual genera un reservorio de genes de resistencia entre los miembros de este ecosistema que pueden ser genéticamente movilizables y por tanto transmisibles (67, 68). Las alteraciones en las proporciones relativas de la comunidad microbiana intestinal se conocen como disbiosis, y pueden conllevar la desregulación del sistema inmune del hospedador e incrementar la susceptibilidad a ciertas patologías. En este sentido, los antibióticos inducen cambios importantes para la inmunidad innata a través de la eliminación de grupos microbianos clave, cuyos ligandos son reconocidos por receptores específicos y que son imprescindibles para el mantenimiento de la función intestinal.

1.1.4.5. Otros factores

Otros factores que han demostrado ejercer influencia sobre la composición de la microbiota intestinal son el estrés o hábitos como el ejercicio físico, el consumo de alcohol, o el tabaquismo. Las bacterias intestinales son sensibles a mediadores del estrés como las hormonas secretadas en el eje hipotálamo-pituitaria-adrenal, y se sabe que responden a los neurotransmisores liberados por el hospedador (69). Los microorganismos intestinales también liberan sustancias neuroactivas que afectan a las características fisiológicas del hospedador, como el ácido gamma amino butírico o el triptófano (70). En cuanto al ejercicio físico, se ha constatado que influye en la microbiota según la intensidad con que se practique, aunque hay que ser prudente a la hora de extraer conclusiones ya que la práctica del ejercicio lleva asociada unos hábitos alimenticios distintos de los de individuos sedentarios, por lo que éstos también podrían influir sobre la composición de la microbiota (71).

1.1.5. Métodos de estudio de la microbiota intestinal.

Aunque los microorganismos del TGI fueron unos de los primeros observados al microscopio por Antony Van Leeuwenhoek (72), hoy en día es imposible cultivar muchos de ellos bajo condiciones de laboratorio (73). Durante los últimos 10 años, el desarrollo de las técnicas de identificación basadas en la secuenciación masiva del ADN ha permitido expandir nuestro conocimiento de la microbiota intestinal, expansión que ha sido limitada precisamente por nuestra incapacidad de crecer en condiciones experimentales muchos de estos microorganismos. Para llevar a cabo el estudio del microbioma humano se parte de muestras de heces o de biopsias de mucosa intestinal de los individuos. Para la caracterización de la

composición microbiana, el primer paso es sembrar las muestras en medios de cultivo selectivos o bien purificar su ADN, dependiendo de si para la aproximación experimental elegida se van a utilizar técnicas dependientes o independientes de cultivo, respectivamente.

1.1.5.1. Técnicas dependientes de cultivo.

Son las técnicas de microbiología clásicas y permiten el aislamiento y estudio de entre un 10%-25% de la microbiota total presente en heces (74). Esta limitación es debida principalmente a que la mayoría de especies bacterianas intestinales son exigentes nutricionalmente y además anaerobias estrictas, sobreviviendo con dificultad a la manipulación propia de un crecimiento sobre placa Petri. Las mejoras de las técnicas para cultivar microorganismos anaerobios han permitido caracterizar algunos de los géneros dominantes de la microbiota intestinal, como son *Bacteroides*, *Clostridium*, o *Bifidobacterium*, pero actualmente aún no se cuenta con las herramientas adecuadas para poder cultivar muchos de los microorganismos intestinales.

Algunos autores han sugerido la necesidad de desarrollar nuevos métodos de cultivo para poder identificar nuevas especies. La culturómica, como estos autores denominan a esta metodología, es una aproximación experimental que persigue el aislamiento de microorganismos a gran escala a través de la combinación de múltiples condiciones experimentales y medios de cultivo (75). Tiene como fin capturar la biodiversidad microbiana de forma viable y funcional a través del diseño de un gran número de medios de cultivo que combinen diferentes atmósferas, temperaturas, pH, nutrientes, minerales, antibióticos o incluso fagos.

1.1.5.2. Técnicas independientes de cultivo.

Las denominadas técnicas ómicas son un conjunto de técnicas que se han desarrollado o bien aplicado en los últimos años al estudio concreto de la microbiota humana, y gracias a las cuales se ha obtenido una visión más amplia y profunda de las funciones que la microbiota desempeña en el hospedador (figura 6). A continuación se resume cada una de estas técnicas ómicas.

(i) Metagenómica.

Con la llegada de las tecnologías de alto poder de procesamiento de secuenciación de ADN (*Next Generation Sequencing*, NGS) el estudio de una comunidad microbiana compleja puede abordarse de forma independiente de cultivo en tres etapas principales: (i) obtención del material genético de la muestra, (ii) la amplificación con cebadores específicos del gen (o de una parte del gen) que codifica el ARN ribosomal bacteriano 16S y posterior

secuenciación de los amplicones obtenidos, o bien (iii) la secuenciación de todo el material genético extraída de la muestra (según hablemos de metagenómica filogenética o total), y (iv) el análisis bioinformático de los resultados previa comparación de las secuencias obtenidas con bases de datos genómicas ya existentes (Ribosomal Database Project, Silva, Greengenes). Cada una de estas etapas puede influir en los resultados finales dependiendo de las diferentes tecnologías utilizadas y del uso de diferentes bases de datos.

La primera técnica molecular aplicada a la ecología microbiana fue la secuenciación del gen ARN ribosomal 16S (ADNr16S), molécula que presenta regiones altamente conservadas y regiones variables entre organismos. A este tipo de identificación se la conoce como *metagenómica filogenética* o del 16S (Figura 5) (76). Este método es rápido, provee con efectividad la determinación del perfil de bacterias de una comunidad, y ya se ha incorporado en laboratorios de microbiología clínica. En cambio, a nivel informativo no va más allá de establecer el abanico de composición bacteriana de la muestra, ya que a menos que se amplifiquen otros genes específicos no ofrece información sobre otros microorganismos potencialmente presentes como virus, hongos o protozoos, ni tampoco ofrece información a nivel funcional. Antiguamente la secuenciación del gen del ADNr16S se realizaba a través del método Sanger. Actualmente la secuenciación se realiza a través de plataformas de secuenciación de alto rendimiento, lo cual ha abaratado enormemente los costes de la técnica y ha aumentado el número de secuencias por muestra, aunque como norma general se obtienen lecturas con una longitud media más corta que con el método Sanger (77).

Como contrapunto a la metagenómica filogenética se encuentra la metagenómica total, o *shotgun metagenomics*. Esta técnica consiste en la secuenciación de todo el material genético aislado de una comunidad microbiana, generándose un perfil genético mucho más amplio y complejo. Esto se debe a que se obtiene información de todos los genes presentes en las muestras problema, incluidos microorganismos no bacterianos. La metagenómica total es una herramienta muy útil a la hora de descifrar el contenido genético y funciones potenciales codificadas en los genomas de los microorganismos presentes en una muestra problema. Sin embargo su utilidad es limitada ya que no proporciona información sobre la actividad real de la microbiota, sino sobre su actividad potencial. El impulso de los proyectos MetaHit y Human Microbiome Project fue esencial para que esta técnica pudiera abaratar sus costes y pudiera ser aplicada a otros estudios del microbioma intestinal y a su influencia en distintos estados fisiológicos y enfermedades (78)

Cabe destacar que existen metodologías bioinformáticas que permiten realizar una inferencia funcional a partir de datos de metagenómica filogenética (79). PICRUSt es una herramienta bioinformática, que predice mediante algoritmos la composición funcional de un

metagenoma, usando datos de genes marcadores y una base de datos de genomas de referencia. De esta manera se puede predecir qué familias de genes están presentes cruzando los datos de genomas conocidos con bases de ontología como KEGG, y así combinar esta información para estimar la composición del metagenoma y por tanto su potencial actividad funcional (80).

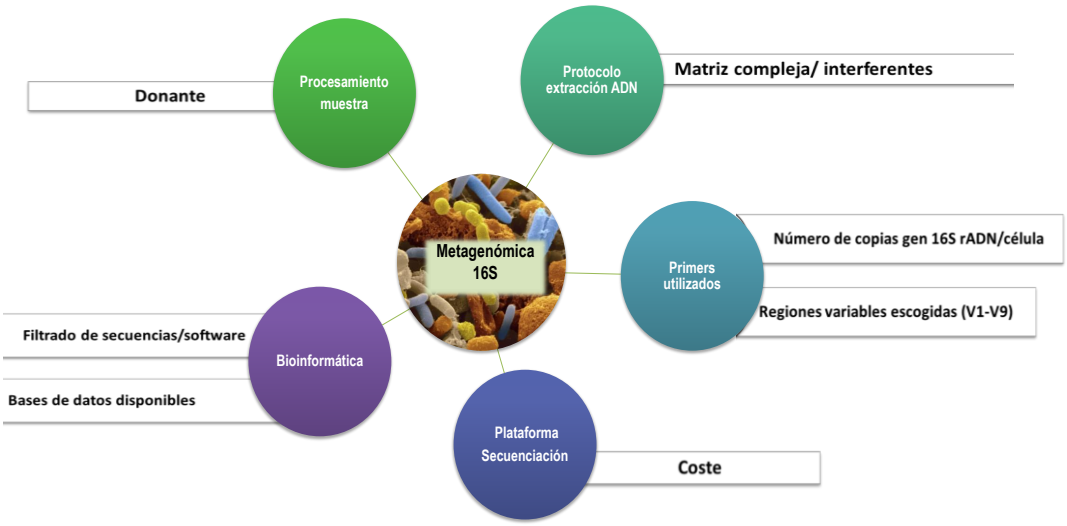

Figura 5. Etapas del análisis de metagenómica filogenética, así como de factores que afectan a cada una de ellas y que establecen puntos críticos en los resultados.

(ii) Metatranscriptómica.

La metatranscriptómica consiste en la secuenciación del ARN (mensajero o microARN) contenido en todas las células de una población. Esta técnica es relativamente novedosa y permite analizar los genes expresados/reprimidos por la microbiota en diferentes condiciones experimentales (81). La metatranscriptómica ha resultado muy útil en la identificación de la regulación y expresión de sistemas biológicos como el microbioma humano, pero su aplicación en la actualidad es aún muy limitada. Además, algunos de los pasos experimentales de esta técnica pueden sesgar los resultados. Por ejemplo todos los procesos de manejo del ARN, molécula altamente inestable, la purificación del ARN que se desee secuenciar (mARN, microARN, etc.), o la retrotrascripción previa a ADN copia (cADN) para la construcción de la librería de secuenciación. Otros problemas técnicos adicionales son el proceso en sí de obtención del ARN a partir de una muestra compleja así como su preservación, la baja calidad del ARN obtenido a partir de microbiotas intestinales humanas, los protocolos de eliminación del RNA ribosomal que representa en torno al 90% del total en este tipo de muestras, la corta vida media del ARN en la célula que limita su detección a respuestas a cambios medioambientales, una base de datos de transcriptomas

insuficiente debido a la abundancia de anotaciones automáticas, y por último la presencia de ARN del hospedador como contaminante que no son eficientemente eliminados con los protocolos actuales (82). Todos estos inconvenientes explican por qué a día de hoy no se ha extendido a gran escala la metatranscriptómica para el estudio de la microbiota intestinal.

(i) Metaproteómica

Las técnicas de proteómica se basan en la identificación y cuantificación de proteínas a gran escala mediante separación multidimensional acoplada a espectrometría de masas, partiendo de digeridos proteicos (proteómica *bottom-up*) o de las proteínas enteras (proteómica *top-down*). Adicionalmente, el patrón de fragmentación de un péptido o de una proteína revela su secuencia de aminoácidos y algunas de sus modificaciones postraduccionales. Las masas de los péptidos o los patrones de fragmentación peptídica (también llamados péptidos hijos) se asocian a las proteínas que los originan mediante algoritmos que comparan estos valores de masas experimentales con los teóricos obtenidos previamente *in silico* a partir de las secuencias de proteínas.

La metaproteómica es el estudio del proteoma colectivo de una comunidad celular (83). Los estudios de proteómica en microorganismos individuales han sugerido que una gran parte de los cambios en respuesta a una condición experimental ocurre a nivel de síntesis o degradación proteica, por lo que medir la concentración de una proteína suele suponer una medida directa de su actividad funcional real. En uno de los estudios pioneros en aplicar la metaproteómica al estudio de la microbiota intestinal, se caracterizó el metaproteoma de un adulto sano en el que se vio que más del 50% de todas las proteínas microbianas estaban involucradas en funciones basales de los microorganismos, como es el proceso de traducción y las vías centrales de producción de energía (84). En otro estudio se comparó el metaproteoma intestinal de pacientes con enfermedad de Crohn frente a individuos sanos, revelándose cambios significativos en abundancias de proteínas en más de 100 familias proteicas, aparte de la ausencia de proteínas involucradas en la producción de AGCC en los individuos enfermos. Esto correlaciona con la baja abundancia de microorganismos productores de butirato, lo que según algunos autores podría contribuir al estado pro-inflamatorio observado en la mucosa intestinal de estos pacientes (85).

(iii) Metabolómica

La metabolómica se basa en la detección a gran escala de metabolitos y pequeñas moléculas de manera directa (86). Esta técnica combina el uso de técnicas de separación analítica de compuestos como la cromatografía líquida o la electroforesis capilar, acopladas a la detección y/o cuantificación por espectrometría de masas, resonancia magnética nuclear u

otras técnicas de identificación. Metodológicamente es más similar a la metaproteómica que a la metagenómica y a la metatranscriptómica, pero sin embargo, y a diferencia de la primera, la metabolómica es compatible con cualquier otro experimento de secuenciación al precisar de relativamente bajos volúmenes de muestra (la metaproteómica requiere de más volumen de muestra y a veces de una ardua preparación de la muestra).

La metabolómica tiene la ventaja de que el rango de moléculas diferentes que se pueden detectar es muy alto, desde carbohidratos hidrofílicos de baja masa molecular, como la glucosa, a lípidos hidrofóbicos de mayor masa molecular, como los triglicéridos, pasando por compuestos naturales más o menos complejos, como los antibióticos o los polifenoles. Dado que las interacciones entre microorganismos o entre microorganismo-hospedador están frecuentemente mediadas por metabolitos secretados, la metabolómica es una herramienta muy útil para entender la actividad funcional de las interacciones existentes en estos ecosistemas. Se piensa que el metaboloma humano, tanto a nivel de fluidos corporales como de tejidos, está ampliamente influenciado por la microbiota, y que muchos de estos metabolitos microbianos tienen relevancia en la salud hasta el punto de que algunos autores los consideran co-metabolitos microbiota-hospedador (87). Por ejemplo, un estudio analizó la concentración de una serie de metabolitos mediante UPLC-MS/MS en un gran número de muestras humanas de individuos sanos, estableciendo nuevos rangos de referencia en orina para 3 co-metabolitos producidos por distintos patógenos, y que tiene interés para futuras investigaciones epidemiológicas (88). Otro estudio ha identificado un grupo de co-metabolitos característicos de individuos obesos, cuya concentración es mucho menor en individuos delgados (89). Por tanto, la utilidad de la metabolómica en la identificación y validación de moléculas diana y de biomarcadores de determinados estados patológicos es destacable (90, 91).

Las bases de datos de metabolómica de acceso público son bastante potentes y presentan un alto grado de curación experimental, a diferencia de las bases de datos correspondientes a otras técnicas ómicas, notablemente las de función de proteínas. Existe una base de datos de acceso libre llamada Metaboloma Humano (HMDB) que contiene información de metabolitos que se encuentran en el cuerpo humano, en distintos tejidos y fluidos (http://hmdb.ca). Uno de los objetivos de HMDB es que el uso de la metabolómica se aplique a la química clínica, al descubrimiento de biomarcadores y al ámbito docente general (92).

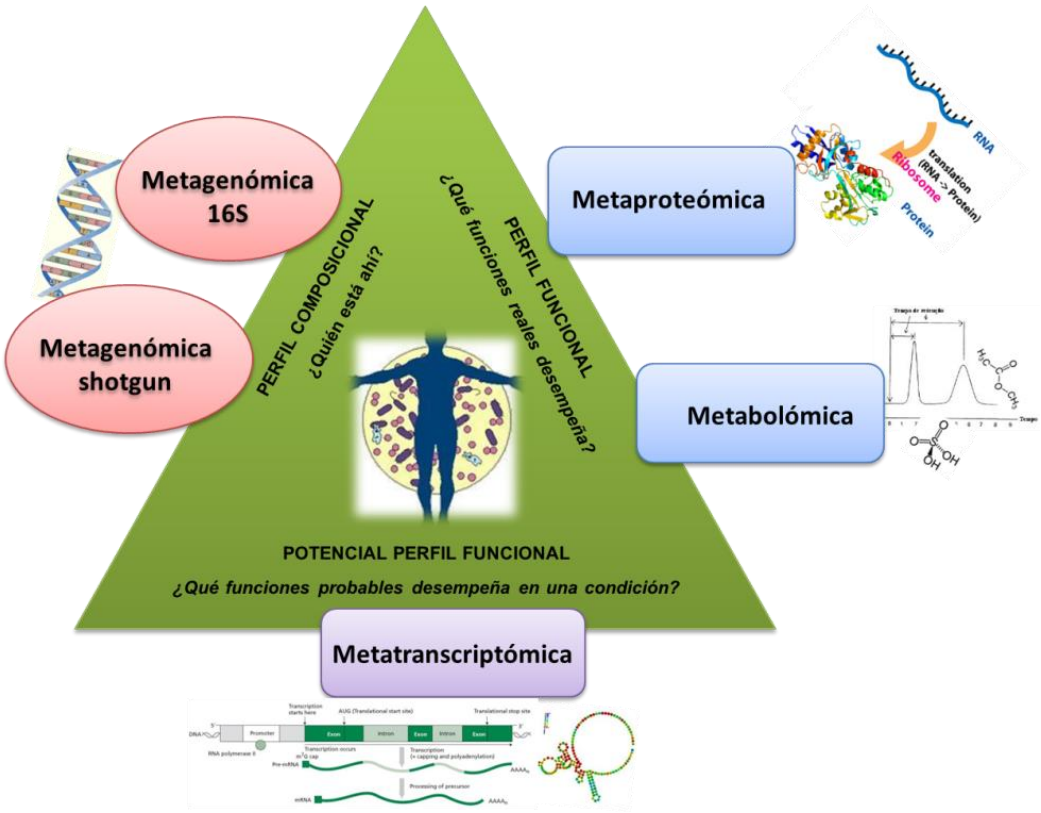

Figura 6. Esquema representativo de las distintas técnicas ómicas contempladas en esta introducción, en el que se muestra qué tipo de moléculas se identifican mediante cada una de las técnicas, así como la información que proporcionan en el caso concreto de la microbiota intestinal.

1.2. Interacción de la microbiota intestinal con el sistema inmune.

El sistema inmune está sometido a una continua interacción con la microbiota, y esta interacción va a condicionar su función. Más aún, la microbiota se va a ver afectada por la respuesta del sistema inmune del hospedador hacia ella, estando esto a su vez condicionado por factores genéticos. En el TGI, los microorganismos están separados del hospedador por una monocapa de células epiteliales, que se encuentran a su vez recubiertas por una capa de mucus. Esta aparente separación determina lo que en ciertos casos se considera como una acción a distancia de la microbiota, y en el que el muestreo de los antígenos microbianos por parte de las células presentadoras de antígenos es crucial para el desarrollo de la respuesta inmune correcta.

1.2.1. Interacción de la microbiota con distintos componentes del hospedador.

De manera esquemática, la interacción física entre la microbiota y el sistema inmune

ocurre a tres niveles. El primer nivel de interacción ocurre en la capa de mucus situada hacia el lumen intestinal, y que está compuesta por dos subcapas: una externa menos densa, altamente colonizada por microbiota, y otra interna más densa y donde están presentes una amplia gama de péptidos antimicrobianos (PAMs) e inmunoglobulina A secretada. Debido a esta distribución física de la mucosa intestinal, en condiciones normales, la capa interna densa de mucus es prácticamente impenetrable a la microbiota intestinal (93, 94).

El segundo nivel de interacción ocurre a nivel de la capa de células epiteliales intestinales que están en contacto con la *lamina propria* (LP) en su parte basolateral y con la subcapa de mucus más densa en su superficie apical. Las células epiteliales se dividen en varios tipos: i) células Goblet que producen la mucina que es el principal componente del mucus, ii) colonocitos y enterocitos especializados en la absorción de nutrientes, iii) células de Paneth especializadas en la producción de PAMs, y iv) células M que capturan antígenos para facilitar su detección por parte de células del sistema inmune presentadoras de antígenos (95). Las células epiteliales juegan un papel importante en el denominado efecto barrera o separación física entre la parte interna del organismo y el ambiente exterior, y para ello se unen entre ellas mediante las denominadas uniones estrechas o *tight junctions,* aislando el interior de la mucosa del mucus y del contenido intestinal.

El tercer nivel de interacción microbiota/hospedador se sitúa a nivel de la *lamina propria*. A este nivel se sitúan los nódulos mesentéricos que en conjunto forman el denominado tejido linfoide asociado al intestino (GALT). En la mucosa se encuentran folículos linfoides aislados denominados placas de Peyer, y que son sitios activos de reclutamiento de células inmunes, así como de activación de células T y B en caso de reconocimiento de patrones moleculares asociados a microorganismos (MAMPs). En primer lugar, estos MAMPs son detectados por células presentadoras de antígenos, principalmente células dendríticas (CD) y macrófagos. Éstas migran a los nódulos mesentéricos donde interaccionan con células T vírgenes y células B, activándolas y dando lugar a la inducción de una respuesta inmune concreta (96).

1.2.2. Influencia de la microbiota intestinal en el desarrollo y homeostasis del sistema inmune.

La microbiota intestinal juega un papel importante en el desarrollo y maduración del sistema inmunológico del hospedador tal y como ha sido evidenciado en ratones "*germ-free*". Estos animales presentan un sistema inmune poco desarrollado comparado con ratones convencionales (97). Se observa, por ejemplo, que poseen un número drásticamente menor de linfocitos intraepiteliales en su mucosa intestinal, un número menor de placas de Peyer y de

menor tamaño, un número menor de criptoplacas, una estructura de las criptas alterada, un reducido número de células de Goblet productoras de mucus y por tanto una capa de mucus de espesor menor que ratones normales, y un ciego de mayor tamaño (98). En estos animales puede inducirse una maduración del sistema inmune bien tras abandonar las condiciones de esterilidad y permitir la colonización por parte de la microbiota, o bien mediante transferencia de una microbiota proveniente de una muestra fecal (99).

El desarrollo correcto de la función inmune depende de la interacción de la microbiota con familias de receptores de reconocimiento de patrones (PRRs) específicos presentes en el hospedador (100). Los PRRs se localizan en la superficie celular y en orgánulos de células epiteliales, CDs y macrófagos, células que están constantemente muestreando el lumen intestinal en busca de los anteriormente citados MAMPs, como son ciertos componentes de la superficie celular bacteriana, (el lipopolisacárido o la flagelina por ejemplo), o ácidos nucleicos de virus y bacterias. Algunos de los PRRs más importantes son los receptores de la familia "Toll-like" (TLRs), los de la familia "NOD-like" (NLRs), las lectinas de tipo C como DC-SIGN (CLRs) y los receptores de tipo "RIG-I-like" (RLRs) (101).

Durante la respuesta immune innata a la microbiota se secretan diversos compuestos a la luz intestinal, como inmunoglobulinas A específicas o péptidos antimicrobianos como Reg IIIγ. Esto tiene como fin limitar el contacto de los microorganismos con el hospedador mediante el control de la carga microbiana constituyendo un sistema de respuesta genérico, ya que todos los microorganismos tienen MAMPs. En cambio, la respuesta inmune adaptativa es más específica dependiendo del microorganismo. La microbiota comensal puede ser dividida, a grandes rasgos, en dos tipos: i) microorganismos que promueven una respuesta efectora, y ii) microorganismos que inducen una respuesta reguladora, directamente relacionada con la homeostasis intestinal (102), y con el desarrollo de tolerancia hacia los microorganismos de la microbiota (103). Ambos tipos de bacterias comparten características estructurales y de función a veces muy similares, y aún no se ha esclarecido por qué unas pueden ser causa de brotes patológicos bajo condiciones fisiológicas específicas mientras que otras con estructuras similares no tienen tal efecto (104).

Las bacterias efectoras actúan induciendo una respuesta principalmente T_h1, T_h2 o T_h17. Estas respuestas son necesarias para luchar contra patógenos extra o intracelulares, pero su exceso o desregulación puede provocar un daño crónico para los tejidos del hospedador (105). Un ejemplo típico de bacterias efectoras son las bacterias filamentosas segmentadas (SFB), inductoras de la respuesta T_h17 en ratones, o *Helicobacter hepaticus* (106, 107). Dependiendo del estado inmunológico del hospedador, el efecto de estas bacterias puede ser positivo o negativo (108, 109). Especies de la familia *Prevotellaceae*, o las especies *Bacteroides fragilis* o *Klebsiella pneumoniae* tienen un marcado perfil efector y exacerban la

respuesta inmune en varios modelos murinos (110). Se piensa que el efecto de estas bacterias está directamente relacionado con su capacidad de colonización de la mucosa intestinal. Por ejemplo las SFB se adhieren fuertemente al epitelio del íleon, algunas especies de la familia *Prevotellaceae* son capaces de colonizar las criptas intestinales, y *B. fragilis* es capaz de penetrar en la capa interior de mucus en el colon (111).

Los microorganismos reguladores actúan induciendo la proliferación de células T reguladoras en el intestino. En ratones "*germ-free*" se observa que la colonización con una microbiota modelo induce la producción y reclutamiento de estas células en la mucosa intestinal, siendo su presencia fundamental para mantener la homeostasis intestinal y un bajo grado de inflamación en la mucosa. Algunas especies bacterianas inmunomoduladoras pertenecen al género *Clostridium* (clústeres IV y XIVa), y ciertos compuestos específicos de la superficie de otros microorganismos como el polisacárido capsular A (PSA) de *B. fragilis* y el péptido STp codificado en un proteína secretada por *Lactobacillus plantarum* han mostrado ser también inflamatorios. El efecto regulador de los clostridios parece mediado por la secreción de AGCC, principalmente butírico (112). En el caso de *B. fragilis* el reconocimiento de la estructura del PSA se lleva a cabo por el receptor TLR2 presente en la membrana de CDs, que promueven una respuesta T_{reg} (113). En el caso del péptido bioactivo STp, éste induce la producción de IL-10 por parte de CDs promoviendo la tolerancia inmune *in vitro* (114). Además, este péptido es capaz de revertir el fenotipo desregulado de CDs de pacientes con colitis ulcerosa, las cuales secretan una gran cantidad de citoquinas pro-inflamatorias y baja de citoquinas anti-inflamatorias (115).

En resumen, la microbiota intestinal está compuesta por especies que a pesar de parecerse estructuralmente generan respuestas específicas diferentes en el diálogo con el sistema inmune del hospedador. Este equilibrio entre especies efectoras y reguladoras es uno de los factores críticos para el correcto desarrollo y función del sistema inmune, y cualquier desplazamiento hacia uno de estos dos tipos de respuesta puede provocar la aparición de un estado patológico.

1.2.3. Estrategias de modulación de la microbiota intestinal: probióticos y prebióticos.

Hoy en día existe un creciente interés en intentar prevenir y tratar enfermedades mediante intervenciones alimentarias que incluyan microorganismos beneficiosos, así como sustratos específicos que promuevan su crecimiento. Estas intervenciones tienen como fin influir favorablemente en la composición de la microbiota intestinal, corrigiendo posibles

disbiosis y disminuyendo el efecto perjudicial de esta en el hospedador (116)

Los probióticos son microorganismos vivos que, cuando se administran en cantidades adecuadas, confieren beneficios en la salud del hospedador (117). Los prebióticos son componentes de los alimentos no digeribles que afectan beneficiosamente al hospedador al estimular selectivamente el crecimiento y/o actividad de uno o un número limitado de especies bacterianas que residen en el colon (118). Ambos pueden ser utilizados como suplementos en alimentación humana, pero muchos tipos de probióticos y prebióticos están presentes en multitud de alimentos en nuestra vida diaria como alimentos fermentados, lácteos, vegetales, frutas y hortalizas. Cuando probióticos y prebióticos son utilizados de forma combinada se denominan simbióticos.

Los probióticos más frecuentemente usados en alimentación humana son especies bacterianas de los géneros *Lactobacillus* y *Bifidobacterium*, aunque también se utilizan cepas de los géneros *Enterococcus*, *Propionibacterium, Streptococcus* y algunas levaduras como *Saccharomyces boulardii* (119). En los últimos años, un gran número de estudios tanto en humanos como en modelos animales han analizado el beneficio de los probióticos sobre la salud humana, centrándose principalmente en la prevención y tratamiento de distintas enfermedades (120). Teóricamente podría llegarse a modular favorablemente una microbiota indígena desbalanceada a través de una intervención nutricional. Rijkers y colaboradores han propuesto catalogar los beneficios de los probióticos en tres grupos de acuerdo a su modo de acción: i) interferencia a través de un mecanismo de competencia con el crecimiento de microorganismos patógenos, ii) mejora de la función barrera de la mucosa intestinal y del sistema inmune intestinal, y iii) influencia extra-intestinal a través del sistema inmune en órganos alejados del TGI (117).

1.3. Disbiosis de la microbiota intestinal y trastornos del sistema inmune.

Se conoce como disbiosis intestinal a cualquier desviación de la composición de la microbiota, que condiciona una serie de interacciones con el hospedador notablemente diferentes a las que se establecen cuando una microbiota "normal" está presente. La disbiosis se establece siempre por comparación de un grupo poblacional específico con un grupo control y sano, ya que a día de hoy no se ha definido aún qué es una microbiota normal debido a la gran variabilidad interindividual.

Un gran número de enfermedades aparecen asociadas a disbiosis intestinales más o menos pronunciadas. Por ejemplo, la diarrea asociada a antibióticos está causada por una alteración directa sobre la microbiota, lo cual resulta en una reducción del procesamiento de carbohidratos no digeribles y un rápido crecimiento de bacterias oportunistas potencialmente

patógenas. Los efectos pueden variar desde episodios de diarrea y malestar leves que cesan cuando se retira el antibiótico, hasta serias complicaciones como megacolon tóxico, perforación intestinal, infección por *Clostridium difficile* u otros patógenos, y muerte. El riesgo de muerte se incrementa en función de las condiciones clínicas del individuo, como edades extremas, presencia de patologías de base y/u hospitalización (121).

Un ejemplo bien estudiado de disbiosis es la que ocurre en la enfermedad inflamatoria intestinal (EII). Una de las hipótesis más fuertes que explican la patogénesis de esta enfermedad es la presencia de una interacción anormal entre la microbiota intestinal y el GALT, lo que conduce a una respuesta aberrante del sistema inmune frente a los microorganismos comensales. La microbiota intestinal, con una disbiosis marcada hacia la pérdida de variabilidad bacteriana y hacia la disminución de miembros del filo Firmicutes, parece inducir un estímulo pro-inflamatorio que además genera daños en la barrera epitelial (122). Cuando se rompe la barrera intestinal, se ven alteradas definitivamente las interacciones microbiota-hospedador, la secreción de IgAs y la respuesta esta vez exacerbada del sistema inmune a la microbiota intestinal (123) (Figura 7). Por todo ello la presencia de un correcto balance en la microbiota intestinal y la ausencia de disbiosis se está empezando a considerar como un factor a tener en cuenta para llevar una vida saludable (124). Sin embargo, y a día de hoy, no está claro si la disbiosis es un factor que precede a la EII, o si más bien es consecuencia del desarrollo de la misma. Esto es aplicable no sólo a la EII, sino a otras muchas enfermedades donde se ha constatado la presencia de disbiosis intestinal.

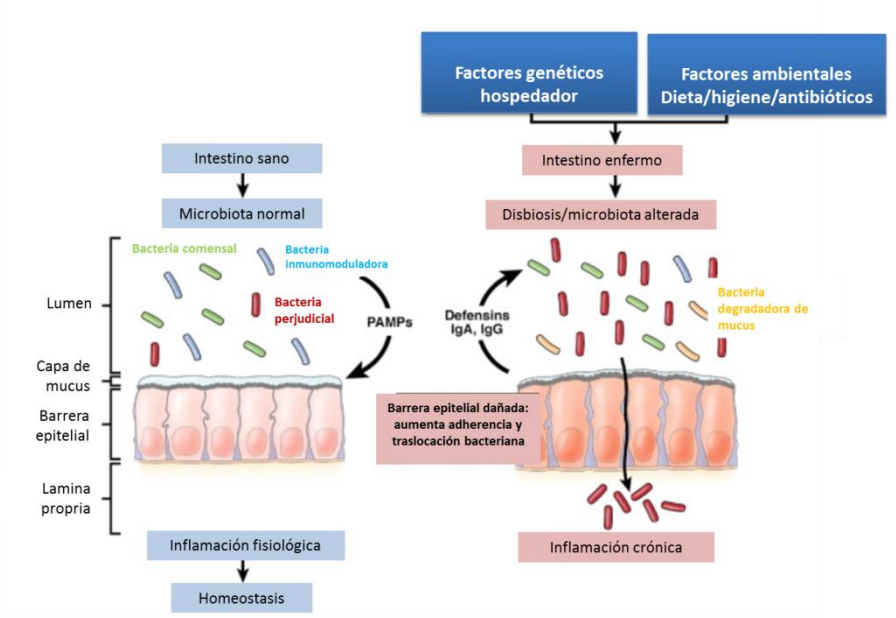

Figura 7. Esquema en el que se compara una situación homeostática de la mucosa intestinal (izquierda) caracterizada por una inflamación fisiológica, y una situación de disbiosis de la microbiota que viene condicionada por diversos factores del hospedador y que conduce a un estado

de salud alterado a través de una inflamación crónica (derecha) Adaptado de .

1.3.1. El papel de la microbiota intestinal en estados fisiopatológicos.

En la literatura científica existe un gran número de evidencias que asocian una gran cantidad de patologías no-infecciosas (tanto gastrointestinales como de otros tipos), a una disbiosis intestinal. Algunas de ellas son enfermedades inflamatorias intestinales crónicas como la Enfermedad de Crohn (EC) y la Colitis Ulcerosa (CU) (que se engloban dentro de la EII), el Síndrome Metabólico, el Síndrome del Intestino Irritable (SII), la aterosclerosis, así como enfermedades de marcado carácter autoinmune como la Diabetes Tipo 1 (DT1), la Artritis Reumatoide (AR), el Lupus Eritematoso Sistémico (LES), la alergia, e incluso el cáncer colo-rectal (CRC). En todas ellas hay estudios que describen la microbiota intestinal a múltiples niveles: composición, función, presencia de biomarcadores asociados a microorganismos, a dieta u otros factores medioambientales, etc. En la tabla 1 del apéndice se muestra un resumen de los trabajos donde se muestran disbiosis de la microbiota intestinal asociadas a distintas condiciones fisiológicas o enfermedades.

1.3.2 Lupus eritematoso sistémico.

El LES es una enfermedad autoinmune multifactorial cuya etiología es todavía desconocida. En la patología de la enfermedad están implicados factores tanto genéticos, algunos ya ampliamente caracterizados (125), como ambientales (126). Su tasa de incidencia es mucho mayor en mujeres que en hombres, ya que está asociado a un fuerte componente hormonal que influye en el inicio y el desarrollo de la enfermedad. La patogénesis del LES se sustenta en una respuesta inmune alterada que se caracteriza por una diferenciación incontrolada de células T, que lleva a la activación/proliferación anormal de células B que producen anticuerpos IgG dirigidos contra moléculas propias del individuo (auto-anticuerpos), como ADN de doble cadena, histonas y otras moléculas. Estos auto-anticuerpos forman inmunocomplejos que se depositan en los tejidos causando una inflamación sistémica en diferentes partes del organismo, siendo típico daño renal.

Durante los últimos años, el empleo de la metagenómica ha evidenciado que la composición de los microorganismos intestinales juega un papel importante en el desarrollo de diversas enfermedades autoinmunes, incluyendo la DT1, la AR e incluso la esclerosis múltiple (127). Sin embargo, la implicación de la microbiota en LES no está del todo clara, principalmente debido a la falta de evidencia en modelos animales.

Está muy bien establecido que los factores genéticos del hospedador influyen en la

susceptibilidad al LES. Sin embargo, la falta de concordancia de síntomas entre individuos gemelos genéticamente idénticos, o entre individuos del mismo grupo étnico pero expuestos a diferentes ambientes, como africanos y afroamericanos, sugiere un papel importante de factores no genéticos en la patología del (128, 129). Parece ser que la mejora de la higiene y la ausencia de ciertos microorganismos contribuye a la incidencia y progresión del LES, además de la presencia de infecciones con algunos virus. Por ejemplo tanto el virus Epstein–Barr (EBV), el citomegalovirus (CMV), y el virus de la hepatitis B (HBV) han sido relacionados con la patogénesis del LES aunque de distinta forma. Mientras que EBV y CMV son considerados desencadenantes del LES (130, 131), existe una evidencia creciente de que infecciones por HBV podría tener un papel protector frente a las apariciones de brotes de LES (132) y otros (133).

Por otro lado, diversos componentes de bacterias tanto Gram-positivas como Gram-negativas han sido relacionadas con la iniciación y mantenimiento del LES a través de la interacción con los TLRs (134). Por ejemplo en el caso de individuos con LES los monocitos liberan el componente soluble CD14 en respuesta al LPS de bacterias Gram-negativas, siendo este proceso muy importante en la amplificación de procesos inflamatorios (135). De hecho, en modelos murinos de LES se ha visto que inyecciones repetidas de LPS resultan en un incremento en la producción de autoanticuerpos y la aparición de glomerulonefritis (136) .

El ácido lipoteicoico (LTA) es un componente mayoritario de la pared celular de bacterias Gram-positivas y es ligando del TLR2, cuya expresión se ve incrementada en células T, células B y monocitos de pacientes con LES. Un incremento de TLR2 lleva asociado incrementos en la secreción de citoquinas IL-17a e IL-17F, asociadas a respuesta inflamatoria en ratones. De hecho, estirpes de ratones a los que se les ha eliminado el gen TLR2 muestran unos síntomas atenuados en esta misma configuración experimental, probablemente debido a la imposibilidad de responder via TLR2 (137).

Existe también una evidencia creciente de que otros receptores como TLR7 y TLR9 juegan un papel importante en el desarrollo del LES (138). Estos dos receptores están involucrados en la activación de CDs y clones de células B autoreactivos a través del reconocimiento de antígenos que principalmente son ARN endógeno de cadena simple (TLR7) y ADN con alto contenido en citosina y guanina (TLR9); ambos autoantígenos se originan a partir de los núcleos celulares. De hecho, uno de los modelos de lupus murino es una estirpe en la que se encuentra duplicado el gen que codifica para el TLR7, y que presenta un exceso de señalización molecular a través de esta vía (139). Por último, existen cada vez más evidencias de la presencia clones de células T autorreactivos en individuos con LES, y que reconocen antígenos microbianos que presentan cierta homología estructural con biomoléculas propias, también conocida como mimetismo molecular, como por ejemplo la

existente entre péptidos microbianos y péptidos propios (140).

1.3.3 Alergia y asma.

El asma y las alergias son trastornos complejos determinados por factores genéticos y medioambientales, que se caracterizan principalmente por inflamación local en la piel y vías respiratorias. La base inmunológica de la enfermedad consiste en un reconocimiento defectuoso de alérgenos ambientales contra los que se inicia una respuesta inmune consistente en una diferenciación anómala de células T naïve hacia T_h2, incremento de citoquinas involucradas en la producción de IgE y la proliferación de mastocitos, basófilos y eosinófilos, lo cual genera inflamación y la aparición de los síntomas propios de esta enfermedad (141).

Como se ha visto que la microbiota intestinal es crucial en el mantenimiento de la homeostasis y la regulación inmune, se piensa que ésta debe tener cierto papel en la patología de la enfermedad (142). Se ha visto que la composición de la microbiota en las vías respiratorias está relacionada con brotes de la enfermedad, del mismo modo que la microbiota de la piel determina manifestaciones en dermatitis atópica (143). Dentro del marco de inflamación crónica y generalizada del individuo, otra posibilidad es que la microbiota intestinal influyera en la microbiota o en la respuesta inmune de otras partes del cuerpo. Aunque existe cierta controversia sobre este tema (144), una hipótesis muy extendida establece que la aparición de alergias podría ser causada por una baja exposición antigénica propia característica de ambientes cada vez menos contaminados, así como la existencia de un menor número de infecciones en la infancia; esto se conoce como la hipótesis de la higiene. (145, 146). La hipótesis de la higiene es reconocida por muchos autores como uno de los factores a tener en cuenta en la aparición de alergias, ya que diversos estudios epidemiológicos han revelado que existe una mayor incidencia en países desarrollados (147).

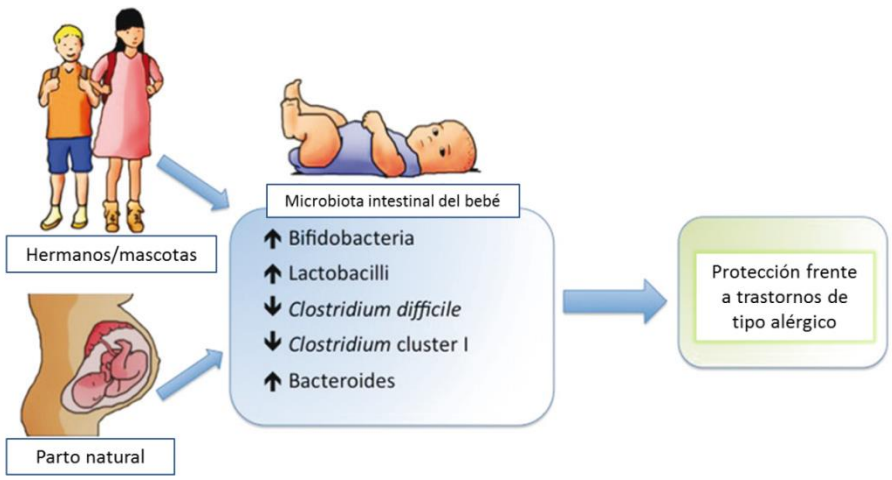

Figura 8. Efectos sobre la microbiota intestinal de factores ambientales directamente relacionados con el desarrollo de alergias en la edad adulta. La ventana de actuación se ha visto que está en la infancia, donde se ha visto que el tipo de nacimiento, y el contacto primario con microorganismos del entorno familiar juegan un papel fundamental en el desarrollo de alergia y asma. Adaptada de Penders y cols, *New insights into the hygiene hypothesis in allergic diseases,* Gut Microbes 2014.

Relacionadas con la alergia se encuentran las enfermedades atópicas, enfermedades de tipo inflamatorio crónico y deslocalizadas producidas por la acción de las IgE. En el caso del eczema atópico, caracterizado por un hinchazón y enrojecimiento continuo de la piel, se ha comprobado que la microbiota intestinal es diferente en niños enfermos con respecto a niños sanos (148). Esta diferencia parece ser debida a distintos mecanismos de colonización neonatal y a la interacción del individuo con su entorno en los primeros meses de vida. Sin embargo, no se han podido asociar claramente grupos taxonómicos concretos con la aparición de alergia o asma, debido principalmente a resultados contradictorios entre distintos estudios (149). En algunos estudios se ha visto un incremento en la abundancia del filo *Proteobacteria* en las vías respiratorias de individuos con asma, y dentro de este grupo se ha reportado una mayor presencia de especies del género *Haemophilus*. También se ha detectado una reducción de especies del filo *Bacteroidetes* y un aumento de especies del filo *Proteobacteria*, de especies del género *Streptococcus* y de la especie *Moraxella catarrhalis* con ciertos tipos de asma (150, 151).

OBJETIVOS DE LA TESIS

Desde las etapas más tempranas de la vida la microbiota intestinal influye positivamente en el correcto desarrollo del sistema inmune. El tracto gastrointestinal adulto alberga una comunidad microbiana con gran diversidad de especies. La mucosa intestinal es uno de los primeros sitios de contacto entre el sistema inmune y microorganismos tanto comensales como patógenos. Estas bacterias y sus metabolitos están en contacto constante con nuestro sistema inmune, estableciéndose un diálogo molecular complejo y dinámico que determina la tolerancia a la microbiota comensal y la respuesta inmune rápida y efectiva frente a patógenos.

Aunque en la mayoría de patologías relacionadas con una función inmune incorrecta se han descrito mutaciones en genes humanos asociadas a una menor o mayor susceptibilidad de desarrollar dichas enfermedades, muchos de estos trastornos han sido asociados con una disbiosis de la microbiota intestinal, es decir un desequilibrio en las abundancias relativas de las poblaciones microbianas intestinales.

En este contexto, en la presente tesis doctoral nos planteamos estudiar la microbiota intestinal humana en grupos de población representativos de dos patologías del sistema inmune, una enfermedad autoinmune prototipo, el lupus eritematoso sistémico (LES), y la enfermedad alérgica respiratoria. En primer lugar pusimos a punto una serie de métodos para

el estudio de la microbiota intestinal, el aislamiento de microbiota fecal, y la identificación bacteriana mediante técnicas independientes de cultivo. Seguidamente aplicamos estas metodologías para obtener los perfiles microbianos propios de cada patología. Finalmente y una vez identificadas las alteraciones microbianas específicas de estos grupos, así como el diferente perfil metabólico de la microbiota asociada al LES, llevamos a cabo estrategias de modulación *in vitro* para el restablecimiento de la microbiota intestinal, mediante la selección de especies de bacterias comensales, que compensasen la respuesta inmune descompensada en estos individuos.

Para llevar a cabo estos objetivos principales, establecimos unos objetivos parciales:

OBJETIVO 1: Optimización de técnicas de estudio y manipulación de la microbiota intestinal humana.

En este primer objetivo pusimos a punto la parte metodológica de estudio y manipulación de la microbiota intestinal a partir de muestras fecales, en lo que respecta al aislamiento de los microorganismos y a la extracción del material genético microbiano.

El trabajo correspondiente a este objetivo se engloba en el capítulo 2 y en el capítulo adicional de la tesis:

Capítulo 2: Desarrollo de técnicas de estudio de la microbiota intestinal: aplicación de gradientes de densidad para el aislamiento de microbiota fecal humana.

Capítulo adicional: Optimización del estudio de la microbiota intestinal mediante análisis del gen 16S rRNA con la plataforma de secuenciación IonTorrent.

OBJETIVO 2: Caracterización de la microbiota intestinal en individuos con trastornos inmunológicos.

En el segundo objetivo de esta tesis aplicamos la metodología puesta a punto en el objetivo 1 para estudias algunas características de la microbiota intestinal en LES y alergia. Este segundo objetivo se enfocó desde dos puntos de vista: perfil de composición microbiana y perfil funcional.

Capítulo 3: Microbiota intestinal de individuos con trastornos inmunológicos. Disbiosis

intestinal asociada a lupus eritematoso sistémico. Composición de la microbiota intestinal de individuos con asma.

Capítulo 4: Perfil metabólico de la microbiota intestinal de individuos con lupus eritematoso sistémico.

OBJETIVO 3: Búsqueda de estrategias de modulación de la microbiota intestinal de individuos con LES.

En el tercer objetivo de esta tesis se planteó la posible corrección de la respuesta inmunológica mediante la suplementación de la microbiota de LES con bacterias comensales Para llevar a cabo este objetivo se aislaron las microbiotas de individuos con LES, se suplementaron con bacterias seleccionadas y se realizaron cocultivos *in vitro* con células dendríticas y linfocitos T vírgenes.

El trabajo realizado para llevar a cabo este tercer objetivo se engloba en la segunda parte del último capítulo de la tesis:

Capítulo 4: Capacidad inmunomoduladora de la microbiota intestinal.

TRABAJO EXPERIMENTAL

EXPERIMENTAL WORK

CAPÍTULO 1

Mecanismos moleculares involucrados en la interacción entre bacterias beneficiosas y el Sistema Inmune.

El estudio de la microbiota intestinal humana puede realizarse desde varios puntos de vista, desde la caracterización de las abundancias relativas de sus subpoblaciones en distintas condiciones fisiológicas hasta el estudio de su función apoyándose en modelos animales. Una gran parte del estudio actual de la microbiota persigue conocer cómo modular la interacción entre ésta y el hospedador para tratar de corregir la disbiosis y las interacciones anómalas que aparecen en el marco de múltiples enfermedades.

En este primer capítulo de la tesis se han compilado los principales mecanismos moleculares a través de los cuales microorganismos probióticos de los géneros *Bifidobacterium* y *Lactobacillus* interaccionan con el hospedador. Además de estos probióticos clásicos, se citan mecanismos moleculares ejercidos por los denominados probióticos emergentes, bacterias comensales que han mostrado conferir beneficios al hospedador, como *Faecalibacterium prausnitzii*, *Roseburia hominis* o *Akkermansia muciniphila*. La bacteria *F. prausnitzii* es incluso considerada como un marcador de salud por algunos autores, y abundancias relativas bajas correlacionan con la aparición de alguna de las formas de Enfermedad Inflamatoria Intestinal.

Los resultados que conforman este capítulo 1 se presentan en el siguiente manuscrito que ya ha sido publicado.

- **Hevia A**, Delgado S, Sánchez B, Margolles A. Molecular Players Involved in the Interaction Between Beneficial Bacteria and the Immune System. Frontiers in Microbiology. 2015; 6:1285.

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Molecular Players Involved in the Interaction Between Beneficial Bacteria and the Immune System

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The human gastrointestinal tract is a very complex ecosystem, in which there is a continuous interaction between nutrients, host cells, and microorganisms. The gut microbiota comprises trillions of microbes that have been selected during evolution on the basis of their functionality and capacity to survive in, and adapt to, the intestinal environment. Host bacteria and our immune system constantly sense and react to one another. In this regard, commensal microbes contribute to gut homeostasis, whereas the necessary responses are triggered against enteropathogens. Some representatives of our gut microbiota have beneficial effects on human health. Some of the most important roles of these microbes are to help to maintain the integrity of the mucosal barrier, to provide nutrients such as vitamins, or to protect against pathogens. In addition, the interaction between commensal microbiota and the mucosal immune system is crucial for proper immune function. This process is mainly performed via the pattern recognition receptors of epithelial cells, such as Toll-like or Nod-like receptors, which are able to recognize the molecular effectors that are produced by intestinal microbes. These effectors mediate processes that can ameliorate certain inflammatory gut disorders, discriminate between beneficial and pathogenic bacteria, or increase the number of immune cells or their pattern recognition receptors (PRRs). This review intends to summarize the molecular players produced by probiotic bacteria, notably *Lactobacillus* and *Bifidobacterium* strains, but also other very promising potential probiotics, which affect the human immune system.

Keywords: *Bifidobacterium*, immunomodulation, *Lactobacillus*, molecular players, probiotic

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THE HUMAN GUT MICROBIOTA AND THE IMMUNE SYSTEM

From the early stages of life, one of the most important roles of the gut microbiota is to contribute to the development of a proper immune system. Normally, humans live in a homeostatic symbiosis with their gastrointestinal microbes, providing them with nutrients and a friendly environment, whereas the microbiota aids in the appropriate development and maintenance of the host's gut mucosa. Epithelial function is influenced by direct host/microbiota interactions and microbial metabolism. The large intestine acts as an anaerobic bioreactor for the enteric bacterial community, which is fueled by host diet components that cannot be processed in the small intestine, as well as by endogenous nutrients, such as host glycans from mucus and cell debris released from epithelial cells. Additionally, the microbiota synthesizes essential amino acids, vitamins, and short chain fatty acids (SCFAs) by degrading a variety of proteins and otherwise non-digestible polysaccharides (Sekirov et al., 2010).

The commensal microbiota ensures the mechanical integrity of the mucosal barrier, thereby offering protection against harmful pathogenic microbes (**Figure 1**). Commensal bacteria can adhere to the intestinal mucus and competitively inhibit the adhesion of enteropathogens; they also produce bacteriocins and SCFAs, compounds that are able to inhibit the growth of other microorganisms. Additionally, some antimicrobial metabolites, such as the defensins secreted in the intestine, contribute to the host's control of these microorganisms (Salzman et al., 2007). Further protection of the host is provided by inducing the mucosal immune system to produce immunoglobulin A, which is released in the intestinal lumen in large amounts and limits local bacterial colonization, thereby preventing bacteria from penetrating the epithelium (Salzman et al., 2007).

A major issue is how the intestine distinguishes between the abundant, normal microbiota and rare pathogens. The immune system fights pathogenic bacteria, but tolerates the presence of commensal species, even though their cellular structures are quite similar and they have common mechanisms of interacting with host immune cell receptors; this phenomenon is called immune tolerance. In this way, our immune cells differentiate between commensals and pathogens. This is carried out by our innate immune system through pattern recognition receptors (PRRs) (**Figure 1**), including Toll-like receptors (TLRs), transmembrane receptors that scan the external milieu of the intestinal lumen, and Nod-like intracellular receptors (NODLR), which guard the cytoplasmic space (Claes et al., 2015; Sellge and Kufer, 2015). Other PRRs have also been described, such as C-type lectin receptors, formylated peptide receptors, retinoic acid-inducible (RIG)-like helicases, and intracellular interleukin-1 (IL-1)-converting enzyme protease-activating factor (Denes et al., 2012; Bufe et al., 2015; Dambuza and Brown, 2015; Yao et al., 2015). PRRs are able to specifically recognize and bind different microbial macromolecular ligands, which are designated as microbial-associated molecular patterns (MAMPs), such as lipopolysaccharide, flagellin and other proteins, bacterial peptidoglycan, viral RNAs, and fungal carbohydrates. As a result, the T cell subset involved in regulating the immune balance is finely tuned by the host and the microbes with which it interacts, and disequilibrium between effector T helper (Th) and regulatory T cells (Treg) leads to impaired immune responses (Noack and Miossec, 2014; Nyirenda et al., 2015; Yousefi et al., 2015). Effector Th cells are derived from progenitor naïve CD4+ T cells via maturational processes

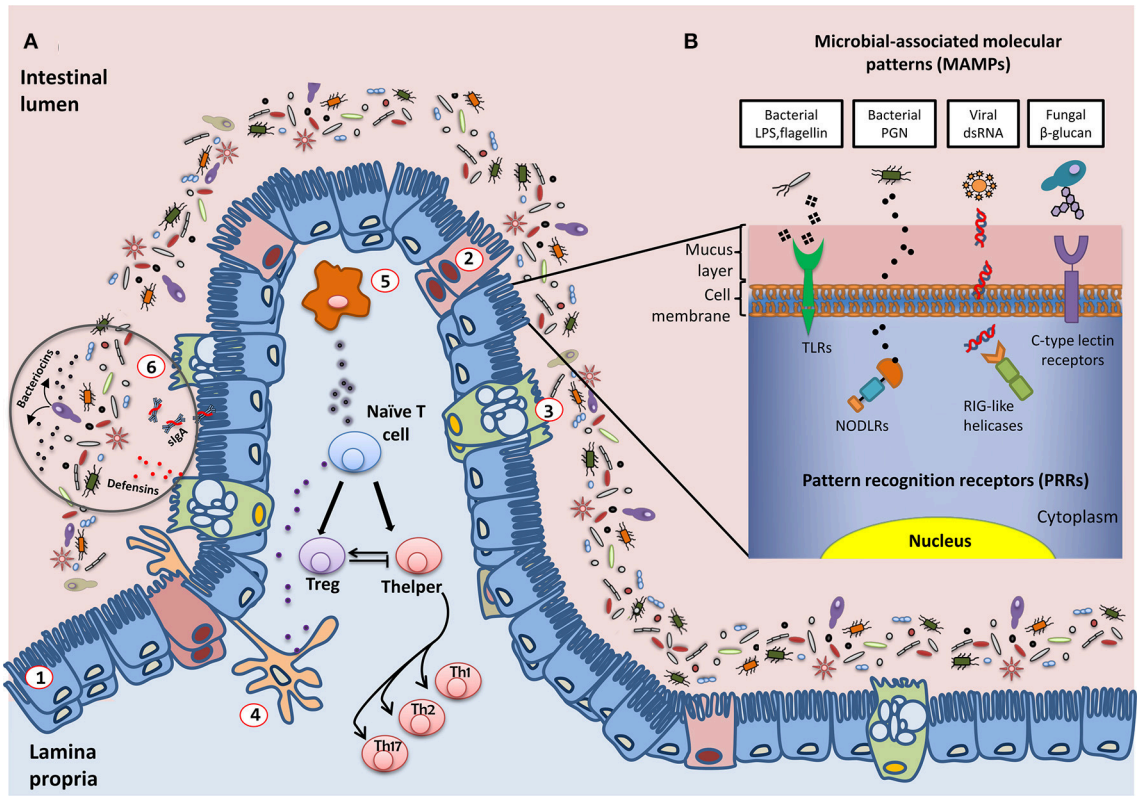

FIGURE 1 | Schematic representation of the interactions established between the intestinal microbiota and the host immune system. (A) General overview of the epithelium in contact with multiple species of microorganisms that constitute the intestinal microbiota: (1) enterocytes; (2) M cells; (3) Goblet cells; antigen presentation cells (APC): (4) dendritic cells and (5) macrophages; (6) defensins, bacteriocins, and secreted IgA (sIgA) also play an important role in controlling the levels of the different populations of microorganisms. A fine-tuned balance of Tcell maturation toward Treg or Thelper cells must be established to assure the tolerogenic response of the host immune system. **(B)** Examples of molecular interactions between microbial antigens and host cells through Pattern Recognition Receptors (PRRs). LPS, lipopolysaccharide; PGN, peptidoglycan; dsRNA, double-strand RNA; TLR, toll-like receptor; NODLR, nucleotide-binding oligomerization domain-like receptors; RIG-like helicases, retinoic acid-inducible gene 1 like helicases.

that are induced by antigenic stimulation. Their function depends on complex interactions with antigen-presenting cells (APCs) in a permissive environment, which is characterized by the antigen type and load, costimulatory molecules, and cytokine signaling. CD4+ T cells may differentiate into different Th phenotypes (mainly Th1, Th2, and Th17) that produce distinct cytokines with different biological functions, or they may evolve into the inducible Treg lineage, which performs immunomodulatory functions (Sakaguchi et al., 2010; Wing and Sakaguchi, 2014). The Th1 subgroup recognizes intracellular pathogens and mainly produces IL-2, interferon (IFN), and tumor necrosis factor alpha (TNFα), thereby supporting typical cellular immunity. Th2 cells, which are essential for eliminating extracellular pathogens such as helminths, express IL-4, IL-5, IL-10, and IL-13, which aid humoral immunity. The Th17 subset, which is involved in fighting Gram-negative bacteria, fungi, and some protozoa, secretes IL-17, which has strong pro-inflammatory effects. Overall, Th responses are accurately balanced to avoid both self-antigen reactivity and excessive reactions to antigens. In fact, dysregulated Th1 responses drive cell-mediated autoimmune disorders, and enhanced Th2 activity is involved in atopy, whereas Th17 cells are probably responsible for chronic tissue inflammation. In contrast, skewing the response away from Treg cells may lead to the onset and/or progression of autoimmune diseases in humans (Eisenstein and Williams, 2009).

PROBIOTICS AND THE IMMUNE SYSTEM

During the last few years, it has been proposed that the intestinal microbiota can be positively modulated by the administration of bacteria or bacterial substrates, and it is likely that, to some extent, this might lead to a significant modulation of the immune system (Dongarrà et al., 2013; Sánchez et al., 2015; Scott et al., 2015). To this end, substantial research efforts are concentrated on using probiotics as potential modulators of gut microbial community. Probiotics are commensal microorganisms that are present in the intestinal tract and in many fermented foods, and they are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). The vast majority of probiotic bacteria are Gram-positive strains, mainly species of the *Lactobacillus* and *Bifidobacterium* genera, although some non-pathogenic strains of *Escherichia coli* and certain yeasts are also considered to be probiotics. Currently, there is an increasing interest in considering some common colonizers of the human gut to be novel probiotics, because of their potential health properties; they are called *emerging probiotics* (Hill et al., 2014; Rodríguez et al., 2015). Some examples are *Faecalibacterium prausnitzii* and *Akkermansia muciniphila.*

Probiotics can exert their beneficial properties in a wide range of ways, including direct cell-to-cell contact in the human gut, by secreting diverse molecules that act as the final mediators of probiotic crosstalk, or through cross-feeding mechanisms. The chemical composition of the molecular effectors is very diverse and consists of proteins that are secreted into the extracellular milieu or localized on the surface of the bacteria, low molecular weight peptides, amino acids, cell-wall polysaccharides or components, bacterial DNA, or SCFAs (Macpherson and Harris, 2004; Turroni et al., 2013). Given the different molecular natures of these molecular effectors, their mechanisms of action are very diverse. Therefore, this review includes only a summary of the molecular bases underlying the immunomodulatory properties of probiotic bacteria (**Figure 2**). In addition, we must consider that genetic differences in the expression of host receptors, the variable composition of the autochthonous microbiota in different individuals, and other host factors that contribute to the response to bacterial signals are likely to explain the variability in responses to probiotics in responding and non-responding individuals (Salonen et al., 2014).

IMMUNOMODULATORY EFFECTORS

A significant number of relevant studies have highlighted the immunomodulatory effects that *Lactobacillus* and *Bifidobacterium* strains exert on the host immune system. For instance, there is evidence that *Bifidobacterium bifidum* LMG13195 and *Bifidobacterium breve* IPLA 20004 enhance intestinal barrier function and preferentially elicit Treg cell differentiation, which induces the expression of anti-inflammatory cytokines, when co-cultured with the human colorectal adenocarcinoma cell line HT29 (López et al., 2012). *Lactobacillus rhamnosus* GG interacts with macrophages in such a way that activated macrophages can discriminate between probiotic and pathogenic bacteria by INF-mediated TLR gene regulation (Miettinen et al., 2008), and the interaction between *Lactobacillus casei* CRL 431 and gut-associated immune cells induces an increase in the number of CD-206 and TLR2 receptors (Aragón et al., 2014).

The mediators of these interactions are largely unknown, although surface and cell-envelope molecules have been identified as some of the main players. Among them, we can distinguish between proteins and other components, such as peptidoglycan (PG), exopolysaccharides (EPS), teichoic acids (TA), and lipoteichoic acids (LTA). Known molecular effectors that mediate immunomodulatory mechanisms are listed in **Table 1**.

Surface Proteins

Cell surface proteins include the S-layer proteins (Slp), which constitute the major surface proteins of some lactobacilli. In *Lactobacillus helveticus* fb213, *Lactobacillus acidophilus* fb116, and *L. acidophilus* fb214, Slp are well studied, and it is likely that they are necessary for lactobacilli survival in the gastrointestinal tract, as they can bind to components of the extracellular matrix, such as collagen and fibronectin, of intestinal cells (Meng et al., 2014; Yadav et al., 2015). Konstantinov and colleagues used an *slpA* knockout mutant of *L. acidophilus* to show that the interaction occurs via the recognition of SlpA by a specific receptor of dendritic cells, denominated DC-SIGN (Konstantinov et al., 2008). Additionally, proteins from structures that are included in the PG layer, such as pili, fimbria, and flagella, are recognized by the host immune

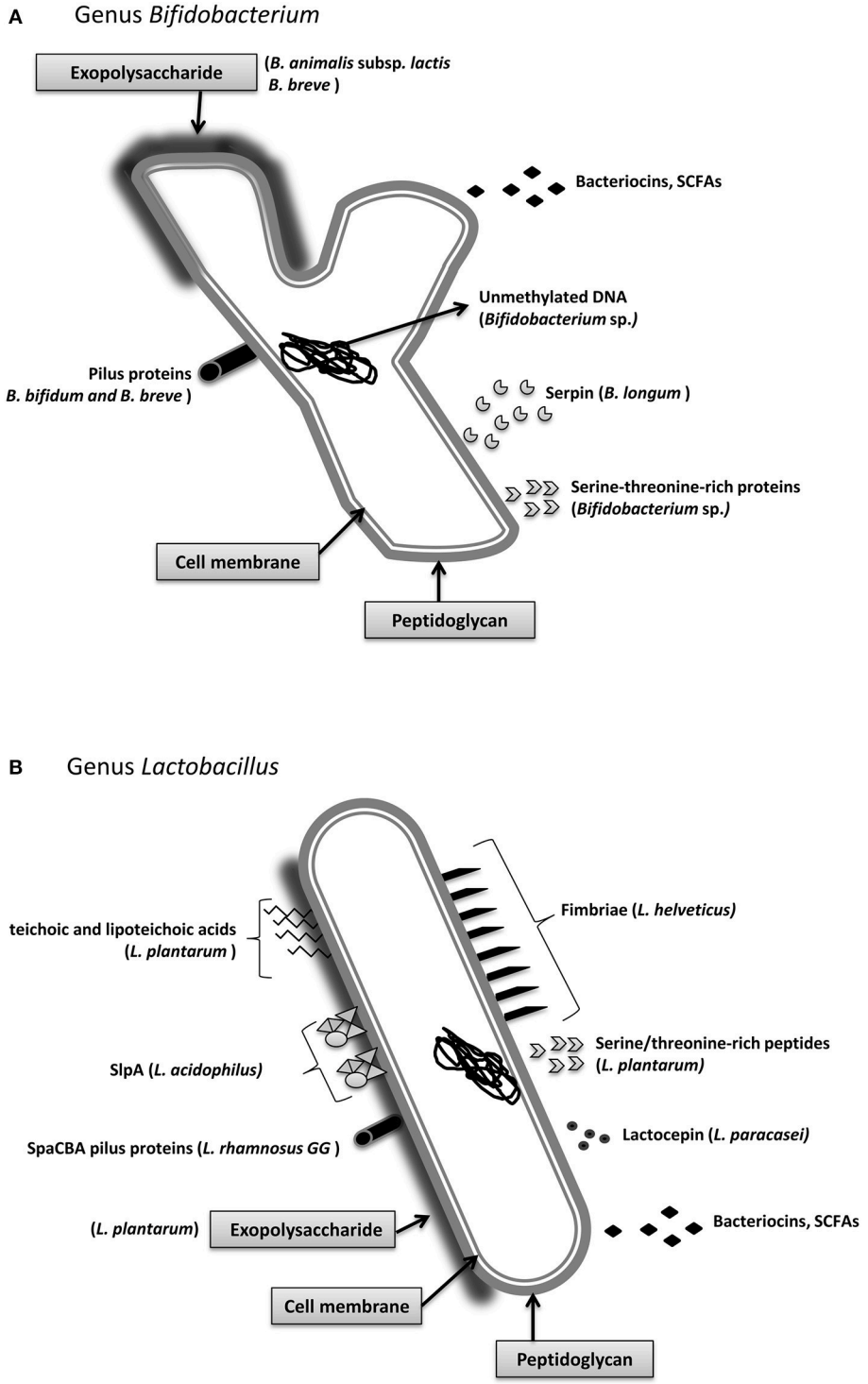

FIGURE 2 | Main molecular effectors that are able to trigger immunomodulatory responses in the host: *Bifidobacterium* (A) and *Lactobacillus* (B). Some of these effectors are species-specific, such as the S-layer protein A from *Lactobacillus acidophilus*, whereas others, such as short chain fatty acids, are secreted by the vast majority of strains. Detailed information about the mechanisms and the molecular effectors is included in Section Immunomodulatory Effectors.

system. Recently, it has been reported that bacterial SpaCBA pilus fibers in *L. rhamnosus* GG may be responsible for its well-known adhesion properties and confer the ability to contact host cells (Reunanen et al., 2012). *B. bifidum* PRL2010 pili have been shown to induce TNF-α production and decrease IL-10 production in the mouse mucosa, as well as to adhere

TABLE 1 | Examples of immunomodulatory effectors produced by classic/emerging probiotics.

Immunomodulatory effector	Species	Probiotic type	Effect on host immune system	References
Surface Layer Protein A (SlpA)	*L. acidophilus*	Classic	Immunomodulation of intestinal dendritic cells	Konstantinov et al., 2008
Pili proteins (SpaCBA)	*L. rhamnosus*	Classic	Contact with mucosal cells	Reunanen et al., 2012
Pili	*B. bifidum*	Classic	Increase TNF-α and decrease IL-10 production	Turroni et al., 2013
	B. breve	Classic	Host colonization	O'Connell Motherway et al., 2011
Fimbriae	*E. coli*	Emerging	Host-colonization	Kleta et al., 2014
	L. plantarum	Classic	Immunomodulation	Murofushi et al., 2015
Serpin	*B. longum*	Classic	Human neutrophil and pancreatic elastase inhibitor	Ivanov et al., 2006
Serine-threonine rich proteins	*Bifidobacterium sp.*	Classic	Intestinal homeostasis	Nezametdinova et al., 2014
	Lactobacillus sp.	Classic	Intestinal homeostasis	Zakharevich et al., 2012
Serine-threonine rich peptide (STp)	*L. plantarum*	Classic	Anti-inflammatory; modulates intestinal dendritic cell function	Bernardo et al., 2012; Al-Hassi et al., 2014
Lactocepin	*L. paracasei*	Classic	Hydrolyzes IP-10	von Schillde et al., 2012
Secreted 15 kDa protein	*F. prausnitzii*	Emerging	Anti-inflammatory	Quévrain et al., 2015
Exopolysaccharides	*B. breve*	Classic	Immunomodulation	Fanning et al., 2012
	*B. lactis**	Classic	Immunomodulation	Hidalgo-Cantabrana et al., 2014
Unmethylated CpG DNA	*Bifidobacterium sp.*	Classic	Induces Th1 response	Ménard et al., 2010
Teichoic/Lipoteichoic acids	*L. plantarum*	Classic	Anti-inflammatory	Grangette et al., 2005
Butyrate	*R. hominis* *F. prausnitzii* *A. muciniphila*	Emerging	Anti-inflammatory	Maslowski et al., 2009

**Synonym of B. animalis subsp. lactis.*

to diverse extracellular matrix proteins (Turroni et al., 2013), while *B. breve* UCC2003 pili are essential for host colonization (O'Connell Motherway et al., 2011). In another recent work, gene complementation studies were used to show that the fimbriae of the probiotic strain *E. coli* Nissle 1917 were involved in the adhesion to porcine intestinal cells, thereby helping to prevent infection with enteropathogenic *E. coli* (EPEC) (Kleta et al., 2014).

Cell Wall Non-proteinaceous Components

Non-proteinaceous cell wall components have different roles in microbe-host crosstalk. It has been shown that the EPS from *Lactobacillus* and *Bifidobacterium* strains can have a modulator role in preventing pathogen invasion, even though the EPS of pathogenic bacteria have been classically viewed as possible virulent factors. Examples of immunomodulatory EPS are those from *B. breve* and *Bifidobacterium animalis* subsp. *lactis* (Fanning et al., 2012; Hidalgo-Cantabrana et al., 2014) or *Lactobacillus plantarum* strains (Murofushi et al., 2015). TAs are linear polymers of ribitol phosphate or glycerol phosphate that are covalently bound to D-alanine, monosaccharides, or amino sugars, and they are attached either to PG (wall TAs) or to the cytoplasmic membrane (membrane TAs or lipoteichoic acids; LTAs). TAs from *L. plantarum* were shown to display anti-inflammatory properties, as shown by the different cytokine production profiles of peripheral blood mononuclear cells (PBMCs) and monocytes exposed to this molecule (Grangette et al., 2005). In addition, mice fed a diet supplemented with *L. plantarum* LTAs or with an LTA-producing strain showed better scores in a colitis model compared with the control group and mice that were fed a *L. plantarum* LTA-deficient strain (Grangette et al., 2005). Although there have been a few promising results, this topic requires further research to clarify the mechanisms of action of the cell wall components of probiotics on the human gut microbiota.

Soluble Compounds

Soluble components that are produced by probiotic bacteria can also affect the bacterial-host interplay. In *Bifidobacterium longum* the secretion of serpin, a serine protease inhibitor, which specifically binds and inactivates human neutrophil and pancreatic elastase, was shown to contribute to gut homeostasis (Ivanov et al., 2006). Additionally, it has been observed that some proteins with characteristic biochemical motifs that are produced by both commensal and pathogenic bacteria can elicit specific functions and affect immune cells of the intestinal lumen. This is the case for a family of serine-threonine rich proteins, which was described in species of *Lactobacillus* and *Bifidobacterium*, with a recently described kinase function (Zakharevich et al., 2012; Nezametdinova et al., 2014). In lactobacilli, a serine-threonine peptide, STp, which is contained in a protein secreted by *L. plantarum*, was shown to be involved in bacterial aggregation (Hevia et al., 2013). Additionally, this peptide can modulate the dendritic cell phenotype of ulcerative colitis (UC) patients (Bernardo et al., 2012; Al-Hassi et al., 2014). It was also demonstrated that the

immunomodulatory effect of *Lactobacillus paracasei* is mediated, at least in part, by the secreted protease lactocepin, which selectively degrades the chemokine IFN-γ-inducible protein 10 (IP-10) that functions in lymphocyte recruitment (von Schillde et al., 2012). There are other examples of non-proteinaceous compounds that can exert certain effects on the host. Some species of *Bifidobacterium* possess unmethylated CpG motifs in their DNA that were able to induce TLR9 activation, which is known to trigger a Th1 orientation of the immune system (Ménard et al., 2010). In contrast, in other studies, it was shown that intragastric and subcutaneous administration of DNA from a probiotic mix ameliorated the severity of colitis in a murine experimental colitis model, whereas a methylated probiotic DNA had no effect (Rachmilewitz et al., 2004).

EMERGING PROBIOTICS, A NOVEL SOURCE OF IMMUNOMODULATORY EFFECTORS

In addition to *Lactobacillus* and *Bifidobacterium*, other microorganisms have received substantial interest among researchers as potentially new, beneficial gut bacteria. Most of them are common colonizers of the human gut under normal conditions. Some of these microbial types are considered to be markers of dysbiosis in intestinal inflammatory diseases, such as UC and Crohn's disease (Manichanh et al., 2006; El Aidy et al., 2013). In these conditions, a loss of microbial diversity and a significant reduction of members of *Clostridium* clusters IV and XIVa have been reported, particularly in bacteria involved in butyrate and propionate metabolism, such as *Ruminococcus*, *Eubacterium*, *Roseburia*, and *Faecalibacterium*. In this section, we will highlight current research on *F. prausnitzii* and *A. muciniphila*, two bacteria that have received much attention during the last few years because of their potential immunomodulatory properties.

F. prausnitzii is a "novel" intestinal bacterium whose immunomodulatory properties have been well characterized *in vitro* and *in vivo*. This anaerobic, Gram-positive bacterium seems to play a role in the maintenance of gut homeostasis, and its population is normally reduced in intestinal inflammatory diseases (Sokol et al., 2008; Cao et al., 2014; Machiels et al., 2014). In 2008, Sokol and colleagues studied the effects of whole bacteria, a cell culture supernatant, bacterial DNA, or membrane-derived fractions *in vitro* using the Caco-2 epithelial colorectal adenocarcinoma cell line and PBMCs, as well as *in vivo* in a mouse model of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis (Sokol et al., 2008). The results showed that *F. prausnitzii* cells exerted anti-inflammatory effects in PBMCs. Furthermore, its culture supernatant reduced IL-8 secretion and abolished the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in Caco-2 cells. Moreover, although no significant improvement of the disease was detected in mice, partial disease scores significantly improved in colitic mice receiving the cell culture supernatants, compared with the non-receiving group. Accordingly, the authors hypothesized that the beneficial effects of *F. prausnitzii* must be executed by a soluble compound that is secreted by the bacteria. In relation to this, recent work showed that *F. prausnitzii* secreted a 15-kDa protein with anti-inflammatory properties. This protein was able to inhibit the NF-κB pathway in intestinal epithelial cells, and it prevented colitis in an animal model (Quévrain et al., 2015). Additional research showed that this bacterium restored physiological parameters and downregulated cytokine profiles in mice with colitis, as well as increased the Treg population to a greater degree than other commensals such as *B. longum* (Qiu et al., 2013; Martín et al., 2015). UC patients have fewer butyrate-producing *Roseburia hominis* and *F. prausnitzii* (Machiels et al., 2014). It is likely that a significant part of their anti-inflammatory action results from the effect of SCFAs in colonocytes, as acetate, propionate, and butyrate modulate the inflammatory responses of immune cells through receptors such as Gpr43 and Gpr41 (Maslowski et al., 2009). However, despite all the information that has recently been discovered about these bacterial groups in healthy and diseased states, and besides butyrate seeming to be the key homeostasis promoter, additional work is required to elucidate the molecular mechanisms through which *F. prausnitzii* interacts with the host gut environment.

A. muciniphila is another common member of the healthy gut microbiota in humans at all stages of age (Collado et al., 2007; Belzer and de Vos, 2012). *A. muciniphila* is a Gram-negative, strictly anaerobic, mucin-degrading microorganism member of the *Verrucomicrobia* phylum, and it was one of the first bacteria shown to utilize mucin, the glycosylated protein layer that covers the gut epithelium, as its sole carbon, nitrogen, and sulfur source. The products derived from mucin degradation are mainly SCFAs that feed colonocyte metabolism and confer health properties to the host. By degrading the mucin of the external mucus layer, *A. muciniphila* helps with the continuous renovation of the protective cover of the mucosae, and it maintains a healthy protective barrier that prevents the entrance of enteropathogens into the epithelium (Lukovac et al., 2014). In addition, when *A. muciniphila* was administered to mice, there were increased intestinal levels of endocannabinoids that control inflammation, the gut barrier, and gut peptide secretion, suggesting an immunomodulatory role for this bacterium (Everard et al., 2013).

CONCLUDING REMARKS

In conclusion, even though much effort has been put into probiotic research during recent decades, the mechanisms underlying the immunomodulatory effects of beneficial intestinal bacteria have scarcely been elucidated. There is compelling evidence that novel bacterial players, other than *Lactobacillus* and *Bifidobacterium*, could play a role in these processes and are much more important than previously thought; however, difficulties in growing some of these bacteria on laboratory- and industrial-scales, and the lack of molecular tools needed to perform functional genomic analyses, seriously hamper the characterization of novel strains. Further research is needed to overcome these culturing and functional characterization difficulties to perform well-designed pre-clinical

and intervention studies that shed new light on the mechanisms responsible for the beneficial effects attributed to these bacteria.

AUTHOR CONTRIBUTIONS

AH, SD, BS, and AM contributed to the conception and design of the work, and to the acquisition, analysis, and interpretation of the data. All authors contributed to the drafting of the manuscript and approved the final version to be published.

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

CAPÍTULO 2

Desarrollo de técnicas de estudio de la microbiota intestinal: aplicación de gradientes de densidad para el aislamiento de microbiota fecal humana.

En este segundo capítulo de la tesis se describe la optimización de la metodología para el análisis de la microbiota fecal, incluyendo su extracción del resto de componentes de las heces. En la mayor parte de estudios de metagenómica no se realiza una separación previa de las células bacterianas, sino que se procesa el conjunto y se extrae el ADN para identificar las distintas poblaciones de microorganismos. Sin embargo a lo largo del desarrollo de esta Tesis doctoral se plantearon diversos experimentos que hicieron necesario obtener una microbiota aislada que fuera viable y representativa de la muestra de heces del donante.

La metodología presentada en este capítulo demuestra que es posible trabajar con microbiota aislada de una muestra fecal, sin perjuicio significativo de las poblaciones microbianas mayoritarias, mediante un sencillo protocolo de centrifugación en gradiente de densidad. Este protocolo se probó en 5 muestras fecales de individuos con LES y en 3 de controles sanos, con y sin aislamiento previo de su microbiota, y mediante metagenómica filogenética (gen del ADN ribosómico 16S) se comprobó que la microbiota extraída era representativa de la microbiota original, manteniendo además su viabilidad tal y como se demostró mediante el conteo absoluto de células vivas y muertas por citometría de flujo.

Los resultados de este capítulo se presentan en forma de un artículo publicado:

- **Hevia A**, Delgado S, Margolles A, Sánchez B. Application of density gradient for the isolation of the fecal microbial stool component and the potential use thereof. Scientific Reports, 2015; 5:16807.

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Application of density gradient for the isolation of the fecal microbial stool component and the potential use thereof

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The idea of considering the gut microbiota as a virtual human organ has led to the concept of fecal microbiota transplantation (FMT), which has recently been extremely successful in the treatment of cases of recurrent *Clostridium difficile* infection. Administration of safe, viable, and representative fecal microbiota is crucial for FMT. To our knowledge, suitable techniques and systematic conditions for separating the fecal microbiota from stool samples have not been thoroughly investigated. In this work we show the potential to separate stool microorganisms from the rest of fecal material using a procedure with a Nycodenz® density gradient, yielding 10^{10} viable bacteria per two grams of feces. This procedure did not affect the original microbiota composition in terms of viability, distribution and proportions, as assessed by a phylogenetic metagenomic approach. Obtaining the fecal microbiota by concentration and separation of the microorganisms from the rest of the stool components would allow the standardization of its recovery and its long-term preservation. FMT or similar microbiota restoration therapies could be used for the treatment of several disorders, or even for aesthetic purposes, so the method described in our work may contribute to the setting of the basis for the development of safe and standardized products.

The human gastrointestinal tract (GI) is a complex ecosystem in which the resident microbiota and nutrients continuously interact with host cells[1]. Gut microbiota is composed of trillions of bacteria, outnumbering the eukaryotic cells of our body in one order of magnitude[2], and the idea of considering our intestinal microbiota as a virtual organ is gaining popularity among the scientific community[3]. Genes provided by our gut microbiota are denominated 'gut microbiome', but sometimes the term 'human microbiome' (theoretically the gene complement of all the microbes inhabiting our body) is used as a synonym[4]. Accounting for nearly 10,000,000 unique genes, notably greater than the "modest" number of 21,000 human genes[5,6], our gut microbiota complement metabolic attributes that are absent in our organism, including the ability to take advantage of otherwise non-metabolizable nutrients, the production of short-chain fatty acids or vitamins, and many others[7]. On the contrary, the human host provides microbiota with nutrients *i.e.*, our GI is a kind of microbial garden where each individual farms its own beneficial microbes.

During the last few years the great advance of high-throughput sequencing technologies and their application in the study of gut microbial communities, is providing growing evidence that gut microbiota has an important impact in the successful maturing of our immune system and in several facets of human physiology, which may include the triggering, progression and establishment of several diseases. Not only are the gut microbiota profiles affected by diet[8,9], age[10], or geography[11], alteration of our intestinal microorganism composition has been linked to some gut and autoimmune disorders such as

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obesity[12], metabolic syndrome[13], rheumatoid arthritis[14], type-1 diabetes[15], inflammatory bowel diseases[16], and systemic lupus erythematosus (SLE)[17].

If we agree that the human microbiota is one more of our organs, the concept of fecal microbiota transplantation (FMT) will quickly arise. In scientific literature, FMT was first described in the late 1950s[18], and can be defined as the 'delivering of processed stools from a healthy individual to the gut of a sick person through enema, colonoscopy or other means'[19]. Notably, FMT had an impressive efficacy (more than 90% success in some cases) in the displacement of recurrent *Clostridium difficile* infection from the intestine of affected individuals who were not responding to antibiotic therapy, and in the re-establishment of a balanced gut microbiota[20]. Very recently, FMT was successfully applied to treat antibiotic induced colitis[21]. Generalization of unregulated FMT in certain populations led the US Food and Drug Administration (FDA) to strictly regulate faeces as a biological drug[22].

These properties of FMT on human gut health have had a high impact in society, including thousands of entries on well-known social networks and blogs, as well as the creation of foundations and other non-profit organizations dedicated to the promotion of FMT application (*e.g.* http://thefecaltransplantfoundation.org/, http://thepowerofpoop.com/, http://www.openbiome.org/). Current methodology for FMT includes processing of fresh donor feces in the same day. However, some protocols have been published in order to separate the intestinal microbiota from the rest of fecal material by microfiltration, allowing for instance its storage[23]. Following this protocol, the intestinal microbiota are firstly microfiltered in the presence of a cryoprotectant and then frozen at −80 °C. This microbial preparation has been shown as effective as a fresh feces preparation for the displacement of *Clostridium difficile*, as evidenced by 16S rRNA gene profiling[24].

Potential applications of FMT, other than recurrent *C. difficile* infections, are numerous but deserve studies on the normalization and standardization of what is the healthy fecal microbiota. Firstly, extracting the microbiota and its separation from the rest of the stool material under controlled conditions could serve to avoid the unappealing nature of feces. Secondly, this could allow the long-term preservation of fecal microbiota, allowing its propagation in bioreactors, even many years after its extraction. In addition, it will facilitate tasks such as stool screening for viruses (HIV, hepatitis and others), parasites and other undesirable microorganisms.

In this work, we describe the application of a fecal microbiota separation procedure by the use of a density gradient. Using 16S rDNA metagenomic profiling we confirmed that the overall microbial community structure remained unaltered after being separated from the stools. Potential applications for this method for the long-term preservation of the intestinal microbiota are also discussed.

Materials and Methods

Ethical Statement. Ethics approval for this study was obtained within the framework of the project AGL2010–14952, from the Spanish Ministry of Economy and Competitiveness ("Towards a better understanding of gut microbiota functionality in some immune disorders"). Final approval was obtained from the Bioethics Committee of CSIC (Consejo Superior de Investigaciones Científicas) and from the Regional Ethics Committee for Clinical Research (Servicio de Salud del Principado de Asturias) in compliance with the Declaration of Helsinki. All determinations were performed with fully informed written consent from all participants involved in the study.

Study subjects. Stool samples from this study were obtained from five Systemic Lupus Erythematosus (SLE) patients and three healthy controls, selected from a previous study in which the gut microbiota dysbiosis associated with SLE was described[17]. Detailed clinical and nutritional data from those participants can be retrieved in the above mentioned study. SLE patients not used antibiotics, glucocorticoids, immunosuppressive drugs, monoclonal antibodies or other immunotherapies during the 6 months prior to sample collection.

Stool samples, microbiota separation and DNA extraction. A part of each stool sample was submitted to a density gradient in order to separate the microbiota from the rest of the fecal material, according to the method of Courtois and colleagues with some modifications[25]. Two grams of feces were homogenized in 18 mL of sterile NaCl 0.9% (w/v), in a laboratory paddle blender (Stomacher Lab Blender 400, Seward Ltd. UK) for 1 min. A solution of Nycodenz® 80% (w/v) (PROGEN Biotechnik GmbH, Heidelberg, Denmark) was prepared in ultrapure water, and sterilized at 121 °C for 15 min. A volume of 10.5 mL of the diluted, homogenized fecal sample was placed on top of 3.5 mL of the Nycodenz® solution, and centrifuged for 40 min at 4 °C (10,000 × g, TST41.14 rotor, Kontron, Milan, Italy). The upper phase, containing soluble debris, was discarded after the centrifugation step, and the layers corresponding to the microbiota extracted with 10.5 mL of PBS (Fig. 1) were collected. Cellular suspensions were kept on ice for 5 minutes, in order to allow non-soluble debris to precipitate, were then washed twice, and stored in aliquots of 1 mL, at −80 °C, until DNA extraction was performed. In all the cases, DNA directly from homogenized stool samples, or from the corresponding separated microbiota fractions was extracted using the QIAamp DNA Stool Mini kit (Qiagen Ltd., Strasse, Germany), as described in a previous work[26].

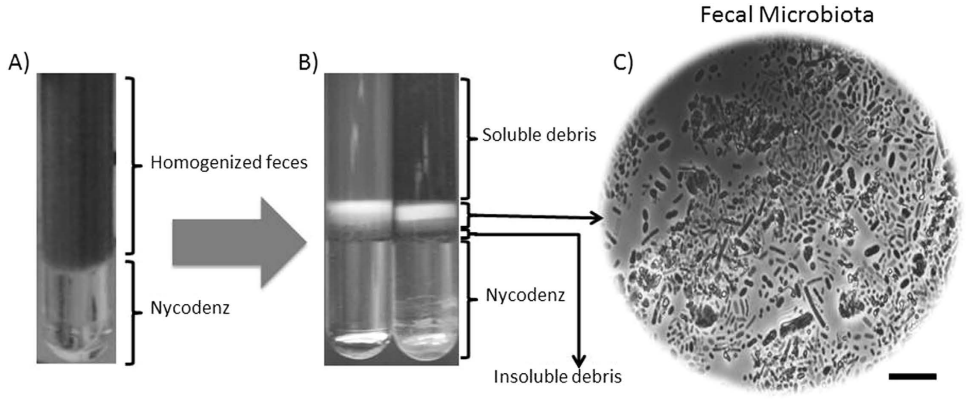

Figure 1. Workflow of the experimental setup used in this work. (**A**) Diluted homogenized fecal samples were loaded on top of a Nycodenz® solution, as described in the material and methods section. (**B**) After centrifugation four layers were formed. Examination of the layer content in a phase-contrast microscope allowed us to determine the presence of one layer, corresponding to the fecal microbiota, between two layers containing soluble (upper) and insoluble (lower) fecal debris all above the Nycodenz. (**C**) Light photography of the microbiota layer, showing a high diversity of microbial sizes and shapes. Bar, 10 μm.

Efficiency and yield of the microbiota separation procedure. In order to evaluate the yield of the microbiota extraction and establish the viability of the microbiota recovered, three additional fecal samples from three healthy donors were analyzed by flow cytometry before and after density gradient extraction. For enumeration of bacteria the samples were measured using a flow cytometer (Cytomics FC500, Beckman-Coulter Inc., Miami, Florida, USA) with the Bacteria counting kit (Invitrogen™, Life Technologies, Thermo Fisher Scientific, Waltham, MA). The absolute counting values in the samples were determined taking a minimum of 2,000 and a maximum of 10,000 fluorescent standard beads, and according to the analysis of the areas corresponding to beads: alive bacteria (stained with Syto9) and dead bacteria (stained with propidium iodide, Sigma, St. Louis, MO). The trigger signal was established at side scatter (SSC) detector (as recommended by the Bacteria counting kit, Invitrogen) and fluorescence signals were collected at FL1 detector (510–550 nm) for Syto9 and FL4 detector (660–700 nm) for propidium iodide. Microfiltered PBS was used as negative control. Additionally, as control of dead microbiota, we treated an aliquot of the fecal samples at 98 °C for 10 min plus 15 min under UV light exposition. The viability of microbiota in each sample was calculated as the percentage of live bacteria within the fecal microbiota before and after the Nycodenz® extraction procedure. The absolute number of bacteria was calculated using the fluorescent beads as internal standard in each sample, following the supplier's recommendations for ratiometric counting. Relative concentrations were expressed as the absolute number of bacteria in relation to grams of total dry fecal matter, which was determined according to FIL-IDF standards[27,28].

16 sRNA gene profiling analysis. Partial 16S rRNA gene amplicons were obtained with primers Probio_Uni and Probio_Rev (targeting the V3 and V4 region) by PCR as described in previous works of our research group[17,26]. Sequence libraries using the Ion Sequencing 200 kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA), were prepared from the purified PCR products and sequenced in an Ion Torrent PGM system at the GenProbio Ltd facilities (http://www.genprobio.com). After sequencing, specific sequence read groups such as low quality and polyclonal sequences were removed by the PGM software. Sequences matching the PGM 3′ adaptor were also automatically trimmed. All PGM quality-approved, trimmed and filtered data were exported as .sff files.

The .sff files were processed using QIIME 1.7.0 with the scripts and procedures described in previous works[26,29]. Only sequence reads with a length of between 150 and 200 bp, as well as with a mean sequence quality score higher than 25 were retained as part of the quality control. Sequences were trimmed at the first base if a low quality rolling 10 bp window was found, and other sequences such as homopolymers (>7 bp), or sequences with mismatched primers were omitted. In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at $\geq$97% sequence homology and chimeric sequences were removed using Chimera Slayer[30]. All reads were classified to the lowest possible taxonomic rank using QIIME and a reference dataset from GreenGenes (version 13.5, May 2013, http://greengenes.secondgenome.com), but in general family level was the lowest taxonomic unit considered throughout the study. OTUs were assigned using uclust by using the script pick_de_novo_otus.py provided with QIIME and exported in BIOM format for downstream analyses[31]. Different alpha diversity metrics (Chao, Observed Species, Shannon and Simpson) were calculated from the BIOM formatted tables using the alpha_diversity.py script provided by QIIME.

Statistical analysis. 16S rRNA gene profiles before or after density gradient extraction were evaluated at four taxonomic levels (Phylum, Class, Order and Family). Samples were ordered according to their microbial profiles using three different and unsupervised multivariate analysis, Principal Component Analysis (PCA), Principal Coordinate Analysis (PCO) and Correspondence Analysis (CA), implemented in the software PAST v3.0[32]. After ordering, samples were classified according to the sample type used for DNA extraction (feces versus microbiota separated on Nycodenz® density gradient). Different statistical tests were conducted on the multivariate data, including One-way ANOSIM and One-way PERMANOVA, each one with 9,999 permutations. In order to assess differences in single taxonomic groups, OTU tables in BIOM format were collapsed at the four taxonomic levels, exported in tab-delimited text format and analyzed using STAMP v2.0.3[33]. Association of taxa to the sample type used for DNA extraction was assessed by running two-sided Welch's tests on every pair of means. The False Discovery Rate correction[34] was finally applied and significant differences in taxa between the two experimental conditions were only considered below a p-value of 0.05 and a q-value below 0.2, as in previous works[17,35]. Finally, a similarity matrix using the Jaccard index was obtained for all the samples at the family level using PAST v3.0. Similarities between samples were represented in dendrograms built with the Simple Linkage method or with the Neighbour Joining algorithm (using 9,999 repetitions).

Results and Discussion

During the last few years the growing interest in understanding the human gut microbiota composition has led to a greater knowledge on how these microbial populations may be altered in the framework of certain diseases, notably those with autoimmune or inflammatory components. Gut microbiota is starting to be considered as a dynamic organ of the human body and, as such, susceptible to be transplanted for therapeutic purposes. In all the reported routes and means of administration of FMT the fecal material (fresh or frozen) is diluted in a saline solution, or lyophilized, usually under non-controlled atmospheres.

Separation of microbes from feces using density gradient based methodologies is not a novel concept, as it has been used to separate bacteria from soil since the 1970s. The first separation protocols consisted on repeated blending-centrifugation steps in different buffers and salt solutions[36,37]. Later on, bacterial separation by centrifugation on modified sucrose gradients[38], or by passage through a cation exchange resin (Jacobsen and Rasmussen, 1992) were proposed[39]. The major limitation of those protocols is that they are time consuming and therefore difficult to implement in routine analysis; this limitation is overcome using Nycodenz resine[25], although no data on bacterial viability is given. In the framework of FMT, separating the GI microbiota from the rest of fecal material offers the advantage of reducing the hygienic concerns due to the unappealing nature of feces.

In this work eight fecal samples from a previous research work addressing the intestinal dysbiosis in the framework of SLE[17], were chosen. These samples were divergent regarding bacterial diversity as reflected in the different values of the Firmicutes to Bacteroidetes ratio (FBR, lower in SLE patients with respect to healthy controls; HC4 = 8.6, HC32 = 8.8, HC33 = 4.5, SLE2 = 1.6, SLE12 = 1.0, SLE13 = 1.6, SLE21 = 1.2, SLE22 = 0.3). Interestingly, changes in FBR have been observed in certain human disorders such as Crohn's disease, human type 2 diabetes, or obesity. The rationale underlying this choice was to assess whether our extraction method could interfere in samples with different FBRs.

In our approach, a homogenized fecal dilution was loaded on top of an 80% w/v Nycodenz® solution (Fig. 1A), and centrifuged at 10,000 × g. This differed from the approach of Rooijers *et al.*[40], which followed the methodology of Murayama *et al.*[41], with different Nycodenz® gradient preparation and different relative centrifuge force values. Microbiota was separated from the rest of the fecal material in a single centrifugation step. As can be seen in Fig. 1B, two layers corresponding to the microbial biomass were observed in the top of the insoluble debris layer. This debris facilitated the task of microorganism recovery once the upper phase of soluble debris had been removed, as it offered a physical barrier avoiding the mixing of the resuspended microbiota with the lower Nycodenz® layer. The different layers were submitted to contrast phase microscopy, and the vast majority of microorganisms were observed in the above mentioned two layers (Fig. 1C).

The approach employed to evaluate the efficiency of the Nycodenz® extraction procedure showed that the viability of fecal microbiota separated with the density gradient was maintained to a good extent as compared with the fresh fecal microbiota (Suppl. Fig. 1). On average, in the microbiota recovered after the Nycodenz® treatment 66.9% of the bacteria were still alive, with values ranging between 71.3 and 60.6% (66.9 ± 5.6) among the three fecal samples analyzed, meanwhile in fresh feces the viability was estimated to range between 85.6–49.9% (68.6 ± 17.9). This gives an idea of the good efficiency of the methodology proposed for the concentration and isolation of viable microbiota, regardless the variation of feces in terms of humidity and fiber content. The concentrations of live bacteria in the three samples analyzed by flow cytometry ranged between 3.2×10^9 and 7.2×10^9 ($5.2 \pm 2.0 \times 10^9$) bacteria/gram of fecal dry matter in the original samples of fresh feces, and between 5.7×10^9 and 9.0×10^9 ($7.0 \pm 1.8 \times 10^9$) per gram of feces after density gradient extraction. This means that all the viable bacteria are extracted from the fecal material, with a yield of around 10^{10} viable bacteria per two grams of fecal sample. No significant differences were found in mean concentrations before and after the treatment. Thus, our results showed minimal variability in the viable microbiota recovered among different individual donors, and no impact of the isolation protocol over the integrity of the fecal bacteria.

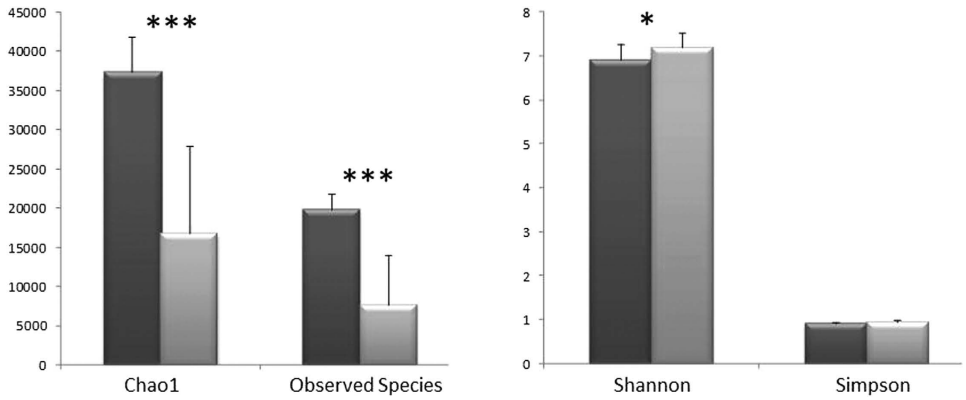

Figure 2. Different alpha diversity indices obtained from the stool samples before (dark gray) or after (light gray) microbiota separation by density gradient. Bars represent the Mean ± Standard Deviation. (*$p < 0.05$; ***$p < 0.001$).

The analysis of the microbiota fractions extracted using the density gradient resulted in an overall decrease of diversity when compared to the results obtained from the DNA extracted directly from feces. This was true when alpha-diversity indices that take into account only species richness (Chao 1, Observed Species) were calculated, but the contrary was observed when using the Shannon index, which takes into account species evenness (Shannon) (Fig. 2). No difference in alpha-diversity was detected using the Simpson index. Overall, this means that although the number of recovered OTUs is lower after Nycodenz® extraction, the proportions between them did not necessarily vary during the extraction process. Care should be taken in the sense that reductions in alpha-diversity might affect FMT effectiveness, as precise bacterial groups important to the balance of dysbiosis could be lost. In this way, further research is needed to determine if the reduction in OTUs/species could be in part due to oxygen exposure during manipulation of the microbiota separation in the density gradient. It is possible that particular microbial types, more susceptible to oxygen, may be protected if the fecal microbiota extraction is performed under strict anaerobic conditions. It will be also interesting to elucidate if Nycodenz® gradients are helpful in selectively removing undesirable molecules/microorganisms from the feces, such as toxins, prions and viruses.

In order to know whether the differences observed in microbial diversity between the samples, with or without Nycodenz® extraction, could be used for clustering purposes, samples were ordered using the taxonomic composition at the Phylum or Family level through different methodologies: Principal Components Analysis (PCA), Principal Coordinates Analysis (PCoA) and Correspondence Analysis (CA) (Fig. 3). When used in an unbiased way, i.e. without providing information on the source of the different microbial profiles, all of the ordering methods were able to cluster samples into separated groups (feces vs extracted microbiota) (Fig. 3). Absence of effect of the extraction method was sustained statistically, *a posteriori*, with the use of non-parametric tests such as One-way ANOSIM and One-way PERMANOVA. In both cases, samples were firstly classified according to their origin and their similarities measured according to Euclidean distances. The p-values obtained did not support the classification of samples into two groups (feces vs extracted microbiota; One-way ANOSIM; p-value 0.733; One-way PERMANOVA; p-value 0.353).

In order to further study the effect of microbial separation by density gradient on the 16S RNA gene profiling, a similarity matrix using the relative family abundances was built by calculating Jaccard distances, a method already used in other metagenomic studies[42]. Samples were clustered using those inter-sample distances according to the Simple Linkage method, or through the Neighbour Joining algorithm, and the corresponding dendrograms obtained (Fig. 4). In both cases, samples in which the DNA was extracted after microbiota separation, clustered with their corresponding feces samples, with the exception of samples LS12 and HD33. In these samples, the effect of the microbiota extraction over the metagenomic profiles was higher, with some groups showing drastic changes at the phylum or family levels (Suppl. Fig. 2). These results confirmed that, as a general rule, the microbial communities extracted using the density gradient centrifugation procedure are representative of those present in the original stool sample.

Separation of microorganisms using a Nicodenz® density gradient was first introduced for the isolation of bacteria from soil by Lindahl and Bakken[43]. This method has also been successfully applied in other biological systems, such as in the description of the intestinal metagenome of the red palm weevil (*Rhynchophorus ferrugineus*)[44], or in the gene expression assessment in dairy matrices[45]. Separating bacteria from certain matrix compounds may be very useful for downstream molecular biology applications, as this step removes many of the components inhibiting PCR, such as humic compounds, or colored substances interfering with blot hybridization protocols[46].

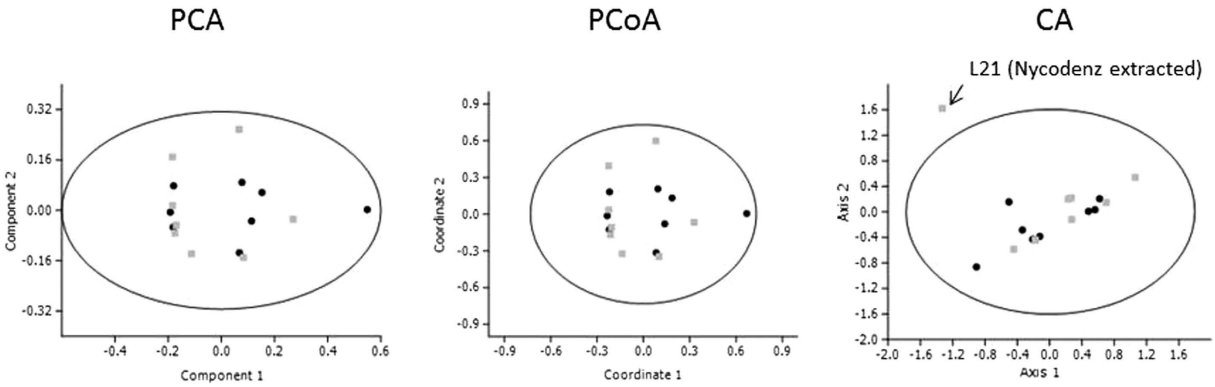

Figure 3. Different non-biased multivariate ordering methodologies were used in order to determine whether the microbial populations obtained directly from homogenized stool samples (black dots), or from the separated fecal microbiota (gray squares) clustered apart according to their composition. Ellipses represent the estimated region where 95% of population points were expected to fall. Analyses were performed at the Phylum and Family levels. PCA: Principal Component Analysis; PCoA, Principal Coordinates Analysis; CA, Correspondence Analysis.

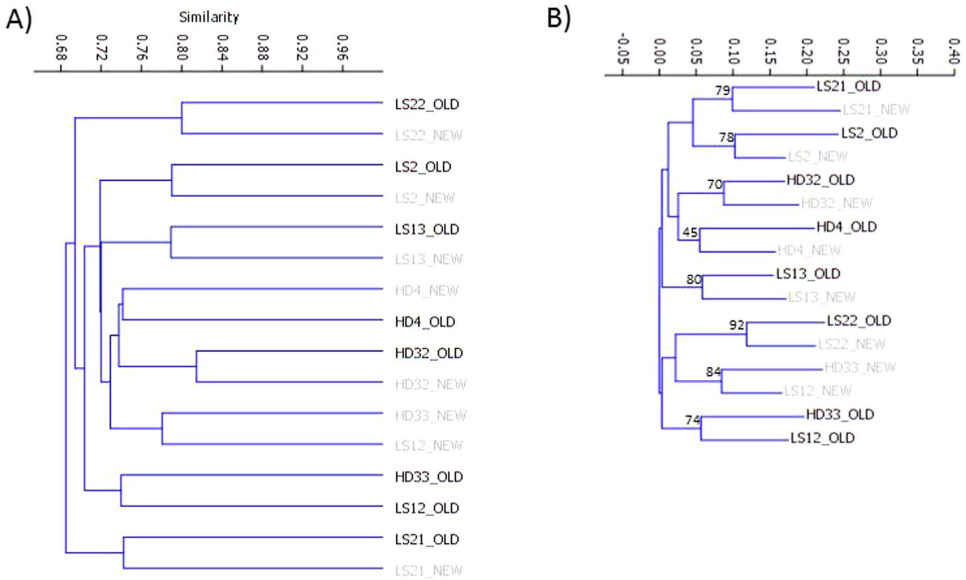

Figure 4. Dendrograms showing similarity of samples according to Jaccard distances; (**A**) clustering through simple linkage; (**B**) clustering using to Neigbour Joining with branch support (10,000 repetitions). NEW and OLD suffixes denote samples where microbiota was or was not extracted in the density gradient, respectively, prior to DNA extraction.

In our work, the application of this methodology was shown to not affect the global variability of the extracted microbiota, and the diversity of bacteria extracted directly from the soil or following Nycodenz® gradient was not significantly different, with the exception of γ-Proteobacteria[25]. However, some taxonomic groups showed significant variations according to the gradient extraction when the totality of the samples was grouped and analyzed (Table 1). In general, 16S rRNA gene profiles from samples in which microbiota was extracted in the Nycodenz® gradient were characterized for higher relative abundances of the Firmicutes phylum, this being due to higher recoveries of the Clostridiales order (Suppl. Fig. 3). Several groups form the Beta, Delta, and Epsilon divisions of the Proteobacteria phyla also showed significant variations according to treatment, although to a lesser extent.

In general, the methodology presented in this work offers a simple and straight-forward method to extract and separate the fecal microbiota from the rest of stool components, allowing further improvements such as performing this process under controlled atmospheric conditions. In addition, this extraction step may eliminate some undesirable compounds of the feces, but this deserves further research. Our approach could be of use in obtaining representative intestinal microbiota free of stool material for

Taxon	Faeces Mean[a] ± SD[b]	Gradient Mean[c]	SD[d]	p[e]	FDR[f]
Phylum					
Firmicutes	58.98 ± 16.17	78.94	13.82	0.02	0.14
Proteobacteria	1.87 ± 1.32	0.18	0.19	0.01	0.11
Class					
Clostridia	57.87 ± 16.00	77.43	13.49	0.02	0.20
Erysipelotrichi	0.29 ± 0.20	0.05	0.03	0.01	0.16
Betaproteobacteria	0.78 ± 0.86	0.01	0.01	0.04	0.17
Deltaproteobacteria	0.13 ± 0.07	0.07	0.04	0.04	0.16
Epsilonproteobacteria	0.00[g] ± 0.00	0.00	0.00	0.02	0.19
Unclassified Proteobacteria Class	0.01 ± 0.01	0.00	0.00	0.03	0.18
Opitutae	0.18 ± 0.20	0.00	0.00	0.03	0.19
Order					
Unclassified Bacteroidetes Order	0.05 ± 0.02	0.03	0.02	0.01	0.01
Clostridiales	57.60 ± 15.90	77.12	13.44	0.02	0.02
Erysipelotrichales	0.29 ± 0.20	0.05	0.03	0.01	0.01
Burkholderiales	0.76 ± 0.84	0.01	0.01	0.04	0.04
Desulfovibrionales	0.13 ± 0.07	0.06	0.04	0.04	0.04
Unclassified Proteobacteria Order	0.01 ± 0.01	0.00	0.00	0.03	0.03
Cerasicoccales	0.16 ± 0.17	0.00	0.00	0.03	0.03
Family					
Odoribacteraceae	0.25 ± 0.11	0.05	0.04	0.00	0.10
Unclassified Clostridiales Family	3.35 ± 1.44	6.57	2.10	0.00	0.16

Table 1. Taxons showing statistical differences in their relative abundances when comparing direct DNA extraction from faeces or after density gradient separation of the microbiota. [a]Original: mean rel. freq. (%). [b]Original: std. dev. (%). [c]Gradient extracted: mean rel. freq. (%). [d]Gradient extracted: std. dev. (%). [e]P-values. [f]False Discovery Rate (30). [g]values > 0.001.

long-term storage purposes. This might also be helpful as the first step for preserving the overall microbial communities, and used in the near future in the design of microbiota-based products/vehicles for FMT and novel intestinal restoration bio-therapies. However, further improvements of this method are needed as, for instance, some *Clostridium*-related OTUs were significantly affected by the Nycodenz® extraction, and some members of this genus such as *C. scindens* can be relevant in the CDI treatment[47]. In addition, animal experiments are needed in order to show that microbiota extracted following this method is effectively engrafted in the host.

Interestingly, this protocol can be scaled up allowing the processing of larger fecal amounts with centrifugal devices with higher capacity. For instance, 430 grams of fecal material can be processed using swinging rotors (up to $30{,}000 \times g$) allocating 4×1000 mL buckets, with an expected yield of 10^{12} viable bacteria.

To sum up, obtaining the representative microbiota from the feces of a healthy donor using the Nycodenz® density gradient described in this work would allow the concentration of intestinal microbiota and keep it separated from the rest of the stool components, whilst maintaining high viability levels. On one hand, Nycodenz® is a safe molecule, in terms of human toxicity, which can easily be removed from samples in the process of microbiota extraction. With respect to other molecules used for density gradient isolation, the X-ray dense-compound Nycodenz® shows advantages, such as a non-inhibitory effect on the activity of most enzymes, compatibility to protein determination assays and, what is relevant for the purpose of this paper, it shows a low toxicity in human beings[41]. On the other hand, density gradient allows for the recovery of representative and viable fecal microbiota and the suppression of non-desirable microorganisms/compounds, as well as facilitating the testing of the samples for hazardous agents. This might allow the development of downstream applications such as microbiota-based therapeutic strategies for microbial intestinal restoration, both in the framework of a given disease or for other applications.

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Author Contributions

A.M. and B.S. conceived the research. A.H., S.D. and B.S. performed the experiments and wrote the main manuscript text. All authors reviewed the manuscript.

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CAPITULO 3

Caracterización de la microbiota intestinal de individuos con trastornos inmunológicos.

En este capítulo de la tesis se aplicó la metodología optimizada en el capítulo anterior para abordar la caracterización de la microbiota intestinal en poblaciones específicas afectadas con dos patologías típicas de humanos: una de naturaleza autoinmune, el lupus eritematoso sistémico (LES), y otra no autoinmune, la alergia respiratoria o asma.

En el caso del LES existían en la bibliografía científica indicios de la implicación de distintos microorganismos en la patología de la enfermedad, pero hasta el momento de realización de esta tesis no había sido claramente establecida la disbiosis en la microbiota intestinal de este colectivo de pacientes. Por ello los resultados presentados en este capítulo son novedosos y establecen una base de trabajo sobre la que se podría trabajar para diseñar terapias nutricionales como diana de actuación para mejorar el tratamiento del LES, enfermedad para la que actualmente no existe cura y que se trata con distintos fármacos inmunosupresores.

Además de esta caracterización poblacional, se realizó una inferencia funcional a partir de los perfiles metagenómicos, lo cual dio como resultados más destacables la presencia de procesos metabólicos potencialmente asociados con pacientes de LES, en concreto una sobre-representación de rutas relacionadas con el metabolismo de glicanos y de procesos metabólicos de fosforilación oxidativa.

En el trabajo de la alergia respiratoria se determinó también el perfil filogenético de la microbiota intestinal de los individuos, y como resultado obtuvimos diferencias significativas en una especie de bifidobacteria, *Bifidobacterium adolescentis,* cuyos niveles disminuyeron a medida que aumentaba el tiempo de diagnóstico de la enfermedad. Si bien no pudimos validar estos resultados mediante otra técnica, se podría tomar como diana esta especie bacteriana en

estudios futuros para intentar modular la microbiota de este colectivo.

Para poder llevar a cabo estos dos trabajos contamos con la colaboración de instituciones en el ámbito sanitario: la Unidad de Alergología del Hospital Universitario Central de Asturias, y la Asociación de Lúpicos de Asturias. También contamos con la colaboración de otras instituciones académicas como el Departamento de Fisiología Animal de la Universidad de Oviedo, y de la Universidad de Parma en Italia para la secuenciación del ADN.

Los resultados que conforman este capítulo se presentan como dos manuscritos ya publicados:

- **Hevia A**, Milani C, López P, Cuervo A, Arboleya S, Duranti S, Turroni F, González S, Suárez A, Gueimonde M, Ventura M, Sánchez B, Margolles A. Intestinal Dysbiosis Associated with Systemic Lupus Erythematosus. MBio, 2014; 5:e01548-14.
- **Hevia A**, Milani C, López P, Donado CD, Cuervo A, González S, Suárez A, Turroni F, Gueimonde M, Ventura M, Sánchez B, Margolles A. Allergic Patients with Long-Term Asthma Display Low Levels of Bifidobacterium adolescentis. PLoS One, 2016; 11:e0147809.

RESEARCH ARTICLE

Intestinal Dysbiosis Associated with Systemic Lupus Erythematosus

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ABSTRACT **Systemic lupus erythematosus (SLE) is the prototypical systemic autoimmune disease in humans and is characterized by the presence of hyperactive immune cells and aberrant antibody responses to nuclear and cytoplasmic antigens, including characteristic anti–double-stranded DNA antibodies. We performed a cross-sectional study in order to determine if an SLE-associated gut dysbiosis exists in patients without active disease. A group of 20 SLE patients in remission, for which there was strict inclusion and exclusion criteria, was recruited, and we used an optimized Ion Torrent 16S rRNA gene-based analysis protocol to decipher the fecal microbial profiles of these patients and compare them with those of 20 age- and sex-matched healthy control subjects. We found diversity to be comparable based on Shannon's index. However, we saw a significantly lower *Firmicutes*/*Bacteroidetes* ratio in SLE individuals (median ratio, 1.97) than in healthy subjects (median ratio, 4.86; $P < 0.002$). A lower *Firmicutes*/*Bacteroidetes* ratio in SLE individuals was corroborated by quantitative PCR analysis. Notably, a decrease of some *Firmicutes* families was also detected. This dysbiosis is reflected, based on *in silico* functional inference, in an overrepresentation of oxidative phosphorylation and glycan utilization pathways in SLE patient microbiota.**

IMPORTANCE **Growing evidence suggests that the gut microbiota might impact symptoms and progression of some autoimmune diseases. However, how and why this microbial community influences SLE remains to be elucidated. This is the first report describing an SLE-associated intestinal dysbiosis, and it contributes to the understanding of the interplay between the intestinal microbiota and the host in autoimmune disorders.**

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Metagenomic studies on gut microbiota burst onto the scientific scene during the last decade, due to the advent of next-generation sequencing techniques. In a very short period of time, microbiologists moved from the study of single, isolated, cultivable microorganisms, specifically, those able to grow under standard laboratory conditions, to the investigation of very complex microbial communities, mainly composed of uncultivable bacteria (1, 2). The first metagenomics reports enabled an overview of the complexity of our gut microbial communities (3, 4). Further studies focused on establishing the correlation between the human gut microbiome, the collective genomes of all microbes inhabiting the gut (5), and different physiological states, including those having an influence on health. Currently, we know that the gut microbiota might affect food and drug metabolism (6), influences human behavior (7), shifts during the course of pregnancy (8), displays age-associated changes (9–12), and possesses distinctive features depending on geographical location (12, 13), among other features. It is also becoming clear that there is a strong link between dietary patterns and the gut microbial profile (14, 15). Furthermore, some links have been established between some disorders (for example, obesity and metabolic syndrome) and an imbalance in the gut microbial ecology, also called dysbiosis (16–18). Remarkably, intestinal dysbiosis has also been associated with autoimmune diseases, such as rheumatoid arthritis, type 1 diabetes, and inflammatory bowel disease (IBD) (19–21).

Systemic lupus erythematous (SLE) is a prototypical autoimmune disease in humans that is characterized by the presence of hyperactive immune cells and aberrant antibody responses to nuclear and cytoplasmic antigens. Genetic, immunological, hormonal, and environmental factors contribute to disease susceptibility (22), and its prevalence varies greatly depending on the population under study, although a prevalence of 2 to 5 cases per 10,000 inhabitants is reportedly considered normal (23). Among the environmental factors, growing evidence suggests that molecular mimicry as a result of viral infection may contribute to the development of lupus (24). Also, some reports have highlighted intestinal infections that may ameliorate SLE symptoms (25), and a marked difference in the specificity of antibodies to bacterial DNA in healthy people and SLE patients has been indicated (26). In fact, there is early evidence of a different abundance of cultivable intestinal bacteria in SLE (27). Remarkably, it has been suggested that novel SLE biomarkers can be potentially found in the

TABLE 1 Demographic, clinical, and immunological features of SLE patients

Subject no.	Age (yrs)	Disease duration (yrs)	Anti-dsDNA titer (U/ml)[a]	Complement C3 (g/liter)[a]	Complement C4 (g/liter)[a]	Clinical and immunological features[b]
SLE1	43	2	0.3	0.93	0.2	MR, PH, HD, ANA
SLE2	68	3	0.7	0.96	0.18	MR, DL, PH, AR
SLE4	35	4	7.7	1.53	0.22	PH, OU, RD, ANA
SLE5	50	5	18	1.67	0.44	PH, OU, HD, ANA, anti-SSa
SLE6	35	3	48	0.81	0.13	MR, OU, AR, HD, ANA, anti-dsDNA, anti-SSa
SLE7	70	3	27	1.74	0.37	PH, OU, HD, ANA, anti-dsDNA
SLE11	54	24	99.1	1.43	0.28	MR, DL, PH, AR, HD, ANA, anti-dsDNA, anti-Sm
SLE12	58	6	13	0.84	0.22	DL, PH, AR, HD, ANA, anti-SSa, RF
SLE13	40	6	0.6	0.83	0.25	MR, OU, ANA
SLE14	40	12	4	0.92	0.18	AR, SE, RD, ANA, anti-SSb
SLE15	51	24	104	0.83	0.14	MR, DL, PH, AR, SE, HD, ANA, anti-dsDNA, anti-SSa, anti-SSb, anti-Sm, anti-RNP, anti-CLP
SLE16	54	24	45	1.76	0.3	PH, AR, ANA, anti-dsDNA, anti-SSa
SLE17	46	13	19	0.8	0.11	MR, DL, PH, OU, AR, ANA, anti-dsDNA, anti-SSa, RF
SLE18	43	12	4.1	1.04	0.16	DL, PH, OU, AR, SE, HD, ANA, anti-SSa, RF
SLE19	34	4	0	1.19	0.25	MR, PH, OU, ANA
SLE20	51	7	5.8	0.67	0.14	PH, OU, ANA, anti-SSa
SLE21	59	11	1.2	1.16	0.17	PH, ANA, anti-dsDNA, anti-SSa, anti-CLP
SLE22	64	11	4.4	1.17	0.25	MR, PH, AR, ANA, anti-dsDNA, anti-SSa
SLE24	46	14	0.4	1.08	0.4	MR, PH, HD, ANA, anti-SSa, RF
SLE26	46	20	38	0.89	0.18	MR, PH, OU, RD, HD, ANA, anti-dsDNA.

[a] At the time of sampling.

[b] Cumulatively registered. Abbreviations: ANA, antinuclear antibodies; anti-RNP, antiribonucleoprotein antibodies; anti-Sm, anti-Smith antigen antibodies; anti-CLP, anticardiolipin antibodies; RF, rheumatoid factor; AR, arthritis; DL, discoid lesions; HD, hematological disorder; MR, malar rash; OU, oral ulcers; PH, photosensitivity; RD, renal disorder; SE, serositis.

human microbiota (28). However, a study of the potential dysbiosis associated with SLE had not been tackled until now. Therefore, in this report we took advantage of next-generation sequencing techniques to explore the potential interplay of the human microbiome and SLE. We have proven, for the first time, that there is a gut microbial dysbiosis associated with SLE.

RESULTS AND DISCUSSION

Despite all the scientific knowledge generated in the last few years, and although few studies published so far support the dysbiosis theory as a key factor promoting chronic inflammation in autoimmune diseases (29–32), there is no scientific work that has taken advantage of next-generation sequencing techniques to explore the potential interplay of the human microbiome and SLE, the prototypical autoimmune disease in humans. Therefore, we designed our work with the aim of answering if there is an SLE-associated intestinal dysbiosis and, if so, which microbial population groups are related to the dysbiosis.

We defined the SLE population group by considering that there is a census of about 300 SLE patients in Asturias (from a total population of about 1,000,000 inhabitants). Thus, we were able to obtain a group of SLE patients from a well-defined geographical location to compare them with a similar group of healthy controls (HC), considering factors such as sex, age, medication (absence of antibiotic, steroid, and immunological treatments during the last 6 months), medical history (presenting a wide variety of clinical SLE manifestations), duration of the disease (2 to 24 years), and absence of flares of disease activity at the time of sampling (systemic lupus erythematosus disease activity index [SLEDAI] score of ≤8 at the time of sample collection). The group of SLE patients included individuals with a large variety of symptoms (Table 1), allowing us to establish correlations between the microbial profile and SLE, which are very likely independent of a specific pattern of symptoms. This variability in the phenotype of the disease is an intrinsic characteristic of SLE (22). We also selected patients with no active disease at the time of sampling, because the clinical manifestations of the disease in this population group are not biased by the pharmacological treatment necessary to treat SLE individuals during disease relapse. Furthermore, mean dietary intakes of energy, macronutrients, micronutrients, fiber, and phytocompounds were recorded, both from patients with SLE and healthy subjects, and we found that there was no significant difference between the 2 groups (Table 2). Also, no significant difference was found between the 2 groups regarding lifestyle-related factors (smoking, alcohol consumption, physical activity, and use of vitamin and mineral supplements [data not shown]). This reduced the possibility that our analysis was affected by factors shown to have an influence on the gut microbial profile, such as age (9), diet (15), or phenolic compound intake (33, 34).

Our work is based on 16S rRNA gene-based data for fecal microbiota and the bioinformatic analysis of the results. In a previous work (35), we optimized protocols to study the human fecal microbial population by using an Ion Torrent PGM sequencing platform. This methodology was applied in the current study, and we obtained an average of 592,305 high-quality reads per fecal sample (see Table S1 in the supplemental material). Rarefaction curves obtained by plotting the Shannon, Chao1, and phylogenetic diversity indexes against the number of sequences (see Fig. S1 in the supplemental material) showed that a large part of the diversity of the samples was detected. The microbiota composition at the phylum and family levels was obtained (Fig. 1; see also Fig. S2 in the supplemental material). Remarkably, even considering the broad heterogeneity of the clinical manifestations of SLE

TABLE 2 General characteristics and mean dietary intake of energy, macronutrients, fiber forms, and phyto-compounds in patients with SLE and healthy controls

Characteristic	SLE patients ($n = 20$)	Healthy controls ($n = 20$)
Female sex (%)	100	100
Age (yrs)	49.2 ± 10.7[b]	46.9 ± 8.6
BMI (kg/m^2)	26.1 ± 5.3	25.2 ± 4.2
Energy (kcal/day)	2,173.1 ± 722.4	1,875.9 ± 332.8
Lipid (g/day)[a]	84.5 ± 41.0	85.4 ± 20.5
MUFA (g/day)[a]	35.3 ± 19.7	35.7 ± 7.6
PUFA (g/day)[a]	17.2 ± 9.7	17.5 ± 9.4
SFA (g/day)[a]	24.9 ± 14.1	25.0 ± 6.0
Protein (g/day)[a]	104.9 ± 27.6	100.6 ± 20.9
Carbohydrates (g/day)[a]	205.0 ± 75.6	203.5 ± 47.0
Dietary fiber (g/day)[a]	24.9 ± 10.4	25.3 ± 9.1
Insoluble fiber (g/day)[a]	16.0 ± 8.6	16.6 ± 7.5
Soluble fiber (g/day)[a]	2.9 ± 1.5	2.8 ± 1.1
Total isoflavones (mg/day)[a]	2.4 ± 2.4	2.5 ± 2.7
Total phenolics (mg/day)[a]	833.2 ± 527.3	916.3 ± 437.8

[a] Model was adjusted for energy and BMI. PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.
[b] Values are means ± SD.

in the individuals under study (Table 1), we obtained a particular type of microbiota for the SLE group. In this regard, the presence of anti–double-stranded DNA (dsDNA) antibodies and other clinical data were organized in a metadata file for all the microbiota profiles. A principal component analysis (PCoA) was performed with both metadata/microbiota profiles, using the variability of the 16S rRNA gene profiling at different taxonomic levels. Sample classification according to the metadata revealed no specific clustering of the samples or correlations with the different clinical features or anti-dsDNA antibodies (data not shown).

In silico analysis of the sequences highlighted the key findings of our work. A high-quality filtering approach was used in order to process the Ion Torrent-generated sequencing data (see Table S1 in the supplemental material); a total of 293,436 unfiltered operational taxonomic units (OTUs) were identified by using uclust for *de novo* OTU picking. Based on each of five alpha-diversity measures (Chao1, PD whole tree, observed species, Shannon, and Simpson indexes), patients and controls were not significantly different (data not shown). Notably, one of the main results was the identification of a clear dysbiosis between the two study groups which was characterized by a higher relative abundance of

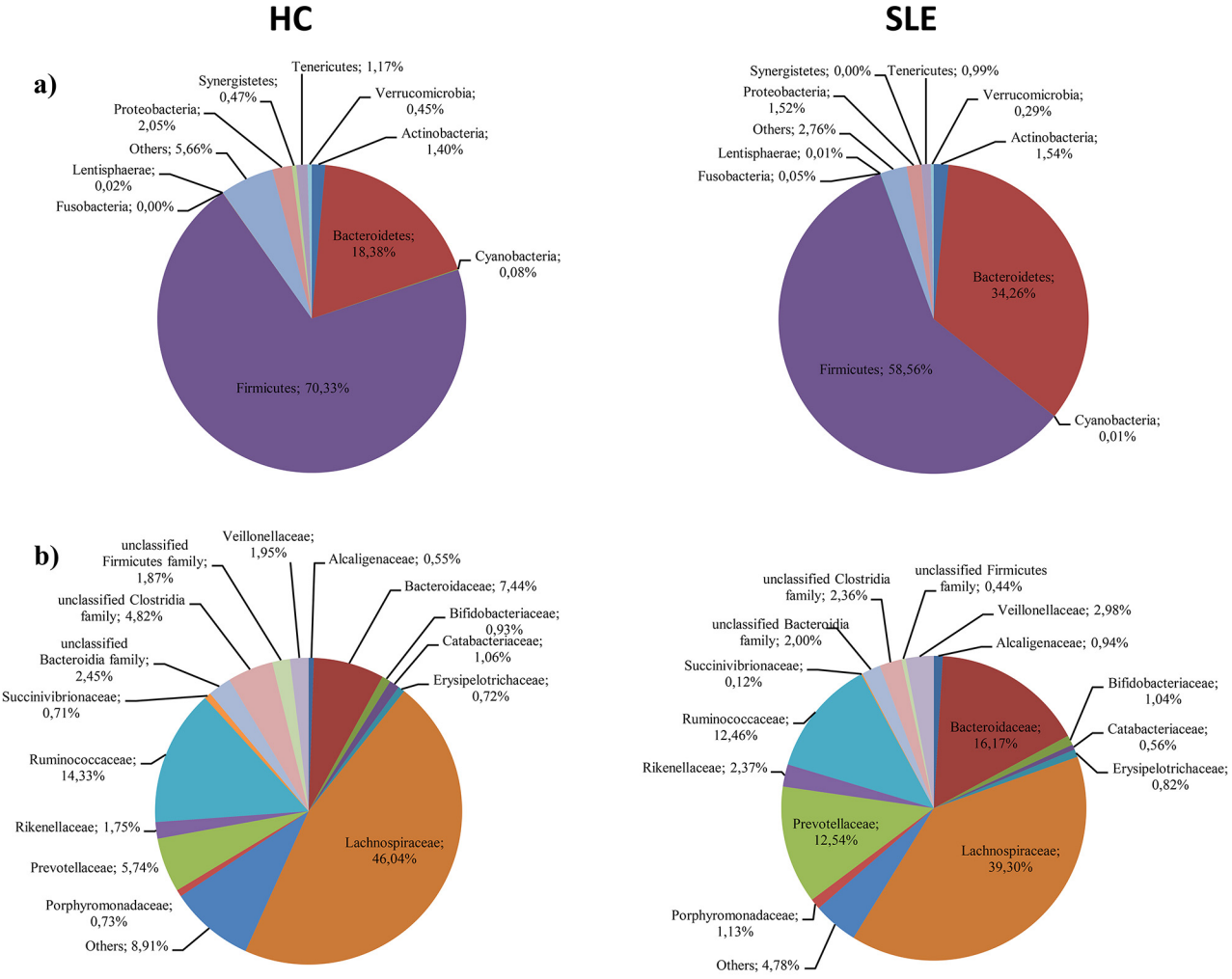

FIG 1 Aggregate microbiota composition in fecal samples from control (HC) and lupus-affected (SLE) subjects at the phylum level (a) and family level (b). In panel b, only taxonomic groups representing >0.5% are shown.

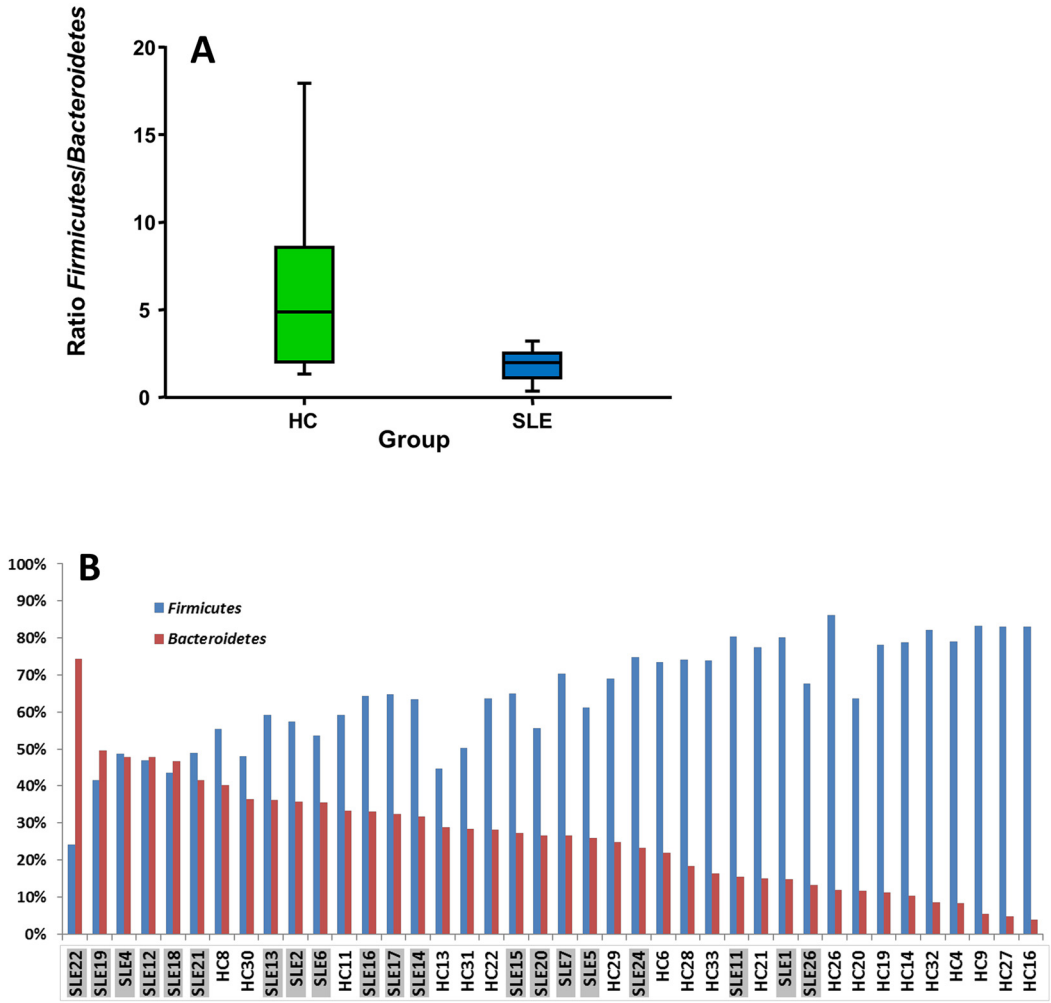

FIG 2 (A) Box plot of *Firmicutes*/*Bacteroidetes* ratios (median ± IQR) in SLE patients versus healthy controls. (B) Percentages of 16S rRNA reads of *Bacteroidetes* (red bars) and *Firmicutes* (blue bars) in the DNA extracted from fecal samples of SLE patients (SLE codes) and healthy controls (HC codes). Ratios are significantly different ($P < 0.002$).

Bacteroidetes in the SLE group. Overall, we detected a significant decrease in the *Firmicutes*/*Bacteroidetes* ratio in SLE individuals: the microbiota of SLE patients, compared with controls, had an almost-2.5-fold-decreased ratio (Fig. 2A) ($P < 0.002$). Looking at the different individuals, a gradient from lower (SLE) to higher (HC) *Firmicutes*/*Bacteroidetes* ratios was observed (Fig. 2B). These 16S rRNA gene-based analyses were corroborated by quantitative PCR (qPCR) analysis. The levels (reported as the log cells/g, with the interquartile range [IQR] in parentheses) of total fecal bacteria were 10.62 (9.46 to 10.80) in SLE patients and 10.35 (10.07 to 10.59) in controls. *Firmicutes* levels in the SLE and control groups reached 9.69 (8.86 to 10.38) and 9.99 (9.68 to 10.31), respectively, while those of *Bacteroidetes* were 10.52 (9.56 to 10.83) and 9.89 (9.59 to 10.23), respectively. No statistically significant differences in these levels of microbial groups were found between SLE patients and controls. However, when the data were expressed as the relative percentages of *Firmicutes* and *Bacteroidetes* with respect to the total bacterial level, a significantly higher ($P < 0.05$) percentage of *Bacteroidetes* was observed in the SLE group. Moreover, when the *Firmicutes*/*Bacteroidetes* ratio was calculated, a statistically significant ($P < 0.01$) decrease in the SLE group with respect to the control group was found (0.94 [0.90 to 0.98] versus 1.01 [0.96 to 1.06], respectively). The differences in the ratios based on qPCR were less pronounced than the differences obtained with the 16S rRNA profiling, likely because the two techniques provide different kinds of information: a relative proportion of sequences (from the 16S rRNA gene-based analysis) versus an absolute quantification of sequences (via qPCR). Thus, the fact that we obtained clear evidence for a significantly lower *Firmicutes*/*Bacteroidetes* ratio in SLE patients when we used two different culture-independent techniques supports the soundness and reliability of our analyses. The phyla *Bacteroidetes* and *Firmicutes* include the most abundant components of the human gut microbiota (36). Dysbiosis between *Firmicutes* and *Bacteroidetes* in the human gut has been described in previous studies in association with some disorders. The ratio between *Firmicutes* and *Bacteroidetes* decreases in human type 2 diabetes compared with controls (37). Also, most studies of the microbiota in people with Crohn's disease report a decrease in the abundance of *Firmicutes* and an increase in *Bacteroidetes* in association with the disease (38). An

opposite situation is observed in obesity, in which the dysbiosis is characterized by an increase in the *Firmicutes*/*Bacteroidetes* ratio (17). Therefore, this specific microbial balance between the more abundant phyla in the human gut seems to be dependent on the physiology of the disorder. In relation to this, it has been reported that this equilibrium is susceptible to modification by shifts in the dietary pattern. Wu et al. (15) reported that *Firmicutes* levels were positively associated with a low-fat/high-fiber diet. Also, dietary interventions, including whole grain in the diet, increase the *Firmicutes*/*Bacteroidetes* ratio (39).

We prepared scatter plots (see Fig. S3A in the supplemental material), and they clearly highlighted a positive association between *Firmicutes* in healthy controls ($P < 0.01$) and *Bacteroidetes* in SLE patients ($P < 0.001$). This association was confirmed at lower taxonomic levels, and normalized abundances of the classes *Bacteroidia* (phylum *Bacteroidetes*) and *Clostridia* (phylum *Firmicutes*) and the orders *Bacteroidales* and *Clostridiales* differed significantly between the SLE patients and the healthy controls (see Fig. S3B). At the family level, *Lachnospiraceae* ($P < 0.05$) and *Ruminococcaceae* ($P < 0.05$) were positively associated with healthy controls (see Fig. S4 and S5 and Table S2 in the supplemental material).

Statistical differences between the two groups (HC and SLE) were calculated by a PERMANOVA test, with the distance data obtained after ordination analysis (PCoA) using PAST v 3.1 (40). In all cases, data from the relative taxa abundances were used, and distances were computed according to the Bray-Curtis similarity index. The groups of SLE and HC differed statistically whenever phylum- or family-level data were used ($P < 0.01$ or $P < 0.02$, respectively).

Unsupervised PCoA of the 16S rRNA sequence data identified the phyla *Bacteroidetes* and *Firmicutes* as the main gradients for SLE and healthy control groups, respectively (Fig. 3A). PCoA at the family level showed that the families *Lachnospiraceae* and *Ruminococcaceae* were located near the healthy controls (Fig. 3B). Interestingly, *Lachnospiraceae* was the most abundant family in the feces of both study groups (Fig. 1; see also Fig. S2 in the supplemental material). Depletion of *Lachnospiraceae* and *Ruminococcaceae* has been associated with *Clostridium difficile* infections and nosocomial diarrhea (41). Also, several studies showed a decrease of *Lachnospiraceae* in IBD patients, and this family has been suggested as a biomarker of disease activity (42–44). However, the functional consequences of the depletion of these bacteria in the previously mentioned intestinal diseases remain to be investigated. Although the amplicons of the 16S rRNA sequences could be relatively short to perform a totally reliable population structure analysis at the genus/species level, it is noteworthy that the relative abundance of sequences assigned to *Bacteroides* spp. were significantly higher in SLE samples ($P < 0.02$). In the control group, we also found a significantly higher ($P < 0.05$) relative abundance of sequences tentatively assigned to *Desulfovibrio*, the most common genus of sulfate-reducing bacteria in the human gut (45). It is worth mentioning that some authors have described how dysbiosis could affect mucosal barrier function and impair immunoregulatory mechanisms, leading to pathological effects in systemic immunity. Using animal models, a direct involvement of components of the microbiota in chronic intestinal inflammation (46) and the protective role of specific commensals in avoiding bacterial translocation (47) have been demonstrated. Finally, we should bear in mind that nonsteroidal anti-inflammatory drugs and the antimalarial treatment of SLE patients could have an influence on the observed dysbiosis.

In our study, we also determined the tentative metagenomes from phylogenetically associated reference genomes. Our aim was to highlight metabolic pathways and shifts associated with the SLE population compared with controls. We inferred the functionality of the different putative metagenomes by using PICRUSt software, which allows the prediction of metabolic pathways from the 16S rRNA reads (48). A functional analysis using the data obtained from the KEGG pathways at level 3 allowed us to detect certain processes potentially associated with either healthy controls or SLE. KEGG levels are the different hierarchical subdivisions in which the functions of a biological system (cell, organism, or ecosystem) are arranged according to information organized in the KEGG database (http://www.genome.jp/kegg/kegg1a.html). Pathways displaying a difference in mean proportions between healthy controls and SLE groups of at least 0.1% are represented in Fig. S6 in the supplemental material. Some glycan degradation pathways are slightly overrepresented in the microbiota of SLE patients, probably due to the higher abundance of *Bacteroidetes* in these samples. *Bacteroidetes*, and specifically the main genus of this phylum, *Bacteroides*, have been shown to display broad glycan-degrading abilities (49). The same occurs with lipopolysaccharide biosynthesis proteins, which is in direct relation to the higher abundance of *Bacteroidetes* in the SLE samples. Remarkably, oxidative phosphorylation processes seem to be associated with SLE patients. This finding indicates that some bacteria able to perform oxidative phosphorylation may be better adapted to the intestinal ecosystem of individuals with SLE, and this could be related to the imbalance in the oxidative stress environment at the intestinal level that has been linked to some autoimmune diseases (48).

In summary, understanding and potentially manipulating immune responses through the action of intestinal microbiota comprise one of the most active fields in probiotic and prebiotic research. Most likely, the dysbiosis defined in this work is the consequence of the altered immune function of SLE patients. At present, the treatment of SLE patients is exclusively performed with drugs, and our findings could indicate that challenging the immune system with the bacteria depleted in SLE, or substances promoting their growth, could influence SLE physiology. To the best of our knowledge, experimental papers about the relationship of the human gut microbiome and SLE have not been published, and this is the first report describing an SLE-associated intestinal dysbiosis. Thus, our results establish the basis to delve deeper into the understanding of the relationship between the human gut microbiota and autoimmune diseases.

MATERIALS AND METHODS

Ethics statement. Ethics approval for this study (reference code AGL2010-14952; grant title "Towards a better understanding of gut microbiota functionality in some immune disorders") was obtained from the Bioethics Committee of CSIC (Consejo Superior de Investigaciones Científicas) and from the Regional Ethics Committee for Clinical Research (Servicio de Salud del Principado de Asturias) in compliance with the Declaration of Helsinki. All determinations were performed with fully informed written consent from all participants involved in the study.

Study subjects. The study sample comprised 20 patients with SLE (SLE codes) and 20 healthy controls (HC codes). SLE patients were selected from the updated Asturian Register of Lupus (Asociación Lúpicos de Asturias, Oviedo, Spain). All of them fulfilled at least four of the American College of Rheumatology criteria for SLE (50). The individuals re-

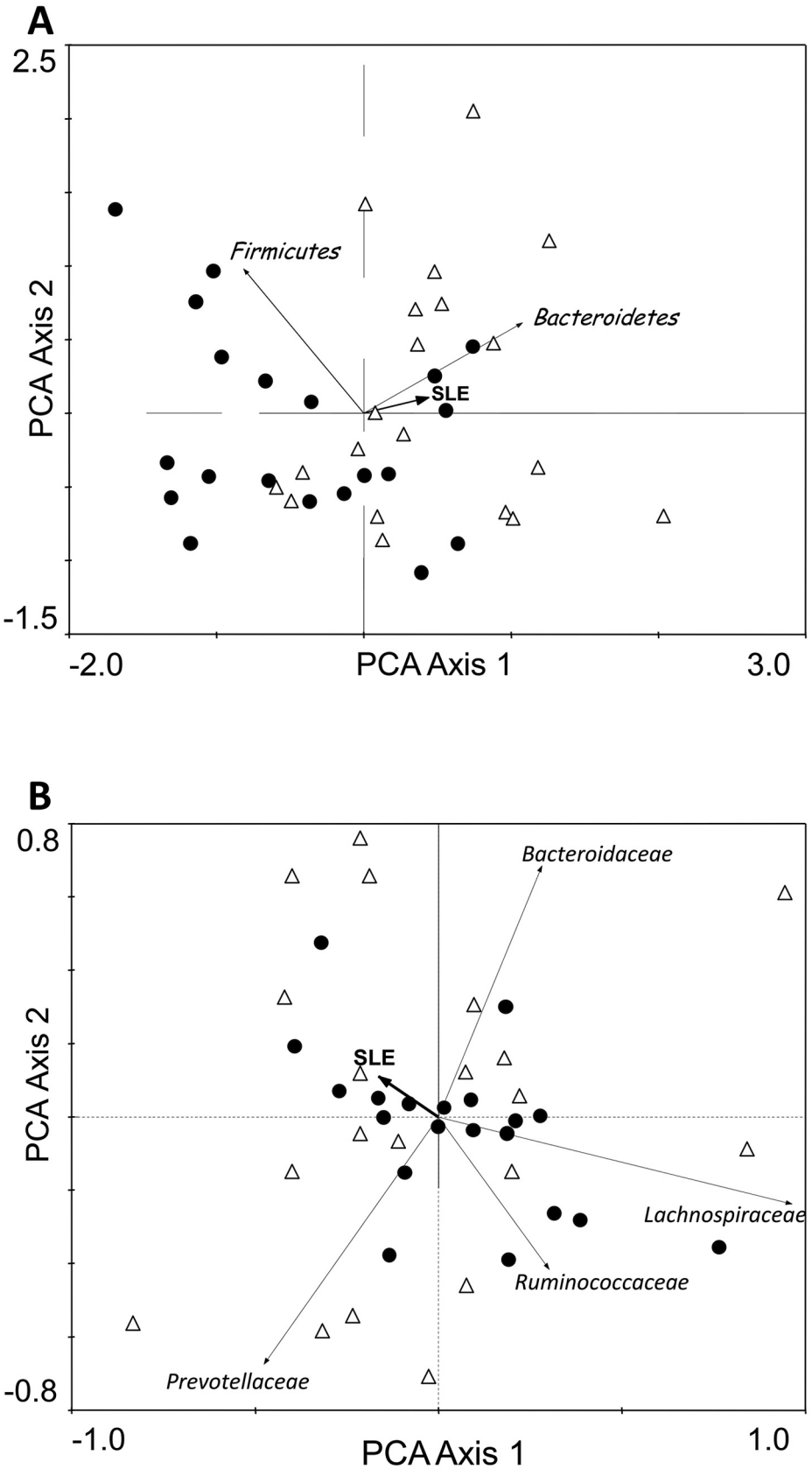

FIG 3 PCoA results for the 16S rRNA profiles at the phylum (A) and family (B) level. The presence or absence of SLE was further included as metadata. HC, closed circles; SLE, open triangles.

cruited for the SLE group were in remission and had not had any immunotherapy or corticoid treatment during the previous months, since those are the treatments that could have the strongest influence on the physiology of the patients. All patients were women of Caucasian origin, 49.2 ± 10.7 years old (mean ± standard deviation [SD]), and had no active disease at the time of sampling (SLEDAI score of ≤8). Information on clinical manifestations was obtained by reviewing clinical records (Table 1). Patients were also asked precise questions regarding treatment received during the previous 6 months. Only those individuals who had not used antibiotics, glucocorticoids, immunosuppressive drugs, monoclonal antibodies, or other immunotherapies were recruited for the study. Eighteen patients were receiving antimalarial treatment, and all of them were regular consumers of nonsteroidal anti-inflammatory drugs. Twenty age-matched healthy women (46.9 ± 8.6 years old) from the same population were recruited as controls.

(i) Nutritional assessment. Variables of macro- and micronutrient intakes were collected by means of a semiquantitative food frequency questionnaire that included 160 items. During a personal interview, carried out within 7 days after the collection of the fecal sample, subjects were asked item by item whether they usually ate the food and, if so, how much they ate. For this purpose, three different serving sizes of each cooked food were presented in pictures to the participants so that they could choose from up to 7 serving sizes (from "less than the small one" to "more than the large one"). For some of the foods consumed, amounts were recorded in household units, by volume, or by measuring with a ruler. Special attention was paid to cooking practices and number and amount of ingredients used in each recipe, as well as questions concerning menu preparation (e.g., type of oil or milk used).

Food intake was analyzed for energy and macro- and micronutrient contents by using the nutrient food composition tables developed by CESNID (51). Total, soluble, and insoluble fiber intake was completed based on Marlett food composition tables (52), and polyphenol content was calculated from the U.S. Department of Agriculture (USDA) Nutrient Database (53).

(ii) Anthropometric measures. The body mass index (BMI) was calculated from the following formula: weight/(height)^2 (in kg/m^2). Height was registered by using a stadiometer with an accuracy of ±1 mm (Año-Sayol, Barcelona, Spain). Subjects were barefoot, in an upright position, and with the head positioned in the Frankfort horizontal plane. Weight was measured on a scale with an accuracy of ±100 g (Seca, Hamburg, Germany).

(iii) Lifestyle-related factors. During the interview, other factors associated with the lifestyle of the subject were registered. Smoking habit, physical activity, alcohol consumption, and supplements use were included in the questionnaire. Smoking status was categorized as nonsmoker (including exsmokers and occasional smokers) or current smoker. Those subjects who reported that they never exercised were categorized as physically inactive. "Regular alcohol consumer" refers to those subjects who declared a regular consumption of alcoholic drinks. Also, the use of vitamin and mineral supplements during the last month was self-reported.

Fecal sample collection and DNA extraction. Fresh fecal material (between 10 and 50 g per person) was collected in a sterile container and immediately manipulated and homogenized within a maximum of 3 h from defecation. During the waiting period, from defecation to homogenization, samples were kept at 4°C. Thirty milliliters of RNAlater solution (Applied Biosystems, Foster City, CA) was added to 10 g of sample, and the mixture was homogenized in a sterile bag, using a stomacher apparatus (IUL Instruments, Barcelona, Spain) with three cycles at high speed, 1 min per cycle. Homogenized samples were then stored at −80°C until use.

For DNA extraction, samples were thawed and the QIAamp DNA stool minikit (Qiagen Ltd., Strasse, Germany) was used as previously described (35).

16S rRNA gene amplification. Partial 16S rRNA gene sequences were amplified from extracted DNA by using the primer pair Probio_Uni (5′-CCTACGGGRSGCAGCAG-3′)/Probio_Rev (5′-ATTACCGCGGCTGCT-3′) (35), which targets the V3 region of the 16S rRNA gene sequence. The PCR conditions used were 5 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C, followed by 10 min at 72°C. Amplification was carried out using a Verity thermocycler (Applied Biosystems). The integrity of the PCR amplicons was analyzed by electrophoresis on an Experion workstation (Bio-Rad, Hertfordshire, United Kingdom).

Ion Torrent PGM sequencing of 16S rRNA gene-based amplicons. The PCR products derived from amplification of specific 16S rRNA gene hypervariable regions were purified by electrophoretic separation on a 1.5% agarose gel and the use of a Wizard SV Gen PCR cleanup system (Promega, Madison, WI), followed by a further purification step involving Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) in order to remove primer dimers. The DNA concentration of the amplified sequence library was estimated through use of the Experion system (Bio-Rad). From the concentration and the average size of each amplicon library, the amount of DNA fragments per microliter was calculated and libraries for each run were diluted to 3E9 DNA molecules prior to clonal amplification. Emulsion PCR was carried out using the Ion OneTouch 200 template kit v2 DL (Life Technologies, Guilford, CA) according to the manufacturer's instructions. Sequencing of the amplicon libraries was carried out on 316 chips by using the Ion Torrent PGM system and employing the Ion Sequencing 200 kit (Life Technologies) according to the supplier's instructions at the DNA sequencing facility, GenProbio s.r.l. After sequencing, the individual sequence reads were filtered with the PGM software to remove low-quality and polyclonal sequences. Sequences matching the PGM 3′ adaptor were also automatically trimmed. All PGM quality-approved, trimmed, and filtered data were exported as SFF files.

Sequence-based microbiota analysis. The SFF files were processed using QIIME (54). Quality control retained sequences had lengths between 150 and 200 bp, a mean sequence quality score of >25, and with truncation of a sequence at the first base if a low-quality rolling 10-bp window was found. Presence of homopolymers of >7 bp and sequences with mismatched primers were omitted. In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA OTUs were defined at ≥97% sequence homology by using uclust (55). Chimeric sequences were removed using ChimeraSlayer (56). Furthermore, OTUs that included less than 10 sequences were filtered using QIIME (54). All reads were classified to the lowest possible taxonomic rank by using QIIME and a reference data set from the Ribosomal Database Project (57). The sequence data features of all the samples are included in Table S1 in the supplemental material.

Different alpha diversity indexes were calculated using QIIME and information from the OTU tables using the alpha_diversity.py script. The different diversity metrics were set by passing the option –s to the script. The following indexes were calculated for every sample and compared between groups by using a two-sided Student's *t* test: Chao1, PD whole tree, observed species, Shannon, and Simpson.

Analysis by qPCR. Quantification of total fecal bacteria, *Firmicutes*, and *Bacteroidetes* by qPCR was performed by using previously described primers and conditions (58–60). Analyses were done in duplicate in a 7500 Fast real-time PCR system (Applied Biosystems) using Sybr green PCR master mix (Applied Biosystems). Standard curves were made with pure cultures, grown under anaerobic conditions at 37°C, of *Escherichia coli* LMG 2092 in Gifu anaerobic medium (GAM; Nissui Pharmaceutical Co., Tokyo, Japan), *Faecalibacterium prausnitzii* DMSZ 17677 in RCM formula (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), and *Bacteroides thetaiotaomicron* DSMZ 2079 in GAM.

Functional inference. The functionality of the different metagenomes, grouped by disease status (healthy control versus SLE), was predicted using the software PICRUSt 0.9.1 (http://picrust.github.io), which has been explained in detail elsewhere (48). In short, this software allows

the prediction of functional pathways from the 16S rRNA reads. First, a collection of closed-reference OTUs was obtained from the filtered reads by using QIIME v 1.7.0 (54) and by querying the data against the IMG/GG reference collection (GreenGenes database, May 2013 version; http://greengenes.lbl.gov). Reverse-strand matching was enabled during the query, and OTUs were picked at a 97% identity. A BIOM-formatted table (biological observation matrix [61]) was obtained with the pick_closed_reference_otus.py script. This table, containing the relative abundances of the different reference OTUs in all the metagenomes, was normalized based on the predicted 16S rRNA copy number by using the script normalize_by_copy_number.py. Final functional predictions, inferred from the metagenomes, were created with the script predict_metagenomes.py. When necessary, tab-delimited tables were obtained with the script convert_biom.py.

Analysis of predicted metagenomes. PICRUSt and QIIME provide a number of scripts that can be useful for analyzing both 16S rRNA gene relative abundances and the predicted metabolic data. Predicted metagenomic contents were collapsed at KEGG pathway level 3 (http://www.genome.jp/kegg/pathway.html) with the categorize_by_function.py script, and the data were analyzed statistically by using STAMP v 2.0.0 (62). STAMP allows data filtering and the application of different statistical tests and corrections, including PCoA. It also generates different graphics, including box plots, error plots, and scatter plots. Data of the KEGG pathway distributions were plotted by using the script summarize_taxa_through_plots.py. Associations of different taxonomic categories to SLE were statistically analyzed with the script otu_category_significance.py.

Statistical analyses. Statistical analysis was performed using IBM-SPSS version 19.0 (IBM SPSS, Inc., Chicago, IL). For descriptive purposes, in Table 2 the mean values are presented as means ± SD on untransformed variables. Differences between SLE patients and controls were compared by using a multivariate linear model, including energy intake and BMI as covariates.

Individuals were ordered according to their sequence data composition by principal component analysis using the taxonomic data at the phylum and family levels. Patterns were extracted using all the variations from the taxonomic data via an indirect method as a model and SLE as metadata. To analyze the associations between inferred metabolic pathways and SLE, metabolic pathways with very low abundance levels (<0.001 in 50% of the samples) were excluded from all analyses. Association of KEGG pathways to SLE were identified by running two-sided Welch tests on every pair of means. This test is a variation of Student's *t* test and is used when equal variance cannot be assumed in both groups. Confidence intervals (95%) were obtained by inverting the Welch's tests. The false discovery rate (FDR) correction (48, 63) was finally applied in all cases, and significant differences between healthy controls and SLE patients were only considered when below a P value of 0.05 or a q value below 0.2. P and q values at the phylum and family levels are included in Table S2 in the supplemental material. In the particular case of the family *Desulfovibrionaceae* (with $P < 0.05$), further statistical analysis was carried out for sequences tentatively assigned to the genus *Desulfovibrio* by using a one-sided *t* test.

In relation to qPCR results, not all the bacterial groups showed normal distribution; therefore, differences in bacterial levels between groups of individuals were analyzed using a nonparametric test (Mann-Whitney U test).

Nucleotide sequence accession number. The raw sequences reported in this article have been deposited in the NCBI Short Read Archive (SRA; study accession number SRP028162).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01548-14/-/DCSupplemental.

Figure S1, DOC file, 0.2 MB.
Figure S2, DOC file, 0.1 MB.
Figure S3, DOC file, 0.3 MB.
Figure S4, DOC file, 0.2 MB.
Figure S5, DOC file, 0.5 MB.
Figure S6, DOCX file, 0.2 MB.
Table S1, PDF file, 0.04 MB.
Table S2, PDF file, 0.4 MB.

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RESEARCH ARTICLE

Allergic Patients with Long-Term Asthma Display Low Levels of *Bifidobacterium adolescentis*

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Abstract

Accumulated evidence suggests a relationship between specific allergic processes, such as atopic eczema in children, and an aberrant fecal microbiota. However, little is known about the complete microbiota profile of adult individuals suffering from asthma. We determined the fecal microbiota in 21 adult patients suffering allergic asthma (age 39.43 ± 10.98 years old) and compare it with the fecal microbiota of 22 healthy controls (age 39.29 ± 9.21 years old) using culture independent techniques. An Ion-Torrent 16S rRNA gene-based amplification and sequencing protocol was used to determine the fecal microbiota profile of the individuals. Sequence microbiota analysis showed that the microbial alpha-diversity was not significantly different between healthy and allergic individuals and no clear clustering of the samples was obtained using an unsupervised principal component analysis. However, the analysis of specific bacterial groups allowed us to detect significantly lower levels of bifidobacteria in patients with long-term asthma. Also, in allergic individuals the *Bifidobacterium adolescentis* species prevailed within the bifidobacterial population. The reduction in the levels on bifidobacteria in patients with long-term asthma suggests a new target in allergy research and opens possibilities for the therapeutic modulation of the gut microbiota in this group of patients.

Introduction

In recent years, growing evidence supporting the "hygiene hypothesis", which states that a lack of early microbial stimulation results in aberrant immune responses to innocuous antigens later in life, is rising [1,2]. It has also been suggested that modifications of the intestinal

microbiota composition that occur as a result of the westernized life-style has disrupted mechanisms that are involved in the development of immunological tolerance [3]. In relation to this, the scientific information about the relationship between allergy and gut microbiota dysbiosis (an imbalance in the gut microbial ecology) is controversial nowadays. While some reports highlight an aberrant microbiota associated with allergic manifestation, such as asthma, rhinitis, and eczema, others did not find any significant differences in the microbial profile, or specific microbial groups, in the gut microbiota of allergic individuals. This could be due to the fact that different allergens can drive allergen-specific responses, to the lack of standardized protocols to analyze the human microbiota, and because the studies were not performed in well-defined population groups. For instance, some studies have indicated an association between the gut microbiota composition and atopic disease, and there is solid evidence that variations of particular intestinal microorganisms might be associated with this physiological condition [4]. Also, a shift of the gut bacterial profiles has been associated with immune disorders in infants and in adults [5–8], as well as in some food allergies, such as milk-hypersensitivity [9,10]. However, despite some positive results, strategies to ameliorate the clinical manifestations of allergy through the modulation of the intestinal microbiota using probiotic microorganisms yielded limited results [11,12].

In this work we performed a cross-sectional study in which we characterized the microbiota of a representative group of adult patients suffering allergic asthma and compared it with a group of healthy controls. We used a 16S rRNA gene-based analysis protocol for this purpose. Significant differences in the bifidobacterial population of asthmatic patients were highlighted.

Materials and Methods

Ethical Statement

Ethics approval for this study (reference code AGL2010-14952; grant title "Towards a better understanding of gut microbiota functionality in some immune disorders") was obtained from the Bioethics Committee of CSIC (Consejo Superior de Investigaciones Científicas) and from the Regional Ethics Committee for Clinical Research (Servicio de Salud del Principado de Asturias) in compliance with the Declaration of Helsinki and we have obtained permission from participants to publish potentially identifying case details. All determinations were performed with fully informed written consent from all participants involved in the study. The study did not interfere with patients' normal care.

Study subjects

The study sample comprised 21 patients with allergic asthma (AL codes; 9 male and 12 female; age 39.43 ± 10.98, and 22 healthy controls (HC codes, 7 male and 15 female, age 39.29 ± 9.21). Patient recruitment was carried out in the allergology consultation of the Central University Hospital of Asturias. Information on clinical manifestations was obtained by reviewing clinical records and by personal interviews. Asthma diagnosis was stablished based on the criteria of the Global Initiative for Asthma—GINA [13]. Inclusion criteria also included confirmed diagnosis of asthma (with or without rhinitis) due to perennial allergens. The diagnosis was stablished when patients had a positive skin test and serum-specific IgE levels greater or equal to 3.5 kU/L, with clinically related symptoms. Common relevant antigens were usually house dust mite (HDM), grasses, dog epithelia and a work place allergen (green coffee). All patients were sensitized to HDM and had a positive Skin Prick Test (SPT) result to at least one of the tested allergens.

Serum total IgE, serum-specific IgE testing and SPT were determined at the same time of fecal sampling (Table 1). All patients were diagnosed as persistent asthma with regular control

Table 1. Relevant demographic and clinical features of AL patients.

Sample	Age (years)	Sex	IgE Titer (IU/ml)	SPT	Relevant causal agent*	Specific IgE (KU/l)	Rhinitis duration (years)	Asthma duration (years)
AL1	40	F	160	HDM	*D. pteronysinus*	17.5	40	40
AL2	27	F	133	HDM	*D. pteronysinus*	32	12	1.5
AL3	24	F	2799	HDM, dog epithelia	Dog epitehlia	>100	-	2.0
AL4	55	M	388	HDM	*D. pteronysinus*	16	12	19
AL5	36	M	187	HDM, grasses	*Lepidoglyphus*	28	3	3
AL6	46	F	715	HDM, grasses, green cafe	Green coffe	45	30	20
AL7	38	M	104	HDM	*Lepidoglyphus*	10	-	38
AL8	49	F	173	HDM	*D. pteronysinus*	20	10	4
AL9	51	F	389	HDM	Cat epithelia	42	33	3.5
AL10	37	M	235	HDM	*D. pteronysinus*	52	32	14
AL11	32	F	608	HDM, grasses	*D. pteronysinus*	75	27	15
AL12	54	F	87	HDM	*D. pteronysinus*	15	52	5
AL13	38	F	62	HDM	*D. pteronysinus*	12	23	11
AL14	57	M	87	HDM	*D. pteronysinus*	12	52	32
AL15	27	M	158	HDM	*D. pteronysinus*	37	7	23
AL16	37	F	76	HDM	*D. pteronysinus*	10	12	4
AL17	50	F	119	HDM	*D. pteronysinus*	34	45	11
AL18	22	M	300	HDM	*D. pteronysinus*	58	-	3
AL19	22	F	89	HDM	*D. pteronysinus*	15	-	7
AL20	42	M	110	HDM	*D. pteronysinus*	7	37	34
AL21	44	M	484	HDM	*D. pteronysinus*	63	30	38

SPT, Skin Prick Test.

HDM, House Dust Mites.

* More relevant agent in perennial symptoms.

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treatment. Most of the patients required daily treatment with inhaled glucocorticosteroid (low or medium dose) alone or with Laba (long-acting beta agonist) (step of treatment 2, 3 or 4 of GINA). Five patients [AL7, AL9, AL11, AL15 and AL16] only required inhaled treatment in a discontinuous way (step 1 or 2 of GINA). All patients excluding AL3, AL7, AL18 and AL19 were suffering perennial rhinitis. AL3, AL7, AL18 and AL19 were suffering intermittent rhinitis or no rhinitis.

The exclusion criteria considered for this study included subjects diagnosed as having autoimmune diseases, inflammatory bowel disease, or other diseases known to affect intestinal function, as well as subjects who received immunosuppressive therapy and/or allergen specific immunotherapy in the last 5 years (excluding patient AL1 who received an incomplete treatment of immunotherapy [6 months only] 20 months before the fecal sample collection). Patients with oral glucocorticoid or antibiotic treatments during the 6 months prior to the sample collection date were also excluded from the study.

Skin Prick Tests

Thirteen allergens representing the most relevant inhalant allergens in allergic rhinitis and asthma in Spain according to previous studies [14] (*Dermatophagoides pteronysinus*, *Lepidoglyphus destructor*, *Betula berrucosa*, *Cypresus sempervirens*, *Platanus hispánica*, *Lolium perenne* and *Plantago lanceolata*, dog and cat dander, *Alternaria alternata*, *Cladosporium herbarum*,

Peniccillium notatum and *Aspergillus fumigatus*) were obtained from Leti S.A. (Spain) or Alk Bello (Spain). For skin prick tests, all allergens were standardized using biological units [15]. Green coffee was tested 1/10 weight/volume in saline serum. A SPT was considered positive when the patient developed a wheal larger than a histamine wheal or larger than 3 mm.

Total IgE and specific IgE determination

InmunoCAP Total IgE and ImmunoCAP Specific IgE (Thermo Fisher) were used to determine the total IgE and specific IgE, respectively. Methods were carried out according to the manufacturer's instructions.

Fecal sample collection and DNA extraction

Fresh fecal material (between 10 and 50 grams per person) was collected in sterile containers and immediately manipulated and homogenized within a maximum of 3 hours from defecation. During the time between defecation to homogenization, samples were kept at 4°C. Thirty ml of RNAlater solution (Applied Biosystems, Foster City, CA) were added to 10 grams of sample and the mixture was homogenized in sterile bags, using a stomacher apparatus (IUL Instruments, Barcelona, Spain) (three cycles at high speed, one minute per cycle). Homogenized samples were then stored at -80°C until use. For DNA extraction, samples were thawed and the QIAamp DNA Stool Mini kit was used (Qiagen Ltd., Strasse, Germany), as previously described [16].

16S rRNA gene amplification and sequencing of 16S rRNA gene-based amplicons

Primer selection, amplicon generation and purification, amplicon library construction and sequencing were performed essentially as described by Hevia et al. [17]. Briefly, the primer pair Probio_Uni / Probio_Rev [16] was used to generate amplicon pools of approximately 200 bp length. The integrity of the PCR amplicons was analyzed by electrophoresis prior to their purification using the Wizard SV Gen PCR Clean-Up System (Promega, Madison, WI), and the Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany). Libraries for each run were diluted to 3E9 DNA molecules prior to clonal amplification. Emulsion PCR was carried out using the Ion OneTouch™ 200 Template Kit v2 DL (Life Technologies, Guilford, CA) and sequencing of the amplicon libraries was carried out on 316 chips using the Ion Torrent PGM system and employing the Ion Sequencing 200 kit (Life Technologies). After sequencing, individual sequence reads were filtered by the PGM software to remove low quality and polyclonal sequences. Sequences matching the PGM 3' adaptor were also automatically trimmed. All PGM quality-approved, trimmed and filtered data were exported as SFF files.

Sequence-based microbiota analysis

The SFF files were processed using QIIME [18]. Quality control retained sequences with an average length of 180 bp (S1 Table), mean sequence quality score >25, with truncation of a sequence at the first base if a low quality rolling 10 bp window was found. Presence of homopolymers >7 bp, and sequences with mismatched primers were omitted. In order to calculate alpha and beta diversity indices, 16S rRNA Operational Taxonomic Units (OTUs) were defined at $\geq$ 97% sequence homology using uclust [19]. Chimeric sequences were removed using ChimeraSlayer [20]. All reads were classified to the lowest possible taxonomic rank using QIIME and a reference dataset from the Ribosomal Database Project [21].

Nucleotide sequence accession number

The raw sequences reported in this article have been deposited in the NCBI Short Read Archive (SRA) (study accession number: PRJNA276631).

Statistical analyses

Statistical analysis was performed using IBM-SPSS version 19.0 (IBM SPSS, Inc., Chicago, IL). Individuals were ordered according to their sequence data composition by Principal Component Analysis using the taxonomic data at the phylum and family level. Patterns were extracted using all the variation from the taxonomic data using an indirect method as model, and allergy as metadata. Significant differences between healthy controls and asthmatic patients were only considered below a p-value of 0.05. The p-values at the phylum, family and genus level are included in S2 Table. Multivariate linear regression was performed in order to identify the clinical and demographical features associated with the levels of *B. adolescentis* in asthmatic individuals. The statistical parameters employed were β (standardized regression coefficient) and R^2 (coefficient of multiple determinations). The conventional probability value for significance (0.05) was used in the interpretation of results.

Results

To shed some light on the potential relationship between the human microbiota and allergy, we analyzed the fecal microbiota of 21 subjects suffering from allergic asthma. We followed strict exclusion criteria trying to minimize the influence of environmental and genetic factors on our analysis. We considered a homogeneous group, with a very specific pathology: confirmed allergic asthma. Clinical history of the patients and the evidence of an IgE mediated disease are the main criteria in our analysis, in order to associate the microbiota profile in a highly selected allergic group. Fig 1 shows the microbiota composition at phylum, family and genus level that was obtained for healthy individuals and asthmatic persons. Rarefaction curves obtained by plotting the Shannon and Chao1 indexes against the number of sequences (S1 Fig) showed that a large part of the diversity of the samples was detected. We did not obtain significant alpha-diversity indexes (Chao1, PD Whole Tree, Observed Species, Shannon and Simpson indexes) between asthmatic and healthy individuals (data not shown). Furthermore, individuals were ordered according to their sequence data composition by Unsupervised Principal Component Analysis (PCA) using the taxonomic data at the phylum, family and genus level. No clear clustering of the samples was obtained at the three different levels analyzed (Fig 2 shows the plot obtained using genus level), suggesting that the characteristics of the microbiota are similar considering the sample sets of sequences in both population groups.

In our study, we organized relevant data of the patients (age, sex, total and specific IgE titers, causal agent, rhinitis, disease duration) in a metadata file for all the microbiota profiles. Both metadata/microbiota profiles were loaded in STAMP v2.0.3 and a PCA analysis performed using the variability of the 16S rRNA gene profiling at different taxonomic levels. Sample classification according to the metadata revealed no specific clustering of the samples or correlation with different clinical features and analytical results. Furthermore, our results do not stablish a link between microbial groups and asthma when the fecal microbiota was analyzed in its entirety, and statistically significant differences between healthy controls and asthmatic patients were only detected for *Faecalibacterium* and *Bifidobacterium* at the genus level (both genera were more abundant in allergic asthma patients; S2 Table). However, when we examined the possible relationship between the bacterial groups present in the fecal samples and the time suffering from asthma, we found that low levels of bifidobacteria correlate with long ailment periods of asthma (Fig 3). Although the amplicons of the 16S sequences could be

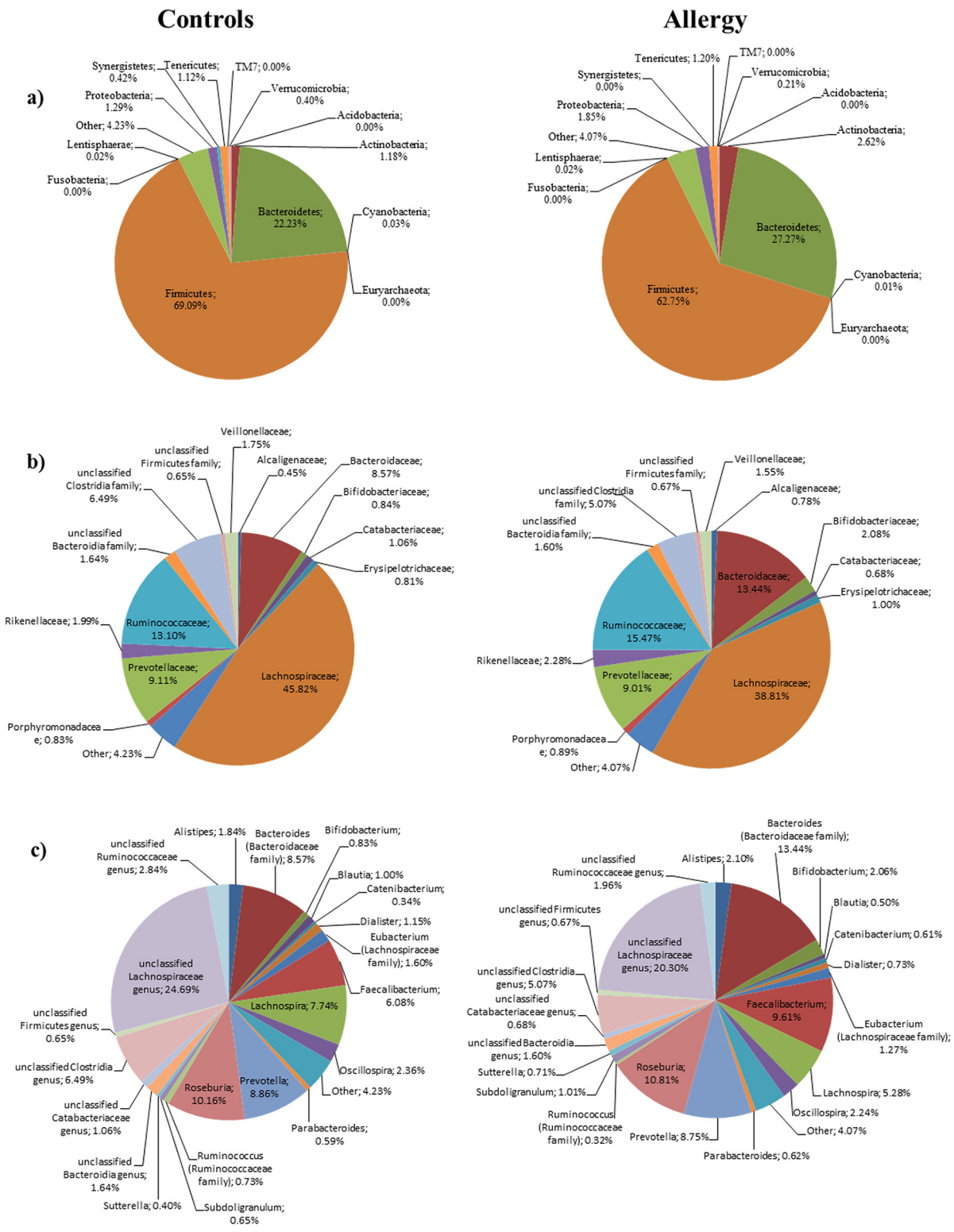

Fig 1. Aggregate microbiota composition in faecal samples from healthy controls and allergic asthma patients at phylum level (panel a), family level (panel b) and genus level (panel c). In panels b and c only taxonomic groups above 0.5% are shown.

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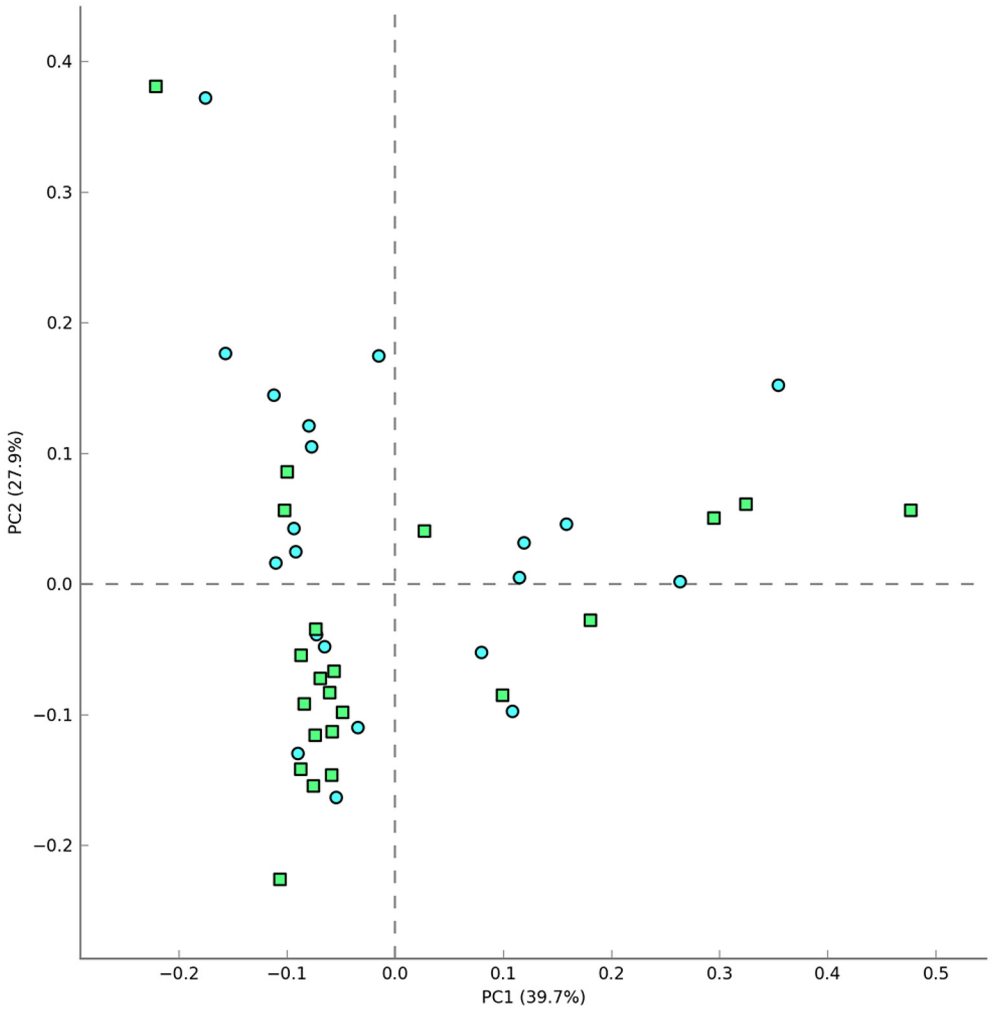

Fig 2. Principal Component Analysis using the 16S rRNA metagenomic profiles and the genus level. Presence/absence of asthma was further included as metadata. (Green squares: healthy controls; blue circles: asthmatic patients).

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relatively short to perform a totally reliable population structure analysis at species level, it is noteworthy that the relative abundance of sequences assigned to *Bifidobacterium adolescentis* was significantly higher in allergic asthma samples. Remarkably, in asthmatic individuals the *B. adolescentis* species prevailed, but in healthy controls a group of other species (*Bifidobacterium longum*, *Bifidobacterium breve* and *Bifidobacterium bifidum*) constituted the majority of the bifidobacterial population. Furthermore, if we subcategorize the allergic asthma patients considering the median for asthma ailment (11 years), the means of the *B. adolescentis* population in the two groups (long-term asthma and short-term asthma groups; $>$ or $<$ 11 years suffering from asthma) are significantly different (ANOVA test, $p<0.002$). Remarkably, our observations were significantly relevant for allergic asthma but not for duration of allergic rhinitis. Furthermore, using a Spearman Ranked Scores analysis, the levels of *Bifidobacterium* and of *B. adolescentis* were negatively correlated with the asthma duration (correlation coefficient -0,84074, p-value: 3,4588E-06 for *Bifidobacterium*; correlation coefficient -0,84682, p-value: 2,494E-06 for *B. adolescentis*). On the other hand, a multivariate linear analysis showed that the time suffering the disease and the IgE titer were identified as *B. adolescentis* predictor

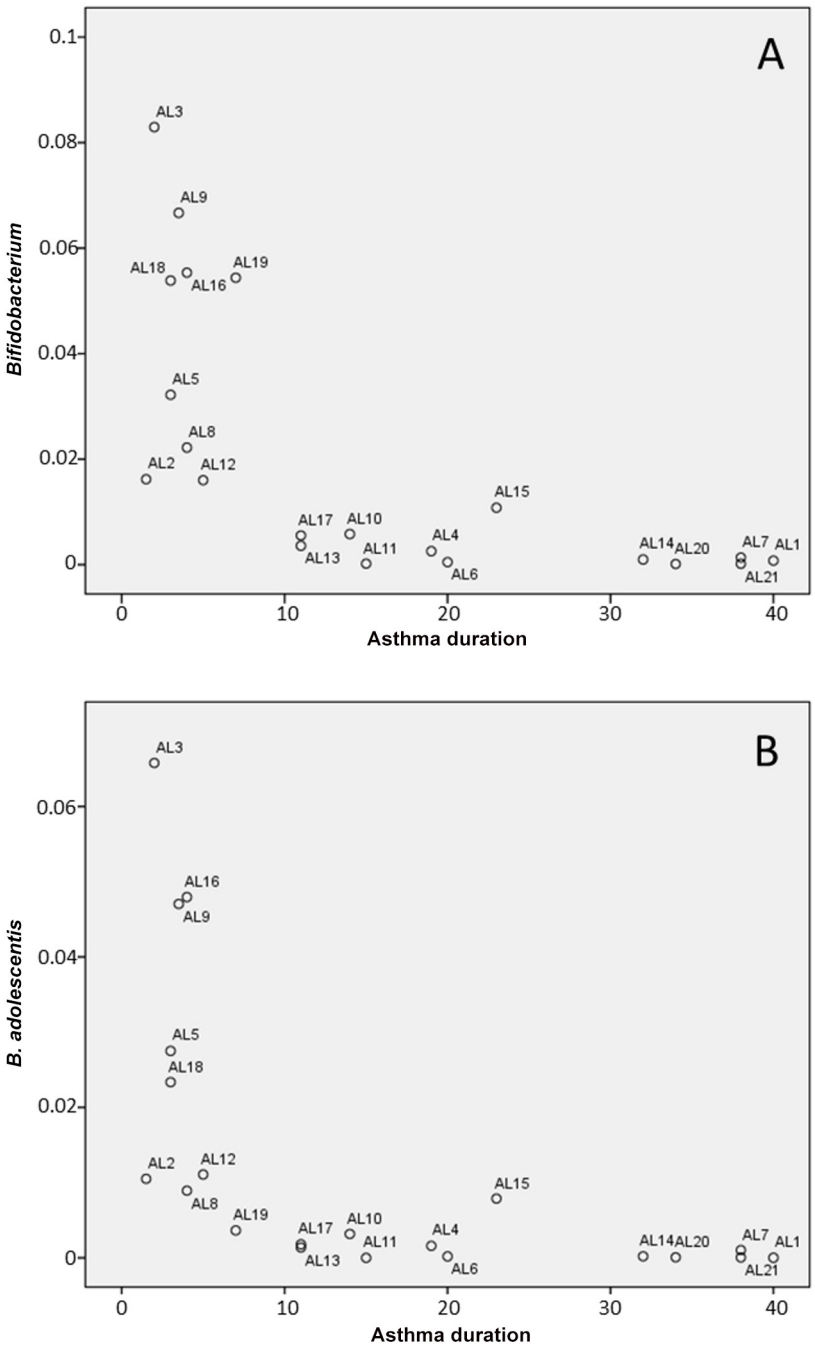

Fig 3. Correlation between the time of asthma ailment and the abundance of bifidobacteria (A) and *B. adolescentis* (B) in allergic asthma patients.

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variables. We found that there is a negative association of *B. adolescentis* with the time suffering the disease (β = -0.457), and a positive association with total IgE levels (β = 0.444), p model <0.001. Other clinical or demographical variables such as sex, age, most relevant causal allergen, specific IgE or time of rhinitis disease did not show any association.

Discussion

The microbiota plays an integral role in the homeostasis of multiple interconnected host metabolic and immune networks. There is solid experimental evidence that gut microbiota regulates our inflammatory immune response [22]. Disturbed gut colonization patterns have been associated with allergic disease but whether microbial variation is the cause or effect of these diseases is still under investigation. Whereas some authors have established a link between specific microbial profiles and allergy, other authors suggest that this should not be considered in broad terms and that it depends on the clinical manifestation of the allergic reaction and of the specific antigens triggering the immune response [9, 23,24].

Previous studies in infant/children populations have shown that lower levels of bifidobacteria are found in feces of allergic subjects compared with non-allergic individuals. However, the present work, performed in a well-defined population of allergic asthma adults, shows that the relative abundance of bifidobacteria is higher in the asthma group, suggesting an influence of this particular pathology on shaping bifidobacterial ecology in the gut. In relation to this, infants of atopic mothers were the only ones to be colonized with *B. adolescentis* and infants from allergic mothers had lower counts of bifidobacteria in feces than infants from non-allergic mothers [25]. Furthermore, Stsepetova et al. [26] indicated a less diverse composition of intestinal microbiota and prevalence of *B. adolescentis* in allergic childrens, and He et al. [27] found that *B. adolescentis* is found more often in the fecal microbiota of allergic childrens than in non-allergic ones. Thus, from previous works it seems that there is an association between *B. adolescentis* and allergy. The novelty of our findings is that, to our knowledge, this is the first time that bifidobacteria in general, and *B. adolescentis* in particular, has been associated with the ailment of allergic asthma. Remarkably, higher levels of *Faecalibacterium* were detected in the asthmatic patients; this finding could be related to the regular treatment with anti-inflammatory drugs of most of the patients, since the presence of *Faecalibacterium* is normally decreased during inflammatory process [28].

In summary, our data suggest that there is not a significant difference between the fecal microbiota profile of allergic subjects suffering from asthma and healthy individuals, neither a different microbial diversity. However, lower bifidobacterial levels are present in asthmatic individuals with long-term asthma, compared with those whose asthma was diagnosed more recently. Our findings suggest that the differences in the bifidobacterial populations in control vs asthma groups, as well as in short-term vs long-term asthma groups, could be explored further in order to develop novel treatments based on gut microbiota modulation. The results obtained also contribute to generate knowledge on the potential role of bifidobacteria in the physiology of allergy, and support the potential use of strains of these genera as probiotics to modulate allergy symptoms.

Supporting Information

S1 Fig. Rarefaction curves generated for 16S rRNA gene sequences obtained from faecal samples of control subjects (HC samples) and allergic asthma patients (AL samples). Panel a represents the rarefaction curves using the Shannon index. Panel b displays rarefaction curves using the Chao1 index.
(DOCX)

S1 Table. Sequence data features.
(DOCX)

S2 Table. Statistically significant differences between healthy controls (HC) and patients (AL) at the phylum, family and genus levels.
(XLSX)

Author Contributions

Conceived and designed the experiments: AH SG AS CDD MG MV BS AM. Performed the experiments: AH CM PL AC SG AS FT MG MV BS AM. Analyzed the data: AH CM PL CDD SG AS FT MG MV BS AM. Contributed reagents/materials/analysis tools: CM FT MG MV BS AM. Wrote the paper: AH CM PL CDD AC SG AS FT MG MV BS AM.

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CAPÍTULO 4

Caracterización de la funcionalidad de la microbiota intestinal de individuos con lupus eritematoso sistémico.

En este capítulo de la tesis se presentan los resultados obtenidos del análisis de la microbiota intestinal de individuos con LES a nivel funcional, es decir, de su actividad metabólica en el ambiente intestinal. La disbiosis de una comunidad microbiana compleja puede estar representada no solo por alteraciones poblacionales sino por diferencias globales en sus actividades a nivel funcional, que se traducen en último término en la producción diferencial de distintos metabolitos con capacidad efectora en el diálogo con el hospedador.

Para ello hicimos uso de la metabolómica como herramienta, lo que nos proporcionó un conjunto de técnicas integradas que en conjunto y a través de una potente base de datos nos permitió cuantificar una gran cantidad de metabolitos de la microbiota fecal de LES y controles sanos. En nuestro estudio observamos diferencias significativas en un grupo de metabolitos entre controles sanos y LES; también observamos diferencias entre individuos sanos con alto y bajo índice de masa corporal, factor que no es determinante en el caso del LES. Esto resalta la categorización que se establece en la enfermedad autoinmune, sobre el índice de masa corporal, como factor dominante en la regulación del metabolismo microbiano gastrointestinal.

Por otro lado en este capítulo también abordamos intentar modular la respuesta inmune de una microbiota intestinal de LES mediante suplementación con distintas especies de microorganismos que aparecieron disminuidos en este grupo, en diferentes concentraciones. Este efecto se evaluó en un modelo *in vitro* utilizando co-cultivos con células dendríticas derivadas de monocitos de sangre periférica y linfocitos T vírgenes.

El enriquecimiento de la microbiota fecal de LES con ciertos microorganismos

comensales o con una cepa de *Bifidobacterium bifidum* corrigió en cierta medida la respuesta inmunológica alterada en LES. Para el futuro quedaría validar en experimentos *in vivo* la aplicación de estas especies bacterianas en la mejora de los síntomas de LES.

Los resultados correspondientes obtenidos en este capítulo se muestran como dos artículos publicados:

- Lopez P, de Paz B, Rodríguez-Carrio J, **Hevia A,** Sánchez B, Margolles A, Suarez A. Th17 responses and natural IgM antibodies are related to gut microbiota composition in systemic lupus erythematosus patients. Scientific Reports. 2016 Apr 5;6:24072.

- Rojo D, **Hevia A**, Bargiela R, López P, Cuervo A, González S, Suárez A, Sánchez B, Martínez-Martínez M, Milani C, Ventura M, Barbas C, Moya A, Suárez A, Margolles A, Ferrer M. Ranking the impact of human health disorders on gut metabolism: Systemic lupus erythematosus and obesity as study cases. Scientific Reports, 2015; 5:8310.

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Ranking the impact of human health disorders on gut metabolism: Systemic lupus erythematosus and obesity as study cases

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Multiple factors have been shown to alter intestinal microbial diversity. It remains to be seen, however, how multiple collective pressures impact the activity in the gut environment and which, if any, is positioned as a dominant driving factor determining the final metabolic outcomes. Here, we describe the results of a metabolome-wide scan of gut microbiota in 18 subjects with systemic lupus erythematosus (SLE) and 17 healthy control subjects and demonstrate a statistically significant difference ($p < 0.05$) between the two groups. Healthy controls could be categorized ($p < 0.05$) based on their body mass index (BMI), whereas individuals with SLE could not. We discuss the prevalence of SLE compared with BMI as the dominant factor that regulates gastrointestinal microbial metabolism and provide plausible explanatory causes. Our results uncover novel perspectives with clinical relevance for human biology. In particular, we rank the importance of various pathophysiologies for gut homeostasis.

Our commensal microbiota is a plastic "organ" comprised of trillions of microbes with symbiotic functional capabilities that directly affect human health. Important studies on the relationship of intestinal microbiota with diseases have linked profound changes in the composition of the population and metabolic functions of the gut microbiota to common human intestinal disorders, such as obesity[1], Crohn's disease and colitis-associated colorectal carcinoma[2], ulcerative colitis and irritable bowel syndrome[1], and *Clostridium difficile*-associated diarrhea[3,4]. Recent studies have also suggested that factors, such as antibiotic treatments[4,5] and diet[6], and subject characteristics, such as age[7], may be involved in alterations in the microbiota. Researchers are beginning to recognize and understand the short- and long-term consequences of these changes[5,7–9].

Although evidence has suggested an additional link between gut microbiota and immune disorders[10], this relationship remains incompletely understood. In a previous work, a relevant intestinal dysbiosis was described in the prototypical auto-immune disease systemic lupus erythematosus (SLE)[11]. This microbial imbalance was characterized by increased *Bacteroidetes* levels and a lower *Firmicutes*/*Bacteroidetes* ratio. Metagenome functional inference highlighted putative metabolic processes that were potentially associated with SLE patients, such as an overrepresentation of glycan metabolism and oxidative phosphorylation. However, although this *in silico* analysis could be correlated with a higher abundance of some specific bacterial groups in lupus (i.e., *Bacteroidetes*), experimental evidence of the overall gut microbiota functionality in SLE patients is lacking. In this report, a metabolome-wide scan of the gut microbiota in patients ($n = 18$; SLE codes) with the prototypical

auto-immune disease SLE[12] and healthy controls (HC codes; $n = 17$) is presented. The aim was to discuss primarily whether SLE plays a role in shaping the metabolism of gastrointestinal microbiota, and if so, to obtain information on the cause and effect relationship between the altered microbial metabolites and the underlying disease. This study should be of relevance because patients with SLE exhibit a marked predisposition to metabolic syndrome[13], atherosclerosis[14], renal and urinary dysfunctions[13,15], and insulin resistance[15], and some of these disorders have been linked to gastrointestinal microbiota[16,17]. These results demonstrate a separation between the chemical compositions of gut microbiota of both the SLE and HC groups, which was not observed by examining the taxa abundance and composition of their corresponding microbiota. In addition, body mass index (BMI) was shown to have a remarkable effect in healthy subjects although having no effect in patients with SLE, which suggests that the auto-immune response is a stronger driver of intestinal dysbiosis than obesity.

Results

Study cohorts. SLE patients ($n = 18$) were recruited from the updated Asturian Register of Lupus. All patients fulfilled at least four of the American College of Rheumatology criteria for SLE[11]. All were women of Caucasian descent (49.1 ± 9.7 years old) with no active disease at the time of sampling (Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score ≤8). Information on the clinical manifestations of the disease was obtained from the individual clinical records (Supplementary Table 1). Only individuals who had not used antibiotics, glucocorticoids, immunosuppressive drugs, monoclonal antibodies, or other immunotherapies in the 6 months prior to enrollment were recruited for the study. Seventeen gender- and age-matched healthy controls (HC codes) were recruited from the same population. To reduce the possibility that our assay was affected by factors known to influence the gut microbial profile, such as age, diet, and medications, patients with similar factors were selected. These factors included gender (all study subjects were women); age; the absence of antibiotics, steroids, and immunological treatments; medical history (presenting with a wide variety of clinical SLE manifestations); disease duration (2–24 years); and an absence of flares of disease activity at the time of sampling (Supplementary Table 1). Additional factors also included the mean dietary intake of energy, macronutrients, micronutrients, fiber, and phyto compounds (Supplementary Table 2) and lifestyle-related factors (smoking, alcohol consumption, physical activity, and use of vitamin and mineral supplements). The complete datasets together with body mass index (BMI) values for each of the investigated individuals are presented in Supplementary Table 3.

Contribution and prevalence of SLE in gut homeostasis. Our study protocol comprised the isolation of metabolites from microorganisms obtained from fecal material followed by a metabolome-wide scan via a combination of mass spectrometry (MS) with liquid chromatography (LC) and capillary electrophoresis (CE) separations. Of 134,312 masses, a total of 955 (LC-MS positive mode: 331; LC-MS negative mode: 549; CE-MS: 75) fulfilled statistical criteria for selection (p value <0.05; Mann-Whitney U test or t-test; Supplementary Table 4). A scatter plot based on principal component analysis scores obtained from this set of compounds revealed a clear separation between the SLE and HC groups (Figure 1). Mass signals were highly similar in all SLE patients regardless of age, BMI, disease duration, dietary intake, lifestyle-related factors, or medical history (Supplementary Tables 1–3). Interestingly, the HC group was divided into two distinct subgroups (Figure 1). A total of 572 of the 134,312 masses (LC-MS positive mode: 155; LC-MS negative mode: 352; CE-MS: 65) caused this separation ($p < 0.05$; Supplementary Table 4). Regardless of age, dietary intake and lifestyle-related factors (Supplementary Tables 2 and 3), HC subjects with BMIs ranging from 20.19 to 24.83 kg/m^2 formed Cluster 1, whereas those with BMIs ranging from 25.24 to 36.92 kg/m^2 formed Cluster 2. This separation was not observed in SLE patients (Figure 1), whose BMIs ranged from 19.95 to 37.91 kg/m^2 (Supplementary Table 3).

Taken together, we demonstrate that the immune status of SLE patients is thus a dominant factor that swiftly regulates the metabolome of the gut microbiota regardless of environmental or individual characteristics. In contrast, in healthy subjects in whom no stronger pressure than BMI exists, BMI (e.g., overweight or obesity) becomes a driving factor determining microbiotal metabolism. The results provided in Figure 1 suggest that the division of the clusters in HC subjects occur within a BMI interval ranging from 24.83 to 25.24 kg/m^2, which corresponds to the upper and lower limits of the two

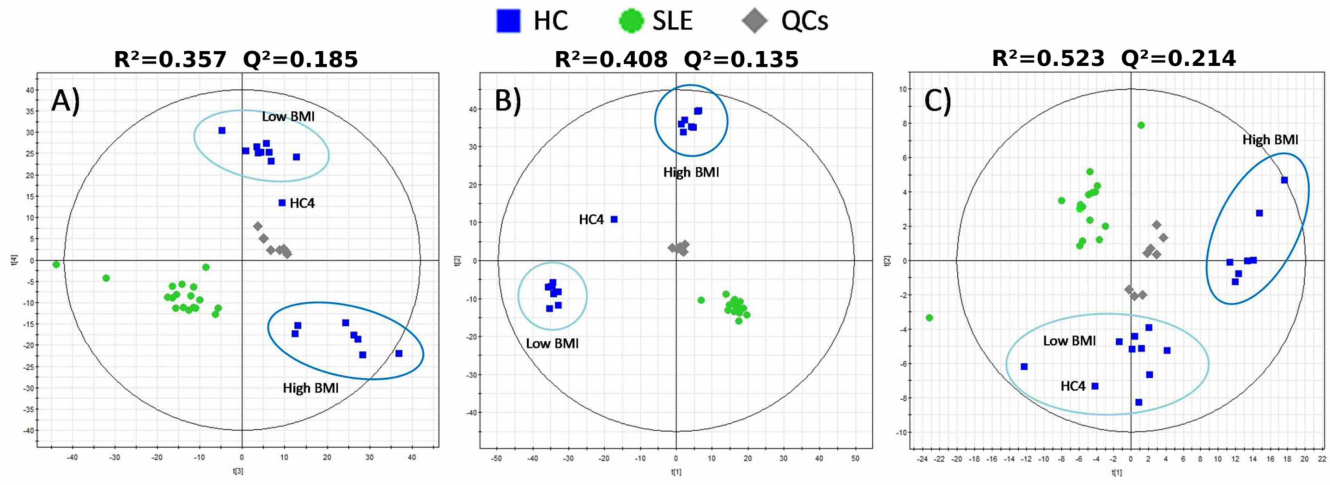

Figure 1 | A principal components analysis (PCA) plot for the models built using the set of filtered data that were present in at least 50% of the quality controls (QCs) and for which the coefficients of variation were less than 30% across the QCs. A) PCA plot based on LC-MS (+) data: 4 components (PC3 vs. PC4 shown; no biological variation described by the first two components). B) PCA plot based on LC-MS (−) data: 5 components (PC1 vs. PC2 shown). C) PCA plot based on CE-MS data: 6 components (PC1 vs. PC2 shown). Statistics (R^2 and Q^2) are provided in the Figure panels. HC4, with a BMI of 26.29 kg/m^2, located within "High" and "Low" BMI groups was rejected prior to statistical analysis. The "High BMI" cluster was formed from the following samples (BMI in kg/m^2 in parentheses): HC6 (27.19), HC8 (27.40), HC19 (25.24), HC20 (28.82), HC26 (30.92), HC29 (36.90), and HC33 (25.95). The "Low BMI" cluster was formed from the following samples: HC11 (20.19), HC13 (24.83), HC14 (24.80), HC16 (22.18), HC21 (23.18), HC22 (23.07), HC28 (21.90), HC30 (22.68), and HC32 (23.13).

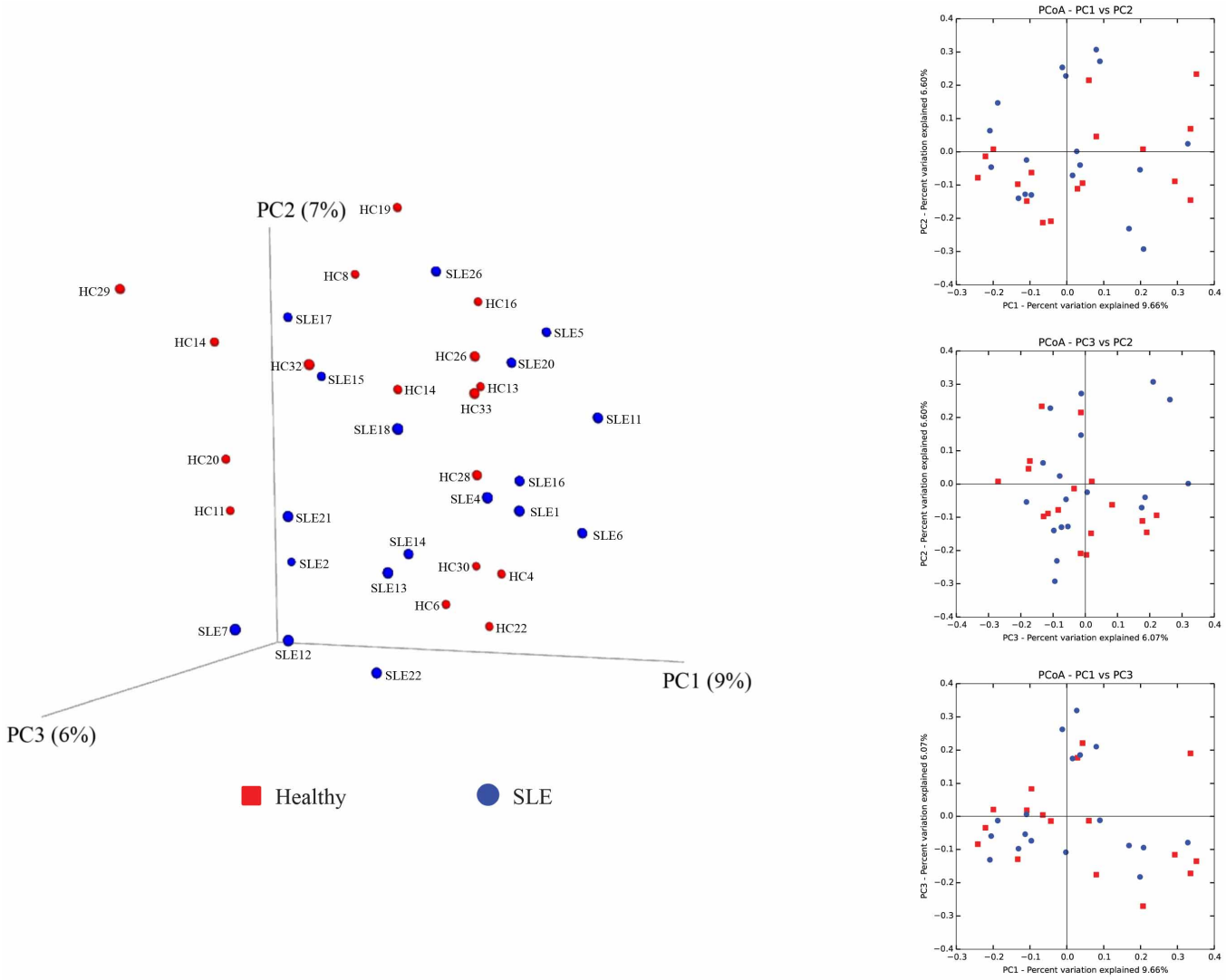

Figure 2 | Principal coordinate analysis (PCoA) of SLE and HC based on taxa abundance and composition of gut microbiota. Three-dimensional and bi-dimensional PCoA representation based on a UniFrac similarity matrix of the composition of SLE and HC sample operation taxonomic units (OTUs) is shown. Percentages shown along the axes represent the proportion of dissimilarities captured by the axes. Total bacterial 16S rRNA data and taxa abundance were determined as reported[11]. The raw sequences reported in this article have been deposited in the NCBI Short Read Archive (SRA) (study accession number: SRP028162). Codes are as shown in Supplementary Table 3.

identified clusters (all samples with BMI $\leq$ 24.83 kg/m^2 clustered together, and samples with BMI $\geq$ 25.24 kg/m^2 formed a separate cluster). Note that lean-normal individuals are typically characterized by a BMI $\leq$ 24.99 kg/m^2, over-weight individuals by a BMI $\geq$ 25 kg/m^2 (from 25.0 to 29.99), and obese individuals by a BMI $\geq$ 30 kg/m^2 (from 30.0 to 40)[18]. Therefore, one could assume that an unknown mechanism triggers the alteration of gut microbiota functionality at the frontier between lean and over-weight host status. This finding is supported by our previous study in a different population of healthy lean and obese individuals showing that the gut microbiota boosts glycosyl hydrolase activities at a similar BMI range (i.e., 24.5–25 kg/m^2)[17]. This result is of particular importance as, to the best of our knowledge, no previous investigation has linked host BMI to gut microbiota metabolic dynamics in the development of obesity.

It should be highlighted that a principal coordinate analysis (PCoA) of SLE and HC subjects, based on 16 rRNA microbiota profiles, did not result in subcategorized clusters, either between SLE and HC or between HC subjects with high and low BMI (Figure 2)[11]. This suggests that the changes induced by SLE or BMI (in healthy controls) become marked at the highest level of the functional hierarchy, i.e., the metabolite level (Figure 1), regardless of the heterogeneities that appear below the functional level, i.e., the level of microbiotal population structure (or 16S rRNA).

Association of SLE with chemical compositions in the gut microbiota: explanatory analysis. As mentioned above, only 955 of 134,312 (or 0.72% of the total) mass features statistically ($p < 0.05$) differed between the SLE and HC groups, suggesting that the impact of SLE on the gut microbiotal metabolite-wide flux distribution and on metabolism itself is moderately low. As we are aware that our study identifies metabolic signatures (955) associated with immune status in SLE compared to HC individuals, mechanisms explaining these associations must be proposed. For this purpose, empirical formulas were assigned to masses that achieved statistical criteria ($p < 0.05$) with a maximum error of 5 ppm using the CEU Mass Mediator (http://biolab.uspceu.com/mediator). We describe each of the major effects linked to key chemical species below.

We first observed that SLE patients exhibited reduced levels of homoserine lactone (HSL) (11.3-fold reduction; $p = 0.001$; Supplementary Table 5). HSL is the degradation product of *N*-acyl-HSLs (AHSL) when metabolized by AHSL lactonases and acylases[19–21].

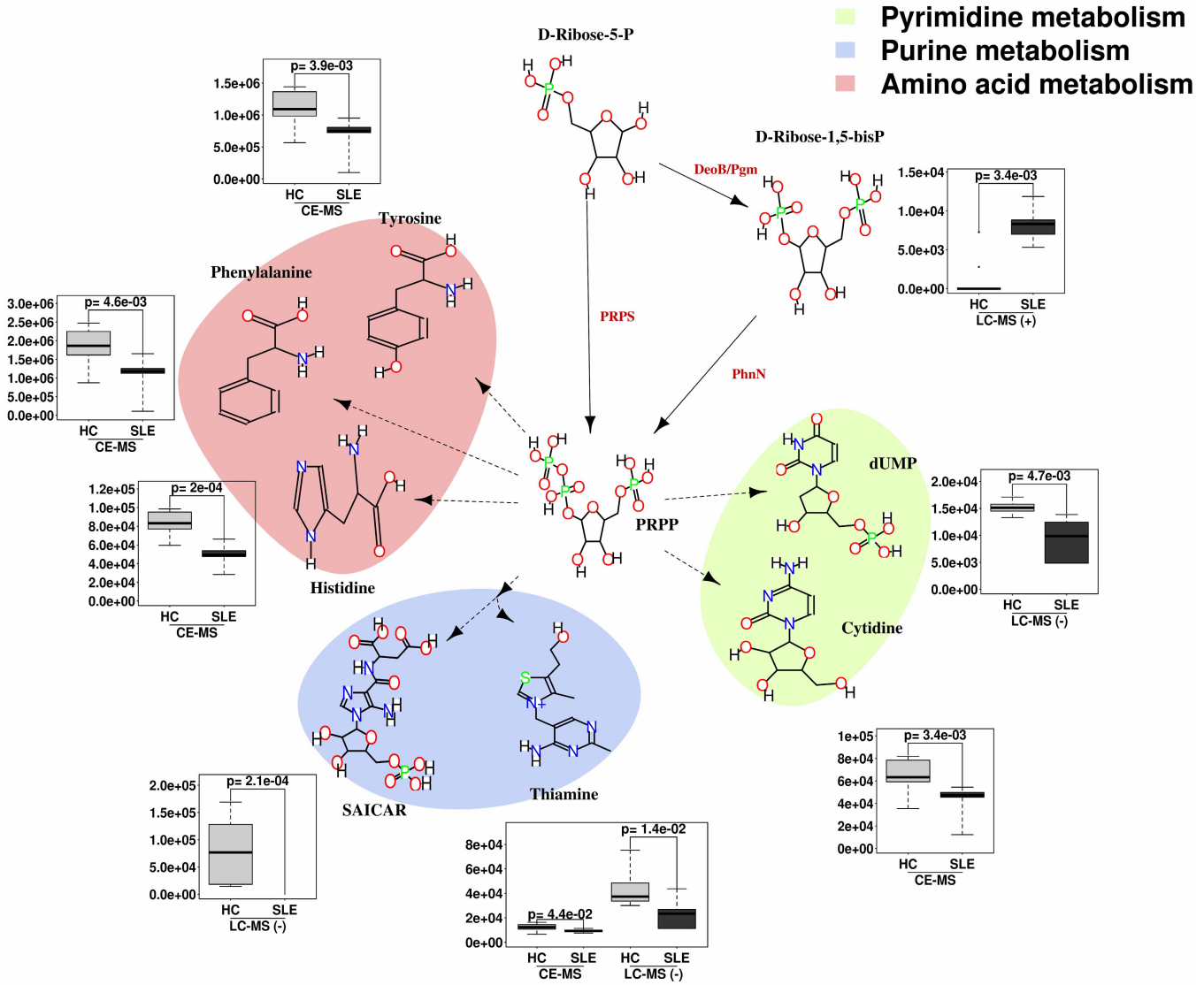

Figure 3 | Box plots of key metabolite abundance levels in SLE patients compared with HC controls. The data and the statistical significances were extracted from the data presented in Supplementary Table 5. The data are presented in the context of the KEGG metabolism pathways and indicate the connection to each of the chemical species.

The reduced accumulation of HSL in SLE patients may be related to an increase in the activity of quorum quenching enzymes that can decrease the pool of AHSL and thus might attenuate quorum sensing and cell-to-cell communication and promote disease progression[19–23].

Compared to HC subjects, SLE patients also exhibited significantly reduced levels of N-acetylmuramic acid (MURNAc) (25.0-fold reduction; $p = 0.00005$) and, to a lesser extent, N-acetylglucosamine (1.5-fold reduction; $p = 0.0004$) (Supplementary Table 5). Both are essential components of the peptidoglycan biopolymer of bacterial cell walls. Peptidoglycans have long been known to promote an inflammatory response; thus, lowering the production of peptidoglycan components, caused by deficiencies in key enzymes[24] and/or the bacteria that express them, has been demonstrated to potentially influence signaling, disease factors and immune responses[16,25,26]. In addition, a series of observations have led to the hypothesis that in patients with rheumatoid arthritis with a genetic basis, normal intestinal microbiota harbor bacteria with cell walls capable of stimulating rheumatoid factor, thus possibly inducing arthritis[16]. It is therefore plausible that SLE induces deficiencies in signaling chemical species, particularly MURNAc, that compose the cell walls of gastrointestinal bacteria and that these deficiencies affect the progression of the disease and its collateral effects.

Significantly increased levels of ribose-1,5-bisphosphate (R1,5-dP; 629.8-fold increase; $p = 0.0034$) were also observed in SLE patients compared with HC subjects (Figure 3). This chemical species is an intermediate in the production of 5-phospho-alpha-D-ribose-1-diphosphate (PRPP), which is required for *de novo* purine and/or pyrimidine biosynthesis and the synthesis of amino acids such as histidine, tyrosine, and phenylalanine. In fact, an absence of 1-(5′-phosphoribosyl)-5-amino-4-(N-succinocarboxamide)-imidazole (SAICAR) ($p = 0.0002$) and slightly reduced production levels of thiamine (1.9-fold reduction; $p < 0.042$), dUMP (1.7-fold reduction; $p = 0.005$), cytidine (1.5-fold reduction; $p = 0.0003$), histidine (1.7-fold reduction; $p = 0.0002$), tyrosine (1.6-fold reduction; $p = 0.004$), and phenylalanine (1.7-fold; $p = 0.005$) were observed in SLE patients compared with healthy controls; note that the production of these chemical species depends on the PRPP concentration.

We reasoned that the decreased activity of R1,5-dP-modifying enzymes may lead to an accumulation of this substrate in SLE patients; this in turn may result in reduced production of metabolites in the consequent metabolic steps. This was further confirmed by targeted metabolomics where the extension of the biochemical production of R1,5-dP from its reaction substrate ribose-5-phosphate (Rib5P) as well as the consequent conversion to PRPP (see Figure 4)

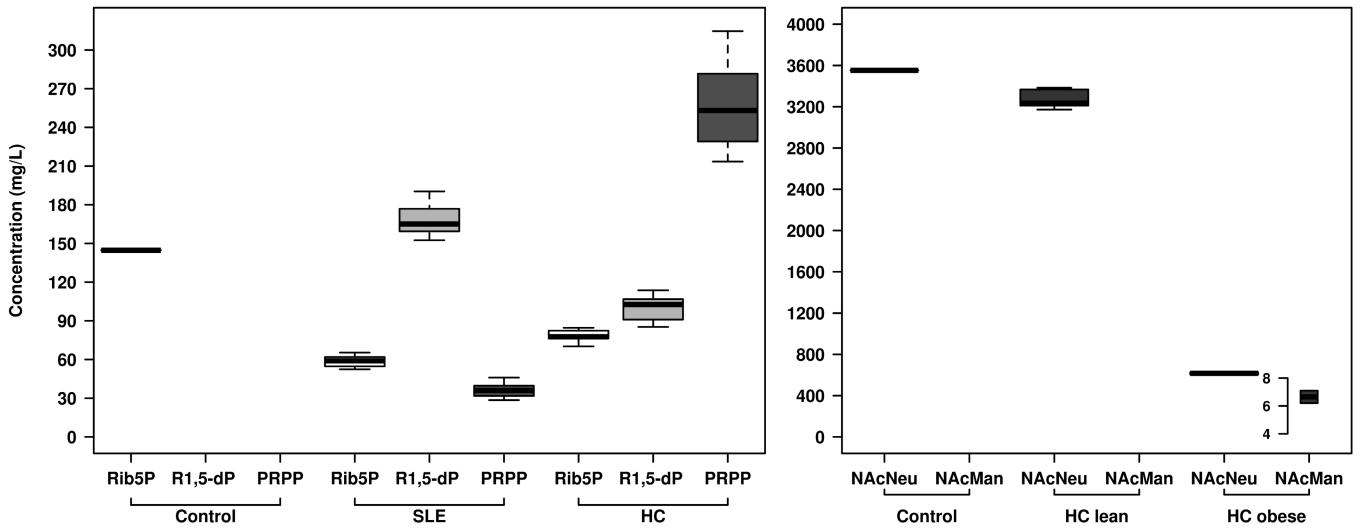

Figure 4 | Box plots of chemical species concentrations in reaction tests containing Rib5P (Panel A) and sialic acid (Panel B). The corresponding peak areas were integrated using MassHunter Quantitative Analysis (B.05.00, Agilent). The final concentration of each analyte per sample was calculated using the interpolation of each peak area in the corresponding calibration curve. Reaction conditions were as follows: 1 mL of 50 mM HEPES pH 7.0, 0.1 mg of total protein, and 0.15 mg of Rib5P (Panel A) or 3.5 mg of sialic acid (Panel B). Reactions were incubated for 15 min at 25°C, after which substrate conversion was examined by LC-QTOF-MS using the conditions described in the Methods section. All SLE and HC samples were tested for substrate conversion in Panel A, whereas data for only the lean and obese HC subgroups are shown in B. Reactions without proteins but with Rib5P or sialic acid were used as controls, and the initial concentrations are shown for comparative purposes.

was evaluated using microbiotal protein extracts. Indeed, the following four presumptive enzymes are implicated in these transformations: *i*) phosphopentomutase (DeoB) and ribose 1,5-bisphosphokinase (PhnN) transforming Rib5P to R1,5-dP; *ii*) phosphoglucomutase (Pgm) metabolizing R1,5-dP to PRPP; and *iii*) ribose-phosphate pyrophosphokinase (PrsA protein), which is implicated in the direct transformation of Rib5P to PRPP. For this transformation, microbial protein extracts from each of the SLE and HC subjects were obtained as previously described[16] for an activity test using a solution containing Rib5P (see Methods section). The extent of Rib5P transformation and the presence of the R1,5-dP and PRPP reaction products were quantified by LC-QqQ-MS (Figure 4). At the end of our assay, transformation of Rib5P was demonstrated to a similar extent in both groups (54 to 40% residual concentration). A higher concentration of R1,5-dP (1.7-fold) and a significantly lower concentration of PRPP (7.0-fold) were observed in SLE patients compared to HC subjects (Figure 4). This confirms that the accumulation of R1,5-dP in SLE patients is most likely due to a lower level of Pgm activity involved in the transformation of Rib5P to PRPP and not to the increased level of DeoB activity that controls R1,5-dP biosynthesis from Rib5P.

Finally, we further noted that SLE patients accumulated mesoporphyrin IX ($p < 0.0008$) and protoporphyrin IX ($p = 0.0004$), which were absent in HC subjects (Supplementary Table 5). The fact that mesoporphyrin IX is an inhibitor of heme synthesis and ferrochelatase activity is consistent with the accumulation of protoporphyrin IX due to the presumptive inhibition of HemH proteins[27]. We suggest that SLE most likely decreases the iron uptake capacity of the gut microbiota and may also inhibit heme synthesis. In agreement with this, the serum ferritin level in SLE patients ($n = 18$) was approximately 1.5-fold lower (according to mean values) than in HC subjects ($n = 17$) (Supplementary Table 3), and thus a relationship between the deficiency in microbiotal iron uptake observed using a metabolome-wide scan in SLE patients and a lower level of serum ferritin could be suggested.

Higher BMIs in healthy controls promote the presence of bacteria possessing the sialic acid catabolic pathway. Only 572 of 134,312 (or 0.43% of the total) mass features were statistically ($p < 0.05$) different between healthy controls with high and low BMI values (Figure 1). Therefore, when both groups were compared, the data indicated that the impact of BMI on the gut microbiotal metabolite-wide flux distribution and metabolism itself was moderately low. However, among differences in other chemical species (Supplementary Table 5), an absence of N-acetylneuraminate ($p = 9.65e^{-7}$; Supplementary Table 5) was strongly associated with HC subjects with BMIs $\geq$ 25.24 kg/m^2 (sub-group "High BMI" in Figure 1). We reasoned that the activities of N-acetylneuraminate lyases, encoded by the sialic acid catabolic gene *nah*A[28], which remove a pyruvate from N-acetylneuraminate as a first step in the catabolism of sialic acid, should be strongly depleted in lean individuals (here, BMI $\leq$ 24.83 kg/m^2). This may result in the accumulation of sialic acid when compared with overweight or obese individuals (sub-group "High BMI").

To confirm this, the transformation of sialic acid into its corresponding product N-acetyl-D-mannosamine was further examined using a target metabolomics approach, in which the conversion of sialic acid was followed using microbiotal protein extracts (see Methods). The results revealed that lean HC patients were not able to metabolize sialic acid, although it was transformed (only 13.8% residual concentration at the end of the assay) to N-acetyl-D-mannosamine in obese HC subjects (Figure 4). This confirms that the catabolism of sialic acid may be strongly diminished in lean individuals, most likely due to the absence or lower activity level of NahA proteins.

Taken together, it is plausible that individuals from both high and low BMI sub-groups may possess colonic bacteria that have the capacity to liberate sialic acid from the mucosa and transport it to bacterial cells. However, individuals with high BMI (here, $\geq$25.24 kg/m^2) may have an additional genomic complement for the sialic acid catabolic pathway (i.e., bacteria that produce NahA) that enables further metabolism of sialic acid, whereas colonic bacteria from individuals with low BMI (here, $\leq$24.83 kg/m^2) may be unable to catabolize sialic acid. Thus, sialic acid tends to accumulate in bacterial cells. This was confirmed by biochemical tests and target metabolomics analyses. These data suggest that BMI (e.g., overweight/obesity) may not alter mucosal carbohydrate bioavailability but rather alters how liberated sugars

are catabolized. We speculate that community members in individuals with high BMIs may efficiently consume mucosal carbohydrates, which in turn may induce growth and self-promoting host inflammation compared with individuals with low BMI. Consistent with this hypothesis, it has been reported recently that bacteria and pathogens that are unable to catabolize sialic acid exhibit impaired expansion[28].

Discussion

We have demonstrated for the first time that the gastrointestinal microbiota can be affected by immune factors. This association was found at the level of the metabolite landscape of gut microbiota (Figure 1) but not at the level of bacterial composition (Figure 2), which suggests that SLE can influence the heterogeneous species inhabiting the gut in such a concerted way that a distinctive metabolic pattern arises. These results demonstrate that deficiencies in the chemical species mediating cell signaling and regulation are among the major effects of SLE. We speculate that lowering quorum sensing, cell-to-cell communication and cell wall biosynthesis, which are known to be of global importance in microbial ecosystems[19–24], may be partially responsible for the concerted mechanism inducing these common metabolic patterns. Such alterations may also act as disease factors by promoting the immune response, as has been suggested in the case of arthritis[24,25]. These alterations may also cause alterations in specific cell-critical systems, such as nucleotide biosynthesis, iron uptake and heme synthesis, without substantial loss of metabolic robustness. However, further experimental evidence is needed to confirm the cause and effect relationship between the altered metabolites and the underlying SLE disease.

We also found that BMI has an effect at the level of the metabolite landscape but not at the level of the microbiota composition based on 16S rRNA gene survey. Interestingly, such changes were only noticeable in healthy controls. The impact of BMI on metabolism itself is also limited, and only select robust effects on the catabolism of sialic acid were revealed. The fact that no effect on the production of regulatory/signaling molecules and cell wall synthesis was observed in HC individuals suggests that different mechanisms may be responsible for generating the distinct metabolic patterns of SLE and HC intestinal microbiota and that a regulatory/signaling response may be one of the major causes linking the altered microbial metabolites and SLE disease, where BMI did not have an effect.

Together, the evidence generated in this study demonstrates that the gut microbiota functionality can be affected by immune and weight factors. Also, it demonstrates that metabolome-wide assessments may be a better indicator than 16S rRNA survey to enable not only the segregation of different diseases and disorders (here, SLE and overweight/obesity) but also the ranking of the effects of the disease/disorder on microbiotal metabolism. As an example, we demonstrated that an immune response, exemplified by SLE, is a dominant factor compared to obesity in controlling the metabolism of the intestinal microbiota. We believe that these findings, for which no previous evidence exists in the scientific literature, potentially open new research avenues for investigating the response mechanisms of human gut microbiota to a single or collective immune, genetic, pathogenic, and dietary pressures, and more importantly, the interaction and relative clinical importance of each of these factors for the progression of different diseases and predispositions to metabolic dysfunctions, such as metabolic syndrome in SLE patients[13].

Only 955 of 134,312 (or 0.72% of the total) and 572 of 134,312 (or 0.43% of the total) mass features were found to significantly differ between the SLE and HC groups and between HC subjects with high and low BMI values, respectively. It is therefore important to evaluate whether or not such subtle differences can be considered within a common range. It is worth noting, however, that no report to date has described the metabolomic profiling of either bacterial fecal extracts or fecal fluids from patients with SLE; therefore, little is known about whether the observed differences between SLE and HC are within a common range. In the case of subjects that are discordant for weight, few examples exist in the literature that have examined fecal fluid metabolomes. Thus, it should be highlighted that a recent report examining the cecal metabolome revealed that only 65 out of a total of 10,515 mass signals (or 0.7% of the total) were significantly associated with a high-fat diet[29]. In a different study, only 22 fecal metabolites have been shown to be differentially produced in monozygotic twin pairs that were discordant for weight[18]. In cases of other pathophysiologies, using metabolite profiling of fecal fluids, it was found that only: *i*) 18 fecal metabolites allowed discrimination between ulcerative colitis and irritable bowel syndrome and healthy control patients[30]; *ii*) 99 allowed discrimination of humanized and gnotobiotic mice, even though they possess quite distinct microbiota (85% of genera and microbial species are different)[31]; *iii*) 43 metabolites were found to differ when comparing human, mouse and rat fecal metabolomes[32]; and *iv*) 22 metabolites allow the segregation of patients with colorectal cancer compared to healthy adults[33]. Therefore, based on bibliographic records in the specialized literature, the subtle differences associated with SLE or BMI reported in the present study can be considered within a common range observed for, or even few times higher than, those observed for reported pathophysiologies.

The further question that arises is whether these subtle differences in gut microbiota functionality are sufficient to have physiological implications. Based on the data reported herein, it is plausible that only selective effects in a number of key metabolites with major biological relevance/significance may be sufficient to induce gut homeostasis or alterations in gut microbiota functionality, even though the impact on the global metabolome is moderate, regardless of the heterogeneities at the population level. The deficiencies observed in chemical species participating in, for example, quorum sensing, cell-to-cell communication and cell wall biosynthesis, which are known to be of global importance in microbial ecosystems, agree with this hypothesis. Having said that, it should be noted that in many cases, minor differences, e.g., at the population level, have been demonstrated to induce strong physiological changes. As an example, it has recently been demonstrated that one or two strains are sufficient to drive major changes in gastrointestinal and host (mouse) metabolic profiles where up to 10^{12} microbial cells or more than 500 species may coexist[31].

The effects of various pathophysiologies in the human gut metabolome have been previously examined[18,29–33]. However, no clear associations between fecal fluid metabolome patterns and individual pathophysiologies (e.g., weight gain or the presence of a disease) were previously observed; this was mainly due to the large inter-individual variation. For example, the examination of cecal metabolomes from at least 10 obese mice (body weight change: from 2 to 8 grams) revealed heterogeneous distributions, and no clear clusters were visible at the BMI level[29]. Such inter-individual variation was not observed in this study, as mass signals within grouped subjects (SLE patients and HC subjects with "low" or "high" BMI) were highly similar regardless of age, disease duration, dietary intake, lifestyle-related factors or medical history. One of the major differences from previous studies is that herein we focused on the isolation of metabolites from microbes isolated from stool material followed by a metabolome-wide scan, rather than examining total fecal fluids. In relation to this examination, the microbiota is the central bioreactor of the gastrointestinal tract, and a dynamic interplay exists with the host and the environment[7]. As a result of metabolic actions and environmental inputs, the gut environment and, in turn, the fluid fecal material contains a complex mixture of metabolites provided through the diet, the host and intestinal bacteria. Such complex mixtures are commonly investigated in metabolomics studies[18,29–33]. Metabolites from intestinal bacteria, rather than dietary and host metabolites, are required to maintain and repair the large intestine and to support human health[34]. Therefore, any

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knowledge related to metabolites that are directly produced or adsorbed (from environmental inputs or the host) by gut microbes, not those present in complex whole fecal fluids, may be of relevance not only for investigating what is happening throughout the gut but also for determining their role in pathophysiologies and human health. We believe this investigation will provide information that can be directly linked to complementary microbial data, i.e., 16S rRNA gene profiles, which is difficult to achieve otherwise if non-microbial metabolites (from the environment or host), which are commonly considered when working with whole fecal material, are investigated. In the present study, metabolites from intestinal bacteria have been shown to be good indicators of gut microbiota functionality under various pathophysiologies, and they may be more effective than fecal fluids as a read-out of pathophysiologically induced alterations. Note that our study relates to metabolite levels inside gut bacterial cells, which may have a different meaning than those in plasma and, to some extent, in fecal fluids.

Finally, further research is required of the mechanisms that generate the distinct and robust gut microbial metabolic profiles discussed herein. This investigation will provide a deeper view on *what the microbiota do* rather than *who they are*. We hypothesize that new reliable clinical information and explanatory and mechanistic plausibility for these associations as well as new sensitive, predictive disease biomarkers of clinical relevance may arise when the microbiotal metabolite landscape rather than heterogeneous species gut composition are investigated.

Methods

Chemicals and reagents. The following reagents were used: acetonitrile (HPLC-MS grade, Sigma-Aldrich, Taufkirchen, Germany), formic acid (MS grade, Sigma-Aldrich, Steinheim, Germany), L-methionine sulfone (Sigma-Aldrich, Taufkirchen, Germany), sodium hydroxide (Panreac, Montcada I Reixac, Spain) and ammonia (Panreac, Castellar del Vallès, Barcelona, Spain). For reference masses, purine and hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine (HP) from Agilent (API-TOF reference mass solution kit) were used. All solutions were prepared using MilliQ® water (Millipore, Billerica, MA, USA).

Sample treatment for metabolite isolation. Fresh stool samples were collected from each subject, frozen immediately, and stored at −80°C until they were processed. A total of 34 samples were metabolome-typed using a combination of untargeted mass spectrometry and two different and complementary separation techniques (liquid chromatography-mass spectrometry [LC-ESI-QTOF-MS] and capillary electrophoresis-mass spectrometry [CE-TOF-MS]). To facilitate this analysis, microbial cells were separated from the fecal material, and the total microbial metabolites were extracted from equal amounts of microbial cells per sample by adapting a previously reported method[4] and including a two-step extraction method that was shown to produce the optimal extraction efficiency for both polar and hydrophobic metabolites.

Briefly, microbial cells were separated from the fecal matrix by mixing 0.4 g of fecal sample with 1.2 mL of phosphate-buffered saline (PBS) solution (1 : 3 w/v feces to PBS ratio); following re-suspension (by 1 min of vigorous vortexing), the samples were then centrifuged at 1,000 *g* at 4°C for 1 min to remove fecal debris. The supernatant (0.6 mL) was transferred to a 2-mL Eppendorf tube and centrifuged at 13,000 *g* at 4°C for 5 min to pellet the cells. Immediately after isolating the microbial cells, the cells were used for MeOH extraction by adding 1.2 mL of cold (−80°C) HPLC-grade MeOH. The samples were then vortex-mixed (for 10 s) again and sonicated for 30 s (in a Sonicator® 3000; Misonix) at 15 W in an ice cooler (−20°C). This protocol was repeated twice more with a 5-min storage at −20°C between each cycle, and the final pellet was removed following centrifugation at 12,000 *g* for 10 min at 4°C. Immediately after the MeOH extracts were obtained, the MeOH solution was stored at −80°C, and the remaining cell pellet was re-suspended in 1.2 mL of cold (4°C) HPLC-grade H_2O and subjected to 3 cycles of sonication for 20 s (in a Sonicator® 3000; Misonix) at 15 W in ice water. The samples were incubated on ice for 2 min between cycles. The final pellet was removed following centrifugation at 12,000 *g* for 10 min at 4°C. Immediately after the H_2O and MeOH extracts were obtained, a mixture was prepared by combining equal amounts (1 mL) of each of the extracts. Once prepared, the final solution was stored in 20-mL penicillin vials at −80°C for use.

Two hundred microliters of the cellular extracts were mixed with 200 μL of acetonitrile to precipitate the proteins. This solution was then centrifuged at 13,000 rpm at 4°C for 10 min to separate any solid impurities. The supernatants were removed and filtered through 0.2-μm nylon syringe filters. Then, 100 μL were transferred to analytical vials for LC-MS analysis. For CE-MS analysis, 70 μL of filtered extract were evaporated to dryness using a Speedvac Concentrator, and each sample was then reconstituted in 70 μL of MilliQ® water containing an internal standard (0.2 mM L-methionine sulfone) and 0.1 M formic acid.

Preparation of quality controls (QCs) for metabolomic fingerprinting. Because the samples interact during the separation technique and MS, it is crucial to employ quality controls (QCs) during LC-MS and CE-MS to ensure analytical reproducibility. Indeed, QC samples are required at the beginning of the sequence to stabilize the system and throughout the analytical runs at periodic intervals of time to monitor variations in signal across time[35]. QC samples were prepared independently for LC-MS and CE-MS by pooling and mixing equal volumes of each sample. After gently vortexing, the mix was also filtered and subsequently transferred to an analytical vial.

Metabolomic fingerprinting by LC-ESI-QTOF-MS. The metabolic profile was achieved using a liquid chromatography system consisting of a degasser, a binary pump, and an autosampler (1290 infinity, Agilent). Samples (0.5 μL) were applied to a reversed-phase column (Zorbax Extend C_{18} 50 × 2.1 mm, 3 μm; Agilent), which was maintained at 60°C during the analysis. The system was operated at a flow rate of 0.6 mL/min with solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The gradient was 5% B (0–1 min), 5 to 80% B (1–7 min), 80 to 100% B (7–11.5 min), and 100 to 5% B (11.5–12 min). The system was finally held at 5% B for 3 min to re-equilibrate the system (15 min of total analysis time). Data were collected in positive and negative ESI modes in separate runs using QTOF (Agilent 6550 iFunnel). Analyses were performed in both positive and negative ion modes. The positive mode was operated in full-scan mode from *m/z* 50 to 1000. The capillary voltage was 3000 V with a scan rate of 1.0 spectrum per second. The gas temperature was 250°C, the drying gas flow was 12 L/min and the nebulizer was 52 psi. The MS-TOF parameters were as follows: fragmentor, 175 V; skimmer, 65 V; and octopole radio frequency (OCT RF Vpp) voltage, 750 V. The negative ion mode was operated in full-scan mode from *m/z* 50 to 1100. The capillary voltage was 3000 V with a scan rate of 1.0 spectrum per second. The gas temperature was 250°C. The drying gas flow was 12 L/min, and the nebulizer was 52 psi. The MS-TOF parameters included the following: fragmentor, 250 V; skimmer, 65 V; and octopole radio frequency voltage, 750 V. During the analyses, two reference masses were used: 121.0509 (detected *m/z* $[C_5H_4N_4 + H]^+$) and 922.0098 (detected *m/z* $[C_{18}H_{18}O_6N_3P_3F_{24} + H]^+$) in positive mode and 112.9855 (detected *m/z* $[C_2O_2F_3\text{-}H]^-$) and 1033.9881 (detected *m/z* $[C_{18}H_{18}O_6N_3P_3F_{24}$ + TFA-H$]^-$) in negative mode. The references were continuously infused into the system, enabling constant mass correction. Samples were analyzed in randomized runs, during which they were incubated in an autosampler at 4°C. The analytical runs for both polarities were set up starting with the analysis of ten QCs followed by the samples; a QC sample was injected in between blocks of five samples until the end of the run.

Metabolomic fingerprinting by CE-TOF-MS. A capillary electrophoresis apparatus (7100 Agilent) coupled to a TOF Mass Spectrometer (6224 Agilent) was employed. The CE mode was controlled by ChemStation software (B.04.03, Agilent), and the MS mode was controlled by Mass Hunter Workstation Data Analysis (B.02.01, Agilent). The separation occurred in a fused-silica capillary (Agilent: total length, 100 cm; internal diameter, 50 μm). All separations were performed in normal polarity with a background electrolyte containing 0.8 M formic acid in 10% methanol (*v/v*) at 20°C. New capillaries were pre-conditioned with a flush of 1.0 M NaOH for 30 min followed by MilliQ® water for 30 min and the background electrolyte for 30 min. Prior to each analysis, the capillary was conditioned with a flush of background electrolyte for 5 min. The sheath liquid (6 μL/min) was MeOH:H_2O (1 : 1) containing 1.0 mM formic acid with two references masses of *m/z* 121.0509 ([C5H4N4 + H]$^+$) and *m/z* 922.0098 ([C18H18O6N3P3F24 + H]$^+$), which enabled correction and higher mass accuracy during MS. Samples were hydrodynamically injected at 50 mBar for 50 s. The stacking was performed by applying the background electrolyte at 100 mBar for 10 s. The separation voltage was 30 kV, and the internal pressure was 25 mBar; the analyses were performed within 35 min. The MS parameters included the following: fragmentor, 100 V; skimmer, 65 V; octopole, 750 V; drying gas temperature, 200°C; flow rate, 10 L/min; and capillary voltage, 3500 V. Data were acquired in positive mode with a full scan from *m/z* 85 to 1000 at a rate of 1.41 scan/s. The analytical run started with an analysis of five QCs followed by the samples; a QC sample was injected in between blocks of five samples until the end of the run.

Metabolomic data treatment. The Feature Extraction tool in the Mass Hunter Qualitative Analysis software (B.05.00, Agilent) was used. The alignment of the raw data was performed using MassProfiler Professional software (B.12.01, Agilent). The variables were then filtered. Data present in at least 50% of the QCs, with coefficients of variation less than 30% across the QCs, were selected, and models were subsequently built using SIMCA-P+ software (12.0.1.0, Umetrics; Figure 1). Based on the PCA and the patient's BMI, sample HC4 was rejected prior to statistical analysis. Subsequently, any missing values were replaced by the mean (if the variable was present in more than 2/3 of the samples per group) or by half of the minimum value (if the variable was present in 1/3 to 2/3 of the samples per group). Missing variables that were present in less than 1/3 of samples per group were denoted as zero. Finally, groups were compared in pairs (SLE vs. HC and HCh [HC with high BMI] vs. HCl [HC with low BMI]) using Mann-Whitney *U* tests or *t*-tests followed by Bonferroni corrections to minimize false positives (corrected p value ≤ 0.05; MATLAB 7.10.0.499). The resulting list of accurate masses that significantly differed

between groups was searched using the CEU Mass Mediator search tool (http://biolab.uspceu.com/mediator; error ± 10 ppm). This procedure was performed independently for each analytical platform.

Note that the robustness of the analytical procedure was demonstrated by the tight clustering of the QCs (Figure 1), showing that the separation between groups was due to actual biological variability and was not random.

16S rRNA microbiota analysis. The QIIME software suite was used to construct a UniFrac similarity matrix of all 16S rRNA profiling samples included in this study based on their operational taxonomic unit (OTU) profiles[11]. This similarity matrix was processed to obtain a three-dimensional principal coordinate analysis (PCoA) where the percentages shown along the axes represent the proportion of dissimilarities between samples captured by the axes (Figure 2). The raw sequences reported in this article have been deposited in the NCBI Short Read Archive (SRA) (study accession number: SRP028162)[11].

Biochemical tests by targeted metabolomics. Protein extracts from each of the SLE patients and HD subjects were obtained and used for activity tests as previously described[17]. The extent of transformation of Rib5P to R1,5-dP and to PRPP was quantified in a solution containing 1 mL of 50 mM 4-(2-hydroxyethyl piperazine-1-ethanesulfonic acid (HEPES), pH 7.0, 0.15 mg/mL ribose-5-phosphate and 0.1 mg/mL total protein. Similarly, transformation of sialic acid (3.5 mg/mL) to N-acetyl-D-mannosamine was performed under similar conditions. Reactions were kept for a total time of 15 min at 25°C, and substrate conversion was examined by LC-QqQ-MS as described below. Reactions without proteins and substrates were used as controls; they were subsequently treated in the same manner as samples.

Sample treatment. Prior to analysis, the samples were diluted 1 : 3 with MilliQ® water (dilution factor 4), and a pool of samples was prepared by extracting 10 µL from each vial. Independently, a mix of standards (Rib5P, R1,5-dP, PRPP, N-acetyl-neuraminic acid and N-acetyl-D-mannosamine and N-acetyl-neuraminic acid; all from Sigma Chemical Co., St. Louis, MO) was prepared from independent solutions of each compound, with a final concentration of 10 ppm (mg/L) in MilliQ® water, and subsequently diluted to 7.5, 5, 2.5 and 1 ppm (mg/L).

LC-QqQ-MS. Each analysis was achieved using a liquid chromatography system consisting of a degasser, binary pump and autosampler (1290 Infinity, Agilent Technologies, Santa Clara, CA, USA) coupled to a triple quadrupole mass spectrometer (6460, Agilent Technologies). A Kinetex® HILIC column (150 × 2.1 mm, 2.6 µm, Phenomenex, Torrance, CA, USA) was maintained at 50°C during the analysis. The system was operated at a flow rate of 0.6 mL/min with solvent A (water with 5 mM of ammonium formate, pH 6.8) and solvent B (acetonitrile). The gradient was as follows: 75% B (0–0.5 min), 75 to 0% B (0.5–5 min), 0% B (5–6 min) and 0 to 75% B (6–7 min), keeping the re-equilibration at 75% B for 4 min (11 min of total analysis time). Data were collected in negative MRM mode. The MS-ESI parameters were optimized as follows: capillary voltage, 2500 V; gas temperature, 200°C; drying gas flow, 8 L/min; nebulizer, 48 psi; and nozzle voltage, 0 V. The MS-QqQ parameters were as follows: fragmentor, 116 V; dwell time, 20 ms; and cell accelerator voltage, 7 V. The *m/z* quantification transition for each compound was as follows: Rib5P (229-97), R1,5-dP (309-97), PRPP (388.9-79), N-acetyl-mannosamine (220.1-59.1) and N-acetyl-neuraminic acid (308.1-87). The analytical run started with the analysis of ten injections of the pool to equilibrate the chromatographic system and was followed by the samples in a randomized order. Samples were maintained at 4°C throughout the run, and 20 µL of each sample were injected.

Study approval. Ethical approval for this study (reference code AGL2010-14952; grant title "Towards better understanding of gut microbiota functionality in some immune disorders") was obtained from the Bioethics Committee of CSIC and from the Regional Ethics Committee for Clinical Research (*Servicio de Salud del Principado de Asturias*) in compliance with the Declaration of Helsinki. All determinations were performed with fully informed written consent from all participants involved in this study. All experiments were performed in accordance with approved guidelines and regulations.

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Author contributions

The study was conceived by M.F. and A.Ma. All authors contributed to the data collection. A.H., D.R., M.M.-M. and M.F. performed the experiments, and R.B. contributed to data analysis. Data interpretation and manuscript preparation were performed by D.R. and M.F. P.L., A.C., S.G. and Ana.S. provided the fecal material and clinical records. B.S. provided biodiversity input. C.M. and M.V. provided 16S rRNA and bioinformatic data analysis. C.B. provided analytic and intellectual input on the metabolome data. Ant.S and A.Mo. provided intellectual input. All authors have critically reviewed and edited the manuscript and have approved its publication.

Additional information

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Th17 responses and natural IgM antibodies are related to gut microbiota composition in systemic lupus erythematosus patients

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Intestinal dysbiosis, characterized by a reduced *Firmicutes*/*Bacteroidetes* ratio, has been reported in systemic lupus erythematosus (SLE) patients. In this study, *in vitro* cultures revealed that microbiota isolated from SLE patient stool samples (SLE-M) promoted lymphocyte activation and Th17 differentiation from naïve CD4$^+$ lymphocytes to a greater extent than healthy control-microbiota. Enrichment of SLE-M with Treg-inducing bacteria showed that a mixture of two Clostridia strains significantly reduced the Th17/Th1 balance, whereas *Bifidobacterium bifidum* supplementation prevented CD4$^+$ lymphocyte over-activation, thus supporting a possible therapeutic benefit of probiotics containing Treg-inducer strains in order to restore the Treg/Th17/Th1 imbalance present in SLE. In fact, *ex vivo* analyses of patient samples showed enlarged Th17 and Foxp3$^+$ IL-17$^+$ populations, suggesting a possible Treg-Th17 trans-differentiation. Moreover, analyses of fecal microbiota revealed a negative correlation between IL-17$^+$ populations and *Firmicutes* in healthy controls, whereas in SLE this phylum correlated directly with serum levels of IFNγ, a Th1 cytokine slightly reduced in patients. Finally, the frequency of *Synergistetes*, positively correlated with the *Firmicutes*/*Bacteroidetes* ratio in healthy controls, tended to be reduced in patients when anti-dsDNA titers were increased and showed a strong negative correlation with IL-6 serum levels and correlated positively with protective natural IgM antibodies against phosphorylcholine.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease triggered by a combination of environmental and genetic factors that result in a breakdown in tolerance towards self-antigens[1]. The subsequent production of autoantibodies by autoreactive B cells constitutes a key pathological factor in SLE, since it leads to the formation and deposition of immune-complexes that cause tissue damage[2]. Likewise, naïve CD4$^+$ cells activated by recognition of such self-antigens can be differentiated into several subsets based on the pattern of cytokines present in the local environment[3]. In addition to the well-known paradigm of Th1/Th2 cell immune response, nowadays much evidence reveals the presence of alterations in Th17 and regulatory T (Treg) cells in SLE disease[4–6]. With regard to Th17cells, some studies support their pivotal role as primary drivers of autoimmune responses in SLE through the secretion of proinflammatory cytokines involved in local inflammation and tissue destruction, including IL-17, IL-22 andIL-23[7,8]. Accordingly, increased circulating levels of IL-17 and IL-17-producing T cells have been recently reported in SLE[9–11]. Moreover, IL-17-producing T cells have also been shown to infiltrate the lungs, skin and kidneys in lupus patients, contributing to organ damage[10,12]. Conversely, Treg cells are essential for preventing autoimmune and inflammatory diseases, since they present a suppressive activity on aberrant effector responses[13]. Naturally occurring Treg cells emerge from the thymus and are primarily characterized by the presence of high levels of CD25 (IL2Rα chain) and FOXP3, a transcription factor required for the development and function of Treg cells[14]. In addition, Treg cells could be expanded or induced in peripheral tissues in response to diverse antigens[15]. Most studies report either reduced numbers or impaired function of circulating Treg cells in SLE patients[16–18].

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Increasing evidence suggests that the composition of the commensal microbiota colonizing the gut affects the differentiation of immune cells present in the gut-associated lymphoid tissues (GALTs)[19]. Specifically, plasmatic cells in the lamina propria are involved in the production of T cell-independent antibodies against components of both commensal and pathogenic bacteria as well as apoptotic cells, named natural IgM antibodies[20]. Interestingly, several studies have reported immunoregulatory functions of natural IgM antibodies inhibiting the inflammatory signaling in innate immune cells and suppressing autoimmune disease[21,22]. On the other hand, after the recognition of bacterial antigens, gut dendritic cells (DCs) may induce the differentiation of naïve $CD4^+$ T cells into different types of effector or regulatory T cells[23–26]. Under physiologic conditions, the normal microbiota presented in healthy individuals favors the maintenance of the intestinal immune homeostasis[27]. Conversely, several studies suggest that alterations in the gut microbiota composition, known as dysbiosis, may be a critical factor in the development of numerous immune-mediated pathologies, probably in disease-susceptible hosts, through the generation of an imbalance between Th and Treg cells[19,28–31]. In this sense, intestinal dysbiosis has been associated with the development of several autoimmune diseases, including inflammatory bowel disease, type 1 diabetes, rheumatoid arthritis and multiple sclerosis[32–38]. In this regard, we have recently described a SLE-associated intestinal dysbiosis characterized by a significantly lower *Firmicutes* to *Bacteroidetes* ratio, the most abundant phyla in the human gut[39] that has been previously described as imbalanced in other disorders[37,38,40]. Since these studies suggest that microbiota could control the Th/Treg axis outside the gut, the immune stimulation by specific bacteria could have a beneficial effect on inflammatory diseases[33]. Thus, it is known that some bacterial strains might induce the generation of Treg cells (iTreg) from naïve precursors[23,41–43]. Specifically, accumulating evidence supports the role of commensal strains of *Bifidobacterium* and *Clostridium* spp. belonging to clusters IV and XIVa in the induction of Treg cells[23,41–43].

The study aims to evaluate the influence of fecal microbiota obtained from SLE patients and healthy controls in the *in vitro* differentiation of Th and Treg populations as well as the possible effect of enriching SLE gut microbiota with bifidobacteria and Clostridia strains known to be inducers of Treg cells. Then, we analyzed the possible relationship between the SLE-associated gut dysbiosis and the presence of immune parameters characteristic of these patients, such as the Treg/Th populations, cytokine levels, disease activity and the production of both pathogenic anti-dsDNA and protective natural IgM anti-phosphoryl choline antibodies.

Results

***In vitro* effect of SLE fecal microbiota on Treg/Th differentiation.** Given the gut dysbiosis recently reported in SLE patients[39], we aimed to evaluate the influence of fecal microbiota obtained from SLE patients (SLE-M) and healthy controls (HC-M) in the *in vitro* differentiation of regulatory T cells (Treg), as well as Th1 and Th17 effector populations from naïve $CD4^+$ T lymphocytes. In addition, to estimate the effect of enriching the gut microbiota with strains able to increase the Treg subset, 5, 10 or 30% of SLE-M were replaced with the same amounts of *Bifidobacterium bifidum* LMG13195 (Bb), a strain known to induce Foxp3 expression[23,41], or with a mixture of two Clostridia strains (Cl) with a putative Treg-inducer effect (*Ruminococcus obeum* DSM25238 and *Blautia coccoides* DSM935)[42,43]. Thus, immature monocyte-derived DCs were treated for 48 h with LPS, as a maturation control, or with the different bacterial preparations and then used to prime $CD4^+CD45RA^+$ naïve T lymphocytes. After 12 days of culture in the presence of IL-2, Treg ($CD4^+CD25^{high}CD127^{low}Foxp3^+$), Th17 and Th1(IFN$\gamma$- and IL-17-expresing cells, respectively) populations were determined by flow cytometry in $CD4^+$ lymphocytes.

As expected, stimulation and expansion with IL-2 induced IL-2Rα (CD25) expression in most $CD4^+$ lymphocytes (Fig. 1A), however the amount of cells presenting elevated CD25 levels ($CD25^{high}$) showed differences among treatments (Fig. 1B). Specifically, SLE-M cultures tended to generate more $CD25^{high}$ cells than HC-M, whereas the lowest levels of this population were obtained after stimulation with the Bb strain. In fact, the upregulatory effect of SLE-M on CD25 expression was reverted after Bb supplementation. Regarding Treg cells ($CD4^+CD25^{high}$ $CD127^{low}Foxp3^+$), no significant differences were observed between HC-M or SLE-M stimulation. However, the proportion of $Foxp3^+$ cells included within the $CD25^{high}$ population was lower in SLE- than in HC-M derived cultures (Fig. 1C), thus suggesting that part of the $CD25^{high}$ cells generated in the presence of SLE-M were activated lymphocytes rather than Treg cells. Unexpectedly, although Bb increased $Foxp3^+$ cells notably, supplementation of SLE-M with this strain did not increase the generation of $Foxp3^+$ cells within the $CD25^{high}$ subset.

Finally, although no significant differences were detected in IL-17 or IFNγ expression between cells treated with both fecal microbiotas, the IL-17/IFNγ ratio was significantly higher in SLE- than in HC-M cultures, whereas the lowest ratio was induced by Cl-conditioned DCs. Moreover, SLE-M supplementation with Cl, but not with Bb, induced a significant dose dependent reduction of the IL-17/IFNγ balance (Fig. 1D). Therefore, *in vitro* stimulation of naïve $CD4^+$ T cells with SLE-M seems to promote lymphocyte activation and Th17 differentiation to a greater extent than HC-M treatment, thus supporting Th17/Treg disturbances in SLE. In addition, enrichment of SLE microbiota with Bb may prevent lymphocyte activation whereas Cl supplementation restores Th17/Th1 balance.

Relationship between immune parameters and fecal microbiota in SLE patients. To know whether the Treg/Th responses elicited *in vitro* by SLE-M could reflect the characteristic immune features of SLE patients, we analyzed Foxp3, CD25, CD127, IL-17 and IFNγ expression in fresh peripheral blood $CD4^+$ lymphocytes as well as the serum levels of a battery of cytokines in 37 SLE patients and 36 HC (Table 1). In addition, since 40 of these individuals (20 SLE and 20 HC) have been previously included in a metagenomic study of fecal microbiota[39] (Table 2), we determined the possible relationship between these immune parameters and the SLE-associated gut dysbiosis.

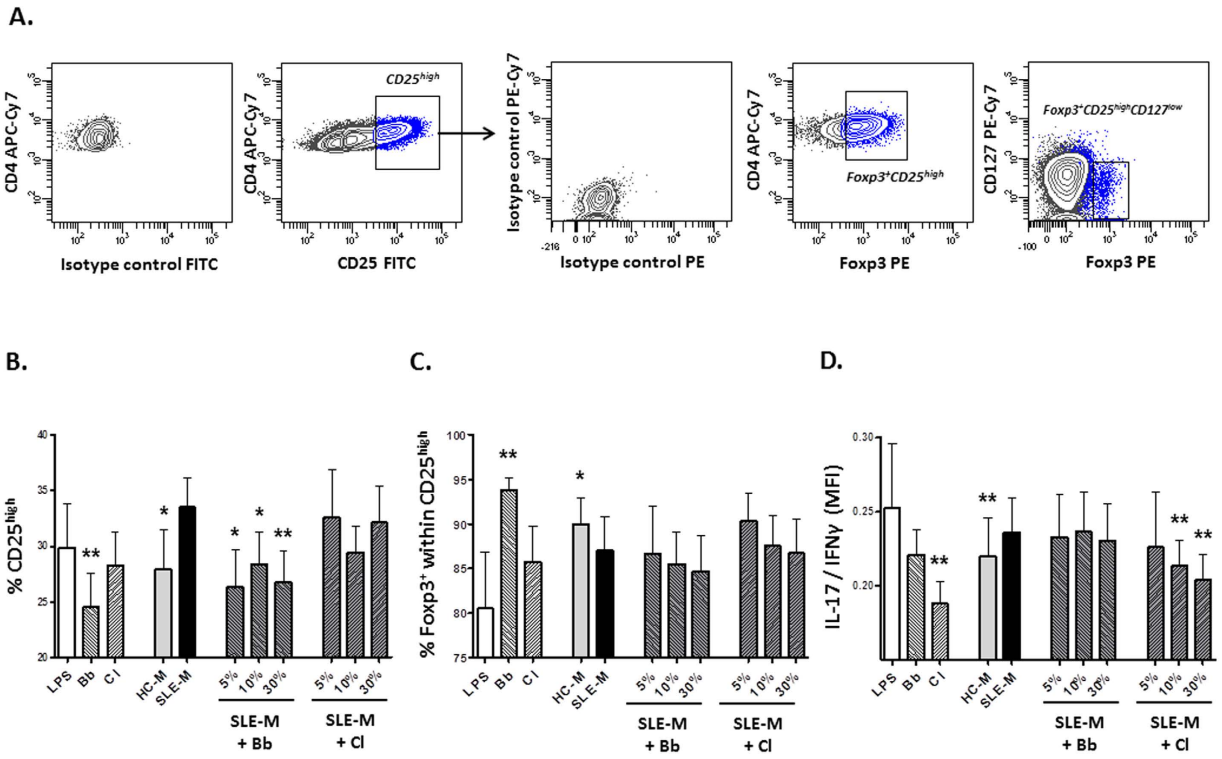

Figure 1. Fecal microbiota isolated from SLE patients influences Treg/Th differentiation. Naïve $CD4^+CD45RA^+$ lymphocytes were co-cultured for 12 days with DC previously maturated with LPS (maturation control), fecal microbiota isolated from healthy controls (HC-M) or SLE patients (SLE-M) as well as with *Bifidobacterium bifidum* LMG13195 (Bb), Clostridia strains (Cl) or SLE-M containing 5, 10 or 30% Bb or Cl. Cultured $CD4^+$ T cells were recovered, stained for Treg markers, IL-17 and IFNγ and analyzed by flow cytometry. (**A**) Sequential gating strategy used to identify Treg cells ($CD4^+CD25^{high}CD127^{low}Foxp3^+$). Positive cells for each marker were determined using fluorescence of cells labelled with the corresponding isotype-matched conjugated irrelevant MAb as a negative control. $CD4^+$ T cells showing the highest CD25 expression were identified as $CD4^+CD25^{high}$. Then, $CD25^{high}$ population was analyzed to determine the proportion of cells expressing Foxp3 ($Foxp3^+$ within $CD25^{high}$) as well as the amount of Treg cells ($CD25^{high}CD127^{low}Foxp3^+$). Density plots correspond to a representative experiment. Analyses of (**B**) $CD25^{high}$population, (**C**) $Foxp3^+$ cells within $CD25^{high}$ population, and (**C**) IL-17/IFNγ expression. Bars represent the mean and SEM of seven independent experiments performed with different blood donors. Statistical differences between SLE-M and the different treatments were evaluated by the Wilcoxon test for paired data. $^*p < 0.1$; $^{**}p < 0.05$.

Results showed an increased frequency of Th17 cells in SLE patients compared to HC, especially in those presenting anti-dsDNA antibodies, whereas no significant differences were observed in the Th1 subset (Fig. 2A). Moreover, $Foxp3^+$ IL-17^+ double positive cells were significantly increased in SLE patients compared with HC, the highest levels of such cells being observed again in those patients presenting anti-dsDNA antibodies (Fig. 2B). No significant differences were detected in the percentage of Treg cells ($CD4^+$ $CD25^{high}$ $CD127^{low}Foxp3^+$), suggesting that a Treg-Th17 trans-differentiation process could be involved in the development of $Foxp3^+$ cells without regulatory activity in SLE patients.

On the other hand, the analysis of fecal microbiota in the HC group revealed a negative correlation between the frequency of *Firmicutes* and the size of the Th17 subset, most notably in $Foxp3^+$ IL-17^+ cells, whereas the opposite occurred with *Bacteroidetes* (Table 3); all these correlations were confirmed by multivariate linear regression analyses adjusted by weight, BMI and blood lipids ($^*p < 0.05$, $R^2 > 0.6$). However, such associations were completely absent in patients, in agreement with our previously described reduction of the *Firmicutes/Bacteroidetes* ratio in SLE.

Regarding cytokine serum levels, SLE patients presented higher amounts of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IFNα, TNFα, GM-CSF, BLyS and leptin than HC, whereas IFNγ showed a clear tendency to reduction (Fig. 3A). Interestingly, none of the increased or unchanged cytokines in SLE displayed significant associations with *Firmicutes* or *Bacteroidetes*, however, IFNγ levels correlated negatively with *Bacteroidetes* and positively with *Firmicutes* and the *Firmicutes/Bacteroidetes* ratio in patients (Fig. 3B). These associations were not detected in HC.

Association between the frequency of *Synergistetes* and the presence of natural antibodies. As we previously reported[39], the proportion of fecal *Synergistetes* did not show significant differences between SLE patients and healthy controls. However, we found a negative correlation trend between this bacterial group and

	SLE patients (N = 37)
Sex (female/male) (n)	36/1
Age, years (mean ± SD)	48.40 ± 12.99
Age at diagnosis, years (mean ± SD)	38.32 ± 13.64
Disease duration, years (mean ± SD)	8.48 ± 6.65
SLEDAI score (mean ± SD)	4.45 ± 3.28
Clinical manifestations, n (%)	
Malar rash	17 (45.95)
Discoid lesions	8 (21.62)
Photosensitivity	21 (56.76)
Oral ulcers	16 (43.24)
Arthritis	22 (59.46)
Serositis	6 (16.22)
Cytopenia	24 (64.86)
Renal disorder	11 (29.73)
Neurological disorder	2 (5.40)
Autoantibodies, n (%)	
ANAs	37 (100.00)
Anti-dsDNA/titer, U/ml (mean ± SD)	18 (48.65)/41.50 ± 61.31
Anti-SSa	19 (51.35)
Anti-SSb	3 (9.68)
Anti-Sm	4 (10.81)
Anti-RNP	4 (10.81)
Treatment, n (%)	
None or NSAIDs	4 (10.81)
Antimalarial drugs	30 (81.08)
Glucocorticoids	10 (27.03)
Immunosuppressive drugs[a]	3 (8.11)

Table 1. Demographic and clinical features of SLE patients. dsDNA: double stranded DNA; NSAID: non-steroidal anti-inflammatory drug. [a]Methotrexate and/or mycophenolate mophetil.

	SLE patients (N = 20)	Healthy controls (N = 20)
Sex (female/male) (n)	20/0	20/0
Age (years)	49.21 ± 10.70	46.92 ± 8.63
BMI (Kg/m^2)	26.17 ± 5.34	25.20 ± 4.20
Weigth (Kg)	64.73 ± 11.23	67.28 ± 14.03
Blood lipids (mg/dL)		
Total cholesterol	195.85 ± 37.22	198.32 ± 34.34
HDL cholesterol	64.80 ± 15.52	63.00 ± 9.09
LDL cholesterol	116.35 ± 38.52	120,32 ± 31.38
Triglycerides	71.55 ± 31.02	73.73 ± 37.33
Disease parameters		
Age at diagnosis (years)	38.24 ± 11.85	
Disease duration (years)	10.40 ± 7.31	
SLEDAI score	4.83 ± 2.89	
Anti-dsDNA titer (U/ml)	22.01 ± 30.37	
Treatment, n		
None or NSAIDs	2	
Antimalarial drugs	18	

Table 2. SLE patients and healthy controls included in the intestinal microbiota analysis. Values represent means ± SD. BMI: body mass index; HDL: high-density lipoprotein; LDL: low-density lipoproteins; dsDNA: double stranded DNA; NSAID: non-steroidal anti-inflammatory drug.

the titer of anti-dsDNA antibodies ($r = -0.386$, $p = 0.084$), not detected with any other of the previously analyzed microbial groups. Likewise, serum levels of IL-6 associated negatively with the amount of *Synergistetes* in SLE patients ($r = -0.738$, $p < 0.001$), thus suggesting a possible relationship between this bacterial group and disease

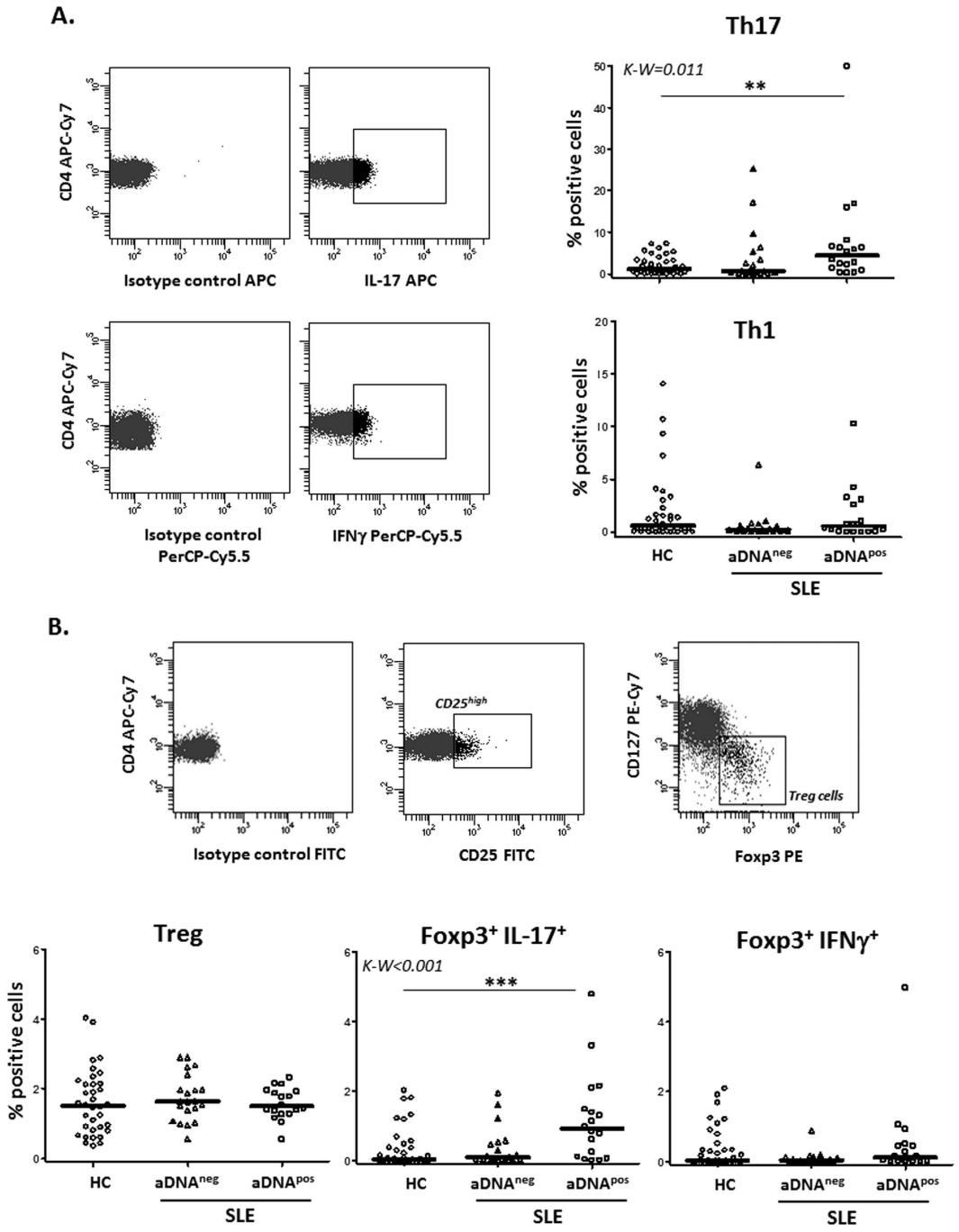

Figure 2. Increased IL-17 producing cells in SLE patients with anti-dsDNA antibodies. Foxp3, CD25, CD127, IL-17 and IFNγ expression was analyzed in fresh peripheral blood $CD4^+$ lymphocytes from SLE patients and HC. (**A**) Dot-plots show cells positive for IL-17 or IFNγ expression, determined attending to the fluorescence of cells labelled with the corresponding isotype-matched conjugated irrelevant MAb as a negative control. Scatter plots represent the percentage of IL-17^+ (Th17) and IFNγ^+ (Th1) $CD4^+$ cells in HC and SLE patients presenting (*pos*) or not (*neg*) anti-dsDNA antibodies (aDNA). Horizontal bars show the median. (**B**) Treg cells were sequentially identified as $CD4^+CD25^{high}CD127^{low}Foxp3^+$ cells. Scatter plots represent the quantity of Treg, $Foxp3^+$ IL-17^+ and $Foxp3^+$ IFNγ^+ cells in HC and SLE patients in function of their anti-dsDNA status, and horizontal bars show the median. Statistical differences among groups were evaluated by Kruskal-Wallis test and Dunn's post test was conducted to determine which groups' pairs had different means. $^{**}p < 0.01$; $^{***}p < 0.001$.

activity or antibody production. Therefore, aiming to expand the knowledge about the possible role played by intestinal *Synergistetes* in the development of humoral immune responses, we quantified serum levels of anti-PC IgM, natural protective antibodies, and anti-PC IgG (lacking this effect) as well as total circulating IgM and IgG.

CD4$^+$ T subsets	*Firmicutes*	*Bacteroidetes*	*Firmicutes/Bacteroidetes*
Th1	r = 0.040	r = 0.183	r = −0.119
	p = 0.862	p = 0.427	p = 0.606
Th17	**r = −0.444***	r = 0.294	r = −0.329
	p = 0.044	p = 0.196	p = 0.145
Foxp3$^+$ IL-17$^+$	**r = −0.561***	**r = 0.465***	**r = −0.487***
	p = 0.008	**p = 0.034**	**p = 0.025**
Foxp3$^+$ IFNγ^+	r = 0.054	r = 0.156	r = −0.089
	p = 0.816	p = 0.500	p = 0.702

Table 3. Associations of *Firmicutes*, *Bacteroidetes* and the *Firmicutes* to *Bacteroidetes* ratio with different CD4$^+$ T cell subsets in healthy controls. Correlation analyses were evaluated by Spearman test and confirmed by multivariate linear regression analyses adjusted by weight, BMI and blood lipids (*$p < 0.05$, $R^2 > 0.6$).

No significant differences were detected in anti-PC antibodies (IgM and IgG) and total IgM between patients and controls, but the amount of total IgG was increased in patients compared to controls (p = 0.027). Interestingly, anti-dsDNA titer correlated negatively with both isotype anti-PC antibodies (IgM: r = −0.508, p = 0.019; IgG: r = −0.460, p = 0.036) and with total IgM (r = −0.501, p = 0.021) but not IgG (r = 0.065, p = 0.780) levels, thus suggesting a detrimental effect of SLE disease activity on naturally occurring protective IgM antibodies.

On the other hand, *Synergistetes* exhibited a positive correlation with total and anti-PC IgM in SLE patients and with total and anti-PC IgM/IgG ratio in patients and controls (Table 4). Interestingly, serum levels of IL-6 in patients showed the opposite associations. All these results suggest that intestinal *Synergistetes* may promote the development of protective natural IgM antibodies, an effect particularly relevant in SLE patients since the increased levels of anti-dsDNA and/or IL-6 could downregulate this bacterial group.

Finally, it is noteworthy that *Synergistetes* correlated negatively with *Bacteroidetes* (r = −0.486, p = 0.022) and positively with the *Firmicutes/Bacteroidetes* ratio (r = 0.443, p = 0.039) in controls, but not in SLE patients (r = 0.075, p = 0.745 and r = −0.136, p = 0.556, respectively), supporting the role of these bacterial groups in SLE.

Discussion

Intestinal dysbiosis has been associated with several immune mediated diseases, including SLE[39] and other autoimmune conditions[32–38], pointing out a possible role of the microbiome in the balance between inflammatory and regulatory responses, either in the intestine or systemically. Therefore, understanding how the gut microbiota shapes immune responses seems to be critical for human health, particularly in chronic inflammatory disorders such as autoimmune diseases. However, to the best of our knowledge, this is the first study evaluating the effect of intestinal microbiota isolated from SLE stool samples on the induction of effector or regulatory T cell responses.

Our findings revealed that the differences in the composition of the fecal microbiota between SLE patients and HC elicited different *in vitro* immune responses. Specifically, DCs stimulated with SLE-M promoted Th17 differentiation from naïve CD4$^+$ T lymphocytes to a greater extent than HC-M conditioned DCs. No differences were detected in the generation of Treg cells, but the enlarged CD25^{high} population obtained with SLE-M treatment tended to include a lower proportion of Foxp3$^+$ cells, supporting their activated rather than regulatory status. Hence, the altered SLE gut microbiota could enhance lymphocyte activation and Th17 differentiation, thus sustaining the preexisting inflammation.

These findings are in accordance with the results of the *ex vivo* analyses, since the frequency of Th17 cells was increased in fresh peripheral blood from SLE patients compared to HC, especially in those presenting anti-dsDNA antibodies, whereas no differences were observed in Treg cells. Interestingly, SLE patients exhibited a higher proportion of Foxp3$^+$ cells producers of IL-17, thus suggesting a possible Treg-Th17 trans-differentiation process that could be responsible for the reduced regulatory activity of Treg cells reported in SLE patients[18]. Similarly, an increased prevalence of circulating IL-17 and Foxp3 double-expressing CD4$^+$ lymphocytes, together with a reduced Treg suppressor ability, have been found in patients with inflammatory bowel disease[44]. The elevated frequency of Treg cells present in the gut compared with other organs[45] is noteworthy, but they have an increased Treg/Th plasticity which could be influenced by inflammatory mediators, specific bacterial strains and other micro-environmental factors[46]. In line with this, the analyses of fecal microbiota revealed a strong negative correlation between the size of IL-17$^+$ Foxp3$^+$, and to a lesser extend Th17, populations and the frequency of *Firmicutes* in healthy controls, suggesting that they could counteract Th17 differentiation. On the other hand, serum levels of IFNγ, the prototypic Th1 cytokine and slightly reduced in SLE, correlated directly in patients with the amount of *Firmicutes* and with the *Firmicutes* to *Bacteroidetes* ratio, this imbalance being the main feature of SLE dysbiosis and independent to disease duration, lifestyle and dietary-related factors[39]. Therefore, we hypothesize that bacterial strains belonging to the *Firmicutes* phylum could be involved in the generation and/or maintenance of functional Treg cells in the gut, avoiding the trans-differentiation into effector Th17 cells in physiological conditions, whereas a diminished proportion of these bacteria in pathological situations may promote Th17 vs Th1 and Treg bias and generate IL-17$^+$ Foxp3$^+$ cells. Thus, enrichment of gut microbiota in Treg-inducing bacteria could be a desirable goal for SLE patients. However, although *Firmicutes/Bacteroidetes* ratio represents the main difference between healthy controls and SLE patients, the specific bacterial groups implicated in the observed Th17 immune response associated to SLE microbiota could not be determined by our approach, even more having into account that *Firmicutes* phyla includes both Th17[47] and Treg[23,41–44] inducing bacteria. Hence,

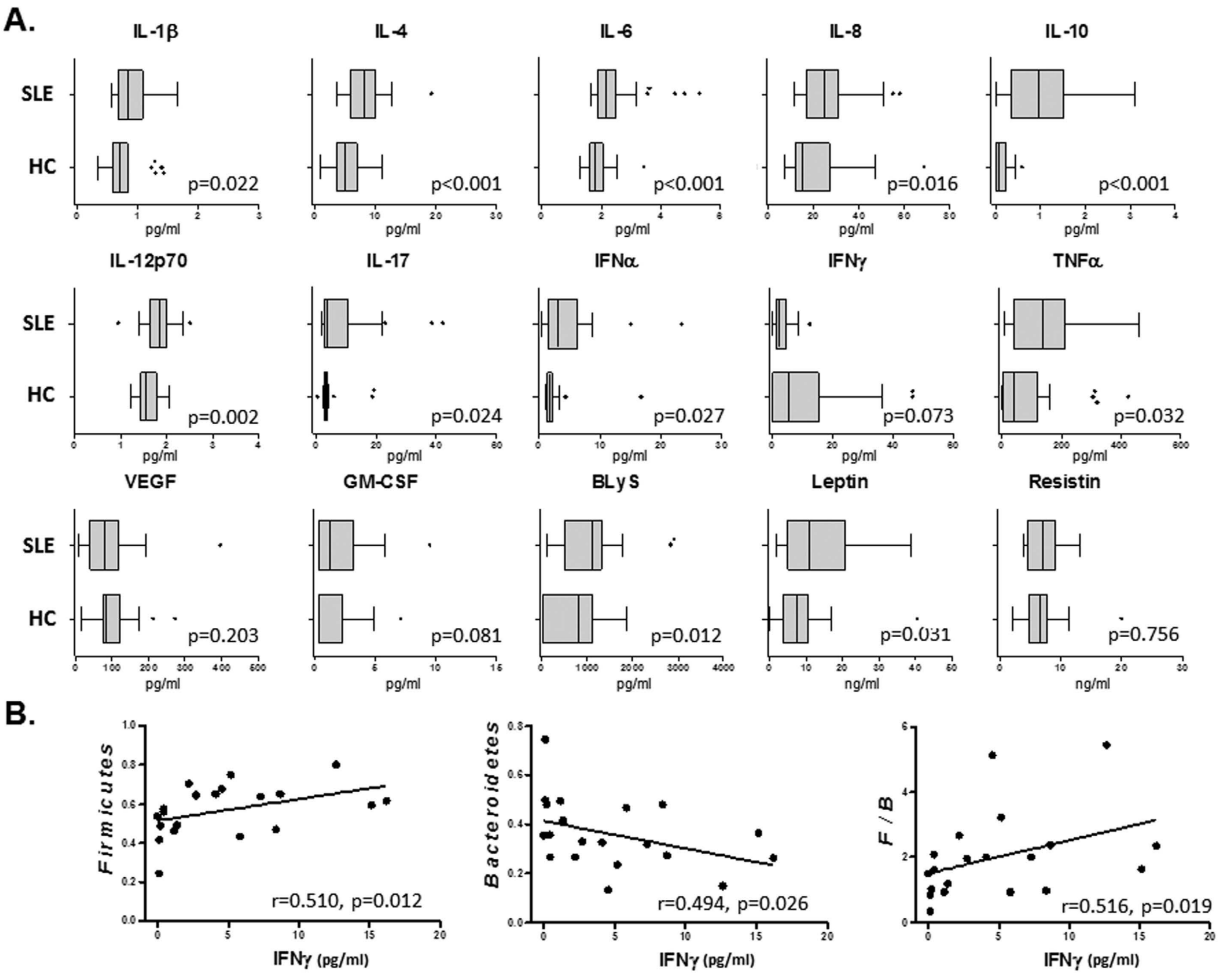

Figure 3. IFNγ serum levels in SLE patients were associated with *Firmicutes* to *Bacteroidetes* ratio. (**A**) Box and whiskers represent median and interquartile range of circulating amounts of cytokines in SLE patients compared to controls. Differences between both groups were assessed by the non-parametric Mann-Whitney *U* test. (**B**) Correlations between serum levels of IFNγ and the frequency of *Firmicutes*, *Bacteroidetes* or the *Firmicutes* to *Bacteroidetes* ratio (F/B) in SLE patients were evaluated using Spearman test.

	Synergistetes		IL-6	
	HC	SLE	HC	SLE
IgM:				
anti-PC	r = 0.404	**r = 0.624***	r = −0.004	**r = −0.473**
	p = 0.062	**p = 0.002**	p = 0.986	**p = 0.030**
total	r = 0.101	**r = 0.824***	r = 0.081	**r = −0.642***
	p = 0.662	**p < 0.001**	p = 0.729	**p = 0.002**
IgG:				
anti-PC	r = −0.156	r = 0.239	r = −0.049	r = −0.038
	p = 0.523	p = 0.286	p = 0.842	p = 0.286
total	r = −0.301	r = −0.078	r = 0.133	r = 0.332
	p = 0.211	p = 0.737	p = 0.586	p = 0.142
IgM/IgG ratio:				
anti-PC	**r = 0.670**	**r = 0.495***	r = −0.005	**r = −0.488**
	p = 0.002	**p = 0.023**	p = 0.983	**p = 0.025**
total	**r = 0.496**	**r = 0.597***	r = −0.082	**r = −0.610***
	p = 0.031	**p = 0.004**	p = 0.737	**p = 0.003**

Table 4. Relationship of fecal *Synergistetes* and IL-6 serum levels with anti-PC and total IgM and IgG antibodies. Correlation analyses were evaluated by Spearman test and confirmed by multivariate linear regression analyses adjusted by weight, BMI and blood lipids (*$p < 0.05$, $R^2 > 0.6$).

the confirmation of such hypothesis would benefit from additional experiments using germ-free mice or fecal microbiota transplant procedures in animal models.

Certain commensal microorganisms have demonstrated a Treg-inducer capability, and therefore have been proposed as potential probiotic strains appropriate for the modulation of an excessive inflammatory response to restore the immune homeostasis at the intestinal mucosa. Hence, in this study we performed *in vitro* analyses aiming to determine the possible effect of enriching SLE gut microbiota with strains known to be able to differentiate naïve T lymphocytes into Treg cells. *Firmicutes* phylum contains several *Clostridium* spp., belonging to clusters IV and XIVa, known to induce Treg cells[23,41–43]. Similarly, bifidobacteria have been also demonstrated to promote the Treg polarization and Th17 reduction *in vivo* using murine models of colitis[48]. Therefore, we used a mixture of two of these Clostridia strains, as well as a *Bifidobacterium* strain with previously demonstrated *in vitro* ability to generate functional Treg cells[23,24]. Unexpectedly, neither was able to increase this population, but results showed other beneficial effects, different for each bacterial treatment. Supplementation of SLE-M with Clostridia induced a significant dose-dependent reduction of the IL-17/IFNγ balance, thus restoring Th1 bias, whereas bifidobacteria enrichment prevented the over-activation of $CD4^+$ lymphocytes, as detected by the reduction of CD25 expression. These effects, however, could be the consequence of an active suppression of effector Th cells. In fact, the observed down regulation of Th17 cells may be explained by a preferential promotion of Treg cells by Clostridia strains, in accordance with the results obtained by Atarashi *et al.*[43] using germ-free mice colonized with different fractions of healthy human microbiota. Likewise, suppressive function of Treg cells generated with the *Bifidobacterium* strain used here results in downregulation of CD25 expression on effector cells[23]. Therefore, in view of these results and the previously reported SLE dysbiosis, it seems reasonable to consider the possible therapeutic benefit of the supplementation with probiotics containing Treg-inducer strains in order to restore the Treg/Th17/Th1 balance in SLE patients.

Another remarkable result is the suggested role played by *Synergistetes*, a little known intestinal bacterium, in SLE. This bacterial group correlated negatively with *Bacteroidetes* and positively with the *Firmicutes* to *Bacteroidetes* ratio in healthy controls, whereas in SLE patients a strong negative correlation was shown with serum levels of IL-6, a proinflammatory and Th17 promoting cytokine increased in SLE patients. Moreover, the amount of *Synergistetes* tended to be reduced when the titer of anti-dsDNA antibodies were increased, characteristic of SLE. These findings led us to evaluate the possible role of intestinal *Synegistetes* in the development of protective humoral immune responses. Natural IgM antibodies against commensal bacteria or neoantigens from apoptotic cells are commonly present in the human circulation from birth and have protective and immunoregulatory functions. Among them, IgM antibodies that recognize phosphorylcholine (PC) are valuable components of the immune system known to increase the phagocytosis of apoptotic cells and inhibit inflammatory pathways in autoimmunity and atherosclerosis[20,22,49]. However, anti-PC IgG did not seem to possess these protective effects. Results in our SLE cohort revealed that the proportion of *Synergistetes* correlated positively with total and anti-PC IgM and IgM/IgG ratio in SLE patients, whereas serum levels of IL-6 exhibited the opposite associations. These data suggest a protective role of intestinal *Synergistetes* promoting the generation of natural IgM antibodies, which could be hampered in SLE patients, especially those with high IL-6 levels. Interestingly, it has been reported that anti-PC IgM antibodies can counteract IL-6 upregulation *in vitro* and *in vivo*[22], probably by the inhibition of MAPK responses to TLR agonists, including lupus immune complexes[50], thus explaining the opposite associations of these natural antibodies with *Synergistetes* and IL-6. Moreover, B1 cells and anti-PC antibodies are increased in IL-6 knockout mice whereas the opposite occurs with B2 cells and IgG levels[51]. In line with these results, the anti-dsDNA titer correlated negatively with anti-PC levels, thus supporting a deleterious effect of disease activity on the amount of natural protective antibodies. In fact, higher anti-PC IgM levels have been associated with lower SLE disease activity[20]. Unfortunately, active patients were not included in our study of the intestinal microbiota to avoid interference with the treatments, so no significant associations were detected between SLEDAI score and *Synergistetes* or anti-PC IgM antibodies.

The involvement of *Synergistetes* in the promotion of natural antibodies, and especially those with an atheroprotective role, could be of clinical relevance for patients with SLE and other autoimmune diseases, since they usually develop premature atherosclerosis and have an increased cardiovascular risk not explained by classical factors. It has been reported that diminished levels of anti-PC IgM antibodies in SLE patients could predict a subclinical cardiovascular disease[21]. Moreover, patients that have suffered a cardiovascular event presented significantly lower levels of these antibodies compared to the rest of patients[21,49]. In fact, therapy based on passive transfer of anti-PC has been used to inhibit atherosclerosis development[52]. Therefore, the identification of intestinal microorganisms able to expand natural humoral responses through the promotion of protective IgM antibodies, as may be *Synergistetes*, could be valuable tools to design clinical interventions in order to improve both disease activity and prevention of cardiovascular complications. In this sense, the analysis of fecal *Synergistetes* in SLE patients with and without cardiovascular events should be of interest.

In summary, our *in vitro* cultures with fecal microbiota isolated from SLE patients suggested that immune responses against intestinal bacteria could be involved in the lymphocyte over-activation as well as in the Treg-Th17 trans-differentiation observed in SLE patients, with the reduced *Firmicutes* to *Bacteroidetes* ratio probably having a role in this process. The altered immune responses associated with the intestinal dysbiosis could be reestablished, at least in part, by the supplementation with beneficial bacterial strains able to induce suppressor responses. In addition, our results revealed a possible role of intestinal *Synergistetes* in the development of natural protective anti-PC IgM antibodies. This could be of special relevance in patients with high IL-6 and/or anti-dsDNA levels, since they present low frequency of these bacteria.

Methods

Ethics approval. Ethics approval for this study was obtained from the Bioethics Committee of CSIC (Consejo Superior de Investigaciones Científicas) and from the Regional Ethics Committee for Clinical Research

(Servicio de Salud del Principado de Asturias), according to the Declaration of Helsinki. All methods were carried out in accordance with the approved guidelines and signed informed written consent was collected from all participants prior to participation in the study.

Generation of monocyte-derived DCs. Human peripheral blood mononuclear cells (PBMCs) were obtained from standard buffy-coat preparations from 7 healthy blood donors (Asturian Blood Transfusion Center, Oviedo, Spain) by centrifugation over Ficoll-Hypaque gradients (Lymphoprep, Nycomed, Oslo, Norway). Monocytes ($CD14^+ \geq 95\%$) were isolated from previously obtained PBMCs by negative selection using the Human Monocyte enrichment kit (EasySep, Stem Cell Technologies, Canada).

Immature DCs were obtained from isolated monocytes by standard procedures. Thus, monocytes were cultured in 24-wellplates at 5×10^5 cells/ml for 7 days at 37 °C and 5% CO_2 in complete RPMI medium (RPMIc) [RPMI 1640 containing 2 mML-glutamine and 25 mM Hepes (Bio Whitaker, Verviers, Belgium), supplemented with 10% heat-inactivated fetal calf serum (FCS) and the antibiotics streptomycin and ampicillin at 100 mg/ml] in the presence of recombinant human (rh) IL-4 (35 ng/ml) and rhGM-CSF (70 ng/ml) (R&D Systems, Abingdon, UK). At days 2 and 5, 0.5 ml of the medium was removed without disturbing the clusters of developing DC and 0.5 ml of freshly made GM-CSF- and IL-4-containing medium was added to the wells, restoring the final volume in each well to 1 ml. At day 7, immature DCs were recovered, washed and resuspended in RPMIc medium at 5×10^5 cells/ml for subsequent maturation.

Stimulation of monocyte-derived DCs with isolated fecal microbiota. Immature monocyte-derived DCs were cultured in RPMIc medium with 1 mg/ml LPS from *E. coli* 0111:B4 (Sigma, St. Louis, MO), as a positive control of maturation, or with different bacterial preparations at a DC:bacteria ratio of 1:10. To this end, a pooled fecal microbiota (M) isolated from either four healthy controls (HC-M) or five SLE patients (SLE-M), previously shown to be representative of both conditions by metagenomics studies[39], were prepared and used in DC cultures. Also, SLE-M were enriched with different proportions of *Bifidobacterium bifidum* LMG13195 (Bb), a strain known to induce Foxp3 expression[23,41], or with a mixture of two Clostridia strains (Cl: *Ruminococcus obeum* DSM25238 and *Blautia coccoides* DSM935, ratio 1:1), which have a putative Treg-inducer effect[42,43]. Thus, 5, 10 or 15% SLE-M were replaced with the same proportions of Bb or Cl and used for DCs stimulation. Cultures with Bb and Cl bacterial preparations were performed as controls. After 48 hours, DCs from the 11 different treatments were harvested, the mature phenotype tested and used to stimulate naïve $CD4^+$ T cells.

Naïve $CD4^+$ T cell stimulation with microbial conditioned-DCs. $CD4^+$ T cells were isolated from PBMCs by negative selection using the Human $CD4^+$ T Cell Enrichment Kit (EasySep), following the manufacturer's instructions, and then naïve $CD45RA^+$ T cells were separated after depletion of $CD45RO^+$ cells (Miltenyi Biotec, Germany). Monocyte-derived DCs maturated with LPS or with the different bacterial preparations were cocultured with purified $CD4^+$ $CD45RA^+$ T cells in 48-well plates at DC:T cell ratio of 1:10. At day 5 and 8, cells incubated with all treatments were expanded with IL-2 (30 U). After 12 days, cells were collected and washed twice before analysis of the Treg/Th17/Th1 phenotype by flow cytometry.

Bacterial strains and growth conditions. *Bifidobacterium bifidum* LMG13195 was grown in de MRS broth (Difco, Detroit, MI) supplemented with 0.05% (w/v) L-cysteine (Sigma). *Ruminococcus obeum* DSM25238 and *Blautia coccoides* DSM935 were cultivated in a combination of Reinforced Clostridial Broth (Merck, Darmstadt, Germany) and Brain-Heart Infusion (Difco), supplemented with 5% (v/v) heat-inactivated FCS (LabClinics, Barcelona, Spain). Cultures were grown at 37 °C in a MG500 anaerobic chamber (Don Whitley Scientific, West Yorkshire, UK) with an atmosphere of 10% (v/v) H_2, 10% CO_2, and 80% N_2. Cultures were harvested by centrifugation, washed three times in phosphate buffered saline (PBS) and resuspended in the same buffer to a concentration of 10^8 bacteria/ml. Bacterial were counted by using a Thoma cell counting chamber (Marienfeld Superior, Germany). Bacterial cells were killed by exposing them to three consecutive cycles of 30 minutes under radiation in a UV chamber (15 W, Selecta, Barcelona, Spain). Plate counting was carried out after UV treatment to corroborate the absence of bacteria able to recover in the proper medium. UV-killed bacterial suspensions were distributed in aliquots, and stored at −80 °C until use. The identity of the strains was confirmed by sequencing the V1 and V2 variable regions of the 16 S rRNA gene using primers plb16 (5′-AGAGTTTGATCCTGGCTCAG-3′) and mlb16 (5′-GGCTGCTGGCACGTAGTTAG-3′)[53].

Stool samples and microbiota separation. One portion of stool sample of 4 healthy controls and 5 SLE patients, previously used in a metagenomic study[39], was submitted to a density gradient centrifugation to separate the microbiota from the rest of the fecal material, according to the method of Courtois *et al.*[54]. Feces were homogenized in sterile NaCl 0.9% (1:9; w/v) for 1 min in a homogenizer (Stomacher Lab Blender 400, WVR, Barcelona, Spain). A solution of Nycodenz® 80% (w/v) (PROGEN Biotechnik GmbH, Heidelberg, DE) was prepared in ultrapure water, and sterilized at 121 °C for 15 min. A volume of 10.5 ml of the homogenized fecal samples was placed on the top of 3.5 ml of the Nycodenz® solution, and centrifuged at 10,000 g for 40 min at 4 °C. The upper phase, containing soluble debris, was discarded and the layers corresponding to the microbiota were kept in ice for 5 min, in order to allow non-soluble debris to precipitate, washed twice in PBS, and stored in the same buffer in aliquots of 1 ml of 10^8 microorganisms/ml at −80 °C. UV-inactivated microbiota was obtained as described in the previous section.

SLE patients and healthy controls. Thirty-seven SLE patients fulfilling at least four American College of Rheumatology (ACR) revised criteria for the classification of SLE[55] were selected from the updated Asturian

Register of Lupus[56,57]. Information on clinical features during the disease course was obtained by reviewing clinical histories (Table 1). Thirty-six sex and age-matched healthy blood donors were used as controls (mean age ± SD: 42.56 years ± 11.39). At the time of sampling, anti-dsDNA titer, SLE disease activity index (SLEDAI) and/or weight, BMI (body mass index) and blood lipids [Triglycerides, HDL (high-density lipoprotein), LDL (low-density lipoproteins) and total cholesterol] were evaluated and patients were asked precise questions regarding the treatment received over the previous 6 months.

Intestinal microbiota analysis. Metagenomic analysis of fecal microbiota were performed in 20 non active SLE patients without antibiotic or immunosuppressive treatment in the last 6 months and 20 age-matched healthy controls as previously described[39]. Faecal DNA extraction, 16 S rRNA amplification sequencing of 16 S rRNA gene-based amplicons and the sequence-based microbiota analysis were reported elsewhere[39]. The raw sequences reported in this article are deposited in the NCBI Short Read Archive (SRA) (study accession number: SRP028162).

Flow cytometric analysis. Phenotypic studies of cultured cells and blood samples were performed after staining with the appropriate monoclonalantibody (mAb). Maturation of cultured DCs was verified after staining for 30 min at 4 °C with anti -CD86 fluorescein isothiocyanate (FITC), -CD80 phycoerythrin (PE), -HLA-DR PE-Cy5, -CD1a FITC mAb, or with the corresponding isotype matched conjugated irrelevant mAb as a negative control (all mAb were supplied by Pharmingen). To determine Treg/Th17/Th1 phenotype both in cultured cells and blood samples [previously lysed with 2 ml BD Lysing Solution (BD Biosciences, San Diego, CA) for 5 minutes and washed twice with PBS], $CD4^+$ lymphocytes were first extracellularly stained with anti-CD4 allophycocyanin-Cy7 (APC-Cy7), anti-CD25 FITC and anti-CD127 PE-Cy7mAb or with the corresponding isotype-matched conjugated irrelevant mAb (all from eBiosciences, San Diego, CA). Then, cells were fixed, permeabilized and intracellularly stained with anti-FOXP3 PE, IFNγ PerCP-Cy5.5 and IL-17 A APC (Foxp3/transcription factor staining buffer set; eBiosciences) or with the corresponding isotype-matched conjugated irrelevant mAb, following the manufacturer's instructions. A minimum of 10,000 $CD4^+$ lymphocytes were acquired on a FACSCanto II flow cytometer (BD) and analyzed using the FlowJo software (Tree Star Inc). The specific fluorescence intensity was quantified as the mean fluorescence intensity (MFI) calculated by subtracting the background of isotype matched control staining from the total fluorescence.

Cytokine determination. Serum samples from SLE patients and HC were collected and maintained at −80 °C until cytokine determination was carried out. IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17 A, IFNα, VEGF and GM-CSF amounts were analyzed by Cytometric Bead Arrays Flex Set using a FACS Canto II flow cytometer (BD Biosciences). For IL-1β, IL-6, IL-10 and IL-12p70, an Enhanced Sensitivity Flex Set was needed. ELISA kits were used for the quantification of TNFα, leptin, resistin (Mini EDK kit, PeproTech), BLyS (Human BAFF Instant ELISA, eBioscience) and IFNγ (OptEIA kit, BD) following the manufacturer's instructions. The lower limits of detection were 48.4 fg/ml for IL-1β, 1.4 pg/ml for IL-4, 68.4 fg/ml for IL-6, 1.2 pg/ml for IL-8, 13.7 fg/ml for IL-10, 12.6 fg/ml for IL- 12p70, 0.3 pg/ml for IL-17 A, 1.25 pg/ml for IFNα, 4.5 pg/ml for VEGF, 0.2 pg/ml for GM-CSF, 3.9 pg/ml for TNFα, 63 pg/ml for leptin, 24 pg/ml for resistin, 130 pg/ml for BLyS and 0.58 pg/ml for IFNγ.

Anti-Phosphorylcholine antibodies quantification. IgG and IgM antibodies against Phosphorylcholine (anti-PC) were quantified in serum samples from patients and controls by an in-house ELISA test, as follows. Microtiter wells (Maxisorp, Nunc) were coated overnight with phosphorylcholine conjugated to bovine serum albumin (PC-BSA) (Biosearch Technologies, Petaluma) and blocked with PBS 2% BSA for 2 hours at 37 °C. A sera pool of healthy controls was used as anti-PC Ab standard. Serum samples and anti-PC standard were diluted in Tris Buffered Saline (TBS) and incubated for 2 hours at room temperature (RT). After washing with TBS/Tween 20 (0.05%), wells were incubated for 2 hours at RT with alkaline phosphatase-conjugated anti-human IgG or IgM (Immunostep, Salamanca, Spain). Finally, plates were washed twice and revealed using p-nitrophenylphosphate as substrate. Absorbance was determined at a wavelength of 405 nm. Quantities of serum anti-PC arbitrary units were calculated for each sample according to the standard curves. Similarly, total IgG or IgM were quantified by conventional ELISA techniques.

Statistical analysis. The Kolmogorov-Smirnov test was used to assess the normal distribution of the data. *In vitro* experiments data were represented by mean ± SEM and differences between culture conditions were assessed by the paired Wilcoxon test. SLE patients and healthy controls cytokine serum levels were expressed as the median value (interquartile range) and non-parametric Mann-Whitney U-test was used to determine differences between both groups. Percentages of Treg/Th1/Th17 cells were compared by using the Kruskal-Wallis test; when a significant test was obtained, Dunn's post hoc tests were conducted to determine statistical differences in pairs of groups. Associations of fecal microbial group's frequencies with T cell subsets, cytokine serum levels and autoantibodies were examined by Spearman's rank correlation test and confirmed by multivariate linear regression analyses adjusted by weight, BMI and blood lipids (triglycerides, HDL, LDL and total cholesterol]. GraphPad Prism 5 software (GraphPad Software, USA) and SPSS 22 statistical software package (SPSS Inc.) were used for all determinations, and a p-value < 0.05 was considered significant.

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Author Contributions

P.L. participated in the study conduct, sample collection, review of clinical manifestations, disease activity and therapy of patients, experimental procedures, data analysis/interpretation and manuscript preparation. B.P. and J.R.-C. performed some experimental procedures and data analysis. B.S., A.H. and A.M. participated in microbiota related design study, experiments and analysis. A.S. contributed to study design/conduct, data interpretation and manuscript preparation.

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CAPÍTULO ADICIONAL

Optimización del estudio de la microbiota intestinal mediante análisis del gen 16S rRNA con la plataforma de secuenciación IonTorrent.

En este capítulo de la Tesis doctoral, que complementa al capítulo 2, nos centramos en desarrollar la metodología óptima para analizar las poblaciones microbianas presentes en la microbiota fecal humana, metodología que se aplicó después para llevar a cabo los estudios del capítulo 3.

Comparamos 4 métodos diferentes de extracción y purificación del material genético de muestras de heces. Para ello se contaminaron a propósito heces de una rata "*germ-free"* con diferentes mezclas de microorganismos Gram positivos y Gram negativos, y una Arqueobacteria; posteriormente se aplicaron los 4 métodos de extracción de ADN. Para la identificación de las distintas poblaciones microbianas se amplificó el gen del ARN ribosomal 16S con distintos pares de oligonucleótidos cebadores, algunos descritos en otros trabajos de metagénomica, y otros diseñados por nosotros para intentar cubrir lo más posible el mayor número de especies bacterianas con el menor tamaño de amplicón posible.

Los resultados que conforman este capítulo se presentan en el siguiente artículo:

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Assessing the Fecal Microbiota: An Optimized Ion Torrent 16S rRNA Gene-Based Analysis Protocol

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Abstract

Assessing the distribution of 16S rRNA gene sequences within a biological sample represents the current state-of-the-art for determination of human gut microbiota composition. Advances in dissecting the microbial biodiversity of this ecosystem have very much been dependent on the development of novel high-throughput DNA sequencing technologies, like the Ion Torrent. However, the precise representation of this bacterial community may be affected by the protocols used for DNA extraction as well as by the PCR primers employed in the amplification reaction. Here, we describe an optimized protocol for 16S rRNA gene-based profiling of the fecal microbiota.

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Introduction

The gut microbiota is presumed to play a key role in human health and disease through its impact on nutrition, pathogenesis and immunology [1]. During the last decade extensive efforts have been made to determine the complexity of the microbial communities residing in the gut (for review see [2]). In fact, (gut) disorders such as inflammatory bowel disease [3,4], irritable bowel syndrome [5], obesity [6,7] and necrotizing enterocolitis [8] have been considered as deviations from a healthy gut microbiota composition.

The capability of high through-put sequencing of 16S rRNA gene sequences by means of Next Generation Sequencing (NGS) technologies has been pivotal in facilitating the discovery of gut microbiota biodiversity [9]. The Ion Torrent PGM instrument represents a recently commercialized bench-top NGS platform and is marketed as being less costly and with a faster turnaround as compared to other NGS techniques such as the 454 and Illumina platforms [10,11]. Application of the Ion Torrent technology to 16S rRNA-based profiling of complex bacterial communities has been achieved for the investigation of the aquatic microbial community structure of the Athabasca river [12], the bacterial and archaeal community dynamics in a covered anaerobic pond that was utilized to treat waste from a piggery [13], and the microbial population residing in human subgingival plaque [14].

Previous studies have pointed out how the obtained biodiversity image of the gut microbiota is affected by various protocols used for DNA extraction, as well as by the particular PCR primers used for amplification of the targeted region of the 16S rRNA gene [15–17], leading to an underestimation of key components of the gut microbiota of infants, in particular bifidobacteria [18]. In fact, based on both culture-based techniques and analysis using species-specific DNA probes, bifidobacteria were considered to represent the dominant component of the neonatal gut microbiota, [19–21], though other microbiota studies have suggested that bifidobacteria are present at low abundance or even absent in the infant gut microbiota [22,23].

These findings reinforce the need for a reliable protocol to investigate the composition of the human gut microbiota. Here, we describe a procedure specifically designed for the Ion Torrent PGM technology to determine the biodiversity of the human gut by means of 16S rRNA gene-based sequence profiling.

Materials and Methods

Subject Recruitment and Fecal Sample Collection

The study was approved by the Ethical Committee of the Regional Asturias Public Health Service (SESPA) and informed written consent was obtained from the mothers. All subjects were healthy and had not received any antibiotic or probiotic in the previous 3 months. Stool samples consisted of 6–10 gr of fresh fecal material, and were immediately frozen upon collection at −80°C until processed for DNA extraction.

Bacterial Strains and Growth Conditions

Ten representatives of abundant microorganisms of the human gastrointestinal tract were used in this study. These include *Bifidobacterium longum* NCIMB 8809, *Collinsella intestinalis* DSM 13280, *Blautia producta* DSM 2950, *Escherichia coli* LMG 2092 and *Klebsiella pneumoniae* CECT 143, which they were grown in de Man-Rogosa-Sharpe (MRS) broth (Difco, Detroit, MI) supplemented with 0.05% (w/v) L-cysteine (Sigma, St. Louis, MO) (MRSC). *Prevotella copri* DSM 18205 and *Blautia coccoides* DSM 935 were cultivated in a combination of Reinforced Clostridial Broth (Merck, Darmstadt, Germany) and Brain-Heart Infusion (Difco), supplemented with 5% (v/v) heat-inactivated fetal bovine serum (LabClinics, Barcelona, Spain). For culturing *Bacteroides thetaiotaomicron* DSMZ 2079, the latter medium was supplemented with 0.005% haemin (Sigma) and 0.005% Vitamin K1 (Sigma). *Faecalibacterium prausnitzii* DSM 17677 was grown in Wilkins-Chalgren Anaerobe broth (Merck), following the recommendations included in the DSMZ medium 339. Finally, an active culture of *Methanobrevibacter smithii* DSM 861, grown in *Methanobacterium* medium (DSMZ 119) was directly supplied by DSMZ.

Cultures were incubated at 37°C in an MG500 anaerobic chamber (Don Whitley Scientific, West Yorkshire, United Kingdom) with an atmosphere of 10% (v/v) H_2, 10% CO_2, and 80% N_2. Taxonomic identity of the microorganisms used for the 16S rRNA gene microbial profiling assays was assessed by sequencing the V1 and V2 variable regions of the 16S rRNA gene using primers plb16 (5′-AGAGTTTGATCCTGGCTCAG-3′) and mlb16 (5′-GGCTGCTGGCACGTAGTTAG-3′) [24].

PCR Primer Design

Primers Probio_Uni/Probio_Rev (Table 1) were assessed for specificity using the ARB software package [25] and the SILVA 108 SSU Reference 16S rRNA gene database release [26]. In order to validate the designed primers, we used an *in silico* approach based on BLASTN matches with corresponding 16S rRNA gene sequences from various bacteria that are commonly found in the gut as well as those that are not expected in this environment, which provided allowed us to evaluate if these primers may also be used to examine the biodiversity of different microbial ecosystems. In order to increase the number of microbial taxa recognized by the primers designed in this study we introduced a certain level of nucleotide degeneracy.

Deliberate Contamination of Faeces from Germ-free Rats and DNA Extraction

Pellets for each strain were extensively washed with PBS, concentrated in a PBS solution and cell counts were calculated in a Neubauer Chamber. Feces from germ-free Fisher 344 rats (males) were deliberately contaminated with three different mixes of microorganisms, named mix P1, mix P2 and mix P3, and homogenised for 1 min using a stomacher (IUL Instruments, Barcelona, Spain). In all cases, *M. smithii* was added at a final concentration of 1.30E9 cells/g faeces. In the first mix (P1), both Gram positive and Gram negative microorganisms were added at a final viable count of 3.84 E9 cells/g feces for each strain. In the second mix (P2) Gram positive numbers were the same as in the P1 mix, but Gram negative bacteria were added at a final concentration of 3.84 E7 cells/g faeces for each strain, resulting in a cell population in which the numbers of each Gram negative strain are 2 logs below the numbers of Gram positive strains. Finally, in the third mix (P3) the number of each introduced Gram positive bacterial strain was 2 logs below the Gram negatives: 3.84 E7 cells/g feces for each Gram positive strain, and 3.84 E9 cells/g feces for each Gram negative strain.

Following the homogenization of the various feces-bacterial mixes, four different DNA extraction procedures were used. i) The DNA extraction protocol using the MOBIO Power Soil DNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA) [recommended by the Human Microbiome Consortium (www.hmpdacc.org)] and from here on referred to as 'MBio DNA-extraction'; ii) The MBio DNA-extraction, which included an initial enzymatic treatment for 1 h (enzymatic mix: Tris 50 mM pH 8.0, 10 mM $MgSO_4$, 5 mg/ml lysozyme and 100 U/ml mutanolysin), from here on referred to as 'MBioEz DNA-extraction'; iii) The QIAamp DNA Stool Mini kit following the manufacturer's intructions

Table 1. Primers used in this study.

Primer name	Adapter sequence	Key	Tag barcode	GAT	Primer Sequence (5′–3′)	Reference
520F	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TGAGCGGAAC	GAT	AYTGGGYDTAAAGNG	[28]
802R	CCTCTCTATGGGCAGTCGGTGAT				TACNVGGGTATCTAATCC	[28]
Probio_Uni	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TTGGAGTGTC	GAT	CCTACGGGRSGCAGCAG	In this study
Probio_Rev	CCTCTCTATGGGCAGTCGGTGAT				ATTACCGCGGCTGCT	In this study
P1	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	TCTATTCGTC	GAT	CCTACGGGAGGCAGCAG	[27]
P2	CCTCTCTATGGGCAGTCGGTGAT				ATTACCGCGGCTGCT	[27]
Bact-8F					AGAGTTTGATCCTGGCTCAG	[22,23]
1391R					GACGGGCGGTGTGTRCA	[22,23]
Bact-1510R					CGGTTACCTTGTTACGACTT	[32]
ENV1					AGAGTTTGATNNTGGCTCAG	[22,27,28,33]
ENV2					CGGNTACCTTGTTACGACTT	[22,27,28,33]

doi:10.1371/journal.pone.0068739.t001

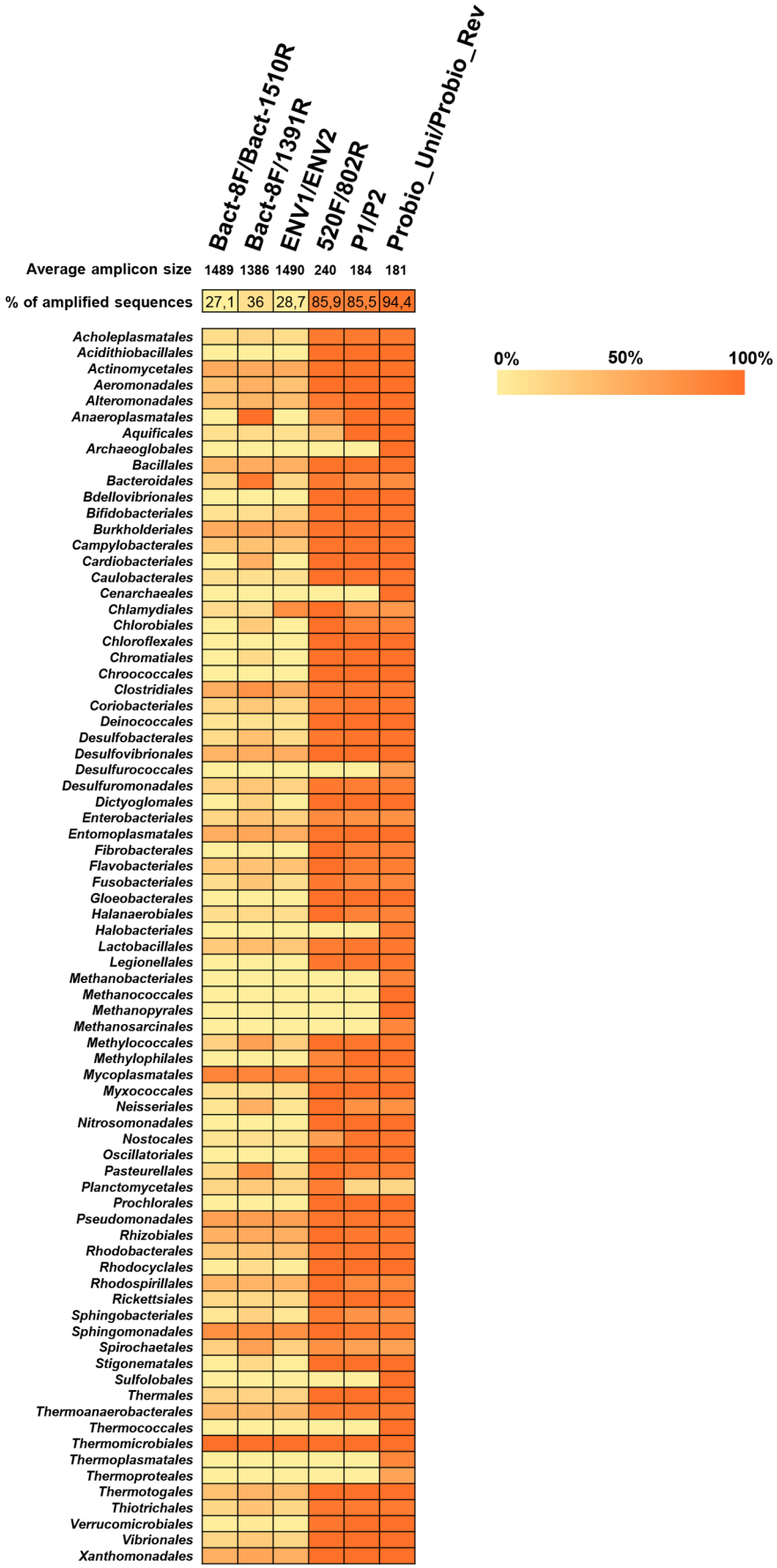
Bact-8F/Bact-1510R
Bact-8F/1391R
ENV1/ENV2
520F/802R
P1/P2
Probio_Uni/Probio_Rev
Average amplicon size
1489 1386 1490 240 184 181
% of amplified sequences
27,1 36 28,7 85,9 85,5 94,4
0%
50%
100%
Acholeplasmatales
Acidithiobacillales
Actinomycetales
Aeromonadales
Alteromonadales
Anaeroplasmatales
Aquificales
Archaeoglobales
Bacillales
Bacteroidales
Bdellovibrionales
Bifidobacteriales
Burkholderiales
Campylobacterales
Cardiobacteriales
Caulobacterales
Cenarchaeales
Chlamydiales
Chlorobiales
Chloroflexales
Chromatiales
Chroococcales
Clostridiales
Coriobacteriales
Deinococcales
Desulfobacterales
Desulfovibrionales
Desulfurococcales
Desulfuromonadales
Dictyoglomales
Enterobacteriales
Entomoplasmatales
Fibrobacterales
Flavobacteriales
Fusobacteriales
Gloeobacterales
Halanaerobiales
Halobacteriales
Lactobacillales
Legionellales
Methanobacteriales
Methanococcales
Methanopyrales
Methanosarcinales
Methylococcales
Methylophilales
Mycoplasmatales
Myxococcales
Neisseriales
Nitrosomonadales
Nostocales
Oscillatoriales
Pasteurellales
Planctomycetales
Prochlorales
Pseudomonadales
Rhizobiales
Rhodobacterales
Rhodocyclales
Rhodospirillales
Rickettsiales
Sphingobacteriales
Sphingomonadales
Spirochaetales
Stigonematales
Sulfolobales
Thermales
Thermoanaerobacterales
Thermococcales
Thermomicrobiales
Thermoplasmatales
Thermoproteales
Thermotogales
Thiotrichales
Verrucomicrobiales
Vibrionales
Xanthomonadales

Figure 1. Heat map showing the order classification rates for optimal choice of primers. Cells are colored on a gradient from 0% to 100% matches of the primer with the 16S rRNA gene target sequences of the indicated microbial order. The size of the amplicons generated by the various primer pairs are indicated. Furthermore, the percentage of the amplification rate from each primer pair on the various microbial taxa here described is indicated at the top of the heat map.
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(Qiagen Ltd., Strasse, Germany), from here on referred to as 'Qia DNA-extraction'; iv) The Qia DNA-extraction protocol including an initial mechanical cell disruption step by inclusion of 0.1 mm zirconium–silica beads (Biospec Products, Bartlesville, OK) and by subjecting the sample to three 1 min pulses at maximum speed in a bead beater (FastPrep FP120 Thermo Savant; Qbiogene, Inc., Illkirch, France) with intervals of 1 min on ice and, subsequently, the mechanical treatment was followed by an enzymatic lysis step for 1 h at 37°C (enzymatic mix: 50 mM Tris-HCl, pH 8.0, 10 mM $MgSO_4$, 5 mg/ml lysozyme and 50 U/ml mutanolysin) from here on referred to as 'QiaEz DNA-extraction'.

Mouse Trial

All animals used in this study were cared for in compliance with guidelines established by the Italian Ministry of Health. All procedures were approved by the University of Parma, as executed by the Institutional Animal Care and Use Committee (Dipartimento per la Sanità Pubblica Veterinaria, la Nutrizione e la Sicurezza degli Alimenti Direzione Generale della Sanità Animale e del Farmaco Veterinario, Italy). Fecal samples were collected after removal of animals from their box and no sacrifice of the animals was performed.

16S rRNA Gene Amplification

Partial 16S rRNA gene sequences were amplified from extracted DNA using primer pair Probio_Uni and/Probio_Rev, which targets the V3 region of the 16S rRNA gene sequence, or employing primer pair P1 and P2 [27], which targets the V3 region of the 16S rRNA gene sequences, or primer pair 520F and 802R [28] corresponding to the V4 region of the 16S rRNA gene sequences. These primers were designed to include at their 5′ end one of the two adaptor sequences used in the Ion Torrent-sequencing library preparation protocol linking a unique Tag barcode of 10 bases to identify different samples. The complete list of the primers used in this study is reported in Table 1.

The PCR conditions used were 5 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 55°C and 90 s at 72°C, followed by 10 min at 72°C. Amplification was carried out by using a Verity Thermocycler (Applied Biosystems). The integrity of the PCR amplicons was analyzed by electrophoresis on an Experion workstation (BioRad, UK).

Ion Torrent PGM Sequencing of 16S rRNA Gene-based Amplicons

The PCR products derived from amplification of specific 16S rRNA gene hypervariable regions were purified by electrophoretic separation on an 1.5% agarose gel and the use of a Wizard SV Gen PCR Clean-Up System (Promega), followed by a further purification step involving the Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) in order to remove primer dimers. DNA concentration of the amplified sequence library was estimated through the Experion system (BioRad). From the concentration and the average size of each amplicon library, the amount of DNA fragments per microliter was calculated and libraries for each run were diluted to 3E9 DNA molecules prior to clonal amplification. Emulsion PCR was carried out using the Ion OneTouch™ 200 Template Kit v2 DL (Life Technologies) according to the manufacturer's instructions. Sequencing of the amplicon libraries was carried out on a 314 chip using the Ion Torrent PGM system and employing the Ion Sequencing 200 kit (Life Technologies) according to the supplier's instructions. After sequencing, the individual sequence reads were filtered by the PGM software to remove low quality and polyclonal sequences. Sequences matching the PGM 3′ adaptor were also automatically trimmed. All PGM quality-approved, trimmed and filtered data were exported as sff files.

Sequence-based Microbiota Analysis

The sff files were processed using QIIME [29]. Quality control retained sequences with a length between 150 and 200 bp, mean sequence quality score >25, with truncation of a sequence at the first base if a low quality rolling 10 bp window was found. Presence of homopolymers >6 bp, and sequences with mismatched primers were omitted. In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥97% sequence homology. All reads were classified to the lowest possible taxonomic rank using QIIME and a reference dataset from the Ribosomal Database Project [30].

OTUs were assigned using uclust [31]. The hierarchical clustering based on population profiles of most common and abundant taxa was performed using UPGMA clustering (Unweighted Pair Group Method with Arithmetic mean, also known as average linkage) on the distance matrix of OTU abundance. This resulted in a Newick formatted tree, which was obtained utilizing the QIIME package.

Nucleotide Sequence Accession Numbers

The raw sequences reported in this article have been deposited in the NCBI Short Read Archive (SRA) (SAMN02009352, SAMN02009353, SAMN02009354, SAMN02009355, SAMN02009356, SAMN02009357).

Results and Discussion

Design of a Suitable PCR Primer Pair for 16S rRNA Gene Sequence Profiling on the Ion Torrent NGS Platform

Previous investigations on taxonomic classification of the human gut microbiota were based on PCR primers that target the 16S rRNA gene. In order to develop a specific and reliable 16S rRNA gene-based primer set that is suitable for Ion Torrent sequencing technology, we selected, based on currently available literature [27,28], primer pairs that generate an amplicon of a maximum size of 200 bp and that target the V4 or V3 hypervariable region of the 16S rRNA gene, corresponding to positions 563–797 and 341–534 (coordinates based on the 16S rRNA gene of *Escherichia coli* strain K-12 substr. MG1655). In addition, a novel primer set, i.e. Probio_Uni/Probio_Rev, was designed for the purpose of this study by modification of the well established primer set P1/P2, originally designed by [27]. Compared to other primer pairs mentioned above, the Probio_Uni/Probio_Rev primer pair perfectly matches with a higher number of human gut microbiota components as detected by an *in silico* analysis against Ribosomal Database Project's (RDP) sequences. As displayed in Figure 1, the Probio_Uni/Probio_Rev primer pair theoretically targets all 16S rRNA gene sequences of the gut microbiota bacterial orders we selected with an average similarity of 94.4% and generating

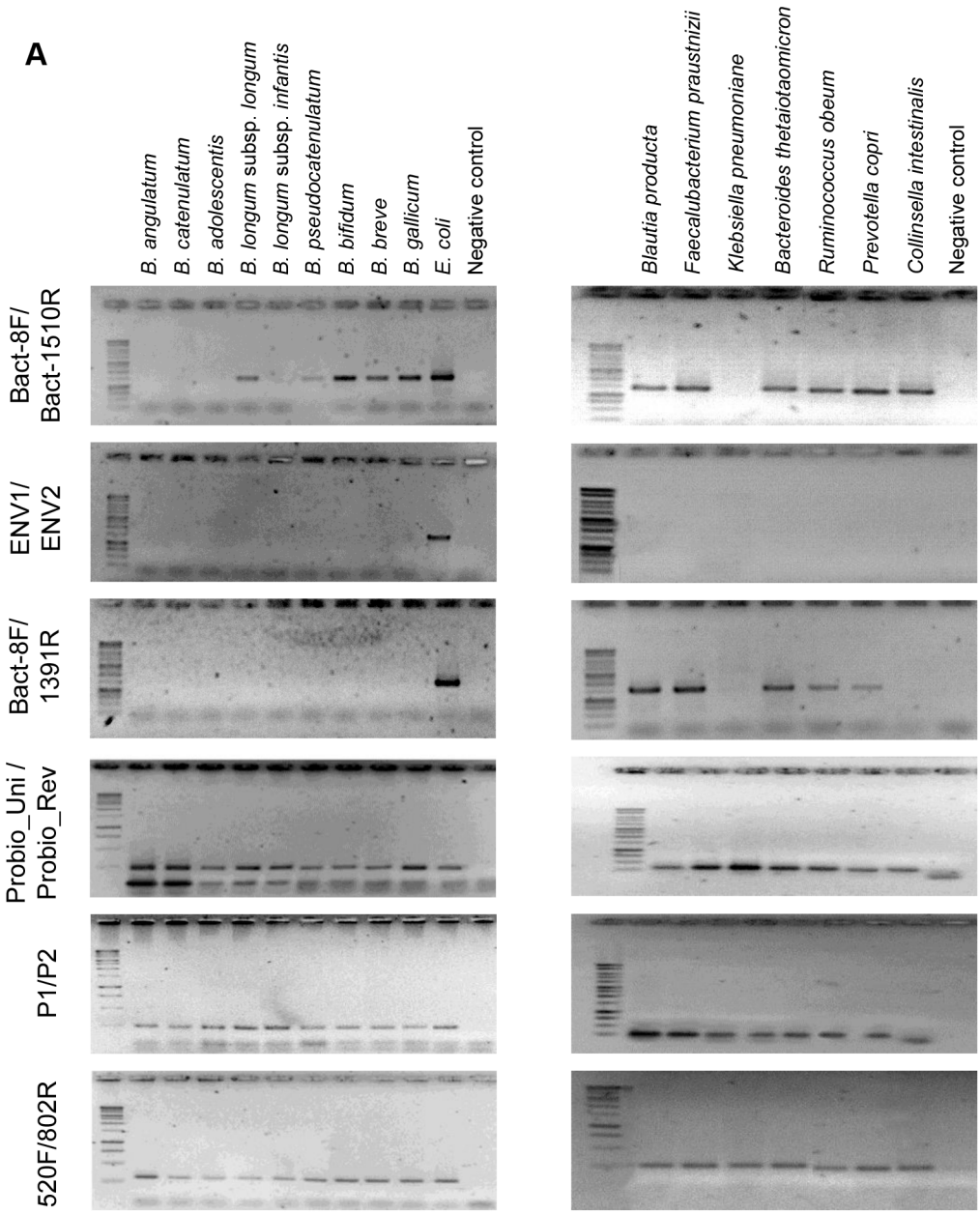
A
B. angulatum
B. catenulatum
B. adolescentis
B. longum subsp. longum
B. longum subsp. infantis
B. pseudocatenulatum
B. bifidum
B. breve
B. gallicum
E. coli
Negative control
Blautia producta
Faecalubacterium praustnizii
Klebsiella pneumoniane
Bacteroides thetaiotaomicron
Ruminococcus obeum
Prevotella copri
Collinsella intestinalis
Negative control
Bact-8F/ Bact-1510R
ENV1/ ENV2
Bact-8F/ 1391R
Probio_Uni / Probio_Rev
P1/P2
520F/802R
B
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
1,00E+01
1,00E+00
Bact-8F/ Bact-1510R
Bact-8F/1391R
ENV1/ ENV2
Enterococcaceae
Enterobacteriaceae
Bacteroides
Atopobium
Lactobacillus
Bifidobacterium

Figure 2. Fecal levels of different microorganisms as analyzed by PCR using different 16S rRNA gene-based primers. Panel a depicts PCR amplification targeting the 16S rRNA gene of different enteric bifidobacteria as well as key intestinal bacteria using the primers described by [22] [27,32,33,43] and the primer set Probio_Uni/Probio_Rev. Panel b. displays the microbial profile of the amplicons generated by the primer sets described by [22,32,33] according to the following taxa: *Bifidobacteriaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Lactobacillales*, *Bacteroidetes* group, and *Atopobium* group.
doi:10.1371/journal.pone.0068739.g002

an optimal Ion Torrent amplicon with an average length of 181 bp. Specifically, compared to the primer pair P1/P2 [27], the Probio_Uni/Probio_Rev primer pair perfectly targets (is fully complementary without mismatches) relevant 16S rRNA gene sequences of *Archaeoglobales*, *Thermococcales*, *Sulfolobales*, *Cenarchaeales*, *Methanococcales* and *Methanopyrales* (Fig. 1).

Development of an Appropriate Methodology for DNA Amplification

Precise assessment of the composition of the human gut microbiota crucially depends on the reliability and specificity of the PCR primers employed to amplify the 16S rRNA gene of

Table 2. Relation between the percentages of 16S rRNA gene sequences obtained using the four different DNA extraction methods and the mixes P1, P2 and P3.

MIX	Species	Cell number	% each strain	16S copy number	relative abundance not normalized				relative abundance normalized			
P1					**MBio**	**MBioEz**	**Qia**	**QiaEz**	**MBio**	**MBioEz**	**Qia**	**QiaEz**
	B. longum	3,84E+09	10,71	4	2,99	57,36	3,39	23,47	1,07	43,68	1,35	20,54
	B. coccoides+B. producta	7,68E+09	21,42	1+	1,61	1	3,5	0,58	2,30	3,05	5,59	2,03
	C. intestinalis	3,84E+09	10,71	2*	31,58	18,47	18,75	17,58	22,57	28,13	14,98	30,78
	F. prausnitzii	3,84E+09	10,71	1	47,14	4,62	42,04	3,61	67,38	14,07	67,19	12,64
	E. coli	3,84E+09	10,71	7	2,32	1,53	6,6	24,4	0,47	0,67	1,51	12,21
	K. pneumoniae	3,84E+09	10,71	8	3,45	2,6	11,37	4,18	0,62	0,99	2,27	1,83
	P. copri	3,84E+09	10,71	1*	2,23	0,41	2,09	0,58	3,19	1,25	3,34	2,03
	B. thetaiotaomicron	3,84E+09	10,71	5	8,38	13,2	11,74	25,47	2,40	8,04	3,75	17,84
	M. smithii	1,30E+09	3,63	2	0,01	0,08	0	0,06	0,01	0,12	0,00	0,11
P2												
	B. longum	3,84E+09	18,59	4	5,64	18,59	3,52	2,15	1,72	7,21	1,11	0,69
	B. coccoides+B. producta	7,68E+09	37,18	1+	2,86	2,43	9,06	6,35	3,49	3,77	11,42	8,17
	C. intestinalis	3,84E+09	18,59	2*	27,11	33,14	32,12	35,76	16,56	25,72	20,25	23,01
	F. prausnitzii	3,84E+09	18,59	1	63,94	39,88	52,87	52,33	78,12	61,90	66,66	67,35
	E. coli	3,84E+07	0,19	7	0,08	3,21	0,63	0,8	0,01	0,71	0,11	0,15
	K. pneumoniae	3,84E+07	0,19	8	0,05	1,26	0,82	1,2	0,01	0,24	0,13	0,19
	P. copri	3,84E+07	0,19	1*	0,03	0,12	0,1	0,17	0,04	0,19	0,13	0,22
	B. thetaiotaomicron	3,84E+07	0,19	5	0,19	0,84	0,75	0,86	0,05	0,26	0,19	0,22
	M. smithii	1,30E+09	6,29	2	0	0	0	0	0,00	0,00	0,00	0,00
P3												
	B. longum	3,84E+07	0,23	4	0,1	0,2	0,05	0,29	0,09	0,26	0,05	0,42
	B. coccoides+B. producta	7,68E+07	0,46	1+	0	0	0,02	0,02	0,00	0,00	0,09	0,12
	C. intestinalis	3,84E+07	0,23	2*	0,32	0,33	0,14	0,2	0,57	0,86	0,31	0,58
	F. prausnitzii	3,84E+07	0,23	1	0,35	0	0,29	0,08	1,24	0,00	1,27	0,47
	E. coli	3,84E+09	22,79	7	14,06	14,33	24,5	48,13	7,10	10,67	15,28	40,15
	K. pneumoniae	3,84E+09	22,79	8	27,77	17,97	36,86	9,24	12,28	11,70	20,12	6,74
	P. copri	3,84E+09	22,79	1*	13,86	1,53	8,75	0,48	49,01	7,97	38,21	2,80
	B. thetaiotaomicron	3,84E+09	22,79	5	42,02	65,4	28,26	41,46	29,72	68,15	24,68	48,42
	M. smithii	1,30E+09	7,71	2	0	0,15	0	0,1	0,00	0,39	0,00	0,29

The sequence of *B. coccoides* and *B. producta* were indistinguishable and were included in the same group.
*Means that only draft genomes are available, thus the 16S copy number is probably underestimated.
+ Means that no sequenced genomes are available, thus the *genera* average 16S copy number was used.
The normalized relative abundance was calculated by division of the relative abundances of every species by their predicted 16S copy number. Results of each sample were normalized so that their sum is 100%.
doi:10.1371/journal.pone.0068739.t002

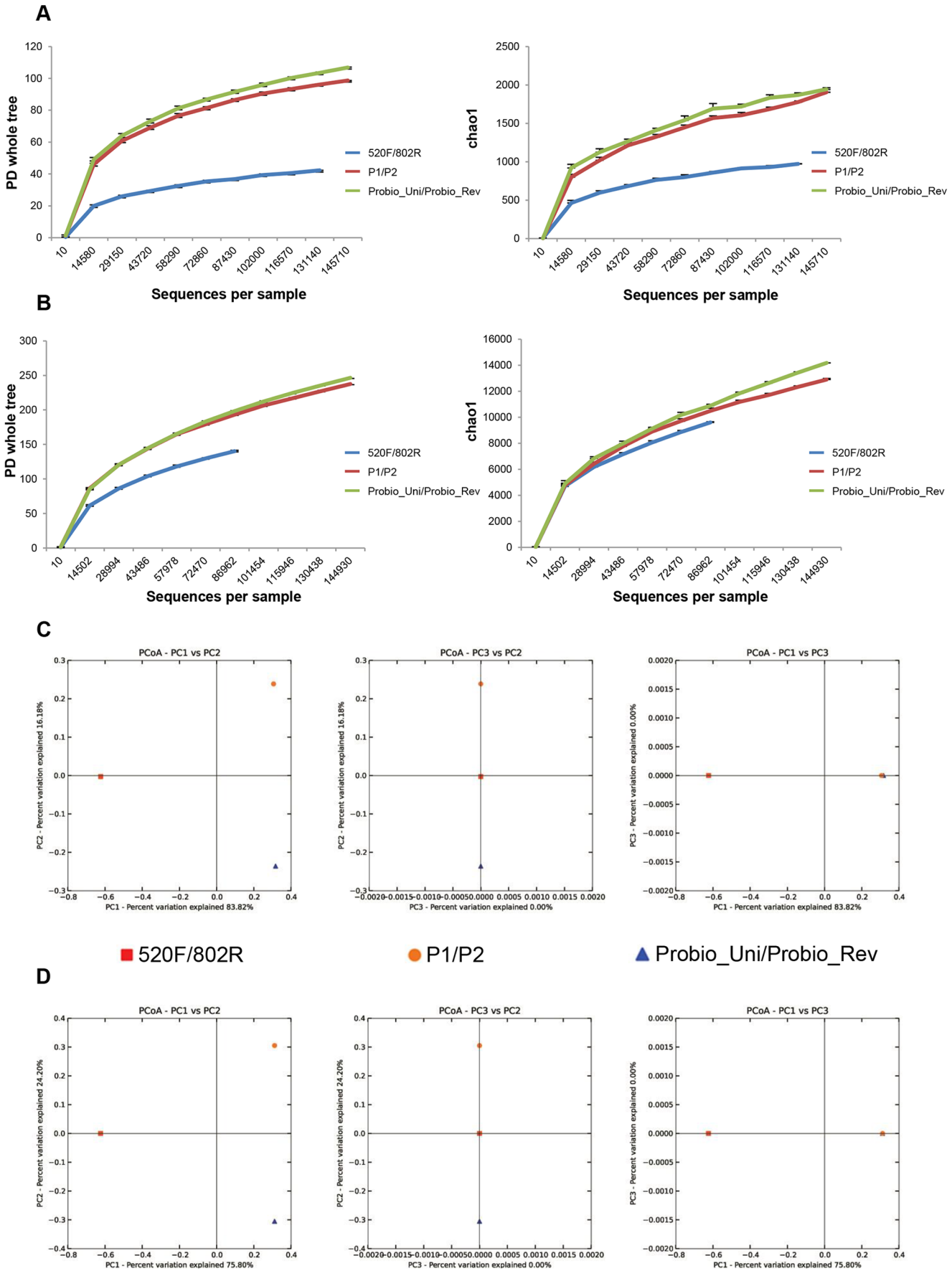
A
PD whole tree
Sequences per sample
520F/802R
P1/P2
Probio_Uni/Probio_Rev
chao1
B
C
PCoA - PC1 vs PC2
PCoA - PC3 vs PC2
PCoA - PC1 vs PC3
PC1 - Percent variation explained 83.82%
PC2 - Percent variation explained 16.18%
PC3 - Percent variation explained 0.00%
D
PC1 - Percent variation explained 75.80%
PC2 - Percent variation explained 24.20%

Figure 3. Rarefaction curves generated for 16S rRNA gene sequences and Principal Coordinate Analysis (PCoA) based on the phylotypes identified from different PCR primer sets as well as from different samples (stool samples of infants and fecal samples of mothers). Panels a and c display the rarefaction curves and the PCoA from stool samples of infants. Panels b and d show the rarefaction curves and the PCoA from fecal samples of mothers. In panels c and d percentages shown along the axes represent the proportion of dissimilarities captured by the axes. Each symbol represents the 16S rRNA gene sequences from each sample which are displayed in a different colour and shape according to the PCR primer pair. In panels a and b the plots depicted on the left represent the rarefaction curves determined using the PD index whereas the plots show on the right constitute the rarefaction curves obtained using the Chao index. A 95% confidence intervals was added to the rarefaction curves.
doi:10.1371/journal.pone.0068739.g003

specific groups of the bacterial community. Comparative assays were performed by amplification reactions using the same samples as templates and either different sets of previously described PCR primers targeting bacterial [32] [22,27,28,32,33], or the newly designed PCR primer pair (Probio_Uni/Probio_Rev) aimed at amplification of the 16S rRNA gene of key gut microbiota members such as those belonging to the genera *Bifidobacterium*, *Bacteroides*, *Collinsella*, *Prevotella*, *Escherichia*, *Blautia*, *Faecalibacterium*, *Klebsiella* and *Methanobrevibacter*. Notably, no or very little PCR-mediated amplification product was obtained when DNA, extracted from commonly encountered human gut bifidobacterial species (*Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium catenulatum/Bifidobacterium pseudocatenulatum*, *Bifidobacterium angulatum* and *Bifidobacterium gallicum*) (for review see [34]), was used as a template and employing Bact-8F/Bact-1510R, ENV1/ENV2 and Bact-8F/1319R primer combinations. The dissection by quantitative real-time qPCR of the microbial composition according to several common intestinal microbiota taxa (e.g., *Bifidobacteriaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Lactobacillales*, *Bacteroidetes* group, and *Atopobium* group) of amplicons obtained from fecal DNA extracted using the method involving an enzymatic lysis based on previously published PCR primers [22,27,28,32,33] yielded very different microbial profiles (Fig. 2). In particular, the microbial taxon *Bifidobacterium* appears to be completely absent when employing primer combinations ENV1/ENV2 and Bact-8F/Bact-1510R [22,33]. This is consistent with these primers exhibiting the lowest level of homology with the corresponding 16S rRNA gene sequences of members of the taxa *Bifidobacteriaceae*, *Lactobacillus* and *Enterobacteriaceae* (Fig. 1). Altogether these results lead us to conclude that the apparent lack of bifidobacterial sequences from certain previously published 16S rRNA gene profiling gut microbiota studies is in part due to PCR primers that were biased against key members of the gut microbiota such as bifidobacteria. Thus, many of the published studies describing the bacterial composition of the infant gut seem to have significantly underestimated the incidence and diversity of these commensals.

Setting up of a Valid Methodology for Sample Processing

Various studies investigating the human fecal microbiota biodiversity have reported highly variable and sometimes contradictory results [22,23,35]. Although these results may have been due to biological variation between individuals, it has been shown that sample preparation methods, such as DNA extraction does play a significant role in explaining the reported variations [16]. In order to address this issue we evaluated four fecal DNA extraction methods, i.e. involving different commercial kits and by introducing an initial enzymatic treatment prior to the actual DNA extraction protocol (see materials and methods), and assessed which of these methods would provide the most accurate representation of microbial populations in fecal samples by means of 16S rRNA gene-based profiling using the Ion Torrent PGM technology. Germ-free fecal samples from rats were mixed with various combinations of nine different microbial strains belonging to the following species: *Bifidobacterium longum*, *Collinsella intestinalis*, *Blautia coccoides*, *Blautia producta*, *Faecalibacterium prausnitzii*, *Escherichia coli*, *Prevotella copri*, *Klebsiella pneumoniae*, *Bacteroides thetaiotaomicron* and the Archeal strain *Methanobrevibacter smithii*, representing some of the most abundant bacterial species in the human gastrointestinal tract, according to several reports [36,37], while *M. smithii* is the most frequently found archeal bacterium in the human gut [38]. Total DNA samples, extracted by different methods from feces contaminated with microbial mixes of these ten microorganisms in different proportions, were used as template for amplification with the primers developed in this study, i.e., Probio_Uni and Probio_Rev. Gnotobiotic feces were used as negative controls in the four extraction procedures, yielding as expected no amplicons. The Ion Torrent sequencing platform, as optimized here, was shown to detect all bacterial strains present in the samples, independent of the relative amount of each strain. However, *M. smithii* was not consistently detected in all samples analyzed. Since the primers Probio_Uni and Probio_Rev match with a high score to the 16S rRNA gene sequences of this archaea, our results suggest incomplete lysis of archaeal cells. In this regard, *Methanobrevibacter* cells are covered by pseudomurein, a polymer that is similar to bacterial peptidoglycan with the exception of the l-N-acetyltalosaminuronic acid linked (β-1,3) to d-N-acetylglucosamine (GlcNAc) and the absence of D-amino acids in the interpeptide bridges [39]. Therefore, it seems that methods focused on pseudomurein digestion are needed for optimizing methanobacteria lysis. Overall, our results point that this microorganism is present in the human intestine in quantities higher than previously reported in some microbiota studies. Notably, our results indicate that several species, such as *F. prausnitzii*, *C. intestinalis* and *B. thetaiotaomicron*, are normally overestimated, whereas others, such as *B. longum*, are underestimated.

In order to determine which of the four tested DNA extraction methods provided the most reliable representation of the fecal bacterial community, we determined the proportion of each microbial group using the percentages of sequences obtained after 16S rRNA gene-based microbial profile analysis and compared this to the known number of microorganisms added to each sample. As previously shown, the evaluation of the abundance of members of the gut microbiota based on 16S rRNA gene profiling might be influenced by the copy number of 16S rRNA gene sequences [40].

Thus, we decided to normalize the number of reads linked to microbial taxon for the copy number of 16S rRNA gene loci present in their genome sequences (Table 2 and Figure S1). The detected difference, expressed in log units, between the number of microorganisms added and the number of sequences, either normalized for the copy number of 16S rRNA gene loci or not normalized, was calculated for each microorganism. The Bray-Curtis dissimilarity indexes were then calculated between the experimental data obtained for each bacterial mix (P1, P2 and P3) and the true levels of added microbes present in these mixes. This approach allows the identification of the best method without introducing subjective biases. In fact, the method showing the lowest mean value is considered to provide the most accurate

Table 3. Quantitative data of the 16S rRNA gene sequence datasets used in this study.

Dataset*	Sample	Number of reads	Number of reads removed because of:						Final read number	Reduced by (%)
			Outside bounds (160–300)	Ambiguous bases	Mean quality <15	Homopolymer runs >6bp	Primer mismatch >1	Low quality window truncation results in <160bp		
M	M520F/802R	171559							103757	39.52%
	MProbio_Uni/Probio_Rev	231660							146688	36.68%
	MP1/P2	239873							152995	36.22%
	TOTAL	**643092**	**130387**	**0**	**0**	**18290**	**25459**	**65516**	**403440**	**37.27%**
I	I520F/802R	180917							133592	26.16%
	IProbio_Uni/Probio_Rev	251632							151790	39.68%
	IP1/P2	186780							145730	21.98%
	TOTAL	**633877**	**37396**	**0**	**8060**	**6029**	**90701**	**59878**	**431112**	**31.99%**

*The M dataset stands for mother's fecal sample dataset; I dataset correspond to the infant's stool sample dataset.

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overall representation of the bacterial communities in feces, based on the fact that the obtained Bray-Curtis dissimilarity index value is the lowest for the situation where the observed discrepancy between the number of microorganisms added and detected is the smallest. Thus, when normalization for the copy number of 16S rRNA gene loci was not considered, we found that the Qia-DNA extraction method yielded the most reliable results out of the four methods tested here, since it provides the most accurate reflection of the fecal microbiota composition. In contrast, the MBioEz-DNA extraction method generated the least accurate results. The Bray-Curtis distances using the four different methods were 0.17 (Qia), 0.23 (QiaEz), 0.29 (MBio) and 0.45 (MBioEz). However, if normalization for the number of 16S rRNA gene copies was taken into account, the Qia and QiaEz-DNA extraction methods showed the best results, with Bray-Curtis distance values being 0.17, 0.16, 0.39, and 0.42 for the Qia, QiaEz, MBio, and MBioEz-DNA extraction methods, respectively. We also noticed that an initial enzymatic treatment to enhance cell lysis increases the representation of some species, such as *B. thetaiotaomicron*. It is worthwhile mentioning that results are expected to be different when alternative DNA extraction protocols are used. In fact, other studies have recently indicated that the Qia-DNA extraction method is not as accurate as alternative bead-beating methods [41,42].

Comparison of Gut Microbiota Profiling using Different Primer Sets

In order to further evaluate the efficacy of different primer sets, including Probio_Uni/Probio_Rev, P1/P2 [27], 520F/802R [43] to delineate the microbiota composition of human fecal samples, we sequenced 16S rRNA gene-based amplicons achieved with either of these primers pairs, using the same DNA stock extracted from two different fecal samples. For this purpose two human fecal DNA samples were used, one retrieved from a three month infant stool sample, which based on previous published data was considered to possess a low level of complexity [18] and another one extracted from a mother's fecal sample assumed to include a diverse composition of microbiota [18,32,43]. In total, 1,276,969 sequence reads, representing 633,877 and 643,092 reads per infant- and mother sample, respectively, were generated on the Ion Torrent PGM machine (Table 3). The decrease in the rate of phylotype detection and the plateauing of various diversity indices for this set of PCR primers demonstrated that a large part of the diversity in these libraries had been detected, even though a higher number of phylotypes was identified for the primer pair Probio_Uni/Probio_Rev as compared to the other PCR primer sets (Fig. 3).

The significance test in UniFrac [44] reported P-Values (Bonferroni corrected) equal to 1e-02 for every pair of samples of both mother and infant sets, meaning that the results are highly significant (if P-values ≤ 0.05 are considered to be statistical significant). This method was used to evaluate if the cluster distribution of the sequences achieved with the different PCR primers differs from random expectations. Principal Coordinate Analysis (PCoA), applied using the UniFrac program, showed that, while the datasets obtained with the P1/P2 and Probio_uni/Probio_rev primer pairs cluster together, the dataset achieved with the 520F/802R primer pair is rather different (Fig. 3). This suggests that the microbial composition assessed by this latter primer set is different than those detected by either P1/P2 or Probio_uni/Probio_rev.

Clustering of de-noised high quality reads generated 18,910 and 2,361 OTUs for the mother and infant samples, respectively. As expected the microbial composition of the two fecal samples

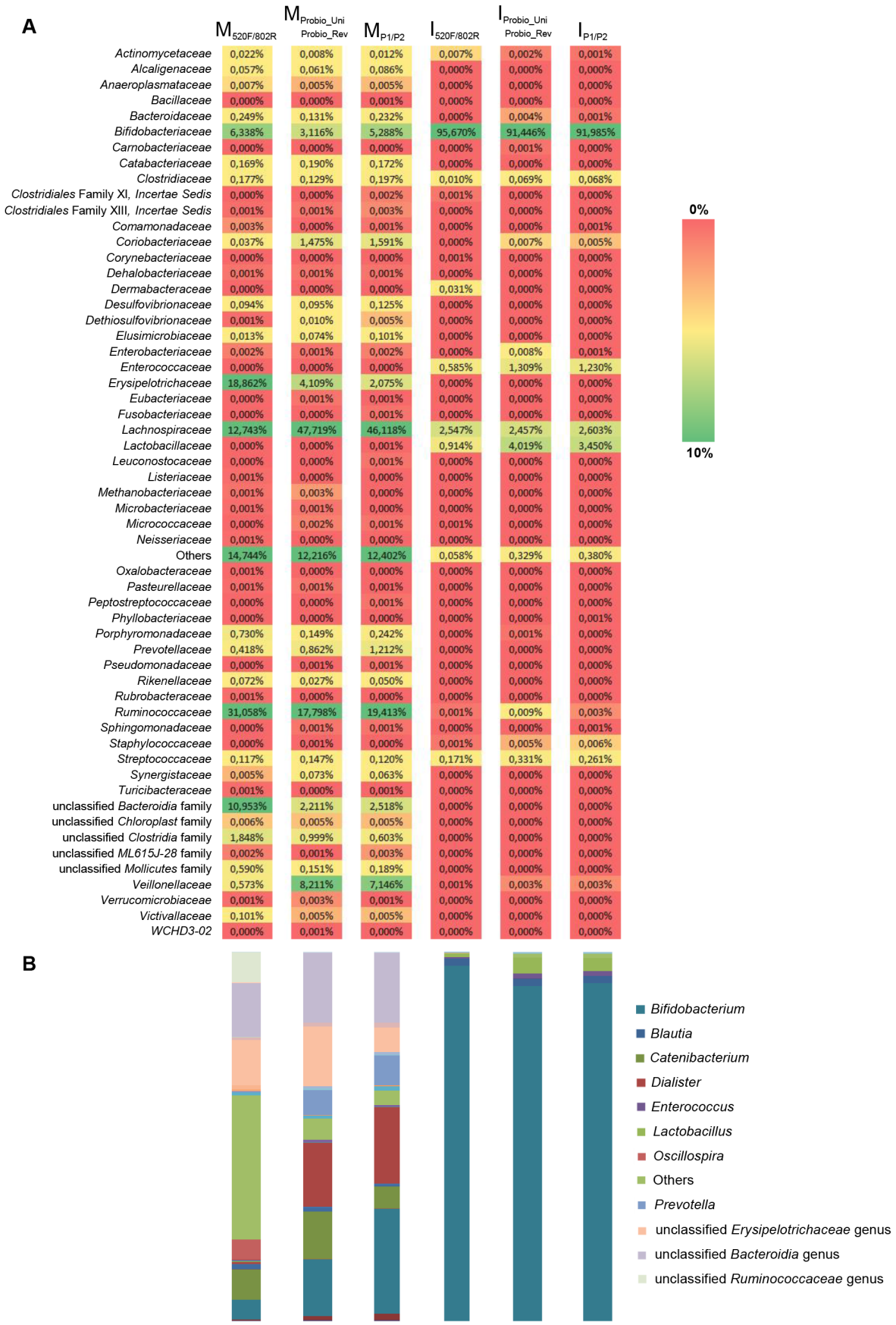
A
M520F/802R
MProbio_Uni Probio_Rev
MP1/P2
I520F/802R
IProbio_Uni Probio_Rev
IP1/P2
Actinomycetaceae 0,022% 0,008% 0,012% 0,007% 0,002% 0,001%
Alcaligenaceae 0,057% 0,061% 0,086% 0,000% 0,000% 0,000%
Anaeroplasmataceae 0,007% 0,005% 0,005% 0,000% 0,000% 0,000%
Bacillaceae 0,000% 0,000% 0,001% 0,000% 0,000% 0,000%
Bacteroidaceae 0,249% 0,131% 0,232% 0,000% 0,004% 0,001%
Bifidobacteriaceae 6,338% 3,116% 5,288% 95,670% 91,446% 91,985%
Carnobacteriaceae 0,000% 0,000% 0,000% 0,000% 0,001% 0,000%
Catabacteriaceae 0,169% 0,190% 0,172% 0,000% 0,000% 0,000%
Clostridiaceae 0,177% 0,129% 0,197% 0,010% 0,069% 0,068%
Clostridiales Family XI, Incertae Sedis 0,000% 0,000% 0,002% 0,001% 0,000% 0,000%
Clostridiales Family XIII, Incertae Sedis 0,001% 0,001% 0,003% 0,000% 0,000% 0,000%
Comamonadaceae 0,003% 0,000% 0,001% 0,000% 0,000% 0,001%
Coriobacteriaceae 0,037% 1,475% 1,591% 0,000% 0,007% 0,005%
Corynebacteriaceae 0,000% 0,000% 0,000% 0,001% 0,000% 0,000%
Dehalobacteriaceae 0,001% 0,001% 0,001% 0,000% 0,000% 0,000%
Dermabacteraceae 0,000% 0,000% 0,000% 0,031% 0,000% 0,000%
Desulfovibrionaceae 0,094% 0,095% 0,125% 0,000% 0,000% 0,000%
Dethiosulfovibrionaceae 0,001% 0,010% 0,005% 0,000% 0,000% 0,000%
Elusimicrobiaceae 0,013% 0,074% 0,101% 0,000% 0,000% 0,000%
Enterobacteriaceae 0,002% 0,001% 0,002% 0,000% 0,008% 0,001%
Enterococcaceae 0,000% 0,000% 0,000% 0,585% 1,309% 1,230%
Erysipelotrichaceae 18,862% 4,109% 2,075% 0,000% 0,000% 0,000%
Eubacteriaceae 0,000% 0,001% 0,001% 0,000% 0,000% 0,000%
Fusobacteriaceae 0,000% 0,000% 0,001% 0,000% 0,000% 0,000%
Lachnospiraceae 12,743% 47,719% 46,118% 2,547% 2,457% 2,603%
Lactobacillaceae 0,000% 0,000% 0,001% 0,914% 4,019% 3,450%
Leuconostocaceae 0,000% 0,000% 0,001% 0,000% 0,000% 0,000%
Listeriaceae 0,001% 0,000% 0,000% 0,000% 0,000% 0,000%
Methanobacteriaceae 0,001% 0,003% 0,000% 0,000% 0,000% 0,000%
Microbacteriaceae 0,001% 0,001% 0,000% 0,000% 0,000% 0,000%
Micrococcaceae 0,000% 0,002% 0,001% 0,001% 0,000% 0,000%
Neisseriaceae 0,001% 0,000% 0,000% 0,000% 0,000% 0,000%
Others 14,744% 12,216% 12,402% 0,058% 0,329% 0,380%
Oxalobacteraceae 0,001% 0,000% 0,000% 0,000% 0,000% 0,000%
Pasteurellaceae 0,001% 0,001% 0,001% 0,000% 0,000% 0,000%
Peptostreptococcaceae 0,000% 0,000% 0,001% 0,000% 0,000% 0,000%
Phyllobacteriaceae 0,000% 0,000% 0,000% 0,000% 0,000% 0,001%
Porphyromonadaceae 0,730% 0,149% 0,242% 0,000% 0,001% 0,000%
Prevotellaceae 0,418% 0,862% 1,212% 0,000% 0,000% 0,000%
Pseudomonadaceae 0,000% 0,001% 0,001% 0,000% 0,000% 0,000%
Rikenellaceae 0,072% 0,027% 0,050% 0,000% 0,000% 0,000%
Rubrobacteraceae 0,001% 0,000% 0,000% 0,000% 0,000% 0,000%
Ruminococcaceae 31,058% 17,798% 19,413% 0,001% 0,009% 0,003%
Sphingomonadaceae 0,000% 0,001% 0,001% 0,000% 0,000% 0,001%
Staphylococcaceae 0,000% 0,001% 0,000% 0,001% 0,005% 0,006%
Streptococcaceae 0,117% 0,147% 0,120% 0,171% 0,331% 0,261%
Synergistaceae 0,005% 0,073% 0,063% 0,000% 0,000% 0,000%
Turicibacteraceae 0,001% 0,000% 0,001% 0,000% 0,000% 0,000%
unclassified Bacteroidia family 10,953% 2,211% 2,518% 0,000% 0,000% 0,000%
unclassified Chloroplast family 0,006% 0,005% 0,005% 0,000% 0,000% 0,000%
unclassified Clostridia family 1,848% 0,999% 0,603% 0,000% 0,000% 0,000%
unclassified ML615J-28 family 0,002% 0,001% 0,003% 0,000% 0,000% 0,000%
unclassified Mollicutes family 0,590% 0,151% 0,189% 0,000% 0,000% 0,000%
Veillonellaceae 0,573% 8,211% 7,146% 0,001% 0,003% 0,003%
Verrucomicrobiaceae 0,001% 0,003% 0,001% 0,000% 0,000% 0,000%
Victivallaceae 0,101% 0,005% 0,005% 0,000% 0,000% 0,000%
WCHD3-02 0,000% 0,001% 0,000% 0,000% 0,000% 0,000%
0%
10%
B
Bifidobacterium
Blautia
Catenibacterium
Dialister
Enterococcus
Lactobacillus
Oscillospira
Others
Prevotella
unclassified Erysipelotrichaceae genus
unclassified Bacteroidia genus
unclassified Ruminococcaceae genus

Figure 4. Number of sequences per phylotype for each PCR primer pair and sample (stool samples of infants and fecal samples of mothers). The y axis displays the OTUs at family level detected in this study; each row represents a different OTU. Increasing darkness of the colour scale corresponds to higher estimated relative abundance. At the bottom of the heatmap a representation of the abundance of different phylotypes at the genus level detected by different PCR primer set for each sample has been displayed.
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displayed a much simpler OTU organization in the infant stool sample as compared to that of the mother. At genus level, sequencing reads could be assigned to 83 main individual taxons, of which 30 were present in all the three PCR-primer dataset in at least one of the two samples (Fig. 4). Furthermore, we analyzed the similarities and differences in the composition of the fecal sample assayed using the three PCR primer sets by hierarchical clustering based on population profiles of the most common and abundant taxa (Fig. 4). The clustering patterns are also reflected in the corresponding bar diagram (Fig. 4), highlighting bias in resolution of the PCR primer sets against specific microbial taxa. Microbiota composition observed with these different primer sets, highlighted a discrepancy with respect the *Enterococcaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Streptococcaceae* and *Veillonellaceae* (Fig. 4). In fact, primer set Probio_Uni/Probio_Rev displayed a higher proportion of members of these bacterial groups compared to the other set of primers, where a lower number or no phylotypes corresponding to the above mentioned microbial taxa were identified (Fig. 4). The latter finding thus reinforces the importance of a reliable PCR primer set for the appropriate delineation of the biodiversity of the human gut.

Conclusions

Appropriate primer selection as well as DNA extraction protocols in microbiota studies using 16S rRNA gene sequencing approach is essential to enable trustworthy representation of the organisms present in an environment such as human gut ecosystem. In this context, dominant infant gut microbiota members such as bifidobacteria are under-represented in many published metagenomic studies of the microbial biodiversity of the infant gut [22] due to technical biases. In fact, the presence of a thick cell wall of bifidobacterial cells as well as a polysaccharide surface layer [45] might render these microorganisms recalcitrant to cell lysis, thus causing a low recovery of their chromosomal DNA. In contrast, other key members of the gut microbiota such as *Faecalibacterium* and *Bacteroides* might be over-represented in most of the current human gut metagenomic studies. Here, we were able to demonstrate that the bias observed against the detection of bifidobacteria was due to the DNA extraction as well as PCR steps. We have shown that erroneous conclusions about the presence/absence as well as relative proportion of bifidobacteria are likely if primers that do not sufficiently complement the target 16S rRNA gene sequence are used. Noticeably, the error frequencies predicted to occur within DNA sequences generated by IonTorrent is equivalent to that estimated for other NGS technologies such as 454, i.e., about 1% [9]. Such a low level of error should not affect the final OTUs prediction, since these are calculated at 97% of nucleotide identity.

In this study we have designed a PCR primer set allowing accurate detection of bifidobacteria as well as other main members of the human gut microbiota, which, in combination with a fast and cheap sequencing approach like the Ion Torrent PGM, is suitable for the investigation of the microbial composition of the human gut microbiota.

Supporting Information

Figure S1 Ratio of 16S rRNA gene sequences obtained after the analysis of the artificially contaminated gnotobiotic fecal samples. Four different DNA extraction procedures (Qia, QiaEz, MBio and MBioEz DNA-extractions) and three different mixes of microorganisms (P1, P2 and P3) were used (see material and methods section). A, B and C represent the results obtained for the three different mixes (P1, P2 and P3, respectively) when the relative abundance of 16S rRNA gene sequences was not normalized. D, E and F represent the results obtained for the three different mixes (P1, P2 and P3, respectively) when the relative abundance of 16S sequences was normalized considering the predicted 16S copy number of the strains. In the center of each panel, the expected result according to the real microbial population present in each sample is depicted, and the graphics on the corners show the results using the four different extraction methods. The sequence of *Blautia coccoides* and *Blautia producta* were indistinguishable and were included in the same group.
(TIF)

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Author Contributions

Conceived and designed the experiments: MV AM DV MG BS RM. Performed the experiments: CM AH EF SD FT GL. Analyzed the data: MV AM. Contributed reagents/materials/analysis tools: MV AM. Wrote the paper: MV AM DV.

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DISCUSIÓN

DISCUSSION

DISCUSIÓN GENERAL

En los últimos años el avance de las técnicas de secuenciación masiva de ADN ha permitido describir de forma muy precisa las comunidades microbianas que habitan en diferentes partes del cuerpo humano, incluido el tracto gastrointestinal (GTI). Los diferentes estudios de caracterización de la microbiota intestinal en distintos grupos de población, tanto sana como con algún tipo de patología, han permitido establecer la composición microbiana intestinal característica en un amplio abanico de situaciones fisiológicas (152). Estos estudios han sido cruciales como punto de partida para comprender la relación entre la microbiota y distintas enfermedades, principalmente de origen (auto)inmune e inflamatorias. El principal objetivo de esta tesis doctoral ha sido caracterizar la disbiosis microbiana intestinal en el contexto de ciertos trastornos del sistema inmune. Para ello hemos caracterizado la comunidad bacteriana intestinal de grupos poblacionales definidos, pacientes de Lupus Eritematoso Sistémico (LES) y alérgicos, con respecto a grupos control debidamente seleccionados en cuanto a parámetros demográficos.

Estudio de la composición de la microbiota intestinal.

En primer lugar, se pusieron a punto metodologías para el estudio de la microbiota intestinal humana. Planteamos distintos experimentos para establecer de la forma más representativa posible los perfiles microbianos de muestras fecales humanas. Para ello se

optimizó un protocolo de aislamiento de ADN de una microbiota fecal compuesta por 10 microorganismos representativos de la microbiota humana.,. Se probaron distintos métodos de extracción y se utilizaron también distintos oligonucleótidos para la amplificación del gen ribosomal 16S. De los resultados obtenidos en esta tesis destaca que los niveles de la arqueobacteria *Methanobrevibacter smithii* aparecieron severamente subestimados utilizando cualquiera de los métodos de extracción. Esto sugiere una lisis incompleta de este grupo microbiano, probablemente debido a que la mayoría de las arqueobacterias poseen una capa paracristalina bidimensional de 5-25 nm de la glicoproteína S-layer que rodea a la célula, y una pared celular compuesta de los polímeros pseudomureina o metanocondroitina, y no peptidoglicano como las bacterias. Estas estructuras la hacen casi insensible a la mayoría de tratamientos antimicrobianos, incluida la lisis celular con enzimas (153, 154) que debería realizarse con otras específicas como las endoisopeptidasas PeiW y PeiP que rompen la pseudomureína de los metanógenos (155).

Para el resto de especies Gram positivas y negativas se obtuvieron resultados variables; por ejemplo los niveles de las especies *Faecalibacterium prausnitzii*, *Colinsella intestinalis* y *Bacteroides thetaiotaomicron* aparecieron incrementados con respecto a las células añadidas cuando se utilizó el kit comercial de Qiagen, mientras que las bifidobacterias se detectaron en niveles inferiores a los añadidos. En cambio, los métodos que incorporaron un paso previo de lisis enzimática con mutanolisina, especialmente efectiva sobre la pared celular de bifidobacterias, sobrevaloraron excesivamente los niveles de estas últimas (156). Nuestros resultados en este trabajo muestran la enorme influencia que tienen los métodos de lisis bacteriana y la secuencia de los oligonucleótidos utilizados en la amplificación del gen del 16S ribosomal en los resultados de metagenómica filogenética. La elección de unos u otros son puntos críticos que introducen sesgos en los resultados sobre la composición bacteriana obtenida de muestras fecales, como ya ha sido reportado en trabajos anteriores (157).

Paralelamente pusimos a punto un protocolo de separación de microbiota total de muestras de heces por centrifugación diferencial en gradiente de densidad. Para ello se optimizó un protocolo de Curtois y colaboradores, en el cual aislaron la comunidad

microbiana del suelo, extrajeron su ADN y demostraron que la separación no afecta significativamente a la diversidad bacteriana, como ocurre en nuestro trabajo (158). Con nuestro protocolo, la extracción de la microbiota de heces no modificaba los niveles relativos de los grupos mayoritarios, aunque sí los de algún grupo específico minoritario (159). Esta metodología sería aplicada posteriormente para realizar los experimentos *in vitro* de co-cultivo de microbiota de LES con células inmunes (160).

Disbiosis de la microbiota intestinal asociada a enfermedades de origen inmune

Las enfermedades inmunológicas son trastornos causados por mecanismos del sistema inmunitario que bien son anormales, deficitarios o están ausentes. Dentro de ellas, las enfermedades autoinmunes son enfermedades en las que el sistema inmune ataca a células o estructuras moleculares del propio organismo. Se clasifican en sistémicas o multiorgánicas (no específicas de órganos), como el caso del LES, artritis reumatoide (AR), celiaquía, psoriasis y esclerosis múltiple, y en síndromes locales (órgano específicas), como la diabetes mellitus tipo 1 (DT1), tiroiditis o anemia hemolítica autoinmune. Algunos autores también incluyen a la EII como una patología autoinmune inflamatoria crónica específica del tracto gastrointestinal (TGI). Por otro lado, las reacciones alérgicas típicas son reacciones de hipersensibilidad tipo 1 provocadas por la re-exposición a un tipo específico de antígeno denominado alérgeno. La diferencia entre una respuesta inmunitaria normal y una hipersensibilidad de tipo 1 radica principalmente en la secreción descontrolada de IgE por parte de las células plasmáticas (147).

En nuestro trabajo sobre LES la disbiosis bacteriana intestinal no se puso de manifiesto mediante la biodiversidad bacteriana entre lúpicos y controles, ya que no observamos diferencias significativas en los índices alfa (Chao1, PD whole tree, especies observadas, Shannon y Simpson). En cambio, sí que se detectaron cambios en las proporciones relativas de los dos filos bacterianos más abundantes en la microbiota intestinal de pacientes y controles sanos: Firmicutes y Bacteroidetes. Nuestro estudio identificó una mayor abundancia relativa de Bacteroidetes en pacientes de LES, así como un ratio Firmicutes/Bacteroidetes (F/B) 2,5 veces menor en LES (ratio mediana 1,97) con respecto a los controles sanos (ratio

mediana 4,86) calculado a partir de las abundancias relativas de las secuencias correspondientes a estos dos filos.

Si comparamos nuestros principales resultados de la disbiosis en LES con lo observado por otros autores en otras enfermedades de origen autoinmune como la DT1 o la AR, se aprecian resultados similares. En la DT1 se observó un incremento en las abundancias relativas de Bacteroidetes y disminución en Firmicutes, y además un descenso en la diversidad microbiana generalizada. Más concretamente la especie *Bacteroides ovatus* es la especie que más aumentó dentro del filo Bacteroidetes, y al igual que en LES el orden *Clostridiales* fue el que más disminuyó su abundancia relativa dentro del filo Firmicutes (161). En la AR la microbiota intestinal presenta menor diversidad en comparación con individuos sanos, principalmente debido a una reducción de los niveles relativos de los géneros *Coprococcus, Akkermansia, Ruminococcus y Pseudobutyrivibrio* (Scher et al. 2014). La AR guarda bastantes similitudes con el LES ya que se caracteriza por una inflamación persistente de las articulaciones debida a la presencia de autoanticuerpos contra moléculas sinoviales, producidos por células B autoreactivas, lo cual genera deformidad, incapacidad funcional, y destrucción progresiva de las articulaciones (162). Los brotes de LES y AR se asocian frecuentemente a la aparición de algunas infecciones, sobre todo víricas, como con el virus de la hepatitis B o el de Epstein Barr (163). También existen casos descritos en AR de infecciones bacterianas asociadas, como con *Yersinia, Shigella, Salmonella* y *Campylobacter*, (164, 165). Todavía está en controversia si estas infecciones son las causantes de brotes, empeoramiento de la progresión, o por el contrario tienen un efector protector contra el LES (140).

Si comparamos nuestros resultados con otros trabajos en los que se ha estudiado la relación entre la composición de la microbiota intestinal y su relación con patologías, vemos que estos últimos reportan diversas alteraciones de la microbiota intestinal, bien como una disminución de la diversidad total de especies medida con distintos índices, o bien como ratios F/B alteradas. Por ejemplo en la enfermedad inflamatoria intestinal (EII), tanto Enfermedad de Crohn como Colitis Ulcerosa, se ha descrito una disminución del ratio F/B, (al

igual que en nuestro estudio de LES). La disbiosis de la EII también se caracteriza por un descenso en la diversidad microbiana y por un incremento en la abundancia relativa de ciertas especies de Proteobacterias (166, 167). Muchas especies pertenecientes al filo *Firmicutes* aparecen disminuidos en EII, y aunque en nuestros resultados de LES no pudimos describir la microbiota a nivel de especie ni género debido a las limitaciones de nuestra metodología, ya que el tamaño del amplicón obtenido en la amplificación de la región variable V3 o V4 del gen ribosomal 16S era de unas 180 pares de bases, sí que asociamos este descenso a las familias *Lachnospiraceae* y *Ruminococcaceae* grupos taxonómicos a los que pertenecen especies de interés como *Roseburia intestinalis* o *Blautia coccoides* de *Lachnospiraceae,* y *Faecalibacterium prausnitzii* de *Ruminococcaceae* (168). Es destacable que numerosos miembros de estas dos familias están poco representados en enfermedades que cursan con procesos inflamatorios intestinales, como la EII (Morgan et al. 2012. Genome Biol 13:R79).Al igual que en LES, la ratio F/B también fue menor en un estudio con una cohorte de enfermos de diabetes tipo 2 con respecto a controles sanos (169). En el caso de la obesidad parece claro que existe una disbiosis en la microbiota intestinal, aunque se detectan ratios F/B mayores que en individuos normopeso, todo lo contrario que en LES (170). Esto mismo se observa en el síndrome metabólico: una reducción de los niveles relativos de representantes del filo *Bacteroidetes* y un incremento en los Firmicutes (171).

Posible implicación de la disbiosis de la microbiota intestinal en LES y alergia

La disbiosis microbiana intestinal en individuos con LES se caracterizó principalmente por una disminución de la ratio F/B. El origen específico del LES no se ha identificado, sí bien se han descrito las características inmunológicas clave de esta enfermedad. De manera general la respuesta inmune aberrante del LES se basa en una hiperreactividad de linfocitos B productoras de auto-anticuerpos que reconocen hasta 100 epítopos celulares propios. Por otro lado, los anticuerpos IgG doblan en concentración a los de la población normal en plasma.

Estudios en diversos modelos animales de lupus han arrojado pistas sobre la etiología del LES. Existen modelos de lupus espontáneo, en los que los animales desarrollan la enfermedad

con mayor o menor rapidez y severidad, que han permitido identificar numerosos loci de susceptibilidad genética a la enfermedad. Los más característicos son la F1 del cruce de ratones NZB/W (mutaciones en loci del MHC), y cepas derivadas de éste como la cepa MRL/lpr (mutación en el gen del receptor FAS). Otro modelo es la cepa BXSB/Yaa (duplicación del gen que codifica para el TLR7) (172, 173). También existen modelos animales de LES inducido por agentes químicos, como el pristano (2,6,10,14-tetrametilpentadecano). Gracias a este modelo se comprobó que los microorganismos participan en la patogénesis del LES, ya que si estos ratones se mantienen en condiciones de esterilidad los títulos de autoanticuerpos son más bajos que con respecto a ratones control (174).

En el modelo murino de lupus BXSB/Yaa, hemos indicado que existe una duplicación de un fragmento del cromosoma X que incluye el gen del receptor del sistema inmune innato TLR7. Esta copia extra es suficiente para amplificar la respuesta de células B al ligando del TLR7, el ARN de cadena sencilla de virus intracelulares, lo cual hace que estos ratones presenten títulos de auto-anticuerpos anti-RNA incrementados (138, 175). Aparte del TLR7, otros receptores TLR que reconocen ligandos microbianos específicos de la microbiota han probado tener efecto directo en el LES. Por ejemplo la expresión del gen que codifica para el receptor TLR9 está incrementada en LES con respecto a individuos sanos, correlacionando estos niveles con los de citoquinas típicamente pro-inflamatorias como IL-6, IFN-γ, y TNF-α (176). Por otro lado tanto mutaciones en los genes que codifican para los receptores TLR2 y TLR4 se relacionan con susceptibilidad al LES y a otras enfermedades autoinmunes a través de modelos animales, como el modelo lpr/lpr (136, 177).

En base a estas evidencias, un posible mecanismo que relacionaría la microbiota con el LES es el mimetismo molecular entre epítopos de la microbiota y epítopos propios reconocidos por células T autoreactivas (178, 179). En la literatura científica hay múltiples ejemplos de proteínas y fragmentos de ADN bacteriano que son fuente de mimetismo molecular, tanto en LES como en enfermedades relacionadas como la Glomerulonefritis (180–182).

Nuestro estudio sobre la disbiosis existente en la microbiota intestinal de individuos con LES, refuerza la hipótesis de que la microbiota intestinal tiene un papel hasta ahora no estudiado con atención. Sin embargo recientemente se ha publicado un estudio en el modelo murino MRL/lpr que contradice en parte nuestros resultados en cuanto a la disbiosis de la microbiota intestinal en LES. En este trabajo los ratones con LES mostraron mayores niveles de familias como *Lachnospiraceae*, *Ruminococcaceae* y *Rikenellaceae,* y una reducción de la familia *Lactobacillaceae* (183), es decir lo contrario de nuestros resultados en humanos, en los que los individuos con LES tienen mayores niveles de Bacteroidetes y menores de Firmicutes (concentradas precisamente en las familias *Lachnospiraceae* y *Ruminococcaceae*). Observaron también diferencias entre sexos, en concreto mayores niveles de *Lachnospiraceae* y *Bacteroidetes* en hembras con lupus, menores de *Bifidobacterium* y *Erysipelotrichaceae* en machos*,* y similares de *Lactobacillaceae.* Además, la diversidad microbiana global era mayor en ratones con LES, comparados con los controles sanos.

Estas diferencias pueden ser debidas a varios factores: la anatomía, fisiología y genética de ratones no es comparable a la humana, y en cuanto a su microbiota intestinal, aunque los filos mayoritarios son como en humanos *Firmicutes* y *Bacteroidetes*, la abundancia relativa de la mayoría de géneros dominantes es bastante diferente a la humana; por ejemplo *Prevotella* está disminuida, *Faecalibacterium* raramente se detecta, y *Lactobacillus*, *Alistipes* y *Turicibacter* son los más abundantes. *Clostridium*, *Bacteroides* y *Blautia* por el contrario, tienen una abundancia relativa similar en ambos organismos (184, 185). Además, el modelo animal que usan estos autores se caracteriza por una mutación en el gen FAS, que codifica un receptor de la familia de los receptores del factor de necrosis tumoral (TNF), y en humanos, la señalización defectuosa por parte de Fas puede llevar a desarrollar un síndrome autoinmune que presenta síntomas similares con el LES, aunque también diferencias, como que no aparece glomerulonefritis en los pacientes (172). Todos estos resultados ponen de manifiesto que la translación de resultados en modelos murinos a humanos para el LES a día de hoy todavía es limitada. De manera similar ocurre en los resultados de microbiota y obesidad. Existen discrepancias entre modelos animales y humanos: por un lado hay autores que

reportan un ratio F/B mayor en humanos con obesidad (171, 186), y otros que observan lo contrario, un ratio reducido ((187)) o sin cambios en las proporciones (12), y por otro lado hay modelos animales murinos que apoyan ambas observaciones: el modelo murino de obesidad *ob/ob,* cuyos ratones no poseen el gen que codifica la hormona leptina reguladora del apetito, desarrollan obesidad y presentan una microbiota con un ratio F/B mayor que controles sanos (188, 189); sin embargo Serino y colaboradores hallaron un ratio menor en ratones obesos ((190)) En estos trabajo se argumentan posibles explicaciones a estos resultados dispares: factores como la heterogeneidad en la edad de los sujetos en humanos, o las diferencias en procesamiento y técnicas de análisis de las muestras.

En contraste con los datos de LES obtenidos durante esta tesis doctoral, no se ha detectado una disbiosis clara en el caso de los individuos con alergia. Sin embargo, sí hemos encontrado una relación entre los niveles de *Bifidobacterium adolescentis* y alergia. En un trabajo previo se ha descrito que los niños de madres alérgicas presentaron menores niveles de bifidobacterias en heces que los hijos de madres no alérgicas (191). En cuanto a los niveles de bifidobacterias hallados en individuos con alergia, hay estudios en niños que muestran bajos niveles de bifidobacterias en heces de los niños alérgicos comparados con no alérgicos (192).

Existen múltiples trabajos que relacionan la microbiota intestinal, la función inmune y el desarrollo de la alergia (193, 194). En un estudio reciente del Proyecto *Gut* norteamericano, con 1879 participantes adultos, vieron la relación de la microbiota fecal con varios tipos de alergias, entre ellas asma (193). Concluyeron que los individuos alérgicos mostraron una menor diversidad de especies, una reducción de miembros del orden *Clostridiales*, y un incremento en el orden *Bacteroidales.* Sin embargo, para el caso concreto del asma, la diversidad (número de especies observadas) de la microbiota fecal no correlacionó con el asma, ni tampoco la composición de poblaciones bacterianas, lo cual está de acuerdo con nuestros resultados.

Algunos autores defienden que alteraciones en la función inmune del tracto respiratorio

pueden estar ligados a la actividad inmunomoduladora de la microbiota intestinal a través del concepto de "respuesta de mucosa única" (195, 196). Esta hipótesis propone que la presentación antigénica en un sitio de la mucosa local, estimula la migración de células linfoides a otros sitios de mucosa alejados, condicionando la respuesta inmune en otras mucosas.

Cambios en la funcionalidad de la microbiota

Cada vez es más evidente, gracias a las técnicas ómicas, que la microbiota intestinal está involucrada en la regulación de muchos procesos metabólicos del hospedador a través de compuestos que producen, secretan al lumen intestinal e interaccionan en el epitelio intestinal. La correlación de metabolitos hallados asociados a perfiles microbianos específicos está siendo posible gracias a técnicas moleculares de análisis y cuantificación directas, e incluso en algún caso están sirviendo para la búsqueda de biomarcadores químicos de enfermedades (80, 90).

Algunos de los metabolitos que encontramos en niveles diferentes entre LES y controles sanos soportan la idea del estrés oxidativo en el ambiente intestinal de estos pacientes. En la microbiota de LES se detectaron niveles más bajos de homoserina lactona, ácido N-acetilmurámico, y N-acetilglucosamina. Estos dos últimos son componentes esenciales del peptidoglicano de la pared celular bacteriana, el cual se sabe tiene propiedades inflamatorias (197). Este resultado contrasta con los menores niveles de Firmicutes hallados en LES, ya que precisamente son microorganismos Gram-positivos, y por tanto con una pared celular más gruesa que los Gram negativos. Por tanto se esperaría a priori una mayor acumulación de estos metabolitos en controles sanos. Por el contrario, los niveles de mesoporfirina IX y de protoporfirina IX, ambas relacionadas con la síntesis del grupo hemo, fueron mayores en la microbiota de LES. Podría sugerirse que en LES la microbiota posee una menor capacidad de incorporar hierro, hecho que correlaciona con los menores niveles séricos de ferritina hallados en LES. De forma interesante, la protoporfirina IX es una molécula relacionada directamente con el estrés oxidativo en bacterias, ya que existe una regulación estricta de la asimilación de

hierro para prevenir un exceso de éste intracelularmente; un ejemplo es *Bifidobacterium animalis* subsp. *lactis,* en la cual se detectaron cambios severos en la expresión de genes que codifican enzimas involucradas en reacciones redox que liberan protoporfirina IX cuando se la sometía a estrés oxidativo (198, 199). Por tanto basándonos en estos datos, podría pensarse que existen compuestos derivados de la microbiota que son diferentes en el marco del LES, y que podrían utilizarse como marcadores de enfermedad, o como dianas sobre las que actuar para revertir el ambiente redox intestinal. Estos resultados también parecen indicar que en la microbiota intestinal de LES existen bacterias mejor adaptadas a un ecosistema intestinal de mayor estrés oxidativo, lo cual podría favorecer el establecimiento de la disbiosis (200).

Estrategias para modificar la respuesta inmune a través de la modulación de la microbiota

Numerosos estudios *in vitro* han generado datos prometedores sobre el uso potencial de microorganismos para corregir distintas situaciones de disbiosis intestinal, como el uso de probióticos. Una definición de probiótico es la de "microorganismos vivos que, cuando se administran en cantidades adecuadas, confieren un beneficio de salud para el hospedador" (117). La mayoría de microorganismos probióticos utilizados actualmente son bacterias del ácido láctico y bifidobacterias, pertenecientes principalmente a los géneros *Lactobacillus* y *Bifidobacterium*.

El efecto inmunomodulador de los probióticos está asociado, de manera general, con un descenso en la producción de citoquinas pro-inflamatorias y un aumento de las anti-inflamatorias, así como de la inducción de respuestas T como la Treg que como hemos visto en nuestros resultados son defectuosas en LES (201, 202). Para alcanzar una situación homeostasis en LES, las respuestas inmunes efectoras y reguladoras deben estar debidamente balanceadas, lo cual podría alcanzarse mediante el uso de probióticos (203).

Los estudios de metagenómica recientes sugieren que ciertas bacterias comensales no estudiadas hasta hace relativamente poco tiempo, podrían representar nuevos candidatos a probióticos. Datos experimentales recientes en modelos animales indican que algunas de estas especies bacterianas han mostrado capacidades para mitigar la inflamación intestinal, inducir

la regulación inmune, o fortalecer la función barrera intestinal. Ejemplos de estos microorganismos son *Akkermansia muciniphila*, *Faecalibacterium prausnitzii* y ciertas especies del género *Roseburia.*

Algunas de estas nuevas especies bacterianas mencionadas ejercen su actividad beneficiosa a través de la producción de ciertos metabolitos. Varios estudios muestran que los AGCCs, y entre ellos, el butirato, se producen de forma mayoritaria por bacterias anaerobias comensales que pertenecen sobre todo al grupo de clostridios, en concreto a los clústers IV y XIVa, destacando el género *Roseburia* y la especie *F. prausnitzii* (204, 205). Sin embargo, en nuestros resultados de cuantificación de metabolitos producidos por parte de la microbiota fecal de LES, no observamos diferencias significativas en AGCCs entre controles sanos y pacientes (206), observación que puede deberse a que bien estos compuestos no se acumulan intracelularmente en las bacterias, o son rápidamente absorbidos por los colonocitos (207).

En EII se han descrito abundancias relativas reducidas de la especie *F. prausnitzii.* Células vivas y metabolitos derivados de esta especie de firmicutes han mostrado efecto anti-inflamatorio consistente en un incremento de la secreción de IL-10 por células dendríticas y una supresión de la secreción de IL-17 en un modelo de CU inducida en ratones (208–210). Esta especie también es capaz de expandir la población de células T reguladoras en células mononucleares aisladas de sangre periférica (211). En general, los AGCCs (acetato, propionato y butirato) generados tras la fermentación de fibra de la dieta por la microbiota intestinal son utilizados como fuente de energía por los colonocitos (212, 213) pero aparte de su función energética también se ha comprobado que contribuyen a la función immune, y por ello podría ser interesante el aumento de las especies productoras tanto en EII como en LES (214–216).

En esta tesis doctoral hemos llevado a cabo estudios *in vitro* con algunos "probióticos emergentes" cuyos niveles estaban alterados en LES con el objetivo de corregir la respuesta inmune (*Ruminococcus obeum* y *Blautia coccoides*). También utilizamos un probiótico clásico que en trabajos anteriores había mostrado potencial inmunoregulador a través de la inducción de la respuesta Treg, *Bifidobacterium bifidum* LMG13195. Para ello enriquecimos

la microbiota fecal aislada de individuos de LES (159), bien con la bifidobacteria o bien con la mezcla de clostridios, y las enfrentamos a células dendríticas derivadas de monocitos de sangre periférica. Posteriormente enfrentamos estas células dendríticas condicionadas a células T vírgenes alogénicas. Comprobamos que ni la suplementación de la microbiota de LES con la bifidobacteria ni con los clostridios hizo aumentar la población de células Treg, pero sí observamos una reducción en la ratio IL-17/IFNγ en la microbiota enriquecida con clostridios, y una menor proliferación de células T efectoras con la bifidobacteria. Todo esto sugiere que estas especies pueden reducir la activación de células T al reducir este ratio de citoquinas proinflamatorias, lo cual podría llevar a pensar en una hipotética estrategia de modulación de la microbiota intestinal en LES. Aunque en esta tesis no se ha planteado este tipo de estudios en humanos, nuestros resultados apoyan el uso de estos microorganismos en la modulación de la respuesta inmune en el LES (160).

¿Es posible una intervención alimentaria para tratar el LES o la alergia?

En un experimento en un modelo de LES murino, se realizó una suplementación con vitamina A/ácido retinoico (217, 218), comprobándose que el tratamiento atenuó los síntomas del lupus, probablemente por un lado promoviendo la inducción de células Treg (219), y por otro restableciendo los niveles de la familia *Lactobacillaceae* que estaban disminuidos en los ratones con LES (183). Actualmente no hay suficiente información sobre el impacto que la alimentación pueda tener en las enfermedades autoinmunes, aunque sí existe algún trabajo sobre cómo afecta la dieta al LES. Por ejemplo en un estudio en el modelo murino de lupus NZB/W F1 se vio que dietas ricas en grasas aceleraban el desarrollo de comorbilidades asociadas al LES, como el síndrome metabólico (220). En otros modelos murinos de LES se ha visto que la ingesta calórica, proteica, y en especial la restricción en grasas, provoca una reducción significativa en la generación de complejos inmunes depositados en el riñón, reduce la proteinuria y prolonga la esperanza de vida en los ratones (221).

Estos estudios con modelos animales sugieren que es posible diseñar una estrategia alimentaria dirigida a retardar la aparición y mejorar la severidad del LES. Nuestras evidencias sugieren que las bacterias productoras de butírico podrían utilizarse junto con una

dieta rica en fibra para aumentar los niveles de Firmicutes en individuos con LES. De esta manera la disbiosis de la microbiota se podría revertir, produciendo teóricamente una inmunomodulación a nivel del GALT, y contribuyendo en último término a reducir la inflamación general presente en esta enfermedad. La alimentación es un factor muy influyente en la composición de la microbiota intestinal; por ejemplo una dieta restrictiva en carbohidratos o lípidos incrementa la población de *Bacteroidetes* en individuos con obesidad que presentan un alto ratio F/B de partida (222). Se sabe que existe una asociación entre la ingesta de frutas como naranjas y manzanas con un descenso en microorganismos incrementados en LES (223). Existen evidencias de que compuestos fenólicos derivados del metabolismo de estas frutas promueven el crecimiento de bifibobacterias, las cuales pueden tener un efecto beneficioso en LES debido a la propiedad inmunomoduladora de algunas de sus cepas, como *B. bifidum* LMG13195 (224). Con estos datos, podemos postular que la disbiosis en LES podría corregirse a través de una dieta baja en proteínas animales y grasas saturadas y suplementada en fibra, de la que se sabe que favorece el crecimiento de los miembros del filo *Firmicutes* (38).

En el caso de la alergia y el asma sí que existen en la literatura estudios y meta-análisis que describen la influencia de la alimentación, bien a través de la microbiota o directamente en el hospedador (225–227). Parece ser que la alimentación de la madre previa al nacimiento puede influir en el desarrollo de asma y alergia en sus hijos, tal como se mostró en el estudio transversal de Bunyavanich y colaboradores, en el que asociaron una alta ingesta de alimentos que contienen alérgenos típicos como cacahuetes, leche y trigo con menor posibilidad de padecer alergia y asma en sus hijos (228). Un meta-análisis realizado en 2011 sobre la relación de la alergia con alimentos y nutrientes concretos, concluyó que a pesar de que la evidencia epidemiológica disponible es escasa y de que no hay ensayos clínicos suficientes, existen datos para asociar las vitaminas A, D y E, oligoelementos como el zinc, alimentos como frutas y vegetales, y una dieta mediterránea como factores que previenen el asma (226).

En cuanto al uso de probióticos para tratar la alergia un meta-análisis realizado en

2013 concluyó efectividad de los probióticos administrados a edades tempranas o bien a las madres en etapa prenatal, asociado a una reducción del riesgo de sensibilización atópica y de los niveles totales de IgE en niños (229). En cambio el mismo meta-análisis concluye que los probióticos no mejoran los síntomas del asma una vez aparecido (229).

En conclusión, este trabajo demuestra la existencia de una disbiosis intestinal en LES. La respuesta inmune en esta situación podría ser corregidamediante suplementación con probióticos clásicos o emergentes. Por tanto, estas poblaciones bacterianas pueden reprsentar los objetivos de futuras terapias nutricionales orientadas a revertir esta disbiosis, y favorecer las respuestas inmunes adecuadas a nivel de mucosa intestinal.

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ANEXOS

ANNEXED

Tabla 1

Condición fisiológica / enfermedad	Disbiosis asociada	Referencia
Enfermedad Inflamatoria Intestinal	Descenso en Firmicutes con un incremento concomitante en Bacteroidetes y Enterobacterias. Descenso global en la diversidad microbiana.	The gut microbiota in IBD. Nature Reviews Gastroenterology & Hepatology (2012) 9:599-608.
Síndrome del colon irritable	Variabilidad en los resultados. Incremento de proteobacterias y de ciertos Firmicutes. Descenso de bifidobacterias, bacteroidetes y de otros grupos específicos de Firmicutes. Evidencias de sobrecrecimiento bacteriano intestinal.	Intestinal microbiota in functional bowel disorders: a Rome foundation report. Gut 2013;62:159-176
Lupus sistémico eritematoso	Incremento de Bacteroidetes, descenso de Firmicutes principalmente de las familias *Lachnospiraceae* y *Ruminococcaceae*	Intestinal dysbiosis associated with Systemic Lupus Erythematosus. mBIO. (2014) 5 no. 5 e01548-14.
Cáncer colorectal	Presencia de *Escherichia coli* PKS+ (productora de colibactina)	The microbiome and cancer. Nature Rev. Cancer (2013) 13:800–812.
Diabetes tipo 1	Incremento de *Bacteroides ovatus* y de la especie no definida de Firmicutes CO19. Pérdida global de diversidad.	Toward defining the autoimmune microbiome for type 1 diabetes. ISMEJ, 5(1):82-9. (2011).
Síndrome metabólico	Descenso en los niveles de bacterias productoras de butirato.	Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology 2012; 143: 913–16, e7.
Artritis reumatoide	Evidencia en modelos animales de implicación del microbioma a través de un incremento de las bacterias filamentosas segmentadas.	The microbiome and rheumatoid arthritis. Nature Reviews Rheumatology (2011) 7:569-578.
Artritis psoriática	Descenso en diversidad microbiana. Descenso relativo de especies de los géneros *Coprococcus, Akkermansia, Ruminococcus, y Pseudobutyrivibrio.*	Decreased Bacterial Diversity Characterizes the Altered Gut Microbiota in Patients With Psoriatic Arthritis, Resembling Dysbiosis in Inflammatory Bowel DiseaseArthritis & Rheumatology. 2015 67:128-139
Enfermedad celíaca	Incremento en Bacteroides and *Clostridium leptum* en todos los estadíos de la enfermedad. Incremento en *Escherichia coli*/*Staphylococcus* sp. y descenso en *Bifidobacterium* sp. en pacientes no tratados.	Specific duodenal and faecal bacterial groups associated with paediatric coeliac disease. J Clin Pathol. 2009 62:264-269.
Asma	Descenso de los géneros *Faecalibacterium, Lachnospira, Rothia, Veillonella* y *Peptostreptococcus*. Incremento del género *Oscillospira*.	Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med. 2015 30:307ra152.
Obesidad	Microbiota de obesos con mayor abundancia relativa de Firmicutes.	An obesity-associated gut microbiome with increased capacity for energy harvest. Nature (2006) 444:1027-1031.
Vejez	Reducción de *Ruminococcus* sp. y *Blautia* sp. Incrementos de *Escherichia/Shigella*.	Gut microbiota composition correlates with diet and health in the elderly. Nature (2012) 488:178-184.
Niños prematuros	Reducción de la familia *Bacteroidaceae* e increment de *Lactobacillaceae*. La administración perinatal de antibióticos se asocia a un incremento en la familia *Enterobacteriaceae*.	Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics. J Pediatr 2015;166:538-44

INFORME SOBRE LA CALIDAD DE LOS ARTÍCULOS

La información sobre las revistas en las que se han publicado los artículos se ha recogido de la Web of Knowledge (https://www.recursoscientificos.fecyt.es/). Se listan a continuación los artículos con: el factor de impacto de cada revista **(FI)**, el cual corresponde al año de publicación del artículo o en el caso de los artículos más recientes, a los últimos datos publicados por Journal Citation Reports (año 2014); el **área SCI** a la que está asociada la revista; el cuartil **(Q)** en el que se encuentra la revista.

1. Hevia A, Delgado S, Sánchez B, Margolles A. Molecular Players Involved in the Interaction Between Beneficial Bacteria and the Immune System. **Frontiers in Microbiology**, 2015; 6:1285.

Area SCI	Q	FI
Microbiology	1	3.989

2. Hevia A, Delgado S, Margolles A, Sánchez B. Application of density gradient for the isolation of the fecal microbial stool component and the potential use thereof. **Scientific Reports**, 2015; 5:16807.

Area SCI	Q	FI
Multidisciplinary Sciences	1	5.578

3. Hevia A, Milani C, López P, Cuervo A, Arboleya S, Duranti S, Turroni F, González S, Suárez A, Gueimonde M, Ventura M, Sánchez B, Margolles A. Intestinal Dysbiosis Associated with Systemic Lupus Erythematosus. **MBio**, 2014; 5:e01548-14.

Area SCI	Q	FI
Microbiology	1	6.786

4. Hevia A, Milani C, López P, Donado CD, Cuervo A, González S, Suárez A, Turroni F, Gueimonde M, Ventura M, Sánchez B, Margolles A. Allergic Patients with Long-Term Asthma Display Low Levels of Bifidobacterium adolescentis. **PLoS One**, 2016; 11:e0147809.

Area SCI	Q	FI
Multidisciplinary Sciences	1	3.234

5. Rojo D, Hevia A, Bargiela R, López P, Cuervo A, González S, Suárez A, Sánchez B, Martínez-Martínez M, Milani C, Ventura M, Barbas C, Moya A, Suárez A, Margolles A, Ferrer M. Ranking the impact of human health disorders on gut metabolism: Systemic lupus erythematosus and obesity as study cases. **Scientific Reports**, 2015; 5:8310.

Area SCI	Q	FI
Multidisciplinary Sciences	1	5.578

6. Lopez P, de Paz B, Rodríguez-Carrio J, Hevia A, Sánchez B, Margolles A, Suarez A. Th17 responses and natural IgM antibodies are related to gut microbiota composition in systemic lupus erythematosus patients. **Scientific Reports**, 2016; 5;6:24072.

Area SCI	Q	FI
Multidisciplinary Sciences	1	5.578

7. Milani C, Hevia A, Foroni E, Duranti S, Turroni F, Lugli GA, Sanchez B, Martín R, Gueimonde M, van Sinderen D, Margolles A, Ventura M. Assessing the Fecal Microbiota: An Optimized Ion Torrent 16S rRNA Gene-Based Analysis Protocol. **PLoS One**, 2013; 8:e68739.

Area SCI	Q	FI
Multidisciplinary Sciences	1	3.534

INFORMACIÓN SUPLEMENTARIA ARTÍCULO:

Hevia A, Delgado S, Margolles A, Sánchez B. Application of density gradient for the isolation of the fecal microbial stool component and the potential use thereof. **Scientific Reports**, 2015; 5:16807.

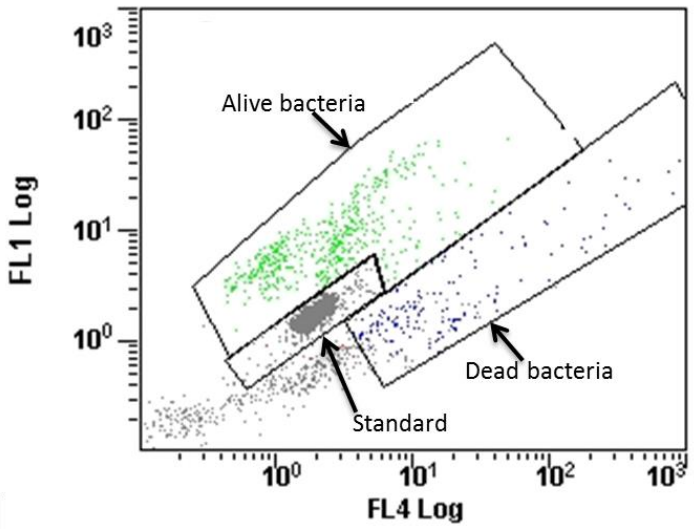
Suppl. Figure 1. Flow cytometry diagrams showing the proportions of alive and dead bacteria in a representative sample before and after Nycodenz extraction. The two diagrams on the bottom represent control samples in which all bacteria were killed.

Suppl. Figure 2. Microbial composition of the different samples used in this study at the Phylum and Family levels, represented as the relative sequence abundances (%). Paired samples (H, homogenized stool sample vs E, microbiota extracted sample) are grouped in Systemic Lupus Erythematosus (SLE) patients and Healthy Controls (HC).

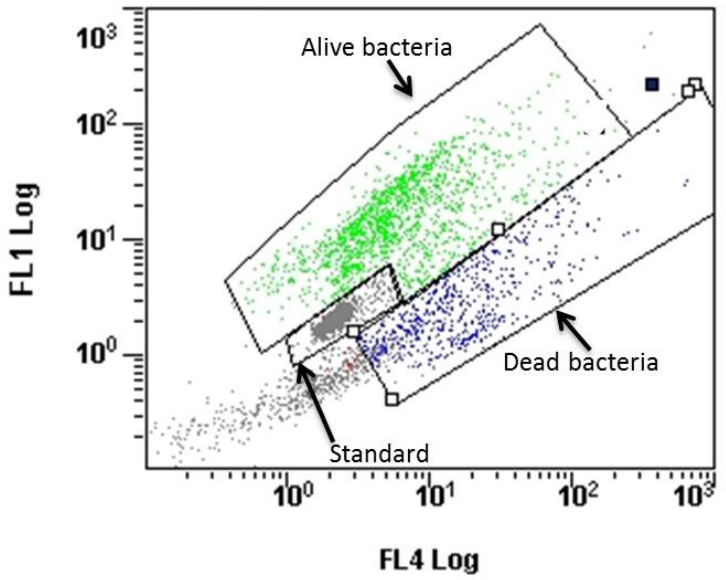
Suppl. Figure 3. Cumulative microbial composition of the samples grouped by experimental condition (DNA extracted directly from feces of after density gradient extraction). Graphics were plotted at the Phylum and Family levels, and data is represented as relative abundances (%). In the case of the Phylum level, an additional graphic excluding the two more abundant taxa (Bacteroidetes and Firmicutes) is shown in order to evaluate changes in minority phyla.

Supplementary Figure 1

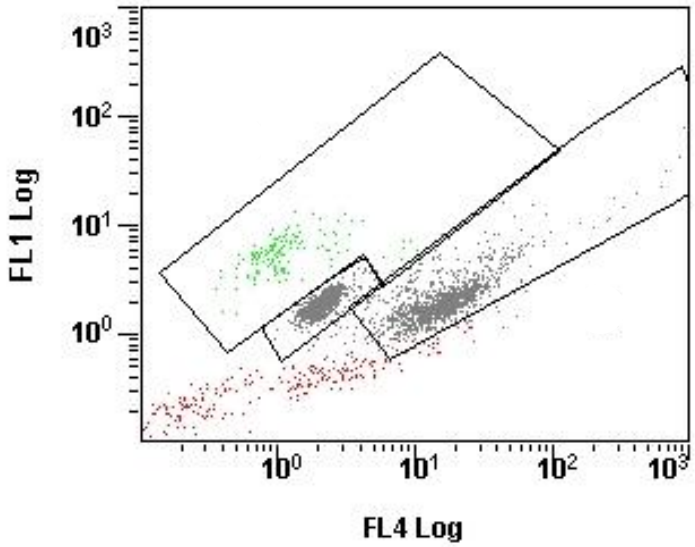
A.1) Fecal microbiota before Nycodenz® separation



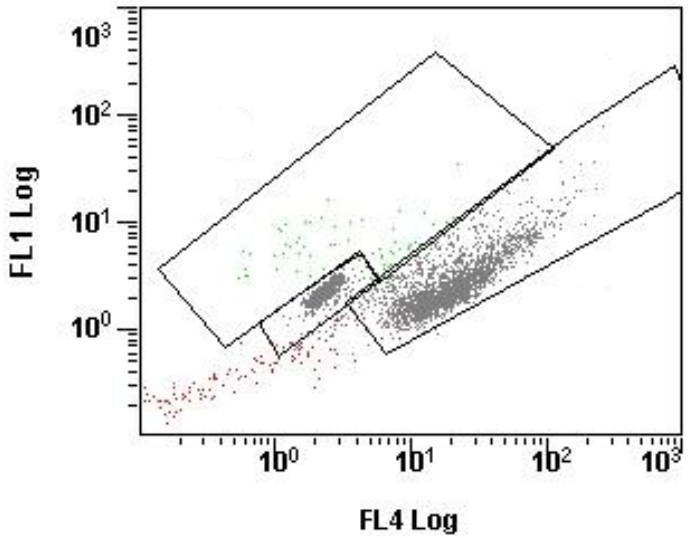
B.1) Fecal microbiota after separation



A.2) Control of dead microbiota before separation.

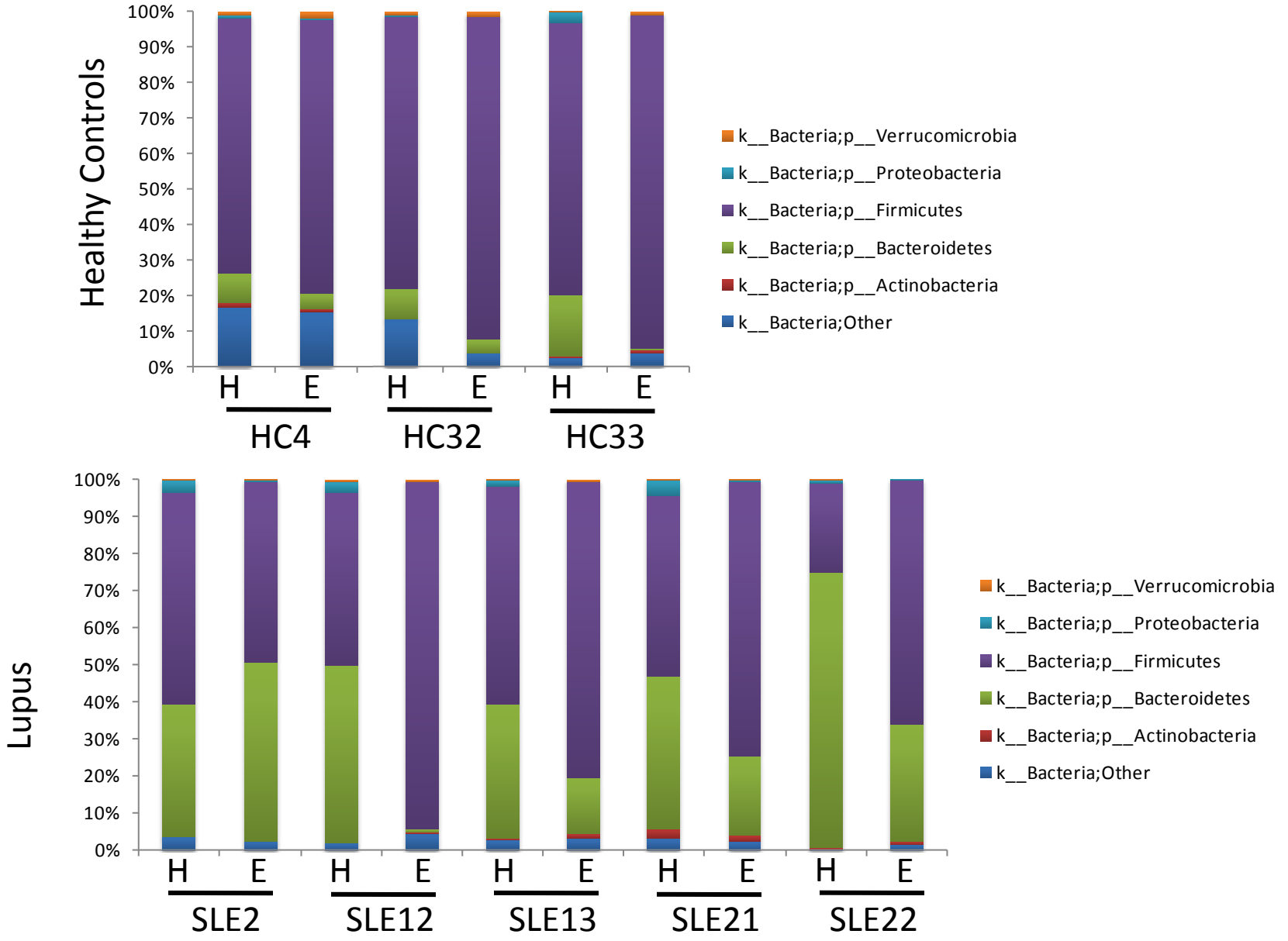


B.2) Control of dead microbiota after separation

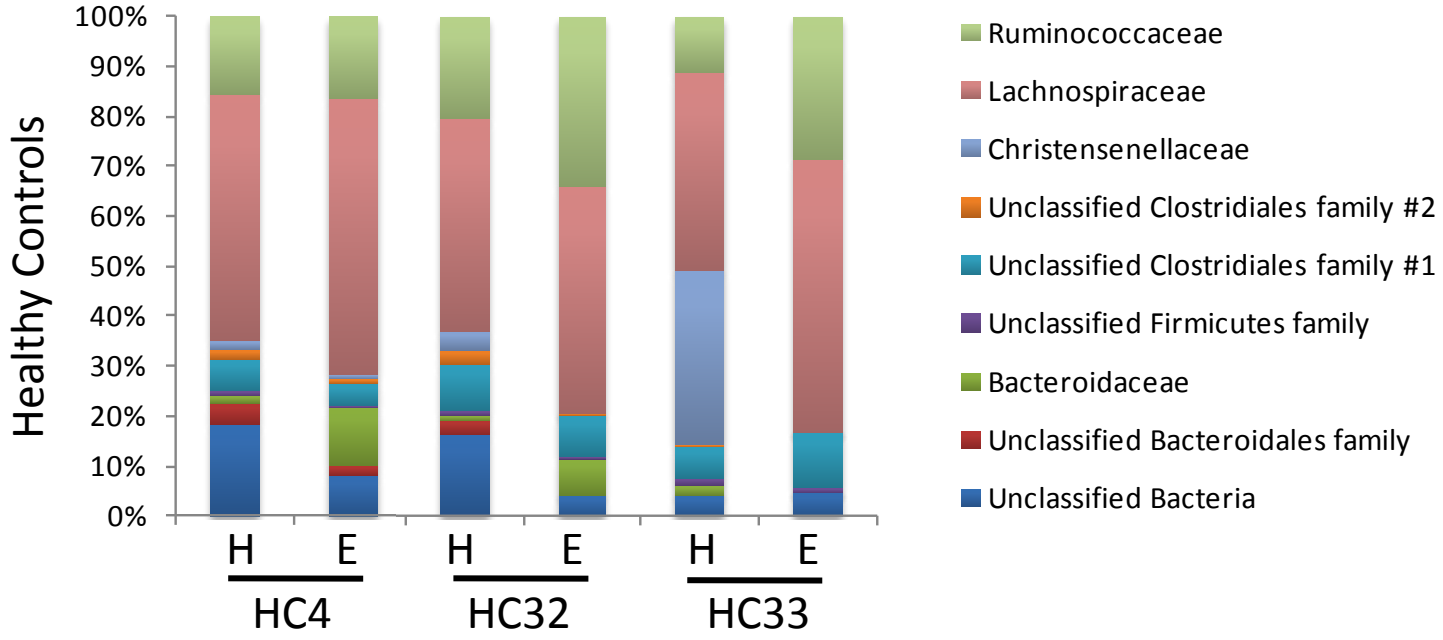


Supplementary Figure 2

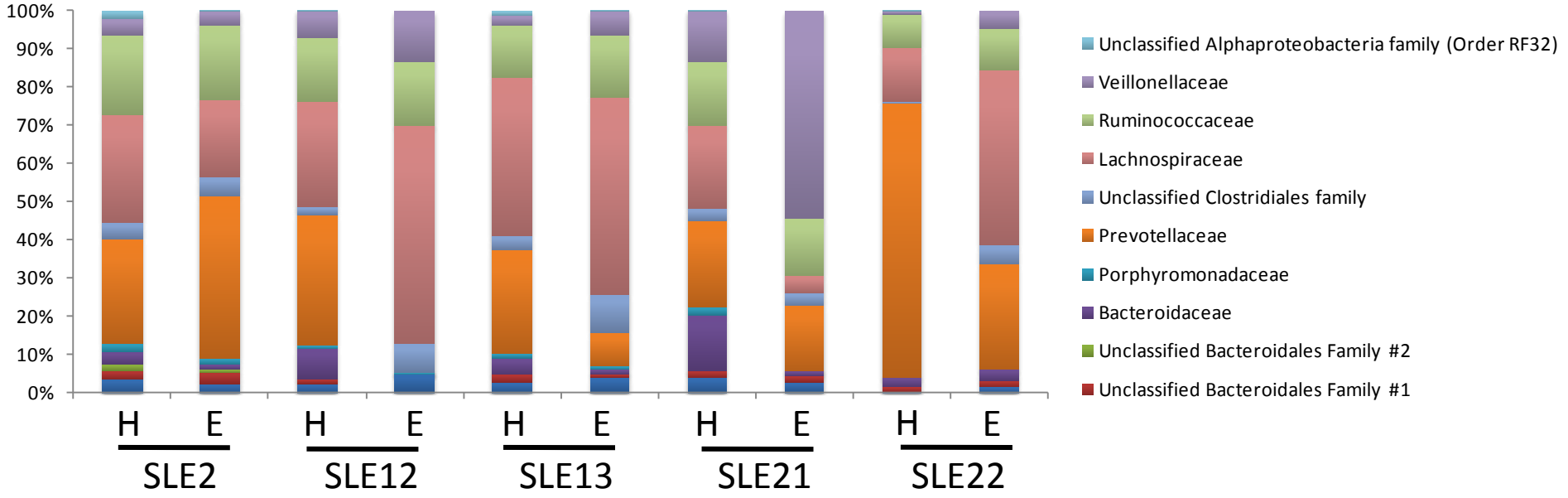
Phylum level



Family level

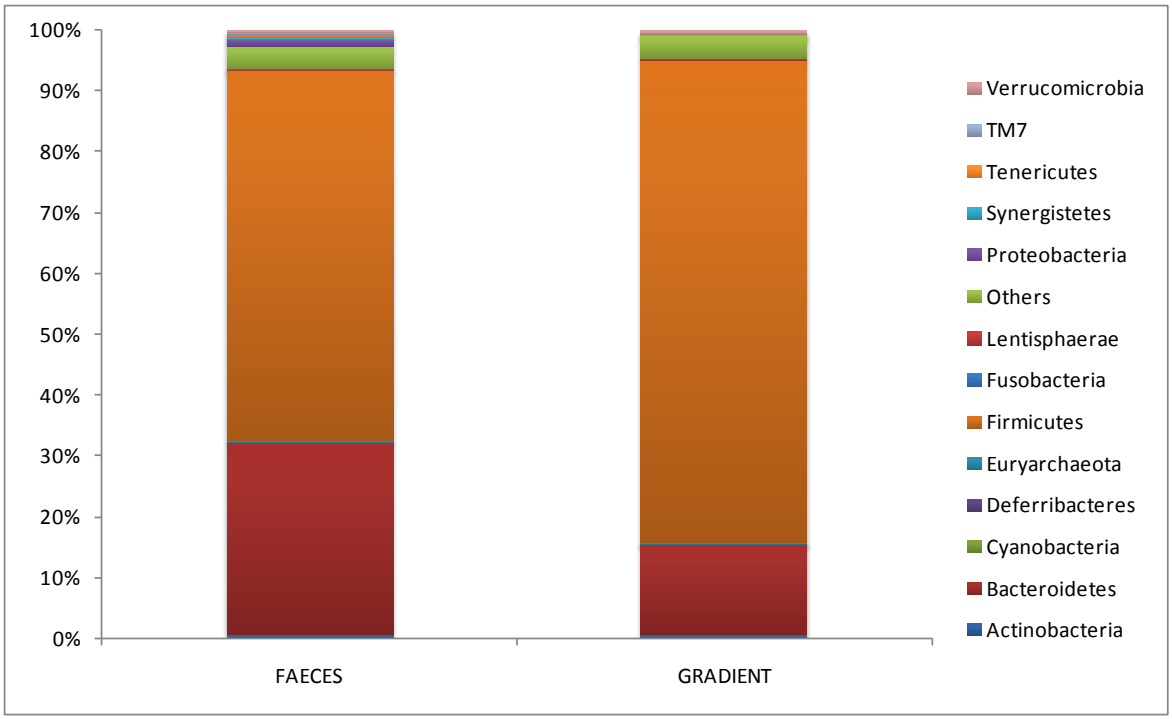


Lupus

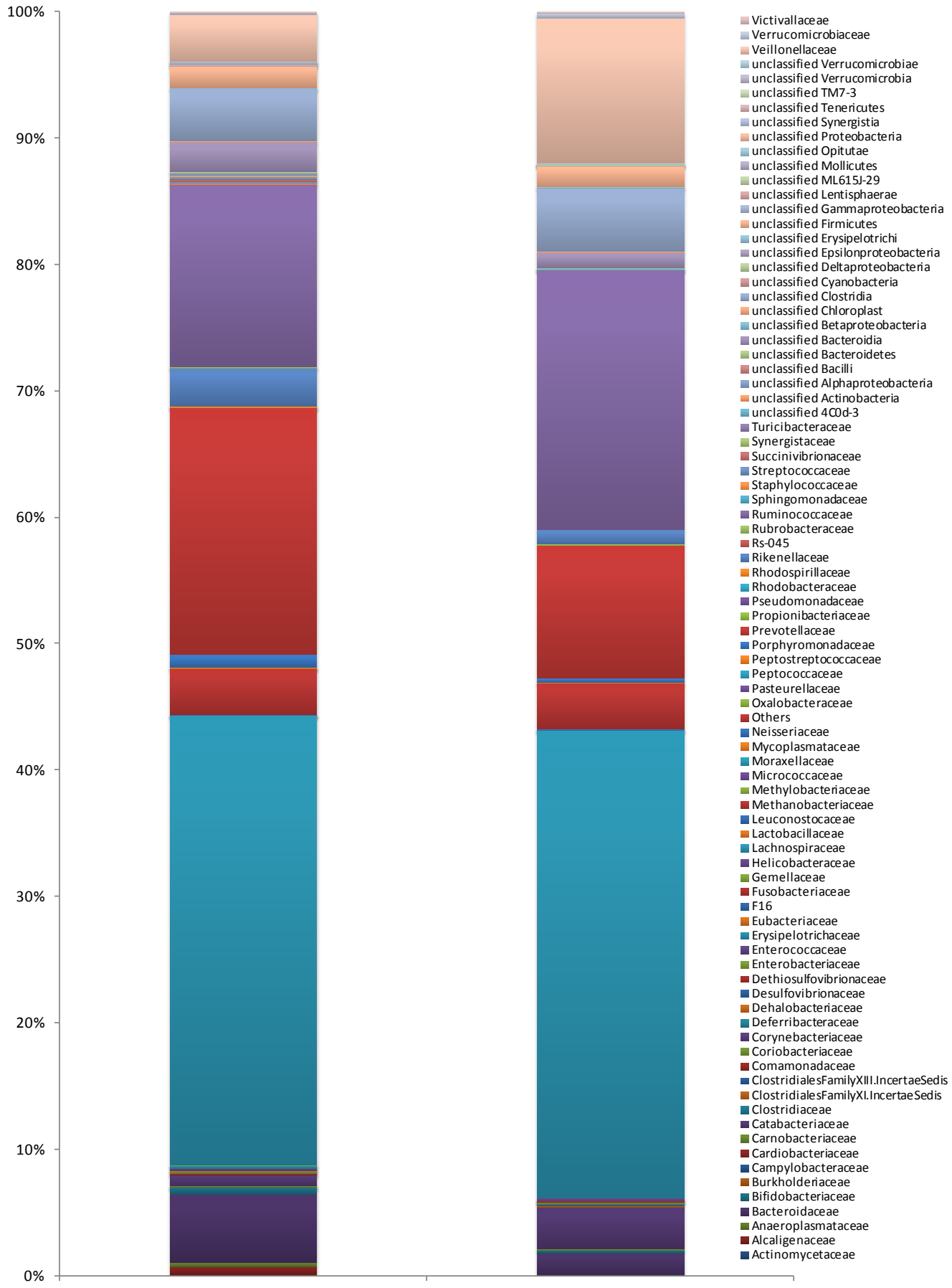


Suppl. Figure 3

Phylum



Family


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
FAECES
GRADIENT
Victivallaceae
Verrucomicrobiaceae
Veillonellaceae
unclassified Verrucomicrobiae
unclassified Verrucomicrobia
unclassified TM7-3
unclassified Tenericutes
unclassified Synergistia
unclassified Proteobacteria
unclassified Opitutae
unclassified Mollicutes
unclassified ML615J-29
unclassified Lentisphaerae
unclassified Gammaproteobacteria
unclassified Firmicutes
unclassified Erysipelotrichi
unclassified Epsilonproteobacteria
unclassified Deltaproteobacteria
unclassified Cyanobacteria
unclassified Clostridia
unclassified Chloroplast
unclassified Betaproteobacteria
unclassified Bacteroidia
unclassified Bacteroidetes
unclassified Bacilli
unclassified Alphaproteobacteria
unclassified Actinobacteria
unclassified 4C0d-3
Turicibacteraceae
Synergistaceae
Succinivibrionaceae
Streptococcaceae
Staphylococcaceae
Sphingomonadaceae
Ruminococcaceae
Rubrobacteraceae
Rs-045
Rikenellaceae
Rhodospirillaceae
Rhodobacteraceae
Pseudomonadaceae
Propionibacteriaceae
Prevotellaceae
Porphyromonadaceae
Peptostreptococcaceae
Peptococcaceae
Pasteurellaceae
Oxalobacteraceae
Others
Neisseriaceae
Mycoplasmataceae
Moraxellaceae
Micrococcaceae
Methylobacteriaceae
Methanobacteriaceae
Leuconostocaceae
Lactobacillaceae
Lachnospiraceae
Helicobacteraceae
Gemellaceae
Fusobacteriaceae
F16
Eubacteriaceae
Erysipelotrichaceae
Enterococcaceae
Enterobacteriaceae
Dethiosulfovibrionaceae
Desulfovibrionaceae
Dehalobacteriaceae
Deferribacteraceae
Corynebacteriaceae
Coriobacteriaceae
Comamonadaceae
ClostridialesFamilyXIII.IncertaeSedis
ClostridialesFamilyXI.IncertaeSedis
Clostridiaceae
Catabacteriaceae
Carnobacteriaceae
Cardiobacteriaceae
Campylobacteraceae
Burkholderiaceae
Bifidobacteriaceae
Bacteroidaceae
Anaeroplasmataceae
Alcaligenaceae
Actinomycetaceae

INFORMACIÓN SUPLEMENTARIA ARTÍCULO:

Hevia A, Milani C, López P, Cuervo A, Arboleya S, Duranti S, Turroni F, González S, Suárez A, Gueimonde M, Ventura M, Sánchez B, Margolles A. Intestinal Dysbiosis Associated with Systemic Lupus Erythematosus. **MBio**, 2014; 5:e01548-14.

Figure S1. Rarefaction curves generated for 16S rRNA gene sequences obtained from faecal samples of control subjects (HC samples, grey lines) and lupus affected subjects (SLE samples, red lines). Panel a represents the rarefaction curves using the Shannon index. Panel b displays rarefaction curves using the Chao1 index. Panel c shows the number of phylotypes identified against the number of sequences per sample.

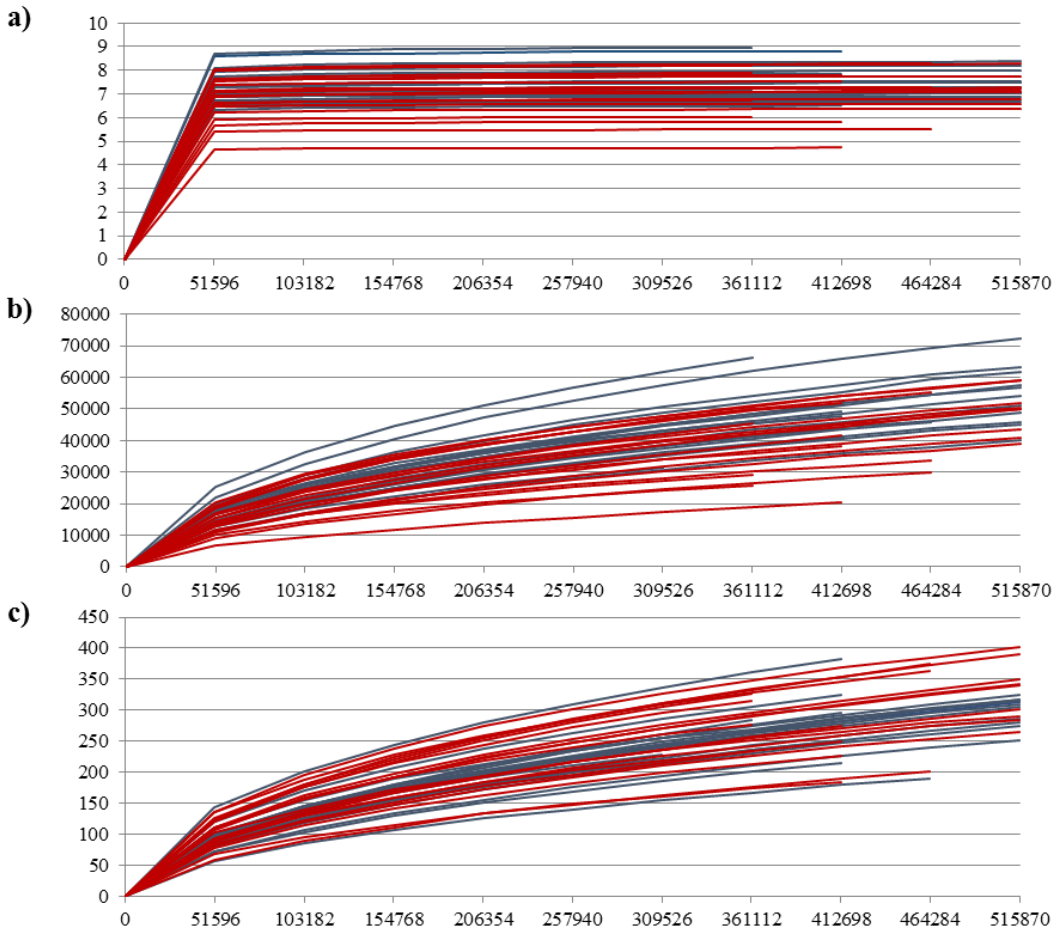


Figure S2. Inter-individual variation in faecal samples of SLE subjects (panel a) and control subjects (panel b) in the proportion of the major microbial phyla/families. The aggregate microbiota composition of control (HC codes) and lupus affected (SLE codes) subjects at the levels of phylum, and family is indicated at the bottom of each inset. Only taxonomic groups above 1% are shown in legends.

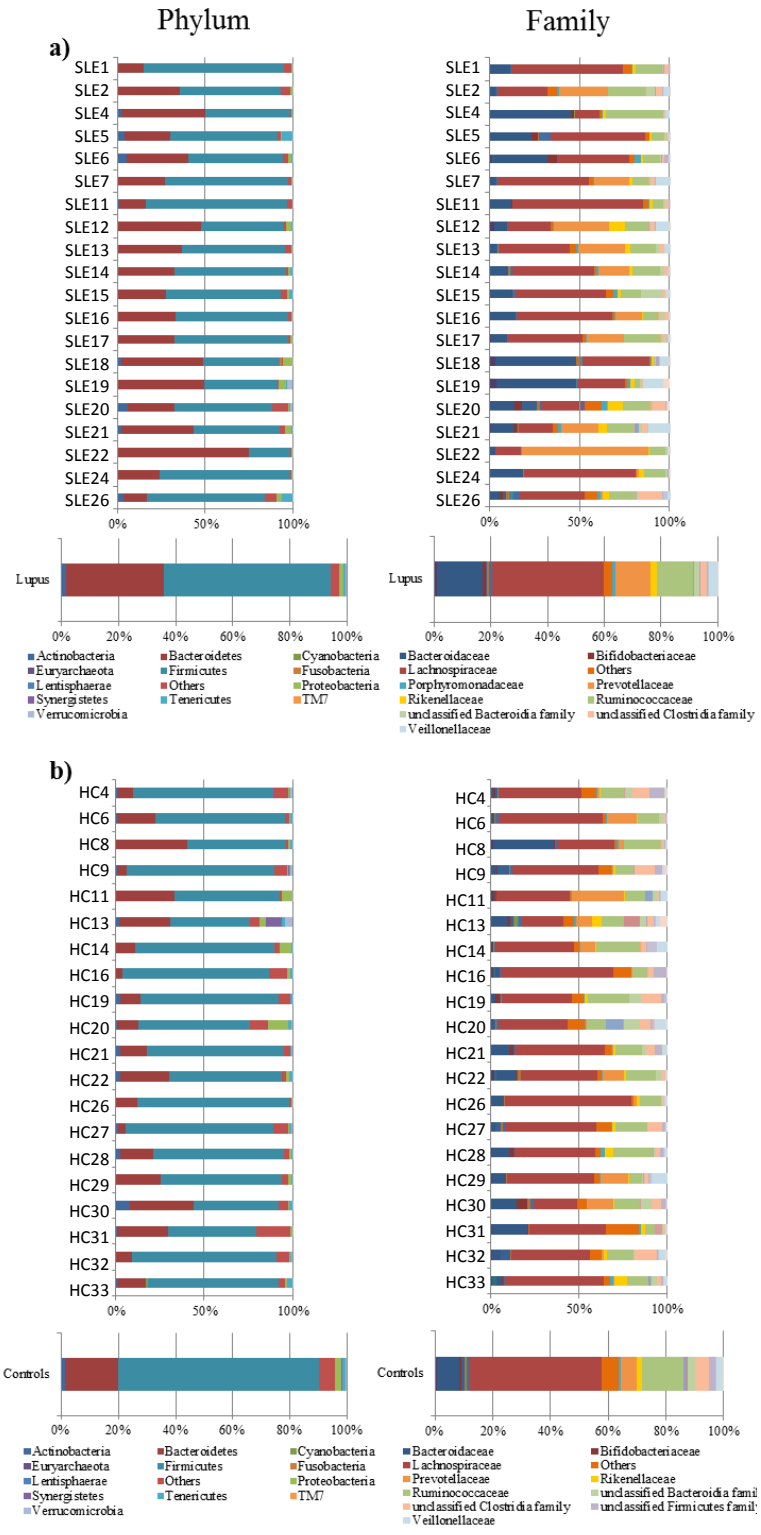


Figure S3. A) Scatter plot at the phylum level in which the relative abundances (mean) of *Firmicutes* and *Bacteroidetes* in healthy controls (x axis), and SLE patients (y axis) are represented. B) The normalized abundances of the classes *Bacteroidia* and *Clostridia*, and the orders *Bacteroidales* and *Clostridiales* differed significantly between the lupus (SLE) and the healthy control (HC) 16S ribosomal DNA sequence data, as represented in the error plots. Tables with relative abundances and sequence metadata were elaborated using QIIME, and analyzed using STAMP v2.0.0. Two-sided Welch's tests were run on every pair of means. Axes represent mean proportions of the taxa in HC (x axis) or SLE (y axis). Vertical and horizontal lines in each taxa represent data spread (2nd and 98th percentile) in the HC group (horizontal lines) and in the SLE group (vertical lines). The statistical fit of the points to the gray-dashed y = x line is represented by the Coefficient of determination in the upper-left part of the plot (R2). The y = x line represents the boundary delimiting taxa that are enriched in the HC (blue dots) or in the SLE group (yellow dots).

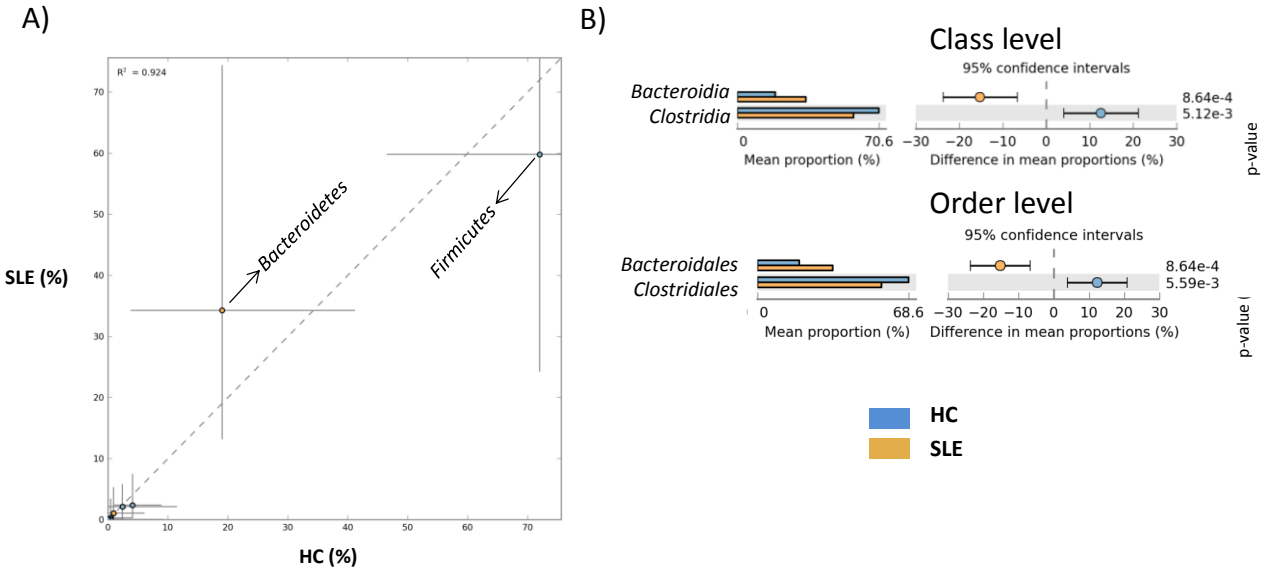


Figure S4. Scatter plot at the family level in which the relative abundances (mean) of the bacterial families in healthy controls (x axis) and lupus patients (y axis) are represented. Families showing the greatest relative abundances and divergence from the diagonal are labeled with arrows.

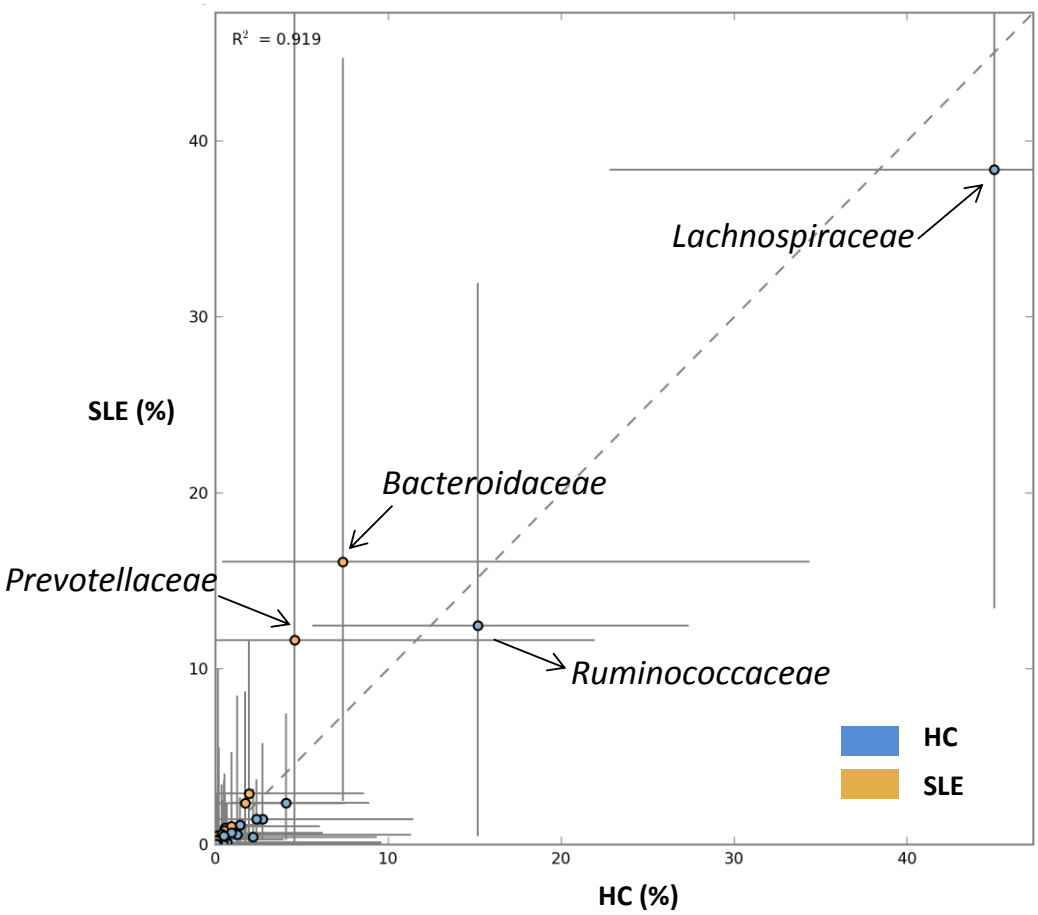


Figure S5. Summaries of the communities present in the different samples analyzed in this study. Plots were obtained at four taxonomic levels: phylum (A), class (B), order (C) and family (D), and the most abundant taxons are labeled with arrows. Data was obtained using QIIME v 1.7.0 with the scripts pick_de_novo_otus.py and summarize_taxa_through_plots.py.

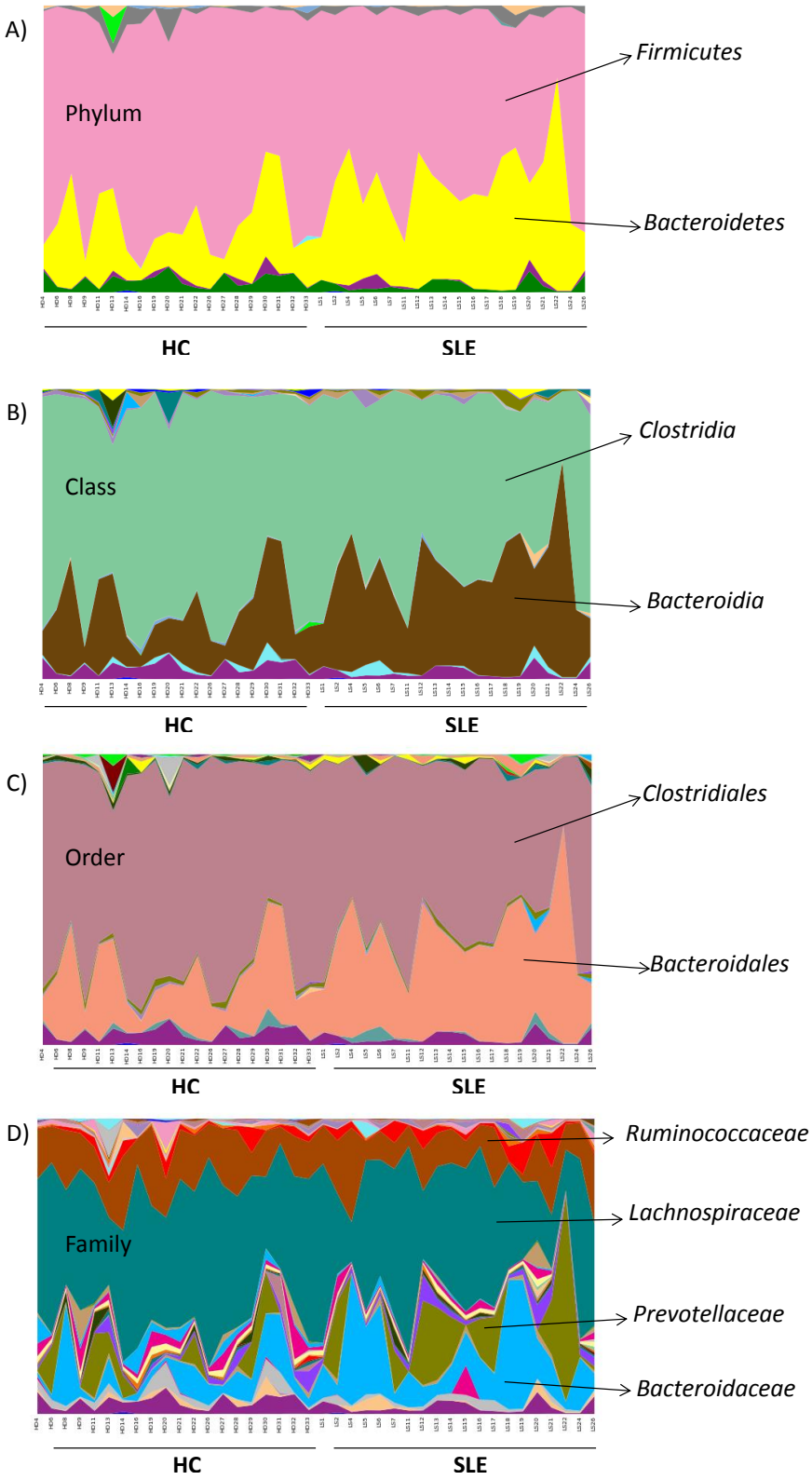
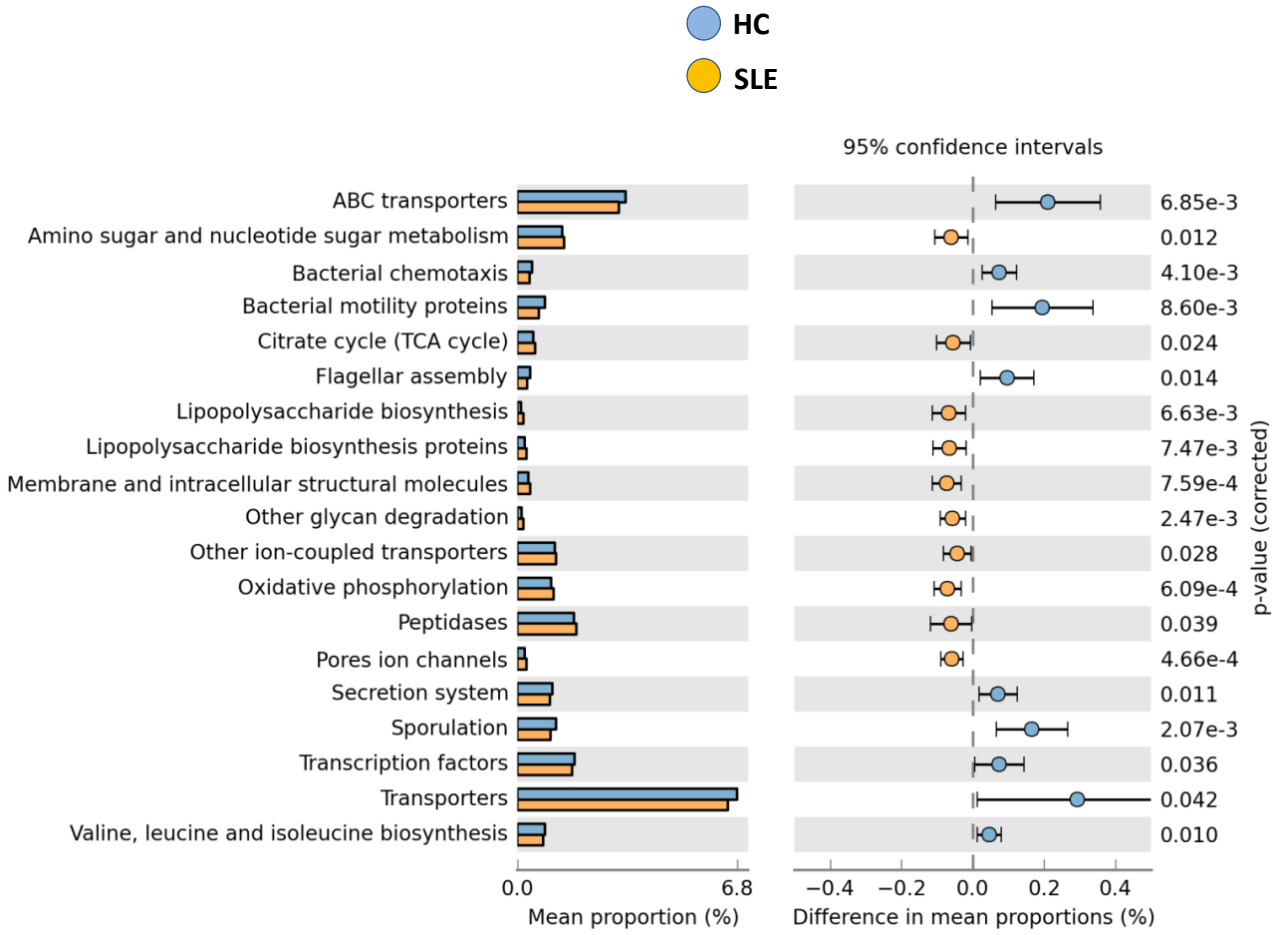


Figure S6. KEGG pathways (hierarchical level 3) whose normalized abundances differed between SLE patients and healthy controls. Functional metagenomes were predicted using PICRUSt scripts, and only pathways statistically significant are shown. In the y-axis, the KEGG pathways are indicated. Mean proportions for each metadata group (healthy controls vs. SLE) are shown together with the confidence intervals of the data. Variations due to every single metagenome on mean proportions are shown by horizontal error bars.



Supplementary Table 1. Sequence data features.

Supplementary Table 2. Statistically significant differences between healthy controls (HC) and patients (LES) at the phylum, family and genus levels.

Dataset	Sample	Number of reads	Number of reads removed because of:						Final read number	Reduced by (%)	average reads length	sequence output (Mbp)
			Outside bounds (150-400)	Ambiguous bases	Mean quality <25	Homopolymer runs >7bp	Primer mismatch >1	Low quality window truncation results in <150bp				
Healthy controls	HC4	705107	117184	0	0	485	49256	22309	515873	26.84%	187.2	96.57
	HC6	516755	64323	0	0	701	30268	16062	405401	21.55%	189.1	76.66
	HC8	452171	83112	0	0	664	19712	17609	331074	26.78%	193.25	63.98
	HC9	800844	154617	0	0	1175	49787	35075	560190	30.05%	186.3	104.36
	HC11	750409	102522	0	0	646	27941	24678	594622	20.76%	192.2	114.29
	HC13	682959	157189	0	0	7249	19937	38886	459698	32.69%	191.1	87.85
	HC14	950453	170255	0	0	7614	35618	26086	710879	25.21%	189	134.36
	HC16	624186	91195	0	0	705	20792	15731	495763	20.57%	186.4	92.41
	HC19	1324073	418738	0	0	2426	35519	33300	834090	37.01%	187.4	156.31
	HC20	1523816	349659	0	0	1469	142882	63653	966152	36.60%	189.6	183.18
	HC21	822090	99350	0	0	855	59218	19277	643390	21.74%	189.3	121.79
	HC22	559020	65118	0	0	1086	33420	14406	444990	20.40%	191.7	85.30
	HC26	647372	154647	0	0	672	23038	23939	445076	31.25%	186.3	82.92
	HC27	766684	297828	0	0	2071	16239	40393	410153	46.50%	184	75.47
	HC28	803461	169323	0	0	4049	30558	24060	575471	28.38%	189.6	109.11
	HC29	837208	354714	0	0	158	30128	21789	430419	48.59%	190.2	81.87
	HC30	784110	86460	0	0	1414	61435	21367	613434	21.77%	193.3	118.58
	HC31	835948	97078	0	0	648	48554	19778	669890	19.86%	191.2	128.08
	HC32	1419470	189686	0	0	1954	90892	38689	1098249	22.63%	188.6	207.13
	HC33	653027	137698	0	0	596	44159	19113	451461	30.87%	188.5	85.10
SLE patients	SLE1	593645	145786	0	0	1825	13490	18748	413796	30.30%	187.1	77.42
	SLE2	1008507	190635	0	0	4002	34536	24177	755155	25.12%	193.7	146.27
	SLE4	675462	245708	0	0	223	31425	11020	387086	42.69%	194.5	75.29
	SLE5	815861	173680	0	0	844	57027	34675	549635	32.63%	189.5	104.16
	SLE6	1609871	237380	0	0	1886	68786	34556	1267263	21.28%	193	244.58
	SLE7	529028	101529	0	0	663	32569	26278	367989	30.44%	190.4	70.07
	SLE11	596855	99732	0	0	1006	25782	23688	446647	25.17%	187	83.52
	SLE12	1203765	222933	0	0	1196	49622	41299	888715	26.17%	195.8	174.01
	SLE13	1184606	301608	0	0	1092	40057	33837	808012	31.79%	192.3	155.38
	SLE14	752639	195841	0	0	1008	23901	36877	495012	34.23%	190.2	94.15
	SLE15	1378807	331578	0	0	3851	49347	36457	957574	30.55%	190.6	182.51
	SLE16	976561	244070	0	0	586	52941	29671	649293	33.51%	190.8	123.89
	SLE17	1091503	248805	0	0	938	98180	37672	705908	35.33%	191	134.83
	SLE18	550336	87101	0	0	777	46112	20734	395612	28.11%	195.9	77.50
	SLE19	719033	132480	0	0	950	61030	24548	500025	30.46%	197.5	98.75
	SLE20	524211	96018	0	0	944	28466	15987	382796	26.98%	192	73.50
	SLE21	827185	82885	0	0	2110	28076	18795	695319	15.94%	197	136.98
	SLE22	552419	74815	0	0	394	22631	8735	445844	19.29%	200	89.17
	SLE24	625567	129481	0	0	130	18311	6806	470839	24.73%	190.7	89.79
	SLE26	609646	96214	0	0	1267	29003	29774	453388	25.63%	190.9	86.55
Average		832117	169974	0	0	1558	42016	26263	592305	28.51%	190.85	113.09

STATISTICS PHYLUM

Taxon	HC: mean rel. freq. (%)	HC: std. dev. (%)	SLE: mean rel. freq. (%)	SLE: std. dev. (%)	p-values	FDR (q-value)
k__Archaea;p__Euryarchaeota	0.00095547	0.002926786	0.000478866	0.001143342	0.514640909	0.701783057
k__Bacteria;Other	4.090374505	2.39494086	2.354716314	1.991595206	**0.020153648**	**0.100768238**
k__Bacteria;p__Actinobacteria	0.943342349	1.396055166	1.050488509	1.503805264	0.821176221	0.821176221
k__Bacteria;p__Bacteroidetes	19.0405042	11.77592807	34.25922595	14.07306427	**0.000892235**	**0.013383519**
k__Bacteria;p__Cyanobacteria	0.108276025	0.366030585	0.01350243	0.031094723	0.274583124	0.58839241
k__Bacteria;p__Firmicutes	72.01472536	12.72287405	59.76869272	13.76609987	**0.007086381**	**0.053147861**
k__Bacteria;p__Fusobacteria	0.001702696	0.004941363	0.053512002	0.229777336	0.338140489	0.563567482
k__Bacteria;p__Lentisphaerae	0.01841236	0.02462489	0.005053462	0.015484691	0.053845864	**0.161537593**
k__Bacteria;p__Proteobacteria	2.430374745	2.825270666	2.115669578	1.476232065	0.670173581	0.718043122
k__Bacteria;p__Synergistetes	0.468439255	1.992149018	0.004363349	0.010011524	0.32266976	0.605005799
k__Bacteria;p__Tenericutes	0.363467696	0.612583384	0.047736342	0.107845781	**0.038613759**	**0.144801595**
k__Bacteria;p__TM7	0.000944838	0.000807362	0.000562165	0.000731924	0.134215563	0.335538907
k__Bacteria;p__Verrucomicrobia	0.459838546	0.899799121	0.291738651	0.753262859	0.536203886	0.618696792
Unassignable;Other	4.55E-06	1.98E-05	1.24E-05	3.78E-05	0.428428301	0.642642451
Unclassified;Other	0.058637399	0.141911232	0.034247251	0.093274049	0.535616609	0.669520761

HC: Healthy Controls
SLE: Lupus Patients

STATISTICS FAMILY

Taxon	HC: mean rel. freq. (%)	HC: std. dev. (%)	SLE: mean rel. freq. (%)	SLE: std. dev. (%)	p-values	FDR (q-values)
k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae	0.000693248	0.002761014	0.000150301	0.000625431	0.098237547	0.257089752
k__Archaea;p__Euryarchaeota;c__Thermoplasmata;o__E2;f__WCHD3-02	0.000262222	0.001143	0.000328565	0.00098628	0.274014884	0.455457172
k__Bacteria;Other;Other;Other;Other	4.090374505	2.39494086	2.354716314	1.991595206	**0.00479849**	**0.147553491**
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae	0.001537408	0.001809878	0.002364265	0.006096793	0.338914151	0.47915449
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae	4.10E-05	7.48E-05	0.000934324	0.003981814	0.395128943	0.4860086
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae	0	0	1.31E-05	5.69E-05	0.397648381	0.465816675
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae	0.002677698	0.005174816	0.002318589	0.004146711	0.193981183	0.372807586
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	3.58E-05	7.51E-05	5.08E-05	0.000180704	0.299520644	0.46051299
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;Other	0.001181464	0.002425025	0.001404417	0.003596106	0.279981161	0.447242634
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae	0.929105296	1.386892334	1.033468543	1.485706595	0.279976539	0.453119925
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;Other	0.000785928	0.00130193	0.000985697	0.001581466	0.31589192	0.47383788
k__Bacteria;p__Actinobacteria;c__Actinobacteria;Other;Other	0.007589169	0.012533348	0.008420644	0.012181369	0.276945058	0.454189895
k__Bacteria;p__Actinobacteria;c__Rubrobacteria;o__Rubrobacterales;f__Rubrobacteraceae	1.55E-05	6.77E-05	3.95E-06	1.72E-05	0.113658783	0.279600607
k__Bacteria;p__Actinobacteria;Other;Other;Other	0.000373055	0.000553924	0.00052426	0.000842884	0.35285501	0.487653553
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__	0.150050107	0.296035403	0.511923347	2.166021982	0.3621288	0.484150461
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Barnesiellaceae]	0.430144977	0.543852355	0.376493186	0.28025986	0.167886773	0.350001239
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae]	0.215665419	0.148572201	0.249835776	0.175083969	0.352244455	0.492341681
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae]	0.933188781	1.584481028	0.66595103	1.143310925	0.132129726	0.290213506
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae	7.372991889	7.975350681	16.06421449	13.4871828	0.471019759	0.471019759
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae	0.559072563	0.514382344	0.93484455	0.869899141	0.448750356	0.463834402
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	4.591465505	6.663744808	11.61931912	16.71920424	0.452093022	0.463395348
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae	1.735064119	1.942603269	2.346906198	2.337303869	0.384309235	0.487319958
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7	0.604648963	2.085570935	0.000446186	0.001034781	0.052842811	0.259986629
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;Other	2.379045143	2.378849649	1.441241304	1.039146527	**0.03033751**	0.207306302
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;Other;Other	0	0	5.22E-06	2.28E-05	0.397648381	0.461422178
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae	0	0	2.21E-05	9.61E-05	0.397648381	0.479517165
k__Bacteria;p__Bacteroidetes;Other;Other;Other	0.069166733	0.123895201	0.04802348	0.032493995	0.114173195	0.275358882
k__Bacteria;p__Cyanobacteria;c__4C0d-2;o__YS2;f__	0.107787767	0.366141215	0.012924576	0.031015643	0.065303782	0.267745505
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__	0.000488259	0.000480003	0.000577853	0.000708174	0.32119233	0.475983814
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae	0	0	2.30E-05	6.96E-05	0.436701424	0.455205722
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae	1.97E-05	6.77E-05	1.78E-05	5.54E-05	0.221028518	0.418253965
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;Other	0	0	1.00E-05	4.36E-05	0.397648381	0.470295681
k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__Gemellaceae	0.001973157	0.007456545	0.000730115	0.001330703	0.114899573	0.266653727
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__	9.66E-05	0.000217864	0.000228166	0.000816769	0.356063762	0.486620475
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae	8.27E-05	0.000178899	0.000149147	0.000385083	0.356897254	0.482399585
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae	0.002743867	0.00864462	0.0014392	0.002097706	0.126063073	0.287143665
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae	0.003595507	0.009716573	0.003254286	0.01347612	0.221233738	0.412299239
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae	0.003758681	0.010139339	0.040629795	0.159240329	0.398481687	0.445574978
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae	0.000198443	0.00048047	0.11742575	0.510767162	0.39769554	0.457164032
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae	0.088194867	0.124394898	0.231672339	0.326377312	0.455766997	0.463300336
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;Other	0.001104518	0.001106856	0.003293408	0.007336486	0.425417279	0.44723355
k__Bacteria;p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae	0.001825222	0.002514904	0.001547263	0.003146952	0.182217668	0.361496341
k__Bacteria;p__Firmicutes;c__Bacilli;Other;Other	0.000679765	0.000792528	0.00226234	0.003056235	0.466648184	0.470473169
k__Bacteria;p__Firmicutes;c__Clostridia;o__;f__	0.124002635	0.120360566	0.152126051	0.187024186	0.336837031	0.487422998
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__	2.182374562	2.62770324	0.425731109	0.680439722	**0.00239998**	**0.147598901**
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Catabacteriaceae	1.270508587	2.658808561	0.560438685	1.839370307	0.08218892	0.22975539
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae	0.138617826	0.179565262	0.118729826	0.15495581	0.170668611	0.349870653
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae	0.00135358	0.001643083	0.000555079	0.000942627	**0.01808988**	**0.185421265**
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae	0.000249439	0.000566057	5.08E-05	0.000177259	**0.03761543**	0.220318926
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	45.04810549	11.28882292	38.37061787	16.12420625	**0.03533527**	0.217311906
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae	0.005646889	0.004696664	0.005469505	0.018179046	0.230364676	0.416689046
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae	0.043919129	0.036857612	0.017101207	0.015431291	**0.001699**	0.208977311
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	15.18315909	5.562864461	12.44290665	7.080094083	**0.04595474**	0.235518034
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	1.953120514	2.199719475	2.88718468	3.463636017	0.397986388	0.453262276
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other	2.73948275	2.772751881	1.436473815	1.308662443	**0.01783323**	**0.199407885**
k__Bacteria;p__Firmicutes;c__Clostridia;o__Coriobacteriales;f__	0.00117466	0.00126875	0.000350191	0.000386268	**0.00301104**	**0.123452454**
k__Bacteria;p__Firmicutes;c__Clostridia;o__Coriobacteriales;f__Coriobacteriaceae	0.440115819	0.44823406	0.462391402	0.670185408	0.260745104	0.45171335
k__Bacteria;p__Firmicutes;c__Clostridia;o__SHA-98;f__	0.009730406	0.012138363	0.00547296	0.013474997	0.074458869	0.261669738
k__Bacteria;p__Firmicutes;c__Clostridia;Other;Other	1.43156991	0.613816183	1.10416131	0.58969964	**0.02424595**	**0.175426613**
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__	0.018882064	0.038272247	0.000328649	0.000945148	**0.01145144**	0.234754597
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__[Coprobacillaceae]	0.499314367	0.446307876	0.487161977	0.859325955	0.227798789	0.418197776
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	0.21298335	0.435500846	0.321319314	1.183471014	0.306824455	0.465918617
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;Other	0.003770134	0.004860595	0.002294812	0.004309175	0.078220116	0.260029035
k__Bacteria;p__Firmicutes;c__RF3;o__ML615J-28;f__	0.033880141	0.095036126	0.11318134	0.459909074	0.364287115	0.481799088
k__Bacteria;p__Firmicutes;Other;Other;Other	0.568491084	0.434617932	0.451962868	0.378250432	0.091345741	0.249678358
k__Bacteria;p__Fusobacteria;c__Fusobacteria;o__Fusobacteriales;f__Fusobacteriaceae	0.000550055	0.001820616	0.053428073	0.229622521	0.398068484	0.449196546
k__Bacteria;p__Fusobacteria;c__Fusobacteria;o__Fusobacteriales;f__Leptotrichiaceae	0.001152641	0.00467496	3.94E-05	0.000110402	0.074386599	0.269104461
k__Bacteria;p__Fusobacteria;c__Fusobacteria;o__Fusobacteriales;Other	0	0	4.45E-05	0.00016627	0.414899687	0.447654925
k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];o__Victivallales;f__Victivallaceae	0.017200283	0.023947924	0.004798667	0.015419299	**0.01586573**	**0.19514846**
k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];Other;Other	0.001212077	0.002161146	0.000254796	0.000734798	**0.01913407**	**0.168106436**
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__RF32;f__	0.382542583	0.846582432	0.584964244	0.761151133	0.370674349	0.485031329
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae	6.84E-05	0.000149412	0	0	**0.01441773**	**0.197042264**
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae	0	0	1.24E-05	5.39E-05	0.397648381	0.48426486
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae	0	0	5.22E-05	0.00022774	0.397648381	0.474861659
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__mitochondria	4.58E-05	0.000104899	5.84E-05	0.000181094	0.287202265	0.44716302
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae	4.55E-06	1.98E-05	0	0	0.078542095	0.241516944
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;Other;Other	0.014666037	0.029023987	0.015622291	0.0213077	0.259880328	0.456646863
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae	0.536335593	0.479343907	0.788497321	0.98517418	0.39893177	0.442059528
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae	1.22E-05	3.71E-05	5.22E-05	0.000139001	0.419276937	0.444578132
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae	0.00013541	0.000198417	0.000274169	0.000479658	0.415526138	0.444432304
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae	1.20E-05	3.72E-05	1.21E-05	5.27E-05	0.23894467	0.425944846
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;Other	0.072929119	0.084528711	0.208066985	0.61371065	0.392097054	0.487150886
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae	0.000160287	0.000589939	4.79E-05	9.03E-05	0.100385397	0.25723758
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;Other;Other	0.036909036	0.047400945	0.083418476	0.217977684	0.38717506	0.485944208
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae	0.223038228	0.335665529	0.111584309	0.09670175	**0.04241068**	0.237114233
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;Other	0.0027039	0.005042331	0.001259669	0.0022397	0.062902454	0.286555625
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;Other;Other	0.003449081	0.00695556	0.001187626	0.001553068	**0.04314912**	0.230753968
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae	0.000377341	0.000340906	0.000476018	0.000681661	0.338805164	0.484570176
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Helicobacteraceae	3.54E-05	0.000132402	0	0	0.061555192	0.291203408
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Succinivibrionaceae	0.676678435	2.21044062	0.112819407	0.490874492	0.069070517	0.274053988
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;Other	0.001266314	0.005112764	0.00030202	0.001316473	0.103487775	0.259775435
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Cardiobacteriales;f__Cardiobacteriaceae	1.22E-05	5.31E-05	6.62E-06	2.89E-05	0.164520858	0.348897682
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae	0.052641193	0.184572655	0.148717577	0.464277008	0.378595393	0.485075348
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae	0.007401785	0.014502244	0.008884138	0.023444755	0.281858531	0.444469222
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae	2.72E-05	8.33E-05	5.63E-06	2.45E-05	0.069284003	0.266310388
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae	5.99E-06	2.61E-05	2.49E-05	6.62E-05	0.414677469	0.45137459
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae	1.22E-05	5.31E-05	0	0	0.078542095	0.247709686
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;Other;Other	0.078540887	0.244764661	0.026069506	0.082772988	0.091690791	0.245173203
k__Bacteria;p__Proteobacteria;Other;Other;Other	0.34036363	1.22169516	0.023253431	0.021028632	0.064766073	0.274697482
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae	0.000153206	0.000406201	0.002370909	0.008789294	0.408193554	0.448283992
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae	0.466187243	1.983481537	0.001979379	0.005307792	0.07630399	0.260705298
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;Other	0.002038428	0.008469333	0	0	0.073163711	0.272701104
k__Bacteria;p__Synergistetes;c__Synergistia;Other;Other	1.09E-05	4.74E-05	1.31E-05	5.69E-05	0.262283084	0.448066936
k__Bacteria;p__Synergistetes;Other;Other;Other	4.95E-05	0.000190066	0	0	0.064379032	0.28280789
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;f__	2.45E-05	0.000106586	0	0	0.078542095	0.235626286
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;f__Anaeroplasmataceae	0.222181267	0.555766607	0.022132571	0.088300803	**0.03260967**	0.211104727
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;Other	3.03E-05	0.000131885	7.89E-06	3.44E-05	0.114788808	0.27151968
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Mycoplasmatales;f__Mycoplasmataceae	0.000146715	0.000639516	0	0	0.078542095	0.224666924
k__Bacteria;p__Tenericutes;c__Mollicutes;o__RF39;f__	0.112168604	0.154927272	0.023181197	0.04263489	**0.00585901**	**0.144131696**
k__Bacteria;p__Tenericutes;c__Mollicutes;Other;Other	0.028916401	0.064021064	0.002414683	0.009930717	**0.02138772**	**0.175379321**
k__Bacteria;p__TM7;c__TM7-3;o__;f__	0.000709616	0.000617005	0.000358624	0.000475186	**0.01364172**	0.209741454
k__Bacteria;p__TM7;c__TM7-3;o__CW040;f__	9.11E-06	3.97E-05	1.10E-05	4.81E-05	0.263383615	0.443783351
k__Bacteria;p__TM7;c__TM7-3;o__CW040;f__F16	9.73E-06	2.92E-05	1.70E-05	5.86E-05	0.325751574	0.476993376
k__Bacteria;p__TM7;c__TM7-3;o__I025;f__Rs-045	2.38E-05	6.02E-05	1.31E-05	5.69E-05	0.136712629	0.295011462
k__Bacteria;p__TM7;c__TM7-3;Other;Other	0.000185087	0.000252143	0.000162444	0.00027581	0.188841428	0.368690408
k__Bacteria;p__TM7;Other;Other;Other	7.46E-06	3.25E-05	0	0	0.078542095	0.230016137
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__[Cerasicoccales];f__[Cerasicoccaceae]	0.07124009	0.134763599	0.016629677	0.029816552	**0.02366214**	**0.181902669**
k__Bacteria;p__Verrucomicrobia;c__Opitutae;Other;Other	0.018052371	0.028390846	0.005195545	0.011579108	**0.01892216**	**0.179032788**
k__Bacteria;p__Verrucomicrobia;c__Verruco-5;o__WCHB1-41;f__RFP12	8.15E-06	3.55E-05	0	0	0.078542095	0.254228362

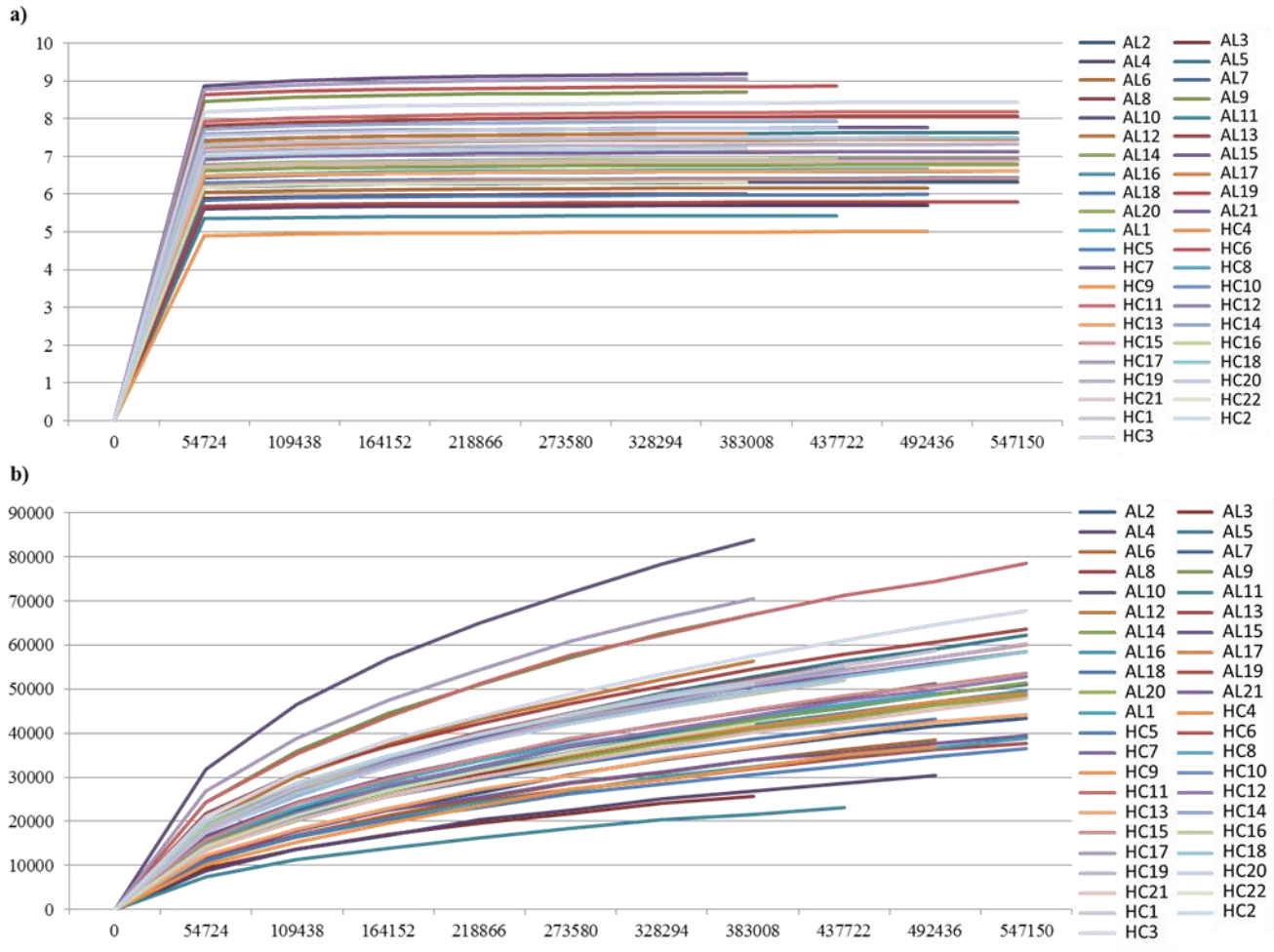
HC: Healthy Controls
SLE: Lupus Patients

k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae	0.363011957	0.855453322	0.267697396	0.753514227	0.170851648	0.344504143
k__Bacteria;p__Verrucomicrobia;Other;Other;Other	0.007525977	0.010775011	0.002216033	0.004202265	**0.01344994**	0.236334735
Unassignable;Other;Other;Other;Other	4.55E-06	1.98E-05	1.24E-05	3.78E-05	0.374183738	0.484469471
Unclassified;Other;Other;Other;Other	0.058637399	0.141911232	0.034247251	0.093274049	0.127527764	0.285198454

INFORMACIÓN SUPLEMENTARIA ARTÍCULO:

Hevia A, Milani C, López P, Donado CD, Cuervo A, González S, Suárez A, Turroni F, Gueimonde M, Ventura M, Sánchez B, Margolles A. Allergic Patients with Long-Term Asthma Display Low Levels of Bifidobacterium adolescentis. **PLoS One**, 2016; 11:e0147809.

S1 Fig. Rarefaction curves generated for 16S rRNA gene sequences obtained from faecal samples of control subjects (HC samples) and allergic asthma patients (AL samples). Panel a represents the rarefaction curves using the Shannon index. Panel b displays rarefaction curves using the Chao1 index.



Dataset	Sample	Number of reads	Number of reads removed because of:						Final reads number	Reduced by (%)	average reads length	Sequence Output (Mbp)
			Outside bounds (150-400)	Ambiguous bases	Mean quality <25	Homopolymer runs >7bp	Primer mismatch >1	Low quality windows truncation results in <150bp				
Healthy controls	HC1	705107	117184	0	0	485	49256	22309	515873	26.84%	187.2	96.57
	HC2	516755	64323	0	0	701	30268	16062	405401	21.55%	189.1	76.66
	HC3	800844	154617	0	0	1175	49787	35075	560190	30.05%	186.3	104.36
	HC4	750409	102522	0	0	646	27941	24678	594622	20.76%	192.2	114.29
	HC5	2043202	542937	0	0	626	81256	59920	1358463	33.51%	196.5	266.94
	HC6	682959	157189	0	0	7249	19937	38886	459698	32.69%	191.1	87.85
	HC7	948930	145829	0	0	1367	60152	26943	714639	24.69%	187.1	133.71
	HC8	624186	91195	0	0	705	20792	15731	495763	20.57%	186.4	92.41
	HC9	651188	80640	0	0	498	28704	13714	527632	18.97%	196.7	103.79
	HC10	588180	158159	0	0	483	21507	22471	385560	34.45%	186.5	71.91
	HC11	1324073	418738	0	0	2426	35519	33300	834090	37.01%	187.4	156.31
	HC12	822090	99350	0	0	855	59218	19277	643390	21.74%	189.3	121.79
	HC13	1260689	160710	0	0	5575	87280	29647	977477	22.46%	196.3	191.88
	HC14	592338	73766	0	0	2497	34182	19793	462100	21.99%	383.9	177.40
	HC15	842264	225177	0	0	197	28266	13183	575441	31.68%	187.6	107.95
	HC16	647372	154647	0	0	672	23038	23939	445076	31.25%	186.3	82.92
	HC17	766684	297828	0	0	2071	16239	40393	410153	46.50%	184	75.47
	HC18	803461	169323	0	0	4049	30558	24060	575471	28.38%	189.6	109.11
	HC19	1419470	189686	0	0	1954	90892	38689	1098249	22.63%	188.6	207.13
	HC20	638109	145072	0	0	777	21445	17367	453448	28.94%	192	87.06
	HC21	977825	148439	0	0	1041	56972	28454	742918	24.02%	196	145.61
	HC22	591244	140515	0	0	567	36224	15913	398025	32.68%	189.7	75.51
Allergic patients	AL1	1572245	139941	0	0	5167	52450	32456	1342231	14.63%	193.7	259.99
	AL2	1059336	130980	0	0	1867	49227	17949	859313	18.88%	193.1	165.93
	AL3	664346	236268	0	0	97	17048	7916	403017	39.34%	197.4	79.56
	AL4	653445	75093	0	0	196	50862	8821	518473	20.66%	193.8	100.48
	AL5	872568	119802	0	0	2277	51997	29505	668987	23.33%	191.3	127.98
	AL6	671449	93278	0	0	580	38027	9885	529679	21.11%	190.9	101.12
	AL7	808550	87389	0	0	1207	54105	20770	645079	20.22%	193.6	124.89
	AL8	709243	115210	0	0	1048	27934	18376	546675	22.92%	384	209.92
	AL9	714325	253623	0	0	303	21664	30939	407796	42.91%	190	77.48
	AL10	692806	202082	0	0	1463	18656	52644	417960	39.67%	184	76.90
	AL11	610199	68204	0	0	184	44709	7942	489160	19.84%	197.7	96.71
	AL12	537164	89365	0	0	1594	23642	22409	400154	25.51%	192.2	76.91
	AL13	811040	157438	0	0	1821	63140	41485	547156	32.54%	186.9	102.26
	AL14	725363	99333	0	0	1073	20764	17652	586541	19.14%	377.1	221.18
	AL15	658803	84063	0	0	1157	41492	28947	503144	23.63%	190.9	96.05
	AL16	1113622	274500	0	0	3057	36169	27199	772697	30.61%	190.4	147.12
	AL17	1363487	283794	0	0	5947	43480	58481	971785	28.73%	189	183.67
	AL18	667831	78075	0	0	535	47619	15217	526385	21.18%	188.2	99.07
	AL19	1115415	159116	0	0	1370	51199	24859	878871	21.21%	191.1	167.95
	AL20	926394	158659	0	0	1045	30000	14130	722560	22.00%	189.3	136.78
	AL21	840647	162457	0	0	227	31866	16731	629366	25.13%	195.3	122.92

S1 Table. Sequence data features.

S2 Table. Statistically significant differences between healthy controls (HC) and patients (AL) at the phylum, family and genus levels.

STATISTICS PHYLUM

Taxon	AL: mean rel. freq. (%)	AL: std. dev. (%)	HC: mean rel. freq. (%)	HC: std. dev. (%)	p-values
k__Archaea;p__Euryarchaeota	3.70386077748e-05	0.000108489836192	0.000312709039372	0.00108724197523	0.2604470518
k__Bacteria;Other	6.05870422531	4.62401751198	6.62187141218	4.97435552262	0.709104399335
k__Bacteria;p__Acidobacteria	0.0	0.0	6.11837988236e-06	2.80379389414e-05	0.328694683236
k__Bacteria;p__Actinobacteria	2.60793086529	2.78737559645	1.17209895121	0.980022383007	0.0391028179736
k__Bacteria;p__Bacteroidetes	27.2592806425	13.7064813434	22.3769365496	16.6680533384	0.310807619313
k__Bacteria;p__Cyanobacteria	0.0810558554009	0.314477740035	0.0362327039543	0.0761188533391	0.541335612068
k__Bacteria;p__Firmicutes	61.3727317943	11.3558195403	67.0275708274	14.8161913187	0.176778272188
k__Bacteria;p__Fusobacteria	0.00337133453232	0.014271191525	0.000162806372424	0.000151069054767	0.326713195975
k__Bacteria;p__Lentisphaerae	0.0146907576604	0.0271090336897	0.0193368181698	0.0258724902553	0.577970109477
k__Bacteria;p__Proteobacteria	2.13320029959	2.44100964427	1.5778504332	1.62986425215	0.39982234791
k__Bacteria;p__Synergistetes	0.00251913518557	0.00371896964471	0.425493811729	1.90722764688	0.321054668887
k__Bacteria;p__Tenericutes	0.171830708045	0.374957875877	0.293521349559	0.458010287368	0.356502170333
k__Bacteria;p__TM7	0.000560228264662	0.000646769052831	0.000968423873558	0.00101116715455	0.130569697518
k__Bacteria;p__Verrucomicrobia	0.249901741444	0.704583573571	0.415257166552	0.866276106235	0.505475901131
Unassignable;Other	0.0	0.0	4.13881965333e-06	1.89664543492e-05	0.328694683236
Unclassified;Other	0.044185373868	0.0988543585404	0.0323757799091	0.0474575463374	0.632234161192

STATISTICS FAMILY

Taxon	AL: mean rel. freq. (%	AL: std. dev. (%)	HC: mean rel. freq. (%)	HC: std. dev. (%)	p-values
k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae	3.70386077748e-05	0.0001084898361	5.07468845853e-05	0.000122011098767	0.705488126223
k__Archaea;p__Euryarchaeota;c__Thermoplasmata;o__E2;f__[Methanomassiliicoccaceae]	0.0	0.0	0.000261962154787	0.00109260968829	0.28433201893
k__Bacteria;Other;Other;Other;Other	6.05870422531	4.62401751198	6.62187141218	4.97435552262	0.709104399335
k__Bacteria;p__Acidobacteria;c__[Chloracidobacteria];o__RB41;f__Ellin6075	0.0	0.0	6.11837988236e-06	2.80379389414e-05	0.328694683236
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae	0.00136354719135	0.0029880553867	0.000789744059396	0.000776463516512	0.413881488307
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae	0.0	0.0	1.93599782033e-05	6.19242868187e-05	0.166664587406
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae	0.00177535533744	0.0024373678050	0.00116080207234	0.00119835005158	0.317790509465
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae	1.19001803353e-05	5.32192243486e-(	5.93274873345e-05	0.000271872701502	0.441263184434
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Pseudonocardiaceae	5.54152533701e-06	2.47824547052e-(	0.0	0.0	0.329256577172
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;Other	0.00162899781731	0.0014697733889	0.000948137876995	0.00115308916522	0.108253378165
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae	2.07187529694	2.54947330566	0.83162684327	0.869984380936	0.0497953174808
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;Other	1.44646361663e-05	4.61071159962e-(	2.16029564426e-05	5.70795214179e-05	0.66124671048
k__Bacteria;p__Actinobacteria;c__Actinobacteria;Other;Other	0.0198469794083	0.0207020923714	0.00790648070318	0.0104266893834	0.0278422293367
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae	0.478953428031	0.938131544732	0.312596753143	0.252392068151	0.450947363493
k__Bacteria;p__Actinobacteria;c__Rubrobacteria;o__Rubrobacterales;f__Rubrobacteraceae	2.51583938049e-05	6.47748711953e-(	2.35941095631e-05	7.00389536786e-05	0.94114174827
k__Bacteria;p__Actinobacteria;Other;Other;Other	0.0324301958237	0.0407965358144	0.016946305552	0.0177505562349	0.129842768627
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__	0.0981310622853	0.398402745615	0.14875358967	0.315268702145	0.655443736868
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Barnesiellaceae]	0.528598410946	0.730219822436	0.426260530208	0.471480887294	0.599380104133
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae]	0.275602354554	0.24891492534	0.239310257433	0.171929697722	0.592120242979
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae]	0.49809727057	1.06296621883	0.66733124991	1.44709427446	0.670901437304
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae	13.3338661827	11.6063755629	8.46660431355	11.5010508703	0.185031416422
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae	0.621604242727	0.534577520426	0.654639057247	0.698401421932	0.865450273226
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	8.27690645517	12.1912652764	8.19367104004	14.9392552124	0.984470303389
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae	2.24943749856	1.72310998026	1.97188576195	1.84837544125	0.621440140025
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7	0.0220187825391	0.0860379515158	0.187291135927	0.637321128823	0.251822936791
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;Other	1.30610551601	0.996184130106	1.37503411129	1.23596824758	0.844788940684
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae	1.73031018982e-05	5.34434583011e-(	0.0	0.0	0.163135183774
k__Bacteria;p__Bacteroidetes;Other;Other;Other	0.0488955633797	0.0406044861008	0.046155502404	0.0412833193537	0.831432119587
k__Bacteria;p__Cyanobacteria;c__4C0d-2;o__YS2;f__	0.0806067073748	0.314555150028	0.0357020133035	0.0760145198062	0.540672968285
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Stramenopiles;f__	0.0	0.0	6.11837988236e-06	2.80379389414e-05	0.328694683236
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__	0.000445600272642	0.0008604951548	0.000519922080101	0.00039277199193	0.726880757474

k__Bacteria;p__Cyanobacteria;Other;Other;Other	3.54775352521e-06	1.58660360996e-(	4.65019079268e-06	2.13098513034e-05	0.851481239768
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae	9.18447973553e-06	4.10742420532e-(	2.24103259858e-05	6.24405977894e-05	0.426100369876
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae	2.06673447438e-05	6.42933251005e-(	0.0	0.0	0.166014407803
k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__Gemellaceae	0.00128198155072	0.0036001382341	0.000273982165559	0.000430550604367	0.227611905577
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__	0.000620100590939	0.0024022104338	0.000104870679127	0.000213814319083	0.350519377398
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae	0.000378313796367	0.0009781404246	2.14982400782e-05	7.01943219193e-05	0.11915299035
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae	0.00190599081855	0.0037861270981	0.000560779999715	0.000635627509133	0.131764958317
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae	0.000365152965061	0.0008208708218	0.00140789357532	0.00435559302015	0.29278352896
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae	0.00267462464595	0.0055940894851	0.00301680435793	0.00455371408605	0.831525833942
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae	8.46696944452e-05	0.0002588539894	0.000147174008139	0.000455481295033	0.590425366577
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae	0.294558787809	0.655987154645	0.0567697117701	0.0689627026619	0.122170352593
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;Other	0.00215540163981	0.0044808253431	0.000759455923324	0.000762112750474	0.183729049859
k__Bacteria;p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae	0.00124615815144	0.0021285911493	0.001252478907	0.00222158510394	0.992622014959
k__Bacteria;p__Firmicutes;c__Bacilli;Other;Other	0.00132916746418	0.0023825007440	0.000331643055263	0.000370397681202	0.0783038799828
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__	0.487659525695	0.719011491063	0.578688877437	0.777175262405	0.698901159555
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Acidaminobacteraceae]	5.54152533701e-06	2.47824547052e-(	0.0	0.0	0.329256577172
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae]	0.0278173708642	0.0222807777494	0.0235872216977	0.0123827924307	0.461085073186
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae]	0.000832552476462	0.0014597685621	0.00176305550436	0.00247374862036	0.14931041921
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae	0.692430445358	1.48420769371	1.67317106849	3.0681434787	0.199117245925
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae	0.228077281358	0.502160081474	0.236237057931	0.223848837845	0.94735549074
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae	0.000872688739923	0.0012698954806	0.00166129615331	0.00214393820407	0.158630381582
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__EtOH8	0.00232043326913	0.0084535422276	0.00132712933742	0.00213268612325	0.614866405957
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae	0.000232821120572	0.0004668658830	0.000268485860796	0.000533846147638	0.820790026245
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae	37.6349926222	13.1810521645	44.8627269122	14.227560196	0.0989407697005
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae	0.00473605182769	0.0055087056962	0.00616205284055	0.00503828114016	0.392884338623
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae	0.000124796069514	0.0002435060857	0.000230284158527	0.00027859250592	0.203458802817
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae	15.2329623528	6.51514767439	12.4555179714	4.72280524063	0.12827320528
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae	1.51984164694	1.24142043226	1.70791454127	2.43245794889	0.755605918933
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other	3.70829251349	1.99835068123	3.98901320884	2.19097698098	0.67019925473
k__Bacteria;p__Firmicutes;c__Clostridia;o__SHA-98;f__	0.00409273498993	0.0060143996101	0.00638688174476	0.00785251994636	0.298701163127
k__Bacteria;p__Firmicutes;c__Clostridia;Other;Other	0.0890575433179	0.0539355187749	0.125580238723	0.0993828601601	0.150630829796
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae	1.00611468311	2.00722321842	0.818752142389	1.01192111484	0.710685501797
k__Bacteria;p__Firmicutes;Other;Other;Other	0.425637988218	0.243563863663	0.4739136984	0.314850969495	0.584999941646

k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae	0.0033463752485	0.0142767846134	0.000125913718243	0.000152850042019	0.325146189652
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae	2.49592838204e-05	8.58772737097e-(	3.68926541805e-05	6.94921289684e-05	0.628527042802
k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];o__Victivallales;f__Victivallaceae	0.014201538928	0.0268342559032	0.0188464660339	0.0255873710907	0.574032994657
k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];o__Z20;f__R4-45B	5.67857881072e-05	0.0002539537647	0.0	0.0	0.329256577172
k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];Other;Other	0.000432432944378	0.0010967759758	0.000490352135886	0.00120843479865	0.873015750051
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__RF32;f__	0.320804279178	0.6161877458	0.476346892463	0.993968844535	0.548843566386
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae	1.79803419124e-05	8.04105335495e-(	3.99444099218e-05	0.000117466785215	0.48735608699
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae	0.0	0.0	4.65019079268e-06	2.13098513034e-05	0.328694683236
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__mitochondria	5.02286189652e-05	0.0001301168480	5.31323151469e-05	0.000109852481512	0.938996105541
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae	0.0	0.0	4.13881965334e-06	1.89664543492e-05	0.328694683236
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;Other;Other	0.0159064343177	0.0379254237949	0.0219057438374	0.03857815248	0.618312792953
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae	0.856754762335	0.929528466593	0.432542293868	0.391600727083	0.0700246170783
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae	0.000214868627368	0.0007445445281	9.83651708604e-06	4.50765841215e-05	0.233096002204
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae	0.000633611264305	0.0016492989120	0.00100600986077	0.00214370018124	0.535480666543
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae	0.0014445779793	0.0026236709208	0.00371537079101	0.00558901934586	0.103714413059
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;Other	0.120644962702	0.202810260576	0.146210338752	0.270012773166	0.732819083014
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae	7.03740316818e-05	0.0001613648504	3.81666474578e-05	8.16837363499e-05	0.429989601901
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;Other;Other	0.0258929196894	0.0281622352274	0.0164663519416	0.0131702237209	0.184099585398
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae	0.0957616619076	0.0819038877573	0.199294916378	0.323285767645	0.16842526368
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;Other	0.0011972876583	0.0014698240268	0.00211596416169	0.0044581840445	0.379279574861
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;Other;Other	0.000731256532952	0.0007411653190	0.00328592790848	0.00775562480527	0.147632370828
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae	0.000388787301383	0.0007170395801	0.000346815769309	0.000386139437424	0.8183078415
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Helicobacteraceae	0.00278849625209	0.01032614419	1.81118404837e-05	5.77864338682e-05	0.244235148463
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;Other	4.59495882611e-05	0.0001712530577	0.0	0.0	0.244185696629
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Succinivibrionaceae	0.418675353249	1.86410071524	0.185901896956	0.845744688869	0.61367082355
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;Other	0.00137232321259	0.0061372159809	0.000252261100329	0.00115600558715	0.430976141146
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Cardiobacteriales;f__Cardiobacteriaceae	2.5819461728e-05	6.33102305203e-(	1.10823388966e-05	5.07856568708e-05	0.417452878842
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae	0.135389835692	0.34138204524	0.0515198079987	0.17483886632	0.333707486751
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae	0.00922526767976	0.013618090983	0.00624278743562	0.0105926119223	0.44017670015
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae	2.94728701312e-05	0.0001004568788	9.88791455577e-06	4.53121169171e-05	0.431708588312
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae	0.000536857817731	0.0022891186747	5.44959722028e-06	2.49731917689e-05	0.311597169474
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae	0.0	0.0	2.25023616225e-05	7.11675285501e-05	0.1621183097
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;Other;Other	0.0277851284474	0.0954173615799	0.0151662336304	0.0531548444861	0.607024551219

k__Bacteria;p__Proteobacteria;Other;Other;Other	0.0968118028327	0.317344812022	0.0153139173962	0.0191811655359	0.265026524463
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae	9.20219639676e-05	0.0003117794771	0.00013055300434	0.000390117102675	0.728054627805
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae	0.0024271132216	0.0037538073404	0.424508027835	1.90345541086	0.321118693212
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;Other	0.0	0.0	0.000855230888907	0.0038013877986	0.314278059577
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;f__Anaeroplasmataceae	0.000199041644878	0.0003897323379	0.135501899381	0.432448991355	0.166358783377
k__Bacteria;p__Tenericutes;c__Mollicutes;o__RF39;f__	0.0366194344298	0.0609080113473	0.0873355760152	0.114715004555	0.0845459000051
k__Bacteria;p__Tenericutes;c__Mollicutes;Other;Other	0.000313760114657	0.0008599026839	0.00366914685055	0.0098572079379	0.134962592146
k__Bacteria;p__Tenericutes;c__RF3;o__ML615J-28;f__	0.132001716437	0.34907423425	0.0651636325236	0.103209212704	0.418998774672
k__Bacteria;p__Tenericutes;Other;Other;Other	0.00269675541936	0.0065625017001	0.0018510947893	0.00295863021294	0.602080136267
k__Bacteria;p__TM7;c__MJK10;o__;f__	3.54775352521e-06	1.58660360996e-(	0.0	0.0	0.329256577172
k__Bacteria;p__TM7;c__TM7-3;o__;f__	0.000307942670212	0.0003376956187	0.000756330261859	0.000908709000794	0.0439944154055
k__Bacteria;p__TM7;c__TM7-3;o__CW040;f__	0.000174155182328	0.0002495351139	0.000112617959598	0.000216709759012	0.405209661232
k__Bacteria;p__TM7;c__TM7-3;o__CW040;f__F16	2.62932728544e-05	6.45100846638e-(	1.27536393486e-05	4.29759642879e-05	0.436675458224
k__Bacteria;p__TM7;c__TM7-3;o__I025;f__Rs-045	8.71066860915e-06	3.89552942791e-(	3.63454032037e-05	7.19638215872e-05	0.133469544342
k__Bacteria;p__TM7;c__TM7-3;Other;Other	3.95787171336e-05	9.16916459258e-(	5.03766095487e-05	9.98388097317e-05	0.72000822149
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__[Cerasicoccales];f__[Cerasicoccaceae]	0.186163342763	0.613517316668	0.066917902095	0.119056753539	0.402527050295
k__Bacteria;p__Verrucomicrobia;c__Opitutae;Other;Other	0.0275076721633	0.0796864029891	0.0197462372606	0.0326597288954	0.68916772102
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae	0.0319909215056	0.0583433916999	0.323045421312	0.825986669971	0.122052288708
k__Bacteria;p__Verrucomicrobia;Other;Other;Other	0.00423980501225	0.0110773396388	0.00554760588413	0.00846763086247	0.674571089891
Unassignable;Other;Other;Other;Other	0.0	0.0	4.13881965334e-06	1.89664543492e-05	0.328694683236
Unclassified;Other;Other;Other;Other	0.0441853738679	0.0988543585404	0.0323757799091	0.0474575463375	0.632234161193

STATISTICS GENUS

Taxon	AL: mean rel. freq. (%)	AL: std. dev. (%)	HC: mean rel. freq.	HC: std. dev. (%)	p-values
k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae;g__Methanobrevibac	3.70386077748e-05	0.0001084898361	5.07468845853e-0	0.00012201109	0.705488126223
k__Archaea;p__Euryarchaeota;c__Thermoplasmata;o__E2;f__[Methanomassiliicoccaceae];g__vadinCA11	0.0	0.0	0.0002619621547	0.00109260968	0.28433201893
k__Bacteria;Other;Other;Other;Other;Other	6.05870422531	4.62401751198	6.62187141218	4.97435552262	0.709104399334
k__Bacteria;p__Acidobacteria;c__[Chloracidobacteria];o__RB41;f__Ellin6075;g__	0.0	0.0	6.11837988236e-0	2.80379389414	0.328694683236
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae;g__	4.9001628569e-06	2.19141944977e-0	1.54737996546e-0	5.12306734196	0.393529656998
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae;g__Actinomyces	0.00135052840633	0.0029927218305	0.0007561180471	0.00076093400	0.397741816481
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae;g__Mobiluncus	0.0	0.0	6.69205498487e-0	3.0666848523e-	0.328694683236
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae;Other	8.11862216266e-06	3.63075820787e-0	1.14601576009e-0	3.95402952758	0.779327775074
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium	0.0	0.0	1.93599782033e-0	6.19242868186	0.166664587406
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Rothia	0.00176396213052	0.0024417216537	0.0011515016907	0.00119222160	0.319681932348
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;Other	1.13932069143e-05	5.09519702841e-0	9.30038158537e-0	4.26197026069	0.887575726976
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium	0.0	0.0	9.88791455577e-0	4.53121169171	0.328694683236
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;Other	1.19001803353e-05	5.32192243486e-0	4.94395727786e-0	0.00022656058	0.467717169394
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Pseudonocardiaceae;Other	5.541525337e-06	2.47824547051e-0	0.0	0.0	0.329256577172
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;Other;Other	0.00162899781731	0.0014697733889	0.0009481378769	0.00115308916	0.108253378165
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae;g__Bifidobacterium	2.01925163414	2.50158002552	0.814028182379	0.85178032713	0.0518285632369
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae;g__Gardnerella	0.0	0.0	9.83651708605e-0	4.50765841215	0.328694683236
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae;g__Scardovia	0.00010581519747	0.0003134925511	4.39229024326e-0	0.00014032816	0.425428123562
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae;Other	0.0525178476028	0.0597690144013	0.0175449014721	0.02452893459	0.0222628974815
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;Other;Other	1.44646361663e-05	4.61071159962e-0	2.16029564426e-0	5.7079521418e-	0.66124671048
k__Bacteria;p__Actinobacteria;c__Actinobacteria;Other;Other;Other	0.0198469794083	0.0207020923714	0.0079064807031	0.01042668938	0.0278422293368
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__	0.00960015139762	0.0221901346768	0.0076351770638	0.00953650450	0.717824495599
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Adlercreutzia	0.00592696243857	0.0070601952816	0.0051084295638	0.00642550711	0.700315518845
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Atopobium	0.00284320610239	0.0088542467974	0.0016038095323	0.00279146930	0.555286850051
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Collinsella	0.310995040375	0.571352107253	0.187611833866	0.17532101941	0.364257661338
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Eggerthella	0.000403984967661	0.0005929803288	0.0016751539753	0.00353987982	0.118924131735
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Slackia	0.0250297048226	0.0507522872003	0.0369719571043	0.07168800088	0.540322296424
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;Other	0.124154377928	0.364790947827	0.0719903920371	0.09616013902	0.541877407939
k__Bacteria;p__Actinobacteria;c__Rubrobacteria;o__Rubrobacterales;f__Rubrobacteraceae;g__Rubrobacter	2.51583938049e-05	6.47748711953e-0	2.35941095631e-0	7.00389536787	0.941141748271
k__Bacteria;p__Actinobacteria;Other;Other;Other;Other	0.0324301958237	0.0407965358144	0.016946305552	0.01775055623	0.129842768627
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__	0.0981310622852	0.398402745614	0.14875358967	0.31526870214	0.655443736867

k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Barnesiellaceae];g__	0.528598410945	0.730219822436	0.426260530208	0.47148088729	0.599380104134
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae];g__Butyricimonas	0.0930700674587	0.125804934219	0.126271833091	0.11486765639	0.383450896689
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae];g__Odoribacter	0.182359008927	0.239693126968	0.112909818954	0.09102176067	0.235528151675
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae];Other	0.000173278168294	0.0003119800830	0.0001286053878	0.00021092866	0.596379109668
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__	0.0133432664184	0.0315601282581	0.0050258260460	0.01171093209	0.278260295464
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__[Prevotella]	0.263733515765	0.818534402827	0.412657871778	1.37581998737	0.674532014813
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__CF231	5.67857881072e-05	0.00025395376472	0.0	0.0	0.329256577172
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__Paraprevotella	0.207212741877	0.269918712735	0.227233526465	0.31073757114	0.826550742789
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];Other	0.0137509607207	0.0420304180488	0.0224140256204	0.06991752113	0.631818182766
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__5-7N15	0.000449753061472	0.0017229150833	0.0002230623458	0.00053888094	0.57886681299
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	13.3028227701	11.5893627299	8.44221250222	11.4848560333	0.18500065453
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;Other	0.0305936595122	0.0271554676294	0.024168748989	0.02917900829	0.469407810043
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Dysgonomonas	0.000113238691919	0.00050641882562	0.0	0.0	0.329256577172
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Paludibacter	0.0	0.0	2.17983888811e-0	9.98927670756	0.328694683236
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	0.608127008738	0.528855384966	0.637095721614	0.68371229386	0.879835944457
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Porphyromonas	3.75015781622e-05	0.00010542756465	0.0003141830706	0.00071896792	0.0951613213636
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;Other	0.0133264937185	0.0238716952488	0.0172073541733	0.02528091783	0.61587575656
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella	8.21507957766	12.14178852	8.14134968141	14.8846362606	0.986190849444
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;Other	0.061826877514	0.0867303318068	0.0523213586282	0.08246769810	0.721243867681
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__	2.18425517486	1.66690696757	1.90082276698	1.79090060799	0.602534889008
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__AF12	5.541525337e-06	2.47824547051e-0	1.14200227259e-0	5.23331185794	0.646595665131
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Rikenella	0.0	0.0	1.17892274755e-0	5.40250272915	0.328694683236
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;Other	0.0651767821732	0.0684288884568	0.0710397857212	0.08224785966	0.804941267269
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7;g__	0.0220187825391	0.0860379515158	0.187291135927	0.63732112882	0.251822936791
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;Other;Other	1.30610551601	0.996184130105	1.37503411129	1.23596824758	0.844788940683
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Capnocytophaga	1.73031018982e-05	5.34434583011e-0	0.0	0.0	0.163135183774
k__Bacteria;p__Bacteroidetes;Other;Other;Other;Other	0.0488955633797	0.0406044861008	0.046155502404	0.04128331935	0.831432119588
k__Bacteria;p__Cyanobacteria;c__4C0d-2;o__YS2;f__;g__	0.0806067073747	0.314555150028	0.0357020133035	0.07601451980	0.540672968285
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Stramenopiles;f__;g__	0.0	0.0	6.11837988236e-0	2.80379389414	0.328694683236
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__;g__	0.000445600272642	0.0008604951548	0.0005199220801	0.00039277199	0.726880757474
k__Bacteria;p__Cyanobacteria;Other;Other;Other;Other	3.54775352521e-06	1.58660360996e-0	4.65019079269e-0	2.13098513035	0.851481239767
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Anoxybacillus	0.0	0.0	3.34602749244e-0	1.53334242615	0.328694683236
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;Other	9.18447973553e-06	4.10742420532e-0	1.90642984934e-0	6.15734820071	0.547556860365

k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus	2.06673447438e-05	6.42933251005e-0	0.0	0.0	0.166014407803
k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__Gemellaceae;g__	0.000977700752275	0.0029023815433	0.0002109962299	0.00036798876	0.254394590233
k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__Gemellaceae;g__Gemella	8.71066860915e-06	3.8955294279e-05	0.0	0.0	0.329256577172
k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__Gemellaceae;Other	0.000295570129832	0.0006916888181	6.29859355873e-0	0.00010506251	0.151823390024
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__;g__	0.000620100590939	0.0024022104338	0.0001048706791	0.00021381431	0.350519377398
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae;g__Abiotrophia	0.000378313796367	0.0009781404246	2.14982400782e-0	7.01943219193	0.11915299035
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae;g__Carnobacterium	0.000300552057512	0.0013037872423	6.11837988236e-0	2.80379389414	0.324680561978
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae;g__Granulicatella	0.00159832067839	0.0035902795929	0.0005546616198	0.00063646060	0.214039674929
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae;Other	7.11808265619e-06	3.18330333774e-0	0.0	0.0	0.329256577172
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Enterococcus	0.000204042291176	0.0006756576637	2.06629892015e-0	5.35704435078	0.240162303828
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Vagococcus	1.13932069143e-05	5.09519702841e-0	0.0	0.0	0.329256577172
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;Other	0.00014971746697	0.0003213774027	0.0013872305861	0.00436041994	0.208588661688
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__	0.0	0.0	1.79152012806e-0	5.66984507321	0.162391115354
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	0.00267462464595	0.0055940894851	0.0029942389658	0.00450104132	0.841789189859
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;Other	0.0	0.0	4.65019079269e-0	2.13098513035	0.328694683236
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae;g__Leuconostoc	1.91363281692e-05	6.62878640439e-0	1.35637273287e-0	4.37931967446	0.753927441683
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae;g__Weissella	0.0	0.0	4.35967777621e-0	0.00019978553	0.328694683236
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae;Other	6.5533366276e-05	0.0002551851150	9.00135030473e-0	0.00024658956	0.756541411861
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__	5.88868378684e-05	0.0001109087451	1.39505723781e-0	6.39295539104	0.124228230377
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Lactococcus	0.00628294891678	0.0220672619096	0.0034204994621	0.00616483957	0.581120152647
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus	0.284971859597	0.628468484225	0.052718489029	0.06828789171	0.115570312828
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;Other	0.00324509245731	0.0080956526846	0.0006167727065	0.00078331853	0.163541448724
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;Other;Other	0.00215540163981	0.0044808253431	0.0007594559233	0.00076211275	0.183729049859
k__Bacteria;p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae;g__Turicibacter	0.00124615815144	0.0021285911493	0.001252478907	0.00222158510	0.992622014958
k__Bacteria;p__Firmicutes;c__Bacilli;Other;Other;Other	0.00132916746418	0.0023825007440	0.0003316430552	0.00037039768	0.0783038799828
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__	0.487659525695	0.719011491063	0.578688877437	0.77717526240	0.698901159555
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Acidaminobacteraceae];g__	5.541525337e-06	2.47824547051e-0	0.0	0.0	0.329256577172
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__	0.00897255416352	0.0118088909499	0.0076899988556	0.00540126809	0.6608217343
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__Mogibacterium	0.000585740499885	0.0009165557131	0.0003989105809	0.00081160703	0.494354623995
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];Other	0.0182590762008	0.0157750324582	0.0154983122611	0.01185429631	0.531821933783
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__1-68	0.000170904420611	0.0003470060252	0.0001917485208	0.00051847204	0.880067009318
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Anaerococcus	0.0	0.0	3.49082479528e-0	0.00011789887	0.189239780316
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Finegoldia	0.0	0.0	2.5685662945e-05	6.50881165081	0.0848848123702

k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Parvimonas	9.92591456489e-05	0.0002344153038	0.0002867331578	0.00037462467	0.061558705138
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Peptoniphilus	7.19650148673e-05	0.0001035790289	7.46435701336e-0	0.00014944513	0.946965273841
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__ph2	1.08364134484e-05	4.84619142058e-0	3.54693528895e-0	7.59469630544	0.221558118793
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__WAL_1855D	0.000444196533544	0.0009137418218	0.0010589421175	0.00164718471	0.146512385418
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];Other	3.53909483419e-05	9.51642105355e-0	5.4924874127e-05	0.00016657871	0.645801847149
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae;g__	0.676052523255	1.48178681376	1.64191710517	3.05474042589	0.204127828921
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae;g__Christensenella	0.000982663231392	0.0010352868912	0.0015816443966	0.00380062475	0.493277015262
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae;Other	0.0153952588712	0.0127844570802	0.029672318928	0.02319696126	0.0194870233008
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__02d06	0.0989501738955	0.365855159963	0.0744184212437	0.12403761860	0.778287697088
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium	0.0592595711518	0.0567421621784	0.0863896246436	0.07982631614	0.215748955662
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Sarcina	7.38889941453e-05	0.0001451077422	0.0001211937446	0.00040560697	0.619950752376
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__SMB53	0.0478027996472	0.073516993804	0.0542688146595	0.08549521148	0.796150069419
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;Other	0.0219908476694	0.0709284699244	0.02103900364	0.03849004082	0.958048345446
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae;g__	0.0	0.0	1.51716795474e-0	4.86607004031	0.167774312226
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae;g__Dehalobacterium	0.000799522011613	0.0011618424867	0.0014419610649	0.00187473036	0.193464231954
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae;Other	7.316672831e-05	0.0001596042546	0.0002041634088	0.00030813870	0.0949912968278
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__EtOH8;g__	0.00232043326913	0.0084535422276	0.0013271293374	0.00213268612	0.614866405957
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__Anaerofustis	0.000151026494225	0.0003012178732	9.83632184873e-0	0.00024199827	0.54198460455
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__Pseudoramibacter_Eubacterium	8.17946263471e-05	0.0002149811688	0.0001305709840	0.00034001962	0.584582733868
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;Other	0.0	0.0	3.9551658223e-05	0.00018124846	0.328694683236
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__	0.000517436194881	0.0005944175591	0.0007780389865	0.00106836799	0.338533945191
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__[Ruminococcus]	0.000746258961009	0.0007891070966	0.0006982693296	0.00064870240	0.833108735476
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Anaerostipes	0.091818906388	0.093603661359	0.176689558677	0.16875057723	0.0533363295922
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia	0.490342116759	0.23603042408	0.964436343121	0.96626519698	0.0393710547528
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Catonella	0.000165269077913	0.0004824919807	4.65780186992e-0	0.00010150906	0.293033499521
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus	0.0616001073121	0.0439313492951	0.119562588503	0.08716913073	0.0108802452198
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Dorea	0.870563655018	1.2243592429	0.999982341666	1.20057327223	0.734453272758
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Epulopiscium	0.00171032844219	0.006987576059	0.0001081745819	0.00031750831	0.317834439488
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Lachnobacterium	0.000216301989611	0.0002803846111	0.0001834406518	0.00020043417	0.669847795074
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Lachnospira	5.14751544592	3.03954350408	7.54356601113	6.4597548304	0.13616107248
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Moryella	0.000294081136015	0.0004644752693	0.0005975569256	0.00108233414	0.249150134524
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Pseudobutyrivibrio	10.435673341	9.02433695956	9.79525384076	6.50033806697	0.796612971739
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia	0.128526398911	0.134080281861	0.131178283694	0.19459535056	0.95958439705

k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Shuttleworthia	0.000513795993256	0.0006279394010	0.0008515155266	0.00094443208	0.18393414035
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;Other	20.4047891791	8.49607245356	25.1287943706	9.02263459595	0.0917682085999
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__	0.00356200143562	0.0054918249814	0.0031618733871	0.00401641785	0.792335590985
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__Peptococcus	5.73614859358e-05	0.0001436257352	0.0008098049801	0.00303381999	0.269011813217
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__rc4-4	0.000349100752994	0.0015612260293	0.0014360575831	0.00344291352	0.199593403657
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;Other	0.000767588153143	0.0017602880618	0.0007543168900	0.00138232302	0.978790114189
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__Filifactor	5.541525337e-06	2.47824547051e-0	2.28379232914e-0	6.09447244573	0.239987156497
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__Peptostreptococcus	0.000119254544177	0.0002449545833	0.0002074462352	0.00026811993	0.277593297272
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__	0.00938062015775	0.0087430948873	0.0149286885837	0.01720020631	0.199342713623
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Anaerofilum	0.000618205179853	0.0008577090662	0.0006630004012	0.00071015921	0.856785595389
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Anaerotruncus	0.00509864212571	0.0050764882280	0.0081453789310	0.00864965498	0.17553505661
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Faecalibacterium	9.82437926058	6.07716768887	6.26946580436	3.08565772781	0.0260512243331
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira	2.6316201151	1.64925425249	3.00000178233	1.80940283797	0.49918558156
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus	0.540854833688	0.382723536687	0.994532712062	1.17587983804	0.105668900043
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;Other	2.22101067595	1.08488185722	2.16778060476	1.38255112055	0.891333594752
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__	4.9001628569e-06	2.19141944977e-0	0.0	0.0	0.329256577172
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Acidaminococcus	0.0731372106295	0.222192806525	0.0101376647051	0.03027777971	0.222767614199
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Dialister	0.687612438468	1.21073109084	1.07734526299	1.96432880674	0.447182980153
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Megamonas	0.00182731283365	0.0077577744229	0.0531286070285	0.23222053998	0.323136448944
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Megasphaera	0.114681266956	0.351592282144	0.0944435005998	0.42035027742	0.867799344839
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Mitsuokella	0.0240911612489	0.0837931461882	0.0689826974748	0.26319815187	0.464254946747
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Phascolarctobacterium	0.445080546436	0.6347110055	0.176150006976	0.26286093216	0.0905463509135
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Selenomonas	1.38903338131e-05	4.48172823063e-0	4.65019079269e-0	2.13098513035	0.409950797352
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Succiniclasticum	0.117203158171	0.518424714217	0.155218624331	0.66453778768	0.838807245122
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella	0.0303473661734	0.0763406417514	0.0094565719483	0.02538991390	0.255768464424
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;Other	0.0258423955258	0.0260683207139	0.0630469550246	0.12952141194	0.210266715683
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other	3.70829251349	1.99835068123	3.98901320884	2.19097698098	0.67019925473
k__Bacteria;p__Firmicutes;c__Clostridia;o__SHA-98;f__;g__	0.00409273498993	0.0060143996101	0.0063868817447	0.00785251994	0.298701163127
k__Bacteria;p__Firmicutes;c__Clostridia;Other;Other;Other	0.0890575433179	0.0539355187749	0.125580238723	0.09938286016	0.150630829796
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__	0.233788928803	0.512150274128	0.221255131894	0.20096705868	0.919382623469
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__[Eubacterium]	0.102035033303	0.241823429478	0.177865749971	0.39781507779	0.463378046984
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Bulleidia	0.00114534400206	0.0042033337660	0.0085631865798	0.03681209020	0.369199160945
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Catenibacterium	0.635973349579	1.74337039049	0.376771003061	0.97904712273	0.564034050275

k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__cc_115	0.00217353109548	0.00414214752756	0.0079432726953	0.02805791690	0.361599271184
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Coprobacillus	0.000843922905819	0.00252046866586	0.0019275768507	0.00380631955	0.287382237893
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Holdemania	0.00262051698864	0.00369006424082	0.0038110490428	0.00591543686	0.442259347152
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__p-75-a5	0.000123035874232	0.00055023315692	0.0	0.0	0.329256577172
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;Other	0.0274110205583	0.061380256293	0.0206151722949	0.02101961697	0.64279781878
k__Bacteria;p__Firmicutes;Other;Other;Other;Other	0.425637988218	0.243563863663	0.4739136984	0.31485096949	0.584999941646
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__	1.89285960358e-05	8.46512549092e-0	0.0	0.0	0.329256577172
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__Fusobacterium	0.000125859799629	0.00033012659913	9.97101961036e-0	0.00014252701	0.746608692842
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;Other	0.00320158685283	0.0142166871269	2.62035221398e-0	7.36226754621	0.329791656726
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae;g__Leptotrichia	6.59032434938e-06	2.9472826478e-05	3.07742742982e-0	6.64801276322	0.139807477894
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae;g__Sneathia	1.8368959471e-05	8.21484841062e-0	6.11837988236e-0	2.80379389414	0.532788825788
k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];o__Victivallales;f__Victivallaceae;g__	0.014201538928	0.0268342559032	0.0188464660339	0.02558737109	0.574032994658
k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];o__Z20;f__R4-45B;g__	5.67857881072e-05	0.00025395376472	0.0	0.0	0.329256577172
k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];Other;Other;Other	0.000432432944378	0.00109677597584	0.0004903521358	0.00120843479	0.873015750051
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__RF32;f__;g__	0.320804279178	0.6161877458	0.476346892463	0.99396884453	0.548843566386
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium	0.0	0.0	1.57973366006e-0	7.23924907511	0.328694683236
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;Other	1.79803419124e-05	8.04105335495e-0	2.41470733211e-0	6.18907363103	0.785413078338
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae;g__Acetobacter	0.0	0.0	4.65019079269e-0	2.13098513035	0.328694683236
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__mitochondria;g__	5.02286189652e-05	0.00013011684802	5.31323151469e-0	0.00010985248	0.93899610554
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingobium	0.0	0.0	4.13881965333e-0	1.89664543492	0.328694683236
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;Other;Other;Other	0.0159064343177	0.0379254237949	0.0219057438374	0.03857815248	0.618312792952
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__Sutterella	0.85603665806	0.928969055061	0.432293830865	0.39142678992	0.0701630024984
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;Other	0.000718104275584	0.00140698460264	0.0002484630033	0.00045733710	0.167638541754
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Lautropia	0.000214868627368	0.00074454452812	9.83651708605e-0	4.50765841215	0.233096002204
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Aquabacterium	0.0	0.0	1.14200227259e-0	5.23331185794	0.328694683236
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Comamonas	0.0	0.0	3.3498418341e-05	0.00012388728	0.228978425484
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;Other	0.000633611264305	0.00164929891204	0.0009610914197	0.00215973845	0.587328194355
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Oxalobacter	0.00142659444804	0.00260358268518	0.0036747187738	0.00557871848	0.106106324066
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;Other	1.79835312637e-05	5.7572397193e-05	4.06520171736e-0	7.89642689819	0.298515957354
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;Other;Other	0.120644962702	0.202810260576	0.146210338752	0.27001277316	0.732819083013
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Eikenella	0.0	0.0	1.60702135185e-0	5.55576681814	0.199238120645
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Neisseria	6.02359538072e-05	0.00014995300744	1.29278300994e-0	4.26149049127	0.187107405068
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;Other	1.01380778746e-05	3.27660938964e-0	9.16860383987e-0	4.20158211132	0.934584041168

k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;Other;Other;Other	0.0258929196894	0.0281622352274	0.0164663519416	0.01317022372(	0.184099585399
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__	0.00220576515232	0.0052668943798:	0.0007334046436!	0.00318326598(	0.289465893899
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__Bilophila	0.0652333042924	0.0669995435658	0.0661163794548	0.06290869800	0.965549571107
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__Desulfovibrio	0.0155373889384	0.0280567029459	0.0845777409764	0.16731036328(	0.0756250724117
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;Other	0.0127852035244	0.0124987351613	0.0478673913025	0.11807918624	0.189935231755
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;Other;Other	0.0011972876583	0.0014698240268(	0.0021159641616(	0.00445818404(	0.379279574861
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;Other;Other;Other	0.000731256532952	0.0007411653190!	0.0032859279084(	0.00775562480(	0.147632370828
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae;g__Campylobacter	0.000388787301383	0.0007170395801(	0.0003468157693(	0.00038613943	0.8183078415
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Helicobacteraceae;g__Flexispira	0.00234203840018	0.0086509638534(	7.89908008891e-(	3.61981324279(	0.241653858292
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Helicobacteraceae;Other	0.000446457851907	0.0016755675189:	1.02127603948e-(	4.68007475635(	0.258134898108
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;Other;Other	4.59495882611e-05	0.0001712530577:	0.0	0.0	0.244185696629
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Succinivibrionaceae;g__Succinivibrio	0.401562679521	1.78882021091	0.183246198956	0.83395678288	0.623209064508
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Succinivibrionaceae;Other	0.0171126737279	0.075286268966	0.0026556979996(	0.01178930547	0.405532348994
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;Other;Other	0.00137232321259	0.0061372159809:	0.0002522611003:	0.00115600558	0.430976141146
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Cardiobacteriales;f__Cardiobacteriaceae;g__Cardiobacterium	2.5819461728e-05	6.33102305203e-0	1.10823388966e-(	5.07856568708(	0.417452878842
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__	0.0437201757473	0.156523801613	0.0187911238428	0.06056173668(	0.51114963722
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Citrobacter	0.000920176324769	0.0026831669758	0.0003903358463(	0.00119361548(	0.424903470269
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Erwinia	0.0	0.0	7.64427576754e-(	3.50304723379(	0.328694683236
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Gluconacetobact	0.00265504483075	0.0118737214497	1.83551396471e-(	8.41138168245(	0.332541734445
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Klebsiella	0.0244093850185	0.0917005501049	0.0007440857228(	0.00225487251(	0.262169479109
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Proteus	1.74213372183e-05	7.79105885581e-0	0.0	0.0	0.329256577172
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Serratia	0.00016319666216	0.0007298376605(	7.64427576754e-(	3.50304723379(	0.352355395192
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Trabulsiella	0.0070496401909	0.0253955383176	0.0007995540676(	0.00201984057(	0.285378381034
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;Other	0.0564547955802	0.176978029022	0.0307610648279	0.11484088814!	0.586907323809
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Actinobacillus	1.22820567984e-05	3.87005700055e-0	4.65019079269e-(	2.13098513035(	0.443172522838
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Aggregatibacter	0.00010275188397	0.0003618895824(	0.0	0.0	0.218738753063
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Haemophilus	0.00701718740906	0.0104349750973	0.0049091674389:	0.00869681232(	0.487541062459
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;Other	0.00209304632993	0.0030930815449(	0.0013289698059:	0.00196760531(	0.354636151859
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter	2.13542479685e-05	9.5499100132e-05	9.88791455577e-(	4.53121169171(	0.629834883381
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Enhydrobacter	8.11862216266e-06	3.63075820787e-0	0.0	0.0	0.329256577172
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas	0.000529739735078	0.0022573221155:	0.0	0.0	0.30646358819
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;Other	7.11808265619e-06	3.18330333774e-0	5.44959722027e-(	2.49731917689(	0.853344526383
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomo	0.0	0.0	1.14200227259e-(	5.23331185794(	0.328694683236

k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Xanthomonas	0.0	0.0	1.10823388966e-0	5.07856568708e	0.328694683236
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;Other;Other;Other	0.0277851284474	0.0954173615799	0.0151662336304	0.05315484448e	0.607024551219
k__Bacteria;p__Proteobacteria;Other;Other;Other;Other	0.0968118028327	0.317344812022	0.0153139173962	0.01918116553e	0.265026524463
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae;g__Pyramidobacter	8.71218011106e-05	0.0003107596264	0.0001264141846e	0.00039099621e	0.722922466006
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae;g__TG5	4.9001628569e-06	2.19141944977e-0	4.13881965333e-0	1.89664543492e	0.906116077133
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae;g__	0.0	0.0	0.0453876564946	0.20774484525e	0.328131866764
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae;g__Cloacibacillus	0.000284260908259	0.0009329267159	0.0011052562770e	0.00312210181e	0.260168095627
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae;g__Synergistes	0.000110113993422	0.0002021242988e	0.0219736603383	0.09879823355e	0.322077977747
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae;g__vadinCA02	0.0	0.0	8.81118908231e-0	4.03779409323e	0.328694683236
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae;Other	0.00203273831992	0.0031997232354e	0.356032643537	1.59662898925	0.321177336109
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;Other;Other	0.0	0.0	0.0008552308889e	0.00380138779e	0.314278059577
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;f__Anaeroplasmataceae;g__	0.0	0.0	0.0002629863792e	0.00087254866e	0.181742359036
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;f__Anaeroplasmataceae;Other	0.000199041644878	0.0003897323379	0.135238913002	0.43157947866e	0.166331624623
k__Bacteria;p__Tenericutes;c__Mollicutes;o__RF39;f__;g__	0.0366194344298	0.0609080113473	0.0873355760152	0.11471500455e	0.084545900005
k__Bacteria;p__Tenericutes;c__Mollicutes;Other;Other;Other	0.000313760114657	0.0008599026839	0.0036691468505e	0.00985720793e	0.134962592146
k__Bacteria;p__Tenericutes;c__RF3;o__ML615J-28;f__;g__	0.132001716437	0.34907423425	0.0651636325236	0.10320921270e	0.418998774672
k__Bacteria;p__Tenericutes;Other;Other;Other;Other	0.00269675541936	0.0065625017001e	0.0018510947893	0.00295863021e	0.602080136267
k__Bacteria;p__TM7;c__MJK10;o__;f__;g__	3.54775352521e-06	1.58660360996e-0	0.0	0.0	0.329256577172
k__Bacteria;p__TM7;c__TM7-3;o__;f__;g__	0.000307942670211	0.0003376956187	0.0007563302618e	0.00090870900e	0.0439944154055
k__Bacteria;p__TM7;c__TM7-3;o__CW040;f__;g__	0.000174155182328	0.0002495351139	0.0001126179595e	0.00021670975e	0.405209661232
k__Bacteria;p__TM7;c__TM7-3;o__CW040;f__F16;g__	2.62932728544e-05	6.45100846638e-0	1.27536393486e-0	4.29759642879e	0.436675458223
k__Bacteria;p__TM7;c__TM7-3;o__I025;f__Rs-045;g__	8.71066860915e-06	3.8955294279e-05	3.63454032037e-0	7.19638215872e	0.133469544342
k__Bacteria;p__TM7;c__TM7-3;Other;Other;Other	3.95787171336e-05	9.16916459258e-0	5.03766095487e-0	9.98388097317e	0.72000822149
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__[Cerasicoccales];f__[Cerasicoccaceae];g__	0.186124317474	0.61334664883	0.0668684464533	0.11903655911e	0.402359429041
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__[Cerasicoccales];f__[Cerasicoccaceae];Other	3.90252887774e-05	0.0001745263970	4.94556416489e-0	0.00011803161e	0.824782979329
k__Bacteria;p__Verrucomicrobia;c__Opitutae;Other;Other;Other	0.0275076721633	0.0796864029891	0.0197462372606	0.03265972889e	0.689167721021
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__Akkermansia	0.0319329271217	0.058257694961	0.322890242782	0.82567183568e	0.122033949507
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;Other	5.79943839182e-05	0.0001715413946	0.0001551785297e	0.00032230044e	0.233873243557
k__Bacteria;p__Verrucomicrobia;Other;Other;Other;Other	0.00423980501225	0.0110773396388	0.0055476058841e	0.00846763086e	0.674571089891
Unassignable;Other;Other;Other;Other;Other	0.0	0.0	4.13881965333e-0	1.89664543492e	0.328694683236
Unclassified;Other;Other;Other;Other;Other	0.0441853738679	0.0988543585404	0.0323757799091	0.04745754633e	0.632234161193

INFORMACIÓN SUPLEMENTARIA ARTÍCULO:

Rojo D, Hevia A, Bargiela R, López P, Cuervo A, González S, Suárez A, Sánchez B, Martínez-Martínez M, Milani C, Ventura M, Barbas C, Moya A, Suárez A, Margolles A, Ferrer M. Ranking the impact of human health disorders on gut metabolism: Systemic lupus erythematosus and obesity as study cases. **Scientific Reports**, 2015; 5:8310.

Supplementary Table 1 Demographics and clinical features of the SLE patients.

Total SLE patients*	**n=18**
Age at diagnosis, median yearts (IQR)	35.00 (15.00)
Disease duration, median years (IQR)	7.00 (9.00)
Clinical manifestations, n (%)	
Malar rash	9 (50.0)
Discoid lesions	6 (33.3)
Photosensitivity	14 (77.8)
Oral ulcers	9 (50.0)
Arthritis	10 (55.6)
Serositis	3 (16.7)
Renal disorder	3 (16.7)
Neurological disorder	0 (0.0)
Haematological disorder	9 (50.0)
Anti-dsDNA, n (%)	9 (50.0)
Titer, median U/ml (IQR)	10.35 (36.45)

*dsDNA: double stranded DNA; IQR: interquartile range

Supplementary Table 2 Summarized general and disease characteristics of the SLE patients and HC individuals. Full details for each subject are provided in Supplementary Table 3.

	SLE patients (n=18)	**HC subjects (n=17)**
Female sex (%)	100	100
Age (year) (mean ± sd)	49.1 ± 9.7	48.5 ± 8.0
Smokers (%)	29	24
Habitual alcohol consumers (%)	35	59
Regular physical activity (%)	35	59
Compsumption of fermented foods (%)	12	35
Use of vitamins and mineral supplements (%)	18	41

Supplementary Table 3 Detailed characteristics of the SLE patients and HC individuals. BMI values are included. Numerical code for column "Health": 0, excellent; 1, good; 2, normal; 3, regular; 4, bad. Numerical code for column "Smoke": 0, no smoke; 1, smoker; 2, ex-smoker.

Code	Age	Sex	BMI (kg/m2)	Medication (relevant for SLE)	Type of medication	Supplements	Type of supplements	Fiber	Health	Smoke	Cigarretes	Alcohol	Diet (type of diet)
HC4	54	WOMEN	26.29	NO		YES	Onagra, isoflavonas, melisa, magnesio	NO	2	0		NO	NO
HC6	53	WOMEN	27.19	NO		NO		NO	0	2		NO	NO
HC8	56	WOMEN	27.40	NO		NO		NO	0	2		YES	YES (hypocaloric)
HC11	36	WOMEN	20.19	NO		NO		NO	2	1	2,00	YES	NO
HC13	46	WOMEN	24.83	NO		NO		NO	3	1	20,00	NO	NO
HC14	59	WOMEN	24.80	NO		NO		NO	2	0		NO	NO
HC16	43	WOMEN	22.18	NO		YES	Calcium	NO	1	1	15,00	YES	NO
HC19	44	WOMEN	25.24	NO		NO		NO	1	0		NO	NO
HC20	58	WOMEN	28.82	NO		NO		NO	2	2		YES	NO
HC21	52	WOMEN	23.18	NO		YES	Primrose oil (2 capsule/day)	NO	1	0		YES	YES (caloric restriction)
HC22	41	WOMEN	23.07	NO		YES	Vitamin A and E (1/year)	NO	1	0		YES	NO
HC26	42	WOMEN	30.92	NO		NO		NO	2	0		NO	YES (hypocaloric - 1700 kcal))
HC28	40	WOMEN	21.90	NO		NO		NO	1	1	1,50	YES	NO
HC29	47	WOMEN	36.90	NO		NO		NO	1	2		YES	NO
HC30	63	WOMEN	22.68	NO		YES	Vitamin D (hidroferol 1/month)	NO	1	0		YES	NO
HC32	39	WOMEN	23.13	NO		YES	Iron (1 pill ferritin/day)	NO	3	0		NO	NO
HC33	52	WOMEN	25.95	NO		YES	Primrose oil (2 capsule/day)	NO	1	2		YES	NO
SLE1	43	WOMEN	21.99	YES	Hydroxychloroquine	YES	Vegetable laxative (Fave de fuca), Chelidon, Heliocare	NO	3	1	13,50	NO	NO
SLE2	68	WOMEN	37.91	YES	Hydroxychloroquine	NO		NO	3	0		NO	NO
SLE4	34	WOMEN	24.09	YES	Hydroxychloroquine	NO		NO	3	2		YES	NO
SLE5	50	WOMEN	35.43	YES	Hydroxychloroquine	YES	Soya-bean leciti	NO	4	2		NO	NO
SLE6	35	WOMEN	25.07	YES	Hydroxychloroquine	NO		NO	3	0			NO
SLE7	70	WOMEN	24.05	YES	Hydroxychloroquine	YES	icapsR, calcium sandoz	NO	2	0		NO	NO
SLE11	54	WOMEN	29.29	YES	Hydroxychloroquine	NO		NO	2	2		NO	YES hypocaloric)
SLE12	58	WOMEN	26.05	YES	Hydroxychloroquine	NO		NO	3	1	20,00	YES	NO
SLE13	40	WOMEN	32.16	YES	Hydroxychloroquine	YES	Vitamin D	NO	3	2		YES	NO
SLE14	40	WOMEN	20.93	YES	Hydroxychloroquine	NO		NO	2	2		YES	NO
SLE15	51	WOMEN	19.95	YES	Hydroxychloroquine	NO		NO	2	1	20,00	YES	NO
SLE16	54	WOMEN	27.17	NO		NO		NO	3	0		NO	NO
SLE17	46	WOMEN	25.23	YES	Hydroxychloroquine	NO		NO	2	1	11,00	NO	NO
SLE18	43	WOMEN	21.15	NO		NO		NO	3	1	40,00	YES	NO
SLE20	51	WOMEN	20.19	YES	Hydroxychloroquine	NO		NO	4	0		NO	NO
SLE21	59	WOMEN	33.99	YES	Hydroxychloroquine	NO		NO	3	0		NO	NO
SLE22	64	WOMEN	27.00	YES	Hydroxychloroquine	NO		NO	4	2		NO	YES (no vegetables)
SLE 26	46	WOMEN	27.22	YES	Hydroxychloroquine	NO		NO	2	0		NO	YES (hypocaloric)

Supplementary Table 3 cont. Detailed characteristics of the SLE patients and HC individuals. BMI values are included. Numerical code for column "Health": 0, excellent; 1, good; 2, normal; 3, regular; 4, bad. Numerical code for column "Smoke": 0, no smoke; 1, smoker; 2, ex-smoker.

Code	Menarche	Childrens	Breastfeeding	Time of breastfeeding	Menopause	Age of menopause	Leucocites	Red blood cells	Hemoglobine	Hematocrit	V.C.M	H.C.M	C.H.C.M
HC4	9	1	YES	3,00	1,00	48	3,45	4,67	14,40	45,60	97,50	30,80	31,60
HC6	16	2	YES	1,00	1,00		3,34	4,77	13,60	41,00	86,00	28,60	33,30
HC8	15	2	YES	6,00	1,00		6,37	4,61	14,20	42,10	91,50	30,90	33,70
HC11	11	0			0,00		10,28	4,45	13,00	39,30	88,30	29,30	33,20
HC13	14	2	NO		2,00		8,03	3,97	13,10	37,10	93,40	32,90	35,20
HC14	14	1	YES	3,50	1,00	52	3,31	4,62	13,30	36,70	79,50	28,80	36,30
HC16	12	0			2,00		7,25	4,68	14,50	43,20	92,40	31,00	33,50
HC19	17	3	YES	12,00	0,00		4,15	3,67	11,50	34,00	92,70	31,20	33,70
HC20	14	3	YES	12,00	1,00	2	6,05	4,92	14,60	44,00	89,40	29,70	33,20
HC21	11	3	YES	7,00	2,00		4,62	5,30	15,50	46,60	87,90	29,30	33,30
HC22	14	0			0,00		7,35	4,88	15,00	44,20	90,60	30,70	33,90
HC26	11	0			0,00	0	5,52	5,05	14,50	42,40	84,00	28,70	34,10
HC28	14	2	YES	8,50	0,00		4,99	4,93	14,40	43,00	87,20	29,30	33,60
HC29	12	2	YES	4	0,00		6,27	4,64	14,00	41,70	89,90	30,20	33,60
HC30	13	2	YES	6,00	1,00		6,90	4,91	15,60	45,70	93,20	31,70	34,00
HC32	13	2	YES	10,00	0,00		5,20	4,54	14,00	41,00	90,20	30,90	34,20
HC33	11	2	YES	10,00	0,00		4,04	4,64	14,10	41,30	88,90	30,40	34,20
SLE1	13						2,90	4,53	13,70	39,10	86,20	30,20	35,00
SLE2	11	3	YES	12,00	1,00	55	3,62	5,05	13,70	41,50	82,30	27,20	33,00
SLE4	14	1	YES	3,00	0,00		4,69	4,74	12,90	41,30	87,10	27,30	31,30
SLE5	13	4	YES	5,00	2,00		2,22	4,70	12,10	38,80	82,50	25,70	31,20
SLE6	12	1	NO		0,00		3,92	4,28	12,40	40,00	93,30	29,00	31,10
SLE7	12	4	YES	16,00	1,00	50	3,56	4,62	13,50	42,70	92,60	29,30	31,60
SLE11	12				1,00	49	5,55	4,88	13,50	41,20	84,30	27,60	32,70
SLE12	12	2	YES	1,50	1,00		3,17	3,96	13,20	39,70	100,40	33,30	33,20
SLE13	14	0			1,00	40	5,51	4,94	13,20	39,40	79,70	26,80	33,60
SLE14	11	0					4,08	4,11	12,90	38,30	93,20	31,30	33,60
SLE15	13	2	NO		1,00	46	5,92	4,61	15,60	46,50	100,90	33,90	33,60
SLE16	12	3	YES	8,00	1,00	48	4,39	4,78	14,20	43,20	90,50	29,60	32,80
SLE17	11	1	NO		2,00		6,87	4,43	14,10	41,10	92,80	31,99	34,40
SLE18	13	2	YES	3,00	0,00		3,61	4,15	13,30	39,10	94,20	32,10	34,00
SLE20	11	1	NO		1,00	45	4,62	4,40	12,90	38,30	87,00	29,40	33,80
SLE21	13	0			1,00	53	5,24	4,50	13,50	38,10	84,60	30,00	35,40
SLE22	10	0			1,00	43	3,78	4,28	12,40	36,10	84,20	29,00	34,50
SLE 26	13	2	YES	3,00	2,00		5,77	5,50	16,00	46,50	82,70	28,40	34,30

Supplementary Table 3 cont. Detailed characteristics of the SLE patients and HC individuals. BMI values are included. Numerical code for column "Health": 0, excellent; 1, good; 2, normal; 3, regular; 4, bad. Numerical code for column "Smoke": 0, no smoke; 1, smoker; 2, ex-smoker.

Code	R.D.W	Plaquets	V.P.M	P.D.W	P.T.C	Neutrofils	Lymphocytes	Monocytes	Eosinophils	Basophils	L.U.C	Neutrofils_Abs	Lymphocytes_Abs
HC4	13,80	245,00	8,00	61,30	0,19	46,30	44,10	4,50	2,10	1,10	1,90	1,59	1,52
HC6	15,00	247,00	7,40	56,60	0,18	39,70	49,60	6,00	1,30	0,50	2,70	1,33	1,66
HC8	12,90	296,00	8,80	53,80	0,26	46,10	44,20	5,50	2,10	0,30	1,80	2,93	2,81
HC11	13,30	188,00	8,70	55,50	0,16	69,40	23,20	3,50	2,80	0,30	0,90	2,13	2,39
HC13	14,30	267,00	7,30	56,20	0,19	73,40	17,00	6,60	1,90	0,20	0,90	5,89	1,36
HC14	13,40	210,00	6,50	53,90	0,13	49,40	37,70	4,20	5,60	0,40	2,60	1,63	1,25
HC16	12,20	376,00	9,00	59,30	0,33	58,10	32,80	4,60	1,60	0,30	2,50	4,21	2,38
HC19	12,40	242,00	8,10	41,40	0,19	51,50	34,50	4,60	6,50	0,60	2,30	2,14	1,43
HC20						55,70	32,00	7,10	4,20	1,02			
HC21						53,60	36,10	7,30	2,33	0,66			
HC22						3,99	32,70	8,39	3,41	1,18			
HC26						65,50	23,70	6,21	3,75	0,81			
HC28						62,70	23,70	7,66	4,78	1,18			
HC29	.	289,00	.	.	.	65,70	24,80	6,65	1,53	1,30	.	.	.
HC30						55,60	37,10	4,62	1,79	0,88			
HC32	12,60	268,00	7,40	52,40	0,19	66,30	23,70	5,70	2,10	0,70	1,50	3,45	1,23
HC33	12,80	304,00	7,10	50,90	0,21	53,90	30,30	5,00	7,80	0,90	2,10	2,18	1,22
SLE1	14,80	181,00	8,70	48,70	0,15	56,70	32,60	8,10	0,30	0,30	2,10	1,64	0,94
SLE2	13,70	182,00	8,40	61,10	0,15	68,90	22,20	5,50	0,90	0,20	2,20	2,50	0,80
SLE4	14,40	219,00	8,40	53,30	0,18	69,50	21,00	6,00	1,90	0,10	1,50	3,26	0,98
SLE5	14,40	234,00	8,10	57,60	0,18	50,60	33,20	8,70	3,50	1,00	3,00	1,12	0,74
SLE6	14,90	198,00	9,10	92,40	0,18	71,30	18,10	4,80	0,90	0,40	4,40	2,80	0,71
SLE7	14,20	165,00	9,50	55,90	0,15	61,90	26,00	5,90	3,80	0,50	1,90	2,21	0,93
SLE11	13,60	190,00	8,20	52,50	0,15	42,30	45,40	5,90	2,90	0,50	3,00	2,35	2,52
SLE12	13,90	194,00	9,60	69,20	0,18	55,40	34,80	5,30	2,70	0,50	1,30	1,76	1,11
SLE13	12,90	270,00	7,20	51,90	0,19	64,80	23,20	5,90	3,70	1,00	1,40	3,57	1,28
SLE14	12,50	271,00	8,60	61,60	0,23	45,90	40,60	6,20	3,10	0,70	3,40	1,87	1,66
SLE15	12,80	243,00	7,80	55,50	0,18	71,20	19,90	6,10	1,30	0,40	1,00	4,22	1,18
SLE16	13,30	180,00	8,60	62,50	0,15	48,50	42,50	2,70	5,10	0,30	0,90	2,13	1,87
SLE17						64,00	25,70	8,00	0,87	1,39			
SLE18						2,57	17,00	8,30	1,64	0,64			
SLE20						58,70	31,20		6,40	1,22			
SLE21	13,40	268,00	7,00	58,30	0,18	71,80	16,60	6,30	3,30	0,60	1,40	3,76	0,87
SLE22	13,00	204,00	6,40	47,90	0,13	40,80	50,30	6,50	0,10	0,10	2,20	1,54	1,90
SLE 26		187,00				53,90	33,40	6,18	5,02	1,50			

Supplementary Table 3 cont. Detailed characteristics of the SLE patients and HC individuals. BMI values are included. Numerical code for column "Health": 0, excellent; 1, good; 2, normal; 3, regular; 4, bad. Numerical code for column "Smoke": 0, no smoke; 1, smoker; 2, ex-smoker.

Code	Monocytes_Ab	Eosinophils_Abs	Basophils_Abs	L.U.C_Abs	Glucose	Uric acid	Ferritin	Transferrin	Folic acid	Vitamin B12	Triglycerides	Cholesterol	HDL cholesterol	Atherogenic index	LDL cholesterol	Albumin
HC4	0,16	0,07	0,04	0,07	86,00	3,60	67,00	230,00	7,00	287,00	55,00	185,00	80,00	2,30	94,00	4,50
HC6	0,20	0,04	0,02	0,10	93,00	3,70	40,00	256,00	8,40	611,00	69,00	259,00	93,00	2,70	153,00	4,80
HC8	0,35	0,14	0,02	0,12	79,00	6,50	181,00	200,00	9,80	553,00	62,00	184,00	55,00	3,30	116,00	5,20
HC11	0,36	0,29	0,03	0,10	85,00	3,10	108,00	307,00	5,40	568,00	110,00	282,00	70,00	4,00	190,00	4,80
HC13	0,53	0,15	0,02	0,08	80,00	2,90	52,00	196,00	2,30	209,00	44,00	180,00	50,00	3,60	121,00	4,30
HC14	0,14	0,19	0,01	0,08	116,00	4,70	149,00	236,00	11,30	556,00	91,00	227,00	62,00	3,60	146,00	4,90
HC16	0,34	0,12	0,03	0,18	80,00	4,30	36,00	221,00	5,70	351,00	68,00	181,00	62,00	2,90	105,00	4,80
HC19	0,19	0,27	0,02	0,10	96,00	4,50	42,00	243,00	5,80	432,00	31,00	154,00	60,00	2,50	87,00	4,50
HC20					98,00	2,40	173,32	217,00	7,69	490,00	73,00	199,00	71,00	2,80	113,00	4,40
HC21					118,00	2,80	31,73	223,00	10,70	783,00	47,00	190,00	80,00	2,38	101,00	4,90
HC22					88,00	2,50	46,71	227,00	6,75	563,00	60,00	182,00	69,00	2,64	101,00	4,40
HC26					98,00	4,50	37,08	200,00	8,65	466,00	132,00	231,00	48,00	4,81	157,00	4,40
HC28					81,00	3,10	10,67	259,00	4,09	516,00	58,00	166,00	65,00	2,55	89,00	4,40
HC29	.	.	.	.	123,00	3,90	42,62	215,00	6,13	540,00	76,00	191,00	63,00	3,03	113,00	4,40
HC30					91,00	2,70	130,14	204,80	22,30	599,00	120,00	181,00	55,00	3,29	102,00	4,50
HC32	0,30	0,11	0,04	0,08	78,00	3,60	21,00	260,00	4,80	693,00	76,00	284,00	71,00	4,00	197,00	5,60
HC33	0,20	0,32	0,04	0,08	100,00	2,70	66,00	245,00	9,20	584,00	57,00	233,00	56,00	4,10	165,00	5,30
SLE1	0,23	0,01	0,01	0,06	81,00	2,50	16,00	292,00	6,60	263,00	47,00	185,00	76,00	2,40	99,00	5,00
SLE2	0,20	0,03	0,01	0,08	103,00	4,00	8,00	317,00	7,30	231,00	52,00	200,00	81,00	2,40	108,00	4,70
SLE4	0,28	0,09	0,01	0,07	86,00	3,60	9,00	215,00	3,90	270,00	35,00	140,00	78,00	1,70	55,00	4,70
SLE5	0,19	0,08	0,02	0,07	92,00	4,20	7,00	340,00	12,20	541,00	51,00	186,00	64,00	2,90	111,00	4,70
SLE6	0,19	0,04	0,01	0,17	90,00	3,50	17,00	270,00	20,00	350,00	29,00	158,00	69,00	2,20	83,00	5,40
SLE7	0,21	0,14	0,02	0,07	93,00	4,30	108,00	222,00	18,60	544,00	65,00	206,00	51,00	4,00	142,00	4,70
SLE11	0,33	0,16	0,03	0,17	88,00	4,70	53,00	252,00	4,30	349,00	56,00	158,00	47,00	3,30	99,00	4,60
SLE12	0,17	0,08	0,01	0,04	86,00	3,80	197,00	201,00	9,70	691,00	45,00	215,00	70,00	3,00	136,00	5,00
SLE13	0,32	0,20	0,06	0,08	82,00	3,40	14,00	261,00	5,60	460,00	67,00	220,00	68,00	3,20	138,00	4,50
SLE14	0,25	0,13	0,03	0,14	90,00	3,60	46,00	241,00	7,30	550,00	42,00	226,00	105,00	2,10	112,00	4,20
SLE15	0,36	0,08	0,03	0,06	67,00	5,40	44,00	237,00	12,40	469,00	143,00	274,00	55,00	4,90	190,00	4,90
SLE16	0,12	0,23	0,01	0,04	80,00	6,00	115,00	227,00	6,10	547,00	92,00	222,00	49,00	4,50	154,00	4,70
SLE17					81,00	4,00	91,42	200,00	6,45	352,00	108,00	200,00	41,00	4,88	137,00	4,40
SLE18					90,00	2,30	30,91	228,40	3,24	165,00	86,00	147,00	57,00	2,58	73,00	4,30
SLE20					98,00	2,90	26,68	244,40	8,06	282,00	87,00	182,00	76,00	2,39	89,00	4,50
SLE21	0,33	0,17	0,03	0,07	83,00	4,20	43,00	236,00	4,50	767,00	76,00	218,00	60,00	3,60	142,00	4,70
SLE22	0,24	0,00	0,00	0,08	85,00	3,00	12,00	304,00	5,70	430,00	117,00	241,00	49,00	4,90	168,00	4,90
SLE 26					98,00	3,30	53,92	181,30	3,02	360,00	141,00	172,00	40,00	4,30	104,00	4,10

Supplementary Table 4 List of raw and statistically (designated as ST) significant masses identified and quantified in a metabolome-wide scan of gut microbiota. For differential quantitative metabolomics, we compared the metabolomes of samples by evaluating peak areas from chromatographic peaks. A list of masses identified by LC-MS using positive and negative polarities and CE-MS following alignment are presented for SLE vs. HC and for HC low (HCl) vs. high (HCh) BMI. The technique (LC-MS positive (+) or negative (-) mode or CE-MS), mass error (in ppm), retention time (RT; as ppm@RT), the *p* value calculated using Mann-Whitney *U* test or *t*-test (denoted "*p* *t*-test or Mann-Whitney *U* test") followed by Bonferroni corrections (designated "p Bonf), and the abundance level per sample (SLE or HC) and per group of samples (average [X] for "HC", "SLE", "HCh", or "HCl" groups) are shown. Statistically significant differences (*p* values) per metabolite identified, and pairwise comparisons among mean values (abundance levels) are provided. Panel abbreviations and content as follows: LC+ raw, LC- raw, and CE raw, list of masses identified in LC-MS using positive (+) and negative (-) polarities and CE-MS, respectively, following alignment; LC+ ST SLE vs. HC, LC- ST SLE vs. HC, and CE ST SLE vs. HC, list of differential/statistically significant masses identified in LC-MS using positive and negative polarities and CE-MS, respectively, in the SLE patients compared with HC subjects; LC+ ST HCh vs. HCl, LC- ST HCh vs. HCl, and CE ST HCh vs. HCl, list of differential/statistically significant masses identified in LC-MS using positive and negative polarities and CE-MS, respectively, in the HCh subjects compared with HCl subjects.

Supplementary Table 5 List of putatively identified (ID) masses of molecules that achieved statistical criteria (Supplementary Table 4) and were responsible for samples/groups separations. The technique (LC-MS positive (+) or negative (-) mode or CE-MS), experimental mass (designated "Mass"), retention time (RT), theoretical mass (designated "Designated mass"), mass error (in ppm), putative name and formula, *p* values calculated using Mann-Whitney *U* test or *t*-test (denoted "*p* *t*-test or Mann-Whitney *U* test") followed by Bonferroni corrections (designated "p Bonf), and the abundance level per sample (SLE or HC) and per group of samples (average [X] for "HC", "SLE", "HCh", or "HCl" groups) are presented. Panel abbreviations and content as follows: LC+ ID SLE vs. HC, LC- ID SLE vs. HC, and CE ID SLE vs. HC, list of putatively identified masses in LC-MS using positive (+) and negative (-) polarities and CE-MS, respectively, that significantly differed in the SLE patients compared with HC subjects; LC+ ID HCh vs. HCl, LC- ID HCh vs. HCl, and CE ID HCh vs. HCl, list of putatively identified masses in LC-MS using positive and negative polarities and CE-MS, respectively, that were significantly different in the HCh subjects compared with HCl subjects.

Por falta de espacio se incluye sólo el enlace a estas Tablas Suplementarias:
http://www.nature.com/articles/srep08310#supplementary-information

INFORMACIÓN SUPLEMENTARIA ARTÍCULO:

Hevia A, Milani C, Foroni E, Duranti S, Turroni F, Lugli GA, Sanchez B, Martín R, Gueimonde M, van Sinderen D, Margolles A, Ventura M. Assessing the Fecal Microbiota: An Optimized Ion Torrent 16S rRNA Gene-Based Analysis Protocol. **PLoS One**, 2013; 8:e68739.

Figure 1: Ratio of 16S rRNA gene sequences obtained after the analysis of the artificially contaminated gnotobiotic fecal samples.