UNIVERSIDAD DE OVIEDO

Programa de Doctorado en Ingeniería Química, Ambiental y Bioalimentaria

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INTERACCIONES ENTRE BIFIDOBACTERIAS Y OTRAS POBLACIONES MICROBIANAS INTESTINALES HUMANAS

TESIS DOCTORAL

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
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RESUMEN (en español)

La microbiota intestinal es un ecosistema dinámico influenciado por factores como la dieta, enfermedades o la edad. Produce vitaminas, así como ácidos grasos de cadena corta (AGCC) a partir de carbohidratos no digeribles de la dieta, y protege frente a patógenos. Tres de los géneros más representativos de la microbiota son *Bacteroides*, *Faecalibacterium* y *Bifidobacterium*. Las interacciones entre estos microrganismos, así como con los compuestos presentes en el ambiente intestinal, y con las células humanas, condicionan en gran parte la influencia que la microbiota ejerce sobre la salud del hospedador. *Bacteroides* se caracteriza por un metabolismo sacarolítico y proteolítico muy amplio, e incluye algunas especies patógenas oportunistas. Por otra parte, a *Faecalibacterium prausnitzii* se le ha atribuido un efecto antiinflamatorio y niveles reducidos de este microorganismo se han relacionado con enfermedades inflamatorias intestinales; esta especie puede utilizar ácido acético producido por otras especies y formar ácido butírico, en un fenómeno conocido como "cross-feeding". Ciertas cepas del género *Bifidobacterium* son utilizadas como probióticos y algunas pueden producir exopolisacáridos (EPS), que son carbohidratos complejos localizados en el exterior celular.

En trabajos previos, nuestro grupo investigador demostró, mediante cultivos fecales, que los EPS de bifidobacterias tienen la capacidad de modular la microbiota intestinal. Aumentaron las poblaciones de bifidobacterias y *Bacteroides*, obteniéndose mayores niveles de ácido propiónico respecto a los cultivos con glucosa. Por otro lado, algunos trabajos han estudiado la interacción entre bifidobacterias y bacterias productoras de ácido butírico.

En este contexto, el objetivo principal de esta Tesis Doctoral fue profundizar en el conocimiento de las interacciones metabólicas entre el género *Bifidobacterium* y otros miembros de la microbiota intestinal. Se llevaron a cabo co-cultivos de diferentes combinaciones de *Bifidobacterium-Bacteroides* en glucosa y EPS de bifidobacterias como carbohidratos fermentables, así como de *Bifidobacterium-Faecalibacterium* con diferentes

fuentes de carbono. Se realizaron recuentos de los microorganismos crecidos en mono y co-cultivo mediante qPCR, se analizaron los metabolitos producidos por cromatografía de gases y cromatografía liquida (CG y HPLC) y el perfil intracelular y extracelular de aminoácidos por UHPLC, se realizaron estudios proteómicos mediante 2D-DIGE y de expresión génica mediante RT-qPCR.

Los datos obtenidos revelaron que las interacciones *Bacteroides-Bifidobacterium* están fuertemente influenciadas por las fuentes de carbono disponibles y por la capacidad metabólica de estos microorganismos. El co-cultivo de *Bifidobacterium longum* y *Bacteroides fragilis* provocó cambios en la producción de enzimas del catabolismo central de carbohidratos en ambos microorganismos y condujo a la sobreproducción de una proteína con función chaperona en *Ba. fragilis*. El crecimiento de *Ba. fragilis* en presencia de EPS, con o sin aminoácidos en el medio de cultivo, condujo a cambios metabólicos y fisiológicos destinados a optimizar la obtención de energía en forma de ATP y a mantener el balance redox intracelular, obteniéndose una producción relativa de ácido propiónico mayor que en cultivos con glucosa. *Ba. fragilis* es capaz de utilizar EPS producidos por bifidobacterias como única fuente de carbono fermentable, dando lugar a un aumento de la producción relativa de ácido propiónico y el mantenimiento de la viabilidad de este microorganismo en etapas tardías de crecimiento. Finalmente se demostró un mecanismo de cross-feeding para la producción de butirato entre *Bifidobacterium adolescentis* y *F. prausnitzii* en presencia de fructooligosacáridos como fuente de carbono.

El presente trabajo pone de manifiesto la especificidad de las interacciones entre los microorganismos intestinales, dependiente tanto de las cepas como de las fuentes de carbono disponibles.

RESUMEN (en Inglés)

The intestinal microbiota constitutes a very dynamic ecosystem influenced by factors like diet, age or disease-status. It accomplishes several functions to the host, such as short chain fatty acid production or protection against pathogens. *Faecalibacterium*, *Bacteroides* and *Bifidobacterium* are three of the most representative bacterial genera of the intestinal microbiota. Interactions between these microorganisms with the carbon sources present in the intestine and with human cells determine the impact of the microbiota on the host health. *Bacteroides* is characterised by a wide ability to use undigested carbohydrates and proteins and some species are considered as opportunistic pathogens. Low levels of *Faecalibacterium prausnitzii* have been related to inflammatory diseases. This species has the ability to use acetic acid released by other microorganisms to produce butyrate, through a phenomenon known as cross-feeding. Some *Bifidobacterium* species have been used as probiotics during the last years. They produce acetic and lactic acids as the main carbohydrate catabolites and also lower amounts of formate, succinate and ethanol. Some species of them are considered as probiotics while some others are able to produce exopolysaccharides (EPS), complex carbohydrates located at the external cell surface

Previous works in our research group demonstrated that bifidobacterial EPS exert a modulatory action on the intestinal microbiota in faecal cultures, resulting in higher counts of *Bifidobacterium* and *Bacteroides* as well as in higher levels of propionate with respect to the cultures with glucose. Furthermore, the interaction between *Bifidobacterium* and butyrate-producing bacteria has been reported previously by other authors.

In this context, the main aim of our work was to study the interactions between *Bifidobacterium* and some other intestinal microbial populations. With this purpose, metabolic features were compared between monocultures and co-cultures of different *Bifidobacterium-Bacteroides* combinations, with glucose and bifidobacterial EPS as fermentable carbon sources. *Bifidobacterium-Faecalibacterium* combinations with different carbon sources were also carried out. Bacterial counts of monocultures and co-cultures were performed by qPCR, metabolite production was analysed by gas and liquid chromatography (GC and HPLC), amino acid levels were determined by UHPLC, differences in protein production between both conditions were retrieved by 2D-DIGE

proteomic analyses, and gene expression studies were performed by RT-qPCR.

Our data indicate that *Bacteroides-Bifidobacterium* interactions are influenced by the strain used and by the carbon source present in the culture medium. Furthermore, we found that the interactions between *Bifidobacterium longum* and *Bacteroides fragilis*, when comparing proteomes of mono-cultures with the co-culture, resulted in changes in the production of enzymes from the central carbohydrate metabolism of both microorganisms and in the overproduction of a chaperone by *Ba. fragilis*. We also demonstrated that bifidobacterial EPS in culture medium with and without amino acids, promoted physiological and metabolic changes in *Ba. fragilis*. These changes pointed to an optimization of ATP production and the maintenance of the intracellular redox balance. As a result, relative propionate production was enhanced with respect to cultures with glucose. *Ba. fragilis* was also able to effectively use bifidobacterial EPS as the sole fermentable carbon source with a concomitant production of AGCC and an improvement in the survival of this microorganism in late phase growth. Finally, we demonstrated a cross-feeding mechanism between *Bifidobacterium adolescentis* and *F. prausnitzii* for butyrate production in fructooligosaccharides as carbon source.

These results evidenced the importance of the strain and the carbon source involved in the pattern of microbial interactions that may occur in the intestinal environment.

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Ahora que la luz al final del túnel está más cerca que nunca, tengo la sensación de que todas las horas de laboratorio intentando desarrollar protocolos, creciendo microorganismos "pejigueros" y luchando incansablemente contra la mínima presencia de oxígeno quedan bastante lejos. Al igual que los "bichitos" en nuestro intestino se necesitan unos a otros para poder sobrevivir y sacar el mayor provecho de todas las situaciones, esta Tesis Doctoral ha sido posible gracias a la aportación, directa o indirecta, de muchas personas que han intentado sacar el mayor provecho de mí y de mi trabajo. Es por eso que deseo agradecer a todas ellas por haber formado parte, de una u otra forma, de estos cuatro años y medio de trabajo.

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Cuatro años y medio dan para 3 localidades en las que vivir, 5 mudanzas, una estancia, mucha gente nueva que conocer, otra que desconocer, son demasiadas cosas para mencionar en un par de páginas, por lo que si alguien se da por olvidado, que se dé también por agradecido.

"Ever tried. Ever failed. No matter.

Try again. Fail again. Fail Better"

- Samuel Beckett "Worstward Ho"

ÍNDICE, LISTA DE TABLAS Y FIGURAS Y ABREVIATURAS

ABREVIATURAS

2D-DIGE: 2 Dimensional Difference Gel Electrophoresis

AGCC/SFCA: Ácidos Grasos de Cadena Corta/ Short Chain Fatty Acids

AGR/BCFA: Ácidos Grasos Ramificados/ Branched Chain Fatty Acids

CG/GC: Cromatografía de gases/ Gas Chromatography

EPS: Exopolisacáridos/ Exopolysaccharides

FAO: Organización de las Naciones Unidas para la Alimentación y la Agricultura

FISH: Fluorescence *in situ* Hybridization

FOS: Fructooligosacáridos/ Fructooligosaccharides

HePS: Heteropolysaccharides

HoPS: Homopolysaccharides

HPLC: High Performance Liquid Chromatography

mCFBM: Medio basal sin fuente de carbono modificado/ Modified Carbon Free Basal Medium

MM: Medio mínimo/ Minimal Medium

PEP: Fosfoenolpiruvato/ Phospoenolpyruvate

qPCR: Quantitative polymerase chain reaction

RT-qPCR: Reverse Transcription-Polymerase Chain Reaction

UHPLC: Ultra High Performance Liquid Chromatograph

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INTRODUCCIÓN

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1. Microbiota intestinal

El cuerpo humano se encuentra colonizado por una gran cantidad de microorganismos, desde las zonas más accesibles al exterior, como la piel, hasta otras menos accesibles, como el tracto gastrointestinal (Consortium, 2012). La colonización de este último es una característica común a lo largo de la escala evolutiva, produciéndose tanto en especies de invertebrados, como el calamar *Euprymna scolopes,* como en vertebrados mamíferos y el ser humano (Kostic y cols., 2013). En general, la complejidad y el número total de microorganismos que colonizan a las especies animales, aumentan a lo largo de la escala evolutiva. Un claro ejemplo de esta coevolución, son los organismos utilizados como modelos experimentales en el estudio de las relaciones microbiota-hospedador (Figura 1).

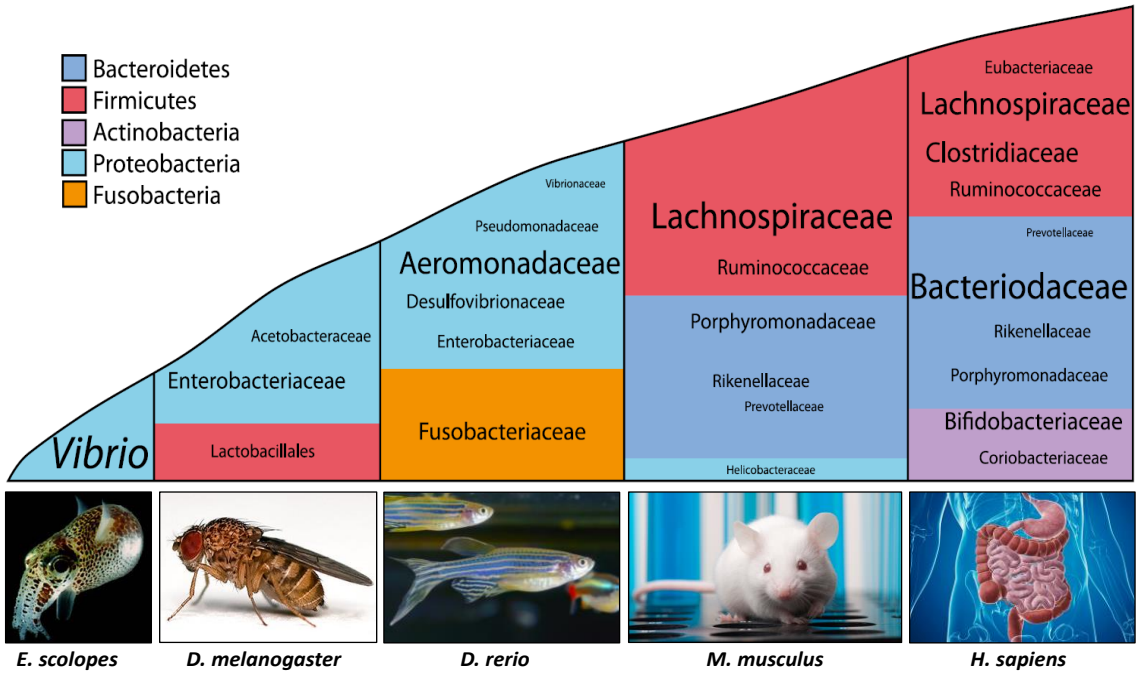

Figura 1. Coevolución de la microbiota intestinal y principales especies animales utilizados en estudios de las interacciones microbiota-hospedador (modificado de Kostic y cols., 2013). Los filos están representados por colores y la abundancia relativa de las familias está representada por el tamaño de la letra.

A lo largo de la evolución, el hospedador y su microbiota han desarrollado una relación de mutualismo. Así, en el primitivo tracto gastrointestinal de *E. scolopes*, sólo se encuentra la especie

microbiana *Vibrio fischeri*, que además de emitir bioluminiscencia, está implicada en el desarrollo del órgano luminoso del calamar. Este órgano ayuda al hospedador a camuflarse simulando la luz de la luna y escapar de los depredadores, mientras que la comunidad de *V. fischeri* recibe una fuente constante de alimento por parte del calamar (Kimbell y McFall-Ngai, 2003). En el caso de la microbiota intestinal humana, sin embargo, su composición y estructura es mucho más diversa y, como consecuencia, las relaciones microbiota-hospedador son de naturaleza mucho más compleja, lo que añade una dificultad considerable a su estudio. Algunos aspectos de la composición de la microbiota intestinal humana y sus funciones se van a tratar en esta introducción.

1.1 Composición de la microbiota intestinal humana

El tracto grastrointestinal está colonizado por miembros de los tres dominios de la vida, eucariotas, arqueobacterias y procariotas, además de virus. Las bacterias tienen una representación mayoritaria llegando a un total de 10^{14} células, lo que supone 10 veces el número de células eucariotas que forman parte del cuerpo humano (Qin y cols., 2010), aunque recientemente, teniendo en cuenta los eritrocitos, y rompiendo el mito, se ha estimado que la proporción real es 1,3:1 (Sender y cols., 2016). La microbiota es un ecosistema dinámico y complejo; edad, dieta, aparición de enfermedades crónicas, tratamiento con antibióticos o el suministro de probióticos son algunos de los múltiples factores que modifican su composición a nivel individual (Salazar y cols., 2014; Tojo y cols., 2014; Flint y cols., 2015; Hu y cols., 2015). Por otro lado, a nivel poblacional, se ha observado que la distribución geográfica, posiblemente debido a su influencia en los factores dietéticos y ambientales, influye en la composición y características de la microbiota. En un estudio reciente, donde se comparaba la microbiota del tejido tumoral y tejido sano adyacente al tumor en pacientes de cáncer colorectal en España y en Estados Unidos, se observó que las muestras se agrupaban mejor según la localización geográfica de los individuos que por la pertenencia a tejido sano o tejido canceroso (Allali y cols., 2015). En otro estudio comparando 1020 individuos sanos de 23 poblaciones diferentes de todo el mundo, se obtuvo una correlación positiva entre la proporción de Firmicutes con la latitud en la que habitaban estas poblaciones (Suzuki y Worobey, 2014). Se ha observado, además, una relación entre la microbiota y el fondo genético del hospedador tanto en modelos animales, en los que la estirpe del animal influye sobre la microbiota que se establece en cada individuo (Zhao y cols., 2013; Ericsson y cols., 2015), como en seres humanos (Khachatryan y

cols., 2008). Por otro lado, parece que la correcta colonización del intestino en los primeros días de vida condiciona notablemente la microbiota residente en el adulto (Cilieborg y cols., 2012).

1.1.1 Colonización y evolución de la microbiota intestinal humana

Mediante el estudio de la carga bacteriana presente en el líquido amniótico y la placenta, se ha concluido que antes del nacimiento el feto ya está en contacto con bacterias (DiGiulio, 2012), encontrándose una colonización microbiana incipiente presente en el meconio (Moles y cols., 2013). No obstante, es en el momento del parto en el que se inicia la exposición a un ambiente de elevada carga microbiana y, por consiguiente, el que determina la colonización del intestino del recién nacido (Dominguez-Bello y cols., 2010). Esta microbiota, inicialmente inestable, va aumentando su diversidad y complejidad hasta llegar, en torno a los 3 años de vida, a una composición similar a la de la edad adulta (Lozupone y cols., 2012). La correcta colonización microbiana del neonato está determinada tanto por el tipo de parto (presentando en general mayores niveles de bifidobacterias y de *Bacteroides* los niños nacidos por parto vaginal que los niños nacidos por cesárea), como por el tipo de alimentación que recibe el recién nacido (siendo mayor el número de bifidobacterias en los niños alimentados con leche materna) (Biasucci y cols., 2010; Martin y cols., 2012). En la edad adulta, donde la variabilidad a nivel de filo es escasa, la microbiota está dominada por los filos Firmicutes y Bacteroidetes, aunque también están presentes los filos Actinobacteria, Proteobacteria y Verrucomicrobia (Yatsunenko y cols., 2012). Al llegar a la senectud, de nuevo se producen cambios en la composición de la microbiota y aumenta la variabilidad entre individuos dependiendo de determinados factores ambientales, como la estancia en residencia geriátrica o la toma de antibióticos (Claesson y cols., 2012; Jeffery y cols., 2015).

1.1.2 Microbiota anaerobia normal

Según se ha comentado anteriormente, no existe mucha variabilidad entre individuos en la composición de la microbiota a nivel de filo, mientras que a niveles taxonómicos inferiores se ha observado una heterogeneidad mucho mayor. Esta conservación de la microbiota a niveles taxonómicos elevados, ha permitido realizar una clasificación de la población humana en tres grandes grupos, llamados "enterotipos", que han sido denominados según el género con mayor abundancia relativa en cada perfil: *Bacteroides*, *Prevotella* o *Ruminococcus* (Arumugam y cols., 2011). La clasificación de la microbiota en enterotipos como grupos discretos es actualmente controvertida.

El replanteamiento de la existencia real de "enterotipos" se basa en que los individuos comparten entre sí una parte muy pequeña de sus comunidades microbianas (Consortium, 2012) y en que un único individuo porta cepas bacterianas que son persistentes en su microbiota pero difieren notablemente de las de otros individuos (Faith y cols., 2013; Schloissnig y cols., 2013). Se debe tener en cuenta también que la microbiota de un individuo puede variar a lo largo del tiempo y el momento de toma de muestra podría introducir un sesgo importante en el resultado. Por ello, la clasificación en enterotipos como grupos discretos, según algunos autores, no sería del todo correcta, debiendo considerarse la microbiota de la población humana como una distribución continua (Knights y cols., 2014).

Dejando a un lado la división taxonómica de la microbiota y centrándonos en la composición genética de ésta, el proyecto europeo MetaHit ha demostrado que aproximadamente el 40% de los genes bacterianos son compartidos por un elevado número de los individuos adultos. Muchos de estos genes son redundantes y se hallan en varias especies bacterianas, de manera que aunque la variabilidad entre individuos es alta en términos taxonómicos, las funciones básicas de la microbiota estarían más conservadas entre ellos (Qin y cols., 2010). Esta mayor conservación a nivel funcional que a nivel de composición bacteriana parece producirse no solo en la edad adulta, sino también en las etapas iniciales de la vida (Ang y cols., 2014).

La distribución de la microbiota a lo largo del tracto gastrointestinal, desde el esófago hasta el estómago es también variable (Figura 2) y se ve fuertemente influenciada por las secreciones liberadas, especialmente, al lumen intestinal. Además de variar en composición, la cantidad de microorganismos aumenta de forma gradual a lo largo del tracto digestivo, oscilando entre 10^2 bacterias por gramo de contenido en esófago y estómago, hasta 10^{12} bacterias por gramo de contenido en el colon (Sekirov y cols., 2010). Cabe destacar que más del 70% de las bacterias del sistema digestivo se encuentran en el intestino grueso, siendo esta el hábitat más poblado del cuerpo humano (Ley y cols., 2006).

La microbiota no sólo tiene una distribución diferencial a lo largo del tubo digestivo, también tiene una distribución axial desde el epitelio hasta el lumen intestinal (Propheter y Hooper, 2015). Hasta la fecha, no se han estudiado en profundidad las causas fisiológicas que influyen en la localización de cada uno de los grupos bacterianos en el intestino. Varios estudios sugieren que cerca del epitelio intestinal se podrían encontrar los productores de ácido butírico, como *Faecalibacterium*

prausnitzii. Las observaciones en las que se apoya esta hipótesis, se basan en que el ácido butírico producido por esta especie, es utilizado por los colonocitos como fuente de carbono, y que, a pesar de ser anaerobio estricto, este microorganismo posee la capacidad de utilizar flavinas y tioles del ambiente como receptores de oxígeno, permitiéndole sobrevivir en las zonas próximas al epitelio, donde la presión parcial de O_2 es más alta (Khan y cols., 2012a). Por otro lado, las bacterias con una capacidad metabólica superior al resto de géneros, como el género *Bacteroides*, se encontrarían en localizaciones más cercanas al lumen, donde se produce la llegada de fuentes de carbono de forma regular. En este sentido, cabe destacar un estudio reciente en el que se combinan técnicas de marcaje fluorescente con técnicas de fijación de muestra poco agresivas y programas informáticos de análisis de imágenes, para determinar la distribución de grupos bacterianos en un modelo de ratón. Así, Earle y colaboradores (2015), siguieron el comportamiento y distribución de un miembro del género *Bacteroides* cuando ratones colonizados con microbiota humana fueron alimentados con una dieta estándar, encontrándose *Bacteroides* cerca del lumen intestinal, y cuando fueron alimentados con una dieta sin fuentes de carbono fermentables por la microbiota, en cuyo caso, *Bacteroides* empezaba a utilizar componentes de la mucosa como fuente de carbono adentrándose en la capa de mucus.

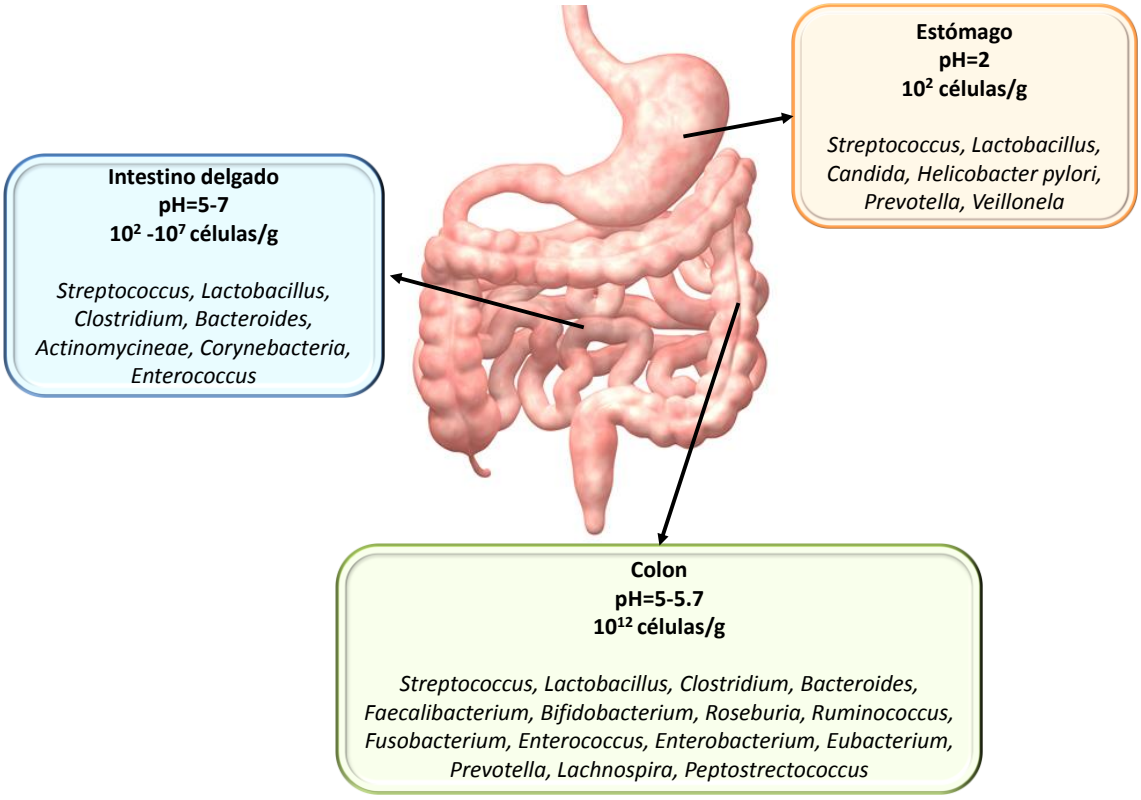

Figura 2. Distribución de la microbiota a lo largo del tracto gastrointestinal humano (Modificado de Jandhyala y cols., 2015)

En este ambiente intestinal hay tres géneros bacterianos que se encuentran en todos los individuos, generalmente de forma mayoritaria: *Bacteroides*, *Faecalibacterium* y *Bifidobacterium.* Estos géneros son los representantes principales de los filos mayoritarios presentes en el colon humano: Bacteroidetes, Firmicutes y Actinobacterias. Miembros de estos tres géneros han sido los utilizados durante el desarrollo de esta Tesis doctoral, por lo que se describen brevemente a continuación.

1.1.2.1 *Bacteroides* spp.

El género *Bacteroides* es uno de los más abundantes en la microbiota intestinal humana, llegando a una proporción del 20-50% de la microbiota total dependiendo de los individuos (Mahowald y cols., 2009). Pertenecen al filo Bacteroidetes y son bacilos Gram-, anaerobios, resistentes a bilis y no forman esporas. Se han descrito 31 especies dentro de este género, aunque existen otras 21 potenciales especies, que aún no han sido convenientemente caracterizadas y también podrían formar parte de este grupo. Poseen una alta capacidad de adaptación a cambios ambientales, especialmente *Bacteroides fragilis,* especie tipo del género. Como consecuencia, es esta especie la que más se aísla en muestras procedentes de infecciones sistémicas y abscesos en cirugía, cáncer o traumatismos del tracto gastrointestinal (Wexler, 2013). Los miembros del género *Bacteroides* tienen la capacidad de utilizar polisacáridos vegetales que provienen de la dieta y no son digeridos por el hospedador. La especie *Bacteroides thetaiotaomicron* tiene una capacidad metabólica muy amplia, siendo altamente competitiva para la utilización de fuentes de carbono disponibles en el intestino, tanto glúcidos como proteínas (Sonnenburg y cols., 2010). Los principales metabolitos producidos a partir del catabolismo de glúcidos por *Bacteroides* son los ácidos acético, propiónico, succínico y láctico, pudiendo, además, utilizar este último como fuente de carbono en determinadas condiciones (Figura 3) (Macy y cols., 1978; Pan y Imlay, 2001).

Respecto a su relación con el hospedador, este género puede comportarse como mutualista o como potencial patógeno. Respecto a su relación mutualista, se ha demostrado que el polisacárido A (PSA), polímero capsular de ciertas cepas de *B. fragilis*, contribuye al desarrollo y maduración del sistema inmune del hospedador, además de promover la función T_{reg}, básica para un buen funcionamiento del sistema inmune (Mazmanian y cols., 2005; Telesford y cols., 2015). Los polímeros de la cápsula también han sido descritos como factores de virulencia, ya que están implicados en la adaptación a diferentes ambientes, como puede ser el torrente sanguíneo, lo que

facilita a este microorganismo la capacidad de producir infecciones sistémicas en determinadas condiciones (Wexler, 2007).

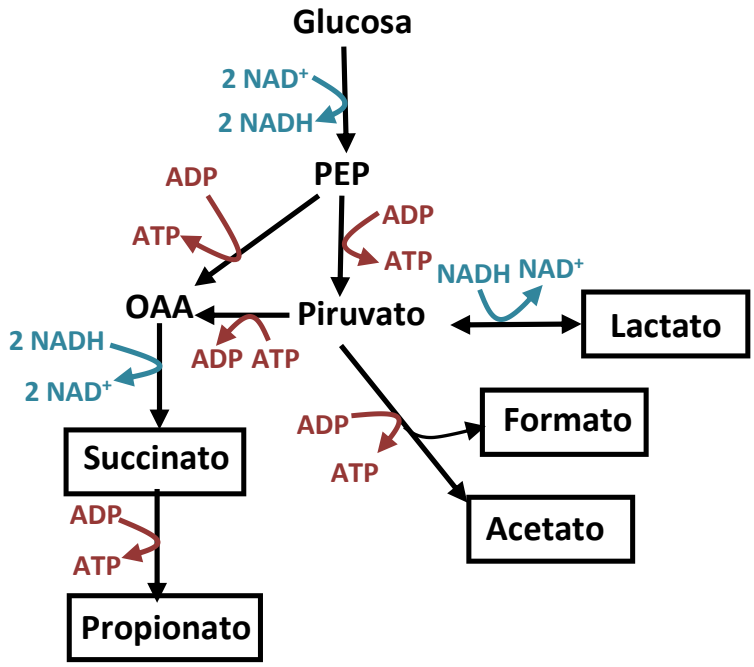

Figura 3. Resumen del metabolismo central de carbohidratos del género *Bacteroides* (Modificado de Pan e Imlay, 2001). PEP, fosfoenolpiruvato. OAA, oxalacetato.

1.1.2.2 *Faecalibacterium*

El género *Faecalibacterium,* anteriormente considerado dentro del género *Fusobacterium*, es monoespecífico, siendo *F. prausnitzii* su único representante. Pertenecen al filo Firmicutes y forman parte del grupo IV de clostridios. Son bacilos Gram-, anaerobios, no formadores de esporas con un contenido G+C entre 47-57% (Duncan y cols., 2002) que está presente en una proporción del 8-12% en la microbiota intestinal de adultos sanos (Walker y cols., 2011). Su catabolismo de carbohidratos se caracteriza por la utilización de acético procedente de otras bacterias y la producción de los ácidos butírico, fórmico y láctico (Figura 4) (Lopez-Siles y cols., 2012).

Aunque es extremadamente sensible al oxígeno, parece que este microorganismo se encuentra en los sustratos más cercanos al epitelio intestinal, donde hay una difusión parcial de oxígeno hacia la mucosa. Experimentos recientes han explicado esta aparente contradicción gracias a la existencia de una lanzadera extracelular en la que están involucrados flavinas y tioles, ambos presentes en el colon de humanos sanos. Estos compuestos sirven como mediadores en el transporte de electrones, que finalmente se transfieren al oxigeno del medio (Khan y cols., 2012a; Khan y cols.,

2012b). El interés en este microorganismo y otros productores de ácido butírico ha aumentado notablemente en los últimos años dada la asociación de niveles bajos de *F. prausnitzii* en la microbiota de individuos con enfermedades inflamatorias intestinales, como puede ser la enfermedad de Crohn (Jia, 2010). Además, recientemente se ha aislado una cepa productora de una matriz polimérica extracelular con la capacidad de atenuar los síntomas en un modelo de colitis inducida por dextrán sulfato sódico (DSS) y de producir una respuesta inmunológica antinflamatoria (Rossi y cols., 2015).

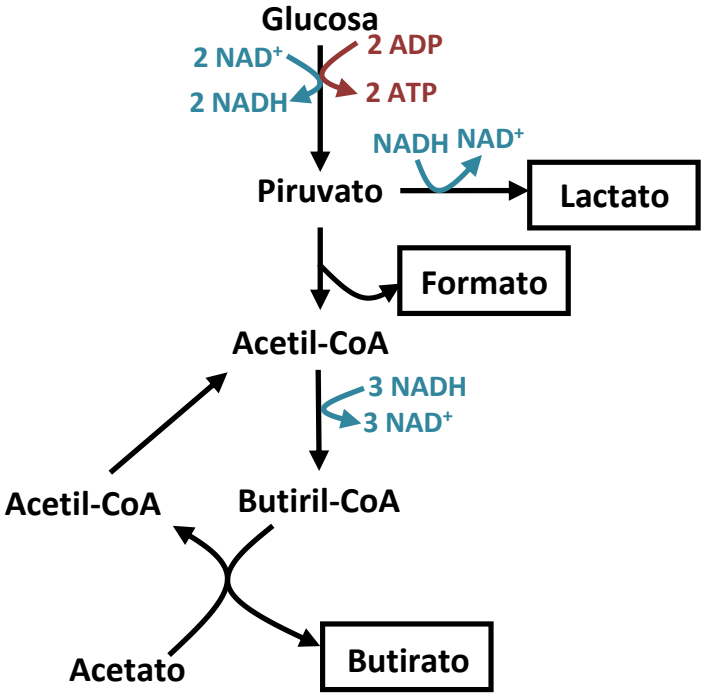

Figura 4. Resumen del metabolismo central de carbohidratos de *Faecalibacterium prausnitzii* (Modificado de Khan y cols. 2012b).

1.1.2.3 *Bifidobacterium spp.*

Es el grupo predominante en la microbiota de lactantes sanos, pero en etapas posteriores de la vida, su número se reduce hasta menos de un 5% de la microbiota intestinal en la vida adulta, llegando incluso a niveles menores en ancianos (Gueimonde y cols., 2010; Arumugam y cols., 2011). Pertenecen al filo Actinobacteria y son bacilos Gram +, anaerobios, no formadores de esporas con un contenido alto en G+C en su ADN (>50%) (De Vuyst y cols., 2014). Las especies de bifidobacterias dominantes en la microbiota humana sufren variación a lo largo de la vida, siendo *B. adolescentis* y *B. longum* las que se mantienen como mayoritarias en adultos sanos; en consecuencia, los genomas de estos dos géneros contienen un alto número de genes relacionados con la

degradación de carbohidratos provenientes de la dieta (Schell y cols., 2002; Turroni y cols., 2012; Duranti y cols., 2014). Las bifidobacterias son capaces de degradar hexosas y lactosa mediante lo que se conoce como vía bífida o ruta de la fructosa-6-fosfato fosfocelotasa; en ella, de forma teórica se producen 1,5 moles de ácido acético y un mol de ácido láctico por cada mol de glucosa consumido. Esta relación difiere, no obstante, dependiendo de la especie y también varía dependiendo de la fuente de carbono disponible (Gonzalez-Rodriguez y cols., 2013; Rios-Covian y cols., 2016). Las bifidobacterias también son capaces de producir de forma minoritaria ácido fórmico, ácido succínico y etanol (Figura 5) (Van der Meulen y cols., 2006).

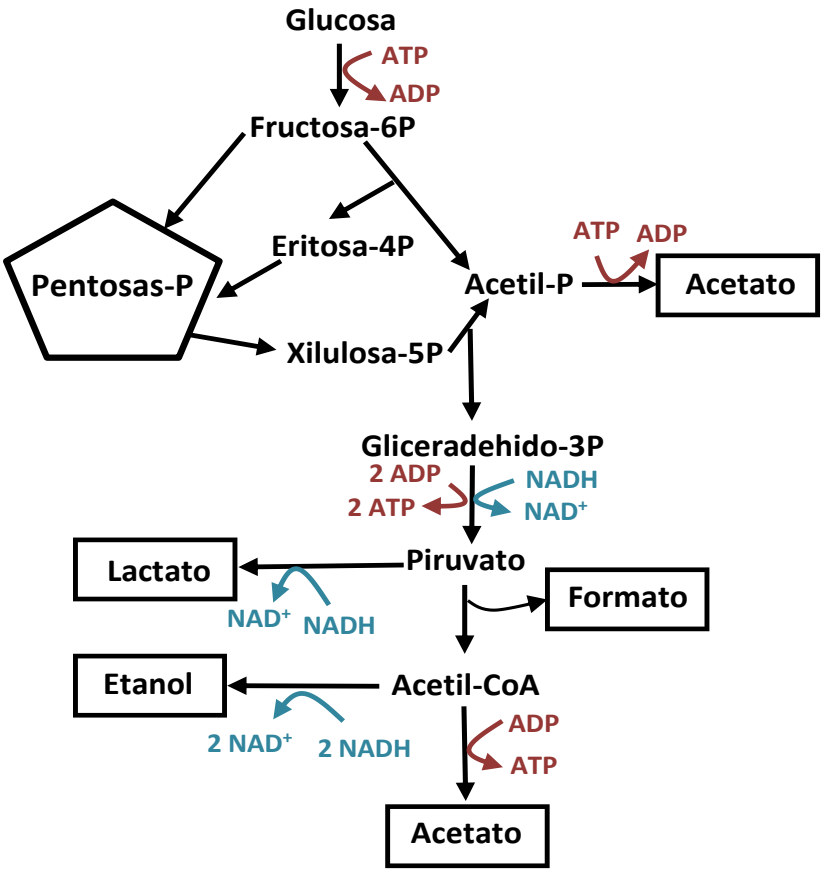

Figura 5. Resumen del metabolismo central de carbohidratos del género *Bifidobacterium* (Modificado de De Vuyst y cols. 2014)

Algunos microorganismos de este género son utilizados como microorganismos probióticos en diversos productos comerciales. La Organización Mundial de la Salud definió los probióticos como "microorganismos vivos que administrados en cantidades adecuadas, proporcionan un beneficio para la salud" (FAO/WHO, 2006). Desde entonces, se han desarrollado múltiples formas de suministrar y comercializar estos microrganismos, bien sea mediante su inclusión en productos lácteos fermentados o suplementos comercializados en forma de pastillas, cápsulas, liofilizados, etc.

Debido a la confusión social surgida en torno al término "probiótico" y su utilización de forma poco precisa para fines comerciales, un comité de expertos en diferentes áreas, relacionadas con los probióticos, se ha reunido recientemente para matizar los aspectos relativos a este concepto (Hill y cols., 2014). En la figura 6 se muestran, de forma global, los posibles mecanismos de acción beneficiosos de los probióticos sobre la salud del hospedador.

Dentro del marco de los probioticos, el género *Bifidobacterium*, junto a *Lactobacillus*, ha sido uno de los más utilizados y algunas de sus especies tienen el estatus QPS ("Qualified Presumption of Safety") (EFSA, 2007). Por otro lado, niveles reducidos de bifidobacterias se han relacionado, en algunos casos, con diferentes estados de enfermedad gastrointestinal, como el síndrome de colon irritable o cáncer colorectal (Tojo y cols., 2014);

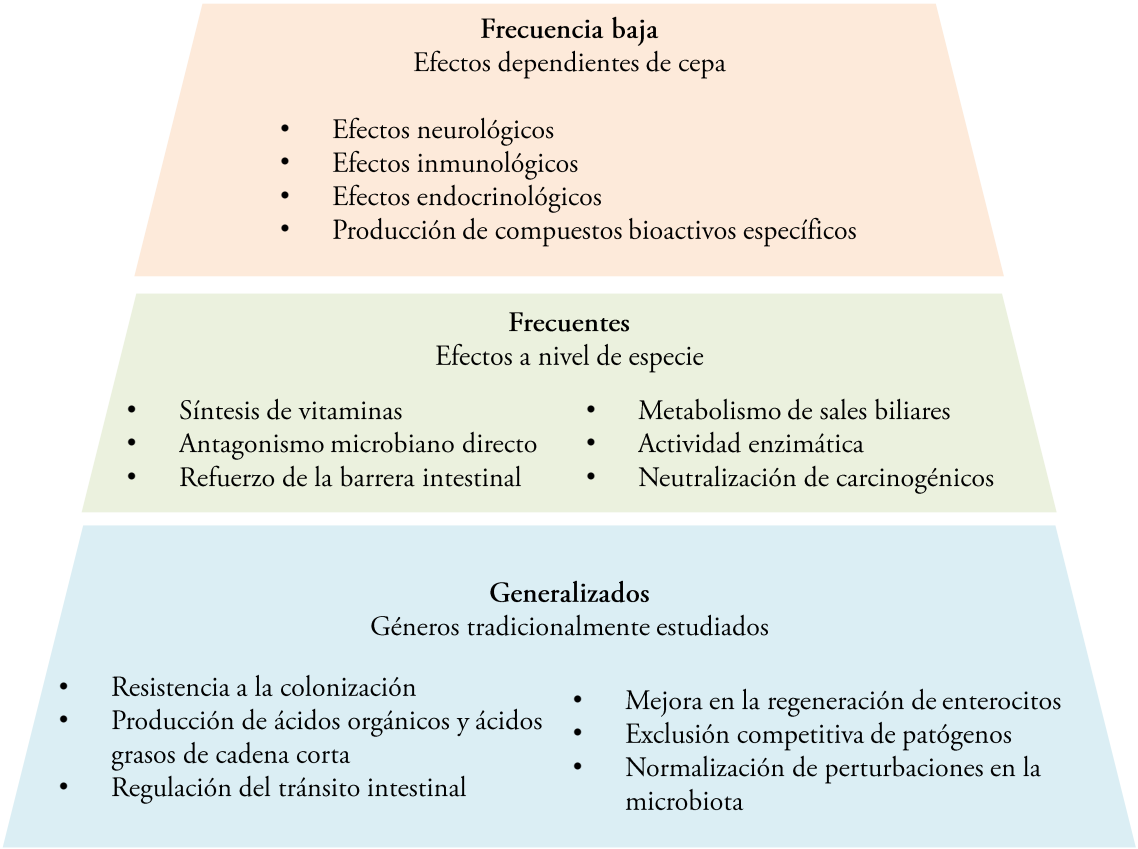

Figura 6. Posible distribución de los mecanismos de acción de los probióticos (modificado de Hill y cols. 2014). Los efectos de los probióticos en el hospedador, pueden darse de manera muy variable, de forma que casi todas las cepas de una especie lo posean o que sólo lo hagan un pequeño número. Por otro lado, las evidencias acumuladas sugieren que se pueden hacer ciertas generalizaciones respecto a los efectos que tienen los probióticos en nuestra salud

Algunas cepas del género *Bifidobacterium* son capaces de producir (EPS), que son polímeros complejos secretados al exterior celular; su función se ha relacionado, entre otras, con la protección de la bacteria frente a factores externos y, además, juegan un papel clave en la formación del biofilm

bacteriano (Hidalgo-Cantabrana y cols., 2014). Estos polímeros pueden ser homopolisacáridos (HoPS), cuando están formados por la unión de moléculas del mismo sacárido; y heteropolisacáridos (HePS), cuando hay diferentes moléculas de sacárido en su composición. Hasta la fecha, en el género *Bifidobacterium* sólo se ha descrito la producción de HePS (Ruas-Madiedo, 2014). Aunque aún no se ha demostrado la producción de estos polímeros en el intestino, se ha demostrado *in vitro* que su síntesis es estimulada por la presencia de sales biliares (Ruas-Madiedo y cols., 2009). Por otro lado, su probable presencia en el intestino, y la habilidad de otros géneros bacterianos de utilizar los EPS producidos por otros microorganismos, han potenciado el estudio de los EPS como potenciales sustratos prebióticos (Grosu-Tudor y cols., 2013). La FAO definió en 2007 los prebioticos como *componentes alimentarios no viables que confiere un efecto beneficioso en el hospedador asociado a la modulación de la microbiota intestinal*. Dada la controversia que se generó respecto a esta definición, algunos autores han propuesto otras como "*ingrediente que, fermentado de forma selectiva, resulta en cambios específicos en la composición y/o actividad de la microbiota intestinal, confiriendo beneficios en la salud del hospedador"* (Roberfroid y cols., 2010).

1.2 Principales funciones de la microbiota intestinal humana

Las cuatro funciones principales que cumple la microbiota intestinal en el cuerpo humano son: metabólica, protectora, inmunomoduladora y función trópica.

La principal acción metabólica de las bacterias intestinales, es la fermentación de componentes de la dieta no digeribles por el hospedador, como son los glúcidos complejos y algunas proteínas, con la consiguiente liberación de ácidos grasos de cadena corta (AGCC) y ácidos grasos ramificados (AGR), principalmente (Flint y cols., 2015). Las rutas metabólicas de síntesis de los principales AGCC y su relación con la salud serán tratadas con más profundidad en el apartado 2.3. Además del catabolismo de glúcidos, otras funciones metabólicas de la microbiota incluyen la síntesis vitamina de K y vitaminas del grupo B que son utilizadas por el hospedador. También ciertos géneros, como *Bacteroides*, están implicados en el metabolismo de aminoácidos y de lípidos; respecto a estos últimos, algunas especies de los géneros *Bifidobacterium* y *Lactobacillus* tienen la capacidad de producir conjugados del ácido linoleico, compuestos que poseen un efecto regulador del apetito (Gorissen y cols., 2010). Por otro lado, la microbiota intestinal también participa en la absorción de calcio, magnesio y hierro y en la eliminación o inactivación de compuestos perjudiciales, como algunos xenobióticos (Jandhyala y cols., 2015).

Uno de los mecanismos principales del efecto protector que ejerce la microbiota intestinal sobre el hospedador se basa en el mantenimiento de la integridad de la barrera intestinal, a través de la producción de compuestos antimicrobianos, la estimulación de la producción de mucus y la activación de genes implicados en el mantenimiento de desmosomas y uniones estrechas del epitelio (Ashida y cols., 2012; Ostaff y cols., 2013). También se han descrito mecanismos específicos de inhibición de patógenos, como la competencia por nutrientes y/o sitios de unión a la mucosa o estimulación del sistema inmune del hospedador (Kamada y cols., 2013).

La microbiota y los metabolitos producidos por su actividad tienen un marcado efecto sobre el sistema inmune. Así, por ejemplo, el butirato, procedente de la fermentación de glúcidos complejos, además de ser utilizado como fuente de carbono por los colonocitos, es capaz de modificar el perfil de producción de citoquinas de los linfocitos T helper (T_H), mejorando la integridad de la barrera epitelial intestinal (Kau y cols., 2011). En la etapa postnatal temprana, la microbiota constituye un enorme estímulo antigénico necesario para el desarrollo tanto del sistema inmune innato como del adaptativo. Tanto es así, que se ha demostrado que la falta de esta exposición a la microbiota en etapas tempranas altera la respuesta inmune del individuo durante el resto de su vida (Olszak y cols., 2012). Durante la vida adulta, la microbiota residente influye en la maduración de los linfocitos T_H del tejido linfoide asociado al intestino y de T_{reg} mediante la presentación de antígenos microbianos por las células dendríticas, lo que contribuye a mantener una homeostasis en la respuesta inmune del hospedador (Weng y Walker, 2013).

A partir de la demostración del desarrollo deficiente de las vellosidades en los tejidos asociados al intestino delgado en ratones axénicos, se ha estudiado el papel que tiene la microbiota en el desarrollo de un intestino funcional y su posible influencia en otros sistemas del organismo, como puede ser el sistema nervioso (Jandhyala y cols., 2015). En los últimos años se ha estudiado la relación de la microbiota con el desarrollo y normal funcionamiento del sistema nervioso, a través del eje intestino-cerebro. Este concepto cobra importancia al tener en cuenta que la instauración de una microbiota definitiva y el desarrollo neuronal coinciden en el tiempo (Burokas y cols., 2015).

2. Interacciones de la microbiota intestinal humana

Son muchos los factores que influyen en la composición de la microbiota humana, y como consecuencia, en la forma en la que ésta afecta al hospedador. A la hora de estudiar las interacciones

microbiota-hospedador, también hay que tener cuenta las distintas relaciones que se establecen entre los propios miembros de la comunidad microbiana, y de estos con los compuestos presentes en el exterior celular. El esquema de la figura 7 resume las principales interacciones en las que la microbiota intestinal está implicada.

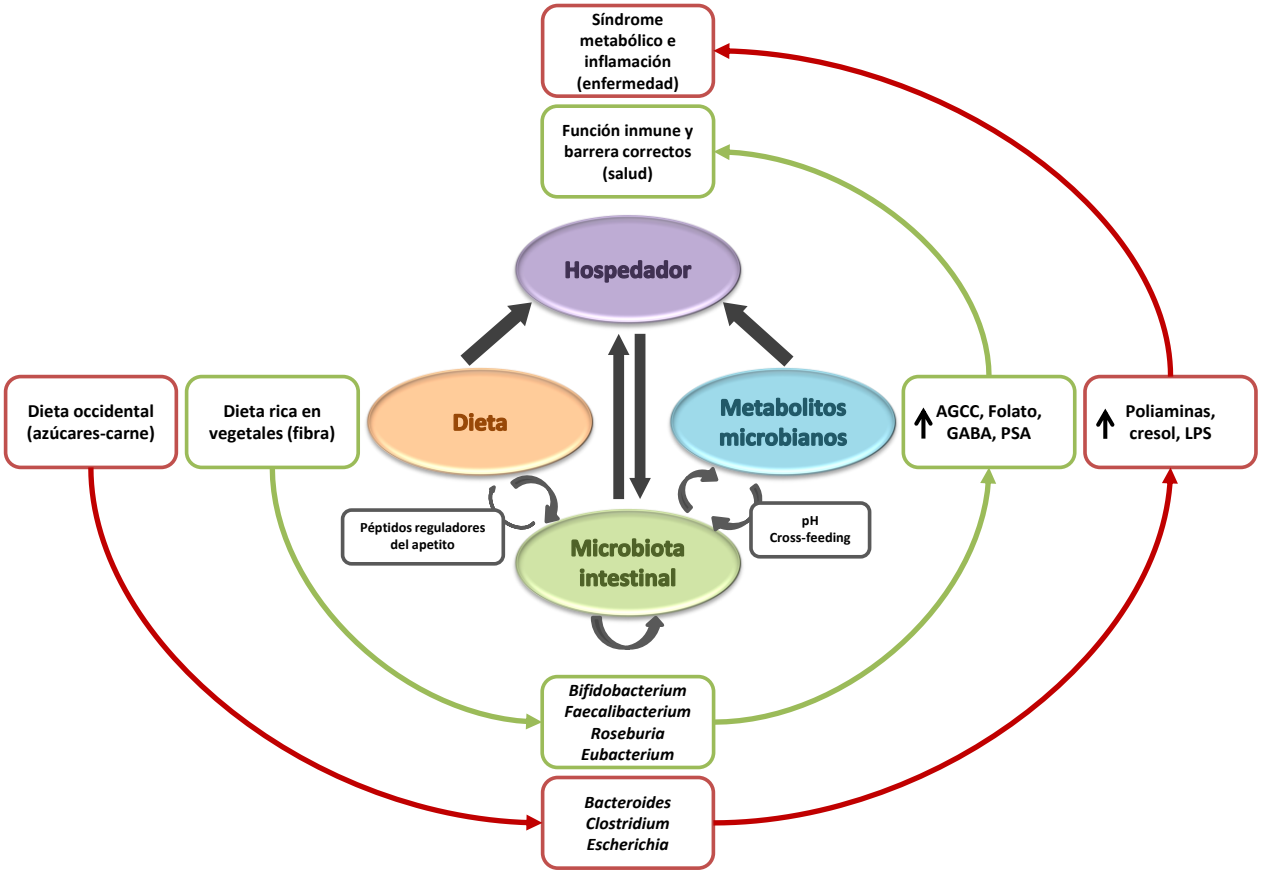

Figura 7. Principales interacciones entre dieta, microbiota, metabolitos microbianos y hospedador (modificado de Tan y O'toole, 2015). Diferencias en la dieta pueden dar lugar a diferentes perfiles bacterianos, y por consiguiente, diferente interacción con el hospedador. GABA, ácido gamma-aminobutírico. PSA, polisacárido A. LSP, lipopolisacárido.

2.1 Interacciones microbiota-hospedador

La interacción microbiota-hospedador y su efecto sobre el estado de salud están muy relacionados con las funciones que las bacterias intestinales cumplen en el organismo humano. Esta interacción, incluye la utilización de metabolitos bacterianos, principalmente AGCC por parte del hospedador, la estimulación del desarrollo del epitelio intestinal o la inmunomodulación mencionados en el apartado 1.2. En estados en los que existe un desequilibrio de la composición y/o funcionalidad de la microbiota intestinal, llamado disbiosis, estas interacciones pueden verse afectadas, con efectos perjudiciales para le hospedador.

Por ejemplo, se ha descrito una reducción de la capa de mucus en estados de enfermedad inflamatoria (Hansson, 2012). Por otro lado, cuando el equilibrio de la microbiota esta alterado, especies como *Bacteroides fragilis* o *Fusobacterium nocrophorum* pueden invadir e infectar los tejidos adyacentes, además de causar abscesos en otras partes del cuerpo. Otro microorganismo asociado con la disbiosis microbiana intestinal, que tiene efectos negativos sobre el hospedador, es *Clostridium difficile,* cuyas infecciones de repetición son difíciles de erradicar (McKenney y Pamer, 2015). Existen otros estados de enfermedad o inflamación relacionados con disbiosis microbiana, como son el síndrome metabólico, obesidad, enfermedad inflamatoria intestinal, diabetes tipo 2 y cáncer colorectal (Zhang y cols., 2015). Otro área de interés es el estudio de la interacción microbiota-hospedador sobre el eje intestino-cerebro, algunos autores han relacionado la abundancia de ciertos grupos bacterianos con la depresión, y en concreto se ha evidenciado que la concentración elevada en heces de ácido isovalérico, producto de la utilización de aminoácidos ramificados por la microbiota, está relacionado con estados de depresión (Szczesniak y cols., 2015).

2.2 Interacciones bacteria-bacteria en la microbiota intestinal

En el campo de la ecología se distinguen diversos tipos de interacciones entre organimos que también son de aplicación en el campo de la microbiota intestinal. Éstas son: parasitismo, comensalismo, neutralismo, amensalismo, competición y mutualismo. Actualmente, el estudio *in vivo* de estas interacciones complicado debido a la falta de técnicas adecuadas; por eso, hasta la fecha, se han estudiado principalmente las interacciones entre grupos concretos tanto *in vitro* como en modelos con ratones gnotobióticos. Con el avance de las técnicas de secuenciación masiva de ADN y la bioinformática, se ha empezado a profundizar en estos fenómenos mediante el desarrollo de modelos matemáticos que nos ayudan a comprender hasta qué punto las interacciones entre los microorganismos del intestino, considerada como una comunidad dinámica, influyen en la estabilidad de las funciones microbianas y la salud del hospedador (Stein y cols., 2013; Coyte y cols., 2015). Un estudio reciente examinó, *in silico*, el tipo de interacciones que pueden ocurrir entre 11 de los principales grupos existentes en la microbiota en las diferentes condiciones del ambiente gastrointestinal. A lo largo del estudio se valoraron diferentes tipos de dieta y diferentes concentraciones de oxígeno en el medio, llegando a la conclusión de que bajo condiciones anóxicas las relaciones especie-especie que se ven más favorecidas son las mutualistas (Heinken y Thiele, 2015). Un ejemplo claro de mutualismo es el fenómeno conocido como sintrofia, en inglés “cross-

feeding". Según su etimología, este concepto se refiere a la asociación de organismos para metabolizar alimentos. Este proceso consiste en la utilización de compuestos generados por un microrganismo por parte de un segundo microorganismo, dichos sustratos pueden ser generados al liberar moléculas simples durante la degradación de moléculas complejas o al liberar productos finales del catabolismo. Este fenómeno, se presenta en el intestino, principalmente, en relación con la utilización de productos de la degradación de glúcidos complejos y en el metabolismo de AGCC, que se tratarán de manera amplia en el apartado 2.3. Cabe destacar que este tipo de interacción no afecta sólo al metabolismo microbiano en sí mismo, si no que podría influir en la distribución de los grupos bacterianos dentro de la comunidad microbiana e incluso en las variaciones que se producen cuando las condiciones ambientales del intestino cambian. A este respecto, en ratones colonizados con microbiota humana se marcó con sondas fluorecentes una especie del género *Bacteroides* y otra especie no patógena del género *Salmonella,* capaz de utilizar el ácido siálico liberado por los miembros del género *Bacteroides* al degradar la capa de mucus. Los ratones fueron sometidos a dos tipos de dieta, una estándar y otra sin carbohidratos accesibles para la microbiota. En abundancia de fuentes de carbono fermentables por la microbiota, *Bacteroides* y *Salmonella* tenían una localización diferente en el ecosistema de la microbiota intestinal; con la falta de fuentes de carbono fermentables, los nichos de estos dos microorganismos aparecían entremezclados (Earle y cols., 2015). Las relaciones de amensalismo, en las que sólo uno de los microorganismos implicados se ve afectado de forma negativa, engloban la producción de compuestos con efecto antimicrobiano que se han tratado brevemente en el apartado 1.2., cuando se discutió el papel protector frente a patógenos de la microbiota intestinal.

En relación con la utilización de compuestos producidos por otros miembros de la comunidad microbiana, los EPS bacterianos podrían tener un papel relevante. En este sentido se ha estudiado el potencial prebiótico de EPS producidos por lactobacilos y bifidobacterias, tanto en cultivo de cepa única (Sonnenburg y cols., 2010; Grosu-Tudor y cols., 2013; van Bueren y cols., 2015) como en cultivos fecales (Cinquin y cols., 2006; Salazar y cols., 2008; Salazar y cols., 2009).

Teniendo en cuenta que en el colon se alcanza una concentración de 10^{12} microorganismos por gramo de contenido fecal, es de esperar que exista una fuerte competencia microbiana, principalmente por las fuentes de carbono presentes en el medio (Sonnenburg y cols., 2010). Se ha estudiado *in vitro* e *in vivo* la competencia por el sustrato, generalmente prebióticos, de manera que

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al seguir la evolución de la microbiota en presencia de la fuente de carbono, se ha podido elucidar qué grupos microbianos son los más competitivos en la utilización de cada fuente de carbono. Por ejemplo, en un estudio de intervención donde se administraba inulina a voluntarios sanos, se constató que los niveles de *F. prausnitzii* y de *B. adolescentis* aumentaban respecto al grupo que estaba tomando placebo (Ramirez Farias, 2009). La competencia entre especies se ha demostrado incluso a nivel genético en modelos con ratones gnotobióticos, en los que se detectó un aumento en la transcripción de genes relacionados con el metabolismo glucídico de *Ba. thetaiotaomicron* en presencia de *B. longum* respecto a ratones monocolonizados con *Bacteroides* (Sonnenburg y cols., 2006). Esta competencia podría ser uno de los motores del dinamismo de la microbiota humana, puesto que cambios en la dieta suponen cambios en el tipo y cantidad de fuentes de carbono que llegan al intestino. De hecho, se ha demostrado que los patrones dietéticos son los principales determinantes del enterotipo de los individuos (Bushman y cols., 2013). Cabe destacar, que la especificidad en el uso de polisacáridos complejos, se ha estudiado en varios géneros, entre ellos, *Bacteroides, Bifidobacterium* y *Lactobacillus*, llegandose a la conclusión de que la especificidad en la capacidad de uso de diferentes fuentes de carbono determina la competitividad, y por lo tanto las interacciones microbianas (Falony y cols., 2009b; Sonnenburg y cols., 2010; Watson y cols., 2013; Scott y cols., 2014). Relacionado con la influencia que tiene la especie, e incluso la cepa en esta interacción, también se han realizado estudios en los que se analizó cómo diferentes cepas de los géneros *Bacteroides* y *Bifidobacterium* interaccionaban en presencia de prebióticos tipo inulina, observándose que la habilidad específica para utilizar ese tipo de sustratos por cada microorganismo influenciaba de forma directa la interacción entre ambos géneros (Van der Meulen y cols., 2006; Falony y cols., 2009a).

Otro mecanismo de interacción entre bacterias dentro de las relaciones bacteria-bacteria desde un punto ecológico, es la producción de moléculas relacionadas con el "quorum sensing", (percepción de quorum o autoinducción). Éste es un mecanismo de comunicación entre bacterias en respuesta a la densidad de población celular mediado por moléculas especiales, llamadas autoinductores. En los últimos años además de detectarse este tipo de moléculas en especies patógenas intestinales como *Listeria monocytogenes* o *Salmonella*, se ha demostrado la presencia de este mecanismo en especies del género *Bifidobacterium* (Bassler y cols., 1997; Sun y cols., 2014).

2.3 Ácidos grasos de cadena corta como mediadores de las interacciones en el intestino

Como se ha mencionado a lo largo de esta introducción, uno de los principales agentes participantes en las interacciones de la microbiota tanto con el hospedador como con otros microorganismos son los AGCC, producidos como metabolitos finales de la fermentación de fuentes de carbono presentes en el intestino. Tanto su síntesis e interacción con la microbiota, como los estudios realizados con estos compuestos y su implicación en la salud del hospedador son tratados en la siguiente revisión que forma parte de la introducción de esta memoria de Tesis Doctoral:

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Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health

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The colon is inhabited by a dense population of microorganisms, the so-called "gut microbiota," able to ferment carbohydrates and proteins that escape absorption in the small intestine during digestion. This microbiota produces a wide range of metabolites, including short chain fatty acids (SCFA). These compounds are absorbed in the large bowel and are defined as 1-6 carbon volatile fatty acids which can present straight or branched-chain conformation. Their production is influenced by the pattern of food intake and diet-mediated changes in the gut microbiota. SCFA have distinct physiological effects: they contribute to shaping the gut environment, influence the physiology of the colon, they can be used as energy sources by host cells and the intestinal microbiota and they also participate in different host-signaling mechanisms. We summarize the current knowledge about the production of SCFA, including bacterial cross-feedings interactions, and the biological properties of these metabolites with impact on the human health.

Keywords: short chain fatty acids, diet, human health, intestinal microbiota, cross feeding

INTRODUCTION

The gut microbiota influences our health and nutritional stage via multiple mechanisms, and a mounting body of evidence recognizes that microbial metabolites have a major influence on host physiology. Short chain fatty acids (SCFA) are volatile fatty acids produced by the gut microbiota in the large bowel as fermentation products from food components that are unabsorbed/undigested in the small intestine; they are characterized by containing fewer than six carbons, existing in straight, and branched-chain conformation. Acetic acid (C2), propionic acid (C3), and butyric acid (C4) are the most abundant, representing 90–95% of the SCFA present in the colon. The main sources of SCFA are carbohydrates (CHO) but amino acids valine, leucine, and isoleucine obtained from protein breakdown can be converted into isobutyrate, isovalerate, and 2-methyl butyrate, known as branched-chain SCFA (BSCFA), which contribute very little (5%) to total SCFA production. The aim of the present mini-review is to summarize the current knowledge about SCFA production, including bacterial cross-feedings interactions, and the biological properties of these metabolites with impact in human health.

MECHANISMS OF SCFA PRODUCTION

Metabolic Routes

The main end products resulting from the CHO catabolism of intestinal microbes are acetate, propionate, and butyrate. Lactate, although is not a SCFA, is also produced by some members of the microbiota, such as lactic acid bacteria, bifidobacteria, and proteobacteria, but under normal physiological conditions it does not accumulate in the colon due to the presence of some species, such as *Eubacterium hallii*, that can convert lactate into different SCFA (Flint et al., 2015).

Acetate is the most abundant SCFA in the colon and makes up more than half of the total SCFA detected in feces (Louis et al., 2007). Two main metabolic routes have been described for acetate production by the gut microbiota (**Figure 1**). The majority of acetate is produced by most enteric bacteria as a result of CHO fermentation. In addition, approximately one-third of the colonic acetate is coming from acetogenic bacteria, which are able to synthesize it from hydrogen and carbon dioxide or formic acid through the Wood–Ljungdahl pathway (Miller and Wolin, 1996; Louis et al., 2014).

Propionate and butyrate metabolism have received much attention during the last years, mainly due to the connection between low levels of butyrate and propionate bacterial producers and some diseases in which inflammatory processes are involved. For instance, butyrate producers are normally low in ulcerative colitis (Machiels et al., 2014) and reduced levels of propionate producers have been detected in children at risk of asthma (Arrieta et al., 2015).

Three different pathways are used by colonic bacteria for propionate formation: succinate pathway, acrylate pathway, and propanodiol pathway (Reichardt et al., 2014) (**Figure 1**). The succinate route utilizes succinate as a substrate for propionate formation and involves the descarboxylation of methylmalonyl-CoA to propionyl-CoA. This pathway is present in several *Firmicutes,* belonging to the *Negativicutes* class, and in *Bacteroidetes*. In the acrylate pathway lactate is converted to propionate through the activity of the lactoyl-CoA dehydratase and downstream enzymatic reactions; this route appears to be limited to a few members of the families *Veillonellaceae* and *Lachnospiraceae* (Flint et al., 2015). In the propanodiol pathway, characterized by the conversion of deoxy-sugars to propionate, the CoA-dependent propionaldehyde dehydrogenase, that converts propionaldehyde to propionyl-CoA, has been suggested as a marker for this route. This metabolic pathway is present in bacteria which are phylogenetically distant, including proteobacteria and members of the *Lachnospiraceae* family (Louis et al., 2014; Reichardt et al., 2014). The relative abundance of *Bacteroidetes* has also been linked to the total fecal propionate concentration, suggesting that the succinate pathway is the dominant route within the gut microbiota (Salonen et al., 2014).

Two different pathways for butyrate production are known in butyrate-producing bacteria (**Figure 1**). The butyrate kinase pathway employs phosphotransbutyrylase and butyrate kinase enzymes to convert butyryl-CoA into butyrate (Louis et al., 2004). This route is not common among members of the gut microbiota and is mainly limited to some *Coprococcus* species (Flint et al., 2015). In contrast, the butyryl-CoA: acetate CoA-transferase pathway, in which butyryl-CoA is converted to butyrate in a single step enzymatic reaction, is used by the majority of gut butyrate-producers (Louis et al., 2010), including some of the most abundant genera of the intestinal microbiota, such as *Faecalibacterium*, *Eubacterium,* and *Roseburia*. Remarkably, the production of butyrate and propionate by the same bacterium is not common and only a few anaerobes, such as *Roseburia inulinivorans* and *Coprococcus catus,* are able to produce both (Louis et al., 2014).

Cross-Feeding Mechanisms

Bacterial cross-feeding has a huge impact on the final balance of SCFA production and the efficient exploitation of the substrates that reach the human gut. These mechanisms consist either in the utilization of end products from the metabolism of a given microorganism by another one, called metabolic cross-feeding (**Figure 1**), and/or the utilization by one microorganism of the energy rich complex CHO breakdown products formed by another one, called substrate cross-feeding (Belenguer et al., 2006; Flint et al., 2007). A recent *in silico* study showed that mutualism cross-feeding interactions were promoted by anoxic conditions, which are more common in the large intestine than in the small one (Heinken and Thiele, 2015).

Microorganisms that are not capable of using complex CHO may proliferate by taking advantage of substrate cross-feeding, using breakdown compounds produced by hydrolytic bacteria. This is the case of some *Bifidobacterium* species that are not able to use inulin-type fructans (ITF) but can grow by cross-feeding of mono- and oligosaccharides released by primary inulin degraders in fecal cultures added with inulin as carbon source (Rossi et al., 2005; Salazar et al., 2009). Other example is the degradation of agaro-oligosaccharides (AO), which is more effective when *Bacteroides uniformis* and *Escherichia coli* are grown in co-culture than in separated monoculture (Li et al., 2014). In the same study the authors suggest the utilization of agarotriose, an intermediate in the degradation of AO, by *Bifidobacterium adolescentis* and *Bifidobacterium infantis.* In another work, it was demonstrated that *Roseburia* sp. strain A2-183 is unable to use lactate as carbon source, but when it is co-cultured with *B. adolescentis* L2-32 in the presence of FOS or starch, produces butyrate (Belenguer et al., 2006).

Although, there are a lot of *in vitro* studies pointing to metabolic cross-feeding it was not until recently that was demonstrated *in vivo* by using stable isotopes of acetate, propionate and butyrate perfused into the caecum of mice (Den Besten et al., 2013a). This study evidenced that the bacterial cross-feeding occurred mainly from acetate to butyrate, at lower extent between butyrate and propionate, and almost no metabolic flux exists between propionate and acetate. *In vitro* utilization of acetate by *Faecalibacterium prausnitzii* and *Roseburia sp.* has been evidenced (Duncan et al., 2002, 2004b). Prediction of metabolic fluxes between *F. prausnitzii* A2-165 and *B. adolescentis* L2-32 in co-culture has been reported in a

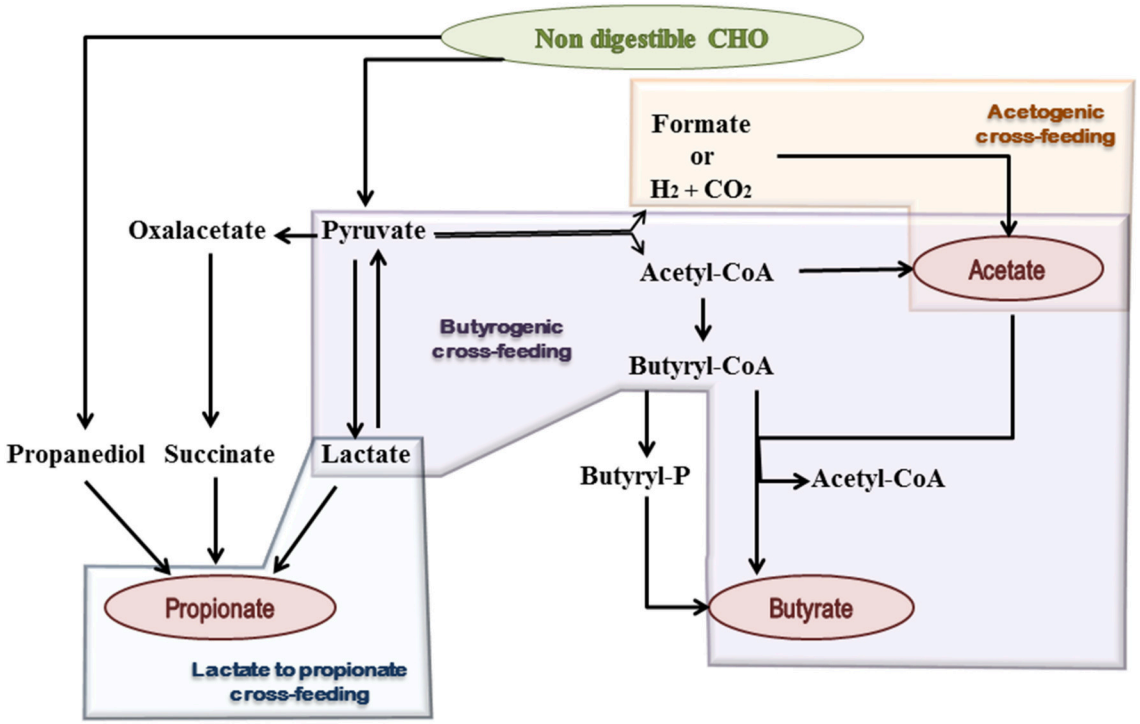

FIGURE 1 | Schematic representation of microbial metabolic pathways and cross-feeding mechanisms, contributing to SCFA formation in the human gut. Shaded geometric shapes summarize routes of formation for each of the three main SCFA: acetate, propionate, and butyrate.

computational model (El-Semman et al., 2014) and was recently demonstrated *in vitro* (Rios-Covián et al., 2015). Moreover, a recent animal study suggests that *F. prausnitzii* is able to use the acetate produced by *Bacteroides thetaiotaomicron in vivo*, this interaction having a significant impact in the modulation of the intestinal mucus barrier (Wrzosek et al., 2013). Although lactate is not a SCFA, it is used by some butyrate and propionate producing bacteria, avoiding metabolic acidosis in the host (El Aidy et al., 2013). Several *in vitro* studies confirm that lactate and/or acetate produced by *Bifidobacterium* when grown in oligofructose, is used by members of *Roseburia, Eubacterium,* and *Anaeroestipes* genera (Duncan et al., 2004a,b; Belenguer et al., 2006; Falony et al., 2006). Members of *Veillonella* and *Propionibacterium* are capable of transforming lactate to propionate *in vitro* (Counotte et al., 1981). H_2 plays an important role in cross-feeding as well. Co-cultures of *Roseburia intestinalis* with the methanogen *Methanobrevibacter smithii* and the acetogen *Blautia hydrogenotrophica,* resulted in a decrease of final H_2 and the production of CH_4 and acetate. The acetate formed is used by *R. intestinalis* to produce butyrate (Chassard and Bernalier-Donadille, 2006). *Ba. thetaiotaomicron* bi-associated mice with *Bl. hydrogenotrophica* showed higher levels of acetate in caecal contents and lower NADH/NAD^+ ratio; the removal of H_2 by *B. hydrogenotrophyca* in this case allows *Ba. thetaoitaomicron* to regenerate NAD+ (Rey et al., 2010).

IMPACT OF DIET ON GUT MICROBIOTA COMPOSITION AND SCFA PRODUCTION

Diet affects the gut microbiota composition and activity, and therefore the profile of SCFA and BSCFA synthesized, this having a deep impact on human health (Brussow and Parkinson, 2014; Louis et al., 2014). The first work linking the long-term diet style with the so-called human "enterotypes" was published in 2011 (Wu et al., 2011) but it has been also demonstrated that short-term diets can alter the human gut microbiome (David et al., 2014). The amount and relative abundance of SCFA may be considered as biomarkers of a healthy status (**Table 1A**). For example, high fiber-low fat and meat diets are characterized by the presence of higher amounts of fecal SCFA than diets with reduced fiber intake (De Filippo et al., 2010; Cuervo et al., 2013; Ou et al., 2013). A reduction in fecal butyrate has been found in patients with colorectal adenocarcinoma (Chen et al., 2013), whereas obesity has been related with increases in total fecal SCFA concentration (Fernandes et al., 2014; Rahat-Rozenbloom et al., 2014) which tend to decrease following an anti-obesity treatment (Patil et al., 2012). These epidemiological data have been further supported by dietary intervention studies carried out with different human populations (**Table 1B**). Prebiotic substrates that selectively promote the growth of beneficial microbiota also induce changes in SCFA production of healthy individuals (Lecerf et al., 2012) and in patients with irritable

bowel syndrome or those receiving enteral nutrition (Majid et al., 2011; Halmos et al., 2015). Interestingly, the consumption of dairy products fermented with beneficial bacteria also modifies the intestinal microbiota toward more butyrate producers in comparison to chemically-acidified milk (Veiga et al., 2014). Finally, dietary intervention studies carried out with different overweight and obese populations seemed to be effective in lowering the high levels of fecal SCFA associated with the obesity status (Salazar et al., 2015).

Although animal and human trials provide the best models for studying the influence of diet on the gut microbiota, *in vitro* fecal cultures constitute simpler approaches for investigating the interactions of diet and food components with the intestinal microbiota. Available *in vitro* models range from simple batch fermentation (Salazar et al., 2009; Arboleya et al., 2013b) to complex multi-stage continuous culture systems. The SHIME (Van Den Abbeele et al., 2010) and SIMGI models (Barroso et al., 2015) simulate the digestion from stomach to colon whereas the EnteroMix (Makivuokko et al., 2005) and the Lacroix models mimic the entire colonic process. TIM-2 reproduces the proximal colon and incorporates a dialysis membrane that simulates absorption of microbial metabolites by the body (Minekus et al., 1999). A microbial bias regarding butyrate and propionate producers occurs with some of these models (Van Den Abbeele et al., 2010), that could be alleviated by incorporating a simulation of the intestinal mucosa surface (Van Den Abbeele et al., 2013a). Labelling substrates with the stable isotope ^{13}C makes possible to link the fermentation with specific members of the microbiota and to quantify production of metabolites (Maathuis et al., 2012) whilst the mathematical modeling is becoming a useful tool to study microbe-diet-host interactions (Shoaie et al., 2015).

When studying *in vitro* the influence of dietary components on microbial composition, the main aim usually is to increase beneficial bacteria and to enhance the production of SCFA whereas minimizing the synthesis of BSCFA. The fermentation of different substrates has been evaluated, ITF being the most studied (Sivieri et al., 2014). Starch (Fassler et al., 2006), arabinans, arabinoxylans (Van Den Abbeele et al., 2013b), galactooligosaccharides (Rodriguez-Colinas et al., 2013), xylitol (Makelainen et al., 2007), and lactulose (Cardelle-Cobas et al., 2009) have been also considered. The influence of polyphenols on the gut microbiota metabolism is currently receiving considerable attention (Valdés et al., 2015). Different microbial fermentation patterns can be obtained depending on physico-chemical characteristics of the substrates, speed of fermentation and the microbial populations involved in the process (initial breakdown of long polymers, direct fermentation of substrates, and cross-feeding interactions; Hernot et al., 2009; Zhou et al., 2013; Puertollano et al., 2014). Probiotics and their extracellular components (exopolysaccharides), can also act as modulators of SCFA microbial formation (Salazar et al., 2009; Van Zanten et al., 2012). In addition, a large number of studies highlight the influence of different foods and long-term diets on the intestinal microbiota activity and specifically, over the pattern of SCFA (Yang and Rose, 2014; Costabile et al., 2015).

The basal microbiota composition has also a profound influence on the final effects exerted *in vitro* by diet on microbial populations and metabolic activity (Arboleya et al., 2013a; Souza et al., 2014). In this regard, it has been found a different response to probiotics and prebiotics by the microbiota of individuals from different groups of age (Arboleya et al., 2013a; Likotrafiti et al., 2014), or between obese and lean people (Yang et al., 2013).

BIOLOGICAL EFFECTS OF SCFA

One of the health effects attributed to the production of SCFA is the concomitant reduction of the luminal pH, which by itself inhibits pathogenic microorganisms and increases the absorption of some nutrients (Macfarlane and Macfarlane, 2012). Acetate has been found to be a key player in the ability of bifidobacteria to inhibit enteropathogens (Fukuda et al., 2011). Moreover, butyrate fuels the intestinal epithelial cells and increases mucin production which may result in changes on bacterial adhesion (Jung et al., 2015) and improved tight-junctions integrity (Peng et al., 2009). Thus, the production of SCFA seems to play an important role in the maintenance of the gut barrier function.

After their production, SCFA will be absorbed and used in different biosynthetic routes by the host (Den Besten et al., 2013b). During the intestinal absorption process part of the SCFA, mainly butyrate, will be metabolized by the colonocytes (Pryde et al., 2002) whilst the rest will be transported by the hepatic vein and go into the liver, where they will be metabolized (Den Besten et al., 2013b). These SCFA will enter diverse CHO and lipid metabolic routes; propionate will mainly incorporate into gluconeogenesis whilst acetate and butyrate will be mostly introduced into the lipid biosynthesis. The involvement of SCFA in energy and lipid metabolism attracted the attention of researchers toward the potential role of SCFA in the control of metabolic syndrome. A reduction in obesity and insulin resistance in experimental animals on high-fat diet after dietary supplementation with butyrate has been observed (Gao et al., 2009). This protective effect of SCFA on the high-fat diet-induced metabolic alterations seems to be dependent on down-regulation of the peroxisome proliferator-activated receptor gamma (PPARγ), therefore promoting a change from lipid synthesis to lipids oxidation (Den Besten et al., 2015). Interestingly although the three main intestinal SCFA have a protective effect on diet-induced obesity, butyrate and propionate seem to exert larger effects than acetate (Lin et al., 2012). Different mechanisms have been proposed to explain these effects, the activation of signaling pathways mediated by protein kinases, such as AMP-activated protein kinase (Gao et al., 2009; Peng et al., 2009; Den Besten et al., 2015) or mitogen-activated protein kinases (MAPK; Jung et al., 2015), being a common observation. Butyrate and propionate, but not acetate, have been reported to induce the production of gut hormones, thus reducing food intake (Lin et al., 2012). Acetate has also been found to reduce the appetite, in this case through the interaction with the central nervous system (Frost et al., 2014). However, in spite of these promising animal data, controlled human intervention studies are still needed before drawing firm conclusions (Canfora et al., 2015).

TABLE 1 | (A) Epidemiological studies, carried out since 2010, showing the impact of diet on SCFA produced by the gut microbiota. The shaded areas indicate a change in the populations analyzed in terms of their health status. D, days; y, year. (B) Intervention studies, carried out since 2010, showing the impact of diet on SCFA produced by the gut microbiota. The shaded areas indicate a change in the populations analyzed in terms of their health status. D, day; w, week; m, month; y, year.

(A)

Subjects, age (*n*)	Parameters determined	Main results	References
•European children, 1–6 y (15) •Burkina Faso (BF) (rural) children (15)	3-d dietary questionnaire (from EU parents) and interview on diet (from BF mothers), fecal samples	**BF children**: ↑SCFA;↑*Bacteroidetes*, ↓*Firmicutes*, ↓*Enterobacteriaceae*; unique *Prevotella*, *Xylanibacter* (lacking in EU)	De Filippo et al., 2010
•Healthy African Americans, 50–65 y (12) •Healthy South Africans (12)	Fresh fecal samples, microbiota and SCFA analysis, cancer biomarkers	**Native Africans**: ↑SCFA, total bacteria, major butyrate-producing groups, dominance of *Prevotella* **African-Americans**: dominance of *Bacteroides*	Ou et al., 2013
•Healthy elderly, 76–95 (32)	Food frequency questionnaire, fecal SCFA analysis	**Correlation fiber and SCFA**: Potato intake with total SCFA and apple with propionate	Cuervo et al., 2013
•Overweight (OWO) (11) •Lean (11)	3-d diet record, fresh fecal sample, SCFA absorption measure	**OWO**: ↑Age-adjusted fecal SCFA concentration, not due to higher absorption rate	Rahat-Rozenbloom et al., 2014
•Overweight (OWO) (42) •Lean (52)	3-d diet records, physical activity questionnaires, fecal samples	**OWO:** ↑ SCFA; dietary intakes and physical activity levels did not differ	Fernandes et al., 2014
•Indian individuals, 21–62 y (20): lean (5), normal (5), obese (5), surgically treated obese (5)	Fresh fecal samples, microbiota, and SCFA analysis	**Obese**: ↑ SCFA,↑*Bacteroides* **Treated-obese**: ↓SCFA ↓*Bacteroides*	Patil et al., 2012
•Advanced colorectal adenoma patients (A-CRA) (344) •Healthy control (344)	Dietary fiber intake, fecal SCFA, and microbiota analysis	**A-CRA group**: ↓SCFA production, ↓butyrate and butyrate-producing bacteria	Chen et al., 2013
•Celiac disease (CD) patients: normal diet, 13–60 y (10) and gluten-free, 21–66 y (11) •Healthy, 24–42 y (11)	Fresh fecal samples, microbiota, and SCFA analysis	**Untreated CD and treated CD**: ↑ SCFA than healthy **Treated CD patients**: ↓*Lactobacillus* and *Bifidobacterium* diversity	Nistal et al., 2012

(B)

Subjects, age (*n*)	Intervention diet (period)	Main outcomes	References
•Healthy African Americans, 50–65 y (20) •Healthy South Africans, 50–65 y (20)	Own diet (2 w) followed by exchange to high-fiber, low-fat African-style (2 w) Own diet (2 w) followed by high-fat, low-fiber Western-style (2 w)	**African style diet**: ↑ butyrate; reciprocal changes in colon cancer risk biomarkers	O'keefe et al., 2015
•Healthy volunteers (23)	Cross-over: high red meat (HRM) diet vs. HRM plus butyrylated high-amylose maize starch (HAMSB) (4/4 w wash-out)	**HRM+HAMSB diet**:↑ excretion of SCFA and microbiota composition changes	Le Leu et al., 2015
•Healthy active volunteers (51)	Parallel-groups: butyrylated high amylose maize starch (HAMSB) vs. low-AMS (28 d)	**HAMSB diet**:↑free, bound and total butyrate and propionate	West et al., 2013
•Healthy volunteers, 20–50 y (17)	Cross-over: whole-grain (WG) vs. refined grain (2/5 w wash out)	**WG diet**: ↑acetate and butyrate	Ross et al., 2013
•Healthy volunteers, 18–85 y (63)	Cross-over: wheat bran extract (WBE) (3 or 10 g WBE) vs. placebo (0 g WBE; 3 w, 2 w wash-out)	**Daily intake of 10 g WBE**:↑bifidobacteria;↑ fecal SCFA and ↓ fecal pH	Francois et al., 2012
•Healthy volunteers, 18–24 y (60)	Parallel-groups: xylo-oligosaccharide (XOS) vs. inulin-XOS mixture (INU-XOS) vs. placebo (maltodextrin; 4 w)	**XOS**: ↑bifidobacteria and butyrate, and ↓acetate **INU-XOS**: ↑SCFA and propionate, and maintain acetate level	Lecerf et al., 2012
•Ulcerative colitis (UC) remission patients (19) •Healthy volunteers (10)	Cross-over: Australian diet vs. plus wheat bran-associated fiber and high amylose-associated resistant starch (8 w)	**Intervention diet**: did not correct the low gut fermentation in patients with UC	James et al., 2015
•Irritable bowel syndrome (IBS) with constipation woman, 20–69 y (32)	Parallel-groups: Milk acidified product (MP) vs. Fermented Milk product (FMP) (4 w)	**FMP**:↑potential butyrate producers, and ↑Total SCFA *in vitro*↑butyrate	Veiga et al., 2014

(Continued)

TABLE 1 | Continued

(B)

Subjects, age (*n*)	Intervention diet (period)	Main outcomes	References
•IBS patients (27) •Healthy volunteers (6)	Cross-over: Australian diet vs. low FODMAP (Fermentable Oligo-, Di-, Mono-saccharides And Polyols) diet (21/21 d wash-out)	**Australian diet**:↑ relative abundance *Clostridium* cluster XIVa (butyrate-producer) **Low FODMAP diet**:↓total bacterial abundance	Halmos et al., 2015
•Cow's milk protein allergy infants (16) Healthy infants (12)	Cross-over:hydrolysed whey protein formula (eHF) without lactose vs. eHF containing 3.8% lactose (2 m)	**Addition of lactose**: ↑SCFA**;** ↑LAB and bifidobacteria; ↓*Bacteroides*/clostridia	Francavilla et al., 2012
•Obese women 18–65 y (30)	Parallel-groups: ITF vs. placebo (maltodextrin) (3m)	**ITF**:↓ total SCFA, acetate and propionate; ↑bifidobacteria	Salazar et al., 2015
•Obese men, 27–73 y (14)	Cross-over: high type III resistant starch (3 w) or high in wheat bran (3 w) and ended with weight-loss (low fat and carbohydrate, high protein, 3 w)	**Diet**: only explain 10% total variance in microbiota; amount of propionate correlated with *Bacteroidetes*	Salonen et al., 2014
•Obese volunteers, 45–77 y (6)	Cross-sectional: strict vegetarian diet (1 m)	↓SCFA;↓*Firmicutes/Bacteroidetes* ratio; ↑*Clostridium* clusters XIVa-IV; ↓*Enterobacteriaceae*	Kim et al., 2013
•Obese men, 21–74 y (17)	Cross-over: high-protein moderate-carbohydrate (HPMC) vs. high-protein low-carbohydrate (HPLC) (maintenance diet 7 d, 4 w)	**HPMC and HPLC diets**: ↑BSCFA (respect maintenance diet) **HPLC diet**: ↓butyrate and ↓*Roseburia/E.rectale*	Russell et al., 2011
•High Metabolic Syndrome risk volunteers (88)	Parallel-groups: High saturated fat (HS) vs. high monounsaturated fat (MUFA)/high glycaemic index (GI) (HM/HGI) vs. high MUFA/low GI (HM/LGI) vs. high carbohydrate (CHO)/high GI (HC/HGI) vs. and high CHO/low GI (HC/LGI) (24 w)	**High carbohydrate diets** (regardless GI):↑saccharolytic bacteria (including *Bacteroides* and *Bifidobacterium*) **High fat diets**:↓bacterial numbers **High saturated fat diet**:↑excretion of SCFA	Fava et al., 2013
•Hospitalized patients under enteral nutrition (41)	Parallel-groups: standard enteral formula vs. standard formula enriched FOS and fiber (12 d)	**FOS/fiber-enriched formula**: ↑butyrate	Majid et al., 2011

It has also been observed that SCFA protect against the development of colorectal cancer (CRC), with most studies focusing on butyrate (Canani et al., 2011; Keku et al., 2015). Butyrate promotes colon motility, reduces inflammation, increases visceral irrigation, induces apoptosis, and inhibits tumor cell progression (Zhang et al., 2010; Canani et al., 2011; Leonel and Alvarez-Leite, 2012; Keku et al., 2015), all of these properties being beneficial in CRC prevention. In cancerous colonocytes, due to the Warburg effect, butyrate accumulates, which increases its activity as inhibitor of histone deacetylation, promoting apoptosis of CRC cells. Interestingly, a recent animal study suggests that the protective effect of dietary fiber upon CRC is dependent on the production of butyrate by the microbiota (Donohoe et al., 2014).

In addition, butyrate and propionate have also been reported to induce the differentiation of T-regulatory cells, assisting to control intestinal inflammation; this effect seems to be mediated via inhibition of histone deacetylation (Donohoe et al., 2014; Louis et al., 2014). This control of intestinal inflammation may result beneficial in terms of gut barrier maintenance, reducing the risk of inflammatory bowel disease or CRC. Unlike what happens with the three main intestinal SCFA, acetate, propionate, and butyrate, little is known about the potential health effects of other SCFA.

CONCLUDING REMARKS

The main role of diet is to provide enough macro- and micronutrients to fulfill daily requirements and well-being. However, during the last decades the association between dietary intake and physiology has been increasingly-recognized, although many of the molecular and immunological aspects by which dietary components could influence human health remain still largely unknown. Bacterial fermentation of CHO and proteins produces SCFA which emerge as major mediators in linking nutrition, gut microbiota, physiology and pathology. Many biological effects seem to be mediated by these bacterial metabolites but a conclusive proof is not available for many of the health claims made for SCFA. Promising *in vitro* and animal studies have been published but they cannot be easily extrapolated to the human situation. The design of improved approaches combining *in vitro*, *in vivo,* and "omics" technologies should be carried out, with emphasis in human intervention trials, to explore the mechanisms of production and action of SCFA, thus opening the possibility to find strategies for developing personalized nutrition.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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ANTECEDENTES Y OBJETIVOS

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En los últimos años se ha ido acumulando una fuerte evidencia científica a favor de la relación existente entre la microbiota intestinal humana y la salud del hospedador. Dado el papel que juegan las bacterias intestinales en nuestro estado de salud, todos los factores que influyen en su desarrollo, estabilidad y actividad metabólica, afectan de forma más o menos directa al hospedador. Uno de los mecanismos más importantes en la relación microbiota-hospedador, es la producción de AGCC (principalmente los ácidos acético, propiónico y butírico) por parte de la microbiota a partir de fuentes de carbono no metabolizadas en el proceso de digestión. El balance final de las concentraciones de estos ácidos que van a estar disponibles para el hospedador depende tanto de la composición a nivel de cepa, que determina los niveles de producción y utilización de estos componentes por la microbiota intestinal, como del tipo de fuentes de carbono fermentables por la microbiota disponibles en el colon y, como consecuencia, de las interacciones que se dan entre todos estos factores.

Las bifidobacterias son un género bacteriano presente en la microbiota humana. La disminución de este grupo respecto a otros, se ha asociado a estados de inflamación y de enfermedad, por lo que se asume que juega un papel importante en el mantenimiento de un estado de salud óptimo. Como resultado, muchas cepas del género han sido utilizadas como probióticos para modular la microbiota a través de la alimentación. Este género bacteriano se caracteriza por ser un productor de ácido acético en altas cantidades, metabolito que puede ser utilizado por géneros productores de ácido butírico. Algunas cepas producen EPS, polímeros que se han caracterizado en algunas de ellas y que tienen la capacidad de modular la microbiota humana. Por otro lado muchas cepas de bifidobacterias son capaces de metabolizar carbohidratos complejos presentes en la dieta, lo que les confiere la oportunidad de competir con otros géneros de la microbiota en este ambiente. Finalmente, se han descrito mecanismos de autoinducción en algunas especies del género, poniendo de manifiesto su capacidad de comunicación entre ellas. Por todo esto, el género *Bifidobacterium* es un buen candidato para profundizar en las interacciones bacteria-bacteria que se dan dentro de la comunidad microbiana colónica.

Estudios previos de nuestro grupo investigador habían puesto de manifiesto, mediante la realización de cultivos fecales, la capacidad de EPS producidos por bifidobacterias para modular la microbiota intestinal, en concreto el EPS E44, aislado de una cepa de *Bifidobacterium longum* de origen fecal, y el EPS R1, aislado de otra cepa de *Bifidobacterium animalis* subsp. *lactis*, procedente de un producto lácteo comercial. En estos cultivos, los géneros bacterianos que se vieron más favorecidos por la presencia de los EPS fueron *Bifidobacterium* y *Bacteroides.* Las interacciones de estos dos grupos microbianos se habían estudiado anteriormente, entre otros, por Falony y colaboradores (2009) en cultivos con fructooligosacáridos tipo inulina como fuente de carbono.

Por otra parte, existen estudios sobre los mecanismos de cross-feeding, o sintrofía, de bifidobacterias con géneros bacterianos productores de ácido butírico, como *Eubacterium, Anaeroestipes y Roseburia*. En concreto, estos géneros metabolizan ácido láctico y/o ácido acético producidos por las bifidobacterias. Sin embargo, hasta el inicio de la presente Tesis Doctoral, el cross-feeding entre las bifidobacterias y *F. prausnitzii*, especie intestinal de importancia clave en estados de salud y enfermedad, no había sido estudiado. De modo similar, se conoce muy poco de los mecanismos de interacción metabólica que se relacionan con producción de ácido propiónico a nivel de microbiota intestinal

En este contexto, el objetivo global de esta Tesis fue profundizar en el conocimiento de las relaciones del género *Bifidobacterium* con otras poblaciones microbianas intestinales humanas mayoritarias como *Bacteroides* y *Faecalibacterium*.

Para ello, se establecieron tres objetivos, correspondientes con los tres capítulos que forman parte de esta tesis:

Capítulo 1: Estudio de la interacción entre los géneros *Bifidobacterium* y *Bacteroides* en presencia de diferentes fuentes de carbono, incluidos EPS aislados de bifidobacterias.

Se incluyen dos publicaciones en las que se aborda el estudio de las relaciones entre los géneros *Bifidobacterium* y *Bacteroides* a dos niveles. En un primer artículo se llevaron a cabo cocultivos de cepas pertenecientes a ambos géneros con diferentes fuentes de carbono, para comprobar la influencia en el crecimiento y metabolismo entre ambos grupos. En el segundo artículo se profundizó a nivel molecular, mediante estudios de proteómica y expresión génica en las interacciones de dos especies concretas, *B. longum* y *Ba. fragilis*.

Manuscrito 1

Rios-Covian, D., Arboleya, S., Hernandez-Barranco, A.M., Alvarez-Buylla, J.R., Ruas-Madiedo, P., Gueimonde, M., and de los Reyes-Gavilan, C.G. (2013). Interactions between *Bifidobacterium* and *Bacteroides* species in cofermentations are affected by carbon sources, including exopolysaccharides produced by bifidobacteria. Appl Environ Microbiol 79, 7518-7524. doi: 10.1128/AEM.02545-13.

Manuscrito 2

Rios-Covián, D., Sánchez, B., Martínez, N., Cuesta, I., Hernández-Barranco, A.M., de los Reyes-Gavilán, C.G. and Gueimonde, G. A proteomic approach towards understanding the cross-talk between *Bacteroides fragilis* and *Bifidobacterium longum* in co-culture. Can Jour Microbiol. 10.1139/cjm-2015-0804 In Press

Capítulo 2: Estudio del papel de *Bacteroides fragilis* en la degradación de EPS aislados de bifidobacterias

Este capítulo incluye dos publicaciones en las que se aborda el estudio del comportamiento de *Ba. fragilis* en presencia de EPS aislados de bifidobacterias. Primeramente se realizó un estudio fisiológico, proteómico y de expresión génica del metabolismo de *Ba. fragilis* en presencia de EPS. En una segunda publicación, se demuestra la utilización efectiva del EPS por *Ba. fragilis* mediante técnicas cromatográficas.

Manuscrito 3

Rios-Covian, D., Sanchez, B., Salazar, N., Martinez, N., Redruello, B., Gueimonde, M., and de Los Reyes-Gavilan, C.G. (2015). Different metabolic features of *Bacteroides fragilis* growing in the presence of glucose and exopolysaccharides of bifidobacteria. Front Microbiol 6, 825. doi: 10.3389/fmicb.2015.00825.

Manuscrito 4

Rios-Covián, D., Cuesta, I., Alvarez-Buylla, J.R., Gueimonde, G., Ruas-Madiedo, P., and de los Reyes-Gavilán, C.G. *Bacteroides fragilis* metabolises exopolysaccharides produced by bifidobacteria. BMC Microbiol. Enviado

Capítulo 3: Interacción metabólica entre *Bifidobacterium adolescentis* y *Faecalibacterium prausnitzii.*

Este capítulo incluye una publicación en la que se desmuestra experimentalmente el cross-feeding entre B*ifidobacterium adolescentis* y *Faecalibacterium prausnitzii:*

Manuscrito 5

Rios-Covian, D., Gueimonde, M., Duncan, S.H., Flint, H.J., and de Los Reyes-Gavilan, C.G. (2015). Enhanced butyrate formation by cross-feeding between *Faecalibacterium prausnitzii* and *Bifidobacterium adolescentis*. FEMS Microbiol Lett 362 (21) doi: 10.1093/femsle/fnv176.

TRABAJO EXPERIMENTAL:

CAPÍTULO 1

ESTUDIO DE LA INTERACCIÓN ENTRE LOS GÉNEROS *Bifidobacterium* Y *Bacteroides* EN PRESENCIA DE DIFERENTES FUENTES DE CARBONO, INCLUIDOS EPS AISLADOS DE BIFIDOBACTERIAS

CAPÍTULO 1

Estudio de la interacción entre los géneros *Bifidobacterium* y *Bacteroides* en presencia de diferentes fuentes de carbono, incluidos EPS aislados de bifidobacterias.

Los géneros *Bacteroides* y *Bifidobacterium* están presentes en la microbiota intestinal de adultos sanos. En estudios previos de nuestro grupo investigador, utilizando EPS aislados de bifidobacteria como carbohidrato fermentable en cultivos fecales, se observó un efecto bifidogénico y un aumento en los recuentos del género *Bacteroides* respecto a los cultivos con glucosa. Además, se produjo un retraso en la producción de ácido acético y se alcanzaron concentraciones de ácido propiónico más elevadas que en cultivos con glucosa, lo que condujo a una disminución de la relación ácido acético/ácido propiónico.

A fin de profundizar en el conocimiento de la relación entre estos dos microrganismos, *Bifidobacterium* y *Bacteroides*, se llevaron a cabo cultivos conjuntos utilizando diferentes fuentes de carbono simples y complejas (glucosa, EPS aislado de bifidobacterias e inulina). Se determinaron los cambios a nivel fisiológico y molecular (proteómica y expresión génica) involucrados en la interacción entre estos géneros microbianos.

El objetivo de este capítulo se aborda en las siguientes publicaciones:

Manuscrito 1

Rios-Covian, D., Arboleya, S., Hernandez-Barranco, A.M., Alvarez-Buylla, J.R., Ruas-Madiedo, P., Gueimonde, M., and de los Reyes-Gavilan, C.G. (2013). Interactions between *Bifidobacterium* and *Bacteroides* species in cofermentations are affected by carbon sources, including exopolysaccharides produced by bifidobacteria. Appl Environ Microbiol 79, 7518-7524. doi: 10.1128/AEM.02545-13.

Manuscrito 2

Rios-Covián, D., Sánchez, B., Martínez, N., Cuesta, I., Hernández-Barranco, A.M., de los Reyes-Gavilán, C.G. and Gueimonde, G. A proteomic approach towards understanding the cross-talk between *Bacteroides fragilis* and *Bifidobacterium longum* in co-culture. Can J Microbiol. 10.1139/cjm-2015-0804. In press

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Interactions between *Bifidobacterium* and *Bacteroides* Species in Cofermentations Are Affected by Carbon Sources, Including Exopolysaccharides Produced by Bifidobacteria

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Cocultures of strains from two *Bifidobacterium* and two *Bacteroides* species were performed with exopolysaccharides (EPS) previously purified from bifidobacteria, with inulin, or with glucose as the carbon source. *Bifidobacterium longum* NB667 and *Bifidobacterium breve* IPLA20004 grew in glucose but showed poor or no growth in complex carbohydrates (inulin, EPS E44, and EPS R1), whereas *Bacteroides* grew well in the four carbon sources tested. In the presence of glucose, the growth of *Bacteroides thetaiotaomicron* DSM-2079 was inhibited by *B. breve*, whereas it remained unaffected in the presence of *B. longum*. *Ba. fragilis* DSM-2151 contributed to a greater survival of *B. longum*, promoting changes in the synthesis of short-chain fatty acids (SCFA) and organic acids in coculture with respect to monocultures. In complex carbohydrates, cocultures of bifidobacterium strains with *Ba. thetaiotaomicron* did not modify the behavior of *Bacteroides* nor improve the poor growth of bifidobacteria. The metabolic activity of *Ba. fragilis* in coculture with bifidobacteria was not affected by EPS, but greater survival of bifidobacteria at late stages of incubation occurred in cocultures than in monocultures, leading to a higher production of acetic acid than in monocultures. Therefore, cocultures of *Bifidobacterium* and *Bacteroides* can behave differently against fermentable carbohydrates as a function of the specific characteristics of the strains from each species. These results stress the importance of considering specific species and strain interactions and not simply higher taxonomic divisions in the relationship among intestinal microbial populations and their different responses to probiotics and prebiotics.

The colon is a complex microbial ecosystem dominated by obligate anaerobes that reach levels up to 10^{11} cells per gram of intestinal content (1, 2). In spite of the huge diversity of strains, up to 87% of the microbial inhabitants of the human colon belong to only two bacterial phyla, *Bacteroidetes* and *Firmicutes*. *Actinobacteria* and other phyla are present at lower levels (3). Within the group of intestinal *Bacteroidetes*, *Bacteroides* spp. account for up to 20% of the human colon microbiota (4). Although a great variety of *Bacteroides* species has been reported among individuals, *Bacteroides thetaiotaomicron* always seems to be present (5, 6). This species is considered a human symbiont that stabilizes the colon ecosystem, but the genus also harbors some notorious opportunistic and pathogenic species, as is the case of *Bacteroides fragilis* (7). Members of the *Bacteroides* genus are saccharolytic microorganisms producing succinic, acetic, lactic, and propionic acids, but they are also capable of proteolytic fermentation (8). Bifidobacteria account for approximately 3% of the adult human microbiota (9) and are frequently identified as probiotics, based on the implied health-promoting benefits attributed to some strains (10). *Bifidobacterium longum* is one of the predominant species in adult humans. This species and *Bifidobacterium breve* are also abundant in the intestine of infants. Bifidobacteria produce lactic and acetic acids as the main metabolic end products of carbohydrate fermentation and smaller amounts of formic acid and ethanol (11). Prebiotics are defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species in the colon, thus improving host health (12). Many of the health-promoting effects attributed to prebiotic substrates are due to their suitability to be fermented by the colonic microbiota producing short-chain fatty acids (SCFA). Bifidobacteria have traditionally been considered the target of prebiotic action, as these substrates can be directly metabolized by these microorganisms; however, some *in vitro* and *in vivo* evidences indicate that the effects could also indirectly involve other members of the human colon microbiota through the utilization of these substrates in combination with bifidobacteria. The most well-studied prebiotics to date are inulin-type fructans (13–15). Some researchers have previously demonstrated different degradation mechanisms of oligofructose and inulin-like fructans by *Bifidobacterium* and *Bacteroides* species in pure cultures, as well as in cocultures (14–16).

Some bifidobacteria are able to produce exopolysaccharides (EPS), which are complex polymers composed of several units of monosaccharides (17). EPS from bifidobacteria may be released *in situ* by microorganisms of this species inhabiting the human colon or may be produced by probiotics present as adjunct cultures in fermented dairy products. Although the synthesis of EPS *in vivo* has not been demonstrated and the amount of polymer released by the producing bacteria would be presumably low, our previous work indicates that bile stimulates the production of EPS by bifidobacteria in *in vitro* simulated gastrointestinal conditions (17,

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18). In addition, EPS could act as fermentable substrates for the human colonic microbiota (19, 20). The fermentation in fecal batch cultures of small amounts of EPS and inulin (0.3%, wt/vol) caused shifts in the synthesis of SCFA related to variations in the levels of some intestinal microbial populations, such as *Bacteroides* and *Bifidobacterium* (20). Therefore, in the present work, we selected strains from two species of *Bifidobacterium* (*B. breve* and *B. longum*) and two species of *Bacteroides* (*Ba. thetaiotaomicron* and *Ba. fragilis*) as a model of study, in order to gain an insight into the influence that the presence of EPS and other carbon sources could exert on the interactions between members of these two intestinal microbial groups, by growing them separately and together.

MATERIALS AND METHODS

Bacterial strains. Two *Bacteroides* and two *Bifidobacterium* strains belonging to different species were used in monocultures and cocultures in this study. *Ba. thetaiotaomicron* DSM-2079 and *Ba. fragilis* DSM-2151 strains were obtained from the DSMZ bacterial culture collection (Braunschweig, Germany). *B. longum* NB667 was from the NIZO food research culture collection (Ede, The Netherlands), and *B. breve* IPLA20004 (also named *B. breve* BM 12/11) was isolated from breast milk (21) and is held in the IPLA's culture collection. Species identity was confirmed by partial amplification of the 16S rRNA gene using primers plb16 and mlb16 (22) and by sequencing and alignment with sequences from reference strains held in the GenBank database. Strains from frozen stocks were reactivated in Gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical Co, Tokyo, Japan) and in MRS broth (BioKar Diagnostics, Beauvais, France) supplemented with 0.25% (wt/vol) of L-cysteine (Sigma Chemical Co., St. Louis, MO) (named GAMc and MRSc) for *Bacteroides* and *Bifidobacterium*, respectively. Strains were incubated overnight at 37°C in an anaerobic cabinet (Mac 1000; Don Whitley Scientific, West Yorkshire, United Kingdom) under a 10% H_2, 10% CO_2, and 80% N_2 atmosphere. To prepare the inoculum stocks, 10 ml of modified carbohydrate-free basal medium (mCFBM; composition specified below) with 1% (wt/vol) glucose was inoculated (1% [vol/vol]) with cultures of *Bifidobacterium* and *Bacteroides* strains and incubated for 16 to 18 h, as indicated before. Cultures were then centrifuged at 12,000 × *g* for 10 min and resuspended in the same volume of mCFBM without a carbon source. Inocula were frozen under liquid N_2 and stored at −80°C until use.

EPS isolation. EPS fractions produced by *Bifidobacterium animalis* subsp. *lactis* IPLA R1, a dairy origin strain (23), and by *B. longum* subsp. *longum* IPLA E44, a fecal isolate from a healthy adult (24), were isolated and purified from the cellular biomass harvested from agar-MRSc agar plates as specified by Ruas-Madiedo et al. (23).

Batch culture fermentation. Uncontrolled-pH batch cultures were performed in the nondefined peptone and yeast extract containing CFBM, previously described by Salazar et al. (20). For the present work, it was modified by the addition of vitamin B_{12} (10 mg liter^{-1}), vitamin K (2 mg liter^{-1}), vitamin B_1 (2 mg liter^{-1}), pyridoxal (1 mg liter^{-1}), calcium pantothenate (2 mg liter^{-1}), folic acid (1 mg liter^{-1}), riboflavin (1 mg liter^{-1}), biotin (1 mg liter^{-1}), nicotinic acid (3 mg liter^{-1}), para-aminobenzoic acid (1 mg liter^{-1}), and a solution (2 ml liter^{-1}) of ferrous citrate (25 mM) and trisodium citrate (75 mM) (mCFBM). The final pH of the medium ranged between 6.7 and 7.0.

Pairwise combinations of *Bifidobacterium* and *Bacteroides* strains, as well as monocultures of strains, were performed in mCFBM with an added 0.3% (wt/vol) of glucose, inulin, or purified EPS E44 or EPS R1 fractions. The corresponding frozen inocula were added (1% [vol/vol]) to 3.5 ml of the culture medium. Trials of cocultures and the corresponding monocultures in different carbon sources were run in triplicate for a period of 72 h at 37°C under anaerobic conditions. Samples were obtained at fixed times for microbial counts and SCFA and organic acid analyses.

The ability to utilize lactic acid by the two *Bacteroides* strains considered in this work was tested in mCFBM, with 0.15% lactic acid (vol/vol) added as the carbon source. Additionally, the ability of *Bacteroides* strains to use the organic nitrogen compounds present in the culture medium was assessed in mCFBM by determining growth and the ability to produce branched-chain fatty acids (BCFA). Cultures were incubated for 72 h in anaerobic conditions as indicated above. At the end of incubation, optical density at 600 nm was determined in cultures, and samples were taken for SCFA and organic acid analyses.

Estimation of bacterial growth by qPCR. Quantification (cell counts ml^{-1}) of *Bifidobacterium* and *Bacteroides* species growing in monoculture and coculture was performed throughout fermentations by quantitative PCR (qPCR) using DNA isolated from batch cultures. Standard curves were obtained by converting 16S rRNA gene copies to cell counts obtained in pure cultures of each strain growing in MRSc in the case of *Bifidobacterium* and GAMc for *Bacteroides*. Primers and conditions were those previously described (25).

Analysis of SCFA, organic acids, and glucose. Cell-free supernatants from cultures were filtered through 0.2-μm-pore-size filters. Identification and quantification of SCFA and BCFA were carried out by gas chromatography-mass spectrometry/flame ionization detector (MS/FID), using a system composed of a 6890N gas chromatograph (Agilent Technologies Inc., Palo Alto, CA, USA) connected with an FID and an MS 5973N detector (Agilent), as described previously (19, 26). A high-performance liquid chromatography (HPLC) system composed of an Alliance 2695 separation module, a photodiode array (PDA) detector (Waters 996), a refractive index detector (Waters 2414), and Empower software (Waters, Milford, MA) was employed. The PDA detector was used for quantification of organic acids at 210 nm, whereas the amount of glucose was analyzed with the refractive index detector. Chromatographic conditions were those indicated previously by Salazar et al. (27). Results of SCFA, BCFA, and organic acids were expressed in millimolar concentrations.

Calculation of carbon recovery. Carbon recovery (CR), expressed in percentages, was calculated by comparing the total amount of carbon recovered in the metabolites analyzed to the total amount of glucose consumed. For *Bacteroides* strains, the production of one mole of CO_2 for every mole of acetic acid formed (+ 1 × [acetic acid] in the equation below) was considered, as well as the uptake of one mole of CO_2 for every mole of succinic acid produced (− 1 × [succinic acid] in the equation) (16, 28–30).

The following equations were used: CR of bifidobacteria ≥ 100 × (3 × [lactic acid] + 2 × [acetic acid] + 1 × [formic acid]/6 × [glucose consumed]) and CR of bacteroides ≥ 100 × (2 × [acetic acid] + 3 × [propionic acid] + 4 × [succinic acid] + 1 × [formic acid] +1 × [acetic acid] − 1 × [succinic acid]/6 × [glucose consumed]).

Statistical analysis. Statistical analyses were performed using the SPSS-PC software, version 19.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) tests were run in monocultures of *Bacteroides* and *Bifidobacterium* for the different SCFA and organic acids. Strains were used as factors, with two categories corresponding to the different species of each genus analyzed. One-way ANOVA was also performed to compare the results of the different parameters by using cocultures versus monocultures and time of incubation as factors. When necessary, a *post hoc* least significant difference (LSD) comparison test was applied to determine statistical differences between categories.

RESULTS AND DISCUSSION

Behavior of *Bifidobacterium* species growing in pure culture. In pure culture, *B. longum* NB667 was able to grow in glucose, inulin, and EPS ($P < 0.05$), whereas *B. breve* IPLA20004 displayed significant growth only in cultures with glucose but not with EPS or inulin (Fig. 1). In monocultures of bifidobacteria, the pH decreased by about 2.5 units throughout fermentation when glucose was the carbon source, whereas it showed little variation in complex carbohydrates (data not shown).

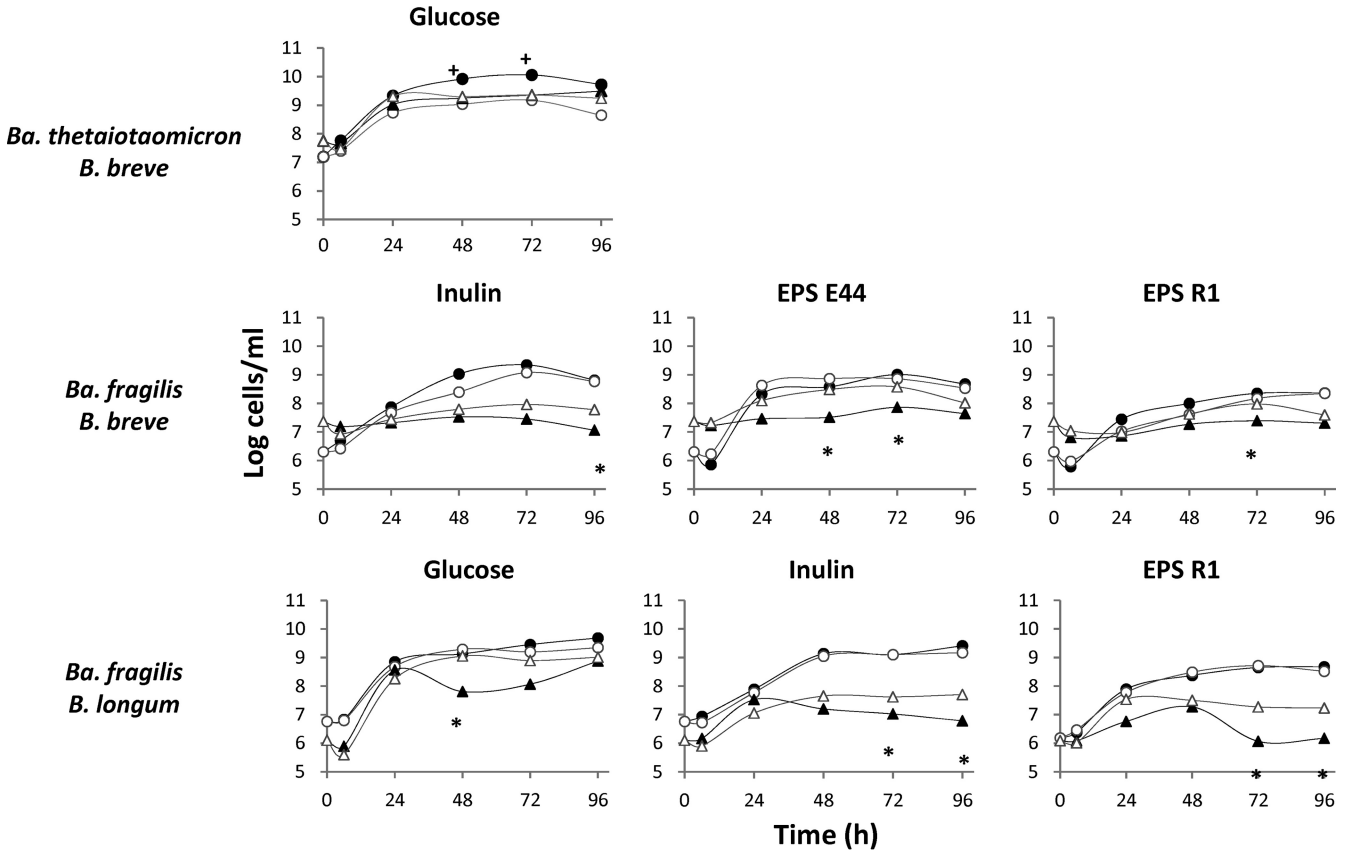

FIG 1 Growth (mean of log cells ml^{-1}) in single culture and in coculture of *Ba. thetaiotaomicron* DSMZ 2079 or *Ba. fragilis* DSMZ 2151 with *B. longum* NB 667 or *B. breve* IPLA 20004 in a basal medium supplemented with 0.3% glucose, inulin, EPS E44, or EPS R1 as a carbon source. ●, *Bacteroides* strain growing in single culture; ○, *Bacteroides* strain growing in coculture; ▲, *Bifidobacterium* strain growing in single culture; △, *Bifidobacterium* strain growing in coculture. The coefficient of variation (standard deviation × 100/mean) of data obtained from the three replicates was about 4.2 to 5.5%. +, significant differences ($P < 0.05$) of *Bacteroides* counts reached in coculture compared to the corresponding monoculture; *, significant differences ($P < 0.05$) of *Bifidobacterium* counts reached in coculture compared to the corresponding monoculture. Among all the possible culture combinations of *Bacteroides* and *Bifidobacterium* strains, the sole combination showing significant variation in the growth of *Bacteroides* and those enhancing significantly the survival of *Bifidobacterium* ($P < 0.05$) are presented.

Glucose was consumed almost completely after 72 h of fermentation in mCFBM cultures of both strains, with a carbon recovery above 90%. Although acetic acid was the most abundant metabolite formed, clear differences between the metabolic profiles of both *Bifidobacterium* strains were found (Table 1). Thus, whereas *B. longum* NB667 formed considerable amounts of lactic acid and smaller amounts of formic acid, *B. breve* IPLA20004 produced more formic than lactic acid.

Glucomannans from yeast extract present in the culture medium interfere with the detection and quantification of EPS and inulin (19), and, therefore, the calculation of the polymer consumed for cultures with added EPS was not possible in our experimental conditions. The fermentation pattern in cultures with complex carbohydrates by *Bifidobacterium* differed from those obtained with glucose and led mainly to the formation of small amounts of acetic acid in cultures of both strains (Table 1). Several authors have previously demonstrated a metabolic shift in the glycolytic pathway toward more acetic and formic acids and ethanol production at the expense of lactic acid in bifidobacteria when growth slows (31–33). In this way, less readily fermentable energy sources lead to more ATP formed per mole of sugar consumed (14, 15, 34). The predominant acetic acid production together with the limited or no increase of *B. breve* and *B. longum* population levels in cultures with EPS and inulin support the conditions of limited access to energy from these carbohydrates.

Behavior of *Bacteroides* species growing in pure culture. *Ba. thetaiotaomicron* DSM-2079 and *Ba. fragilis* DSM-2151 grew well in the carbon sources tested (Fig. 1). The pH during incubation decreased more in glucose (1.5 to 1.9 units) than in cultures with EPS (0.3 to 0.7 units). In inulin as the carbohydrate source, *Ba. fragilis* was able to promote a more pronounced pH decrease than *Ba. thetaiotaomicron* (1 pH unit compared to 0.1), which is in line with the higher SCFA production by *Ba. fragilis* than by *Ba. thetaiotaomicron* (Table 1). About 60 to 70% of the glucose was consumed after 72 h of fermentation in pure cultures of both strains (Table 1). In spite of this, CR at this time was nearly 100% in cultures of *Ba. thetaiotaomicron* and higher than this value in cultures of *Ba. fragilis*. A reason for this may be the fermentation of carbohydrates different from glucose and/or organic nitrogen compounds present in the culture medium. In this respect, it is known that members of the genus *Bacteroides* can ferment proteins and amino acids producing BCFA (35–37). We corroborated that *Ba. thetaiotaomicron* DSM-2079 and *Ba. fragilis* DSM-2151 grew slowly in mCFBM, producing BCFA (mainly isobutyric and

TABLE 1 SCFA and organic acid concentrations and glucose consumption obtained in uncontrolled-pH monocultures of *Bifidobacterium* and *Bacteroides* species at 72 h of incubation with glucose, inulin, EPS E44, and EPS R1 as carbon sources[a]

Carbon source	Species	Glucose consumption (mM)	Concn (mM)					Carbon recovery (%)
			Acetic acid	Propionic acid	Lactic acid	Succinic acid	Formic acid	
Glucose	Control (0 h)	0	3.77 ± 1.29	0.48 ± 0.01	0.08 ± 0.13	0.09 ± 0.01	0.05 ± 0.07	NA
	Ba. thetaiotaomicron	8.91 ± 1.41	10.94 ± 1.81	3.74 ± 0.50*	–	6.18 ± 0.73*	1.83 ± 0.31	97 ± 12
	Ba. fragilis	7.08 ± 1.24	12.58 ± 0.77	10.28 ± 1.00	–	3.24 ± 0.30	1.33 ± 0.20	132 ± 27
	B. breve	11.29 ± 1.22	29.24 ± 4.65	–	1.96 ± 0.55*	–	9.50 ± 1.26*	97 ± 14
	B. longum	12.88 ± 2.38	27.21 ± 4.31	–	10.15 ± 0.85	–	1.75 ± 0.13	102 ± 10
Inulin	Control (0 h)	NA	2.02 ± 0.49	0.46 ± 0.01	–	0.09 ± 0.02	–	NA
	Ba. thetaiotaomicron	NA	4.86 ± 0.80*	2.25 ± 0.40*	–	1.26 ± 0.12	–	NA
	Ba. fragilis	NA	9.55 ± 1.44	9.66 ± 1.47	–	1.22 ± 0.21	–	NA
	B. breve	NA	2.95 ± 0.51	–	–	–	–	NA
	B. longum	NA	3.10 ± 0.76	–	–	–	–	NA
EPS E44	Control (0 h)	NA	1.29 ± 0.14	0.45 ± 0.00		0.10 ± 0.02	–	NA
	Ba. thetaiotaomicron	NA	7.40 ± 1.59	6.29 ± 1.92		1.79 ± 0.32	–	NA
	Ba. fragilis	NA	6.12 ± 1.09	6.50 ± 0.32		1.72 ± 0.30	–	NA
	B. breve	NA	3.74 ± 1.06*	–	–	–	–	NA
	B. longum	NA	1.97 ± 0.18	–	–	–	–	NA
EPS R1	Control (0 h)	NA	1.33 ± 0.22	0.45 ± 0.00		0.10 ± 0.03	–	NA
	Ba. thetaiotaomicron	NA	7.02 ± 1.74*	5.58 ± 1.96		1.45 ± 0.79	–	NA
	Ba. fragilis	NA	3.71 ± 0.51	4.09 ± 0.93		1.45 ± 0.24	–	NA
	B. breve	NA	3.08 ± 0.61*	–	–	–	–	NA
	B. longum	NA	1.68 ± 0.23	–	–	–	–	NA

[a] Initial glucose level in the culture medium was 12.04 ± 1.38 mM. *, significant differences between strains from the same genus ($P < 0.05$); –, no detection or detection below the quantification limit; NA, not applicable.

isovaleric acids) and SCFA (experimental data not shown). This provides a rationale for the high CR values obtained in pure cultures of *Ba. thetaiotaomicron* and *Ba. fragilis*.

Different fermentation patterns were evidenced between *Ba. thetaiotaomicron* DSM-2079 and *Ba. fragilis* DSM-2151 regarding the production of SCFA (Table 1). Although acetic acid was the most abundant metabolite produced from glucose, in cultures of *Ba. thetaiotaomicron*, it was followed in abundance by succinic and then propionic acids, whereas *Ba. fragilis* produced clearly more propionic than succinic acid (Table 1). In complex carbon sources (inulin and EPS), the metabolic profile also differed between *Bacteroides* strains (Table 1). Propionic acid was the most abundant metabolite produced by *Ba. fragilis*, followed by acetic acid, whereas *Ba. thetaiotaomicron* produced more acetic than propionic acid. Previous studies by other authors indicated that the fermentation product profile from carbohydrates by *Bacteroides* greatly differed depending on the substrates. Succinic acid was generally the main metabolite produced at short generation times, whereas the proportions of acetic and propionic acids increased at long generation times or with less readily fermentable carbohydrates (28, 30, 38). Our results confirm these observations, as the proportion of propionic to succinic acid was higher in complex carbon sources than in glucose.

Interaction of *Bacteroides* and *Bifidobacterium* species in coculture. Decreases in pH paralleled the increases in SCFA concentrations in cocultures of *Bifidobacterium* and *Bacteroides*. In inulin, cocultures with *Ba. fragilis* reached higher concentrations of SCFA and succinic acid than cocultures with *Ba. thetaiotaomicron* (Table 2), thus leading to more pronounced pH decreases in the former.

Bacterial levels and metabolite production by pairwise combinations of *Bifidobacterium* and *Bacteroides* strains incubated with the different carbon sources were compared with the results obtained from pure cultures of the corresponding strains. In general, *Bacteroides* reached higher population levels than *Bifidobacterium* in cocultures, and the presence of bifidobacteria seems not to affect the growth of *Bacteroides* (data not shown). The only exception to this was the delayed growth at prolonged incubation times of *Ba. thetaiotaomicron* cocultured with *B. breve* when glucose was used as the carbon source (Fig. 1). A possible explanation for this inhibition could be the production under such conditions of antimicrobial compounds by *B. breve* (39) or the outcompetition at prolonged incubation times of bifidobacteria by using carbon sources still available in the culture medium and not consumed by *Ba. thetaiotaomicron*. Relating to this, we have previously reported on the inhibition by *B. longum* of other Gram-positive bacteria growing in combined culture (40, 41). Coculture with *Ba. thetaiotaomicron* did not improve the poor growth displayed by bifidobacteria in pure cultures with complex carbon sources (data not shown). In contrast, the survival of *Bifidobacterium* increased in the presence of *Ba. fragilis* in most carbohydrate sources so that cocultivation of both microorganisms resulted in higher population levels of *B. breve* and *B. longum* at late stages of incubation than those obtained in the corresponding monocultures (Fig. 1).

Both *Bifidobacterium* and *Bacteroides* species can produce acetic and formic acids. *Bacteroides* is able to form succinic acid, whereas this compound is not synthesized or is produced in very small amounts by bifidobacteria in any condition. Finally, while *Bacteroides* is a propionic acid producer, the metabolic pathway for the synthesis of propionic acid is not present in bifidobacteria

TABLE 2 SCFA and organic acid concentrations in uncontrolled-pH cocultures of *Bifidobacterium* with *Bacteroides* strains at 72 h of incubation with glucose (initial levels of 12.04 ± 1.38 mM), inulin, EPS E44, or EPS R1 as carbon sources[a]

Carbon source	Sugar consumption or SCFA and organic acid formation	Concn (mM) of glucose consumed or SCFA and organic acids formed				
			Ba. thetaiotaomicron		*Ba. fragilis*	
		Control (0 h)	*B. breve*	*B. longum*	*B. breve*	*B. longum*
Glucose	Glucose consumption	0	11.20 ± 1.43	12.91 ± 1.83	7.55 ± 0.71 ↓ B	11.33 ± 0.81 ↑ Ba
	Acetic acid	3.77 ± 1.29	24.16 ± 3.15 ↑ Ba	18.20 ± 4.63 ↓ B	19.61 ± 2.24 ↑ Ba ↓ B	20.88 ± 2.92 ↑ Ba ↓ B
	Propionic acid	0.48 ± 0.01	0.83 ± 0.26 ↓ Ba	3.89 ± 0.50	6.37 ± 0.62 ↓ Ba	5.83 ± 0.47 ↓ Ba
	Lactic acid	0.08 ± 0.13	0.27 ± 0.10	3.05 ± 1.94 ↓ B	0.00 ± 0.00 ↓ B	5.71 ± 1.18 ↓ B
	Formic acid	0.05 ± 0.07	8.83 ± 1.27 ↑ Ba	2.48 ± 0.19 ↑ Ba ↑ B	4.75 ± 0.66 ↑ Ba ↓ B	1.86 ± 0.21 ↑ Ba
	Succinic acid	0.09 ± 0.01	1.04 ± 0.68 ↓ Ba	6.88 ± 0.74	2.95 ± 0.29	2.45 ± 0.30 ↓ Ba
Inulin	Acetic acid	2.02 ± 0.49	3.79 ± 0.34	4.81 ± 0.92	8.21 ± 1.40 ↑ B	8.48 ± 1.10 ↑ B
	Propionic acid	0.46 ± 0.01	1.41 ± 0.06	2.55 ± 0.46	5.34 ± 0.42 ↓ Ba	9.31 ± 1.10
	Succinic acid	0.09 ± 0.02	0.95 ± 0.15	1.25 ± 0.14	1.05 ± 0.18	1.37 ± 0.04
EPS E44	Acetic acid	1.29 ± 0.14	8.52 ± 2.16 ↑ B	8.47 ± 2.47 ↑ B	9.03 ± 1.66 ↑ Ba ↑ B	6.96 ± 0.99 ↑ Ba ↑ B
	Propionic acid	0.45 ± 0.00	4.78 ± 1.97	7.24 ± 1.81	6.07 ± 0.85	5.96 ± 1.94
	Succinic acid	0.10 ± 0.02	1.44 ± 0.06 ↓ Ba	2.12 ± 0.22	1.55 ± 0.14	1.52 ± 0.32
EPS R1	Acetic acid	1.33 ± 0.22	7.49 ± 1.99	7.78 ± 1.67 ↑ B	4.81 ± 0.47 ↑ Ba ↑ B	4.91 ± 0.76 ↑ Ba ↑ B
	Propionic acid	0.45 ± 0.00	3.51 ± 0.94	6.57 ± 1.00	3.39 ± 0.49	4.07 ± 0.77
	Succinic acid	0.10 ± 0.03	0.96 ± 0.80	1.99 ± 0.17	0.96 ± 0.35	1.44 ± 0.22

[a] ↑ Ba and ↓ Ba indicate significantly higher or lower levels ($P < 0.05$), respectively, of a given metabolite in coculture than in the corresponding monoculture of the *Bacteroides* strain. ↑ B and ↓ B indicate significantly higher or lower levels ($P < 0.05$), respectively, of a given metabolite in coculture than in the corresponding monoculture of the *Bifidobacterium* strain. Glucose consumption is indicated for cocultures with this sugar as the carbon source.

(15, 28, 29, 32). Therefore, the metabolic contribution of microorganisms in coculture was inferred from the levels of propionic and succinic acids produced by *Bacteroides*, as well as from the levels of other common metabolites from carbohydrate fermentation (SCFA and organic acids) synthesized by both bacteria. With glucose as the carbon source, acetic acid reached levels in cocultures of *Ba. thetaiotaomicron* and *B. breve* similar to those in pure cultures of the bifidobacteria, whereas considerably smaller amounts of propionic and succinic acids were obtained from cocultures than in the monocultures of *Bacteroides* (Table 2). This suggests an impairment of the metabolic activity of *Ba. thetaiotaomicron* in the presence of *B. breve* as a consequence of its growth inhibition. In the remaining *Bacteroides* and *Bifidobacterium* combinations, acetic acid attained intermediate levels between the lower concentration reached by the monocultures of *Bacteroides* and the higher level of the monocultures of bifidobacteria. Specifically, in cocultures of *Ba. thetaiotaomicron* DSM-2079 and *B. longum* NB667, the production of propionic and succinic acids was similar to that in the monoculture of *Bacteroides*, suggesting that the metabolic activity of *Ba. thetaiotaomicron* probably remained unaffected under such conditions; however, in combined cultures of *Ba. fragilis* and bifidobacteria, lower propionic concentrations, and similar or lower levels of succinic acid than in *Bacteroides* monocultures, were obtained. This pointed to a probable slowdown of the metabolic activity of *Ba. fragilis* when bifidobacteria were present. On the other hand, lower lactic acid levels were obtained in most cocultures in glucose with respect to the monocultures of the corresponding *Bifidobacterium* strain, as was previously reported in coculture fermentations of bifidobacteria and bacteroides with inulin-type fructans (16). *Ba. thetaiotaomicron* DSM-2079 and *Ba. fragilis* DSM-2151 growing alone contributed scarcely both to the consumption of lactic acid present in the culture medium (14 to 16%) and to the production of formic acid (experimental data not shown). These findings, together with the increase of formic acid in cocultures of *Ba. thetaiotaomicron* and *B. longum* with respect to the corresponding monocultures, point to shifts in the metabolism of lactic and formic acids by one or both microorganisms when they are growing together.

With complex carbon sources, acetic acid was generally the most abundant metabolite produced in cocultures, followed by propionic acid and smaller amounts of succinic acid. Propionic and succinic acid levels in cocultures were similar to levels attained in the corresponding monocultures of *Bacteroides* for most pairwise combinations of strains, indicating that the metabolism of *Bacteroides* was probably not affected by the presence of bifidobacteria. In the presence of EPS, cocultures with *Ba. thetaiotaomicron* displayed levels of acetic acid close to the concentrations reached by monocultures of *Bacteroides*. In contrast, higher production of acetic acid was obtained in cocultures of bifidobacteria and *Ba. fragilis* DSM-2151 with EPS as the carbon source than in the corresponding monocultures of *Bifidobacterium* and *Bacteroides*, thus indicating an enhancement of the production of this acid in cocultures with *Ba. fragilis*. Therefore, the behavior of bifidobacterium strains in the same substrate appears to be influenced by the growth and metabolic characteristics of the *Bacteroides* strain present in the same environment. Falony et al. (16) indicated that the capacity of several *Bifidobacterium* strains to compete with *Ba. thetaiotaomicron* for the use of inulin-type fructans was dependent on the ability of the bifidobacteria to degrade fructose and oligofructose in addition to inulin. Using germfree mice colonized with *Ba. thetaiotaomicron* and *B. longum*, an expansion in the diversity of polysaccharides targeted for degradation by *B. thetaiotaomicron* has been observed in the presence of the bifidobacteria, demonstrating an adaptation for substrate utilization by both species in response to one another (42).

In short, differences in growth and metabolic characteristics of *Bifidobacterium* and *Bacteroides* strains can influence their joint behavior against EPS and other fermentable carbohydrate sources available in the growth environment. The results presented here stress the importance of considering specific species and strains, and not simply high taxonomic divisions, in the relationship among intestinal microbial populations. Variations at the level of species or strain composition among individuals or human population groups could condition a different response of their intestinal microbiota to specific diets or probiotic and prebiotic interventions.

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A proteomic approach towards understanding the cross-talk between *Bacteroides fragilis* and *Bifidobacterium longum* in co-culture

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Abstract: **A better understanding of the interactions among intestinal microbes is needed in order to decipher the complex cross-talk that takes place within the human gut. *Bacteroides* and *Bifidobacterium* genera are among the most relevant intestinal bacteria and it has been previously reported that co-culture of these two microorganisms affect their survival. Therefore, co-cultures of *Bifidobacterium longum* NB667 and *Bacteriodes fragilis* DSMZ2151 were performed with the aim of unravelling the mechanisms involved in their interaction. To this end we applied proteomic (2D-DIGE) analyses and quantified by chromatographic techniques the bacterial metabolites produced during co-incubation. Co-culture stimulated the growth of *B. longum* retarding that of *B. fragilis*, with concomitant changes in the production of some proteins and metabolites of both bacteria. The combined culture promoted up-regulation of the bifidobacterial pyruvate kinase and down-regulation of the *Bacteroides* phoshoenolpyruvate carboxykinase, two enzymes involved in the catabolism of carbohydrates. Moreover, *B. fragilis* FKBP-type peptidyl-prolyl cis/trans isomerase, a protein with chaperone-like activity, was found to be over-produced in co-culture, suggesting the induction of a stress response in this microorganism. This study provides mechanistic data to deepen our understanding on the interaction between Bacteroides and Bifidobacterium intestinal populations.**

***Keyword: Bifidobacterium*, *Bacteroides*, cross-talk, interaction, co-culture.**

The intestinal tract constitutes one of the habitats displaying higher bacterial diversity in the human body (Human Microbiome Project Consortium 2012) This complex microbial ecosystem plays an essential role in the maintenance of human health and the disruption of its homeostasis has been linked to several health disorders (Clemente et al. 2012). In spite of its large bacterial diversity, recent studies have indicated that at high phylogenetic level, this ecosystem is dominated by a limited number of bacterial phyla. Among them, Bacteroidetes and Firmicutes are clearly the most abundant in adults, with other phyla such as Actinobacteria, Proteobacteria or Verrumicrobia, being also present at lower levels (Eckburg et al. 2005).

Among Bacteroidetes, the genus *Bacteroides* represent the most abundant population, constituting about a 20-50% of the colon microbiota (Eckburg et al. 2005). These microorganisms have a predominantly saccharolytic metabolism and are able to ferment host and dietary-derived glucans to succinic, acetic, lactic and propionic acids as the main metabolic end-products (Rios-Covian et al. 2013). The ability of these microorganisms to break-down complex carbohydrates, such as indigestible plant polysaccharides, makes *Bacteroides* a key player within the intestinal ecosystem (Sonnenburg et al. 2005). Different species of this genus can be present in the intestine, *Bacteriodes*

thetaiotaomicron being the most frequently found in humans (Eckburg et al. 2005). The genus *Bacteroides* also harbors species that, although often present in the intestinal ecosystem, are important opportunistic pathogens, such as *Bacteroides fragilis* (Chaudhry and Sharma 2011).

The genus *Bifidobacterium* is the main representative of the Actinobacteria phylum within the human colon, accounting for up to 3% of the adult microbiota (Matsuki et al. 2002). Bifidobacteria metabolize sugars by the fructose-6-phosphate phosphoketolase pathway, which leads to the production of acetic and lactic acids as main metabolic end-products (Margolles et al. 2011). This genus is among the dominant intestinal bacteria found in the intestine of breast-fed infants, their levels drop later on with weaning and remains stable during the adulthood (Tojo et al. 2014). Diverse species of the genus populate the human gut and *Bifidobacterium longum* is one of the most abundant. Alterations on the levels and/or species composition of the intestinal bifidobacterial populations have been related with different disease states (Tojo et al. 2014)

Whereas *Bacteroides* and *Bifidobacterium* are among the most relevant intestinal microorganisms, the potential interactions between these two bacteria, and of them with complex carbohydrates that may be present in the gut, have attracted the attention of researchers (Adamberg et al. 2014; Falony et al. 2009; Rios-Covian et al. 2013; Rios-Covian et al. 2015). In a previous work we observed that the co-culture of *B. fragilis* DSMZ2151 with *B. longum* NB667 enhanced the survival of the later microorganism (Rios-Covian et al. 2013). In the present study we performed co-culture experiments and proteomic analyses in order to advance a step further on the understanding of such interactions and the potential mechanisms involved.

With this aim, *B. longum* NB667 (NIZO food research culture collection, EDE, The Netherlands) and *B. fragilis* DSMZ2151 (DSMZ bacterial collection, Braunschweig, Germany) were incubated in mono-culture and co-culture. Bacterial strains were recovered in MRSc and GAMs broths, respectively, and mono-culture and co-culture experiments were carried out in mCFBM medium supplemented with 0.3 % (w/v) glucose as described previously (Rios-Covian et al. 2013). Mono-cultures were inoculated at 1% (v/v) with overnight cultures of *B. fragilis* or *B. longum* in mCFBM whereas in co-cultures each of the two strains was inoculated at this percentage. Two samples were collected at T1 (14 ± 1 hours of incubation, mid-late exponential growth phase, corresponding with OD_{600} 0.75 ± 0.05 for mono-cultures and 1.05 ± 0.05 for the co-culture), and T2 (18.5 ± 1 h of incubation, early-stationary growth phase, corresponding with OD_{600} 1.25 ± 0.1 for mono-cultures and co-culture). Bacterial growth (cell counts mL^{-1}) was estimated by qPCR using previously described primers and conditions (Arboleya et al. 2012).

Cell-free supernatants of mono-cultures and co-cultures were acidified with HCl to pH 2.5±0.5 and the SCFA produced during incubation were quantified in the supernatants by Gas Chromatography using a system composed of a 6890 gas chromatograph (Agilent Technologies Inc., Palo Alto, CA, USA) connected with a FID detector (Agilent) as described previously (Salazar et al. 2008). Organic acids and glucose were analyzed by HPLC consisting on an Alliance 2695 separation module and a photo diode array (PDA) detector (Waters 996) at 210 nm for organic acids, a refractive index detector for glucose (Waters 2414), and Empower software (Waters, Mildford, MA, USA). Chromatographic conditions were those published elsewhere (Salazar et al. 2009). Concentrations are expressed in millimolar (mM).

Differences in the proteome of each strain in mono-culture with respect to co-culture at both T1 and T2, were assessed separately by two-dimensional difference gel electrophoresis (2D-DIGE) as described previously (Rios-Covian et al. 2015). Mono-cultures were labelled with Cy3 dye whereas the co-culture was labelled with Cy5 dye (Supplementary Fig. 1). Another gel was performed with a synthetic mixture of both monocultures labeled with Cy3 and the proteins from co-culture labeled with Cy5, no new spots were detected as a consequence of cocultivation. As a result, in gels of monoculture *vs.* co-culture, spots detected in the co-culture that were not present in the mono-culture were not considered in the study as putatively belonging to the other microorganism present in the co-culture. Gels were analyzed with the 2D Imagemaster (GE Healthcare) software. During the analysis, overlapping spots were excluded. As proteins marked in the co-culture belong to two different microorganisms, an average abundance ratio between proteins in the co-culture relative to the mono-culture was calculated for each strain, representing the average of the abundance ratio obtained for all the spots available in the co-culture for this microorganism. All ratios were normalized by this value and *t* tests were run between mono-culture and co-culture. Spots displaying a significant change on their production (fold-change higher than 2 or lower than -2) were excised from gels and identified as previously described (Rios-Covian et al. 2015).

The relative expression of genes coding for proteins with significant changes in their production on the basis of the 2D-DIGE experiments were further analyzed. RNA was extracted from 10 mL of cultures taken at the end of the incubation (T2) using the RNeasy mini kit (Qiagen) as reported by Ulve et al. (2008). Gene expression was determined by reverse transcription qPCR (RT-qPCR) using the ΔΔ Ct method as described previously (Gueimonde et al. 2007). Primers used in the present study were: F 5'-ATCACTGAACCGACTCCGACA-3'and R 5'-GCAATGATAAGAATGCAGCACC-3 for *B. fragilis* phosphoenolpyruvate carboxykinase; F 5'-AAAATCTCCTGGTTCAGTTCGC-3' and R 5'-AGGCAACTTCAGCTTCCAGG-3' for *B. fragilis* FKBP-type peptidyl-prolyl cis-transisomerase; and F 5'-GGATCTCTTCGAGGTTCTCC-3' and R 5'-CACGAGATCATGGACGAGGAA-3' for *B. longum* pyruvate kinase. The 16S rRNA gene was employed as endogenous control by using previously described primers (Arboleya et al. 2012) F 5'-GATTCTGGCTCAGGATGAACGC-3' and R 5'-CTGATAGGACGCGACCCCAT-3' for *Bifidobacterium* and F 5'-GAGAGGAAGGTCCCCCAC-3' and R 5'-CGCKACTTGGCTGGTTCAG-3' for *Bacteroides*.

One way ANOVA statistical tests were run for the analyses of values obtained for the different parameters considered between mono-cultures and co-cultures using the IBM SPSS software, version 22.00 (IBM, Armonk, New York, USA).

The results obtained corroborated previous data (Rios-Covian et al. 2013) indicating that co-incubation of *B. longum* NB667 with *B. fragilis* DSMZ2151 stimulated the growth of the former microorganism, resulting in significantly higher levels ($p<0.05$) of *B. longum* in co-culture than in the corresponding mono-culture (9.07 vs 8.11 log cells mL^{-1}) at the end of the incubation (Table 1). In contrast, the growth of *B. fragilis* was delayed by the presence of the bifidobacteria, as denoted by the significantly lower ($p<0.05$) bacterial levels obtained in the co-culture at the first sampling point (T1; representing mild-late exponential phase) (8.66 *vs.* 8.93 log cells mL). This difference, however, disappeared later (T2; early-stationary phase), with *B. fragilis* population reaching similar levels in co-culture than in the corresponding mono-culture (8.93 *vs.* 8.89 log cells mL^{-1}) (Table 1).

As expected from the bacterial growth patterns, the consumption of glucose was higher in the co-culture than in mono-cultures, these differences achieving statistical significance ($p<0.05$) at the later sampling point analyzed (Table 1). The enhanced consumption of glucose in co-culture led to higher concentrations of acetate and lactate ($p<0.05$), which suggests a predominant production by the bifidobacteria. These data are in good agreement with the increased bifidobacterial growth observed for *B. longum* when it was grown with *B. fragilis*. Moreover, in accordance with the delayed growth of *B. fragilis* in co-culture, the levels of propionate, succinate and formate (mainly produced by *Bacteroides* with no or very limited production by bifidobacteria) were lower in such conditions than in the corresponding mono-culture (Table 1). These results are in agreement with previous reports (Rios-Covian et al. 2013).

To further elucidate the mechanisms involved in this microbe-microbe interaction, the protein pools of *B. longum* NB667, *B. fragilis* DSMZ2151 and its co-culture were analyzed and compared by means of 2D-DIGE. At this point, it is important to underline that the proteomic 2D-DIGE analysis of bacterial co-cultures possess a serious methodological limitation because several spots cannot be analyzed as a consequence of the same gel-migration properties of some proteins of both microorganism, therefore overlapping in the gel (supplementary Fig. 1). In spite of this limitation the proteomic analyses allowed us to identify some spots differentially produced in co-culture with regard to the corresponding mono-cultures. By using as a threshold the occurrence of statistically significant ($p<0.05$) changes in production for values greater or lower than two-fold, three spots were found to be affected (Table 2). One of these spots corresponded to the enzyme pyruvate kinase of *B. longum* which was found to be over-produced at both sampling times (T1 and T2). Moreover, RT-qPCR analyses of the expression of the corresponding gene (*pyk*) at the end of the incubation period (T2) confirmed this observation with more than six-fold induction of the gene expression (Table 2). This enzyme, that catalyzes the conversion of phosphoenolpyruvate to pyruvate, constitutes one of the key steps of sugar metabolism in bifidobacteria (de los Reyes-Gavilán et al. 2005) (Fig. 1). This suggests a stimulation of the catabolism of carbohydrates by *B. longum* when co-cultured with *B. fragilis*, thus providing an explanation for its improved growth and the shift on its carbohydrate metabolism in co-culture. Interestingly, the pyruvate kinase has been previously found to be over-produced by *B. longum* (Sanchez et al. 2005; Sanchez et al. 2007) and other microorganisms (Len et al. 2004) under stress conditions. As regarding proteins of *B. fragilis*, the proteomic results evidenced three spots with significant changes in their production in co-cultures with respect to mono-cultures, at least in one of the time points analyzed (Table 2). Two of these

spots, likely isoforms, were identified by MALDI TOF/TOF tandem mass spectrometry as a FKBP-type peptidyl-prolyl cis/trans isomerase. This enzyme catalyzes the cis/trans isomerization of peptide bonds adjacent to proline residues (Janowski et al. 1997). In bacteria, this enzyme plays an important role in native protein folding and re-naturalization of denatured proteins and, therefore, it has chaperone-like activity (Jo et al. 2015). Different proteins involved in protein folding and turnover have been found to be up- or down-regulated in *B. fragilis* when grown in complex carbohydrates, such as bifidobacterial exopolysaccharides (Rios-Covian et al. 2015). Moreover, chaperones production in bacteria is often affected by stress factors (Ruiz et al. 2011). In this context, our results suggest that the presence of *B. longum* in the co-culture promotes a response in *B. fragilis* leading to a modification on the production of this chaperone. However, the direction of this modification remains unclear as according to the proteomic results there is an up-regulation of the protein production at both sampling times (T1 and T2) but, on the contrary, the expression analyses at the end of incubation (T2) indicate a repression of the gene expression (Table 2). To this regard, discrepancies between protein and gene expression analyses are not un-common (Aakko et al. 2014; Wu et al. 2011). It must be pointed out that we employed two different techniques, each with its own performance and limitations. RT-qPCR quantifies the mRNA, whose life-span is relatively short, whilst proteomics quantify the protein accumulated in the cell at a given time, including the effect of post-translational regulation. These differences may account for the apparently contradictory results obtained.

The second *B. fragilis* protein found to be affected by the co-incubation with *B. longum* was the phoshoenolpyruvate carboxykinase, a key enzyme in the catabolism of *Bacteroides*. This enzyme, catalyzing the conversion of phosphoenolpyruvate to oxalacetate (Figure 1), was found to be under-produced at T1 by proteomics and was found down-regulated at the end of incubation (T2) by the RT-qPCR experiments (Table 2). This down-regulation suggests that co-cultivation with *B. longum* promotes a shift on the central sugar catabolism of *B. fragilis*, which may be focused to the use of complex carbohydrates and proteins in order to outcompete *B. longum*. Supporting this hypothesis are our previous results showing down-regulation of the phosphoenolpyruvate carboxykinase and an activation of the catabolism of amino acids by *B. fragilis* when grown in media containing bacterial exopolysaccharides (complex carbohydrates that promote slow growth) as carbon source with respect to glucose (Rios-Covian et al. 2015). *Bacteroides* is well adapted to the use of complex polysaccharides and an expansion on its ability to degrade these substrates has been observed when gnotobiotic animals mono-colonized by *Bacteroides* were compared with those co-colonized by *Bacteroides* and *B. longum* (Sonnenburg et al. 2006). Changes observed by us on the *Bacteroides* metabolism in co-culture with *B. longum* could account for the delayed bacterial growth and reduced metabolites production observed in such conditions.

To sum up, the results of this study provide new insights into the physiological and molecular mechanisms that determine the interaction of *B. fragilis* and *B. longum*, two important intestinal microorganisms, when growing together. *B. fragilis* in the co-culture seems to stimulate the growth and metabolic activity of *B. longum* when glucose is available as a carbon source. The contrary seems to be true for *B. fragilis*, whose growth was inhibited and the metabolites production reduced in the presence of *B. longum*. The proteomic data allowed identifying the central sugar catabolism pathways as key regulators of this interaction, underlining the role of metabolic interactions among microorganisms as drivers of the cross-talk between these two intestinal bacteria. Increasing our understanding on the microbe-microbe interactions among intestinal bacteria will allow the development of high efficacious and specific microbiota-modulation strategies for improving human health.

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Table 1.Glucose consumption, SCFA and organic acid concentrations (mM) and bacterial counts (log cells mL^{-1}) in co-cultures of *B. fragilis* DSMZ2151 and *B. longum* NB667 at mid-late exponential (T1) and early-stationary (T2) phases of growth.

Time	Culture	Glucose consumption	Acetate	Propionate	Lactate	Succinate	Formate	Bacterial counts	
								Bacteroides	*Bifidobacterium*
T1	DSM2151	0.91 ± 0.06^{a}	3.24 ± 0.01^{a}	0.82 ± 0.01^{b}	0.77 ± 0.04^{a}	0.92 ± 0.03^{c}	2.51 ± 0.10^{b}	8.93±0.16*	-
	NB667	3.35 ± 1.18^{b}	8.91 ± 1.02^{b}	-	3.46 ± 0.37^{c}	0.20 ± 0.03^{a}	0.97 ± 0.13^{a}	-	8.59±0.77
	Co-culture	3.58 ± 0.41^{b}	7.99 ± 0.07^{b}	0.61 ± 0.01^{a}	2.77 ± 0.10^{b}	0.80 ± 0.02^{b}	2.20 ± 0.06^{b}	8.66±0.09*	8.79±0.19
T2	DSM2151	1.86 ± 0.30^{a}	4.06 ± 0.09^{a}	1.08 ± 0.06^{b}	4.59 ± 0.30^{a}	0.86 ± 0.16^{b}	2.81 ± 0.06^{c}	8.93±0.09	-
	NB667	8.70 ± 0.13^{b}	16.70 ± 0.34^{b}	-	8.46 ± 0.23^{b}	0.21 ± 0.01^{a}	0.57 ± 0.03^{a}	-	8.11±0.32*
	Co-culture	10.26 ± 0.03^{c}	18.39 ± 0.26^{c}	0.32 ± 0.01^{a}	10.51 ± 0.17^{c}	0.66 ± 0.02^{b}	0.93 ± 0.05^{b}	8.89±0.41	9.07±0.29*

Different letter within the same-time point indicate significant differences among cultures. Asterisks indicate significant differences between mono-culture and co-culture. - not determined/not detected (below detection limit)

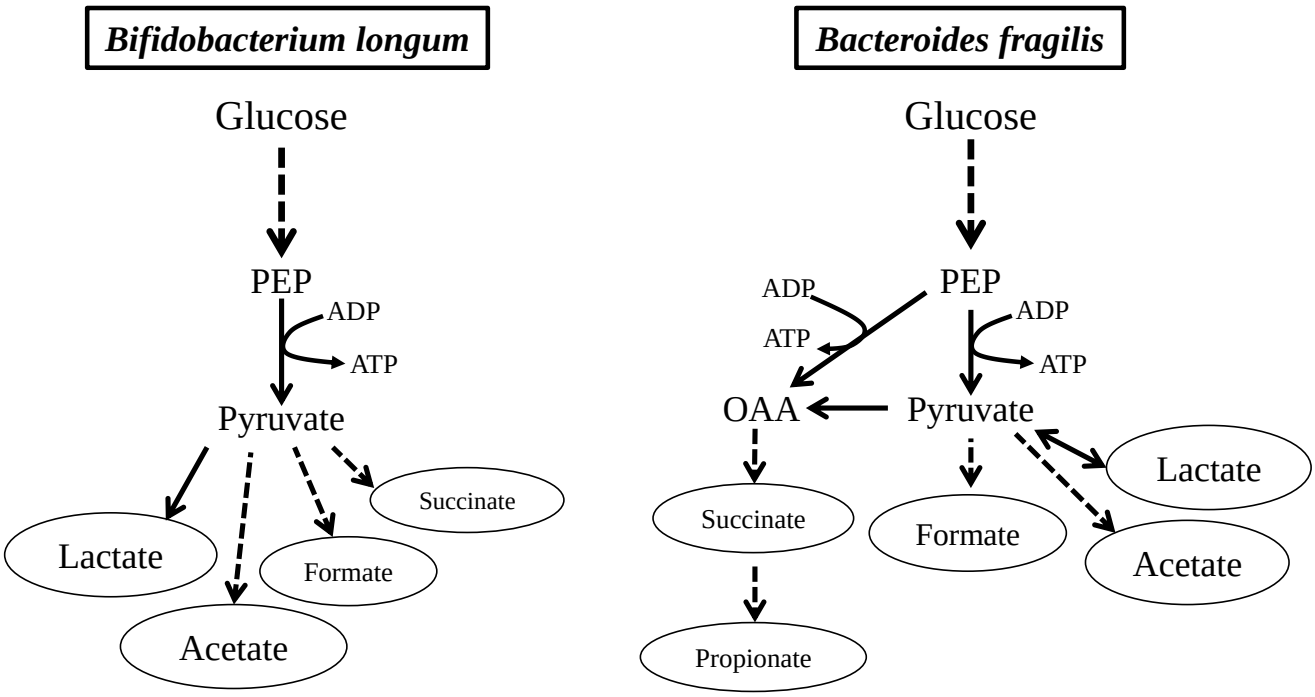

Figure 1 Schematic representation of *B. fragilis* and *B. longum* metabolism. Final metabolites are rounded. Dark arrows indicate one reaction step, dashed arrows indicate more than one reaction. Phospoenolpyruvate, PEP; Oxalacetate, OAA.1, pyruvate kinase; 2, phospoenolpyruvate carboxykinase.

Table 2. Identification of proteins affected by co-cultivation of *B. fragilis* DSMZ2151 and *B. longum* NB667 and expression of the corresponding genes

COG orthology	Spot no.[a]	Putative function[b]	GI number[c]	Mass[d]	pI[d]	MASCOT score	No. of peptides matched[e]	Coverage	Protein change fold[f]		Gene expression change fold[g]
									T1	T2	T2
Energy production and conversion	Bac25	phosphoenolpyruvate carboxykinase	gi|53715725	59333	5.73	333	15	25	-2.15	ND	-3.06±1.47
Posttranslational modification, protein turnover, chaperones	Bac42	FKBP-type peptidyl-prolyl cis-transisomerase	gi|547946109	51380	4.69	153	15	28	3.76	2.02	-2.84±0.80
	Bac44	FKBP-type peptidyl-prolyl cis-transisomerase	gi|547946109	51380	4.69	155	11	19	2.99	ND	
Carbohydrate transport and metabolism	Bif8	Pyruvate kinase	gi|312133003	52310	5.24	525	29	49	ND	2.95	6.55±1.25
	Bif9	Pyruvate kinase	gi|312133003	52310	5.24	587	34	54	ND	4.54	
	Bif10	Pyruvate kinase	gi|312133003	52310	5.24	527	33	49	2.22	2.17	

[a]Spot numbers refer to the proteins labelled in 2D-DIGE gels. *B. fragilis*, Bac; *B. longum*, Bif.
[b]Putative functions were assigned from the NCBI gene database
[c]GI number in the NCBInr database for *B. fragilis* DSMZ2141 and *B. longum* NB667
[d]As given by the NCBInr databse for *B. fragilis* DSMZ2151 and *B. longum* NB667. Molecular masses are expressed in kilodaltons
[e]Number of tryptic peptides observed that contributed to the percentage of amino acid coverage.
[f]Normalized change fold for each protein production derived from cells grown in mono-culture with respect to the protein derived from cells grown in co-culture.ND, ratio below 2.
[g]Gene expression change fold for each gen derived from cDNA obtained from cells grown in mono-culture with respect to the cDNA obtained from cells grown in co-culture.16s RNA gene was used as housekeeping gene.

CAPÍTULO 2

ESTUDIO DEL PAPEL DE *Bacteroides fragilis* EN LA DEGRADACIÓN DE EPS AISLADOS DE BIFIDOBACTERIAS

CAPÍTULO 2

Estudio del papel de *Bacteroides fragilis* en la degradación de EPS aislados de bifidobacterias.

El género *Bacteroides* se caracteriza por la capacidad de utilización de una gran variedad de fuentes de carbono, desde carbohidratos complejos hasta proteínas y aminoácidos no digeribles por el hospedador. Los EPS son polímeros complejos secretados al exterior celular por microorganismos y que se han relacionado en varios estudios con la protección de la célula y la formación del biofilm bacteriano. Por ese motivo, su producción dentro del entorno intestinal es muy probable, aunque no se ha demostrado aún y podrían influir en la producción de metabolitos por la microbiota intestinal.

Como se ha demostrado en el capítulo anterior, tanto *Ba. thetaiotaomicron* como *Ba. fragilis* son capaces de crecer en presencia de EPS, presentando una pauta de crecimiento diferente en comparación con glucosa, que indica una influencia de estos carbohidratos complejos en el metabolismo microbiano. Decidimos, por tanto, llevar a cabo un estudio metabólico en profundidad de los cambios producidos en *Ba. fragilis* en presencia tanto de EPS E44 como de EPS R1, dado que este microorganismo mostró un comportamiento ligeramente diferente con cada uno de los EPS empleados en este estudio. Primeramente realizamos el estudio en medio no definido, en el que se encontraban, además de EPS, multitud de compuestos, pero no carbohidratos simples, simulando unas condiciones parecidas a las del colon humano. En un segundo paso, el estudio se realizó en un medio mínimo con EPS como única fuente de carbono a fin de demostrar la utilización efectiva de estos polímeros como sustrato fermentable por *Ba. fragilis*.

El objetivo de este capítulo se aborda en las siguientes publicaciones:

Manuscrito 3

Rios-Covian, D., Sanchez, B., Salazar, N., Martinez, N., Redruello, B., Gueimonde, M., and de Los Reyes-Gavilan, C.G. (2015). Different metabolic features of *Bacteroides fragilis* growing in the presence of glucose and exopolysaccharides of bifidobacteria. Front Microbiol 6, 825. doi: 10.3389/fmicb.2015.00825.

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Different metabolic features of *Bacteroides fragilis* growing in the presence of glucose and exopolysaccharides of bifidobacteria

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Bacteroides is among the most abundant microorganism inhabiting the human intestine. They are saccharolytic bacteria able to use dietary or host-derived glycans as energy sources. Some *Bacteroides fragilis* strains contribute to the maturation of the immune system but it is also an opportunistic pathogen. The intestine is the habitat of most *Bifidobacterium* species, some of whose strains are considered probiotics. Bifidobacteria can synthesize exopolysaccharides (EPSs), which are complex carbohydrates that may be available in the intestinal environment. We studied the metabolism of *B. fragilis* when an EPS preparation from bifidobacteria was added to the growth medium compared to its behavior with added glucose. 2D-DIGE coupled with the identification by MALDI-TOF/TOF evidenced proteins that were differentially produced when EPS was added. The results were supported by RT-qPCR gene expression analysis. The intracellular and extracellular pattern of certain amino acids, the redox balance and the α-glucosidase activity were differently affected in EPS with respect to glucose. These results allowed us to hypothesize that three general main events, namely the activation of amino acids catabolism, enhancement of the transketolase reaction from the pentose-phosphate cycle, and activation of the succinate-propionate pathway, promote a shift of bacterial metabolism rendering more reducing power and optimizing the energetic yield in the form of ATP when *Bacteroides* grow with added EPSs. Our results expand the knowledge about the capacity of *B. fragilis* for adapting to complex carbohydrates and amino acids present in the intestinal environment.

Keywords: *Bacteroides fragilis*, exopolysaccharides, *Bifidobacterium*, glucose, metabolism, probiotics

Introduction

The microbes in our body reach levels of up to 100 trillion (10^{12}) cells, the majority of which reside in the colon and are anaerobes (Qin et al., 2010). The adult human distal gut microbiota is dominated by two phyla, the Firmicutes and the Bacteroidetes, the genus *Bacteroides* accounting for 20–50% in most individuals (Rigottier-Gois et al., 2003; Mahowald et al., 2009). This group of microorganisms appears during the first few days of life in the intestine of full-term neonates

(Arboleya et al., 2015) and remains at low levels (10^7 cells per gram of intestinal content) in breast-fed infants, increasing after weaning (Mackie et al., 1999). *Bacteroides* species are usually considered as symbionts or mutualists in the human intestine (Hooper and Gordon, 2001). However, in certain circumstances some species can act as opportunistic pathogens (Wexler, 2007). Indeed, the relatively large genome of *Bacteroides* enables these microorganisms to behave both as beneficial and harmful bacteria depending on the host environmental conditions. In this way, the capsular polysaccharide of certain strains of *Bacteroides fragilis* can contribute to the development and maturation of the host immune system (Mazmanian et al., 2005) but is also an important virulence determinant of this bacterium (Wexler, 2007). *Bacteroides* is an anaerobic, bile-resistant, non-spore-forming, and Gram-negative rod. It is a saccharolytic-versatile microorganism able to use dietary or host-derived glycans according to the nutrient availability (Sonnenburg et al., 2005). Members of *Bacteroides* can incorporate amino acids from the external environment (Smith and Macfarlane, 1998). Succinic, acetic, lactic, and propionic acids are produced by *Bacteroides* in variable proportions during fermentation (Rios-Covian et al., 2013). Then, these organic acids and short chain fatty acids (SCFAs) can be utilized by other intestinal microorganisms through cross-feeding mechanisms (Scott et al., 2008) or be partly reabsorbed through the large intestine, thus serving as an energy source for the host (Hooper et al., 2002).

Exopolysaccharides (EPSs) are complex carbohydrate polymers which can be produced by many microorganisms, as is the case of some *Bifidobacterium* strains (Ruas-Madiedo et al., 2007). The intestine is the normal habitat of most species of *Bifidobacterium*, some of whose strains are considered as probiotics and are being included in functional foods (Masco et al., 2005). The synthesis of EPS by bifidobacteria *in vivo* has not yet been demonstrated. However, previous evidence indicates that the presence of bile stimulates the *in vitro* production of EPS by bifidobacteria (Ruas-Madiedo et al., 2006, 2009). In addition, it has been unveiled that in the presence of EPS a relative increase of propionic acid proportions occurs as a result of the metabolic activity of colonic microbiota (Salazar et al., 2008).

The microbiota composition and its functionality in the gastrointestinal ecosystem have been intensively studied, but the dynamics of such microbiota at the metabolic level is not yet well-known. Indeed, in spite of relevant studies on the mechanisms of virulence and pathogenicity of *B. fragilis*, still little is known about the physiology of this microorganism and the adaptation of its metabolism to the gut ecosystem (Wexler, 2007). Although the effective consumption by *B. fragilis* or other intestinal microorganisms of bacterial EPSs has not been unequivocally demonstrated yet, we have recent evidence showing differential growth and metabolic patterns by *B. fragilis* depending on the carbon sources present in the external environment. Thus, a shift toward propionic acid production was found when *B. fragilis* was incubated with bifidobacterial EPS whereas in glucose, acetic acid was the most abundant metabolite formed (Rios-Covian et al., 2013). Therefore, the aim of the present work was to gain insight into *B. fragilis* metabolism grown in the presence of different carbohydrates, including EPSs produced by bifidobacteria, and to examine the results obtained in the light of the role that this bacterium plays as symbiont and opportunistic pathogen in the human gut.

Materials and Methods

Bacterial Culture Conditions

Frozen stocks of *B. fragilis* DSM2151 (DSMZ bacterial pure collection, Braunschweig, Germany) were reactivated in Gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 0.25% (w/v) L-cysteine (Sigma Chemical Co., St. Louis, MO, USA) (named GAMc) and incubated at 37°C for 24 h in an anaerobic cabinet (Mac 1000; Don Whitley Scientific, West Yorkshire, UK) under a 10% H_2, 10% CO_2, and 80% N_2 atmosphere. The pH-free liquid batch cultures of *B. fragilis* were performed in 50 mL of a non-defined peptone and yeast-extract containing basal medium (BM) previously used by us in human fecal cultures (Salazar et al., 2008) and which was subsequently adapted for *Bacteroides* co-cultivation with bifidobacteria (Rios-Covian et al., 2013). The medium had the following composition: peptone (2 g/L), yeast extract (2 g/L), NaCl (100 mg/L), K_2HPO_4 (40 mg/L), KH_2PO_4 (40 mg/L), $MgSO_4$ (10 mg/L), $CaCl_2.H_2O$ (10 mg/L), $NaHCO_3$ (2 g/L), L-cysteine (2 g/L), bile salts (0.5 g/L), hemin (4 mg/L), Tween 80 (2 mL/L), $FeSO_4$ (50 μM), and $Na_3C_3H_5O(COO)_3$ (150 μM). A vitamin solution was added, resulting in final concentrations of: vitamin B_{12} (10 mg/L), vitamin K (2 mg/L), vitamin B_1 (2 mg/L), pyridoxal (1 mg/L), calcium pantothenate (2 mg/L), folic acid (1 mg/L), riboflavin (1 mg/L), biotin (1 mg/L), nicotinic acid (3 mg/L), para-aminobenzoic acid (1 mg/L). The BM was supplemented with 0.3% (w/v) glucose, or bifidobacterial EPS E44 or R1, as specified previously (Rios-Covian et al., 2013).The final pH of the medium ranged between 6.7 and 7.0. A culture of *B. fragilis* incubated in BM without external carbohydrates added was used for comparison in part of the study. The estimate of growth was obtained by measuring the optical density of cultures at 600 nm (OD_{600}). Culture media were inoculated with 1% (v/v) of an overnight culture of *B. fragilis* in BM with 1% (w/v) glucose resulting in initial population levels of around 10^6 CFU/mL.

EPS Isolation

Exopolysaccharide fractions were obtained from *Bifidobacterium animalis* subsp. *lactis* IPLA R1, a dairy origin strain (Ruas-Madiedo et al., 2006), and from *Bifidobacterium longum* IPLA E44, a fecal isolate from a healthy adult donor (Delgado et al., 2006). EPSs were isolated and purified from the cellular biomass of the producing strains harvested from agar-MRS plates supplemented with 0.25% (w/v) L-cysteine (Sigma Chemical Co.; agar-MRSc) as specified by Salazar et al. (2009b). Briefly, the biomass was collected with ultrapure water and mixed with one volume of 2 M NaOH and gently stirred overnight at room temperature; then, cells were removed by centrifugation and EPSs from supernatants were precipitated with two volumes of

absolute cold ethanol for 48 h at 4°C. After centrifugation at 10,000 × *g* for 30 min at 4°C, the EPS fraction was resuspended in ultrapure water, and dialyzed against water for 3 days at 4°C in dialysis tubes of 12- to 14-kDa molecular mass cutoff.

The isolated and purified EPS E44 and R1 fractions contained less than 2.37 and 2.03% (w/w) protein respectively, implying a final protein contribution to the culture medium of about 0.007 and 0.006% (w/v) for EPS fractions E44 and R1, respectively.

Analysis of SCFA and Organic Acids

Cell-free supernatants from cultures were filtered (0.2 μm). Quantification and identification of SCFA and branched chain fatty acids (BCFAs) was carried out by gas chromatography-mass spectrometry/flame injection detector (MS/FID) using a system composed of a 6890N GC (Agilent Technologies, Inc., Palo Alto, CA, USA) connected with a FID and a MS spectrometry 5973N detector (Agilent Technologies) as described previously (Salazar et al., 2008). Identification and quantification of organic acids was carried out on an HPLC chromatographic system composed of an Alliance 2690 module injector, a PDA 996 photodiode array detector, a 410 differential refractometer detector and Empower software (Waters, Milford, MA, USA). Chromatographic conditions were those indicated previously by Salazar et al. (2009a). Results of SCFA (acetic, propionic), BCFA (isobutyric, isovaleric), and organic acids (lactic, formic, pyruvic, succinic) concentrations were expressed in millimolar (mM). Concentrations of these compounds in media before inoculation (time 0) were subtracted from concentrations in cultures with each carbohydrate added at fixed sampling points during incubation. The sum of acetic, propionic, isobutyric, isovaleric, succinic, formic, pyruvic, and lactic acids was calculated. The molar proportion of each compound was obtained as the concentration percentage with respect to the total SCFA + BCFA + organic acids.

Amino Acids Analyses

Quantitative determination of amino acids in cell-free supernatants from cultures and in cell-free extracts (CFEs) was carried out by ultra-HPLC (UHPLC) using the method described in Redruello et al. (2013). The chromatographic system consisted of an H-Class Acquity UHPLC coupled to a PDA detector set at 280 nm (Waters). To obtain the CFEs, 10 mL of cultures were centrifuged at 6,500 × *g* for 5 min and concentrated in 1 mL of PBS. Resuspended bacterial pellets were twice broken by sonication for 30 s at 75 W and kept on ice for 1 min between sonication treatments. Cellular rests and unbroken cells were removed by centrifugation at 16,000 × *g* for 10 min at 4°C. CFE were ultra-filtered through a 3-kDa centricon following the manufacturer's instructions (Millipore, Billerica, MA, USA). One-hundred milliliters of filtered CFE or supernatants were derivatized as described in Redruello et al. (2013) and then one microliter was immediately injected into the chromatographic system. Total protein concentration of the CFE was determined using the Pierce® BCA Protein Assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA) according to the manufacturer's instructions. Results of amino acid concentrations in supernatants are expressed in millimolar (mM), whereas results of amino acid concentrations in CFEs are expressed in mmol/g of total protein. Amino acid levels of the media before inoculation (time 0) were subtracted from those in cultures containing each carbon source.

Proteomic Analyses

Differences in the proteome of *B. fragilis* growing in the presence of EPS E44 or EPS R1, with respect to glucose, were assessed separately by two-dimensional difference gel electrophoresis (2D-DIGE) as specified by Hidalgo-Cantabrana et al. (2013). In short, CFE from each culture were firstly obtained. Proteins were precipitated by the methanol-chloroform method according to Wessel and Flugge (1984), and resuspended in solubilization buffer (Destreak rehydration solution; GE Healthcare Biosciences, Uppsala, Sweden). Standard 2D gels were conducted first for each condition and were stained with Blue Silver Coomassie (Candiano et al., 2004) in order to obtain reference maps and spot-picking for mass spectrometry analyses. 2D-DIGE was then performed for each EPS and glucose cultures. The manufacturer's instruction of minimal dye protocol was adopted. Cultures of *B. fragilis* grown with glucose were labeled with Cy3 whereas cultures with each of the EPSs E44 and EPS R1 were labeled with Cy5. Gels were scanned in a Typhoon 9400 scanner (GE Healthcare) at a resolution of 100 μm and analyzed with the 2DImageMaster software (GE Healthcare). *t*-test were run between samples in each gel, and spots displaying statistical differences were excised from Coomassie stained gels and sent to the company Inbiotec (Leon, Spain) for digestion and identification by MALDI-TOF/TOF using standard protocols.

Gene Expression Analyses

Gene expression was determined by reverse transcription qPCR (RT-qPCR). Ten mL of *B. fragilis* cultures were collected at late exponential phase of growth, cells were mixed with RNA protective bacterial reagent (Qiagen GmbH, Hilden, Germany) and stored at −80°C until use. RNA extraction from cells was performed by phenol/chloroform treatment combined with physical lysis followed by the use of the RNeasy mini kit (Qiagen) as indicated by Ulve et al. (2008). The cDNA was obtained by reverse transcription of 1-μg total RNA using the kit "High Capacity cDNA Reverse Transcription" (Life Technologies, Alcobendas, Madrid). Real-time PCR was performed in an ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems) and expression levels were calculated by the ΔΔCt method as described previously (Gueimonde et al., 2007). The primers used in this study are listed in Supplementary Table S1, the 16S rRNA gene was used as endogenous control.

α-Glucosidase Activity

Two mL of culture were concentrated to 1 mL of PBS and CFE was obtained as described in the amino acid analysis section. Total protein determination was as indicated in the same section. The α-glucosidase activity was assayed both in culture supernatants and CFEs by determining the release of *p*-nitrophenol from the substrate *p*-NP α-D glucopyranoside, as described previously (Noriega et al., 2004). Specific activity in

both CFEs and supernatants was calculated relative to the amount of cell protein present in 1 mL of culture and was expressed as U/mg.

Estimation of the Redox Balance

The intracellular redox balance was determined by fluorescence spectroscopy. The fluorescence emissions of buffered cell suspensions at an OD_{600} of 0.6 in 50 mM Tris-HCl buffer pH 7.0 were monitored in an Eclipse fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA, USA). The intensity values corresponding to NAD(P)H were calculated from the 413-nm emission at an excitation wavelength (λex) of 316-nm, whereas for FAD the intensity values were calculated from the 436-nm emission at a λex of 380-nm, as described by Ammor et al. (2004). The redox ratio was deduced from the NAD(P)H- and FAD-related measurements using the equation: redox ratio = FAD intensity/(FAD intensity + NAD(P)H intensity) (Kirkpatrick et al., 2005). Intensity obtained from the suspension buffer was subtracted from those obtained from the samples.

Statistical Analysis

Statistical analyses were performed using the SPSS-PC software, version 19.0 (SPSS Inc., Chicago, IL, USA). One-way analyses of variance (ANOVA) were run to compare different parameters in culture media added with bifidobacterial EPS (E44 or R1) or glucose or in media without carbohydrates added externally. When appropriate, a *post hoc* least significant difference (LSD) comparison test was applied to determine differences among the different conditions assayed. In the case of the α-glucosidase activity, *t*-tests were run between samples in the different carbohydrates. Experiments were carried out in triplicate.

Results

Growth Pattern

Growth pattern of *B. fragilis* varied depending on the culture conditions (**Table 1**). The maximum OD_{600} (OD_{600} max) was significantly higher in cultures with glucose than in the other conditions whereas the lowest values for this parameter were obtained in cultures without carbohydrates added ($P < 0.05$). Considerably lower pH values were obtained in glucose, thus reflecting more acid production and a more active metabolism and growth with this sugar. For most of the experiments performed in this work, we have collected samples when cultures reached their OD_{600} max, which corresponds to late exponential phase (**Table 1**).

TABLE 1 | Parameter values of *Bacteroides fragilis* cultures grown in the presence of glucose, EPS E44, EPS R1 or without carbohydrate source added (WCS) at OD_{600} max.

	Glucose	E44	R1	WCS
OD_{600} max	1.16 ± 0.21^{c}	0.61 ± 0.06^{b}	0.47 ± 0.07^{b}	0.23 ± 0.02^{a}
Time to reach OD_{600} max (h)	21.71 ± 0.49	23.21 ± 0.28	24.44 ± 0.24	20.6 ± 0.53
pH at OD_{600} max	5.37 ± 0.14^{a}	6.92 ± 0.12^{b}	6.92 ± 0.04^{b}	6.87 ± 0.09^{b}

Different letters indicate significant differences between cultures with different carbohydrates ($P < 0.05$).

SCFA and Organic Acids Production Profile

Molar proportions of the different SCFA and organic acids produced by *B. fragilis* during growth were clearly different in cultures with additional glucose than in cultures with EPS or without external carbohydrates added (**Figure 1**). Acetic acid was the most abundant SCFA produced in the presence of glucose (33.23%) whereas under other culture conditions the highest molar proportions corresponded to propionic acid (42.31% in cultures with EPS E44, 40.68% in cultures with EPS R1, and 44.23% in cultures without external carbohydrates added). A clear decrease of succinic and formic acids production occurred in cultures with EPS (7.88 and 9.39% for succinic acid and 7.48 and 9.85% for formic acid in EPS E44, and EPS R1, respectively) and without external carbohydrates added (12.19 and 5.22% for succinic and formic acids, respectively) as compared to glucose (18.62% for succinic acid and 20.19% for formic acid, respectively; $P < 0.05$); this happens at the expense of an increase in molar proportions of propionic acid (from 23.13% in glucose to > 40% in the other conditions) and BSCFA (0.22% in glucose in contrast with >5.5% in EPS and without carbohydrates added; $P < 0.05$). Lactic acid appeared in cultures with additional glucose but it was not detected under other conditions. A considerably higher propionic to succinic acid ratio was obtained in cultures with EPS or without carbohydrates (5.68 ± 1.28, 4.43 ± 0.73, and 3.62 ± 0.56 for EPS E44 and R1 and for cultures without carbohydrates added vs. 1.33 ± 046 in glucose; $P < 0.05$). In contrast, *B. fragilis* growing in glucose presented higher acetic to propionic acids ratio (1.44 ± 0.17 in glucose vs. 0.87 ± 0.02 and 0.83 ± 0.08 for EPS E44 and R1, and 0.63 ± 0.04 for cultures without carbohydrates added; $P < 0.05$; **Figure 1**). The production of total SCFA plus organic acids was twice as high in cultures with glucose (19.7 ± 1.06 mM) than in cultures with EPS (8.12 ± 1.85 and 7.96 ± 0.95 mM for EPS E44 and R1, respectively) and was around twofold higher in the presence of these polymers than in media without the addition of carbohydrates (4.65 ± 0.39 mM).

α-Glucosidase Activity

Cell-free extract of *B. fragilis* incubated in the presence of EPS E44 and R1 displayed higher α-glucosidase activity than in the presence of glucose, where the amount of this enzymatic activity was negligible ($P < 0.05$; **Figure 2**). No remarkable α-glucosidase activity was found in the supernatants of cultures, neither in supernatants nor CFE of cultures incubated in the absence of carbohydrates added. This indicated that the α-glucosidase activity was mainly intracellular and its production was dependent on the presence of EPS in the culture medium.

Amino Acids and Ammonia Profiles

In culture supernatants of *B. fragilis* grown in the presence of EPS or in medium without additional carbohydrates, the levels of total free amino acids hardly increased, or even decreased

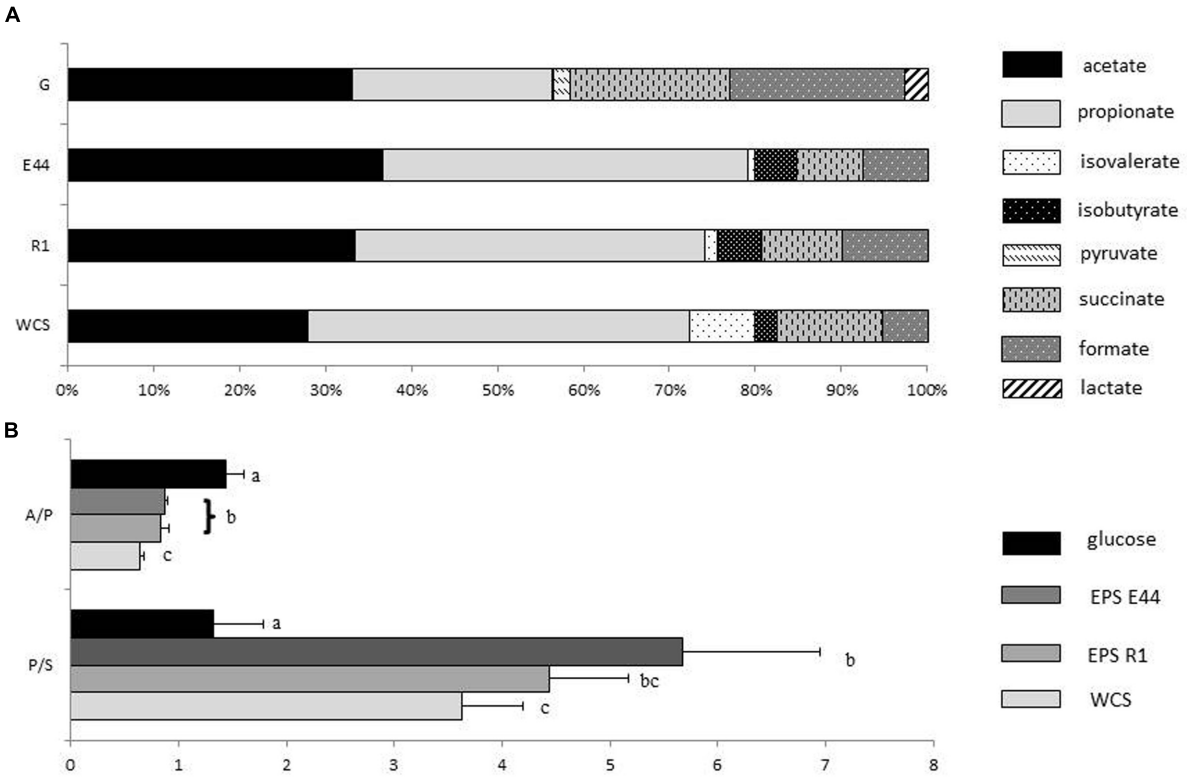

FIGURE 1 | (A) Molar proportions of SCFA, and organic acids produced by *Bacteroides fragilis* grown in glucose (G), EPS E44, and EPS R1 and without carbohydrates added (WCS). Acetate ■; propionate ■; isovalerate ■; isobutyrate ■; pyruvate ■; succinate ■; formate ■; lactate ■. **(B)** Acetate to propionate ratio (A/P) along with propionate to succinate ratio (P/S) of *Bacteroides fragilis* grown in glucose, EPS E44, EPS R1, and WCS. Glucose ■, E44 ■, R1 ■, WCS ■. Letters indicate significant differences among cultures with the different carbohydrates ($P < 0.05$). Error bars represent standard deviations. Total metabolite production in glucose: 19.7 ± 1.06, EPS E44: 8.12 ± 1.85, EPS R1: 7.96 ± 0.95, and WCS:4.65 ± 0.39 mM.

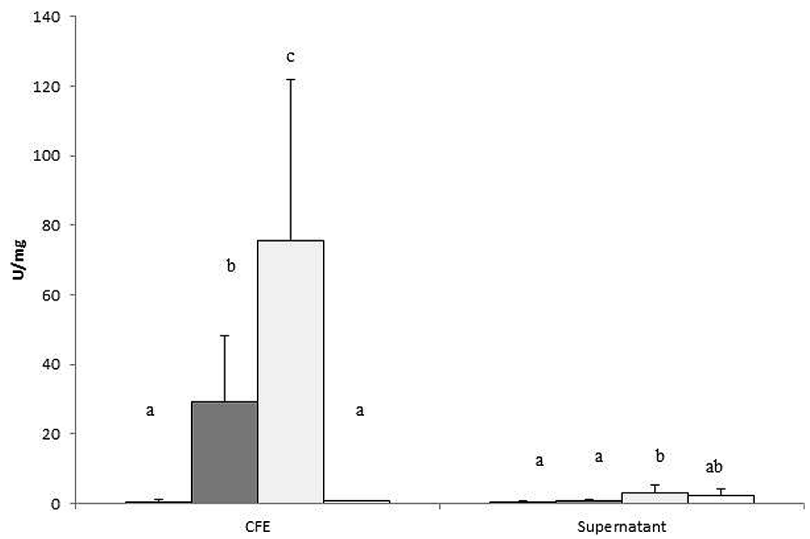

FIGURE 2 | α-glucosidase activity was determined in cell-free extract (CFE) and supernatants of *B. fragilis* grown in glucose (black bars), EPS E44 (dark gray bars), EPS R1 (light gray bars) and without carbon source added (WCS; white bars), and was referred to the amount of protein present in 1 mL of cell culture and expressed as U/mg protein. Different letters indicate significant differences among cultures with the different carbohydrates ($P < 0.05$). Error bars represent standard deviation.

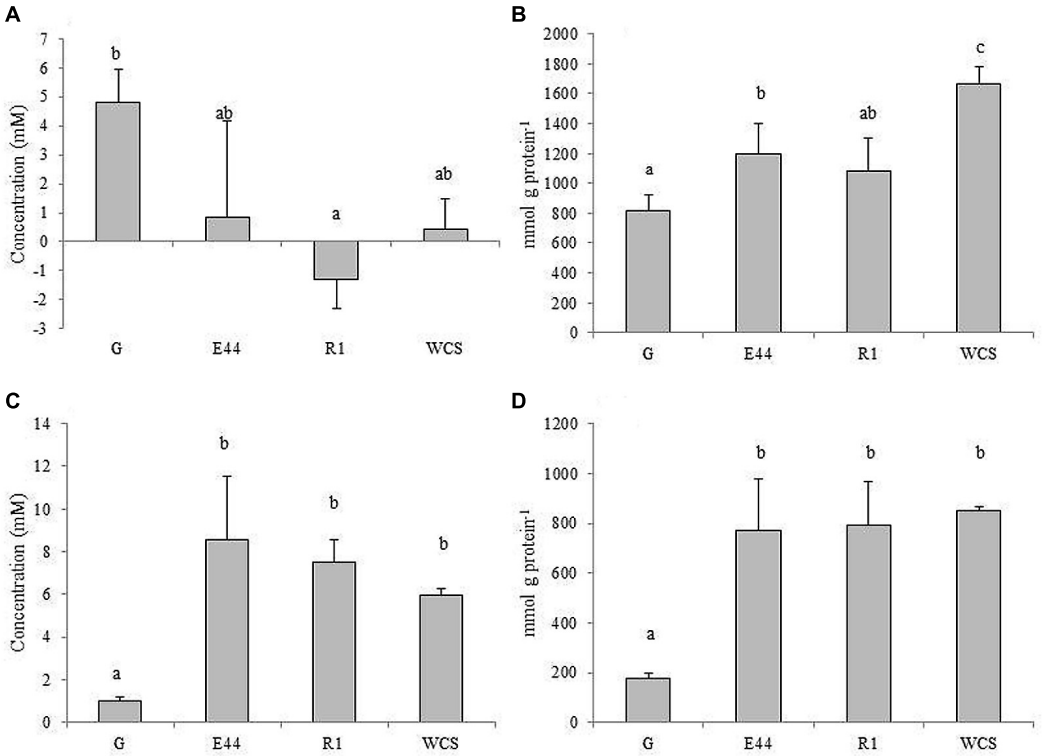

FIGURE 3 | Total amino acid concentrations in culture supernatants (A), and CFEs (B) along with ammonia levels in supernatants (C) and CFEs (D) of *B. fragilis* DSMZ2151 grown in glucose (G), EPS E44, EPS R1 and in medium without carbohydrate source (WCS). Different letters indicate significant differences among cultures with the different carbohydrates ($P < 0.05$). Error bars represent standard deviation.

after incubation, whereas with glucose the concentration of total amino acids clearly rose for the same conditions ($P < 0.05$; **Figure 3A**). In CFE, although levels of free amino acids per mg of cellular protein augmented in all cases, the most pronounced increases occurred in the absence of exogenous carbohydrates. Cultures in the presence of additional EPS displayed intermediate concentrations with respect to the other two conditions (**Figure 3B**). Levels of ammonia increased more in supernatants of cultures with EPS or without carbohydrates added than in cultures with glucose ($P < 0.05$; **Figure 3C**). Intracellular content of ammonia followed the same trend as the supernatants, with a higher accumulation in CFE of *B. fragilis* grown in the absence of carbohydrates and in media amended with EPS, than with glucose ($P < 0.05$; **Figure 3D**). All these factors taken together indicated that, proportionally, more amino acids are taken up by cells, and a concomitant release of ammonia took place when *B. fragilis* was grown in medium supplemented with EPS or in the absence of carbohydrates, as compared with glucose. Intracellular accumulation of amino acids seems to occur to a higher extent in media with EPS or without carbohydrates than in the medium containing glucose ($P < 0.05$).

In order to better understand these phenomena, the profile of amino acid concentrations was analyzed by UHPLC (Supplementary Table S2). Regardless of the culture conditions, specific amino acids were removed to variable extents, from the external medium, whereas others seemed to accumulate. The intracellular level of the different amino acids also differed. Notably, the carbohydrates present in the culture medium affected the profile of some amino acids differently. Thus, glutamic acid, glycine, threonine, GABA, tyrosine, and ornithine accumulated in the supernatants of cultures in the presence of glucose, whereas they were removed or accumulated at significantly lower levels in the presence of one or the two EPSs or in the absence of additional carbohydrates ($P < 0.05$). Conversely, with EPS or without exogenous carbohydrates, lysine and histidine, along with other amino acids, were found in supernatants at significantly higher concentrations than in cultures with glucose ($P < 0.05$). In addition, the intracellular content of six amino acids (aspartic acid, glutamic acid, tyrosine, valine, isoleucine, and lysine) appeared significantly augmented in the absence of carbohydrates added compared to cultures supplemented with glucose or EPS ($P < 0.05$). This indicated a shift in the amino acid metabolism by *B. fragilis* influenced by the type of carbohydrates available in the culture medium.

Further experimentation focused on *B. fragilis* growing in media in the presence of EPS or glucose as we aimed to ascertain the metabolic differences under these two conditions.

Proteomic Analysis of *B. fragilis* Growing in the Presence of Different Carbohydrates

The protein pool of *B. fragilis* was analyzed by means of 2D-DIGE and the proteome of the microorganism growing in the

TABLE 2 | Identification of *B. fragilis* DSMZ 2151 proteins affected by carbohydrate sources.

COG orthology	Spot no.[a]	Putative function[b]	GI number[c]	Mass[d]	pI[d]	MASCOT score	No. of peptides matched[e]	Cov.	Change fold[f]	
									E44	R1
Carbohydrate transport and metabolism	78	Transketolase	gi| 265763039	72.4	5.5	594	26	52	ND	2.48
	79	Transketolase	gi| 265763039	72.4	5.5	401	21	42	2.32	4.04
	81	Alpha-glucosidase	gi| 265762646	82.1	5.7	743	30	54	7.08	2.97
	87	Pyruvate phosphate dikinase	gi| 53713829	100.6	5.4	564	31	39	3.46	2.37
	89	Pyruvate phosphate dikinase	gi| 53713829	100.6	5.4	410	37	39	3.19	4.39
	90	Pyruvate phosphate dikinase	gi| 53713829	100.6	5.4	629	36	52	3.04	3.86
	105	Pyruvate phosphate dikinase	gi| 60682047	100.6	5.5	840	35	49	3.26	2.67
	101	Galactokinase	gi| 53712943	43.5	5.2	728	22	52	ND	2.29
Lipid transport and metabolism	82	Methylmalonyl-CoA mutase, large subunit	gi| 53715084	79.2	5.6	458	23	43	4.56	2.51
Amino acid transport and metabolism	3	Oxidoreductase (diaminopimelate dehydrogenase)	gi| 60682941	32.5	6.2	938	25	90	−2.06	−2.84
	9	NAD(P)H-utilizing glutamate dehydrogenase	gi| 60682871	49.1	6.1	526	19	53	−6.2	−7.37
	10	NAD(P)H-utilizing glutamate dehydrogenase	gi| 60682871	49.1	6.1	461	16	42	−6.17	−7.60
	11	NAD(P)H-utilizing glutamate dehydrogenase	gi| 60682871	49.1	6.1	430	17	44	−5.50	−7.19
	28	Ketol-acid reductoisomerase	gi| 53715042	38.2	5.2	1100	14	58	ND	−2.48
	62	Acetolactate synthase, large subunit, biosynthetic type	gi| 392698324	61.7	5.1	209	12	25	−2.45	−2.15
Post-translational modification, protein turnover, chaperones	12	Serine protease	gi| 53714036	54.7	6.5	296	14	30	−2.12	−2.30
Translation, ribosomal structure and biogenesis	36	Translation elongation factor 1A (EF-1A/EF-Tu)	gi| 319900910	43.7	5.3	260	8	23	ND	−4.76
	37	Elongation factor Tu	gi| 53715484	43.8	5.2	354	11	32	−2.96	−6.87
	83	Elongation factor G	gi| 265766981	80.4	5.7	489	24	38	2.59	2.83
	84	Elongation factor G	gi| 53715151	80.4	5.6	775	37	51	2.55	2.82
	103	Elongation factor G	gi| 53715468	77.9	5.1	712	23	44	ND	−2.87
	95	50S ribosomal protein L14	gi| 301161079	13.2	9.9	160	6	58	-	−2.03
	99	Translation initiation inhibitor	gi| 53714265	13.1	5.0	88	3	25	-	3.60
Cell wall, membrane, envelope biogenesis	110	Major outer membrane protein OmpA	gi| 53715321	43.3	8.7	361	15	37	ND	4.77
Inorganic ion transport and metabolism	108	Ferritin	gi| 53714336	18.1	5.0	692	12	71	2.35	2.00
Function unknown	39	Hypothetical protein BF2494	gi| 60681974	45.8	5.2	545	18	43	2.30	3.28
	41	Hypothetical protein BF2494	gi| 60681974	45.8	5.2	590	18	42	2.87	4.04
	43	Hypothetical protein BF2494	gi| 60681974	45.8	5.2	574	18	40	2.90	3.75
	92	Hypothetical protein BSHG_4061	gi| 383118833	16.3	5.1	163	8	60	−3.74	ND
	107	Hypothetical protein BF2537	gi| 53713828	20.1	6.3	533	13	79	ND	2.24

[a]*Spot numbers refer to the proteins labeled in 2D-DIGE gels.*
[b]*Putative functions were assigned from the NCBI gene database.*
[c]*GI number in the NCBI database for B. fragilis DSMZ 2151.*
[d]*As given by the NCBInr database for B. fragilis DSMZ2151. Molecular masses are expressed in kilodaltons.*
[e]*Number of tryptic peptides observed contributing to the percentage of amino acid coverage.*
[f]*Normalized change fold for each protein production derived from cells grown in EPS E44 or R1 with respect to the protein derived from cells grown in glucose. ND, ratio below 2; -, spot not detected.*

presence of each of the two EPS was compared with the proteome of the microorganism in glucose (**Table 2**). We focused only on spots corresponding to proteins displaying statistical differences ($P < 0.05$) and production ratios in EPS greater or lower than twofold compared to glucose alone, in at least one of the two polymers. According to this criterion, the production of 10 proteins was found downregulated in cultures with one or both EPSs whereas 13 proteins were upregulated. Cultures of *B. fragilis* with additional EPS R1 presented more variation in the protein-production levels with respect to glucose than cultures with EPS E44. Thus, three proteins were underproduced in the presence of the EPS R1, but not with EPS E44, whereas a unique protein

was downregulated with EPS E44 but not with EPS R1; four proteins were found upregulated only in the presence of EPS R1, but not with EPS E44 whilst the contrary was never observed. The presence of some protein spots with the same GI number and molecular mass, but differing in their experimental isoelectric points, suggested the presence of isoforms. Proteins and enzymes whose production was affected by the carbohydrates available in the culture medium belong to several functional COG categories (**Table 2**).

Figure 4 depicts a schematic representation of the main affected metabolic pathways of *B. fragilis* and the proteins involved. As shown in **Table 2**, several enzymes related to the metabolism of carbohydrates were upregulated in the presence of one or two EPS: alpha-glucosidase and galactokinase-enzymes related to the release of 1→4 α-D-glucose from complex carbohydrates and with the phosphorylation of D-galactose facilitating its entry into several metabolic routes, respectively; transketolase-enzyme from the pentose phosphate pathway that catalyzes the transfer of aldehyde or ketonic groups between monosaccharides of different carbon residues; and the pyruvate phosphate dikinase-catalyzing the interconversion of phosphoenolpyruvate (PEP) and pyruvate. The overproduction of α-glucosidase was consistent with the higher enzymatic activity found in CFE of *B. fragilis* grown with EPS added (**Figure 2**).

Several proteins participating in the metabolism of different amino acids, some of which catalyze redox reactions, were underproduced in cultures of *B. fragilis* when incubated with EPS fractions: diaminopimelate dehydrogenase–oxidoreductase participating in one of the two redundant pathways leading to the formation of lysine and diaminopimelic acid; NAD(P)H-dependent glutamate dehydrogenase–oxidoreductase participating in the assimilation of ammonia to form glutamate; ketol-acid reductoisomerase and acetolactate synthase-two enzymes, oxidoreductase and transferase respectively, involved in the biosynthesis of isoleucine, valine, and leucine. Concomitantly, several proteins related to the translation and elongation steps of protein synthesis, including ribosomal proteins and elongation factors, were underproduced in the presence of one or both EPS. At the same time a translation initiation inhibitor was overproduced in the presence of EPS R1 (**Table 2**). These metabolic changes suggest a modification of the metabolism of amino acids, proteins, and peptides in *B. fragilis* when the microorganism is grown in the presence of bifidobacterial EPS fractions as compared to glucose.

Other proteins whose production was affected by carbohydrates available in the culture medium include: (i) the large subunit of the methylmalonil-CoA mutase-enzyme participating in the synthesis of propionate from succinate, the production of which was enhanced with EPS fractions, (ii) the major outer membrane protein OmpA which was overproduced only with EPS R1, and (iii) ferritin which was overproduced in the presence of both EPSs (**Table 2**). Additionally, several hypothetical proteins of unknown function in *B. fragilis* were differentially produced in the presence of one or two EPS in the culture medium. Possibly, proteins BF2494, BF2537, and BSGH_4061 according to BLAST homology with membrane proteins with TRP domain, a histidine kinase membrane protein and heat-shock protein respectively, in other *Bacteroides* species (data not shown).

Changes in Gene Expression by RT-qPCR

The relative expression of genes coding for some proteins selected on the basis of the 2D-DIGE experiments was further analyzed by qRT-PCR (Supplementary Figure S1). The genes for the NAD(P)H-dependent glutamate dehydrogenase (*gdhB*) and the acetolactate synthase (*ilvB*) showed downregulation in the presence of both EPSs, whereas the gene coding for the major outer membrane protein OmpA (*ompA*) and the pyruvate phosphate dikinase (*ppdK*) displayed increased expression under the same conditions. These results are in agreement with the differential protein production found by proteomic analyses. The transketolase gene (*tktB*) appeared upregulated in the presence of the EPS E44 and slightly downregulated with EPS R1, which partly supports the behavior found for the corresponding protein in 2D-DIGE experiments. However, in spite of the clearly higher molar proportions of propionate obtained by us in cultures of *B. fragilis* in the presence of EPS E44 and R1 and the enhanced production of the methyl-malonyl-CoA mutase enzyme in such conditions, only a moderate overexpression and a slight underexpression of the corresponding gene (*mutB*) were respectively obtained in cultures of *B. fragilis* with EPS E44 and EPS R1 at the OD_{600} max of cultures. Attempts to analyze the expression of *mutB* at earlier points during growth proved unsuccessful due to the lack of sufficient RNA for RT-qPCR determinations (data not shown).

In addition, we assessed the expression of three genes playing a key role in the entry of PEP/pyruvate into the succinate-propionate pathway in *Bacteroides* (**Figure 4** and Supplementary Figure S1) and which did not display detectable changes in 2D-DIGE gels. One of them, the PEP carboxykinase (*pckA*) is responsible for the carboxylation of PEP to oxaloacetate with ATP formation. Pyruvate carboxylase (*pyc*) catalyzes the synthesis of oxaloacetate by carboxylation of pyruvate with ATP consumption, and malate dehydrogenase (*mdh*) catalyzes the formation of malic acid from pyruvate with consumption of reducing power. *PckA* was underexpressed in the presence of both EPS, whereas *pyc* was overexpressed and a slight overexpression or underexpression was respectively found for *mdh* with EPS E44 and R1.

Intracellular Redox Balance

The redox ratio was significantly lower ($P < 0.05$) in cultures added with EPS E44 and R1 than in cultures in the presence of glucose, indicating a more reduced intracellular state of *B. fragilis* in the former two conditions. The NAD(P)H-associated fluorescence was significantly higher ($P < 0.05$) in cultures of *B. fragilis* ran with EPS fractions than in cultures with glucose whereas no significant differences ($P > 0.05$) were found in the fluorescence associated to FAD between cultures with EPS and glucose (**Table 3**). This indicated that the lower redox ratio found in our cultures when *B. fragilis* was grown in the presence of EPS was mainly due to an increase of the pool of intracellular NAD(P)H rather than to variations in FAD intracellular levels.

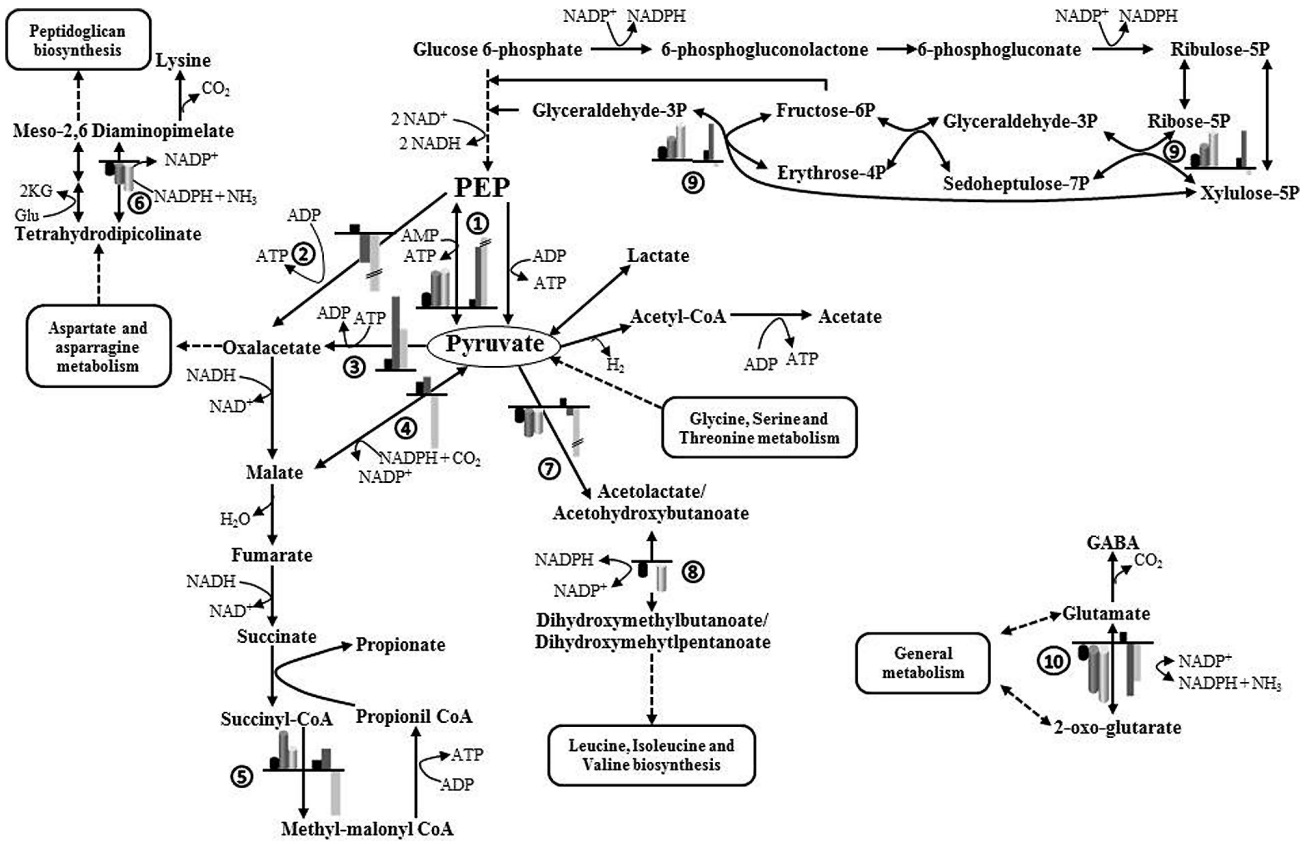

FIGURE 4 | Schematic representation of the main metabolic pathways affected by the availability of carbohydrates added to the culture medium (glucose, EPS E44, and EPS R1) during growth of *B. fragilis*. Production ratios of enzymes catalyzing different steps in the presence of EPS with respect to glucose are indicated by cylindrical bars. Fold changes in the expression of genes in the presence of EPS with respect to glucose are indicated by rectangular bars. Black bars, glucose; dark gray bars, EPS E44; light gray bars, EPS R1. Ratios greater than sevenfold are shown with a height break. Enzymes: (1) pyruvate phosphate dikinase, (2) PEP carboxykinase, (3) pyruvate carboxylase, (4) malate dehydrogenase, (5) methyl-malonylCoA mutase, (6) diaminopimelate dehydrogenase, (7) acetolactate synthase, (8) ketol-acid reductoisomerase, (9) transketolase, (10) NADPH-dependent glutamate dehydrogenase. 2KG, 2-oxoglutarate; Glu, glutamate; PEP, phosphoenolpyruvate.

TABLE 3 | Intracellular redox ratios of NAD(P)H- and FAD fluorescence associated with *B. fragilis* grown in the presence of additional glucose, EPS E44 and EPS R1.

	Glucose	EPS E44	EPS R1
Redox ratio	0.70 ± 0.03^{c}	0.59 ± 0.06^{b}	0.46 ± 0.09^{a}
Fluorescence (NAD(P)H)	2.89 ± 0.99^{a}	7.07 ± 0.93^{b}	9.21 ± 0.90^{c}
Fluorescence (FAD)	6.30 ± 1.71	10.38 ± 2.97	8.31 ± 3.48

Letters indicate significant differences between cultures with different carbohydrates ($P < 0.05$). The redox ratio was calculated according to the formula: $\frac{FAD^{+}}{FAD^{+}+NAD(P)H}$.

Discussion

The majority of *B. fragilis* metabolic studies go back 2–3 decades ago, and in those studies the influence of external nutritional conditions were not considered. Under the culture conditions used in the present study, *B. fragilis* was able to grow in the presence of glucose and bifidobacterial EPS, the pH decrease in the culture medium being more pronounced with glucose than with the bacterial polymers. The slow growth of *B. fragilis* in the presence of EPS and the need of enough biomass and comparable cell counts for proteomic analyses, prompted us to choose late exponential phase for most of the experiments in this study (growth curves shown in Rios-Covian et al., 2013).

Molar proportions of propionic acid in cultures of *B. fragilis* in the presence of EPS or in the absence of additional carbohydrates were considerably higher than in cultures with glucose, which occurred with a concomitant reduction in the proportion of succinic, formic, and lactic acids. These results essentially confirmed our previous findings and those of other authors indicating that the ratio of propionic to succinic acid in cultures of *Bacteroides* at advanced stages of growth is higher in complex carbon sources or under carbohydrate-shortage conditions than in medium with glucose (Kotarski and Salyers, 1981; Rios-Covian et al., 2013; Adamberg et al., 2014). In addition, the higher production of SCFA and organic acids in cultures with EPS compared to cultures without carbohydrate supplementation suggests a possible utilization of these polymers as fermentable substrates (Rios-Covian et al., 2013).

The α-D-glucosidase is one of the most abundant glycoside hydrolases produced by *B. fragilis*; the enzyme, located in the periplasmic space (Berg et al., 1980), catalyzes the hydrolysis of terminal, non-reducing (1→4) linked α-D glucose from complex carbohydrates. We found that the α-glucosidase activity was virtually absent both in culture supernatants and in CFEs when grown in glucose or in the absence of additional carbohydrates, but was present in CFEs from *B. fragilis* cultured in the presence of EPSs. The EPS 44 fraction contains two polymers of different molar mass which are composed of glucose and galactose in proportion 1:1 (Salazar et al., 2009a) whereas EPS R1 is formed by three polymers of different molar mass which are composed by glucose, galactose, and rhamnose in proportions 1:1:1.5 (Ruas-Madiedo et al., 2010). Since both EPS fractions contain glucose in their composition (Salazar et al., 2009a; Ruas-Madiedo et al., 2010), α-glucosidase may be involved in the utilization of these EPS fractions by *B. fragilis*. Proteomics confirmed the overproduction of the α-glucosidase in the presence of both EPSs and also revealed that galactokinase, another enzyme related to the metabolism of carbohydrates, was overexpressed in the presence of EPS R1. The overproduction of these two enzymes suggests an expansion in the metabolic ability of *B. fragilis* to take advantage of complex carbohydrates when the microorganism is grown in the absence of readily fermentable carbohydrates. To this respect it is worth mentioning that galactose is one of the most abundant monosaccharides in intestinal mucin (Robbe et al., 2003) whereas starch (backbone of 1→4 α-D-glucose) is the main source of complex carbohydrates in the human diet.

Amino Acids Metabolism

Bacteroides can produce BCFA from amino acids and proteins (Macfarlane et al., 1991; Smith and Macfarlane, 1998) and recent data showed that *B. fragilis* can release them in the presence of EPS (Rios-Covian et al., 2013). Remarkably, isovaleric and isobutyric acids, originating from the catabolism of leucine and valine respectively, were formed in *B. fragilis* cultures with EPS but not glucose. Our experimental results also point to an enhanced consumption of the pool of amino acids present in the culture medium and a higher release of ammonia in the presence of EPS as compared to glucose. The high removal of asparagine from supernatants and the absence of residual asparagine and alanine in CFEs indicated the efficient conversion of asparagine to aspartic acid rendering alanine that is finally transformed to pyruvic acid, under all culture conditions tested. An enhanced removal of threonine from the culture medium also occurred in the presence of EPS, whereas glycine accumulated at lower levels than in the presence of glucose. Glycine and serine are intermediate in the degradation of threonine toward their incorporation at the level of pyruvate; therefore these three amino acids may serve as a source of pyruvate for *B. fragilis* grown in the presence of EPS. Moreover, the enzymes ketol-acid reductoisomerase and acetolactate synthase, that participate in the biosynthetic pathway of isoleucine, leucine (amino acids removed from the culture medium by *B. fragilis* under all conditions) and valine from pyruvate were found to be underproduced in EPS as compared to glucose. These facts suggest that in *B. fragilis* the metabolism of some amino acids rendering pyruvate, a key intermediate of central catabolism, was activated whereas the synthesis of those using pyruvate as a precursor may be partly inhibited in the presence of bacterial EPS. Interestingly, threonine is a major component of the human intestinal mucin, together with asparagine and serine (Aksoy and Akinci, 2004). This result points out an adaptation of *B. fragilis* metabolism for improving utilization of intestinal mucin, as previously reported by Adamberg et al. (2014) for *Bacteroides thetaiotaomicron* subjected to amino acid starvation.

A clear underproduction of the enzyme NAD(P)H-dependent glutamate dehydrogenase was found in the presence of EPS. *B. fragilis* possesses two distinct glutamate dehydrogenases that play a fundamental role in nitrogen assimilation (Yamamoto et al., 1987; Baggio and Morrison, 1996). The NAD(P)H-dependent glutamate dehydrogenase catalyzes the assimilation of ammonia by reductive amination of α-ketoglutarate to form L-glutamate, whereas the NADH-dependent glutamate dehydrogenase catalyzes the reverse reaction. The NADH-dependent enzyme displays a basal activity that increases at high organic nitrogen concentration whereas in such conditions the NAD(P)H-dependent enzyme was inhibited (Abrahams and Abratt, 1998). In addition, glutamic acid is a metabolic intermediate in the synthesis of different amino acids and other compounds, its concentration in CFE being the highest found by us under all conditions assayed. It is then plausible that the catabolic reaction in the direction of ammonia and α-ketoglutarate formation from glutamate would predominate in cultures with EPS, as compared to cultures performed with glucose. The α-ketoglutarate formed could then serve as a substrate for transamination reactions in *B. fragilis* (Abrahams and Abratt, 1998). On the other hand, GABA was present in significantly lower levels in the culture supernatants of *B. fragilis* incubated with additional EPS, as compared with the cultures in the presence of glucose. GABA is formed from glutamate in a single reaction step mediated by a glutamate decarboxylase enzyme. An acid resistance mechanism has been described in some intestinal bacteria in which a molecule of extracellular glutamate is antiported with an intracellular proton and converted to GABA that is subsequently exchanged for another extracellular glutamate (Cotter et al., 2001). Likely, under mild acidic conditions occurring in cultures with EPS as compared to glucose, the glutamate decarboxylase pathway is not being used by *B. fragilis,* thus GABA is not formed.

Aspartic acid is a precursor in the biosynthetic pathway of lysine and meso-diaminopimelic acid. Lysine takes part of the pentapeptide bridge of the peptidoglycan in Gram-positive bacteria, and is replaced by meso-diaminopimelic acid in Gram negatives. *B. fragilis* has two redundant biosynthetic pathways for the synthesis of meso 2,6-diaminopimelate: one is catalized by the oxidorreductase diaminopimelate dehydrogenase with consumption of ammonia and NADPH and the other occurs in two steps, catalyzed by an aminotransferase and an epimerase, respectively. The diaminopimelate dehydrogenase was found underproduced by proteomic analyses when *B. fragilis* was grown in the presence of EPS, thus favoring in such conditions the alternative route which generates a molecule of α-ketoglutarate from glutamate to form 2,6-diaminopimelate (Hudson et al.,

2011). The α-ketoglutarate could then serve as substrate for cellular transamination reactions, as commented before. Our results also support the idea that the amino acids are more efficiently removed by *B. fragilis* from the culture medium under shortage conditions of readily fermentable carbohydrates, as would be the case when this microorganism is incubated with EPS. In this respect, the strong influence that available carbohydrates exert on peptide and amino acids metabolism in *Bacteroides* and other intestinal bacteria is known (Macfarlane et al., 1991; Smith and Macfarlane, 1998; Adamberg et al., 2014).

Finally, some proteins involved in translation, ribosomal structure, and biogenesis were underproduced whereas a translation initiation inhibitor was upregulated in *B. fragilis* in the presence of EPS as compared to glucose, indicating a slowdown of the protein synthesis in this bacterium. Under these conditions the intracellular pool of amino acids can be maintained by transamination reactions involving α-ketoacids and amino acids collected from the culture medium.

Energetics, Metabolism, and Redox Balance

Using proteomics we could not find variations in the production of glycolytic enzymes. However, overproduction of pyruvate phosphate dikinase was confirmed by proteomics and gene expression analyses. This enzyme catalyzes the interconversion of PEP and pyruvate, with ATP formation in the forward direction. Although pyruvate phosphate dikinase functions in the gluconeogenesis direction in several organisms, the pyruvate formation seems to be favored at moderate acidic conditions in *Bacteroides symbiosus* (optimum pH in the forward direction 6.6 and 7.2-7.8 in the reverse direction; Reeves, 1971), as may also occur in some parts of the human colon ecosystem (Vertzoni et al., 2010). The dominant route of microbial propionate synthesis in the gut is the succinate–propionate pathway via formation of methyl-malonyl-CoA, which is present in *Bacteroides* (Reichardt et al., 2014). In our case, it seems that when *B. fragilis* is growing in glucose, a significant part of PEP would be converted to oxaloacetate, malate, and pyruvate being then formed by decarboxylation of oxaloacetate (Macy et al., 1978). However, we found that the gene coding for PEP carboxykinase (catalyzing carboxylation of PEP to oxaloacetate) was under-expressed when *B. fragilis* was grown in EPS as compared to glucose; in such conditions the pyruvate carboxylase (catalyzing carboxylation of pyruvate to oxaloacetate) was over-expressed and the malate dehydrogenase displayed slight variation in its expression in cultures with EPS when compared to cultures with glucose. Taking into account that an increase in the degradation of amino acids leading to pyruvate and a partial inhibition of the synthesis of amino acids from pyruvate seems to occur in *B. fragilis* cultured in the presence of EPS, a relative accumulation of pyruvate and a relative shortage of PEP originating from carbohydrates may happen under such conditions. Therefore, it is plausible that in the presence of EPS a significant part of oxaloacetate would be formed from pyruvate. The pentose-phosphate pathway generates pentoses and reducing power in the form of NADPH. In the presence of glucose, the succinate-propionate pathway keeps the intracellular redox balance of NAD^+/NADH + H^+ through the reoxidation of two moles of NADH generated in the conversion of glucose to PEP in glycolysis. However, under conditions of carbohydrate shortage and enhanced amino acid removal from the medium, as we propose occurs when *B. fragilis* grows in a BM with additional EPS, a redox imbalance may happen due to insufficient reducing power supply from glycolysis. Under such conditions, the main input for intermediates of the succinate–propionate pathway would be the amino acids incorporated at the level of pyruvate and therefore a relative discompensation between NADH/NADPH may occur. The flux enhancement through the pentose-phosphate pathway leads to NADPH synthesis and overexpression of transketolase in cultures with EPS, providing the additional reducing power needed to maintain the cellular redox balance while accomplishing essential metabolic functions. The lower redox ratio associated with higher levels of NADH that we found in cells of *B. fragilis* grown with EPS, agrees with this hypothesis. Moreover, a metabolic redirection of NADH to NADPH has been shown in *Pseudomonas fluorescens* under oxidative stress (Singh et al., 2008). The metabolic ability to inter-convert both nicotinamide dinucleotides, although not yet demonstrated in *Bacteroides*, may ensure keeping the intracellular NADH and NADPH pools as an adaptation to external stimuli.

The Unknown Balance between Beneficial and Detrimental Effects in the Intestinal Ecosystem

A ferritin was overproduced in *B. fragilis* grown in the presence of EPS as compared to glucose. Ferritin-like proteins sequestrate iron in response to the presence of oxygen, playing an important role in the pathogenicity of this bacterium (Rocha and Smith, 2013). On the other hand, the major outer membrane protein OmpA was overproduced in cultures of *B. fragilis* grown with EPS R1. In other microorganisms, OmpA functions as a porin that enables the passage of nutrients and different compounds into the cell (Reeves et al., 1996, 1997). OmpA has also been implicated in cell functions related with pathogenicity (Soulas et al., 2000; Smith et al., 2007). In *B. fragilis*, the involvement of OmpA1 in maintaining cell structure as well as in the release of cytokines by murine splenocytes has been demonstrated (Magalashvili et al., 2008; Wexler et al., 2009). Whether OmpA overproduction is related with an improved ability of *B. fragilis* to obtain nutrients from the environment, and whether this is related with beneficial or detrimental effects for the host is, at present, unknown.

Conclusion

The results presented provide an insight into the physiological and molecular mechanisms that allow *B. fragilis* to adapt to an environment where EPSs are the main source of carbohydrates. Protein expression with different functions was modulated, and the intracellular and extracellular pattern of given amino acids, redox balance, and α-glucosidase activity were affected by the external environment. Three main events, namely the activation of amino acid catabolism, enhancement of the transketolase reaction from the pentose-phosphate pathway and the activation of the succinate–propionate pathway for propionic formation,

suggest a metabolic shift in this bacterium toward the generation of more reducing power, and to optimize the ATP yield. These changes would represent a metabolic adaptation of *B. fragilis* to take advantage of carbohydrates and proteins available in the intestinal environment, such as mucin. Recent results from our group indicated that in the presence of EPS, propionic acid production increased in human fecal cultures (Salazar et al., 2008), whereas the growth of bifidobacteria was enhanced by *B. fragilis* (Rios-Covian et al., 2013). Both effects are generally considered as health promoters in the human gut, but other potentially harmful effects from the couple EPS/*B. fragilis* cannot be ruled out and deserve further investigation. Our work has revealed a number of intriguing topics for future research regarding the relationship of probiotics and prebiotics with the symbiont or mutualistic microbial populations of the human intestinal ecosystem.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00825

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Bacteroides fragilis metabolises exopolysaccharides produced by bifidobacteria

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Abstract: **Background.** *Bacteroides fragilis* is the most frequent species at the human intestinal mucosal surface, it contributes to the maturation of the immune system although is also considered as an opportunistic pathogen. Some *Bifidobacterium* strains produce exopolysaccharides (EPS), complex carbohydrate polymers that promote changes in the metabolism of *B. fragilis* when this microorganism grows in their presence. To demonstrate that *B. fragilis* can use EPS from bifidobacteria as fermentable substrates, purified EPS fractions from two strains, *Bifidobacterium longum* E44 and *Bifidobacterium animalis* subsp. *lactis* R1, were added as the sole carbon source in cultures of *B. fragilis* DSMZ 2151 in a minimal medium. Bacterial counts were determined during incubation and the evolution of organic acids, short chain fatty acids (SCFA) and evolution of EPS fractions was analysed by chromatography.

Results. Growth of B. fragilis at early stages of incubation was slower in EPS than with glucose, but the presence of the polymer increased microbial survival later on. A shift in the metabolism of B. fragilis occurred from early to late stages of incubation optimizing cell energy production while keeping the intracellular redox balance. The amount of the two peaks with different molar mass of the EPS E44 clearly decreased along incubation whereas a consumption of the polymer R1 was not so evident.

Conclusions. This report demonstrates that *B. fragilis* can consume some EPS from bifidobacteria, with a concomitant release of SCFA and organic acids, suggesting a role for these biopolymers in bacteria-bacteria cross-talk within the intestine.

***Keyword: Bacteroides fragilis, Exopolysaccharides, Bifidobacterium, MALLS, Heteropolysaccharides*.**

BACKGROUND

The colonic microbiota is a complex community whose metabolic activity influences our health and nutritional status through diverse pathways [1]. Microbiota of adult healthy people is dominated by the phyla Firmicutes and Bacteroidetes, even though the composition at the species level is highly variable among individuals [2, 3]. Members of this microbiota are able to degrade complex polysaccharides and, therefore, genes involved in the degradation and consumption of these compounds are widespread among the genomes of microbiota-resident species [4, 5]. In this respect, the genus *Bacteroides* has the ability to use a wide range of carbohydrates, this ability varying as depending on the species considered [6]. *Bacteroides* is an anaerobic, bile-resistant, non-spore forming, and Gram negative rod [7] that accounts for up to 20-50% of the total microbiota in most individuals [8]. *Bacteroides fragilis* is the most frequent species at the mucosal surface [9] and can contribute to the development and maturation of the host immune system [10]. This species has an extraordinarily good adaptability to environmental changes due to its capacity to regulate the cell surface [11]. Consequently *B. fragilis* is the clinical isolate most frequently found in systemic infections, this microorganism being then considered as an opportunistic pathogen [7].

Exopolysaccharides (EPS) are complex carbohydrates located outside the cell; some *Bifidobacterium* strains, as well as many other microorganisms, are able to produce these polymers [12]. The synthesis of these compounds in the intestine has not been demonstrated yet. However, it has been proven *in vitro* that the presence of bile, which is released to the small intestine during digestion, stimulates the production of EPS by bifidobacteria [13]. EPS could be constituted either by a unique type of monomer, named as homopolysaccharides (HoPS), or by more than one monosaccharide type, then known as

heteropolysaccharides (HePS). All bifidobacterial EPS characterized until present are HePS [14].

Differential growth of members from the genus *Bacteroides* in the presence of EPS isolated from *Lactobacillus* and *Bifidobacterium* strains has been previously reported [6, 15, 16]. Particularly, two EPS fractions isolated from *Bifidobacterium longum* E44 and *Bifidobacterium animalis* subsp. *lactis* R1, have shown the capacity to act as fermentable substrates by the intestinal microbiota, thus promoting the increase of *Bacteroides* populations in faecal cultures [15, 17]. Specific changes have been demonstrated as well in the metabolism of *B. fragilis* when grown in the presence of EPS E44 and R1 as compared with cultures in glucose [18]. Although all these studies suggest that *B. fragilis* can use bifidobacterial EPS as fermentable substrates, this had not yet been effectively proven. In the present work, we assessed this question by testing the ability of *B. fragilis* DSMZ 2151 to growth in a minimal medium added with EPS from bifidobacteria as the sole carbon and energy source.

METHODS

Bacterial strains and culture media. Frozen stocks of *B. fragilis* DSMZ 2151 (DSMZ bacterial pure collection, Braunschewig, Germany) were reactivated in Gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 0.25% (w/v) L-cysteine (Sigma Chemical co., St. Louis, MO, USA) (named GAMc) and incubated overnight at 37ºC in an anaerobic cabinet (Mac 100; Don Whitley Scientific, West Yorkshire, UK) under a 10% H_2, 10% CO_2, and 80% N_2 atmosphere. pH free batch cultures of *B. fragilis* were performed in a modified minimal medium (MM) previously used in *B. fragilis* metabolism studies [19]. Briefly, the medium contained per litre: $(NH_4)_2SO_4$, 1 g; KH_2PO_4, 0.9 g; NaCl, 0.9 g; $CaCl_2 \cdot 2H_20$, 26.5 mg; $MgCl \cdot 6H_20$, 20 mg; $MnCl_2 \cdot 4H_20$, 10 mg; $FeSO_4 \cdot 7H_20$, 4 mg; $CoCl_2 \cdot 6H_20$, 1 mg; resazurin, 1 mg; vitamin B_{12}, 10 µg; vitamin K_1, 2 mg; and haemin, 4 mg. MM was supplemented with 0.5% (w/v) of glucose, EPS E44 or EPS R1 and the final pH of the medium ranged between 7.6 and 7.9. MM was inoculated with a 1% (v/v) overnight culture of *B. fragilis* in GAMc in a final volume of 10 mL. A culture of *B. fragilis* inoculated in MM without carbon source added was used as a negative control. Potential changes over time in the characteristics of the EPS fractions during incubation not due to the microbial action were monitored in uninoculated MM added with EPS. Bacterial growth was monitored by counting in agar-GAMc plates at 0, 24, 48, 72 and 144 h of incubation. Experiments were run in triplicate using the same inoculum in all conditions.

EPS isolation. The EPS fractions were obtained from the strains *B. animalis* subsp. *lactis* IPLA R1 [20], a dairy origin strain, and *B. longum* IPLA E44 [21], a faecal isolate from a healthy adult faeces. Cellular biomass was harvested with ultrapure water from agar-MRS (Biokar, Allone, France) plates with 0.25% (w/v) L-cysteine (Sigma) and incubated for 3 days at 37 ºC under anaerobic conditions [22]. In brief, EPS was separated from the cellular biomass by gently stirring overnight with one volume of 2 M NaOH at room temperature. Then, cells were removed by centrifugation and EPS were precipitated from the supernatant with two volumes of absolute cold ethanol for 48 h at 4º C. After centrifugation at 10,000 x *g* for 30 min at 4º C, the EPS fraction was resuspended in ultrapure water and dialyzed against water during 3 days in 12- to 14-kDa molecular weight cut off dialysis tubes (Sigma). The protein content of the polymers was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions. Finally, EPS fractions were freeze-dried .The EPS E44 and R1 fractions contained 2.25% and 1.99% protein, respectively.

Metabolite analysis and EPS molar mas distribution and quantification. Organic acids (lactic, succinic and formic) formed during incubation were analysed by HPLC. Cell-free supernatants from cultures were filtered (0.2 µm) and quantified using an Alliance 2695 module injector, a PDA 966 photodiode array detector, a 2414 differential refractometer detector and the Empower software (Walters, Mildford, MA), following the chromatographic conditions described previously [22]. The weight average molar mass (M_w) distribution of EPS fractions was determined in the same equipment by size-exclusion chromatography (SEC) using two different columns placed in series, TSK-Gel G3000 PW_{xL} and TSK-Gel G5000 PW_{xL} (Supelco-Sigma) and the multiangle laser light scattering (MALLS) detector DawnHeleos II (Wyatt Europe GmbM, Dembach,Germany) as described previously [22]. The EPS peaks were detected and quantified with the refractive index detector, using standards of dextran (Fluka-Sigma) of different molar masses; the presence of proteins was monitored through a PDA detector set at 220 nm [17]. Short chain fatty acids (SCFA; acetic and propionic) were quantified in the supernatants by Gas Chromatography (GC) using a system composed of a 6890N gas chromatograph (Agilent Technologies Inc., Palo Alto, CA, USA) connected with a FID detector (Agilent) and a mass spectrometry (MS) 5973N detector (Agilent) as

described previously [15]. Concentrations were expressed in millimolar (mM).

Statistical analysis One way ANOVA statistical tests was run to compare the evolution of the different parameters analysed along time or among cultures with the different carbon and energy sources by means of the IBM SPSS software, version 22.00 (IBM, Armonk, New York, USA). SNK post-hoc test was used when required.

RESULTS

The metabolite production and growth pattern of *B. fragilis* DSMZ 2151 was dependent on the carbon source present in the culture medium. The final pH attained with glucose (6.52±0.16) was lower than with EPS E44 and EPS R1 (7.62±0.02 and 7.66±0.01 respectively). The pH values remained unchanged in MM without carbon source added (7.73 ± 0.05) whereas bacterial counts increased 1.3 log units from 0 to 24 h of incubation in such conditions, probably due to the metabolic inertia of the inoculum (Table 1). At 24 h of incubation microbial counts reached were 1.22 log ufc mL^{-1} higher in glucose and 0.6-0.8 greater in EPS than in the control MM medium without any carbon source added (p<0.05) (Table 1). From that point on, the time course of *B. fragilis* growth was different depending on the carbon source, and microbial population levels remained generally higher in EPS than in glucose at late stages of growth (from 48 h of incubation) (Table 1). Thus, microbial counts in glucose continuously decreased from 24 h to the end of incubation (p<0.05). In contrast, levels of *B. fragilis* with both EPS remained largely unchanged from 24 to 72 h. From that time population levels decreased with both polymers, this decrease being much more pronounced with EPS R1 than with EPS E44.

The production of fermentation end-products from the catabolism of carbohydrates by *B. fragilis* was notably higher in glucose than in EPS and also in medium with the different EPS than in the medium without carbohydrates added all along the incubation period considered in the study (p<0.05) (Table 1). Glucose was depleted in the culture medium after 24 h (data not shown) and metabolite levels remained without noticeable variations until 144 h of incubation. In contrast, total metabolites produced in medium with both EPS increased continuously until the end of fermentation, reaching clearly higher levels at the end of the incubation period in the culture with EPS E44 than in EPS R1 (p<0.05). We analysed more in depth the evolution pattern of the different organic acids and SCFA formed during incubation (Table 1). The acetic to propionic acids ratio displayed higher values in glucose than in both EPS (p<0.05) whereas, conversely, the propionic to succinic as well as the acetic to lactic acid ratios showed a trend (p<0.1) to display higher values in cultures with EPS than with glucose. Regarding levels of the different microbial metabolites in cultures with EPS, the acetic, propionic and succinic acids increased until 24 h, decreased from 24 to 48-72 h, and increased again until the end of incubation. On the opposite, the concentration of lactic acid reached the maximum value at 48 h and then experienced a continuous decrease until the end of incubation (p<0.05). As a consequence, at 48 h the acetic to lactic acid ratio reached its minimum value whereas the acetic to propionic ratio attained its maximum in cultures with EPS E44.

Since the only carbon source available in our experimental conditions were either glucose or EPS, we examined the evolution of the EPS fractions over time, looking for detectable variations in the amount and/or physicochemical characteristics of the polymer peaks that could demonstrate their consumption. Peaks of EPS E44, purified from the cellular biomass of *Bif. longum* E44, were according to that previously reported [22] and consisted in two polymers, one of 5×10^6 Da (E44 P1) representing 18.8% of the total mass, and a second one of 9×10^3 Da (E44 P2), representing 81.2 % (Figure S1). We ruled out any change in the molar mass distribution of the EPS E44 fraction along incubation in uninoculated MM (data not shown). Interestingly, the incubation of EPS with *B. fragilis* in the MM resulted in a significant decrease in the amount of the higher molar mass peak (E44 P1) of E44 from 48 h to 144 h of incubation (p<0.05) (Figure 1A and 1B) and of the smaller molar mass peak (E44 P2) between 48 and 72 h of incubation, remaining stable from 72 to 144 h (p<0.05) (Figure 1A and 1B). Molar mass distribution of EPS R1 was also in accordance with that previously reported by us [23], and consisted of three peaks; one of 1×10^6 Da (R1 P1), another one of 2.35×10^4 Da (R2 P2) and a small one of 4×10^3 Da (R3 P3). This last peak overlapped in our analysis with an UV absorption peak and was then excluded from our study (Figure S1). The total amount of the higher molar mass R1 polymer (R1 P1) did not vary significantly during incubation, independently on the presence of *B. fragilis* (Figure 1D). The amount of the medium size (R1 P2) suffered an apparent depletion along incubation, which reached statistical significance at late incubation times (48, 72 and 144 h, p <0.05); however, the final amount of this polymer was similar to the one found in the MM without *B. fragilis*, with values at 144 not differing significantly (p>0.05) between the culture medium inoculated or not with this bacterium (Figure 1C and 1D) (statistical data not shown).

DISCUSSION

The use of bacterial EPS by *Bacteroides thetaiotaomicron* has been described before with HoPS produced by some lactic acid bacteria and *Streptococcus* spp. [6, 16, 24]. Although the use of HePS from bifidobacteria had not been definitely demonstrated yet, we have previously identified changes in the metabolism of *B. fragilis* in the presence of these polymers in an undefined medium [18]. Results from the current study demonstrated an effective growth of this specie in the presence of HePS. As compared to glucose, *Bacteroides* population levels attained with our EPS at early stages of growth were lower, but the presence of these polymers contributed to increase the survival of the microorganism at late states of growth, this last phenomenon being more pronounced with EPS E44 than with EPS R1. This was in accordance with the different growth patterns obtained depending on the carbon source used. Besides, a differential metabolic activity of *B. fragilis* in the presence of EPS as compared to glucose was evidenced. *B. fragilis* remained metabolically active in cultures with EPS for a longer period of time, with the highest activity corresponding to the cultures with the polymer E44. Variations obtained in metabolic profiles of *Bacteroides* cultures as depending on the carbon sources were similar to those indicated previously by us using an undefined medium [18]. Additionally, higher propionic to succinic acid ratios with complex carbon sources relative to glucose, similar to that found in the current study, have been previously reported [25]

In the present study we observed a clear shift in the metabolism of *B. fragilis* during the time course of fermentation in the presence of EPS with respect to glucose, which was more pronounced with the polymer E44 than with R1; this was mainly supported by variations in the relative production of acetic, propionic and succinic acids, and by a decrease from 48 h of incubation in the levels of lactic acid. In *Bacteroides* the formation of acetate from pyruvate and the production of propionate through the succinate/propionate pathway provide the cell with extra energy in the form of ATP whilst this last via also allows regeneration of NAD+ spent during glucolysis. This resulted *a priori* more advantageous in energetic terms than regeneration of NAD+ by conversion of pyruvate into lactate [18, 19]. Therefore, it seems that the shift in the metabolism of *B. fragilis* occurring from early to late phases of growth in cultures with EPS is mainly directed to the optimization of cell energy production while keeping the intracellular redox balance. Although *B. fragilis* has the capacity to metabolize moderate amounts of lactic acid [26] as well as amino acids [27], our work was performed in a minimal medium, so that no amino acid sources were available and the scarce consumption of lactic acid that may occur does not explain the growth and metabolic activity of the bacterium in such conditions.

The results from SEC-MALS chromatography indicated that *B. fragilis* was able to use the EPS E44 produced by *Bif. longum* as a fermentable substrate. The chemical structure of EPS E44 has not been elucidated yet but it is known that EPS E44 contains glucose and galactose in its composition [22]. It is then possible that the saccharolytic enzymatic machinery of *B. fragilis* could include enzymes able to participate in the degradation of this complex substrate. On the other hand, changes in growth and metabolic patterns occurring at late stages of growth in EPS E44 could be related with the cessation of consumption of the smaller peak that found beyond 72 h of incubation, and hence with a scarcity of carbon source available from this time. Our results are not conclusive about the possible degradation of the EPS R1 fraction by *B. fragilis.* Even though there were no significant changes in the EPS peaks, we could not rule out changes in the amount of the smallest polymer, not considered in our study because the overlapping with a protein peak. Variations in this small polymer may provide a possible explanation for the increased metabolic activity evidenced in cultures of *B. fragilis* until 72 h of incubation in the presence of the EPS R1 fraction as compared to the control in MM without carbon sources added. The EPS R1 fraction is formed by glucose, galactose and rhamnose [23] and only the chemical structure of the high molar mass peak has been determined to date, which is composed by 50% rhamnose [28]. Although we know that the presence of both EPS stimulates the production of α-glucosidase by *B. fragilis*, the high molar mass polymer of the EPS R1 fraction lacks the α-linkages targeted by this enzymatic activity [28]. This together with the inability of *B. fragilis* to ferment rhamnose [27], could pose difficulties for the use as fermentable substrate of the EPS R1 by this microorganism.

The ability of *B. fragilis* to use bifidobacterial EPS may provide this microorganism with a long-term available complex carbon source, thus enhancing its survival and conferring it a selective advantage in environments where nutrients are scarce, such as the case of the human large intestine. *Bacteroides* plays an important role in the utilization of indigestible dietary compounds and complex polymers secreted by other microorganisms [6]. Some EPS and capsular polysaccharides are involved in adhesion to eukaryotic cells, biofilm formation and protection of several species against the gastrointestinal stressing factors [14]. In this way the ability of *Bacteroides* to degrade these

polymers may confer this microorganism a role in the regulation of microbial relationships in the gut ecosystem. Fermentation of EPS in faecal cultures lead to an increase in propionic acid production [17], most likely due to the metabolic activity of members from the genus *Bacteroides*.

CONCLUSIONS

The present work is the first report demonstrating that *B. fragilis* is able to use some EPS produced by a bifidobacteria as substrate for growth, which resulted in a partial polymer consumption and the concomitant release of metabolic end products from its fermentation. By extrapolating these findings to the human gut, it may be hypothesized that the feeding relationship between microbial EPS and *Bacteroides* could have an impact in the SCFA production balance in the gut, which is ultimately related with the human health.

AUTHORS' CONTRIBUTION

DRC participated in the design of the experiment and carried out the bacterial cultures, data analysis and interpretation of results, and drafted the manuscript. IC carried out the HPLC analysis. JRA performed the GC analysis. PRM participated in the analysis and interpretation of MALLS data. MG participated in the study design and data interpretation. CGR participated in the study design and data interpretation. All authors revised and approved the final manuscript.

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COMPETING INTEREST

The authors declare that they have no competing interests

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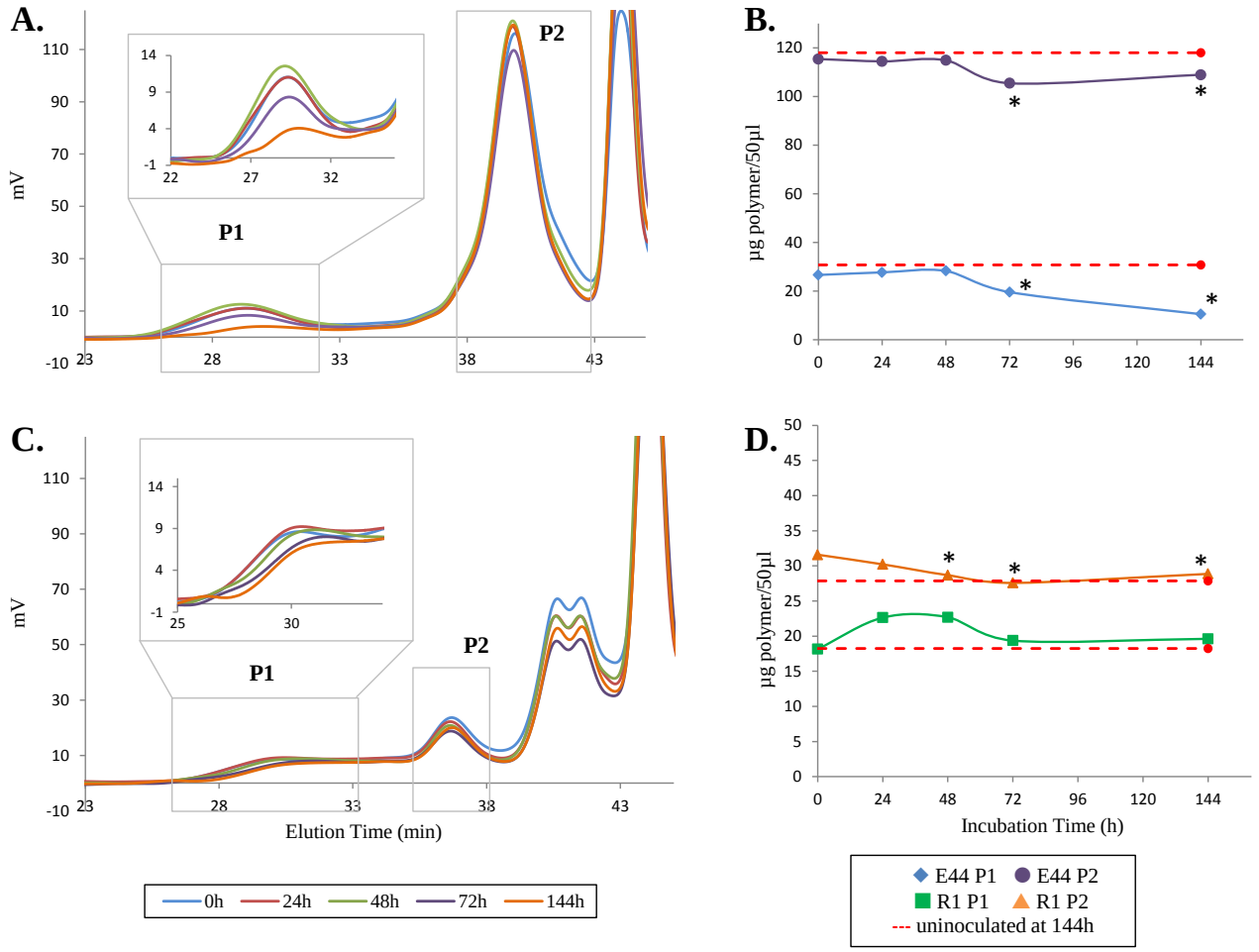

Figure 1 Time course of the bifidobacterial EPS degradation by *B. fragilis*. Refractive index chromatogram for degradation of EPS E44 peaks along time (A). Evolution of the total amount of EPS E44 peaks along time (B). Refractive index chromatogram for degradation of EPS R1 peaks along time (C). Evolution of the total amount of the two biggest peaks of EPS R1 fraction along time (D). Significant reduction of the amount of EPS in the corresponding peak with respect to time 0 is indicated by asterisks (p<0.05). Dashed red lines indicate the amount of polymer for each peak after 144 hours of incubation in the absence of *B. fragilis*.

Table 1. Growth and metabolic parameters of *B. fragilis* grown in glucose, EPS or without carbon source.

Carbon source	Time (h)	Acetic acid	Propionic acid	Lactic acid	Succinic acid	Formic acid	Total metabolites*	Acetic / Propionic	Propionic / Succinic	Acetic / Lactic	Bacterial counts^{+}
Glucose	24	13.00 ± 0.41^{D}	4.35 ± 0.63^{C}	14.81 ± 0.27^{B}	$3.46\pm0.19^{a\,B}$	7.33 ± 0.15^{D}	$42.95\pm1.40^{a\,C}$	3.02 ± 0.35^{C}	1.25 ± 0.12^{A}	0.88 ± 0.01^{A}	$9.58\pm0.07^{d\,C}$
	48	13.28 ± 0.74^{C}	4.69 ± 0.21^{C}	17.11 ± 1.26^{B}	$3.75\pm0.27^{a\,B}$	6.86 ± 0.38^{D}	$45.73\pm1.22^{ab\,D}$	2.83 ± 0.08^{D}	1.25 ± 0.13^{A}	0.78 ± 0.07^{A}	$8.47\pm0.16^{c\,B}$
	72	13.80 ± 0.53^{C}	5.04 ± 0.47^{D}	16.15 ± 1.01^{B}	$4.37\pm0.18^{b\,C}$	7.59 ± 0.12^{C}	$47.11\pm0.41^{b\,C}$	2.75 ± 0.18^{D}	1.15 ± 0.09^{A}	0.86 ± 0.09	$7.76\pm0.06^{b\,A}$
	144	13.66 ± 0.26^{D}	4.96 ± 0.32^{D}	15.27 ± 0.87^{B}	$4.56\pm0.52^{b\,B}$	7.26 ± 0.45^{C}	$46.02\pm1.96^{ab\,D}$	2.76 ± 0.15^{C}	1.09 ± 0.06^{A}	0.90 ± 0.05^{A}	$7.46\pm0.17^{a\,B}$
EPS E44	24	$1.24\pm0.06^{b\,B}$	$0.78\pm0.08^{b\,A,B}$	$0.45\pm0.07^{b\,A}$	0.12 ± 0.02^{A}	$0.43\pm0.04^{a\,B}$	$3.01\pm0.11^{a\,B}$	$1.60\pm0.10^{a\,B}$	6.78 ± 0.27^{D}	$2.83\pm0.53^{a\,A}$	$9.17\pm0.12^{c\,B}$
	48	$0.93\pm0.08^{a\,A,B}$	$0.39\pm0.08^{a\,A}$	$0.95\pm0.05^{c\,A}$	-	$1.27\pm0.24^{b\,C}$	$3.56\pm0.22^{b\,C}$	$2.44\pm0.30^{b\,C}$	ND	$0.98\pm0.12^{a\,A}$	$8.91\pm0.02^{b\,C}$
	72	$1.89\pm0.10^{c\,B}$	$0.95\pm0.13^{b\,B}$	$0.77\pm0.08^{c\,A}$	-	$1.67\pm0.46^{b\,B}$	$5.31\pm0.34^{c\,B}$	$2.01\pm0.19^{ab\,C}$	ND	2.48 ± 0.38^{a}	$9.05\pm0.05^{bc\,B}$
	144	$5.53\pm0.08^{d\,C}$	$2.75\pm0.13^{c\,C}$	$0.26\pm0.15^{a\,A}$	0.13 ± 0.05^{A}	$1.40\pm0.31^{b\,B}$	$10.07\pm0.39^{d\,C}$	$2.01\pm0.12^{ab\,B}$	25.22 ± 11.55^{B}	$26.24\pm12.29^{b\,A}$	$8.68\pm0.14^{a\,C}$
EPS R1	24	$1.70\pm0.11^{a\,C}$	$1.37\pm0.12^{b\,B}$	$0.16\pm0.10^{b\,A}$	$0.26\pm0.05^{b\,A}$	$0.73\pm0.25^{a\,C}$	$4.22\pm0.09^{a\,B}$	$1.25\pm0.03^{a\,B}$	$5.40\pm0.54^{b\,C}$	$14.03\pm7.32^{a\,B}$	$9.01\pm0.09^{b\,B}$
	48	$1.40\pm0.22^{a\,B}$	$0.82\pm0.12^{a\,B}$	$0.49\pm0.09^{c\,A}$	$0.15\pm0.01^{a\,A}$	$1.92\pm0.12^{c\,B}$	$4.81\pm0.37^{b\,B}$	$1.70\pm0.08^{b\,B}$	$5.64\pm0.55^{b\,B}$	$2.86\pm0.12^{a\,B}$	$9.17\pm0.09^{b\,D}$
	72	$2.08\pm0.10^{b\,B}$	$1.44\pm0.16^{b\,C}$	$0.12\pm0.09^{b\,A}$	$0.26\pm0.00^{b\,B}$	$1.35\pm0.18^{b\,B}$	$5.25\pm0.09^{b\,B}$	$1.45\pm0.11^{a\,B}$	$5.62\pm0.62^{b\,B}$	25.26 ± 19.65^{a}	$9.15\pm0.13^{b\,B}$
	144	$3.13\pm0.18^{c\,B}$	$1.70\pm0.09^{c\,B}$	$0.02\pm0.01^{a\,A}$	$0.53\pm0.02^{c\,A}$	$1.27\pm0.26^{b\,B}$	$6.65\pm0.34^{c\,B}$	$1.85\pm0.20^{b\,B}$	$3.21\pm0.29^{a\,A}$	$>100^{b\,B}$	$6.73\pm0.21^{a\,A}$
WCS	24	$0.21\pm0.05^{a\,A}$	$0.26\pm0.03^{a\,A}$	-	$0.11\pm0.07^{a\,A}$	$0.09\pm0.03^{a\,A}$	$0.72\pm0.12^{a\,A}$	0.79 ± 0.12^{A}	2.33 ± 0.43^{B}	ND	$8.36\pm0.15^{c\,A}$
	48	$0.27\pm0.05^{a\,A}$	$0.34\pm0.03^{b\,A}$	-	$0.21\pm0.01^{b\,A}$	$0.17\pm0.03^{b\,A}$	$1.01\pm0.05^{a\,A}$	0.79 ± 0.10^{A}	1.66 ± 0.21^{A}	ND	$8.10\pm0.06^{c\,A}$
	72	$0.35\pm0.06^{a\,A}$	$0.36\pm0.00^{b\,A}$	-	$0.20\pm0.03^{b\,A}$	$0.17\pm0.04^{b\,A}$	$1.08\pm0.02^{a\,A}$	0.99 ± 0.16^{A}	1.83 ± 0.29^{A}	ND	$7.84\pm0.11^{b\,A}$
	144	$0.66\pm0.22^{b\,A}$	$0.51\pm0.05^{c\,A}$	-	$0.23\pm0.05^{b\,A}$	$0.26\pm0.03^{c\,A}$	$1.68\pm0.35^{b\,A}$	1.28 ± 0.30^{A}	2.30 ± 0.36^{A}	ND	$6.61\pm0.19^{a\,A}$

Metabolite concentrations are expressed in mM, bacterial counts are expressed in ufc mL^{-1} Columns for the same carbon source with different lower case letter superscripts indicate significant differences along time (p>0.05). Different capital letters superscripts indicate significant differences among carbon sources at the same sampling point (p<0.05). * Sum of acetic, propionic, lactic, succinic, formic and pyruvic acids. $^{+}$ Counts at 0 h were 7.02± 0.08 log ufc mL^{-1}. WCS, without carbon source. -, under the detection limit. ND, Not determined.

CAPÍTULO 3

INTERACCIÓN METABÓLICA ENTRE *Bifidobacterium adolescentis* Y *Faecalibacterium prausnitzii*

CAPÍTULO 3

Interacción metabólica entre *Bifidobacterium adolescentis* y *Faecalibacterium prausnitzii*

Faecalibacterium prausnitzii es el principal productor de ácido butírico en el intestino humano. Niveles bajos de este microrganismo se han relacionado con estados de inflamación en varias enfermedades intestinales. Aunque posee una maquinaria metabólica escasa para la utilización de las fuentes de carbono que llegan al intestino y es muy sensible al oxígeno, *F. prausnitzii* es uno de los microorganismos mayoritarios en adultos sanos y se cree que su localización es especialmente cercana al epitelio intestinal. Uno de los factores que ayudarían a su supervivencia en estas condiciones, *a priori* desfavorables, es su capacidad de utilizar ácido acético liberado por otros microorganismos para la producción de ácido butírico.

Estudios anteriores habían demostrado un aumento de *B. adolescentis* y de *F. prausnitzii* en adultos sanos como respuesta a la administración de inulina durante un periodo de tiempo prolongado. Además, un estudio *in silico* reciente, predijo la existencia de flujos de metabolitos entre estos dos microrganismos crecidos en glucosa como fuente de carbono. En este contexto, nos planteamos demostrar esta interacción y para ello evaluamos diferentes cepas y fuentes de carbono para encontrar las condiciones idóneas para la reproducción del fenómeno de cross-feeding.

El objetivo de este capítulo se aborda en una publicación:

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Research Letter

RESEARCH LETTER – Physiology & Biochemistry

Enhanced butyrate formation by cross-feeding between *Faecalibacterium prausnitzii* and *Bifidobacterium adolescentis*

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One sentence summary: The article provides the first experimental demonstration of enhanced butyrate formation by a cross feeding mechanisms betweeen *Faecalibacterium prausnitzii*, a comensal bacteria of the human colon, with *Bifidobacterium adolescentis*, one of the most abundant bifidobacteria from the adult's intestinal microbiota.

Editor: Michael Cox

ABSTRACT

Cross-feeding is an important metabolic interaction mechanism of bacterial groups inhabiting the human colon and includes features such as the utilization of acetate by butyrate-producing bacteria as may occur between *Bifidobacterium* and *Faecalibacterium* genera. In this study, we assessed the utilization of different carbon sources (glucose, starch, inulin and fructooligosaccharides) by strains of both genera and selected the best suited combinations for evidencing this cross-feeding phenomenon. Co-cultures of *Bifidobacterium adolescentis* L2–32 with *Faecalibacterium prausnitzii* S3/L3 with fructooligosaccharides as carbon source, as well as with *F. prausnitzii* A2–165 in starch, were carried out and the production of short-chain fatty acids was determined. In both co-cultures, acetate levels decreased between 8 and 24 h of incubation and were lower than in the corresponding *B. adolescentis* monocultures. In contrast, butyrate concentrations were higher in co-cultures as compared to the respective *F. prausnitzii* monocultures, indicating enhanced formation of butyrate by *F. prausnitzii* in the presence of the bifidobacteria. Variations in the levels of acetate and butyrate were more pronounced in the co-culture with fructooligosaccharides than with starch. Our results provide a clear demonstration of cross-feeding between *B. adolescentis* and *F. prausnitzii*.

Keywords: *Faecalibacterium prausnitzii*; *Bifidobacterium adolescentis*; butyrate; acetate; starch; fructooligosaccharides (FOS); probiotics

INTRODUCTION

Nutrient cross-feeding is an important and integral component of the dynamic and functional complex microbial ecosystem found in the human colon (Belenguer *et al.* 2006; Flint *et al.* 2012). The large intestine is colonised by a dense microbial community comprised mainly of Bacteroidetes, Firmicutes and Actinobacteria. A key function of these predominantly anaerobic

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microbes is to ferment dietary residues that escape digestion by host enzymes (Flint *et al.* 2012). Collectively, the bacterial species that co-exist within this dense community possess an elaborate array of enzymes, including glycosyl-hydrolases, which allows the cells to degrade complex carbohydrates. For example, non-digestible carbohydrates such as resistant starches, non-starch polysaccharides (NSP) and oligosaccharides, including fructooligosaccharides (FOS), are major sources of energy for colonic bacteria (Flint *et al.* 2012). Intermediate carbohydrate breakdown products and certain fermentation products serve as carbon and energy sources for cross-feeding bacteria (Duncan, Louis and Flint 2004; Belenguer *et al.* 2006) and are mainly fermented to short-chain fatty acids (SCFA) and gases (Duncan *et al.* 2007). The major fermentation products detected in the colon are acetate, propionate and butyrate often in the ratio of around 10:2:1 depending on dietary intakes. Acetate is utilised by butyrate producers that employ the dominant butyryl CoA:acetate CoA transferase route (Duncan, Louis and Flint 2004; Louis *et al.* 2010). One of the most dominant bacterial species detected in the healthy human large intestine is *Faecalibacterium prausnitzii* (Flint *et al.* 2015); this microorganism is a butyrate producer that employs this route for butyrate formation and representative strains have been reported to grow poorly in the absence of acetate (Duncan *et al.* 2002). *F. prausnitzii* is considered important in health promotion, as in addition to forming butyrate it also possesses other anti-inflammatory attributes (Sokol *et al.* 2009).

It is interesting to speculate why *F. prausnitzii* is so successful in the healthy colon despite the fact that there is a diverse range of carbohydrates consumed in our diets and *F. prausnitzii* grows poorly on non-digestible carbohydrates (Lopez-Siles *et al.* 2012), which are a major carbon source for colonic anaerobes (Cummings and Macfarlane 1991). Its competitive prowess may, at least in part, be due to its ability to utilise end products of fermentation in the colon suggesting that it may therefore be a successful cross-feeder.

Some of the best studied bacteria from the human colon belong to the *Bifidobacterium* genus. *Bifidobacterium* has long been considered a beneficial microorganism and reduced *Bifidobacterium* levels have been linked to different disease states (Tojo *et al.* 2014). Therefore, there is a long-standing interest in promoting bifidobacteria in the colon either directly through probiotic approaches or indirectly by prebiotics (Olano-Martin, Gibson and Rastell 2002; Cardelle-Cobas *et al.* 2009; Arboleya *et al.* 2011; Tojo *et al.* 2014). Different *Bifidobacterium* species can be found in the human gut (Salazar *et al.* 2015) and *Bifidobacterium adolescentis* is one of the most frequently found in adults (Matsuki *et al.* 2004).

Furthermore, there is considerable interest in using dietary and prebiotic strategies to modulate the gut microbiota as this has the potential to influence several aspects of host health. It should be noted however that introduction of a prebiotic supplement will impact not only on one target bacterial species, which traditionally has included *Bifidobacterium* species, but also on others through cross-feeding interactions (Ramirez-Farias *et al.* 2009; Scott *et al.* 2014).

Here, we explore the distinct ability of strains belonging to the species *F. prausnitzii* and the genus *Bifidobacterium* to grow in different carbon sources with a focus on the interactions that occur between *B. adolescentis*, one of the most abundant *Bifidobacterium* species in the colon, with representative *F. prausnitzii* isolates when provided with FOS or starch in the growth medium. These investigations demonstrated clear cross-feeding and enhanced butyrate formation when certain of these strains were in co-culture.

MATERIAL AND METHODS

Bacterial strains

The study included three *F. prausnitzii* strains and five strains belonging to the genus *Bifidobacterium*. *B. adolescentis* L2–32 and *F. prausnitzii* strains A2–165, L2–6 and S3L/3 were originally isolated from adult human stool samples (Barcenilla *et al.* 2000; Louis *et al.* 2004). The strain *Bifidobacterium breve* IPLA 20006 was previously isolated from breast milk, and the strains *Bifidobacterium bifidum* IPLA 20015 and *Bifidobacterium pseudocatenulatum* IPLA 20026 from infant faeces (Solís *et al.* 2010). In addition, a *Bifidobacterium longum* strain (IPLA 20027) isolated in TOS agar medium (MERK, Germany) from the stool of a healthy 90-year-old woman was also included.

Growth media, monoculture and co-culture conditions

All strains used in this study were recovered in M2GSC medium (Miyazaki *et al.* 1997) from frozen stocks and grown overnight at 37°C under anaerobic conditions with O_2-free CO_2 using the Hungate tube method. For the substrate fermentation and SCFA growth boosting tests, strains were grown in YCFA medium (Lopez-Siles *et al.* 2012) as described above with and without SCFA supplementation. When appropriate, an SCFA solution was added to the medium obtaining a final concentration of 33 mM acetate, 9 mM propionate and 1 mM each of *iso*-butyrate, *iso*-valerate and valerate. Single carbon sources were added before autoclaving to give a final concentration of 0.2% (w/v) and the final pH was adjusted to 6.5 ± 0.2. Carbon sources used were glucose (Fischer Scientific, USA), soluble starch from potato (Sigma-Aldrich, USA), inulin from two different sources (dahlia and chicory) and FOS P95 (Beneo, Belgium). Growth in cultures was monitored spectrophotometrically by measuring the OD_{650} and growth rate (h^{-1}) was determined at exponential growth (Pirt 1975).

To study the interaction between *Bifidobacterium* and *F. prausnitzii*, co-culture combinations of selected strains in specific carbon sources were carried out. 100 μL of an M2GSC overnight culture of each strain were added to 7.5 mL of YCFA medium with and without SCFA and supplemented with the appropriate carbon source. Co-culture tubes were inoculated with 100 μL of each strain. Culture conditions were the same as described above and growth was monitored by determining OD_{650}. Samples for microbiological and SCFA analyses were taken at 0, 8 and 24 h of incubation.

Carbohydrate fermentation profiles

Fermentation profiles of *Bifidobacterium* strains were obtained in API 50 CH strips (BioMerieux, France) following the manufacturer's instructions. Strips were incubated at 37°C in an anaerobic Chamber (Mac 1000; Don Whitley Scientific, West Yorkshire, UK).

SCFA and lactate determinations

SCFA and lactate content in batch cultures were determined by capillary gas chromatography analysis following conversion to *t*-butylmethylsilyl derivatives (Richardson *et al.* 1989). The lower limit for reliable detection of SCFA changes was 0.2 mM.

One-way analysis of variance (ANOVA) using the IBM SPSS software version 22.0 (IMB, Armonk, New York, USA) was run to compare the levels of SCFA between monocultures and co-cultures at 8 and 24 h of incubation as well as between both incubation times for the same culture. *Post hoc* comparison was

Table 1. Carbohydrate fermentation profiles obtained with API 50 CH strips of different *Bifidobacterium* strains used in this study.

Carbon source	*B. adolescentis* L2–32	*B. pseudocatenulatum* IPLA 20026	*B. bifidum* IPLA 20015	*B. longum* IPLA 20027	*B. breve* IPLA 20006
L-Arabinose	+	+	–	–	–
D-Ribose	+	+	–	+	+
D-Xylose	+	+	–	–	–
D-Mannose	–	–	–	+	+
D-sorbitol	+	–	–	–	–
MDM	–	–	–	+	–
MDG	+	+	–	+	–
NAG	–	–	+	+	–
Arbutin	+	+	–	+	–
Esculin	+	+	–	+	+
Salicin	+	+	–	+	+
D-Celobiose	+	–	–	+	–
D-Maltose	+	+	–	+	+
D-Melobiose	+	+	–	+	+
D-Sacharose	+	+	–	+	+
D-Trehalose	–	–	–	+	–
D-Rafinose	+	+	–	–	+
Starch	+	–	–	–	–
Glycogen	+	–	–	–	–
Gentiobiose	+	–	+	+	–
D-Turanose	+	+	–	–	+
D-Lyxose	–	–	–	+	–
D-Tagatose	–	–	–	+	–
5KG	–	+	–	+	–

+ Positive result; – Negative result; MDM, Methyl-αD-Mannopyranoside; MDG, Methyl-αD-Glucopyranoside; NAG, N-Acetylglucosamine; 5KG, potassium 5-Ketogluconate.

achieved when appropriate by a least significant difference test (LSD).

RESULTS

Regarding bifidobacteria, a preliminary test of the fermentation capability of different carbohydrates was carried out in API 50 CH strips, in order to ascertain whether or not they were able to ferment a range of carbohydrates. All strains fermented D-galactose, D-glucose, D-fructose and D-lactose to a variable extent whereas differential fermentation profiles were obtained among strains for the carbohydrates indicated in Table 1.

B. adolescentis L2–32 was the only one able to ferment starch whereas none of the microorganisms tested were able to ferment inulin. Further tests to confirm growth in glucose, inulin and FOS were carried out in Hungate tubes with YCFA medium with and without SCFA added (Table 2). In this medium, *B. bifidum* IPLA 20015 did not grow in any carbon source tested except glucose, whereas *B. longum* IPLA 20027 and *B. breve* IPLA 20006 displayed optimal growth with FOS P95 but grew poorly with starch. *B. pseudocatenulatum* IPLA 20026 and *B. adolescentis* L2–32 grew well on starch, and to a lesser extent in FOS P95. In view of the slightly better growth of *B. adolescentis* L2–32 with FOS P95 and starch and that the presence of SCFA did not improve its behaviour against in the later carbon source we selected *B. adolescentis* L2–32 for further experiments.

Growth of *F. prausnitzii* strains with glucose was reported to be stimulated by the presence of acetate in the growth medium, which contributes to butyrate formation via the butyryl CoA:acetate CoA-transferase route (Duncan *et al.* 2002; Duncan, Louis and Flint 2004). Table 2 shows that growth of the *F. prausnitzii* strains in different carbon sources improved when media were supplemented with SCFA (including 30 mM acetate). Growth of *F. prausnitzii* L2–6 with glucose and of S3L/3 with FOS P95 was poorer than growth of the other two *Faecalibacterium* strains with the same carbon source, as observed previously and starch promoted little or no growth of any of the three strains (Lopez-Siles *et al.* 2012). The inclusion of SCFA improved growth of *F. prausnitzii* strains with both types of inulin (dahlia or chicory origin) as carbon source.

We decided to investigate whether or not acetate supplied by a *Bifidobacterium* strain would stimulate the growth and metabolic activity of *F. prausnitzii* in co-culture. *B. adolescentis* L2–32 was able to ferment FOS P95 in pure culture, producing mainly acetate together with lower concentrations of formate and lactate (Figs 1 and 2). *F. prausnitzii* strain S3L/3 grew poorly on FOS in monoculture (Table 2). Separate co-cultures of this strain with *B. adolescentis* L2–32 resulted in an overall stimulation of bacterial growth and an increase in butyrate concentration of around 8 mM after 24 h (Figs 1 and 2); butyrate concentrations were significantly higher in such co-cultures than in the corresponding *F. prausnitzii* monoculture ($P < 0.05$). A decrease in acetate levels occurred in co-culture of *F. prausnitzii* S3L/3 with *B. adolescentis* L2–32 from 8 to 24 h of incubation and with acetate levels at 24 h being even lower than in the corresponding *B. adolescentis* monoculture ($P < 0.05$) (Fig. 2).

A similar experiment was performed involving *F. prausnitzii* A2–165 and *B. adolescentis* L2–32 with starch as substrate. *B. adolescentis* L2–32 was able to ferment this complex carbohydrate in pure culture whereas *F. prausnitzii* A2–165 did not grow in pure culture with this substrate (Table 2). Thus, we studied the ability of *F. prausnitzii* to use the acetate produced by *B. adolescentis* L2–32, which could result in enhanced growth of both microorganisms. Less butyrate was formed (2.9 mM) in the co-culture

Table 2. Growth of *F. prausnitzii* and *B. adolescentis* strains in YC medium with (YCFA-) or without (YC-) added SCFA. Glucose (G), starch (S), inulin from dahlia (Id), inulin from chicory (Ic) or FOS (P95) were included as carbon source as indicated. Basal medium with no carbohydrates added was used for comparison.

	F. prausnitzii A2–165		*F. prausnitzii* L2–6		*F. prausnitzii* S3L/3	
Media	OD_{650} 24 h	Growth rate (h^{-1})	OD_{650} 24 h	Growth rate (h^{-1})	OD_{650} 24 h	Growth rate (h^{-1})
YCFAG	0.83 ± 0.03	0.47 ± 0.06	0.24 ± 0.08	0.46 ± 0.07	0.77 ± 0.05	0.13 ± 0.03
YCG	0.39 ± 0.05	0.38 ± 0.04	0.36 ± 0.03	0.28 ± 0.08	0.30 ± 0.01	0.36 ± 0.06
YCFAS	0.08 ± 0.00	–	0.20 ± 0.01	ND	0.19 ± 0.01	–
YCS	0.06 ± 0.01	–	0.12 ± 0.01	–	0.11 ± 0.01	–
YCFAId	0.30 ± 0.02	0.37 ± 0.03	0.32 ± 0.01	0.22 ± 0.03	0.18 ± 0.00	–
YCId	0.21 ± 0.01	0.19 ± 0.05	0.11 ± 0.01	–	0.08 ± 0.01	–
YCFAIc	0.37 ± 0.01	0.52 ± 0.11	0.34 ± 0.01	ND	0.20 ± 0.01	–
YCIc	0.09 ± 0.03	–	0.05 ± 0.00	–	0.11 ± 0.01	–
YCFAP95	0.91 ± 0.03	0.31 ± 0.05	0.63 ± 0.01	0.27 ± 0.03	0.24 ± 0.01	ND
YCP95	0.39 ± 0.08	0.16 ± 0.07	0.27 ± 0.01	0.27 ± 0.09	0.14 ± 0.00	–
YCFA	0.09 ± 0.01	–	0.20 ± 0.01	ND	0.16 ± 0.01	–
YC	0.09 ± 0.01	–	0.15 ± 0.01	–	0.06 ± 0.00	–

	B. adolescentis L2–32		*B. pseudocatenolatum* IPLA 20026		*B. bifidum* IPLA 20015		*B. longum* IPLA 20027		*B. breve* IPLA 20006	
Media	OD_{650} 24 h	Growth rate (h^{-1})	OD_{650} 24 h	Growth rate (h^{-1})	OD_{650} 24 h	Growth rate (h^{-1})	OD_{650} 24 h	Growth rate (h^{-1})	OD_{650} 24 h	Growth rate (h^{-1})
YCFAG	0.63 ± 0.05	0.76 ± 0.06	0.13 ± 0.06	–	0.61 ± 0.01	0.17 ± 0.02	0.65 ± 0.04	0.72 ± 0.08	0.22 ± 0.02	ND
YCG	0.59 ± 0.07	0.65 ± 0.06	0.16 ± 0.03	–	0.61 ± 0.01	0.27 ± 0.09	0.34 ± 0.01	0.06 ± 0.02	0.10 ± 0.02	–
YCFAS	0.63 ± 0.00	0.55 ± 0.07	0.79 ± 0.03	0.23 ± 0.04	0.00 ± 0.00	–	–0.01 ± 0.00	–	0.02 ± 0.00	–
YCS	0.97 ± 0.06	0.46 ± 0.04	0.73 ± 0.01	0.19 ± 0.04	0.01 ± 0.01	–	0.03 ± 0.01	–	0.02 ± 0.01	–
YCFAId	0.07 ± 0.03	–	0.07 ± 0.00	–	0.06 ± 0.02	–	0.08 ± 0.01	–	0.06 ± 0.01	–
YCId	0.01 ± 0.00	–	0.05 ± 0.01	–	0.02 ± 0.00	–	0.04 ± 0.02	–	0.06 ± 0.01	–
YCFAIc	0.05 ± 0.00	–	0.03 ± 0.00	–	0.03 ± 0.00	–	0.04 ± 0.00	–	0.03 ± 0.00	–
YCIc	0.05 ± 0.01	–	0.05 ± 0.01	–	0.05 ± 0.02	–	0.06 ± 0.01	–	0.03 ± 0.01	–
YCFAP95	0.44 ± 0.10	0.29 ± 0.06	0.35 ± 0.12	0.55 ± 0.06	0.06 ± 0.00	–	0.74 ± 0.05	0.42 ± 0.04	0.38 ± 0.00	ND
YCP95	0.37 ± 0.19	0.25 ± 0.10	0.33 ± 0.12	0.47 ± 0.10	0.04 ± 0.01	–	0.50 ± 0.01	0.45 ± 0.02	0.35 ± 0.02	ND
YCFA	0.06 ± 0.00	–	0.04 ± 0.00	–	0.02 ± 0.01	–	0.03 ± 0.01	–	0.00 ± 0.00	–
YC	0.05 ± 0.01	–	0.04 ± 0.02	–	0.01 ± 0.01	–	0.03 ± 0.00	–	0.04 ± 0.02	–

– means OD values lower than 0.2; ND, not done.

of *F. prausnitzii* A2–165 and *B. adolescentis* L2–32 than in the other previous strains combination using FOS P95 as fermentable substrate (Fig. 2). With starch as carbon source acetate levels in *B. adolescentis* L2–32 monoculture decreased from 22 to 18 mM in the co-culture ($P < 0.05$) (Fig. 2) but no major differences were observed in the overall growth of the co-culture with respect to the monocultures (Fig. 1).

Overall, co-cultures of *F. prausnitzii* S3L/3 and *B. adolescentis* L2–32 with FOS P95 showed a significant decrease of acetate and a concomitant increase of butyrate from 8 to 24 h of incubation ($P < 0.05$). However, both acetate and butyrate increased moderately ($P < 0.05$) between 8 and 24 h in the case of co-cultures of *F. prausnitzii* A2–165 and *B. adolescentis* L2–32 with starch.

DISCUSSION

Acetate requirement for optimal growth of *F. prausnitzii* when using glucose as carbon source has been demonstrated previously (Duncan *et al.* 2002; Duncan, Louis and Flint 2004). In the present study we have shown similar requirements of acetate when the carbon source present in the medium was FOS.

The relationship between prebiotics, bifidogenic effects and higher butyrate production has been reported previously. In this regard, several cross-feeding experiments have been described between members of the *Bifidobacterium* genus and other butyrate-producing colonic bacteria, such as members of the genera *Eubacterium*, *Anaerostipes* and *Roseburia* (Duncan, Louis and Flint 2004; Kanauchi *et al.* 1999; Belenguer *et al.* 2006; Falony *et al.* 2006), but no previous experimental evidence was available demonstrating cross-feeding between bifidobacteria and *Faecalibacterium* strains. Higher butyrate production was previously reported during *in vitro* faecal cultures when FOS was added as carbon source to the system (Vitali *et al.* 2012). On the other hand, populations of *B. adolescentis* and *F. prausnitzii* were found to increase in an intervention study, after the administration of inulin (Ramirez-Farias *et al.* 2009). Moreover, recently a computational model has been developed to predict the fluxes and SFCA production in co-cultures of *F. prausnitzii* A2–165 and *B. adolescentis* L2–32 (El-Semman *et al.* 2014). However, in spite of these previous data this is the first study providing direct experimental evidence of a potential interaction between *Bifidobacterium* and *Faecalibacterium* strains through cross-feeding mechanisms using *in vitro* co-cultures of both microorganisms.

F. prausnitzii requires a carbohydrate energy source for growth and butyrate formation, while *B. adolescentis* does not produce butyrate. The observed higher values for acetate at 8 h than at 24 h together with the butyrate production in co-cultures on

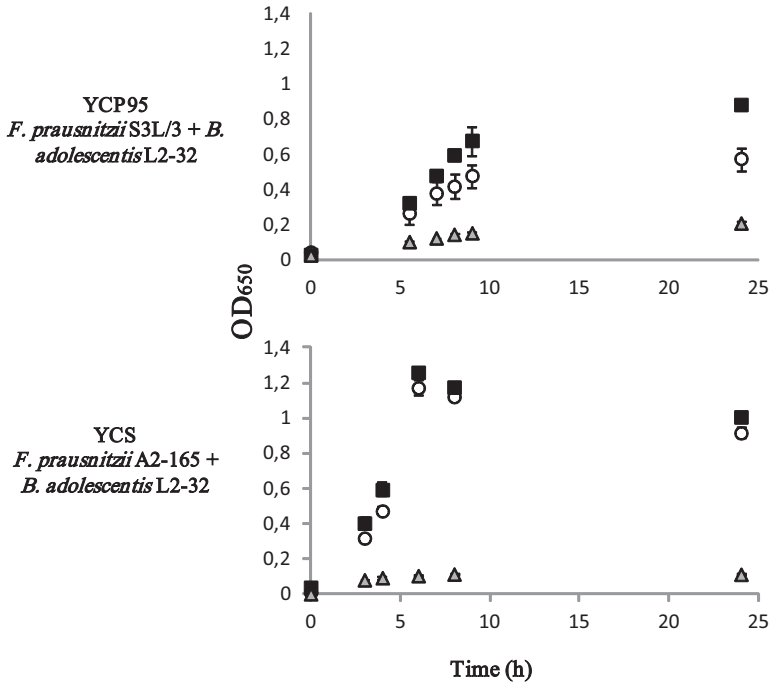

Figure 1. Growth of *F. prausnitzii* and *B. adolescentis* in monoculture and co-cultures, determined by OD_{650}: grey triangles, *F. prausnitzii* strain, white circles, *B. adolescentis* L2–32 and black squares, co-culture. Strains and carbon source used in each experiment are indicated in the left side of each panel.

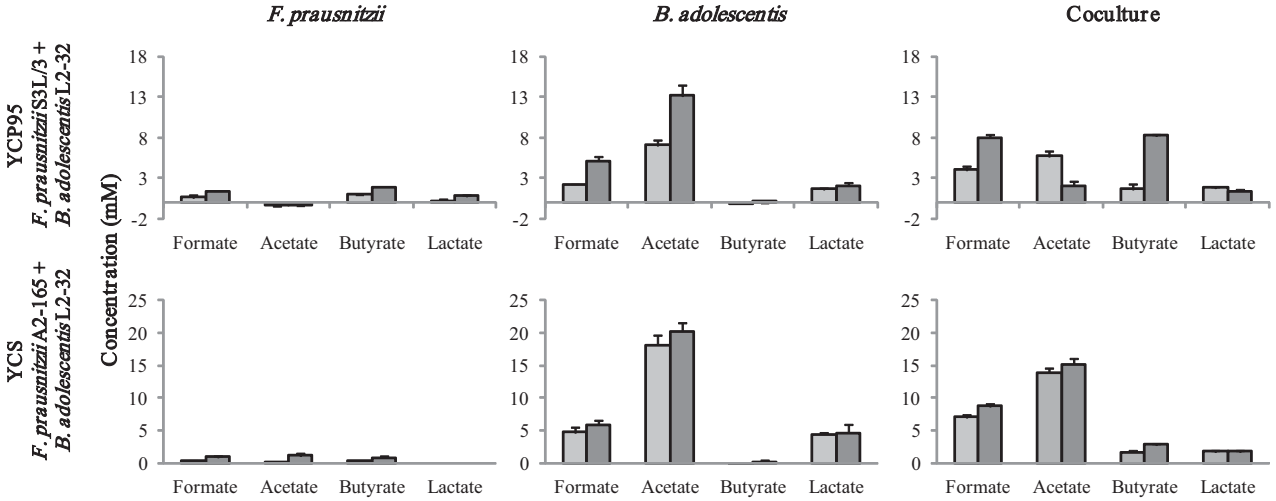

Figure 2. SCFA and lactate production by *F. prausnitzii* and *B. adolescentis* in mono-culture and co-cultures. Strains and carbon sources used in each experiment are indicated in the left side of each panel. Values are changes in concentrations calculated by subtracting the initial values at 0 h. Light grey bars, 8 h of incubation and dark grey bars, 24 h of incubation.

YCFAP95 FOS medium with no SCFA, suggest that *F. prausnitzii* growth benefits from the acetate that is being supplied by the second species, *B. adolescentis*, which is also competing for the P95 FOS present in the culture medium. In cultures of *F. prausnitzii* A2–165 using starch as carbon source, butyrate production was lower even though the production of acetate by L2–32 was higher in starch. This suggests a more limited ability of the strain *F. prausnitzii* A2–165 to compete with *B. adolescentis* for starch breakdown products as it needs available carbon source and acetate for optimal growth. The stimulation of butyrate production in the co-culture must, therefore, be partly attributed to the ability of the *F. prausnitzii* strains to compete for the substrate and the partial consumption of the acetate formed by the *Bifidobacterium* strain. This suggests that these cross-feeding mechanisms are less effective in medium containing starch as the carbon source. On the other hand, differences in the metabolism of mono and oligosaccharide constituents of FOS and starch by *B. adolescentis* and *Faecalibacterium* could also influence such cross-feeding processes.

Our results point to mechanisms of synergy that may take place between these two microorganisms in which, *F. prausnitzii* is able to use the acetate produced by *B. adolescentis* thereby boosting butyrate formation and therefore supply to the colonic mucosa. In addition, cross feeding of partial breakdown

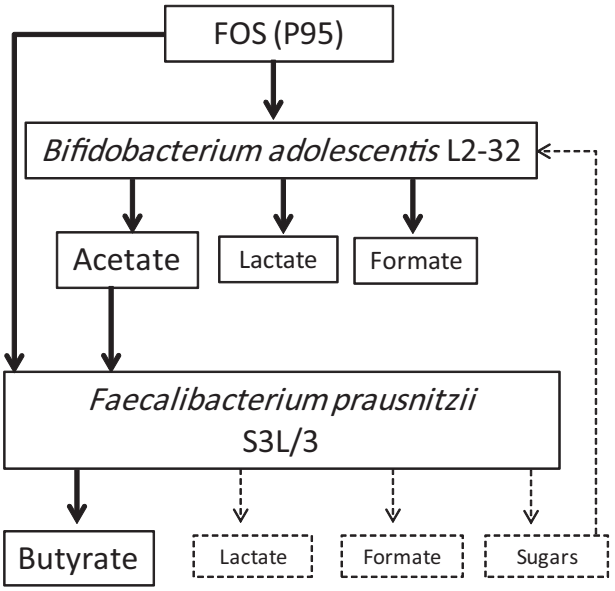

Figure 3. Schematic representation of the proposed interaction between *B. adolescentis* L2–32 and *F. prausnitzii* S3L/3 strain using FOS P95 as carbon source. FOS P95 is used by both microorganisms, but with low efficiency. The synergistic mechanism consists of consumption by *F. prausnitzii* of the acetate produced by *B. adolescentis*. The growth of *B. adolescentis* may be enhanced by carbohydrate residues released in the breakdown of the FOS chain by *F. prausnitzii*. Continuous arrows demonstrate outcomes from the present work. Discontinuous arrows indicate events that may occur but that have not been demonstrated in the present work.

products is likely to be highly significant *in vivo*. *B. adolescentis* may therefore take advantage of the breakdown products of FOS P95 formed by *F. prausnitzii* and the hypothetical mechanism of this synergy is presented in Fig. 3. Such cross feeding may benefit growth of *F. prausnitzii*, thus helping to explain the abundance of this species in the healthy human gut.

F. prausnitzii is a key player in the maintenance of intestinal and systemic host health. A decrease in *F. prausnitzii* and butyrate levels defines microbiota dysbiosis in patients suffering inflammatory bowel disease (Machiels *et al.* 2014; Lopez-Siles *et al.* 2015). Remission of inflammatory parameters has been obtained in animal models following the administration of *F. prausnitzii* or its metabolic products (Zhang *et al.* 2014; Rossi *et al.* 2015). Other studies have also highlighted the relevance of metabolic interactions between *Bacteroides thetaiotaomicron* and *F. prausnitzii* on the physiology of the colonic epithelium whereby *F. prausnitzii* is able to use the acetate produced by *B. thetaiotaomicron* with the subsequent modulation of the intestinal mucus barrier by modification of goblet cells and mucin glycosylation (Wrzosek *et al.* 2013). The relevance for the host physiology of the potential interactions between *F. prausnitzii* and probiotics or other members of the intestinal microbiota is therefore an area of great interest.

In the intestinal environment acetate is normally available at high concentrations, but this may not always be the case, for example, in special gut microenvironments, or following a period of substrate deprivation or antibiotic treatment (Hamer *et al.* 2008). Under these circumstances, the interactions revealed in the present work could have an important impact on *F. prausnitzii* populations and butyrate production in the colon. These interactions are also of interest as they suggest mechanisms by which probiotic bifidobacteria and prebiotic administration might influence gut metabolism and promote butyrate production.

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Conflict of interest. None declared.

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DISCUSIÓN

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1. Consideraciones iniciales

El cuerpo humano está colonizado por numerosas comunidades microbianas localizadas en diferentes ambientes, desde la piel o la boca, hasta la vagina o el intestino (Consortium, 2012). En los últimos años, cada vez son más las evidencias científicas que relacionan la microbiota intestinal humana con la salud (Louis y cols., 2014; Bubnov y cols., 2015; Conlon y Bird, 2015; Flint y cols., 2015). En concreto, la microbiota que coloniza el tracto gastrointestinal va aumentando tanto en diversidad como en número desde el esófago, hasta el colon, donde se concentra la mayor parte de microorganismos (Qin y cols., 2010). El desarrollo de las tecnologías de alto poder de procesamiento capaces de manejar multitud de datos sobre secuencias de ADN, ARN y proteínas (genómica, metagenómica, proteómica, transcriptómica, metabolómica, etc.…) han permitido profundizar en el conocimiento de la microbiota humana como comunidad, particularmente en lo que se refiere a la microbiota intestinal, caracterizando y comparando los microorganismos y su funcionalidad en diferentes estados de salud o enfermedad, a lo largo de la vida, así como la influencia de factores externos (dieta, tratamientos médicos, estilo de vida, etnia, etc…) en este ecosistema microbiano. A partir del conocimiento de la composición y funcionalidad de la microbiota de personas sanas y las alteraciones observadas en estados de enfermedad o próximos a ella (disbiosis), se ha impulsado el empleo racional de estrategias nutricionales, principalmente mediante el uso de probióticos y prebióticos; estas estrategias se dirigen a modular la microbiota e intentan revertir las alteraciones observadas, tomando como patrón la microbiota de una población sana control del mismo entorno.

Por otro lado, las interacciones que aparecen entre los miembros de la comunidad microbiana intestinal, y de éstos con las fuentes de carbono complejas disponibles y procedentes en su mayoría de carbohidratos no digeribles de la dieta, son determinantes en la producción de metabolitos, principalmente AGCC, con importantes efectos en la salud (Rios-Covian y cols, 2016). El estudio de estas interacciones, considerando la microbiota como un ecosistema, es

complejo y presenta actualmente importantes limitaciones metodológicas y técnicas. Es posible el planteamiento teórico de interacciones entre miembros de la microbiota intestinal a partir de estudios *in silico* basados en datos genómicos, metagenómicos y de inferencia funcional (Stein y cols., 2013; Coyte y cols., 2015; Heinken y Thiele, 2015; Turroni y cols., 2015). Recientemente, un estudio *in vivo* demostró la existencia de flujos de transformación entre los principales AGCC producidos por la microbiota intestinal, acético, propiónico y butírico, destacándose la formación de ácido butírico a partir de ácido acético como la interconversión predominante, probablemente por mecanismos de cross-feeding, en el ciego de ratones perfundidos con AGCC marcados con isótopos estables (den Besten y cols., 2013).

Hasta el momento las investigaciones sobre interacciones microbianas en el ecosistema intestinal se han dirigido principalmente a la búsqueda de microorganismos inhibidores de patógenos, siendo aún escasos los estudios que se centran en las interacciones entre miembros de la microbiota comensal. En este sentido, la mayor parte de la experimentación llevada a cabo con microorganismos mutualistas intestinales se ha realizado *in vitro* utilizando cepas y fuentes de carbono específicas (Falony y cols., 2009; Sonnenburg y cols., 2010; Scott y cols., 2014). Sólo un reducido número de trabajos se ha adentrado en los mecanismos moleculares que explican las interacciones que ocurren entre los grupos microbianos estudiados (Ruiz y cols., 2009; Riviere y cols., 2015; Turroni y cols., 2015).

Las bifidobacterias son miembros subdominantes de la microbiota intestinal humana, algunas de sus cepas son consideradas como probióticos y gozan del status QPS otorgado por la EFSA. En este contexto, en esta Tesis Doctoral nos planteamos el estudio, a nivel fisiológico y molecular, de algunas de las interacciones de miembros del género *Bifidobacterium* y de determinados componentes celulares (exopolisacáridos) y metabolitos producidos (AGCC) por los mismos, con otros microrganismos comensales de la microbiota intestinal humana.

2. Consideraciones sobre los modelos en co-cultivo para el estudio de interacciones microbianas

El procedimiento experimental que hemos utilizado en esta Tesis Doctoral para el estudio de las interacciones entre microorganismos ha sido el co-cultivo discontinuo de las cepas seleccionadas. Inicialmente valoramos las ventajas e inconvenientes de la utilización de cultivos compartimentalizados *vs.* no compartimentalizados como modelo para el estudio de las

interacciones célula-célula. En los cultivos compartimentalizados las células de los microorganismos se encuentran en secciones diferentes, existiendo solamente intercambio de medio de cultivo y moléculas pequeñas, por debajo del punto de corte (cut-off) de la membrana de separación. Esta compartimentalización puede llevarse a cabo mediante placas transwell, cámaras de difusión en cultivos discontinuos o continuos, o mediante un sistema de jeringuillas unidas por un tubo en el que se colocan membranas porosas que evitan el paso de los microorgansimos (Ruiz y cols., 2009; Avendaño-Perez y cols., 2015; Saraoui y cols., 2016) (Figura 8). Los cultivos compartimentalizados permiten obtener la biomasa de forma independiente para cada microorganismo, de manera que las técnicas de recuento y obtención de componentes celulares se simplifican. Sin embargo, se suprime el contacto directo célula-célula y se dificulta la difusión homogénea de los metabolitos y los componentes extracelulares (principalmente cuando existen densidades microbianas altas) que debe ser forzado de forma mecánica, principalmente en cultivos discontinuos. Todo ello introduce factores exógenos que podrían modificar las respuestas celulares. En contraposición, la principal ventaja de los cultivos no compartimentalizados es el contacto directo célula-célula, que reproduce mejor las condiciones fisiológicas reales; por otro lado, no se requieren manipulaciones externas, con lo que se evita la introducción de factores exógenos en el sistema. Sin embargo, es necesario recurrir a medios de cultivo selectivos y/o diferenciales ó a técnicas moleculares como FISH o qPCR para el recuento diferencial de los diferentes microorganismos presentes en el co-cultivo (Belenguer y cols., 2006; Salazar y cols., 2009; Li y cols., 2014). Se dificulta también la discriminación entre los metabolitos y componentes celulares de los diferentes microorganismos.

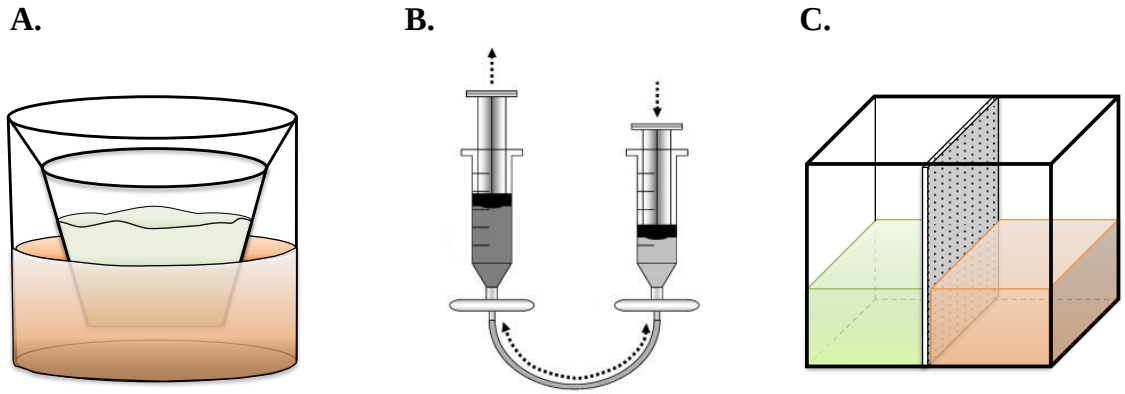

Figura 8. Sistemas de compartimentalización para co-cultivos. A, Placas transwell. B, Sistema de jeringuillas. C, Cámara de difusión

En nuestro caso, después de valorar la importancia del contacto célula-célula y la dificultad que podía suponer para el intercambio de medio de cultivo y nutrientes, la viscosidad del medio de

cultivo con las fuentes de carbono complejas a utilizar en el presente trabajo (EPS de bifidobacterias e inulina) se optó por los co-cultivos no compartimentalizados. Los recuentos diferenciales se realizaron mediante qPCR. No obstante, la coincidencia parcial de tipos de AGCC y ácidos orgánicos producidos por ambos microorganismos en estudio así como la identidad de proteínas y enzimas, han podido conducir a una subestimación de los cambios reales ocurridos.

3. Interacciones del género *Bifidobacterium* con microorganismos del ambiente intestinal.

En el presente trabajo hemos considerado las interacciones de las bifidobacterias con dos de las principales poblaciones microbianas intestinales: el género *Bacteroides* y la especie *Faecalibacterium prausnitzii*. Ambos se encuentran entre los microorganismos más abundantes de la microbiota intestinal de individuos sanos. El género *Bacteroides* tiene una amplia capacidad para degradar carbohidratos complejos y es productor de ácido propiónico mientras que *F. prausnitzii* es consumidor de ácido acético y carbohidratos y produce ácido butírico.

3.1 Interacciones con el género *Bacteroides*

3.1.1 Interacciones microorganismo-microorganismo

Hemos determinado mediante co-cultivos *Bifidobacterium* / *Bacteroides* la influencia de la presencia de un microorganismo sobre el crecimiento microbiano y actividad metabólica del otro (Manuscrito 1). Se seleccionaron cepas pertenecientes a dos especies diferentes de cada género. *Ba. thetaiomicron* es un microorganismo ubicuo de la microbiota intestinal humana con una extensa capacidad de degradación de carbohidratos complejos mientras que *Ba. fragilis* posee una alta capacidad de adaptación a cambios ambientales, pudiendo actuar como comensal y patógeno oportunista. En cuanto al género *Bifidobacterium,* la especie *B. longum* es una de las más abundantes en el intestino de individuos sanos y *B. breve* es una de las especies dominantes en el intestino de neonatos. Hemos comparado el comportamiento en co-cultivo de microorganismos de ambos géneros, en todas las combinaciones posibles, cuando se utilizan EPS de bifidobacterias como carbohidratos disponibles, respecto al comportamiento en inulina, substrato con efecto prebiótico reconocido, y glucosa, azúcar no disponible en el colon y no considerada como prebiótico. Se evidenciaron diferencias en el comportamiento entre ambos géneros dependiendo de la fuente de

carbono añadida al medio de cultivo. Así, mientras que el crecimiento de *Bifidobacterium* en fuentes de carbono complejas (inulina y EPS) fue escaso, *Bacteroides* mostró un crecimiento eficiente en todas las fuentes de carbono ensayadas. Más allá de los rasgos diferenciales del comportamiento a nivel de género en monocultivos, las interacciones en co-cultivo estuvieron profundamente influenciadas por las características específicas de las cepas de cada especie utilizadas. Así, el crecimiento de *Bacteroides* en co-cultivo no se vio afectado de forma notable por la presencia de bifidobacterias, salvo en el caso de la inhibición de *Ba. thetaiotaomicron* en co-cultivo con *B. breve* en presencia de glucosa. Este hecho podría deberse tanto a la síntesis de algún compuesto inhibidor por parte de *B. breve* como a una mayor competencia en la utilización de la fuente de carbono por parte de la bifidobacteria que limitaría su utilización y crearía un ambiente desfavorable para *Ba. thetaiotaomicron* (efecto Jameson) (Avendaño-Perez y cols., 2015). Por otro lado, el co-cultivo con *Ba. fragilis*, condujo a un ligero retraso en el crecimiento y una ligera disminución de la actividad metabólica de este microorganismo, sin embargo, mejoró claramente la supervivencia e incrementó la actividad metabólica de *B. breve* en las tres fuentes de carbono complejas ensayadas así como de *B. longum* en inulina, EPS R1 y glucosa en etapas tardías de crecimiento. Estos resultados pusieron de manifiesto una variación en el comportamiento de los microorganismos ensayados en las fuentes de carbono fermentables en función de las características metabólicas concretas de las especies acompañantes en el co-cultivo. Estudios previos de otros autores habían evidenciado la influencia tanto positiva (Oliveira y cols., 2012; Riviere y cols., 2015) como negativa (Marquet y cols., 2009; Sánchez y cols., 2013) en el crecimiento de bifidobacterias de la presencia de otros microorganismos en el medio de cultivo. Teniendo en cuenta la complejidad y diversidad de la microbiota intestinal y la composición específica de cepas de cada individuo (Schloissnig y cols., 2013), nuestros resultados ponen de manifiesto la influencia que la presencia de especies y cepas concretas puede tener en la relación entre poblaciones microbianas intestinales y contribuirían a explicar, por tanto, las diferencias que parecen constatarse en algunos estudios a nivel individual y poblacional en cuanto al grado de eficacia de intervenciones nutricionales concretas (van Baarlen y cols., 2011; Grześkowiak y cols., 2012; Zeevi y cols., 2015).

A la vista de las diferencias en crecimiento y actividad metabólica detectadas en co-cultivos de las diferentes especies y cepas de *Bifidobacterium* y *Bacteroides*, decidimos profundizar en los mecanismos de interacción entre ambos géneros a nivel molecular. Para ello analizamos las variaciones en la síntesis de proteínas y expresión génica en co-cultivo respecto a la situación de los

correspondientes monocultivos (Manuscrito 2). Escogimos como modelo de estudio la combinación *B. longum* NB667 /*Ba. fragilis* DSM2151, dado que fue en la que se obtuvo una mayor supervivencia de la bifidobacteria. Por otra parte, fue necesario realizar el estudio en glucosa para asegurar suficiente biomasa y proteína en fase exponencial con el fin de poder llevar a cabo el estudio proteómico mediante electroforesis en gel diferencial (2D-DIGE), siendo el co-cultivo *B. longum* NB667 /*Ba fragilis* DSM2151 el único que mostró variaciones notables respecto al monocultivo en el comportamiento de la bifidobacteria en este carbohidrato.

La metodología que seguimos, con marcaje de ambos monocultivos con el mismo fluoróforo y del co-cultivo con un fluoróforo diferente (Figura 9), impidió la diferenciación de las proteínas de ambos microorganismos cuando las condiciones de migración en los geles bidimensionales fueron idénticas, de manera que un número considerable de spots tuvieron que ser descartados del estudio (Figura 9). A pesar de las limitaciones de la técnica, se observaron diferencias claras de producción y expresión génica para tres enzimas. Los co-cultivos afectaron al catabolismo de carbohidratos en ambos microorganismos, causando una sobreproducción de la enzima piruvato quinasa en *B. longum* y provocando una disminución de la formación de fosfoenolpiruvato carboxiquinasa en *Bacteroides*. Sobre las implicaciones de los cambios de expresión de la fosfoenolpiruvato carboxiquinasa en el metabolismo general de *Ba. fragilis* se discutirá más adelante. Detectamos también cambios de expresión en la proteína peptidil/prolil cis/trans isomerasa tipo FKPB, una chaperona relacionada con el plegamiento de proteínas, lo que sugiere la activación de un mecanismo de respuesta a estrés en *Ba. fragilis* en presencia de *B. longum*.

En *B. longum* la piruvato quinasa cataliza la conversión irreversible de fosfoenolpiruvato a piruvato con la formación de una molécula de ATP. Este enzima también se ha encontrado sobreproducido en bifidobacterias en condiciones de estrés gastrointestinal (Sanchez y cols., 2005; Sanchez y cols., 2007), así como en *Lactobacillus delbrueckii* en cultivos conjuntos con *B. longum* (Sánchez y cols., 2013). La sobreexpresión de este enzima, con la consiguiente producción de ATP podría otorgar a la bifidobacteria una mayor capacidad competitiva y, por lo tanto contribuir a la mayor supervivencia mostrada en el co-cultivo respecto al monocultivo.

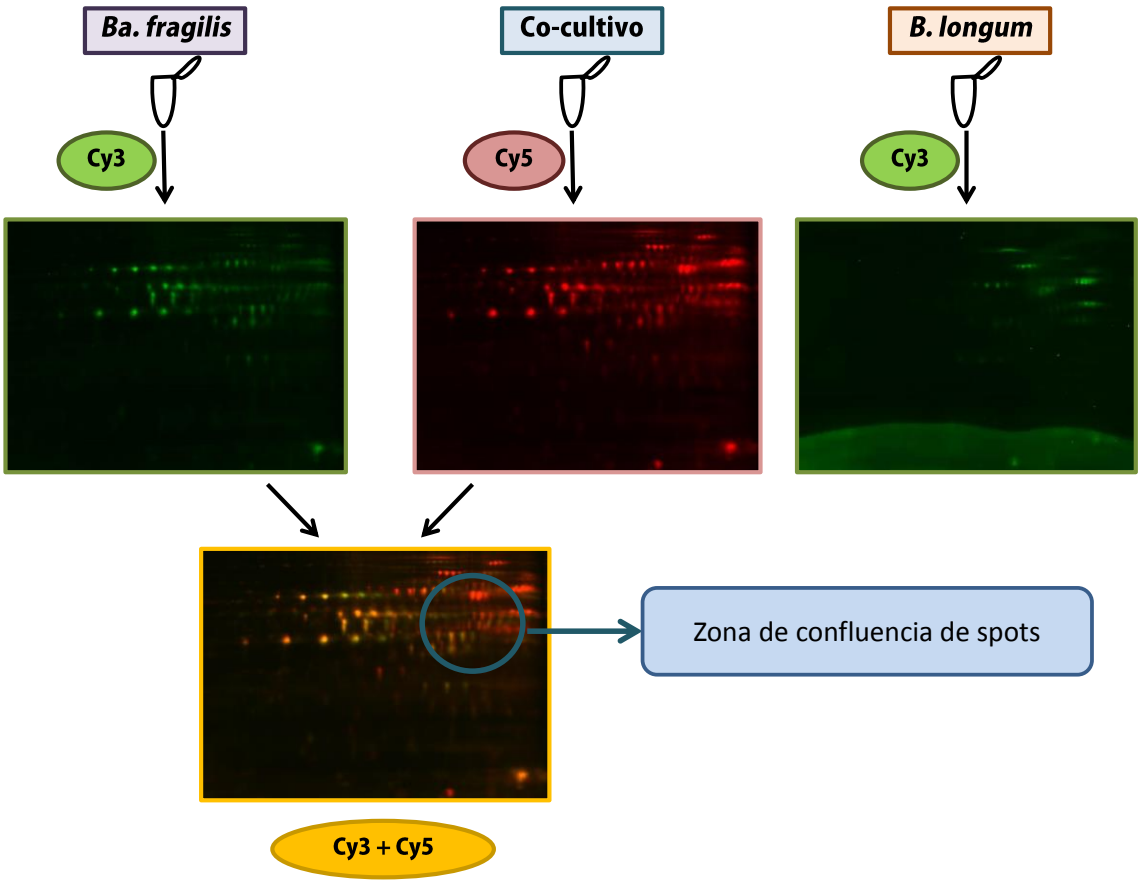

Figura 9. Esquema de la metodología 2D-DIGE en la realización de co-cultivos. En la zona marcada como zona de confluencia, la mayoría de spots de ambos microorganismos confluían, de manera que no pudieron ser analizados por el software informático. Como consecuencia, una gran parte de las proteínas de ambos microorganismos no fueron analizadas.

3.1.2 Interacciones de *Bacteroides* con EPS producidos por bifidobacterias

3.1.2.1 *Bacteroides fragilis* modifica su metabolismo en presencia de EPS aislados de bifidobacterias

Estudios previos de nuestro grupo de investigación mostraron que la presencia de EPS de bifidobacterias, en cultivos fecales, producía un incremento de la producción de ácido propiónico como consecuencia de la actividad metabólica de la microbiota intestinal, con un incremento de la población de microorganismos del género *Bacteroides* (Salazar y cols., 2008; Salazar y cols., 2009). Por otra parte, aunque aún no se ha demostrado la síntesis de EPS en el ambiente intestinal, hemos probado *in vitro* que la presencia de bilis, liberada al intestino delgado durante la digestión, estimula la síntesis de EPS por *B. animalis* (Ruas-Madiedo y cols. 2009). Es posible, por tanto, que los EPS producidos por bifidobacterias y otros microorganismos del ambiente intestinal puedan estar disponibles para las poblaciones microbianas sacarolíticas intestinales, entre las que *Bacteroides* es predominante. Por otra parte, algunos estudios muestran la resistencia de EPS de bifidobacterias a la

digestión gastrointestinal (Salazar y cols. 2009), de manera que los polímeros producidos por los cultivos iniciadores en algunos alimentos fermentados podrían llegar al colon, constituyendo una fuente adicional de carbohidratos fermentables para la microbiota intestinal. Los cultivos de *Ba. thetaiotaomicron* y *Ba. fragilis* realizados a lo largo de esta Tesis en medio basal con aminoácidos (mCFBM) y con EPS como único carbohidrato añadido mostraron un claro incremento de los niveles de ácido propiónico y una disminución de la relación de los ácidos acético/propiónico a lo largo de la fermentación, con respecto al patrón de producción de AGCC obtenido en los mismos cultivos utilizando glucosa como carbohidrato fermentable. Estos datos indican que el aumento de ácido propiónico previamente observado por nosotros en cultivos fecales adicionados con EPS (Salazar y cols. 2008; Salazar y cols. 2009) podría deberse en gran parte a la actividad metabólica de los microorganismos del género *Bacteroides* que son componentes mayoritarios de la microbiota intestinal.

Con el fin de conocer más en profundidad, a nivel fisiológico y molecular, los mecanismos de interacción de *Bacteroides* con los EPS de bifidobacterias, hemos estudiado los eventos que tienen lugar durante el cultivo de *Ba. fragilis* DSMZ2151 en presencia de los EPS E44 y R1 (Manuscrito 3). Hemos obtenido datos sobre la producción de AGCC y ácidos orgánicos, síntesis de proteínas celulares de diferentes grupos funcionales, expresión de genes y producción de proteínas del metabolismo central y relacionados con la degradación e incorporación de carbohidratos del medio, composición intra y extracelular de aminoácidos en los cultivos, niveles de NAD(P)H y FAD y balance redox intracelular. El análisis global de todos estos datos nos ha permitido formular la siguiente hipótesis general sobre los cambios que deben tener lugar en el metabolismo de *Ba. fragilis* para posibilitar su adaptación a un medio en el que los EPS, y adicionalmente los aminoácidos del medio, son las únicas fuentes de carbono disponibles. En presencia de EPS, nuestros datos sugieren una activación del catabolismo de aquellos aminoácidos cuyo esqueleto carbonado se incorpora a nivel de piruvato (metabolismo de glicina, serina y treonina) y a una represión de la síntesis de los que utilizan como precursor el ácido pirúvico (biosíntesis de leucina, isoleucina y valina), potenciándose asimismo la formación de α-cetoglutarato a partir de ácido glutámico, probablemente para favorecer las reacciones de transaminación a partir de este substrato y así mantener el pool intracelular de aminoácidos. Por otra parte, parece ocurrir también una expansión de la capacidad metabólica de *Ba. fragilis* para aprovechar los carbohidratos complejos presentes en el medio de cultivo. Se potencia la conversión preferente de PEP a piruvato en detrimento de su

paso directo a oxalacetato, a pesar de que esta última vía (conversión de PEP a oxalacetato) resulte más ventajosa *a priori* desde el punto de vista energético. Estos cambios irían encaminados a dar entrada al ácido pirúvico proveniente del catabolismo de aminoácidos a la vía del succinato-propionato. La potenciación de esta vía y el aumento de la conversión de ácido succínico en ácido propiónico, resulta energéticamente más favorable que la liberación de ácido succínico simplemente, al producirse una molécula extra de ATP en la formación de ácido propiónico (Figura 4, Manuscrito 3; Figura 10A). En presencia de glucosa y fuentes de carbono fácilmente asimilables, la vía del succinato-propionato mantiene el balance redox celular a través de la reoxidación de los dos moléculas de NADH generadas en la conversión de glucosa a PEP durante la glucolisis. Sin embargo, la escasa actividad de la vía glucolítica en ausencia de fuentes de carbono rápidamente fermentables, como ocurre en los cultivos en presencia de EPS, junto con la potenciación de la vía del succinato-propionato conducirían a un desbalance redox con un estado celular progresivamente más oxidado. Dicho desbalance redox sería compensado por el metabolismo de *Ba. fragilis* mediante dos estrategias: 1) inhibición de las rutas de síntesis de aminoácidos en las que intervienen reacciones redox con consumo de equivalentes reductores, y de las reacciones de elongación para la síntesis de proteínas 2) activación de la vía de las pentosas fosfato, permitiendo la regeneración de poder reductor con formación de NAD(P)H.

En relación con lo que se acaba de comentar, la inhibición de la producción de la enzima fosfoenolpiruvato carboxiquinasa que habíamos observado cuando *Ba. fragilis* se cultiva en presencia de *B. longum* (Manuscrito 2) sugiere un redireccionamiento, también en este caso, del catabolismo de *Bacteroides* hacia la formación de piruvato, de forma parecida a lo que ocurre en presencia de fuentes de carbono complejas y de metabolización lenta. Esta adaptación proporciona a *Ba. fragilis* una expansión de su capacidad metabólica para la obtención de energía a partir de fuentes de carbono alternativas en condiciones de competencia por nutrientes, como pueden ser aminoácidos u otras fuentes de carbono y energía de metabolización lenta, según acabamos de discutir previamente.

3.1.2.2 *Bacteroides fragilis* utiliza EPS aislados de bifidobacterias.

Hasta el momento, los medios de cultivo utilizados en esta Tesis Doctoral para los estudios metabólicos y de interacción fueron medios no definidos en los que se encontraban en proporción variable multitud de compuestos orgánicos que pueden ser utilizados por la maquinaria enzimática y metabólica de *Bacteroides* como fuente de carbono en ausencia de otros substratos más fácilmente

fermentables. Este diseño experimental nos ha permitido conocer los eventos metabólicos que tiene lugar en ausencia de carbohidratos fácilmente fermentables, pero en presencia de otros compuestos, así como de carbohidratos complejos, situación parecida a la del colon humano. El mayor crecimiento de *Ba. fragilis* y la mayor cantidad de metabolitos formados en medio de cultivo no definido (mCFBM) suplementado con EPS respecto al mismo medio sin fuente de carbono añadida, sugerían la utilización de los EPS como substratos fermentables, aunque las condiciones de nuestros ensayos no nos posibilitaban la validación de esta hipótesis. Por tanto, a fin de demostrar que *Ba. fragilis* puede metabolizar EPS producidos por bifidobacterias, realizamos incubaciones en medio mínimo utilizando fracciones purificadas de los EPS E44 y R1 (Manuscrito 4). En estos cultivos con EPS en medio mínimo ocurrió un incremento gradual de las proporciones molares de ácido propiónico a expensas de la disminución progresiva de la proporción molar de ácido láctico a lo largo de la incubación (Figura 10B), lo que pone de manifiesto un redireccionamiento gradual del catabolismo de *Ba. fragilis* hacia la síntesis de ácido propiónico y una adaptación del metabolismo microbiano a la obtención de energía a partir de fuentes de carbono de fermentación lenta.

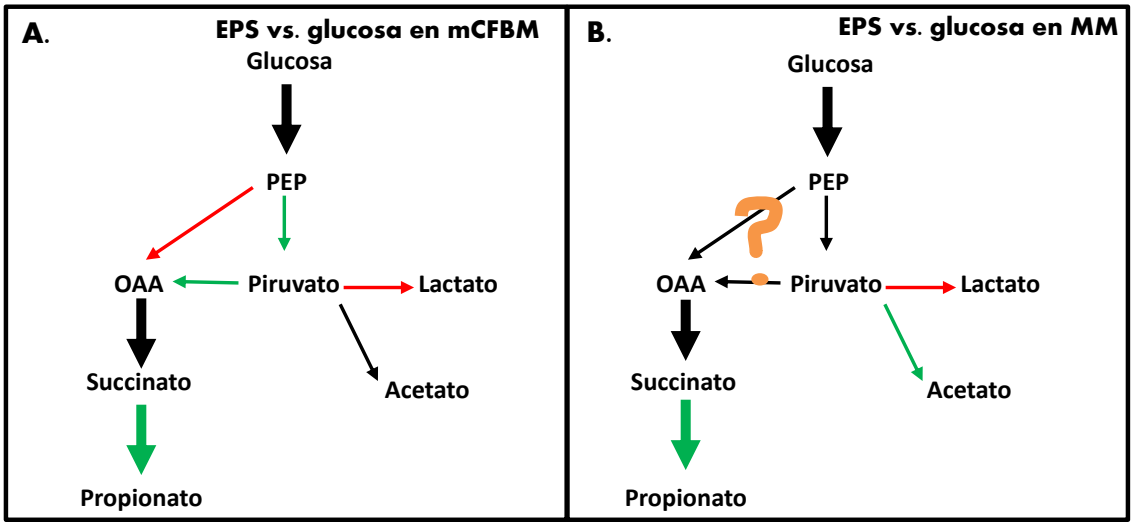

Figura 10. Resumen de los principales cambios en el metabolismo de *Ba. fragilis* en presencia de EPS. Las flechas rojas indican la inhibición de la expresión del gen que codifica el enzima que cataliza la reacción, menor producción de dicho enzima o menor producción del metabolito resultante. Las flechas verdes indican sobreexpresión del gen que codifica el enzima que cataliza la reacción, mayor producción de dicho enzima o mayor producción del metabolito resultante. MM, medio mínimo. mCFBM, medio basal con aminoácidos. PEP, fosfoenolpiruvato. OAA, oxalacetato.

Además, en estas condiciones se produjo una clara disminución a lo largo de la incubación de los dos picos de diferente masa molar que constituyen la fracción EPS E44, coincidiendo con un aumento en la liberación de AGCC y ácidos orgánicos. En cuanto a la fracción de EPS R1, aunque

no pudimos demostrar la degradación de los dos picos de mayor masa molar, no podemos descartar una utilización del pico de menor masa molar, que no pudimos analizar debido al solapamiento producido con el conjunto de proteínas presentes en las muestras en las condiciones cromatográficas utilizadas. El crecimiento de *Ba. fragilis* en etapas iniciales a expensas de EPS como única fuente de carbono fermentable fue menor que con glucosa pero contribuyó a mantener la viabilidad de *Ba. fragilis* en etapas tardías de crecimiento con respecto al patrón de comportamiento de este microorganismo en glucosa. Todo ello, supone la demostración de que *Ba. fragilis* puede utilizar algunos EPS de origen microbiano como fuente de carbono para su crecimiento, posibilitándole su supervivencia en condiciones de escasez de nutrientes. Nuestro trabajo constituye la primera demostración de dicha utilización como fuente de carbono fermentable de EPS microbianos por *Ba. fragilis*.

En *Bacteroides*, la formación de acetato a partir de piruvato y la producción de propionato por la vía del succinato-propionato sirven para proveer energía en forma de ATP a la célula, permitiendo además esta última vía la regeneración del NAD+ consumido durante la glucolisis, con el fin de estabilizar el balance redox celular. Aunque la regeneración de NAD+ puede obtenerse también mediante la conversión de ácido pirúvico a ácido láctico, la vía del succinato-propionato resulta energéticamente más favorable que la simple regeneración de NADH por producción de ácido láctico. Se observa a lo largo de todo nuestro estudio, y de forma más clara en los cultivos en medio mínimo, una relación inversa entre la abundancia relativa de los ácidos propiónico y láctico en los cultivos de *Ba. fragilis*. Así, los cultivos con glucosa presentaron menores niveles de ácido propiónico y mayores niveles de ácido láctico en medio mínimo respecto a medio basal con aminoácidos. Estas diferencias metabólicas podrían atribuirse a una formación preferente de ATP por síntesis de ácido acético y a la regeneración de NAD+ mediante formación de ácido láctico a partir de los carbonos procedentes de la glucolisis en MM (Figura 11A). Sin embargo, en medio basal con aminoácidos estaría ocurriendo también una entrada de carbonos procedentes de aminoácidos a nivel del piruvato, que conducirían a una activación de la vía del succinato-propionato con el consiguiente aumento de la proporción de ácido propiónico sintetizado y la disminución subsecuente de la conversión de piruvato a lactato (Figura 11B). Cuando se compara la producción de los diferentes metabolitos en MM y mCFBM con EPS como única fuente de carbono fermentable, de mano, las proporciones molares de lactato y acetato que se obtienen con MM son mayores que en mCFBM, en el que hay aminoácidos disponibles para *Bacteroides*,

mientras que la proporción molar de propionato es menor (Figuras 11C y 11D; Manuscritos 3 y 4). Este resultado refleja la vía preferente de obtención de energía y regeneración de NAD+ por *Ba. fragilis* dependiendo de la actividad de la vía glucolítica y el grado de regeneración de poder reductor dependiendo de las fuentes de carbono alternativas presentes en el medio.

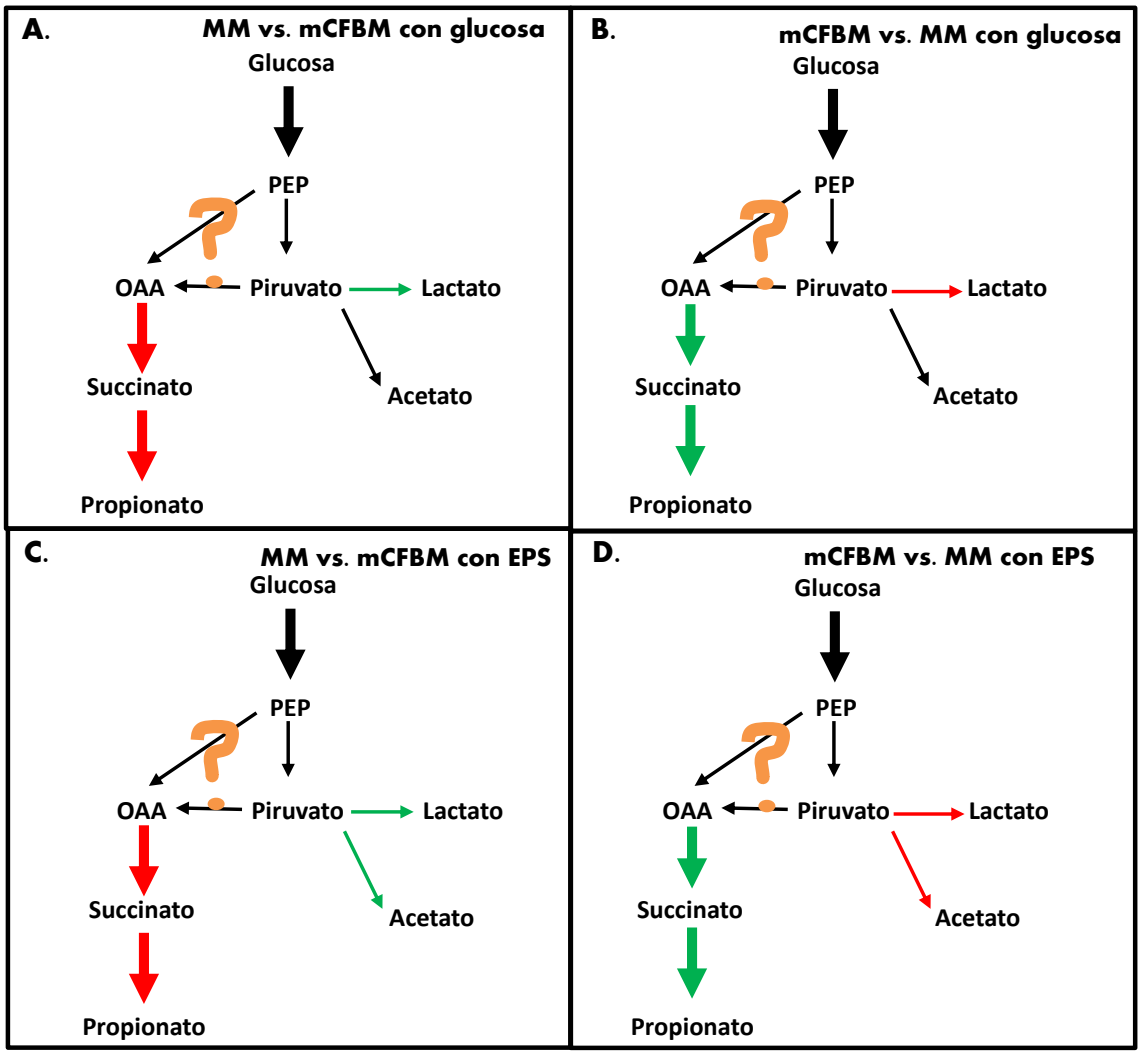

Figura 11. Resumen de los principales cambios en el metabolismo de *Ba. fragilis* en medio no definido con aminoácidos (mCFBM) y en medio mínimo sin aminoácidos (MM). Las flechas rojas indican menor producción relativa del metabolito resultante. Las flechas verdes indican mayor producción relativa del metabolito resultante. . PEP, fosfoenolpiruvato. OAA, oxalacetato.

3.1.3 Papel del género *Bacteroides* en la síntesis de ácido propiónico en el ambiente intestinal.

Bacteroides es capaz de utilizar EPS de bifidobacterias como substrato fermentable, con la consiguiente liberación de metabolitos entre los que el ácido propiónico se encuentra en alta proporción. Esta capacidad metabólica contribuye a mantener su viabilidad durante periodos

prolongados de tiempo en medio de cultivo. Extrapolando nuestros datos al ecosistema del colon humano, se podría plantear la hipótesis de que una relación trófica entre los EPS microbianos y *Bacteroides* podría influir sobre el balance de AGCC producidos en el intestino; además, la escasez de fuentes de carbono fácilmente fermentables y la presencia de carbohidratos complejos no digeribles de la dieta en el colon podrían favorecer la producción de ácido propiónico por parte de *Bacteroides,* predominando sobre la producción de ácido láctico y succínico.

Son tres las rutas metabólicas descritas en la microbiota intestinal que conducen a la síntesis de ácido propiónico, siendo mayoritaria la vía de la metil-malonil-coA mutasa, utilizada por los miembros del género *Bacteroides* (Reichardt y cols., 2014). *Veillonella parvula*, *Phascolarctobacterium succinatutens* y *Phascolarctobacterium faecium* son otros miembros de la microbiota intestinal capaces de producir ácido propiónico por esta vía. El primero de ellos puede sintetizar ácido propiónico a partir de ácido láctico y succínico mientras que los miembros del género *Phascolarctobacterium* son capaces de sintetizar ácido propionico a partir de succinato únicamente. Considerando que *Bacteroides* y bifidobacterias pueden producir ácido láctico y ácido succínico, la posibilidad de que al balance total de ácido propiónico producido por la microbiota intestinal pudieran contribuir también interacciones tipo cross-feeding (metabólico o de substrato) entre varios microorganismos, está escasamente explorada. Existen también otras rutas metabólicas por las que la microbiota intestinal produce propionato. *Coprococcus catus* es capaz de utilizar lactato para la producción de propiónico por la vía del acrilato. La tercera vía, del propanediol, es utilizada por *Roseburia inulinivorans* y *Ruminococcus obeum* para producir ácido propiónico a partir de ramnosa y fucosa (Reichardt y cols., 2014).

Hasta la fecha, no se conoce ningún miembro de la microbiota intestinal capaz de incorporar y metabolizar ácido propiónico, por lo que se considera que todo el ácido propiónico producido por la microbiota intestinal podría estar disponible para el hospedador (Chambers y cols., 2015).No obstante, un estudio reciente en el que se infusionaban AGCC marcados con isótopos estables en el intestino de ratones, puso de manifiesto la formación de ácido butírico a partir de ácido propiónico, siendo en todo caso esta vía de producción de butirato claramente minoritaria en relación con la producción a partir de ácido acético (den Besten y cols., 2013). El desarrollo de nuevas técnicas de análisis y el estudio de microorganismos no cultivables actualmente en condiciones de laboratorio, podrían ayudar a descifrar y comprender mejor las interacciones

microbianas que tienen lugar en el ecosistema intestinal en relación con la producción de ácido propiónico y el papel de *Bacteroides* y otros microorganismos intestinales en dichas interacciones (Salazar y cols., 2009; Lopez-Siles y cols., 2012).

La disponibilidad en el colón de ácido propiónico tiene una gran influencia sobre la salud humana (Chambers y cols., 2015). Entre los efectos fisiológicos del ácido propiónico en el organismo se encuentra su capacidad saciante, en cuyo mecanismo de acción interviene la liberación de péptido YY y de péptido 2 tipo glucagón por parte de las células intestinales, relacionados con la ingesta de alimentos. En un estudio reciente en el que se administraba inulina conjugada con propionato durante 24 semanas a 60 adultos con sobrepeso con el fin de observar su efecto a largo plazo, se constató una reducción de la ganancia de peso respecto a un grupo control al que se le administro inulina sin conjugar (Chambers y cols., 2015). Por otro lado, son varios los estudios que apuntan a un efecto hipolipemiante del ácido propiónico mediante la inhibición de la síntesis de ácidos grasos en el hígado y, como consecuencia, una disminución de ácidos grasos libres circulantes en sangre (Al-Lahham y cols., 2010).

3.2 Cross-feeding entre *Bifidobacterium adolescentis* y *Faecalibacterium prausnitzii*. Papel de los mecanismos de interacción metabólica microbiana en la síntesis de butirato en el colon.

Los mecanismos de cross-feeding entre bifidobacterias y otros miembros de la microbiota intestinal para la producción de ácido butírico a partir de los ácidos láctico y acético se encuentra bastante más estudiado que las posibles interacciones para la producción de ácido propiónico y son varios los microorganismos para los que se ha descrito su participación en mecanismos de cross-feeding con bifidobacterias (Tabla 1). Ello se debe, en gran parte, a la asociación demostrada entre el efecto bifidogénico y butirogénico desde los primeros estudios acerca de la influencia de probióticos y prebióticos sobre las poblaciones de bifidobacterias intestinales (Ramirez Farias, 2009; Riviere y cols., 2015). El trabajo de Ramirez-Farias y colaboradores (2006) proporcionó el primer dato sobre la posible existencia de mecanismos de cross-feeding a nivel intestinal entre *F. prausnitzii* y *B. adolescentis*; en dicho trabajo se observó un aumento de los recuentos de ambos microorganismos en la microbiota fecal de individuos que habían ingerido inulina, en comparación con el grupo placebo. Más recientemente, un estudio *in silico* de los genomas de dos cepas de ambos

microorganismos (*B. adolescentis* L2-32 y *F. prausnitzii* A2-165) permitieron desarrollar un modelo matemático de interacción para predecir los flujos de nutrientes entre ambos, en presencia de glucosa como fuente de carbono, sugiriéndose en dicho trabajo que ambos microorganismos poseían en conjunto la información genética necesaria para posibilitar las interacciones tipo cross-feeding (El-Semman y cols., 2014). El trabajo desarrollado en esta tesis, utilizando las mismas cepas que en el modelo matemático que acabamos de mencionar, demostró experimentalmente y por primera vez la utilización por parte de *F. prausnitzii* del ácido acético producido por *B. adolescentis*, en presencia de fructooligosacáridos como fuente de carbono, lo que contribuyó a potenciar la producción de ácido butírico por *F. prausnitzii* (Manuscrito 5).

Tabla 1. Microorganismos capaces de utilizar metabolitos producidos por miembros del género *Bifidobaterium* mediante mecanismos de cross-feeding

Microorganismo	Metabolitos utilizados	Metabolitos producidos	Referencia
Roseburia intestinalis	Ac. acético	Ac. butírico	(Belenguer y cols., 2006; Falony y cols., 2006)
Eubacterium hallii	Ac. acético y láctico	Ac. butírico	(Belenguer y cols., 2006)
Anaerostipes caccae	Ac. acético y láctico	Ac. butírico	(Belenguer y cols., 2006; Falony y cols., 2006)
Desulfovibrio piger	Ac. láctico	Ac. acético	(Marquet y cols., 2009)
Coprococcus catus	Ac. acético	Ac. butírico y propiónico	(Reichardt y cols., 2014)
Eubacterium rectales	Ac. acético	Ac. butírico	(Riviere y cols., 2015)
Faecalibacterium prausnitzii	Ac. acético	Ac.butírico	Esta Tesis

La maquinaria metabólica de *F. prausnitzii* es poco efectiva en cuanto a la utilización de los carbohidratos complejos que llegan al intestino procedentes de los alimentos, siendo además un microorganismo extremadamente sensible al oxígeno. A pesar de ello, constituye uno de los grupos mayoritarios de la microbiota intestinal humana, asociándose su descenso con estados de inflamación relacionados con disbiosis (Galecka, 2013). Una de las claves para la supervivencia de estos microorganimos en el ambiente intestinal es su capacidad para utilizar el ácido acético producido por otros miembros de la microbiota, como es el caso de bifidobacterias. En estados de enfermedad donde los niveles de *F. prausnitzii* se ven seriamente reducidos, el refuerzo de los mecanismos de cross-feeding a través de estrategias nutricionales (algunas de ellas mediante la

administración de combinaciones apropiadas de probióticos-prebióticos) pueden contribuir a restaurar los niveles de *Faecalibacterium* y, por consiguiente los niveles de butirato.

La mayoría de los géneros microbianos productores de ácido butírico presentes en el intestino, emplean la ruta de la butiril-CoA:acetato CoA transferasa. Existe una segunda ruta, de la butirato quinasa, exclusiva de algunas especies del género *Coprococcus* (Flint y cols., 2015). Las funciones que cumple el ácido butírico en el organismo humano comprenden desde el mantenimiento de la homeostasis del epitelio intestinal, hasta su implicación en el control de la respuesta inmunitaria de las células T reguladoras presentes en el colon (Zhang y cols., 2016). Además, está relacionado con una mayor sensibilidad a la insulina, y como consecuencia a un aumento del gasto metabólico. Además, promueve la oxidación de ácidos grasos y la termogénesis en músculo, hígado y tejido adiposo (Kasubuchi y cols., 2015). Dado que la microbiota es la principal fuente de butirato para nuestro organismo, el desarrollo de estrategias para recuperar unos niveles adecuados es esencial para el tratamiento de enfermedades inflamatorias intestinales y enfermedades relacionadas con la obesidad.

4. Consideraciones finales y perspectivas de futuro

En la presente Tesis doctoral hemos utilizado los microorganismos del género *Bifidobacterium* como eje central para el estudio de algunas de las interacciones bacterianas que tienen lugar entre los componentes de la microbiota intestinal, sus metabolitos y los nutrientes del entorno (Figura 12). Hemos puesto de manifiesto un aumento de la supervivencia de *B. longum* en presencia de *Ba. fragilis* en fuentes de carbono simples y complejas. Por otra parte, hemos demostrado que *Ba. fragilis* es capaz de utilizar los EPS producidos por bifidobacterias como fuente de carbono, adaptando su metabolismo a dicha fuente de carbono; la utilización de estos polímeros le permite aumentar su viabilidad en dichas condiciones. La adaptación del metabolismo de *Ba. fragilis* a la presencia de EPS y de otras fuentes de carbono complejas, como la inulina, conducen a un considerable aumento de la proporción relativa de ácido propiónico producido. Finalmente hemos demostrado experimentalmente, por primera, vez un mecanismo de cross-feeding entre *B. adolescentis* y *F. prausnitzii* en el que este último microorganismo en presencia de fructooligosacáridos es capaz de utilizar eficazmente el ácido acético producido por la bifidobacteria, consiguiéndose un considerable aumento de la síntesis de ácido butírico.

En los últimos años, la caracterización y descripción de poblaciones microbianas mediante técnicas de secuenciación de última generación ha estado en auge, lo que ha relegado a un segundo plano los estudios metabólicos y fisiológicos de los microorganismos considerados de forma separada o en grupos de interacción discretos. Teniendo en cuenta que cada individuo presenta una microbiota única, en la que se pueden encontrar cepas con características únicas y diferenciadas respecto a las de otro individuo, el estudio de las interacciones entre los miembros de la microbiota y de éstos con las fuentes de carbono es esencial para llegar a completar el conocimiento, por el momento más avanzado, sobre la composición y diversidad genética de este ecosistema. Un conocimiento más amplio del metabolismo de los miembros de la microbiota, de su relación con otros microorganismos del mismo entorno y de la verdadera especificidad de estas interacciones es crucial para establecer estrategias de modulación de la microbiota intestinal en diferentes estados de disbiosis. Este conocimiento podría ser una herramienta útil a la hora de diseñar dietas o intervenciones con probióticos y prebióticos dirigidos a poblaciones específicas. En el presente trabajo hemos demostrado que, más allá de los comportamientos generales de los microorganismos a nivel de género y en función de la fuente de carbono disponible, la elección de la cepa y fuente de carbono concretas condicionan en gran medida los resultados obtenidos. Todo ello, junto a los resultados ya obtenidos por otros autores apuntan a la necesidad de un diseño a nivel de grupo de población, o más probablemente personalizado, para obtener el máximo grado de eficacia en las intervenciones nutricionales y en el empleo de probióticos y prebióticos.

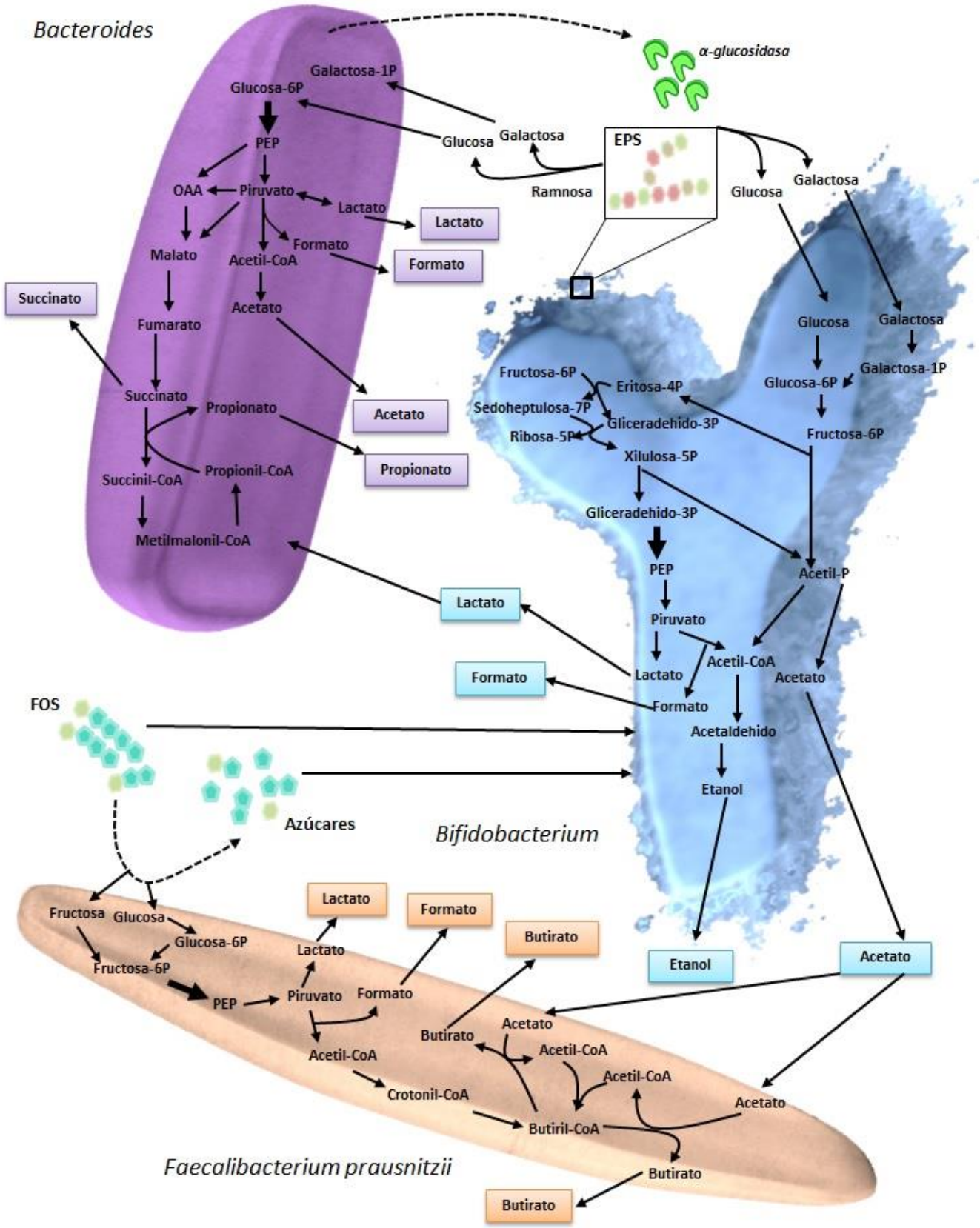

Figura 12. Resumen de las principales interacciones microbianas estudiadas en la presente Tesis Doctoral. PEP, fosfoenolpiruvato. OAA, oxalacetato. EPS, exopolisacárido

CONCLUSIONES

CONCLUSIONS

CONCLUSIONES

1. Las interacciones en co-cultivo entre miembros de los géneros *Bifidobacterium* y *Bacteroides* están fuertemente influenciadas por las características específicas de las cepas utilizadas y por las fuentes de carbono disponibles. En general, en fuentes de carbono complejas (inulina y exopolisacáridos de bifidobacterias-EPS), la proporción molar de ácido propiónico obtenida en los co-cultivos fue mayor que en glucosa.

2. La presencia de *Bacteroides fragilis* DSMZ2151 aumentó la supervivencia de *Bifidobacterium longum* y *Bifidobacterium breve* en co-cultivo. En concreto, el cultivo conjunto de *B. fragilis* DSMZ2151 y *B. longum* NB667 estimuló el crecimiento de la bifidobacteria en etapas tempranas de crecimiento, y causó cambios en la síntesis de enzimas relacionadas con el metabolismo central de hidratos de carbono de ambos microorganismos. La sobreproducción de la proteína con actividad chaperona FKBP-type peptidyl-prolyl cis/trans isomerasa por *B. fragilis* en co-cultivo sugiere la activación de un mecanismo de respuesta a estrés en este microorganismo.

3. El cultivo de *Bacteroides* en presencia de EPS de bifidobacterias condujo a un claro incremento en la proporción molar de ácido propiónico y a una disminución de la relación de los ácidos acético/propiónico a lo largo de la fermentación, con respecto al patrón de producción de estos mismos compuestos en cultivos utilizando glucosa como fuente de carbono.

4. El análisis global de los datos obtenidos a partir de cultivos de *Ba. fragilis* DSMZ2151 crecido en presencia EPS de bifidobacterias y su comparación con el comportamiento en glucosa, nos ha permitido formular una hipótesis general sobre los cambios a nivel fisiológico y molecular que deben tener lugar en el metabolismo de *Ba. fragilis* para posibilitar su adaptación a un medio en el que los EPS, y adicionalmente los aminoácidos del medio, son las fuentes de carbono disponibles.

5. En medio complejo con aminoácidos disponibles, es probable que ocurra una activación del catabolismo de los aminoácidos con la incorporación masiva de esqueletos carbonados a nivel de piruvato, y la activación de la vía del succinato-propionato que potencia la formación de propionato

y provee a la célula de energía en forma de ATP. El déficit de poder reductor generado por la utilización preferente de esta vía y la baja actividad glucolítica que ocurre en presencia de fuentes de carbono de fermentación lenta como los EPS, podría compensarse mediante la activación de la ruta de las pentosas fosfato para proveer a la célula de poder reductor extra en forma de NAD(P)H, a fin de mantener el equilibrio redox intracelular.

6. *Ba. fragilis* es capaz de utilizar de forma efectiva el EPS E44 aislado de *B. longum* como fuente de carbono y energía. En presencia de EPS como única fuente de carbono fermentable, se produjo una adaptación del metabolismo de *Bacteroides* hacia el progresivo incremento de la proporción de ácido propiónico sintetizado. La utilización de EPS posibilitó también a *Ba. fragilis* el mantenimiento de la viabilidad celular a tiempos prolongados de incubación.

7. Hemos demostrado experimentalmente un mecanismo de cross-feeding entre *Faecalibacterium prausnitzii* y *Bifidobacterium adolescentis*. La cepa *F. prausnitzii* S3L/3 fue capaz de utilizar el ácido acético producido por *Bifidobacterium adolescentis* L2-32 a partir de la fermentación del oligosacárido FOS P95, produciéndose un claro incremento de la producción de ácido butírico en co-cultivo respecto al monocultivo de *F. prausnitzii*.

Perspectiva final. Los resultados obtenidos en esta Tesis Doctoral indican que en el comportamiento de los microorganismos de la microbiota intestinal tienen una fuerte influencia los microorganismos del entorno y las fuentes de carbono disponibles. Todo ello, junto a los resultados ya obtenidos por otros autores apuntan a un diseño a nivel de grupo de población, o incluso personalizado, para obtener el máximo grado de eficacia en las intervenciones nutricionales y en el empleo de probióticos y prebióticos dirigidos a corregir disbiosis en la microbiota intestinal.

CONCLUSIONS

1. The interactions between members of *Bifidobacterium* and *Bacteroides* genera are strongly influenced by the strains and the carbon source present in the culture medium. In general, higher propionate levels were obtained in complex carbon sources (inulin and bifidobacterial EPS) than in glucose.

2. The presence of *Bacteroides fragilis* DSMZ2151 resulted in a higher survival of *Bifidobacterium longum* and *Bifidobacterium breve* in co-culture. In particular, co-cultures of *Ba. fragilis* with *Bifidobacterium longum* NB667, resulted in a better growth of the bifidobacteria at early stages of incubation and promoted changes in the central carbohydrate catabolism in both microorganisms. The overproduction of the FKBP-type pepetidyl-prolyl cis/trans isomerase of *B. fragilis*, a protein with chaperone function, in co-culture suggests the induction of a stress response in this microorganism.

3. The incubation of *Ba. fragilis* in the presence of EPS promoted a clear increase in the molar proportion of propionic acid and a decrease in the acetic/propionic acids ratio along fermentation with respect to the results obtained in glucose.

4. The data obtained in the cultures of *Ba. fragilis* DSM2151 grown with bifidobacterial EPS, in comparison with the cultures grown with glucose, allowed us to suggest a general hypothesis on changes at physiological and molecular levels that may occur in the metabolism of this microorganism to cope with environmental conditions in which EPS, and additionally amino acids from the culture medium, are the carbon sources available.

5. In complex medium with amino acids available, an activation of the catabolism of these compounds may occur through the conversion to pyruvate and the activation of the succinate-propionate pathway, which enhanced the propionate formation and provides the cell with energy in the form of ATP. A deficiency in reducing power, probably generated by the preferential use of the succinate-propionate pathway and by the scarce glucolytic activity, occurs in the presence of slowly

fermentable carbon sources, as is the case of EPS. This metabolic imbalance may be counteracted by the activation of the pentose phosphate pathway, which provides the cell with reducing power as NAD(P)H, thus keeping the intracellular redox balance..

6. *Ba. fragilis* was able to effectively use EPS E44 isolated from *B. longum* as carbon and energy source. This resulted in a metabolic adaptation of *Ba. fragilis* towards the progressive increase in the proportion of propionic produced. The use of EPS also enabled this microorganism to keep its cellular viability at long incubation times.

7. There is a cross-feeding mechanism between *Faecalibacterium prausnitzii* and *B. adolescentis*. *F. prausnitzii* S3L/3 was able to utilize acetate produced by *B. adolescentis* L2-32 during the co-cultivation in FOS P95 as carbon source. Butyrate production was enhanced in the co-culture with respect to the monoculture of *F. prausnitzii*.

Concluding remark. The results obtained in this PhD Thesis confirm that the behaviour of the intestinal microbiota populations is influenced by the presence of other microorganisms and the carbon sources available in the environment. These results, along with previous studies by other authors, point to a personalised design in the administration of pre- and probiotics, as well as the design of nutritional interventions in order to obtain the maximum eficiency for correcting dysbiosis of the intestinal microbiota.

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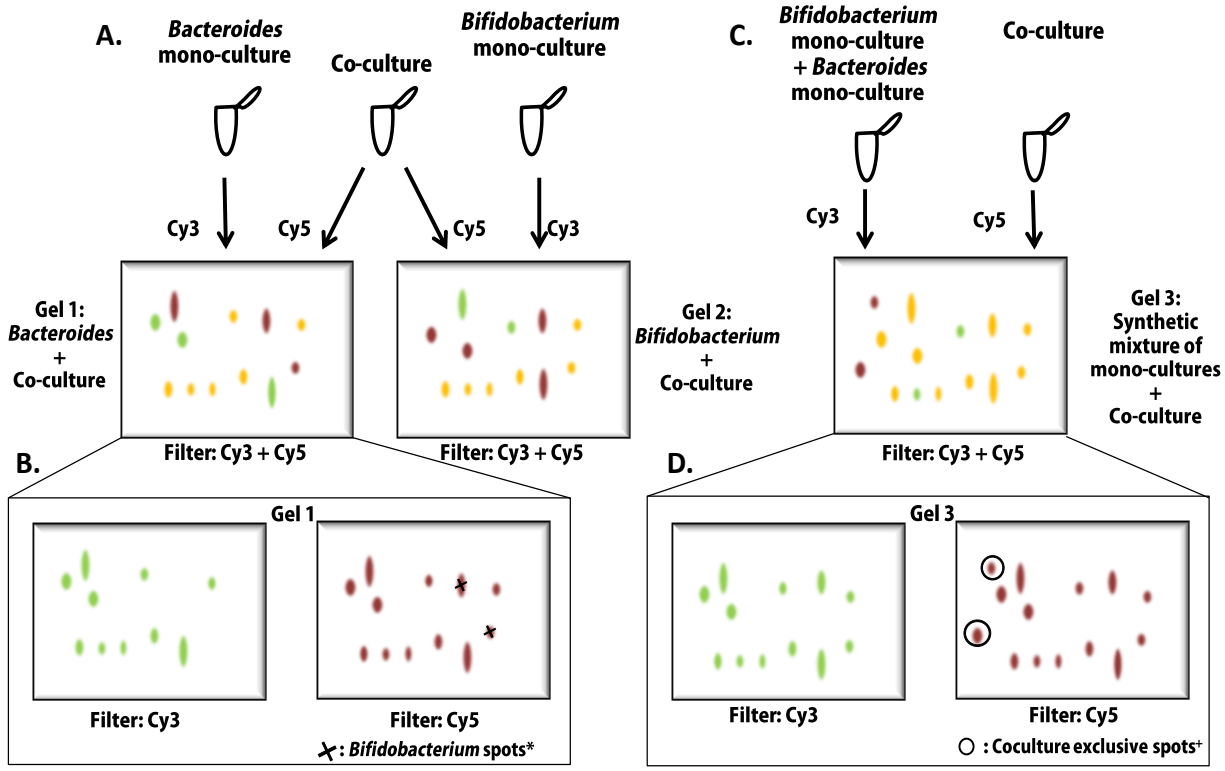
ANEXOS

ANEXOS

1. Material suplementario Manuscrito 2



Supplementary Figure 1. Methodology used in this study. Comparison of mono-culture with co-culture was performed in separated gels for each microorganism. Green spots mean higher production of protein in the mono-culture, red spots mean higher production of protein in the co-culture, and yellow spots mean that the protein production was similar in both conditions. *Spots detected in the co-cultures but not in the respective mono-culture were removed from this study, as putatively belonging to the other microorganism present in the co-culture. + To check the production of new proteins as a consequence of co-cultivation, a new gel was performed with a synthetic mixture of both mono-cultures marked with Cy3 and the co-culture marked with Cy5. In the present study, both samples showed the same spots, consequently, no new spots were produced due to the co-

2. Material suplementario Manuscrito 3

Table S1. Targeted genes and primers used for RT-qPCR in this study. Annealing temperature (Tm) was 60ºC.

Target gene	Primer name	Primer Sequence 5'-3'
Glutamate dehydrogenase (*gdhB*)	BFgdhF	GTGATGCGGAAATCATGCGT
	BFgdhR	TGACGCCACAATTCAAGCA
Phosphoenol pyruvate kinase (*pck*)	BFpepkF	ATCACTGAACCGACTCCGACA
	BFpepR	GCAATGATAAGAATGCAGCACC
Acetolactate synthetase *(ilvB)*	BFilvF	CGTGCATTCCCAGCATACC
	BFilvR	TTGGGACTGTCGGCTTTGC
Methylmalonil-CoA mutase (*mutB*)	BFmmmF	CCCTTACCGGTTTTCACACATT
	BFmmmR	TCAGGCCGAAGTGGACAAG
Membrane protein OmpA (*ompA*)	BFOmpAF	CGGTACGCGGAGCAATGT
	BFOmpAR	GTGGCAGAAGTTGCCGAACA
Pyruvate phosphate dikinase (*ppdK*)	BFpyrKF	GCACGTTCCTCTTCGCTCA
	BFpyrKR	TAAGATCGGTTCCCAGCGTT
Transketolase (*tktB*)	BFtktF	GGATCAGTGCTGTAGGAGTCGA
	BFtktR	TGGAAGAGACAACCATTGCG
Pyruvate carboxylase (*pyc*)	BFpyCF	CGGAGTCTCTTGCGCATTCT
	BFpyCR	AAGAGGTACTTTCGCCGCTT
Malate dehydrogenase (*mdh*)	BFmdhF	TGACCCGCCAACTCTACGA
	BFmdhR	CTTCGGCAAATACCACAC

Table S2. Changes in aminoacid profile during incubation of *B. fragilis* with glucose (G), EPS (E44 and R1) and without carbohydrate source added to the culture medium (WCS). Arrows up and down indicate increase or decrease, respectively, of amino acid concentration as regards to the initial levels, during incubation. Different letters indicate significant differences among cultures in different carbohydrate sources ($P < 0.05$).

Amino acid	Carbohydrate source	Supernatant		Cell-free extracts
		mM	% consumption	(mmol / g protein)
Aspartic	G	-0.76±0.08^{a}	-52.33±12.65↓^{a}	11,83±5,31^{a}
	E44	-0.56±0.07^{b}	-38.09±4.90↓^{ab}	35.84±26.45^{a}
	R1	-0.44±0.06^{b}	-32.68±4.93↓^{b}	37.03±11.98^{a}
	WCS	0.12±0.02^{c}	9.00±1.78↑^{c}	91.27±11.82^{b}
Glutamic	G	0.14±0.11^{b}	3.49±3.23↑^{b}	139.11±42.62^{a}
	E44	0.27±0.05^{b}	6.15±1.67↑^{b}	166.06±31.72^{a}
	R1	-0.09±0.02^{a}	-2.17±0.52↓^{a}	184.27±38.88^{a}
	WCS	-0.09±0.04^{a}	-2.15±1.01↓^{a}	285.30±15.12^{b}
Asparagine	G	-1.04±0.14	-100.00±0.00↓	0.00±0.00
	E44	-0.99±0.16	-100.00±0.00↓	0.00±0.00
	R1	-1.02±0.21	-100.00±0.00↓	0.00±0.00
	WCS	-0.94±0.00	-100.00±0.00↓	0.00±0.00
Serine	G	-0.28±0.44^{b}	-11.28±-19.64↓^{b}	7.42±9.66
	E44	-1.05±0.12^{a}	-54.68±18.66↓^{a}	12.28±21.27
	R1	-0.63±0.25^{ab}	-33.41±16.58↓^{ab}	0.80±1.60
	WCS	-0.20±0.05^{b}	-10.50±2.75↓^{b}	9.81±11.45
Histidine	G	0.11±0.01^{a}	31.72±8.60↑^{a}	0.60±1.19
	E44	0.21±0.03^{b}	89.98±15.29↑^{c}	0.90±1.56
	R1	0.18±0.05^{ab}	75.61±6.32↑^{bc}	1.69±1.96
	WCS	0.14±0.01^{ab}	59.49±4.44↑^{b}	0.00±0.00
Glycine	G	1.21±0.34^{b}	84.07±33.00↑^{b}	90.24±16.82
	E44	0.51±0.10^{a}	34.60±7.04↑^{a}	92.03±24.68
	R1	0.40±0.04^{a}	28.83±2.11↑^{a}	97.91±23.37
	WCS	0.38±0.04^{a}	27.81±2.98↑^{a}	114.48±11.11
Threonine	G	1.64±0.07^{c}	133.27±10.36↑^{c}	15.78±13.67
	E44	-0.33±0.15^{a}	-27.62±18.20↓^{a}	2.10±3.63
	R1	-0.27±0.06^{a}	-22.35±5.41↓^{a}	5.39±4.97
	WCS	0.22±0.04^{b}	17.58±2.88↑^{b}	0.00±0.00
Arginine	G	0.27±0.22	28.58±20.48↑^{a}	1.78±2.28^{a}
	E44	0.54±0.14	65.33±7.60↑^{b}	28.22±4.33^{ab}
	R1	0.40±0.06	50.38±15.68↑^{ab}	29.11±11.28^{b}
	WCS	0.41±0.03	54.09±4.25↑^{ab}	12.15±11.31^{ab}
GABA	G	0.52±0.22^{b}	521.51±283.81↑^{b}	66.04±33.62^{b}
	E44	0.02±0.01^{a}	16.88±14.39↑^{a}	0.00±0.00^{a}
	R1	-0.05±0.11^{a}	14.11±15.40↑^{a}	0.49±0.57^{a}
	WCS	0.08±0.00^{a}	81.61±1.99↑^{a}	0.00±0.00^{a}
Alanine	G	1.35±0.45	28.43±13.36↑	0.00±0.00

	E44	0.56±0.30	10.80±6.50↑	0.00±0.00
	R1	0.58±1.02	8.50±13.80↑	0.00±0.00
	WCS	0.27±0.14	6.03±3.15↑	0.00±0.00
Proline	G	1.38±1.23	50.43±50.30↑	109.99±41.22[a]
	E44	0.99±0.44	20.38±8.43↑	251.05±14.75[b]
	R1	0.17±0.39	3.41±8.17↑	238.07±48.66[b]
	WCS	0.16±0.10	3.60±2.31↑	366.59±19.34[b]
Tyrosine	G	0.23±0.15[b]	35.16±25.85↑[b]	122.93±12.22[a]
	E44	0.01±0.08[ab]	0.83±10.43↑[a]	99.77±14.73[a]
	R1	0.01±0.10[ab]	0.90±11.80↑[a]	112.96±25.47[a]
	WCS	-0.03±0.03[a]	-3.62±3.39↓[a]	220.81±17.88[b]
Valine	G	0.15±0.09	8.66±6.03↑	101.67±5.64[a]
	E44	0.07±0.15	3.28±9.86↑	80.95±34.37[a]
	R1	-0.03±0.03	-1.77±1.18↓	105.64±19.56[a]
	WCS	0.06±0.04	3.74±2.38↓	185.28±2.78[b]
Methionine	G	-0.05±0.01	-12.65±3.72↓	1.77±0.81
	E44	0.01±0.06	-0.02±15.06↓	7.20±0.02
	R1	-0.03±0.00	-5.49±3.84↓	7.53±1.33
	WCS	-0.01±0.00	-1.42±0.67↓	6.82±3.01
Tryptophan	G	-0.01±0.01[a]	-3.87±6.84↓[a]	0.00±0.00
	E44	0.03±0.02[b]	15.15±11.19↑[b]	0.00±0.00
	R1	0.02±0.01[b]	12.57±6.14↑[b]	0.22±0.44
	WCS	0.01±0.01[ab]	4.92±3.28↑[ab]	0.00±0.00
Isoleucine	G	-0.22±0.16	-12.18±7.88↓	58.51±2.38[a]
	E44	-0.25±0.19	-16.26±15.49↓	43.69±28.92[a]
	R1	-0.27±0.04	-16.36±2.91↓	63.36±12.58[a]
	WCS	-0.11±0.03	-7.08±2.25↓	110.71±6.83[b]
Leucine	G	-0.71±0.27	-21.61±5.87↓	32.32±13.33
	E44	-0.67±0.39	-22.70±17.28↓	30.79±32.46
	R1	-0.73±0.19	-22.81±2.70↓	50.97±19.89
	WCS	-0.29±0.07	-10.06±2.39↓	77.70±25.53
Phenylalanine	G	-0.14±0.05	-9.81±3.66↓	25.24±4.77
	E44	-0.10±0.07	-7.99±7.04↓	23.21±20.13
	R1	-0.17±0.09	-11.58±3.22↓	36.06±10.15
	WCS	-0.06±0.03	-4.97±2.53↓	49.83±9.39
Ornithine	G	0.20±0.13[c]	226.93±159.69↑[b]	14.45±11.61[b]
	E44	0.01±0.02[b]	7.60±17.85↑[a]	0.00±0.00[a]
	R1	0.08±0.03[bc]	65.14±26.49↑[a]	11,99±3,18[ab]
	WCS	-0.37±0.01[a]	-60.43±1.35↓[a]	0.00±0.00[a]
Lysine	G	0.39±0.10[a]	34.94±10.18↑[a]	39.96±7.33[a]
	E44	1.01±0.25[b]	79.89±8.65↑[b]	85.79±26.98[ab]
	R1	0.67±0.26[ab]	57.00±27.36↑[ab]	105.53±21.72[b]
	WCS	0.82±0.05[b]	72.87±4.65↑[b]	132.88±10.83[c]

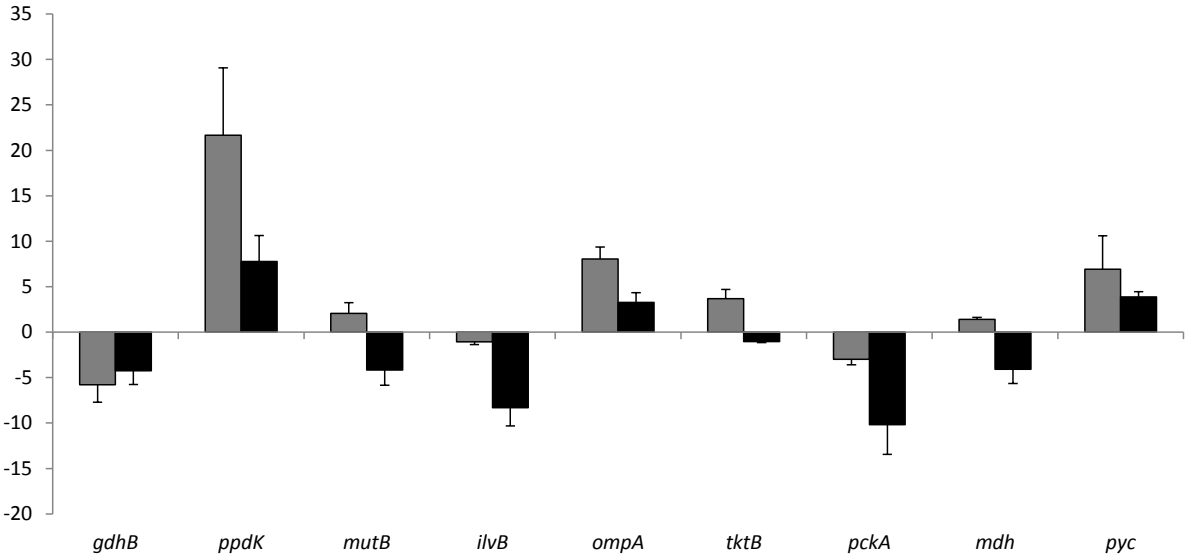


Figure S1. Relative expression levels of different genes in cultures of *B. fragilis* grown in the presence of bifidobacterial EPS fractions with respect to the cultures in glucose. Glutamate dehydrogenase (*gdhB)*, pyruvate phosphate dikinase (*ppdK)*, methyl-malonyl CoA mutase (*mutB*), acetolactate synthetase (*ilvB)*,membrane protein OmpA (*ompA)*, transketolase (*tktB*), phosphoenolpyruvate carboxykinase (pckA), malate dehydrogenase (*mdh*) and pyruvate carboxylase (*pyc*) genes. Grey bars, changes in gene expression levels in the presence of EPS E44 with respect to glucose. Black bars, changes in gene expression levels in the presence of EPS R1 with respect to glucose.

3. Material suplementario Manuscrito 4

Table S1. Molar proportions of metabolites produced by *B. fragilis* in glucose, EPS E44 and EPS R1 as carbon source and without carbon source

Carbon source	Time (h)	Acetic acid	Propionic acid	Lactic acid	Succinic acid	Formic acid
Glc	24	30.26±0.11	10.10±1.15	34.49±0.51	8.06±0.18	17.08±0.80
	48	29.03±1.11	10.25±0.20	37.40±2.20	8.22±0.76	15.02±1.16
	72	29.29±1.09	10.69±0.93	34.29±2.14	9.27±0.40	16.12±0.40
	144	29.72±0.95	10.78±0.25	33.18±1.33	9.88±0.73	15.78±0.68
EPS E44	24	41.05±0.73	25.80±1.85	14.87±2.94	3.81±0.41	14.43±0.94
	48	26.15±2.78	10.95±2.66	26.70±0.64	1.51±0.47	35.65±4.91
	72	35.71±3.74	18.03±3.57	14.50±1.20	0.54±0.51	31.17±6.92
	144	54.96±1.37	27.40±2.17	2.52±1.41	1.25±0.53	13.87±2.56
EPS R1	24	40.43±3.39	32.49±3.31	3.71±2.39	6.09±1.15	17.28±5.44
	48	29.06±2.49	17.08±1.36	10.17±1.12	3.04±0.19	40.19±4.35
	72	39.57±2.02	27.50±2.95	2.37±1.68	4.89±0.02	25.67±3.28
	144	47.01±1.41	25.63±2.65	0.34±0.11	7.98±0.14	19.04±3.02
SFC	24	29.20±3.22	36.67±1.90	ND	16.08±3.82	12.19±3.03
	48	26.48±3.51	33.58±2.13	ND	20.39±1.54	16.61±3.36
	72	32.55±5.49	33.02±1.14	ND	18.32±2.59	15.73±3.18
	144	38.46±4.43	30.66±3.28	ND	13.41±1.27	15.60±1.21

ND- Not determined because there were values of lactic under 0.

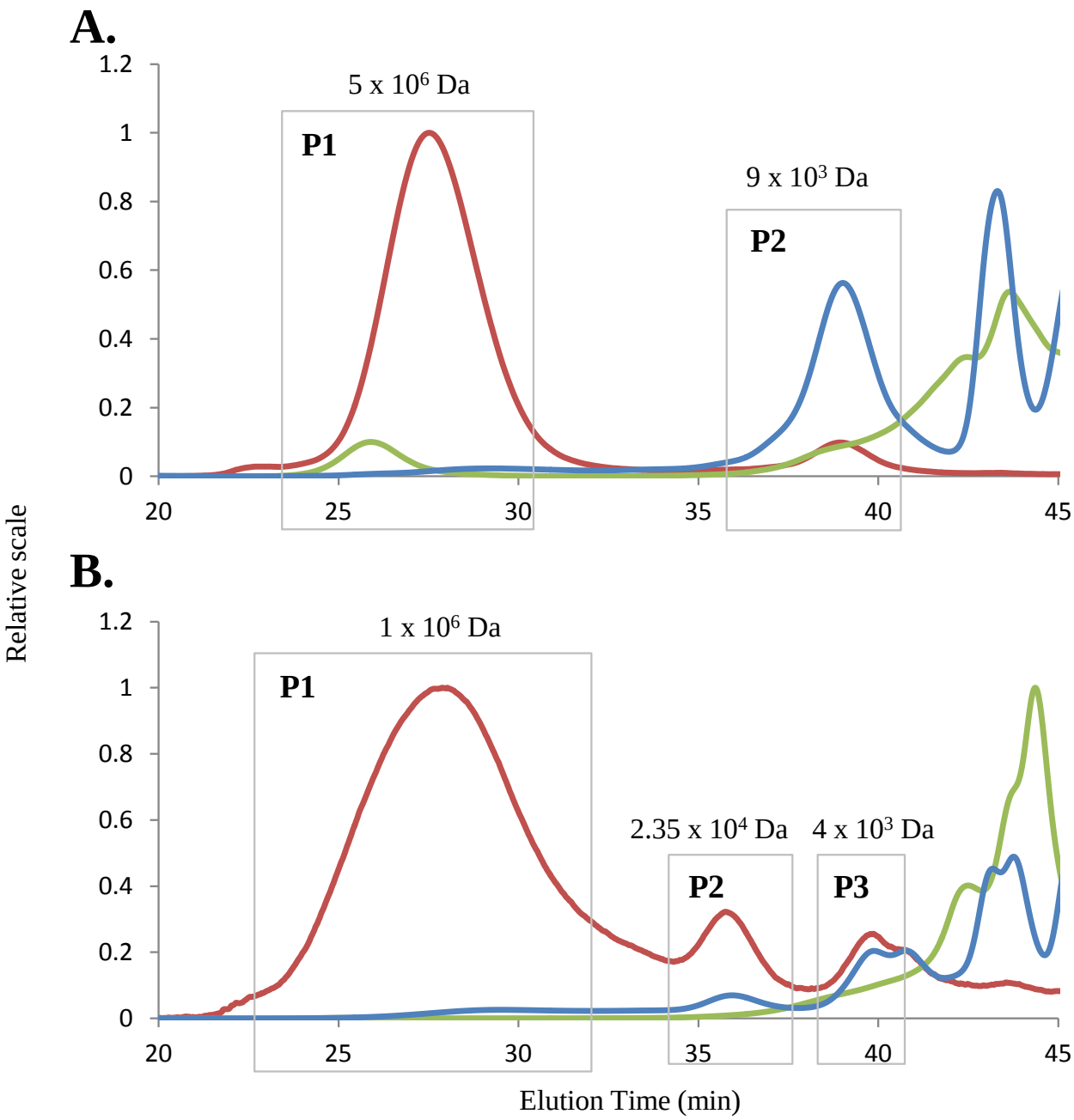


Figure S1. Size exclusion chromatography (SEC-MALS) analysis of the EPS E44 (A) and EPS R1 (B) fractions purified from the cell biomass of *Bifidobacterium longum* E44 and *Bifidobacterium animalis* subsp. *lactis* R1, respectively. Refractive index detector (blue line) for detection and quantification of EPS peaks, PDA detector (green line) set at 280 nm to identify the presence of proteins, and the multiangle laser light scattering (MALS) for molar mass distribution of the EPS fractions (red line).

4. Informe sobre la calidad de los artículos

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Manuscrito	Revista	Area	Q	FI	Citas
Manuscrito 0	Frontiers in Microbiology	Microbiology	Q1	3,989	0
Manuscrito 1	Applied and environmental Microbiology	Microbiology	Q1	3,952	10
Manuscrito 2	Canadian Journal of Microbiology	Biotechnology and Applied Microbiology	Q3	1,220	In press
Manuscrito 3	Frontiers in Microbiology	Microbiology	Q1	3,989	0
Manuscrito 4	BMC Microbiology	Microbiology	Q2	2.729	Enviado
Manuscrito 5	FEMS Letter in Microbiology	Microbiology	Q3	2,121	1