Departamento de Biotecnología

Identificación de componentes de la microbiota intestinal potencialmente beneficiosos frente a la obesidad

MEMORIA DE TESIS PRESENTADA POR

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CERTIFICA:

que la Licenciada en Biotecnología Eva Mª Gómez del Pulgar Villanueva ha realizado bajo su dirección en el Instituto de Agroquímica y Tecnología de Alimentos el trabajo que lleva por título "Identificación de componentes de la microbiota intestinal potencialmente beneficiosos frente a la obesidad", y autoriza su presentación para optar al grado de Doctor.

Y para que así conste, expido y firmo el presente certificado en Valencia, a 16 de octubre de 2018.

Dra. Yolanda Sanz Herranz

A mi familia,

A ti.

RESUMEN

La obesidad es un problema de salud a nivel mundial que incrementa el riesgo de sufrir un amplio rango de comorbilidades, incluyendo la diabetes tipo II y las enfermedades cardiovasculares. Estas patologías pueden estar precedidas por el síndrome metabólico, definido como una agrupación de marcadores de riesgo cardiometabólico, incluyendo la obesidad abdominal, el desequilibrio en la homeostasis de la glucosa, la dislipidemia y la hipertensión. La obesidad y el sobrepeso causan cada vez más muertes cada año y se estima que aproximadamente un cuarto de la población adulta sufre síndrome metabólico. Debido a estos motivos, es necesario encontrar estrategias para reducir la obesidad y sus co-morbilidades y que puedan ser implementadas en los sistemas de salud públicos. El objetivo general de la tesis ha sido avanzar en el conocimiento de la influencia de la composición y funciones de la microbiota intestinal y su genoma (microbioma) en la obesidad y alteraciones metabólicas asociadas e identificar bacterias intestinales que, junto a cambios en la dieta, puedan contribuir a establecer estrategias de intervención más eficaces para controlar estos desórdenes.

El capítulo primero de la tesis se centra en el estudio de la seguridad y habilidad para metabolizar distintas fuentes de fibra de *Bacteroides uniformis* CECT 7771, una cepa que demostró capacidad para reducir las alteraciones metabólicas asociadas a la obesidad inducida por la dieta en estudios pre-clínicos previos. Para ello, hemos llevado a cabo la secuenciación del genoma y el análisis del transcriptoma de la bacteria en respuesta a diferentes fuentes de carbono (glucosa, salvado de trigo, pectina, mucina, goma arábiga e inulina). Se ha observado que *B. uniformis* CECT7771 es capaz de fermentar todos los sustratos evaluados y crecer más rápido en presencia de aquellas fuentes de carbono de estructura más sencilla (salvado de trigo rico en arabinoxilanos (AXOS) y glucosa). Además, *B.uniformis* CECT 7771 posee la capacidad de degradar los O-glicanos de la mucina y producir butirato y disminuir la producción de ácidos grasos de cadena larga y proteínas transportadoras de acilo (ACPs), implicados en la síntesis de lipopolisacárido (LPS) bacteriano causante de endotoxemia. Cuando crece en presencia de pectina, *B. uniformis* CECT 7771 incrementa la producción y secreción de GABA, lo cual podría tener un impacto en el eje intestino-cerebro. *B. uniformis* CECT 7771 no ha mostrado signos de toxicidad o patogenicidad tras el análisis exhaustivo de los genes codificados en su genoma, ni tras su administración por vía oral en un modelo de toxicidad sub-crónica en ratas.

En este modelo, la bacteria produce un perfil de citocinas anti-inflamatorio comparado con el grupo placebo y muestra un efecto dosis-dependiente en la disminución de los valores de la alanina aminotransferasa (ALT), sugiriendo un efecto hepato-protector.

En el segundo capítulo de la tesis se ha llevado a cabo un estudio de intervención en humanos con sobrepeso y síndrome metabólico en el que se evaluó, en un primer lugar, los efectos de la suplementación dietética con fibras enriquecidas en AXOS y ácidos grasos poliinsaturados (PUFAs) durante 4 semanas en la composición de la microbiota intestinal y en los parámetros bioquímicos (medidas antropométricas, metabolismo de glucosa, perfil lipídico, marcadores de inflamación, etc.). Los resultados demuestran que la ingesta de AXOS modifica la microbiota, ejerciendo un efecto bifidogénico y un aumento de especies bacterianas productoras de butirato, aunque no hemos visto una mejora en los parámetros bioquímicos analizados. En el caso de la intervención con PUFAs, no hemos detectado cambios en la composición de la microbiota ni en los parámetros bioquímicos evaluados. En segundo lugar, hemos llevado a cabo un estudio multi-ómico en un subgrupo de sujetos de la intervención con AXOS a fin de profundizar en los efectos de este tipo de fibra. El análisis metagenómico nos ha permitido identificar cambios en la configuración de la microbiota intestinal, incluyendo un aumento de *Ruminococcus gnavus* y *Prevotella* spp. así como una disminución de *Parabacteroides, Paraprevotella* y *Rikenella*. También hemos detectado un incremento de metagenes implicados en la producción de tetrahidrofolato y derivados del ácido fólico, los cuales llevan a cabo una función protectora frente a la hiperhomocisteinemia; la vitamina K2, implicada en la correcta absorción del calcio; y el GABA, cuya baja concentración en el organismo se asocia a estados de ansiedad y desbalance energético y que favorece la secreción de insulina y regula los niveles de ceramidas en plasma. El análisis conjunto del metagenoma y el metaboloma nos ha permitido establecer correlaciones positivas entre la abundancia de *Prevotella* y las concentraciones de ácidos grasos de cadena corta y succinato, así como entre aumentos de *Eubacterium rectale* y reducciones en metilaminas, que podrían estar implicadas en la mejora del metabolismo de glucosa y reducción del riesgo de sufrir patologías crónicas. Además, el análisis lipidómico demuestra una reducción de las ceramidas en plasma, que sugieren mejoría del metabolismo de la glucosa. La integración de datos multi-omicos sugieren, globalmente, que la ingesta de AXOS podría contribuir especialmente al mantenimiento de la homeostasis de la glucosa en individuos con sobrepeso.

RESUM

L'obesitat és un problema de salut a nivell mundial que incrementa el risc de patir un ampli rang de comorbilitats, incloent la diabetis tipus II i les malalties cardiovasculars. Estes patologies poden estar precedides per la síndrome metabòlica, definit com una agrupació de marcadors de risc cardiometabólic, incloent l'obesitat abdominal, el desequilibri en l'homeostasis de la glucosa, la dislipidemia i la hipertensió. L'obesitat i la sobrecàrrega causen cada vegada més morts cada any i s'estima que aproximadament un quart de la població adulta patix síndrome metabòlica. A causa d'estos motius, és necessari trobar estratègies per a reduir l'obesitat i les seues comorbilitats i que puguen ser implementades en els sistemes de salut públics. L'objectiu general de la tesi ha sigut avançar en el coneixement de la influència de la composició i funcions de la microbiota intestinal i el seu genoma (microbioma) en l'obesitat i alteracions metabóliques associades i identificar bacteris intestinals que, junt amb canvis en la dieta, puguen contribuir a establir estratègies d'intervenció més eficaços per a controlar estos desordes. El capítol primer de la tesi es centra en l'estudi de la seguretat i habilitat per a metabolitzar distintes fonts de fibra de *Bacteroides uniformis* CECT 7771, una cepa que va demostrar capacitat per a reduir les alteracions metabòliques associades a l'obesitat induïda per la dieta en estudis preclínics previs. Per a això, hem dut a terme la seqüenciació del genoma i l'anàlisi del transcriptoma del bacteri en resposta a diferents fonts de carboni (glucosa, segó de blat, pectina, mucina, goma aràbiga i inulina). S'ha observat que *B. uniformis* CECT7771 és capaç de fermentar tots els substrats avaluats i créixer més ràpid en presència d'aquelles fonts de carboni d'estructura més senzilla (segó de blat ric en arabinoxilans (AXOS) i glucosa).

A més, *B.uniformis* CECT 7771 posseïx la capacitat de degradar els O-glicans de la mucina i produir butirat i disminuir la producció d'àcids grassos de cadena llarga i proteïnes transportadores d'acil (ACPs), implicades en la síntesi de lipopolisacárid (LPS) bacterià causant d'endotoxemia. Quan creix en presència de pectina, *B. uniformis* CECT 7771 incrementa la producció i secreció de GABA, la qual cosa podria tindre un impacte en l'eix intestí-cervell. *B. uniformis* CECT 7771 no ha mostrat signes de toxicitat o patogenicidad després de l'anàlisi exhaustiva dels gens codificats en el seu genoma, ni després de la seua administració per via oral en un model de toxicitat subcrònica en rates. En este model, el bacteri produïx un perfil de citoquines antiinflamatori comparat amb el grup placebo i mostra un efecte

dosi- dependent en la disminució dels valors de l'alanino aminotransferasa (ALT), suggerint un efecte hepato-protector.

En el segon capítol de la tesi s'ha dut a terme un estudi d'intervenció en humans amb sobrecàrrega i síndrome metabòlica en què es va avaluar, en un primer lloc, els efectes de la suplementació dietètica amb fibres enriquides en AXOS i àcids grassos poliinsaturats (PUFAs) durant 4 setmanes en la composició de la microbiota intestinal i en els paràmetres bioquímics (mesures antropomètriques, metabolisme de glucosa, perfil lipídic, marcadors d'inflamació, etc.). Els resultats demostren que la ingesta d'AXOS modifica la microbiota, exercint un efecte bifidogénic i un augment d'espècies bacterianes productores de butirat, encara que no hem vist una millora en els paràmetres bioquímics analitzats. En el cas de la intervenció amb PUFAs, no hem detectat canvis en la composició de la microbiota ni en els paràmetres bioquímics avaluats.

En segon lloc, hem dut a terme un estudi multi-ómic en un subgrup de subjectes de la intervenció amb AXOS a fi d'aprofundir en els efectes d'este tipus de fibra. L'anàlisi metagenómic ens ha permés identificar canvis en la configuració de la microbiota intestinal, incloent un augment de *Ruminococcus gnavus* i *Prevotella* spp. així com una disminució de *Parabacteroides*, *Paraprevotella* i *Rikenella*. També hem detectat un increment de metagens implicats en la producció de tetrahidrofolat i derivats de l'àcid fòlic, els quals duen a terme una funció protectora enfront de l'hiperhomocisteinemia; la vitamina K2, implicada en la correcta absorció del calci; i el GABA, la baixa concentració del qual en l'organisme s'associa a estats d'ansietat i desbalanç energètic i que afavorix la secreció d'insulina i regula els nivells de ceramides en plasma. L'anàlisi conjunta del metagenoma i el metaboloma ens ha permés establir correlacions positives entre l'abundància de Prevotella i les concentracions d'àcids grassos de cadena curta i succinat, així com entre augments d'*Eubacterium rectale* i reduccions en metilamines, que podrien estar implicades en la millora del metabolisme de glucosa i reducció del risc de patir patologies cròniques. A més, l'anàlisi lipidómic demostra una reducció de les ceramides en plasma, que suggerixen millora del metabolisme de la glucosa. La integració de dades multi-omices suggerixen, globalment, que la ingesta d'AXOS podria contribuir especialment al manteniment de l'homeostasis de la glucosa en individus amb sobrecàrrega.

ABSTRACT

Obesity is a global health problem that increases the risk of suffering a wide range of comorbidities, including type II diabetes and cardiovascular diseases. These pathologies can be preceded by the metabolic syndrome, defined as a grouping of cardiometabolic risk markers, including abdominal obesity, the imbalance in glucose homeostasis, dyslipidemia and hypertension. Obesity and overweight cause more and more deaths every year and it is estimated that approximately a quarter of the adult population suffers metabolic syndrome. Due to these reasons, it is necessary to find strategies to reduce obesity and its co-morbidities that can be implemented in public health systems. The general objective of the thesis has been to advance in the knowledge of the influence of the composition and functions of the intestinal microbiota and its genome (microbiome) in obesity and associated metabolic alterations and to identify intestinal bacteria that, together with changes in the diet, can help to establish more effective intervention strategies to control these disorders.

The first chapter of the thesis focuses on the study of the safety and ability to metabolize different fiber sources of *Bacteroides uniformis* CECT 7771, a strain that demonstrated the ability to reduce the metabolic alterations associated with diet-induced obesity in previous pre-clinical studies. For this, we have carried out the genome sequencing and the transcriptome analysis of the bacteria in response to different carbon sources (glucose, wheat bran, pectin, mucin, gum arabic and inulin). It has been observed that *B. uniformis* CECT7771 is able to ferment all evaluated substrates and grow faster in the presence of those carbon sources of simpler structure (wheat bran rich in arabinoxylans (AXOS) and glucose).

In addition, *B.uniformis* CECT 7771 has the ability to degrade the mucin O-glycans, produce butyrate and decrease the production of long chain fatty acids and acyl transport proteins (ACPs), involved in the synthesis of bacterial lipopolysaccharide (LPS) that causes endotoxemia. When grown in the presence of pectin, *B. uniformis* CECT 7771 increases the production and secretion of GABA, which could have an impact on the gut-brain axis. *B. uniformis* CECT 7771 has shown no signs of toxicity or pathogenicity after exhaustive analysis of the genes encoded in its genome, or after oral administration in a model of sub-chronic toxicity in rats. In this model, the bacterium produces an anti-inflammatory cytokine profile compared to the

placebo group and shows a dose-dependent effect in the decrease of the alanine aminotransferase (ALT) values, suggesting a hepato-protective effect.

In the second chapter of the thesis it was done an intervention study in humans with overweight and metabolic syndrome to evaluate at first the effects of dietary supplementation with fibers enriched in AXOS and polyunsaturated fatty acids (PUFAs) during 4 weeks in the composition of the intestinal microbiota and in the biochemical parameters (anthropometric measurements, glucose metabolism, lipid profile, markers of inflammation, etc.). The results show that the intake of AXOS modifies the microbiota, exerting a bifidogenic effect and an increase of bacterial species that produce butyrate, although we have not seen an improvement in the biochemical parameters analyzed. In the case of the intervention with PUFAs, we have not detected changes in the composition of the microbiota or in the biochemical parameters evaluated. Secondly, we have carried out a multi-omic study in a subgroup of subjects of the intervention with AXOS in order to delve into the effects of this type of fiber. The metagenomic analysis has allowed us to identify changes in the configuration of the intestinal microbiota, including an increase in *Ruminococcus gnavus* and *Prevotella* spp. as well as a decrease of *Parabacteroides*, *Paraprevotella* and *Rikenella*. We have also detected an increase in metagenes involved in the production of tetrahydrofolate and folic acid derivatives, which perform a protective function against hyperhomocysteinemia; vitamin K2, involved in the correct absorption of calcium; and GABA, whose low concentration in the body is associated with states of anxiety and energy imbalance and that favors the secretion of insulin and regulates plasma ceramide levels.

The joint analysis of the metagenome and the metabolome has allowed us to establish positive correlations between the abundance of *Prevotella* and the concentrations of short chain fatty acids and succinate, as well as between increases of *Eubacterium rectale* and reductions in methylamines, which could be involved in the improvement of glucose metabolism and reduction of the risk of suffering chronic pathologies. In addition, lipid analysis demonstrates a reduction of plasma ceramides, which suggest improved glucose metabolism. The integration of multi-omic data suggests, overall, that the intake of AXOS could contribute especially to the maintenance of glucose homeostasis in overweight individuals.

ÍNDICE

INTRODUCCIÓN

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1. Conceptos generales

El conjunto de microorganismos (bacterias, arqueas, virus y algunos eucariotas como hongos y levaduras [1]) que colonizan el tracto gastrointestinal (TGI) se denomina "microbiota" y ha co-evolucionado con su hospedador creando una intrincada relación de mutuo beneficio [2, 3]. Recientemente se ha calculado que la relación "bacterias (del cuerpo humano): células humanas" se aproxima a una proporción 1:1 [4]. La capacidad codificante del genoma microbiano (microbioma) se ha estimado que es unas 100 veces superior a la del genoma humano, aportando actividades metabólicas adicionales que influyen en su fisiología [2, 5]. La microbiota intestinal está integrada por 5-7 filos bacterianos principalmente de los 52 reconocidos en la Tierra. En los adultos, los Firmicutes y Bacteroidetes son el filo dominante (~ 90% del total de bacterias) seguido por las Actinobacterias y Proteobacterias (<1-5% del total de bacterias). El filo Bacteroidetes está compuesto principalmente por los géneros Bacteroides y Prevotella mientras que el filo Firmicutes tiene representación de más de 200 géneros diferentes [6]. Las Proteobacterias incluyen la conocida familia de las Enterobacteriaceae, que representa ~0.1% del total de bacterias de la microbiota. El filo Actinobacteria incluye el género Bifidobacteria, cuya abundancia varía sustancialmente en función de la edad del individuo [6].

Entre las diferentes funciones fisiológicas en las que interviene la microbiota intestinal cabe destacar el refuerzo de la integridad de la barrera intestinal [7], la obtención de energía a partir de la dieta y la síntesis de micronutrientes como las vitaminas [8], la protección frente a patógenos [9], la regulación del sistema inmune del hospedador [10] y la diferenciación y desarrollo del epitelio intestinal y las estructuras linfoides [11]. La capacidad para llevar a cabo estas funciones puede verse truncada por la alteración de la configuración de la microbiota, proceso conocido como "disbiosis" [12].

Debido a las características fisiológicas del intestino delgado [13], sólo aquellas bacterias que sean capaces de tolerar un pH ácido, que tengan un metabolismo anaerobio facultativo, un rápido crecimiento y sean capaces de adherirse al epitelio o mucus intestinal son las que predominan en esta zona [14]. Se observa abundancia de especies bacterianas de la clase

Proteobacteria y del género *Clostridium* en estudios en humanos [15] al igual que especies del orden Lactobacillales y la clase Proteobacteria en estudios en ratones [16]. En el intestino delgado destaca la sobreexpresión de genes relacionados con degradación de carbohidratos sencillos y metabolismo de aminoácidos [14].

Por otro lado, el ciego y colon presentan una mayor densidad y diversidad bacteriana debido a un pH menos ácido y tiempos más prolongados de tránsito intestinal [13, 14, 17]. Las bacterias de esta zona son capaces de fermentar la fibra de la dieta y especialmente los polisacáridos más complejos no digeridos por otras bacterias o por las enzimas digestivas humanas [17]. Por otro lado, dichas bacterias también serían capaces de fragmentar y metabolizar compuestos derivados del propio hospedador y ricos en carbohidratos complejos (moco intestinal rico en glicoproteínas) [18]. Bacterias pertenecientes a las familias Prevotellaceae, Lachnospiraceae, Ruminococcaceae, Bacteroidaceae y Rikenellaceae suelen ser las dominantes en el intestino grueso de humanos [14].

En ambos tramos se observan células especializadas, llamadas *goblet*, secretoras de moco, cuyo principal componente es la mucina Muc2 [13], que recubre el epitelio intestinal y lubrica el intestino facilitando el tránsito y la expulsión de antígenos. Esta capa supone una interfase entre el lumen intestinal y los tejidos del hospedador. En el intestino delgado, donde la absorción de nutrientes es muy elevada y la capa de moco está más suelta y es más penetrable [19], las bacterias no suelen habitar debido a la presencia de compuestos antimicrobianos [14, 20], producidos por las células *Paneth*. La capa de moco es más extensa y tiene mayor volumen en el intestino grueso. Allí las bacterias pueden penetrar en ella y degradar los polisacáridos de las mucinas utilizando esta barrera física como fuente de alimento, como es el caso de las especies *Bacteroides caccae*, *Bacteroides thethaiotaomicron*, *Bacteroides fragilis, Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Ruminococcus gnavus*, *Ruminococcus torques*, *Bifidobacterium bifidum* y *Bifidobacterium longum*, entre otras [21]. Ver figura 1.

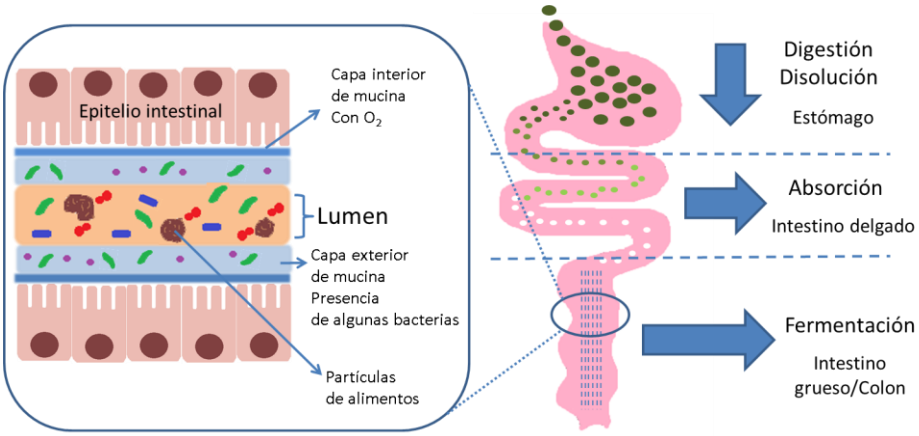

Figura 1. Microambientes del intestino grueso. El epitelio intestinal se encuentra recubierto de una capa interior de mucina fina desprovista de bacterias en un buen estado de salud. Otra capa de mucina exterior, con presencia de algunas bacterias degradadoras de moco intestinal como *Akkermansia muciniphila,* conecta con el lumen intestinal. La microbiota es mucho más diversa en el lumen o fluido intestinal. En él, existe otro microambiente constituído por algunas bacterias que crecen adheridas a partículas alimenticias sólidas.

1.1 Factores ambientales y endógenos que modulan la microbiota intestinal

La microbiota intestinal está modulada principalmente por una combinación de factores ambientales, como la dieta, la ingesta de medicamentos o el estilo de vida, junto con otros factores con menor influencia establecidos por el propio hospedador como su genética [22].

El modo de parto [23], el tipo de lactancia [24] y la edad gestacional [25] son factores importantes que modulan la microbiota intestinal durante los primeros estadios de la vida; cuando la microbiota intestinal alcanza la madurez es más estable pero siguen existiendo factores que influyen en su configuración. Ver figura 2.

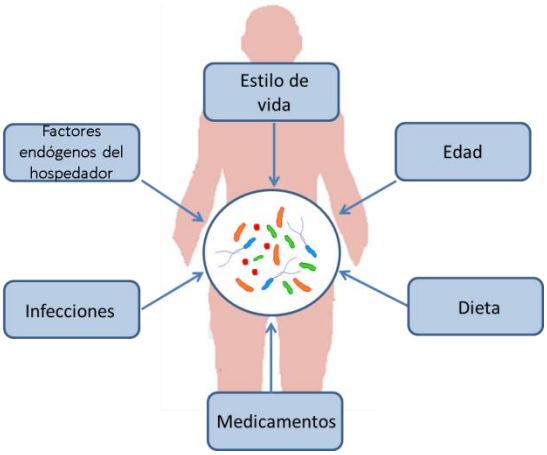

Figura 2. Factores que modulan la microbiota intestinal.

1.1.1 Estilo de vida y dieta

La localización geográfica [26, 27], hábitos de higiene y salud diarios (fumar) [28], la actividad física, así como diferentes condiciones psico-sociales del individuo (estrés) [29], son factores que afectan a la composición de la microbiota humana, como varios estudios han reflejado. La dieta es el principal factor ambiental que modula la configuración de la microbiota [30]. Cambios en la dieta pueden ser detectados hasta 24 h tras la alteración de la dieta habitual [31] y dependiendo de si son a corto plazo o a largo plazo, pueden tener diferentes efectos en la microbiota [32]. Aunque algunos grupos bacterianos son influenciados por intervenciones cortas, cambios más profundos en la estructura, como por ejemplo en los "enterotipos", no se han observado [33] y posiblemente requerirían de intervenciones más prolongadas en el tiempo [32].

Existen estudios que evidencian diferencias en la composición de la microbiota intestinal de personas con dietas basadas en frutas y vegetales y dietas basadas en consumo de carne. La dieta basada en consumo de vegetales se enriquece con bacterias con capacidad para degradar la fibra (p. ej. *Faecalibacterium prausnitzii*, *Roseburia*), mientras que aquellas basadas en el consumo de carne enriquecen la microbiota en especies con actividad proteolítica y tolerantes a los ácidos biliares (p. ej. especies de los géneros *Bacteroides*, *Alistipes*), así como también aumentan la expresión de genes relacionados con la síntesis de vitaminas y metabolitos relacionados con el consumo de proteínas [34].

También se ha observado que la ingesta de dietas occidentalizadas ricas en azúcares refinados y grasas suponen a menudo condiciones extremas para la microbiota, pudiendo con ello romper el equilibrio y la homeostasis característicos entre la microbiota y el hospedador, produciendo una pérdida de la diversidad bacteriana [30]. Estas dietas pueden provocar el enriquecimiento de la microbiota en bacterias patógenas que favorecen un estado pro-inflamatorio en el TGI, relacionado con el riesgo de desarrollar enfermedades como la obesidad, el cáncer [35], etc.

La absorción de los lípidos procedentes de la dieta es facilitada por la acción de las sales biliares, cuya función como agentes surfactantes es necesaria para aumentar el área de superficie de las micelas de grasa de los alimentos y así favorecer su degradación y absorción. La presencia de ácidos biliares en el intestino modula su ambiente fisicoquímico y contribuye también a modificar la estructura de la microbiota y su composición [36]. Los ácidos biliares primarios como el taurocolato, promueven la germinación de esporas endógenas producidas por las bacterias de la propia microbiota. Esto permite que la recolonización se produzca por estas bacterias endógenas tras procesos de disbiosis y desequilibrio [37]. Además, su conversión de ácidos biliares primarios a secundarios tiene un papel importante en la expansión de determinados taxones bacterianos proinflamatorios que pueden estar implicados en enfermedades como la cirrosis [38].

1.1.2 Medicamentos

Muchos medicamentos y xenobióticos utilizados en la actualidad tienen impacto en la composición de la microbiota intestinal. También, en muchos casos, la propia microbiota afecta a la eficacia de los medicamentos [39]. La berberina, por ejemplo, es un alcaloide usado en China desde la antigüedad al cuál se le atribuyen propiedades antibióticas y que además se ha observado que modula la microbiota y tiene propiedades antidiabéticas [40, 41]. Algunos efectos secundarios de los medicamentos en el sistema digestivo pueden ser ocasionados precisamente por esos cambios en la composición de la microbiota, pero esto a su vez, podría ser la razón de la eficacia de los mismos [42]. Un ejemplo de ello es la metformina, un fármaco empleado en el tratamiento de la diabetes tipo 2 y que también altera la composición de la microbiota en animales y humanos, por lo que se sugiere que este podría ser uno de sus mecanismos de acción [43, 44].

Los antibióticos, xenobióticos que no tienen dianas específicas en el hospedador, modifican la microbiota intestinal y la expresión génica del microbioma [45], como se ha podido observar en numerosos estudios tanto a corto como a largo plazo. Aquellos de gran espectro de acción reducen la diversidad bacteriana mientras expanden algunos taxones bacterianos en detrimento de otros. Así, selecciona bacterias resistentes a los mismos, incrementando la transferencia horizontal de estos genes de resistencia y permitiendo la intrusión de bacterias potencialmente patógenas oportunistas que se establecen en nichos del TGI que quedan vacíos por los efectos de los antibióticos [46]. No obstante, algunos de los antibióticos producidos por la microbiota intestinal actúan como moléculas mediadoras o señalizadoras naturales que regulan las interacciones entre distintas bacterias y dan estructura y forma a la composición de la comunidad microbiana del TGI [47].

Los antibióticos como los macrólidos producen un aumento de Bacteroides y Proteobacterias y disminuyen los Firmicutes y las Actinobacterias, disminuyendo en términos generales la diversidad bacteriana [48]. La exposición a algunos antibióticos da lugar a recuperaciones rápidas, pero sólo parciales de la composición inicial de la microbiota e incluso a veces efectos persistentes hasta 4 años tras su exposición [49], como es el caso de la clindamicina, con un fuerte efecto sobre bacterias anaerobias, que reduce la población de Bifidobacterias y Lactobacilos [50]. Tras su uso, parece haber sólo recuperación (meses después del tratamiento) de los Lactobacilos pero no de las Bifidobacterias. La vancomicina, en cambio, disminuye la abundancia de Firmicutes [51] . Otros antibióticos que en principio tienen relativamente poco efecto en bacterias anaerobias, se ha visto que sí que afectan a la composición de la microbiota intestinal, como es el caso de la ciprofloxacina [52].

Otro de los efectos que se ha visto recientemente asociados al tratamiento con antibióticos es que la reducción de la microbiota ocasionada por su efecto también afecta a la producción de ácidos biliares secundarios y el metabolismo de la serotonina en el colon, disminuyendo la motilidad del TGI en ratones [53].

La exposición a antibióticos durante la infancia puede también tener efectos duraderos y predisponer al sujeto a una variedad de enfermedades en su etapa adulta [54]. Incluso a dosis sub-terapéuticas en la infancia, se ha visto que los antibióticos producen en ratones una alteración de la masa del tejido graso y una modificación en la producción de metabolitos por la

microbiota y en el metabolismo de los ácidos grasos hepáticos, entre otros efectos [55].

1.1.3 Infecciones

Algunos estudios han demostrado que la composición y diversidad de la microbiota intestinal se encuentra alterada cuando se dan algunas infecciones bacterianas y víricas [56-58]. Un ejemplo de ello son las infecciones enteropatógenas de *Citrobacter rodentium*, que llega a reducir la abundancia relativa de *Lactobacillus* [59] o algunos estudios que han demostrado la reducción de la riqueza y diversidad microbiana de individuos con infecciones por *Clostridium difficile*, causando disbiosis en comparación con los individuos sanos [60].

1.1.4 Factores endógenos del hospedador

El genotipo del hospedador es uno de los factores que influyen en la colonización y composición de la microbiota, aunque de forma cuantitativamente menos relevante que los factores ambientales.

Un ejemplo de ello es la codificación de una α1,2-fucosiltransferasa (determinada por el genotipo del gen FUT2, que puede ser secretor o no secretor) responsable de la expresión de antígenos ABO en la mucosa del TGI. La capa de mucosa, cuya composición se ha descrito previamente como formada por mucina altamente glicosilada, puede variar su O-glicosilación en base al tipo de glicosil-transferasa expresada y según su localización en el aparato de Golgi de las células *Goblet* [61], afectando así a la composición de la microbiota intestinal. Cuando el genotipo del hospedador de FUT2 es no secretor, la composición de la microbiota varía hacia un perfil relacionado con la enfermedad de Crohn [62].

Otros factores endógenos que modulan la microbiota son las barreras que el TGI establece para limitar su exposición al sistema inmune del hospedador. Estas barreras pueden ser físicas, como el epitelio y las capas de mucosa; bioquímicas, como algunos enzimas y proteínas antimicrobianas e inmunológicas, como las proteínas IgA y el epitelio asociado a las células del sistema inmune [63].

La microbiota puede ser modulada por el sistema inmune del hospedador, que intenta evitar la invasión de bacterias patógenas oportunistas en los tejidos estratificando y compartimentando a la microbiota endógena. Para

ello, las células *Paneth,* células Goblet y enterocitos producen sustancias antimicrobianas como histatinas, α-defensinas, proteínas de unión a lipopolisacáridos (LPS), lisozimas, lectinas, etc. en la barrera de las mucosas [63, 64]. Algunas de estas moléculas son expresadas constitutivamente mientras que otras son expresadas en respuesta a señales e interacciones con receptores específicos de determinados microorganismos y se basan en su mayoría en ataques enzimáticos a la pared celular de algunas bacterias o en la destrucción de membranas externas de las mismas [63]. La inmunoglobulina A secretora (SIgA) es un componente del sistema inmune del hospedador que interactúa con las bacterias en la capa de moco más externa y que limita la exposición de las células epiteliales a las mismas. Además parece estar implicada en la formación de biofilms bacterianos [65]. Su función es muy importante para mantener el equilibrio de la microbiota en el TGI, puesto que se ha visto que en ratones deficientes para la expresión de IgA se produce disbiosis microbiana y una activación del sistema inmune a consecuencia de ello [66].

1.2 Funciones de la microbiota intestinal en el hospedador

La microbiota intestinal es considerada como un órgano en sí mismo con una extensa capacidad metabólica y elevada plasticidad funcional. Sus funciones protectoras, inmunológicas y metabólicas en relación con la salud han sido ampliamente estudiadas, aunque el conocimiento de que se dispone se basa fundamentalmente en estudios animales. Ver figura 3.

Una de las principales funciones de la microbiota es colaborar con su maquinaria enzimática en la nutrición y metabolismo del hospedador. Los carbohidratos, especialmente oligo y polisacáridos, que escapan a las enzimas digestivas del hospedador suelen ser fermentados por bacterias pertenecientes a los géneros *Bacteroides, Faecalibacterium, Roseburia* o *Bifidobacterium*, produciendo con ello ácidos grasos de cadena corta o SCFA (del inglés *short chain fatty acids*) como acetato, propionato y butirato, producidos en proporción 3:1:1 [67], que son fuente de energía para los colonocitos del hospedador [68], y también están implicados en numerosos procesos como la expresión génica (tanto butirato como propionato actúan como histonas deacetilasas [69, 70]), quimiotaxis, diferenciación y proliferación celular y apoptosis [71]. Además, el butirato también tiene

propiedades antiinflamatorias y anticancerígenas [69, 70] y puede prevenir la acumulación de subproductos tóxicos como el D-lactato [72].

La microbiota intestinal también participa en el metabolismo de proteínas con su maquinaria enzimática, produciendo péptidos cortos, aminoácidos y derivados y ácidos grasos de cadena corta y ramificada [73]. Muchos de los aminoácidos producidos son convertidos en moléculas señalizadoras y neuroactivas. Por ejemplo, el glutamato es convertido en ácido ɣ-aminobutírico (GABA) por la acción de las glutamato decarboxilasas [74] y la L-histidina en histamina por la histamina decarboxilasa de las bacterias [75]. Componentes de la microbiota también sintetizan vitaminas como la B_{12} que es producida por bacterias ácido-lácticas [76, 77] y el folato, que es producido por las *Bifidobacterias* [78]. La vitamina K, riboflavina, biotina, ácido nicotínico, ácido pantoténico, piridoxina y tiamina también son productos del metabolismo de bacterias intestinales [79]. Algunas especies del género *Roseburia* son capaces de sintetizar precursores del ácido linoleico conjugado, el cual posee propiedades antidiabéticas, antiobesogénicas e hipolipidémicas, entre otras [80]. Los polifenoles ingeridos con la dieta, como por ejemplo la isoflavona de la soja, pueden ser degradados por algunas bacterias como *Slackia isoflavoniconvertens* la cual produce subproductos bioactivos como el equol y/o O-desmetilangolensina (O-DMA), con efectos endocrinos y de atenuación de síntomas de la menopausia [81].

La microbiota intestinal ejerce una función importante en el desarrollo de las defensas del hospedador, de naturaleza inmune y no inmune. Las bacterias comensales regulan la expresión de los genes de producción de mucinas (MUC-2 y MUC-3) por las células *Goblet* del epitelio intestinal y modifican los patrones de transglicosilación, lo cual puede afectar a la adhesión, colonización e invasión de bacterias [82]. La microbiota modula los tejidos linfoides asociados al intestino (GALT, por el término en inglés (*gut-associated lymphoid tissues*)) que consiste en una variedad de células (linfocitos, células dendríticas (CDs), macrófagos, etc.) agrupada en estructuras organizadas como las Placas de Peyer, nódulos linfoides mesentéricos (NLM), y folículos linfoides aislados [83]. Las bacterias son reconocidas mediante el contacto de sus patrones moleculares asociados a microorganismos (PMAMs) como pueden ser el peptidoglicano, lipopolisacáridos o flagelos, con receptores de reconocimiento de patrones moleculares (RRP) presentes en las células *Paneth* del hospedador [84] y células presentadoras de antígenos como macrófagos y CD presentes en el epitelio intestinal, los cuáles disciernen entre microorganismos patógenos e

inocuos. Estos receptores son principalmente de tipo Toll y Nod (TLR y NLR). La unión de PMAMs a estos receptores activa rutas de señalización (factor nuclear (FN)-κB, protein kinasas activadas por mitógeno (MAPKs), y factores reguladores del interferón (IRFs)) cuya función es activar la expresión de genes inflamatorios, incluyendo aquellos que codifican para producción de citoquinas y sus receptores, proteínas inmunomoduladoras y asociadas a estrés, entre otras [82]. Estas moléculas también contribuyen al reclutamiento de otras células del sistema inmune (linfocitos, basófilos, neutrófilos, CD, y células natural killer (NK)) promoviendo así la respuesta para la eliminación de patógenos.

Por ejemplo, *L. casei* CRL 431 (Gram positiva), induce un aumento de células TLR2+, mientras que *E. coli* 13-7 (Gram negativa) induce un aumento de células TLR4+ y, con ello, inducen la producción de diferentes tipo de citoquinas por parte de las células del sistema inmune (IL-10 anti-inflamatoria e IL-12 pro-inflamatoria, respectivamente) modulando así la respuesta del sistema inmune [85].

Además, algunos compuestos producidos por la microbiota contribuyen también a la modulación y desarrollo del sistema inmune en el intestino, como por ejemplo, los SCFA, los cuales contribuyen al desarrollo de las células Treg [86, 87] e influyen también en la producción de citoquinas, como la IL-18 [71].

Estudios recientes muestran indicios sobre el efecto beneficioso de la microbiota intestinal en áreas diferentes al TGI, incluyendo órganos y tejidos implicados en el metabolismo (hígado, tejido adiposo) y el cerebro a través del llamado eje intestino-cerebro [88]. En este último caso, se ha sugerido que los metabolitos producidos por la microbiota, como los ácidos grasos de cadena corta, pueden actuar sobre tejidos periféricos modificando el metabolismo y como compuestos neuroactivos (GABA, serotonina, etc.) que modulan la función del sistema nervioso, afectando al comportamiento, emociones y estado de ánimo del hospedador [89].

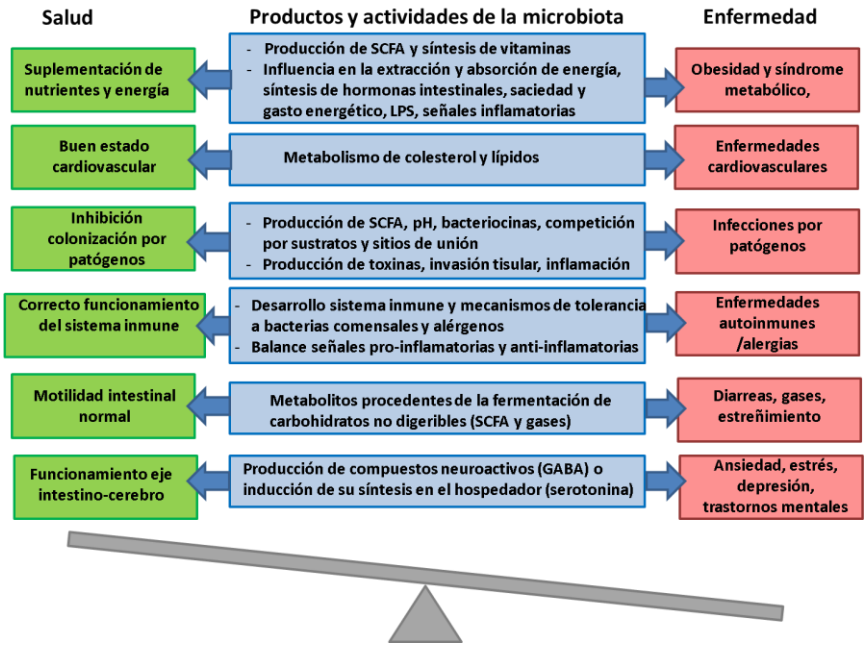

Figura 3. Influencia de la microbiota intestinal en la salud y enfermedad. El resultado del balance de moléculas y actividades llevadas a cabo por el conjunto de la microbiota determinará su contribución al estado de salud del huésped (Adaptada de Flint *et al*., 2012).

1.3 Técnicas empleadas para el estudio de la microbiota intestinal.

1.3.1 Técnicas clásicas de microbiología

El intestino humano es un sistema abierto, con volumen constante y un recambio permanente de nutrientes, en el que los microorganismos tienen que crecer a una determinada velocidad para sobrevivir en dicho entorno. En un colon saludable, se encuentran densidades de 10^{11} células bacterianas/gramo de contenido intestinal [90].

El cultivo de estas bacterias *in vitro* es complicado debido a que la mayoría de las bacterias que forman parte de la microbiota intestinal son anaerobias estrictas. Por este motivo, su crecimiento *in vitro* debe llevarse a cabo en completa anaerobiosis empleando para ello generadores de anaerobiosis,

tubos *"Hungate"* o cámaras de anaerobiosis. Además, los medios de cultivo se deben suplementar con compuestos presentes en el medio natural (intestino) de las bacterias: fluidos del rumen, filtrados de heces, SCFA, que son utilizados como sustratos por las bacterias, así como con compuestos reductores como la cisteína-hidroclorhídrica o el tioglicolato sódico [91, 92].

Sin embargo, la mayoría de las secuencias de ADN bacteriano obtenidas en trabajos de estudio de la microbiota por técnicas de secuenciación se corresponden con secuencias de las especies más abundantes en la microbiota y que ya han sido previamente cultivadas. Sólo las especies menos abundantes son aquellas con menos probabilidad de ser cultivadas, lo cual indica que quizá el principal problema es que no se llevan a cabo esfuerzos suficientes para el aislamiento *in vitro* de las bacterias y no tanto la imposibilidad de cultivarlas [90, 92]. Actualmente se ha introducido una nueva técnica denominada "culturómica" que consiste en la aplicación de condiciones de alto rendimiento para el cultivo de la microbiota humana acopladas al empleo MALDI-TOF (*matrix-assisted laser desorption/ ionization–time of flight*) o secuenciación del gen del ARNr 16S para la identificación de colonias aisladas [93].

Además, la información generada por las nuevas tecnologías de secuenciación masiva puede ser empleada para diseñar nuevos medios de cultivo que permitan el crecimiento de bacterias que previamente no se hayan podido aislar por no conocer sus requerimientos nutricionales [94].

Otra forma más sofisticada de simular *in vitro* el sistema natural del TGI es mediante el empleo de fermentadores (en *batch* o continuos) [95], cada vez más avanzados, como el sistema de fermentadores propuesto hace unos años por la Dra. Emma Allen-Vercoe, en los cuales conseguían una simulación aproximada del ambiente intestinal para poder obtener comunidades microbianas representativas para su manipulación experimental [96]. Aunque éstos se asemejen más al sistema natural que el cultivo de bacterias en tubos de ensayo o placas Petri con medio agar, son sistemas que siguen teniendo desventajas como la falta de un sistema inmune que interactúe con las bacterias o, en el caso de los fermentadores en *batch*, la acumulación de metabolitos que en condiciones normales son absorbidos por el intestino por lo que no refleja del todo la realidad del sistema *in vivo*.

A pesar de sus limitaciones, las técnicas de microbiología clásicas son todavía muy necesarias en esta área de investigación porque el cultivo de las bacterias intestinales en laboratorio, su aislamiento y la comprobación de su

bioseguridad son completamente necesarios para poder explotar una cepa con fines terapéuticos[90].

1.3.2 Técnicas de secuenciación de segunda generación (Next Generation Sequencing)

El estudio de la microbiota intestinal ha alcanzado hitos sin precedentes durante la década pasada a consecuencia del gran desarrollo que las técnicas de secuenciación de ADN de alto rendimiento (*High-Throughput DNA Sequencing Technologies*) han sufrido [97]. Esto ha hecho que se pueda obtener una visión mucho más amplia del total de bacterias presentes en una muestra, sin necesidad de cultivar previamente a las bacterias *in vitro,* siendo más fácil su estudio [97]. Con la emergencia de estas técnicas, proyectos como el Human Microbiome Project (HMP) [98] o el MetaHIT [99] han contribuído a aumentar los datos de libre acceso de los que se disponía sobre la microbiota intestinal.

Una de las formas de conocer la composición de la microbiota en una muestra es mediante el estudio de genes marcadores bacterianos como el del ARN de la subunidad ribosomal pequeña (ARNr 16S en bacterias y arqueas y ARNr 18S en eucariotas) [100]. Este gen tiene la peculiaridad de poseer 9 regiones hipervariables que difieren en longitud, posición y discriminación taxonómica entre diferentes grupos bacterianos [101]. La región 4 (V4) es normalmente la más informativa [102]. De esta forma, tras llevar a cabo una extracción de ADN bacteriano de la muestra, un segmento de dichos genes es amplificado empleando oligonucleótidos dirigidos a hibridar con secuencias altamente conservadas. Se genera así una mezcla de "amplicones" que serán posteriormente secuenciados de forma masiva [97]. Para estas secuenciaciones se emplean tecnologías de secuenciación de segunda generación como la plataforma Illumina MiSeq, que genera secuencias de corta longitud capaces de ser agrupadas por identidad de secuencia en Unidades Taxonómicas Operacionales (*Operational Taxonomic Units*, OTUs, del inglés), que serán una aproximación razonable a la identidad de las especies bacterianas teóricas que componen la muestra. Cuando las 1500 pb del gen del ARNr 16S son secuenciadas, es posible "descifrar" la identidad de la especie bacteriana. Sin embargo, debido a la típica corta longitud de las lecturas generadas por estas tecnologías de segunda generación, lo más común es llegar a identificar secuencias de ADN a nivel de género, por lo que raramente se analizan las categorías taxonómicas de especie [103].

Una opción para estudiar el genoma de toda la comunidad microbiana (metagenoma) o de una determinada bacteria intestinal aislada es la secuenciación del genoma completo mediante la técnica "*shotgun*". El ADN (meta)genómico se fragmenta aleatoriamente y tras la secuenciación de dichos fragmentos, las lecturas de ADN se mapean frente a genomas de referencia. En el caso de genomas de bacterias puras de los que no existan genomas de referencia, se lleva a cabo un ensamblaje del genoma *de novo* [104].

La transcriptómica es el estudio de los transcritos de ARN en un determinado organismo, mientras que el término metatranscriptómica hace referencia al estudio combinado de los transcritos producidos por toda una comunidad microbiana. La metatranscriptómica permite identificar qué genes se están expresando en un determinado momento y bajo unas determinadas condiciones ambientales en una muestra compleja [97]. A menudo no existen genomas de referencia para mapear los transcritos, antes de llevar a cabo el análisis de los transcritos, por lo que es necesario llevar a cabo un ensamblaje *de novo* del genoma o los genomas [105] para usarlos como genomas de referencia, y a pesar de ello, muchos de los genes transcritos pueden ser todavía de función desconocida.

Por último, la metabolómica permite el estudio de las funciones de la microbiota intestinal o de aislados concretos basándose en el análisis de los metabolitos producidos por las bacterias o bien por reacciones coordinadas entre las bacterias intestinales y el hospedador. De esta forma se pueden identificar los productos finales del metabolismo conjunto de los dos organismos en estudios in vivo [106], a partir del análisis de muestras de cultivos puros o de muestras biológicas como heces, orina o plasma sanguíneo, mediante técnicas como espectrometría de masas o resonancia magnética nuclear [107].

2. Microbiota intestinal, dieta y obesidad

2.1 Obesidad

La obesidad está considerada como la epidemia del siglo XXI [108]. En la actualidad, la mayoría de las personas vive en países donde la obesidad y sobrepeso causa más muertes al año que la desnutrición [109]. La Organización Mundial de la Salud - OMS (*World Health Organization*, WHO)

establecía a finales del año 2017 que la incidencia de la obesidad en el mundo se había casi triplicado desde 1975, habiendo, en 2016, más de 650 millones de personas adultas obesas (13% de la población mundial) y más de 1.9 billones de personas adultas con sobrepeso (39% de la población mundial) [109]. La obesidad también afecta de forma alarmante a adolescentes y niños. Más de 340 millones de niños/adolescentes entre 5-19 años y 41 millones de niños de menos de 5 años padecían obesidad o sobrepeso en 2016 [109] y estas cifras continúan en aumento según la WHO (2017).

La obesidad se define como un aumento de la ingesta en relación al gasto energético que conlleva un incremento del peso corporal y una excesiva acumulación de tejido graso, normalmente acompañado por un estado de inflamación sistémica de bajo grado [110].

Para evaluar y determinar la composición corporal existen técnicas de antropometría, incluyendo el índice de masa corporal (IMC), definido como el peso dividido por la talla al cuadrado ($kg \cdot m^{-2}$) o la circunferencia de la cintura entre otros. Según los valores obtenidos del IMC, la OMS establece que aquellas personas con un IMC igual o superior a 25 Kg/m^2 tienen sobrepeso y cuando el IMC es igual o superior a 30 Kg/m^2 tienen obesidad [109]. Un IMC por encima de 40 Kg/m^2 es indicativo de obesidad mórbida [109]. Este índice es calculado igualmente para hombres y mujeres, pero en el caso de los niños se calcula basándose en la desviación estándar del IMC y teniendo en cuenta la edad [111].

2.1.1 Etiología

La principal causa de la obesidad es el estilo de vida sedentario [112] y el exceso en el consumo de comida altamente energética y porciones cada vez mayores, por encima de las necesidades de la persona en relación a su gasto energético [113], aunque la etiología es mucho más compleja [110, 114].

La heredabilidad del IMC es alta y se estima que puede llegar a ser del 40-70% [115]. En la actualidad, se reconocen algunas formas monogénicas relacionadas con la obesidad, como las mutaciones en MC4R (que codifica para el receptor de la melanocortina 4), genes que codifican para la leptina (*Lep*) y su receptor (*Lepr*), la pro-opiomelanocortina (*Pomc*), etc [116]. Algunos estudios de asociación a gran escala (*Large-scale genome wide association studies*, GWAS) han permitido encontrar más de 300 *loci* relacionados con la obesidad, entre ellos el descubrimiento del locus FTO y sus variantes, donde cada alelo aumenta el riesgo de obesidad entre 1.2-1.32

veces [110]. Aun así, todas estas variantes no explicarían más del 5% de la variación en el IMC [117]. De hecho, se sabe que la obesidad depende en gran medida de factores ambientales como el estatus socioeconómico, el comportamiento de la persona, las interacciones genotipo-fenotipo, las horas dedicadas al descanso y sueño [118], la práctica de deporte o actividad física, el consumo de fármacos [119], la composición de la microbiota intestinal [120], la epigenética [115], la temperatura ambiente [118] y la calidad de los alimentos ingeridos [121], entre otros factores [110].

Existen también factores a tener en cuenta en el periodo prenatal [122] que pueden predisponer al individuo a un desarrollo posterior de la obesidad como la ganancia de peso excesiva en la madre durante la gestación y la sobrenutrición [123] o desnutrición [124] durante la vida fetal. La lactancia prolongada también se ha asociado a una prevalencia menor de sobrepeso en edad adulta del niño [125].

La interacción entre los genes y los factores ambientales previamente mencionados regulan el balance energético asociado a la ganancia o pérdida de peso y a muchos otros procesos fisiológicos. El balance energético es controlado por una serie de mecanismos centrales y de señales periféricas (hormonas y neuropéptidos) que proceden de las células del tejido adiposo, estómago, páncreas, intestino y microbiota intestinal. Cuando este balance energético se desregula se puede alterar el control del apetito, la termogénesis, adipogénesis y metabolismo de la glucosa y los lípidos [118] y puede derivar todo en un proceso de inflamación crónico asociado a la obesidad.

2.1.2 Patofisiología de la obesidad y comorbilidades asociadas

El proceso de desarrollo de la obesidad es lento y conlleva efectos anatómicos, puesto que se aumenta la proporción de grasa acumulada y disminuye la masa muscular, en comparación con personas que no sufren sobrepeso u obesidad. La secreción de insulina en estado de ayuno y tras la ingesta de glucosa aumenta linealmente conforme aumenta el IMC [126]. Además, las citoquinas producidas por las células del sistema inmune infiltradas en el tejidos adiposo, en parte como respuesta a la apoptosis de adipocitos, contribuyen a un estado de resistencia a la insulina, característico de los pacientes con obesidad [114]. Ver figura 4.

Una de las comorbilidades más frecuentes de la obesidad es el síndrome metabólico, que a su vez constituye un factor de riesgo para el desarrollo de

enfermedad cardiovascular y diabetes de tipo 2. El síndrome metabólico se diagnostica cuando al menos 3 de los siguientes 5 criterios están presentes en el paciente [127]:

- Obesidad visceral
- Hipertrigliceridemia
- Niveles bajos de colesterol HDL
- Elevada presión sanguínea
- Niveles elevados de azúcar en sangre

Por último, la obesidad también se ha relacionado con algunos desórdenes mentales, como por ejemplo la ansiedad y el estrés [128]. Además, algunos psicofármacos, pueden causar un aumento sustancial de peso como efecto secundario [119].

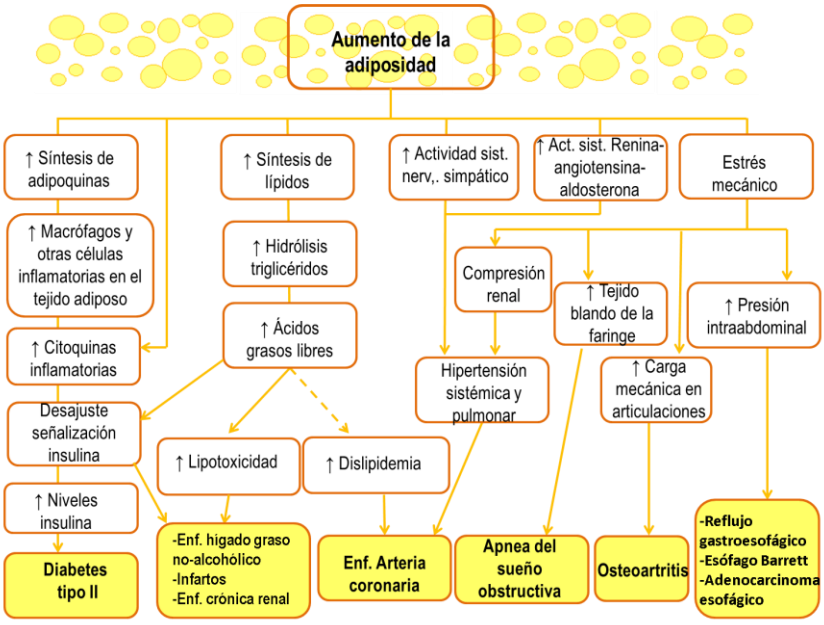

Figura 4. Comorbilidades asociadas al aumento de adiposidad (obesidad). Las flechas discontinuas indican una asociación indirecta (Adaptada de Heymsfield *et al*., 2017).

2.1.3 Tratamientos

El tratamiento escogido depende del grado de severidad de la enfermedad en el paciente, siendo la primera elección los cambios en el estilo de vida (p. ej. dietas, ejercicio), en segundo lugar el tratamiento con medicamentos, que también se complementa con dieta y ejercicio, y por último lugar, cuando el IMC supera los 40 kg/m^2, se considera la opción de realizar una cirugía bariátrica al paciente.

La modificación del estilo de vida implica cambios en los hábitos alimenticios y un incremento de la actividad física, ambos implicando poco coste y mínimos riesgos para la salud del paciente. Normalmente suele haber un seguimiento dietético por parte de un nutricionista experimentado, donde normalmente suele perderse de media un 5-8% del peso total en un periodo de 6 meses [129]. El principal inconveniente de estas prácticas es la recuperación de peso tras la finalización de un tratamiento o seguimiento, ya que un seguimiento a largo plazo a menudo no es posible [129].

El uso de la cirugía bariátrica se ha incrementado un 70% entre los años 2000 y 2010 [130] debido al incremento de los pacientes que sufren de obesidad mórbida. Estos métodos son hoy por hoy los que mayores logros tienen, aunque también suponen un alto coste y con muchos riesgos asociados debidos a la cirugía. Además, un porcentaje de los pacientes recupera peso con el tiempo tras la operación [114]. Cambios en los sabores y preferencias por las comidas, la señalización vagal, la actividad hormonal gastrointestinal, los ácidos biliares circulantes y la microbiota intestinal, son algunos de los posibles mecanismos que intervienen en esa pérdida de peso e incluso en la remisión de la diabetes [131].

2.2 Relación entre microbiota intestinal, obesidad y dieta

2.2.1 Asociaciones entre la microbiota intestinal y la obesidad

Existen numerosos estudios que demuestran asociaciones positivas y negativas entre algunos componentes de la microbiota y parámetros como el peso corporal, el consumo energético, la adiposidad, la glicemia, la intolerancia a la glucosa, la resistencia a la leptina, la resistencia a la insulina, la inflamación o la permeabilidad intestinal [132]. Por ejemplo, *Akkermansia*

muciniphila, *Faecalibacterium prausnitzii* y los géneros de *Lactobacillus* y *Bacteroides* han sido asociados a una atenuación de co-morbilidades asociadas a la obesidad mientras que otras bacterias de las familias Lachnospiraceae y Desulfovibrionaceae parecen estar implicadas en el proceso de la obesidad, aunque los resultados no son consistentes en todos los estudios [132].

Algunos estudios han demostrado que el fenotipo obeso puede ser desarrollado en animales libres de gérmenes (delgados) con un trasplante fecal de la microbiota procedente de los animales o humanos obesos, indicando que la microbiota puede ser un agente causal de la enfermedad, aunque su configuración sea también consecuencia de la dieta que siguen los sujetos obesos [120, 133-135]. Ver figura 5.

Los mecanismos por los cuales la microbiota intestinal podría estar involucrada en la patogénesis de la obesidad a través de la dieta podrían resumirse en:

(i) La capacidad para extraer energía de la dieta y aumentar la absorción de ésta por el hospedador. La microbiota de animales obesos parece tener mayor capacidad para extraer energía de la dieta ingerida posibilitando la digestión de compuestos no digeribles por las enzimas digestivas humanas (polisacáridos complejos), aunque la ingesta de estos animales sea inferior, y aumentando su absorción y acumulación en tejidos periféricos mediante la regulación de genes del hospedador implicados en estos procesos [120]. Ver Figura 5. A.

(ii) La capacidad de alterar las secreciones del sistema enteroendocrino. La fermentación de las fibras de la dieta por parte de la microbiota intestinal genera SCFAs, que poseen efectos beneficiosos frente a la obesidad (que serán comentados en apartados posteriores), pero su producción está alterada en sujetos obesos [120, 136-138]. Entre otros efectos, estos compuestos activan receptores específicos para nutrientes que estimulan la secreción intestinal de hormonas implicadas en la sensación de saciedad como el péptido YY (PYY) o el péptido 1 similar al glucagón (GLP-1), por lo que son importantes para la regulación del apetito y la ingesta [132].

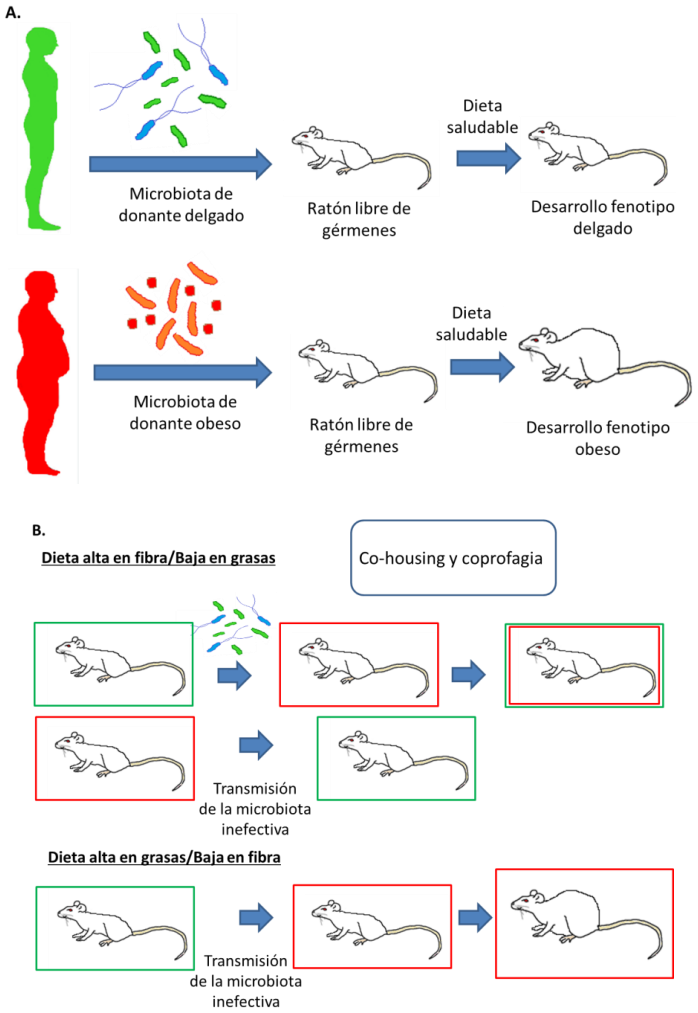

Figura 5. A. Cuando los ratones libres de gérmenes son colonizados con la microbiota de un individuo humano, desarrollan el fenotipo del donante. **B**. Cuando se produce coprofagia por *co-housing* de ratones con ambos tipos de microbiota, si siguen una dieta saludable (rica en fibras y baja en grasas), los ratones que recibieron microbiota de individuo obeso serán también colonizados por la microbiota del individuo sano y no desarrollarán fenotipo obeso. En cambio, la microbiota del individuo obeso no coloniza con efectividad a los ratones previamente inoculados con microbiota del individuo delgado, por lo que dichos animales permanecen con fenotipo delgado. El tipo de dieta está también implicado en el fenotipo desarrollado de modo que la infiltración de la microbiota de los sujetos delgados en los potencialmente obesos protege frente a la obesidad sólo en el contexto de una dieta saludable [135] (Adaptada de Walker y Parkhill, 2013).

(iii) La capacidad de generar inflamación intestinal e incrementar la permeabilidad intestinal. La microbiota intestinal puede contribuir a aumentar la inflamación y permeabilidad intestinal y subsecuentemente la inflamación sistémica del hospedador [139]. Las citoquinas inflamatorias también pueden pasar la barrera hematoencefálica [140], estimular a la microglía y/o neuronas aferentes vagales e inducir así una inflamación central y resistencia a insulina. Además, modelos animales libres de gérmenes que reciben microbiota de animales obesos incrementan su permeabilidad intestinal e inflamación, como se ha visto en algunos estudios [141]. También la barrera hematoencefálica es más permeable en animales libres de gérmenes que en los convencionales y la función se recupera cuando son recolonizados o bien cuando se administran SCFAs [140].

2.2.2 Influencia de la dieta en la microbiota y sus implicaciones en la obesidad

Fibra dietética. La fibra dietética no es digerida por las enzimas humanas y llega a las zonas del colon proximal y distal donde puede ser fermentada por la microbiota intestinal presente, constituyendo su principal fuente de energía y generando ácidos orgánicos (como el ácido láctico o succínico y SCFAs (como el acetato, propionato y butirato). Por todo ello, la cantidad y calidad de la fibra consumida por la dieta se considera uno de los principales determinantes de la composición y función de la microbiota intestinal [142].

Los efectos que su consumo tiene en el hospedador varían dependiendo del tipo de fibra ingerida, lo cual está directamente relacionado con sus propiedades fisicoquímicas y estructurales (p. ej. viscosidad, solubilidad) e indirectamente relacionado con la composición de la microbiota de cada individuo. La fibra soluble e insoluble, a diferencia de los carbohidratos digeribles, son capaces de reducir la respuesta glicémica postpandrial debido a que se digieren de forma lenta [143]. Además también contribuyen a aumentar la saciedad y su ingesta está recomendada para la pérdida o mantenimiento de peso, puesto que su contenido energético es de ~1.91 kcal/g (8 kJ/g) en comparación con otros macronutrientes como los carbohidratos digeribles (~4.06 kcal/g), las proteínas (~4.06 kcal/g), y grasas (~8.84 kcal/g)[144]. El consumo de dietas ricas en fibra (a niveles por encima de las recomendaciones) se ha asociado con una reducción del riesgo de enfermedades coronarias y diabetes tipo 2, así como con la mejora del mantenimiento del peso corporal [145-148].

Las bacterias intestinales poseen múltiples rutas de generación de SCFAs a partir de las fibras [8] (ver figura 6), donde comúnmente el fosfoenolpiruvato resulta el intermediario glicolítico por excelencia, aunque los grupos bacterianos pertenecientes a *Bifidobacterium* spp. sólo poseen una única ruta (ruta derivada de la fructosa-6-fosfato) [149]. Las bacterias de los filos Bacteroidetes y Firmicutes son aquellas con mayor capacidad/versatilidad para llevar a cabo la degradación de los diferentes tipos de fibra comúnmente ingerida con la dieta, aunque las especies *Bifidobacterium* (Actinobacteria) también poseen gran versatilidad para el catabolismo de oligosacáridos [150].

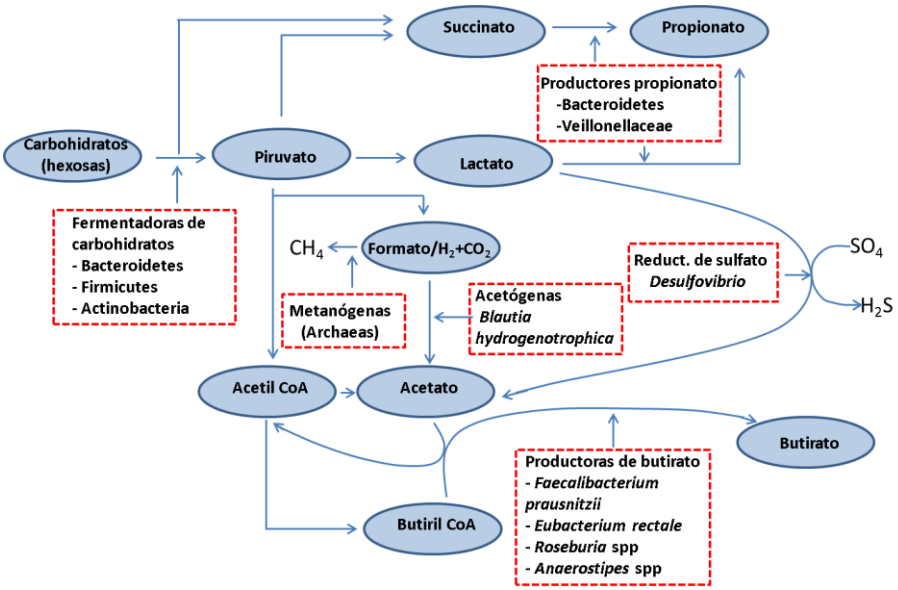

Figura 6. Esquema del metabolismo de los ácidos grasos de cadena corta (SCFA) con los principales grupos y especies bacterianas implicados en su producción (Adaptada de Flint *et al*., 2012).

Los SCFAs son absorbidos en un 95% por los colonocitos [151] que los emplean como fuente de energía en el siguiente orden de preferencia: butirato>propionato>acetato [152]. Mientras que el butirato es el principal sustento de dichas células, el propionato y acetato pueden pasar a la circulación sanguínea y llegar a los tejidos periféricos donde pueden ser empleados como sustratos para la gluconeogénesis y lipogénesis. El acetato

puede ser sustrato para la síntesis de colesterol [153, 154] y el propionato puede inhibirla, así como también, mediante la liberación de péptidos anorexigénicos, puede contribuir a la disminución del nivel de los triglicéridos hepáticos y la ingesta de comida [155-157].

Los efectos del butirato, procedente de la fermentación de la fibra dietética, en la fisiología del hospedador son muy variados. Se ha observado que sus niveles están reducidos en pacientes con enfermedad inflamatoria intestinal [158-160] y que se aumenta la producción de GLP-2, asociado a la mejora de la integridad de la mucosa intestinal con función barrera, mejorando la proliferación de las células de las criptas del colon, la elongación de los vellosidades y reduciendo la apoptosis [161]. También se ha visto que el butirato y propionato ejercen funciones protectoras frente a la obesidad inducida por la dieta y la resistencia a la insulina en modelo de ratón mediante la inducción de GLP1, que induce saciedad y mejora el metabolismo de la glucosa, y reduciendo la inflamación [156].

Grasas. Existen relativamente pocos estudios en los que se haya analizado la metabolización de grasas de la dieta por la microbiota y su repercusión en la salud, pero se sabe que existen bacterias en rumiantes capaces de metabolizar los ácidos grasos poli-insaturados de cadena larga (PUFAs, del inglés *poly-unsaturated fatty acids*) [162] así como en los roedores [163, 164]. Algunas bacterias de género *Achromobacter, Acinetobacter, Alcaligenes, Bacillus, Enterococcus, Staphylococcus, Lactobacillus, Propionibacterium, Serratia marcescens, Pseudomonas, Proteus vulgaris* o *Enterobacter* [165-167], presentes en la microbiota intestinal humana poseen lipasas que pueden contribuir a la degradación de lípidos, aunque no sean bacterias predominantes.

Los resultados de los estudios de intervención nutricional no sugieren importantes efectos de las grasas en la microbiota intestinal o en la salud metabólica mientras que los estudios observacionales sí que indican asociaciones entre la microbiota y efectos en la salud. Es importante remarcar que muchos de los ensayos de intervención tienen muy corta duración (3-6 semanas) y pequeño tamaño, lo que puede ser la razón por la que no se observan efectos claros del consumo de determinados tipos de grasas en la microbiota y en la salud metabólica.

Las dietas ricas en grasas saturadas en general disminuyen la diversidad microbiana [168, 169] y los efectos que pueden tener en la microbiota y el

metabolismo han sido estudiados en varias intervenciones en las cuales se ha administrado ácidos grasos monoinsaturados (MUFAs, del inglés *mono-unsaturatted fatty acids*), obteniéndose un aumento de Parabacteroides, Prevotella, Turicibacter y géneros de la familia Enterobacteriaceae en adultos con riesgos cardiovasculares [170]. También se han observado disminuciones de la circunferencia de la cintura a raíz de las intervenciones con MUFAs en pacientes con diabetes tipo 2 (DT2) [169].

En los estudios observacionales se observó que la ingesta de grasas totales y grasas saturadas disminuye la abundancia bacteriana, diversidad o riqueza [171]. Especies como *Clostridium bolteae* se ven aumentadas [172] así como también lo hace la razón de Firmicutes/Bacteroidetes [173], algunos géneros del cluster XI *Clostridium* en pacientes con DT2 [174] y *Eubacterium rectale* y *Clostridium coccoides* en mujeres pre-menopáusicas produciendo un aumento de la grasa corporal [175], mientras que otras especies como *Faecalibacterium prausnitzii* disminuyen [172], así como también *Prevotella* [173] y el cluster IV de *Clostridium* en individuos con DT2 [174]. Los estudios observacionales también indican que la ingesta de MUFAs parece aumentar especies del género *Blautia* [176] y la ingesta de PUFAs aumenta la presencia de *Bacteroidetes* [177] y disminuye la de *Tenericutes* [176].

También se han observado aumentos en el número de bacterias reductoras de sulfato y generadoras de ácido sulfhídrico, como *Bilophila wadsworthia* como consecuencia de la ingesta de dietas ricas en grasas [178]. Otros cambios observados en el contexto de dietas ricas en grasas son aumentos de *Alistipes* y reducciones de *Roseburia* o *Ruminococcus bromii* [34].

Los ácidos grasos saturados incorporados a las dietas occidentalizadas promueven la diferenciación y proliferación de células del sistema inmune como linfocitos T helper 1 y/o T helper17 y la reducción de SCFAs en el intestino, promoviendo un ambiente inflamatorio que en parte es consecuencia de modificaciones en la microbiota [179]. En modelos animales se ha demostrado que algunas bacterias del filo Firmicutes, que aumentan en la microbiota de individuos que ingieren dietas ricas en grasa, contribuyen a la absorción de lípidos a nivel intestinal [180], mientras que otras bacterias de filo Bacteroidetes, como *Bacteroides uniformis* CECT 7771, reducen la absorción de grasas por los enterocitos [181], explicando su mecanismos de acción en la obesidad.

Algunos estudios indican que las dietas ricas en grasas aumentan la concentración de bacterias Gram-negativas que poseen lipopolisacárido (LPS) o bien su translocación, incrementando la activación del sistema inmune y la inflamación [182, 183]. Esto puede conducir a la pérdida de la integridad de barrera intestinal, como se ha explicado en el punto (iii) de la sección 2.2.2 favoreciendo la inflamación sistémica [184].

La presencia de bacterias como *Akkermansia muciniphila* parecen revertir los efectos de la dieta rica en grasas en la pérdida de la función barrera y producción de moco en modelos animales [185], reduciendo las alteraciones metabólicas asociadas a la obesidad como por ejemplo la resistencia a la insulina [186].

Proteínas. Las proteínas procedentes de la dieta son digeridas en primer lugar en el intestino delgado por las enzimas pancreáticas y las peptidasas de los enterocitos. Los enterocitos colaboran en la absorción y posterior liberación de oligopéptidos y aminoácidos al sistema circulatorio portal [187]. Aproximadamente el 10-12% de las proteínas de la dieta pasan al intestino grueso, donde tiene lugar una proteólisis adicional por parte de la microbiota intestinal [142]. Este porcentaje aumenta de forma proporcional al aumento de la ingesta de proteína en la dieta. A diferencia de los enterocitos, los colonocitos no absorben los productos generados a partir de la degradación de las proteínas por lo que estos compuestos se emplean como fuente de energía por las bacterias intestinales o se secretan finalmente en las heces [187]. Normalmente la mayor parte de la fermentación de aminoácidos tiene lugar en el colon distal. Aquí apenas hay carbohidratos ya que éstos han sido previamente fermentados por ser una fuente de energía para las bacterias preferente a las proteínas [188].

Las bacterias intestinales que llevan a cabo proteólisis en el colon han sido estudiadas principalmente durante los años 1980-1990, y pertenecen principalmente a los géneros *Bacteroides, Clostridium, Propionibacterium, Fusobacterium, Streptococcus, Lactobacillus, Peptostreptococcus, Actinomyces, Peptococcus, Ruminococcus, Bacillus, Staphylococcus, Megasphaera, Acidaminococcus* y algunas Enterobacterias [187, 189, 190]. En la actualidad, es necesario llevar a cabo estudios en mayor profundidad, mediante nuevas técnicas que permiten analizar nuevas bacterias que no habían sido previamente detectadas por métodos de cultivo o sondas o primes específicos utilizando técnicas convencionales.

Los metabolitos generados a partir de la fermentación de aminoácidos por parte de la microbiota son muy diversos: sulfuro de hidrógeno, amonio, compuestos aromáticos (fenol, p-cresol, indol), poliaminas (putrescina, cadaverina), SCFAs, ácidos grasos ramificados (isobutirato), ácidos orgánicos (formato, lactato, succinato), etanol, gases (H_2, CO_2, CH_4), y compuestos potencialmente neuroactivos (p. ej. ácido ɣ-aminobutírico, serotonina, histamina) entre otros [187]. Muchos de estos compuestos son también producidos por el propio hospedador (co-metabolitos) y esto dificulta a menudo comprender la implicación de la microbiota en su generación y sus efectos. [191, 192].

Existen estudios que demuestran que las dietas ricas en proteínas reducen el peso corporal, la tensión arterial, los niveles de triglicéridos y masa adiposa además de disminuir el riesgos cardiometabólicos [193-195], todo ello atribuido a alteraciones en el metabolismo energético y la disminución del apetito, con lo que se reduce la asimilación energética y se contribuye a la pérdida de peso [196]. En este sentido, algunos compuestos producidos por bacterias intestinales como el indol o los SCFAs, podrían contribuir a los efectos beneficiosos de las proteínas ya que están implicados en la inmunidad, la saciedad y la motilidad intestinal [197-199]. Además, otros productos de la fermentación bacteriana de proteínas, como el ácido ɣ-aminobutírico o la serotonina, podrían regular la ansiedad, saciedad o inmunidad [199-201] aunque el efecto fisiológico en el huésped y en sus tejidos y ambiente intestinal todavía está siendo objeto de estudio.

Por otra parte, las dietas ricas en proteínas también presentan efectos adversos para la salud del hospedador, puesto que se ha relacionado con enfermedades intestinales como cáncer colorrectal, colitis ulcerosa y daños renales [202]. Por ejemplo, se ha visto que cuando las bacterias metabolizan la L-carnitina de la carne roja se produce óxido-trimetil amina-N en el hígado, que puede conllevar al desarrollo de aterosclerosis en roedores [203]. El fenol y p-cresol pueden causar daño en las células epiteliales, modificando las uniones intercelulares disminuyendo la integridad de la barrera de la mucosa intestinal [204, 205].

3. Estrategias dietéticas basadas en la modulación del ecosistema intestinal para combatir la obesidad

3.1 Prebióticos

Desde hace más de 20 años, una clase de compuestos, denominados prebióticos, ha sido reconocida por su habilidad para modular la composición o actividad de la microbiota intestinal en beneficio del hospedador. La Asociación Científica Internacional para Probióticos y Prebióticos (ISAPP) se ha encargado de revisar periódicamente el concepto de prebiótico, en base al avance en las tecnologías disponibles para el estudio de la microbiota y en cuanto al conocimiento de su modo de acción. La primera definición de prebiótico fue en 1995, como "ingrediente alimenticio no digerible que afecta beneficiosamente al hospedador estimulando el crecimiento y actividad de una o un número limitado de bacterias residentes en el colon"[206].

A finales de 2017, el panel actual de la ISAPP llevó a cabo la última actualización del concepto de prebiótico, que fue definido como un "*Sustrato selectivamente utilizado por microorganismos del hospedador confiriéndole beneficios para la salud*" [207].

Debido a los avances tecnológicos, en la actualidad se ha llegado a identificar un mayor rango de bacterias intestinales capaces de utilizar prebióticos como sustratos para la fermentación, aparte de los ya previamente conocidos como las bifidobacterias y lactobacilos, y otras rutas metabólicas, por lo que estos grupos bacterianos ya no son los únicos grupos que se usan como indicadores de la actividad prebiótica. [208, 209]. No obstante, si la modificación de la composición de la microbiota intestinal producida por el posible probiótico no se lleva a cabo por un proceso selectivo derivado de su utilización por los microorganismos que confieren el beneficio, entonces no se trata de un prebiótico [207]. Así se descarta los antibióticos, minerales, vitaminas o bacteriófagos como prebióticos.

Aunque la mayoría de los prebióticos se administran oralmente, también pueden ser administrados localmente en otras zonas como la vagina o la piel. Sus efectos beneficiosos se extienden al TGI (estimulación del sistema inmune y barrera frente a patógenos), al cardiometabolismo (reducción de niveles de lípidos y resistencia a la insulina), salud mental (metabolitos que

afectan a la función cerebral y cognición) y huesos (biodisponibilidad de minerales), entre otros [207].

Los prebióticos establecidos como tal en la actualidad son constituidos principalmente por carbohidratos no digeribles, pero algunas sustancias como los polifenoles [210], podrían encajar en la definición actual de prebiótico (ver figura 7). La clasificación de las fibras como prebióticos es también complicada porque existe variabilidad en el efecto producido entre individuos, además de que las bacterias que resulten estimuladas por un determinado prebiótico pueden no estar presente o no estarlo en concentraciones suficientes en un determinado individuo que la consuma y ser ello la causa de que el efecto no sea observado. También es necesario establecer la dosis adecuada de suplementación del prebiótico [211].

Los prebióticos más estudiados hasta la fecha son los oligosacáridos no-digeribles de tipo fructano (FOS) y galactano (GOS), preferentemente metabolizados por bifidobacterias [212]. Esto se debe a que poseen enlaces de unión degradables por β-fructanosidasas y β-galactosidasas, ambas enzimas predominantes en el genoma de las bifidobacterias.

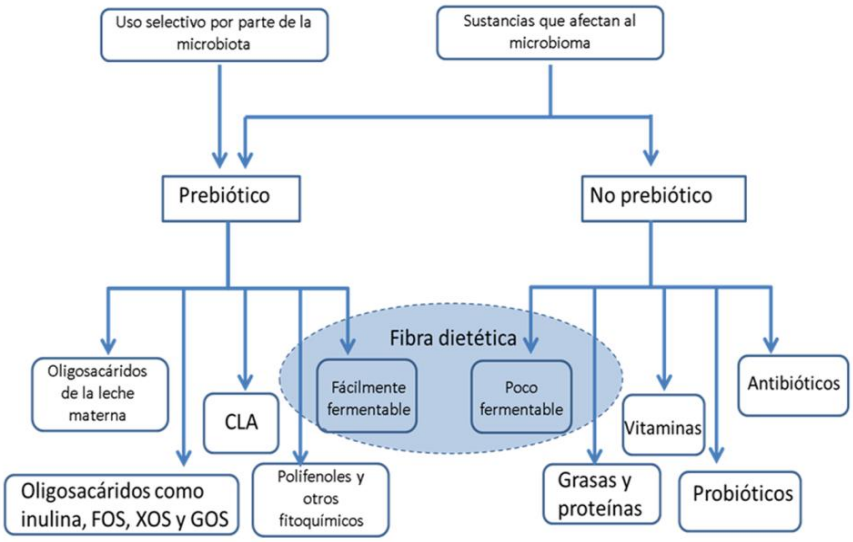

Figura 7. Distinción de sustancias prebióticas (reconocidas y potencial candidatos) según las evidencias hasta la fecha. CLA, ácido linoleico conjugado; FOS, fructooligosacaridos; GOS, galactooligosacaridos; XOS, xilooligosacaridos. Adaptado de Gibson *et al*., 2017.

3.2 Probióticos

Los probióticos se definen como organismos vivos que administrados en dosis adecuadas confieren efectos beneficiosos en el hospedador [213].

3.2.1 Probióticos clásicos

La mayoría de los probióticos comercializados en la actualidad son bacterias ácido-lácticas y bifidobacterias, comúnmente llamados "probióticos clásicos" [214], que son microorganismos con larga historia de uso en alimentación y considerados seguros QPS (Qualified presuption as safe según la EFSA en la Unión Europea) o GRAS (*Generally Recognized As Safe* en EEUU). Los mecanismos por los cuales estas bacterias ejercen diferentes beneficios para la salud incluyen la mejora de la función barrera intestinal, la exclusión competitiva de patógenos y modulación de los sistemas inmune y neuroendocrino local y sistémicamente, lo cual ha permitido proponer aplicaciones de estos probióticos para el tratamiento de diarreas debidas a la toma de antibióticos, alergias, enterocolitis necrotizante, síndrome del intestino irritable y obesidad y comorbilidades asociadas [215], aunque el grado de evidencia sobre su efectividad en humanos varía dependiendo de la condición y de las cepas. En particular, la evidencia de eficacia de los probióticos existentes para combatir la obesidad y sus comorbilidades en humanos es escasa y los resultados no son tan contundentes como en los ensayos en modelos animales. Algunos ejemplos de estudios clínicos son aquellos que han demostrado la eficacia de *Lactobacillus gasseri* SBT2055 en la disminución de la adiposidad abdominal y peso corporal [216], o la tendencia a disminuir la concentración de triglicéridos, malondialdehído e interleucina IL-6 en pacientes con DT2 a los cuáles se les administra una mezcla probiótica de diferentes lactobacilos (*L. acidophilus*, *L. bulgaricus*, *L. bifidum*, and *L. casei*) [217]. En otro ensayo clínico llevado a cabo por Rajkumar y colaboradores, se observó una mejora del perfil lipídico, se redujo el colesterol y los triacilglicéridos, la proteína C-reactiva y los niveles de colesterol LDL y se aumentó el HDL y la sensibilidad a la insulina, administrando una mezcla probiótica de bifidobacterias, lactobacilos y *S.thermophilus* [218]. Otros ejemplos de estudios clínicos donde se hace uso de probióticos clásicos para combatir la obesidad, DT2, síndrome de resistencia a la insulina o enfermedad de hígado graso no alcohólico han sido revisados en mayor profundidad por Saéz-Lara y colaboradores [219].

Aunque se piensa que los probióticos son una estrategia para corregir la disbiosis asociada a ciertas enfermedades, recomponiendo así la microbiota intestinal "saludable", no existen estudios clínicos que prueben directamente que la ingesta de probióticos clásicos produzca cambios sustanciales en la microbiota y que esto sea la causa de la mejora de la mayoría de patologías estudiadas en humanos [220]. Además, en general la magnitud de los efectos de probióticos clásicos es modesta. Esto puede ser debido a la falta de una selección racional de los mismos para aplicaciones clínicas concretas, así como al hecho de que no siempre son empleadas bacterias mayoritarias en el ecosistema intestinal sino que su selección se ha basado en criterios de seguridad y aptitud tecnológica que facilitaban su comercialización. Se observa, por tanto, una creciente necesidad de mejorar las estrategias de selección de cepas como potenciales probióticos basándose en criterios de cribado racionales y acordes a su finalidad nutricional o clínica.

3.2.2 Probióticos de nueva generación

Los estudios moleculares del microbioma humano han permitido descubrir nuevos componentes de la microbiota intestinal que podrían tener un papel funcional importante para la salud del hospedador, pudiendo ser el origen de una nueva generación de probióticos diferentes a los estudiados hasta el momento y que podrían mejorar la eficacia de las formulaciones descritas hasta la fecha.

Hace unos años, los trasplantes fecales eran la opción elegida como terapias alternativas cuando otras terapias habían sido infructíferas, normalmente para casos de infecciones recurrentes por *Clostridium difficile* [221], aunque también se ha empleado de forma experimental en un estudio como estrategia en el tratamiento del síndrome metabólico [222]. Debido a los problemas de seguridad inherentes a estos tratamientos por la posible presencia de patógenos o resistencias a antibióticos y factores de virulencia, los usos de esta técnica son bastante restringidos. La eficacia de este método indica que la presencia de determinados grupos de bacterias (muchos de los cuales son desconocidos o poco estudiados) están directamente asociados con la causa de la enfermedad y su manipulación puede contribuir a restaurar el buen estado de salud del hospedador [214]. Por ello, sería interesante profundizar en el estudio del efecto de estas nuevas bacterias de forma controlada.

Un ensayo llevado a cabo por Atarashi *et al*. demostró que la fracción bacteriana resistente a cloroformo procedente de heces humanas promovía la acumulación de células T reguladoras mientras que la fracción sensible al cloroformo era la responsable de la acumulación de células Th17. Las 17 cepas responsables de la inducción de células T reguladoras pertenecían a la clase Clostridia, concretamente los clusters IV, XVIa y XVIII y eran capaces de generar SCFAs y contribuir a la estimulación del sistema inmune con sus antígenos bacterianos [223, 224].

Faecalibacterium prausnitzii, una especie perteneciente al filo Firmicutes, y al cluster IV del género *Clostridium* y que representa un 5% de la microbiota intestinal, es una de las principales bacterias productoras de butirato en el intestino humano [225]. A esta especie se le ha atribuído propiedades antiinflamatorias ya que apenas induce la producción de citoquinas pro-inflamatorias por parte del hospedador (IL-12 y IFN-ɣ) y por el contrario induce una elevada secreción de citoquinas anti-inflamatorias como IL-10 [226, 227]. Bien las células o su sobrenadante reducen la severidad de la inflamación inducida químicamente en bajo grado [228], aguda [227] o crónica [229] en modelos murinos, dada su capacidad para producir péptidos inhibidores de NF-κB, IL-8 [227] y un aumento de la producción de células T reguladoras [226]. *F. prausnitzii* también produce metabolitos que ayudan a conservar la función barrera intestinal [228] y contribuye al restablecimiento de los niveles de serotonina (neurotransmisor que afecta a la motilidad intestinal) en modelos murinos tratados con células o su sobrenadante [228].

Akkermansia muciniphila pertenece al filo de Verrucomicrobia, representa un 3-5% del total de la microbiota en individuos sanos [230] y coloniza mayormente las mucosa intestinales ricas en glicanos participando en el intercambio de nutrientes y comunicación con el hospedador [231]. Su abundancia disminuye en ratones ob/ob y en aquellos alimentados con dietas altas en grasas [185]. Cuando los animales son tratados con prebióticos, los niveles de *A. muciniphila* se restauran y consecuentemente mejoran algunos parámetros metabólicos e inflamatorios [232]. La cepa *A. muciniphila* Muc^T (ATTC BAA-835) mejora los valores de algunos parámetros relacionados con la obesidad y modula el control de la homeostasis de la glucosa y la saciedad [185]. Además, aumenta el grosor de la capa de moco epitelial restaurando así la función de permeabilidad intestinal en el hospedador [185, 233]. Ratones normales a los cuales se les administró la bacteria durante 5 semanas redujeron la ganancia de peso y la masa adiposa y mejoraron los

parámetros bioquímicos como la tolerancia a la glucosa y sensibilidad a la insulina así como disminuyeron los niveles de inflamación [234]. Así mismo, también se han observado efectos similares en ratones obesos [235]. Otro estudio mostró que el tratamiento de la DT2 con metformina puede tener como base de su eficacia un aumento de la abundancia de *A. muciniphila* [236].

Bacteroides uniformis es una especie bacteriana comensal perteneciente al filo Bacteroides que se encuentra en elevadas cantidades en la microbiota normal del ser humano [237]. Algunos estudios han demostrado que la ingesta de leche materna parece incrementar la prevalencia de *B. uniformis* en las muestras de heces de niños sanos [238] y estudios epidemiológicos demuestran que la lactancia materna reduce el riesgo de obesidad y DT2 [239]. La cepa *Bacteroides uniformis* CECT 7771 fue aislada a partir de muestras de heces de niños alimentados con leche materna y seleccionada de entre otras cepas de *B. uniformis* por su bajo potencial inflamatorio *in vitro* y fue testada *in vivo* en modelo murino de obesidad inducida por dieta rica en grasa. En modelos murinos, *B. uniformis* CECT 7771 reduce la ganancia de peso, la esteatosis y colesterol hepáticos y los niveles de triglicéridos, aumenta el número de adipocitos de pequeño tamaño, reduce el colesterol sérico y los niveles de glucosa, insulina y leptina. También reduce la absorción de grasa y mejora la tolerancia oral a glucosa. Además, mejora la función de macrófagos y células dendríticas alteradas en la obesidad y restaura parcialmente la microbiota intestinal alterada de los ratones obesos, de forma que la bacteria podría estar implicada en la mejora del estado de salud del hospedador por mecanismos directos e indirectos [181]. Se necesitan más estudios para poder elucidar los mecanismos moleculares que podrían estar implicados en las propiedades beneficiosas de esta cepa como potencial probiótico de nueva generación.

3.2.3 Proceso de desarrollo de un probiótico.

El desarrollo de un nuevo probiótico incluye una sucesión de pasos que pueden comprender desde el aislamiento de la bacteria hasta su caracterización y estudios de su seguridad y efectividad preclínicos y clínicos antes de poder comercializarse.

El origen de los aislados con potencial probiótico puede ser a partir de alimentos consumidos por el ser humano [240] o del propio ser humano, bien de las heces o muestras intestinales de individuos sanos [240, 241] o de otras

muestras biológicas como la leche materna [242]. Esto garantiza un mayor éxito y más probabilidades de que el aislado en cuestión sea funcionalmente relevante y, por ejemplo, que sea capaz de colonizar al menos de forma transitoria el TGI humano [240, 243].

Aunque no existen pautas específicas sobre los criterios de selección de las cepas que puedan ser potenciales probióticos, gran parte de los desarrollos realizados hasta ahora y especialmente con probióticos clásicos se ha basado en las pautas sugeridas por la FAO/WHO [244]. En este texto se indica que la cepa debe ser correctamente identificada, debido a que las propiedades beneficiosas de una determinada bacteria son específicas de la cepa y dosis administrada, aunque algunas cepas podrían compartir propiedades a nivel de género [213]. No obstante, salvo que esto se demuestre, se requiere de la combinación de ensayos y métodos de caracterización fenotípicos y genotípicos y el uso de técnicas de biología molecular para la caracterización de los potenciales probióticos a nivel de cepa [245].

Debe llevarse a cabo estudios *in vitro* para una selección preliminar (ver figura 8) y posteriormente estudios *in vivo* de las cepas seleccionadas para testar sus potenciales propiedades probióticas en animales modelo [246]. Para que una cepa sea considerada como potencial probiótico no es necesario que todos los criterios sean cumplidos, esto puede variar dependiendo de su finalidad [247].

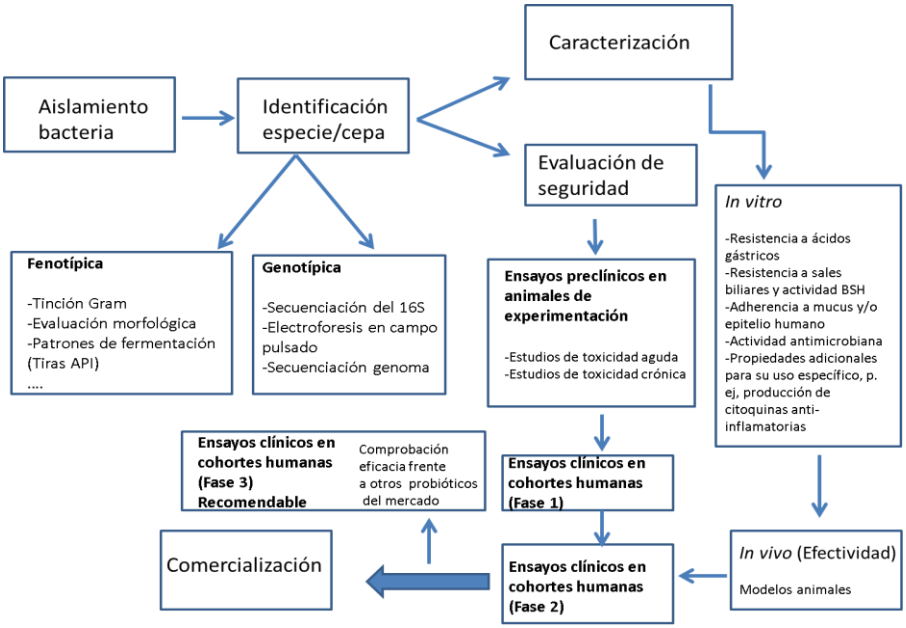

Figura 8. Pasos a seguir para el desarrollo de nuevos probióticos desde su aislamiento hasta su aplicación y comercialización.

En cuanto a la seguridad de los probióticos, normalmente no se exigen estudios específicos de toxicidad para cepas que pertenecen a especies con estatus GRAS o QPS [248, 249]. La seguridad está basada en 4 consideraciones:

- La taxonomía para la cual es buscada la calificación de QPS
- La relación taxonómica del microorganismo con los clasificados como QPS y el nivel de conocimiento que se tiene sobre él para poder tomar una decisión sobre su estatus de seguridad
- La patogenicidad que pueda mostrar el microorganismo
- La finalidad o uso que se desee hacer del microorganismo; bien entrar en la cadena alimentaria o bien ser utilizado para producir otros productos que entren en la cadena alimentaria.

Cepas pertenecientes a *Lactobacillus*, *Bifidobacteria* y *Streptococcus* tienen un largo historial como para ser reconocidas en su mayoría como QPS y GRAS [240], pero evaluaciones exhaustivas deben realizarse para poder obtener esta categoría en nuevas clases de bacteria que pretendan constituir una nueva generación de probióticos. Estos estudios de seguridad incluyen una inequívoca clasificación taxonómica y la secuenciación de su genoma para

descartar la presencia de factores de virulencia y resistencias a antibióticos transmisibles, tests *in vitro* para confirmar el perfil de sensibilidad y resistencia a antibióticos y posible producción de metabolitos adversos y ensayos de toxicidad sub-crónica en modelos animales. También es deseable disponer de estudios de seguridad en humanos (ver figura 8).

3.3 Simbióticos

La combinación de probióticos y prebióticos que aumenten sus efectos beneficiosos de manera sinérgica se denomina simbiótico [250].

Hasta la fecha, el uso de algunos simbióticos para el tratamiento de la obesidad en estudios clínicos ha demostrado ciertos efectos positivos. Por ejemplo, los simbióticos que se muestran en la Tabla 1 han reducido el IMC en mujeres, han disminuido el tejido adiposo y los niveles de leptina y han incrementado la abundancia de especies pertenecientes a la familia *Lachnospiraceae* en heces. Los tratamientos parecen contribuir a disminuir el z-score del IMC y la circunferencia de cintura en niños, así como también disminuir los niveles de colesterol total, colesterol LDL y triglicéridos en suero (Ver tabla 1 a continuación).

En este ámbito, las investigaciones que se realizan actualmente están orientadas a la identificación de prebióticos que favorezcan el crecimiento y funciones metabólicas deseables de otras bacterias comensales del tracto intestinal de forma que se pueda aumentar su especificidad y potenciar y diversificar los efectos sobre la salud, como se expondrá en el contenido de mi tesis doctoral.

Tabla 1. Estudios llevados a cabo con simbióticos en humanos para el tratamiento de la obesidad y sus comorbilidades, como la resistencia a insulina y diabetes tipo 2.

Sujetos	Simbiótico	Duración	Observaciones	Ref.
153 hombres y mujeres obesos	*L. rhamnosus* CGMCC1.3724, 6 X10^8 CFU, e inulina	36 semanas	↓ peso y ↓ de niveles de leptina. ↑ de Lachnospiraceae	[251]
70 niños y adolescentes con alto IMC	*L. casei*, *L. rhamnosus, S. thermophilus*, *B. breve, L. acidophilus*, *B. longum*, *L. bulgaricus* y FOS	8 semanas	↓ del z-score del IMC y circumferencia de cintura	[252]
77 niños obesos	*L. acidophilus*, *L. rhamnosus*, *B. bifidum*, *B. longum*, *E. faecium*, y FOS	4 semanas	Cambios en medidas antropométricas. ↓ colesterol total, colesterol LDL y niveles de estrés oxidativo sérico	[253]
38 sujetos con síndrome de resistencia a insulina	*L. casei*, *L. rhamnosus*, *S. thermophilus, B. breve, L. acidophilus, B. longum, L. bulgaricus*, y FOS	28 semanas	Mejora de los niveles de glucosa sérica en ayunas y de la resistencia a insulina	[254]
54 pacientes con diabetes tipo 2	*L. acidophilus, L. casei, L. rhamnosus, L. bulgaricus, B. breve, B. longum, S. thermophilus*, 10^9 CFU,y 100 mg FOS	8 semanas	↑ HOMA-IR y niveles de glutatión, ↓ proteína C reactiva en suero	[255]
81 pacientes con diabetes tipo 2	*L. sporogenes*, 1X10^8 CFU y 0.07 g inulin por 1 g	8 semanas	↓ niveles insulina sérica, HOMA-IR, y función del modelo homeostático de células β	[256]
78 pacientes con diabetes tipo 2	*L. sporogenes*, 1X10^8 CFU y 0.07 g inulina por 1 g	8 semanas	↓en perfil lipídico en suero (TAG, TC/HDL-C), ↑colesterol HDL	[257]
20 pacientes con diabetes tipo 2	*L. acidophilus* 10^8 CFU/mL, *B. bifidum* 108 CFU/mL y 2 g oligofructosa	2 semanas	↑ colesterol HDL, ↓glicemia en ayunas	[258]
62 pacientes con diabetes tipo 2	*L. sporogenes* (1 × 10^7 CFU), 0.04 g inulina por 1 g	6 semanas	Efectos significativos en insulina sérica, hs-CRP, ácidos urico y niveles en plasma de glutatión total	[259]

OBJETIVOS

OBJETIVOS

El objetivo general de la tesis ha sido avanzar en el conocimiento de la influencia de la composición y funciones de la microbiota intestinal y su genoma (microbioma) en la obesidad y alteraciones metabólicas asociadas e identificar bacterias intestinales que, junto a cambios en la dieta, puedan contribuir a establecer estrategias de intervención más eficaces para controlar estos desórdenes.

Para llevar a cabo el objetivo general antes descrito, en la presente tesis doctoral se han abordado los siguientes objetivos parciales:

1. Determinar las interacciones entre la dieta, la obesidad y la microbiota intestinal, en cuanto a su composición y funciones, mediante estudios de intervención en humanos.

2. Identificar y optimizar el crecimiento de las bacterias asociadas con un fenotipo metabólico saludable en humanos, con el propósito de desarrollar posibles cepas probióticas que puedan reducir la obesidad y el riesgo de sufrir patologías asociadas.

3. Identificar posibles mecanismos de acción de bacterias que puedan constituir una nueva generación de probióticos para el tratamiento de la obesidad y sus co-morbilidades, mediante el análisis de su genoma y transcriptoma en presencias de distintas fuentes de fibra dietética *in vitro*, y evaluar su seguridad en modelos animales.

RESULTADOS Y DISCUSIÓN

CAPÍTULOS DE LA TESIS

Capítulo 1: *Bacteroides uniformis* CECT 7771 como potencial probiótico de nueva generación. Optimización de su crecimiento, posibles mecanismos de acción y seguridad.

- The Glycolytic Versatility of *Bacteroides uniformis* CECT 7771 and Its Genome Response to Oligo and Polysaccharides.

- Safety assessment of *Bacteroides uniformis* CECT 7771, a symbiont of the infant's gut microbiota.

Capítulo 2: Estudio de las interacciones entre la composición estructural y funcional de la microbiota intestinal, la dieta y la obesidad y sus co-morbilidades.

- Arabinoxylan oligosaccharides and polyunsaturated fatty acid effects on gut microbiota and metabolic markers in overweight individuals with signs of metabolic syndrome: a randomized cross-over trial

- A multi-omics approach to unraveling the microbiome-mediated effects of arabinoxylan-oligosaccharides in overweight humans.

CAPÍTULO 1

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Bacteroides uniformis CECT 7771 como potencial probiótico de nueva generación. Optimización de su crecimiento, posibles mecanismos de acción y seguridad.

- The Glycolytic Versatility of *Bacteroides uniformis* CECT 7771 and Its Genome Response to Oligo and Polysaccharides.

- Safety assessment of *Bacteroides uniformis* CECT 7771, a symbiont of the infant's gut microbiota.

The glycolytic versatility of *Bacteroides uniformis* CECT 7771 and its genome response to oligo and polysaccharides

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Originality-Significance Statement

This study encompasses the genome assessment of *Bacteroides uniformis* strains and reveals such strains exhibit an unprecedented repertoire of genes associated with glycan utilization outnumbering those previously known for archetypal species such as *B. thetaiotaomicron*. We have sequenced the *B. uniformis* CECT 7771 genome with cutting-edge technology such as nanopore sequencing, which permitted an almost complete genome assembly and outlining functional features of the *B. uniformis* strains unknown at date, and we used a large amount of genetic information derived from transcriptome analysis to assess the genome response against a different types of oligo- and polysaccharides and complex mixtures of them. Utilization of the *B. uniformis* CECT 7771 symbiont for treatment of obesity, its co-morbidities, and behavioral disorders represents the development of a new generation of probiotic strains with more potential than classical lactic-acid bacteria (LAB) or bifidobacteria thanks to its great glycolytic potential. Moreover, our study clearly indicate that combination of this potential probiotic strain with appropriate dietary fiber could enhance its healthy effect by increasing significantly the production of pivotal compounds such as those derived from the butanoate metabolism.

Abstract

Bacteroides spp. are dominant components of the gut microbiota and prosper in glycan enriched environments but knowledge of the machinery of human symbiont species for utilization of dietary and endogenous sources of glycans and their byproducts is limited. We have used nanopore-based technology to sequence the *B. uniformis* CECT 7771 genome, a human symbiont with a proven pre-clinical efficacy on metabolic and immune dysfunctions in obesity animal models. We have also used massive sequencing approaches to distinguish the genome expression patterns in response to carbon sources of different complexity during growth. At genome-wide level, our analyses globally demonstrate that *B. uniformis* strains exhibit an expanded glycolytic capability when compared with other *Bacteroides* species. The dietary fibers used as carbon source exerted different effects on *B. uniformis* CECT 7771 activating different molecular pathways and, therefore, allowing the production of different metabolites with potential impact on gut and mental health. The genome and transcriptome analysis of *B. uniformis* CECT 7771, in response to different carbon sources, shows its high versatility to utilize both dietary and endogenous glycans along with the production of potential beneficial end products for both the bacterium and the host, pointing to a mechanistic basis for a mutualistic relationship.

Introduction

The human gastrointestinal tract (GIT) is colonized by hundreds of microbial species exhibiting certain patterns of temporal succession and ecological niche (Koenig *et al.*, 2011; Stearns *et al.*, 2011).The number of studies concerning the inventory of microbiome functions and species inhabiting our GIT has reached a colossal magnitude, yielding valuable information of the bacterial genomes present in the human gut (Qin *et al.*, 2010; Li *et al.*, 2014). Diet is thought to be one of the main environmental factors modulating the gut microbiota to which gut microbes quickly respond (David *et al.*, 2014). A vast amount of human studies indicate that plant-derived foods and especially dietary fiber beneficially impact bowel function and metabolic health, effects which could be partly mediated by gut microbiota (reviewed in (Benitez-Paez *et al.*, 2016b)).

Most of the recent population human studies investigating the relationship between gut microbiota and lifestyle indicate that *Prevotella* spp., which are members of the Bacteroidetes phylum, are those bacteria associated with the intake of fiber-rich diets (De Filippo *et al.*, 2010; Yatsunenko *et al.*, 2012; Ou *et al.*, 2013). Notwithstanding, *Bacteroides* spp., which are also dominant members of the phylum Bacteroides in humans, are also known to be thrive in environments enriched in oligo and polysaccharides derived from plants (Matijasic *et al.*, 2014). Their *sus* genes (from starch utilization system) have been characterized as the cornerstone for binding and importing of polysaccharide compounds (Reeves *et al.*, 1997) and they are co-regulated with other genes encoding enzymes involved in glycan degradation in so called PULs (polysaccharide utilization loci) (Martens *et al.*, 2009).

Bacteroides tethaiotaomicron is considered as the archetype for studies of *sus* genes and PULs given the large number of genes encoded in its genome circumscribed to be involved in oligo and polysaccharide degradation and conferring this species high versatility to degrade a wide variety of glycan compounds (Xu *et al.*, 2003). Moreover, the genome sequence of other *Bacteroides* species has permitted to expand the knowledge of the glycolytic potential of other species such as *B. cellulosyliticus* (McNulty *et al.*, 2013). Likewise, reconstruction of genes for carbohydrate degradation from gut metagenomic data has indicated that *Bacteroides uniformis* also exhibits an important glycolytic capability (Tasse *et al.*, 2010). Strikingly, the fibrolytic potential of *B. uniformis* was supported by a recent metagenomic study,

which indicates that its dietary fiber degrading role is not restricted to the colon, but it extends to distal regions of the small intestine such as ileum (Patrascu *et al*., 2017).

A past study investigating the colonization pattern of *Bacteroides* species in the full-term newborns' gut suggested that *B. uniformis* abundance is promoted by breast-feeding compared to formula feeding (Sanchez *et al*., 2011). This finding suggest the potential of *B. uniformis* to thrive in the infant's gut from the early postnatal stage, presumably at expenses of the oligosaccharides present in the human milk (German *et al*., 2008). Our group has previously demonstrated *in vivo* the safety and effectiveness of *B. uniformis* CECT 7771 to ameliorate the metabolic and immune dysfunctions associated with obesity in mice (Gauffin Cano *et al*., 2012; Fernandez-Murga and Sanz, 2016).

The objective of the present study was to uncover the potential glycolytic activity of *B. uniformis* CECT 7771 compared to other strains of the same species and to other *Bacteroides* species by an extensive comparative genome analysis. Secondly, we analyzed the genome response and metabolic output as a function of the carbon source available in the growth medium. To this end, we exposed *B. uniformis* CECT 7771 to a variety of oligo and polysaccharides in controlled anaerobic cultures and analyzed the genomic response by a genome-wide exploratory gene expression analysis, quantitative PCR, and metabolite quantification analysis of end-products to identify the molecular pathways activated as a consequence of oligo- or polysaccharide fermentation.

Methods

Cells and culture media

Bacteroides uniformis CECT 7771 was isolated from stools of a healthy infant included in a prospective observational study carried out in a sub-group of 75 full-term newborns with at least one first-degree relative with celiac disease belonging to the Proficel study cohort (Sánchez et al., 2011). *Bacteroides uniformis* CECT 7771 was grown for 48 hours in plates of Schaedler anaerobe medium (Oxoid) supplemented with 100 mg/L kanamycin, 7.5 mg/L vancomycin, and 0.5 mg/L vitamin K_3. Bacterial growth was setup at 37 °C under anaerobic conditions into a Whitley DG250 Anaerobic Workstation (don Whitley Scientific, Inc., Shipley, UK). Single colonies were used to inoculate10 mL of modified Schaedler broth (10 g/L triptone soy broth, 2.43 g/L caseine pancreatic peptone, 0.43 g/L soy peptone, 2.15 g/L meat extract, 5 g/L yeast extract,5 g/L glucose, 0.75 g/L Tris-HCl, 0.4 g/L L-cysteine, 0.01 g/L hemin and 0.5 mg/L vitamin k_3). The L-cysteine, hemin and vitamin K were filtered individually by using 0.22 µm disposable filters (Millipore) and then were added to the autoclaved media. Ten mL of an overnight culture of the strain studied was used for genomic DNA isolation.

To evaluate the effects of different carbon sources on *B. uniformis* CECT 7771 growth, overnight cultures (0.7-0.8 OD_{600}) of this bacterial strain were diluted 1/20 in fresh pre-warmed and oxygen-depleted modified Schadler media containing 0.5% w/v of the different carbon sources including glucose(Scharlau, Cat#GL01271000), inulin (Sigma, Cat#I2255), wheat bran extract (WBE) (Cargill, Antwerp, Belgium), gum arabic (Sigma, Cat#30888), pectin (Sigma, Cat#P9135), or type II mucin from porcine stomach (Sigma, Cat#M2378) and growth kinetics were monitored for 10 hours. Each carbon source stock was sterilized by filtration using 0.22 µm disposable filters, thus avoiding the alteration of their original structure and chemical composition. The OD_{600} was measured at one hour intervals for each sample until stationary phase. An aliquot of respective cultures was collected at exponential growth phase ($OD_{600} \sim 0.4$) for RNAseq analysis. The cells were then pelleted by centrifugation at 3,000 x *g* for 15 minutes at 4°C. The supernatant was aspired off, filtered using 0.22 µ filters and immediately stored at -80°C for further metabolite analyses and the cell pellet was stored at -80°Cfor RNA extraction. Three independent experimental trials were included in the analyses.

DNA and RNA isolation

DNA and RNA from cell cultures were isolated using the MasterPure™Gram Positive DNA Purification Kit (Epicentre) with slight variations over manufacturer's instructions. Briefly, a cell lysis step was improved by incubating each cell suspension with 500 mg Lysozyme (Sigma, Cat #62970) and 20 U Mutanolysin (Sigma, Cat #9901) for 60 minutes at 37°C. For RNA isolation samples were incubated with 2 U DNase I (Epicentre) at 37°C for 60 minutes instead of the RNase A treatment.

Genomic DNA sequencing

B. uniformis CECT 7771 DNA was sequenced using MinION portable DNA sequencer and flowcells based on the R7.3 and R9.4 pore chemistries. The R7.3 run consisted of a DNA library prepared with the Genomic DNA Sequencing Kit SQK-MAP006 (Oxford nanopore technologies) and 6 μg genomic DNA according to manufacturer's instructions. The genomic DNA was sheared with gTUBE™ (Covaris) to get fragments of about 10Kbp, the sheared DNA was then repaired with the PreCR® Repair Mix (New England Biolabs) followed by purification using Agencourt AMPure XP beads (Beckman Coulter). DNA attached to AMPure beads was washed twice with freshly prepared 70% ethanol and magnetic rack. The DNA was eluted in 46 μL nuclease-free water and quantified by using Qubit 3.0 fluorometer and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

One microgram of repaired DNA was further processed using the NEBNext Ultra II End Repair/dA-Tailing Module (New England Biolabs) followed by a new round of washing in magnetic rack as previously done. The DNA was eluted from magnetic beads using 38 μL nuclease-free water and adapter ligation step was conducted by adding 10 uL Adapter Mix, 2 uL HP adapter, and 50 uL Blunt/TA Master Mix (New England Biolabs) mixing by inversion between each sequential addition. After 15 min incubation at room temperature 1 μL HP Tether was added to ligation reaction and incubation was extended for 10 min.

The adapter-ligated genomic DNA was recovered with Dynabeads® MyOne Streptavidin C1 beads (Thermo Fisher Scientific) and a magnetic rack, and Elution Buffer (SQK-MAP006 kit). Approximately, 205 ng DNA library were recovered and they were loaded into a brand new, sealed R7.3 flowcell

previously fitted to the MinION™ and primed twice with 71 μL premixed nuclease free water, 75 μL 2x running buffer, and 4 μL fuel mix.

The initial sequencing mix was prepared with 56 μL nuclease-free water, 75 μL 2X running buffer, 4 μL fuel mix, and 15 μL DNA library (~ 120 ng). A standard 48-h sequencing protocol was initiated using MinKNOW™ v0.50.2.15. The base-calling was performed through data transference using the Metrichor™ agent v2.36.2 and 2D Basecalling workflow v1.62. During the sequencing run, the remained aliquot of DNA library was loaded in equally conditions after 24h of initial input. The R9.4 run consisted of a DNA library prepared with the 1D Genomic DNA Sequencing Kit SQK-LSK108 (Oxford nanopore technologies) and 3 μg genomic DNA according to manufacturer's instructions. Briefly, the DNA repair kit used was NEBNext FFPE RepairMix (New England Biolabs) and the final DNA library purification was performed with AMPure beads instead Dynabeads®. The sequencing mix was obtained by combining 37.5 μL running buffer, 25.5 μL LLB beads, and 12 μL DNA library (~ 240 ng) and loaded into the spot-on port of R9.4 flowcell previously mounted into aMinION™ MkIb sequencer. A standard 48-h sequencing protocol was initiated using MinKNOW™ v1.1.17. The base-calling was performed through data transference using the Metrichor™ agent v2.43.1 and 1D BasecallingFLO-MIN106 450bs workflow v1.121. During the sequencing run, the remained aliquot of DNA library was loaded in equally conditions after 12h of initial input. This sequencing run was extended for 24h in sum.

RNA sequencing

The total RNA obtained was quantified by using the Qubit 3.0 fluorometer and Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific). Thus, thirty micrograms total RNA were obtained by pooling respective replicates of different culture conditions in equimolar quantities (10 μg per replicate). RNA pools were sent to Eurofins Genomics GmbH (Ebersberg, Germany) to produce cDNA libraries with an insert size of ~ 400 bp with prior rRNA depletion using RiboZeroTM Magnetic Kit Gram-Negative Bacteria (Epicentre). The six cDNA libraries (glucose, WBE, pectin, inulin, gumarabic, and mucin) were pooled and sequenced in one Illumina HiSeq2500 channel with chemistry v4 and configuration 2x125 paired-end reads.

Data analysis

Quality assessment of fast5 files and conversion to fastq and fasta formats was performed using the poRe (Watson *et al*., 2014) package v0.17. The *B. uniformis* CECT 7771 genome assembly was performed with 2D reads from R7.3 chemistry (32,764 reads), the high quality set of 1D reads from R9.4 chemistry (10,598 reads), and the Canu assembler v1.3 (Koren *et al.*, 2017) with options *genomeSize*=4.5m, *minReadLength*=500, *corMinCoverage*=5. A genome sequence refinement was performed with the RNAseq data derived from Illumina HiSeq2500 consisting of approximately 275 million paired-end reads, which were mapped against the draft genome of *B. uniformis* CECT 7771 using *bowtie2* (Langmead and Salzberg, 2012) for aligning and *samtools* v1.3.1 (Li *et al*., 2009) set of algorithms for indexing, sorting, and pileup of mapped reads. A consensus sequence was recovered and further processed to allow super-scaffolding. This last step was completed by performing *de novo* transcriptome assembly using the entire set of RNAseq paired-end reads and the *velvet* v1.2.10 and *oases* v0.2.09 assemblers using 63 as k-mer parameter (Zerbino and Birney, 2008; Schulz *et al*., 2012). Thus, the entire set of contigs assembled (31,576 in total) were mapped against the draft genome of *B. uniformis* CECT 7771 obtaining a new and more refined consensus sequence. Finally, a blast-based comparison among refined scaffolds of *B. uniformis* CECT 7771 was carried out to detect strong similarity among edges. This information was used to perform manual assembly of the 11 initial scaffolds generated from Canu into 6 superscaffolds. The genome assembly of the *B. uniformis* CECT 7771 was submitted to the European Nucleotide Archive (ENA) where it is publicly available under primary accession number PRJEB19372 (ENA WGS accession: FZQS01000001-FZQS01000006).

Comparative genomics against the *B. uniformis* ATCC 8492 (ENA WGS accession: AAYH02000000), *B. uniformis* CL03T00C23 (ENA WGS accession: AGXY01000000), *B. uniformis* dnLKV2 (ENA WGS accession: ASSO01000000), and *B. uniformis* 3978-T3i (ENA WGS accession: JNHO01000000) was assessed with alignment algorithms implemented in Mauve v2.3.1 (Darling *et al.*, 2010) and BRIG (Alikhan *et al*., 2011). Furthermore, genome-wide average nucleotide identity approach implemented in JSpecies v1.2.1 (Richter and Rossello-Mora, 2009) was used to reconstruct phylogenetic relationships among *B. uniformis* species and other *Bacteroides* such as *Bacteroides vulgatus* ATCC 8482 (ENA accession: CP000139.1), *Bacteroides fragilis* NCTC 9343 (ENA accession: CR626927.1), *Bacteroides thetaiotaomicron* VPI 5482 (ENA accession: AE015928.1), *Bacteroides caccae* ATCC 43185 (ENA WGS

accession: AAVM02000000), and *Bacteroides stercoris* ATCC 43183 (ENA WGS accession: ABFZ02000000). Values from ANIm and tetranucleotide distribution were used to reconstruct a UPGMA dendrogram (http://genomes.urv.cat/UPGMA) using the RSMD distance coefficient and the iTOL web server (Letunic and Bork, 2016) was employed to draw the respective rooted trees.

Gene prediction and functional annotation were achieved by using Prodigal v2.6 (Hyatt *et al*., 2010), tRNAscan v1.4 (Lowe and Eddy, 1997), RNAmmer v1.2 (Lagesen *et al*., 2007), CRISPR Recognition Tool (CRT) (Bland *et al*., 2007), KEGG Automatic Annotation system (Moriya *et al*., 2007), CAZy database (Lombard *et al*., 2014), SMART database (Letunic *et al*., 2014), and CAT server (Park *et al*., 2010). Venn diagrams were designed in *jvenn* server (Bardou *et al*., 2014).

Assessment for presence of virulence factors, antibiotic resistance genes, and toxins was done by executing local blast to compare all the ORFs predicted against the annotated proteins present in the VFDB (Chen *et al*., 2016), MvirDB (Zhou *et al.*, 2007), and ARDB (Liu and Pop, 2009) databases.

Processing of RNAseq data was done according to previous studies (Benitez-Paez *et al*., 2016a). Briefly, the quality filtering and trimming was performed using FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Read mapping was assisted by Blast algorithm (Altschul *et al*., 1990) and selecting alignments >50% of read length (>70 nt) and 100% identity. Read counts were normalized using RPKM and a genome-wide exploratory differential expression among transcriptomes generated from usage of different carbon sources was measured with GFOLD (Feng *et al*., 2012) using glucose-derived expression as baseline feature and followed by further evaluation with qPCR methods. In order to increase the stringency for detecting more probable signals of differential expression, we only selected genes with GFOLD score ≤-1.5 or ≥1.5 (log_2 fold-change) despite than any gene with GFOLD score different than zero would be indicative of up- or down-regulation. Sequence information supporting the six *B. uniformis* CECT 7771 transcriptome analyses was submitted to the ENA where it is publicly available under primary accession number PRJEB19372. Hierarchical clustering of genes differentially expressed in different culture conditions was achieved by using euclidean distance and average linkage methods (Metsalu and Vilo, 2015).

Quantitative PCR

The genes BUNIF7771_0387, BUNIF7771_0544, BUNIF7771_0548, BUNIF7771_1668, BUNIF7771_1883, BUNIF7771_3473, BUNIF7771_3732, and BUNIF7771_4131 were selected from the exploratory RNAseq analysis to assess specific changes in expression by qPCR. The gene-specific oligonucleotides used for this aim are presented in the Supplementary Table 1.

The cDNA was synthesized using 5µg of total and non-pooled RNA remaining from that used for the RNAseq approach (three replicates per treatment), and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The qPCR reactions were set in 96-well plates using the SYBR Green I Master Mix (Roche Lifesciences), 0.5 µM of forward oligonucleotide, 0.25 µM of reverse oligonucleotide, and 1 µL of the cDNA reaction. All treatment samples were set in triplicate in the plate and amplified in a LightCycler 480 II with the following cycling profile: initial incubation at 95° for 5 min and 40 cycles of 10 s at 95°, 20 s at 63°, and 15 s at 72°. Finally, the melting curve was set from 65 to 97° with a ramp rate of 0.11°/s.

The expression level for each gene was measure according to the ΔΔCt method, using the expression of the 16S rRNA gene as calibrator, and expression of glucose samples as reference. RQ values were finally obtained with calculation of $2^{-\Delta\Delta Ct}$ for all samples and replicates. Differential expression was assessed by the one-sided t-test with Welch's correction supporting pairwise comparisons between gene expression under glucose and remaining treatments.

The copy number of the pBU7771 extrachromosomal element was calculated by absolute quantification using the primers pBU7771-F and pBU7771-R (Supplementary Table 1). The single-stranded DNA (ssDNA), fully covering the region to be amplified (111 nt) was obtained from Isogen Life Science B.V (Utrecht, The Netherlands) where it was synthesized, PAGE-purified, and quantified, and used in molecule titration during qPCR. The number of plasmid molecules per ng DNA was obtained and divided by theoretical number of *B. uniformis* CECT 7771 genomes presented in 1 ng DNA (5.16Mb) (http://cels.uri.edu/gsc/cndna.html), this ratio was used to infer the number of plasmids per cell.

Metabolite quantification

The gamma-amino butyric acid (GABA) concentration in supernatants obtained from glucose, mucin and pectin cultures was measured by LC/MS approach. Briefly, 150 μL of the supernatants collected at $OD_{600} \sim 0.4$ were send to the Central Service for Experimental Research (SCSIE) at the University of Valencia. The GABA (Sigma, Cat#A2129) standards were prepared in milliQ grade water. Both the standards and samples were processed for protein precipitation by diluting 1:4 with acetonitrile in 200 μL reaction volume, then samples were centrifuged at 10,000 x *g* for 10 minutes and 50 μL of supernatant were loaded in the ACQUITY® TQD (Waters Corporation. Milford, MS, USA) LC instrument coupled to an electrospray ionization source (ESI). GABA separation was achieved in a HILIC Kinetex column (Phenomenex®) using 0.1% formic acid:75% acetonitrile at 23ºC. The area under curve (AUC) values of respective standards at 1 mM, 0.1 mM, 0.01 mM, and 0.001 mM GABA were used to calculate GABA concentration in glucose, mucin, and pectin supernatants. Statistical analysis was assessed by comparing values obtained from three independent supernatant replicates using one-way ANOVA with pairwise comparisons and bonferroni correction.

Results

The genome of Bacteroides uniformis CECT 7771

Following an extensive sequence analysis of the genomic information retrieved from a third generation sequencing platform, the MinION™, we could initially assemble the *B. uniformis* CECT 7771 genome which consisted of ~ 5.16Mbp with a N50 of 1.32Mbp arranged in 11 scaffolds. The MinION™ data is characterized by producing error-prone DNA reads with a per-base accuracy above 85% in 2D reads (Ip et al., 2015), therefore we assumed further and hybrid approaches to get genome sequence refinement. After refinement steps with RNAseq data, using individual reads and assembled into longer transcript contigs, derived from six different experiments to assess the transcriptional profile under different carbon sources, we obtained a high quality genome assembly consisting of 5.16 Mpb with a N50 2.44Mbp and arranged in 5 super-scaffolds representing the chromosomal genetic information of *B. uniformis* CECT 7771. This genome information was submitted to the European Nucleotide Archive (ENA) and can be accessed with identifiers FZQS01000001 to FZQS01000005.

Additionally, we found an extra-chromosomal element with 2,746bp in length that seems to encode neither virulence nor antibiotic resistance genes (ENA accession FZQS01000006). By absolute quantification approaches we found the pBU7771 plasmid is present in very-low copy number reaching a proportion of 1.86 plasmids per cell.

We have predicted that in the current version of the *B. uniformis* CECT 7771 genome there are 5,226 ORFs, 4 ribosomal RNA operons, and 67 tRNAs encoded. Moreover, we found one CRISPR region characterized to have 11 repeats consisting of the following DNA sequence: GTTGTGATTTGCTTTCATTTTAGTATCTTTGAACCATTGGAAACAGC and 10 spacers with an average length of 30nt. This last feature was confirmed by the fact that the downstream of this CRISPR region are contiguously located the BUNIF7771_0521 and BUNIF7771_0522 ORFs encoding the Cas2 and Cas1 CRISPR-associated endonucleases, respectively. Ribosomal RNA sequence information of *B. uniformis* CECT 7771 is publicly available in the ENA under accession numbers LT745888, LT745889, and LT745890 for 5S, 16S, and 23S molecules, respectively. An initial comparison with other genome assemblies from *B. uniformis* strains is presented in Table 1.

Table 1.Genome assembly comparison among different strains of *B. uniformis*.

Feature	*B. uniformis* CECT 7771	*B. uniformis* ATCC 8492	*B. uniformis* CL03T003C23	*B. uniformis* 3978-T3i	*B. uniformis* dnLKV2
Sequencing platform	MinION™ HiSeq 2500	454 GS20	HiSeq2000	HiSeq2500	HiSeq2000
Assembly length	5.16Mbp	4.72Mbp	4.96Mbp	5.05Mbp	4.84Mpb
Scaffolds	6	33	8	47	11
N50	2.44Mbp	0.29Mbp	4.92Mbp	1.01Mbp	0.95Mbp
Plasmids	1	0	0	0	0
RNA ribosomal operons[1]	4	4[2]	4	6	3
GC%	46.7	46.4	45.9	46.4.	46.3

The assembly statistics for *B. uniformis* ATCC 8492, CL03T003C23, 3978-T3i, and dnLKV2 strains were recovered from information available at ENA for respective assembly accessions (see methods).

[1] Number of RNA operons estimated through analysis of respective assemblies with RNAmmer v1.2.

[2] Calculated from the maximum number of 16*S* and 5S rRNA genes found given that only one 23S rRNA gene was found.

The genome assembly presented in this study fully fits the features observed for previously sequenced *B. uniformis* strains. Notably, the draft genome of *B. uniformis* CECT 7771 share similar GC content with its counterparts being 46.7% and constitute the largest genome assembly of any *B. uniformis* published with the lowest number of scaffolds reconstructed. In this regard, it seems that the hybrid assembly strategy based on long read sequencing approach followed by refinement with massive short read data resulted in a valid methodology to sequencing the *B. uniformis* CECT 7771 genome retrieving more sequence information useful to distinguish interesting features of *B. uniformis* strains. Among such features we highlighted the presence of 4 complete ribosomal RNA operons whose number was not consensual from previous assemblies. Besides, we reported for the first time the presence of a plasmid element in a *B. uniformis* strain of

2,746bp in length. Using this element as query in a Blast searching against the NCBI's non-redundant nucleotide database we observed that this element was commonly found in other *Bacteroides* species consisting of an extrachromosomal DNA element that was also recovered from metamobilome studies in rat cecum (Jorgensen *et al*., 2014) with a high sequence similarity to a plasmid element borne by *B. fragilis* IB143 (Smith *et a*l., 1995). Notwithstanding, none of the three ORFs predicted to be encoded in this element match with any antibiotic resistance, virulence, or pathogenic gene annotated in the ARDB, MvirDB, and VFDB databases.

Comparative genomics approaches based on whole-genome alignment were used to identify distinctive features of *B. uniformis* CECT 7771 compared to other strains belonging to the same and to other species. The average nucleotide identity and tetranucleotide distribution across the genome indicated that *B. uniformis* CECT appears to be very related to other *B. uniformis* strains but its genomic structure also differs from that of other *Bacteroides* species (Figure 1 and Supplementary Table 2).A more detailed comparison among different *B. uniformis* strains has led to identify genomic regions exclusively present in the genome of *B. uniformis*CECT 7771, named specificity regions hereinafter (SR1 to SR5, Figure 2). Despite the low level of functional annotation recovered when ORFs encoded into the major SR regions were analyzed by KAAS server (Moriya *et al*., 2007), we could identify interesting features such as presence of endopeptidases (SR1 and SR3), DNA methylases (SR1, SR2, and SR3), a type-IV restriction endonuclease (SR3), a two-component system (SR1), and polysaccharide metabolism associated enzymes (SR2) for importing, degradation and synthesis.

The members of the phylum Bacteroidetes are well known components of the gut microbiota thriving in environments enriched in glycans. The *sus* genes, an archetypal locus of membrane proteins for binding and importing polysaccharides in Bacteroidetes species, are fundamental part of the PULs. The number of these loci specialized in glycan degradation in *Bacteroides* spp. and *Prevotella* spp. species outnumbers the number of PULs found in species of other phyla such as Firmicutes (White *et al*., 2014).

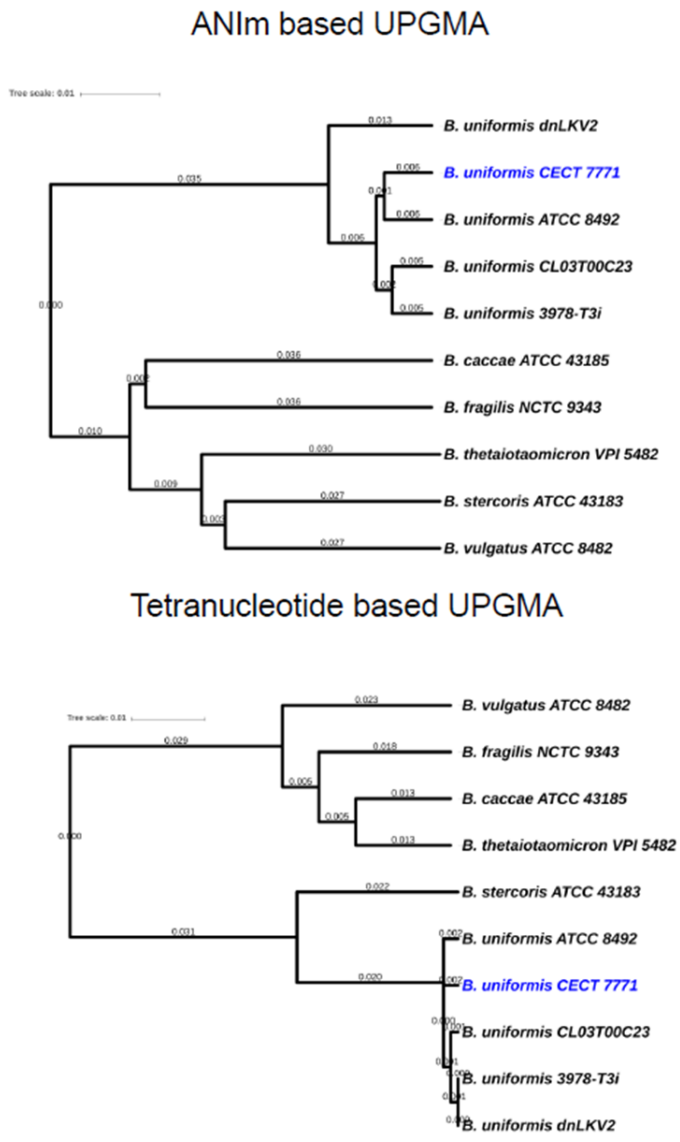

Figure 1. Phylogenetic relationships among *Bacteroides* species. The genome information of all species and strains presented in the figure was used for comparative analysis using algorithms implemented in the Jspecies tool (Richter and Rossello-Mora, 2009). Genetic distance based on the Average Nucleotide Identity is presented in the left UPGMA dendrogram. The relationships based on tetranucleotide distribution is presented in the UPGMA right dendrogram. Branch lengths are based on the RSMD distance coefficient.

Therefore, we wanted to survey the number and classes of enzymes involved in binding, degradation, modification, and synthesis of glycans in the *B. uniformis* CECT 7771 genome. Using the CAT server (Park *et al*., 2010), we retrieved annotation of ORFs encoding enzymes homologues to reference genes present in the CAZy database (Lombard *et al.*, 2014). A first comparison was accomplished at strain level using the genome information from *B. uniformis* strains listed in Table 1.

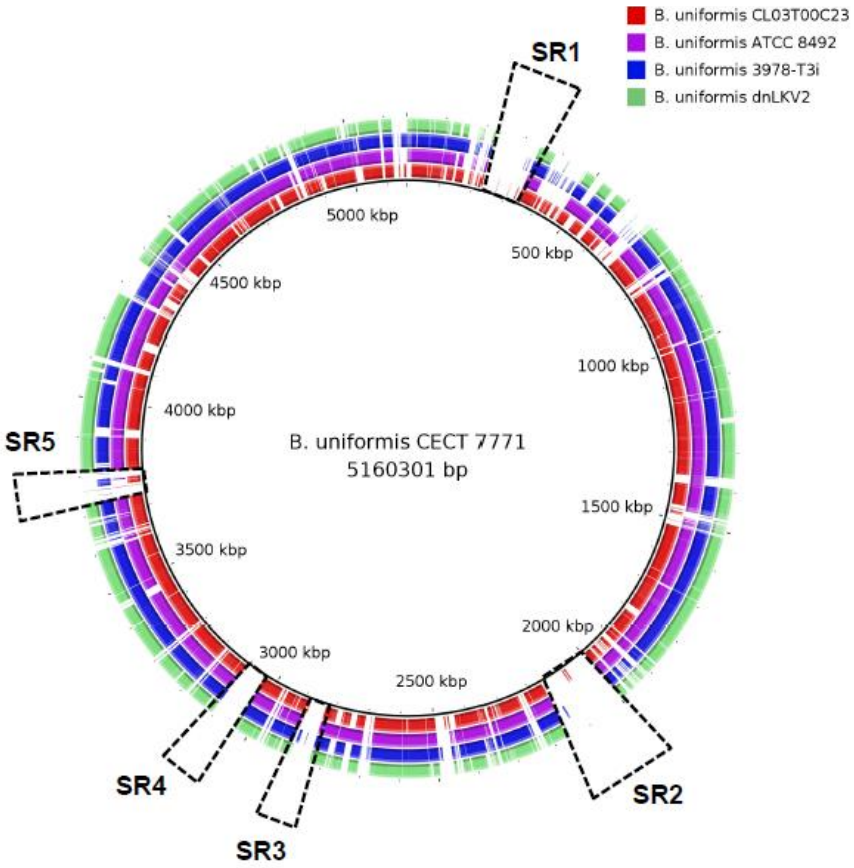

Figure 2. Comparative analysis of *Bacteroides uniformis* strains. Circular representation of *B. uniformis* CECT 7771 (black inner line), *B. unformis* CL03T00C23, (red), *B. unformis* ATCC 8495 (purple), *B. unformis* 3978-T3i(blue), and *B. unformis* dnLKV2 (green) genomes. They are compared using whole-genome and blast-based alignment. Genomic regions exclusively found in*B. uniformis* CECT 7771 are highlighted with dashed rectangles.

Consequently, we have recovered a total of 622, 626, 605, 624, and 671 CAZy genes for *B. uniformis* CECT 7771, *B. uniformis* ATCC 8492, *B. uniformis* CL03T00C23, *B uniformis* dnLKV2, and *B. uniformis* 3978-T3i, respectively. Those genes were grouped into non-redundant CAZy family domains, then families were split in case of multi-domain proteins, and finally compared via Venn diagrams to disclose function uniqueness in every single strain (Figure 3A). We found similar number of families in all strains and the assembled *B. uniformis* CECT 7771 genome encodes 115 different CAZy functional familes whereas other strains encode up to 120. Interestingly, the five strains share in average 88% (103 / 117) of CAZy families and up to 90% (122/135) in case of pairwise comparisons

When strain-specific functions were explored, we found that all strains showed unique features encoded in their respective chromosomes (Supplementary Table 3). Although the glycosidehydrolase (GH) function was predominantly recovered in a set of unique genes in each strain, the presence of glycosyl transferase (GT) activities was only observed in *B. uniformis* CECT 7771 and 3978-T3i strains. The GT catalyzes the transference of sugar moieties from an activated donor to a specific substrate in an enzyme-dependent manner. In addition to the GT genes exclusively present in the genomes of strains of *B. uniformis*, the strains the *B. uniformis* CECT 7771 and 3978-T3i strains exhibited the largest number of GT enzyme encoded genes (30) when compared with counterparts (25 genes per strain on average). Therefore, the gain of GT genes would be indicative of the higher capability of these strains to synthesizing disaccharides, oligosaccharides, polysaccharides, and even glycolipids. Accordingly, *B. uniformis* CECT 7771 could represent an important and unexplored reservoir of GT functions for production of natural compounds with an ample range of biotechnology applications.

On the other hand, comparisons at species level were done using similar analysis as the previous one but with the genomic information of the annotated genes in the CAZy database and available for *Bacteroides* species such as: *B. xylanisolvens* XB1A, *B. cellulosilyticus* WH2, *B. thetaiotaomicron* VPI5482, *B. vulgatus* ATCC8482, and *B. fragilis* NCTC9343. Strikingly, the number of CAZy genes encoded in other *Bacteroides* species is lower than detected in *B. uniformis* strains (Figure 3B). The above observation reveals a new degree of specialization of *B. uniformis* strains that outnumbers the CAZy genes found in other *Bacteroides* species even in *B. thetaiotaomicron,* the archetype for characterization of PULs and *B. cellulosilyticus* previously

described to encode a expanded glycolytic potential (McNulty *et al.*, 2013). In the same line of though, almost the 23% (27/115) of *B. uniformis* CECT 7771 CAZy genes cannot be identified in other species. Moreover, the number of CAZy genes present in *Bacteroides* spp. genomes seem to be indicative of their role as commensals or potential pathogens since the number of these genes found in *B. vulgatus* and *B. fragilis*, considered as pathobionts (Wexler, 2007), are abnormally lower than in other species, being particularly notable in case of *B. fragilis* with almost half of genes found in *B. uniformis* strains.

Finally, a searching for potential antibiotic resistance and virulence genes was completed by comparing the full set of ORFs predicted to be encoded in the *B. uniformis* CECT 7771 genome according to the proteins annotated in the ARDB, MvirDB, and VFDB databases. We detected presence of a β-lactamase protein encoded in the ORF BUNIF7771_3570 (99% sequence identity against the AAA66962 ARDB entry) and presence of the TetQ protein encoded in the ORF BUNIF7771_4507 (90% sequence identity against the AAS83507 ARDB entry), both proteins with homologues in other *B. uniformis* genomes analyzed in above sections. An additional KEGG based functional analysis of ORFs predicted to be encoded by the *B. uniformis* CECT 7771 genome has revealed the presence of a total of 24 genes corresponding to 11 different multidrug efflux pumps, one D-alanyl-D-alanine dipeptidase (*vanX,* K08641) associated with vancomycin resistance, and a couple of genes associated with cationic antimicrobial peptide resistance.

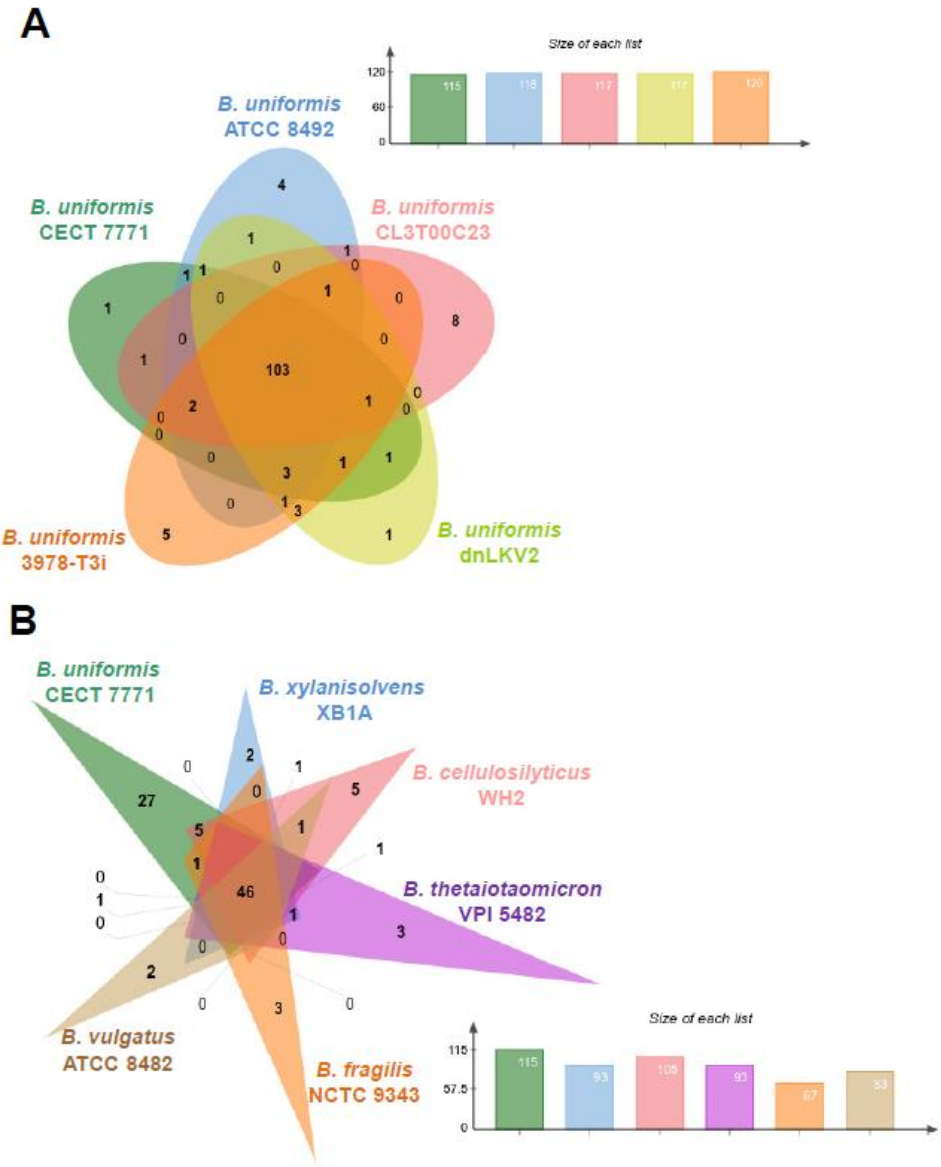

Figure 3. *B. uniformis* CECT 7771 and carbohydrate metabolism. (A) Venn diagram showing the strain-specific and shared CAZy families among five *B. uniformis* strains. (B) Venn diagram showing the species-specific and shared CAZy families among six *Bacteroides* species. The number of non-redundant CAZy familes present in respective genomes is showed below respective Venn diagrams following the color nomenclature.

B. uniformis CECT 7771 growth fitness depending on the carbon source utilization

In the light of the wide repertoire of genes dedicated to polysaccharide degradation identified in *B. uniformis* CECT 7771 genome, we wanted to investigate those expressed in response to complex carbon sources that can be present in the intestinal tract (GIT) such as mucin, a heavily glycosylated secretion product of the intestine epithelial cells.

To do so, the ability of *B. uniformis* CECT 7771 to grow in the presence of glucose, gum arabic, WBE, inulin, mucin, or pectin as primary carbon source was evaluated. Growth patterns differed (Figure 4A) essentially regarding the the log phase and the yield at stationary phase. To quantify the ability to growth in the different carbon sources we also calculated the doubling time during the exponential growth phase (Figure 4B). In general, we observed that all carbon sources except WBE increase the doubling time compared to glucose; this effect was especially significant for gum arabic ($p < 0.022$). Notably, WBE showed no fitness cost and, conversely, this carbon sourceboosted the *B. uniformis* CECT 7771 growth reducing the doubling time from 91 to 62 min ($p < 0.057$).The opposite effects of gum arabic and WBE on growth fitness could be explained by their composition, whereas gum arabic consist of a complex mixture of branched polymers of galactose (39-42%), rhamnose (12-16%), arabinose (24-27%), and glucuronic acid (15-16%) (Ali et al., 2009), the WBE is principally composed of AXOS (>69%), which are a mixture of low molecular weight xylo- and arabinoxylo-oligosaccharides (Cargill, Antwerp, Belgium). The complex polysaccharides contained in gum arabic, inulin, and pectin seem to be more difficult to be fermented by *B. uniformis* CECT 7771 probably by lacking the proper de-branching enzymes useful in the first hydrolytic stages to oligosaccharide level. Therefore, *B. uniformis* CECT 7771 is more specialized in the utilization of oligosaccharides than in very complex carbohydrates, which could also be related to the strain origin (isolated from stools of a breast-fed infant).

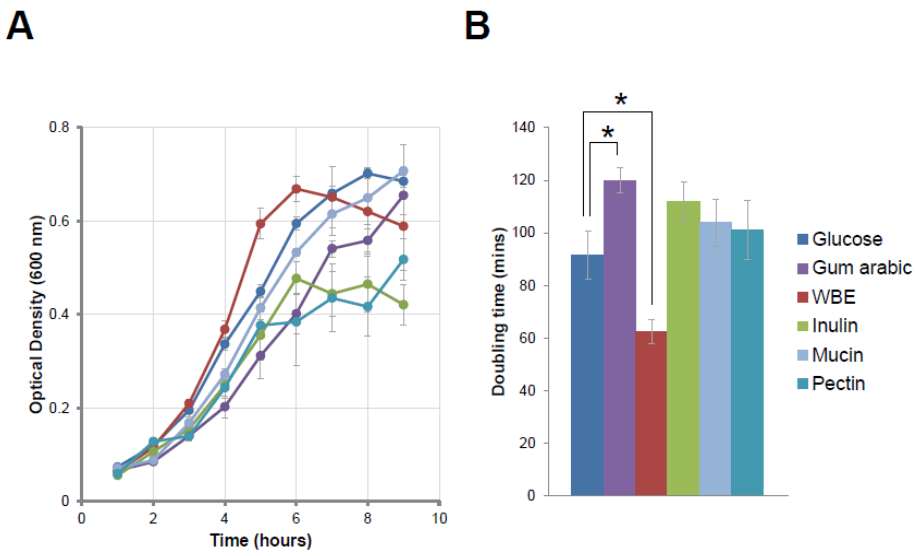

Figure 4. Growth fitness of *B. uniformis* CECTC 7771 in different carbon sources. (A) Growth curve comparison between *B. uniformis* CECT 7771 cultures using glucose (blue line), gum arabic (purple), WBE (red), inulin (green), pectin (baby blue), or mucin (blue-green) as carbon source. Growth was monitored measuring OD600 at 60 min intervals. The OD600 values are presented as a mean of three independent replicates (±SEM). (B) Doubling times calculated during the exponential growth phase by linear regression. The values are present as a mean of three independent replicates (±SEM). The stars indicate differential growth rates when compared to glucose used as reference ($p < 0.022$ for gum arabic and $p < 0.057$ for WBE) supported on pairwise *t*-test comparison with Welch's correction.

Genome response of B. uniformis CECT 7771to different carbon sources

With the aim to complement the study above, we also did a genome-wide expression analysis in the presence of gum arabic, WBE, inulin, mucin, and pectin. An exploratory expression analysis was done using a RNAseq approach with RNA pools from respective replicates obtained in different and independent experiments. Using comparative expression algorithms implemented in GFOLD (Feng *et al.*, 2012) we could discern a set of genes more probable to be differentially regulated under the respective conditions.

By setting an increased threshold for selection (see methods) we obtained a total of 633 genes with a high probability to be differentially expressed among all conditions (Figure 5A).

Interestingly, the gum arabic and mucin seem to induce and attenuate the expression of a larger set of genes in *B. uniformis* CECT 7771. Moreover, both carbon sources similarly modify the expression of 152 out of 633 (24%) genes differentially expressed in all conditions indicating they would induce a similar response. This is consistent with what graphically retrieved when a hierarchical clustering approach was used to study the particular gene expression patterns for the 633 genes with plausible differential expression (Figure 5B). Globally, the exposure of *B. uniformis* CECT 7771 to gum arabic and mucin appears to have a deep impact generating more transcriptional activity across its genome conversely to what observed in the response to WBE and inulin, which trend to trigger more concrete expression signals confined to a few and well-delimited gene clusters (Supplementary Figure 1).

Gum arabic. The exploratory gene expression pattern derived from gum arabic fermentation suggest that 220 genes are significantly up-regulated whereas only 30 are down-regulated. The functional annotation of respective set of genes, using the KEGG Modules, suggests that gum arabic triggers a higher expression of genes involved in central metabolic functions such as reductive pentose phosphate and citrate cycles (M00167 and M00173, respectively). Additionally, gum arabic would induce over-expression of a wide variety of genes involved in starch and sucrose metabolism (ko:00500 pathway) like endoglucanases (K01179), glucosidases (K01182, K05349), and debranching enzymes (K01196). Notwithstanding, other glycan metabolic pathways (e.g. galactose - ko:00052, fructose and mannose - ko:00051, and other glycan degradation - ko:00511) seem to be also up-regulated by the significantly higher number of reads mapped against galactosidase (K01190, K12111), fucosidase (K15923), epimerase (K01785), mannosidase (K01218), isomerase (K01813), arabinofuranosidase (K01209), and epimerase (K01785) genes.

Importantly, we disclosed a potential over-expression of the phosphate butyryltransferase (K00634) and butyrate kinase (K00929) genes, involved in production of the short-chain fatty acid (SCFA) butyrate. As a consequence of the great number and variety of genes activated and involved in carbohydrate catabolic and anabolic pathways a wide range of amino acid metabolism pathways were seen to be probably up-regulated as well, except

for metabolic pathways involving sulfur-containing amino acids (e.g. cysteine and methionine) which appear to be down-regulated.

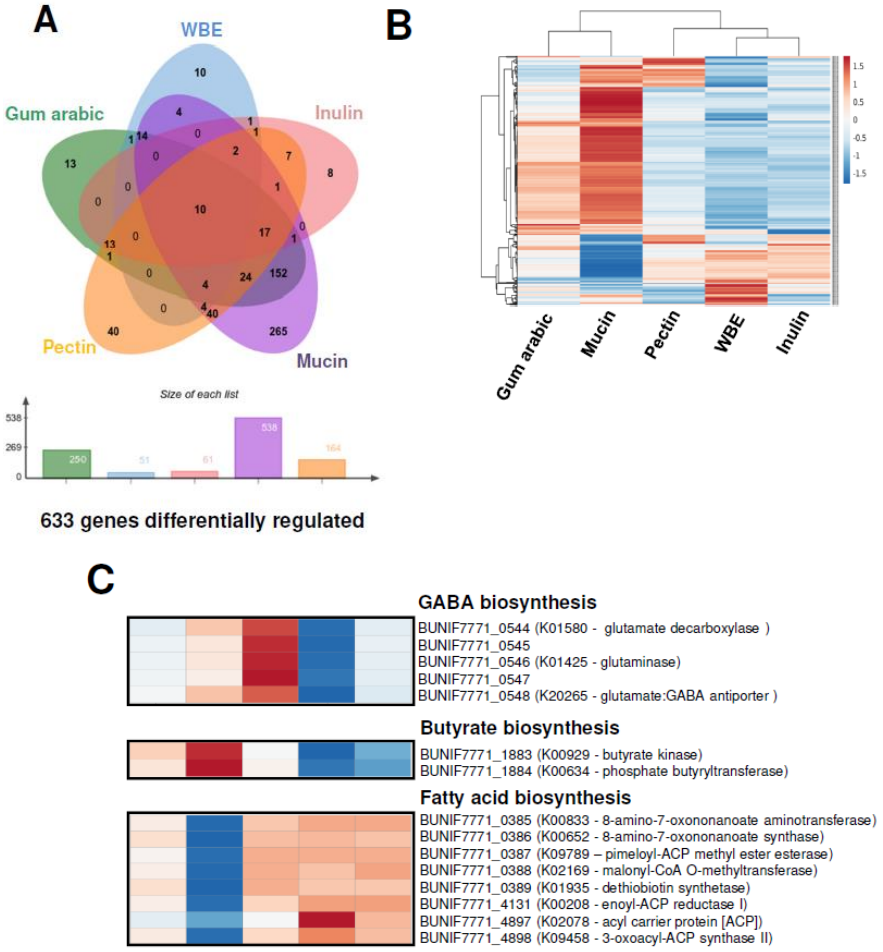

Figure 5. Expression patterns in the *B. uniformis* CECT 7771 genome. (A) Venn diagram showing the number of genes differentially expressed in *B. uniformis* CECT 7771 cultures using gum arabic, WBE, inulin, pectin, and mucin as carbon source. The expression pattern in glucose media was used as normalizer. The total number of genes showing differential expression in different conditions are depicted below the Venn diagram. (B) Heatmap with expression values obtained from the exploratory transcriptome analysis of the 633 genes showing differential expression in all condition tested. Hierarchical clustering was assessed for the treatments and genes using euclidean distance and average linkage methods implemented in ClustVistweb server.

Globally, it seems that sulfur relay system is weakened with gum arabic given that expression of other important enzymes for this process as the tRNA-uridine 2-sulfurtransferase (K00566) and cysteine synthase A (K01738) are attenuated as well. This results could partially explain the growth phenotype observed for *B. uniformis* CECT 7771 in gum arabic given that trafficking and delivering of sulfur is indispensable for biosynthesis of Fe/S clusters in proteins, enzyme cofactors, and tRNAs (Mueller, 2006).

The ample range of functions disclosed to be boosted by gum arabic is consistent with its complex nature based on a mixture of very different branched polysaccharides. Notably, when the specific set of genes differentially expressed in gum arabic cultures (see Figure 5A, N= 13) was analyzed, from the functional point of view, we disclosed genes encoding proteins and enzymes highly related with rhamnose metabolism such as L-rhamnose-H+ transport protein (K02856), L-rhamnose isomerase (K01813), and rhamnulokinase (K00848). These results makes sense since the complex mixture of the gum arabic includes rhamnose (12-16%) (Ali *et al*., 2009) and this appears to be part of no other carbon sources employed in this study.

WBE. The expression pattern associated to WBE fermentation was the most compact and well-defined in terms of the low number of clustered genes under differential expression when compared against glucose response (see Supplementary Figure 1). As expected and given its composition, we observed trends of over-expression in genes associated with metabolism of arabinoxylans. Thus, we found that a higher number of DNA reads were mapped against xylulokinase (K00854), L-arabinose isomerase (K01804), xylose isomerase (K01805), alpha-N-arabinofuranosidase (K01209), beta-D-xylosidase (K15920), and arabinoxylanarabinofuranohydrolase (K15921) genes. By contrast, we detected that lysine biosynthesis (ko:M00527) could be attenuated by down-regulation of the diaminopimelate epimerase (K01778) and LL-diaminopimelate aminotransferase (K10206) genes. Moreover, biosynthesis of other amino acids may be also decreased given that glutamate (K00265, K00266), glutamine (K01915), and asparagine (K01953) synthases appeared to be down-regulated as well. Among the set of genes specifically associated with WBE (see Figure 5A, N= 10) we found an over-expression pattern for those encoding ABC transporter proteins (K02003, K02004, K2005), outer membrane protein (K012538), and evidently genes encoding enzymes for xylan metabolism as beta-glucosidase (K05349), xylose isomerase (K01805), and beta-D-xylosidase (K15920).

Inulin. Similarly to WBE, inulin induced differential expression in a low number of genes. The functional annotation of genes with a tendency to be up-regulated in response to inulin was scarce. In consequence, we could not distinguish any molecular functions particularly related to this carbon source, except for the orthologues K02004/K02003/K01990, and K01190 which encodes respective genes for ABC sugar transporters and beta-galactosidase proteins comprising general functions into the carbohydrate metabolic pathway. Interestingly, inulin also trends to limit the expression of genes related to glutamate (K00266), glutamine (K01915), and asparagine (K01953) biosynthesis as previously seen with WBE.

Pectin. Opposite to seen in RNAseq data derived from WBE and inulin experiments, pectin in culture medium produced significant transcriptional activity in several genes across the *B. uniformis* CECT 7771 genome. In sum, 118 genes showed a tendency to be up-regulated and 46 to be down-regulated. Over-expression signals from the 6-phosphofructokinase 1 (K00850), biphosphoglycerate mutase (K01834), and the fructose-bisphosphate aldolase (K11645) indicates that glycolysis and gluconeogenesis pathways could be even more active in presence of pectin than glucose. Likewise, the more abundant expression of genes related with galactose metabolism (ko:00052) such as the beta-galactosidase (K01190), UDP-glucose 4-epimerase, and the same 6-phosphofructokinase 1 (K00850) fits with the primary composition of the pectin consisting of galacturonic acid polymers. Other genes involved in carbohydrate metabolism whose expression would be induced by pectin include the ABC permeases and transporters (K01990, K02003, K02004), glycogen phosphorylase (K00688), alpha-N-arabinofuranosidase (K01209), UDP-N-acetylglucosamine 4,6-dehydratase (K15894), glycosyltransferase EpsJ (K19427), and the genes encoding enzymes involving in polysaccharide biosynthesis as the heptose III glucuronosyltransferase (K019354) and Fuc2NAc and GlcNAc transferase (K13007).

Remarkably, pectin would also activate the butanoate metabolism (ko:00650) by over-expression of the phosphate butyryltransferase (K00634), butyrate kinase (K00929), and glutamate decarboxylase (K01580) genes involved in production of pivotal metabolites for human colon function as butyrate and GABA (Bourassa *et al.*, 2016; Mazzoli and Pessione, 2016).

The over-expression pattern observed for the glutamate:GABA antiporter (K20265) would be a further indication that pectin does induce production

and releasing of GABA to the extracellular media by *B. uniformis* CECT 7771. The genes encoding the glycosyltransferase EpsJ, Fuc2NAc and GlcNAc transferase and the glutamate decarboxylase and glutamate:GABA antiporter appear to be specifically involved in the response to pectin fermentation by *B. uniformis* CECT 7771.

On the other hand, signals for gene expression attenuation would affect predominantly the alanine, aspartate and glutamate (ko:00250) and nitrogen (ko:00910) metabolism as well as arginine (ko:00220) and lysine (ko:00300) biosynthesis by down-regulation of the glutamate synthase (NADPH/NADH) large chain (K00265), glutamate synthase (NADPH/NADH) small chain (K00266), aspartate-ammonia ligase (K01914), glutamine synthetase (K01915), asparagine synthase (K01953), diaminopimelate epimerase (K01778) and LL-diaminopimelate aminotransferase (K10206) genes.

Mucin. The expression patterns observed in the *B. uniformis* CECT 7771 genome as a consequence of the mucin glycan utilization comprised up-regulation and down-regulation of 423 and 115 genes, respectively. As a result, most of the genes positively and negatively regulated by mucin appear to be specific to this condition as well as the metabolic pathways potentially altered and regarding this expression pattern.

By far, this was the most variable and complex gene expression response when compared with gum arabic, WBE, inulin, and pectin. Functional categorization of genes with a trend to be over-expressed include genes involved in glycolysis and galactose degradation as well as other genes involved in keratan sulfate, chondroitin sulfate, heparan sulfate, dermatan sulfate, D-galacturone, and D-glucuronate degradation. The genes globally up-regulated and involved in molecular pathways like the amino sugar and nucleotide sugar metabolism (ko:00520), the starch and sucrose metabolism (ko:00500), the galactose metabolism (ko:00052), the fructose and mannose metabolism (ko:00051), and the glycosaminoglycan degradation (ko:00531), and representing the so called PULs (Polysaccharide Utilization Loci) of *B. uniformis* CECT 7771 for mucin glycan degradation are listed in Supplementary Table 4.

Similarly to that observed in gum arabic and pectin cultures, the amino acid metabolism is tightly connected with sugar degradation pathways, therefore, we also detected up-regulation of genes involved in glycine-serine-threonine (ko:00260), alanine-aspartate-glutamate (ko:00250), phenyalanine

(ko:00360), tryptophan (ko:00380), and arginine (ko:00220) metabolism. Analogously to that found in gum arabic and pectin cultures, mucin glycans induced the highest increased expression signal for genes involved in butyrate production (K00929, K00634) (see Figure 5C).

Outstandingly, the genes with a down-regulation pattern of expression indicate that biosynthesis of long-chain fatty acids is mainly affected (Figure 5C). Consequently, the genes involved in the biotin (K00652, K00833, K01935), acyl-CoA (K01897), and pimeloyl-ACP biosynthesis (K02169, K09789) as well as the *fabD* (K00645), *fabF* (K09458), and *fabI* (K00208) orthologues associated with the initiation an elongation of fatty acids were determined to have a lower expression level in mucin than glucose media. Additionally, lysine and branched-chain amino acids biosynthesis seem to be also attenuated by degradation of mucin glycans. Globally, the nitrogen metabolism appear to be also constrained given the lower expression dedicate to glutamate-glutamine (K00262, K00265, K00266, K01915) and ornithine biosynthesis (K00145, K00821).

Altogether, the expression signals observed indicate that multiple pathways of carbohydrate degradation are activated in *B. uniformis* CECT 7771, which could be explained by the great variety of oligosaccharides attached to mucus proteins. Therefore, is plausible to assign a mucin degrader role to *B. uniformis* CECT 7771 that seems to be further supported by the up-regulation of the orthologue *clpB* encoding the ATP-dependent Clp protease (K03695).

qPCR validation of differential gene expression

Despite the consistency among the metabolic functions up- and down-regulated depending on the carbon source used to grow *B. uniformis* CECT 7771, we validated the changes in expression of a set of 8 genes by qPCR, using replicated and non-pooled total RNA samples remaining from the exploratory whole-transcriptome experiment. These genes were selected for their possible role in the host physiology. In particular we analyzed the relative expression of following genes: BUNIF7771_0544 (K01580 - glutamate decarboxylase), BUNIF7771_0548 (K20265 - glutamate:GABA antiporter), BUNIF7771_1883 (K00929 - butyrate kinase), BUNIF7771_0387 (K09789 – pimeloyl-ACP methyl ester esterase), BUNIF7771_3732 (K01915 - glutamine synthase), BUNIF7771_4131 (K00208 - enoyl-ACP reductase I), BUNIF7771_1668 (K01813 - L-rhamnose isomerase), and BUNIF7771_3473

(K01805 - xylose isomerase). When we compared the respective and relative gene expression to that found in the presence of glucose, we corroborated almost all the gene expression patterns inferred from the transcriptome approach (Supplementary Figure 2).

GABA production by B. uniformis CECT 7771

Once we corroborated the expression pattern observed for *B. uniformis* CECT 7771 genes associated with GABA production by qPCR, we further analyzed the GABA concentration in culture supernatants from mucin and pectin conditions that showed a clear over-expression of glutamate decarboxylase (BUNIF7771_0544) and glutamate:GABA antiporter (BUNIF7771_0544) genes (Supplementary Figure 2). When compared the respective concentrations of GABA, determined by a LC/MS approach, we observed that mucin and pectin altered the GABA production pattern observed in the glucose-containing media by increasing its concentration in the extracellular media 45% and 63%, respectively (Figure 6).

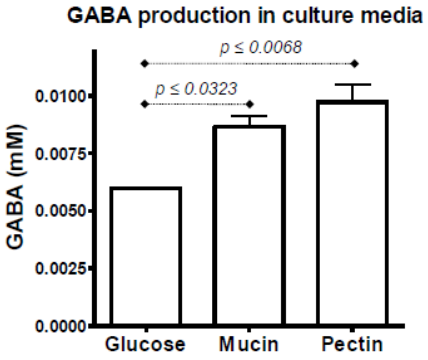

Figure 6. Quantification of GABA production in culture supernatants. Culture supernatants from conditions where over expression of glutamate decarboxylase (BUNIF7771_0544 - K01580) and glutamate:GABA antiporter (BUNIF7771_0548 - K20265) genes was inferred from the exploratory RNAseq and corroborated by qPCR were subject of LC-MS analysis in order to determine the GABA concentration at log phase of respective cultures (OD_{600} ~ 0.4). The comparisons were made using three independent replicates per condition and statistical analysis was assessed through pairwise *t*-test with bonferroni correction.

This last data, obtaining by direct quantification of the metabolic output of a selected set of genes, with a over-expression pattern inferred by our exploratory RNAseq approach, demonstrated conclusively the impact of the

carbon sources in production of pivotal metabolites for human health by symbionts, and reinforce the idea of designing potential synbiotic products based on the combined administration of *B. uniformis* CECT 7771 strains with pectin and/or mucin-derived glycans.

Conclusions

Using nanopore-based technology we could obtain reliable data to assembly a draft genome of the strain *B. uniformis* CECT 7771. The assembly and the primary sequence of the superscaffolds obtained were refined with paired-end transcriptome data permitting the prediction of more than 5,200 ORFs to be encoded in this human symbiont strain. Although genome information of other *B. uniformis* strains are publicly available in different biological databases, we presented for the first time the comparative analysis of such genomes describing their glycolytic capability.

Our results indicate that *B. uniformis* strains have the largest repertoire of CAZy genes among *Bacteroides* species, and this set of genes outnumbers those observed in the archetype species *B. tethaiotaomicron* and the highly versatile species *B. cellulosyliticus*. To proof the glycan degradation versatility inferred *in silico* from the *B. uniformis* CECT 7771 genome we demonstrated that it was able to use all carbon sources tested with different growth fitness cost. Similarly, the genome response to all different glycans used induced different expression patterns with characteristic metabolic outputs. Our data indicates that *B. uniformis* CECT 7771 is able to utilize the *O*-glycans covalently attached to mammal mucin proteins as well as previously reported for *B. tethaiotaomicron* (Sonnenburg *et al*., 2005) and *B. fragilis* (Roberton and Stanley, 1982). Consequently, it is expected that *B. uniformis* be a mucin-degrader bacteria and that predominantly colonizes the mucosal surface and, therefore, be tightly interacting with the host immune system and modulating it as well. Our data also indicate that the wide repertoire of glycans being *O*-linked to mucus layer proteins at the intestinal epithelium could be enough to promote the growth and persistency of *B. uniformis* in absence of dietary fiber. The fact that mucin glycans induce a higher production of butyrate in the growth medium by up-regulating the genes responsible for its production in *B. uniformis* CECT 7771 clearly disclose a metabolic circuit, which could be the cornerstone of a mutualistic relationship between the bacterium and the host, given that butyrate reciprocally induces production of mucin in colorectal cells (Jung *et al*., 2015). Besides, it is expected that *B. uniformis* CECT 7771 be able also to modulate the host immune response, as a consequence of the mucin glycan utilization, by attenuating the production of long-chain fatty acids and expression of the acyl carrier protein (ACP), fundamental components of the LPS (lipopolysaccharide) biosynthesis pathway in Gram-negative bacteria (Masoudi et al., 2014; Emiola *et al.*, 2015).

Our study also shows that the use of pectin by *B. uniformis* CECT 7771 enhances the butanoate metabolism, therefore increasing the production of butyrate and specially the production of the inhibitory neurotransmitter GABA, which could contribute to strengthen the gut barrier and play a role in mental health, respectively (Dinan and Cryan, 2017; Sandhu *et al*., 2017).

Globally, our results shed light on the molecular basis of mutualism between the humans and an important component of the gut microbiota, such as *Bacteroides uniformis.* The ample repertoire of glycolytic activities observed in this mucin-degrader symbiont could have been the result of the need to consume primarily endogenous nutrients to survive in the infant's gut, which seem to exert a deep impact on *B. uniformis* metabolism, even when dietary glycans are absent, regarding the production of pivotal molecules for gut and possibly mental health maintenance.

Author Contributions

EGdP performed the cell cultures, isolation of nucleic acids, and qPCR experiments. ABP sequenced, assembled, and annotated the bacterial genome as well as performed transcriptome analysis. ABP and YS designed and directed the study. All authors contributed to manuscript writing.

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Conflict of interest

The authors have no conflict of interest to declare.

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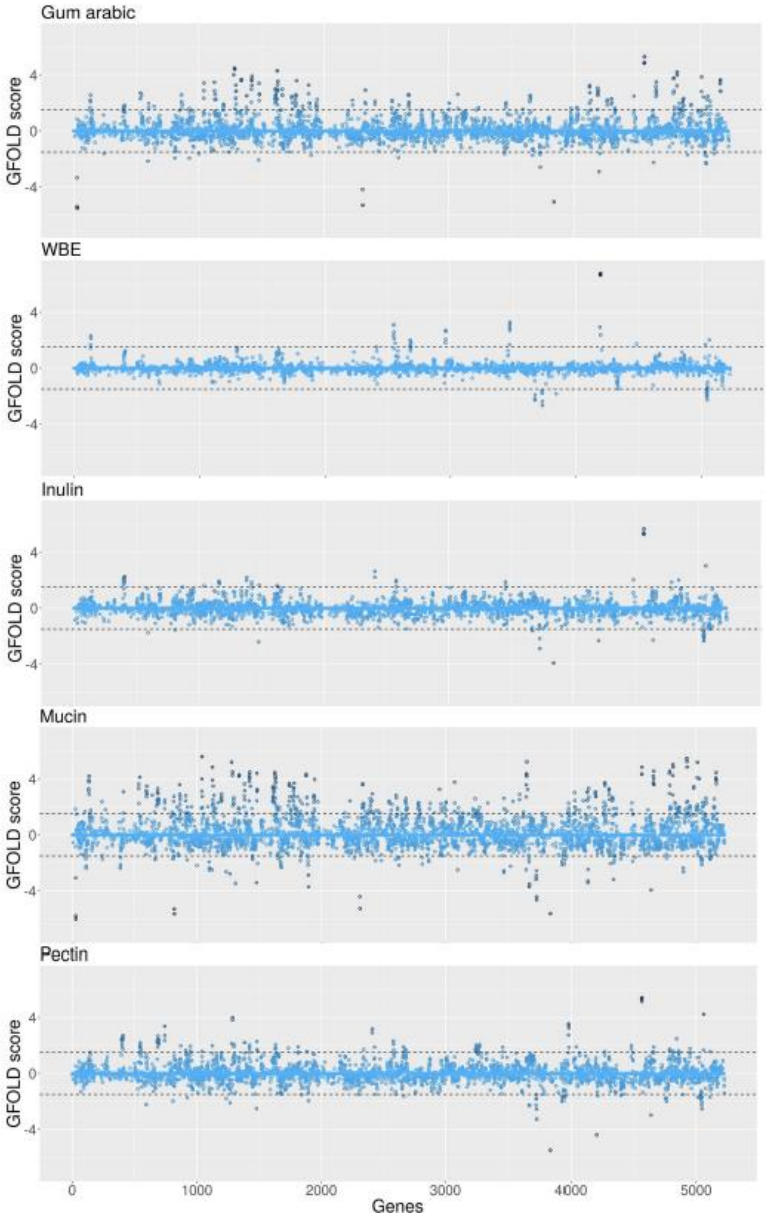
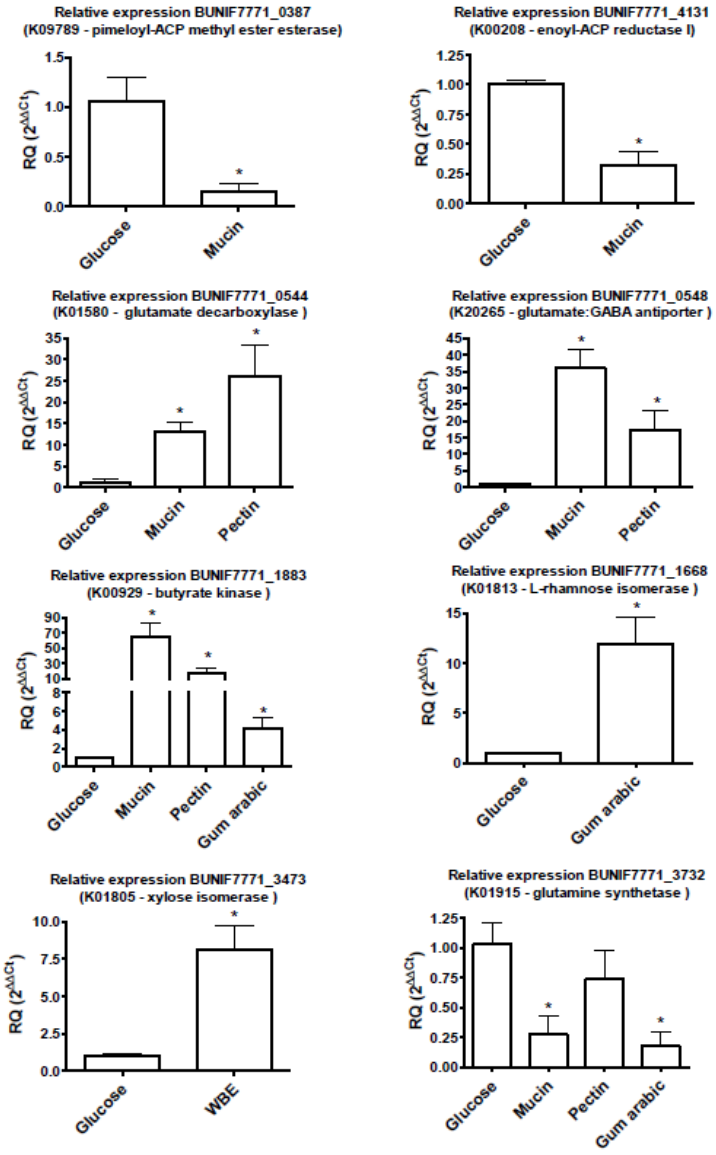


Supplementary Figure 1. Expression patterns across the *B. uniformis* CECT 7771 genome. GFOLD scores obtained for every single gene predicted to be encoded by the *B. uniformis* CECT 7771 genome were plotted for all culture conditions tested in this study. The dashed lines indicated the threshold for up- (≥1.5) or down-regulation (≤-1.5). The more distant GFOLD values are from zero, the darker the dots become.



Supplementary Figure 2. Gene expression assessment by qPCR. A set of 8 different genes with differential expression patterns in different culture conditions were evaluated to corroborate the RNAseq analysis. In this case, non-pooled RNA samples were used, the expression of 16S was used as normalizer (endogenous control), and the glucose sample was used as reference. Eight panels are presented with respective expression data in terms of RQ ($2^{-\Delta\Delta Ct}$) for all genes selected. The respective gen tag (BUNIF7771_XXXX) and the gene functional annotation according to KEGG Orthology are showed as headers. * $p < 0.05$.

Table S1. Primers for qPCR analysis.

Primer name	Primer sequence 5'-3'	PCR product
BU-387F	GGAATTCCCGAAAAGACCTTCCG	
BU-387R	GTAGGCATTGCTGTCTTTGCACAT	99bp
BU-544F	TATGCAACCAAGCTGATGAACGAAG	
BU-544R	AGAGGTTGGCCACGATGTTGATAC	112bp
BU-548F	TGGATTTCCACAGCAGCTTCTTCC	
BU-548R	TCTTTTACGTGGATACCGCCCATT	115bp
BU-1668F	AACATTTCGAAAGCTGGATGCAATGG	
BU-1668R	GTCCGGATGAGCCAGAGAAAGAT	113bp
BU-1883F	GGAGGCCAATGACATTCCCTTTAAG	
BU-1883R	AGCATATCGTCCAACATGGCATCG	114bp
BU-3473F	CGAAGTGAACCACGCTACTTTGG	
BU-3473R	GTTCTGGTAGTCACCACGGTTG	112bp
BU-3732F	ATGTTCGAGTCCATCGGTGTCATG	
BU-3732R	AGCACGCGGGCTTCAATCTGTATT	104bp
BU-4131F	GGTGCTCTGAATGAGCAGTCCAT	
BU-4131R	CATACGGACAGCAACCGGTGTAT	102bp
pBU7771F	GCCCCCTTAACCCCCTGTCA	
pBU7771R	CACTCCCCGGCTTCACTGCT	111bp
16S-F	CACGGGTGAGTAACACGTATCC	
16S-R	GCATCCCCATCGATAACCGAAA	132bp

Table S2. Average Nucleotide Identity (ANI) analysis among different strains of *B. uniformis*.

	***B. uniformis* CECT 7771**	***B. uniformis* ATCC 8492**	***B. uniformis* CL03T00C23**	***B. uniformis* 3978-T3i**	***B. uniformis* dnLKV2**	***B. stercoris* ATCC 43183**	***B. caccae* ATCC 43185**	***B. fragilis* NCTC 9343**	***B. vulgatus* ATCC 8482**	***B. thetaiotaomicron* VPI 5482**
***B. uniformis* CECT 7771**	100 100									
***B. uniformis* ATCC 8492**	99.6 98.4	100 100								
***B. uniformis* CL03T00C23**	99.7 98.1	99.9 98.4	100 100							
***B. uniformis* 3978-T3i**	99.7 98.2	99.9 98.5	99.9 98.4	100 100						
***B. uniformis* dnLKV2**	99.6 97.3	99.9 97.7	99.9 98.0	99.9 97.9	100 100					
***B. stercoris* ATCC 43183**	95.3 86.8	94.4 86.7	95.2 85.4	94.9 86.5	94.7 85.5	100 100				
***B. caccae* ATCC 43185**	85.5 84.1	85.3 85.1	86.0 83.5	85.8 83.4	85.8 84.2	90.1 84.4	100 100			
***B. fragilis* NCTC 9343**	83.3 84.3	83.3 86.2	84.2 84.0	83.9 84.3	83.8 83.8	87.2 84.5	93.8 83.9	100 100		
***B. vulgatus* ATCC 8482**	91.1 92.4	90.6 93.7	91.3 90.9	91.0 92.5	91.1 87.4	92.9 92.4	94.4 88.1	93.0 85.7	100 100	
***B. thetaiotaomicron* VPI 5482**	88.1 88.3	87.9 87.3	88.5 86.4	88.3 86.7	88.2 85.6	90.7 88.2	96.7 85.12	96.5 84.3	95.8 88.6	100 100

Genome-wide comparisons among *B. uniformis* genomes and reference *B. stercoris, B. fragilis, B. caccae, B. vulgatus,* and *B. thetaiotaomicron* genomes. Two different comparisons were made using tetranucleotide distribution correlation (top numbers) and average nucleotide identity (ANI) based on MUMmer algorithm (bottom numbers) (Richter and Rossello-Mora, 2009).

Table S3. Strain-specific CAZy functions in *B. uniformis*

Strain	CAZy family	Function[1]	EC number[1]
***B. uniformis* CECT 7771**	GT64	heparan α-N-acetylhexosaminyltransferase	2.4.1.224
***B. uniformis* ATCC 8492**	CE12	pectin acetylesterase	3.1.1.-
	GH81	endo-β-1,3-glucanase	3.2.1.39
	GH115	xylan α-1,2-glucuronidase	3.2.1.131
	CBM67	L-rhamnose binding activity	NA
***B. uniformis* CL03T00C23**	AA5	galactose oxidase	1.1.3.9
	GH123	β-N-acetylgalactosaminidase	3.2.1.53
	GH10	endo-1,4-β-xylanase	3.2.1.8
	GH67	xylan α-1,2-glucuronidase	3.2.1.131
	CBM3	cellulose-binding activity	NA
	CE6	acetyl xylan esterase	3.1.1.72
	CBM26	starch-binding activity	NA
	PL1	pectate lyase	4.2.2.2
***B. uniformis* dnLKV2**	GH106	α-L-rhamnosidase	3.2.1.40
***B. uniformis* 3978-T3i**	GT31	N-acetyllactosaminide β-1,3-N-acetylglucosaminyltransferase	2.4.1.149
	GH4	α-glucosidase α-galactosidase	3.2.1.20 3.2.1.22
	GT56	lipid II Fuc4NAc transferase	2.4.1.-
	CE2	acetyl xylan esterase	3.1.1.72
	GT25	β-1,3-glucosyltransferase β-1,2-glucosyltransferase β-1,2-galactosyltransferase β-1,4-galactosyltransferase	2.4.1.-

[1] The function annotation and Enzyme Classification (EC) numbers were obtained from respective family information at CAZy database.

Table S4. Functional analysis of sugar metabolism genes over-expressed in mucin-containing media

Gene tag	KEGG id	GFOLD score	Functional annotation
BUNIF7771_0102	K00616	1.65	E2.2.1.2, talA, talB; transaldolase [EC:2.2.1.2]
BUNIF7771_0131	K00700	3.15	GBE1, glgB; 1,4-alpha-glucan branching enzyme [EC:2.4.1.18]
BUNIF7771_0132	K01187	3.31	malZ; alpha-glucosidase [EC:3.2.1.20]
BUNIF7771_0411	K06158	3.09	ABCF3; ATP-binding cassette, subfamily F, member 3
BUNIF7771_0412	K06158	1.57	ABCF3; ATP-binding cassette, subfamily F, member 3
BUNIF7771_0595	K02039	3.17	phoU; phosphate transport system protein
BUNIF7771_0596	K02036	2.73	pstB; phosphate transport system ATP-binding protein [EC:3.6.3.27]
BUNIF7771_0597	K02038	3.01	pstA; phosphate transport system permease protein
BUNIF7771_0598	K02037	3.49	pstC; phosphate transport system permease protein
BUNIF7771_0599	K02040	2.94	pstS; phosphate transport system substrate-binding protein
BUNIF7771_0685	K11645	3.05	fbaB; fructose-bisphosphate aldolase, class I [EC:4.1.2.13]
BUNIF7771_0689	K00850	2.59	pfkA, PFK; 6-phosphofructokinase 1 [EC:2.7.1.11]
BUNIF7771_0690	K03455	2.42	TC.KEF; monovalent cation:H+

			antiporter-2, CPA2 family
BUNIF7771_0692	K00688	2.25	PYG, glgP; glycogen phosphorylase [EC:2.4.1.1]
BUNIF7771_0693	K00688	2.48	PYG, glgP; glycogen phosphorylase [EC:2.4.1.1]
BUNIF7771_0871	K01990	1.71	ABC-2.A; ABC-2 type transport system ATP-binding protein
BUNIF7771_1128	K16147	2.51	glgE; starch synthase (maltosyl-transferring) [EC:2.4.99.16]
BUNIF7771_1138	K01183	2.83	E3.2.1.14; chitinase [EC:3.2.1.14]
BUNIF7771_1139	K02429	1.82	fucP; MFS transporter, FHS family, L-fucose permease
BUNIF7771_1333	K12373	1.89	HEXA_B; hexosaminidase [EC:3.2.1.52]
BUNIF7771_1342	K00845	1.86	glk; glucokinase [EC:2.7.1.2]
BUNIF7771_1379	K05349	2.00	bglX; beta-glucosidase [EC:3.2.1.21]
BUNIF7771_1483	K01201	3.11	GBA, srfJ; glucosylceramidase [EC:3.2.1.45]
BUNIF7771_1610	K01787	3.38	RENBP; N-acylglucosamine 2-epimerase [EC:5.1.3.8]
BUNIF7771_1611	K16139	2.68	uidB, gusB; glucuronide carrier protein
BUNIF7771_1612	K03292	2.48	TC.GPH; glycoside/pentoside/hexuronide: cation symporter, GPH family
BUNIF7771_1614	K01218	2.79	gmuG; mannan endo-1,4-beta-mannosidase [EC:3.2.1.78]
BUNIF7771_1628	K01218	3.39	gmuG; mannan endo-1,4-beta-mannosidase [EC:3.2.1.78]

BUNIF7771_1634	K01179	2.12	E3.2.1.4; endoglucanase [EC:3.2.1.4]
BUNIF7771_1779	K00864	2.09	glpK, GK; glycerol kinase [EC:2.7.1.30]
BUNIF7771_1781	K00615	3.70	E2.2.1.1, tktA, tktB; transketolase [EC:2.2.1.1]
BUNIF7771_1798	K01190	1.80	lacZ; beta-galactosidase [EC:3.2.1.23]
BUNIF7771_1799	K01206	1.91	FUCA; alpha-L-fucosidase [EC:3.2.1.51]
BUNIF7771_1882	K03931	1.55	ygjK; putative isomerase
BUNIF7771_1944	K01132	2.52	GALNS; N-acetylgalactosamine-6-sulfatase [EC:3.1.6.4]
BUNIF7771_1945	K01195	1.70	uidA, GUSB; beta-glucuronidase [EC:3.2.1.31]
BUNIF7771_1946	K05349	3.96	bglX; beta-glucosidase [EC:3.2.1.21]
BUNIF7771_2331	K01179	3.59	E3.2.1.4; endoglucanase [EC:3.2.1.4]
BUNIF7771_2572	K01208	2.17	cd, ma, nplT; cyclomaltodextrinase / maltogenic alpha-amylase / neopullulanase [EC:3.2.1.54 3.2.1.133
BUNIF7771_2678	K01209	2.42	abfA; alpha-N-arabinofuranosidase [EC:3.2.1.55]
BUNIF7771_2685	K01785	1.86	galM, GALM; aldose 1-epimerase [EC:5.1.3.3]
BUNIF7771_2686	K00849	1.76	galK; galactokinase [EC:2.7.1.6]
BUNIF7771_2687	K00849	1.78	galK; galactokinase [EC:2.7.1.6]

BUNIF7771_3073	K01960	3.76	pycB; pyruvate carboxylase subunit B [EC:6.4.1.1]
BUNIF7771_3242	K15894	1.51	pseB; UDP-N-acetylglucosamine 4,6-dehydratase [EC:4.2.1.115]
BUNIF7771_3244	K15897	1.76	pseG; UDP-2,4-diacetamido-2,4,6-trideoxy-beta-L-altropyranose hydrolase [EC:3.6.1.57]
BUNIF7771_3263	K19354	1.55	waaH; heptose III glucuronosyltransferase [EC:2.4.1.-]
BUNIF7771_3273	K01784	1.96	galE, GALE; UDP-glucose 4-epimerase [EC:5.1.3.2]
BUNIF7771_3471	K00854	1.85	xylB, XYLB; xylulokinase [EC:2.7.1.17]
BUNIF7771_3474	K08138	1.86	xylE; MFS transporter, SP family, xylose:H+ symportor
BUNIF7771_3649	K05546	3.58	GANAB; alpha 1,3-glucosidase [EC:3.2.1.84]
BUNIF7771_3781	K00874	1.53	kdgK; 2-dehydro-3-deoxygluconokinase [EC:2.7.1.45]
BUNIF7771_4139	K00845	1.98	glk; glucokinase [EC:2.7.1.2]
BUNIF7771_4190	K01179	2.99	E3.2.1.4; endoglucanase [EC:3.2.1.4]
BUNIF7771_4195	K05349	2.80	bglX; beta-glucosidase [EC:3.2.1.21]
BUNIF7771_4258	K06859	1.98	pgi1; glucose-6-phosphate isomerase, archaeal [EC:5.3.1.9]
BUNIF7771_4260	K01809	2.34	manA, MPI; mannose-6-phosphate isomerase [EC:5.3.1.8]

BUNIF7771_4301	K01196	3.29	AGL; glycogen debranching enzyme [EC:2.4.1.25 3.2.1.33]
BUNIF7771_4303	K07405	2.54	E3.2.1.1A; alpha-amylase [EC:3.2.1.1]
BUNIF7771_4785	K15923	2.97	AXY8, FUC95A, afcA; alpha-L-fucosidase 2 [EC:3.2.1.51]
BUNIF7771_4786	K05349	4.40	bglX; beta-glucosidase [EC:3.2.1.21]
BUNIF7771_4787	K01190	4.56	lacZ; beta-galactosidase [EC:3.2.1.23]
BUNIF7771_4788	K01811	3.54	xylS, yicI; alpha-D-xyloside xylohydrolase [EC:3.2.1.177]
BUNIF7771_4924	K01134	2.34	ARSA; arylsulfatase A [EC:3.1.6.8]
BUNIF7771_5029	K15921	1.75	xynD; arabinoxylan arabinofuranohydrolase [EC:3.2.1.55]
BUNIF7771_5163	K03332	1.54	fruA; fructan beta-fructosidase [EC:3.2.1.80]

Safety assessment of *Bacteroides uniformis* CECT 7771, a symbiont of the infant's gut microbiota

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Background

The *Bacteroides uniformis* CECT 7771 strain was isolated from a healthy breastfed infant and demonstrated to ameliorate metabolic disorders in a pre-clinical mouse model of obesity. The safety of this strain was also confirmed preliminarily with an acute toxicity study in mice from which adverse effects could not be observed. The objective of this study was to confirm the safety of the oral use of this strain, in a sub-chronic animal trial of 90-days.

Methods and findings

A safety study has been conducted in Wistar rats (males and females, n = 50) divided into 5 experimental groups (n = 10), each were administered either a dose of *B. uniformis* CECT 7771 (10^8 CFU/day, 10^9 CFU/day or 10^{10} CFU/day) or *B. longum* ATCC 15707T (10^{10} CFU/day) used as a control strain with qualified presumption of safety (QPS) status or placebo (vehicle) for 90 days. Signs of toxicity, morbidity and mortality were recorded daily while weight and food intake were recorded weekly. Biochemical parameters related to pancreatic, liver and kidney functions were analysed as well as inflammatory immune markers. Bacterial translocation to peripheral tissues was assessed and histological/histometric analysis was performed in colon sections. Adverse effects were not observed regarding the general health status with food intake at any of the doses tested. The biochemical parameters showed no differences relating to the treatment, except for alanine aminotransferase, whose levels were reduced as the dose of *B. uniformis* CECT 7771 was increased, indicating a potentially beneficial role in liver function. The ratio of anti-inflammatory to pro-inflammatory cytokines suggested that *B. longum* ATCC 15707^T administered at 10^{10} CFU/day and *B. uniformis* CECT 7771 at 10^9 CFU/day could exert anti-inflammatory effects after long-term administration. The fecal microbiota analysis showed that *B. uniformis* CECT 7771 successfully colonize the intestine tract of rats after oral administration and modulates the gut microbiota with no adverse effects like reducing the richness and diversity.

Conclusions

The results indicate that the oral consumption of *B. uniformis* CECT7771 during a sub-chronic 90-day study in rats does not raise safety concerns. The effects of the bacterial strain on markers of liver function and inflammation suggest that it can confer potential benefits. However, further studies are required to confirm the safety of this strain and its products thereof in humans.

Introduction

Knowledge of the gut microbiota configuration and its role in health and disease has greatly increased in recent times by the development and use of next-generation sequencing technologies (NGS) [1]. These techniques have helped to unravel the structure and function of the microbiota in different body sites and associations between its configuration and health or disease. It has also contributed to understanding how interactions between the microbiota, the environment (e.g. diet, antibiotic intake, stress, etc.) and the host (e.g. age, genetics, etc.) may impact on health outcomes. This information has also led to identifying microbiome-based biomarkers for disease risk as well as possible health-promoting bacteria (usually known as probiotics) and, therefore, helping to promote products for future clinical and nutritional applications.

Until now, "classical" probiotics, which are mainly composed of *Lactobacillus* sp. and *Bifidobacterium* sp., have been used for the treatment of diarrhoea, chronic inflammatory bowel disorders (e.g. colitis) or antibiotic-associated diarrhoea, among others [2]. Most of these bacteria have been isolated from fermented foods or from biological samples from humans or animals within the food chain. These have a long history of use as foods because they are consumed as part of the diet and have QPS status [3]. Nonetheless, the use of NGS techniques has allowed the identification of other human intestinal bacteria, which represent a larger proportion of the indigenous components of the microbiota and that could play an important and distinct role in both gastrointestinal and extra-intestinal pathologies [4-6]. Although these new species and strains are normal inhabitants of the human gut, unlike traditional probiotics [7, 8], they lack a history of safe use for human consumption. Therefore, a specific safety assessment must be completed guaranteeing their unequivocal safety status according to their intended use [9, 10].

The *phylum* Bacteroidetes represents between 20 - 40 % of the colonic bacteria, being considered one of the most abundant *phylum* of the human gut microbiota. This phylum includes the *Bacteroides* genus, that consists of 5 - 18 % of the total microbiota, a percentage greater than that represented by the well-known *Lactobacillus* and *Bifidobacterium* [11, 12]. It can be expected that members of this genus play an important role in the gut environment and human physiology. Nevertheless, the research on the potential role of members of this genus to act as probiotics is only in the initial stages. Some species of the genus *Bacteroides* have already shown the ability to outcompete pathogens [13], attenuate auto-immunity by optimizing the Th1/Th2 balance and by inducing T reg cell differentiation [14] and influence positively on satiety and glucose metabolism [5, 15]. These effects have been mediated, at least in part by host-microbe interactions triggered by

structural bacterial components, like the polysaccharide A (PSA) of *Bacteroides fragilis* [16], or bacterial metabolites like short chain fatty acids (SCFAs: acetate, butyrate and propionate) [17]. SCFAs are mainly derived from the fermentation of complex carbohydrates carried out directly by *Bacteroides* sp. or via *cross-feeding* mechanisms between *Bacteroides* with other intestinal bacteria, such as *Faecalibacterium prausnitzii*, *Roseburia* sp., and *Anaerostipes* sp.[18]. However, not all *Bacteroides* sp. are commensals and, for example, some strains of *Bacteroides fragilis* can act as opportunistic pathogens that encode enterotoxins and can be involved in acute diarrheal disease and inflammatory bowel disease [19].

Bacteroides uniformis CECT 7771 was originally isolated from faeces of healthy breastfed infants [20], which have been demonstrated to have a higher abundance of this *Bacteroides* species compared to formula-fed infants. This strain was selected among other *Bacteroides* species and strains due to its ability to induce an anti-inflammatory cytokine profile in *in vitro* macrophage cultures and to ameliorate the metabolic dysfunction of diet-induced obesity in mice. The oral administration of *B. uniformis* CECT 7771 reduced body weight gain and improved lipid metabolism, diminishing liver steatosis, serum cholesterol and triglyceride levels in obese mice. This strain also reduced leptin levels and improved glucose metabolism since it decreased fasting concentrations of serum glucose and insulin, therefore, improving glucose tolerance [5].

Previously, we performed a preliminary evaluation on the safety and tolerability of the oral administration of *B. uniformis* CECT 7771 in a short-term acute study (6 days) in normal and immunocompromised mice, which showed no adverse effects at a dose of 10^9 CFU/day [21].

The aim of this study was to progress the evaluation of the safety of oral administration of *B. uniformis* CECT 7771 in a sub-chronic (90-days) study in rats by conducting an exhaustive examination of possible adverse effects, including assessments of body weight and fat, food intake, bacterial translocation, intestinal tissue histology, and biochemical and immune markers.

Materials and methods

Bacterial strain and culture conditions

Bacteroides uniformis CECT 7771 strain was isolated from stools of healthy infants [20] and deposited in the Spanish Culture Collection (CECT). The bacteria were grown in a Schaedler medium broth (without hemin) at 37 °C in an anaerobic chamber (Bactron 300-2, Shellab). Cells were harvested by centrifugation (6000 g for 10 min) and washed in a phosphate buffered saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4). Bacteria were then re-suspended in 10 % sterile skimmed milk for animal trials (Scharlau). Aliquots of these suspensions were immediately frozen in liquid nitrogen and stored at -80 °C until use. After freezing and thawing, live cells numbers were determined by colony-forming unit (CFU) counting on Schaedler agar medium plates after 48 h incubation at 37 °C. Cell viability resulted in 98.3 %. One fresh aliquot was thawed daily to avoid differences in culture viability along the study.

Sub-chronic (90-day) oral toxicity study in rats

The sub-chronic toxicity study was designed based on the specifications of the OECD/OCDE Guideline to perform a repeating dose, 90-day oral toxicity study (408) [22], in 9 week-old male and female Wistar rats (Envigo RMS, S.L. Sant Feliu de Codines, Barcelona). During the adaptation period (7 days), animals of the same sex were housed in cages in groups of 2-3 animals per cage to create 5 groups (n = 10: 5 males and 5 females per group). They were held in a temperature-controlled (23 °C) room with a 12 h light/dark cycle with 40 – 50 % relative humidity. The rats were rotated between uncleansed cages for 1 week, until all the rats had been exposed to a similar environment, to favour the establishment of a uniform gut microbiota in the rats as a baseline [23].

Animals were fed *ad libitum,* with an unlimited supply of a conventional laboratory diet and water. These were divided into different experimental groups that received daily and additionally (to the conventional diet), one of the following supplements: 1) a control group receiving a dose of placebo (10 % [w/v] skimmed milk) (Control); 2) a group receiving a dose of $1x10^{10}$ CFU *B. longum* ATCC 15707^{T} (*B. longum*) that represent a strain with a Quality Presumption of Safety (QPS) status [24]; 3) a group receiving a dose of $1x10^{8}$ CFU *B. uniformis* CECT 7771 (hereinafter B.Unif8); 4) a group receiving a dose of $1x10^{9}$ CFU *B. uniformis* CECT 7771 (B.Unif9); 5) a group receiving a daily dose of $1x10^{10}$ CFU *B. uniformis* CECT 7771 (B.Unif10).

To reduce the stress of the animals, the bacterial doses were administered through incorporation with a piece of sweet jelly (Transgel®, Charles River) to avoid the use of a gavage as the method of administration.

Signs of toxicity, morbidity and mortality were recorded twice daily and individual body weights were recorded once a week. Food consumption was assessed weekly by registration of food weight data at the beginning and the end of each week.

After the 90-day intervention, animals were anaesthetized; blood was collected by an aortic puncture from each rat, which was then immediately killed by cervical dislocation. Large and small intestine, liver and mesenteric lymph nodes (MLN) were removed and stored for different analyses as described below. All procedures involving animals were specifically approved by the ethics committee of the University of Valencia (Animal Production Section, Central Service of Support to Research [SCSIE], University of Valencia, Spain) and authorized by Dirección General de Agricultura, Ganadería y Pesca (Generalitat Valenciana" (authorization number: 2017/VSC/PEA/00125). A study design scheme is illustrated in Figure 1.

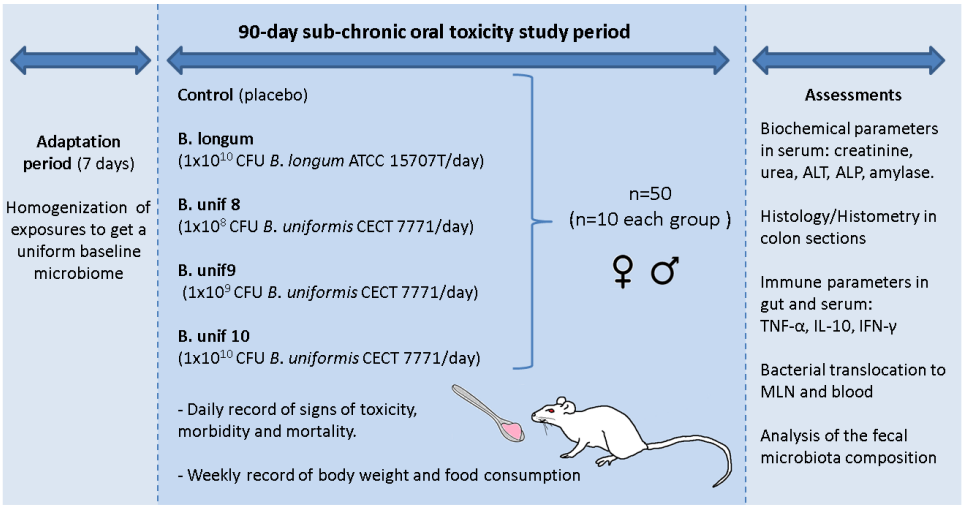

Figure 1. Schematic representation of the study protocol to evaluate the safety of *Bacteroides uniformis* CECT 7771 in a sub-chronic (90-day) oral toxicity study in rats.

Bacterial translocation

Bacterial translocation was analysed in samples of blood and MLN. Samples were homogenized in buffered peptone water (1 g/ml). 100 µl of the resulting homogenates were plated for counting bacterial growths, using Schaedler agar media (Oxoid, UK) for Bacteroides and Wilkins-Chalgren anaerobe agar media (Oxoid, UK) for total anaerobe quantification, after incubation at 37 ºC in an anaerobic chamber for 3 days.

In the case of an incidence of bacterial translocation (the event of positive growth on agar plates), bacterial colonies were recovered, and the bacterial DNA was extracted using the MasterPure Gram-positive DNA purification kit (Epicentre). Then, the possible presence of *Bacteroides uniformis* CECT 7771 in the plates was assessed by PCR, using specific oligonucleotides for the glutamate decarboxylase gene of this specific strain [17]. PCR reactions consisted of 0.5 µM forward oligonucleotide (BU-544F: 5'-TATGCAACCAAGCTGATGAACGAAG-3'), 0.25 µM of reverse oligonucleotide (BU-544R-r: 5'-AGAGGTTGGCCACGATGTTGATAC-3'), 0.25 mM of dNTPs and 20 ng of the DNA sample. The PCR program was as follows: an initial incubation at 98 °C for 3 min and 25 cycles of 20 s at 95 °C, 30 s at 63 °C, and 1 min at 72 °C. Once the cycles had finalized, the reactions were incubated for 5 min at 72 °C and the products visualized in an agarose gel.

Determination of cytokine concentrations

The cytokines IL-10, IFN-ɣ and TNF-α were quantified in serum and jejunum samples, using simplex Luminex kits for each immune parameter and ProcartaPlex Basic Rat kits (eBioscience, Vienna, Austria) in a Luminex 100 IS™ (Luminex Corporation. Austin, TX, USA). For jejunum samples, first, the tissue sections were homogenized in a RIPA buffer containing a cocktail of protease inhibitors (Complete, Mini tablets, Roche life science, Mannheim Germany), using a TissueRuptor® device (Qiagen), keeping the samples in ice during the procedure. Then, the samples were centrifuged (16,000 g for 10 min at 4 °C) and, then, the resulting supernatant was used for cytokine determinations. These measurements were carried out in duplicate for each sample.

Biochemical parameters analysis

Biochemical parameters were quantified in serum obtained by blood centrifugation (1,000 g for 10 min at room temperature). The following enzymatic assay kits were used: alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase assay kits (BioVision Incorporated, Milpitas, USA) and urea and creatinine assay kits (Sigma-Aldrich (Merck KGaA), Darmstadt, Germany). These

measurements were carried out in duplicate for each sample and following the manufacturers' instructions.

Histology and histometry

Sections of the colon from each animal were collected immediately after the sacrifice and were immediately fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 24 h at 4 °C. After that, the colon sections were dehydrated in a graded series of ethanol, cleared with xylene and embedded in paraffin to obtain serial microtome sections (3 μm-thick). Then, the samples were stained with haematoxylin/eosin (HE) solutions to evaluate the structural aspects of the colon (crypts, villi, colonocytes, epithelial, etc.).

For histometry evaluations, the determination of the number of goblet cells per intestinal villi and the depth of intestinal crypts of Lieberkün (10 fields measured per sample) in the HE-stained sections was carried out. In addition, the lymphoid area of individual follicles containing the Peyer's patches was examined, as a determinant of the status of the gut-associated lymphoid tissue (GALT), in the HE-stained colon sections.

Measurements were taken from images obtained using a light microscope fitted with a Nikon, Olympus, Eclipse 90i, UK camera using NIS Elements BR 2.3 research software (Kingston; Surrey, KT2 5PR, England).

Statistical analysis

All data were analysed using GraphPad Prism 5.01 (Graph Pad Software Inc., San Diego, CA, USA). Results are expressed as means ± SEM. All data sets were first analysed to check if data were or were not normally distributed by using the KS normality test. For parametric data sets, two-way analysis of variance (ANOVA) was applied for comparison of means and Tukey's multiple comparison test was used as a *post hoc* test for pairwise comparisons. For non-parametric data, a Kruskal-Wallis test was performed for comparison of medians and Dunn's multiple comparison test was used as a *post hoc* test for pairwise comparisons. Significance was defined as $p \leq 0.05$ in all analysis.

Analysis of the fecal microbiota

DNA extraction and PCR amplification. Fecal samples were collected at the end-point of the intervention period. Approximately, 200 mg stool were used for DNA extraction using the Power Fecal DNA isolation kit (MoBio) following the manufacturer's instructions. A diluted aliquot of the fecal DNA was prepared at ~20 ng/μL to be used in PCR reactions. Approximately 20 ng DNA (1μL diluted

DNA) was used to amplify the V3-V4 hypervariable regions from the bacterial 16S rRNA gene by a 25-cycle PCR program consisting of the following steps: 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 20 s. The PCR reaction was performed using Phusion High-Fidelity Taq Polymerase enzyme (Thermo Scientific) and 6-mer barcoded primers, which target a wide range of bacterial 16S rRNA genes: S-D-Bact-0341-b-S-17 (CCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC).

Sequencing and data analysis

Dual-barcoded amplicons consisted of approximately 500-bp fragments were purified from triplicate reactions per sample using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK). Amplicon DNA was quantified using a Qubit 3.0 fluorometer and the Qubit dsDNA HS Assay Kit (LifeTechnologies, Carlsbad, CA, USA). Samples were mixed by combining equimolar quantities of amplicon DNA (100 ng per sample) and sequenced in an Illumina MiSeq platform with 2×300 PE configuration (CNAG, Barcelona, Spain).

Raw data were delivered in fastq files and pair ends with quality filtering were assembled using Flash software [25].

Sample de-multiplexing was carried out using sequence information from the respective DNA barcodes and the *Mothur* (v1.36.1) [26]. The chimera detection and removal was performed with the *uchime* algorithm [27] implemented in *Mothur*. The taxonomy assessment of DNA reads was complete with a subset of 4456 reads per sample, randomly selected after multiple shuffling (10,000X), and the RDP classifier v 2.12 [28]. The alpha and beta diversity approaches were completed in the *qiime* suite of analysis (v1.9.1) [29] by using the OTU-picking approach. Consequently, the alpha diversity descriptors such as Chao's richness, Shannon's diversity, Simpson's evenness, and Simpson's reciprocal index were computed using the OTU information recovered for all samples. Bray-Curtis and Binary Jaccard dissimilarity indexes were used as metrics to determine distances among samples.

All the statistical evaluation of microbiota data was performed in R v3.4.4.3 (http://cran.r.project.org). Non-parametric Kruskal-Wallis and Mann-Whitney U tests were applied. Treatment was used as unique categorical variable in beta diversity analysis based on the Bray-Curtis and Binary Jaccard dissimilarity indexes and PERMANOVA analysis was performed with the q*iime* suite of analysis. Significant results were selected when corrected p-values were ≤ 0.05.

Results

General health, food intake and weight of the animals

During the experimental procedure, there was no noticeable change in normal activity, hair lustre or behaviour in any of the groups of rats. Animals did not suffer from diarrhoea or other sickness related to treatment. At the end of the study, all animals were alive and healthy in all experimental groups. There were no statistically significant differences in body weight gain or loss or feed intake between different groups (Supplementary figure 1. A and B) nor when males and females were analysed separately (Supplementary figure 2).

Concerning tissue weight, there were no significant differences in the weight of livers between groups (Supplementary figure 1. C), nor when males and females were studied separately (Supplementary figure 3). The colouration, size and appearance of the analysed tissues were normal, and no differences were observed between experimental groups.

Bacterial translocation

Positive bacterial growth was observed after plates were incubated at 37 °C in anaerobic conditions in some samples of NLM homogenates and blood of experimental and placebo groups.

After analysing the identity of the bacteria recovered from positive growth plates by species-specific PCR, no colony was identified as *B. uniformis* CECT 7771, since the fragment corresponding to the glutamate decarboxylase gene was not detected when compared to a positive control using a sample from a pure culture of the strain (data not shown). This finding indicates that the recovery of bacteria in plates was due to cross-contamination, but not translocation of the bacterial strain administered orally during the study.

Biochemical parameters analysis

To detect possible deleterious effects on different organs due to the oral administration of *B. uniformis* CECT 7771, biochemical parameters related to pancreatic, liver and kidney functions were analysed.

ALT levels were significantly reduced in the B_Unif9 and B_Unif10 groups compared to the control group ($p<0.05$ and $p<0.01$, respectively) (Figure 2. A). This pattern may indicate a dose-effect of *B. uniformis* CECT 7771 on ALT levels. When the serum values of ALT of males and females were analysed separately, it was observed that in males, the highest dose of *B. uniformis* CECT 7771 significantly reduced the levels of the enzyme compared to the control group

receiving the placebo, and those receiving *B. longum* ATCC 15707^{T} (P<0.05 in both comparisons) (Figure 2. C). A similar trend was observed for females, but the difference was not significant (Figure 2. B).

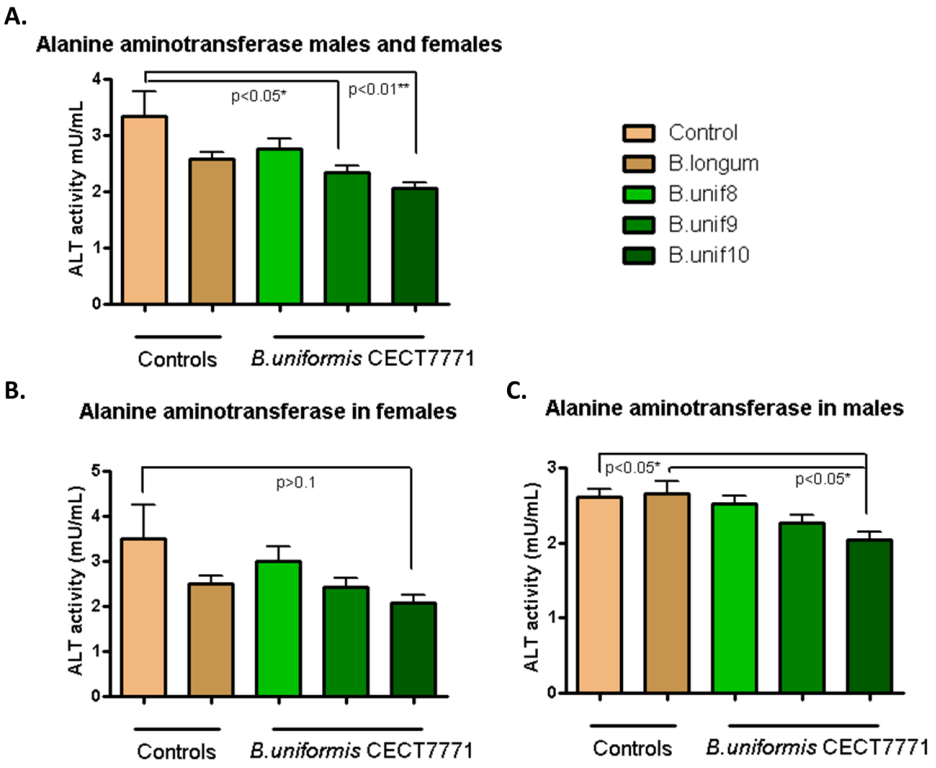

Figure 2. ALT activity measured in serum in all animal participants of the study, and in males and females analysed separately at the end of the safety assessment of *B. uniformis* CECT 7771. **A**, ALT activity (mU/mL) at the end of the study for all groups (50 animals, n = 10 per group); **B**, ALT activity (mU/mL) at the end of the study for females in all groups (25 animals, n = 5 per group); **C**, ALT activity (mU/mL) at the end of the study for males in all groups (25 animals, n = 5 per group). Measurements were made in duplicate. Statistical significance was considered when p-values < 0.05.

ALP concentrations (Supplementary figure 4. A), creatinine concentration (Supplementary figure 4. B) and urea levels (Supplementary figure 4. C) had no significant differences in all the groups studied, neither, if males and females of each group were considered independently (Supplementary figure 5).

The analysis of amylase activity showed similar values for all the groups (Figure 3. A). When males and females were considered independently, it was observed that females' differences were more remarkable, where amylase activity was significantly decreased in the B_Unif9 group of rats, compared to the *B. longum* group and the B_Unif8 ($p<0.05$ for both comparisons), but the differences were not consistent with the existence of a dose-response relationship (Figure 3. B). No differences were observed between all groups for males (Figure 3. C).

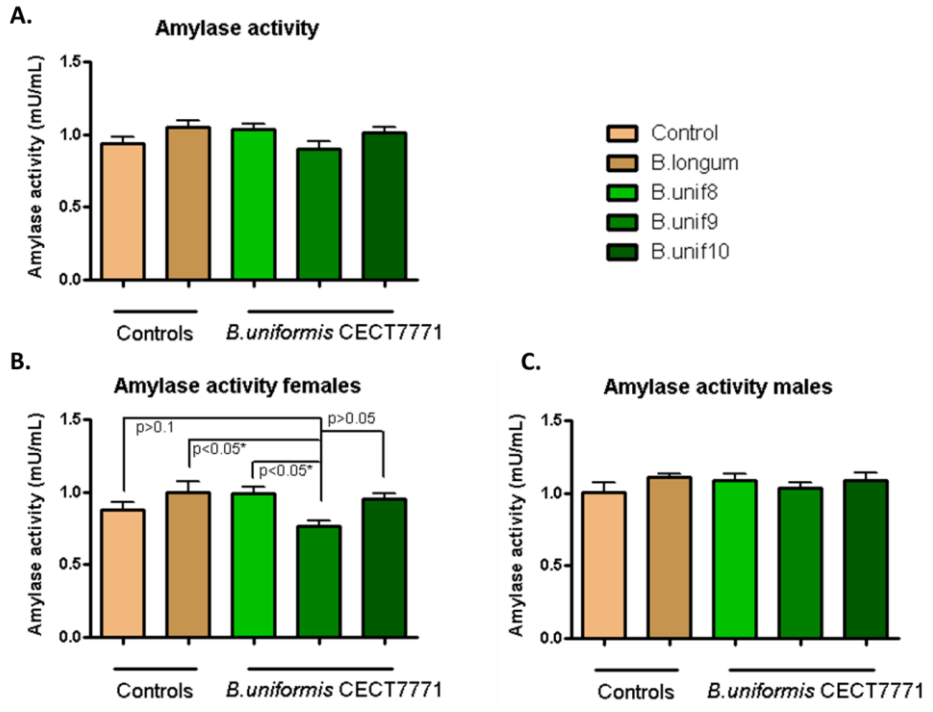

Figure 3. Amylase activity measured in serum in all animal participants of the study, and in males and females analysed separately at the end of the safety assessment of *B. uniformis* CECT 7771. **A**, amylase activity (mU/mL) at the end of the study for all groups (50 animals, n = 10 per group); **B**, amylase activity (mU/mL) at the end of the study for females in all groups (25 animals, n = 5 per group); **C**, amylase activity (mU/mL) at the end of the study for males in all groups (25 animals, n = 5 per group). Measurements were made in duplicate. Statistical significance was considered when a p-value < 0.05.

Determination of cytokine concentrations

The concentration of the anti-inflammatory cytokine IL-10 in serum was significantly reduced in the groups; *B. longum* and B_Unif9, compared to the control group ($p<0.05$ and $p<0.01$, respectively), while there were no statistically significant differences between the control group, B_unif8 and B_Unif10 ($p>0.05$

and p>0.1 respectively) (Figure 4. A). When males and females were analysed independently, it was observed that the IL-10 concentration had a significant increase (p<0.05) in the B._Unif10 group compared to the B_Unif9 group, in the female's serum (Supplementary figure 6. A). The IL-10 concentration of male's serum samples was statistically increased in the B_Unif10 group (p<0.05), compared to the groups *B. longum* and B_Unif8 (Supplementary figure 7. A).

The IL-10 concentration of jejunum samples showed similar levels in control and B_Unif10 groups, while the levels of the B_longum and B_unif9 groups were significantly lowered followed nearly by B_Unif8 (Figure 4. B), following similar patterns of the IL-10 concentration of serum samples. When males and females were analysed independently, it was observed that in the female's jejunum samples, statistical differences of IL-10 concentration were found between control and B_Unif9 group (p<0.01) and between B_Unif9 and B_Unif10 groups (p<0.01) (Supplementary figure 6. B). In the males' jejunum samples, the tendency of the cytokine concentrations was similar to those of the serum samples. B_longum group and B_Unif9 group had levels significantly reduced compared to control group (p<0.05) and compared to B_Unif10 group (p<0.01 and p<0.01, respectively) (Supplementary figure 7. B).

IFN-γ concentrations in serum samples were statistically significantly higher in the control group compared to *B. longum* and B_Unif9 groups (p<0.01) (Figure 4. C). When males and females were analysed independently, no significant differences were observed in females' serum IFN-γ concentrations (Supplementary figure 6. C) but the patterns were alike when considering both genres analysed together. In males' serum samples, IFN-γ was statistically reduced in the B_longum experimental group (p<0.05) compared to the control group (Supplementary figure 7. C).

IFN-γ concentration in jejunum samples was lower in *B. longum* and B_Unif9 groups than in the control group (p<0.01). A significant reduction in B_Unif9 was observed compared to B_Unif8 group (p<0.05). Differences were not detected between the B_Unif10, B_Unif8 group and the control group (Figure 4. D). When males and females were analysed separately, a reduction was found in the cytokine concentration in females' jejunum samples in all the study groups compared to the control group, but this was only statistically significant for the B_Unif9 group (p<0.01) (Supplementary figure 6. D). No differences were observed in jejunum males' samples for all the groups studied (Supplementary figure 7. D).

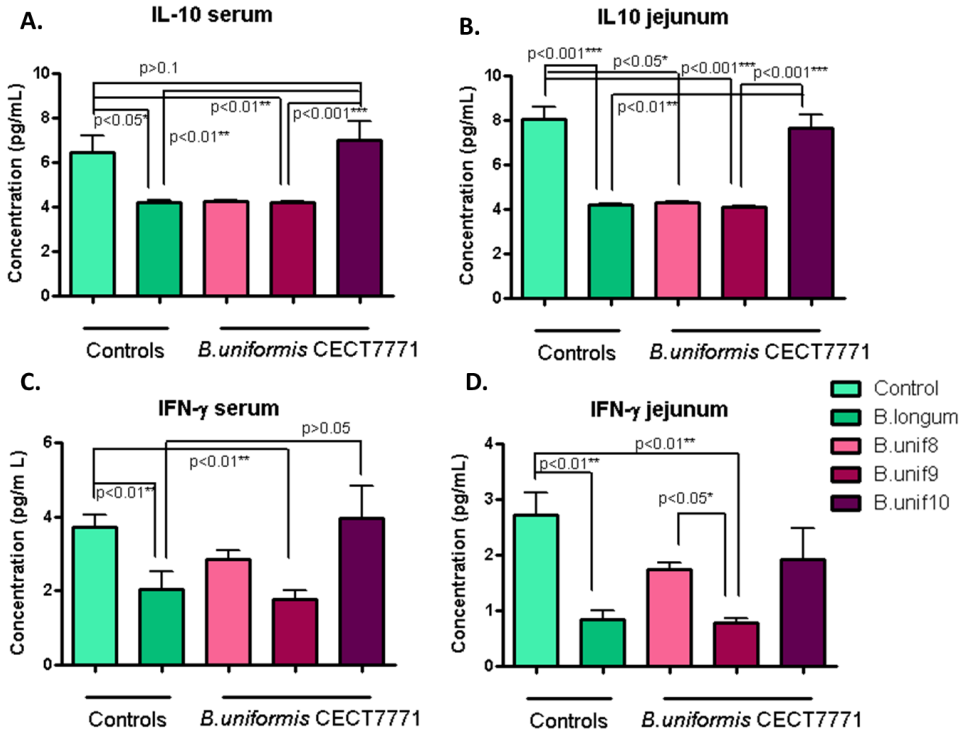

Figure 4. IL-10 and IFN- γ concentrations in serum and jejunum samples in all animal groups at the end of the safety assessment of *B. uniformis* CECT 7771. **A**, IL-10 serum concentration (pg/mL) at the end of the study for all groups (50 animals, n = 10 per group); **B**, IL-10 jejunum concentration (pg/mL) at the end of the study for all groups (50 animals, n = 10 per group); **C**, IFN-γ serum concentration (pg/mL) at the end of the study for all groups (50 animals, n = 10 per group); **D**, IFN- γ jejunum concentration (pg/mL) at the end of the study for all groups (50 animals, n = 10 per group). Measurements were made in duplicate. Statistical significance was considered when a p-value < 0.05.

As IL-10 was measured as a representative anti-inflammatory cytokine and IFN-γ as a pro-inflammatory cytokine, which regulates each other, when the ratio IL-10/IFN-γ was estimated to obtain more definitive information about the balance between anti- and pro-inflammatory responses due to the treatment [30-32], we observed significantly higher ratios in the groups
B. longum and B_unif9 in serum samples, compared to B_Unif8 group ($p<0.05$) (Figure 5. A). Groups B_Unif8 and B_Unif10 had similar levels to the control as it can be observed for the p-values ($p>0.05$).

When males and females IL-10/IFN-γ serum ratios were analysed separately, it was appreciated that in females, the ratio tended to be higher in the B_Unif9 group

than in the others, but no statistically significant differences were found (Supplementary figure 8. A). In males, the group *B_longum* showed the highest ratio, followed closely by the B_Unif9 group, although it was no significant differences compared to the other groups (Supplementary figure 8. B).

The ratios of IL-10/IFN-γ for jejunum samples had similar patterns to the serum samples, being significantly higher for groups B_Unif9 compared to control and B_Unif8 groups ($p<0.01$ for both comparisons) and followed nearly by B_longum group, although this group was only significantly higher compared to B_Unif8 group ($p<0.05$) (Figure 5. B).

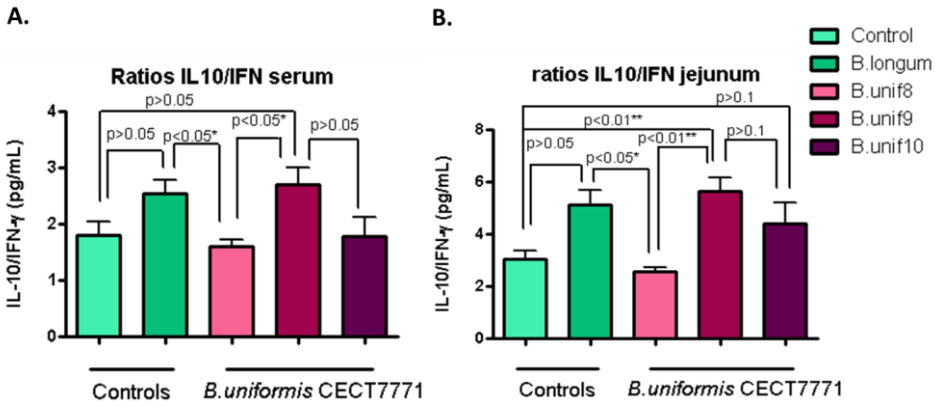

Figure 5. IL-10/IFN- γ ratios in serum and jejunum samples in all animal groups at the end of the safety assessment of *B. uniformis* CECT 7771. **A**, IL-10/IFN- γ ratios (pg/mL) in serum samples at the end of the study for all groups (50 animals, n = 10 per group); **B**, IL-10/IFN-γ ratios (pg/mL) in jejunum samples at the end of the study for all groups (50 animals, n = 10 per group). Measurements were made in duplicate. Statistical significance was considered when a p-value < 0.05.

When males and females were considered separately, it was observed that the females' jejunum ratios, the B_Unif9 group had the highest values (statistically significant compared to the control group, $p<0.05$), (Supplementary figure 8. C), followed by the B_Unif10 group. In the case of the males' jejunum samples, the highest values were detected in the *B. longum* group compared to control group ($p<0.05$), followed by B_Unif9 group, which was not significantly different from the control group ($p>0.1$) (Supplementary figure 8. D).

Cytokine levels of TNF-α were below the detection limit in serum and jejunum in all mouse groups for both males and females (data not shown).

Colon mucosal histology effects

The effect of the intervention in colon tissue structures is shown in Table 1. There were no significant differences in the parameters measured between the experimental groups when considering both males and females.

Table 1. Lieberkün crypts depth (µm) and a number of Goblet cells per crypt in all animal groups, males and females (25 animals, n = 5 per group), at the end of the safety assessment of *B. uniformis* CECT 7771. Samples were assessed from colon sections and measurements were taken in 10 fields for each sample. Statistical significance was considered when a p-value < 0.05.

Parameter	**Gender**	**Groups**				
		Control	**B.longum**	**B.unif8**	**B.unif9**	**B.unif10**
Lieberkühn crypts depth (µm)	*males*	218.49±15.49	208.79±18.20	211.08±9.75	197.66±16.60	205.09±10.83
	females	201.98±25.38	221.60±9.68	203.63±15.76	203.89±23.98	195.82±6.31
Number of goblet cells/crypt	*males*	19.05±4.07	20.91±3.42	18.03±1.59	19.21±3.75	17.83±3.27
	females	14.13±3.88	18.56±3.22	16.77±3.83	20.01±2.99	15.49±3.55

Statistical analyses were also performed for males and females of each experimental group separately. The female's numbers of goblet cells were slightly increased in B_unif9 and B_longum group compared to control ($p>0.05$ and $p>0.1$). (Table 1) and in the case of the crypt depth, no significant changes were observed between groups (Table 1). For males, no differences were found for crypt depth or number of Goblet cells (Table 1).

For all animals, the Lieberkühn crypts had a uniform and constant appearance (Figure 6) as well as the number of goblet cells in the crypts.

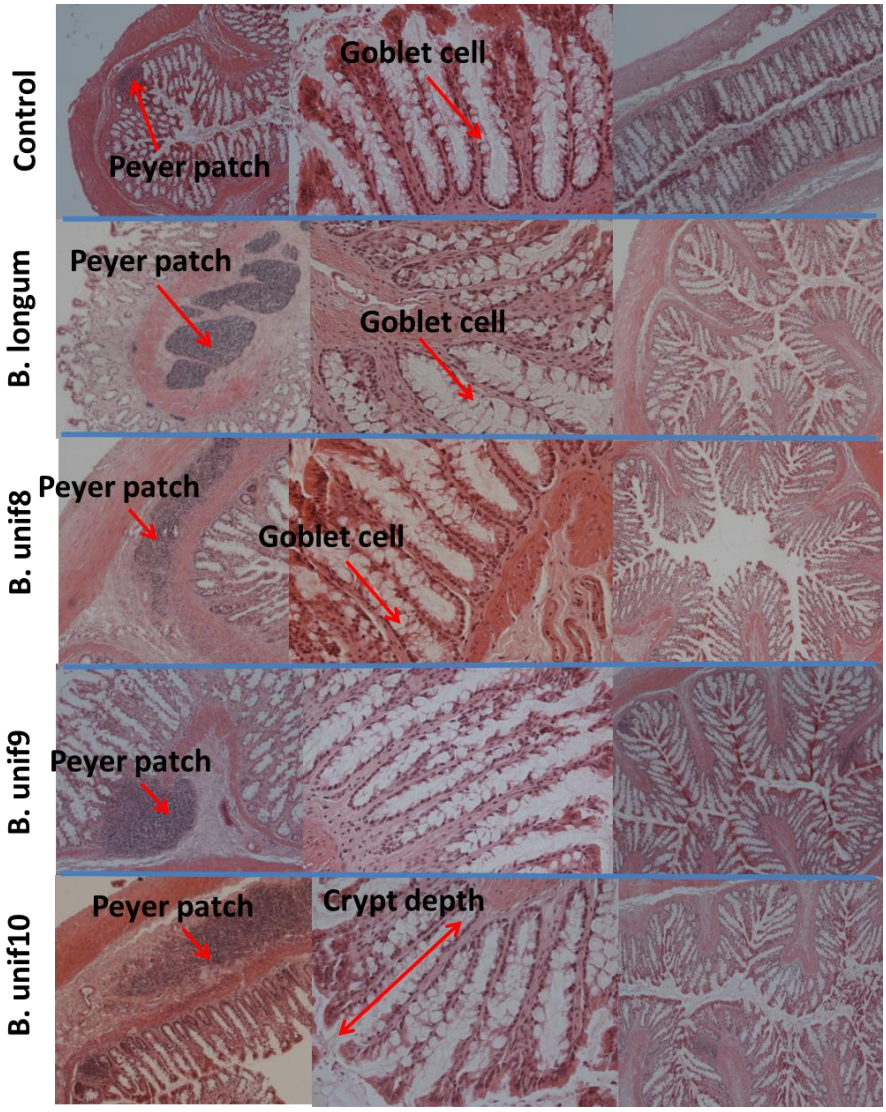

Figure 6. Histology of colon sections from rats of all experimental groups (n=50). Control group received a daily dose of 10% skimmed milk (n=10); B_longum group received a daily dose of 10^{10} CFU of *B. longum* ATCC 15707^{T} (n=10); B_Unif8 group received a daily dose of 10^{8} CFU of *B. uniformis* CECT 7771 (n=10); B_Unif9 group received a daily dose of 10^{9} CFU of *B. uniformis* CECT 7771 (n=10) and B_Unif10 group received a daily dose of 10^{10} CFU of *B. uniformis* CECT 7771(n=10). These measurements were taken in 10 fields for each sample. Photomicrographs 10X and 20X of representative HE-stained slides are shown, indicating *Goblet* cells, crypt depth and Peyer's patches in each group.

Effect of the intervention on the faecal microbiota composition

The intervention with the *B. uniformis* CECT 7771 in rats did not result in major changes in the number of bacterial species in the faecal microbiota. Accordingly, differences in the five common ecological descriptors used to assess alpha diversity, including the observed richness, Chao's richness, Shannon's diversity, Simpson's evenness, and Simpson's reciprocal index, were not detected when all groups were compared. However, the beta diversity analysis revealed significant changes in the structure of the microbial community as a result of the bacteroides administration. Accordingly, permanova test on distances obtained from Bray Curtis dissimilarity index (Pseudo-F= 1.197, p-value= 0.054) and Binary-Jaccard index (Pseudo-F=1.099, p-value=0.005) indicates that the different treatments induce different shifts in the gut microbial community of rats. We also found that microbial species belonging to the Christensenellaceae family were significantly increased by B_longum, B_unif10, and B_unif9 administration compared to control ($p < 0.05$). Additionally, member of the Bacillaceae family were found to be more abundant in B_longum and B_unif10 groups as well ($p < 0.05$). At genus level, we found that *Christensenella* species were more abundant in B_longum, B_unif9, and B_unif10 groups when compared to controls (p=0.017, p=0.05, and p=0.033, respectively) (Figure 7. A). The proportion of reads assigned to *Bacillus* species was observed to be significantly higher in B_longum and B_unif10 groups ($p < 0.05$) (Figure 7. B).

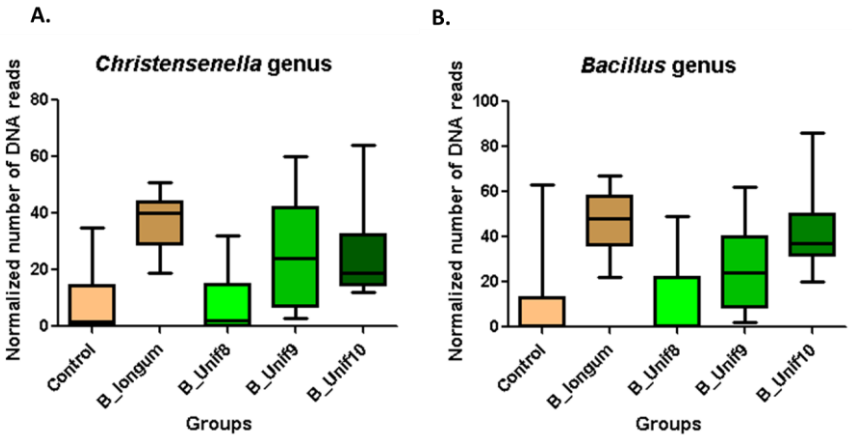

Figure 7. Normalized number of DNA reads for *Christensenella* and *Bacillus* genera for each group of rats. **A**, Normalized number of DNA reads for *Christensenella* genus for each experimental group of animals; **B**, Normalized number of DNA reads for *Bacillus* genus for each experimental group of animals.

At the OTU level, we detected consistent results with the taxonomy assessment at family and genus level. For instance, OTUs related to *Bifidobacterium* genus were more abundant in the B_longum group compared to the control group, showing more than 4 log2 fold-change. *Christensenella* associated OTUs were more abundant in the B_longum and B_Unif10 groups than in controls. Interestingly, in those groups a significantly increased in *Akkermasia muciniphila*-associated OTUs was also observed. Moreover, we observed an increase in *Bifidobacterium* associated OTUs in the B_Unif10 group as well. As expected, B_Unif9 and B_Unif10 groups presented an increased in OTUs closely related to *Bacteroides uniformis* species. Bacteroidales family S24-7 was also increased in these groups.

Discussion

Most of the commercialised probiotics available are still restricted to the genera *Lactobacillus* or *Bifidobacterium* (lactic acid bacteria), isolated from human or animal biological samples or derived from fermented foods, which have been widely consumed in many different countries and cultures due to its generally recognized safety status [33, 34]. The identification of new components of the intestinal microbiota, thanks to the development of more powerful technologies in genome and metagenome sequencing, as well as the advances in culturing methodologies, have increased the possibilities of evaluating the functional role of new indigenous intestinal bacteria that can constitute the so-called "second or next generation probiotics" [35]. Some of these new potential probiotics belong to the genera Bacteroides which may show different beneficial effects depending on the species and strain [36, 37]. Particularly, some of these species of Bacteroides are related to a lean phenotype in observational human studies [38, 39], although contradictions exist in the literature [40].

Breastfeeding has demonstrated a reduced risk for developing obesity and type-2 diabetes [41] and also seems to increase the abundance of *B. uniformis* in the faecal microbiota of healthy infants compared to those formula-fed[1, 20]. Therefore, a strain of this species, *B. uniformis* CECT 7771, was selected among the other human infant *Bacteroides* species and strains for its anti-inflammatory potential *in vitro* and was evaluated in mice with high-fat diet induced obesity [5].

The oral administration of *B. uniformis* CECT 7771 improved diet-induced metabolic dysfunction in obese mice. Given the potential use of this bacterial strain for obesity treatment in humans, a first evaluation of the safety of this strain was performed after acute oral administration to mice for 6 days [21]. As adverse effects were not observed in the acute study, we have completed the toxicological assessments by performing a 90-day study in Wistar rats.

The oral sub-chronic toxicity assessment showed that rats treated with high doses (up to $1x10^{10}$ CFU/day) of the potential probiotic strain *B. uniformis* CECT 7771 were healthy after daily oral administration for 90 days. A dose of $1x10^{10}$ CFU/day in rats is equivalent to $1.62x10^{9}$ CFU/day in humans when normalized by body surface area, that is 10 times higher than the effective dose for the probiotic. Throughout the study, no adverse effects were observed in body weight, food intake or other general indicators of the animals' health status (behaviour, lustre, etc.) because of *B. uniformis* CECT 7771 oral administration.

One of the issues that should be assessed, to indicate bacterial safety, is the possibility of bacterial translocation from the intestinal lumen to MLN and other

tissues that may constitute a risk for systemic infection (bacteraemia). Therefore, the possibility of translocation of the strain was studied and analysed as an indication of potential infection and pathogenicity [42]. Although most *Bacteroides* spp. are considered commensals, some strains of *B. fragilis* are opportunistic pathogens and have been involved in bacteraemia [43], which also justified the previous evaluation of the safety of *B. uniformis* CECT 7771 in an acute toxicity study where none translocation events could be associated with the treatment [21]. Likewise, no translocation of *B. uniformis* CECT 7771 to MLN or blood was detected in the Bacteroides-treated groups in the present study. Although some bacterial counts were found in MLN, these were detected in both controls and experimental groups, suggesting that this was due to cross-contamination as often described in other studies [44]. In addition, specific PCR reactions for the detection of the *Bacteroides uniformis* CECT 7771 glutamate decarboxylase gene in the isolated colonies were all negative, confirming that there was no translocation of the bacterium administered.

Biochemical parameters were observed in serum to detect potential adverse sub-clinical effects of *B. uniformis* CECT 7771 on the function of different organs. Urea and creatinine were used as markers of kidney function, since high levels of urea or creatinine may indicate renal failure, but adverse effects of *B. uniformis* CECT 7771 were not detected. The ALT and ALP activities are indicators of liver function [45, 46] and amylase of the pancreatic function. While ALP levels remained similar in all groups, ALT activity decreased with an increasing dose of the strain tested, which may indicate improvements in the liver function [46]. Further studies would, however, be necessary to confirm this hypothesis. In relation to serum amylase, the group that received the dose of $1x10^9$ CFU of *B. uniformis* CECT 7771 was the one that presented the lower levels of the enzyme in females, with no relevant changes in males.

As expected, cytokine concentrations were generally low (IL-10 and IFN- γ) and TNF-α was below the detection limit in all experimental groups. This indicates that there is no induction of an inflammation state due to bacterial administration. The IL-10/IFN- γ ratio was used as an indicator of the balance between the anti- and pro-inflammatory response to the treatment. The results suggest that *B. uniformis* at 10^9 CFU/day as well as *B. longum* at 10^{10} CFU/day induced an anti-inflammatory cytokine profile, particularly in the intestine. Similar response trends could be identified in male and female when considered separately, although there were subtle differences most probably due to the modest sample size of the sub-groups rather than the different sex. None of the treated groups had ratio values statistically lower than the control group receiving the placebo, indicating that bacteria administration did not increase the inflammatory response in any case.

The sub-chronic administration of *B. uniformis* CECT 7771 to rats slightly increased the number of goblet cells in B_unif9 and *B. longum* groups, when considering all animals or females groups alone. Similar results were obtained as in the previous acute safety assessment of *B. uniformis* CECT 7771 performed by our group [21], as well as in the case of strains of *Bifidobacterium* and *Propionibacterium* investigated by other authors [47, 48]. The increase of goblet cells numbers could improve the mucus production and coating, which constitutes a first defence barrier against pathogens, before the activation of the immune system.

The analysis of the composition of rats' faecal microbiota showed no changes in richness and diversity between groups. Reductions in this parameter has often been associated with different disorders such as inflammatory bowel diseases [49] and obesity and co-morbidities in other studies [50]. Notwithstanding, we observe changes in the global structure of the microbiota of between groups according to the beta diversity analysis. These differences were probably due to the differential abundances detected in particular taxonomy clades among groups. For example, *B. longum* ATCC 15707T intake induced an increase in *Bifidobacterium* species, which indicate that this bacterial species could transiently colonize or reach the large intestine. A similar effect was observed in the B_Unif9 and B_Unif10 groups where a significant increase of *B. uniformis* associated OTUs was detected as a result of the treatments. This last observation suggests that these bacterial dose of *B. uniformis* CECT 7771 may be necessary for a successful transient colonization of the rat intestine (10^9 and 10^{10} CFU/day) since differences were not detected at a lower concentration. Interestingly, *Christensenella* and *Akkermansia muciniphila* species were increased in B_Unif10 group. Both species have been proven to exert beneficial effects in pre-clinical studies related to obesity or associated with health metabolic phenotypes in human observational studies [51, 52]. Our results suggest a potential interaction among such species that could also contribute to some of the beneficial effects attributed to B. uniformis CECT 7771 in disease models [5]. Therefore, future studies should be conducted to demonstrate the metabolic cross-talk among those microbial species.

In conclusion, the results of the safety evaluation of sub-chronic oral administration of *B. uniformis* CECT 7771 to rats for 90 days did not raise safety concerns. No adverse effects were recorded regarding general health indicators, gut mucosal histology, bacterial translocation, organ function or immune markers.

Analysis of faecal microbiota indicate that *B. uniformis* CECT 7771 as well as the bacterium use as QPS control (*B. longum* ATCC 15707T) can reach the large intestine and modulate the gut microbiota composition but with no adverse effects on richness and diversity. The results also indicate improvements in markers related to mucosal protection (e.g. increased goblet cell numbers and anti-/pro-

inflammatory cytokine ratio) and liver function as a result of *B. uniformis* CECT 7771 administration. Further studies are required to confirm the safety and effectivity of this bacterial strain in humans.

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Author's contributions

EMGP and YS designed the study; EMGP conducted experimental work; ABP and EMGP did the statistical and bioinformatic analyses; EMGP draft the paper; all authors critically reviewed the manuscript and approved the final version.

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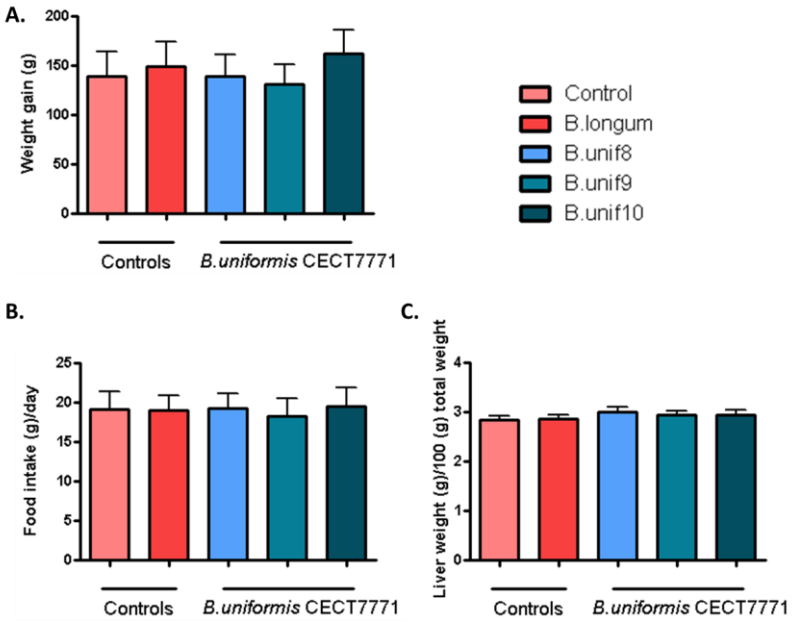
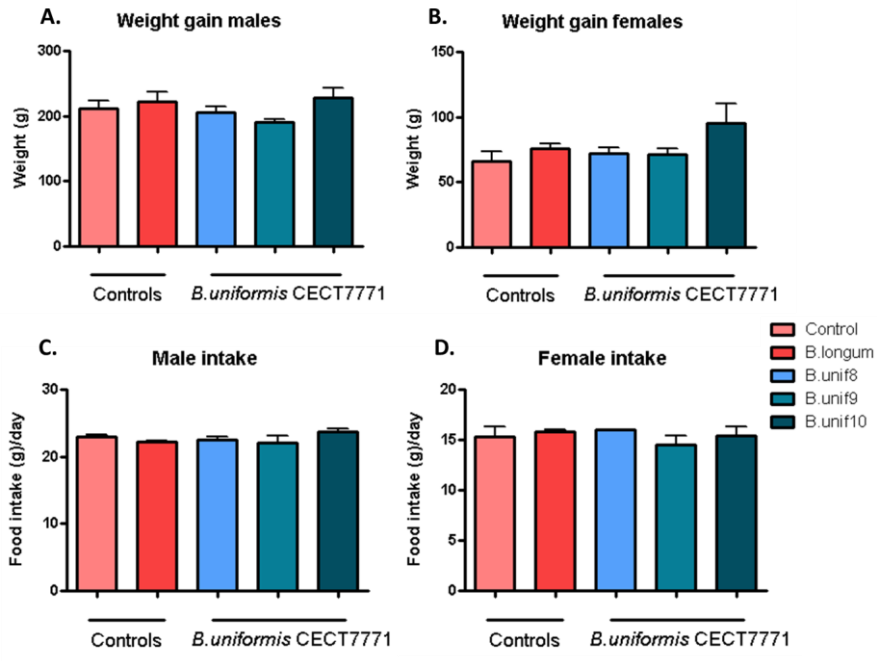
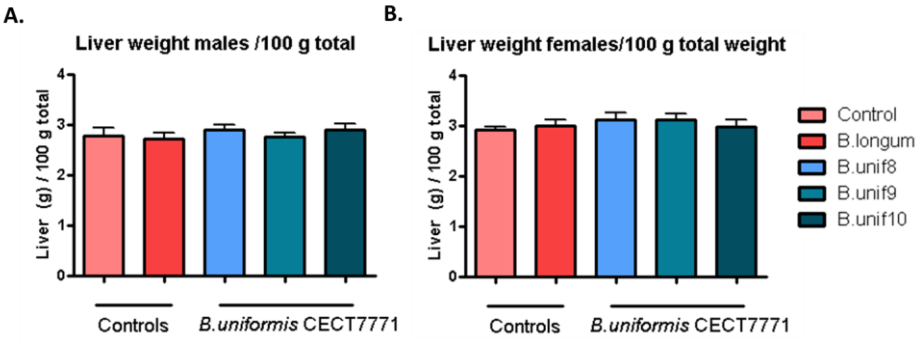


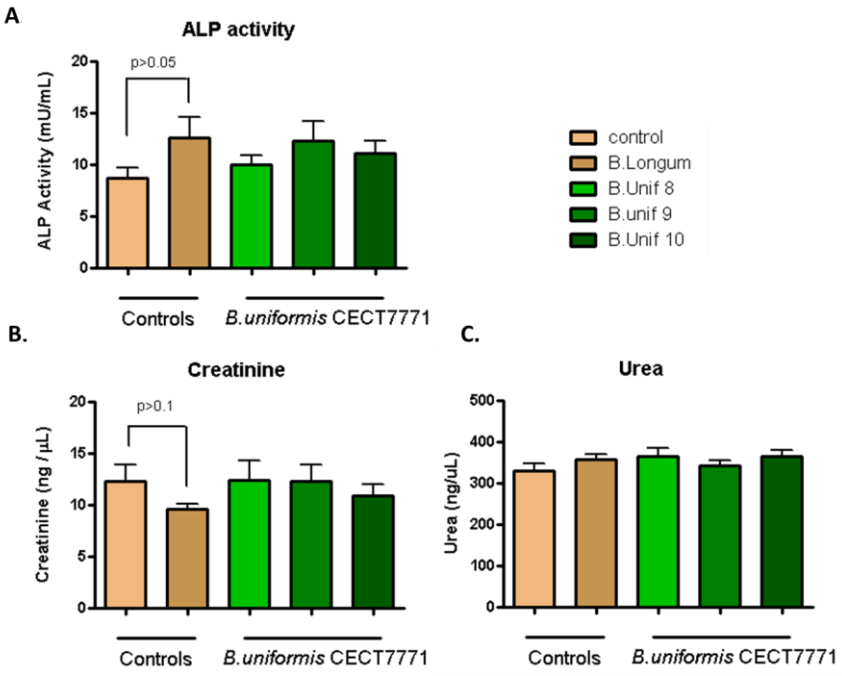
Supplementary figure 1. Body weight gain, food intake and liver tissue weight during the safety assessment of *B. uniformis* CECT 7771. **A,** total body weight gain (g) during the 90 days of assay for all animal participants of the study (50 animals, n = 10 per group); **B,** daily food intake (g) during the 90 days of assay for all animal participants of the study (50 animals, n = 10 per group); **C**, liver weight (g) normalized by 100 g of body weight at the end of the study for all animal participants in the experiment (50 animals, n = 10 per group).



Supplementary figure 2. Body weight gain and food intake during the safety assessment of *B. uniformis* CECT 7771 for males and females analysed separately. **A,** total body weight gain (g) during the 90 days of assay for male participants of the study (25 animals, n = 5 per group); **B,** total body weight gain (g) during the 90 days of assay for female participants of the study (25 animals, n = 5 per group); **C,** daily food intake (g) during the 90 days of assay for male participants of the study (25 animals, n = 5 per group); **D**, daily food intake (g) during the 90 days of assay for female participants of the study (25 animals, n = 5 per group).

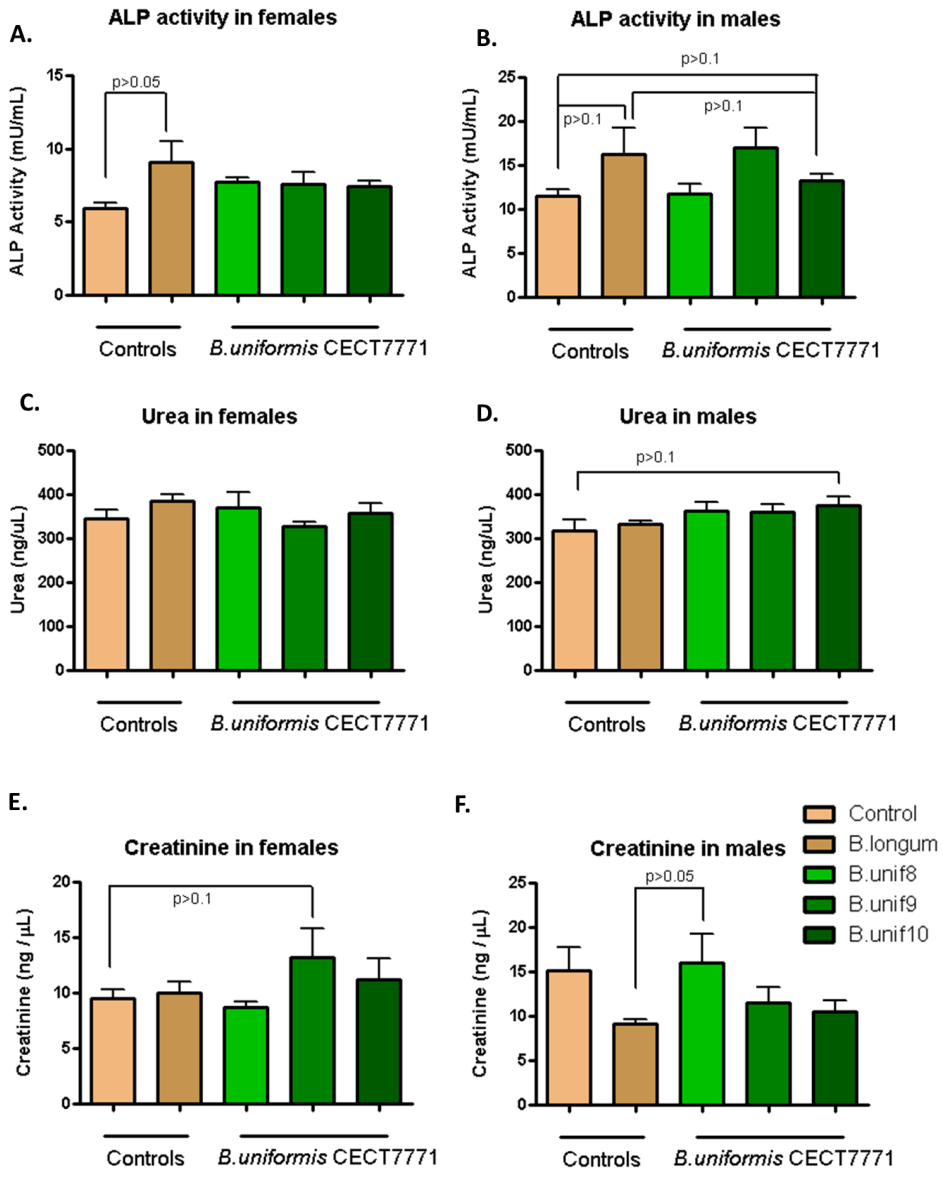


Supplementary figure 3. Liver weight normalized by 100 g of body weight at the end of the study for males and females separately. **A**, liver weight (g) normalized by 100 g of body weight at the end of the study for males (25 animals, n = 5 per group); **B**, liver weight (g) normalized by 100 g of body weight at the end of the study for females (25 animals, n = 5 per group).



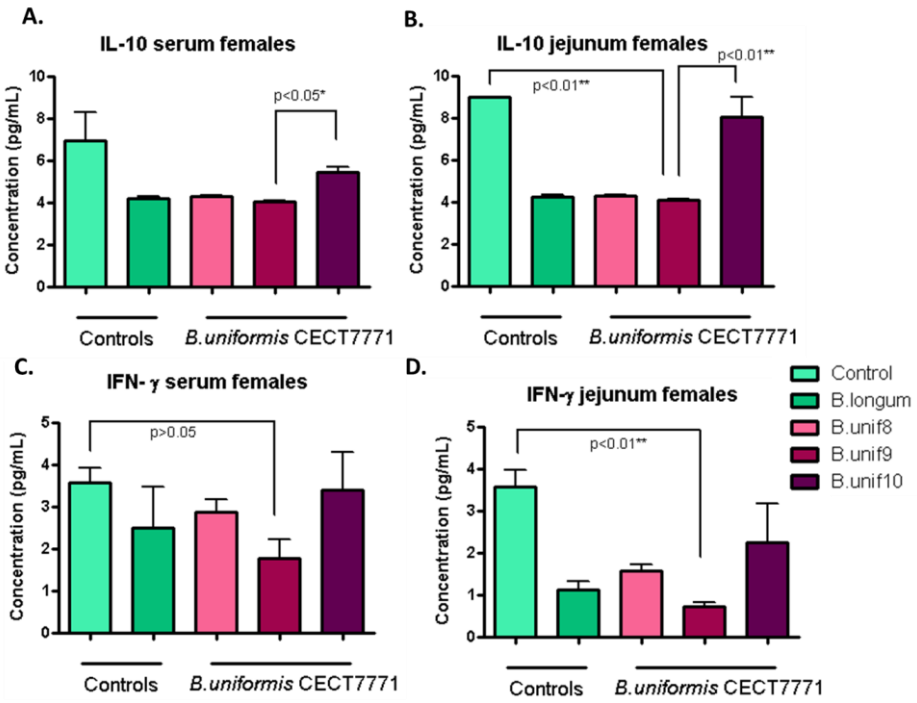
Supplementary figure 4. ALP activity, creatinine and urea concentrations measured in serum in all animal participants of the study at the end of the safety assessment of *B. uniformis* CECT 7771. **A**, ALP activity (mU/mL) at the end of the study for all groups (50 animals, n = 10 per group); **B**, creatinine concentration (ng/µL) at the end of the study for all groups (50 animals, n = 10 per group); **C**, urea concentration (ng/µL) at the end of the study

for all groups (50 animals, n = 10 per group). Measurements were made in duplicate. Statistical significance was considered when a p-value < 0.05.

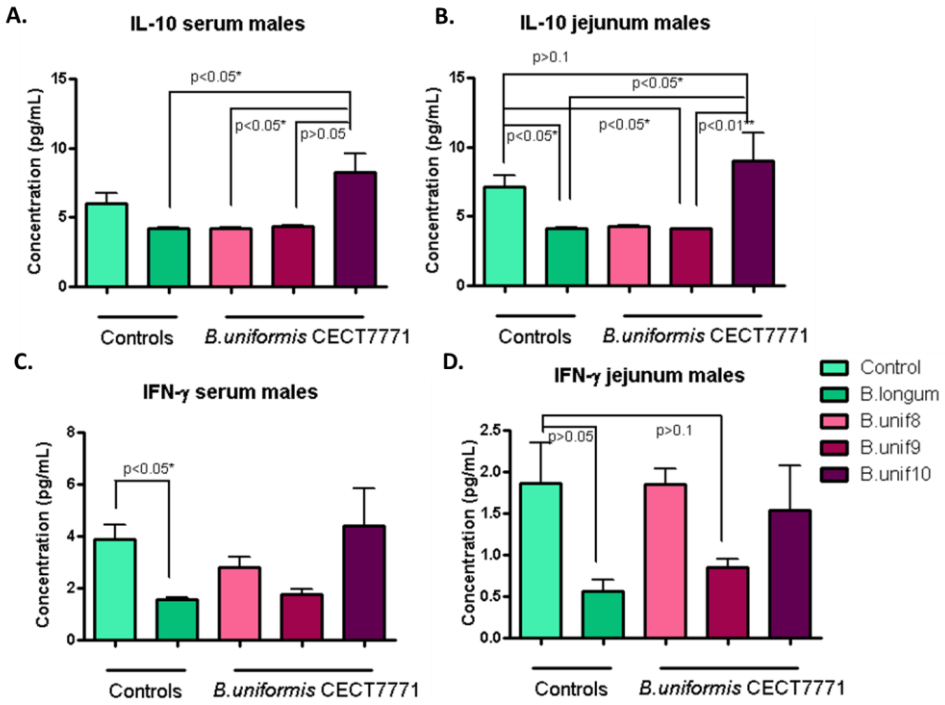


Supplementary figure 5. ALP activity, creatinine and urea concentrations measured in serum in male and female participants of the study at the end of the safety assessment of *B. uniformis* CECT 7771. **A**, ALP activity (mU/mL) at the end of the study for females in all groups (25 animals, n = 5 per group); **B**, ALP activity (mU/mL) at the end of the study for

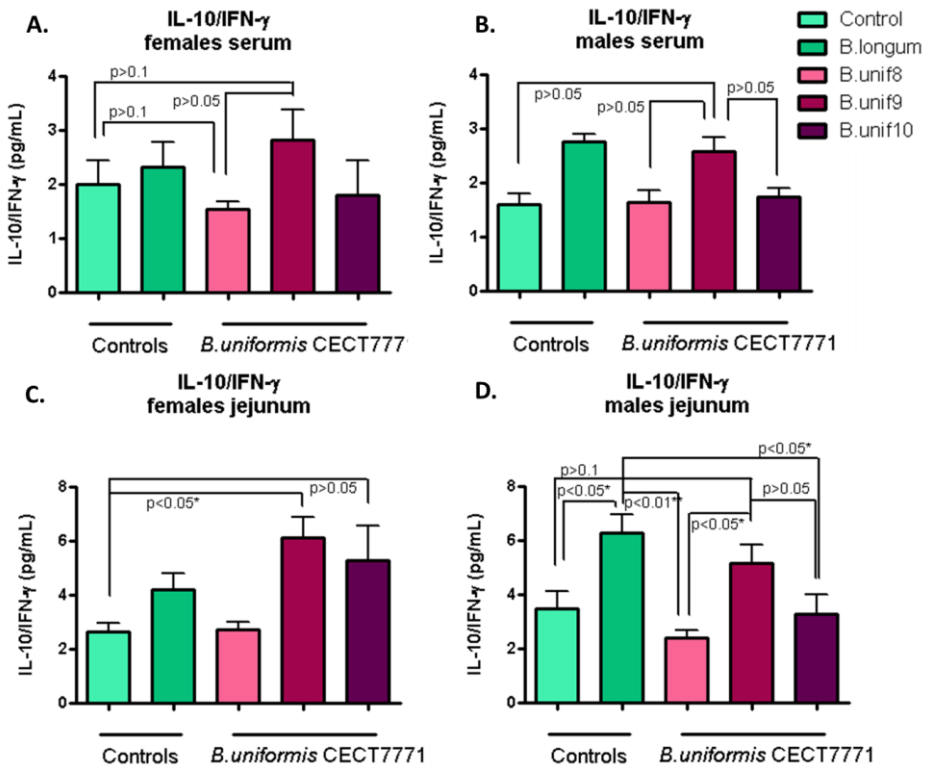
males in all groups (25 animals, n = 5 per group); **C**, urea concentration (ng/µL) at the end of the study for females in all groups (25 animals, n = 5 per group); **D**, urea concentration (ng/µL) at the end of the study for males in all groups (25 animals, n = 5 per group); **E**, creatinine concentration (ng/µL) at the end of the study for females in all groups (25 animals, n = 5 per group); **F**, creatinine concentration (ng/µL) at the end of the study for males in all groups (25 animals, n = 5 per group). Measurements were made in duplicate. Statistical significance was considered when a p-value < 0.05.



Supplementary figure 6. IL-10 and IFN- γ concentrations in serum and jejunum samples in females for all animal groups at the end of the safety assessment of *B. uniformis* CECT 7771. **A**, IL-10 serum concentration (pg/mL) at the end of the study for females in all groups (25 animals, n = 5 per group); **B**, IL-10 jejunum concentration (pg/mL) at the end of the study for females in all groups (25 animals, n = 5 per group); **C**, IFN- γ serum concentration (pg/mL) at the end of the study for females in all groups (25 animals, n = 5 per group); **D**, IFN- γ jejunum concentration (pg/mL) at the end of the study for females in all groups (25 animals, n = 5 per group). Measurements were made in duplicate. Statistical significance was considered when a p-value < 0.05.



Supplementary figure 7. IL-10 and IFN- γ concentrations in serum and jejunum samples in males for all animal groups at the end of the safety assessment of *B. uniformis* CECT 7771. **A**, IL-10 serum concentration (pg/mL) at the end of the study for males in all groups (25 animals, n = 5 per group); **B**, IL-10 jejunum concentration (pg/mL) at the end of the study for males in all groups (25 animals, n = 5 per group); **C**, IFN- γ serum concentration (pg/mL) at the end of the study for males in all groups (25 animals, n = 5 per group); **D**, IFN- γ jejunum concentration (pg/mL) at the end of the study for males in all groups (25 animals, n = 5 per group). Measurements were made in duplicate. Statistical significance was considered when a p-value < 0.05.



Supplementary figure 8. IL-10/IFN- γ ratios in serum and jejunum samples for males and females separately in all animal groups at the end of the safety assessment of *B. uniformis* CECT 7771. **A**, IL-10/IFN- γ ratios (pg/mL) in serum samples at the end of the study for females in all groups (25 animals, n = 5 per group); **B**, IL-10/IFN- γ ratios (pg/mL) in serum samples at the end of the study for males in all groups (25 animals, n = 5 per group); **C**, IL-10/IFN- γ ratios (pg/mL) in jejunum samples at the end of the study for females in all groups (25 animals, n = 5 per group); **D**, IL-10/IFN- γ ratios (pg/mL) in jejunum samples at the end of the study for males in all groups (25 animals, n = 5 per group). Measurements were made in duplicate. Statistical significance was considered when a p-value < 0.05.

CAPÍTULO 2

CAPÍTULO 2

Estudio de las interacciones entre la composición estructural y funcional de la microbiota intestinal, la dieta y la obesidad y sus co-morbilidades.

- Arabinoxylan oligosaccharides and polyunsaturated fatty acid effects on gut microbiota and metabolic markers in overweight individuals with signs of metabolic syndrome: a randomized cross-over trial.

- A multi-omics approach to unraveling the microbiome-mediated effects of arabinoxylan-oligosaccharides in overweight humans.

Arabinoxylan oligosaccharides and polyunsaturated fatty acid effects on gut microbiota and metabolic markers in overweight individuals with signs of metabolic syndrome: a randomized cross-over trial

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(Under review in ***Clinical Nutrition***)

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Abbreviations:

ALAT, alanine aminotransferase; ApoB, apolipoprotein B; ASAT, aspartate aminotransferase; AX, arabinoxylan; AXOS, arabinoxylan oligosaccharides; BMI, body mass index; BP, blood pressure; BW, body weight; CHO, cholesterol; CID, clinical investigation day; CPM, counts per minutes; DHA, docosahexaenoic acid; DNA, deoxyribonucleic acid; EPA, eicosapentaenoicacid; E%, energy percentage; HDL, high density lipoprotein; HOMA-β, homeostatic model assessment - beta-cell function; HOMA-IR, homeostatic model assessment -insulin resistance; hsCRP, high sensitive C-reactive protein; LDA, Linear Discriminant Analysis; LDL, low density lipoprotein; LMM, Linear Mixed Model; OTU, Operational Taxonomic Units; PC, principal coordinate; PCoA, Principal Coordinate Analysis; PCR, Polymerase chain reaction; PUFA, polyunsaturated fatty acids; REE, resting energy expenditure; rRNA, ribosomal ribonucleic acid; SFA, saturated fatty acid; TG, triglycerides; VAS, visual analogue scale; VLDL, very low density lipoprotein; WBE, wheat bran extract

Clinical trial registry (https://www.clinicaltrials.gov/): NCT02215343

Ethical committee: H-4-2014-052

The Danish Data Protection Agency: 2013-54-0522

Abstract

Background & Aims: Gut microbiota composition has been linked to obesity and the metabolic syndrome. Knowledge of specific nutrients and doses required to obtain a dietary modulation of gut microbiota that beneficially influence components of the metabolic syndrome is lacking. The aim of this study was to investigate diet-induced effects on the gut microbiota and on metabolic markers in overweight individuals with indices of the metabolic syndrome. Furthermore, to understand if the diet-induced modulation of the gut microbiota mediates changes in metabolic risk markers.

Methods: A twelve-week study was conducted as a randomized cross-over trial with two diet periods separated by a washout period (3×4 weeks). The dietary intakes of interest were wheat bran extract, rich in arabinoxylan oligosaccharides (AXOS) (15 g/d AXOS) and polyunsaturated fatty acids (PUFA) provided in fish oil capsules (3.6 g/d n-3 PUFA). Before and after intervention periods, weighted dietary records, fecal and blood samples as well as anthropometric data were collected. Gastrointestinal symptoms were evaluated weekly during the diet periods. Gut microbiota composition was analyzed by 16S ribosomal RNA gene amplification of its V3-V4 hypervariable regions.

Results: Twenty-seven participants completed the study (90%). Intake of AXOS induced changes in the gut microbiota composition resulting in a higher abundance of Bifidobacterium and butyrate producing bacterial species. Beta-diversity analysis indicated that the structure of the gut microbiota changed as a result of the AXOS intervention. No changes in metabolic markers were observed after any of the interventions.

Conclusions: AXOS intake has bifidogenic effects and also increases butyrate producers in the gut microbiota; even though this type of dietary fiber did not modulate lipid or glucose metabolic parameters related to metabolic syndrome. PUFA intake did not induce any notable effect on the gut microbiota composition or metabolic risk markers.

Introduction

Obesity is a global health problem (1) and presents a major health risk, as it can lead to a wide range of diseases including type II diabetes and cardiovascular diseases. The increase in health risk is often attributed to the metabolic syndrome that is a cluster of metabolic risk markers including abdominal obesity, impaired glucose metabolism, dyslipidemia and hypertension (2). Worldwide, it has been estimated that approximately one-fourth of the adult human population has the metabolic syndrome (3) and that 3.4 million deaths were caused by overweight and obesity in 2010 (4). Thus, effective strategies to reduce obesity and obesity-related morbidity and mortality are needed in order to be implemented by the public health systems.

Gut microbiota has been associated with obesity (5-7) as well as type II diabetes (8;9) and cardiovascular disease (10). Thus a change in the gut microbiota composition may have the potential to confer improvements in host health and to reduce risk for obesity-associated chronic metabolic diseases. Fecal microbiota transplantation has been suggested to change microbiota composition with subsequent improvement in metabolic markers in an experimental human study (11).

Another more accessible possibility is to modulate the gut microbiota by changing the diet (12;13). In the long term, it may improve health and lower the risk for development of type II diabetes and cardiovascular disease, but our knowledge is still too limited to establish microbiota-based dietary recommendations. In recent years, a vast amount of studies clearly indicate that diet is one of the main environmental factors modulating the gut microbiota. In particular, dietary fiber has been shown to exert a deep impact on gut microbiota structure and function, increasing the abundance of those bacteria specialized in the utilization of fiber as an energy source. Dietary fiber such as wheat bran extract (WBE) is rich in arabinoxylan oligosaccharides (AXOS), which are conceived as prebiotics given their ability to stimulate the growth of bifidobacteria (14). In fact, the AXOS breakdown to arabinose and xylose monomers occurs by microbes such as *Bifidobacterium* species (14). Indeed, previous dietary interventions with AXOS have shown to increase the abundance of the *Bifidobacterium* genus in the human gut microbiota (15-18). In this regard, the AXOS based dietary supplementation deserves special consideration for health maintenance given that bifidobacteria are regarded as healthy microbes with the potential to improve host health, partly via generation of short-chain fatty acids (SCFAs), such as butyrate via cross-feeding mechanisms with butyrate producing bacteria (19). In acute studies intakes of AX and AXOS have shown that overnight AXOS intake may improve glucose metabolism and AX intake reduces the postprandial glucose peak (20;21).

On the other hand, it is known that intake of polyunsaturated fatty acids (PUFA) especially long-chain n-3 PUFAs such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are beneficial for human health (22), as reflected in dietary recommendations (23). Interestingly, a limited number of human studies have indicated that the specific fat subtype could affect microbiota composition (24; 25). The possible link between PUFA intake, gut microbiota composition and metabolic markers remains to be investigated.

We hypothesize that a change in dietary intake can modulate the gut microbiota and, thereby, contribute to improving lipid or glucose metabolic dysfunctions in overweight and obese individuals. With this study we aimed to test how two dietary interventions, WBE with a high AXOS content and fish oil rich in long chain n-3 PUFA, modulate the gut microbiota and metabolic risk markers in overweight individuals who in addition to central obesity fulfil one criterion for the metabolic syndrome.

Materials and Methods

Study design

The study had a cross-over design with two diet periods of each 4 weeks separated by a 4-week washout period (Figure 1). After inclusion, at the screening visit, the participants were randomized to the sequence of the two diets (allocation ratio of the two sequences was 1:1) and all visits were planned to be conducted within a window of ±3 days. The computer-based randomization list was generated at *randomization.com*. Due to the study design blinding of project staff and participants was not possible.

Data were collected on 4 clinical investigation days (CID) during the study: baseline (week 0), after first dietary intervention period (week 4), after washout (before second diet period) (week 8), and after the second dietary intervention period (week 12). Furthermore, the participants had two dietician consultations (week 2 and week 10) where body weight (BW) (non-fasting) and diet were evaluated to ensure BW maintenance. The dietician called the participants in the remaining weeks (week 1, 3, 9 and 11) (Figure 1). Prior to each CID, the participants consumed a standardized dinner in the evening followed by a fasting period of minimum 8 hours. The study was conducted at the Department of Nutrition, Exercise and Sports, University of Copenhagen from August 2014 to June 2015. The study is registered at Clinical Trial (NCT02215343), conducted according to the guidelines laid down in the Declaration of Helsinki and was carried out in accordance with the ethical standards of the responsible regional committee on human experimentation in Denmark, registered asH-4-2014-052, and the Danish Data Protection Agency (2013-54-0522).

Study participants

The participants were recruited through the web-pages (*http://forsøgsperson.dk* and *http://nexs.ku.dk*), social media and newspapers. Informed consent was obtained after the participant had obtained written and spoken information. Participants received either 4,000 d.kr (~$600) or five meetings with a dietician as compensation for their participation.

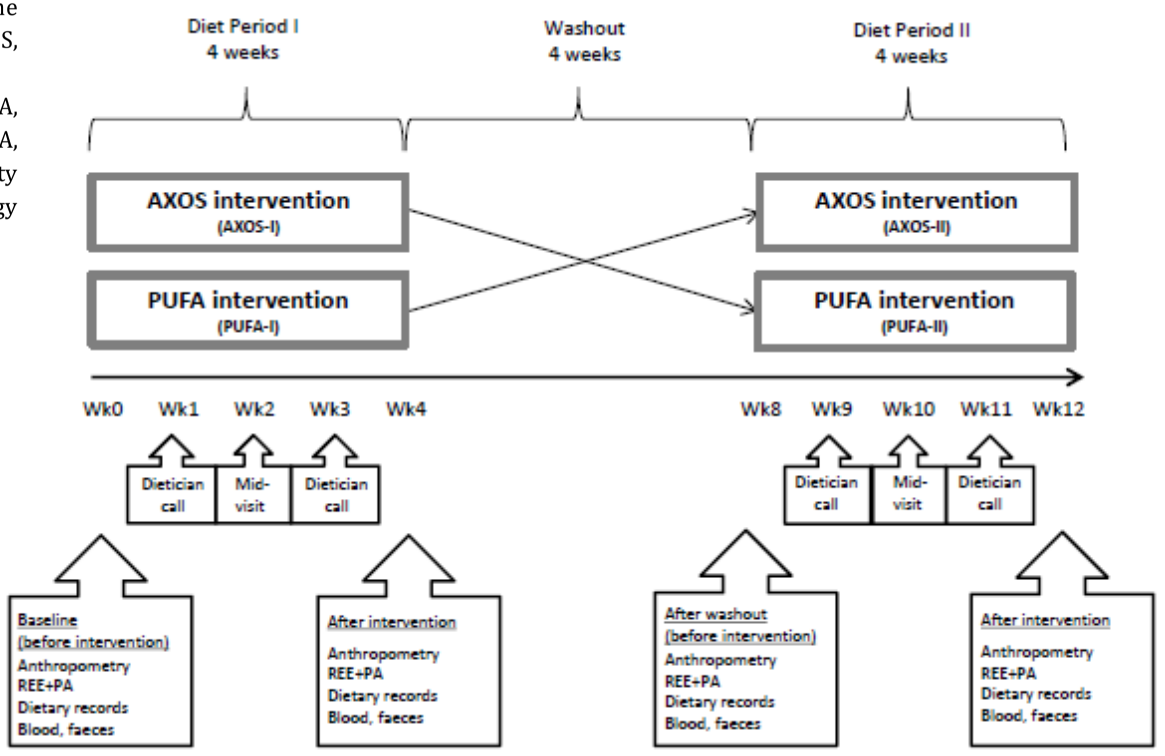

Figure 1. Diagram of the study design AXOS, arabinoxylan oligosaccharides; PA, physical activity; PUFA, polyunsaturated fatty acids; REE: resting energy expenditure, Wk., week

Eligible men and women were 18-60 years and a body mass index (BMI) of 25-40 kg/m^2 at screening. Furthermore, participation required a waist circumference ≥94 cm for men and ≥80 cm for women plus at least one of the following criteria for metabolic syndrome (3); raised triglycerides (TG) (≥1.7 mmol/L), reduced high density lipoprotein (HDL) cholesterol (CHO) (men: <1.03 mmol/L, women: <1.29 mmol/L), raised fasting plasma glucose (≥5.6 mmol/L) or raised blood pressure (BP) (systolic BP ≥130 mmHg or diastolic BP ≥85 mmHg). At screening, blood measurements were evaluated from a finger prick test (Lipid Pro™, infopia Co., Ltd). Additionally, a hemoglobin concentration ≥7 mmol/L was a requirement for inclusion. Women were required to be non-pregnant, non-lactating and not planning pregnancy during the study.

Exclusion criteria were: use of antibiotics three months prior to and during the study, medication related to dyslipidemia, type II diabetes or elevated BP. Furthermore, individuals were not allowed to take dietary supplements with pro- and/or prebiotics, fiber or fish oil six weeks prior to study start. Individuals with special dietary habits including vegetarians and vegans or a known food allergy for components of the study supplements (e.g. wheat, milk etc.) were excluded. Other exclusion criteria were smoking and BW change of ±3 kg two months prior to study start. Excluded were also elite athletes or those with intensive physical training (>10 hours of strenuous physical activity per week) as well as those donating blood one month before study start. Additionally, individuals with gastrointestinal and liver diseases, chronic inflammatory disorders (excluding obesity), psychiatric disorders including treatment required depression, surgical treatment of obesity as well as abdominal surgery were excluded. Individuals unable to comply with the procedures required by the study protocol were excluded.

Intervention

The goal of the AXOS period was to reach an intake of approximately 30 g fiber per day. AXOS was delivered partly as a powder supplement to dissolve in water twice a day and partly as 4 biscuits/crackers per day, nutritional information is provided in Table 1. By providing 15 g WBE per day, 11.2 g of total fiber was administrated to the participants' of which 10.4 g corresponded to AXOS fiber (Table 1). The remaining fiber intake was obtained from the participants' habitual diet and ensured by supervision from a dietician. The goal of the PUFA period was to reach a daily PUFA intake of approximately 10 E% by increasing the intake of PUFA including n-3 fatty acids and lowering saturated fatty acid (SFA) intake. The participants' diet was supplemented with fish oil capsules (~228 kJ/d) containing 3.6 g/dn-3 PUFA (1.32 g/d DHA and 1.86 g/d EPA). Furthermore, the dietician provided individual dietary advices based on the habitual intake of the

participants. During the two diet periods the participants were instructed to maintain their BW. Thus, the dietician guided the participants to iso-calorically substitute food items from their habitual diet with the dietary supplements and to avoid products containing pro- and prebiotics. The participants had weekly contact with the dietician.

Table 1: Characterization of WBE and AXOS supplements consumed during the AXOS intervention.

	WBE (per 100g)	**Powder (5g WBE)**	**Crackers (per piece)**	**Biscuits (per piece)**	**Total daily intake[1]**
Energy (kJ)	812[2]	41[2]	125	162	655
Protein (g)	0.7	0.04	0.6	0.5	2.3
Fat (g)	0	0	0.7	1.8	5.0
Carbohydrates (g)	19.7	1.0	4.8	4.8	21.2
Of which sugar (g)	3.8	0.2	0.7	1.7	5.2
Fiber (g)	72.0[3]	3.6	1.0	1.0	11.2
WBE (g)	100	5	1.3	1.2	15.0
AXOS[4] (g)	69	3.5	0.9	0.8	10.4

[1]Total daily intake: 2 powder, 2 crackers and 2 biscuits
[2]Calculated value
[3]Measured by methods approved by Association of the Official Analytical Chemists(AOAC)2009.01
[4]Average degree of polymerization was 5, Arabinose/Xylose ratio was 0.24, ash content 0.20% and moisture 3.4%
AXOS, arabinoxylan oligosaccharides; WBE, wheat bran extract

Outcomes

The primary outcome of the interventions was to detect changes in the gut microbiota composition. Secondary outcomes to obtain were changes in fasting glucose, insulin and blood lipid concentration (total CHO, LDL-CHO, HDL-CHO, VLDL-CHO, TG), and in the inflammatory markers such as high sensitive C-reactive protein (hsCRP), white blood cell, aspartate aminotransferase (ASAT), and alanine aminotransferase (ALAT). In addition, BP, energy expenditure, and gastrointestinal function were included as exploratory outcomes.

Anthropometry: Participants voided their bladder before anthropometric measurements were collected. BW was measured with the participant in their underwear by a digital scale (Lindells, Malmo, Sweden) approximated to the nearest 0.1 kg. Height was measured twice at screening to the nearest 0.5 cm using a wall mounted stadiometer (Hultafors, Sweden) and the average of the two measurements was recorded. BMI was calculated as BW (kg) divided by squared height (m). Waist and hip circumferences were measured twice with a non-elastic tape measure on the skin with a precision of 0.5 cm, from which an average was calculated. Waist circumference was measured halfway between the lowest rib and iliac crest and the measurement was taken when the participant exhaled. Hip circumference was measured as the largest circumference in the area around the buttock. Sagittal diameter was measured with the participant in a lying position with an abdominal caliper (Holtain-Kahn) with a precision of 0.1 cm when the participant exhaled. Fat mass and lean body mass were determined in underwear by a dual-energy x-ray absorptiometry (DXA) scan (GE Lunar Prodigy).

Blood pressure: After 25 minutes resting in lying position, BP was measured with an automatically inflated cuff (A&D Instruments LTD, Saitama, Japan). BP was measured on the left arm three times if the two last measurements differed with ≤5 mmHg. If the last two measurements differed by >5 mmHg, an additional measurement was performed. The average was calculated from the last two measurements.

Microbiota analysis: Fecal collection took place prior to all CIDs. On one of the three days of dietary record, the participant collected a morning fecal sample, and the collection was kept cold and delivered to the Department within 3 hours. The fecal sample was weighted and a smaller portion was transferred to the EasySampler® kit for stool collection (GP Medical Devices, Denmark) and stored at -80 ˚C. The fecal deoxyribonucleic acid (DNA) was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with a prior step of bead beating in 2 mL micro centrifuge tubes containing 0.1 mm diameter glass beads, ~200 mg faces, and 1 mL InhibitEX buffer. Bead beating was carried out in a Mini-Bead Beater apparatus (BioSpec Products, Bartlesville, USA) with two cycles of shaking during 1 min and incubation on ice between cycles. The fecal DNA was measured by UV methods (Nanodrop, Thermo Scientific, Wilmington, USA) and an aliquot of every sample was prepared at 20 ng/µL with nuclease-free water for polymerase chain reaction (PCR). The V3-V4 hypervariable regions of the 16S ribosomal ribonucleic acid (rRNA) gene were amplified using 20 ng DNA (1 µL diluted aliquot) and 25 PCR cycles consisting of the following steps: 95ºC for 20 sec., 55ºC for 20 sec., and 72ºC for 20 sec. Phusion High-Fidelity Taq Polymerase (Thermo Scientific, Wilmington, USA) and the 6-mer barcoded primers, S-D-Bact-0341-b-S-17

(CCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC) which target a wide range of bacterial 16S rRNA genes (26), were used during PCR. Dual barcoded PCR products, consisting of ~500bp, were purified from triplicate reactions with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK) and quantified through Qubit 3.0 and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Samples were multiplexed by combining equimolar quantities of amplicon DNA (100 ng per sample) and sequenced in an Illumina MiSeq platform with 2x300 PE configuration (Eurofins Genomics GmbH, Ebersberg, Germany). Raw data were delivered in fastq files and pair ends with quality filtering were assembled using *Flash* software (27). Sample de-multiplexing was carried out using sequence information from the respective DNA barcodes and *Mothur v1.36.1* suite of analysis (28). After assembly and barcodes/primers removal, the sequences were processed for chimera removal using *Uchime* algorithm (29) and SILVA reference set of 16S sequences (30). Alpha diversity was calculated with *Mothur v1.36.1* using default parameters and average method in the clustering step. Consequently, the Chao's richness, Shannon's evenness and Simpson's reciprocal index were computed using a high quality and a normalized subset of 17,750 sequences per sample, randomly selected after shuffling (10,000X) of the original dataset. Taxonomic assessment was performed using the Ribosomal Database Project (RDP) classifier v2.12 (31). The Operational Taxonomic Unit (OTU)-picking approach was performed with the normalized subset of 17,750 sequences and the *uclust* algorithm implemented in USEARCH v8.0.1623(32). Beta-diversity was evaluated using Principal Coordinate Analysis (PCoA) and Bray-Curtis dissimilarity index.

Blood biochemistry: Venous blood samples were drawn at the CIDs after an overnight fast. Blood samples for analyses of insulin, ASAT and ALAT, hsCRP and lipid profile (total CHO, VLDL-CHO, LDL-CHO, HDL-CHO, TG, ApoB) were collected in serum tubes and kept at room temperature for 20 minutes to coagulate. Plasma samples for glucose (in fluoride tube) were put directly on ice and immediately centrifuged. All samples were centrifuged at 2500 x *g* for 10 min at 4ºC and stored at -80ºC until processing. Samples for whole blood analyses of hemoglobin and white blood cell were collected in ethylenediaminetetraacetic acid (EDTA) tubes and concentrations were immediately measured (SysmexKX-21, Sysmex Corporation, Kobe, Japan). Insulin was measured by chemiluminescentimmunometric assay (IMMULITE 2000 INSULIN, Siemens Healthcare Diagnostics Inc.) on the IMMULITE2000 INSULIN Analyzer (Siemens Healthcare Diagnostics Products Ltd., UK). Samples with an insulin concentration below the detection limit (14.4 pmol/L) were set to 7.2 pmol/L. Glucose was

measured by enzymatic hexokinase method on the Pentra 400 Analyzer (HORIBA ABX, Montpellier, France). The homeostatic model assessment was used to quantify insulin resistance (HOMA-IR) and beta-cell function (HOMA-β) from measurements of fasting insulin and glucose concentrations. HOMA-IR was calculated as: *(insulin (μU/mL)×glucose (mmol/L))/22.5* and HOMA-β as: *(20×insulin (μU/mL))/(glucose (mmol/L)- 3.5*) (33). HsCRP was measured by immunoturbidimetric method on the Pentra 400 Analyzer (HORIBA ABX, Montpellier, France). ASAT and ALAT were measured on the Pentra 400 Analyzer (HORIBA ABX, Montpellier, France). Lipid profile was analyzed on an auto-analyzer platform DIMENSION VISTA® (Siemens Healthcare Diagnostics Inc., USA). Total CHO and TG were measured by enzymatic methods. LDL-CHO and HDL-CHO were analyzed by the same method but with a disintegration of the other lipoproteins prior to the enzymatic reactions as included in the test scheme. ApoB concentration was measured by nephelometry. Specific antibodies form immunocomplexes with the ApoB proteins, which result in scattering light. Concentration of very low density lipoprotein (VLDL)-CHO was calculated from the values above. All lipids were measured in mg/dL but converted to mmol/L by multiplying with 0.0259 for total-CHO, LDL-CHO, HDL-CHO and VLDL-CHO and multiplying with 0.0113 for TG. ApoB concentration was multiplied with 0.01 for obtaining concentration in g/L.

Breath hydrogen: Fasting breath hydrogen was measured by a hand-held non-invasive Gastro$^+$Gastrolyzer (Bedfont Scientific Ltd, Kent, England).

Energy Expenditure: At all CIDs resting energy expenditure (REE) was measured twice after a minimum of 30 minutes of resting by a ventilated hood system (Jaeger Oxycon PRO, ViasysHealtcare GmbH, Hoechberg, Germany). Each measurement lasted 25 minutes and was separated by a 10-minute rest period. The standardized dinner from the study kitchen at the Department provided prior to each CID contained 3 or 4 MJ, depending on the estimated energy requirements of the participant, and had a macronutrient distribution of 16 E% protein, 31 E% fat and 53 E% carbohydrate. Participants were not allowed to consume alcohol and asked tolimit physical activity 48 hours prior to the REE measurement.

Dietary records and physical activity: Prior to the four CIDs (week 0, 4, 8, 12) the participants reported all ingested foods in a 3-day dietary record including information on brand names, cooking and processing. Whenever possible, foods were weighed otherwise household measures were applied. Content of energy, macro and micro nutrients were calculated as an average from the 3-day dietary records. The dietary records were assessed using a computer database of foods from the National Food Agency of Denmark (Dankost Pro, National Food Agency of Denmark, Søborg, Denmark). During the same 3 consecutive days (3 entire 24-

hour periods) physical activity was measured using a waist-worn accelerometer (ActiGraph GT3X+, Pensacola, FL, USA). Participants were only allowed to take the accelerometer off during showering and swimming and these non-wear activities were recorded in a diary including sleeping and wake-up time. The participants were instructed to maintain their normal physical activity habits during the study. Data were reintegrated into 60 sec. epochs and analyzed using Actilife v6 software. Before analysis self-reported sleeping and non-wear times were removed and the remaining time was scored in ActiLife6 to evaluate physical activity. Only data for participants with at least two days of measurements, defined as a minimum of 600 min wear time per day, were considered valid for analysis. Total tri-axial physical activity (counts per minutes (CPM)) was expressed as a vector magnitude of the total tri-axial counts from monitor wear-time, divided by measured monitor wear-time. Sedentary time, light physical activity, and Moderate-to-Vigorous physical activity (MVPA) were defined as ≤99 vertical CPM, 100–2019 vertical CPM, and ≥2020 vertical CPM, respectively (34).

Compliance: For compliance measurements dietary records of daily supplement intake were obtained. The compliance evaluation was based on the number of days during the diet period where the participants did not consume 100% of the provided supplement. Compliance was evaluated as very good (≤4 days), good (>4 and ≤8 days), bad (>8 and ≤12 days) or very bad (>12 days) during the diet period. The compliance degree was reduced one level if information about intake of supplement was missing.

Adverse events: During the two diet periods the participants filled out a weekly 100 mm visual analogue scale (VAS). The VAS was anchored with “no symptoms” (0 mm) and “extreme symptoms” (100 mm) and the following symptoms were evaluated; stomach pain, abdominal distension, flatulence, constipation, diarrhea, nausea, oily faces, wind break and frequent rectal tenesmus. During the 3-day diet registration, the participants recorded all time points of defecation and evaluated stool consistency using the Bristol stool scale. Average defecation frequency was calculated as the number of stools divided by the 3 days of recording. Average stool consistency was calculated as the sum of Bristol stool scale divided by the total number of registered stools. The Bristol composite measure i.e. a parameter of defecation frequency and stool consistency was calculated as the sum of Bristol stool scale divided by the 3 days of recording. Concomitant medication and adverse events were registered at all CIDs and all mid-visits. An adverse event was evaluated by intensity (mild, moderate or severe) and the project staff evaluated whether the adverse event was related to the intervention (plausible, likely, perhaps, unlikely, impossible).

Statistical analyses

The number of participants was calculated before study start according to previous studies with AXOS interventions (16; 17). Sample size calculation was based on the expected primary outcome "increase in amount of bifidobacteria" during the AXOS intervention period, however based on another analysis method than used in the current study. By including 30 participants (24 completers), this study would have a statistical power of 80% to detect a difference of 0.35 log10 cell/g dry weight feces (SD of 0.6), allowing for a 20% dropout at a 0.05 significance level.

The level of significance was set to $p \leq 0.05$. Statistical analyses on metabolic, physical activity, gastrointestinal symptoms, anthropometry, taxonomy categories, and dietary outcomes were performed using SPSS v24. The effects of the dietary intervention on all outcomes were analyzed using a linear mixed model (LMM) with repeated measures. The model included a treatment (AXOS vs. PUFA) × time (before vs. after intervention) interaction and adjustment for age, gender, and order of treatments as fixed effects. In addition, adjustment for recruiting BMI was included. Data not normally distributed were log-transformed before analysis by LMM. Data are presented as means ±SD unless stated otherwise. Categorical data are presented by numbers and percentages. To investigate the effect of the treatment we compared the before versus after points, within and between treatments (AXOS and PUFA). Additionally to LMM methods, statistical analyses on microbiome outcomes were also performed in R v3.2.3 (http://cran.r.project.org). Non-parametric Wilcoxon Rank-Sum test for the paired samples, the Wilcoxon Signed-Rank test for the unpaired samples, and Linear Discriminant Analysis (LDA) were performed to measure differences among fecal microbial communities at different taxonomic levels as a result of the different interventions with AXOS or PUFAs (36). Structural changes in the gut microbial community associated with diet were assessed by beta diversity analysis based on Bray-Curtis dissimilarity index and permutation based test (Permanova) using *qiime* suite of analysis (37). Pairwise Spearman's rank correlation coefficient between principal coordinate (PC) and OTU abundance were conducted to investigate particular changes in OTU abundances during the diet period. Similarly, pairwise Spearman's rank correlations between OTU abundance (OTUs with a LDA score > 3) and biochemical parameters were determined. The *post hoc* False Discovery Rate (FDR) was used to adjust for multiple comparisons in the correlation tests.

Results

Thirty participants were included in the study. Three participants did not complete the study; one dropped out before study start, one dropped out for personal reasons during the study and one was excluded after the first diet period because the time frame of the study, thus getting the effective number of 28 and 27 participants for AXOS and PUFA interventions, respectively (Figure 2).

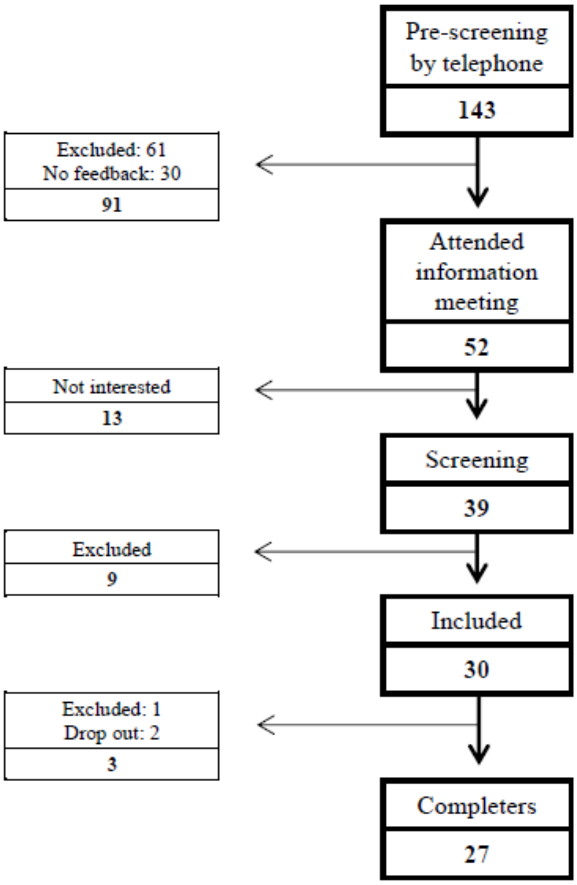

Figure 2: Flow chart of the recruiting process.

Five participants could not manage the visit window of ±3 days, thus four had a longer washout period and one had a longer second diet period. The characteristics of the 29 participants who completed the baseline visit are shown in Table 2. A comparison of participants in the two interventions (AXOS-I and PUFA-I) during the first diet period showed no baseline differences (Supplementary Table 1).

Table 2: Baseline characteristics for the participants who completed the baseline visit (N=29)

	Median	Q1 ; Q3
Age (y)	46.0	34 ; 53
Women (%)[2]	72.4	-
Anthropometric		
Body weight (kg)	84.6	79.6 ; 90.8
BMI (kg/m²)	30.0	27.4 ; 31.7
FM (kg)	35.5	29.4 ; 39.4
LBM (kg)	46.6	41.7 ; 55.7
Fat percent, whole body (%)	40.7	35.4 ; 45.5
Waist circumference (cm)	94.3	90.3 ; 102
Hip circumference(cm)	112	108 ; 115
Sagittal height (cm)	21.8	20.4 ; 23.8
Blood pressure		
Systolic (mmHg)	120	108 ; 128
Diastolic (mmHg)	77.0	71.5 ; 82
Pulse (beats/min)	60	55 ; 63
Lipid profile[3]		
Total CHO (mmol/L)	5.07	4.32 ; 5.42
HDL-CHO (mmol/L)	1.34	1.11 ; 1.59
LDL-CHO (mmol/L)	3.03	2.35 ; 3.61
VLDL-CHO (mmol/L)	0.57	0.41 ; 0.73
TG (mmol/L)	1.18	0.88 ; 1.44
Glucose metabolism[3]		
Glucose (mmol/L)	5.50	5.28 ; 5.68
Insulin (pmol/L)	41.3	18.3 ; 62.0
HOMA-IR	1.47	0.63 ; 2.34
HOMA-β	51.3	33.2 ; 91.6
Metabolic syndrome[3]		
MetS score 0/1/2/3/4 (n)[4]	11/9/5/2/1	
Inflammation markers[3]		
hsCRP(mg/L)	1.57	0.67 ; 2.82
Hb (mmol/L)	8.45	8.25 ; 9.20
WBC (10^9/L)	5.20	4.60 ; 5.95
Liver markers[3]		

ASAT (U/L)	21.0	17.5 ; 25.0
ALAT (U/L)	21.0	16.0 ; 29.0

[1]Data are given for baseline visit (week 0) as median with Q1 ; Q3.

[2]Data is given as percentage

[3]N=28

[4]Data is given as number of participants with either 0, 1, 2, 3 or 4 of the risk markers for metabolic syndrome beyond the increased waist circumference.

ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; BMI, body mass index; CHO, cholesterol; FM, fat mass; Hb, hemoglobin; HDL, high density lipoprotein; HOMA-β, homeostatic model assessment - beta-cell function; HOMA-IR, homeostatic model assessment- insulin resistance; hsCRP, high sensitive C-reactive protein; LBM, lean body mass; LDL, low density lipoprotein; MetS, metabolic syndrome; TG, triglycerides; VLDL, very low density lipoprotein; WBC, white blood cell count.

At baseline, the participants had a fiber intake of 24.5 ±12.0 g/d and this was increased to 31.2 ±7.94 g/d during the AXOS intervention (Table 3). The self-reported compliance showed that of the 28 participants, who completed the AXOS intervention, 21 had a very good compliance, 6 had a good compliance and 1 participant (randomized to PUFA during the first period and AXOS during the second period) had a very bad compliance. The baseline intakes of total fat, SFA, monounsaturated fatty acid (MUFA) and PUFA are shown in Table 3. During the PUFA intervention, PUFA intake increased from 6.19 E% to 7.77 E%. The self-reported compliance showed that of the 27 participants, 25 had a very good compliance and 3 had a good compliance.

Table 3: Dietary intake at baseline, after washout and after each intervention (mean ±SD)

	Baseline (N=29)	**After AXOS (N=28)**	**After PUFA (N=27)**	**Treatment *p*[1]**	**Time *p*[1]**	**Treatmnt × Time *p*[1]**
Energy intake (kJ/d)	8,843 ±2,771	8,836 ±2,383	8,859 ±2,799	0.673	0.796	0.807
Carbohydr. (E%)	45.8 ±6.82	48.0 ±6.53	43.5 ±7.36	0.561	0.864	0.333
Protein (E%)	17.7 ±4.43	17.4 ±4.01	17.3 ±4.43	0.392	0.999	0.370
Fat (E%)	35.0 ±5.41	33.3 ±4.68	36.8 ±4.37	0.202	0.703	0.616
PUFA (E%)	6.19 ±1.70	5.29 ±1.45	7.77 ±1.88	0.002	0.229	0.004
MUFA (E%)	11.9 ±3.13	11.2 ±2.39	11.4 ±2.91	0.360	0.689	0.342
SFA (E%)	12.0 ±3.14	11.8 ±2.61	11.9 ±3.20	0.473	0.851	0.780
Fiber (g/d)	24.5 ±12.0	31.2 ±7.94	20.9 ±6.97	0.008	0.009	0.001

[1]Based on LMM analysis and adjusting by covariates (recruiting BMI) and fixed effects (age, gender, and order of treatments).

AXOS, arabinoxylan oligosaccharides, E%, energy percentage; MUFA, mono unsaturated fatty acids; PUFA, poly unsaturated fatty acids; SFA, saturated fatty acids.

The results from LMM analysis on anthropometric measurements, blood pressure, blood biochemistry and metabolism are found in Table 4. Neither AXOS nor PUFA intakes had any effect on these outcomes, even when outcomes were analyzed separately in the first or second periods of respective interventions. However, flatulence was significantly associated with AXOS intake (Table 4).

Table 4: Outcome related to time points; baseline, after washout and after each intervention (mean ±SD)

	Baseline (N=29)	After AXOS (N=28)	After PUFA (N=27)	Treatment p^1	Time p^1	Treatment × Time p^1
Anthropometric						
Body weight (kg)	88.0 ±13.7	88.7 ±13.8	88.8 ±14.2	0.931	0.929	0.995
WC(cm)	96.5 ±8.82	97.3 ±8.80	96.6 ±9.18	0.511	0.663	0.617
HC (cm)	111 ±6.31	111 ±6.45	111 ±6.45	0.776	0.762	0.741
Sagittal height (cm)	22.0 ±2.53	22.0 ±2.38	21.9 ±2.65	0.776	0.944	0.948
Blood pressure						
Systolic (mmHg)	120 ±15.1	121 ±16.6	117 ±14.8	0.900	0.456	0.118
Diastolic (mmHg)	77.9 ±9.51	77.0 ±9.90	74.4 ±10.4	0.424	0.304	0.486
Pulse (beats/min)	59.3 ±8.14	60.0 ±7.20	60.1 ±7.93	0.644	0.703	0.916
Lipid profile						
Total CHO (mmol/L)	5.05 ±0.94[2]	5.17 ±1.01[4]	5.01 ±0.85[4]	0.321	0.984	0.933
HDL-CHO(mmol/L)	1.40 ±0.40[2]	1.37 ±0.35[4]	1.44 ±0.46[4]	0.687	0.984	0.548
LDL-CHO (mmol/L)	3.06 ±0.87[2]	3.17 ±0.90[4]	2.94 ±0.85[4]	0.352	0.891	0.712
ApoB (g/L)	0.95 ±0.24[2]	0.96 ±0.26[4]	0.93 ±0.25[4]	0.453	0.899	0.882
VLDL-CHO (mmol/L)	0.59 ±0.21[2]	0.64 ±0.24[4]	0.63 ±0.18[4]	0.935	0.147	0.754
Triglycerides (mmol/L)	1.24 ±0.47[2]	1.38 ±0.61[4]	1.11 ±0.43[4]	0.129	0.971	0.150
Glucose						

metabolism						
Glucose (mmol/L)	5.48 ±0.41[2]	5.56 ±0.44[4]	5.61 ±0.38[4]	0.945	0.117	0.318
Insulin (pmol/L)	43.6 ±30.3[2]	48.5 ±34.9[4]	50.3 ±34.1[4]	0.953	0.965	0.598
HOMA-IR	1.54 ±1.08[2]	1.76 ±1.33[4]	1.82 ±1.24[4]	0.983	0.892	0.483
HOMA-β	64.9 ±46.9[2]	67.5 ±47.2[4]	69.4 ±49.1[4]	0.935	0.954	0.725
Inflammation markers						
hsCRP (mg/L)	2.71 ±3.31[2]	2.77 ±5.43[4]	2.73 ±2.99[4]	0.848	0.525	0.352
Hb (mmol/L)	8.66 ±0.76[2]	8.59 ±0.84[4]	8.66 ±0.83[4]	0.425	0.874	0.936
WBC (10^9/L)	5.38 ±1.34[2]	5.41 ±1.18[4]	5.29 ±1.58[4]	0.398	0.505	0.680
Liver markers						
ASAT (U/L)	29.8 ±37.3[2]	23.6 ±8.03[4]	23.9 ±9.02[4]	0.772	0.473	0.792
ALAT (U/L)	31.1 ±33.6[2]	25.3 ±16.5[4]	27.0 ±17.3[4]	0.437	0.431	0.754
ASAT/ALAT	1.05 ±0.31[2]	1.10 ±0.39[4]	1.05 ±0.39[4]	0.663	0.552	0.998
Other						
Flatulence	17.04 ±17.05	30.23 ±19.45	17.46 ±22.35	0.033	0.064	0.103
Bristol	3.78 ±1.34	4.15 ±1.18	3.5 ±1.23	0.037	0.681	0.303
Breath hydrogen (ppm)	20.2 ±25.1	31.9 ±32.0	22.2 ±44.9	0.113	0.559	0.252
PA vector (CPM)	575 ±189	547 ±177	618 ±205	0.393	0.449	0.214
REE (kJ/d)	6,317 ±1,181	6,418 ±1,133	6,388 ±1,209	0.646	0.584	0.599
Respiratory quotient	0.805 ±0.03	0.811 ±0.05	0.802 ±0.04	0.806	0.560	0.544

[1]Based on LMM analysis and adjusting by covariates (recruiting BMI) and fixed effects (age, gender, and order of treatments).
[2]N=28,
[3]N=26
[4]N=27
[5]Data is given as number of participants with either, 0, 1, 2, 3 or 4 numbers of risk markers for metabolic syndrome beyond the increased waist circumference.
ALAT, alanine aminotransferase; ApoB, apolipoprotein B; ASAT, aspartate aminotransferase; AXOS, arabinoxylan oligosaccharides; CHO, cholesterol; CPM, counts per minutes; Hb, hemoglobin; HC, hip circumference; HDL, high density lipoprotein; HOMA-β, homeostatic model assessment - beta-cell function; HOMA-IR, homeostatic model

assessment- insulin resistance; hsCRP, high sensitive C-reactive protein; LDL, low density lipoprotein; MetS, metabolic syndrome; REE, resting energy expenditure; PA, physical activity, PUFA, poly unsaturated fatty acids; VLDL, very low density lipoprotein; WBC, white blood cell count; WC, waist circumference.

Adverse events were registered throughout the study. None of the adverse events were characterized as serious. Two adverse events (a knee operation and a food poisoning during the washout period) were reported as severe in intensity. More than half of the adverse events (54.5 %) were evaluated as unlikely or impossible related to the interventions. The majority of adverse events were seasonal diseases such as sore throat (5 events), common cold (13 events), influenza (4 events) and fever (1 event) in addition to headache (10 events) and gastrointestinal symptoms (28 events). Flatulence was reported more frequently during AXOS intake, compared to PUFA intake, and vice versa for reflux. Otherwise none of the adverse events occurred more frequently during a specific diet period.

The diet-induced microbial community changes were analyzed by comparing the different diet periods of the respective interventions individually to discern a possible carry over effects (Figure 1). We performed the comparisons at several taxonomy levels including phylum and family distribution and OTUs to identify the possible bacterial species modified by the respective diets. At phylum level, we found that AXOS intake only increased the proportion of Actinobacteria in the combined data from both diet periods of the AXOS intervention (LDA = 4.13, $p < 0.0012$) and such effect was basically due to the response in the AXOS-I group (LDA = 4.62, $p < 0.0015$) (AXOS intake during first diet period) given than no effect was observed in the AXOS-II group (Figure 3A), this last result and others described below regarding the distribution of families were similarly obtained following the LMM analysis (results not shown).

We did not detect differences in microbiota composition at baseline between the AXOS-I and PUFA-I participants and a comparative analysis of the microbiota after the washout period (i.e. before the second diet period) between the AXOS-I and PUFA-I subjects did not reveal differences either. The results of further analysis to determine the effects of AXOS on lower taxonomic bacterial categories are reported only for the AXOS-I participants since for the AXOS-II participants no differences were detected. At family level, AXOS increased abundance of the Bifidobacteriaceae (LDA = 4.41, $p <0.0014$) and Coriobacteriacea (LDA = 4.22, $p < 0.0041$) families of the Actinobacteria phylum, whereas the abundances of Rikenellaceae (LDA = 4.37, $p < 0.0238$) and Porphyromonadaceae (LDA = 3.91, $p < 0.0450$) belonging to the phylum Bacteroidetes were reduced (Figure 3B). However, AXOS intake did not lead to significant changes in any alpha-diversity parameter analyzed (Chao's richness, Simpson's reciprocal index, Shannon

evenness). Abundance analysis of OTUs showed that 11 phylotypes increased following AXOS intake (Table 5). As expected, three OTUs were assigned to the genus *Bifidobacterium* and the remaining OTUs were identified as potential members of bacteria groups that include butyrate producers such as *Eubacterium rectale, Eubacterium hallii, Faecalibacterium prautsnitzii, Dorea longicatena, Blautia luti* and *Blautia wexlerae* (all from the phylum Firmicutes and order Clostridales). We also detected decreased abundance of three OTUs that could not be properly identified at genus or species level, but appear to be phylotypes associated with the Ruminococcaceae and Erysipelotrichaceae families of the phylum Firmicutes (Table 5). Beta-diversity analyses were also conducted to evaluate significant shifts in the microbial communities as a result of the AXOS consumption. Using the Bray-Curtis dissimilarity index as descriptor in a PCoA, we depicted a uniform pattern of variation in all subjects after AXOS intake (Figure 4). Graphically, the microbial composition shifts towards the lower left corner of the PCoA plot. This was further supported by a permutation based analysis, which indicated that from all categorical variables analyzed (i.e. gender) only AXOS intake explained the changes in the microbial community structure (Permanova = 1.90, $p < 0.0111$). In order to disclose additional OTUs driving the shift in the microbial structure in response to AXOS, we performed linear correlations among OTU abundances and PC values. Several phylotypes were enriched or reduced in response to AXOS intake (Supplementary Table 2).

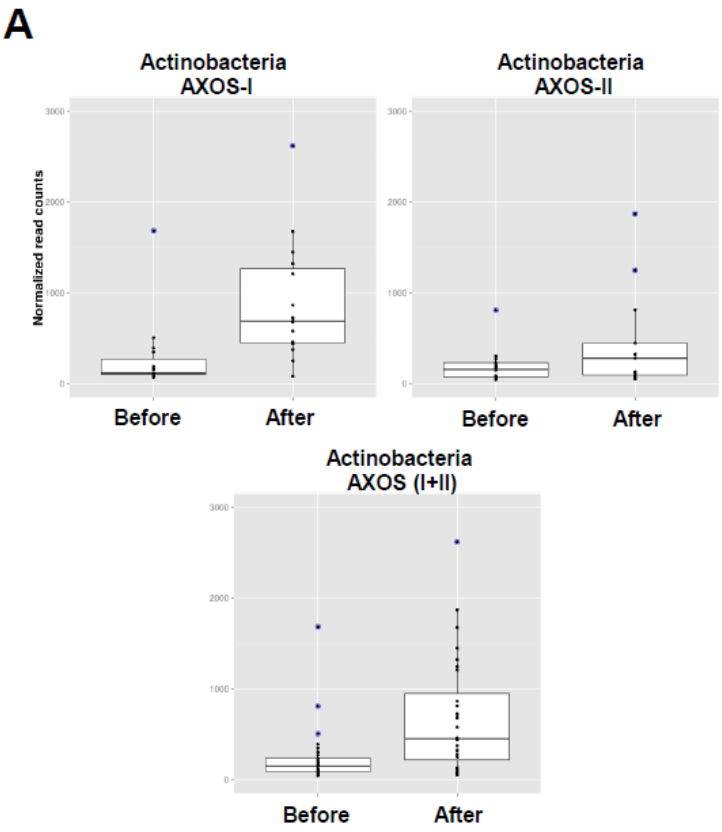

Figure 3: Gut microbiota change during AXOS intervention (N=28). **A)** AXOS intake increased abundance of phylum Actinobacteria (AXOS I+II, $p < 0.0016$; AXOS I , $p < 0.0015$; AXOS II, $p < 0.1825$). Normalized read count (before versus after AXOS intervention) was analyzed by non-parametric LDA analysis.

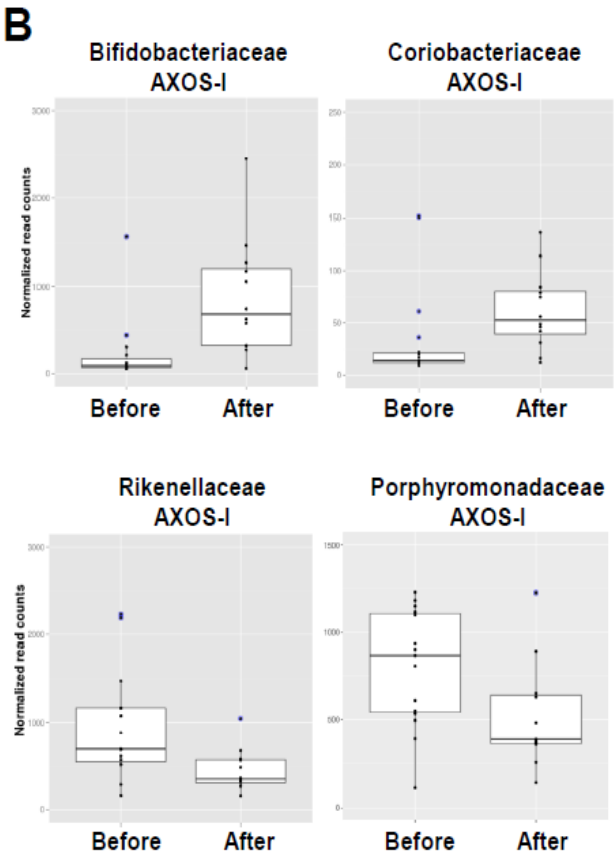

Figure 3 (continuación): Gut microbiota change during AXOS intervention (N=28). **B)** AXOS intake during first diet period increased abundance of Bifidobacteriaceae ($p < 0.0014$) and Coribacteriaceae ($p < 0.0041$) families of Actinobacteria and decreased abundance of Rikenellaceae ($p < 0.0238$) and Porphyromonadaceae ($p < 0.0450$) families of Bacteroidetes. Changes in abundance (before versus after intervention) was analyzed by non-parametric LDA analysis. AXOS, arabinoxylan oligosaccharides; LDA, Linear Discriminant Analysis

The PUFA intervention did not result in detectable microbiota changes at phylum or family levels, in abundances of OTUs or in alpha-diversity parameters, neither using pooled samples from both diet periods or separately. Multidimensional analysis showed a heterogeneous response to the PUFA intervention among the subjects that drastically differed from the more homogeneous response that was observed following the AXOS intervention (at least in the first diet period) (Figure 4). Beta diversity analysis (using samples of single or both diet periods) based on Bray-Curtis dissimilarity index showed no

shifts in the microbial community structure when paired samples were compared before and after the PUFA intervention (Permanova = 0.56, p<0.9601).

Table 5: OTU changes as a result of the AXOS intervention in the first diet period (n=15)

OTU	Blast 16S database[1]	id%[2]	LDA score	*p*-value[3]
Increased abundance				
4	*Eubacterium rectale*	100	4.34	0.029
5	*Faecalibacterium prausnitzii*	99	4.09	0.033
14	*Bifidobacterium faecale, Bifidobacterium stercoris, Bifidobacteriuma dolescentis*	100	3.93	0.044
26	*Blautia wexlerae*	100	3.80	0.001
770	*Bifidobacterium angulatum, Bifidobacterium merycicum, Bifidobacterium pseudocatenulatum, Bifidobacterium catenulatum*	99	3.67	0.019
27	*Fusicatenibacter saccharivorans*	100	3.60	0.036
52	*Bifidobacterium longum*	100	3.31	0.008
534	*Ruminococcus obeum*	99	3.30	0.012
44	*Dorea longicatena*	99	3.26	0.008
78	*Eubacterium hallii*	99	3.21	0.036
54	*Blautia luti*	99	3.19	0.019
Decreased abundance				
751	*Clostridium methylpentosum*	94	3.15	0.035
764	*Anaerotruncus colihominis*	92	3.10	0.035
688	*Erysipelothrix rhusiopathiae*	85	3.09	0.035

[1]Bacterial species/strain matching the OTU sequence according to best hit in a Blast-based search.

[2]Percentage of sequence identity supporting the taxonomic assignation of the respective OTU through the Blast-based search (alignment length percentage were 100 for all the OTUs presented in the table).

[3]Changes in OTU abundance in the microbiota of subjects before to after the first AXOS intervention was compared by non-parametric LDA (only OTUs with a LDA-score above 3are shown in the table). Differences were considered statistically significant at p-values < 0.05.

AXOS, arabinoxylan oligosaccharides; LDA, Linear Discrimination Analysis; OTU, Operational Taxonomic Unit.

Given that AXOS modified the gut microbiota by increasing the abundance of potential beneficial bacterial species, correlations between the OTU abundances

and physiological and biochemical data were analyzed for the AXOS intervention during the first diet period (AXOS-I). More than 170 correlations between OTU abundance and blood biochemistry parameters, based on Spearman's *rho* parameter and FDR ≤ 0.1, were found. Notably, there were a large proportion of positive correlations (~60%) between OTUs abundance and concentration of insulin, TG, LDL-CHO and VLDL-CHO, ApoB, and total CHO. Focusing on those OTUs that repeatedly showed correlations with markers related to similar functions, we could identify three OTUs that exhibited the largest number of positive correlations with biomarkers of lipid metabolism (VLDL, ApoB, total CHO, TG), liver function (ALAT), and glucose metabolism (insulin, HOMA-IR, HOMA-β). Those OTUs were identified as, *Paraprevotella clara* (OTU93, 98% identity), *Eubacterium contortum* (OTU435, 100% identity) and a Lachnoclostridium member of the Lachnospiraceae family (OTU278, >95% identity). Other species showing positive correlations specifically with plasma lipid concentrations (except for HDL-CHO) included *Prevotellamassilia timonensis* (OTU138, 100% identity) and *Mitsuokella jalaludinii* (OTU263, 99% identity). Strikingly, the OTU116, whose identity could be not well solved by Blast or SINA-based comparisons, showed the largest amount of negative correlations with concentrations of lipid metabolic biomarkers such as ApoB (*rho* -0.59; p< 0.005), total CHO (-0.60; p< 0.005) and LDL-CHO (-0.62; p< 0.005).

Moreover, we found a large set of tentative microbial species (OTUs) positively associated with glucose metabolic markers such as fasting insulin, HOMA-IR and HOMA-β values, although we obtained reliable identifications only in few cases including *Intestinimonas butyriciproducens* (OTU172, 100% identity), *Desulfovibrio piger* (OTU97, 99% identity) and *Coprobacter fastidiosus* (OTU99, 99% identity).

We also found negative correlations between the abundances of OTUs and concentrations of glucose or insulin (essentially lower HOMA values) for *Dialister succinatiphilus* (OTU102, 100% identity), *Turcibacter sanguinis* (OTU249, 99% identity) and *Alloprevotella* spp. (OTU289, >95% identity). Additionally, we detected positive correlations between HDL-CHO concentration and the abundances of *Eubacterium coprostanoligenes* group (OTU151, > 95% identity) and a Clostridium from Family XIII *Ihubacter* spp. (OTU926, > 95% identity).

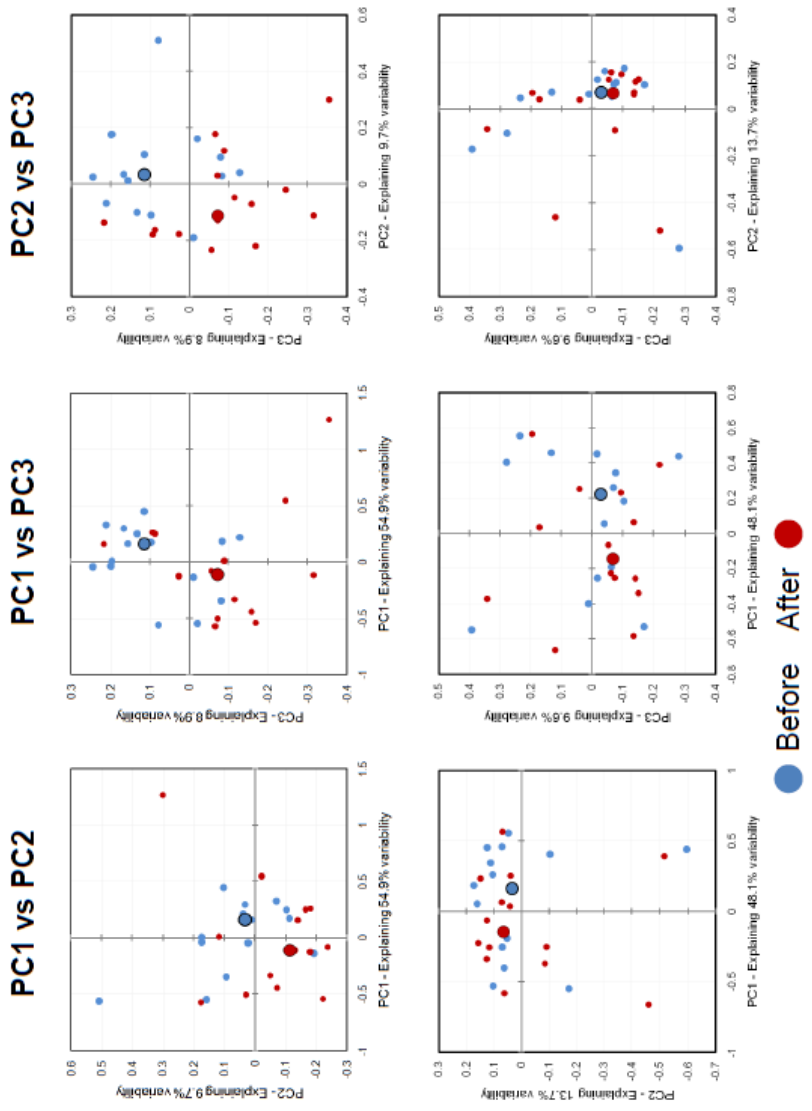

Figure 4: Change in beta-diversity visualized by plots from Principal CoordinateAnalysis. Comparison among the three main principal coordinate PC1, PC2, and PC3 are shown for AXOS intervention (upper part) and PUFA intervention (lower part). Small blue filled circles correspond with samples before intervention and small red filled circles correspond with samples after intervention. Greater filled circles correspond with respective centroids calculated from median of the PCs plotted.

Discussion

The present study reports that AXOS intake increased the abundance of the genus *Bifidobacterium* confirming previous results obtained in human controlled interventions (15-18). Moreover, we found that AXOS intake also increased the abundance of butyrate producing bacteria by the use of massive 16S sequencing methods, that enable the evaluation of the gut microbiota composition as a whole instead of quantifying a restricted number of taxonomy groups targeted by specific primers or probes (e.g. qPCR and hybridization approaches) (15-18). For the first time, we have reported that AXOS intake reduces significantly the proportion of both Rikenellaceae and of Porphyromonadaceae species, which has been associated with inflammatory processes in patients with cirrhosis (35). In particular, the OTUs analysis showed an increased abundance of the species *B. adolescentis* and *B. longum*, which have been shown to be able to hydrolyze AXOS in an *in vitro* study (36). Additionally, members of the genera *Faecalibacterium, Ruminococcus, Dorea,* and *Eubacterium* increased during the AXOS intervention. An increase in bifidobacteria may increase acetate production, which in turn can be metabolized by butyrate producing bacteria, thus stimulating their growth (19). This cross-feeding process could explain the increased abundance of bacteria belonging to the Clostridia class, particularly *E. rectale, F. prausnitzii* and *E. hallii* found in our study. Furthermore, the observed shift in the entire gut microbial community following AXOS intake was associated with increased abundances in *Roseburia, Coprococcus* and *Anaerostipes* species*,* which are butyrate producers as well (19). The remaining OTUs (all belonging to phylum Firmicutes and order Clostridales) that increased in abundance were *Blautia* and *Fusicantenibacter* genera, which have not previously been reported to change in response to AXOS intake (15-18;37).

It was shown that 10 g/d AXOS increased the abundance of the species *B. longum* and *D. longicatena*, but did not affect fasting glucose metabolism, as observed in previous AXOS interventions (16-18). Conversely, the increasing in abundance of the species *B. longum* and *D. longicatena*, as a consequence of the regular consumption of dietary fiber, has been previously associated with reduced insulin resistance (38). Although AXOS has a lower viscosity than AX, which reduces postprandial glucose (39), beneficial dose-dependent effects on overnight glucose metabolism has been already suggested (20). For the lipid profile, we found no major effects in any parameter analyzed, similarly to what reported in other AXOS intervention studies (16;17;40). By contrast, another study showed that 15 g/d AX consumption over 6 weeks decreased the fasting serum glucose, TG, and the apolipoprotein A1 concentrations, compared to placebo treatment (41). The above differences regarding the glucose and lipid metabolism could be related

to the duration of the study and the specific type of dietary fiber used in the intervention. In fact, García *et al.* (41) observed changes in glucose and lipid metabolism by using a 6-week long intervention and using AX, whereas others studies reporting no effects were done with AXOS administration from 2 up to 4 weeks.

Prior to the study initiation, a limited number of human studies indicated that fat type could affect microbiota composition (24; 25) and the main support for our initial hypothesis was evidence from animal studies. No effect of PUFA intake on the gut microbiota composition was observed in our study. A recent human study by Pu *et al.* (42) investigated effects of MUFAs, PUFAs, and canola oil-enriched diets on the microbiota and found that few microbial changes occurred at genus level without effects on higher taxonomic levels after 30 days of dietary intervention. On the other hand, a particular response of *Bifidobacterium, Oscillospira, Lachnospira*, *Coprococcus,* and *Faecalibacterium* was observed in a recent human intervention using PUFAs administered in drinks or capsules during 8-week periods with 12-week washout (43). In our study, the lack of effect on the gut microbiota may be explained by several reasons that should be considered for future research. First, the increase in PUFA intake did not cause a reduction in SFA intake as we aimed for. Second, another possibility is that the change in PUFAs was too small to exert measurable effects on microbial composition in relatively short time. Via the fish oil supplement, we provided a dose of EPA and DHA (1.32 and 1.86 g/d, respectively) lower to that reported to have adverse events (5 g/d) (44) and lower to that showing changes in certain microbial genera (2 g/d EPA and 2 g/d DHA) (43). However, it resulted in a much smaller difference in PUFA intake (1.84 percentage point) compared to the difference between monounsaturated fatty acid (MUFA) and PUFA interventions in the study by Pu *et al.* (42) where PUFA intake differed by 7.2-9.4 percentage points (6.7-9.1 E% vs 16.3 E%). Third, duration is important to consider given that interesting results have been obtained following longer intervention with an ample washout period (43). Although studies have shown that extreme dietary changes can change the gut microbiota composition within a few days, the response to some nutrients could be slower and depending on the concentration and overall dietary intake pattern. Pu *et al.* observed effects after a 30-day intervention (with a 4 weeks washout period) but the changes in fat quality were larger, total fat intake was constant and all consumed meals were provided during the intervention periods (42). Fourth, our sample size calculation was based on bifidogenic fiber effects as data from human interventions with PUFA intake on gut microbiota modulation were not available when the study was designed. Thus, the power to observe effects on gut microbiota composition in relation to PUFA intake could be too low.

On the other hand, different studies have suggested that individuals, depending on their initial gut microbiota, could respond differently to a dietary intervention. Zeevi *et al.* showed that an algorithm including information on gut microbiota composition could predict postprandial glycemic response to a wide range of foods consumed in real-life settings (45). Thus, the glycemic response to a food was affected by the individual gut microbiota composition and surprisingly, what normally is accepted as healthy and unhealthy food did not cause the same glucose response in all individuals. Currently, there is no definition that can distinguish individuals as responders or non-responders to a specific dietary intervention but it is possible that interventions with few participants, as in our study, have a skewed or limited distribution of responders and non-responders which makes it more difficult to observe both microbial and metabolic effects.

A limitation of our study was that the participants were less metabolically challenged than we aimed for. This problem seems to be related to screening methods as fewer participants had low HDL-CHO concentration in their venous blood sample at baseline compared to HDL-CHO concentration measured by the finger prick test at screening. This may also explain why dietary effects on metabolic markers were limited. Effect observed in the AXOS-I group but not in the AXOS-II group could be explained by carry-over effects. We did not observe any carry-over effects as no differences between AXOS-I and PUFA-I groups were observed after the washout period, however, the existence of such differences cannot be completely disregarded, taking the sample size into account.

In conclusion, intake of AXOS changed the gut microbiota composition. Higher abundance of bifidobacteria and butyrate producing bacteria were the main contributors to this change. Multiple correlations were established between specific OTUs and biochemical markers that could be beneficial for metabolic health (e.g. lower HOMA, higher HDL) and should be further explored since limitations in the duration of this study could have precluded the detection of significant beneficial effects on these end-points. PUFA intake did not affect gut microbiota composition or any metabolic marker. Further studies are needed to disentangle the role played by the individual's microbiota in predicting the health related effects in response to dietary interventions.

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Statement of Authorship

LK, LKB, AA, JV, YS and LHL designed the study. JV designed the WBE study products. LK, LKB, EMGP, ABP, GL, and SM, conducted the experimental research. LK, LKB, ABP, PB, SR, YS and LHL analyzed data. LK and ABP wrote the paper, LK and ABP have the primary responsibility for final content. All authors critically reviewed the manuscript and approved the final manuscript.

Conflict of Interest Statement and Funding sources

All the authors declare to have no conflict of interest.

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Availability of data and material

The raw fasta sequences generated from the 16S amplicon sequencing of fecal DNA are publicly available at the MG-RAST server (Meyer et al, 2008. BMC Bioinformatics. 2008;9:386. doi: 10.1186/1471-2105-9-386) upon the project accession number mgp84629.

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Supplemental Table 1: Baseline characteristics for the participants who initiated the trial with either the AXOS or PUFA intervention[1]

	AXOS-I(n=15)		PUFA-I (n=14)		P[2]
	Median	Q1 ; Q3	Median	Q1 ; Q3	
Age (y)	50	34 ; 53	45	26 ; 55	0.51
Women (%)[3]	80.0	-	64.3	-	0.43
Anthropometric					
BW (kg)	86.7	81.7 ; 92.8	84.2	74.7 ; 90.8	0.53
BMI (kg/m²)	29.3	27.7 ; 31.7	30.2	26.7 ; 31.9	0.69
FM (kg)	37.0	32.2 ; 39.4	31.0	27.7 ; 39.7	0.21
LBM (kg)	46.9	42.0 ; 50.9	46.1	40.9 ; 56.8	0.86
Fat percent, whole body (%)	41.7	38.2 ; 45.7	38.8	34.7 ; 44.2	0.29
Waist circumference (cm)	94.8	91.3 ; 101.8	94.0	88.0 ; 101.5	0.68
Hip circumference(cm)	113	109 ; 115	110	104 ; 114	0.33
Sagittal height (cm)	22.4	20.5 ; 23.9	21.5	20.3 ; 23.8	0.63
Blood pressure					
Systolic (mmHg)	119	105 ; 131	123	112 ; 128	0.33
Diastolic (mmHg)	78	75 ; 84	76	70 ; 82	0.22
Pulse (beats/min)	62	55 ; 69	57	54 ; 63	0.34
Lipid profile[4]					
Total CHO (mmol/L)	5.15	4.19 ; 5.46	4.90	4.55 ; 5.35	0.75
HDL-CHO (mmol/L)	1.49	1.29 ; 1.63	1.23	1.09 ; 1.37	0.07
LDL-CHO (mmol/L)	3.06	1.89 ; 3.39	3.00	2.38 ; 3.72	0.60
VLDL-CHO (mmol/L)	0.56	0.41 ; 0.75	0.60	0.47 ; 0.73	0.87
TG (mmol/L)	1.12	0.82 ; 1.36	1.25	0.89 ; 1.47	0.48
Glucose metabolism[4]					
Glucose (mmol/L)	5.59	5.35 ; 5.83	5.39	5.02 ; 5.61	0.21
Insulin (pmol/L)	49.0	23.2 ; 69.9	30.4	17.8 ; 57.4	0.42
HOMA-IR	1.71	0.83 ; 2.54	1.11	0.56 ; 1.89	0.33
HOMA-β	63.1	32.0 ; 93.0	40.6	35.6 ; 73.1	0.64
Metabolic syndrome[4]					
MetS score 0/1/2/3/4 (n)[5]	5/5/3/1/0		6/4/2/1/1		1.00
Inflammation markers[4]					
hsCRP(mg/L)	1.66	1.00 ; 2.37	1.43	0.51 ; 3.26	0.78
Hb (mmol/L)	8.40	7.70 ; 9.10	8.85	8.30 ; 9.30	0.26
WBC (10^9/L)	5.60	4.10 ; 6.90	4.90	4.70 ; 5.50	0.22
Liver markers[4]					

ASAT (U/L)	21.0	19.0 ; 25.0	21.5	17.0 ; 27.0	0.96
ALAT (U/L)	21.0	14.0 ; 27.0	21.5	17.0 ; 34.0	0.73

[1]Data are given for baseline visit (week 0) as median with Q1 ; Q3 for the 29 participants randomized to start with either AXOS (AXOS-I) or PUFA (PUFA-I).

[2] Level of significance from Mann–Whitney U test comparing AXOS-I and PUFA-I.

[3]Data is given as percentage and difference between arms are analysed by Fishers exact test.

[4]N=28

[5]Data is given as number of participants with either 0, 1, 2, 3 or 4 of the risk markers for metabolic syndrome beyond the increased waist circumference and difference between arms are analysed by Fishers exact test.

ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; BMI, body mass index; BW, body weight;CHO,cholesterol; FM, fat mass;Hb, hemoglobin; HDL, high density lipoprotein; HOMA-β, homeostatic model assessment - beta-cell function; HOMA-IR, homeostatic model assessment- insulin resistance; hsCRP, high sensitive C-reactive protein;LBM, lean body mass; LDL, low density lipoprotein; MetS, metabolic syndrome; TG, triglycerides; VLDL, very low density lipoprotein; WBC, white blood cell count.

Supplemental Table 2: OTU changes related to the shift in beta-diversity as a result of the AXOS intervention (n=15) during the first diet period (AXOS-I).

OTU	Blast species (Alignment lenght%, id%)[1]	PC	Spearman's ρ	p-value	p-value (FDR)
Negative correlation (species driving significant changes in the gut microbial community after intervention)					
727	*Eubacterium rectale* (100, 93)	PC2	-0.7662	<0.0001	0.0009
551	*Clostridium hylemonae* (100, 97)	PC2	-0.7339	<0.0001	0.0022
27	*Fusicatenibacter saccharivorans* (100, 100)	PC2	-0.7038	<0.0001	0.0054
26	*Blautia wexlerae* (100, 100)	PC2	-0.7059	<0.0001	0.0063
887	*Ruminococcus faecis* (100, 97)	PC2	-0.6646	<0.0001	0.0100
238	*Eubacterium rectale* (100, 92)	PC2	-0.6507	<0.0001	0.0140
435	*Eubacterium contortum* (100, 100)	PC2	-0.6444	0.0001	0.0153
54	*Blautia luti* (100, 99)	PC2	-0.6353	0.0002	0.0167
293	*Clostridium oroticum* (100, 98)	PC2	-0.6375	0.0002	0.0167
366	*Faecalibacterium prausnitzii* (100, 96)	PC2	-0.6246	0.0002	0.0212
86	*Coprococcus eutactus* (100, 99)	PC3	-0.6218	0.0002	0.0307
286	*Ruminococcus gnavus* (100, 100)	PC2	-0.6084	0.0004	0.0315
1001	*Eubacterium rectale* (100, 94)	PC2	-0.6036	0.0004	0.0334
607	*Coprococcus catus* (100, 96)	PC3	-0.6086	0.0004	0.0370
109	*Clostridium leptum* (100, 92)	PC1	-0.6628	<0.0001	0.0372
4	*Eubacterium rectale* (100, 100)	PC2	-0.6009	0.0006	0.0405
345	*Blautia faecis* (100, 98)	PC2	-0.5869	0.0007	0.0433
801	*Faecalibacterium prausnitzii* (100, 96)	PC2	-0.5797	0.0008	0.0494
63	*Anaerostipes hadrus* (100, 100)	PC2	-0.5683	0.0011	0.0601
5	*Faecalibacterium prausnitzii* (100, 99)	PC2	-0.5760	0.0011	0.0601
699	*Ruminococcus faecis* (100, 97)	PC2	-0.5614	0.0012	0.0613
1066	*Blautia schinkii*, *Blautia producta*, *Blautia coccoides*, *Blautia hansenii* (100, 97)	PC2	-0.5632	0.0012	0.0613
385	*Bacteroides uniformis* (100, 93)	PC2	-0.5567	0.0014	0.0653
811	*Clostridium colinum* (100, 93)	PC2	-0.5528	0.0015	0.0654
207	*Adlercreutzia equolifaciens* (100, 99)	PC2	-0.5434	0.0019	0.0774
405	*Intestinimonas butyriciproducens*, *Acetivibrio cellulolyticus*, *Clostridium alkalicellulosi* (100, 92)	PC1	-0.6149	0.0003	0.0848
390	*Fucophilus fucoidanolyticus* (100, 88)	PC1	-0.6196	0.0003	0.0848
502	*Clostridium innocuum* (100, 99)	PC2	-0.5376	0.0022	0.0853
61	*Clostridium spiroforme* (100, 93)	PC2	-0.5291	0.0026	0.0881
760	*Ruminococcus gnavus* (100, 96)	PC2	-0.5300	0.0026	0.0881
527	*Clostridium spiroforme* (100, 91)	PC2	-0.5305	0.0026	0.0881
534	*Ruminococcus obeum* (100, 98)	PC2	-0.5331	0.0024	0.0881
962	*Adlercreutzia equolifaciens* (100, 96)	PC2	-0.5335	0.0024	0.0881
635	*Fusicatenibacter saccharivorans* (100, 96)	PC2	-0.5238	0.0030	0.0959
174	*Roseburia hominis* (100, 97)	PC2	-0.5217	0.0031	0.0977
632	*Gemmiger formicilis* (100, 93)	PC2	-0.5194	0.0033	0.0999
Positive correlation (species associated with the pre-intervention state)					
1	*Bacteroides dorei* (100, 100)	PC3	0.8042	<0.0001	0.0007
1073	*Bacteroides vulgatus* (100, 97)	PC3	0.7574	<0.0001	0.0007
220	*Prevotella copri* (100, 97)	PC1	0.7611	<0.0001	0.0012
1126	*Bacteroides caccae* (100, 97)	PC3	0.7209	<0.0001	0.0026
9	*Oscillibacter ruminantium* (100, 94)	PC2	0.6740	<0.0001	0.0100

902	*Pseudoflavonifractor capillosus* (100, 92)	PC2	0.6661	<0.0001	0.0100
891	*Bacteroides thetaiotaomicron* (100, 99)	PC3	0.6753	<0.0001	0.0120
318	*Bacteroides vulgatus* (100, 97)	PC3	0.6520	<0.0001	0.0215
185	*Flavonifractor plautii* (100, 100)	PC3	0.6351	0.0001	0.0307
720	*Bacteroides xylanisolvens* (100, 96)	PC3	0.6292	0.0002	0.0307
19	*Alistipes onderdonkii* (100, 100)	PC3	0.6223	0.0002	0.0307
258	*Bacteroides cellulosilyticus* (100, 97)	PC3	0.6144	0.0003	0.0343
817	*Intestinimonas butyriciproducens* (100, 90)	PC2	0.5955	0.0005	0.0390
106	*Bacteroides plebeius* (100, 97)	PC3	0.5873	0.0006	0.0608

OTUs correlated with a shift in beta-diversity were analyzed by linear correlations (Spearman's rho) among PC values and OTU abundance. P-values and post hoc FDR adjusted p-values are presented. A negative correlation indicatesan increase in OTU abundance while PC values decrease, thus a driver of the shift in microbial community.

[1]Bacterial species/strain matching the OTU sequence. Alignment length and percentage of sequence identity supporting the taxonomy assignation of the respective OTU through Blast-based search is presented within parenthesis.

FDR, False Discovery Rate; PC, Principal Coordinate; PCoA, Principal Coordinate Analysis; OTU, OperationalTaxonomic Units

A multi-omics approach to unraveling the microbiome-mediated effects of arabinoxylan-oligosaccharides in overweight humans

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Abstract

Background. Long-term consumption of dietary fiber is generally considered beneficial for weight management and metabolic health, but the results of interventions vary greatly depending on the type of dietary fibers involved. This study provides a comprehensive evaluation of the effects of a specific dietary fiber consisting of a wheat-bran extract enriched in arabinoxylan-oligosaccharides (AXOS) in a human intervention trial.

Methods. An integrated multi-omics analysis has been carried out to evaluate the effects of a 4-week randomized intervention trial with an AXOS enriched-diet in overweight individuals with indices of metabolic syndrome. Gut microbiome and virome analyses were performed by shotgun DNA sequencing in feces, in-depth metabolomics using nuclear magnetic resonance in fecal, urine and plasma samples, and massive lipid profiling using mass spectrometry in fecal and serum/plasma samples. These data were integrated with the study metadata.

Results. In addition to its bifidogenic effect, we observed that AXOS boost the proportion of *Prevotella* species, particularly *P. copri*. The functional metagenome analysis showed increases in the presence of bacterial genes and pathways involved in vitamin/co-factor production, glycan metabolism, and neurotransmitter biosynthesis as a result of AXOS intake. Furthermore, lipidomics analysis revealed reductions in plasma ceramide levels, suggesting improvements in glucose metabolism. Finally, on combining the microbiome-metabolomics data, we observed associations between *Prevotella* abundance and SCFAs and succinate concentration in feces, and identified a potential protective role of *Eubacterium rectale* against production of methylamines, the abundance of which was positively associated with plasma levels of phosphatidylcholine precursors.

Conclusions. The integration of metagenomics, lipidomics and metabolomics data indicates that consumption of AXOS as main source of dietary fiber orchestrates a wide variety of changes in the gut microbiome and the host-microbe (co)metabolic products with potential impact on glucose homeostasis.

Trial registration. This study was registered under ClinicalTrials.gov identifier NCT02215343.

Background

The World Health Organization (WHO) reports that 1.9 billion adults are overweight, 650 million of whom are obese [1], making the obesity epidemic and the resulting metabolic complications an important health concern. The current rise in obesity prevalence in low- and middle-income countries, particularly in urban settings, and in children and adolescents further underlines the importance of prevention and treatment of obesity [1, 2]. Obesity is the result of a long-term imbalance between energy intake and expenditure, mainly caused by over-nutrition and sedentary lifestyle [3]. Obesity is characterized by chronic low-grade inflammation and impairment of the lipid and glucose metabolism, thus increasing the risk of developing comorbidities such as type-2 diabetes (T2D), cardio-vascular disease (CVD), and mental diseases [4, 5]. Different epidemiological studies support the notion that regular consumption of enriched dietary-fiber food is inversely correlated with weight gain, thus potentially reducing the risk of developing T2D and CVD [6-10]. Interestingly, A recent umbrella review of systematic reviews reports that dietary fiber intake has a convincingly protective effect against CVD including coronary artery disease and CVD-related death and that there is suggestive evidence of disease risk reduction for several type of cancers, T2D, and stroke [11]. Accordingly, dietary fiber consumption appears to be a feasible long-term and cost-effective strategy to prevent obesity and its co-morbidities.

Dietary fiber comprises a diverse group of structurally complex carbohydrates with varying effects on human metabolism and the gut microbiota [12]. Arabinoxylans (AX) are cell wall components that constitute a major part of the dietary fiber fraction of cereal grains, and are an important fiber source in the human diet [13]. The main products of AX enzymatic hydrolysis are arabinoxylan-oligosaccharides (AXOS) and xylan-oligosaccharides (XOS), which have a recognized role as prebiotics because of their ability to increase the abundance of bifidobacteria and some butyrate producers in the gut [14-16]. The bifidogenic effect of AX-derived oligosaccharides has shown in human dietary interventions where abundance of *Bifidobacterium* species has been increased in feces as a consequence of the intake of 4 g/day XOS for three weeks [17] or 2-10 g/day AXOS during a similar period of time [18-20]. Although no major impact on blood biochemical, physiological, and anthropometrical parameters has been observed following AXOS/XOS intake, most likely due to the short duration of the studies, the increases in fecal moisture [17] and short-chain fatty acids (SCFA) concentrations [19], and in postprandial ferulic acid concentration [20], altogether confirm utilization of this type of dietary fiber by gut microbiota and its potential impact on metabolic health.

In a previous study, including a randomized cross-over intervention evaluating the effects of AXOS and polyunsaturated fatty acids (PUFA) in overweight subjects with indices of metabolic syndrome (MetS), we reported that AXOS exert a bifidogenic effect and increase the abundance of butyrate-producing bacteria using a 16S rRNA gene sequencing approach [21]. In the current paper, we aimed to further characterize the microbiome, metabolome and lipidome responses to AXOS intake to gain insight into the possible role of the microbiota as mediator of dietary effects on metabolic health. To this end, we have analyzed the biological samples of the group of responders to AXOS consumption (who showed significant changes in their gut microbiota) from our previous study (Kjølbæk et al. [21]). A multi-omics approach has been applied, including shotgun DNA sequencing metagenomics of feces, nuclear magnetic resonance (NMR) metabolomics conducted in feces, plasma and urine, and Mass Spectrometry-based (LC-MS) lipidomics in plasma and feces.

Methods

Study design

The present study is based on a randomized cross-over trial with two diet periods separated by a washout period (3×4 weeks) conducted in 30 overweight and obese individuals (body mass index [BMI] of 25-40 kg/m^2) with MetS index (an increased waist circumference plus at least one of the criteria for MetS [22]) described in detail elsewhere [21]. The dietary fiber ingredient provided was a wheat bran extract, enriched in arabinoxylan oligosaccharides (AXOS) (10.5 g/d AXOS). Before and after intervention periods, weighted dietary records, fecal, urine and blood samples as well as anthropometric measurements were collected. The present study integrates the metadata and multi-omics functional analysis of biological samples of the subset of responders (n = 15) to AXOS intervention from the initial trial [21], the baseline characteristics of this cohort of samples is described in Table S1. Responders were defined as the group of participants included in the same dietary intervention period and showing significant changes in their microbiota composition, as a whole, as a result of the dietary intervention with AXOS assessed by16S rRNA gene sequencing [21].

Sampling

Urine and feces. Collection of fecal and urine samples took place at home prior to the clinical investigation day (CID). The urine sample was collected in the morning and the fecal sample was collected as close to the urine sample as possible. Both samples were kept cold after collection and delivered to the Department within 3 hours after collection of feces. At the Department, the samples were weighed and the density of the urine sample was measured to calculate the volume. For the metabolomic analysis of urine, an aliquot of 4 mL urine was used and 45 µL 0.1% sodium azide was added. For fecal analysis, an aliquot of the sample was transferred to the EasySampler® kit for stool collection (GP Medical Devices, Denmark) for metagenomic analyses. Hereafter, the fecal sample was homogenized with miliQ water 1:1 and an aliquot was used for metabolomic analyses. For lipidomic analysis, 500µL methanol was added per 500 mg homogenized fecal sample. All aliquots were stored at -80 ˚C.

Blood samples. Prior to the CID, the participants consumed a standardized dinner in the evening followed by a fasting period of at least 8 hours. All blood samples were collected in the fasting state. Lipidomic analyses were conducted in plasma and serum. For plasma analysis, blood was collected in EDTA tubes and put directly on ice and for serum analysis, blood was kept at room temperature for 20

minutes to coagulate before centrifugation. For metabolomic analysis, blood for plasma analyses was collected in heparin tubes and put directly on ice. Afterwards, all blood samples were centrifuged at 2500g for 10 min at 4ºC to obtain respective aliquots of serum and plasma and stored at -80ºC. Sampling procedure for plasma and serum samples presented in the main paper -for example, glucose, insulin, lipids, inflammation markers, etc.- is found elsewhere [21].

Metagenomics approach

DNA extraction and shotgun sequencing. The fecal DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with a prior step of bead beating in 2 mL micro centrifuge tubes containing 0.1 mm diameter glass beads, ~200 mg feces, and 1 mL InhibitEX buffer. Bead beating was carried out in a Mini-Bead Beater apparatus (BioSpec Products, Bartlesville, USA) with two cycles of shaking during 1 min and incubation on ice between cycles. The fecal DNA was quantified through Qubit 3.0 and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and 1.5 ug DNA of every sample was sent to be multiplexed and sequenced in a plate of the HiSeq2500 platform with 2x125 paired-end configuration (Eurofins Genomics GmbH, Ebersberg, Germany). The NEBNext® Ultra™ DNA library Prep Kit for Illumina® was used according to manufacturer's instructions with 400-500 bp insert size and low PCR-cycling (5 cycles).

Metagenome data analysis. Approximately 0.5Tb raw data were delivered in fastq files. Paired-end fastq files were used to assemble the fecal metagenome of each individual at two different time points by using *Velvet* assembler v 1.2.10 [23] with *k-mer length* 61, *-exp_cov* auto, and *-ins_length* 200 parameters, followed by an assembly refinement step using the *Metavelvet* extension [24] with the *-ins_length* 200 *-ins_length_sd* 50 configuration. The assembled contigs larger than 200 nt in length were retained and the prediction of potential ORF encoded in such fragments from respective metagenomes was assisted by *FragGeneScan* v1.30 [25], with the *-complete*=0 and *-train*=complete configuration. Peptide sequences obtained from the ORF prediction in all metagenomes were concatenated and clustered at 70% sequence identity using *cdhit* algorithm with *-c* 0.7, *-G* 1, *-M* 10000, *-B* 1, and *-g* 1 parameters [26, 27]. For read mapping against the non-redundant peptide database compiled from the 30 metagenomes assembled, we used the *Usearch* v8.0.1623 algorithm with the following parameters: *-usearch_local, -id* 0.7, *-strand* both, and *-maxaccepts* 1. Differential abundance of coding metagenes was assessed by using negative binomial distribution methods implemented in *edgeR* [28] and determining a false discovery rate (FDR) for selection <0.1. For taxonomy aims, we mapped the remaining set of reads with no

hits after comparison against the non-redundant coding database. Consequently, we mapped those reads against the reference Silva database (Release 128) and read alignments were filtered to retain those expanding beyond 80% of the read length (≥ 100 nt) with ≥99% sequence identity. Differential abundance in phylum, family and genus distribution was evaluated with Linear Mixed Models (LMM) by using time points as fixed effects and subject-specific information (gender, age, and BMI) as random effects. Additionally, we assessed the taxonomy distribution of the coding metagenes with differential abundance by using Blast and the NCBI non-redundant protein database. Species identification was based on hits with best alignment length and score. Cases were defined as "uncertain" when equal scoring was obtained in comparison with more than one hit belonging to different microbial species.

Functional analyses. Preliminary analysis to assess the function of differentially abundant metagenes was completed by submitting the amino acid sequence of metagenes with significant increasing and decreasing abundance, as a consequence of the AXOS intake, to the KEGG Automatic Annotation Server (KAAS) [29]. Advanced functional enrichment analysis was performed by annotating the full set of non-redundant coding sequences, obtained in the global metagenome assembly, against the Pfam database [30] through the WebMGA server [31]. Functional enrichment of Pfam functions in samples after AXOS intake was evaluated by hypergeometric Fisher's exact test with correction for multiple testing using the Benjamini-Hochberg method.

Biochemical and lipidomic data. Plasma biochemical markers previously assessed [21] were re-analyzed in the present study for the selected subset of subjects. The Shapiro-Wilk normality test was estimated for all variables and, accordingly, paired and one-sided *t-test* or Wilcox Signed-Rank test were performed, respectively, in order to establish if the AXOS intervention improved any of the biochemical parameters measured. To establish possible relationships between changes in the abundance of bacterial genera and markers of glucose metabolism (HOMA-IR and fasting insulin) a logistic regression model was applied, using the *glm* [family="binomial"(link="logit"))] function of R v3.4.3; the HOMA-IR index was used as binary outcome (1 = improved and 0 = non-improved) and the changes in the relative abundance of different bacterial genera (Δ_{genus} = Log_{10} normalized reads at end-point - Log_{10} normalized reads at start-point) as explanatory variable. Lipidomics data were analyzed using Wilcox Signed-Rank test for paired samples with Benjamini-Hochberg correction for multiple comparisons when appropriate.

Human virome analysis. Raw fastq sequences were processed using the ViromeScan software to taxonomically characterize the virome directly from metagenomic reads [32] using the *-d human_DNA* parameter to detect only DNA viruses with humans as a natural host. The relative abundance was used to perform statistical analyses and comparisons among samples before and after the AXOS intervention. Alpha-diversity was computed for each sample considering the number of viral species detected within each metagenome. Significance testing was performed using the R package stats and non-parametric Wilcox Signed-Rank test for paired samples. When appropriate, *p* values were adjusted for multiple comparisons using the Benjamini-Hochberg correction. A false discovery rate (FDR) <0.05 was considered as statistically significant. All the statistics and plots for metagenomics and functional approaches were obtained and designed on R v3.4.3.

Plasma and fecal lipidomics

Quantification of plasma lipid species. Lipids were quantified by direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode using the analytical setup and strategy described previously [33, 34]. Lipid extraction was preformed according to the method of Bligh and Dyer [35], in the presence of not naturally occurring lipid species as internal standards. The following lipid species were added as internal standards: phosphatidylcholine (PC) 14:0/14:0, PC 22:0/22:0, phosphatidylethanolamine (PE) 14:0/14:0, PE 20:0/20:0 (di-phytanoyl), phosphatidylinositol (PI) 17:0/17:0, lysophosphatidylcholine (LPC) 13:0, LPC 19:0, sphingosine based ceramides (Cer) d18:1/14:0, Cer 17:0, D7-FC, cholesteryl ester (CE) 17:0 and CE 22:0. A fragment ion of *m/z* 184 was used for PC, sphingomyelin (SM) [34], and LPC [36]. Neutral loss fragments were used for the following lipid classes: PE and PI with a loss of 141 and 277, respectively [37, 38]. PE-based plasmalogens (PE P) were analyzed according to the principles described by Zemski-Berry [39]. Cer and hexosylceramides (HexCer) were analyzed using a fragment ion of m/z 264 [40]. Free cholesterol (FC) and (CE) were quantified using a fragment ion of *m/z* 369 after selective derivatization of FC [33]. Quantification was achieved using two non-naturally occurring internal standards (IS) for each lipid class (except for PI, SM was calculated using PC IS and PE-P were calculated using PE IS) and calibration lines generated by standard addition of a number of naturally occurring species to plasma. Calibration lines were generated for the following naturally occurring species: PC 34:1, 36:2, 38:4, 40:0 and PC O-16:0/20:4; SM d18:1/16:0, 18:1, 18:0; LPC 16:0, 18:1, 18:0; PE 34:1, 36:2, 38:4, 40:6 and PE P-16:0/20:4; Cer d18:1/16:0, 18:0, 20:0, 24:1, 24:0; FC, CE 16:0,18:2,18:1,18:0. De-isotoping and data analysis for all lipid classes was performed by self-programmed Excel Macros

as described previously [34, 41]. Lipid species were annotated according to the recently published proposal for shorthand notation of lipid structures that are derived from mass spectrometry [42]. Glycerophospholipid species annotation was based on the assumption of even numbered carbon chains only. SM species annotation is based on the assumption that a sphingoid base with two hydroxyl groups is present.

Quantification of bile acids. Both plasma and fecal bile acids were quantified by LC-MS/MS using stable isotope dilution analysis. Fecal samples were homogenized as described below using 10-fold dilution of the samples.

Quantification of fecal lipid species. Fecal samples were homogenized in 70% 2-propanol, using gentleMACS dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as described previously [43]. Fecal sterols and 5α/β-stanols were quantified by liquid chromatography-high-resolution mass spectrometry (LC-MS/HRMS) after derivatization to N,N-dimethylglycine esters [43]. Fecal fatty acids were quantified by gas chromatography coupled to mass spectrometry (GC-MS) after preparation of fatty acid methyl esters [44].

Metabolomics

Sample preparation. In order to reduce the water signal, urine samples (1 mL) were freeze-dried and reconstituted in 650 µL NMR phosphate buffer (sodium phosphate 0.2 M, pH 7.4, sodium 3-(tri-methylsilyl)-propionate-2,2,3,3-d4 (TSP) (Sigma-Aldrich) 1mM, 80 % D_2O, 20 % H_2O). TSP served as NMR reference. Feces (200 mg) were homogenized in 800 µL of NMR buffer for 5 min at 25 Hz in a tissue lyser (Qiagen). Plasma samples (350 µL) were mixed with D_2O (350 µL). All homogenized samples were centrifuged (10 min, 4°C, 13 000 rpm) and transferred to 5 mm NMR tubes (Bruker, UK) for analysis by NMR spectroscopy.

Spectra acquisition. The NMR experiments were carried out in the Chemical Analysis Facility (CAF, University of Reading) using a Bruker AV700 NMR instrument equipped with a 5mm inverse CryoProbe™, for increased sensitivity. A standard one-dimensional NOESY-PR-1D experiment was performed on all three types of samples, using a standard preset pulse sequence (noesy1d90°). Additionally, a Carr-Purcell-Meiboom-Gill (CPMG) experiment was applied to plasma samples, where simple presaturation of the water peak was used. This experiment reduced the signal contribution from albumin and lipoproteins present in plasma and highlighted signals from smaller molecules. All samples were analyzed at 300º K, 65k data point spectrum (spectral width 14705 Hz) was obtained by recording 128 scans (8 dummy scans).

Data processing and statistical analysis. Phase and baseline of the spectra were corrected using MestreNova software (version 10.0m MestreLab Research). NMR spectra were referenced to TSP peak for urine and fecal water samples and to glucose (at δ 5.223 ppm) for plasma samples. The processed spectra were digitalized and transferred to Matlab (version R2017a) for the statistical analysis. The residual water signal was removed and all spectra were normalized to the total spectral area for feces and urine. Plasma spectra were not normalized. Relative spectra were mean centered and scaled to unit variance. For the first stage of the analysis (unsupervised), principal component analysis (PCA) was used. The next, supervised stage of the analysis, involved the orthogonal projection to latent structure discriminant analyses (O-PLS-DA) (no orthogonal components used), to compare the changes in metabolite profiles between the two time points. The comparison was made between the baseline (start point) and the end point of AXOS intervention.

NMR spectra were used as a matrix of variables X, and time point vector Y (0-baseline, 1-end point) as a predictor. This analysis was used to construct a model identifying metabolites differentiating between the two time points. The internal validation of the model was evaluated using the following parameters: the goodness of fit (R2Y), showing what percentage of variation is explained by the model, and goodness of prediction (Q2Y), the percentage of Y predicted after 7-cross validation. For the correlation of metabolome with microbiome data, the metagenomics readings (normalized using "reads per million" approach) were used as a predictor Y using the OPLS analysis.

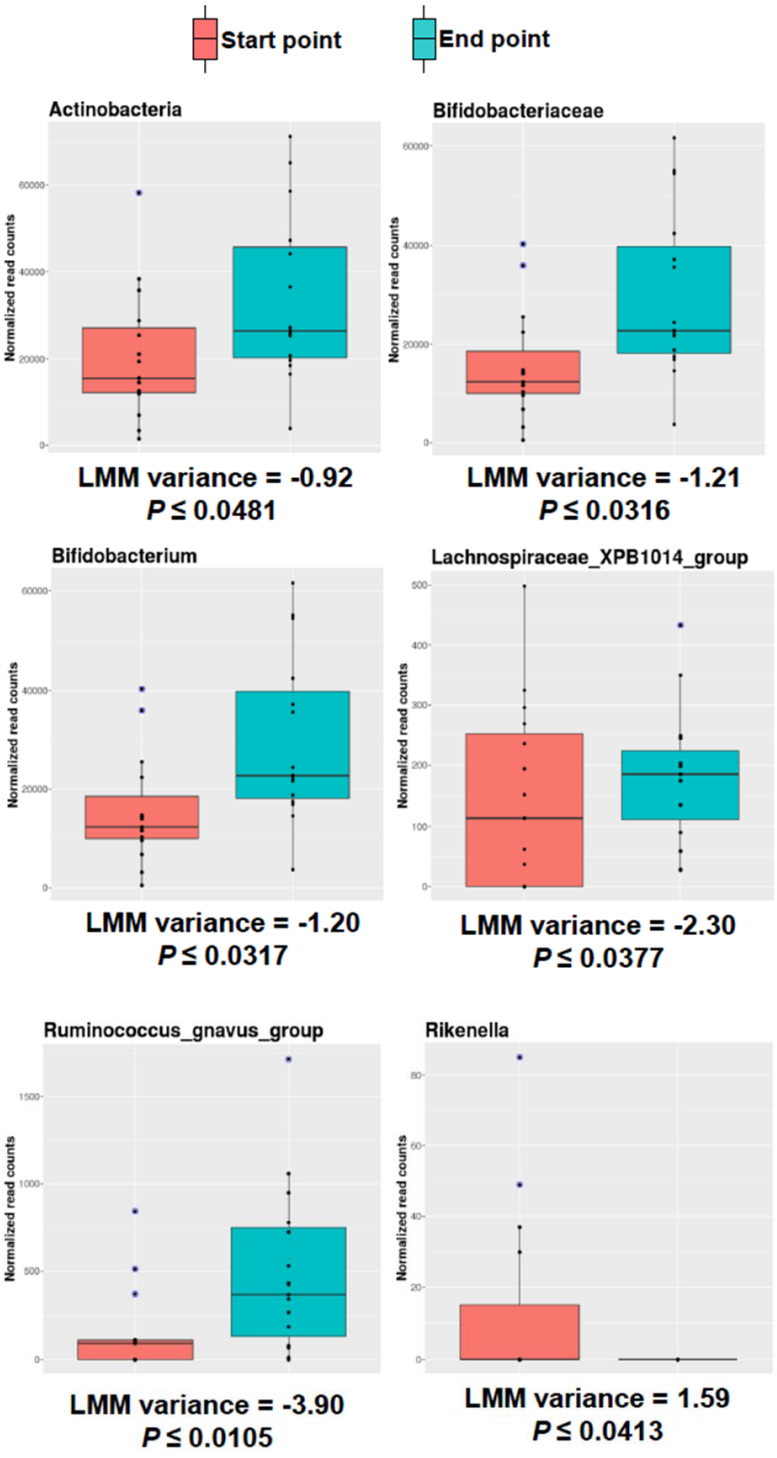
Results

Human gut metagenome's response to AXOS consumption

Taxonomic analysis. The new taxonomic analyses performed on metagenomic shotgut sequencing data indicates that AXOS intake increased the abundance of Actinobacteria ($p \leq 0.0481$), Bifidobacteriaceae ($p \leq 0.0316$), and *Bifidobacterium* ($p < 0.0317$) taxonomy groups comparing samples at baseline with those after the intervention. In addition to this characteristic bifidogenic effect, the abundance of members of the *Ruminococcus gnavus* group ($p \leq 0.0105$) and of the Lachnospiraceae_XPB1014 group ($p \leq 0.0171$) were also increased in samples after the AXOS intervention. Conversely, the AXOS intake reduced the proportion of *Rikenella* ($p \leq 0.0413$), *Parabacteroides* ($p \leq 0.0367$), and *Paraprevotella* ($p \leq 0.0428$) species (Figure 1).

The metagenome analysis was based on the processing of more than 3.2 billion reads (106 million PE reads average per sample), which produced approximately 10.2 million encoding genes assembled. A non-redundant coding metagene database was obtained by clustering protein sequences predicted from sequencing reads, thus retrieving ~1.7 million metagenes as a reference for the assessment of the effects of AXOS intake on the gut microbiome. We found that 3,230 metagenes (~0.19% of the full dataset) exhibited differential abundance, of which 852 were under-represented and 2,378 were over-represented after the intervention.

In order to confidently disclose the taxonomy categories corresponding to those genes, we performed a simple Blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) search against the non-redundant protein database at the NCBI (release May, 2017) making taxonomy assignments of query sequences with alignments of at least 70% amino acid sequence identity, when compared with the respective top matches. Regarding the under-represented set of metagenes, we were able to assign taxonomy categories for 31% of them with certainty, and identify species of the genera *Bacteroides, Alistipes, Eubacterium, Roseburia, Prevotella,* and *Raoutella* (Figure 2A). Of all the species, the metagenes of *Bacteroides* species were those most negatively influenced by AXOS intake, particularly *B. massiliensis* and *B. eggerthii*. Additionally, species such as *Alistipes obesi, Eubacterium siraeum, Roseburia inulivorans, Prevotella buccalis, Prevotella multisaccharivorax* were also reduced by AXOS intake.


Start point
End point
Actinobacteria
Normalized read counts
LMM variance = -0.92
P ≤ 0.0481
Bifidobacteriaceae
Normalized read counts
LMM variance = -1.21
P ≤ 0.0316
Bifidobacterium
Normalized read counts
LMM variance = -1.20
P ≤ 0.0317
Lachnospiraceae_XPB1014_group
Normalized read counts
LMM variance = -2.30
P ≤ 0.0377
Ruminococcus_gnavus_group
Normalized read counts
LMM variance = -3.90
P ≤ 0.0105
Rikenella
Normalized read counts
LMM variance = 1.59
P ≤ 0.0413

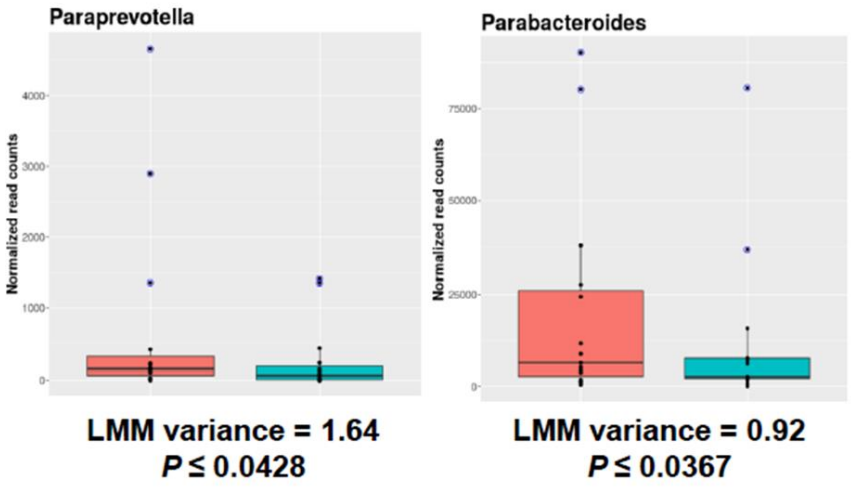


Figure 1. Gut microbiota components significantly influenced by AXOS intake. The distribution of normalized reads belonging to the six taxonomy categories with differential abundance after AXOS consumption is depicted in boxplots. Red boxes represent start point samples (baseline before the intervention) whereas green boxes represent the endpoint samples (at the end of the intervention). Blue data points indicate outliers. The Linear Mixed Model (LMM) estimate and p-values are shown respectively. LMM indicates the variance obtained using log-transformed data.

The analysis of the over-represented set of metagenes allow the identification of *Bifidobacterium species,* with *B. adolescentis, B. longum, B. catenulatum*, and *B. angulatum* as the most predominant (Figure 2B). We observed that more than one third of the over-represented metagenes corresponded to *Prevotella* species, particularly *P. copri* (Figure 2B). We detected and quantified DNA reads for the six *Prevotella* phylotypes present in the SILVA database release 128 (Supplementary information file Figure S1). Interestingly, each individual phylotype showed no significant distribution, including the "Prevotella_9" corresponding to *P. copri*, but with a notable increase in reads of "Prevotella_1" and "Prevotella_9". Globally, the sum of reads for all *Prevotella* phylotypes showed a marginal significance when combined ($p < 0.0501$).

The analysis of the over-represented set of metagenes also enable the identification of additional bacterial species, such as *Bacteroides stercoris*, *Bacteroides ovatus*, *Blautia obeum*, *Blautia wexlerae*, *Eubacterium rectale*, *Eubacterium hallii*, *Roseburia faecis*, and *Adlercreutzia equolifaciens*, which were increased after AXOS intervention (Figure 2B).

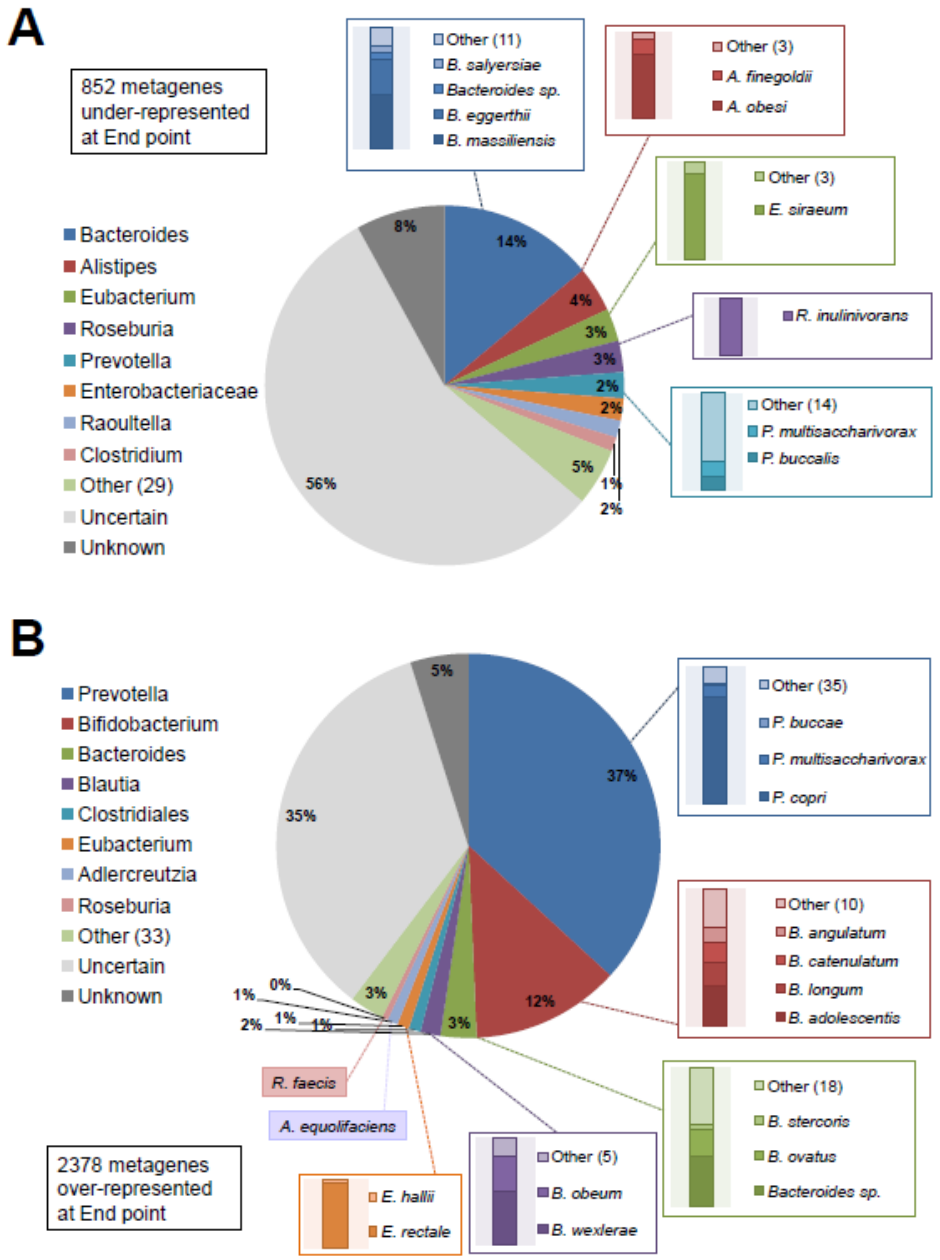


Figure 2. Taxonomy assessment of metagenes with differential abundance as a result of the AXOS intervention. A - Genus and species distribution of the under-represented metagenes in the metagenome of samples after AXOS intervention. B - Similar analysis as A for the over-represented metagenes. Pie-charts indicate the distribution at genus level whereas bar-plots indicate the distribution of the main species, the color legend is maintained accordingly. "Unknown" is used for taxonomic categories showing an identity score lower

than 70% and no taxonomy defined at genus level. Those with a score identity higher than 70% and equal matching with several species from the different genus are shown as "Uncertain". Numbers inside parenthesis show the number of genus or species additionally to those graphically declared.

Functional analysis. The set of metagenes under-represented as a consequence of AXOS intake exhibits a wide range of metabolic and cellular functions according to the annotation with KEGG. Of these, some metagenes encode potential pathogenic features, such as biofilm formation (ko:02026, ko:02025, ko:05111), beta-lactam resistance (ko:01501), bacterial secretion systems for virulence factor release (ko:03070), flagellar assembly (ko:02040), cationic antimicrobial peptides (CAMP resistance) (ko:01503), bacterial chemotaxis (ko:02030), monobactam biosynthesis (ko:00261), and lipopolysaccharide biosynthesis (ko:00540) (Supplementary information file Table S2).

When we analyzed the over-represented set of metagenes, we found a lower level of functional annotation than for the under-represented set of metagenes (24% and 43%, respectively). We then evaluated the function of those metagenes present in the species predominantly influenced by AXOS, such as *Prevotella copri* and *Bifidobacterium* sp., which roughly account for 50% of the over-represented metagenes (Figure 2B). As shown in Figure 3, the AXOS consumption led to an expansion of the collection of genes dedicated to vitamin and cofactor biosynthesis as well as of genes specialized in transport, biosynthesis and degradation of glycans. Notably, we also found a significant increase of genes involved in the biosynthesis of the neurotransmitter gamma-aminobutyric acid (GABA) and precursors of aromatic amino acids (shikimate and chorismate) with potential roles in the gut-brain communication. Moreover, the Venn analysis indicated that the functions amplified to a greater extent by AXOS intake (at least present in 3 out of the 4 groups of over-represented metagenes) consisted of those involved in the biosynthesis of Vitamin K2, the derivative Vitamin B9 tetrahydrofolate, Vitamin B2 and the coenzyme-A biosynthesis. All the above were equally found to be over-represented in metagenes from *Prevotella copri* and *Bifidobacterium* species (Figure 3).

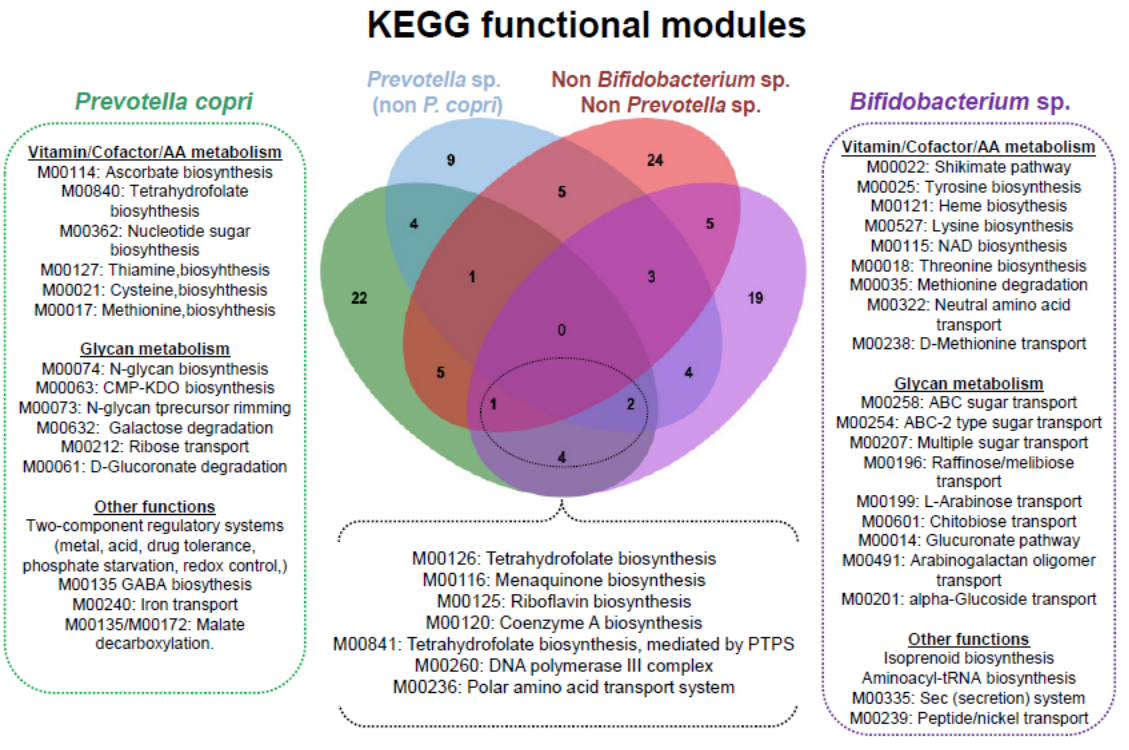


Figure 3. Gain-of-function in the gut microbiome as a consequence of AXOS ingestion. Over-represented metagenes from *Bifidobacterium* sp. (N = 315), *Prevotella copri* (N = 782), non-copri *Prevotella sp.* (N = 415), and non-*Bifidobacterium*/non-*Prevotella* (N = 866) species were mapped to the KEGG database and then assigned to the respective functional modules of such repository. A Venn analysis displays the taxonomy-specific functions gained and those exacerbated by the simultaneous presence in at least 3 out of the 4 groups of bacterial species analyzed (dashed-line circle).

To investigate the bacterial cellular functions boosted by AXOS consumption, we conducted a domain-enrichment analysis using the Pfam annotation system. This showed that the over-represented metagenes were almost exclusively dedicated to glycan metabolism, with several metagenes exhibiting a wide range of particular functions for this fundamental cell process (Table 1). We observed over-representation of functions such as outer-membrane proteins for carbohydrate binding and transport relying on OMP-, TonB- and SusD-like proteins. Moreover, we also identified a vast amount of proteins enriched in carbohydrate binding modules (CBMs) characteristic of glycosyl hydrolase enzymes such as beta-galactosidases, of which central domains were likewise enriched in the over-represented metagenes. More general functions were also found in several metagenes that encode ATPases with no defined molecular roles but apparently related to sugar transport function according to associations inferred from functional and phylogenetic information. Increases in the presence of metagenes encoding for carboxypeptidases and protein domains associated with similar functions (PD40/WD40-like) suggested that AXOS could increase the activity of specific pepetidases of the gut metagenome. An increased presence of several metagenes associated with the prokaryote innate immune response, such as restriction modification systems, was also observed (Table 1).

Table 1. Functional enrichment analysis in the over-represented metagenomes

Domain[1]	Annotation[1]	OR[2]	FDR[3]
PF12875	DUF3826, putative sugar binding domain	98.8	0.0456
PF12128	DUF3584, AAA-type ATPase	69.7	0.0049
PF00686	CBM_20, starch binding domain	43.9	0.0001
PF01637	ATPase_2, ATPase domain	18.4	2.07^{-6}
PF03175	DNA_pol_B_2, DNA polymerase type B	16.4	0.0268
PF15495	Fimbrillin_C, Major fimbrial subunit protein	16.4	0.0268
PF13734	Inhibitor_I69, Spi protease inhibitor	15.3	0.0331
PF16738	CBM26, Starch binding module 26	14.9	0.0342
PF12008	EcoR124_C, Type I restriction and modification enzyme	13.4	0.0456
PF03422	CBM_6, Carbohydrate binding module (family 6)	13.0	0.0035
PF08011	PDDEXK_9, PD-(D/E)XK nuclease superfamily	12.1	4.13^{-8}
PF14905	OMP_b_brl_3, Outer membrane protein beta-barrel family	11.3	2.51^{-8}
PF07676	PD40, WD40-like Beta Propeller Repeat	10.9	0.0021
PF07495	Y_Y_Y, domain of unsaturated carbohydrate periplasmic sensor	10.0	3.12^{-7}
PF16353	DUF4981, Beta-galactosidase activity associated domain	9.7	0.0125
PF05272	VirE, Virulence-associated protein E like protein	9.6	0.0391
PF04313	HSDR_N, Type I restriction enzyme R protein	8.8	0.0058
PF00593	TonB_dep_Rec, TonB dependent receptor	8.5	1.93^{-30}
PF09820	AAA-ATPase_like, Predicted AAA-ATPase	8.3	7.43^{-7}
PF16355	DUF4982, Beta-galactosidase activity associated domain	8.2	0.0261

PF13715	CarboxypepD_reg_2, Carboxypeptidase regulatory-like domain	7.8	1.93^{-30}
PF02929	Bgal_small_N, Beta galactosidase small chain	7.7	0.0044
PF07715	Plug, TonB-dependent receptor	7.6	7.46^{-30}
PF13173	AAA_14, AAA ATPase domain	7.1	9.34^{-8}
PF13620	CarboxypepD_reg, Carboxypeptidase regulatory-like domain	6.8	1.64^{-20}
PF00150	Cellulase, Glycosyl hydrolase family 5	6.7	0.0039
PF12771	SusD-like_2, Starch-binding associating with outer membrane	6.5	0.0272
PF02837	Glyco_hydro_2_N, Glycosyl hydrolase family 2	6.2	5.77^{-7}
PF07944	Glyco_hydro_127, Beta-L-arabinofuranosidase GH127	6.2	0.0336
PF13635	DUF4143, ATPase activity associated domain	5.7	0.0049
PF00703	Glyco_hydro_2, Glycosyl hydrolase family 2	5.5	2.19^{-5}
PF02836	Glyco_hydro_2_C, Glycosyl hydrolase family 2 C-ter	5.2	0.0001
PF04616	Glyco_hydro_43, Glycosyl hydrolase family 43	5.2	0.0011
PF14322	SusD-like_3, Starch-binding associating with outer membrane	4.8	6.86^{-9}
PF01915	Glyco_hydro_3_C, Glycosyl hydrolase family 3 C-ter	4.8	0.0043
PF14310	Fn3-like, Fibronectin type III-like domain	4.6	0.0110
PF07980	SusD_RagB, SusD family	4.5	8.57^{-8}
PF01580	FtsK_SpoIIIE, cell division protein	4.5	0.0433
PF07494	Reg_prop, Two component regulator propeller	4.5	0.0439
PF02321	OEP, Outer membrane efflux protein	4.3	0.0017
PF00933	Glyco_hydro_3_N, Glycosyl hydrolase family 3 N-ter	3.9	0.0220
PF09479	Flg_new, Listeria-Bacteroides repeat domain	2.9	0.0003
PF04851	ResIII, Type III restriction enzyme	2.8	0.0252
PF01381	HTH_3, Helix-turn-helix transcriptional regulator	0.2	0.0023
PF12844	HTH_19, Helix-turn-helix transcriptional regulator	0.1	0.0035
PF13560	HTH_31, Helix-turn-helix transcriptional regulator	0.1	0.0132
PF13443	HTH_26, Cro/C1-type HTH DNA-binding domain	0.1	0.0342
PF00395	SLH, S-layer homology domain - glycoproteins of bacterial surface	0.0	0.0146

[1] Domain identifier and functional annotation according to information retrieved from Pfam database (http://pfam.xfam.org/).

[2] Odds ratio (OR) calculated from the Fisher's exact test under conditional Maximum Likelihood Estimate (MLE) approach.

[3] False discovery rate (FDR) obtained after multiple testing correction of all domains detected (N = 7,711 domains)

Human gut metavirome's response to AXOS intake. We also used the metagenome information to evaluate the presence of virus sequences in the raw data retrieved from shotgun sequencing of fecal DNA. By using the ViromeScan approach [32], we were able to evaluate the relative abundance of approximately 22 virus families. In general terms, we found a predominant tendency towards a decrease of reads matching human virus sequences after the AXOS intervention, although the differences did not reach statistical significance. Interestingly, our viral characterization on the gut samples led to the detection of Megavirales and other giant viruses (> 200 nm in diameter), that were only recently identified in human stool and other human samples [45]. The clinical or biological significance of the presence of these viruses in the human gut remains to be determined, for this reason, our data paves the way to search for giant viruses in the human gut and to establish the impact that diet has on them.

AXOS impact on markers of glucose homeostasis and gut microbiota

A new evaluation in the set of responders indicates that AXOS intake did not result in significant changes in most of the metabolic health outcomes and biomarkers analyzed (anthropometry, inflammatory, lipid or glucose biomarkers) probably due to the limited sample size and short study duration. Nevertheless, we observed that the mean value of fasting insulin as well as of the insulin resistance index (HOMA-IR) were slightly reduced (Figure 4A, B, C). Although this reduction was only observed in a proportion of the subjects analyzed (~47%), it is suggestive of a potential improvement in glucose metabolism probably linked to changes in certain microbiota signatures. Using a logistic regression analysis we found that changes in the HOMA-IR tended to be related to three different taxonomy groups, whose abundance was increased as a result of the intervention (Figure 4D). In particular, the "Lachnospiraceae_AC2044_group" (Log-odds = 1.59, p = 0.0571), "Eubacterium_ruminantium_group" (Log-odds = 1.25, p = 0.0575), and "Lachnospiraceae_XPB1014_group" (Log-odds = 1.16, p = 0.0737) tended to predict the amelioration of HOMA-IR.

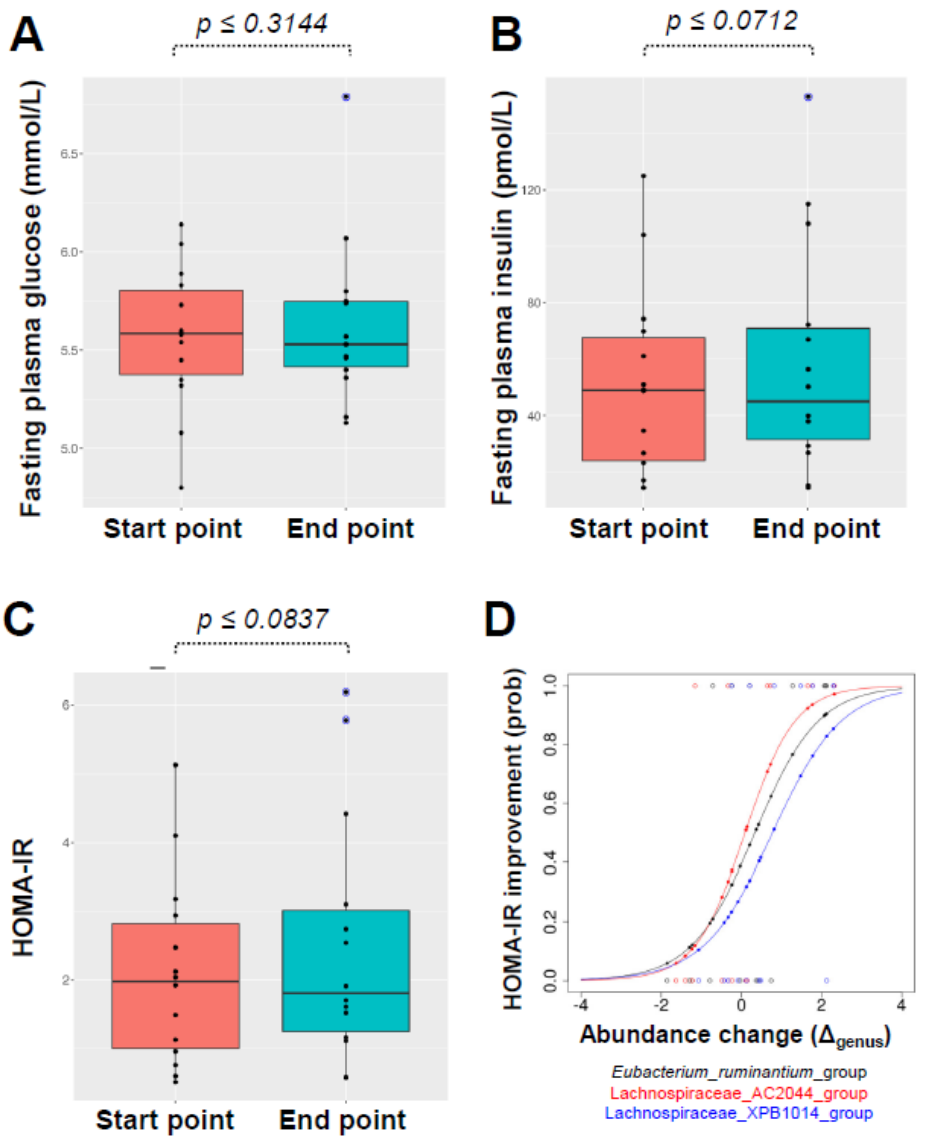


Figure 4. Glucose metabolism and relationship with gut microbiome. The fasting plasma glucose and insulin concentration are depicted in boxplot manner for samples before (Start point) and after (End point) the intervention (A and B panels, respectively). Similarly, the HOMA-IR values derived from the integration of the above biochemical parameters are shown (C). A logistic regression suggesting the gut microbiota groups potentially related to improvements in the HOMA-IR, based on their abundance changes across the intervention, is shown in panel D. The p-values are shown on top of the respective boxplots.

AXOS effects on plasma and fecal lipidome

By using MS-based lipidomics approaches, we were able to quantify 9 lipid classes and more than 150 species according to the chain length and double bond content both in plasma and fecal samples. We found no significant changes in the lipid profiles from feces in response to the intervention. In plasma, we detected a significant increase in one out of 18 analyzed cholesteryl ester species, CE 20:0, and a decrease in two hexoxylceramides and one ceramide (Table 2). For the latter, a quasi-consensus signal of reduction in plasma ceramide levels was observed in 8 out of the 9 features evaluated, thus obtaining a global trend for a decrease in the total ceramide content in plasma samples ($p \leq 0.0788$). We found no correlations between microbiome signatures and this lipidomic feature.

Table 2. Changes in lipid profiles from plasma samples

Lipid species	Lipid subspecies	Start point (µmol/L median)	End point (µmol/L median)	Delta values	p-value[1]
Cholesteryl esters	CE 20:0	1.088	1.348	0.260	0.0014
Ceramides	HexCer d18:1/16:0	0.733	0.570	-0.163	0.0011
	HexCer d18:1/24:1	1.077	0.836	-0.263	0.0011
	Cer d18:1/23:0	0.8835	0.794	-0.09	0.0120
	Cer d18:1/24:0	3.031	2.899	-0.132	0.0901
	Cer d18:1/24:1	1.516	1.457	-0.059	0.1673
	Cer d18:1/20:0	0.173	0.18	0.007	0.4897
	Cer d18:1/22:0	0.988	0.975	-0.014	0.4899
	Cer d18:1/18:0	0.205	0.196	-0.009	0.576
	Cer d18:1/16:0	0.507	0.503	-0.004	0.8017
	Total Cer	**7.504**	**7.423**	**-0.081**	**0.0788**

[1] p-values are based on comparison using Wilcox Signed-Rank test and corrected by multiple testing using the Benjamini-Hochberg method.

Cer = Ceramide, HexCer = hexosylceramide, CE = Cholesteryl ester.

Metabolomic profiling upon AXOS consumption

The NMR-based metabolomic analysis of the metabolite profile before and after the AXOS intervention indicated that there were only changes in the urine hippuric acid concentration (Figure 5). This increase was more prominent in a sub-group of participants, which could be attributed to higher abundance of *Prevotella_6* associated species ($R^2Y=0.5951$, $Q^2Y=0.1320$) and negatively correlated with *Ruminococcaceae UCG.012* abundance ($R^2Y=0.7190$, $Q^2Y=0.2795$). Additional correlation patterns suggested that urine dimethylamine (DMA) levels were associated with proportions of *Peptoclostridium* species ($R^2Y=0.6010$, $Q^2Y=0.2679$). In fecal samples, positive correlations between *Prevotella_2* abundance and the concentration of acetate, propionate and succinate were identified ($R^2Y=0.7357$, $Q^2Y=0.1818$). Finally, application of a supervised model of analysis allowed partial discrimination between plasma samples before and after the intervention (start and end points). Signals from residual very-low-density lipoprotein (VLDL) and triglycerides (TAGs) fractions, including fatty acids and glyceryls of lipids, were to some extent increased after the intervention. Although this could be indicative of a trend for dyslipidemia, the VLDL profile in plasma measured by classical methods indicates no major changes in this type of lipids during the intervention ($p \leq 0.2099$). On the other hand, the abundance of *Eubacterium rectale,* a microbial species boosted by AXOS intake (see Figure 2B and reference [21]), was positively correlated with concentration of phosphocholine and sn-glycero-3-phosphocholine in plasma ($R^2Y=0.5468$, $Q^2Y=0.2928$).

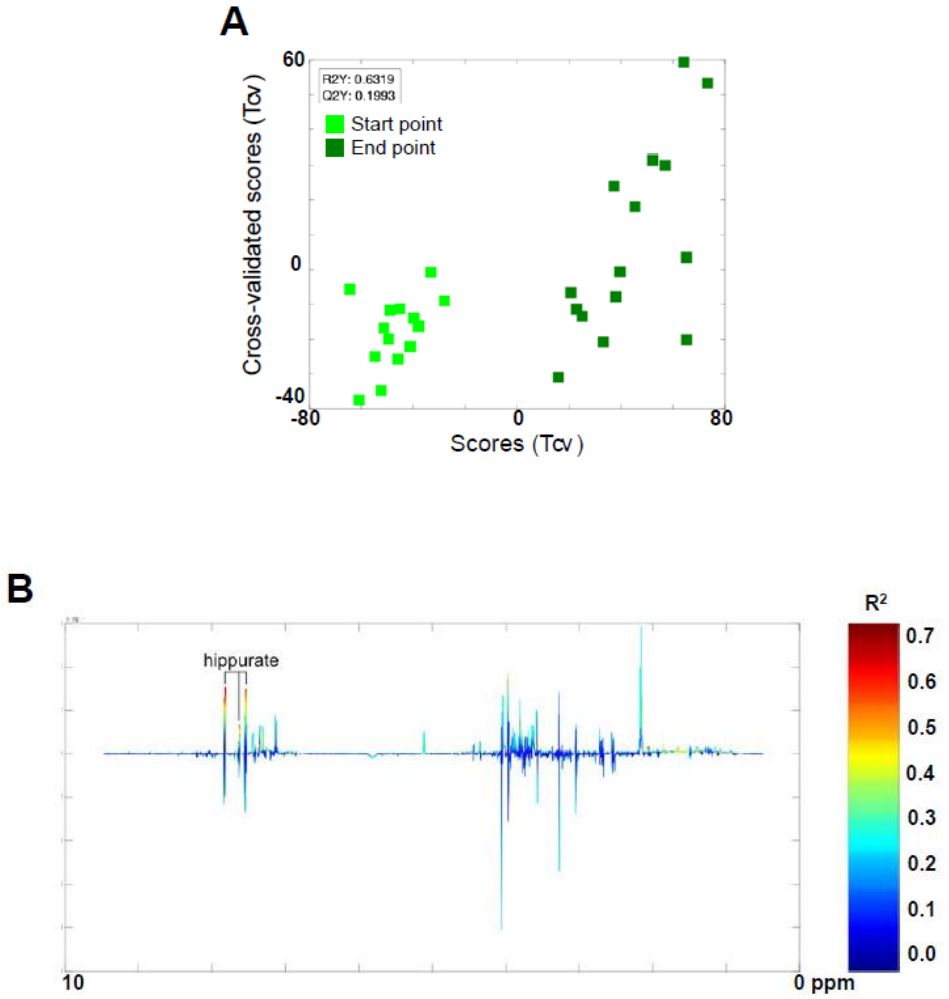


Figure 5. Effects of the dietary intervention on urine metabolome. Urinary metabolome was analyzed by 1H-nuclear magnetic resonance. Orthogonal projection to latent structure discriminant analyses models were used to compare the changes in the urinary metabolome at start and end points. **A**- For this pairwise comparison, the plot of the scores (T) compared with cross-validated scores (Tcv) is shown. **B**- Loading plot is color-coded according to the correlation coefficient (R^2) with Y (predictor vector coding time of the intervention). Q2, goodness of prediction; R2, goodness of fit.

Discussion

Using a multi-omics approach, we have performed an in-depth characterization of the microbiome, lipidome and metabolome response to a specific source of fiber (AXOS). In previous intervention trials, there has been controversy on the effect of AXOS on health outcomes with the microbiome assessments being limited to taxonomic features [18, 19, 21, 46, 47]. The present study aimed at providing a more exhaustive analysis of the AXOS-driven metabolic changes with a particular focus on gut microbiota-induced changes after a 4-week randomized dietary intervention. In agreement with previous findings, we found that a bifidogenic effect of AXOS was evident when the metagenome was analyzed using shotgun sequencing [18-20, 48].

In this study, we report new microbiome effects resulting from AXOS intake, including increases in the abundance of *Ruminococcus gnavus* and Lachnospiraceae_XPB1014 taxonomy groups, the latter related to *Eubacterium, Blautia* and *Roseburia* species (Blastn search against the non-redundant 16S NCBI database, 91-94% sequence identity). We have described the impact on *Blautia* species, and other butyrate/propionate producing bacteria, based on V3-V4 16S amplicon sequencing data and the combination of multivariate and linear analyses of OTUs in a previous paper [21]. Moreover, we observed that AXOS intake significantly reduced *Parabacteroides, Paraprevotella,* and *Rikenella* species. Similarly, reductions in *Parabacteroides* proportions have been linked to improvement of glucose homeostasis in obese mice after dietary supplementation with capsaicin according to another study [49]. This effect produced by AXOS on *Paraprevotella* species was also observed after the Roux-en Y gastric bypass (RYGB)-like and metformin treatments in ZDF rat model of obesity and T2D [50].

Upon assembling and predicting the set of bacterial genes present in the gut microbiome of responders included in the present study, we also found that AXOS intake increased the proportion of *Prevotella* species, and particularly of *Prevotella copri*. When searching the SILVA database (release 128), we found that of the six different phylotypes present in the database only two tend to be increased after AXOS intake (Prevotella_1 and Prevotella_9). However, a change in this taxonomy category ($p < 0.0501$) was detected as a result of the intervention when all *Prevotella* phylotypes were combined and considered in the analysis. These findings could lead to question the proper identification of this complex group of bacteria. In fact this issue has been reported previously in human studies where distinctive correlation patterns between dietary components and different *Prevotella* oligotypes were described [51].

The role of the species *P. copri* in the amelioration of glucose intolerance seems to be contradictory according to the existing scientific evidence. While the early metagenomic assessment on gut microbiome of T2D patients indicated that *P. copri* is linked to an increased production of branched-chain amino acids (in plasma and gut) associated with insulin resistance and to a higher risk of T2D [52], other animal studies reported that *P. copri* has positive impact on glucose homeostasis, given the activation of the intestinal gluconeogenesis via succinate production [53-55]. Therefore, it is difficult to attribute a healthy or harmful role to *P. copri* in glucose homeostasis in the light of the existing evidence, and it is likely that the reported effects also depend on the specific strains tested as well as on other components of the gut microbiota involved in host-microbe and microbe-microbe interactions and on the experimental conditions.

In our particular case, AXOS intake increased a large number of metagenes encoded by *P. copri* and tended to improve the HOMA-IR index, suggesting that AXOS intake can ameliorate the glucose metabolism, via modification of metagenomic functions, in overweight individuals with signs of MetS, in agreement with other studies in humans [47, 56]. Nevertheless, this slight improvement in HOMA-IR values was not explained by a change in abundance of any *Prevotella* phylotype, including those closely related to *P. copri*. By contrast, we unveiled an association trend between the HOMA-IR and three different taxonomy groups, including the Lachnospiraceae_XPB1014 group related to *Eubacterium, Blautia* and *Roseburia* species. Special attention should be given to the species from the Lachnospiraceae_XPB1014_group since these were found to be augmented by AXOS intake (Figure 1). It is likely that several components of the microbiota involved in the generation of SCFAs by cross-feeding mechanisms could explain potential AXOS effects on glucose metabolism. Such effects could be mediated, for example, by the conversion of succinate into SCFAs like propionate, which can trigger intestinal gluconeogenesis and improve glucose metabolism [54].

From a functional point of view, we observed that AXOS consumption increased the abundance of genes involved in carbohydrate metabolism as well as vitamin and cofactor biosynthesis. The expansion of the set of glycosyl hydrolases and enzymes related to carbohydrate metabolism, essentially in *P. copri* and *Bifidobacterium* species, could be involved in the generation of organic acids, including SCFAs, through cross-feeding mechanisms with potential beneficial effects on gut and metabolic health [16]. Theoretically, microbiome-mediated increases in the production of vitamins and cofactors could also bring host benefits (e.g. Vitamin K2 in bone physiology altered in obese individuals [57, 58]), although direct evidence of vitamin production and host utilization remains to be shown.

The AXOS intake increased the representation of metagenes involved in the biosynthesis of tetrahydrofolate (THF, the active form of folic acid), which may also have potential metabolic benefits. Indeed, some obese individuals show folate-deficiency, which could lead to hyperhomocysteinemia related to atherosclerosis risk [59]. In line with this, administration of folic acid and derivatives seems to confer protection against hyperhomocysteinemia-induced oxidative stress [60]. AXOS intervention also increased the capacity of the gut microbiome for aromatic amino acid production (Shikimate pathway, precursor or tryptophan, phenylalanine, and tyrosine, and Tyrosine biosynthesis), which theoretically could increase the generation of derivative neurotransmitters (e.g. serotonin, dopamine, epinephrine, nor-epinephrine).

An augmented capacity for GABA biosynthesis was also detected as a consequence of AXOS intake. Altogether this could result in additional effects on the communication within the gut-brain axis [61]. Different studies based on mice models indicate that low concentrations of GABA are associated with anxiety-like behavior, insulin resistance, energy expenditure imbalance, and weight gain in overweight and obesity [62-65]. Particularly, one animal study indicate that GABA act as activator of insulin secretion given that GABA-like immunoreactive cells in pancreas of rats is reduced in diabetes [66]. A recent study in obese rats also indicated that GABA supplementation reduces serum ceramide concentrations [64]. Therefore, theoretically, the AXOS-driven increased abundance of microbial species encoding GABA production hold promise for the mediation of metabolic and mental health effects. Our findings also show that AXOS intake significantly decreases the concentration of several ceramide species in plasma. Previous reports have also shown an association between the increased levels of ceramides and impaired glucose homeostasis in MetS and T2D patients [67-69].

Finally, the metabolomics approach was useful to trace the utilization of AXOS by gut microbiota through presence of hippuric acid in urine, a microbial metabolite derived from degradation of polyphenols associated with dietary fiber [70]. Our data suggest that hippurate production could be related to the abundance of *Prevotella* species increased by AXOS intake. Additionally, acetate, propionate, and succinate concentration in feces was also positively related to the abundance of *Prevotella* species, which could bring benefits to metabolic health as explained above [54]. The plasma metabolomic profiling indicates that phosphatidylcholine biosynthesis could be increased by AXOS intake via the butyrate-producer *Eubacterium rectale*, which showed positive correlations with phosphatidylcholine precursors. In turn, this could decrease bioavailability of choline for the production of methylamines (DMA, TMA) by other gut microbes [71], such as *Peptoclostridium* species that showed a positive correlation with the DMA urine concentration in

our study. TMA is the precursor of TMAO, which is produced in the liver, kidney and other tissues. Increased plasma levels of TMAO have been associated with enhanced risk of developing atherosclerosis, T2D and chronic kidney disease. Therefore, this could be a mechanism by which AXOS-microbiome mediated effects could confer protection against the impairment of glucose metabolism, [72], as well as against a wide variety of chronic diseases [73], reducing the availability and exposure to methylamines oxide forms.

Conclusions

Using a multi-omics functional approach, we have characterized in depth the effects of AXOS intake on the microbiome, lipidome and metabolome and, thereby, tentatively identified possible microbiome and non-microbiome mediated dietary effects on metabolic health. We have shown that additionally to the well-recognized bifidogenic effect of AXOS, this type of dietary fiber increases the abundance of *Prevotella* and lachnoclostridial species (butyrate producers) along with increases in organic acids (propionate and succinate). Furthermore, AXOS decreases plasma ceramide levels via a microbiome-independent mechanism, which altogether could contribute to an improved glucose metabolism. The direction of other functional metagenomic changes induced by AXOS were related to the production of neuroactive (GABA) and choline metabolites, suggesting potential additional effects that could reduce the risk of developing chronic metabolic conditions. Studies with a larger sample size and longer duration are warranted to confirm whether the metabolome and lipidome profiles resulting from AXOS intake and the induced microbiome configuration are translated into metabolic health outcomes, aiding in the validation of the biomarkers of dietary exposure and function tentatively identified in the present study.

List of abbreviations

AXOS: arabinoxylan oligosaccharides, AX: arabinoxylans, CBM: carbohydrate binding modules, CE: cholesteryl ester, Cer: ceramides, CVD: cardio-vascular disease, DMA: dimethylamine, ENA: European Nucleotide Archive, FC: free cholesterol, FDR: false discovery rate, GABA: gamma-aminobutyric acid, HexCer: hexosylceramides, HOMA IR: homeostatic model assessment for insulin resistance, KAAS: KEGG automatic annotation server, KEGG: Kyoto Encyclopedia for Genes and Genomes, LMM: linear mixed models, LPC: lysophosphatidylcholine, MetS: metabolic syndrome, MS: mass spectrometry, NCBI: National Center for Biotechnology Information, NMR: nuclear magnetic resonance, O-PLS-DA: orthogonal projection to latent structure discriminant analyses, ORF: open reading frame, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PE-P: PE-based plasmalogens, PI: phosphatidylinositol, PCA: principal component analysis, PUFA: poly-unsaturated fatty acids, SCFA: short-chain fatty acids, SM: sphingomyelin, T2D: type-2 diabetes, THF: tetrahydrofolate, TMA: trimethylamine, TSP: sodium 3-(tri-methylsilyl)-propionate-2,2,3,3-d4, WHO: World Health Organization, XOS: xylan oligosaccharides.

Declarations

Ethics approval and consent to participate.

The study is registered at Clinical Trial (NCT02215343), conducted according to the guidelines laid down in the Declaration of Helsinki and was carried out in accordance with the ethical standards of the responsible regional committee on human experimentation in Denmark, registered as H-4-2014-052, and the Danish Data Protection Agency (2013-54-0522).

Availability of data and material.

The raw fastq sequences generated from the shotgun sequencing of fecal DNA are publicly available at the ENA under the project accession number PRJEB25727.

Competing interests.

The authors have no conflict of interest to declare.

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Authors' contributions.

LK, LKB, AA, LHL and YS designed the study; ABP, LK, EMGP, LKB, JB, SM, GL and LHL conducted experimental approaches; ABP, JB, SR, PB, and SC analyzed data; ABP and YS wrote the paper, ABP has the primary responsibility for final content; all authors critically reviewed the manuscript and approved the final version.

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Table S1. Characteristics for the participants involved in the multi-omics assessment (N=15)[1]

	Median	Q1 ; Q3
Age	50	36 ; 52.5
Women (%)	80	-
Anthropometric		
Body weight (kg)	86.7	81.8 ; 91.4
BMI (kg/m²)	29.3	27.9 ; 31.2
FM (kg)	26.1	18.7 ; 33.2
LBM (kg)	56.9	51.4 ; 68.7
Fat percent, whole body (%)	30.0	21.5 ; 38.3
Waist circumference (cm)	94.8	92.1 ; 100.8
Hip circumference (cm)	112.5	109.1 ; 114.9
Sagittal height (cm)	22.4	20.6 ; 23.8
Blood pressure[2]		
Systolic (mmHg)	118.5	105.5 ; 126.0
Diastolic (mmHg)	78.0	75.5 ; 83.5
Pulse (beats/min)	62.0	55.3 ; 66.0
Lipid profile[2]		
Total CHO (mmol/L)	5.17	4.25 ; 5.48
HDL-CHO (mmol/L)	1.48	1.32 ; 1.61
LDL-CHO (mmol/L)	3.06	2.10 ; 3.36
VLDL-CHO (mmol/L)	0.56	0.41 ; 0.72
TG (mmol/L)	1.12	0.87 ; 1.34
Glucose metabolism[2]		
Glucose (mmol/L)	5.59	5.38 ; 5.81
Insulin (pmol/L)	48.9	24.1 ; 67.7
HOMA-IR	1.98	1.00 ; 2.82
HOMA-β	73.0	37.7 ; 106.8
Inflammatory markers[2]		
hsCRP (mg/L)	1.66	1.05 ; 2.27
Hb (mmol/L)	8.40	7.80 ; 9.05
WBC (10^9/L)	5.60	4.33 ; 6.78
Liver markers[2]		
ASAT (U/L)	21.0	19.0 ; 24.3
ALAT (U/L)	21.0	14.8 ; 26.5

[1]Data are given for baseline (Start point) as median with Q1 ; Q3 distribution.

[2] N=14

ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; BMI, body mass index; CHO, cholesterol; FM, fat mass; Hb, hemoglobin; HDL, high density lipoprotein; HOMA-β, homeostatic model assessment - beta-cell function; HOMA-IR, homeostatic model assessment- insulin resistance; hsCRP, high sensitive C-reactive protein; LBM, lean body mass; LDL, low density lipoprotein; TG, triglycerides; VLDL, very low density lipoprotein; WBC, white blood cell count.

Table S2. KEGG metabolic pathways associated with the down-represented metagenes

KEEG pathway (ko number and description)	Number of metagenes annotated
ko01100 Metabolic pathways	71
ko01110 Biosynthesis of secondary metabolites	25
ko01120 Microbial metabolism in diverse environments	25
ko02010 ABC transporters	20
ko01130 Biosynthesis of antibiotics	20
ko02020 Two-component system	17
ko02024 Quorum sensing	16
ko00230 Purine metabolism	15
ko01230 Biosynthesis of amino acids	15
ko01200 Carbon metabolism	14
ko00970 Aminoacyl-tRNA biosynthesis	13
ko00520 Amino sugar and nucleotide sugar metabolism	11
ko03440 Homologous recombination	10
ko00500 Starch and sucrose metabolism	9
ko00620 Pyruvate metabolism	9
ko00250 Alanine, aspartate and glutamate metabolism	9
ko03430 Mismatch repair	8
ko00720 Carbon fixation pathways in prokaryotes	8
ko03030 DNA replication	8
ko00240 Pyrimidine metabolism	7
ko00680 Methane metabolism	7
ko00550 Peptidoglycan biosynthesis	7
ko00051 Fructose and mannose metabolism	6
ko04112 Cell cycle - Caulobacter	6
ko00030 Pentose phosphate pathway	6
ko03018 RNA degradation	5
ko00650 Butanoate metabolism	5
ko03420 Nucleotide excision repair	5
ko03410 Base excision repair	5
ko00010 Glycolysis / Gluconeogenesis	5
ko02026 Biofilm formation - Escherichia coli	5
ko01210 2-Oxocarboxylic acid metabolism	4
ko00710 Carbon fixation in photosynthetic organisms	4
ko00910 Nitrogen metabolism	4

ko00020 Citrate cycle (TCA cycle)	4
ko00511 Other glycan degradation	4
ko00220 Arginine biosynthesis	4
ko00640 Propanoate metabolism	4
ko01501 beta-Lactam resistance	4
ko00052 Galactose metabolism	3
ko03070 Bacterial secretion system	3
ko02025 Biofilm formation - Pseudomonas aeruginosa	3
ko00630 Glyoxylate and dicarboxylate metabolism	3
ko00270 Cysteine and methionine metabolism	3
ko00460 Cyanoamino acid metabolism	3
ko00450 Selenocompound metabolism	3
ko00260 Glycine, serine and threonine metabolism	3
ko03010 Ribosome	3
ko00760 Nicotinate and nicotinamide metabolism	3
ko00300 Lysine biosynthesis	3
ko00670 One carbon pool by folate	2
ko05111 Biofilm formation - Vibrio cholerae	2
ko00860 Porphyrin and chlorophyll metabolism	2
ko00430 Taurine and hypotaurine metabolism	2
ko00513 Various types of N-glycan biosynthesis	1
ko00730 Thiamine metabolism	1
ko00770 Pantothenate and CoA biosynthesis	1
ko02040 Flagellar assembly	1
ko00290 Valine, leucine and isoleucine biosynthesis	1
ko00940 Phenylpropanoid biosynthesis	1
ko04724 Glutamatergic synapse	1
ko00600 Sphingolipid metabolism	1
ko00750 Vitamin B6 metabolism	1
ko00471 D-Glutamine and D-glutamate metabolism	1
ko01503 Cationic antimicrobial peptide (CAMP resistance)	1
ko00564 Glycerophospholipid metabolism	1
ko00261 Monobactam biosynthesis	1
ko04979 Cholesterol metabolism	1
ko01051 Biosynthesis of ansamycins	1
ko02030 Bacterial chemotaxis	1
ko01523 Antifolate resistance	1
ko00920 Sulfur metabolism	1

ko00531 Glycosaminoglycan degradation	1
ko00480 Glutathione metabolism	1
ko00791 Atrazine degradation	1
ko00540 Lipopolysaccharide biosynthesis	1

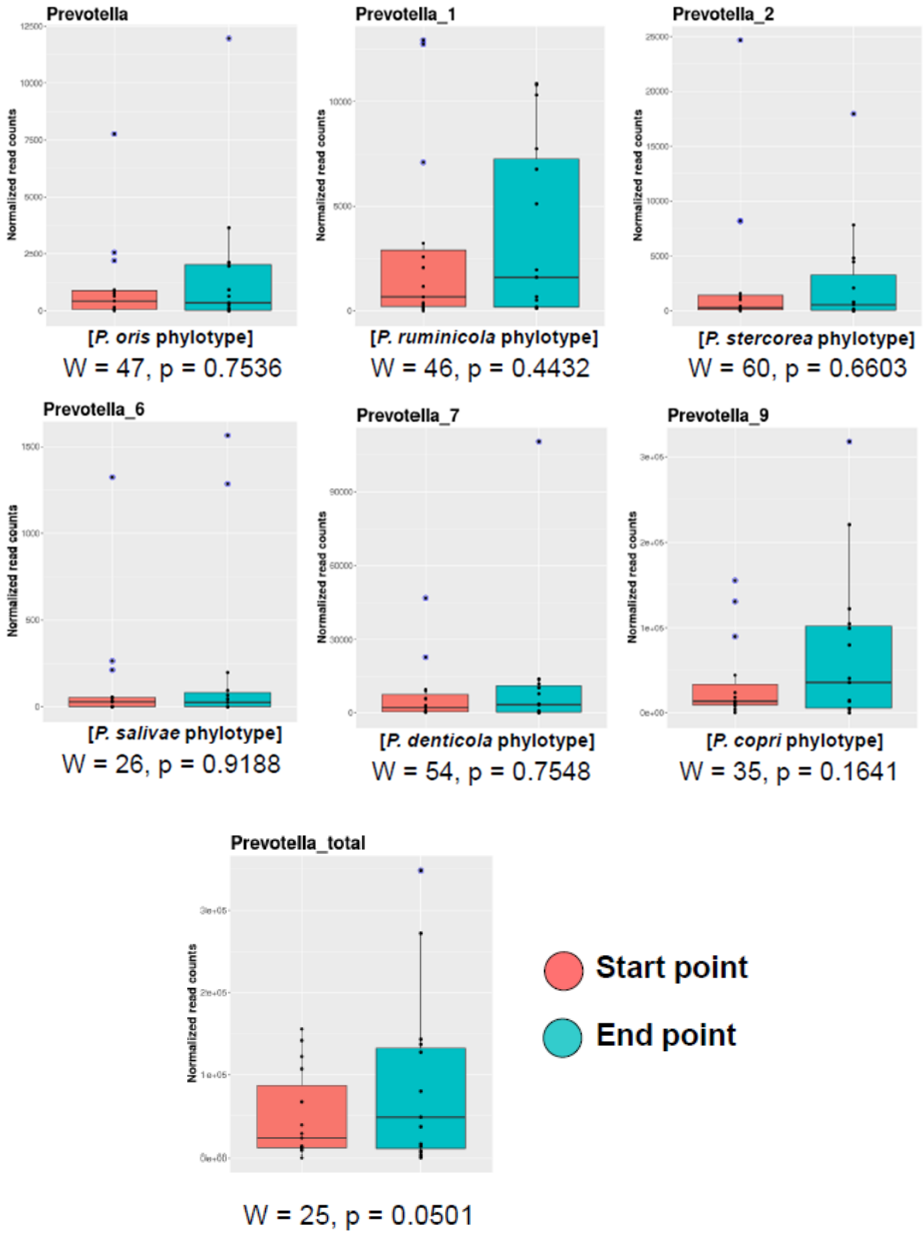

Figure S1. Distribution of *Prevotella* phylotypes according to the SILVA database. The DNA read distribution for the six different *Prevotella* phylotypes found to be annotated in the SILVA database (release 128) is drawn in boxplot. The identity for each phylotype was deduced from the direct comparison of the database reads against the non-redundant 16S NCBI database, thus selecting the matches with top score and sequence identity to assign the taxonomy at the species level. Red boxes show the distribution in start point samples

whereas the green boxes indicate the distribution in the end point samples. Blue data points indicate the outliers and the Wilcox Signed-Rank estimates (W) and p-values are shown below boxplots, respectively.

DISCUSIÓN GENERAL

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Los resultados de esta tesis doctoral, presentados en los dos capítulos previos, responden al objetivo general de avanzar en el entendimiento de cómo la composición y funciones de la microbiota intestinal influye en el desarrollo de la obesidad y co-morbilidades asociadas, así como identificar las bacterias intestinales que, junto a cambios en la dieta, puedan contribuir a establecer estrategias de intervención más eficaces para controlar estos desórdenes.

La figura 9 muestra una representación esquemática de los diferentes estudios llevados a cabo durante la tesis y los principales resultados y contribuciones científicas obtenidos en su conjunto.

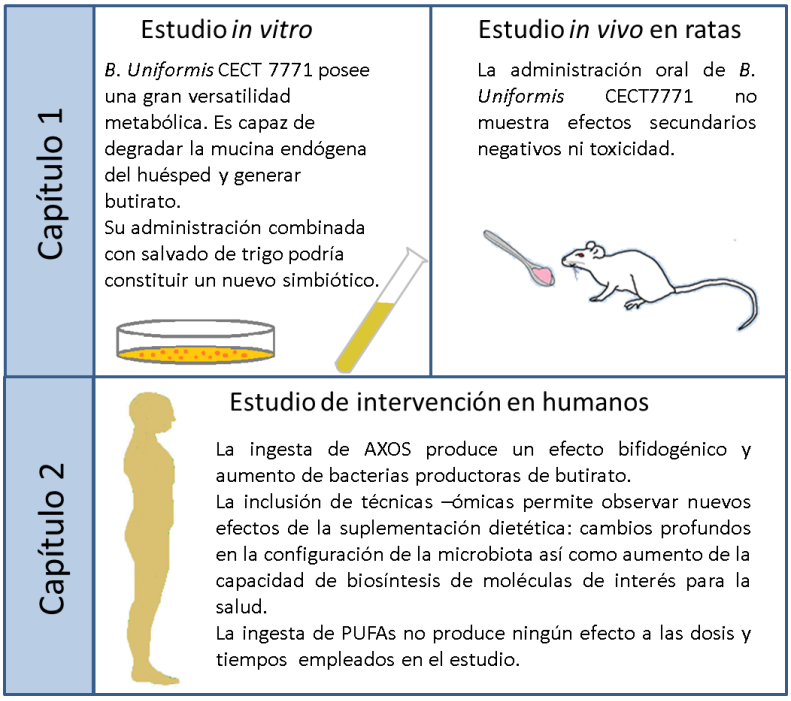

Figura 9. Representación esquemática de los estudios incluidos en la tesis doctoral.

El estudio y caracterización del genoma de *Bacteroides uniformis* CECT 7771, así como de su respuesta *in vitro* a una gran variedad de oligo- y poli-sacáridos, muestra su gran versatilidad metabólica y confirma sus posibles futuras aplicaciones en el tratamiento de la obesidad y co-morbilidades.

El estudio de nuevas cepas bacterianas diferentes a los probióticos clásicos (bacterias acido-lácticas y bifidobacterias) está en auge y es cada vez más

importante, ya que gracias a las nuevas tecnologías de secuenciación se ha podido vislumbrar un gran número de especies bacterianas todavía por cultivar, estudiar y caracterizar que pueden ser beneficiosas para la salud humana. El primer capítulo de la tesis se centra en el estudio *in vitro* e *in vivo* de una cepa del género *Bacteroides* cuyos efectos anti-obesogénicos han sido ya previamente demostrados en un estudio pre-clínico [181].

El primer estudio del capítulo 1 deriva de la necesidad de optimizar el crecimiento de *B.uniformis* CECT 7771, además de profundizar en el conocimiento de sus mecanismos de acción mediante el estudio de su genoma y transcriptoma *in vitro* en presencia de diferentes fuentes de carbono y fibra para el hospedador.

Tras observar los resultados del primer estudio podemos concluir que la función que desempeña una bacteria es enormemente dependiente del tipo de sustrato que utiliza, ya que se pueden activar diferentes rutas metabólicas, por lo que estos descubrimientos son interesantes para potenciar determinadas actividades o funciones llevadas a cabo por una misma bacteria, dependiendo de la dieta. Por ejemplo, la toxicidad producida por el LPS de bacterias Gram negativas podría reducirse mediante alimentos o ingredientes basados en los polisacáridos complejos presentes en la mucosa intestinal de mamíferos, ya que se ha visto que por ejemplo *B. uniformis* CECT 7771 al fermentarlos, reduce la síntesis de ácidos grasos de cadena larga implicados en la síntesis de LPS y disponibles para otras bacterias el ecosistema. Por otra parte, hemos observado que la utilización conjunta de *B.uniformis* CECT 7771 y pectina o mucina, podría contribuir a mejorar la integridad de la barrera intestinal y ejercer efectos beneficiosos en el metabolismo, ya que a través de su fermentación *B.uniformis* CECT 7771 genera butirato, que ejerce mejora la función barrera, ejerce efectos anti-inflamatorios y regula la producción de péptidos endocrinos (GLP1) que mejoran el metabolismo de la glucosa e inducen saciedad.

Este primer estudio también muestra que la especie *B. uniformis* posee el repertorio de genes registrados en la base de datos de CAZy (del inglés Carbohydrate-Active enZYmes Database) más extenso de su género, superando el número de genes de otras especies tipo como *B. thethaoiotaomicron* o *B. cellulosyliticus*. Entre los genes para degradación de carbohidratos de *B. uniformis* CECT 7771 se encuentran algunos que permiten la degradación de los azúcares complejos presentes en las proteínas componentes del moco del epitelio intestinal (denominados *O*-glicanos), mostrando que esta bacteria, posiblemente, es capaz de interactuar directamente con el hospedador desarrollando una relación de mutualismo, ya que los *O*-glicanos del epitelio del hospedador serían empleados por la bacteria como fuente de carbono y a la vez esto conllevaría la producción de butirato, que es empleado como fuente de energía por los colonocitos,

produciéndose una retroalimentación positiva dado que el butirato induce una mayor producción de mucina por parte de estos. La capacidad para obtener nutrientes del propio hospedador sin depender de la ingesta de otras fuentes de carbono provenientes de alimentos ingeridos por la dieta muestra su temprana relación simbiótica establecida con el ser humano posiblemente durante el período de lactancia.

Este estudio también nos ha permitido conocer en mayor profundidad los mecanismos moleculares que podrían mediar los efectos beneficiosos observados en los estudios *in vivo* en un modelo de obesidad previamente descritos por Gauffin *et al*. (2012). La degradación del moco intestinal reduce la producción de LPS, siendo este mecanismo uno de los posibles responsables de la reducción de la inflamación asociada a la obesidad. Además, el aumento de la expresión de genes relacionados con la síntesis de butirato por el consumo principalmente de los O-glicanos podría, en parte, explicar también los posibles mecanismos moleculares subyacentes a muchos de los efectos positivos observados en dicho estudio. El butirato ejerce un efecto inhibidor de las deacetilasas de histonas (DACHs), que se considera un mecanismo epigenético por el que se regula la función de células del sistema inmune y se reduce la inflamación [260], que podrían explicar los efectos antiinflamatorios de esta bacteria en el modelo animal. El butirato suprime los efectos pro-inflamatorios de la lámina propia de los macrófagos [261] y la diferenciación de células dendríticas a partir de células madre de la médula ósea mediante vías de acción como inhibidor de DACHs y promueve la síntesis de células T reguladoras [262, 263]. Esto facilita una mayor tolerancia a la microbiota comensal beneficiosa por parte del sistema inmune del hospedador, así como a otros estímulos pro-inflammatorios [260], pudiendo ser otro de los mecanismos responsables de la mejora del estado inflamatorio asociado a la obesidad. Este efecto además, podría estar potenciado por la restauración de la integridad de la barrera epitelial también debida a la acción del butirato [263]. Los efectos positivos de SCFAs como butirato y propionato han sido también relacionados con la mejora del metabolismo de la glucosa en ratones a través por ejemplo de la inducción de GLP-1 [264], lo que podría explicar en parte los efectos observados en la homeostasis de la glucosa en el estudio de Gauffin *et al*. [265].

La capacidad de producción de GABA por *B. uniformis* CECT 7771 al fermentar algunas fuentes de carbono, como la pectina, también podría ejercer efectos beneficiosos en el metabolismo, por ejemplo reduciendo los niveles de glucosa, como algunos estudios indican [266]. No obstante, sería necesario hacer estudios in vivo administrando conjuntamente esta cepa bacteriana con fuentes de pectina para demonstrar esta hipótesis derivada de un estudio in vitro.

El estudio del transcriptoma de *Bacteroides uniformis* CECT 7771 inducido por la presencia de diferentes fuentes de carbono permite evaluar posibles sinergias entre la bacteria y los sustratos para optimizar su uso como simbióticos.

Este estudio del primer trabajo del capítulo 1 también tuvo como objetivo evaluar qué fuente de carbono (glucosa, salvado de trigo, pectina, mucina, inulina o goma arábiga), podría promover el crecimiento de la bacteria. Este hecho es muy importante desde el punto de vista de su producción a gran escala así como para la posible generación de un "simbiótico", fomentando la sinergia entre una bacteria de interés y un sustrato que utilice preferente.

Esto permitiría reducir los costes de la producción industrial. De hecho, los resultados del estudio concluyen que aquellas fuentes de carbono más sencillas (preferentemente salvado de trigo y después glucosa) permiten reducir los tiempos de duplicación de *B. uniformis* CECT 7771. Mediante la introducción de salvado de trigo rico en AXOS en la formulación de los medios de cultivo puede acortarse el tiempo de producción, llegando al final a concentraciones de biomasa en fase estacionaria con valores similares a los obtenidos con los medios de cultivo comercializados en la actualidad para las bacterias del género *Bacteroides*.

Además, dado que el producto utilizado (AXOS) está comercialmente disponible, su uso como prebiótico o formulado como simbiótico sería factible. Para avanzar en esta dirección, sería interesante comprobar previamente si el efecto observado *in vitro* se observa también *in vivo* y en qué medida se podría reducir la dosis de *B. uniformis* CECT 7771 mediante la suplementación de AXOS, sin perder efectividad o incluso aumentándola. Esto podría reducir tanto su coste, como ampliar su efectividad y dianas de acción.

***Bacteroides uniformis* CECT 7771 no muestra signos de toxicidad ni patogenicidad de acuerdo con el análisis de su genoma y la evaluación del efecto *in vivo* de su administración oral sub-crónica en un ensayo de toxicidad en rata.**

B. uniformis CECT 7771 está protegida bajo patente, por lo que para poder acercarse más a la posibilidad de comercialización de la misma se debe evaluar tanto su efectividad como su seguridad. Con este fin, en primer lugar, secuenciamos el genoma de la cepa bacteriana empleando la tecnología basada en nanoporos, refinada con los datos obtenidos del análisis del transcriptoma obtenido en diferentes condiciones de cultivo *in vitro*. Los datos obtenidos suponen un conocimiento más detallado de las capacidades metabólicas de la

bacteria y la evaluación de su potencial glucolítico para degradar diferentes fuentes de carbono. Además, la información genética obtenida a partir de la secuenciación de su genoma ha contribuido al análisis exhaustivo de su bioseguridad. Esto ha permitido analizar la presencia o no de resistencias a antibióticos, genes de virulencia y la presencia de elementos móviles transponibles en el genoma, una característica no deseada en cepas para las cuales se pretende demostrar su seguridad al carecer del estatus de QPS [267]. En el genoma de *B. uniformis* CECT 7771 se observó un elemento plasmídico, pBU7771, de 2,746 pb encontrado en otras especies del género *Bacteroides* y cuyas 3 ORFs (Open Reading Frames) predichas no codifican resistencias a antibióticos, genes de virulencia u otros genes patogénicos. Al determinar el número de copias del plásmido (aproximadamente 2 copias por célula), se puede inferir que existe una gran probabilidad de que haya progenie de *B. uniformis* CECT 7771 sin este elemento, haciendo que las funciones codificadas en él sean prescindibles para la fisiología de la bacteria. No obstante, se deberán llevar a cabo estudios adicionales para entender la verdadera función de este tipo de elemento genético característico del género *Bacteroides*.

Estos resultados se han complementado con el segundo estudio de este capítulo de tesis, centrado en la evaluación de la seguridad de *B. uniformis* CECT7771 *in vivo*. Dado que la finalidad última del estudio de nuevas bacterias candidatas para ser utilizadas como probióticos es su comercialización como suplemento para mejorar alguna patología o reducir su riesgo, es necesario comprobar la seguridad de las mismas en estudios pre-clínicos en animales antes de realizar los estudios clínicos en humanos. Anteriormente, tras el estudio llevado a cabo en nuestro grupo por Gauffin *et al*., [181] donde se evaluaba la efectividad de *B. uniformis* CECT 7771 en un modelo de obesidad, se llevó a cabo un estudio de seguridad en ratones a los cuales se les administró la cepa durante 6 días para observar posibles efectos adversos de una exposición aguda a la bacteria [268]. Este estudio fue preliminar, puesto que posteriormente se requiere llevar a cabo otros estudios que demuestren la ausencia de efectos adversos tras una administración prolongada en el tiempo [269], evaluando la relación dosis-efecto, para asegurar que no existen efectos nocivos o perjudiciales debidos a su ingesta o exposición a largo plazo. Por ello, se diseñó un segundo estudio con estas características donde la dosis administrada fue desde 10^8 ufc hasta 10^{10} ufc al día (equivalente a $1.62 \cdot 10^9$ ufc/día en una persona cuando se normaliza por área de superficie, lo cual supone una dosis 10 veces más elevada que la dosis efectiva de probiótico para una persona) y administrado diariamente por un periodo de 90 días. El estudio no reveló efectos secundarios en ninguno de los aspectos evaluados (peso, ingesta de comida, comportamiento, parámetros bioquímicos, inflamación, translocación bacteriana o

análisis histológico), indicando que su administración continuada no entraña riesgos en animales. Se requeriría, no obstante, su valoración en humanos.

En este mismo estudio, la administración de la bacteria *B. uniformis* CECT 7771 durante 90 días a ratas (no obesas) mostró un efecto dosis-dependiente en la disminución de los valores de la alanina aminotransferasa (ALT), en comparación con los grupos control. Valores altos de ALT en sangre se atribuyen a problemas o trastornos hepáticos, por lo *B. uniformis* CECT 7771 podría presentar características protectoras de la función hepática, de amplio interés en el tratamiento de enfermedades virales y asociadas a la dieta.

En estudios previos llevados a cabo por nuestro grupo se observó que ratones con obesidad inducida por dieta mejoraban los signos de esteatosis hepática recibiendo una dosis de *B. uniformis* CECT 7771 de 10^9 ufc/día durante 7 semanas. Además, en dicho estudio también se observó una reducción de la acumulación de lípidos en hígado en el grupo de ratones no obesos que recibió la bacteria, aumentando el número de hepatocitos sin esteatosis y con bajo grado de esteatosis [181]. Este grupo de animales tiene características similares a los grupos de ratas que recibieron *B. uniformis* CECT 7771 en el estudio de toxicidad sub-crónico incluído en la presente tesis. Con ello se refuerza la hipótesis de que la ingesta de *B. uniformis* CECT 7771 puede ejercer un efecto beneficioso en la salud hepática.

La razón IL-10/IFN-ɣ fue significativamente mayor en el grupo de ratas que consumió diariamente 10^9 ufc de *B. uniformis* CECT 7771, al igual que el grupo de ratas al cual se le administró *B. longum* ATCC 15707^T a 10^{10} ufc/día. Valores altos de esta razón indican un perfil más anti-inflamatorio, que podría constituir un efecto beneficioso de la bacteria a largo plazo en animales no obesos. Este efecto fue similar al del control *B. longum* ATCC 15707^T, pero requiriendo una dosis una unidad logarítmica superior a la del bacteroide.

Estudios adicionales serían, no obstante, necesarios para establecer si la capacidad antiinflamatoria de *B. uniformis* CECT 7771 está relacionada con los efectos positivos en hígado.

La microbiota intestinal es modulada por el consumo diario de arabinoxilanos (AXOS) durante 4 semanas en individuos que padecen sobrepeso y síndrome metabólico, induciendo un efecto bifidogénico y un incremento de especies bacterianas productoras de butirato.

En el primer estudio del segundo capítulo de la tesis llevamos a cabo una intervención en humanos con sobrepeso y síndrome metabólico con AXOS y

PUFAs con diseño cruzado. Los primeros análisis de microbiota realizados por secuenciación masiva del gen del ARN 16S bacteriano mostraron que la ingesta de AXOS produjo un aumento de la población de bifidobacterias en la microbiota intestinal, coincidiendo con resultados previamente obtenidos [270-273]. Además, a diferencia de otros estudios previos, se observó un aumento de especies productoras de butirato como *E. rectale, F. prausnitzii, E. hallii, Dorea sp., Roseburia* sp. o *Anaerostipes* sp. Ante tales resultados, planteamos la hipótesis de que el consumo de AXOS por parte de la microbiota intestinal podría estar induciendo un fenómeno de alimentación cruzada o *cross-feeding* donde los AXOS son en primer lugar degradados principalmente por las bifidobacterias, induciendo la producción de acetato como subproducto de la fermentación de las fibras y que posteriormente constituye una fuente de alimento para otras bacterias cuyo subproducto es el butirato.

Además, observamos que el consumo de AXOS podría tener efectos beneficiosos derivados de una disminución de la abundancia de las familias Rikenellaceae y Porphyromonadaceae, asociadas a procesos inflamatorios [274].

La ingesta de AXOS y PUFAS no induce cambios significativos en los parámetros bioquímicos analizados tras la intervención de 4 semanas con las dosis establecidas.

A pesar de la modificación de la composición de la microbiota intestinal observada tras la ingesta de AXOS y las múltiples correlaciones positivas y negativas obtenidas entre algunas OTUs y parámetros bioquímicos, no se obtuvo una mejora de los valores finales de dichos parámetros (perfiles lipídicos, metabolismo de la glucosa, marcadores de inflamación). La ingesta de PUFAs no produjo efectos significativos en la composición de la microbiota, ni en los parámetros bioquímicos.

Sin embargo, otros estudios similares sí que han obtenido mejoras en algunos parámetros bioquímicos. Por ejemplo, se observó disminución de glucosa sérica, triglicéridos y ApoA1, tras la ingesta de xilooligosacáridos (XOS) [275] y un efecto de mejora de respuesta a la glucosa y sensibilidad a la insulina dependiendo de la dosis de AXOS ingerida [276], lo cual es un indicativo de que tanto el tiempo de la intervención, como la dosis y la naturaleza de las fibras son un factor muy importante a tener en cuenta en el diseño de los experimentos. En estos estudios el tipo de fibra era similar pero las dosis administradas superiores y los tiempos de intervención más prolongados. Debido a que en el estudio de PUFAs el tamaño muestral de la población de la intervención fue calculado en base a los estudios

previos con AXOS (pues todavía no existían estudios publicados de intervención con PUFAs), podría ser necesario un mayor tamaño muestral para poder ver cualquier efecto que pudiera tener lugar. Aunque no se han podido demostrar en el estudio efectos de "*carry-over"* significativos por períodos de lavado o "*wash-out"* inapropiados, podrían también considerarse como causa de la falta de efectos observados, así como que las complicaciones metabólicas de los individuos que participaron en las intervenciones eran más leves de lo deseado para poder ver algún efecto del consumo de AXOS y PUFAS. Por último, algunos estudios han demostrado que los efectos de las intervenciones dietéticas pueden estar altamente influenciados por la composición inicial de la microbiota de cada individuo, por lo que consecuentemente, algunos individuos pueden responder mejor que otros a los cambios en la dieta o incluso no responder en algunos casos, lo cual puede dificultar la observación de efectos beneficiosos debidos a las intervenciones [277].

Los resultados obtenidos en la intervención con AXOS mediante técnicas –ómicas permite identificar nuevos efectos de la suplementación dietética sobre el microbioma y el metabolismo.

El segundo estudio del capítulo 2 de la tesis se centró en un análisis más exhaustivo mediante técnicas -omicas de las muestras del primer grupo de sujetos del estudio cruzado al que se administró AXOS (AXOS I) y que respondió de forma significativa a la intervención mostrando cambios notables en la abundancia de las bifidobacterias. Este incluyó el análisis del metagenoma y del estudio de metabolitos producidos en heces, orina y plasma mediante el estudio del metaboloma y el lipidoma. Este estudio se diseñó para poder resolver parte de las controversias que se habían generado en publicaciones previas cuando se estudiaba el efecto del AXOS en estudios de intervención en humanos.

El estudio del metagenoma aportó una visión más completa de la variación de la composición taxonómica debida al consumo de AXOS. A parte del efecto bifidogénico y aumento de especies de la familia Lachnospiraceae (*Eubacterium, Blautia, Roseburia*) (observados también en el primer estudio) y *Ruminococcus gnavus*, se llegó a detectar una disminución de abundancia de *Parabacteroides, Paraprevotella* y *Rikenella* y un aumento de especies del género *Prevotella* (especialmente *P. copri*), alrededor de la cual hay bastante controversia, y cuyo hallazgo como género aumentado por el consumo de AXOS en este estudio podría suscitar interés.

Aunque algunos estudios indican asociación de *P.copri* con efectos adversos en la salud [278, 279], recientemente se ha observado que la respuesta a dietas ricas en fibra son dependientes de la proporción *Prevotella/Bacteroides*, donde la mayor prevalencia de Prevotella se asocia a mayores pérdidas de peso y grasa corporal en un estudio de intervención dietética durante 24 semanas [280].

Tanto el género Prevotella como el género Bacteroides son grandes fermentadores de fibra, pero el perfil y proporción de SCFAs producidos por ambos es diferente, por lo que sus proporciones podrían tener efectos fisiológicos diferentes [281].

Admitiendo la teoría anterior, el hecho de que la ingesta de AXOS estimule el crecimiento de *Prevotella* en detrimento de otros fermentadores de fibras del orden Bacteroidales (*Paraprevotella, Parabacteroides, Rikenella*) podría ser considerado como beneficioso. No obstante, además de la abundancia relativa de estos géneros, también puede haber grandes diferencias funcionales dependiendo de las especies e incluso de las cepas por lo que otros estudios han indicado que estas conclusiones son cuestionables [282].

P. copri ha sido previamente asociada al consumo de dieta mediterránea [283] aunque se ha visto que el efecto beneficioso de las fibras de la dieta son dependientes del enterotipo predominante de cada individuo [284]. En el estudio de Kovatcheva-Datchary y colaboradores, sólo aquellos individuos que mostraban un aumento de *Prevotella* eran aquellos que mejoraban la tolerancia a glucosa con el consumo de fibra en la dieta. Para comprobar si *Prevotella* era la responsable de la mejora, se administró *P. copri* DSM18205 a ratones (sin modificaciones de la dieta) y se observó también una mejora de la respuesta a glucosa. Otro estudio llevado a cabo por Zhu y colaboradores mostraba que el repertorio de genes en las cepas del género *Prevotella* era muy extenso, siendo posiblemente una de las causas por las que se den diferentes respuestas a la dieta y la implicación de ello en la salud en diferentes individuos [285]. De hecho, en otro estudio se obtuvieron conclusiones negativas sobre la relación de *P. copri* y la resistencia a insulina, la intolerancia a la glucosa y el aumento de los niveles circulantes de ácidos grasos ramificados (BCAAs) [286].

El estudio funcional del metagenoma tras la ingesta de AXOS permitió obtener más información de la intervención, como el aumento de genes relacionados con el metabolismo de carbohidratos (principalmente pertenecientes a *Bifidobacterias* y *P. copri*) y síntesis de vitaminas y cofactores. Los AXOS indujeron un aumento de la biosíntesis de tetrahidrofolato y otros derivados del ácido fólico o vitamina B9, cuya función protectora contra la hiperhomocisteinemia ha sido previamente estudiada [287] y también se vió aumentada la capacidad de biosíntesis de

menaquinona o vitamina K2, importante para la correcta absorción del calcio y que por lo tanto, puede sugerir un importante papel del consumo de fibras AXOS como suplementos dietéticos para prevenir otros problemas de salud.

Se observó asimismo un aumento de los genes dedicados a la producción de aminoácidos aromáticos, como el triptófano y la tirosina, implicados posteriormente en la producción de neurotransmisores como la dopamina, adrenalina, noradrenalina y serotonina. Además, se observó también un aumento de la capacidad de biosíntesis de GABA, el cual puede tener efectos en la comunicación del eje intestino-cerebro [288] y cuya baja concentración en el organismo ha sido asociada a estados de ansiedad y desbalance energético (ganancia de peso, sobrepeso y obesidad) [29, 289, 290]. No obstante, para confirmar esta hipótesis se tendría que demostrar que un aumento en la síntesis de GABA producido por la ingesta de AXOS, mediado por la microbiota, se traduce en un efecto fisiológico sobre alguna de estas alteraciones, mediante estudio de intervención diseñado con tal finalidad.

Además, el GABA posee efectos beneficiosos como promotor de la secreción de insulina y funciones en la regulación de los niveles de ceramidas en plasma, la cual disminuyó durante la intervención con AXOS, por lo que se puede deducir de los resultados obtenidos en los análisis de lipidómica. Esta disminución de los niveles de ceramidas en plasma podría mejorar la homeostasis de la glucosa, reflejado así en los niveles de HOMA-IR para una buena proporción (47%) de los sujetos incluidos en este periodo de la intervención. Esta tendencia a reducir los niveles de HOMA-IR parece asociada también con un aumento de especies de los géneros *Eubacterium*, *Blautia* y *Roseburia* tras la ingesta de AXOS.

Los análisis de metabolómica también mostraron un aumento de la concentración de ácido hipúrico en orina, y de acetato, propionato y succinato en muestras de heces, resultantes de la degradación de AXOS por especies como las pertenecientes al género *Prevotella*. El consumo de AXOS también se asoció a una reducción de la exposición del hospedador a formas oxidadas de metilaminas, debido al uso de los metabolitos precursores por algunas bacterias como *Eubacterium rectale* para la producción de fosfatidilcolina, disminuyendo así su biodisponibilidad.

CONCLUSIONES

CONCLUSIONES

1. Las cepas de la especie *Bacteroides uniformis* poseen el más amplio repertorio de genes CAZy del género *Bacteroides*. La cepa *B. uniformis* CECT 7771 es capaz de degradar todas las diferentes fuentes de carbono evaluadas (glucosa, salvado de trigo rico en arabinoxilanos (AXOS), inulina, goma arábiga, mucina y pectina), mostrando diferentes patrones de expresión génica para cada una de ellas y generando diferentes metabolitos. La velocidad de crecimiento de *B. uniformis* CECT 7771 es mayor en presencia de fuentes de carbono estructuralmente más sencillas (AXOS y glucosa). *B. uniformis* CECT 7771 es capaz de degradar los *O*-glicanos de la mucina y producir butirato y disminuir la producción de ácidos grasos de cadena larga y proteínas transportadoras de acilo (ACP), implicados en la síntesis de LPS bacteriano causante de endotoxemia. *B. uniformis* CECT 7771, creciendo en presencia de pectina, incrementa la producción y secreción de GABA y, como simbiótico, podría tener un impacto en el eje intestino-cerebro.

2. *Bacteroides uniformis* CECT 7771 no muestra signos de toxicidad o patogenicidad tras el análisis exhaustivo de los genes codificados en su genoma y su administración por via oral en un modelo de toxicidad sub-crónica en ratas. *B. uniformis* CECT 7771 parece inducir un perfil de citocinas anti-inflamatorio comparado con el grupo placebo y muestra un efecto dosis-dependiente en la disminución de los valores de la alanina aminotransferasa (ALT), sugiriendo un potencial efecto hepato-protector.

3. El consumo de AXOS (10,4g/día) durante 4 semanas modifica la configuración de la microbiota intestinal en individuos que padecen sobrepeso y síndrome metabólico, induciendo un efecto bifidogénico y un incremento de especies bacterianas productoras de butirato, que en su conjunto podrían ejercer un efecto beneficioso para la salud metabólica de los individuos, aunque en este estudio no llegaron a detectarse efectos significativos en los parámetros bioquímicos y clínicos analizados.

4. La ingesta de ácidos grasos poliinsaturados (PUFAS; 3,6 g/dia) durante 4 semanas en individuos que padecen sobrepeso y síndrome metabólico no induce cambios significativos en la microbiota ni en los parámetros bioquímicos y clínicos analizados.

5. El estudio metagenómico de las muestras de los individuos con sobrepeso y síndrome metabólico sometidos a una intervención con AXOS durante 4 semanas ha permitido identificar profundos cambios en la configuración de la microbiota intestinal, incluyendo un aumento de *Ruminococcus gnavus* y *Prevotella* spp. y una

disminución de *Parabacteroides, Paraprevotella* y *Rikenella.* También se ha detectado un incremento de metagenes implicados en la producción de tetrahidrofolato y derivados del ácido fólico, con función protectora frente a la hiperhomocisteinemia, la vitamina K2, implicada en la correcta absorción del calcio; y el GABA, cuya baja concentración en el organismo se asocia a estados de ansiedad y desbalance energético y que favorece la secreción de insulina y regula los niveles de ceramidas en plasma.

6. El análisis conjunto del metagenoma y el metaboloma en la misma intervención (5) ha permitido establecer correlaciones positivas entre la abundancia de *Prevotella* y las concentraciones de ácidos grasos de cadena corta y succinato, así como entre aumentos de *Eubacterium rectale* y reducciones en metilaminas, que podrían estar implicadas en la mejora del metabolismo de glucosa y reducción del riesgo de sufrir patologías crónicas. Además, el análisis lipidómico demuestra una reducción de las ceramidas en plasma, que sugieren mejoría del metabolismo de la glucosa. La integración de datos multi-omicos sugieren que la ingesta de AXOS podría contribuir especialmente al mantenimiento de la homeostasis de la glucosa en individuos con sobrepeso.

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ABREVIATURAS de la Introducción y Discusión General

Abreviaturas

TGI	Tracto gastrointestinal	Gastrointestinal tract
LPS	Lipopolisacáridos	Lipopolysaccharides
SIgA	Inmunoglobulina A secretora	Secretor immunoglobulin A
SCFA	Ácidos grasos de cadena corta	Short chain fatty acids
GABA	Ácido gamma-aminobutírico	Gamma-aminobutyric acid
O-DMA	O-desmetilangolensina	O-desmetylangolensin
GALT	Tejidos linfoides asociados al intestino	Gut associated lymphoid tissues
CD	Células dendríticas	Dendritic cells
NLM	Nódulos linfoides mesentéricos	Mesentheric lymphoid nodules
PMAMs	Patrones moleculares asociados a microorganismos	Microorganisms associated molecular patterns
RRP	Receptores de reconocimiento de patrones	Pattern recognition receptors
TLR	Receptores tipo TOLL	TOLL-like receptors
NLR	Receptores tipo NOLL	NOLL-like receptors
FN	Factor nuclear	Nuclear factor
MAPK	Proteína kinasa activada por mitógeno	Mitogen activated protein kinase

NK	Células "Natural killer"	Natural killer cells
IL-10	Interleucina 10	Interleukin 10
IL-12	Interleucina 12	Interleukin 12
Treg	Célula T reguladora	T regulatory cell
ADN/DNA	Ácido desoxirribonucleico	Deoxyribonucleic acid
MALDI-TOF	Desorción/ionización láser asistida por matriz - tiempo de vuelo	Matrix-assisted laser desorption/ionization - time of flight
ARNr 16S	Ácido ribonucleico ribosomal 16S	16S ribosomal ribonucleic acid
HMP	Proyecto del Microbioma Humano	Human Microbiome Project
ARNr 18S	Ácido ribonucleico ribosomal 18S	16S ribosomal ribonucleic acid
OTUs	Unidades taxonómicas operacionales	Operational Taxonomic Units
pb	Pares de bases	Base pairs
OMS/WHO	Organización Mundial para la Salud	World Health Organization
IMC	Ïndice de masa corporal	Body mass index
GWAS	Estudios de asociación del genoma completo	Genome Wide Association Studies
PYY	Péptido YY	YY peptide
GLP-1	Péptido 1 similar al glucagón	Glucagón like peptide 1
GLP-2	Péptido 2 similar al glucagón	Glucagón like peptide 2
MUFAs	Ácidos grasos	Monounsaturated fatty

	monoinsaturados	acids
PUFAs	Ácidos grasos poliinsaturados	Polyunsaturated fatty acids
DT2	Diabetes tipo 2	Type 2 diabetes
ISAPP	Asociación Científica Internacional para probióticos y prebióticos	International Scientific Association for Probiotics and Prebotics
FOS	Fructooligosacáridos	Fructooligosaccharides
GOS	Galactooligosacáridos	Galactooligosaccharides
CLA	Ácido linoleico conjugado	Conjugated linoleic acid
XOS	Xilooligosacáridos	Xylooligosaccharides
QPS	Presunción de seguridad cualificada	Qualified Presumption of Safety
GRAS	Generalmente reconocido como seguro	Generally Recognized as Safe
EFSA	Autoridad Europea de Seguridad Alimentaria	European Food Safety Authority
IL-6	Interleucina 6	Interleukin 6
LDL	Lipoproteína de baja densidad	Low density lipoprotein
HDL	Lipoproteína de alta densidad	High density lipoprotein
Th17	Células T 17 auxiliares	T helper 17 cells
IL-12	Interleucina 12	Interleukin 12
IFN-ɣ	Interferón gamma	Interferon gamma
NF-κβ	Factor nuclear κβ	Nuclear factor κβ
IL-8	Interleucina 8	Interleukin 8

TAG	Triacilglicérido	Triacylglyceride
Hs-CRP	Proteína C reactiva de alta sensibilidad	High sensitivity C reactive protein
HOMA-IR	Evaluación por modelo homeostático para resistencia a la insulina	Insulin resistance homeostatic model assesment
CECT	Colección Española de Cultivos Tipo	Spanish Culture Collection
FAO/WHO	Organización de las Naciones Unidas para la Alimentación y la Agricultura	Food and Agriculture Organization of the United Nations
BSH	Hidrolasa de sales biliares	Bile salt hydrolase
CFU	Unidades formadoras de colonias	Colony forming units

ANEXO

Impact of dietary fiber and fat on gut microbiota remodeling and metabolic health

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Abstract

Background. Scientific evidence suggests that diet plays a role in obesity and its comorbidities, partly via its interactions with the individual's gut microbiota. Likewise, the individual's microbiota influences the efficacy of dietary interventions to reduce body weight. However, we require a better understanding of the key components of the gut microbiota that are responsive to specific diets and of their effects on energy balance in order to use this information in practice.

Scope and Approach. This review provides an up-to-date description of the influence of dietary fibers and fat on gut microbiota and the mechanisms presumably mediating their effects on metabolic health. We also discuss the main knowledge gaps and the need to gain greater understanding of the role of diet-microbe interactions in obesity and the associated comorbidities.

Key Findings and Conclusions. Dietary fibers are major drivers of gut microbiota composition and function, stimulating the dominance of bacteria able to utilize these substrates as energy source, although effects vary depending on both the type of fiber and the individual's microbiota. However, the key bacteria and the primary and secondary metabolic pathways mediating specific fiber-induced effects on the metabolic phenotype remain unclear, and this information is necessary to personalize fiber-based interventions.

The literature also shows that gut microbiota contributes to the adverse consequences of high-fat diets on the metabolic phenotype; however, little is known about the effects of dietary fat type. Further progress is expected from translational approaches integrating controlled dietary intervention human trials, combining functional omics technologies and physiological/clinical endpoints, and mechanistic studies in experimental models. This will ultimately help us to progress towards establishing informed microbiome-based dietary recommendations and interventions, which can contribute to tackling the obesity epidemic and its comorbidities.

Introduction

Obesity has reached pandemic dimensions affecting a vast number of people worldwide. In 2014, approximately 39% of adults (1.9 billion) were overweight and 13% of these (600 million) were obese. Moreover, 42 million children under the age of 5 were reported as overweight or obese in 2013(World Health Organization, 2015). It is well known that obesity is not only associated with populations in high-income countries, but the prevalence is continuously growing in low- and mid-income countries, particularly in urban settings (World Health Organization, 2015). Obesity is a result of an unbalance between energy intake and expenditure, to which over-nutrition and a sedentary lifestyle are major contributors (Coppinger, Jeanes, Dabinett, Vogele, & Reeves, 2010). Obesity is associated with a state of chronic low-grade inflammation, which partly explains the insulin resistance phenotype observed in many obese individuals. In turn, insulin resistance is a component of the metabolic syndrome that often precedes the development of type 2 diabetes (T2D) and cardiovascular disease (CVD) (Jia, DeMarco, & Sowers, 2016). This metabolic inflammation is characterized by infiltration of macrophages and lymphocytes in peripheral tissues. This is accompanied by an increased production of pro-inflammatory cytokines, adipokines, acute-phase proteins and other immune mediators as a consequence of the activation of several signalling pathways, including the nuclear factor kappa B (NFκB)/Inhibitor of the κ kinase (IKK), c-jun N-terminal kinase (JNK), protein kinase R (PKR) and theToll-Like receptors (TLRs) (Gregor & Hotamisligil, 2011). Adipose tissue from obese individuals is considered to be the main contributor to obesity-related metabolic inflammation, with the highest accumulation of infiltrating macrophages and tissue concentrations of cytokines, with similar events occurring in the liver and central nervous system, contributing to systemic insulin resistance (Johnson & Olefsky, 2013).

In the last decade, an increasing number of studies have reported that obesity is associated with alterations in gut microbiota structure, suggesting that specific microbial taxa could be contributing factors to the obesity epidemic, although results are not fully consistent across human observational studies (Sanz, Rastmanesh, & Agostoni, 2013). Animal studies have provided information about the mechanisms by which gut microbiota could play a role in obesity, including contribution to nutrient digestion and absorption and to regulation of immune and neuro-endocrine functions (Moya-Perez, Neef, & Sanz, 2015). Experimental models have also demonstrated that gut microbiota can transmit the obesity-associated metabolic phenotype of its original human host when transferred to a germ-free recipient, providing a first evidence of causality (Turnbaugh, *et al.*, 2006). Furthermore, a unique fecal transplantation study in humans has also

demonstrated that the transference of feces from a lean donor into subjects with metabolic syndrome beneficially influence glucose metabolism, confirming the causal role of gut microbiota (Vrieze, *et al*., 2010). Nonetheless, the role of gut microbiota in obesity seems largely dependent on diet-microbe interactions due to the fact that diet is a major modifiable factor influencing gut microbiota composition and function (De Filippis, *et al*., 2015; Flint, Duncan, Scott, & Louis, 2015). Indeed, experimental models revealed that such interactions contribute to obesity, for example, by increasing lipid absorption or aggravating adipose tissue inflammation independently of adiposity in the context of diets rich in saturated lipids (Caesar, Tremaroli, Kovatcheva-Datchary, Cani, & Backhed, 2015; Semova, *et al*., 2012). Furthermore, dietary reprograming of microbiota ameliorates development of metabolic dysfunction despite susceptible genotypes (Ussar, *et al*., 2015). Nevertheless, our understanding of how diet-microbe interactions influence energy balance, eating behavior and obesity in humans is still insufficient to transform this information into practical solutions to tackle obesity-associated disorders.

This review discusses the most recent data regarding the potential role of dietary fiber and fat in remodeling gut microbiota composition and function and, thereby, in programming metabolic health. It also addresses the main limitations that must be overcome to progress our understanding of the microbiome's role in the chain of events causing obesity. Only on gaining a better understanding of the above, will we be able to speed up the translation of this information into informed microbiome-based dietary interventions and recommendations.

1. Impact of dietary fiber on human physiology

1.1. Dietary fiber: role in metabolic health and as main fuel for gut microbiota.

Dietary fiber is generally defined as non-digestible carbohydrates plus lignin, which include structurally different components including non-starch polysaccharides, resistant oligosaccharides (e.g. fructo-oligosaccharides [FOS], galacto-oligosaccharides [GOS]) and resistant starch (EFSA NDA Panel, 2010). Prebiotics are defined as dietary fibers that modify the composition and/or metabolic activity of gut microbiota, thereby conferring a benefit to the host (G. R. Gibson, 2004; G. R. Gibson, Probert, Loo, Rastall, & Roberfroid, 2004). According to this definition, a wide variety of food ingredients can be classified as prebiotics such as GOS, FOS and longer inulin-derived fructans, xylo-oligosaccharides (XOS) and arabinoxylan oligosaccharides (AXOS); however this is based mainly on their impact on gut microbiota rather than on robust evidence of their effects on health-

related endpoints (Hutkins, *et al*., 2016). Dietary fiber is not digested by human enzymes and thus it reaches proximal colonic regions, where it constitutes the main energy source for obligate anaerobic bacteria, whose fermentative activity leads to the generation of organic acids (lactic, succinic acid) and short-chain fatty acids (SCFA) (acetate, propionate and butyrate). Consequently, the quantity and quality of fiber is considered to be one of the main dietary determinants of gut microbiota composition and function (Scott, Gratz, Sheridan, Flint, & Duncan, 2013). The current recommendations on dietary fiber intake (25 g per day for adults) are based on their well-known role in regulating bowel habits (frequency of defecation), including native chicory inulin considered to be prebiotic (Hutkins, et al., 2016). In addition, there is evidence for a role of dietary fiber and some prebiotics (inulin and oligofructose) in the reduction of dietary glycemic responses and glycemic load, with favorable effects on metabolic risk factors. Furthermore, consumption of fiber-rich diets with fiber intake above recommendations is associated with a reduced risk of coronary heart disease and type 2 diabetes as well as improved weight maintenance (Bes-Rastrollo, Martinez-Gonzalez, Sanchez-Villegas, de la Fuente Arrillaga, & Martinez, 2006; EFSA NDA Panel, 2010; S. Liu, *et al*., 2000; Ludwig, *et al*., 1999; Ye, Chacko, Chou, Kugizaki, & Liu, 2012). Dietary fiber is thought to positively influence metabolic health through multiple mechanisms, although effects cannot be generalized as they vary depending on the type of fiber. The mechanisms of action include direct effects related to its physicochemical and structural properties (e.g. indigestibility, viscosity, etc.) and indirect effects mediated by the individual's gut microbiota. For example, compared to digestible carbohydrates, insoluble and soluble fibers reach distal portion of colon with no major degradation by human enzymes leading to a significant reduction in postprandial glycemic responses due to their slower digestion (EFSA, 2014). Consequently, consumption of fiber improves the glucose metabolism as a whole, which have direct impact on satiety and tip the balance towards oxidation instead storage metabolism (reviewed in (Koh-Banerjee & Rimm, 2003)). Moreover, dietary fiber is considered to be very useful for weight loss/maintenance aims given its low energetics estimated to be ~1.91 kcal/g (8 kJ/g) in comparison with other macronutrients as digestible carbohydrates, (~4.06 kcal/g), proteins (~4.06 kcal/g), and fat (~8.84 kcal/g) (Menezes, et al., 2016). Soluble viscous fibers may also exert beneficial metabolic effects by their ability to form gels that delay gastric emptying, inhibit nutrient absorption and bile acid (BA) binding; altogether this may contribute to a decreased postprandial glycemic response and a reduction in body cholesterol stores due to increased synthesis of new BAs from cholesterol in the liver (Dikeman & Fahey, 2006). In addition, dietary fiber is thought to mediate other effects (e.g. satiety and anti-inflammatory effects) through activation of the fermentative activity of gut

bacteria, and the generation of potentially beneficial metabolites (e.g. SCFAs), as explained in greater detail in section 3.

1.2. Evidence of the influence of dietary fiber on gut microbiota from observational studies.

The role of non-digestible carbohydrates in the gut microbiota is well exemplified by the differences in the infant's gut microbiota between breast-fed and formula-fed infants and between infant formula supplemented or not with oligosaccharides, which mainly stimulate the growth of bifidobacteria (Closa-Monasterolo, *et al*., 2013; Hascoet, *et al*., 2011). These effects have also been well-established by comparing the gut microbiota of individuals from different geographical regions that consume rural diets (Africa and South America) rich in dietary fiber or Western diets (Europe and North America) rich in animal protein and fat(De Filippo, *et al*., 2010; Yatsunenko, *et al.,* 2012). Acomparison of the microbiota between European and African children, consuming a fiber-rich diet, showed that the latter have reduced abundance of Firmicutes and increased abundance of Bacteroidetes, particularly the *Prevotella* and *Xylanibacter* genera, known to have genes specialized in cellulose and xylan utilization, with parallel increased fecal concentrations of SCFAs. In contrast, Enterobacteriaceae species (Proteobacteria) were reduced in African compared with European children (De Filippo, *et al*., 2010). Another large study including healthy children and adults also revealed important differences in bacterial communities and functional gene repertoires between US subjects from metropolitan areas and those from countries with a rural lifestyle (Amazonas of Venezuela and Malawi), finding the genus *Prevotella* to be abundant in humans with a diet rich in corn and cassava and in US children not following a full western diet (Yatsunenko, *et al*., 2012). A more recent study comparing African Americans and rural South Africans, found that animal protein and fat intake was 2-3 times higher in Americans whereas carbohydrate and fiber (mainly resistant starch) intake was higher in Africans. The same authors also reported diet-associated microbiota and metabolite changes that were related to colon cancer risk. While the American microbiota was dominated by *Bacteroides*, the African microbiota was dominated by *Prevotella* and higher levels of starch degraders, carbohydrate fermenters, and butyrate producers. Moreover, the American microbiota had higher levels of potentially pathogenic Proteobacteria (*Escherichia* and *Acinetobacter*) and BA deconjugators (Ou, *et al*., 2013). A recent Dutch population-based metagenomic study involving 1,135 subjects has associated higher diversity, functional microbiome richness and abundance of Bacteroidetes with higher intake of fruits and vegetables (source of dietary fiber), higher concentrations of high-density lipoprotein (HDL) and lower

concentrations of fecal chromogranin A (Zhernakova, *et al*., 2016). The total amount of carbohydrates in the diet was also positively associated with *Bifidobacterium* but negatively associated with *Lactobacillus* and microbiome diversity (Zhernakova, *et al*., 2016). All in all, these observational studies reveal that long-term consumption of fiber-rich diets promotes the dominance of fiber-degraders of the phylum Bacteroidetes and Actinobacteria (*Bifidobacterium*spp.) and, more consistently, of *Prevotella* spp. and reductions in Proteobacteria; nevertheless, *Bacteroides* spp. seem to be adapted to both fiber-rich diets and diets rich in animal protein and fat, probably due to their versatile metabolic capabilities. Notwithstanding, these observational data only provide associations but not causal relationships between specific dietary habits and the predominance of specific bacterial taxa, which limits their value in practice. Furthermore, other relevant environmental factors such as hygiene, geography, and ethnicity that could be involved in the respective gut microbiota profile observed are not well assessed.

A recent experimental study in animal models also suggests that the lack of dietary fiber leads to a substantial loss in gut microbiota diversity, which influences the ability of gut bacteria to be transferred from parents to offspring. It also revealed that simply restoring fiber consumption was not enough to reverse these effects since some bacterial groups failed to return to their previous levels (Sonnenburg, *et al*., 2016). These results have led to hypothesize that long-term dietary changes in industrialized countries could have altered the host-microbiota partnership and microbiome functionality, with an adverse long-term impact on health that could be transmitted from generation to generation (Sonnenburg, *et al.*, 2016). Notwithstanding, evidence from systematic studies in humans is required to confirm this hypothesis.

1.3. Evidence for the influence of dietary fiber on gut microbiota from intervention studies.

A summary is given in Table 1 of recent representative human dietary interventions investigating how most common types of dietary fibers contribute to remodeling the gut microbiota. The responsiveness and effects of dietary fibers may differ depending on the individual's gut microbiota profile (Korpela, *et al*., 2014), suggesting the need to work towards defining more specific and personalized dietary interventions and recommendations.

Table 1. Summary of dietary fiber interventional studies with gut microbiota assessments in humans.

Fiber	Study Design	Subjects	Time	Gender	Population	Effects on gut microbiota[1]	Ref.
Maize-derived WG cereal	DB, R, PC, CO	32	3 weeks	Females (21) Males (11)	European UK	↑*Bifidobacterium*	(Carvalho-Wells, *et al.*, 2010)
WG wheat cereal	DB, R, PC, CO	31	3 weeks	Females (16) Males (15)	European UK	↑*Bifidobacterium, Lactobacillus*	(Costabile, *et al.*, 2008)
WG barley	R, CO	28	4 weeks	Females (17) Males (11)	USA	↑*Blautia,Bifidobacterium, Roseburia, Dialister* ↔*Dialister*-plasma IL-6levels ↔*Eubacterium*-plasmaglucose/insulin	(Martinez, *et al.*, 2013)
Inulin	DB, R, PC, CO	32	4 weeks	Females	European Switzerland	↑*Bifidobacterium*	(Petry, *et al.*, 2012)
Inulin (Agave)	DB, R, PC, CO	29	3 weeks	NA	USA	↑*Bifidobacterium* ↓*Desulfovibrio* ↔*Faecalibacterium* - fecal butyrate	(Holscher, *et al.*, 2015)
Inulin / FOS	DB, R, PC	31	8 weeks	Females	European Spain	↑*Bifidobacterium, Lactobacillus*	(Garcia-Peris, *et al.*, 2012)
Inulin-type fructans	DB, R, PC	30	12 weeks	Females	European Belgium	↑*Bifidobacterium, Faecalibacteriumprausnitzii* ↓*Bacteroides, Propionibacterium* ↔*Bifidobacterium* - plasma LPS levels ↔*Faecailbacterium* - plasma LPS levels ↔*Bacteroides* - Fat mass	(Salazar, *et al.*, 2015)
Inulin /	DB, R,	22	12	Females	European	↓*Faecalibact*	(Majid, *et*

Oligofructose	PC		days (mean)	(9) Males (13)	UK	*erium, Bacteroides, Prevotella*	*al.*, 2014)
Inulin / Oligofructose	DB, PC	30	12 weeks	Females (44)	European Belgium	↑*Bifidobacterium, Faecalibacterium prausnitzii*	(Dewulf, *et al.*, 2013)
Inulin / Oligofructose	DB, R, PC	252	16 weeks	Females (123) Males (129)	European Spain	↑*Bifidobacterium*	(Closa-Monastero lo, *et al.*, 2013)
B-GOS	DB, R, PC, CO	45	6 weeks	Females (29) Males (16)	European UK	↑*Bifidobacterium* ↓*Clostridium histolyticum, Desulfovibrio, Bacteroides*	(Vulevic, *et al.*, 2013)
GOS	DB, R, PC, CO	31	3 weeks	Females	European The Netherlands	↑*Bifidobacterium*	(Whisner, *et al.*, 2013)
GOS	DB, R, PC	163	>16 weeks	NA	European Italy	↑*Bifidobacterium*	(Giovanni ni, *et al.*, 2014)
XOS	R, PC	22	3 weeks	Females (7) Males (15)	Taiwan	↑*Bifidobacterium*	(Chung, *et al.*, 2007)
AXOS	R, PC, CO	20	3 weeks	Females (14) Males (6)	European Belgium	↑*Bifidobacterium*	(Cloetens, *et al.*, 2010)
AXOS	DB, R, PC, CO	63	3 weeks	Females (30) Males (33)	European Belgium	↑*Bifidobacterium*	(Francois, *et al.*, 2012)
AXOS	DB, R, PC, CO	65	3 weeks	Females (35) Males (30)	USA	↑*Bifidobacterium*	(Maki, et al., 2012)
RS3	R, CO	14	3 weeks	NA	European Scotland	↑*Ruminococcusbromii, Eubacterium rectale*	(Walker, *et al.*, 2011)
RS2, RS4	DB, CO	10	3 weeks	Females (5) Males (5)	USA	↑ *Bifidobacterium adolescentis, Eubacterium rectale, Ruminococcusbromii, Parabacteroides distasonis* ↓ *Faecalibacteriumprautsnitzii,*	(Martinez, *et al.*, 2010)

						Doreaformici generans	
RS	R, CO	46	4 weeks	Females (30) Males (16)	Australia	↑*Ruminococcusbromii*	(Abell, *et al.*, 2008)

[1] Gut microbiota changes expressed in terms of abundance. ↑ indicates higher proportions of a determined bacterial genus after intervention, and ↓ indicates the inverse effect. ↔ indicates direct correlations among bacterial abundance and metabolic parameters studied, being negative or positive, respectively.DB= Double-blind; Single-Blind = SB; R= randomized; PC = Placebo-controlled; CO = Cross-over; NA = No information was explicitly available for gender distribution into the intervention groups.

1.3.1. Effects of wholegrain (WG)-rich foods. Wholegrain cereals are composed of starch-rich endosperm, germ, and bran with high plant-fiber content. During harvesting and food processing, these components must preserve their relative proportions as in the intact kernel (HEALTHGRAIN Consortium - http://www.healthgrain.org). Rice, wheat, maize, oats, and barley are the main whole grains consumed worldwide and some of them have been proven to reduce the risk of certain diet-related diseases such as obesity and CVD. A controlled cross-over study showeda bifidogenic effectupon consumption of 48 g/day maize-based WG breakfast cereals during 21 days (Carvalho-Wells, *et al*., 2010). This effect was observed exclusively for the intervention period and not sustained after completion of the WG diet, strongly indicating that WG fiber is predominantly used by *Bifidobacterium* spp. (Carvalho-Wells, *et al*., 2010). Similar results were obtained by Costabile and coworkers who reported increased bifidobacteria and lactobacilli in feces after daily consumption of WG wheat breakfast cereals (48 g/day) in comparison with non-WG cereal (Costabile, *et al*., 2008). More recent results have shown that a four-week dietary intervention with 60 g/day WG barley flakes in healthy adults induced a significant increase in the genus *Blautia* and a less pronounced increase in the abundance of the genera *Roseburia, Bifidobacterium* and *Dialister* (Martinez, *et al.*, 2013). Additionally, this study showed that WG barley, brown rice and specially the combination of WG barley and brown rice reduced plasma interleukin-6 (IL-6) and postprandial glucose. Interestingly, *Eubacterium rectale* was significantly more abundant in volunteers showing improvements in postprandial blood glucose and insulin response, whereas abundance of *Dialister* species was associated with the highest improvements in IL-6 levels (Martinez, *et al.*, 2013).

1.3.2. Resistant starch (RS). Starch is the major component of the plant-derived foods and comprises an important part of the human diet. The starch is referred as

resistant when it cannot be hydrolyzed by digestive enzymes of the human GIT. The RS can be classified into several types (RS1 to RS5) according to the physical or chemical reasons to be indigestible. The RS1 is contained inside whole grains and is physically inaccessible for digestion; the RS2 is also native starch but remains indigestible by its compact structure; the RS3, also known as retrograde starch, is obtained by slow re-crystallization prior to heat disruption on water; the RS4 is the chemically modified starch by cross-linking or esterification; and the RS5 is a mixture of starch with lipids with high stability (Ma & Boye, 2016). Early studies about the RS impact on gut microbiota indicated that administration of controlled diet including 22 g/day RS induces changes in gut microbiota mainly in the clostridia cluster including members of the *Ruminococcus* genus (Abell, Cooke, Bennett, Conlon, & McOrist, 2008). Interventions with 50-60 g/day RS3 increased the abundance of several *Ruminococcus* spp. and especially *Ruminococcus bromii* and *Eubacterium rectale* (Walker, *et al*., 2011). Similar results were obtained when 33 g/day RS2 or RS4 were administrated in baked crakers to volunteers during 3 weeks. In this case, increased proportions of *Bifidobacterium adolescentis* and *Parabacteroides distasonis* were found to be induced particularly by RS4 intake, whereas increased proportions of *Ruminococcus bromii* and *Eubacterium rectale* were induced by RS2 consumption (Martinez, Kim, Duffy, Schlegel, & Walter, 2010). In addition, RS intake of has been found to improve lipid metabolism in individuals with metabolic syndrome and help to control waist circumference and fat mass in non metabolic syndrome individuals (Nichenametla, *et al*., 2014). These beneficial effects of RS on metabolic aspects are thought to be at least partially mediated by the microbiota induced changes but direct evidence still has to be provided.

1.3.3. Inulin and FOS. Inulin and FOS, also called oligofructose or oligofructans, are types of fructo-polysaccharides that consist of several β-linked D-fructosyl residues with a D-glucose group at end of the extended saccharide chain. These differ in the polymerization degree, which may range from 2 to 60 fructose units. FOS are usually produced by degradation of inulin obtained primarily from artichoke and chicory plants. These are used in the food industry as sweeteners, texture modifiers and fibers. A number of intervention studies have shown that the effects of inulin and FOS on gut microbiota composition can be associated with modifications on health related outcomes or subrogated biomarkers (Table 1). In adults and infants, it is generally reported that inulin and FOS intake increases the number of bifidobacteria, sometimes associated with changes in metabolic products (e.g. lactate) (Closa-Monasterolo, *et al.*, 2013; Garcia-Peris, *et al.*, 2012; Petry, Egli, Chassard, Lacroix, & Hurrell, 2012). In some studies, inulin or FOS-induced microbiota changes have also been correlated with indicators of metabolic health. For example, a three-month double-blind placebo-controlled intervention

with a mixture ofinulin/oligofructose or maltodextrin (8 g twice daily in powder to be dissolved in warm drinks) in obese women, showed increased abundances of *Bifidobacterium* spp. and *Faecalibacterium prausnitzii,* which correlated to reduced serum LPS (lipopolysacchraide) levels. Additionally, the researchers observed reductions of *Bacteroides intestinalis*, *Bacteroides vulgatus* and *Propionibacterium* spp., which correlated to modest changes in fat mass. Additionally, they found reductions in plasma LPS, fecal acetate and propionate concentrations, and fasting insulinemia (Dewulf, *et al*., 2013; Salazar, *et al.*, 2015). A recent study has evaluated the role of agave inulin showing a dose-dependent bifidogenic effect. The consumption of 5 or 7.5 g/day agave inulin in chocolate chews, primarily promoted the presence of *B. adolescentis, B. breve, B. longum*, and *B. pseudolongum* (Holscher, *et al*., 2015). Positive correlations were also detected between fecal butyrate concentrations and the dose of fiber, and between fecal butyrate concentration and *Faecalibacterium* abundance. These effects could be explained by cross-feeding interactions disclosed between bifidobacteria and *Faecalibacterium* (Moens, Weckx, & De Vuyst, 2016). Interestingly, a depletion of *Desulfovibrio* species was also identified as a consequence of agave inulin consumption (Holscher, *et al*., 2015), which could be of clinical relevance because increased *Desulfovibrio* species have been related to obesity and the associated endotoxemia (Xiao, et al., 2014; Zhang-Sun, Augusto, Zhao, & Caroff, 2015; Zhang, *et al*., 2009).

1.3.4. GOS

GOS are mainly produced through transgalactosylation reactions mediated by β-galactosidases using lactose or derivatives as substrate. GOS are often used to supplement infant formula due to their chemical and structural resemblance to human milk oligosaccharides. In infant formula, GOS have been shown to exert a bifidogenic effect (Giovannini, *et al*., 2014). In adults, the six-week administration of 5.5 g/day GOS powder mixture dissolved in water to subjects with metabolic syndrome has been shown to reduce levels of *Clostridium histolyticum, Desulfovibrio* spp. and *Bacteroides* spp.(Vulevic, Juric, Tzortzis, & Gibson, 2013). These changes were accompanied by increases in *Bifidobacterium* spp. and reductions in inflammatory markers, including fecal calprotectin and plasma C-reactive protein (CRP) and in some metabolic parameters (e.g. plasma insulin, total cholesterol and triglycerides in males).

1.3.5. Xylans and arabinoxylans. Arabinoxylans (AX) from cereals are cell wall components that constitute a major part of the dietary fiber fraction of cereal grains and thus, an important fiber source in the diet (McCleary, 2003). Enzymatic hydrolysis of AX either in the production of processed foods or by bacteria in the colon yields arabinoxylanoligosaccharides (AXOS) and xylooligosaccharides (XOS),

both of which are proposed to be prebiotic fibers (Broekaert, *et al*., 2011). Additionally to the well known bifidogenic effect of AX, a fact in which is based its prebiotic potential (reviewed in (Riviere, Selak, Lantin, Leroy, & De Vuyst, 2016)), other AX-degrading bacteria in the human colon belong to the genera *Roseburia* and *Bacteroides* and include the butyrate producing *Roseburia intestinalis* (Chassard, Goumy, Leclerc, Del'homme, & Bernalier-Donadille, 2007). These data are of interest since a higher relative abundance of butyrate-producing bacteria and *Bacteroides* spp. has been reported in healthy individuals compared to patients with T2D or pre-diabetic subjects in some studies (reviewed in (Sanz, Olivares, Moya-Perez, & Agostoni, 2015)). Human intervention trials have also shown increased fecal abundance of *Bifidobacterium* spp. following intake of 4 g/day XOS during three weeks (Chung, Hsu, Ko, & Chan, 2007) and from 2.14 to 10 g/day AXOS (Cloetens, et al., 2010; Francois, *et al.*, 2012; Maki, *et al.*, 2012). Furthermore, a higher abundance of this genus has been reported in normal weight subjects compared to obese and T2D subjects in some observational studies *(Schwiertz, et al.,* 2010; Wu, *et al.*, 2010).

2. Microbiome components involved in the utilization of dietary fiber

Dietary intake of fibers may lead to enrichment and altered expression of microbial genes which encode proteins/enzymes of metabolic pathways involved in the utilization of dietary fiber and the production of potentially beneficial metabolites (e.g. SCFAs). It is necessary to identify and characterize these pathways in order to understand the components of the microbiota and the microbiome that may underlie health effects associated with dietary fiber intake. Members of the phyla Bacteroidetes and Firmicutes are specialized in the utilization of complex carbohydrates and are the main producers of SCFAs. Butyrate and propionate are the two most thoroughly investigated SCFAs in terms of their potential role in metabolic health. The production of these SCFAs may require the participation of different bacterial genera and species via cross-feeding mechanisms. For example, *Bacteroides thetaiotaomicron* can directly produce propionate and acetate, which then can be used by *Eubacterium halli* to produce butyrate (Mahowald, *et al*., 2009). Similar cross-feeding mechanisms have been described between some *Bifidobacterium* spp. and *Faecalibacterium prausnitzii* leading to increased butyrate production (Rios-Covian, Gueimonde, Duncan, Flint, & de los Reyes-Gavilan, 2015). Figure 1 shows the pathways identified for bacterial production of butyrate by genomic and metagenomic analysis of the human gut microbiota (Mahowald, *et al*., 2009; Reichardt, *et al*., 2014; Vital, Howe, & Tiedje, 2014). A conventional genetic signature to explore both the enrichment and variability of butyrate producers is via analyzing the butyryl-CoA:acetate CoA-transferase gene (*BCoAT* gene) encoding the respective enzyme responsible for the last step in butyrate production. Quantitative approaches indicate *BCoAT* gene

enrichment in gut microbiota from individuals with a high intake of plant fiber, which is indicative of increased colonic butyrate production (Hippe, *et al.*, 2011; Louis, Young, Holtrop, & Flint, 2010; Remely, *et al.*, 2014; Vital, Gao, Rizzo, Harrison, & Tiedje, 2015).

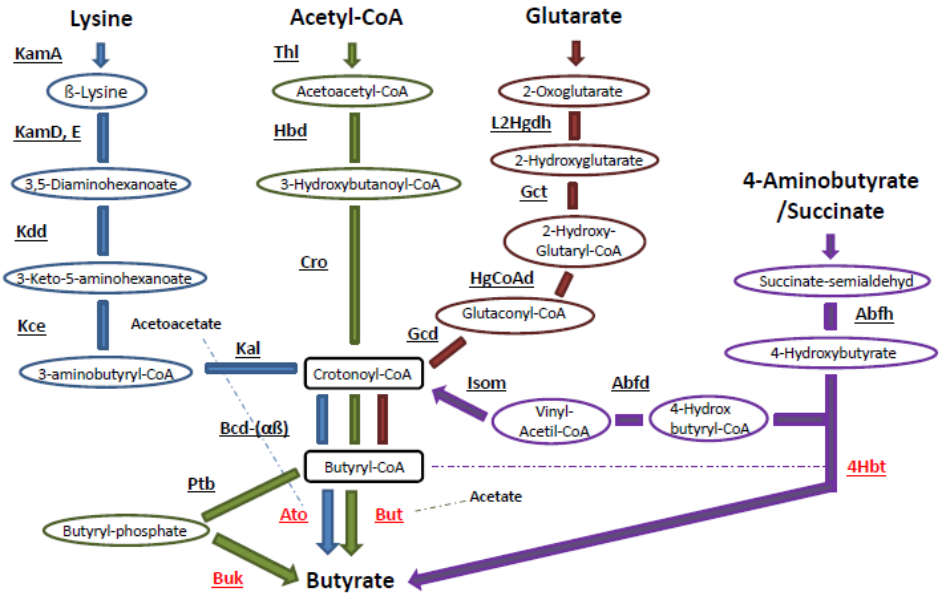

Figure 1.The bacterial butyrate synthesis pathways (adapted from (Vital, *et al*., 2014)). Vital and coworkers have reconstructed four different pathways for butyrate synthesis through and an extensive metagenomic approach. Protein names and major substrates are shown across the different biosynthetic pathways. Genes/proteins responsible of the last step of butyrate production, and frequently used as biomarkers for gut microbiota studies, are highlighted in red. They are known as: 4Hbt, butyryl-CoA:4-hydroxybutyrate CoA transferase; But, butyryl-CoA:acetate CoA transferase; Ato, butyryl-CoA:acetoacetate CoA transferase (α, β subunits); and Buk, butyrate kinase.

Additionally to genes encoding enzymes of pathways responsible for SCFA production, the detection of other genes involved in the uptake and degradation of complex polysaccharides could be useful to define the active bacteria and their mode of action in response to fiber intake. Pioneer studies regarding characterization of proteins involved in the utilization of complex carbohydrates by anaerobe gut bacteria have revealed the essential role of polypeptides encoded by Sus genes, extensively studied in *B. thetaiotaomicron* (Reeves, Wang, & Salyers,

1997). The Sus products were originally described as outer membrane proteins able to bind complex starch. Notwithstanding, the genetic context of their encoding genes has enabled the inclusion of glycoside hydrolases (GH) enzymes in the Sus repertoire of proteins, which collectively work to produce small oligosaccharides that are more easily imported by bacteria. Consequently, Sus genes have become useful to detect different polysaccharide utilization loci (PULs) in other Bacteroides species by comparative genomics approaches, allowing them to be studied in response to a wide variety of complex polysaccharides (reviewed in (White, Lamed, Bayer, & Flint, 2014)). Nowadays, research on carbohydrate utilization by gut bacteria is conceived as a cornerstone to understand their physiology and potential interactions and bidirectional communication with the host in health and disease. In this regard, the Carbohydrate Active Enzymes (CAZy) database (http://www.cazy.org/) is one of the most complete repositories describing the families of structurally-related catalytic and carbohydrate-binding functional domains of enzymes that bind, degrade, modify or create glycosidic bonds (Lombard, Golaconda Ramulu, Drula, Coutinho, & Henrissat, 2013). Hierarchical classification of CAZy comprises 4 main families such as the Glycoside Hydrolase (GH, with 135 subfamilies reported at Nov 2016), the Glycosyltransferase (GT, with 101 subfamilies), the Polysaccharide Lyase (PL, with 24 subfamilies), and the Carbohydrate Esterase (CE, with 16 subfamilies) family. All GH reported are classified according to the functional modules they contain, with the aim to determine sites of action (exo or endo-acting enzymes) or type of cleavage (α- or β-glycosilases). Members of the phyla Bacteroidetes and Firmicutes are characterized by encoding the largest set of GH in their genomes, thus exhibiting a remarkable versatility for the utilization of different polysaccharides as carbon source (White *et al* 2014). These features convert species of such bacterial phyla into key players for degradation of complex polysaccharides in the human colon. Proof of this can be found in the studies performed in Flint's lab with *Ruminoccocus bromii* in which this bacteria was observed to present a specialized extracellular polypeptide complex, known as amylosome (Ze, *et al*., 2015). It was also found to be an indispensable member of the human gut microbiota, having a direct effect on energy recovery from a central component of diet, i.e., RS (Ze, Duncan, Louis, & Flint, 2012). However, *Bifidobacterium* (Actinobacteria) species are also well-known fiber fermenters. Although Bifidobacteria have fewer GHs encoded in their genomes than Bacteroidetes, they also exhibit a great versatility for the uptake and catabolism of oligosaccharides. This versatility is well exemplified in genome-wide expression analyses, which have disclosed a wide variety of genes appearing to respond specifically to different carbon sources (Andersen, *et al*., 2013; O'Connell, *et al.*, 2013). In this context, we have recently described the genome response of *B. pseudocatenulatum* CECT 7765, a strain isolated from breast-fed babies, during utilization of lactulose-derived

oligosaccharides. An exhaustive inventory of GH enzymes present in the genome of this species have a set of open reading frames (ORFs) that seem to control the uptake and degradation of this digestion-resistant oligosaccharide (Benitez-Paez, Moreno, Sanz, & Sanz, 2016).

Although GHs and related proteins appear to be the key traits to infer versatility of gut microbes for utilization of polysaccharides and their contribution to the production of fermentation end-products such as SCFAs, little is known about the effects of fiber fermentation on secondary metabolic pathways and the generation of other nutrients (e.g. amino acids and vitamins) and bioactive compounds. Some *in vitro* studies have reported that oligosaccharide fermentation also increases amino acid synthesis (Benitez-Paez, *et al*., 2016; Sulek, *et al*., 2014). In particular, our study revealed that the utilization of GOS by *B. pseudocatenulatum* CECT 7765, using bacteria cultures, increased the production and extracellular accumulation of branched-chain amino acids such as leucine (Benitez-Paez, *et al*., 2016). Additional studies are, however, needed to understand the effects of the interplay between dietary fiber and amino acid metabolism in the large intestine and fully understand the metabolites resulting from the activity of the gut microbiota and their potential consequences on health beyond the well-known SCFAs.

3. Effects of dietary fiber on metabolic health mediated by gut microbiota

There is a wealth of human intervention studies with dietary fibers, but only a few of them have assessed the relationship between microbiota-induced changes and endpoints related to physiological functions and metabolism. Further studies are also needed that directly assess the effects of fiber-induced microbiota changes on metabolic outcomes, for example via fecal transplantation or via inoculation of specific bacterial consortia from humans into animal models. Consequently, there is still a large degree of uncertainty about to what extent the effects attributed to dietary fibers on metabolic health are mediated by gut microbiota in humans, and which are the key species involved. Nonetheless, considerable mechanistic data are available from other animal study approaches, as summarized below.

3.1. Gut barrier integrity, metabolic endotoxemia and inflammation

Obesity and particularly the intake of a high-fat diet (HFD) are thought to lead to a leaky gut and metabolic endotoxemia (increased serum LPS levels) in animal models and to some extent in humans. This is assumed contributing to the low-grade chronic inflammation leading to metabolic dysfunction and disease (metabolic syndrome and T2D). In fact, LPS is a potently inflammatory bacterial

antigen linked to common metabolic diseases (Conlon & Bird, 2015). LPS is an endotoxin consisting of three parts; lipid A, the oligosaccharide core and the O-antigen, with the lipid A causing endotoxicity. LPS is normally present in the human gut (≥1 g) and under normal conditions it does not cause negative health effects. In healthy humans the normal/low plasma concentration of LPS is 1-200 pg/ml, but increased levels have been found in subjects with obesity and diabetes (Erridge, Attina, Spickett, & Webb, 2007; Moreira, Texeira, Ferreira, Peluzio Mdo, & Alfenas Rde, 2012). LPS binds to TLR4 via CD14 on, for example, the membrane surface of immune cells leading to activation of genes that codify pro-inflammatory cytokines (e.g. TNF-α and IL-6) involved in metabolic inflammation. Experimental models of obesity have shown prebiotic-induced increases in bifidobacteria and *Akkermansia* spp. associated with reduced endotoxemia and systemic inflammation (Cani, *et al*., 2007; Schneeberger, *et al*., 2015). These effects can be partly explained by the ability of those bacteria to ferment glycans leading to SCFA production and promoting local decrease of pH, which may modulate gut microbiota composition and inhibit the growth of enterobacteria, which may be a source of LPS (Delzenne, Neyrinck, & Cani, 2013; Everard, *et al*., 2013). This effect could also be related to the role of SCFAs in strengthening the gut barrier function, which also reduces LPS translocation via different mechanisms, including modulation of expression and localization of tight-junction proteins, induction of endocrine peptide production (GLP-2) and modification of the intestinal levels of endocannabinoids (Everard, *et al.*, 2013).

SCFAs also play an anti-inflammatory role by regulating the size and function of the colonic regulatory T cells (Treg), specifically inducing Foxp3+IL-10–producing Tregs (Smith, *et al*., 2013). SCFAs may also interact with peroxisome proliferator-activated receptor (PPAR) γ, thereby inhibiting pro-inflammatory signal transduction pathways (e.g. nuclear factor-kappa B [NF-κB]) leading to reduction of downstream cytokine/chemokine production (IL-6, IL-8, and MCP-1) in intestinal epithelial cells and metabolic tissues (e.g. adipose tissue) (Mastrofrancesco, *et al*., 2014). Activation of PPARγ also seems to be crucial in orchestrating Treg accumulation and function in the adipose tissue, which play an important role in preventing inflammation and insulin resistance (Cipolletta, Cohen, Spiegelman, Benoist, & Mathis, 2015). Butyrate as well as other SCFAs, protects against the liver inflammation process associated with steatosis by inhibiting the NF-κB activation and downregulating expression of TLR4 receptor (Mattace Raso, *et al*., 2013). The molecular mechanisms underlying SCFA modulation of NF-κB activity have recently been disclosed as related to JNK and p38 kinases, which control NF-κB activity (Haghikia, *et al*., 2015). However, we cannot discard additional mechanisms to control NF-κB function involving

acetylation/deacetylation of histones and the RelA (p65) monomer itself (Davie, 2003; Glozak, Sengupta, Zhang, & Seto, 2005).

3.2. Enteroendocrine secretion and appetite

In obese animals fed inulin-type fructans, there is an increase in plasma anorexigenic peptides (peptide YY and glucagon-like peptide - GLP-1) and a decrease in the orexigenic peptide ghrelin, which increases satiety (reviewed in (Delzenne, *et al*., 2013)). In addition, supplementation with fructans in HFD-fed mice modulates neuronal activation within the arcuate nucleus, which can help to control food intake (Anastasovska, *et al.*, 2012). These effects on anorexigenic peptide secretion could be mediated by interactions of SCFAs with G-protein receptors such as FFAR2 (GPR41) and FFAR3 (GPR43), which could explain induction of satiety and increased insulin sensitivity (Blaut, 2014). Also in humans, prebiotic interventions with fructans have led to increases in anorexigenic peptides and/or decreases in orexigenic (ghrelin) peptides (Cani, Joly, Horsmans, & Delzenne, 2006; Cani, et al., 2009; Parnell & Reimer, 2009; Verhoef, Meyer, & Westerterp, 2011), but effects on satiety have not always been consistent (Peters, Boers, Haddeman, Melnikov, & Qvyjt, 2009).

3.3. Adiposity, lipid and glucose metabolism

Reduced adiposity in rodents due to dietary supplementation with inulin-type fructans or AX has also been attributed to the role of SCFAs in modulating PPARγ expression via interaction with the G-protein coupled receptor protein FFAR3 (Delzenne, Neyrinck, Backhed, & Cani, 2011). Interestingly, den Besten and co-workers found that SCFAs decrease PPARγ expression, thus promoting activity of the uncoupling protein 2 (UCP2) and, thereby, stimulating oxidative metabolism in liver and adipose tissue, insulin sensitivity and weight loss (den Besten, *et al.,* 2015). Studies with inulin-type fructans have also shown they can decrease hepatic accumulation of triglycerides and/or cholesterol in liver tissue. These effects have been associated with a decrease in sterol-response-element-binding protein-dependent cholesterogenesis, lipogenesis, or changes in PPARα-driven fatty acid oxidation (reviewed in (Delzenne, *et al*., 2013)). The majority of studies show prebiotic administration also leads to improved fasting or postprandial glycemia due to the very low digestion rates of prebiotics compared with digestible carbohydrates (for review see (Roberfroid, *et al*., 2010)). In addition, SCFA-stimulation of GLP-1 secretion can also mediate an improvement in glucose metabolism, reducing obesity-related hepatic insulin resistance.

In humans, intervention studies with fructans have reported modest effects on body weight and fat mass in obese adults, but simultaneous changes in microbiota

were not considered to have any correlation (Genta, *et al*., 2009; Parnell & Reimer, 2009). Nevertheless, there are also reports of a lack of effect on body weight in obese children (Liber & Szajewska, 2014). On the other hand, a rapid improvement in glucose tolerance has been observed for individuals consuming WG barley the night prior to analysis. These results were thought to be caused by the high amount of soluble dietary fiber and resistant starch contained in barley kernels, which facilitated bacterial fermentation in the colon overnight and produced significantly higher levels of SCFAs. This was indirectly measured from breath H_2 excretion (Nilsson, Granfeldt, Ostman, Preston, & Bjorck, 2006). Moreover, recent results of this dietary intervention model indicate that the fiber-associated improvement of glucose metabolism is also associated with an increase in *Prevotella* spp. (Kovatcheva-Datchary, *et al*., 2015).

4. Impact of dietary fat on gut microbiota and associated metabolic endpoints

Globally, an increase in dietary fat content is usually paralleled with a decrease in carbohydrates, including dietary fiber content, thus making it difficult to attribute the observed changes, at physiology or gut microbiota levels, exclusively to one of the macronutrients whose proportion is being increased. Consequently, a decreased abundance of butyrate-producing bacteria and lower fecal SCFA excretion following a HFD is most likely caused by a decrease in dietary carbohydrate intake. Therefore, major conclusions derived from future animal or human studies including HFD interventions must be addressed carefully in order to consider confounding effects regarding the proportions and energetics or other macronutrients administrated.

4.1. Evidence from animal studies

The role of gut microbiota in HFD-induced obesity was suggested through animal experiments involving germ-free mice fed a HFD, which were protected from obesity compared to conventionally raised mice (Rabot, *et al*., 2010), thus highlighting the role of microbiota in HFD-induced obesity. Furthermore, a study in mice by Hildebrandt and coworkers showed that changes in the gut microbiota composition were caused by dietary fat content rather than the degree of obesity, suggesting that fat directly impacts on microbiota regardless of the metabolic phenotype (Hildebrandt, *et al.,* 2009). Gut microbiota transferred to germ-free mice from conventionally raised mice resulted in weight gain and a higher relative abundance of Firmicutes and a lower abundance of Bacteroidetes when mice were fed a HFD compared to a low-fat chow diet from 16 weeks of age (Turnbaugh,

Backhed, Fulton, & Gordon, 2008). Although differences established at phylum level are of limited value since each phylum comprise many different species which may potentially play many different functions, a common trait for HFD-feding seems to be that it increases the Firmicutes:Bacteroidetes ratio *(de Wit, et al.,* 2012; Hildebrandt, *et al*., 2009; Lam, *et al*., 2012; Turnbaugh, *et al*., 2008), although there is not complete consistency across studies (Lecomte, e*t al.,* 2015); this would also be due to experimental and environmental differences. A recent 16-week study in mice fed a HFD reports that the abundance of *Akkermansia muciniphila* was progressively and drastically decreased while other groups including *Bifidobacterium* spp. and *Lactobacillus* spp. showed a transient decrease. In contrast the abundance of *Roseburia* spp. and *Bilophila wadsworthia* increased after 12 and 16 weeks upon HFD, respectively (Schneeberger, *et al.*, 2015). Interesting, *B.wadsworthia* have been linked to insulin resistance and inflammation in humans (Brahe, *et al.*, 2015).

Animal studies have revealed different mechanisms by which HFDcould exert adverse effects, partly mediated by the microbiota, on the host metabolic phenotype. For example, diets rich in saturated fat may contribute to inflammation, a hallmark of metabolic dysfunction leading to metabolic syndrome and T2D, by promoting the expansion of pathobionts, reducing the proportion of protective bacteria, and promoting a leaky gut that in turn facilitates the translocation of bacterial products (e.g. LPS) causing immune activation (Caesar, *et al*., 2015; Delzenne, *et al*., 2011; Devkota, *et al*., 2012). In a recent study, HFD-induced microbiota changes were correlated with obesity-related inflammatory and metabolic biomarkers (Schneeberger, *et al*., 2015). *Akkermansia muciniphila* was the species showing the clearest inverse associations with inflammatory markers in the adipose tissue and also with biochemical/hormonal parameters in circulation (i.e., insulin, glucose, triglycerides and leptin).

However, as the majority of the dietary fat is absorbed in the small intestine and does not serve as an energy source for gut microbes, the effect of fat on gut microbiota must be partly mediated by indirect mechanisms. Increased fat intake also leads to increases in fat quantities and of BAs reaching the colon, and particularly the concentration and composition of BAs modulates the gut microbiota exerting antimicrobial effects (Islam, *et al*., 2011; Ridlon, Kang, Hylemon, & Bajaj, 2014). Primary BAs (e.g. cholic acid [CA] and chenodeoxycholic acid [CDCA] in humans and beta-muricholic acid [β-MCA] in mice) are sterol compounds synthesized from cholesterol in the liver, conjugated with taurine and glycine, and then secreted into the small intestine to emulsify lipids to facilitate their digestion and absorption. The majority of BAs are reabsorbed (enterohepatic recycling), but as increased fat intake leads to increased BA secretion, theoretically more BAs will escape enterohepatic recycling, and hence reach the large intestine.

During the transit to the large intestine, primary BAs undergo deconjugation, oxidation of hydroxyl groups at C-3, C-7, andC-12, and 7α/β-dehydroxylation reactions mediated by intestinal bacterial enzymes, yielding secondary BAs such as deoxycholic acid (DCA), lithocholic acid (LCA), and β-muri-deoxycholicacid. Bacterial bile salt hydrolases (BSH), e.g. produced by *Clostridium* spp, catalyze the first reaction on secondary BAs and this is a step necessary for the subsequent7α/β-dehydroxylation (Degirolamo, Rainaldi, Bovenga, Murzilli, & Moschetta, 2014). Overall, the amount and composition of BAs are strongly influenced by gut microbiota and *vice versa,* and BA biotransformation has important biological consequences due to their role in dietary lipid absorption and as signaling molecules, modulating cholesterol and triglyceride metabolism and glucose and energy homeostasis (Degirolamo, *et al*., 2014; Staels & Prawitt, 2013). Secondary BAs have strong antimicrobial activity (e.g. damage of the bacterial cell membrane by interaction with phospholipids) due to their amphipathic properties. For example, DCA has 10 times the bactericidal activity of CA (Islam, *et al*., 2011), therefore an increase in the proportion of secondary BAs following HFD very likely affects the microbiota composition. A rat study, evaluating the effect of adding CA at different doses compared with controls (no CA added), demonstrated adose-dependent increase of fecal BA and DCA (Islam, *et al.*, 2011). Furthermore, a dose-dependent decrease in fecal SCFA concentration was observed along with a reduction in total bacterial count and an increase in Firmicutes at the expense of primarily Bacteroidetes.

Dietary saturated fat compared to poly-unsaturated fatty acids (PUFAs) was also reported to favor taurine conjugation of hepatic BAs, which caused an expansion of δ-Proteobacteria-type pathobionts, in particularly *B. wadsworthia* which is a sulfite-reducing bacterium exerting a cytotoxic effect on epithelial cells and activating Th1-type inflammatory response (Devkota, *et al*., 2012).

Studies in rodent models of HFD-induced obesity have also shown that saturated fat reduces the mucus layer, which acts as the first barrier separating the immune system from microbial and antigen interactions that may activate an inflammatory response. This effect was parallel to a reduction in the abundance of *Akkermansia* spp., while administration of this bacterium reversed it, increasing mucus layer thickness, and thus suggesting a microbiota-mediated effect (Everard, *et al*., 2013). Other animal studies have reported correlations between HFD-induced changes in the microbiota and alterations in the expression of tight junction-related proteins, and in gut permeability. In mice a HFD has been shown to reduce the expression of the tight-junction-related protein zonula occludens (ZO)-1 mRNA (Cani, Delzenne, Amar, & Burcelin, 2008) associated leading to increased gut permeability measured by transepithelial resistance (Lam, *et al*., 2012). Additionally, decreased transepithelial resistance (i.e. increased gut

permeability) was associated a drop in the abundance of *Lactobacillus* spp. and augmented abundance of *Oscillibacter* spp. (Lam, *et al*., 2012).

Animal studies also show that when a HFD is supplemented with either prebiotics (Cani, *et al*., 2007; Everard, e*t al.*, 2013; Serino, *et al.*, 2012) or antibiotics (Cani, Bibiloni, *et al.*, 2008) the HFD-induced alterations in gut microbiota and metabolism are partially reversed, indicating that gut microbiota partly mediate the consequences of HF feeding.

A few studies have investigated the effects of different dietary fatty acids (Lam, *et al*., 2012; Lappi, *et al*., 2013; Simoes, *et al*., 2013). In mice, it has been shown that n-6 high fat diets do not increase insulin resistance, intestinal permeability and fat accumulation to the same degree as saturated fatty acid diets, which is possibly due to a lower increase in H_2S-producing bacteria (Lam, *et al.*, 2012). Likewise, lower decreases in Bacteroidetes have been found under diets rich in n-3 or n-6, compared to diets rich in saturated fatty acids(T. Liu, Hougen, Vollmer, & Hiebert, 2012).

4.2. Evidence from human studies

Only a few human intervention studies have investigated the effects of HFD compared to low-fat diets (LFD) or the type of fat (saturated fat versus PUFAs) in relation to changes in gut microbiota and the metabolic consequences. As found in animal studies, total bacterial counts decrease in humans who consume a HFD (35-38 E%), compared to a LFD (23-27 E%) over 24 weeks (Fava, *et al*., 2013). Moreover, low/moderate-fat intake appears to induce a higher abundance of *Bacteroides* spp. and/or *Bifidobacterium* spp*.,* compared to high-fat intake in human intervention trials (Brinkworth, Noakes, Clifton, & Bird, 2009; Fava, *et al*., 2013). An energy-restricted HFD (58 E%), compared with an isocaloric moderate-fat diet (28 E %) was shown to increase the total number of anaerobes in the moderate-fat group, but not in the high-fat group, but the ratio between anaerobe:aerobe remained unchanged in each group (Brinkworth, *et al*., 2009). Additionally, a study comparing high-fat and moderate-fat *ad libitum* diets (66 E% vs. 35 E%) over 4 weeks did not report any effect on the gut microbiota in terms of total bacterial count; however, the methodology used to study microbiota abundance was based on a limited number of species(Duncan, *et al*., 2007).

As stated above, an increase in the intake of dietary fat is usually at expenses of a decrease in that of simple or complex carbohydrates, making it difficult to attribute the observed effectexclusively to one of the macronutrients. O'Keefe and coworkers (O'Keefe, *et a*l., 2015) compared the effects on gut microbiota in a cross-over study with a 2-week diet period administering either African- or American-

food. The switch from a rural African to an American-diet (52% fat, 21% carbohydrate, 27% protein, and 12% fiber) decreased the abundance of butyrate-producing bacteria and the production of acetate, propionate and butyrate (O'Keefe, *et al*., 2015). Similarly, Duncan and coworkers observeda higher abundance of *Roseburia* and *Eubacterium* and higher fecal excretion of butyrate in humans following a moderate fat diet compared to high-fat intake, with these changes in the gut microbiota and derived metabolites being positively correlated with carbohydrate intake (Duncan, *et al.*, 2007).

O'Keefe and coworkers also measured BA excretion and observed that the high-fat diet of Americans was associated with increased expression of microbial genes coding for the enzyme related to converting primary BAs to secondary BAs, whereas a dietary switch to a lower-fat diet reduced the abundance of these bacteria. Furthermore, excretion of the secondary BAs LCA and DCA was increased by the HFD. Also short-term consumption of diets composed entirely of animal (rich in fat and protein) or plant products (rich in fiber) can rapidly alter gut microbial composition (David, *et al*., 2014). An animal-based diet increased the abundance of bile-tolerant microorganisms, including *Alistipes*, *Bilophila*, and *Bacteroides* species. By contrast this diet decreased the abundance of Firmicutes, including genus and species specialized in the utilization of polysaccharides (*Roseburia, Eubacterium rectale*, and *Ruminococcus bromii*). Furthermore, the animal-based diet increased the abundance of *B. wadsworthia* and secondary BAs. These findings support the observations in rodent models comparing diets rich in PUFA or saturated fat (D. L. Gibson, *et al*., 2015; Schneeberger, *et al.*, 2015), suggesting similar mechanisms of action and similar metabolic effects.

The relationship between PUFAs and the microbiota are even less well understood. A recent study in women with obesity and metabolic syndrome who consumed inulin-type fructans for 3 months reported that PUFA-derived metabolites were associated with *Bifidobacterium* spp*., Eubacterium ventriosum*, and *Lactobacillus* spp*.*, and negatively correlated with serum cholesterol (Druart, *et al.,* 2014). However, another human intervention study found that supplementation with n-3 fatty acids (180 mg EPA and 120 mg DHA) for 6 weeks did not induce changes in the gut microbiota although it decreased insulin resistance and CRP (Rajkumar, *et al*., 2014). Unfortunately, amelioration of these metabolic parameters could not be directly associated with one specific fatty acid since only a mixture was tested. Therefore, further studies are needed to gain greater understanding of how the quality of dietary fat influences gut microbiota composition and function, and potential mediated effects on metabolism in humans.

Concluding remarks

Fiber is an instrumental dietary component that can be used to remodel gut microbiota composition and function to potentiate the beneficial effects of healthy diets on body weight management and metabolism. However, efforts are still needed to identify the optimal functional partnership between key bacterial species and types of fibers, considering the specificities of the individual's microbiota. Fermentation of dietary fiber generates SCFAs, which presumably articulate beneficial effects in the context of obesity; yet many other secondary metabolic products resulting from diet-microbe interactions have yet to be discovered. Gut microbiota appears to contribute to the adverse consequences of high-fat diets on the metabolic phenotype, aggravating the associated low-grade inflammation and increasing energy absorption; however, further studies are needed to understand the potential effects of the quality of dietary fat on the gut microbiota and secondary metabolic process, such as those involving bile acids and their signaling roles. Additional efforts must be conducted to identify the specific components of the gut microbiota, at species and strain level, influenced by different types of dietary fibers and fats and to understand their roles and mechanisms of action in humans to facilitate the use of this information in nutritional practice. This ambitious goal is expected to be accomplished by developing translational research approaches that integrate controlled dietary interventions in humans, combining functional omics technologies and physiological/clinical endpoints, and mechanistic studies in experimental models colonized with specific dietary-driven human microbiotas.

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Lista de Publicaciones

Esta tesis doctoral comprende cuatro artículos; uno de ellos publicado en una revista indexada (1er artículo del capítulo 1 de la tesis), otros dos en revisión enviados a revistas indexadas (artículos del capítulo 2 de la tesis) y otro que será enviado en breve para su publicación (2º artículo del capitulo 2 de la tesis). Además, durante el período de desarrollo de la tesis doctoral se ha contribuído también en las siguientes publicaciones científicas, aparte de las incluídas en la presente tesis:

Jesús Sanchis-Chordà; **Eva María Gómez del Pulgar**; Joaquín Carrasco-Luna; Alfonso Benítez-Páez; Yolanda Sanz; Pilar Codoñer-Franch. *Bifidobacterium pseudocatenulatum* CECT 7765 supplementation improves inflammatory status in insulin-resistant obese children. European Journal of Nutrition. 2018 Sep 24. doi: 10.1007/s00394-018-1828-5

Ali Alehosseini; **Eva María Gómez del Pulgar**; María José Fabra; Laura Gomez Gomez-Mascaraque; Alfonso Benitez-Paez; Mahboobe Sarabi-Jamab; Behrouz Ghorani; Amparo López-Rubio. Agarose-based freeze-dried capsules prepared by the oil-induced biphasic hydrogel particle formation approach for the protection of sensitive probiotic bacteria. August 2018. Food Hydrocolloids 87 DOI: 10.1016/j.foodhyd.2018.08.032.

Ali Alehosseini; **Eva María Gomez del Pulgar**; Laura G. Gómez-Mascaraque; Marta Martínez-Sanz; Maria José Fabra; Yolanda Sanz; Mahboobe Sarabi-Jamab; Behrouz Ghorani; Amparo Lopez-Rubio. Unpurified Gelidium-extracted carbohydrate-rich fractions improve probiotic protection during storage. LWT-Food science and technology. 96, pp. 694 - 703. Elsevier, 06/2018.

María Cuevas-Tena; **Eva María Gómez del Pulgar**; Alfonso Benítez-Páez; Yolanda Sanz; Amparo Alegría; María Jesús Lagarda. Plant sterols and human gut microbiota relationship: An in vitro colonic fermentation study. Journal of functional foods. 44, pp. 322 - 329. 05/2018.

A. Moratalla; I. Gómez-Hurtado; A. Moya-Pérez; P. Zapater; G. Peiró; J.M González-Navajas; **E.M Gómez del Pulgar**; J. Such; Y. Sanz; R. Francés. *Bifidobacterium pseudocatenulatum* CECT7765 promotes a TLR2-dependent anti-inflammatory response in intestinal lymphocytes from mice with cirrhosis. European Journal of Nutrition. 55 - 1, pp. 197 - 206. 06/02/2015.

Kevin Portune; Alfonso Benítez-Páez; **Eva María Gómez del Pulgar**; Víctor Cerrudo; Yolanda Sanz. Gut microbiota, diet and obesity-related disorders - the

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Capítulo de libro: Yolanda Sanz; Kevin Portune; **Eva María Gómez del Pulgar**; Alfonso Benítez-Páez. Targeting the microbiota: considerations for developing probiotics as functional foods. The gut brain-axis. Dietary, probiotic, and prebiotic interventios on the microbiota. pp. 17 - 30. Elsevier, 2016.

Patente: Sanz Y.; Benítez-Páez, A; **Gómez del Pulgar, Eva Mª**; Francés, R.; Gómez-Hurtado, I.; Piñero, Paula; Juanola, Oriol. "Cepa de *Bifidobacterium longum* sub. *infantis* y uso de la misma". Nº Solicitud: 201830961. Fecha de solicitud 05-10-2018. Entidades titulares: CSIC, Fundación para el Fomento de la Inestigación Sanitaria y Biomédica de la Comunidad Valenciana (FISABIO),Universidad Miguel Hernández de Elche y Consorcio Centro de Investigación Biomédica en Red (CIBER)

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"Nada en este mundo debe ser temido…. solo entendido. Ahora es tiempo de entender más, para temer menos".-**Marie Curie**.