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Influencia de la microbiota intestinal en la obesidad

TESIS DOCTORAL

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Dª Yolanda Sanz Herranz, Investigador científico del Instituto de Agroquímica y Tecnología de Alimentos del Consejo Superior de Investigaciones Científicas,

Certifica:

Que la tesis Doctoral titulada **"Influencia de la microbiota intestinal en la obesidad"**, de la que es autora la licenciada Yolanda Arlette Santacruz López, ha sido realizada en el Instituto de Agroquímica y Tecnología de los Alimentos (IATA-CSIC) bajo su dirección y que reúne las condiciones necesarias para optar al grado de Doctor por la Universidad Politécnica de Valencia.

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*"La vida es una unión simbiótica y cooperativa
que permite triunfar a los que se asocian.''*

*Lynn Margulis
(1938-2011)*

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RESUMEN

La obesidad es el resultado del desequilibrio entre la ingesta y el gasto energético, provocando un aumento en el peso y la grasa corporal, así como mayor predisposición a desarrollar enfermedades crónicas. Se ha observado que en el desarrollo de la obesidad no solo interviene la dieta, sino también factores genéticos, endocrinos, psicológicos y ambientales. En las últimas décadas la microbiota intestinal también se ha incluido entre los factores que pueden tener una influencia clave en la obesidad y el metabolismo. La prevalencia de la obesidad especialmente en niños y adolescentes cada vez es mayor, constituyendo uno de los primeros problemas de salud pública a nivel mundial. Por ello, es necesario seguir avanzando en la identificación de los factores que pueden contribuir a la obesidad y en el desarrollo de estrategias alternativas que permitan disminuir su prevalencia y comorbilidades.

El objetivo global de la tesis ha sido la identificación de los cambios en la composición de la microbiota intestinal asociados a la obesidad y la valoración de la función de componentes específicos de la microbiota en la obesidad y las alteraciones metabólicas e inmunológicas asociadas en un modelo animal

El primer objetivo específico fue determinar la influencia de una intervención para el tratamiento de la obesidad sobre la microbiota intestinal de adolescentes obesos y con sobrepeso. Mediante las técnicas de PCR a tiempo real o cuantitativa (q-PCR) e hibridación *in situ* con sondas fluorescentes (FISH), se cuantificaron los principales grupos bacterianos del tracto intestinal.

Los adolescentes que experimentaron mayor perdida de peso, presentaron aumentos de las concentraciones o proporciones relativas de los grupos *Bacteroides* o *Bacteroides - Prevotella* y *Lactobacillus,* así como reducciones del grupo *E. rectale - C.coccoides*. La microbiota de los adolescentes que experimentaron una pérdida de peso significativa se caracterizó por presentar mayores concentraciones iniciales y finales de los grupos *B. fragilis*, *C. leptum* y *B. catenulatum* y menores de los grupos *C. coccoides*, *Lactobacillus*, *Bifidobacterium, B. breve* y *B. bifidum,* lo que sugiere que la microbiota del individuo puede influir en la eficacia de las intervenciones basadas en cambios en el estilo de vida para tratar la obesidad.

En adolescentes obesos se detectó un aumento de la proporción de IgA asociada a bacterias intestinales, relacionado con las proporciones de *C. histolyticum* y *E. rectale - C. coccoides,* que se redujeron tras la intervención para tratar la obesidad, lo que sugiere que algunos componentes de la microbiota posiblemente implicados en la obesidad o sus metabolitos influyen en el sistema inmunitario del hospedador.

La obesidad también se asocia a complicaciones durante el embarazo, incrementando el riesgo de salud del niño al nacer y el de padecer obesidad y otras patologías relacionadas. Por ello se planteó también como objetivo evaluar la composición de la microbiota intestinal de mujeres embarazadas con sobrepeso por qPCR y su relación con parámetros bioquímicos y dietéticos. Las mujeres con sobrepeso presentaron menores concentraciones de los grupos *Staphylococcus, Enterobacteriaceae* y *E. coli,* y reducciones en las de los

géneros *Bifidobacterium* y *Bacteroides*. El aumento de *E. coli* y la reducción de *Bifidobacterium* spp. y *Akkermansia muciniphila* también se asoció a la ganancia excesiva de peso durante el embarazo. Además, los aumentos en la concentración de colesterol sérico se relacionaron con aumentos en *Staphylococcus* spp., los aumentos en el colesterol HDL y reducciones en el colesterol total se relacionaron con aumentos en *Bacteroides* spp., los aumentos en ácido fólico se relacionaron con aumentos en *Bifidobacterium* spp., los niveles de ferritina y transferrina reducida mostraron relaciones opuestas con las concentraciones de enterobacterias y *Bifidobacterium* spp. Por todo ello, se puede afirmar que la microbiota intestinal esta relacionada con el peso corporal, con la ganancia de peso y los parámetros metabólicos durante el embarazo, lo cual puede ser relevante para la salud de la madre y del hijo.

Después de haber estudiado la relación que existe ente la microbiota intestinal y el metabolismo, el siguiente objetivo que se planteó fue estudiar el efecto de la cepa *B. pseudocatenulatum* CECT 7765 como potencial probiótico en un modelo animal de obesidad. En animales con obesidad inducida con una dieta rica en grasa, se observó que la administración de esta bacteria reducía el colesterol, los triglicéridos, la glucosa, la leptina, la esteatosis hepática y el tamaño de los adipocitos con respecto al grupo obeso no tratado con la cepa. Asimismo, mejoró la función de células inmunocompetentes (macrófagos y células dendríticas) y moduló la composición de la microbiota, reduciendo sus propiedades inflamatorias. Por tanto, esta cepa mejora las alteraciones metabólicas e inmunológicas asociadas a la obesidad inducida por la dieta en un modelo murino.

RESUM

L'obesitat és el resultat del desequilibri entre la ingesta i la despesa energètica, provocant un augment en el pes i el greix corporal, així com major predisposició a desenvolupar malalties cròniques. S'ha observat que en el desenvolupament de l'obesitat no solament intervé la dieta, sinó també factors genètics, endocrins, psicològics i ambientals. En les últimes dècades la microbiota intestinal també s'ha inclòs entre els factors que poden tindre una influència clau en l'obesitat i el metabolisme. La prevalença de l'obesitat especialment en xiquets i adolescents cada vegada és major, constituint un dels primers problemes de salut pública a nivell mundial. Per això, és necessari seguir avançant en la identificació dels factors que poden contribuir a l'obesitat i en el desenvolupament d'estratègies alternatives que permeten disminuir la seua prevalença i les malalties associades a l'obesitat.

L'objectiu global de la tesi ha estat la identificació dels canvis en la composició de la microbiota intestinal associats a l'obesitat i la valoració de la funció de components específics de la microbiota en l'obesitat i les alteracions metabòliques i immunològiques associades a un model animal.

El primer objectiu específic va ser determinar la influència d'una intervenció per al tractament de l'obesitat sobre la microbiota intestinal d'adolescents obesos i amb sobrepès. Mitjançant les tècniques de PCR a temps real o quantitativa (q-PCR) i hibridació in situ amb sondes fluorescents (FISH), es van quantificar els principals grups bacterians del tracte intestinal. Els

adolescents que van experimentar major pèrdua de pes, van presentar augments de les concentracions o proporcions relatives dels grups *Bacteroides o Bacteroides-Prevotella i Lactobacillus,* així com reduccions del grup *E. rectale - C. coccoides.* La microbiota dels adolescents que van experimentar una pèrdua de pes significativa es va caracteritzar per presentar majors concentracions inicials i finals dels grups *B. fragilis, C. leptum i B. catenulatum* i menors dels grups *C. coccoides, Lactobacillus, Bifidobacterium, B. breve i B. bifidum,* la qual cosa suggereix que la microbiota de l'individu pot influir en l'eficàcia de les intervencions basades en canvis en l'estil de vida per tractar l'obesitat.

En adolescents obesos es va detectar un augment de la proporció de IgA associada a bacteris intestinals, relacionat amb les proporcions de *C. histolyticum i E. rectale- C. coccoides*, que es van reduir després de la intervenció per tractar l'obesitat, la qual cosa suggereix que alguns components de la microbiota possiblement implicats en l'obesitat o els seus metabòlits influeixen en el sistema immunitari de l'hoste. L'obesitat també s'associa a complicacions durant l'embaràs, incrementant el risc de salut del xiquet en néixer i el de patir obesitat i altres patologies relacionades. Per això es va plantejar també com a objectiu avaluar la composició de la microbiota intestinal de dones embarassades amb sobrepès per qPCR i la seva relació amb paràmetres bioquímics i dietètics. Les dones amb sobrepès van presentar menors concentracions dels grups *Staphylococcus, Enterobacteriaceae i E. coli*, i reduccions en les dels gèneres *Bifidobacterium i Bacteroides*. L'augment d'*E.coli* i la reducció de *Bifidobacterium* spp. i *Akkermansia muciniphila*

també es va associar al guany excessiu de pes durant l'embaràs. A més, els augments en la concentració de colesterol sèric es van relacionar amb augments en *Staphylococcus* spp., els augments en el colesterol HDL i reduccions en el colesterol total es van relacionar amb augments en *Bacteroides* spp., els augments en àcid fòlic es van relacionar amb augments en *Bifidobacterium* spp. i els nivells de ferritina i transferrina reduïda van mostrar relacions oposades amb les concentracions d'enterobacteris i *Bifidobacterium* spp. Per tot això, es pot afirmar que la microbiota intestinal està relacionada amb el pes corporal, amb el guany de pes i els paràmetres metabòlics durant l'embaràs, la qual cosa pot ser rellevant per a la salut de la mare i del fill.

Després d'haver estudiat la relació que existeix entre la microbiota intestinal i el metabolisme, el següent objectiu que es va plantejar va ser estudiar l'efecte del cep *B. pseudocatenulatum* CECT 7765 com a potencial probiòtic en un model animal d'obesitat. En animals amb obesitat induïda amb una dieta rica en greix, es va observar que l'administració d'aquest bacteri reduïa el colesterol, els triglicèrids, la glucosa, la leptina, l'esteatosi hepàtica i la grandària dels adipòcits pel que fa al grup obès no tractat amb el cep. Així mateix, va millorar la funció de cèl·lules inmunocompetents (macròfags i cèl·lules dendrítiques) i va modular la composició de la microbiota, reduint les seues propietats inflamatòries. Per tant, aquest cep millora les alteracions metabòliques i immunològiques associades a l'obesitat induïda per la dieta en un model murino.

SUMMARY

Obesity is the result of imbalance between intake and energy expenditure, causing an increase in weight and body fat and more likely to develop chronic diseases. It has been observed that in the development of obesity not only involving the diet, but genetic, endocrine, psychological and environmental. In recent decades the intestinal microbiota has also been included among the factors that may have a key influence on obesity and metabolism. The prevalence of obesity especially in children and adolescents is increasing, making it one of the first public health problems worldwide. Therefore, further progress is needed in identifying the factors that may contribute to obesity and the development of alternative strategies for reducin the prevalence and comorbidity.

The overall objective of the thesis has been the identification of changes in the composition of the intestinal microbiota associated with obesity and assessment of the role of specific components of the microbiota in obesity and associated metabolic and immunological in an animal model.

The first specific objective was to determine the influence of an intervention for the treatment of obesity on the intestinal microbiota of obese and overweight adolescents. Using the techniques of real time PCR or quantitative (q-PCR) and in situ hybridization with fluorescent probes (FISH), quantified the major groups of bacteria of the intestinal tract. Adolescents who experienced greater weight loss, showed increases in concentrations or relative

proportions of the Bacteroides group and *Bacteroides-Prevotella* and *Lactobacillus*, and reductions in group *E. rectale – C. coccoides*. The microbiota of adolescents who experienced a significant weight loss was characterized by higher initial concentrations and final group *B. fragilis*, *C. leptum* and *B. catenulatum* and lower group *C. coccoides, Lactobacillus, Bifidobacterium, B. breve* and *B. bifidum*, suggesting that the microbiota of the individual can influence the effectiveness of interventions based on changes in lifestyle to treat obesity.

In obese adolescents was an increase in the proportion of IgA associated with intestinal bacteria, related to the proportions of *C. histolyticum* and *E. rectale - C coccoides*, which were reduced after surgery to treat obesity, suggesting that some components of the microbiota possibly involved in obesity or its metabolites influence the host immune system.

Obesity is also associated with complications during pregnancy, increasing the risk of child health at birth and suffer from obesity and other related pathologies. The question arose also as to evaluate the composition of the intestinal microbiota of pregnant women with overweight by qPCR and its relationship with biochemical and dietary parameters. Overweight women had lower concentrations of the groups Staphylococcus, *Enterobacteriaceae* and *E. coli*, and reductions in the genera *Bifidobacterium* and Bacteroides. E. increasing coli and reduction of *Bifidobacterium* spp. and *Akkermansia muciniphila* also associated with excessive weight gain during pregnancy. In addition, increases in serum cholesterol concentration were associated with

increases in Staphylococcus spp., Increases in HDL cholesterol and reductions in total cholesterol were associated with increases in Bacteroides spp., Increases in folic acid were associated with increases in *Bifidobacterium* spp., levels of ferritin and transferrin showed opposing relationships with low levels of *Enterobacteriaceae* and *Bifidobacterium* spp. Therefore, we can say that the intestinal microbiota is related to body weight, with weight gain and metabolic parameters during pregnancy, which may be relevant to the health of the mother and child.

After studying the relationship that exists between the intestinal microbiota and metabolism, the next goal that was raised was to study the effect of strain *B. pseudocatenulatum* CECT 7765 as a potential probiotic in an animal model of obesity. Induced obesity in animals with a diet rich in fat, it was observed that the administration of the bacteria lowered cholesterol, triglycerides, glucose, leptin, hepatic steatosis and adipocyte size compared to untreated obese group with strain. Also improved the function of immunocompetent cells (macrophages and dendritic cells) and modulated the composition of the microbiota, reducing inflammatory properties. Therefore, this strain improved metabolic and immunologic alterations associated with obesity induced by diet in a murine model.

ÍNDICE

1. OBESIDAD

1.1 Prevalencia de la obesidad y su impacto en la salud pública

La obesidad es uno de los principales problemas de salud a nivel mundial, y debido al rápido aumento de su prevalencia en los últimos años, se ha considerado una pandemia (Blancas-Flores, 2009). En los países industrializados ha pasado a ser el principal desorden nutricional (Wang, 2010), y una de las principales causas de muerte y discapacidad, amenazando muchos de los logros sanitarios alcanzados durante el último siglo (Gómez Ambrosi, 2008). Se estima que afecta a 400 millones de individuos y en niños podrían llegar a ser los 15 millones.

Cada vez son más los estudios que se están desarrollando sobre la obesidad debido a que las causas que conducen su aparición son complejas (Angelakis, 2012). La variación de la ganancia de peso sugiere que la obesidad no sólo esta influenciada por la ingesta calórica como antiguamente se consideraba, sino también por factores ambientales, genéticos, neurológicos, endocrinos y la actividad física (Schnitzler, 2012; Angelakis, 2012). La obesidad es consecuencia de un desequilibrio positivo y prolongado entre la ingesta y el gasto energético, que conlleva a un incremento excesivo del peso y grasa corporal (Monereo, 2004), está asociada a un mayor riesgo de sufrir diabetes mellitus tipo 2, hipertensión, hipercolesterolemia, enfermedades asociadas a hígado graso del tipo no alcohólico, artritis, enfermedad de Alzhaimer, algunos tipos de cáncer, mortalidad por enfermedades coronarias

(Ferrante, 2007), además de incrementar el riesgo de apoplejía, problemas respiratorios, apnea del sueño y osteoartritis (Amber, 2007).

La proporción de grasa corporal puede ser medida de forma directa mediante diversas técnicas, como la hidrodensitometría, pletismografía, tomografía computarizada, resonancia magnética, absorciometría dual energética de rayos X o bioimpedanciometría (Martínez, 2010). Dichas técnicas no son comúnmente utilizadas en la práctica diaria para el diagnóstico rutinario de la obesidad, debido a que requieren equipos específicos y especializados, su utilización es más en la investigación. Sin embargo se puede recurrir a técnicas más accesibles, fáciles y reproducibles, basadas en la antropometría, como son el índice de masa corporal (IMC) (Sweeting, 2007), la medición del perímetro de cintura (PC), el cociente de cintura-cadera, la medición de pliegues cutáneos o el índice nutricional (Martínez, 2010).

El índice de masa corporal (IMC), que es la relación del peso en kilogramos sobre la talla al cuadrado en metros, es uno de los principales criterios antropométricos para realizar el diagnóstico del sobrepeso y la obesidad, debido a que el peso está estrechamente relacionado con la grasa corporal (Gómez-Barrado, 2011). La definición de sobrepeso y obesidad según la Sociedad Española para el Estudio de la Obesidad (SEEDO, 2000), en función del IMC se presenta en las tablas 1 y 2.

Tabla 1. Clasificación del Índice de Masa Corporal

IMC (kg/m²)	Estado corporal
< 20	Delgadez
20 – 25	Peso normal
25 – 30	Exceso de peso
30 – 40	Obesidad moderada
> 40	Obesidad mórbida

Fuente: modificada de SEEDO, 2000

Tabla 2. Grados de obesidad

Grado	% :(Peso real / Peso ideal) x100
Normalidad	90 -110
Sobrepeso	110 – 120
Obesidad Leve	120 – 140
Obesidad moderada	140 – 160
Obesidad intensa	160 – 200
Obesidad mórbida	> 200

Fuente: modificada de SEEDO, 2000

Desde la década de los ochentas la Organización Mundial de la Salud (OMS) ha ido advirtiendo de la importancia de abordar el problema de la obesidad de forma prioritaria, Esto hace que el abordaje del tratamiento de la obesidad sea una de las prioridades de la sanidad pública internacional (Angelakis, 2012). Las estimaciones del coste económico que supondría el

tratar las enfermedades crónicas inducidas por el sobrepeso y obesidad, indican que podrían llegar a ser una carga insostenible para los sistemas de salud pública (James, 2010). La OMS, estima que el cuidado de las patologías de los adultos con sobrepeso y obesidad representa el 6% del gasto general en salud de los países europeos (Branka, 2010). Los costes indirectos relacionados con la discapacidad laboral en los individuos y las adaptaciones que debe tener la sociedad para dichos pacientes pueden llegar hasta un 4% del gasto bruto de una nación, como es el caso en China, en donde se estima que doble este porcentaje en 15 años (Popkin, 2007).

1.2 Obesidad Infantil

La obesidad en la edad infantil cada vez más se incrementa debido a los cambios de estilo en las civilizaciones occidentales (Troiano, 1998). El número de ingesta de comida al día, los ayunos prolongados, la baja ingesta de frutas, vegetales y granos, así como el alto consumo de carbohidratos y alimentos fritos, han demostrado una asociación con el sobrepeso y obesidad en escolares (Bernardo, 2012). Algunos autores resaltan la importancia del entorno en su desarrollo, debido a que en este trastorno nutricional influyen arraigadas y profundas normas sociales, difícilmente modificables (Oda, 2008).

Es difícil calcular de forma cuantitativa la prevalencia de la obesidad infantil a nivel mundial, debido a que no existe una definición aceptada internacionalmente (Zhao, 2011). Sin embargo, encuestas realizadas en 144

países en el 2010, indican que 43 millones de niños (de los cuales 35 millones pertenecen a países desarrollados) en edad preescolar, tienen sobrepeso u obesidad, y 92 millones están en riesgo de tener sobrepeso. La prevalencia mundial de la obesidad infantil aumentó de un 4.2% en 1990 a un 6,7% en 2010. De continuar así, se espera que en 2020 aumente a un 9.7% o 60 millones de niños con sobrepeso u obesidad (Raj, 2012).

Se considera que aproximadamente, el 70% de los actuales adolescentes obesos, en edad adulta tendrán obesidad mórbida. Lo cual producirá un aumento en la incidencia en enfermedades crónicas en la siguiente generación adulta y por lo tanto una mayor incidencia de mortandad (Zhao, 2011).

La prevalencia de la obesidad y sobrepeso en España es del 26.3% en individuos de 2-24 años (Aranceta, 2007), y las cifras siguen aumentado. Un estudio realizado en Holanda mostró que solo el 34% de los padres de adolescentes obesos tenían conocimiento de las causas que pueden provocar la obesidad, un 61% acerca del sobrepeso y un 49% de lo que es una dieta saludable (Booij, 2008). Otro estudio realizado en ese mismo país demostró que el 50% de los padres que tenían hijos obesos no reconocían el sobrepeso de sus hijos adolescentes, y al 87% de los padres no se preocupaban por el peso de sus hijos (Jansen, 2006). Los estudios realizados por Rosenbaum y Leibel (1998), con gemelos y hermanos adoptados generaron evidencias de que hay una fuerte influencia genética sobre el desarrollo del peso y composición corporal.

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Cuando se valora el peso de los padres de niños obesos el 50% de ambos progenitores son obesos. El riesgo de obesidad infantil se multiplica por 4 si un padre es obeso y por 8 si ambos padres lo son. Se ha visto que la distribución de la grasa es idéntica de padres a hijos, con lo cual se puede decir que el genotipo influye en esta relación, Dicha relación se confirmó cuando se estudiaron a niños adoptados y gemelos univitelinos y bivitelinos en donde la distribución de grasa que presentaban tenía mayor relación con la carga genética que con la convivencia diaria (Pufal, 2012). Es por ello la necesidad de una máxima divulgación de las buenas prácticas alimentarias y sus consecuencias, así como el saber la clasificación de sobrepeso y obesidad de acuerdo a cada etapa de la vida. Kroon (2010), en un estudio longitudinal identificó que el IMC cambia específicamente entre los 2 y 6 años de edad, siendo uno de los principales condicionantes del sobrepeso en la edad adulta, por lo tanto, el cambio de peso antes de los 2 años es muy importante lo cual se confirmó cuando se observó que el sobrepeso que se presentaba a los 2 años de edad seguía permanente hasta los 30 años, por lo cual se propone que una prevención antes de los 2 años puede ser un factor limitante para evitar la obesidad en adultos.

Recientes estudios epidemiológicos indican que la programación de la obesidad en la adolescencia puede ocurrir como resultado de la nutrición maternal durante el periodo prenatal y otros factores que impactan al ambiente intrauterino (Larson, 2011). David Barker (1989), propuesó la hipótesis del origen fetal de la obesidad, que sugiere que las alteraciones en el estado nutricional de la madre pueden dar como resultado una adaptación metabólica

permanente en el feto, aumentando el riego de padecer enfermedades cardiovasculares, diabetes y obesidad a lo largo de su vida.

La clasificación del IMC es correcta para personas adultas, en donde la correlación peso-grasa esta estrechamente relacionada. Sin embargo, en la población infantil no se debería utilizar dicha clasificación debido a que el porcentaje de grasa corporal con respecto a la talla, no permite discriminar la distribución de la grasa y la masa magra en todo el cuerpo en estas etapas de crecimiento y maduración. Por lo que en individuos menores de 18 años se debe valorar por percentiles según edad, sexo y población (Gómez Barrado, 2011).

Examinando los estándares históricos para definir el sobrepeso en adolescentes se observa que la distribución del IMC cada vez es más desigual. La parte inferior de la distribución se ha desplazado relativamente poco mientras que la parte superior se ha ampliado considerablemente. Este hallazgo sugiere que muchos niños pueden ser más susceptibles genética o socialmente a la influencia de los cambios del entorno. La definición de la obesidad infantil sigue siendo un problema porque la mayoría de las definiciones utilizan una cierta variante del IMC, sin embargo ninguno de los métodos empleados están ampliamente disponibles y/o son fácilmente aplicables a la situación clínica (Gómez-Barrado, 2011). La definición de obesidad en la clínica se realiza indirectamente mediante el IMC y el perímetro de la cintura (PC), recurriendo a puntos de corte fijos para ambos, los cuales están bien establecidos en la edad adulta, pero que subestiman la prevalencia real cuando se usan en la etapa

infanto-juvenil (Reilly, 2011). No existe consenso acerca de los puntos de corte para el sobrepeso y la obesidad en el niño y adolescente. Para el IMC se establece el percentil 95, tal y como recomienda el grupo europeo de obesidad infantil, o en otros estudios el percentil 97, y para el PC está bien establecido el percentil 90 como punto de corte (Gómez Barrado, 2011). Para la población infantil se utiliza el IMC z-score, que se refiere a cuánto se desvía la media del IMC de la población en estudio y en qué dirección respecto de la media de IMC de una distribución normal (Sardón, 2006).

Los principales problemas físicos en la obesidad infantil son complicaciones ortopédicas y apnea del sueño (Zhao, 2011). En cuanto a los problemas psicológicos que se pueden presentar son síntomas depresivos y baja autoestima (Parsons, 1999). Diversos estudios indican que existe una relación entre el sobrepeso u obesidad en la maduración sexual temprana en las niñas, en cuanto a los varones, los resultados son muy divergentes (Bernardo, 2012).

1.3 Etiopatogenia de la obesidad y enfermedades asociadas

A pesar de que la obesidad es un tema de gran interés y cada vez se realizan más estudios sobre ella, aún no es del todo clara su etiología. Su origen se relaciona con un conjunto complejo de factores tanto del tipo biológico, conductuales y ambientales, siendo difícil en cada caso particular valorar la importancia relativa de cada uno de ellos. Según la distribución de grasa corporal, la obesidad se clasifica en central o androide, (comúnmente

llamada tipo manzana), con predominio de tejido graso en la región intrabdominal o periférica, y ginoide (comúnmente llamada tipo pera), con acumulo de tejido adiposo fundamentalmente en la región fémoro-glútea. Esta distribución varía según el género y la raza, siendo en la infancia predominantemente mixta. La diferencia en la distribución de la masa grasa corporal por géneros, fue descrita en 1940 y explicada parcialmente por las diferencias hormonales; los andrógenos se relacionan con un incremento del tejido adiposo visceral, asociándose con factores de riesgo cardiovascular, mientras que los estrógenos se relacionan con el tejido adiposo periférico o subcutáneo (Sweeting, 2007).

Durante la pubertad, acontecen cambios en la distribución de grasa corporal influenciados por la leptina, que inducen la secreción de GnRH (hormona liberadora de gonadotrofinas) y de gonadotropinas. Además, la leptina se correlaciona muy positivamente con el IMC, trasmitiendo información sobre la energía almacenada disponible al cerebro. La concentración sérica de leptina, se incrementa en las mujeres durante la pubertad, pero disminuyen en los varones después de iniciarse la misma, en el estadio 2 de maduración gonadal, originando el característico patrón androide y ginoide de distribución de grasa característico del adolescente y del adulto (Muñoz, 2007).

La mayoría de los niños obesos, presentan dicha enfermedad como consecuencia de una ingesta energética excesiva y/o un gasto calórico reducido y menos del 5% tienen una enfermedad como factor causal de la obesidad, por

ello en la infancia se pueden considerar dos grandes formas de obesidad desde el punto de vista etiológico (Martínez, 2010):

1.- <u>Obesidad nutricional, simple o exógena, idiopática o esencial</u>: Es la más frecuente, un 95% de los casos es de esta naturaleza. Su etiopatogenia no se conoce perfectamente.

2.- <u>Obesidad secundaria o sindrómica</u>: Forma parte de la sintomatología de afecciones conocidas como síndromes dismórficos, endocrinopatías y lesiones del sistema nervioso central.

Las seis lesiones monogénicas principales que producen desordenes endócrinos y que conllevan obesidad en humanos, son:

a) Deficiencia congénita de leptina
b) Defectos en el receptor de leptina
c) Defectos en la pro-hormona convertasa I
d) Deficiencia en la proopiomelanocortina (POMC)
e) Defectos en el receptor de la melanocortina-4
f) Defectos en el receptor gamma-2 en el peroxisoma proliferador activado

A corto plazo el control de la ingesta está principalmente controlado por las vías de señalización que emanan del tracto gastrointestinal, incluidos los péptidos orexigénicos (grelina) que activan las señales de hambre y los

péptidos anorexigénicos (coleocistocinina, péptido similar al glucagón 1 (GLP-1) y péptido tirosina-tirosina (PYY 3-36) que generan señales de saciedad. En el control de la ingesta a largo plazo parece ser primordiales las señales que emanan del tejido adiposo (leptina) y el páncreas (insulina) liberadas en respuesta a la ingesta de alimentos y los depósitos de grasa corporal (Konturek, 2004).

Hasta el momento se ha demostrado la relación de 32 genes del genoma humano con el IMC, sin embargo en un estudio que realizó Speliotes (2010), con 249,796 voluntarios observaron que de 18 loci que tenían relación con la obesidad, la contribución en el IMC de la población solo se presentó en un 2% de los casos. Es por ello que se deben considerar otros factores en el aumento del IMC, no solo el genético.

La obesidad también se considera un estado de inflamación crónica leve, caracterizado por una elevada producción de citoquinas y adipocinas proinflamatorias que contribuyen a alteraciones metabólicas de forma permanente (Sanz, 2009). La concentraciones de mediadores inmunológicos, como el factor de necrosis tumoral alfa (TNF-$\square$), la interleucina IL-6, la IL-1β, la proteína quimioatrayente MCP1 y la leptina, suelen estar elevadas en la sangre periférica de los sujetos obesos, mientras que las de adiponectina, que parece tener efectos antiinflamatorios en la obesidad y mejorar la sensibilidad a la insulina, están reducidas (Tilg, 2006). Se ha visto que TNF-$\square$ está implicado en el desarrollo de la resistencia a la insulina porque incrementa de forma excesiva la fosforilación de serina en el sustrato receptor de la insulina

intracelular-1 (IRS-1), provocando su inactivación (Hotamisligil, 1996). La resistencia a la insulina provoca una hiperinsulinemia y un cúmulo de lípidos en el hígado y en el tejido adiposo (Cani, 2007).

Desde el punto de vista energético, la masa del cuerpo puede ser dividida en dos diferentes compartimentos (Martínez, 2010):

(1) La masa corporal magra, en la cual la energía se almacena como carbohidratos y proteínas.

(2) La masa de grasa corporal, en la cual la energía se almacena como triacilglicerol.

El tejido adiposo es el órgano que más triacilglicerol almacena, en él, los adipocitos tienen una media de 1 microgramo de triacilglicerol por célula. Durante el desarrollo y crecimiento en la infancia, el peso y la composición corporal presentan continuos cambios fisiológicos. En el caso del tejido adiposo, la curva de producción de las células adiposas en la primera etapa de la vida, presentan dos picos máximos: el primero es desde el nacimiento hasta el primer año de vida y el segundo antes de comenzar la pubertad, en donde el incremento de grasa es mayor, ya que podría ser un detonante condicional para el inicio de esta etapa de desarrollo (Wabitsh, 2000).

1.4 Prevención y Tratamiento

Prevención

A nivel internacional, las estrategias preventivas que la OMS ha tomado, fue realizar en mayo de 2004, en la 57ª Asamblea Mundial de la Salud, una "Estrategia Mundial sobre Régimen Alimentario, Actividad Física y Salud". Sus objetivos principales eran "reducir los factores de riesgo y de morbilidad, promover la concienciación y conocimiento general, y fomentar los planes políticos de actuación y seguimiento de la investigación científica".

En 2005, el Ministerio de Salud y Consumo Español ideó la Estrategia para la Nutrición, Actividad física y Prevención de la Obesidad (estrategia NAOS) que tiene como objetivo: "fomentar una alimentación saludable y promover la actividad física para invertir la tendencia ascendente de la prevalencia de la obesidad y con ello, reducir sustancialmente, la morbilidad y mortalidad atribuible a las enfermedades crónicas" (Martínez, 2010). Dentro de este marco se estableció en septiembre de 2005 el código PAOS (Autorregulación de la Publicidad de los Alimentos) dirigida a menores, en el que se subscribieron 33 empresas españolas. Posteriormente, en mayo del 2007, en la Comisión de la Comunidad Europea celebrada en Bruselas, se estableció la estrategia europea sobre nutrición, sobrepeso y obesidad cuyo objetivo es: la promoción de la dieta saludable y la actividad física: una dimensión europea para la prevención del sobrepeso, la obesidad y las enfermedades crónicas. Las intervenciones preventivas sobre la obesidad infantil todavía no han demostrado mejorar la prevalencia de la misma,

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aunque, sí se muestran mejoras en el conocimiento y comportamiento poblacional (Summerbell, 2005).

Las estrategias preventivas deben incluir la recomendación de fomentar hábitos alimentarios y de actividad física adecuados. Estos deben incluir el control por parte de los progenitores del consumo proteico elevado, sobretodo en la etapa inicial de la vida, reducir las comidas de alta densidad energética, ricas en ácidos grasos saturados y trans, "comidas rápidas" y grandes porciones alimentarias, el incremento de fibra, frutas, verduras, y establecer un horario regular de comidas, sin prescindir del desayuno (Cañete, 2007). El éxito de estas medidas dependerá fundamentalmente de la implicación familiar creando un entorno propicio para el niño (McGovem, 2008).

Tratamiento

Los tratamientos tradicionales basados en las dietas hipocalóricas y el aumento de la actividad física han tenido cierto éxito en el control de la obesidad. Sin embargo, por lo general estas estrategias dan lugar a reducciones de peso limitadas y temporales (Sanz, 2009), o solo la perdida de un 10% del peso inicial (Jubbin, 2012). Se sabe que en la obesidad al involucrarse múltiples factores, genéticos, endocrinos, psicológicos y de estilo de vida, se deben planear una estrategia más estructurada y global para que sean efectivas.

El tratamiento de los niños obesos debe ser precoz e individualizado, tras estudiar las comorbilidades que pueden presentarse en la infancia. El tratamiento debe estar basado en partes básicas, las cuales son motivación,

dieta y ejercicio. El clínico debe indagar y conocer los hábitos de vida del niño, que generalmente son el reflejo de los familiares procurando una alimentación saludable, ajustando la energía ingerida a las necesidades reales, y promocionado la actividad física, siendo fundamental para ello el apoyo familiar (Dalmau, 2007). Tanto la hipertrofia del tejido subcutáneo como el incremento de grasa visceral son modificables a través de los cambios nutricionales y la actividad física. Se ha demostrado que la modificación de los hábitos de vida en la infancia, apoyados por la familia, provocan un modesto efecto sobre la obesidad, siendo menor sin la implicación familiar (disminución del IMC 1,5 k/m^2 y 0,4 k/m^2, respectivamente) (Young, 2007).

Con la adopción y mantenimiento de estilos de vida saludables se consigue una mejoría en los parámetros metabólicos, con descenso del tamaño adipocitario y cambios en la expresión génica, descendiendo el estado inflamatorio subyacente a la obesidad (Skilton, 2006). Esto conlleva menor riesgo o mejoría de comorbilidades como la diabetes Mellitus tipo 2, dislipemia (disminuyendo el nivel de triglicéridos y de LDL-colesterol e incrementando el HDL colesterol e Hipertensión arterial) (Rector, 2007). En contraste, la disminución de grasa subcutánea utilizando una liposucción, no altera los niveles plasmáticos de la proteína C reactiva (PCR), interleucina IL-6, TNF-α, ni adiponectina, y por lo tanto, no repercute sobre la sensibilidad a la insulina ni sobre el riesgo cardiovascular (Klein, 2004). Por lo cual no beneficia de manera significativa a las alteraciones metabolicas presentes.

En casos especiales, se puede recurrir a la terapia farmacológica y/o quirúrgica, con unas indicaciones muy precisas en la edad pediátrica. La experiencia en la infancia de fármacos antiobesidad es muy limitada (Martínez, 2010). La Agencia de Administración de Fármacos y Alimentos (FDA) no aprueba el uso de los mismos en niños ni adolescentes jóvenes, por lo que se reserva a pacientes adolescentes en los que el crecimiento se encuentre finalizado. Las indicaciones son niños obesos en los que la dieta y el ejercicio físico han fracasado o niños con sobrepeso, con comorbilidades severas y antecedentes familiares de diabetes mellitus 2 o enfermedades cardiovasculares precoz (August, 2008).

Finalmente, la cirugía bariátrica se contempla como la última opción terapéutica, siendo las indicaciones en la infancia muy restrictivas, debido a que la pérdida brusca de peso puede influir negativamente en el crecimiento. Además, generalmente las comorbilidades de la obesidad en la infancia son menos graves y el pronóstico de la misma es mejor (Dalmau, 2007).

Debido a que no se recomienda utilizar estrategias quirúrgicas ni farmacológicas en menores de edad, dado que las modificaciones metabólicas pueden generar daños colaterales, se debe de recurrir a terapias menos agresivas pero efectivas.

2. MICROBIOTA INTESTINAL

En décadas pasadas se creía que todos los microorganismos podían ser agentes causales de infecciones en cualquier parte que se encontraran en el hospedador, sin embargo, actualmente se sabe que la relación microorganismo - hospedador en muchos casos presenta una interacción de comensalismo no patogénico o mutualismo (Dethlefsen, 2007).

Tradicionalmente, el término más empleado para hacer referencia a las comunidades microbianas que se desarrollaban en el cuerpo de un hospedador sano era el de "flora" o "microflora (Langa, 2006). Actualmente se utiliza la palabra microbiota, para denotar al conjunto microorganismos presentes en el hospedador, y el termino microbioma, para referirse al conjunto de genes microbianos (Backhed, 2004; Vrieze, 2010). La microbiota intestinal es heterogénea y se estima que puede estar integrada por más de 1000 especies bacterianas distintas. El número de células microbianas en el lumen es 10 veces mayor que las células eucariotas del organismo lo que representaría alrededor de 1 kg del peso corporal (Scarpellini, 2010). Además, el microbioma es 100 veces mayor que el genoma humano, por lo cual, la microbiota intestinal puede ser considerada como un órgano virtual exteriorizado, metabólicamente adaptable y flexible, así como rápidamente renovable, que contribuye al metabolismo y tiene un papel importante en la obtención de energía a partir de la dieta (Francois-Pierre, 2009). En diversos estudios se ha observado que los animales libres de gérmenes muestran alta susceptibilidad a infecciones,

indicando que los simbiontes intestinales suponen una importante barrera para la colonización de patógenos potenciales, a este proceso se le ha llamado "exclusión competitiva", en donde ambos grupos o microorganismos compiten por el mismo nicho ecológico, nutrientes y sitios de adhesión (Bik, 2009).

2.1 Colonización y composición de la microbiota intestinal.

El tracto gastrointestinal del feto en el útero, se considera prácticamente estéril, pero en el momento de nacer es rápidamente colonizado con microorganismos de la madre y del ambiente hospitalario (Palmer, 2007), después de 3 o 4 semanas del nacimiento la microbiota intestinal comienza ha estabilizarse. Sin embargo, algunos estudios han demostrado la existencia de bacterias en muestras de líquidos amnióticos y sangre de cordón umbilical obtenidos de madres/neonatos sanos, en el meconio de niños nacidos tanto por parto como por cesárea (Langa, 2006).

Los primeros colonizadores en el momento de nacer son bacterias anaerobias facultativas, estreptococos y coliformes, posteriormente predominan las bacterias anaerobias estrictas de los géneros *Clostridium, Bacteroides* y *Bifidobacterium* (Mackie, 1999). Las bacterias que predominan en las primeras semanas del nacimiento (*Streptococcus, Enterococcus, Staphylococcus, Lactobacillus)* están también muy relacionadas con los grupos bacterianos representativos en la leche materna. Estas bacterias pueden producir un ambiente reductor favorable para la colonización de bacterias anaerobias (*Bifidobacterias, Bacteroides*, y *Clostridium*) (Langa, 2010). La

introducción de la alimentación complementaria también influye en la microbiota que va evolucionando hasta los 2-4 años de edad en los que alcanza una composición más estable que permanece con pocos cambios hasta la edad adulta (Scarpellini, 2010; Vrieze, 2010). En el establecimiento de la microbiota influyen muchos factores, como el tipo de parto, el ambiente, el tipo de lactancia y posiblemente hasta el genotipo (Mackie, 1999).

La microbiota intestinal es cuanti y cualitativamente diferente en cada individuo, depende de la edad, factores ambientales y de la dieta (Scarpellini, 2010), al parecer las poblaciones dominantes suelen permanecer estables a lo largo del tiempo (Zoetendal, 2001). Sin embargo, la composición microbiana puede sufrir alteraciones por el suministro de antibióticos (Deethlefsen, 2008). La edad también conlleva cambios en la composición de la microbiota y por ejemplo, el número de *Clostridium* spp. aumenta a lo largo de la vida mientras que el de *Bifidobacterium* spp. disminuye. En ancianos es más frecuente el aislamiento de *Clostridium difficile*, mohos y enterobacterias que en individuos jóvenes (Langa, 2006). Aunque la asociación entre el tipo de dieta y los distintos grupos microbianos aún no está clara, la microbiota de comunidades occidentales (caracterizada por una ingesta alta en grasa y proteínas de origen animal y un bajo contenido en fibra), parece contener mayores niveles de *Bacteroides* spp. y *Clostridium* spp. y con menores niveles de bacterias lácticas en comparación con la de comunidades orientales (Hayashi, 2002).

Alrededor de 50 filos bacterianos han sido descritas en la microbiota humana, pero solo tres son dominantes en el adulto: Firmicutes, Bacteroidetes

y Actinobacteria (Zoetendal, 2006). Los Firmicutes es el phylum más abundante y contiene alrededor de 200 géneros, incluyendo *Lactobacillus*, *Mycoplasma*, *Bacillus* y *Clostridium*. El filo Bacteroidetes, incluye aproximadamente 20 géneros y el filo Actinobacteria se presentan en menor proporción (Vrieze, 2010). En la tabla 3 se describe a detalle los grupos representativos en las principales partes del tracto gastrointestinal.

Tabla 3. Microbiota del tracto gastrointestinal

Localización	Grupos bacterianos	Concentración (UFC)
Esófago	*Streptococcus, Prevotella, Veillonella*	10^1 - 10^3 /ml
Estómago	*Bacteroides, Enterobactericeae, Lactobacillus, Streptococcus*	10^7 /ml
Duodeno	*Bacteroides, Enterobactericeae, Lactobacillus*	10 /ml
Yeyuno + Íleo	*Bacteroides, Clostridium, Lactobacillus, Enterobacterias, Enterococcus, Streptococcus*	10^9 /g
Colon	*Bacteroides, Bifidobacterium, Clostridium, Enterobactericeae, Enterococcus, Eubacterium, Lactobacillus, Metanobacterias, Pseudomonas, Estafilococcus, Streptococcus,*	10^{14} /g

Fuente: Modificada de Holzapfel, 2006

2.2 Microbiota Intestinal y Obesidad:

La microbiota que coloniza el intestino humano, se considera un nuevo factor implicado en la obesidad y en las enfermedades asociadas debido a su influencia en las funciones metabólicas e inmunológicas del hospedador (Sanz, 2009). Se ha visto que el proceso de colonización intestinal aumenta la capacidad del hospedador, tanto para extraer energía de la dieta como para almacenarla en los adipocitos por diversos mecanismos (Backhed, 2004). La colonización del intestino en animales libres de gérmenes también provoca un aumento en la expresión en el hígado de dos enzimas claves implicadas en la ruta de biosíntesis de novo de ácidos grasos, la acetil-CoA carboxilasa y la sintasa de ácidos grasos, así como de los factores de transcripción ChREBP y SREBP-1, que están involucrados en la respuesta lipogénica de los hepatocitos a la insulina y glucosa (De Graaf, 2008).

Backhed y su equipo (2004), realizaron un estudio colonizando el tracto digestivo de ratones libres de gérmenes, con microbiota autóctona de ratones alimentados con una dieta normal. Después de 10 a 14 días observaron un incremento significativo en la grasa corporal sin tener una mayor ingesta del alimento. Este cambio abarco diferentes mecanismos:

1) fermentación microbiana de polisacáridos de la dieta, que no pueden ser digeridos por las enzimas del hospedador.
2) Por consiguiente, incremento de la absorción intestinal y en el íleon de monosacáridos y ácidos grasos de cadena corta.

3) Conversión de los ácidos de cadena corta a lípidos más complejos en el hígado

4) Regulación microbiana de los genes del hospedador que promueven la deposición de los lípidos en los adipocitos.

La endotoxemia metabólica que se caracteriza por un aumento a niveles séricos de LPS y se asocia a dietas ricas en grasa, la resistencia a la insulina y la diabetes en modelos animales sometidos a una dieta rica en grasas se considera que puede ser un factor inflamatorio causante del aumento de peso corporal (Cani, 2007; Siebler, 2008).

En un estudio de Basseri y colaboradores (2012), demostraron que la principal bacteria responsable de las elevadas cantidades de metano en el colon es *Methanobrevibacter smithii* y que existe una estrecha correlación entre la cantidad de metano en la cavidad oral y el aumento del IMC en animales. Proponen dos mecanismos de acción del metano:

(1) En un estudio *in vivo* en roedores, el gas disminuyo el transito en la porción distal del intestino en un 59%, esta disminución de movimientos podrían contribuir a un incremento en la cantidad de bacterias en el colon,

(2) Las bacterias metanogénicas aceleran la fermentación de polisacáridos y carbohidratos, incrementando la producción de ácidos de cadena corta que son absorbidos por el intestino y sirven como una fuente adicional de energía, esta energía extra puede ser precursora del aumento de peso en el hospedador (Basseri, 2012).

Las principales funciones de la microbiota intestinal son del tipo metabólicas, tróficas, y reguladoras del sistema inmunitario (Hooper, 2001). La presencia de una microbiota intestinal no es esencial para la supervivencia del organismo, pero se ha observado que en ratones libres de gérmenes (germ-free, GF), requieren un 30% más de energía en su dieta, suplementos de vitamina K y ciertas vitaminas del complejo B en comparación a animales con una microbiota normal. En animales con una microbiota autóctona estas vitaminas son producidas por diferentes géneros bacterianos, incluyendo a los *Bacteroides* y *Eubacterium* (Bik, 2009).

2.2.1 Funciones metabólicas

La función metabólica de la microbiota intestinal es esencial para la actividad bioquímica global del organismo, ya que interviene en la obtención de energía de la dieta, digestión y síntesis de nutrientes así como en la generación de compuestos absorbibles y en la producción de vitaminas (Reid, 2003; Bik, 2009).

Los mamíferos no pueden degradar los polisacáridos presentes en la pared celular de las plantas por si solos, sin embargo, la microbiota intestinal presenta enzimas que pueden degradar ese tipo de carbohidratos. La fermentación microbiana de alimentos no digeribles, pueden proveer aproximadamente el 10% de la energía diaria en los omnívoros y hasta un 70% en los herbívoros (Delzenne, 2007).   Esto se corroboró al secuenciar el genoma de *Bacteroides thetaiotaomicron,* en donde se relacionaron 400

enzimas para el transporte, unión y digestión de azúcares complejos, como el almidón (Hooper, 2002).

Como se puede observar en la figura 1, la degradación de los polisacáridos (xilano, pectina, almidón, manano e inulina) en la mucina del hospedador permite la producción de productos intermediarios (lactato, succinato, etc), gases (dióxido de carbono, hidrógeno y metano), etanol, ácido láctico y ácidos grasos de cadena corta (SCFAs, por sus siglas en ingles, short chain fatty acids) que incluyen al acetato, butirato y propionato, los cuales representan la principal fuente de energía de los colonocitos (Sanz, 2008; De Palma, 2011).

El hidrógeno que es producido durante la fermentación de los polisacáridos, puede activar el metabolismo y aumentar la proliferación de bacterias degradadoras de polisacáridos (Tilg, 2011). El acetato puede contribuir a la síntesis de colesterol y lípidos en el hígado por la activación de la acetil-S CoA sintetasa 2 del citosol, mientras que el propionato puede inhibir la síntesis de lípidos provenientes del acetato, al menos esto se ha observado en hepatocitos de roedores (Collins, 1994), Ambos se han visto involucrados en la regulación del metabolismo hepático de la glucosa reduciendo la glucemia postprandrial y la respuesta insulínemica (Verter, 1995; Lasaga, 2010). El butirato ejerce un efecto trófico sobre la mucosa, constituye la principal fuente de energía para los enterocitos y regula el crecimiento y/o diferenciación celular. También se le ha atribuido efectos anti-inflamatorios y anticarcinogénicos aunque su efectividad *in vivo* se desconoce.
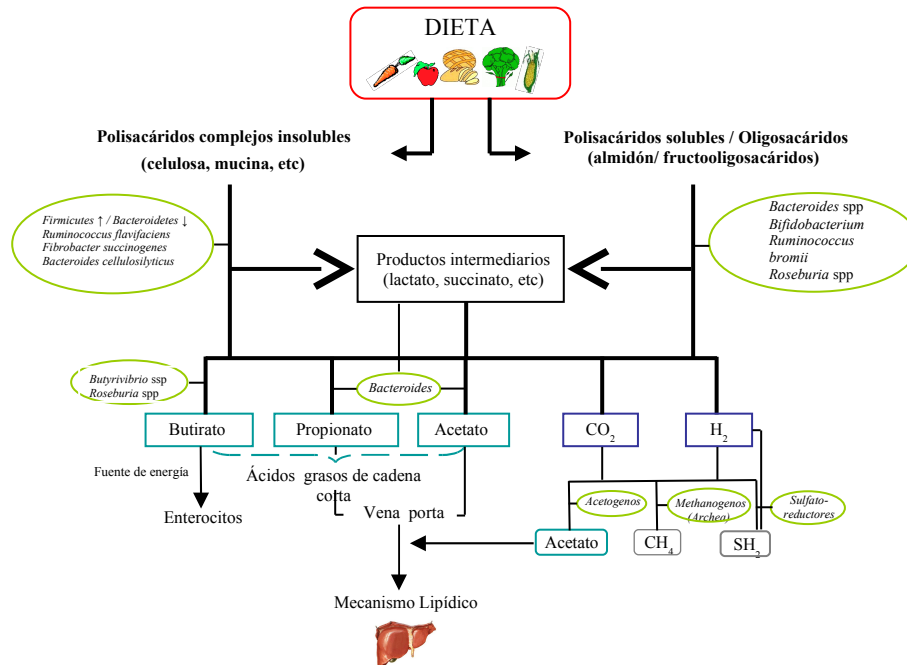
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Figura 1. Diagrama esquemático de las principales rutas metabólicas de

poli y oligosacáridos en el ecosistema gastrointestinal

(Fuente: Modificado de Sanz, 2008).

Además tanto el propionato como el butirato aumentan la sensación de saciedad, por lo cual podrían contribuirla control del apetito. La actividad metabólica de la microbiota también puede contribuir a la generación de compuestos perjudiciales para la salud, como se da en el caso de la degradación bacteriana de las proteínas que puede generar algunos metabolitos como amoniaco, aminas, índoles y fenoles (De Palma, 2011).

2.2.2 Funciones inmunológicas

El epitelio intestinal, el sistema inmune y la microbiota intestinal representan un sistema morfo-funcional responsable del balance dinámico así como de la integridad de la función de barrera del intestino del hospedador (Langa, 2006).

En el epitelio intestinal se encuentran diferentes tipos de células, enterocitos, células globet secretoras de mucus, células enteroendocrinas y células de Paneth que secretan péptidos antimicrobianos y proteínas (Sanz, 2009; Winkler, 2007). Consiste de una única capa de células unidas por uniones intercelulares estrechas que forman una capa impermeable a la mayoría de macromoléculas entre el contenido de la luz intestinal y el organismo (De Palma, 2011).

La integridad de la mucosa intestinal depende de diferentes factores: físicos, fisiológicos, inmunológicos, la dieta, la edad, el uso de antibióticos y la composición de la microbiota intestinal residente (De Palma, 2011). Se ve atacada constantemente por la presencia de antígenos externos y microorganismos patógenos y es un sitio activo para la supresión inmune de reacciones nocivas innecesarias, también se encarga de generar respuestas protectoras para mantener la homeostasis inmune del hospedador (Yuan, 2004).

La microbiota intestinal regula muchos aspectos de la inmunidad innata y la adquirida hecho se ha observado que en animales libres de gérmenes, el tejido linfoide asociado a la mucosa intestinal (GALT, por sus siglas en inglés: Gut associated lymphoid tissue) es inmaduro, presentan ausencia de placas de Peyer, reducción del número de células plasmáticas, bazo y nódulos linfáticos anormales y vellosidades intestinales inmaduras. Tan solo con la colonización de especies de *Bacteroides* se pueden reparar las deficiencias mencionadas (Hooper, 2004). Las continuas interacciones entre el sistema inmunitario y la microbiota intestinal son las responsables del estado de "inflamación controlada" existente en el tracto gastrointestinal, necesario para la generación rápida de una respuesta inflamatoria frente a patógenos (Neish, 2000).

Los receptores de reconocimiento celular de las células del sistema inmunitario innato, como los receptores Toll Like (TLR), constituyen el punto de partida de la inmunidad que se activa en respuesta a los estímulos

microbianos o derivados de la dieta (proteínas, lípidos) e informa a las células inmunocompetentes para que respondan adecuadamente a éstos (Sanz, 2008). De este modo, componentes de bacterias patógenas y las comensales, son reconocidos desencadenando diversas respuestas.

Tras su activación por un ligando, los TLR interactúan con diferentes proteínas adaptadoras que activan la transcripción de distintos sistemas efectores y la síntesis de diversas citoquinas y mediadores inmunológicos de la inflamación. Se ha demostrado que tanto los lipopolisacáridos (LPS) de las bacterias Gram-negativas como los ácidos grasos saturados de la dieta pueden activar el TLR4 e inducir la síntesis de citoquinas TNF alfa IL-6, IL-1B, y quimocinas proinflamatorias relacionadas con la inducción de resistencia a la insulina y el aumento de la adiposidad, tanto en adipocitos, como en macrófagos, por rutas similares (Tsukmo, 2007). No obstante, todavía no se sabe con precisión si la inflamación es una causa o consecuencia de alteraciones como la resistencia a la insulina (Hotamisligil, 2006). En la figura 2 se muestra la activación de los TLR.

La cirugía bariátrica es uno de los procedimientos más eficaces para tratar la obesidad mórbida, mejora las condiciones metabólicas e inflamatorias, pero se sabe poco de su relación con la microbiota intestinal. Zhang (2009), realizó un estudio antes y después de someter a pacientes a la cirugía bariátrica, observó que antes de la cirugía los recuentos de *Bacteroides, Prevotella* y *Archea*, estaban elevados, las cuales son productoras y captadoras de hidrógeno, respectivamente. La transferencia del hidrógeno aumenta la

energía de absorción por el intestino, lo cual repercute en un aumento en la producción de ácidos grasos de cadena corta que son absorbidos por el intestino (Samuel, 2006).

Otro estudio demostró que después de la cirugía las cantidades de los grupos de bacterias metanogénicas y *Prevotellacaea* se redujeron y disminuyeron las cantidades de *Firmicutes* (Zhang, 2009). En estudios realizados con ratas no obesas con cirugía bariátrica, se observó que los *Firmicutes* y los *Bacteroidetes* disminuyen su cantidad inicial en comparación
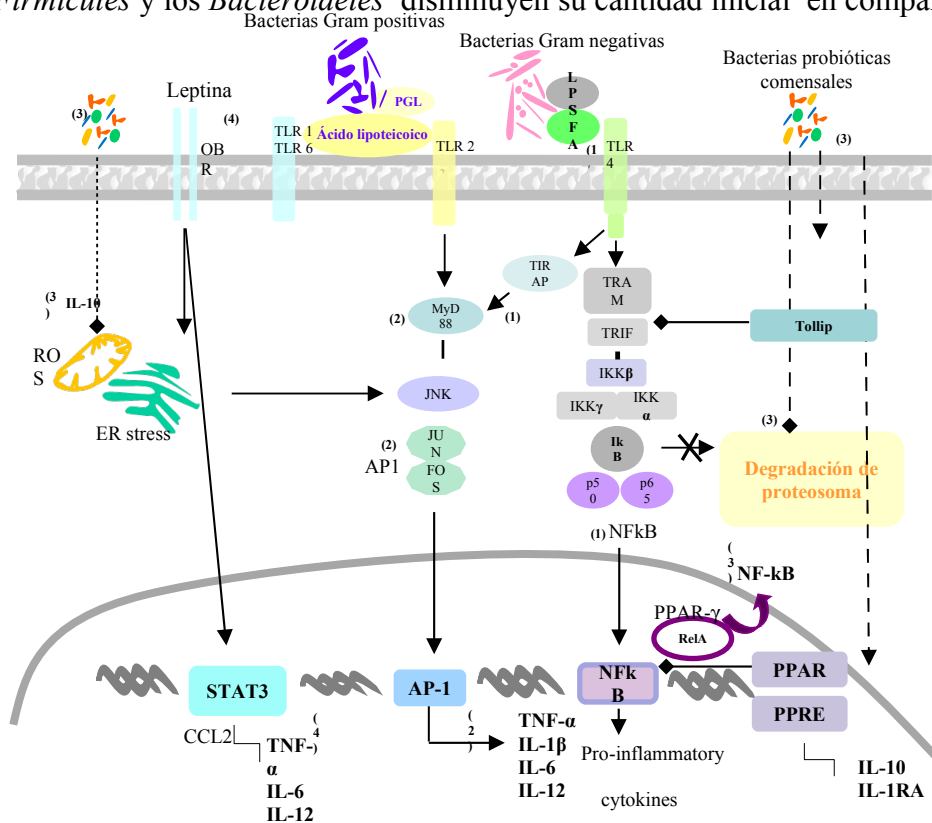

Figura 2. Diagrama de las rutas de señalización generadas por los componentes bacterianos, los ácidos grasos saturados y adipoquinas en células epiteliales y del sistema inmune innato

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produciendo una activación o regulación negativa de las rutas proinflamatorias, relacionadas con la obesidad y la resistencia a la insulina (Sanz, 2008).

al grupo control. Sin embargo las *Proteobacteria* aumentaban hasta 52 veces su cantidad inicial (Li, 2011). Las intervenciones quirúrgicas en el tracto gastrointestinal tienen profundos efectos en la composición de la microbiota intestinal y de los ácidos grasos de cadena corta. No obstante, la cirugía puede inducir una desnutrición (Kootte, 2012).

2.3 Cambios de la microbiota intestinal en individuos obesos

La obesidad se ha asociado a aumentos en la abundancia relativa de Firmicutes y reducciones proporcionales en la abundancia de Bacteroidetes, mediante la comparación de la composición de la microbiota intestinal de ratones genéticamente obesos (ratones deficientes en leptina ob/ob) y delgados. (Sanz, 2009). En un estudio realizado por Ley (2005), al analizar secuencias del 16S rRNA de ratones obesos y delgados observaron que los cambios que ocurrían en la microbiota intestinal eran a nivel de división. La microbiota del ciego de los ratones obesos presentó una diferencia significativa del 50% de reducción en Bacteroidetes con respecto a los ratones delgados y era significativamente diferente con respecto a Firmicutes. Esta relación también se ha asociado a la obesidad en humanos. En un estudio con humanos obesos que se sometieron durante un año a una dieta hipocalórica, se observó un incremento significativo en las proporciones de Bacteroidetes, paralelo a la pérdida de peso (Ley, 2006). Turnbaugh (2009), comparó la microbiota de

gemelos obesos y delgados, demostrando una menor proporción de Bacteroidetes y mayor en *Actinobacteria* en los individuos obesos. El incremento en el ratio Firmicutes/Bacteroidetes en ratones obesos *ob/ob* puede ayudar a promover el incremento de adipocitos o podría representar una respuesta adaptativa al almacenamiento.

En otros estudios sobre la microbiota de ratones genéticamente obesos y delgados también se ha establecido una relación entre la obesidad y una mayor proporción de *Archea* (Ley, 2005). En estudios mas recientes también se ha detectado una reducción de *Bifidobacterium* y un aumento de *Halomonas* y *Sphingomonas* en la microbiota intestinal de ratas Zucker genéticamente obesas (fa/fa) en comparación con el grupo control (Waldram, 2009).

Patrice Cani (2009), demostró que una dieta alta en grasas disminuye el número de bifidobacterias e incrementa el LPS en plasma, también observó que la modulación de la microbiota intestinal mediante antibióticos o con una dieta de oligofructosa reduce la intolerancia a la glucosa, diminuye la ganancia de peso e inhibe la inflamación en ratones obesos. Estos resultados sugieren que los cambios en la microbiota pueden ser los responsables del incremento de endotoxinas en el plasma por los una dieta rica en grasa, lo cual puede contribuir a desencadenar obesidad y diabetes mellitas (Membrez, 2008). Otra ruta alternativa para la inflamación, es la disminución de butirato en plasma, ya que este compuesto tiene propiedades antiinflamatorias (Säemann, 2000). Los dos principales grupos bacterianos productores de butirato son *Roseburia/E.rectale* y especies de *F.prausnitzii*. Se ha visto que una dieta rica

en carbohidratos no digeribles estimula el crecimiento de estos grupos bacterianos y por consiguiente se tiene un aumento de butirato en plasma (Gao, 2009). Al final de este apartado puede añadir un párrafo que resuma el posible uso de probióticos en obesidad.

Los beneficios que se pueden derivar de la modificación de la microbiota intestinal han generado desde hace décadas, un gran interés por el desarrollo de probióticos para la mejora o prevención de ciertas patologías (Langa, 2006). En este contexto y en base a la las funciones que las bacterias intestinales desempeñan en el metabolismo y la regulación del sistema inmune, también se ha propuesto en los últimos años el uso de bacterias probióticas y prebióticos como alternativa para prevenir o controlar la obesidad y comorbilidades, como la diabetes, cuyos resultados más destacados se discutirán en los capítulos de la tesis doctoral.

**EXPOSICIÓN GENERAL DEL PROBLEMA A INVESTIGAR
Y OBJETIVOS**

La obesidad se ha convertido en las últimas décadas en una de las principales causas de muerte y discapacidad (Gómez Ambrosi, 2008). Es una enfermedad crónica derivada de un desequilibrio entre la ingesta y el gasto energético, que provoca un incremento del peso y grasa corporal, y está asociada a un mayor riesgo de sufrir otras enfermedades como la diabetes Mellitus tipo 2 y la esteatosis hepática, así como de mortalidad por enfermedades coronarias. Además, la obesidad se considera asociada a un estado de inflamación de bajo grado relacionado con alteraciones crónicas del metabolismo. Esto hace que el abordaje del tratamiento de esta enfermedad suponga un serio problema público (González, 2008). La obesidad en la adolescencia constituye un antecedente metabólico principalmente para la enfermedad cardiovascular en el adulto y la diabetes tipo 2. La prevalencia de la obesidad y sobrepeso en España es del 26.3 % en individuos de 2-24 años (Aranceta, 2007).

La microbiota intestinal comensal y su genoma (microbioma), que posee una capacidad codificante muy superior a la del genoma humano, contribuyen de forma significativa al metabolismo global del organismo; por esto, se ha considerado que actúa como un órgano equivalente al hígado (Bäckhed, 2004). La microbiota puede aportar nuevos nutrientes, favorecer su digestión y modular la expresión de genes del hospedador implicados en el

metabolismo de macronutrientes y la deposición de lípidos (Sanz, 2008a). Además, la microbiota comensal regula muchos aspectos de la inmunidad innata y adquirida, por lo que su composición también se ha asociado estrechamente con patologías inflamatorias.

En los últimos años la composición de la microbiota se ha empezado a relacionar con la obesidad y su modificación se ha propuesto como alternativa para su prevención o control, aunque todavía existen pocos datos conclusivos al respecto. Por este motivo, en la presente tesis doctoral se planteó avanzar en el análisis de la microbiota intestinal y su asociación con la obesidad en diversos grupos de población y su interrelación con la dieta, y estudiar el efecto del uso de posibles probióticos en modelos animales de obesidad.

Objetivos específicos:

1.- Determinar la influencia de un programa multidisciplinar para tratamiento de la obesidad en adolescentes con sobrepeso, basado en una dieta con restricción calórica y aumento de la actividad física, sobre la composición de su microbiota intestinal y su relación con parámetros dietéticos, bioquímicos e inmunológicos, mediante el uso de PCR a tiempo real (qPCR) e hibridación *in situ* con sondas fluorescentes.

2.- Determinar la composición de la microbiota intestinal de mujeres embarazadas normo peso y con sobrepeso y su relación con el peso corporal, la ganancia de peso durante el embarazo y los parámetros dietéticos y bioquímicos, mediante qPCR.

3.- Evaluar los efectos de la administración oral de la cepa *Bifidobacterium pseudocatenulatum* CECT 7765 en las disfunciones inmunológicas y metabólicas asociadas a la obesidad en un modelo animal alimentado con una dieta rica en grasa.

Interplay between weight loss and gut microbiota composition in overweight adolescents

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Running head: Weight loss and gut microbiota

ABSTRACT

The aim of this study was to determine the influence of an obesity treatment program on the gut microbiota and body weight of overweight adolescents. Thirty-six adolescents (14-15 years), classified as overweight according to the International Obesity Task Force body mass index (BMI) criteria, were submitted to a calorie-restricted diet (10-40%) and increased physical activity (15-23 kcal/kg body weight/wk) program over 10 weeks. Gut bacterial groups were analyzed by quantitative real-time PCR before and after the intervention. A group of subjects (n=23) experience more than 4.0 kg weight loss and showed significant BMI (*P*= 0.030) and BMI z-score (*P*= 0.035) reductions after the intervention, while the other group (n=13) showed less than 2.0 kg weight loss. No significant differences in dietary intake were found between both groups. In the high weight-loss group, *Bacteroides* and *Lactobacillus* counts increased (*P*=0.015 and *P*=0.002, respectively), whereas *Clostridium coccoides* and *B. longum* counts decreased (*P*=0.001 and *P*=0.044, respectively) after the intervention. *Bacteroides* and *C. leptum* counts were significantly higher (*P*=0.004 and *P*<0.001, respectively) while levels of *C. coccoides*, *Lactobacillus, Bifidobacterium* and *B. breve* were significantly lower (*P*<0.001, *P*<0.001 and *P*=0.001, respectively) in the high weight-loss group than in the low weight-loss group before and after the intervention. These findings indicate that calorie restriction and physical activity have an impact on gut microbial composition related to body weight loss, which also seen to be influenced by the individual´s microbiota

INTRODUCTION

Obesity is viewed as one of the major current public-health problems and its impact is highest in children, contributing to significant morbidity in adulthood (1). The development of metabolic complications, associated with obesity during childhood, has repercussions in adulthood, increasing the risk of type-2 diabetes and premature cardiovascular diseases (2). A link is thought to exist between obesity, chronic low-grade inflammation, insulin resistance and endothelial dysfunction (3,4). The risk factors for childhood obesity include diet, low socioeconomic status, parental obesity, rapid infancy weight gain, and decreased physical activity (5). Obesity prevention programs based on changes in school and community environments can decrease childhood weight gain to a limited extent (5). Therefore, further studies on dietary and host factors with an impact on energy balance are needed to improve the intervention strategies and measures for obesity control over time.

Recent reports have suggested that gut microbiota is an important factor affecting energy disposal and storage in adipocytes (6,7). The gut microbiota is also known to be involved in modulation of host immunity, and the inflammatory status associated with obesity in mice (8,9). However, the precise mechanisms by which alterations in microbiota affect obesity and associated disorders are still unclear.

It has been reported how diets based on a high protein intake and/or low carbohydrate intake, or high fat intake may alter microbial composition

and activity in the large intestine and thus exert an impact on gut health (6,8-10). Nevertheless, knowledge of the interactions between energy intake and specific microbial populations, and their influence on body weight, are limited to small-scale clinical trials (7). Specific studies in obese adolescents, who represent a high-risk population group, are lacking.

The objective of this work was to determine the influence of a multidisciplinary obesity treatment program, comprising a calorie-restricted diet and physical activity, on the structure of the fecal microbiota of overweight and obese adolescents and its relation to dietary intake and weight loss by analyzing the main gut bacterial groups and *Bifidobacterium* species by quantitative real-time PCR.

METHODS AND PROCEDURES

Subjects and anthropometric measures

Subjects for the study were selected according to their BMI (weight (kg)/(height (m)^2). Childhood overweight (including obesity) was defined according to the International Obesity Task Force cut-offs for BMI (11). BMI z-scores were calculated as a function of the subject's obesity degree when compared with BMI local reference standards (12). Body weight (kg) was estimated without shoes and with light clothing, and measured to 0.05 kg by using a standard beam balance. Skinfold thicknesses was measured on at the left side of the body to the nearest 0.1 mm using a Holtain skinfold caliper at

triceps, biceps, subcapular, suprailiac, thigh, and calf, as previously described (12). All the anthropometric variables were measured in order, three times and averaged. For all the anthropometric measurements, intra-observer reliability was >95% and inter-observer reliability was >90%.

The characteristics of the 36 selected adolescents (18 female and 18 male; mean age: 14.5 years) to be submitted to the obesity-treatment program are shown in Table 1. None of the volunteers were treated with antibiotics for at least 1 month before the intervention study and also, during the study. The study was conducted in accordance with the ethical rules of the Helsinki Declaration (Hong Kong revision, September 1989), following the EEC Good Clinical Practice guidelines (document 111/3976/88 of July 1990) and current Spanish law which regulates clinical research in humans (Royal Decree 561/1993 regarding clinical trials). Informed consent was obtained from all adolescents and their parents, and the study was approved by the local Ethics Committees**.**

Intervention

Over a 10-week period, the participants were subjected to the intervention based on an energy-restricted diet (a 10-40% reduction) established according to both obesity degree and regular physical activity (13). The maximum energy intake was 1,800 kcal/day for females and 2200 kcal/day for males. The physical activity program was determined by

Table 1 Clinical characteristics of the studied subjects

Total subjects	$N = 36$
Age (years)	14.5 (13.0–15.0)
BMI	
Before intervention	32.8 (29.4–35.2)
After intervention	30.6 (27.5–33.3)
Weight (kg)	
Before intervention	90.5 (81.8–102.2)
After intervention	84.4 (75.3–97.1)
Weight loss (kg)	4.7 (1.7–7.2)
Weight loss (%)	5.8 (2.2–8.6)
BMI z-score	
Before intervention	3.09 (2.31–4.08)
After intervention	2.71 (1.72–3.49)
Low weight–loss group	$N = 13$
Age (years)	14.5 (13.0–15.0)
BMI	
Before intervention	30.7 (26.4–36.3)
After intervention	30.2 (26.2–35.9)
Weight (kg)	
Before intervention	85.9 (69.4–101.6)
After intervention	84.4 (68.2–100.7)
Weight loss (kg)	1.4 (0.75–1.8)
Weight loss (%)	1.3 (0.85–2.25)
BMI z-score	
Before intervention	2.95 (1.6–4.03)
After intervention	2.74 (1.5–3.93)

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Total subjects	**N = 36**
Age (years)	14.5 (13.0—15.0)
BMI	
Before intervention	32.8 (29.4—35.2)
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Weight loss (%)	1.3 (0.85—2.25)
BMI z-score	
Before intervention	2.95 (1.6—4.03)
After intervention	2.74 (1.5—3.93)
High weight—loss group	**N = 23**
Age (years)	14.5 (14.0—15.0)
BMI	
Before intervention	33.1 (30.0—35.0)[a]
After intervention	31.1 (27.5—32.9)[a]
Weight (kg)	
Before intervention	92.3 (83.8—102.5)
After intervention	84.7 (77.6—95.4)
Weight loss (kg)	6.9 (4.8—9.3)
Weight loss (%)	7.5 (5.8—9.3)
BMI z-score	
Before intervention	3.22 (2.57—4.16)[a]
After intervention	2.67 (1.73—3.30)[a]

[a]Data are shown as medians and interquartile range (IQR). Statistical differences before and after intervention were calculated by using the Mann-Whitney U-test at $P < 0.050$.

accelerometry and exercise prescribe at least 1 h of moderate-to-vigorous intensity 3 or 5 days per week, depending of the individual physical activity level. The energy expenditure was estimated in metabolic equivalent values (14) for each activity and the frequency and intensity of the activities of the exercise program (walking, biking, running, swimming, etc.). The energy expenditure range obtained was from 15-23 kcal/kg body weight per week. Diet energy content was set from the resting energy expenditure calculated with the Schofield equation multiplied by 1.3 as physical activity factor (13). Energy restriction was calculated in function of the subject obesity degree: 10% restriction when the subject had a BMI between 0 and 2 s.d. above the mean, 20% with BMI between 2 to 3 s.d above the mean, 30% between 3 to 4 s.d. above the mean, 30% between 3 and 4 s.d., and 40% if the subject had a

BMI >4 s.d. above the mean according to BMI local reference standards. Macronutrient distribution was 50% of energy from carbohydrates, 30 % from fat and 20 % from proteins. Energy distribution during the day was: breakfast: 20 % of daily energy; morning snack: 10-15 % of daily energy; lunch: 30-35 % of daily energy; afternoon snack: 5-10 % of daily energy; dinner: 20-25 % of daily energy.

Dietary assessment

Food diary records were kept for 72h (2 weekdays and 1 weekend day) both before the start of the study (baseline intakes) and after the intervention (week 10). Detailed information on how to record food and drink consumed using common household measures was provided. Food diary records were returned to their dietician, and analyzed for energy, water and nutrient contents based on the CESNID food-composition database of Spanish foods (14).

Fecal and DNA sample preparation

Fecal samples were kept immediately after collection at -20 ºC and stored until analyzed. Samples were diluted 1: 10 (w/v) in PBS (pH 7.2), homogenized and one aliquot was used for DNA extraction by using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany).

Microbial analysis by quantitative real-time PCR (qPCR)

Specific primers targeting different bacterial genera and species were used to characterize the fecal microbiota by qPCR (Table 2), essentially as described previously (15-19). Briefly, PCR amplification and detection were

performed with an ABI PRISM 7000-PCR sequence detection system (Applied Biosystems,Warrington,UK).

Table 2 Oligonucleotide primers used in this study

Target bacterial group/species	Sequence (5′↔3′)	Annealing temperature (°C)	References
Total bacteria	TGGCTCAGGACGAACGCTGGCGGC CCTACTGCTGCCTCCCGTAGGAGT	61	16
Bacteroides fragilis group	ATA GCC TTT CGA AAG RAA GAT CCA GTA TCA ACT GCA ATT TTA	50	16,17
Clostridium coccoides group	AAA TGA CGG TAC CTG ACT AA CTT TGA GTT TCA TTC TTG CGA A	50	16,17
Clostridium leptum group	GCA CAA GCA GTG GAG T CTT CCT CCG TTT TGT CAA	50	16,17
Escherichia coli	GTTAATACCTTTGCTCATTGA ACCAGGGTATCTAATCCTGTT	62	18
Lactobacillus group	GGAAACAG(A/G)TGCTAATACCG CACCGCTACACATGGAG	61	19,20
Bifidobacterium	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	55	16,17
Bifidobacterium longum	TTCCAGTTGATCGCATGGTC TCSCGCTTGCTCCCCCGAT	55	16,17
Bifidobacterium bifidum	CCACATGATCGCATGTGATTG CCGAAGGCTTGCTCCCAAA	55	16,17
Bifidobacterium breve	CCGGATGCTCCATCACAC ACAAAGTGCCTTGCTCCCT	55	16,17
Bifidobacterium adolescentis	CTCCAGTTGGATGCATGTC TCCAGTTGACCGCATGGT	55	16,17
Bifidobacterium catenulatum group	CGGATGCTCCGACTCCT CGAAGGCTTGCTCCCGAT	55	16,17

Each reaction mixture of 25 µL was composed of SYBR® Green PCR Master Mix (SuperArray Bioscience Corporation, Foster City, CA), 1 µL of each of the specific primers at a concentration of 0.25 µmol/ml, and 1 µL of template DNA. Bacterial concentration from each sample was calculated by comparing the Ct values obtained from the standard curves. Standard curves were created using serial tenfold dilution of pure cultures of DNA, corresponding to 10^2 to 10^9 cells from the culture collection as determined by microscopy counts using 4´,6-diamidino-2-phenylindole. The following strains were used as references: *Bacteroides fragilis* DSMZ 2451, *Clostridium coccoides* DSMZ 933, *C. leptum* DSMZ 935, *Lactobacillus casei* ATCC 393, *E. coli* CECT 45, *Bifidobacterium longum* subsp. *longum* CECT 4503, *B. bifidum* LMG 11041, *B. breve* LMG 11042, *B. pseudocatenulatum* CECT

5776, *B. adolescentis* LMG 11037. The strains were obtained from the Spanish Collection of Type Cultures (CECT) and the German Collection of Microorganisms and Cell Cultures (DSMZ).

Statistical analyses

Statistical analyses were done using the SPSS 11.0 software (SPSS Inc, Chicago, IL). Due to non-normal distribution, microbial data are expressed as medians with interquartile ranges (IQRs) and differences in bacterial populations were determined by applying the Mann–Whitney U-test and the Wilcoxon signed-rank test. Correlations among variables were calculated by using the Spearman´s correlation test. Differences in clinical and anthropometric data were also determined by applying the Mann–Whitney U-test. Dietary composition (means and standard deviations) was calculated for crude (unadjusted) nutrients from the 72 h dietary registers and data were averaged for the analysis. All dietary variables submitted to log-transformation showed fit normal distribution. Repeated-measures ANOVA adjusted for sex and age was used to examine differences in group mean intake before (baseline) vs. after the intervention. In every case, P-values <0.050 were considered statistically significant.

RESULTS

Subjects and obesity intervention program

The studied subjects, 50 % female (18/36) and 50 % male (18/36), were 14.5 years old (13.0-15.0 years), and maintained an apparently good health status during the study. Clinic and anthropometric characteristics did not differ significantly among subjects at recruitment time particularly regarding weight (P=0.266), BMI (P=0.221) and BMI-z-score (P=0.138) and, therefore, they were comparable (Table 1). The subjects showed marked differences in weight loss after intervention and, accordingly, subdivided into two groups as low weight-loss group (<2.0 kg of weight loss, n=13) and high weight-loss group (>4.0 kg of weight loss after intervention, n=23). The median of weight loss after 10 weeks under the intervention program for the first group was of 1.4 (0.75-2.00) kg, corresponding to 1.3% (IQR 0.85 – 2.25%) of body weight. This group did not showed significant differences in BMI (P=0.545) weight (P=0.801), and BMI z-score (P= 0.579) before and after the dietary intervention. In the second group, the median of weight loss after 10 weeks under the intervention program was of 6.8 (4.8-9.0) kg, corresponding to 7.5% (IQR 5.8-9.3 %) of body weight, without detecting significant differences between male (P= 0.204) and female (P= 0.083). In this group significant differences in BMI (P= 0.030) and BMI z-score (P= 0.035) were detected before and after the intervention.

Dietary data before and after the intervention of the low weight and high weight-loss groups are shown in Table 3. No interaction between time

(before and after intervention) per sex or age-group was observed. No significant differences in dietary intake of energy, macronutrients, or on food groups level were found between groups before and after the intervention program. The consumption of probiotic foods i.e., yogurt was almost one portion per day (0.9 portion in both groups, one portion in Spain is equivalent to 125g). None of the subjects consumed pre- or probiotics as supplements. The main sourceof carbohydrates in order of increasing intakes per day were cereal, potatoes, fruits, and diary products. The main fiber sources of this population were vegetables, cereals, fruits and legumes.

In both adolescent groups, the dietary intervention mainly resulted in a significant reduction ($P< 0.05$) in intake of total energy (63.8 % mean reduction; s.d. 1.2) and macronutrients including proteins (74.5 % mean reduction, s.d. 27.2), fat (51.8 % mean reduction; SD 3.8), polyunsaturated fatty acids (PUFA) (48.7% mean reduction, s.d. 12.5), carbohydrates (71.6% mean reduction, SD 3.9), simple carbohydrates (73.3% mean reduction; s.d. 0.8), and complex carbohydrates (70.6% mean reduction; s.d. 7.2). The reduction in complex carbohydrate intake was significantly and negatively correlated (R=-0.334; *P*= 0.050) to changes in *B. fragilis* group as a result of the intervention. Likewise, reduction in PUFA intake was almost significantly and negatively correlated (R=-0.313, *P*=0.063) to changes in *Lactobacillus* group counts.

Table 3 Daily energy and nutrient intake before (baseline) and after the intervention.

	Low weight–loss group (>2.0 kg)				High weight–loss group (>4.0 kg)			
	Before intervention (n = 13)		After intervention (n = 13)		Before intervention (n = 23)		After intervention (n = 23)	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Energy (kcal)[a,b]	2,121.67	617.00	1,428.55	216.83	2,377.62	617.56	1,460.62	376.52
Water (g)[a]	1,325.40	377.84	1,109.62	290.99	1,912.94	657.90	1,999.0	708.51
Protein (g)[a,b]	101.30	23.47	75.59	11.66	109.82	29.92	77.04	19.56
Energy from protein (%)[a,b]	18.90	3.08	22.58	2.59	18.62	3.34	21.96	2.40
Plant protein (g)[a,b]	29.28	8.13	20.58	4.26	26.37	7.88	22.44	6.97
Plant protein (%)[b]	5.58	2.06	5.64	1.25	5.31	1.89	6.38	1.26
Animal protein (g)[b]	72.32	22.55	54.10	9.97	76.56	28.67	54.67	15.37
Animal protein (%)	12.80	3.11	16.21	2.41	13.43	6.54	15.77	2.37
Fat (g)[a,b]	91.66	45.82	51	16.32	99.39	38.25	52.11	17.66
Energy from fat (%)[b]	38.11	9.25	34.43	8.20	40.44	5.55	31.85	5.41
Saturated fat (g)[a,b]	26.96	18.06	17.93	4.69	36.37	15.73	15.44	6.50
Energy from saturated fat (%)[b]	11.67	3.70	10.96	2.34	13.30	3.20	9.58	2.18
MUFAs (g)[a,b]	42.41	22.59	22.96	9.76	42.05	16.38	21.94	9.14
Energy from MUFAs (%)[b]	17.65	6.27	15.52	5.72	16.62	3.35	13.45	3.68
PUFAs (g)[a,b]	13.22	6.35	7.03	3.64	16.82	8.01	7.25	2.47
Energy from PUFAs (%)[b]	5.77	1.25	4.91	1.71	6.82	2.40	4.25	0.93
Cholesterol (mg)[a,b]	332.01	114.01	257.97	67.94	371.80	165.12	215.16	118.79
CH (g)[a,b]	223.03	55.40	153.71	33.32	226.29	63.37	163.63	48.75
Energy from CH (%)[b]	43.30	8.36	45.03	7.38	41.89	5.62	47.28	5.07
Simple CH (g)[a,b]	99.85	32.80	61.99	27.63	108.36	43.83	80.91	20.37
Energy from simple CH (%)[b]	16.54	6.32	21.23	7.35	18.23	5.17	22.86	6.10
Complex CH (g)[a,b]	114.63	38.16	74.17	21.33	114.80	32.94	81.60	36.73
Energy from complex CH (%)	23.88	5.77	24.6	5.54	21.29	13.61	23.96	5.34
Dietary fiber (g)	18.31	7.78	17.9	4.63	17.47	7.60	21.38	7.66

CH, carbohydrates; MUFA, Monounsaturated fatty acids; PUFA , Polyunsaturated fatty acids
[a] Significant (P < 0.050) difference within the low-weight loss group between baseline and after the intervention,
[b] Significant difference within the high weight-loss group (based on age and sex adjusted ANOVA for repeated measurements of log-transformed dietary data).

Influence of intervention in fecal bacterial group composition

Interindividual differences on fecal microbiota composition for all studied adolescents were 0.77 (IQR 0.39-1.70) for *B.fragilis* group, -0.36 (IQR -0.82 to 0.29) for *Bifidobacterium*, -0.65 (IQR -0.98 to 0.27) for *C.coccoides*

group, 0.02 (IQR -0.50 to 0.45) for *C.leptum* group, 0.10 (IQR -0.38 to 0.49) for *E.coli*, and 0.43 (IQR 0.09-0.83) for *Lactobacillus* group.

The intervention in whole adolescents population (n=36) resulted in increased counts of *B. fragilis* group (*P*=0.001) and *Lactobacillus* group (*P*=0.030) and decreased counts of *C. coccoides* (*P*=0.028). No significant differences were found in the other bacterial groups analyzed. *B. fragilis* group (R=0.55, P<0.001) and *C.leptum* group (R=0.52, P<0.001) counts after the intervention significantly correlated with higher weight loss (kg), while the opposite correlation were found for the *E.coli* (R=-0.26, P=0.025), *C.coccoides* group (R= -0.61, P < 0.001), *Lactobacillus* group (R= -0.40,   P = 0.001), and *Bifidobacterium* (R= -0.37, P=-0.001) counts.

Changes in bacterial counts as a result of intervention were also evaluated by considering separately the high and the low weight-loss groups (Tables 4 and 5). Significant differences were not found in any of the analyzed groups before and after intervention in the low weight-loss group (n=13 and <2.0 kg of weight loss Table 4), while significant differences were found in the high weight-loss group (n= 23 and >4.0 kg of weight loss Table 5). In this last group, *B. fragilis* group and *Lactobacillus* counts significantly increased (*P*=0.001 and *P*=0.007, respectively), while those of the *C. coccoides* group significantly decreased (*P*=0.001) after 10 weeks of intervention. Moreover, the ratio of *Bifidobacterium* to *Clostridium group* counts increased significantly after the intervention (*P*=0.022) when compared to the ratio

recorded beforehand, while the ratio of *Bifidobacterium* to *B. fragilis* counts decreased (*P*=0.001).

Table 4 Bacterial counts in fecal samples of low weight-loss (<2.0 kg) group of adolescents before and after intervention

| Bacterial group | Bacterial counts[a] (log cells/g fecal sample), *n* = 13 | | | | | | | | Mann–Whitney[a] U-test |
| | Before intervention | | | | After intervention | | | | |
	Pr[b]	Mean	Median	IQR	Pr[b]	Mean	Median	IQR	*P* value
Total bacteria	13	13.2	12.9	12.8–13.9	13	13.2	13.1	12.8–13.4	0.975
Bacteroides fragilis	13	6.2	6.2	5.8–7.0	13	6.3	6.2	5.8–6.9	0.957
Clostridium coccoides	13	10.0	10.0	9.8–10.2	13	9.9	10.0	9.7–10.2	0.978
Clostridium leptum	13	8.2	8.0	7.9–8.5	13	8.4	8.3	7.9–8.8	0.446
Lactobacillus	13	7.9	7.8	7.6–8.1	13	7.9	7.9	7.7–8.1	0.723
Escherichia coli	13	6.7	6.5	6.0–7.7	13	6.6	6.5	6.0–7.1	0.624
Bifidobacterium	13	9.2	9.2	8.8–9.5	13	8.9	9.0	8.4–9.6	0.514
Bifidobacterium longum	13	7.1	7.0	6.8–7.4	13	7.0	6.9	6.3–7.7	0.644
Bifidobacterium breve	13	4.8	4.8	4.4–5.2	13	4.5	4.5	4.3–4.7	0.110
Bifidobacterium bifidum	13	9.1	9.0	8.8–9.4	13	8.9	8.9	8.3–9.7	0.640
Bifidobacterium adolescentis	13	8.1	8.0	7.8–8.4	13	8.0	7.9	7.3–8.7	0.650
Bifidobacterium catenulatum	13	5.8	5.8	5.5–6.2	13	5.5	5.5	5.3–5.7	0.103

Data are shown as medians and interquartile range (IQR) of cell number per gram of fecal samples. [b]Prevalence (Pr) reflects the number of positive amplifications by quantitative real –time PCR from total samples (n=13).*Statical differences between bacterial counts before and after intervention were calculated by using the Mann-Whitney U-test and established at P<0.050

When subjects of high weight-loss group were classified according to gender, certain significant differences were found between the two groups. In females, *B. fragilis* group significantly increased (*P*=0.002) after the intervention while *C. coccoides* counts decreased (*P*=0.023), which was in accordance with the results obtained when considering the total high weight-loss group of adolescents.

Table 5 Bacterial counts in fecal samples of high weight-loss (>4.0 kg) group of adolescents before and after intervention

| Bacterial group | Bacterial counts[a] (log cells/g fecal sample), n = 23 | | | | | | | | Mann–Whitney U-test |
| | Before intervention | | | | After intervention | | | | |
	Pr[b]	Mean	Median	IQR	Pr	Mean	Median	IQR	P value
Total bacteria	23	14.8	14.6	14.0–15.6	23	14.5	14.8	13.1–16.1	0.450
Bacteroides fragilis	23	7.5	7.6	6.7–8.2	23	8.6	8.6	8.1–9.3	0.001*
Clostridium coccoides	23	8.7	8.6	8.3–9.0	23	7.9	7.7	7.4–8.5	0.001*
Clostridium leptum	23	9.5	9.6	8.7–9.9	21	9.5	9.7	9.1–10.0	0.666
Lactobacillus	23	6.4	6.4	5.9–6.9	23	6.9	7.0	6.3–7.1	0.007*
Escherichia coli	23	6.3	6.3	5.8–6.8	23	6.4	6.3	6.1–7.0	0.231
Bifidobacterium	23	8.3	8.1	7.7–8.6	23	8.2	8.2	7.4–8.6	0.692
Bifidobacterium longum	23	7.1	7.2	6.3–7.9	23	6.4	6.2	5.3–7.3	0.044*
Bifidobacterium breve	15	3.5	3.3	3.0–3.6	11	3.2	3.1	3.0–3.5	0.237
Bifidobacterium bifidum	19	5.9	5.6	4.5–7.1	17	5.6	5.6	4.3–7.1	0.490
Bifidobacterium adolescentis	23	7.6	7.9	6.8–8.8	23	6.9	7.0	6.0–8.1	0.082
Bifidobacterium catenulatum	22	7.6	7.7	6.7–8.5	23	7.2	7.6	6.3–8.4	0.594

[a]Data are shown as medians and interquartile range (IQR) of cell number per gram of fecal samples. [b]Prevalence (Pr) reflects the number of positive amplifications by quantitative real –time PCR from total samples (n=23).*Statical differences between bacterial counts before and after intervention were calculated by using the Mann-Whitney U-test and established at P<0.050

Lactobacillus group increased but the differences were not statistically significant. In males, *Lactobacillus* and *B. fragilis* groups increased significantly (*P*=0.001 and *P*= 0.033, respectively) after the intervention, whereas a significant (*P*=0.007) reduction was found in the *C. coccoides* group, as was detected for the total high weight-loss group of adolescents.

Significant correlations between bacterial counts after the intervention and weight loss were found in the high weight-loss group (Figure 1). Increased levels of *B.fragilis* group (R= 0.27, *P*=0.055) and *Lactobacillus* significantly correlated (R=0.55, *P* < 0.001) with weight loss (kg), while the

opposite correlation (R=-0.37, *P*=0.010) was found for the *E. coli* group (Figure 1). Similar correlations were recorded between *Lactobacilllus* (R= 0.53, *P*=0.008) and *B.fragilis* group (R=0.44, *P*=0.036) levels, and body weight-loss percentages. The reductions in BMI *z*-scores as a result of the intervention were also significantly correlated with increased levels of *Lactobacillus* group (R= 0.62, *P*= 0.001) and *B fragilis* group (R= 0.46, *P*= 0.025). Reduced *C. coccoides* group levels were related to weight loss, (R=-0.611, P=0.001). The correlation between the reduction in *Bifidobacterium* to *C.coccoides* group ratio and weight loss was significantly (R=-0.25, *P*=0.030, as well as the correlation between the reduction in *Bifidobacterium* to *B.fragilis* group ratio and weight loss a result of the intervention to weight loss was also almost significantly (R=-0.62, *P* <0.001) as a result of the intervention.
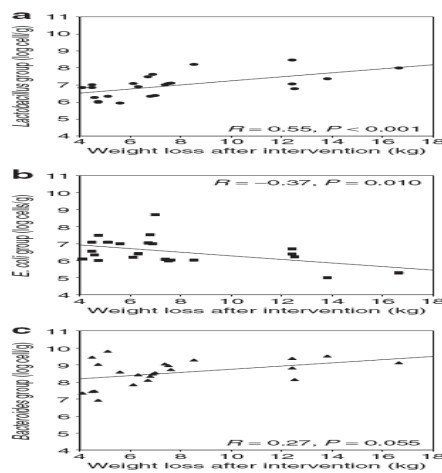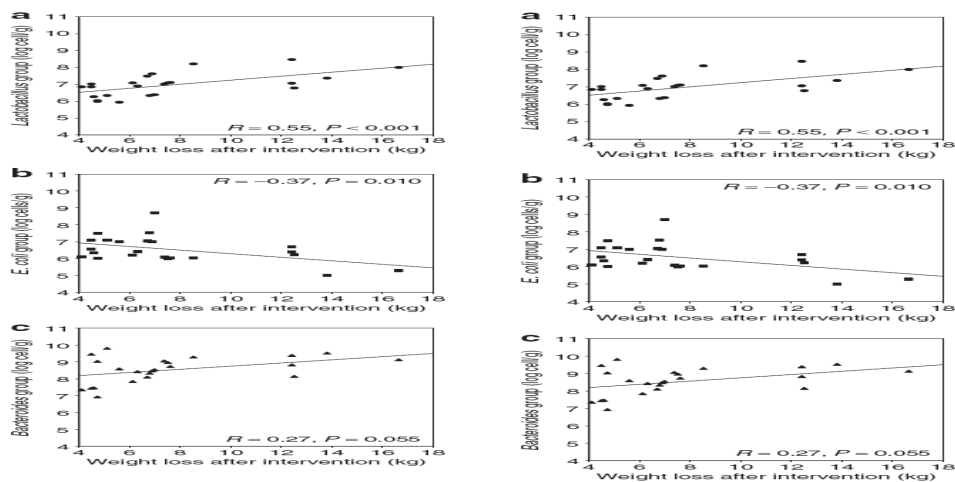
Figure 1 Correlations between fecal bacterial counts and weight loss after intervention in the high weight-loss group (n=23;>4.0 kg weight loss) of adolescents. Lines showed the Pearson correlation (liner adjustment). (a) Lactobacillus group vs weight loss, (b) Escherichia coli vs weight loss, and (c) Bacteroides fragilis group vs weight loss

No significant differences were found in the other *Bifidobacterium* species analyzed. *B. breve* (R= -0.55, *P*-value < 0.001), and *B. bifidum* (R=-

0.69, *P*-value< 0.001) counts after the intervention significantly correlated correlated with lower weight loss (kg), while no correlations were found in the other species. Changes in Bifidobacterium species counts as a result of the intervention were also evaluated by considering separately the high and the low weight–loss groups (Table 4 and 5).

Bifidobacterium species counts showed significant differences as a result of the intervention in the high weight-loss group (table 5), while not in the low weight-loss group of adolescents (table 4). In the high weight-loss group, all *Bifidobacterium* species analyzed decreased after the dietary intervention, although only the changes in *B. longum* counts were significant (*P*=0.044). Similar trends were found when comparing *Bifidobacterium* species composition in males or females. However only *B. adolescentis* counts decreased significantly after intervention (*P*=0.037) in males, whereas no significant differences were found in females. Significant correlations were not detected between *Bifidobacterium* species counts and either weight loss, BMI, or BMI z-score.

Differences in fecal microbiota composition between the low weight-loss and high weight-loss groups of adolescents

The differences in fecal microbiota composition between low and high weight-loss groups of adolescents before and after the intervention are shown in Table 6. Before the intervention, total bacteria, *B.fragilis* group and *C. leptum* counts were significantly higher (P<0.001, *P*=0.004 and *P*<0.001, respectively), while those of *C. coccoides* group, *Lactobacillus group* and

Bifidobacterium were significantly lower (*P*<0.001, *P*<0.001 and *P*=0.001, respectively) in the high weight-loss group than in the low weight-loss group. The ratio of *B.fragilis* group to *C. coccoides* was also significantly higher (*P*< 0.001) in the high weight-loss group. The same trend was detected for *Bifidobacterium* to *C. coccoides* ratio but the differences were not significant (*P*= 0.140). After 10 weeks of intervention, similar differences on microbiota were found between the low weight and the high weight-loss groups. Total bacteria, *B.fragilis* group and *C. leptum* counts were significantly higher (*P*=0.015, *P*=0.001 and *P*<0.001, respectively) while counts of the *C. coccoides* group, *Lactobacillus* group and *Bifidobacterium* were significantly lower (*P*<0.001, *P*<0.001 and *P*=0.008, respectively) in the high weight-loss than in the low weight-loss group. In addition, *B. fragilis* group*, Bifidobacterium* and *Lactobacillus* group to *C. coccoides* group ratios were significantly higher (*P*< 0.001, *P*<0.001 and *P*=0.034, respectively) in the high weight-loss than in the low weight-loss group.

In relation to *Bifidobacterium* species composition, *B. breve* and *B. bifidum* group counts were significantly higher in the low weight-loss than in the high weight-loss group before (*P*=0.001 and *P*< 0.001, respectively) and after intervention (*P*<0.001 for both groups) whereas *Bifudobacterium catenulatum* group levels were higher in high weight-loss group (*P*=0.030 and *P*=0.036, before and after intervention, respectively).

Table 6. Bacterial counts in fecal samples of low and high-weigth-loss groups of adolescents, before and after intervention.

Bacterial group	Low weight–loss group (<2.0 kg) (n = 13)				High weight–loss group (>4.0 kg) (n = 23)				Mann–Whitney U-test
	Pr[a]	Mean	Median	IQR	Pr	Mean	Median	IQR	P value
Bacterial counts[b] before intervention (log cells/g fecal sample)									
Total bacteria	13	13.2	12.9	12.8–13.9	23	14.8	14.6	14.0–15.6	<0.001*
Bacteroides fragilis	13	6.2	6.2	5.8–7.0	23	7.5	7.6	6.7–8.2	0.004*
Clostridium coccoides	13	10.0	10.0	9.8–10.2	23	8.7	8.6	8.3–9.0	<0.001*
Clostridium leptum	13	8.2	8.0	7.9–8.5	23	9.5	9.6	8.7–9.9	<0.001*
Lactobacillus	13	7.9	7.8	7.6–8.1	23	6.4	6.4	5.9–6.9	<0.001*
Escherichia coli	13	6.7	6.5	6.0–7.7	23	6.3	6.3	5.8–6.8	0.123
Bifidobacterium	13	9.2	9.2	8.8–9.5	23	8.3	8.1	7.7–8.6	0.001*
Bifidobacterium longum	13	7.1	7.0	6.8–7.4	23	7.1	7.2	6.3–7.9	0.845
Bifidobacterium breve	13	4.8	4.8	4.4–5.2	15	3.5	3.3	3.0–3.6	0.001*
Bifidobacterium bifidum	13	9.1	9.0	8.8–9.4	19	5.9	5.6	4.5–7.1	<0.001*
Bifidobacterium adolescentis	13	8.1	8.0	7.8–8.4	23	7.6	7.9	6.8–8.8	0.468
Bifidobacterium catenulatum	13	5.8	5.8	5.5–6.2	22	7.6	7.7	6.7–8.5	0.030*
Bacterial counts[b] after intervention (log cells/g fecal sample)									
Total bacteria	13	13.2	13.1	12.8–13.4	23	14.5	14.8	13.1–16.1	0.015*
Bacteroides fragilis	13	6.3	6.2	5.8–6.9	23	8.6*	8.6	8.1–9.3	0.001*
C. coccoides	13	9.9	10.0	9.7–10.2	23	7.9*	7.7	7.4–8.5	<0.001*
C. leptum	13	8.4	8.3	7.9–8.8	21	9.5	9.7	9.1–10.0	<0.001*
Lactobacillus	13	7.9	7.9	7.7–8.1	23	6.9*	7.0	6.3–7.1	<0.001*
E. coli	13	6.6	6.5	6.0–7.1	23	6.4	6.3	6.1–7.0	0.972
Bifidobacterium	13	8.9	9.0	8.4–9.6	23	8.2	8.2	7.4–8.6	0.008*
B. longum	13	7.0	6.9	6.3–7.7	23	6.4*	6.2	5.3–7.3	0.062
B. breve	13	4.5	4.5	4.3–4.7	11	3.2	3.1	3.0–3.5	<0.001*
B. bifidum	13	8.9	8.9	8.3–9.7	17	5.6	5.6	4.3–7.1	<0.001*
B. adolescentis	13	8.0	7.9	7.3–8.7	23	6.9	7.0	6.0–8.1	0.063
B. catenulatum	13	5.5	5.5	5.3–5.7	23	7.2	7.6	6.3–8.4	0.036*

[a]Prevalence (Pr) reflects the number of positive amplifications by quantitative real –time PCR from total samples (n=13 or 23).

[b]Data are shown as medians and interquartile range (IQR) of cell number per gram of fecal samples.

*Statical differences between bacterial counts for each group (high-and low weight adolescent groups)before and after intervention were calculated by using the Mann-Whitney U-test and established at P<0.050.

DISCUSSION

This study shows for the first time that an intervention based on both a reduction in energy intake and an increase in energy expenditure has an important impact on the composition of the gut microbiota of overweight adolescents related to body weight loss. *B. fragilis* group and *Lactobacillus* group seem to be the gut bacterial most amenable to dietary intervention on the basis of the relationships established between the shifts of these bacterial counts and complex carbohydrate and PUFA intakes during the intervention. The *Bacteroides* genus has been shown to have high ability to utilized complex carbohydrates, wich may explain the aforementioned correlation (21). A possible correlation between PUFA intake and *Lactobacillus* group count reductions was also detected, suggesting that PUFA intake may favor the prevalence of *Lactobacillus* group in the gut microbiota. In previous studies, PUFAs have been shown to be utilized by *Lactobacillus*, leading to changes in bacterial fatty acids and suggesting a potential role of *Lactobacillus* as regulators of PUFA absorption *in vivo* (22).In addition, PUFAs have positively influenced the adhesion of Lactobacillus to the jejuna mucosa of gnotobiotic piglets, indicating the intake of these fatty acids may influence the intestinal levels of this bacterial group (23).

Nevertheless, the extent to which these bacterial group counts may change and influence weight loss do not seem to depend only on the diet because significant differences in bacterial counts but not in dietary intakes, were detected between the high weight-loss and the low-weight loss groups during the intervention. Thus, these findings suggest that the individual's gut

microbiota is an additional factor contributing together with lifestyle to body weight regulation.

In response to the intervention, levels of the *B.fragilis* group significantly increased and correlated to weight loss and BMI z-score reductions, while those of the *C. coccoides* group, which comprises the *Clostridium* cluster XIVa including members of other genera such as *Coprococcus, Eubacterium, Lachnospira,* and *Ruminococcus* (17), decreased and correlated to weight loss in the whole adolescent population and the high weight-loss group. These findings were in agreement with the results previously obtained in the same population using fluorescents in situ hybridization technique, which showed that proportions of C. hystolyticum and Eubacterium rectal-C.coccoides groups dropped and those of the Bacteroides – Prevotella group increased after the intervention in those adolescents that los > 4 kg (24). In other studies, the fecal microbiota of obese adult subjects also showed a significant increase in *Bacteroidetes* and a proportional decrease in *Firmicutes* (which included *Clostridium* genus) after following either fat or carbohydrate-restricted low-calorie diet, which led to weight loss over a year (7). Thus, the association between the *B.fragilis* group and *C. coccoides* group with energy intake and body weight changes confirmed in this in different short term intervention study by using different molecular detection techniques resembles that previously established with the broad phyla *Bacteroidetes* and *Firmicutes* in a human long-term intervention study(7).

In this study, the ratio of *Bifidobacterium* to *C. coccoides* group counts significantly increased as a result of the intervention in the high weight-loss group. A significant reduction of this ratio was also evident in children who developed atopic diseases later, indicating that the relative proportions of these bacterial groups may precede the development of immune-related disorders (25). Thus, a reduction in calorie intake and an increase in energy expenditure may also have a beneficial overall impact on these bacterial populations and their relationship to the proinflammatory status linked to obesity. However, the intervention led to reductions in *B. longum* group and *B. adolescentis* counts in the whole adolescent population as well as to reductions in *B. longum* and *B. adolescentis* counts in the high weight –loss group and in male of this group respectively. A reduced dietary intake of carbohydrates by obese adult subjects was shown to be associated with reductions in *Bifidobacterium* counts in previous studies (10), which could also partly explained the reductions of this bacterial groups in the studied adolescents. In fact, genomic and physiological studies have shown that species such a *B. longum and B. adolescentis* may actively participate in the utilization of complex polysaccharides in the colon (21). In general, beneficial effects have previously been attributed to *Bifidobacterium* in connection with obesity. In obese mice models fed with a high fat-content diet, increases in *Bifidobacterium* caused by administering a high fermentable oligosaccharide was positively correlated with the normalization of inflammatory status, improved glucose tolerance and glucose-induced insulin secretion (8, 9). In addition, reductions in *Bifidobacterium* populations have been shown to precede the development of overweight (26).

It is likely that relative proportions of *Bifidobacterium* to other bacterial groups, like those detected in this study in relation to *Clostridium*, rather than absolute numbers have a meaning in the context of obesity. In general, although some of the reported differences in bacterial counts associated to body weight loss were small, from the biological point of view, these could be important in the long term by themselves and because they may lead to changes in the relative proportions of other intestinal bacteria competing for the same ecological niche, which may exert a mild but sustained effect on energy metabolism.

Interestingly, significant increases in *Lactobacillus* group counts in the whole adolescent population and in the high weight-loss group were detected after the intervention, in agreement with the trend previously detected by fluorescent *in situ* hybridization analyses although the differences were not significant (24). In this study, the increase in *Lactobacillus* group counts was correlated with weight loss and BMI *z*-score reductions in the high weight-loss group, pointing to role for this bacterial group in body-weight management. Until now, information about the impact of different diets on *Lactobacillus* group levels was scarce. In a recent human study *Lactobacillus* group levels were not significantly modified after following different diets: high-protein and low-carbohydrate diet or a high-protein and moderate-carbohydrate diet (10). In mice fed with a high fat-content diet no significant differences were found in *Lactobacillus* group levels as compared to controls (8, 9).

The gut microbiota of adolescents also appeared to be different between subjects showing high weight-loss and low weight-loss during the intervention and, apparently, this feature was not related to significant dietary intakes. The adolescent group, which showed higher counts of total bacteria, *B. fragilis* group, *C. leptum* group and *B. catenolatum* group and lower *C. coccoides*, *Lactobacillus, Bifidobacterium* and *B. breve* counts in their fecal microbiota, was the one that experienced the highest weight loss under the intervention. In addition, *B. fragilis* group*, Bifidobacterium* and *Lactobacillus* group to *C. coccoides* group ratios were higher in the high weight-loss group than in the low weight-loss group. Thus, *Bacteroides* and *C. coccoides* group counts of the individual's microbiota seems to oppositely influence the ability of the host to loss weight under the same dietary intervention in agreement with the detected correlations between these bacterial groups and weight loss. The opposite influences that seem to exert these bacterial groups on body weight are in agreement with previous reports in obese mice models and in a small-scale trial with adult human subjects (6-7). In this context although increase counts of *C.leptum* group which includes certain members of genera *Clostridium, Ruminicoccus, Eubacterium* and *Faecalibacterium* that belong to *Clostridium* cluster IV (17), also seemed to favor weight loss, this trend was not confirmed when comparing the bacterial counts of this group before and after the intervention in the high weight-loss group. In addition reduced *B.bifidum* and *B.breve* counts and increased *B.catenulatum* counts seemed to favor weigh loss, but these trends were not confirmed by the changes detected before and after the intervention in the high weight-loss group. Therefore, further studies are needed to draw conclusions about the role of specific

Bifidobacterium species in obesity and weight management. In addition, the possibility that the low weight-loss group did not respond to the intervention due to failure to comply completely with the diet cannot be completely disregarded, because it is well recognized that obese patients have difficulty to accurately record their own food intake.

In summary, an association of specific bacterial groups with obesity and body weight loss has been reported in adolescents, pointing to a role played by *B. fragilis, Lactobacillus* and *Clostridium* groups, as well as by the relative proportions of *B. fragilis, Bifidobacterium* and *Lactobacillus groups* to *C. coccoides group*. The obtained results have also indicated that the interactions between the gut microbiota and body weight may be sensitive to lifestyle intervention to different extent depending on the individual's microbiota structure.

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Shifts in clostridia, bacteroides and immunoglobulin-coating faecal

bacteria associated with weight loss in obese adolescents

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Running title: Gut microbiota associated with weight loss.

ABSTRACT

To evaluate the effects of a multidisciplinary obesity treatment programme on fecal microbiota composition and immunoglobulin-coating bacteria in overweight and obese adolescents and their relationship to weight loss. Thirty-nine overweight and obese adolescents (BMI mean 33.1 range 23.7-50.4; age

mean14.8; range 13.0-16.0). BMI, BMI z-scores and plasma biochemical parameters were measured before and after the intervention. Fecal microbiota was analyzed by fluorescent *in situ* hybridization. Immunoglobulin-coating bacteria were detected using fluorescent-labelled F(ab´)2 antihuman IgA, IgG and IgM.Reductions in *C. histolyticum* and *E. rectale-C. coccoides* proportions significantly correlated with weight and BMI z-score reductions in the whole adolescent population. Proportions of *C.histolyticum, C.lituseburense* and *E.rectale-C.coccoides* dropped significantly whereas those of the *Bacteroides-Prevotella* group increased after the intervention in those adolescents who lost more than 4 kg. Total fecal energy was almost significantly reduced in the same group of adolescents but not in the group that lost less than 2.5 kg. IgA-coating bacterial proportions also decreased significantly in participants who lost more than 6 kg after the intervention, paralleled to reductions in *C. histolyticum* and *E. rectale-C. coccoides* populations. *E. rectale-C. coccoides* proportions also correlated with weight loss and BMI z-score reduction in participants whose weight loss exceeded 4 kg. Specific gut bacteria and an associated IgA response were related to body weight changes in adolescents under lifestyle intervention. These results suggest interactions between diet, gut microbiota and host metabolism and immunity in obesity.

INTRODUCTION

Obesity and the associated metabolic disorders, such as diabetes and metabolic syndrome, have become major public-health issues in adult and paediatric populations worldwide.[1-3] Obesity results from a positive energy balance and is characterized by a state of chronic, low-grade inflammation with abnormal cytokine and acute-phase inflammatory protein production.[4] Treatments based on calorie restriction, exercise and behavioural changes have succeeded to some extent to control obesity, but usually yield limited and transient weight loss.[5] More efficient strategies to control obesity and tackle its metabolic consequences are, therefore, urgently needed. In this context, it is essential to identify interactions between the environmental factors and host mechanisms involved in energy regulation with a view to developing additional intervention strategies. [3,6]

The environmental factors accounting for the dramatic rise in obesity in recent decades are not fully understood. Breastfeeding seems to be a protective factor against obesity later in life, whereas increased energy intake in formula or mixed-fed infants seems to be detrimental.[7] In recent studies, lack of breastfeeding, high early energy intake and high intake of sugar-sweetened beverages have also been shown to contribute to obesity in adolescents.[8] In addition, shifts in the composition of gut microbiota in response to dietary factors, such as total quantity and quality of carbohydrate and fat intake, have been reported.[9] In fact, the microbes populating the gut are currently being investigated as potential environmental factors involved in obesity.[6,9,10] Gut

microbiota is viewed as a metabolic organ that plays a pivotal role in the physiology of energy homeostasis.[11] Commensal bacteria contribute to the digestion of nutrients otherwise inaccessible to humans, such as complex polysaccharides.

The microbial fermentation of undigested dietary compounds in the large intestine can provide up to 10-15% of human daily energy supply.[11] Gut microbes are known to be involved in the absorption of monosaccharides and short-chain fatty acids, as well as in their conversion to complex lipids in the liver and their storage in adipocytes.[6] In addition, commensal bacteria colonizing the gut or in transit may also regulate the signalling pathways that link obesity with inflammation by interacting with the epithelium and host immune system.[12] So far, some studies associated obesity with an increase in the proportion of *Firmicutes* and a reduction in *Bacteroidetes*, in mice obesity models and adult human participants through small-scale intervention studies[10] but other results were controversial.[13] In mice fed on a high-fat diet, increases in *Bifidobacterium* levels achieved by intake of prebiotics were correlated with normalization of inflammatory status and endotoxaemia.[12] Notable differences in microbiota composition have also been shown between exercised and sedentary rats.[14] Nevertheless, the associations between specific gut bacteria and human host metabolism and immunity in relation to obesity remain largely uncharacterized.

The objective of this study was to evaluate the effects of a multidisciplinary obesity treatment programme (including energy-restricted

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diet and increased physical activity) on fecal microbial composition and immunoglobulin-coating bacteria in overweight or obese adolescents and assess their relationship to biochemical parameters and weight loss. Thus, stronger links between gut microbes and human obesity can be established.

METHODS

Participants and experimental design

Participants for the study were selected according to their body mass index (BMI) [weight (kg)/[height (m)^2] and classified as overweight or obese according to the International Obesity Task Force criteria defined by Cole *et al.*[1] during the course of the EVASYON study. The current study was designed to develop a multidisciplinary obesity-treatment programme adapted to Spanish primary health care centres and was assessed by Paediatric services in five cities around Spain. The treatment programme included nutritional and individual diet counselling, including calorie restriction and increased physical activity, as well as group therapies aim at changing behaviour, providing support and encouraging adolescents to change lifestyle and follow treatment recommendations.

A total of 39 overweight or obese Spanish adolescents (20 females and 19 males; mean age 14.8 years) were included in this study, and their characteristics are shown in Table 1. BMI *z*-scores were calculated as a function of the participant's obesity degree when compared with BMI local reference standards.[2] Over a 10-week period, the participants followed an

energy-restricted diet (a 10-40% reduction) established according to both obesity degree and regular physical activity determined by accelerometry.[15] The maximum energy intake was 1800 kcal/day for females and 2200 kcal/day for males. The physical activity program was established to increase energy expenditure by 15-23 kcal/kg body weight per week. None of the volunteers were treated with antibiotics during the study.

Table 1. Clinical characteristics of the studied subjects

Characteristics[a]	Total subjects n = 39	
Age (years)	14.4 (13.0–16.0)	
Diet (kcal day^{-1})	1762 (1300–2200)	
Weight loss (kg)	4.3 (0.8–16.7)	
Energetic expenditure per week	15–23 kcal kg^{-1} body weight	
	Before intervention	*After intervention*
BW (kg)	91.7 (62.0–145.0)	87.4 (62.8–131.2)
BMI (kg/m^2)	33.1 (24.8–50.7)	31.5 (23.7–50.4)
BMI z-score	3.4 (0.9–9.5)	2.9 (0.7–9.4)

[a] Data are expressed as mean value (range), BMI, body mass index, BW, body weight.

Energy food intake

To determine the intake of energy food diary records were kept for 72h (2 weekdays and 1 weekend day) both before the start of the study (baseline intakes) and after the intervention (week 10). Detailed information on how to record food and drink consumed using common household measures was

provided. Food diary records were returned to their dietician, and analyzed for energy contents based on the CESNID food-composition database of Spanish foods.[16]

Biochemical analyses

Fasting plasma glucose, total cholesterol, triglycerides, and HDL cholesterol were measured by enzyme-colorimetric automated methods (Roche, Neuilly sur Seine Cedex, France). LDL cholesterol was calculated by the Friedwald equation. Fasting plasma insulin was measured by the LINCOplex KIT Human Gut Hormone Panel (CAT-HGT-68K, Linco Research-St Charles, MO - USA).

Fecal sample collection and preparation for microbiological analyses

Fecal samples were collected at baseline and after 10 weeks of the intervention, frozen immediately after collection at -20 ºC, and stored until analysed. Feces were diluted 1:10 (w/v^{-1}) in PBS (pH 7.2) and homogenized in a Lab Blender 400 stomacher (Seward Medical London, UK) for 5 min. After low-speed centrifugation (2000 *g*, 2 min), the supernatant was collected. For bacterial quantification, cells were fixed by adding 4% paraformaldehyde solution (Sigma, St Louis, MO, USA) and incubated overnight at 4 ºC. After fixation, bacteria were washed two times in PBS by centrifugation (12000 *g* for 5 min). Finally, cell pellets were suspended in a PBS/ethanol mixture (1:1) and stored at −80 °C until analyzed as described earlier.[17]

Fluorescent *in situ* hybridisation for microbiological analysis

The bacterial groups present in feces were quantified by fluorescent in *situ* hybridization (FISH) using group-specific probes (MOLBIOL, Berlin, Germany). The specific probes and controls used in this study, as well as the hybridization conditions, are shown in Table 2. The EUB 338 probe, targeting a conserved region within the bacterial domain, was used as a positive control,[16] and the NON 338 probe was used as a negative control to eliminate background fluorescence.[17] Control probes were covalently linked at their 5 ´end either to indocyanine dye Cy3 or to fluorescein isothiocyanate (FITC). Specific cell enumeration was performed by combining each of the group-specific FITC-probes with the EUB 338-Cy3 probe as previously described.[17] Briefly, fixed cell suspensions were incubated in the hybridization solution (10 mM Tris-HCl, 0.9 M NaCl, pH 8.0 and 10% SDS) containing 4 ng/μl^{-1} of each fluorescent probe at appropriate temperatures, overnight. Then, hybridized cells were pelleted by centrifugation (10 000 *g* for 5 min) and resuspended in 500μl PBS solution for flow-cytometry analysis. The proportion of each bacterial group was expressed as a ratio of cells hybridizing with the FITC-labelled specific probe to cells hybridizing with the EUB 338-Cy3 probe.[17]

Table 2. Oligonucleotide probes and hybridization conditions used in the analysis of intestinal bacterial by fluorescent in *situ* hybridization

Probe	Target bacterial group	Sequence (5′–3′)	Hybridization conditions (°C)	References
EUB338	Domain bacteria	GCTGCCTCCCGTAGGAGT	50	[18]
NON338	Negative control	ACATCCTACGGGAGGC	50	[19]
Bif164	*Bifidobacterium*	CATCCGGCATTACCACCC	50	[20]
Chis150	*Clostridium histolyticum*	TTATGCGGTATTAATCT(C/T) CCTTT	50	[21]
Clit135	*Clostridium lituseburense*	GTTATCCGTGTGTACAGGG	50	[21]
Erec0482	*Eubacterium rectale/Clostridium coccoides*	GCTTCTTAGTCAGGTACCG	50	[21]
Lab158	*Lactobacillus/Enterococcus*	GGTATTAGCA(C/T)CTGTTTCCA	45	[22]
Bac303	*Bacteroides/Prevotella*	CCAATGTGGGGGACCTT	45	[23]
Enter1432	*Enterobacteriaceae*	CTTTTGCAACCCACT	50	[24]
Ecol1513	*Escherichia coli*	CACCGTAGTGCCTCGTCATCA	50	[25]
Rrec584	*Roseburia* subcluster	GGGACGTTGTTTCTGAGT	50	[26]
SRB687	Sulphate-reducing bacteria	TACGGATTTCACTCCT	50	[27]

Immunoglobulin-coating bacterial analysis

Bacterial cells from 20 µl of the supernatant obtained after low-speed centrifugation were collected (10 000 *g* for 5 min). The pellet was resuspended in 60 µl 1% (w/v^{-1}) BSA/PBS, containing 1% (v/v^{-1}) FITC-labelled F(ab′)2 antihuman IgA, IgG or IgM (CALTAG Laboratories, Burlingame, CA, USA). Another aliquot of each sample was pelleted and resuspended in 60 µl 1% (w/v^{-1}) BSA/PBS and used as control. After 30 min incubation, suspensions were washed two times with PBS. Bacterial pellet was finally resuspended in 500 µl PBS and mixed with 20 µl propidium iodine (100mg l^{-1}) to label total bacteria before flow-cytometry detection.[28]

Flow cytometry

Flow-cytometry detections were performed using an EPICS XL-MCL flow cytometer (Beckman Coulter, Florida, USA) as described earlier.[17] This instrument is equipped with two light scatter detectors that measure forward (FSC) and side scatter (SSC) and fluorescence detectors that detect

appropriately filtered light at green (FL1, 525 nm) and red-orange (FL3, 620 nm) wavelengths. The event rate was kept at the lowest setting (200-300 events per second) to avoid cell coincidence. A total of 15 000 events were recorded in a list mode file and analyzed with the System II V.3 software (Beckman Coulter). The proportion of each bacterial group was expressed as a ratio of cells hybridizing with the FITC-labelled specific probe to cells hybridizing with the universal EUB 338-Cy3 probe.[17] Immunoglobulin coating of faecal bacteria was expressed as a ratio of bacterial cells labelled with FITC-labelled F(ab´)2 antihuman IgA, IgG or IgM to the total cell population hybridizing with propidium iodine.[28]

Fecal energy determination

Energy content of feces was determined by calorimetry as described elsewhere[29] using an Automatic Adiabatic Bomb Calorimeter (Gallenkamp, Leicestershire, UK). Fecal samples were dried by lyophilization and samples of 1.5 g dry weight were analysed in duplicated. Gross energy content of faecal samples was defined as the amount of heat developed by the total combustion of a unit of dry weight sample.

Statistical analyses

Statistical analyses were done using the SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). Results are expressed as median values and ranges determined in duplicate. Total bacteria, Gram-positive and Gram-negative bacteria were calculated by adding the proportions of the corresponding groups

detected by specific probes, which do not overlap. Thus, total Gram-positive bacteria was calculated by adding the proportions obtained with the probes Chis150, Erec0482, Bif164, Clit135 and Lab158 and total Gram-negative was calculated by adding the proportions obtained with the probes Bac303, Ent1432 and SBR687. Differences in bacterial populations immunoglobulin-coating bacteria, fecal energy and biochemical parameters detected before and after the intervention programme were determined using the Mann–Whitney *U*-test of non-normal data distribution. The Spearman´s correlation test was used to calculate the correlations between bacterial count changes and either weight loss or biochemical changes as a result of the intervention. In every case, a *P*-value <0.05 was considered statistically significant.

Statement of ethics

We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research. Informed consent was obtained from all adolescents and their parents, and the study was approved by the local Ethics Committees.

RESULTS

Participants

Clinical characteristics did not differ significantly between the two groups of adolescents (A and B) at recruitment time (Table 1). Base line values of BMI and BMI z-score of group A were 30.7 (26.4-36.3) and 2.95 (1.6-4.03), respectively and those of group B were 33.1 (30.0-35.0) and 3.22 (2.57-4.16), respectively. The subjects, 51% female (20/39) and 49% male (19/39), were 14.4 years old (13.0-16.0 years), maintained an apparently good health status and did not consume antibiotics during the study (Table 1).

Most of the participants (n = 26) experienced significant (P = 0.050) weight loss from 4.1 to 16.6 kg (mean decrease of 7.6 kg) after 10 weeks of following the intervention programme. Some of them (n =13) did not experience remarkable weight loss (mean decrease of 1.1 kg; range 0.8-2.4 P=0.798). These two groups showed significant differences in their weight loss (P<0.001) and BMI z-score reduction (P<0.001) and, accordingly, were subdivided for comparisons of their fecal bacterial populations into groups A (> 4 kg, mean 7.6 kg weight loss) and B (<2.5 kg, mean 1.1 kg weight loss). BMI, and, BMI z-score (P=0.033 and P=0.039, respectively) detected before and after the dietary intervention was also significantly different in group A but not in group B. Group A was further subdivided into other two groups that also displayed significant differences in weight loss (P<0.001), one (group A1) integrated by participants with a weight loss of 4-6 kg (corresponding to a 5.5 % decrease in body weight) and the other (group A2) integrated by those

participants with a weight loss exceeding 6 kg (corresponding to 9.4 % decrease in body weight) for comparisons of fecal immunoglobulin coating bacteria as indicated below.

The dietary intervention resulted in a significant reduction ($P<0.050$) in total energy intake in both adolescent groups from 2284 (2739-1549) to 1429.4 (1049-1782) kcal/day^{-1} in group A and from 2159 (1926-2414) to 1416 (1296-1508) kcal/day^{-1} in group B. No significant differences in dietary energy intake were found between both adolescent groups before and after the intervention program.

Microbiota composition and energy of feces from adolescents

A follow-up study was made of the shifts in composition of fecal microbiota of the participants under study during the weight loss intervention programme. The results of the fecal microbiota analyses before and after intervention by FCM-FISH techniques are shown in Figures 1 and 2, and Table 3.

In the whole adolescent population, the intervention programme led to reductions in *Clostridium histolyticum* proportions, which correlated with weight loss (Figure 1a; r=0.43; P =0.009), as did reductions in *E. rectale-C. coccoides* proportions (Fig 1b; r=0.50, P=0.001). Similar correlations were found between *C. histolyticum* and *E. rectale-C. coccoides* proportions and BMI *z*-scores (r= 0.41; P=0.012 and r=0.39; P=0.014, respectively).
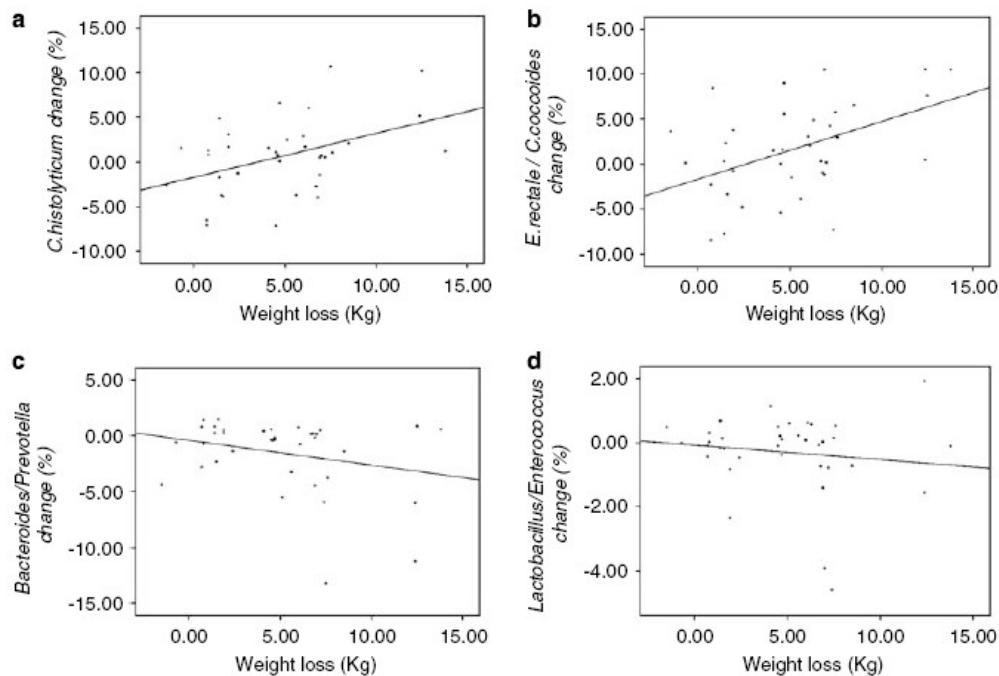
Figure 1. Correlation between changes in different bacterial groups (% before - % after intervention) and weight loss (kg before - kg after diet). Spearman's correlations: *C. histolyticum* changes and weight loss; $r = 0.43$, $P = 0.009$ (a) *E.rectale-C. coccoides* changes and weight loss; $r = 0.50$, $P = 0.001$ (b) *Bacteroides-Prevotella* changes; $r = -0.28$, $P = 0.083$ (c) *Lactobacillus-Enterococcus* changes and weight loss; $r = -0.15$, $P = 0.365$ (d).

Bacteroides proportions increased as a result of the intervention and almost reached significant levels of correlation with weight loss (Figure 1c; $r=-0.28$; $P =0.083$). Although increases in *Lactobacillus-Enterococcus* proportions were also parallel to reductions in weight (Figure 1d; $r =-0.15$; $P =0.361$) and BMI z-scores ($r = -0.29$; $P=0.074$), correlation was not significant. Shifts in *Bifidobacterium, C. lituseburense*, *Enterobacteriaceae*, *Escherichia coli*, *Roseburia* and sulphate-reducing bacterial groups were neither significantly correlated with weight loss nor with BMI z-score reductions.

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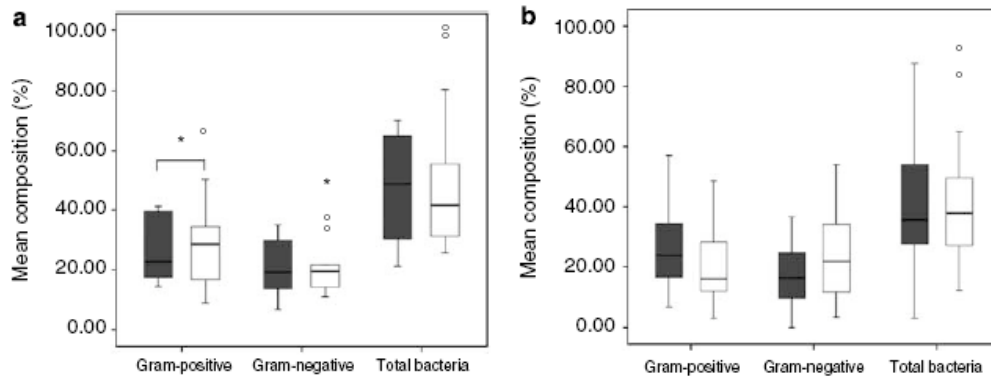
Figure 2. Overall bacterial composition in two adolescents groups: group A with >4 kg weight loss (A); group B with <2.5 kg weight loss (B). total Gram-positive bacteria was calculated by adding the proportions obtained with the probes Chis150, Erec0482, Bif164, Clit135 and Lab158 and total Gram-negative was calculated by adding the proportions obtained with the probes Bac303, Ent1432 and SBR687. *Significant differences ($P < 0.05$) between median values before (grey) and after (white) intervention programme by using the Mann-Whitney U test.

The composition of the fecal microbiota of two groups of adolescents A and B, which displayed significant differences in weight loss (> 4 kg in group A versus < 2.5 kg in group B) and BMI z-score reduction after intervention, was compared at base line, revealing the presence of significantly higher levels (P=0.008) of *Lactobacillus* proportions in the group A than in group B (Table 3). In group A, Gram-positive bacterial populations, estimated by adding the proportions of corresponding groups targeted by the probes, were significantly lower (P=0.046) after the intervention, whereas significant differences in Gram-negative bacteria and total bacteria were not detected (Figure 2a). In contrast, in group B no differences were detected in Gram-positive, Gram-negative or total bacteria proportions present in feces before and after the intervention (Figure 2b).

Table 3. Bacterial composition[a] of faecal samples as assessed by fluorescence in situ hybridization

Bacterial group	Group A Adolescents with >4.0 kg weight loss (n=26)			Group B Adolescents with <2.5 kg weight loss (n=13)		
	Before intervention	After intervention	P-value	Before intervention	After intervention	P-value
Bifidobacterium	8.31 (20.48–0.51)	7.85 (19.58–1.2)	0.898	8.72 (25.48–1.49)	7.14 (15.67–4.23)	0.497
C. histolyticum	5.38 (13.04–2.02)	2.95 (13.12–0.47)	0.011*	6.04 (13.34–1.44)	7.31 (12.52–4.14)	0.573
C. lituseburense	2.53 (17.3–0.33)	1.45 (17–0.16)	0.049*	2.73 (28.04–0.42)	1.98 (18.61–0.52)	0.538
E.rectale/C.coccoides	7.51 (19.4–1.53)	4.55 (20.57–0.51)	0.033*	5.98 (14.23–1.37)	8.02 (21.33–0.76)	0.978
Lactobacillus/Enteroccoccus	1.01 (3.15–0.16)	1.31 (5.64–0.08)	0.604	0.57 (2.88–0.24)	0.65 (2.79–0.1)	0.663
Bacteroides/Prevotella	2.51 (6.92–1.13)	3.09 (16.14–0.93)	0.047*	1.83 (4.30–0.22)	1.77 (7.10–0.34)	0.681
Enteric group	7.27 (22.17–0.43)	7.96 (23.21–0.74)	0.833	6.44 (14.58–2.60)	6.78 (17.59–1.64)	0.682
E. coli	4.48 (17.96–0.11)	5.12 (26–0.1)	0.503	2.30 (11.57–0.20)	1.97 (7.76–0.56)	0.457
Roseburia	6.01 (15.75–2.8)	8.36 (19.49–2.02)	0.304	4.78 (11.55–1.56)	5.34 (12.67–2.97)	0.383
Sulphate-reducing bacteria	7.41 (20.61–0.15)	6.76 (22.45–0.3)	0.749	4.79 (17.07–0.87)	5.62 (15.12–1.46)	0.758

[a] Data were expressed as median proportions of bacterial cell hybridizing with specific-group probes to total bacteria hybridizing with EUB probe 338 and ranges. * Significant differences (P<0.050) between median values of different bacterial group proportions before and after diet were established by using Mann-Whitney *U*-test

In group A, which experienced important weight loss (> 4 kg) and BMI *z*-score reductions (mean decrease 1.05; range 1.86-0.38), *C. histolyticum, C. lituseburense* and *E. rectale-C. coccoides* proportions decreased significantly after the intervention programme (*P*=0.011, *P*=0.049, and *P*=0.033, respectively), whereas those of *Bacteroides-Prevotella* group were significantly increased (*P*=0.047). *Lactobacillus-Enterococcus*, *Enterobacteriaceae*, *E. coli,* and *Roseburia* groups showed slight increases whereas *Bifidobacterium* and sulphate-reducing bacteria tended to decrease but these changes were not statistically significant. Reduced *C. Histolyticum and E. rectale-C. coccoides* proportions significantly correlated with percentage of body weight loss (*r*= 0.48; *P*=0.020 and *r*= 0.41; *P*=0.036, respectively) and those of *E. rectale-C. coccoides* with BMI z-score reductions (*r*= 0.36;

P=0.020 and *r*= 0.41; *P*=0.036, respectively). In the adolescents group B, who did not experience a significant weight loss, none of the analyzed bacterial groups showed statistically significant differences before and after the intervention programme (Table 3). No correlations were detected between bacterial proportions and either body weight or BMI *z*-score reductions.

Fecal energy content before (5.43 (5.11-5.90) kcal g^{-1}) and after (5.16 (4.94-5.28) kcal g^{-1}) the intervention was almost significantly reduced (*P*=0.055) in group A of adolescents. In contrast, fecal energy content before (5.42 (5.04-5.66) kcal g^{-1}) and after (5.33 (5.2-5.42) kcal g^{-1}) the intervention was not significantly different (*P*=0.513) in group B of adolescents.

Biochemical parameters and correlations with faecal microbiota

Biochemical parameters in both groups of adolescents before and after the intervention are shown in Table 4. No significant differences were found in the analyzed biochemical parameters between both adolescent groups A and B before the intervention (base line) except for LDL levels, which were higher in group B of adolescents (P=0.034).

Serum HDL-cholesterol values were significantly higher (*P*=0.031) before than after the intervention in group B of adolescents but did not correlate with any bacterial group changes. Slight changes in serum glucose concentration correlated with slight changes in *E. rectale-C. coccoides* as a result of the intervention in group B of adolescents (*r*=0.683, *P*=0.030). Serum

glucose (*P*=0.029) and total cholesterol (*P*=0.012) concentration significantly dropped in group A of adolescent after the intervention.

Table 4. Biochemical parameters determined in plasma of adolescents before and after the intervention

Parameters	Group B Adolescents with <2.5 kg weight loss (n = 13)					Group A Adolescents with >4.0 kg weight loss (n = 26)				
	Before intervention		After intervention			Before intervention		After intervention		
	Median	Range	Median	Range	P-value	Median	Range	Median	Range	P-value
Glucose (mg per 100 ml)	85.5	83.5–90.0	83.0	78.0–87.0	0.309	87.5	83.0–99.7	83.0	76.7–90.2	0.029*
Total cholesterol (mg per 100 ml)	141.0	121.0–158.0	141.0	129.0–152.0	0.977	152.5	133.0–163.0	132.5	123.7–147.0	0.012*
Triglycerides (mg per 100 ml)	70.0	54.5–88.0	71.0	59.0–100.0	0.562	84.0	57.5–121.5	73.5	50.0–106.5	0.527
HDL cholesterol (mg per 100 ml)	48.0	40.0–82.0	45.0	36.0–49.0	0.236	46.0	40.5–50.0	45.0	39.0–63.0	0.836
LDL cholesterol (mg per 100 ml)	73.0	70.5–101.0	49.0	40.5–75.5	0.031*	79.0	52.2–98.7	75.5	65.0–86.2	0.391
Insulin (pg ml^{-1})	344.2	529.0–664.0	504.6	349.0–760.0	0.555	471.6	417.1–772.0	421.5	263.0–632.0	0.139

*Statistical differences before and after the intervention were calculated using the Mann-Whitney U-test and established at P< 0.050

. Changes in glucose and cholesterol significantly correlated with changes in the enteric group proportions (*r*=-0.547, *P*=0.006 and *r*=-0.462, *P*=0.035, respectively). In addition, changes in glucose significantly correlated with changes in total Gram-negative bacteria (*r*=-0.538, *P*=0.012). LDL-cholesterol was reduced after the intervention although not significantly and correlated to changes in *C. lituseburense* proportions (*r*=-0.508, *P*=0.019).

Immunoglobulin-coating bacteria in faeces from adolescents

Immunoglobulin-coating bacteria were detected in feces of adolescents who experienced the greatest loss (> 4 kg; group A) in body weight (Figure 3). Overall, higher percentages of IgA, IgM and IgG-coating bacteria were detected in feces of adolescents before the intervention than after it. The proportions of IgA-coating bacteria were significantly reduced (*P*=0.034) after

the intervention programme in group A1 of participants, who lost over 6 kg, whereas these differences were not significant in group A2 of participants with weight reductions between 4 and 6 kg (Figure 3).
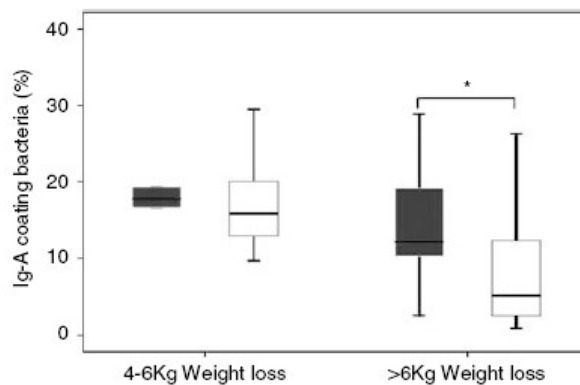

Figure 3. Mean percentage of fecal bacteria coated with IgA. *Significant differences ($P < 0.05$) between median values of different groups (A1 group of adolescents with weight loss 4-6 kg and A2 group of adolescents with weight loss > 6 kg) before (black) and after (white) intervention programme by using the Mann-Whitney *U*-test.

Group A1 also revealed significant reductions in proportions of *C. histolyticum,* and *E. rectale-C. coccoides* groups *(P=0.046* and *P=0.044, respectively)* whereas changes in group A2 did not reach statistical significance ($P<0.050$), indicating that these bacteria exerted the greatest influence on IgA-coating bacterial changes. In addition, reduced *E. rectale-C. coccoides* proportions in group A1 significantly correlated with the percentage of body weight loss and BMI *z*-score reduction ($r= 0.56$; $P=0.023$ and $r= 0.53$; $P=0.035$, respectively). Group A1 also showed significantly lower proportions of total Gram-positive bacterial populations ($P=0.034$) after the intervention programme, whereas no significant differences in Gram-negative bacteria and

total bacteria were detected. Therefore, changes in IgA secretion against the gut microbiota could be explained by specific changes in its composition associated with weight loss.

Discussion

Diet is considered to be one of the main environmental factors shaping the composition of the gut microbiota within a host and affecting their functional relationships. This study has demonstrated that a weight-loss intervention programme, based on a calorie restricted diet and increased physical activity, induced changes in the gut microbiota structure of obese adolescents and that some of these changes correlated with weight loss and BMI z-score reductions. Therefore, the relative abundance of specific gut bacteria seems to be susceptible to lifestyle intervention and may be an additional element for consideration in weight management strategies.

Reduced *E. rectale-C. coccoides* and *C. histolyticum* proportions were significantly correlated with weight loss and BMI z-score reduction in the total population. By contrast, *Bacteroides* proportions increased as a result of the intervention programme, although their correlations with weight loss and BMI z-scores did not reach statistical significance. In previous human studies in adults, decreases in *Firmicutes* division, which include *Clostridium* clusters, as well as increases in *Bacteroidetes* division have also been correlated with the percentage of body weight loss; however, a very limited number of participants were included in the corresponding study.[10] Therefore, the present report has confirmed that the abundance of these two bacterial groups in the distal gut

could be linked to human weight loss although controversial results have also been reported recently.[13]

Specific fecal bacterial proportions differed significantly in group A of adolescents, who experienced a remarkable weight loss (> 4 kg) representing on average 8.1 % of their body weight, as a result of the intervention. Accordingly, previous studies have indicated that *Firmicutes* and *Bacteroides* changes are associated with weight-loss percentage. These changes were only evident when the individuals had lost at least between 2 and 6% of their body weight, without finding a relationship to the type of diet (either fat or carbohydrate restricted).[10] Overall, total Gram-positive bacterial populations were significantly reduced and Gram-negative bacteria slightly increased after the intervention programme in group A of adolescents. *C. histolyticum, E. rectale-C. coccoides* and *C. lituseburense* were identified as the main contributors to the overall reduction in Gram-positive bacteria, whereas *Bacteroides* group contributed to the increase in total Gram-negative bacteria.

These gut microbes could play a role in obesity together with diet by affecting either host metabolism,[6] or the signalling pathways that link inflammation with obesity.[12] *E. rectale-C. coccoides* group includes clostridia cluster XIV, which integrates the main butyrate-producing bacteria in the distal colon.[29] These bacteria are responsible for generating butyric acid from carbohydrate fermentation, which fuels epithelial cells covering up to 70% of their energy needs.[30] The reduction of these bacterial groups by intervention in obese adolescents could contribute to reducing the overall ability of the gut

microbiota to harvest energy from the diet, which could account for up to 10-15% of our daily caloric supply.[31] Nevertheless, butyrate generation by gut microbes has generally been associated with beneficial effects, including satiety promotion, rather than with obesogenic features,[32] indicating that more complex mechanisms related to fatty-acid metabolism could link *Firmicutes* and *Clostridium* clusters with obesity.

C. histolyticum proportions were also reduced after the intervention programme in adolescents and these shifts were correlated with weight loss. This group belongs to clostridia cluster II, which are highly proteolytic and produce acetate as the main end product of metabolism.[33] Increased levels in colonic and serum acetate, which may stimulate lipid synthesis, have also been associated with microbial gut colonization.[34,35] In addition, *C. histolyticum* produces proteases that are cytotoxic for cells and tissues and could be pathogenic factors in the gut environment. The prevalence of this clostridial group could also increase protein fermentation in the colon with the subsequent generation of toxic compounds such as sulphur-containing metabolites.[11] *C. lituseburense* group, which is included in clostridia cluster XI, was also decreased after the intervention in group A of adolescents. This is a heterogeneous phylogenetic cluster but comprises opportunistic pathogens, such as *C. difficile*, and its abundance together with that of *C. histolyticum* group could modify the potential virulence of the gut microbiota in obese patients, which in turn was improved in adolescents showing the highest weight loss.[33] In contrast to *Clostridium* groups, *Bacteroides* were increased in individuals showing a significant weight loss (< 4 kg) after the dietary

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intervention, which may be related to changes in the type of short-chain fatty acid generated and colonic pH increases.[36] Increased *Bacteroides* populations could contribute to generating propionate, which has been shown to inhibit lipid synthesis from acetate and may favour a lean phenotype.[35,37]

The study has also showed almost significant changes in total fecal energy in group A of adolescents after the intervention whereas not in group B. Although the fecal energy only reflect part of the energy supply that could be due to the colonic microbiota, the obtained results could partly explain the detected differences in weight loss between the two adolescent groups (A and B) paralleled to microbiota changes. In fact, differences in total fecal energy between both adolescent groups could not be related to differences in total dietary energy intake. Therefore, these and previous results point for a role of gut bacteria other than common probiotic genera in weight management and, therefore, the current dietary strategies used to modulate the gut microbiota based on the administration of lactobacilli, bifidobacteria and prebiotics that favour their predominance could be questioned in obesity control.

Nevertheless, a recent study has not found correlations between *Bacteroides* populations and obesity by comparing obese and non-obese partiucipants and there was not significant relationship between changes in the percentage of *Bacteroides* in feces and weight loss under reduced calorie diets.[13] Significant shifts detected in *Roseburia-E. rectale* groups detected with the probe Rrec482 and *Bifidobacterium* were related to reductions in carbohydrate intake but regardless weight loss.[13] By contrast, the present study

has not shown significant reductions in these bacterial groups in feces of participants submitted to energy intake reduction, including carbohydrate restrictions (approximately 28% reduction), but confirmed previous relations between *Clostridium* and *Bacteroides* groups with weight loss under dietary intervention.[10] Therefore, the possibility that weight loss depends on both the diet and its interactions with gut microbiota could not be completely disregarded.

Glucose and LDL-cholesterol reductions detected in group A of adolescents were also correlated with shifts in total Gram-negative bacteria and *C. lituseburense*. Although further studies should be carried out to confirm these trends, the results also suggest interactions between diet, gut bacteria and host's metabolism as proposed earlier.[10]

Obesity and related disorders, such as the metabolic syndrome, are also associated with a chronic low-grade inflammation even at early ages, exemplifying the link between metabolism and immunity.[4] In this study, gut microbiota has been identified as a factor stimulating host immunity to different extents depending on weight loss. Elevation of IgA coating bacteria before the intervention programme could be an indication of low-grade inflammation triggered by the gut microbiota before intervention, as this microbiota is characterized by increased levels of opportunistic pathogens when compared with that detected after the diet in group A1 of adolescents.[38]

The decrease in IgA-coating bacteria detected after the intervention was particularly associated with reductions in *C. histolyticum* and *E. rectale-C. coccoides* proportions. The reductions in butyrate-producing bacteria of *E. rectale-C. coccoides* group, as a result of the intervention programme, could also be responsible for reducing energy availability for immune cells leading to reducing IgA-producing cells and mucosal IgA concentrations.[39,40] Our results suggest that the increased host immune response trigger by the gut microbiota can be modified in overweight adolescents by a lifestyle intervention, confirming that there is a relationship between gut microbiota and host immunity in obese human subject.

The limitations of the study include the relative small sample size of subgroups due to differences in weight loss responses of the whole population group and the short duration of the intervention, which could reduce the significance of the detected changes. However, this short-term study confirmed and complemented the results of a previous long-term study (1 year) by using different molecular techniques that target specific bacterial groups.

All in all, this study has provided stronger links between specific bacterial groups and body weight in adolescents under a lifestyle intervention. This study also suggest a role for the gut microbiota in this disorder related to both host metabolism and immunity, evidenced by shifts in the bacteria driving the main metabolic pathways in the colon and showing different pathogenic features, although direct evidence should still be provided.

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Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant woman

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Running title: Pregnant gut microbiota and overweight

ABSTRACT
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Obesity is associated with complications during pregnancy and increased health risks in the newborn. The objective of this study was to establish possible relationships between gut microbiota, body weight, weight gain, and biochemical parameters in pregnant woman. Fifty pregnant women were classified according to their body mass index (BMI) in normal weight (n 34) and overweight (n 16) groups. Gut microbiota composition was analyzed by quantitative real-time PCR in faeces and biochemical parameters in plasma at 24 weeks of pregnancy. Reduced numbers of *Bifidobacterium* and *Bacteroides* and increased numbers of *Staphylococcus, Enterobacteriaceae* and *E. coli* were detected in overweight compared to normal weight pregnant women. *E. coli* numbers were higher in women with excessive weight gain than in woman with normal weight gain during pregnancy, while *Bifidobacterium* and *Akkermansia muciniphila* showed an opposite trend. In the whole population, increased total bacteria and *Staphylococcus* numbers were related to increased plasma cholesterol levels. Increased *Bacteroides* numbers were related to increased HDL cholesterol and folic acid levels, and reduced triglyceride levels. Increased *Bifidobacterium* numbers were related to increased folic acid levels. Increased *Enterobacteriaceae* and *E. coli* numbers were related to increased ferritin and reduced transferrin, while *Bifidobacterium* levels showed the opposite trend. Therefore, gut microbiota composition is related to body weight, weight-gain and metabolic biomarkers during pregnancy, which might be of relevance to the management of woman and infant's health.

INTRODUCTION

The prevalence of obesity is rapidly increasing worldwide, constituting an important health issue. Obesity is the result of a positive imbalance between energy intake and energy expenditure over a long period and is related to the development of other disorders such as diabetes, dyslipaemia and cardiovascular diseases. Obesity is also associated with complications during pregnancy and at the delivery for women and with increased health risks in newborn [1-3].

There are several genetic and environmental factors such as diet, cultural behaviour, and socioeconomic status, which influence obesity [4,5]. In addition, recent reports suggest that the nature and composition of the intestinal microbiota are altered in obesity [6,7]. Lean individuals have more *Bacteroidetes*, while obese individuals have more *Firmicutes*, including *Clostridium* clusters, in their intestinal microbiota [6,7]. It has been proposed that such bacterial composition improved the ability of the host to extract energy from the diet and to store this energy in the adipose tissue [7]. Gut microbiota has also been related to body weight and body weigh loss under a lifestyle intervention in humans [8,9]. Although obesity is an important health issue during pregnancy, the relationships between the gut microbiota composition and obesity has been scarcely studied in pregnant women [10].

The aim of the present study was to analyse the microbiota composition of pregnant women and establish its possible relationships with body weight,

weight gain and biochemical parameters to progress in the understanding of the role of the microbiota in the health status of pregnant woman.

EXPERIMENTAL METHODS

Study participants

The pregnant women were recruited at 20 weeks of pregnancy at the Clinical University Hospital "San Cecilio" de Granada, Spain. Women were classified according to their pre-pregnancy Body Mass Index (BMI) into two groups, overweight women (n=16) with BMI>25 and normal weight women (n=34) with BMI<25 (Table 1). Signed informed consent was obtained from the studied women after a full explanation of the study was given by a member of the team at the first visit. Participants were assured of anonymity and confidentiality. After the visit at the first trimester, women were examined by the obstetrician again at 24 (2nd trimester) and 34 weeks (3rd trimester) and clinical parameters were recorded. At 24 weeks of pregnancy, faecal and blood samples were obtained for microbiological and biochemical analysis. Data on weight before pregnancy was used to calculate weight gain during pregnancy. Normal weight gain ranges were from 11.5 to 16.0 kg for normal weight women (BMI 19.8–25.0) and from 7.0 to 11.5 kg for overweight women (BMI >25), respectively, over pregnancy according to the Institute of Medicine (IOM) criteria [11]. Total weight gains above these values, >16 kg for normal-weight women and >11.5 kg for overweight women, were considered

excessive weight. Data on gestation time and birth weights of the newborns were also collected. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethics committee of the Hospital involved in the study. Written informed consent was obtained from all subjects before their inclusion in the study.

Dietary assessment

Food diary records of pregnant women were kept for 72h (2 weekdays and 1 weekend day) at 24 weeks of pregnancy. Detailed information on how to record food and drink consumed using common household measures was provided. Food diary records were returned to their dietician, and analyzed for energy, water and nutrient contents based on the CESNID food-composition database of Spanish foods[12].

Biochemical parameters

Fasting plasma glucose, total cholesterol, HDL cholesterol, triglycerides, urea, creatinine, uric acid, bilirubin and iron were measured by enzyme-colorimetric automated methods for clinical chemistry (Modular analytics EVO, Roche, Neuilly sur Seine Cedex, France). LDL cholesterol was calculated using the Friedwald's formula [13]. Ferritin, transferrin, folate and thyroid - stimulating hormone (TSH) levels were measured by using the automatic analyser Elecsys 2010 with modular analytics E170 (Roche, Neuilly sur Seine Cedex, France).

Table 1. Clinical characteristics of the studied subjects

Characteristics	Normal-weight group (n 34)		Overweight group (n 16)		P*
	Median	IQR	Median	IQR	
Women					
Age (years)	31·0	27·7–34·2	29·0	28·0–33·5	0·646
Height before pregnancy (cm)	163·0	158·0–167·0	162·0	158·0–166·0	0·967
Weight (kg)					
Before pregnancy	58·0	53·2–64·5	73·0	70·0–84·0	>0·001
First trimester	62·5	58·1–66·2	75·4	71·4–86·4	>0·001
Second trimester (24 weeks)	66·4	60·7–72·3	77·3	73·5–88·6	>0·001
Third trimester (34 weeks)	70·4	66·0–75·5	81·2	78·0–100·0	>0·001
Weight gain over pregnancy (kg)	11·7	8·8–14·3	10·0	6·2–11·4	0·120
BMI (kg/m²)					
Before pregnancy	23·0	20·8–24·3	28·7	26·3–31·2	>0·001
First trimester	23·3	21·0–25·0	29·0	27·8–32·7	>0·001
Second trimester (24 weeks)	24·0	22·7–25·7	30·0	26·8–33·2	>0·001
Third trimester (34 weeks)	26·6	25·1–28·2	30·8	28·9–35·5	>0·001
Newborns					
Duration of gestation (weeks)	39·0	38·5–40·0	39·5	39·0–41·0	0·165
Birth weight (kg)	3·20	3·1–3·4	3·50	3·2–4·0	0·028

Median values and interquartile ranges (IQR), *Significant differences were calculated using the Mann-Whitney U-test. A significant between was considered at P<0.050

The transferrin saturation index was calculated using the following formula: TSI (%) = (ferritin (ug/ml).100)/(transferrin (mg/dl)x1.24).

Sample preparation and DNA extraction

Faecal samples were frozen immediately at -20ºC and kept until processing. Faeces (1g) were diluted 1: 10 (w/v) in PBS (pH 7.2), homogenized and used for DNA extraction. DNA from pure cultures of reference bacterial strains and faecal samples were extracted using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration of DNA was determined with a Nanodrop-1000 spectrophotometer (Nanodrop, Wilmington, DE).

Analysis of faecal microbiota composition

Quantitative real time PCR (qPCR) was used to characterize the microbiota by using of specific primers targeting different bacterial groups and the SYBR® Green PCR Master Mix (SuperArray Bioscience Corporation, Frederick, MD, USA), as previously described [9,10]. PCR amplification and detection were performed with an ABI PRISM 7000-PCR sequence detection system (Applied Biosystems, Warrington, UK). Bacterial concentration from each sample was calculated by comparing the Ct values obtained from standard curves. Standard curves were created using serial 10-fold dilution of pure culture DNA corresponding to 10^2 to 10^9 cell equivalents/ml (genome equivalents/ml). Conversion of the amount of bacteria DNA in samples determined by qPCR to theoretical genome equivalents required the assumption that the genome size and 16S rRNA gene copy number for each bacterial group analyzed was similar. The following genome sizes were used in the study: 2.3 Mb for *Bifidobacterium* (using *B. longum* as standard), 2.9 Mb for *Lactobacillus* (*L. casei*), 5.2 Mb for *Bacteroides* (*B. fragilis*), 4 Mb for *C. coccoides* group, 3.3 Mb for *C. leptum* group, 4.6 Mb for *Enterobacteriaceae* and *E. coli*, 2.8 Mb for *Staphylococcus* (*St. aureus*) and 2.7 Mb for *Akkermansia muciniphila*. Genome sizes were obtained from NCBI data base (Genome project). Standard curves were created using the following reference strains: *Bifidobacterium longum* subsp. *longum* CECT 4503, *Bacteroides fragilis* DSMZ 2451; *Clostridium coccoides* DSMZ 933; *C. leptum* DSMZ 935; *Staphylococcus aureus* CECT 86; *Lactobacillus casei* ATCC 393; *E. coli* CECT 45 and *Akkermansia muciniphila* strain Muc^T (ATCC BAA-835^T).

Statistical analyses

Statistical analyses were done using the SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). Data distribution was analysed by applying the Kolmogorov-Smirnov test and creating a Gaussian. Due to non-normal distribution, microbial data are expressed as medians with interquartile ranges (IQR). The Mann-Whitney U-test was applied for comparisons between bacterial numbers of normal and overweight women and between women with excessive and normal weight gain over pregnancy. Differences in prevalence of bacterial groups were established by applying the Chi-square test. Correlations between variables were determined by applying the Spearman's rank correlation. A $P < .050$ was considered statistically significant for all tests.

RESULTS

Body weight, body mass index, and weight gain over pregnancy

Clinical characteristics of the studied women at recruitment time were similar in both groups (Table 1) except for BMI and body weight. The body weight of the overweight women was significantly higher than that of normal weight women during pregnancy, although no significantly differences ($P=.120$) in weight gain were detected between the groups over time. BMI was significantly different ($P < .050$) between normal weight and overweight women and increased in both groups over pregnancy. The infants were born at

term and the infant's birth weight of the overweight women were higher than those of normal weight women (*P*= .028).

Dietary intakes

Dietary data of normal weight and overweigh pregnant women at 24 weeks of pregnancy are shown in Table 2. No significant differences in dietary intake of energy, macronutrients or on food group level were found between both groups of women. Only the intake of fiber was slightly higher (*P*= .057) in normal weight than in overweight woman. When women were grouped according to the total weight gain over pregnancy into two groups (excessive and normal weight gain), no significant differences in dietary intake of energy, macronutrients or on food group level were found between the two groups. No correlations were found between dietary intakes, body weight and body weight gain.

Biochemical parameters

Biochemical parameters of pregnant women at 24 weeks subdivided according to their BMI in normal and overweight women are shown in Table 3. Bilirubin, iron and folic acid levels were significantly higher in normal than in overweight women (*P*=.021, *P*= .021 and *P*= .042, respectively).

Table 2. Daily energy and nutrient intake in normal and overweight women at 24 weeks of pregnancy.

	Normal-weight group (18 < BMI < 25 kg/m²) (n 34)		Overweight group (BMI > 25 kg/m²) (n 16)		
	Median	IQR	Median	IQR	P *
Energy (kJ)	8·86	7·85–10·16	8·06	6·68–9·74	0·430
Water (g)	8·40	6·47–11·17	8·02	7·27–12·31	1·000
Protein (g)	82·14	73·0–99·0	86·54	75·0–103·0	0·610
Protein (%)	16·31	14·7–17·7	17·53	15·2–20·1	0·265
Plant protein (g)	25·04	22·7–28·7	23·22	16·4–26·5	0·265
Plant protein (%)	4·73	4·4–7·2	4·90	3·5–5·1	0·458
Animal protein (g)	56·85	48·3–71·7	61·25	50·6–74·8	0·546
Animal protein (%)	11·77	9·9–12·7	13·15	10·3–15·7	0·063
Fat (g)	91·35	75·4–105·0	85·00	62·5–105·0	0·458
Energy from fat (%)	40·28	35·8–44·7	40·31	33·5–44·3	0·926
Saturated fat (g)	30·36	27·1–40·4	26·71	20·1–42·0	0·577
Energy from saturated fat (%)	13·77	12·2–14·9	13·33	10·0–17·6	0·963
MUFA (g)	37·20	30·0–48·5	34·34	27·4–41·0	0·577
Energy from MUFA (%)	16·23	14·0–20·0	16·00	15·1–17·7	0·889
PUFA (g)	13·88	11·2–16·4	12·00	9·2–16·7	0·458
Energy from PUFA (%)	5·6	4·8–7·3	5·56	4·4–6·5	0·642
Cholesterol (mg)	279·70	228·5–414·8	352·46	222·1–478·5	0·458
CHO (g)	227·70	200–262·0	197·00	171·8–262·7	0·330
Energy from CHO (%)	43·06	38·0–48·3	42·43	38·0–45·1	0·610
Simple CHO (g)	125·20	111·8–153·3	102·12	79·0–139·5	0·150
Energy from simple CHO (%)	22·02	19·5–27·0	21·23	18·6–23·2	0·280
Complex CHO (g)	101·51	87·5–110·8	98·00	74·8–120·2	0·889
Energy from complex CHO (%)	18·60	17·0–24·1	20·45	17·0–23·4	0·816
Dietary fibre (g)	19·81	16·2–24·3	16·75	13·6–19·0	0·057

Median values and interquartile ranges (IQR). CHO, Carbohydrates
*Statistical significant differences were calculated by using the Mann-Whitney *U*. A significant difference between the groups was considered at *P* <0.050

HDL cholesterol was higher (*P*= .050) in normal than in overweight women, whereas total cholesterol and triglycerides levels were significantly higher in overweight than in normal weight women (*P*= .019 and *P*= .034, respectively). Moreover, increased levels of triglycerides (R=0.30, *P* =.033) and total cholesterol (R=0.43, *P* =.002) and reduced levels of bilirubin (R=-0.36, *P* = .019) and iron (R=-0.33, *P* = .019) correlated to overweight women. When women were grouped according to the total weight gain over pregnancy into two groups (excessive and normal weight gain), correlations with some biochemical parameters were also detected. Increased levels of total cholesterol (R=0.33, *P* = .020) and ferritin (R=0.45, *P* = .001) correlated with women with excessive weight gain over pregnancy.

Table 3. Biochemical parameters recorded at 24 weeks of pregnancy of normal-weight (BMI < 25 kg/m^2) and overweight women. (IBM > 25 kg/m^2)

Biochemical parameter	Reference value	Normal-weight group (n 34)		Overweight group (n 16)		P*
		Median	IQR	Median	IQR	
Glucose (mg/l)	650−1100	765	697−810	770	635−900	0·840
Urea (mg/l)	100−500	200	175−256	197	147−227	0·288
Creatinine (mg/l)	5−12	6	5−7	5	5−6	0·072
Uric acid (mg/l)	24−70	31	27−37	33	29−37	0·493
Bilirubin (mg/l)	0−10	2	2−3	1	1−3	0·021
Cholesterol (mg/l)	1200−2200	2330	2067−2560	2590	2300−2810	0·019
TAG (mg/l)	500−1700	1480	1190−1860	1920	1630−2250	0·034
HDL-cholesterol (mg/l)	450−650	770	697−955	660	580−830	0·050
LDL-cholesterol (mg/l)	500−1500	1350	997−1500	1300	990−1380	0·580
Total protein (g/l)	65−87	70	66−71	70	67−71	0·502
Albumin (g/l)	35−50	40	37−41	38	36−40	0·395
Fe (µg/l)	450−1500	795	637−1057	600	530−950	0·021
Ferritin (ng/ml)	30−400	19·0	10·5−31·2	20·0	16·3−33·7	0·356
Transferrin (mg/l)	2120−3600	3585	3207−4130	3500	3050−3970	0·288
Saturation transferrin index (%)	17·1−30·6	18·7	14·0−25·0	16·5	11·6−23·0	0·362
Folic acid (ng/ml)	3·1−17·5	15·3	10·6−18·5	10·5	7·3−17·0	0·042
TSH (µUI/ml)	0·3−4·2	1·4	0·9−1·7	1·6	1·0−1·6	0·368

Median values and interquartile ranges (IQR) TSH, thyroid-stimulating hormone

* Statistical differences were calculated using the Mann-Whitney *U* test. A significant difference between the group was considered at *P* <.050.

Microbiota composition in normal and overweight women

The bacterial numbers detected in faecal samples of normal- and overweight women are shown in Table 4. *Bifidobacterium* and *Bacteroides* numbers were significantly higher (*P* > .001 and *P* = .035, respectively) in normal weight women than in overweight women, whereas *Enterobacteriaceae* (*P* = .001), *E. coli* (*P* = .005) and *Staphylococcus* (*P* = . 006) numbers were lower in normal weight than in overweight women. *C. coccoides* group numbers were slightly higher in overweight women than in normal weight woman, but not significantly (*P* = .088). The ratio of *Bifidobacterium* to *C. coccoides* group was significantly higher (*P* < .001) in normal weight than in overweight women. The ratio of *Bifidobacterium* to both

Clostridium groups (*C. coccoides* plus *C. leptum*) was also significantly higher (*P* < .001) in normal weight than in overweight women. Increased numbers of *Bifidobacterium* (R=- 0.56, *P* < .001) and *Bacteroides* (R= -0.34, *P* = .020) correlated to normal weight women, while a different trend was found for *Staphylococcus* (R=0.67, *P* = .003), *Enterobacteriaceae* (R=0.46, *P* < .001) and *E. coli* (R=0.40, *P* = .004) (Fig 1). An increased ratio of *Bifidobacterium* to *C. coccoides* correlated to lower BMI (R=-0.60, *P* < .001). Similarly, an increased *Bifidobacterium* to *C. coccoides* plus *C. leptum* ratio was positively related to normal weight women (R=-0.54, *P* < .001).

Table 4. Bacterial numbers in faecal samples (cell equivalents (genome equivalents) per g faeces) analysed by quantitative real-time PCR at 24 weeks of pregnancy.

| | Normal-weight women (n 34) | | | Overweight women (n 16) | | | |
| | | Bacterial numbers (log genome equivalents/g faeces) | | | Bacterial numbers (log genome equivalents/g faeces) | | |
Microbial groups	Prevalence†	Median	IQR	Prevalence†	Median	IQR	P*
Total cell counts	34/34	9·85	9·40–10·24	16/16	9·89	9·40–10·02	0·630
Bifidobacterium	34/34	9·10	8·53–9·52	16/16	8·36	7·74–8·57	>0·001
Lactobacillus group	34/34	7·48	7·35–7·60	16/16	7·70	7·40–7·78	0·053
Clostridium coccoides group	34/34	8·52	7·78–8·87	16/16	8·75	8·29–9·12	0·088
Clostridium leptum group	30/34	8·40	8·04–8·78	14/16	8·35	7·37–8·66	0·313
Bacteroides	34/34	6·88	6·21–7·23	16/16	6·20	6·00–6·66	0·035
Enterobacteriaceae	34/34	6·37	6·10–6·76	16/16	7·23	6·65–7·90	0·001
Escherichia coli	34/34	5·17	4·68–5·70	16/16	6·20	5·50–7·14	0·005
Staphylococcus	8/34	4·40	3·94–4·74	9/16	5·78	4·83–6·37	0·006
Akkermansia muciniphila	34/34	8·35	7·56–9·00	16/16	8·50	7·10–9·45	0·763

Median values and interquartile ranges (IQR)) * Significant differences were calculated by using Mann-Whitney *U* test. A Significant difference between the groups was considered at *P* <.050. † Prevalence reflects the number of positive amplifications from total samples analysed by PCR (n=number of samples analysed)

***Microbiota composition according to weight gain over pregnancy***

Faecal microbiota composition of woman showing normal or excessive weight gain over pregnancy is shown in Table 5. *E. coli* numbers were significantly higher (*P*= .045) in women with excessive weight gain than in

women with normal weight gain over pregnancy. A similar trend was found for *Enterobacteriaceae* numbers although the differences were not significant (P = .142). Contrary to this tendency, *Akkermansia muciniphila* and *Bifidobacterium* numbers were higher (P = .020 and P = .078, respectively) in women with normal weight gain than in those with excessive weight gain.

Table 5. Bacterial numbers in faecal samples (cell equivalents) per g faeces) analysed by quantitative real-PCR according to recommend weight gain over pregnancy.

Microbial groups	Normal weight gain (n 36)†		Excessive weight gain (n 14)		P*
	Prevalence‡	Bacterial numbers (log genome equivalents/g faeces) Median (IQR)	Prevalence‡	Bacterial numbers (log genome equivalents/g faeces) Median (IQR)	
Total cell counts	36/36	9·90 (9·51–10·25)	14/14	9·73 (9·18–10·00)	0·218
Bifidobacterium	36/36	8·92 (8·27–9·44)	14/14	8·46 (8·13–8·22)	0·078
Lactobacillus group	36/36	7·48 (7·39–7·64)	14/14	7·56 (7·35–7·76)	0·440
Clostridium coccoides group	36/36	8·71 (8·07–8·97)	14/14	8·35 (8·15–8·67)	0·315
Clostridium leptum group	32/36	8·42 (8·16–8·78)	12/14	8·17 (7·20–8·68)	0·268
Bacteroides	36/36	6·42 (6·06–7·03)	14/14	6·64 (6·20–7·36)	0·331
Enterobacteriaceae	36/36	6·55 (6·21–6·86)	14/14	6·84 (6·16–8·04)	0·142
Escherichia coli	36/36	5·26 (4·70–5·94)	14/14	6·25 (5·06–8·03)	0·046
Staphylococcus	10/36	4·50 (4·33–5·74)	7/14	4·46 (4·08–5·62)	0·527
Akkermansia muciniphila	36/36	8·54 (7·90–9·50)	14/14	8·12 (6·52–8·60)	0·020

*Significant differences were calculated using Mann-Whitney U test. A significantly difference between the groups was considered at P < .050.
† Normal weight gains over pregnancy according to the Institute of Medicine are < 16.0 kg (BMI < 25 kg/m^2) and < 11.5 kg (IBM > 25 kg/m^2)
Prevalence reflects the number of positive amplifications from total samples analysed by PCR (n=number of samples analysed)
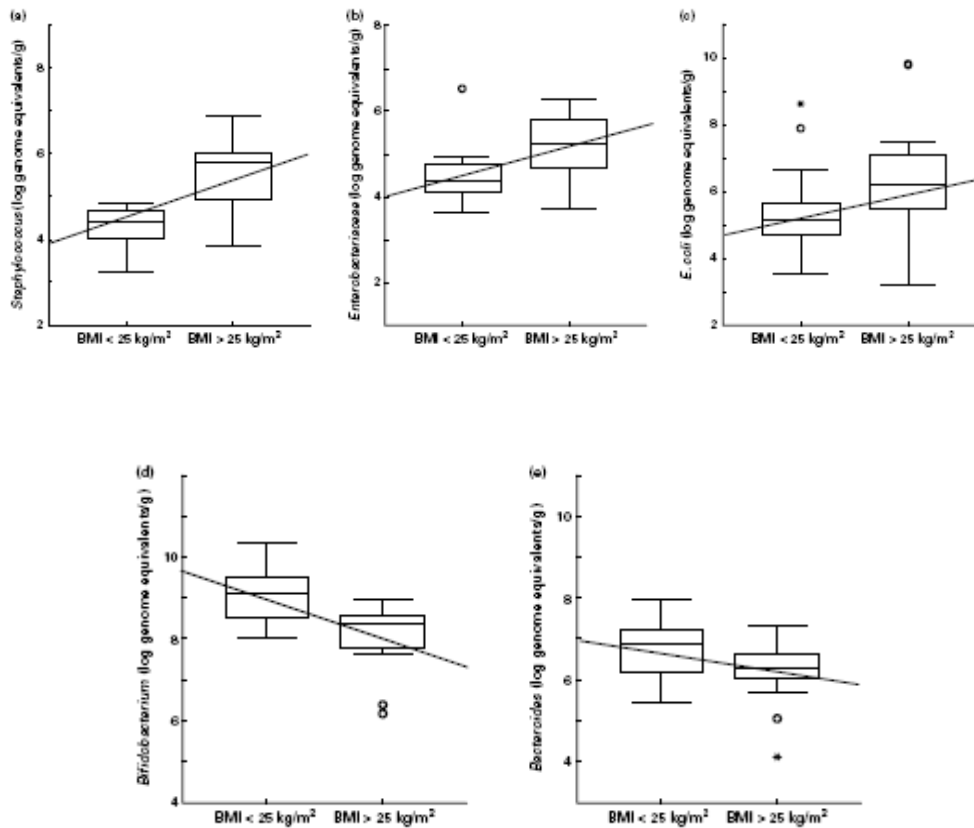
Fig. 1 Relationships between numbers of faecal bacterial groups and weight. Data represent the positive samples. The line in the box is the median (50% percentile), with the lower line the lower 25% border (25% percentile) and the upper line the 75% (75% percentile) border. The end of the upper vertical line is the maximum data value, outliers not considered. The end of the lower vertical line is the lowest value, outliers not considered. The separate dots or asterisks indicate outliers. Lines showed the Spearman correlation (linear adjustment). (a) *Staphylococcus* (R-0.67; *P*=0.003); (b) *Enterobacteriaceae* (R0.46; *P*<0.001); (c) *Escherichia coli* (R0.40; *P*=0.004); (d) *Bifidobacterium* (R=0.56; P<0.001); (e) *Bacteroides* (R-0.34; *P*=0.020)

The prevalence of *C. leptum* group and *Staphylococcus* was higher in women with excessive weight-gain than in women with normal weight gain over pregnancy (P = .545 and P = .124). Increased numbers of *Bifidobacterium* (R=-0.31, P = .029)*, Bacteroides* (R=-0.36, P= .019) and *A. muciniphila* (R= -0.34, P = .017) correlated significantly to normal weight gain over pregnancy (Fig 2). Opposite, increased numbers of *Enterobacteriaceae* (R=0.28, P = .050) and *E. coli* (R=0.42, P = .002) correlated with excessive weight gain over pregnancy (Fig. 2).

Relationships between microbiota composition and dietary intakes

In the whole women population, only increased numbers of total bacteria correlated to reduced energy (R= -0.71 P< .001), animal protein (R= -0.66, P= .001), cholesterol (R= -0.57, P= .007) and PUFA (R= -0.52 P< .015) intakes. The same trend was detected between total bacteria and energy (R=-0.78 P< .001 and R=-0.07 P= .002), animal protein (R= -0.61 P< .015 and R= -0.75 P= .001), and cholesterol (R= -0.52, P< .043 and R=- 0.58 P= .018) intakes in the normal weight group and in the normal weight gain group.
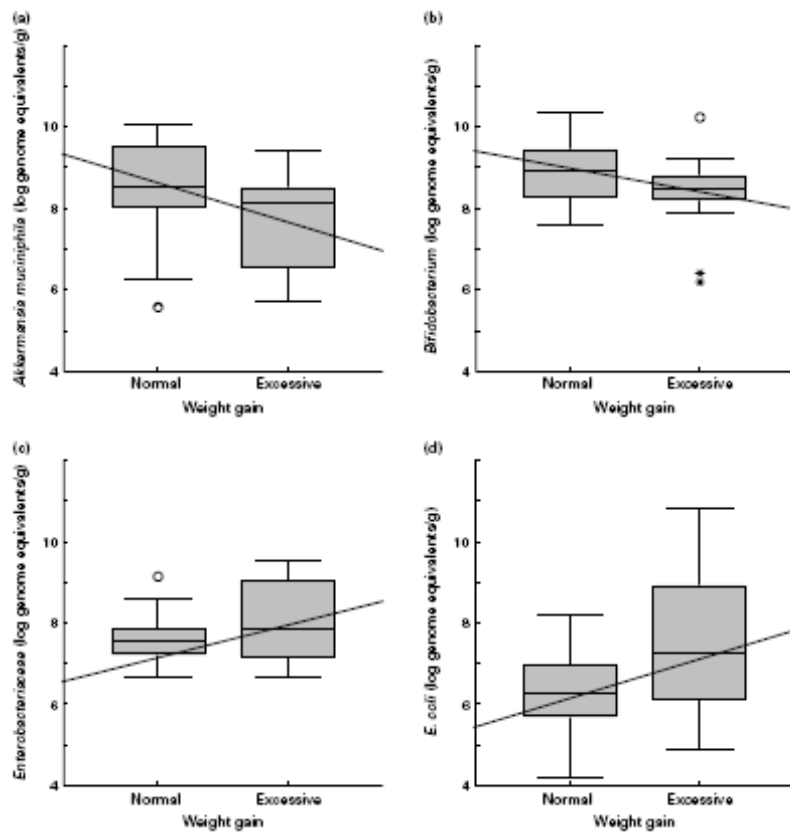
Figure 2. Relationships between numbers of faecal bacterial groups and weight gain over pregnancy. Data represent the positive samples. The line in the box is the median (50% percentile), with the lower line the lower 25% border (25% percentile) and the upper line the 75% (75% percentile) border. The end of the upper vertical line is the maximum data value, outliers not considered. The end of the lower vertical line is the lowest value, outliers not considered. The separate dots or asterisks indicate outliers. Lines showed the Spearman correlation (linear adjustment).(a) *Akkermansia muciniphila* (R-0.34; P= 0.017); (b) *Bifidobacterium* (R-0.-31; P=0.029); (c) *Enterobacteriaceae* (R-0.28; P=0.050); (d) *Escherichia coli* (R-0.42;P=0.002)

Relationships between microbiota composition and biochemical parameters

In the whole women population, total bacterial positively correlated to cholesterol (R=0.350, *P* =.013, respectively). Increased numbers of *Staphylococcus* were related to increased levels of cholesterol (R=0.68, *P* = . 003). Increased numbers of *Enterobacteriaceae* and *E. coli* counts were significantly correlated to increased levels of ferritin (R=0.324, *P*= .023 and R=0.425, *P* = .002) and saturation transferrin index (R=0.302, *P* = .035 and R=0.439, *P* = .002) and reduced levels of transferrin (R=-0.353, *P* = .013 and R=-0.341, *P*= .017). In contrast, increased numbers of *Bifidobacterium* were related to reduced levels of ferritin (R=-0.420, *P* = .003) and saturation transferrin index (R=-0.388, *P*= .006) and to increased levels of transferrin (R=0.348, *P*= .014). In addition, increased numbers of *Bifidobacterium* were related to increased levels of folic acid (R=0.308, *P*= .032). Increased numbers of *Bacteroides* were related to increased levels of HDL cholesterol (R=0.518, *P* < .001) and folic acid (R=0.333, *P* = .020) and to reduced levels of triglycerides (R=-0.371, *P* = .009).

In normal weight women, increased numbers of total bacteria correlated to increased levels of cholesterol (R=0.383, *P*=.025), while in overweight women the correlations were not significant. In normal weight gain women, increased levels of total bacteria were related to increased levels of total cholesterol (R=0.390, *P* = .019), HDL cholesterol (R=0.335, *P* = .046) and folic acid (R=0.338, *P* = .044). Increased numbers of *Staphylococcus* correlated with increased levels of total cholesterol (R=0.881, *P* < .001).

Moreover, increased numbers of *Bacteroides* correlated with higher levels of HDL cholesterol (R=0.620, *P* = .002). In women with excessive weight gain over pregnancy, increased numbers of *Bifidobacterium* were related to increased levels of HDL cholesterol (R=0.572, *P* = .042) and reduced levels of total triglycerides (R=-0.682, *P* = .010). Increased *Bacteroides* numbers were related to reduced levels of triglycerides (R=-0.809, *P* = .001).

Relationships between maternal microbiota composition and infant's birth weight

In the whole women population, significant positive correlations were found between *E. coli* (R=0.331, *P* = .039) and *C. coccoides* (R=0.323, *P* = .045) numbers and infant's birth weight were found. In overweight women, positive correlation were also found between *E. coli numbers* and infant's birth weight (R=0.673, *P* = .035). In excessive weight gain women, significant negative correlations were found between numbers of *Lactobacillus* group and infant's birth weight (R=-0.917, *P* = .001).

DISCUSSION

This study reports differences in the intestinal microbiota of normal weight and overweight pregnant women, associated with body weight and weight gain over pregnancy, suggesting that the intestinal microbiota is a relevant target to weight management in pregnancy. Moreover, newborns form overweight pregnant woman had higher birth weight than those from normal weight pregnant women, suggesting the transference of the mother's features to their newborns. In this context, the results can also be of relevance to the transference of the aberrant microbiota to the newborns, which use the mother's microbiota as inoculums for microbiota development [14]. In this context, a positive relationship between the maternal intestinal *E. coli* numbers and infant's birth weight was demonstrated, which could be related to infant's body weight regulation. In contrast, in excessive weight gain women increased *Lactobacillus* numbers were related to reduced infant's birth weight, suggesting a positive role of this bacterial group in infant's body weight regulation.

In the present study, increased numbers of *Bacteroides*, which belong to *Bacteroidetes* phylum, were detected in normal weight compared to overweight women. In previous studies, the faecal microbiota of lean human subjects was characterized by having increased numbers of *Bacteroidetes* compared to that of obese subjects. Moreover, weight loss under dietary intervention was associated with increases in *Bacteroidetes* and *Bacteroides*

fragilis group numbers in adults and adolescents [6,8,9]. Therefore, the association of *Bacteroidetes* with a lean phenotype established in previous studies has also been confirmed in pregnant woman included in this study. Nevertheless, *Bacteroides* numbers were significantly higher in overweight than in normal weight women and associated with excessive weight gain over pregnancy in the only previous study carried out in pregnant women[10]. These results contradict all previous findings on the role of *Bacteroides* in obesity and highlight the importance of the new evidence provided by this study in this regard.

Increased numbers of *Bifidobacterium* were also related to normal weight women compared to overweight women, and a similar trend was detected in women with normal weight gain compared to those with excessive weight gain over pregnancy. This is in agreement with recent studies, which showed that levels of *Bifidobacterium* were reduced in infants who developed overweight at 7 years old, compared to normal weight children[14]; however, this association was not established in the previous study conducted in pregnant women[10]. In animal models, a role has also been attributed to *Bifidobacterium* in obesity. Obese Zucker rats (*fa/fa*) and mice fed a high fat diet showed reduced *Bifidobacterium* counts [15, 16]. Moreover, the administration of prebiotics to mice fed a high fat diet increased the intestinal *Bifidobacterium* numbers, which positively correlated with improved glucose tolerance and glucose-induced insulin secretion and with the normalization of the inflammatory tone [16.]

In addition, the ratio of *Bifidobacterium* to either *C. coccoides* or to *C. coccoides* plus *C. leptum* group numbers was also significantly higher in normal weight than in overweight women, suggesting a negative role of *Clostridium* in obesity. In agreement, obese human subjects were shown to have increased numbers of *Firmicutes* in their faecal microbiota as compared to lean subjects [6]. Moreover, weight loss under dietary intervention has also been associated with reduction in *Firmicutes* or *C. coccoides* and *C. histolyticum* group proportions [6, 8, 9]. Altogether, these results confirm that increases in the relative abundance of members of *Firmicutes* and, in particular, of some *Clostridium* clusters is associated with excessive body weight.

Staphylococcus numbers were also increased in overweight compared to normal weight women in agreement with a previous study conducted in pregnant woman [10]. Moreover, children becoming overweight at 7 years old showed a greater number of *Staphylococcus aureus* in faeces during infancy [14]. In addition, *Enterobacteriaceae* and *E. coli* were significantly higher in overweight than in normal weight women and also in women with excessive weight gain over pregnancy. Increased levels of Gram-negative bacteria, which could include *Enterobacteriaceae* and *E. coli*, could be related to the endotoxaemia and inflammatory tone associated with obesity as evidenced in animal models [16].

Total cholesterol and triglycerides levels were significantly higher in overweight than in normal weight women and increased cholesterol levels

correlated with excessive weight gain over pregnancy, as expected. In addition, folic acid was significantly lower in overweight than in normal weight women, which is a nutrient involved in the correct differentiation of the neural tube during foetal organogenesis. In fact, obesity is a risk factor for neural tube defects [17]. Moreover, iron levels were also lower in overweight than in normal weight women and increased levels of ferritin correlated to higher weight gain in the whole population and in the excessive weight gain group. It has been described a relationship between obesity and iron deficiency, which can be reflected in reduced plasma levels of iron and transferrin and increased plasma levels of ferritin and saturation transferrin index [18, 19, 21, 22]. The iron deficiency associated with obesity has a multifactorial aetiology and could be due to impairment of intestinal iron uptake and iron release from stores, and to inadequate iron bioavailability because of inflammation. In particular, abnormal ferritin concentrations have been explained by the chronic low-grade inflammation associated with obesity, metabolic syndrome and gestational and type 2 diabetes [20, 21, 22]. Increases in serum ferritin concentrations early in gestation also constitute a risk of gestational diabetes, partly mediated by the maternal fat mass and obesity [22].

This study also reports interesting relationships between biochemical parameters and specific intestinal bacterial groups in pregnant women. While *Bacteroides* numbers seemed to have a positive effect on plasma biomarkers of lipid metabolism, *Staphylococcus* numbers seemed to have a negative effect particularly on plasma cholesterol. Cholesterol and other sterols have been shown to stimulate the growth of at least *S. aureus*[23]; however, in this study no

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correlation was found between cholesterol intake and *Staphylococcus* numbers, which could explain a link with plasma cholesterol levels. Other mechanisms have been proposed to justify the influence of the intestinal microbiota on lipid metabolism, including generation of different short-chain fatty acids and regulation of the host gene expression [6, 7, 24, 25] but the specific relationships found in the present study remain to be elucidated.

Bifidobacterium numbers were positively related to plasma folic acid levels in the whole population, which may be due to the ability of some strains of this genus to synthesise and secrete folates in the human intestinal environment, providing a complementary endogenous source of this vitamin [26]. This metabolic trait of *Bifidobacterium* strains could contribute to improving the nutritional status of the pregnant woman and the foetus.

Enterobacteriaceae/E. coli and *Bifidobacterium* showed inverse relationships with transferrin, saturation transferrin index and ferritin, as well as with body weight in the whole population. Increases in serum transferrin saturation index, because of a transferrin decrease and ferritin increase, have been associated with a decrease of antibacterial activity of serum against enterobacteria, such as *Salmonella enterica*, which could contribute to favouring the survival of this bacterial group [27]. In fact, infections are one of the conditions that can depress transferrin levels. The possibility that the overgrowth of *Enterobacteriaceae* in the gut environment might favour their translocation to some extent and cause a similar effect could not be disregarded. By contrast, the administration of inulin to pigs led to increased

Lactobacillus and *Bifidobacterium* numbers and to up-regulating the expression of genes encoding for iron transporters in the enterocytes, which suggest a connexion between these bacterial groups and/or the prebiotic, and improved iron absorption [28]. Therefore, the relative abundance of *Bifidobacterium* and *Enterobacteriaceae* may differently influence iron metabolism and, in turn, exert opposite effects on the nutritional status of pregnant woman. Unlikely the present study, a previous report on pregnant woman microbiota did not provide any data on biochemical parameters and their possible associations with the microbiota[10].

In summary, specific bacterial groups are oppositely related to overweight and weight gain during pregnancy, pointing for a beneficial role of *Bacteroides* and *Bifidobacterium* in body weight regulation. In addition, novel associations between these bacterial groups and beneficial changes in metabolic biomarkers are provided, suggesting a connexion between the gut microbiota and the host metabolism. Altogether, these findings open new possibilities for the management of body weight and of the nutritional status of pregnant women through modulation of the intestinal microbiota, which may have consequences on later infant's health and deserve further investigations.

ACKNOWLEDGEMENTS

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Bifidobacterium CECT 7765 improves metabolic and immunological dysfunction associated with obesity in high-fat diet fed mice

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Running head: *Bifidobacterium* CECT 7765 roles in obese mice

ABSTRACT

Objective: To evaluate the effects of oral administration of *Bifidobacterium pseudocatenulatum* CECT 7765 on metabolic and immune dysfunction in mice with high-fat diet (HFD) induced obesity.

Design: Adult (age 6–8 week) male wild-type C57BL-6 mice were fed a standard diet (SD) or HFD, supplemented or not with *B. pseudocatenulatum* CECT 7765 for seven weeks (n≥ 6/group). The following parameters were assessed: animal weight, serum levels of cholesterol, triglyceride, glucose and leptin, liver steatosis, white adipose tissue weight and adipocyte size, lipid micelles per enterocyte, functions of immunocompetent cells (macrophages and dendritic cells [DCs]) and composition and pro-inflammatory properties of the faecal microbiota.

Results: *B. pseudocatenulatum* CECT 7765 administration reduced serum cholesterol, triglyceride and glucose levels by 36, 25 and 35 %, respectively, in obese mice. This bacterial strain also induced an increase in serum leptin levels in SD-fed mice but a decrease in HFD-fed mice. The administration of *B. pseudocatenulatum* CECT7765 significantly reduced liver steatosis and the number of larger adipocytes (2000 to 4000 μm2) in HFD-fed mice. These effects were associated with reductions in the number of fat micelles in enterocytes, suggesting reductions in dietary fat absorption. The strain also increased the macrophage oxidative burst, the ability of macrophages and DCs to induce cytokines (TNF-α) in response to pathogenic bacteria (LPS), and the ability of DCs to present antigens and to induce T lymphocyte proliferation. The bacterial strain also restored the composition of the gut microbiota of HFD-fed mice, increasing *Bifidobacterium* and reducing *Enterobacteriaceae* numbers, which altogether led to reducing inflammatory signals coming from the gut.

Conclusion: *B. pseudocatenulatum* CECT7765 was shown to ameliorate both metabolic and immunological dysfunctions related to obesity in HFD-fed mice.

INTRODUCTION

Obesity constitutes a global epidemic nowadays, although its adverse consequences on health have only been seriously considered for the past 10 years.[1] Obesity is the result of a metabolic energy imbalance and is also characterized by immunological dysfunction and chronic, low-grade inflammation.[2]

Recent scientific evidence supports the notion that the gut microbiota is involved in body weight regulation by influencing metabolic and immunologic functions.[3] Obesity has also been related to phylum and group-specific changes in the microbiota. Although not all results are consistent[4], Ley *et al.*[5-6] showed that obesity correlates with a decrease in the relative proportion of *Bacteroidetes* to *Firmicutes* while weight loss in obese human subjects subjected to dietary intervention correlated to an increase in *Bacteroidetes*[5] or the *Bacteroides-Prevotella* group[7] proportions. In addition, reduced *Bifidobacterium* numbers were associated with overweight in pregnant woman by comparisons with normal weight woman.[8] In genetically obese *fa/fa* rats, lacking the leptin receptor, associations between decreased *Bifidobacterium* numbers and obesity have also been reported, suggesting that *Bifidobacterium* may play a role in defining an obese or lean phenotype.[9] Therefore, dietary modulation of the intestinal microbiota has been proposed as a way of improving the efficacy of weight-loss treatments[3,5,10,11]. Nevertheless, there is limited knowledge of the obesity-related benefits of bacteria commercialized

as probiotics (*Lactobacillus* and *Bifidobacterium*), particularly, in relation to their potential effects on associated immunological alterations.

The objective of this study was to evaluate the effects of oral administration of *Bifidobacterium pseudocatenulatum* CECT 7765 on both metabolic and immunological cell functions in mice with high-fat diet induced obesity.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The following *Lactobacillus* and *Bifidobacterium* strains were studied: *L. casei* ATCC 393, *L. casei* IATA-2E11, *L. plantarum* CECT 4185, *L. plantarum* 299v (Probi, Lund, Sweden), *L. plantarum* IATA-L1, *L. reuteri* IATA-LACA8, *B. catenulatum* LMG 110437, *B. pseudocatenulatum* CECT 5776, *B. pseudocatenulatum* CECT 7765, *B. longum* IATA-F1, and *B. longum* BB536 (Morinaga Milk Industry, Zama, Japan). These strains were obtained from the American Type Culture Collection [ATCC], Spanish Type Culture Collection (CECT), Belgian Co-ordinate Collections of Microorganisms (BCCM/LMG), Comercial Química Massó SA, Barcelona, Spain (BB536) or isolated in our laboratory from commercial products (299v) or from stools of

breast-fed infants and identified by sequencing of amplified 16S rDNA regions with the primers 27f, 530f, U-968f previously described[12-14].

Bacteria were grown in MRS broth (Scharlau, Barcelona, Spain) supplemented with 0.05% (w/v) cysteine (MRS-C Sigma, St. Louis, MO), and incubated at 37ºC for 22 h under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK). Cells were harvested by centrifugation (6.000 g for 15 min), washed twice in phosphate buffered saline (PBS, 130 ± sodium chloride, 10 mM sodium phosphate, pH 7.4), and re-suspended in PBS for *in vitro* stimulation of immunocompetent cells or in 10% skimmed milk for oral administration to mice. Aliquots of these suspensions were frozen in liquid nitrogen and stored at -80 °C until used. The number of live cells after freezing and thawing was determined by colony-forming unit (CFU) counting on MRS-C agar after 48 h incubation. For each strain tested, more than 90% cells were alive upon thawing and no significant differences were found during storage time (2 months). One fresh aliquot was thawed for every new experiment to avoid variability in the viability of cultures.

Ability of different bacterial strains to induce cytokine production by macrophages

For evaluating the immunological properties of different *Lactobacillus and Bifidobacterium* strains, the RAW 264 macrophage cell line, obtained from the American Type Culture Collection (Rockville, MD, USA), was cultured overnight into 24-well flat-bottom polystyrene microtiter plates

(Corning, Cultek, Madrid, Spain) at a concentration of $1x10^5$ cells per ml in Dulbeco's Modified Eagles Medium (DMEM) (SigmaTM– St. Louis, MO/USA). Media were changed before stimulation and, then, cells were incubated in the presence of 100 µl of a cell suspension ($1x10^7$ ufc/ml) of each strain for 24 h. Purified LPS from *Salmonella enterica* serotype Typhimurium (Sigma Chemical Co, Madrid, Spain) was used at a concentration of 1 µg/ml as a positive control. Non-stimulated Raw 264.7 cells were also evaluated as controls of basal cytokine production. The cell culture supernatants were collected and stored at −20 ℃ until used for cytokine determination. TNF-α and MCP-1 were quantified by ELISA Ready SET Go! Kit (BD Bioscience, San Diego, CA, USA). Every parameter was assayed by triplicate in two independent experiments.

Animals, diets and experimental design

Adult (age 6–8 week) male wild-type C57BL-6 mice were purchased from Harlan Laboratories. In the adaptation period (7 days), all animals were housed in each stainless-steel cage in a temperature-controlled (23 ℃) room with a 12-h light/dark cycle and 40–50% relative humidity and they were fed a standard diet (SD). Then, mice were randomly divided into four groups (n≥6 mice per group) as follows: (1) a control group, that continued receiving the (SD; (2) an obese group, that was switched to a high-fat diet (HFD); (3) a group that continued receiving the SD and a daily dose of $5x10^8$ CFU *B. pseudocatenulatum* CECT 7765 by gavage; and (4) an obese group that was switched to the HFD and a daily dose of $5x10^8$ CFU *B. pseudocatenulatum*

CECT 7765 by oral gavage. This regime was maintained for seven weeks. The SD (CA.170481 - AIN-76A Purified Diet-Rats/Mice,) and the HFD (TD.06414 - Adjusted Calories Diet - 60/Fat, Harlan Laboratories, Madison, WI 53744-4220) were provided by Harlan Laboratories (Madison, WI 53744-4220). The HFD provided 18.4 % kcal as protein, 21.3 % kcal as carbohydrate and 60.3 % kcal as fat (5.1 kcal/g), whereas the SD provided 18.8 % kcal as protein, 68.8 % kcal as carbohydrate and 12.4 % kcal as fat (3.8 kcal/g). Mice had free access to sterile water. Experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of University of Valencia (Central Service of Support to Research [SCSIE], University of Valencia, Spain) and the protocol was approved by its Ethic Committee *(*Body weight was measured once a week and, at the end of study, animals were fasted for 16 h, anaesthetized, bled by aortic puncture and sacrificed by cervical dislocation. For analysis of metabolic parameters, blood samples were collected in tubes containing EDTA and centrifuged to obtain plasma, which was kept at -20 °C. Faeces were collected initially and at the end of the experimental period (seven weeks) for microbiological analyses. The liver, white adipose (perirenal and epididymal) and small intestinal tissues were excised and rinsed with saline solution, then fixed in 10% neutral formalin buffered solution for histological analysis.

Histology of liver, white adipose and small intestine tissues

Paraffin-embedded tissues were sectioned to a thickness of 4-5 μm and fixed to glass slides. Slides were deparaffinized and stained with

haematoxylin-eosin. The severity of steatosis was determined in 100 hepatocytes of two liver tissue sections per mouse and scored as follows: grade 0 when fat was not detected in hepatocytes; grade 1 when fat occupied less than 30% of hepatocytes; grade 2 when fat occupied between 30 and 60 % of hepatocytes; grade 3, when fat occupied more than 60 % of hepatocytes. Adipocyte cell sizes were measured in 100 cells of two sections of epididymal adipose tissue per mouse[15]. Adipocyte cell sizes were expressed as area ranges using the following ranges: < 2000, 2000-4000,4000-6000 and 6000- 7000 μm^2.

The ratio of fat micelles to enterocyte was determined in 100 cells from two sections of small intestine tissue of each mouse by counting ten 100X light microscope fields. All parameters were measured with a NIKON Eclipse 90i Microscopic, using the NIS Elements BR 2.3 basic research software (Kingston, Surrey, KT2 5PR, UK). All histology analyses were conducted by an experienced histologist in a blind fashion.

Analysis of metabolic parameters

Serum leptin and insulin concentration was determined by enzyme-linked immunosorbent assay (ELISA) (BD Bioscience, San Diego, CA, USA). Biochemical parameters were also quantified in plasma using enzymatic assay kits for glucose (Glucose Liquid Kit; Química Analítica Aplicada SA, Spain), cholesterol (Cholesterol Liquid kit, Química Analítica Aplicada SA, Spain) and triglyceride (Triglyceride Liquid kit, Química Analítica Aplicada SA, Spain).

Isolation and cytokine production by peritoneal macrophages

Peritoneal cells were collected by washing the peritoneal cavity of different mouse groups, with 5 ml of sterile cold Dulbeco's Modified Eagles Medium (DMEM) (SigmaTM– St. Louis, MO/USA), containing 10% inactivated (56 °C for 30 min) foetal bovine serum (FBS) (Gibco, Barcelona, Spain), 100 µg/ml streptomycin and 100 U/ml penicillin (SigmaTM– St. Louis, MO/USA). Isolated macrophages were plated into flask (Corning, Cultek, Madrid, Spain) at a concentration of $1x10^6$ cells per ml in DMEM and incubated for 2 h at 37 °C in an atmosphere containing 5% CO_2, and non-adhered cells were washed out with warm PBS. To evaluate the effects of different stimuli, adhered macrophages were cultured overnight into 24-well flat-bottom polystyrene microtiter plates (Corning, Cultek, Madrid, Spain) at a concentration of $1x10^5$ cells per ml in DEMEN and media were changed before stimulation.

To evaluate the different response to a common stimulus, macrophages from different mouse groups were incubated in the presence of purified LPS from *Salmonella enterica* serotype *Typhimurium* (Sigma Chemical Co, Madrid, Spain) at a concentration of 1 µg/ml. Non-stimulated peritoneal macrophages were also evaluated as controls of basal cytokine production. To evaluate the immune properties of faecal samples related to the microbiota, macrophages from control mice were incubated in the presence of faecal samples (30 µl of 10-fold dilution) from the different mouse groups for 24 h. Faecal samples used as stimuli were collected from six mice in each

experimental group at the end of the study, diluted 10-fold in PBS and homogenized for 3 min. The cell culture supernatants were collected and stored at −20 ºC until used for cytokine determination. TNF-α and IL-10 were quantified by ELISA Ready SET Go! Kit (BD Bioscience, SanDiego, CA, USA). Every parameter was assayed in triplicate in two independent experiments.

Bactericidal activity of peritoneal macrophages

The bactericidal activity of peritoneal macrophages was analysed according to Vieira *et al.*[16] Cells were washed with serum free DMEM and nitroblue tetrazolium (NBT − SigmaTM− St. Louis, MO/USA) at 0.5 mg/ml together with a bacterial extract (Stimulant, No. 840-15-SigmaTM− St. Louis, MO/USA) in an equivalent concentration of McFarland Scale 2 in Lab-tek chamber slide w/cover (Nalge Nunc International, USA). After 1 h of incubation at 37 ºC in 5% CO2 atmosphere, the cells were washed with PBS, then fixed with 4% paraformaldehyde and observed with an optic microscope (Kingston, Surrey, KT2 5PR, UK). One hundred cells per mouse were counted and the percentage of NBT positive cells was determined. Every parameter was assayed in triplicate in two independent experiments.

Isolation and cytokine production by bone marrow-derived dendritic cells

Dendritic cells (DCs) were generated from bone marrow as described previously.[17] Cells were seeded at a concentration of 1×10^6 (90–94% DCs) in

1ml of culture medium without rm GM-CSF in 24-well plates (Corning, Cultek, Madrid, Spain) and incubated in the presence of different stimuli at 37 °C under 5% CO2 for 24 h. To evaluate the different response to a common stimulus, DCs from different mouse groups were incubated in the presence of purified LPS from *Salmonella enterica* serotype *Typhimurium* (Sigma Chemical Co, Madrid, Spain) at a concentration of 1 μg/ml. Non-stimulated peritoneal macrophages were also evaluated as controls of basal cytokine production. To evaluate the immune properties of faecal samples related to the microbiota, DCs from control mice were incubated in the presence of faecal samples (30 μl of 10-fold dilution) from the different mouse groups. Faecal samples were obtained and prepared as described above. The cell culture supernatants were collected and stored at −20 ºC until used for cytokine determination (TNF-α and IL-10) as described above. Every parameter was assayed in triplicate in two independent experiments.

Interactions between DCs and T CD4+ lymphocytes

CD4+ T lymphocytes were isolated from mouse spleens. To do so, spleens were excised, suspended in complete medium and passed through a stainless steel wire mesh, and the crude cell suspension obtained was washed once. CD4+ T cells were immunomagnetically isolated by positive selection with "CD4+ (L3T4) microbeads" (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions. CD4+ T cells (purity exceeded 95%) were used for mixed lymphocyte reaction. Isolated DCs were incubated for 24 h in the presence of 1 μg/ml LPS from *S. typhimurium* (Sigma

Chemical Co, Madrid, Spain). Aliquots of mature DCs from different mouse groups were plated in triplicate with allogeneic CD4+ T cells (TL) at 1:1, 1:2, 1:4, TL / DC cell ratios, in 0.2 ml culture medium in 96-well flat-bottomed plates (Corning, Cultek, Madrid, Spain) at 37 ºC for 72 h. Lymphocyte proliferation was measured with the cell proliferation ELISA BrdU-colorimetric assay (Roche, Diagnostic, Germany). Individual cultures of DCs, and TL stimulated with or without ConA, used as mitogen, were used as controls.

Samples and microbial analysis by quantitative PCR (qPCR)

Faecal samples were weighed, diluted 1:5 (w/v) in PBS (pH 7.2), homogenized by thorough vortex shaking and stored at -20 ºC until analysed. One aliquot of this dilution was used for DNA extraction using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany). Specific primers (Table 1)[21-22] targeting different bacterial genera and species were used to characterize the composition of the microbiota by qPCR using LightCycler® 480 SYBR Green I Master (Roche, USA) with a an ABI PRISM 7000-PCR sequence detection system (Applied Biosystems, UK), as described previously[23].

Statistical analyses

Statistical analyses were carried out using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Biochemical parameter data were normally distributed and significant differences were determined by applying a One-

Way ANOVA with post hoc Tukey's test or Fisher's Least Significant Difference (LSD) test. The rest of data were non-normally distributed and the differences were determined by applying the Mann-Whitney U tests. In every case, P-values <0.05 were considered statistically significant.

Table 1. Oligonucleotide primers used in this study.

Bacterial groups	Sequence (5'–3')	(pb)	Ann. (ºC)	Ref
Total bacteria	TGGCTCAGGACGAACGCTGGCGGC CCTACTGCTGCCTCCCGTAGGAGT	200	59	(1)
Bifidobacterium	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	550	55	(1,2)
Bacteroides	ATA GCC TTT CGA AAG RAA GAT CCA GTA TCA ACT GCA ATT TTA	287	55	(1,2)
Clostridium coccoides group	AAA TGA CGG TAC CTG ACT AA CTT TGA GTT TCA TTC TTG CGA A	440	50	(1,2)
Clostridium leptum group	GCA CAA GCA GTG GAG T CTT CCT CCG TTT TGT CAA	239	50	(1,2)
Enterobacteriaceae	CATTGACGTTACCCGCAGAAGAAG CTCTACGAGACTCAAGCTTGC	195	63	(3)
Lactobacillus group	AGCAGTAGGGAATCTTCCA ATTYCACCGCTACACATG	340	61	(4, 5)

RESULTS

Strain selection based on in vitro ability to induced cytokine production by macrophages.

The results of the effects of different *Lactobacillus and Bifidobacterium* strains on induction of cytokine production by Raw264.7 macrophages are shown in Table 2. All strains induced the production of significantly higher amounts of the pro-inflammatory cytokine TNF-α and the chemokine MCP-1 than the non-stimulated cells although the magnitude of the effect was strain-dependent. The only exception was the strain *B. pseudocatenulatum* CECT 7765 that did not significantly induce TNF-α production above control levels. *B. pseudocatenulatum* CECT 7765 and *B. longum* BB536 also induced significantly lower production of MCP 1 than the other strains studied. *B. pseudocatenulatum* CECT 7765 was finally selected on the basis of its lower pro-inflammatory potential.

Body and total, epididymal and perirenal adipose tissue weight gain in obese mice

The HFD-fed mice experienced significant weight gain as compared to the SD-fed mice as of the first two weeks of treatment (Figure 1-A). In the 7th week, the relative body weight increase of HFD-fed mice was 31.0 ± 10.2 % , whereas that of SD-fed mice was 17.7 ± 8.0 %. The administration of *B. pseudocatenulatum* CECT7765 did not significantly alter body weight in either of the groups (SD and HFD). The weight of total and epididymal adipose

tissues was greater at statistically significant (P=0.016, P=0.019) levels in obese mice than in lean mice (Figure 1-B). The administration of *B. pseudocatenulatum* CECT7765 did not exert a statistically significant effect on total adipose tissue weights, only was observed a slight increase of epididymal adipose tissue weight in SD group (Figure 1-B).

Metabolic parameters in obese mice

Serum levels of biochemical markers of metabolic relevance are shown in Table 3. The HFD induced a significant increase (P=0.05) in serum cholesterol, triglyceride and glucose levels as compared with the SD. The administration of *B. pseudocatenulatum* CECT7765 to HFD-fed mice led to a significant (P=0.05) reduction in serum cholesterol, triglyceride and glucose levels of 36, 25 and 35 % as compared to the levels reached in the HFD-fed mouse group not receiving the bifidobacterial strain. Mice receiving HFD became markedly hyperleptinemic (P=0.001) compared to SD-fed mice (Figure 2-A). The administration of *B. pseudocatenulatum* CECT 7765 induced a significant increase (P=0.001) in leptin levels in SD-fed mice while it significantly (P=0.035) reduced the leptin levels in HFD-fed mice (Figure 2-A). In addition, a significant (P=0.01) increase of insulin was observed in HFD compared to SD-fed mice (Figure 2-B), but *B. pseudocatenulatum* CECT 7765 induced a significant decrease (P=0.004) of insulin levels in HFD mice.

Table 2. Ability of different *Lactobacillus* and *Bifidobacterium* strains to induce cytokine production by RAW264.7 macrophages.

Bacterial strains	Cytokine production	
	TNF-α (pg/ml) Mean (SD)*	MCP 1 (pg/ml) Mean (SD)*
Control	630.0 (56.5)	207.1 (62.0)
LPS (1µg/ml)	9,987.5 (992.4) [a, b]	3,462.0 (104.8) [c, d]
L. casei ATCC 393	2,420.0 (124.7) [a, b]	1,012.6 (19.0) [c, d]
L. casei IATA-2E11	2,121.0 (138.5) [a, b]	807.9 (20.5) [c, d]
L. plantarum CECT 4185	2,849.5 (73.3) [a, b]	1,173.9 (18.2) [c, d]
L. plantarum 299v	2,725.0 (113.1) [a, b]	1,012.6 (19.0) [c, d]
L. plantarum IATA-L1	5,907.0 (83.1) [a, b]	3,187.1 (26.8) [c, d]
L. reuteri IATA-LACA8	3,281.0 (80.8) [a, b]	1,297.5 (88.3) [c, d]
B. catenulatum LMG 110437	6,126.0 (434.1) [a, b]	2,575.4 (42.9) [c, d]
B. pseudocatenulatum CECT 5776	8,110.0 (215.9) [a, b]	3,069.7 (108.6) [c, d]
B. pseudocatenulatum CECT 7765	788.0 (39.2)	378.2 (25.7) [c]
B. longum IATA-F1	7,552.5 (10.9) [a, b]	3081. 9 (13.5) [c, d]
B. longum BB536	1,593 (15.0) [a, b]	443.7 (85.3) [c]

*Results are expressed as mean and standard deviation (SD) of duplicate measures determined in three independent experiments. Significant differences were established at *P*<0.05 by applying ANOVA and *post hoc* Fisher's Least Significant Difference (LSD) test. [a]Significant differences in TNF-α production in relation to the control (DEMEN); [b]Significant differences in TNF-α production in relation to *B. pseudocatenulatum* CECT 5776; [c]Significant differences in MCP 1 production in relation to the control (DEMEN); [d]Significant differences in MCP 1 production in relat
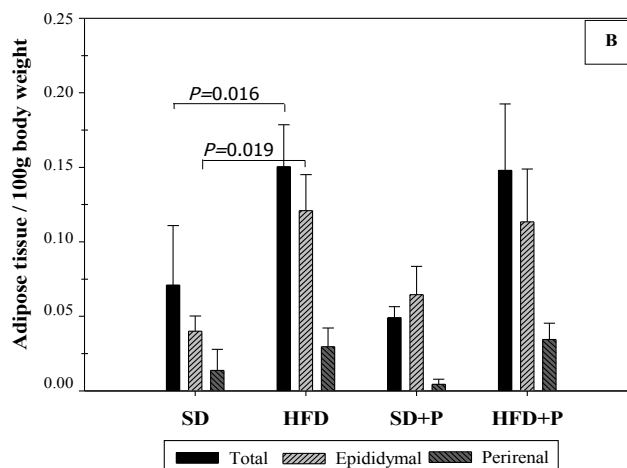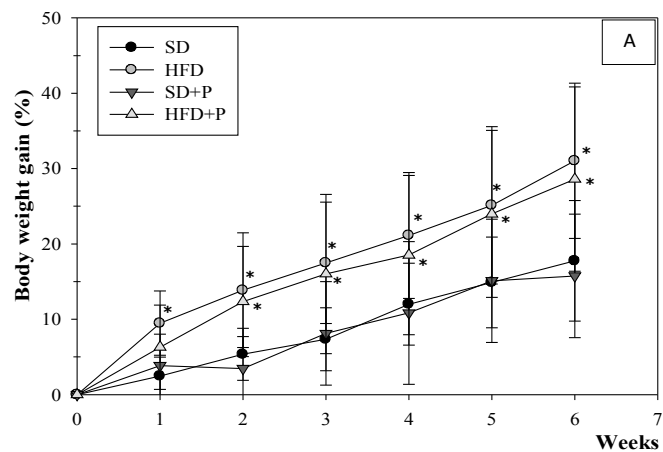
Figure 1. Body weight gain (A) and relative adipose tissue weight (B) in mice fed standard diet or high-fat diet supplemented or not with *B. pseudocatenulatum* CECT7765. SD: standard diet group (control) (n=12); SD+P: standard diet group receiving a daily dose of 5.0 x10^{8} CFU/mouse of *B. pseudocatenulatum* CECT7765 by gavage for seven weeks (n=6); HFD: high-fat diet group (n=12); HFD+P: high-fat diet group receiving a daily dose of 5.0 x10^{8} CFU/mouse ... Mice were weighed we ... expressed as adipose t ... epididymal and periren ... statistically significant d ...
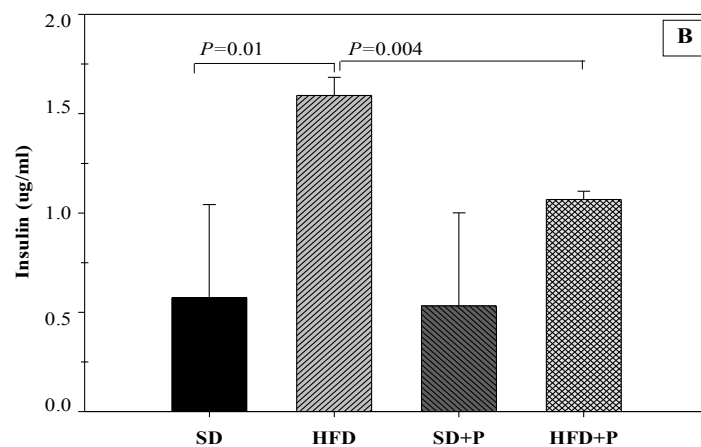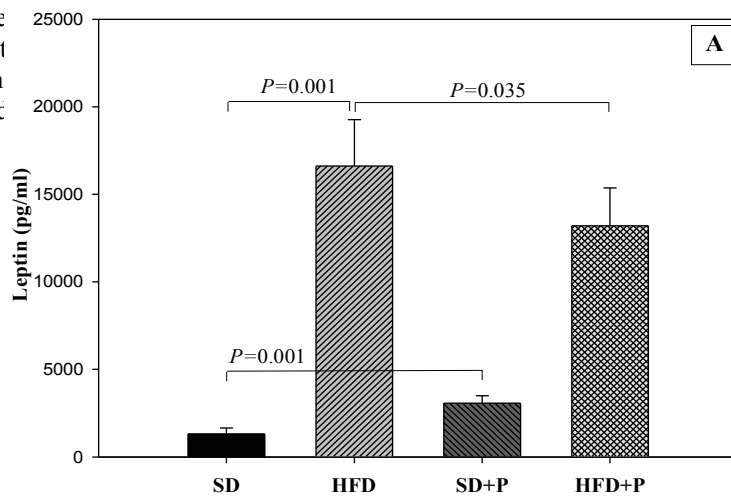
Figure 2. Determination of leptin (A) and insulin (B) levels in mice fed a standard diet (SD) or a high-fat diet (HFD), supplemented or not with *B. pseudocatenulatum* CECT7765. SD: standard diet group (control) (n=6); SD+P: standard diet group receiving a daily dose of 5.0 x10^8 CFU/mouse of *B. pseudocatenulatum* CECT7765 by gavage for seven weeks (n=6); HFD: high-fat diet group (n=6); HFD+P: high-fat diet group receiving a daily dose of 5.0x10^8 CFU/mouse of *B. pseudocatenulatum* CECT7765 by gavage for seven weeks (n=6). Data are expressed as means ± SD and statistically significant differences are established at *P*<0.05.

Hepatic steatosis, adipocyte size, and fat absorption by enterocytes in obese mice

Steatosis and significant increases in the number of hepatocytes with grades 3 (*P*=0.008) and 2 (*P*=0.018) steatosis resulted from the HFD, while hepatocytes with no steatosis or grade-1 (*P*=0.013) steatosis decreased (Figure 3-A). *B. pseudocatenulatum* CECT 7765 significantly reduced steatosis in HFD-fed mice, but did not influence this parameter significantly in mice fed the SD. *B. pseudocatenulatum* CECT7765 administration reduced the number of hepatocytes with grade-3 steatosis (*P*=0.013) and increased those with grades 1 (*P*=0.018) and 2 (*P*=0.018) steatosis in HFD-fed mice.

The effects of the diets, supplemented or not with *B. pseudocatenulatum,* on adipose tissue (epididymal and perirenal) weight and adipocyte size in epididymal adipose tissue are shown in Figures 1B and 4, respectively. The HFD induced increases in on adipose tissue (epididymal and perirenal) weight (P=0.019 and P=0.016, respectively) but the administration of *B. pseudocatenulatum* CECT7765 did not significantly modified these effects. The HFD induced significant increases in the adipocytes in the size ranges 2000-4000 (P=0.018) and 4000-6000 μm2 (P=0.018) and reductions in those ≤2000 μm2 (P=0.018). *B. pseudocatenulatum* CECT7765 administration in SD-fed mice did not induce significant modifications in adipocyte size, while in HFD-fed mice it led to a significant decrease (P=0.018) in the number of larger adipocytes (2000 to 4000 μm2) and an increase in the number of smaller adipocytes (<2000 μm2) (Figure 4-A)

C   D   E
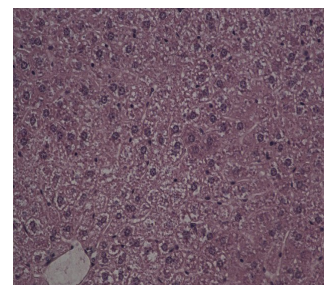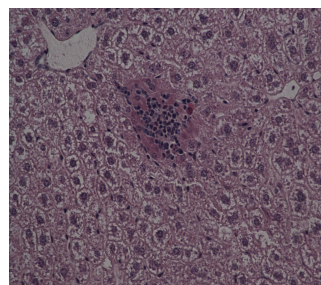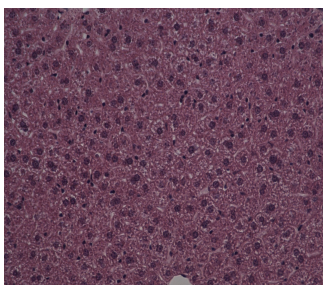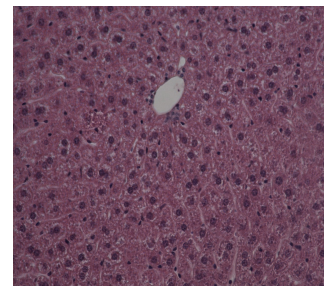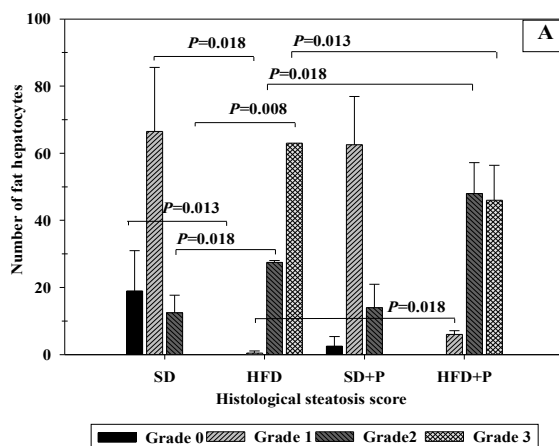
Figure 3. Determination of hepatic steatosis (hepatic histology) in mice fed standard diet or high-fat diet, supplemented or not with *B. pseudocatenulatum* CECT7765. SD: standard diet group (control) (n=6); SD+P: standard diet group receiving a daily dose of 5.0 x10^8 CFU/mouse of *B. pseudocatenulatum* CECT7765 by gavage for seven weeks (n=6); HFD: high-fat diet group (n=6); HFD+P: high-fat diet group receiving a daily dose of 5.0x10^8 CFU/mouse of *B. pseudocatenulatum* CECT7765 by gavage for seven weeks (n=6).The fat vacuoles were measured in 100 hepatocytes of two liver tissue sections per mouse and scored for the severity of steatosis according to the following criteria: For grade-0 steatosis, no fatty hepatocytes; grade-1 steatosis, fat occupying less than 30% of the hepatocyte; grade-2 steatosis, fat occu[...]in less than 30 to 60 % of the [...]to[...] and [...]osis, fat occupying more than 60 % [...] and statistically significant differe[...] representative HE-stained slides are [...]p and (F) HFD+P group.
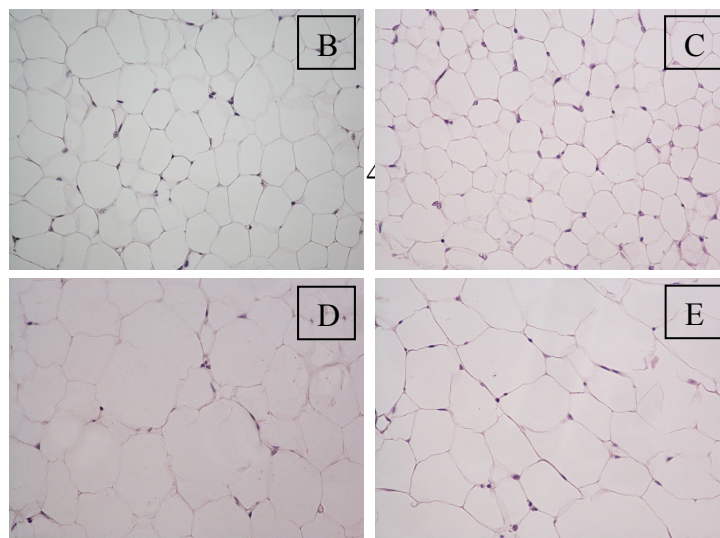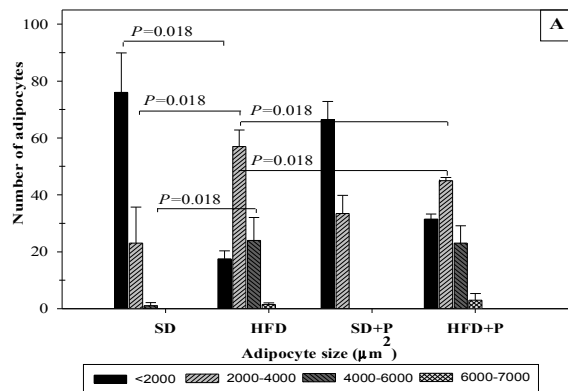
Figure 4. Distribution of adipocyte size in epididymal adipose tissue in mice fed a standard diet or a high-fat diet, supplemented or not with *B. pseudocatenulatum* CECT7765. SD: standard diet group (control) (n=6); SD+P: standard diet group receiving a daily dose of 5.0 x10^8 CFU/mouse of *B. pseudocatenulatum* CECT7765 by gavage for seven weeks (n=6); HFD: high-fat diet group (n=6); HFD+P: high-fat diet group receiving a daily dose of 5.0x10^8 CFU/mouse of *B. pseudocatenulatum* CECT7765 by gavage for seven weeks (n=6). Adipocyte cell sizes are expressed as area ranges as follows: <2000, 2000-4000,4000-6000 and 6000-7000 μm^2. Data are expressed as means ± SD and statistically significant differences are established at *P*<0.05. Photomicrographs 20X of representative HE-stained slides are shown. (B) SD group, (C) SD+P group, (D) HFD group and (E) HFD+P group.

The effects of the HFD and *B. pseudocatenulatum* CECT7765 administration on the number of fat micelles per enterocyte, indicating dietary fat absorption, are shown in Figure 5. The HFD induced a significant increase (*P*=0.018) in the number of fat micelles in the enterocytes, whereas the administration of *B. pseudocatenulatum* CECT7765 reduced (*P*=0.018) these numbers.
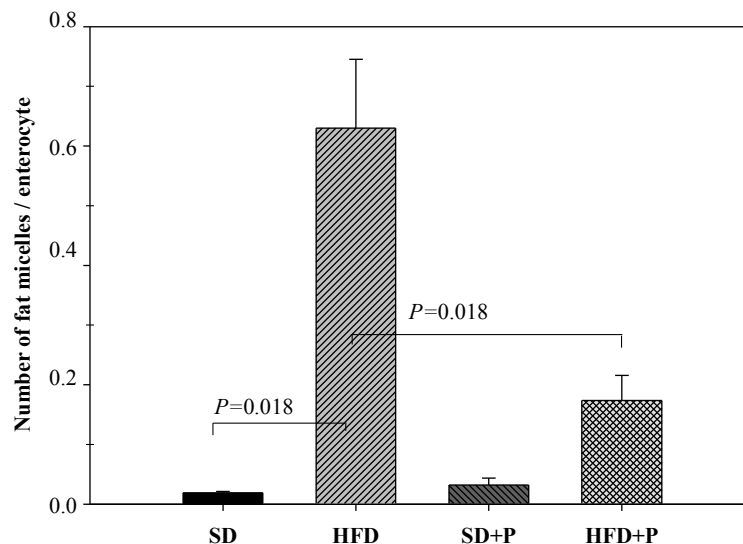
Figure 5. Number of fat micelles per enterocyte in mice fed a standard diet or a high-fat diet, supplemented or not with *B. pseudocatenulatum* CECT7765. SD: standard diet group (control) (n=6); SD+P: standard diet group receiving a daily dose of 5.0 x10^8 CFU/mouse of *B. pseudocatenulatum* CECT7765 by gavage for seven weeks (n=6); HFD: high-fat diet group (n=6); HFD+P: high-fat diet group receiving a daily dose of 5.0x10^8 CFU/mouse of *B. pseudocatenulatum* CECT7765 by gavage for seven weeks (n=6). Data are expressed as means ± SD and statistically significant differences are established at *P*<0.05.

Macrophage functionality

The results of cytokine production by LPS-stimulated peritoneal macrophages of SD and HFD-fed mice with and without *B. pseudocatenulatum* CECT7765 supplementation are shown in Figure 6-A. Cytokine TNF-α production by peritoneal macrophages stimulated with LPS was lower (*P*=0.021) in HFD-fed mice than in SD-fed mice (Figure 6-A). However, *B. pseudocatenulatum* CECT7765 administration significantly increased the ability of LPS-stimulated macrophages to produce TNF-α in HFD (*P*=0.021) while did not exerted a significant effect in SD-fed mice

(Figure 6-A). The HFD did not exert a significant effect on the ability of LPS-stimulated macrophages to produce the anti-inflammatory cytokine IL-10 (Figure 6-A). *B. pseudocatenulatum* CECT7765 administration induced a significant increase in IL-10 production by stimulated macrophages in SD-fed mice (*P*=0.019) but not in those fed a HFD (Figure 6-A).
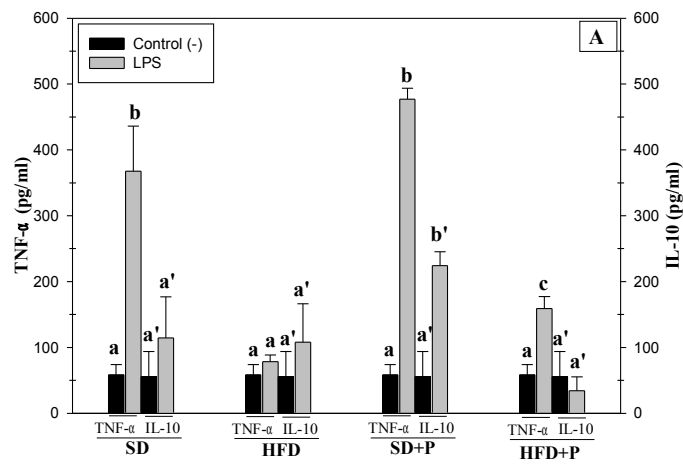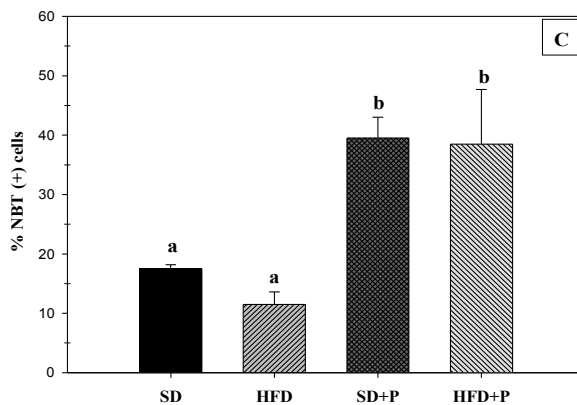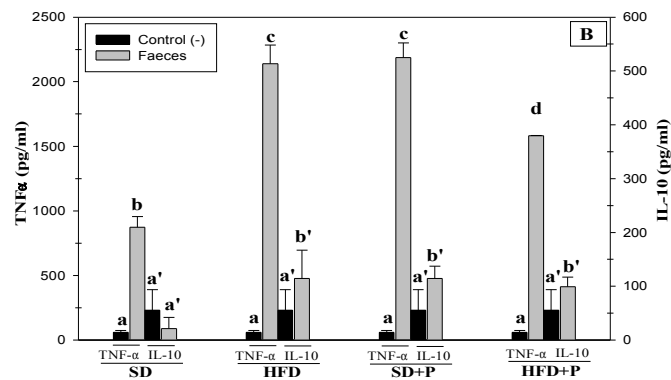

Figure 6-A

Figure 6. Influence of different stimuli on cytokine production and phagocytosis function in peritoneal macrophages of mice fed standard diet or high-fat diet supplemented or not with *B. pseudocatenulatum* CECT7765. SD: standard diet group (control) (n=6); SD+P: standard diet group receiving a daily dose of 5.0 x10^8 CFU/mouse of *B. pseudocatenulatum* CECT7765 by gavage for seven weeks (n=6); HFD: high-fat diet group (n=6); HFD+P: high-fat diet group receiving a daily dose of 5.0x10^8 CFU/mouse of *Bifidobacterium* CECT7765 by gavage for seven weeks (n=6). In the cytokine production study, peritoneal macrophages from different mouse groups were stimulated with purified lipopolysaccharide (LPS) from *Salmonella enterica* serotype Typhimurium (Figure 6-A) and macrophages from control mice were stimulated with faecal samples from different mouse groups (Figure 6-B). Non-stimulated peritoneal macrophages were evaluated as controls of basal cytokine levels. In the phagocytosis study (Figure 6-C), evidence of oxygen-radical production by macrophages was determined by the NBT test after *in vitro* interaction with a bacterial extract. Data are expressed as mean ± SD of duplicate measurements determined in two independent experiments. Statistically significant differences were established at *P*<0.05. Means in the bars with different letters were significantly different between mouse groups (a-c for SD vs. HFD and vs. SD+B, a'-d' for HFD vs. HFD+B).
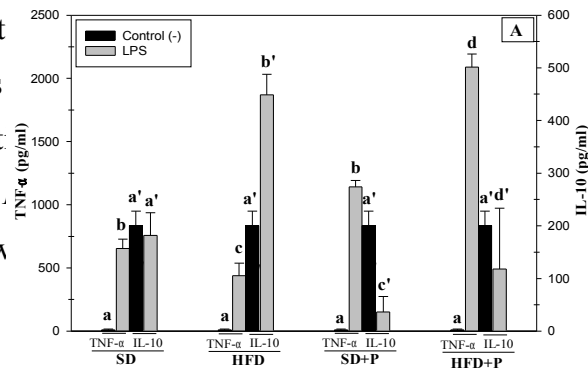
The oxidative burst in peritoneal macrophages after *B. pseudocatenulatum* CECT7765 intake was also studied to analyse effects on phagocytosis function (Figure 6-C), with results indicating this function was boosted in macrophages of both SD (*P*=0.01) and HFD-fed mice (*P*=0.001) (Figure 6-C).

Dendritic cell functionality

In obese mice, *B. pseudocatenulatum* CECT 7765 administration increased LPS-stimulated TNF-α production in DCs (*P*=0.021), which was

reduced by the HFD (*P*=0.021) (Figure 7-A). In HFD-fed mice, production of IL-10 by LPS-stimulated DCs was significantly increased (*P*=0.01), while this effect was partially reversed by the administration of *B. pseudocatenulatum* CECT7765 (*P*=0.034) (Figure 7A). In SD-fed mice, the administration of *B. pseudocatenulatum* CECT7765 also reduced (*P*=0.02) the ability of LPS-stimulated DCs to produced IL-10. The influence of HFD-induced obesity and oral administration of *B. pseudocatenulatum* CECT7765 on the ability of matured DCs to prime a T-cell proliferative response are shown in Figure 7-C. Dendritic cells f   SD-f b i   d b t i d   i   i   cant increase in T-cell proliferat   wn) for all the DC:T cell ratios   )Cs to induce a T-cell proliferat   )n was restored (*P*=0.046) by   ation, and the highest effects w
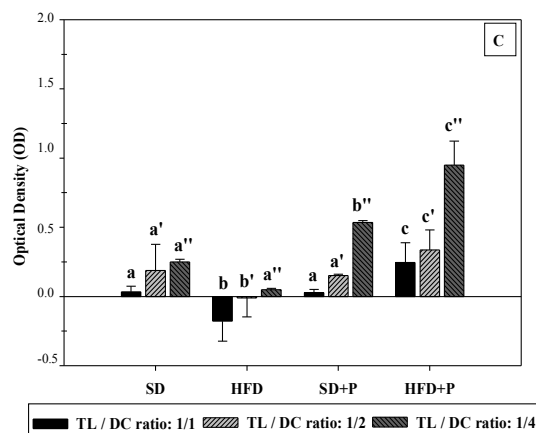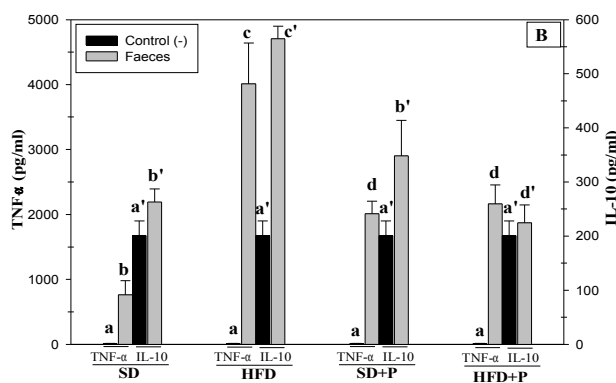
Figure 7 (A, B, C)

Figure 7. Influence of different stimuli on cytokine production and activation of T-lymphocyte proliferation by dendritic cells (DCs) generated from mice fed standard diet or high-fat diet, supplemented or not with *B. pseudocatenulatum* CECT7765. SD: standard diet group (control) (n=6); SD+P: standard diet group receiving a daily dose of 5.0×10^8 CFU/mouse of *B. pseudocatenulatum* CECT7765 by gavage for seven weeks (n=6); HFD: high-fat diet group (n=6); HFD+P: high-fat diet group receiving a daily dose of 5.0×10^8 CFU/mouse of *B. pseudocatenulatum* CECT 7765 by gavage for seven weeks (n=6). In the cytokine production study, DCs from different mouse groups were stimulated with purified lipopolysaccharide (LPS) from *Salmonella enterica* serotype Typhimurium (Figure 1-A) and DCs from control mouse groups were stimulated with faecal samples from different mouse groups (Figure 7-B). Non-stimulated DCs were evaluated as controls of basal cytokine levels. In the lymphocyte proliferation study (Figure 7-C), matured DCs were used for priming a T-cell proliferative response at the following LT/CD ratios: 1:1, 1:2, 1:4. Lymphocyte proliferation was measured with the cell proliferation ELISA BrdU-colorimetric assay. Data are expressed as means ± SD of duplicate measures determined in two independent experiments. Statistically significant differences were established at *P*<0.05.

Microbiota composition and inflammatory properties

The composition of the faecal microbiota in SD and HFD-fed mice is shown in Table 4. The HFD led to reductions in numbers of *Lactobacillus* (P=0.025), *C. coccoides* (P=0.013) and *C. leptum* (P=0.004) groups and *Bifidobacterium* (P=0.004), and to increases in *Enterobacteriaceae* (P=0.025). In HFD-fed mice, *B. pseudocatenulatum* CECT7765 administration increased the numbers of total *Bifidobacterium* spp. (P=0.011) and reduced those of *Enterobacteriaceae* (P=0.007), while in SD-fed mice only total numbers of *Bifidobacterium* spp. (P=0.05) increased.

To evaluate whether these changes in the microbiota composition could modify the inflammatory signals coming from the gut in the different mice groups, assessment was made of faecal samples ability to induce cytokine production by immunocompetent cells *in vitro* (Figures 6-B and Figures 7-B). Faecal samples from HFD-fed mice induced higher TNF-α production (P=0.021) than those from SD-fed mice by macrophages (Figure 6-B) and DCs from control mice (P=0.021) (Figure 7-B), indicating that the HFD induced an increase in the pro-inflammatory signals coming from the intestine. Meanwhile, *B. pseudocatenulatum* CECT7765 administration significantly reduced the production of this pro-inflammatory cytokine by studied immune cells (P=0.043 in macrophage, P=0.021 in DCs), demonstrating its ability to reduce the inflammatory properties of the gut content (Figures 6-B and 7-B). Faecal samples of HFD-fed mice also triggered greater production (P=0.05) of the anti-inflammatory cytokine IL-10 than those from SD-fed mice, which

could be due to the activation of regulatory mechanisms to counteract an inflammatory response, while *B. pseudocatenulatum* CECT7765 administration reduced (P=0.02) this effect only in DCs (Figures 6-B and 7-B). For mice fed SD supplemented with *B. pseudocatenulatum* CECT7765, faecal samples significantly increased (P=0.031) the synthesis of IL-10 in comparison with control mice in macrophages and a similar trend was detected in DCs.

DISCUSSION

This study supports the hypothesis that intervention in the gut ecosystem by administration of specific bacterial strains, such as *B. pseudocatenulatum* CECT7765, could ameliorate both metabolic and immunological dysfunctions underlying obesity.

The oral administration of *B. pseudocatenulatum* CECT7765 to HFD-fed mice induced a decrease in adipocyte size, which could help to curb obesity developing. The largest adipocytes are those that produce the growth factors inducing adipogenesis and maturation of pre-adipocytes into adipocytes, and a

higher proportion of pro-inflammatory mediators contributing to obesity development [24]. Both HFD-induced liver steatosis and obesity have previously been reported [25], predisposing hepatocytes to oxidative stress and activating inflammatory pathways [26]. *B. pseudocatenulatum* CECT7765 administration was also able to reduce fat accumulation in hepatocytes. Moreover, this strain significantly reduced the fat micelles inside enterocytes, which is a potential mechanism whereby adipocyte hypertrophy and liver steatosis could be reduced.

Higher serum leptin concentrations were observed in HFD than in SD-fed mice. Under certain physiological conditions, leptin, a hormone produced predominantly in adipocytes, reduces food intake, increases energy expenditure and inhibits lipogenesis.[27-29] However, obesity often manifests with leptin resistance-associated hyperleptinemia, leading to increased hunger and reduced energy expenditure.[30] *B. pseudocatenulatum* CECT7765 administration decreased leptin levels in obese mice while it increased them in non-obese mice. Reduced leptin levels in obese mice might be beneficial and indicate improved leptin function, which could contribute to the regulation of body-fat distribution and prevention of excessive triglyceride accumulation in peripheral tissues.[31] In the obesity model, it also is likely that the greater proportion of smaller adipocytes induced by *B. pseudocatenulatum* CECT7765 administration could be responsible for reducing plasma leptin concentrations [32] since leptin expression and release may partially depend on adipocyte size.[33] By contrast, in non-obese mice the increased leptin levels induced by *B. pseudocatenulatum* CECT7765 are expected to be beneficial for body weight

maintenance due to its anorexigenic role. [27-29] In this case, the increased leptin levels detected after the administration of the bifidobacterial strain could be related to a slight increase in epididymal adipose tissue weight, since it has been reported that the serum level of leptin correlates with adipose tissue weight or adipocyte size in rodents.[33] However, we can only speculate about this idea because the differences were not statistically significant and the exact mechanism by which the bifidobacterial strain can modulate leptin levels remains to be determined. *B. pseudocatenulatum* CECT7765 supplementation also reduced the HFD-induced increase in serum levels of cholesterol, triglycerides, insulin and glucose. Specific probiotic strains have been shown to ameliorate obesity-related metabolic alterations, such as insulin resistance [25, 31, 34] and increased cholesterol [35-37], as well as triglyceride levels in rodents and also humans in some cases [38-39]; however, reported effects seem to be strain dependent[5 4022] The most likely mechanism by which some *Lactobacillus* and *Bifidobacterium* strains reduce serum cholesterol is by deconjugation of bile acids. This could lead to a reduction in serum cholesterol either by increasing its utilization in *de novo* synthesis of bile acids or by reducing cholesterol solubility and thereby their intestinal absorption and uptake by the low-density lipoprotein receptor pathway in the liver. [41] However, the observed effects could also be due to inhibition of hepatic cholesterol synthesis and/or redistribution of cholesterol from plasma to the liver through the action of short-chain fatty acids, the end products of carbohydrate fermentation by the microbiota in the gut. [42] The effects of reduced triglyceride levels could also be due to the liporegulatory effects of leptin. Direct peripheral actions by leptin

have been implicated in depleting fat content through increased fatty acid oxidation as well as suppressed lipogenesis in peripheral tissues.[43]

This study is the first to demonstrate that the bifidobacterial strain administration improves the immunological dysfunction associated with obesity in mice. Oral administration of *B. pseudocatenulatum* CECT7765 increased the oxidative burst not only in the control but also in HFD fed mice, indicating an improvement in this defence mechanism against pathogens in the context of obesity. It is known that macrophage function is impaired in obese mice, with reduced phagocytic capacity and oxidative burst, which has been linked to increased susceptibility to infections in obese subjects.[44] Our study also demonstrated that cytokine production (TNF-α) by peritoneal macrophages and DCs in response to a pathogenic bacterial stimulus (LPS) is boosted by *B. pseudocatenulatum* CECT7765, which could improve the ability of immunocompetent cells to produce appropriate inflammatory responses in face to infection. This study also confirms that a HFD impairs DC function and that *B. pseudocatenulatum* CECT7765 could restore it, improving the ability of DCs to present antigens and stimulate T-lymphocyte proliferation. Macia *et al.*[45] also demonstrated a functional deficiency of DCs in *ob/ob* mice, suggesting that this damage could be generalized to the more frequent forms of obesity. This DC dysfunction could lead to impaired antigen-specific immune responses of adaptive immunity, which could be involved in the high incidence of infection reported in obese patients and in hypo-responsiveness to vaccination.[2]

The present study also reports differences in the gut microbiota between non-obese and obese HFD-fed mice, which are in agreement with previous studies [46-47], and may partly be related to diet [48]. Our results show that obese mice harboured a significant reduction in gene copy numbers of *Lactobacillus* and *Clostridium* groups and, particularly, *Bifidobacterium* genus, which is partially in agreement with previous studies. [9,46-47] In contrast, other authors found reductions in Bacteroidetes abundance and increases in Firmicutes in *ob/ob* mice [6,48]. *B. pseudocatenulatum* CECT7765 administration increased total *Bifidobacterium* and reduced *Enterobacteriaceae* numbers. These changes in the microbiota composition could contribute to reducing the inflammatory signals coming from the gut, which could affect other peripheral tissues involved in obesity.

Taking the results as a whole, this pre-clinical study supports the notion that dietary strategies targeting the gut ecosystem with specific bacterial strains could be effective to control metabolic disorders and the associated immunological dysfunction, although definitive evidence should be provided by human trials.

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DISCUSIÓN GENERAL

En un primer estudio se evaluó la influencia en la microbiota y la relación con la pérdida de peso de un programa multidisciplinario para tratar la obesidad en adolescentes, basado en una dieta con restricción calórica y aumento de la actividad física. En este estudio se observó que la reducción del peso corporal estaba asociada a un aumento de las concentraciones o proporciones relativas de los grupos *Bacteroides* o *Bacteroides-Prevotella* y *Lactobacillus*, y a reducciones del grupo *E. rectale-C. coccoides* en la microbiota intestinal de aquellos adolescentes que experimentaron pérdidas de peso significativas, pero no así en los que no presentaron pérdida significativa de peso. En este estudio, la aplicación de técnicas distintas (qPCR y FISH) para analizar la microbiota intestinal dio lugar a similares resultados, lo que aportó mayor consistencia a las asociaciones establecidas entre la microbiota y la pérdida de peso.

Los grupos bacterianos más susceptibles al cambio de dieta fueron *Bacteroides fragilis* y *Lactobacillus*, que mostraron correlaciones negativas con la ingesta de carbohidratos y los ácidos grasos poli-insaturados (PUFA) ingeridos, sugiriendo su posible manipulación a través de la misma. Esto tiene relación con lo reportado en trabajos previos en donde se obtuvo que el género bacteroides presentó una alta habilidad para utilizar los carbohidratos complejos (Stappenbeck, 2002). En otros estudios también se ha establecido una estrecha relación entre el grupo de *Lactobacillus* y la absorción de PUFA *in situ*, los cuales han demostrado influir positivamente en la adhesión de los

Lactobacillus en la mucosa del jejuno en cerdos gnotobióticos. La ingesta de los PUFA podría ayudar a mantener los niveles de este grupo bacteriano dentro del tracto digestivo (Kankaanpää, 2004; Bomba, 2003). Sin embargo son escasos los estudios que comprueban el impacto de las dietas bajas en grasas y carbohidratos con las concentraciones de *Lactobacillus* spp.

Con respeto a las especies del género *Bifidobacterium,* los recuentos de *B. longum* y *B. adolescentis* se redujeron en el total de la población de adolescentes obesos tras la intervención. Estudios fisiológicos y genómicos indican que estas especies participan activamente en la utilización de polisacáridos complejos en el colon (Belenguer, 2006), cuya ingesta se redujo en la intervención y podría ser consecuencia de su disminución. De acuerdo con nuestro resultados, Duncan et al. (2007) también observaron una reducción en los recuentos de *Bifidobacterium* spp. al disminuir la ingesta de carbohidratos en un grupo de adultos obesos.

En cuanto al grupo de *Lactobacillus* se detectaron aumentos en la microbiota de toda la población de adolescentes después del tratamiento y este aumento presentó una correlación con la pérdida de peso y el *z*-score del IMC, asociándose por tanto a la regulación del peso corporal de modo positivo. Sin embargo, la función de cepas del genero *Lactobacillus* en la obesidad sigue estando en debate. En estudios de intervención con cepas consideradas probióticas en adultos no se han observado efectos adversos sino incluso moderadamente positivos (Kadooka, 2010) y en niños tan sólo se ha descrito que el crecimiento y desarrollo no están afectados o que se favorece

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alcanzando valores normales, en situaciones especiales como grupos de población carencias nutricionales o mayor susceptibilidad a infecciones (Weizman, 2006; Dupont, 2010).

El incremento en el grupo de *Bacteroides* asociado a la pérdida de peso en adolescentes podría estar implicado en la mejora de parámetros metabólicos, por ejemplo contribuyendo a la generación de propionato, que podría ser un inhibidor de la síntesis de lípidos a partir de acetato (Pouteau, 2003; Walker, 2005). En un estudio anterior, Ley et al (2006) también indicaron que la pérdida de peso en adultos sometidos a una dieta con restricción en hidratos de carbono o en lípidos estaba asociada a reducciones en la proporción del filo Bacteroidetes, lo cual concuerda con nuestros resultados. Sin embargo, esta asociación positiva no ha sido confirmada por otros estudios como el de Duncan et al. (2008) que no encontró relación entre el género *Bacteroides* y la obesidad, al comparar un grupo de individuos obesos y uno control sometidos a una dieta baja en calorías.

En adolescentes con sobrepeso también se observó que la disminución de los grupos *E. rectale* - *C.coccoides* y *Clostridium histolyticum* se correlacionaba con la pérdida de peso en toda la población de adolescentes y en el grupo que experimentó una pérdida significativa de peso. En estudios previos se indicó que cambios en las proporciones relativas de los filos Firmicutes (al que pertenecen los clostridios y Bacteroidetes estaban asociados a la obesidad y pérdida de peso en adultos, de forma similar a nuestro estudio. Estos cambios eran evidentes cuando la pérdida de peso era por lo menos de

ente un 2 y un 6% del peso corporal, sin encontrar una relación con el tipo de dieta (Ley, 2006). En este contexto, se ha observado que el cluster XIV al cual pertenecen *E. rectale- C. coccoides* es uno de los principales implicados en la fermentación colónica y la producción de butirato en la parte distal del colon (Egran, 2001). La reducción de ese grupo bacteriano, consecuencia de la intervención para tratar la obesidad, podría contribuir a reducir la habilidad de la microbiota intestinal para obtener la energía de la dieta por medio de la fermentación, que puede llegar a suponer el 10 o 15% del aporte de calorías diario. No obstante, la posible función de los ácidos grasos de cadena corta en la obesidad es todavía controvertida y por ejemplo, al butirato se le ha atribuido un efecto positivo por contribuir a la formación de péptidos (péptido similar al glucagón-1) que inducen sensación de saciedad.

Asimismo, el estudio en adolescentes con sobrepeso demostró que la microbiota del individuo puede influir en la eficacia de las intervenciones basadas en cambios en el estilo de vida para tratar la obesidad. En concreto, la microbiota de los adolescentes que experimentaron una pérdida de peso significativa se caracterizó por presentar mayores concentraciones iniciales y finales de los grupos *B. fragilis* y *C. leptum* y *B. catenulatum* y menores de los grupos *C. coccoides, Lactobacillus* y *Bifidobacterium, B. breve* y *B. bifidum*, en comparación con los que no experimentaron una pérdida de peso significativa.

En el estudio en adolescentes también se evaluó la relación entre la microbiota y el sistema inmune debido a que la obesidad se asocia con una

inflamación crónica de bajo grado, cada vez más frecuenta a edades tempranas. Con esta finalidad, se determinaron las inmunoglobulinas IgA, IgG e IgM unidas a bacteria en heces, como un primer indicador de la capacidad de la microbiota intestinal de estimular el sistema inmune de la mucosa. En los adolescentes con sobrepeso se detectó un aumento especialmente de la proporción de IgA asociada a bacterias intestinales, relacionado con las proporciones de *C. histolyticum* y *E. rectale-C. coccoides,* que se redujeron tras la intervención en aquellos individuos que experimentaron mayor reducción de peso. Estos resultados sugieren que algunos componentes de la microbiota, posiblemente implicados en la obesidad o sus metabolitos, influyen en el sistema inmunitario del hospedador. Entre los integrantes del grupo *C. histolyticum* existen especies citotóxicas (De Graaf, 2008), cuyos niveles incrementados podrían contribuir a la activación del sistema inmunitario en sujetos obesos. Por otro lado, la reducción del número de grupos bacterianos productores de butirato, como los miembros del grupo *E. rectale-C. coccoides* tras la intervención, podría estar relacionado con una reducción del aporte de energía necesario para las células del sistema inmune y, por consiguiente, la reducción de las células productoras de IgA y de la concentración de IgA en la mucosa (Marschan, 2008; Peuranen, 2004)

En cuanto el estudio realizado en mujeres embarazadas, el sobrepeso se asoció a aumentos en las concentraciones de los grupos *Staphylococcus, Enterobacteriaceae* y *E.coli,* y reducciones en las de los géneros *Bifidobacterium* y *Bacteroides*. El aumento de *E. coli* y la reducción de *Bifidobacterium* spp. y *Akkermansia muciniphila* también se asoció a la

ganancia excesiva de peso durante el embarazo. Por tanto, la asociación entre los niveles de *Bacteroides* spp. y el peso corporal se confirmó tanto en adolescentes como en embarazadas, aunque su influencia en la regulación del peso corporal sigue siendo controvertida como se ha indicado anteriormente. En cuanto a las mujeres embarazadas se sugiere una relación positiva del grupo *Lactobacillus* como posible responsable de la regulación de la ganancia de peso por el hijo.

En el estudio realizado en embarazadas también se correlacionando los parámetros bioquímicos con los cambios de microbiota. El aumentos en la concentración de colesterol sérico se relacionó con aumentos en *Staphylococcus* spp., y el algunos estudios sugieren que el colesterol puede estimular el crecimiento de algunas especies de estafilococos como *S. aureus* (Shine, 1993). Incrementos en el número de *Bacteroides* spp., ser correlacionaron con aumentos en el colesterol HDL y reducciones en el colesterol total. Aumentos en el número de *Bifidobacterium* spp se correlacionaron con aumentos en ácido fólico, que podrían relacionarse con la habilidad de algunas cepas de sintetizar folatos en el intestino (Strozzi, 2008). Por último, los niveles de ferritina and transferrina reducida mostraron relaciones opuestas con las concentraciones de enterobacterias y *Bifidobacterium* spp. En este contexto, aumento en el índice de saturación de transferrina en el suero, debido a una reducción en transferrina y a un aumento en ferritina, se han asociado con una reducción de la actividad antibacteriana del suero frente a enterobacterias (Jolivet-Gougeon, 2008), aunque la repercusión de este fenómeno en la microbiota intestinal se desconoce.

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A fin de tener evidencias más directas sobre el posible efecto de una bacteria potencialmente probiótica en la obesidad, se seleccionó una cepa del género *Bifidobacterium* y se administró a un modelo animal de obesidad inducida por la dieta. La administración oral de *B. pseudocatenulatum* CECT 7765 mejoró las alteraciones bioquímicas e inmunológicas asociadas a la obesidad inducida por una dieta rica en grasa en ratones. La administración de esta cepa redujo la esteatosis hepática y el tamaño de los adipocitos y las concentraciones de glucosa en suero y de lípidos (colesterol y triglicéridos) en suero y en el hígado, la concentración de leptina en suero y mejoró las funciones de células inmunocompetentes (macrófagos y células dendríticas) alteradas por la obesidad.

En previos estudios se ha publicados que ciertas bacterias probióticas pueden reducir los parámetros bioquímicos colesterol, triglicéridos, LDL-colesterol, HDL-colesterol en modelos de animales y en humanos (Kiessling, 2002; Kekkonen, 2008). El mecanismo por el cual algunas cepas de *Lactobacillus* y *Bifidobacterium* pueden disminuir el colesterol podría ser por su capacidad para desconjugar las sales biliares (Begley, 2006). No obstante, los efectos observados también podrían deberse a la inhibición de la síntesis hepática de colesterol y a la redistribución de colesterol del plasma al hígado a través de la acción de los ácidos cortos de cadena corta, que son los productos finales de la fermentación de los carbohidratos por la microbiota intestinal. La cepa evaluada también redujo la absorción de lípidos de la dieta por los enterocitos, lo que podría contribuir a la reducción de lípidos observada en

suero así como a la reducción en la reducción de la esteatosis hepática y el tamaño de los adipocitos.

Pese a que en condiciones fisiológicas la síntesis de leptina reduce la ingesta de alimentos, incrementa el gasto energético e inhibe la lipogénesis (Ahima, 1996; Schwartz, 1994; Zhang, 2000) la obesidad a menudo se manifiesta con hiperleptinemia y resistencia a la leptina (El-Haschimi, 2000). En el modelo animal de obesidad, la cepa administrada también disminuyó los niveles de leptina en los ratones obesos lo que puede indicar una mejora de su función, relacionada por ejemplo con la reducción del tamaño de los adipocitos.

El estudio en el modelo animal de obesidad demuestra por primera vez que la administración oral de una bacteria potencialmente probiótica, como *B. pseudocatenulatum* CECT7765, mejora la disfunción inmunológica asociada a la obesidad en ratones. La administración de esta cepa mejoró la función de macrófagos en la fagocitosis, tanto en animales obesos como controles, lo que refleja una mejora en el mecanismo de defensa frente a patógenos. En sujetos obesos se ha demostrado que los macrófagos presentan una disminución en su capacidad de fagocitosis y respuesta oxidativa, lo cual aumenta su susceptibilidad a infecciones (Zhou, 2009). Además, la administración de esta cepa incrementó la capacidad de macrófagos y células dendríticas para producir TNF-$\square$ en respuesta a un estímulo de patógenos como el LPS, lo que sugiere también una posible mejora de la respuesta defensiva frente a infecciones. Este estudio también confirma que una dieta rica en grasa altera la función de las células dendríticas, reduciendo por ejemplo su habilidad para

presentar antígenos y estimular la proliferación de los linfocitos T tal como se ha descrito en artículos previos (Wolowczuk, 2008; Macia, 2006). Sin embargo, la cepa administrada también mejora esta función en animales controles y obesos.

La administración de *B. pseudocatenulatum* CECT7765 al modelo de obesidad también restableció parcialmente las alteraciones de la microbiota intestinal detectadas en animales obesos, aumentando la abundancia del grupo *C. coccoides* y del género *Bifidobacterium* y reduciendo la de la familia *Enterobaceriaceae.* Además, se demostró que estos cambios en la microbiota atenuaban sus propiedades inflamatorias, que se han relacionado con el aumento de peso, la resistencia a la insulina, la endotoxemia metabólica (Cani, 2007). En ratas obesas Zucker (*fa/fa*) y ratones alimentados con una dieta alta en grasa también se han detectado reducciones en el número de *Bifidobacterium* spp. con respecto al control (Waldram, 2009; Cani, 2007), coincidiendo con nuestros resultados.

Los estudios realizados en humanos en el marco de esta tesis doctoral presentan algunas limitaciones, como el tamaño relativamente reducido de la población estudiada y la corta duración de las intervenciones, que podrían reducir el grado de significación de las asociaciones detectadas entre la microbiota, el peso corporal, los parámetros bioquímicos y la dieta. No obstante, las asociaciones identificadas y, algunas de ellas, confirmadas por otros autores han aportado información clave para avanzar en el desarrollo de estudios que permitan demostrar de forma directa una relación causal entre

determinadas bacterias y la obesidad y alteraciones metabólicas relacionadas. En este contexto, la evaluación preclínica del efecto de una cepa del género *Bifidobacterium* en un modelo de obesidad en esta tesis doctoral, ya ha aportado evidencia directa de su posible aplicación práctica, aunque esta posibilidad deberá ser confirmada finalmente mediante la realización de adecuados estudios en humanos.

Conclusiones

1.- La reducción del peso corporal, como consecuencia de una intervención basada en la reducción del aporte calórico de la dieta y el aumento de la actividad física para tratar la obesidad, se asoció a un aumento de las concentraciones o proporciones relativas de los grupos *Bacteroides* o *Bacteroides-Prevotella* y *Lactobacillus,* así como a reducciones del grupo *E. rectale-C. coccoides* en la microbiota intestinal de aquellos adolescentes que experimentaron pérdidas de peso significativas.

2. La microbiota de los adolescentes que experimentaron una pérdida de peso significativa se caracterizó por presentar mayores concentraciones iniciales y finales de los grupos *B. fragilis* y *C. leptum* y *B. catenulatum* y menores de los grupos *C. coccoides*, *Lactobacillus* y *Bifidobacterium, B. breve* y *B. bifidum,* lo que sugiere que la microbiota del individuo puede influir en la eficacia de las intervenciones basadas en cambios en el estilo de vida para tratar la obesidad.

3. En adolescentes obesos se detectó un aumento de la proporción de IgA asociada a bacterias intestinales, relacionado con las proporciones de *C. histolyticum* y *E. rectale - C. coccoides,* que se redujeron tras la intervención para tratar la obesidad, lo que sugiere que algunos componentes de la microbiota posiblemente implicados en la obesidad o sus metabolitos influyen en el sistema inmunitario del hospedador.

4.- El sobrepeso durante el embarazo se asoció a aumentos en las concentraciones de los grupos *Staphylococcus, Enterobacteriaceae* y *E.coli,* y reducciones en las de los géneros *Bifidobacterium* y *Bacteroides*. El aumento de *E. coli* y la reducción de *Bifidobacterium* spp. y *Akkermansia muciniphila* también se asoció a la ganancia excesiva de peso durante el embarazo.

5. En mujeres embarazadas se detectaron asociaciones entre algunos de los grupos bacterianos de la microbiota intestinal y parámetros bioquímicos que pueden tener influencia en la salud de la madre y el hijo. Aumentos en la concentración de colesterol sérico se relacionaron con aumentos en *Staphylococcus* spp., aumentos en el colesterol HDL y reducciones en el colesterol total ser relacionaron con aumentos en *Bacteroides* spp., aumentos en ácido fólico se relacionaron con aumentos en *Bifidobacterium* spp. y los niveles de ferritina and transferrina reducida mostraron relaciones opuestas con las concentraciones de enterobacterias y *Bifidobacterium* spp.

6. La administración de *Bifidobacterium pseudocatenulatum* CECT 7765 a ratones con obesidad inducida por la dieta mejoró las alteraciones bioquímicas e inmunológicas asociadas a la obesidad inducida por una dieta rica en grasa. La administración de esta cepa redujo la esteatosis hepática y el tamaño de los adipocitos y las concentraciones de glucosa en suero y de lípidos (colesterol y triglicéridos) en suero y en el hígado, y mejoró las funciones de células inmunocompetentes (macrófagos y células dendríticas) alteradas por la obesidad.

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7. La administración de *Bifidobacterium pseudocatenulatum* CECT 7765 a ratones con obesidad inducida por la dieta restableció parcialmente la composición de la microbiota intestinal y redujo sus propiedades inflamatorias, lo que favorecería el control de la inflamación asociado a la obesidad y patologías relacionadas.

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