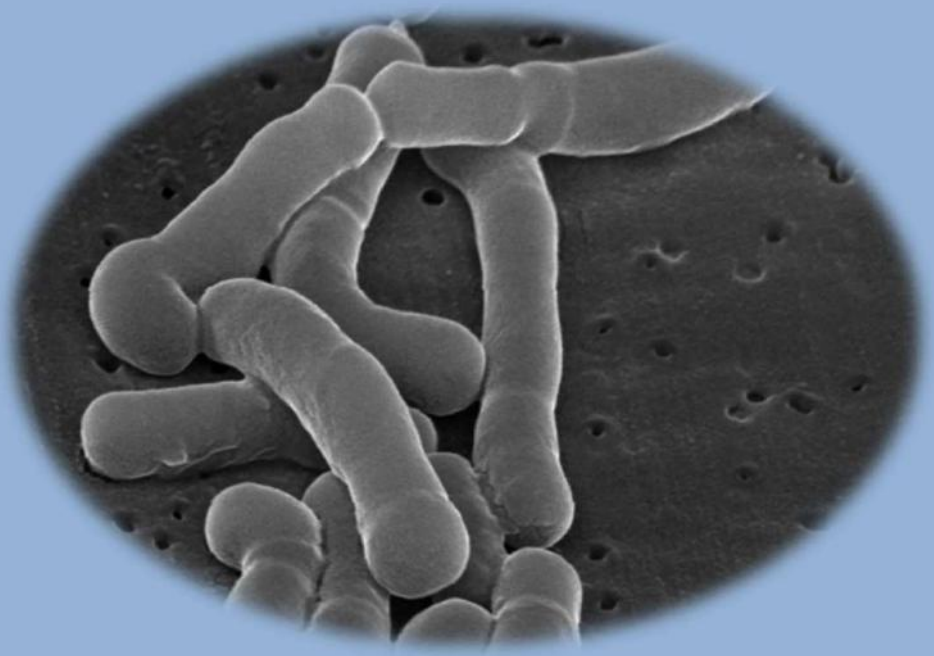
universidad de león

FACULTAD DE VETERINARIA
Programa de Doctorado "Medicina, Sanidad y Producción Animal y Ciencia de los Alimentos"

PROBIÓTICOS Y PREBIÓTICOS PARA APLICACIÓN ESPECÍFICA EN LA TERCERA EDAD



LORENA VALDÉS VARELA

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FACULTAD DE VETERINARIA

PROBIÓTICOS Y PREBIÓTICOS PARA APLICACIÓN ESPECÍFICA EN LA TERCERA EDAD

Memoria presentada por la Licenciada en Veterinaria **Dña. Lorena Valdés Varela** y dirigida por los doctores **D. Miguel Gueimonde Fernández**, **Dña. Patricia Ruas Madiedo** y **Dña. Mercedes López Fernández** para optar al grado de *Doctor por la Universidad de León*, dentro del programa de doctorado "*Medicina, Sanidad y Producción Animal y Ciencia de los Alimentos*".

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CSIC
Consejo Superior de Investigaciones Científicas

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Este trabajo ha sido realizado en el **Instituto de Productos Lácteos de Asturias (IPLA-CSIC)**.

A mis padres
A Betty y Ayss
A Héctor

"Nunca olvidas a alguien importante en tu vida, solo aprendes a vivir sin él"
Anónimo

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“El precio del éxito es trabajo duro, dedicación al trabajo que estamos haciendo, y la determinación de que, ganemos o perdamos, hemos aplicado lo mejor de nosotros mismos a la tarea que tenemos entre manos”

Vince Lombardi

“El final de un trabajo o una relación puede parecer como la noche más oscura, pero no es más que la temporada de invierno. El tiempo de renovación y renacimiento que precede a la nueva plantación. El comienzo del gran ciclo siguiente”

Jonathan Lockwood Huie

ÍNDICE

Listado de abreviaturas ii
Listado de figuras iii
Resumen v

1-INTRODUCCIÓN

1.1. Problemática del envejecimiento de la población 1
1.2. Cambios fisiológicos y nutricionales asociados al envejecimiento 4
1.2.1. Alteraciones del sistema digestivo 4
1.2.2. Inmunosenescencia 6
1.2.3. Alteraciones en la función cognitiva 7
1.2.4. Alteraciones nutricionales 8
1.3. Cambios en la microbiota intestinal durante el envejecimiento 9
1.3.1. Microbiota intestinal: composición y función 9
1.3.2. Alteraciones asociadas al envejecimiento 15
1.4. *Clostridium difficile* como agente infeccioso en la tercera edad 16
1.4.1. Fisiología y taxonomía 16
1.4.2. *C. difficile* como patógeno 18
1.4.2.1. Epidemiología 18
1.4.2.2. Factores de virulencia 20
1.4.2.3. Patogenia 22
1.4.2.4. Cuadro clínico 26
1.4.3. Tratamientos terapéuticos actuales y emergentes 26
1.5. Alimentos funcionales: probióticos y prebióticos 29
1.5.1. Definiciones 29
1.5.2. Microorganismos más comúnmente utilizados en alimentos funcionales 31
1.5.3. Sustratos prebióticos más comúnmente utilizados en alimentos funcionales 33
1.5.4. Aplicaciones de los alimentos funcionales 35
1.5.4.1. Alimentos funcionales probióticos 35
1.5.4.2. Alimentos funcionales prebióticos 40
1.5.5. Probióticos y prebióticos en productos desarrollados para la tercera edad 42

2-OBJETIVOS 45

3-TRABAJO EXPERIMENTAL

Capítulo 1: Selección de probióticos, prebióticos y simbióticos adecuados para la modulación de la microbiota intestinal de personas de edad avanzada. 47

Artículo 1. **Valdés L.**, Salazar N., González S., Arboleya S., Ríos-Covian D., Genovés S., Ramón D., de los Reyes-Gavilán C.G., Ruas-Madiedo P. y Gueimonde M. (2016). Selection of potential probiotic bifidobacteria and prebiotics for elderly by using *in vitro* faecal batch cultures. European Food Research and Technology, en prensa ("online" doi: 10.1007/s00217-016-2732-y). **49**

Artículo 2. **Valdés-Varela L.**, Ruas-Madiedo P. y Gueimonde M. (2016). *In vitro* fermentation of different fructo-oligosaccharides by *Bifidobacterium* strains for the selection of synbiotic combinations. (Manuscrito enviado al International Journal of Food Microbiology). **59**

Capítulo 2: Selección de probióticos, prebióticos y simbióticos frente a *Clostridium difficile*. **71**

Artículo 3. **Valdés L.**, Gueimonde M. y Ruas-Madiedo P. (2015). Monitoring in real time the cytotoxic effect of *Clostridium difficile* upon the intestinal epithelial cell line HT29. Journal of Microbiological Methods, 119, 66-73. **73**

Artículo 4. **Valdés-Varela L.**, Alonso-Guervos M., Garcia-Suárez O., Gueimonde M. y Ruas-Madiedo P. (2016). Screening of bifidobacteria and lactobacilli able to antagonize the cytotoxic effect of *Clostridium difficile* upon intestinal epithelial HT29 monolayer. Frontiers in Microbiology, 7, 577. **81**

Artículo 5. **Valdés-Varela L.**, Hernández-Barranco A.M., Ruas-Madiedo P. y Gueimonde M. (2016). Effect of *Bifidobacterium* upon *Clostridium difficile* growth and toxicity when co-cultured in different prebiotic substrates. Frontiers in Microbiology, 7, 738. **93**

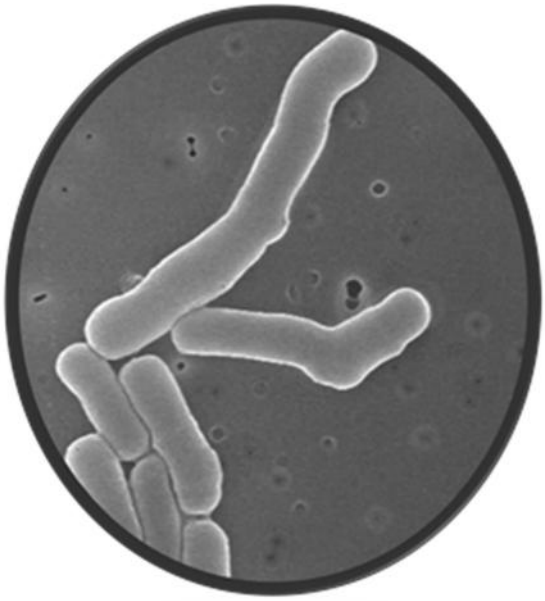
4-DISCUSIÓN GENERAL **103**

5-CONCLUSIONES **121**

6-BIBLIOGRAFÍA **123**

7-ANEXOS

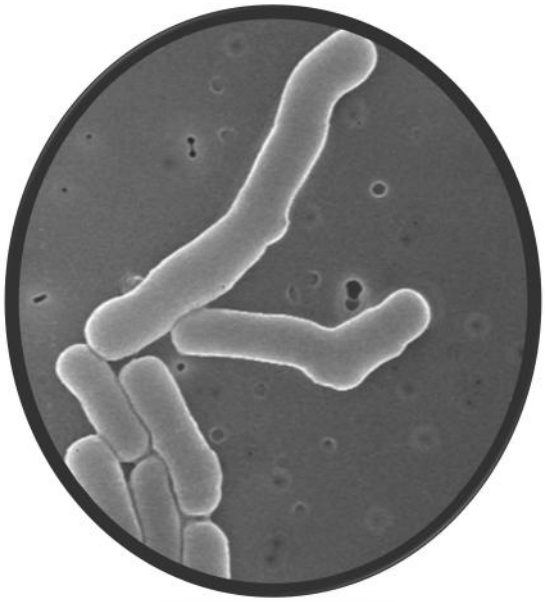
Información suplementaria artículo 1 **147**
Información suplementaria artículo 2 **155**
Información suplementaria artículo 3 **159**
Información suplementaria artículo 4 **161**
Información suplementaria artículo 5 **164**



LISTADO DE ABREVIATURAS

Act: "Actilight".
AGCCs: Ácidos grasos de cadena corta.
ATCC: "American Type Culture Collection" (Colección Americana de Cultivos tipo).
BCCM/LMG: "Belgian Coordinated Collection of Microorganisms/LMG Bacteria Collection".
BHI: "Brain heart infusión".
BI/NAP1/027: Cepa de *Clostridium difficile* caracterizada como grupo BI por análisis por restricción de endonucleasas; como tipo NAP1 por electroforesis en gel de campo pulsado y finalmente 027 como el ribotipo correspondiente.
CDT: "*C. difficile* transferase" (toxina binaria de *Clostridium difficile*)
Células NK: Células "natural killer" (células asesinas naturales).
CFBM: "Carbohydrate free basal medium" (medio basal libre de carbohidratos).
CI: "Cell index" (unidad arbitraria que indica variaciones de impedancia).
CSLM: "Confocal scanning laser microscopy" (microscopio confocal laser de barrido).
DMEM: "Dulbecco´s Modified Eagle Medium".
DP: "Degree of polymerization" (grado de polimerización).
DSMZ: "Deutsche Sammlung von Mikroorganismen und Zellkulturen" **(**Colección Alemana de Cultivos Tipo).
EC50: "Half maximal effective concentration" (concentración de una sustancia que induce la mitad del máximo efecto adverso).
EFSA: "European Food Safety Authority" (Autoridad Europea de Seguridad Alimentaria).
EII: Enfermedad inflamatoria intestinal.
EMP: Vía Embden-Meyerhof-Parnas.
FAO: "Food and Agriculture Organization of the United Nations" (Organización de las Naciones Unidas para la Agricultura y la Alimentación).
FC: "Fold-change" (nivel de cambio).
FDA: "Food and Drug Administration" (Agencia de Medicamentos y Alimentos de EE.UU.).
FFn: Número "n" de unidades de fructosa unidas entre sí por enlaces β-(2-1).
FID; "Flame injection detector" (detector de ionización en llama).
FMT: "Fecal microbiota transplantation" (transplante de microbiota fecal).
FOS: Fructooligosacáridos.
FSB: "Foetal serum bovine" (suero fetal bovino).
GAM: "Gifu anaerobic medium" (medio para el cultivo de bacterias anaerobias).
GFn: Número "n" de unidades de fructosa unidas a una glucosa terminal mediante enlaces glicosídicos β-(2-1).
Glc: Glucosa.
GOS: Galactooligosacáridos.
HMO: "Human milk oligosaccharides" (oligosacáridos de la leche materna).
IACD: Infección asociada a *Clostridium difficile*.
IEC: "Intestinal epitelial cell" (células del epitelio intestinal).
ILSI: "International Life Sciences Institute" (Instituto Internacional de Ciencias de la Salud).
Inu: Inulina.
IPLA: Instituto de Productos Lácteos de Asturias.
LCTs: "Large clostridial toxins" (grandes toxinas clostridiales).
LDL: "Low density lipoprotein" (lipoproteína de baja densidad).
LOAEL: "Lowest observed adverse effect level" (concentración más baja de una sustancia en la que se observan efectos adversos).
MM: "McCoy´s medium".

MRSc: Medio de Man, Rogosa y Sharpe suplementado con cisteína.
MS: "Mass spectrometry" (espectrometría de masas).
NCBI: "National Center for Biotechnology Information" (Centro Nacional para la Información Biotecnológica).
NCFS: "Neutralized cell-free supernatants" (sobrenadantes neutralizados sin células).
NDA: "Panel on Dietetic Products, Nutrition and Allergies, EFSA" (Panel sobre productos dietéticos, nutrición y alergias de la EFSA).
NOAEL: "No observed adverse effect level" (concentración más alta de una sustancia en la que no se observan efectos adversos).
OMS: Organización Mundial de la Salud.
ONU: Organización de las Naciones Unidas.
PaLoc: Locus de patogenicidad.
Proporción A/L: Proporción ácido acético/ácido láctico.
Proporción F/L: Proporción ácido fórmico/ácido láctico.
QPS: "Qualified presumption of safety" (presunción cualificada de seguridad).
RCM: "Reinforced clostridial medium" (medio reforzado para clostridios).
RTCA: "Real time cell analyser" (analizador de células en tiempo real).
scFOS: "Short-chain fructooligosaccharides" (fructooligosacáridos de cadena corta).
Syn: "Synergy 1".
TcdA: Toxina A producida por *Clostridium difficile*.
TcdB: Toxina B producida por *Clostridium difficile*.
Tox-S: "*C. difficile*-free toxigenic supernatant" (sobrenadantes toxigénicos de *C. difficile* sin células).
WCS: "Without carbon source added" (sin fuente de carbono añadida).

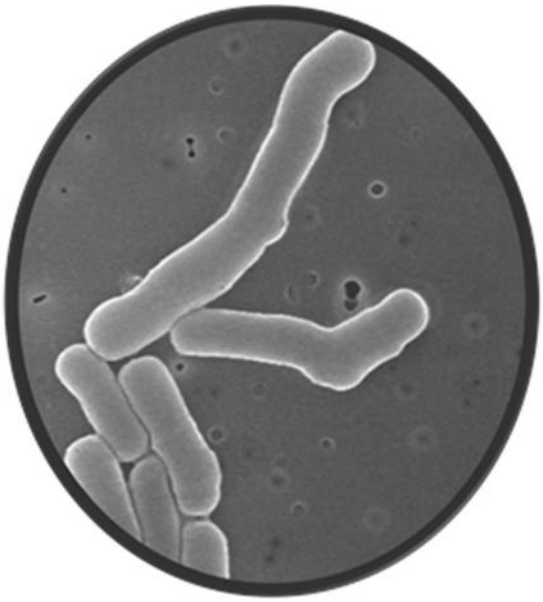


LISTADO DE FIGURAS

ÍNDICE DE FIGURAS

Figura	Leyenda	Página
Figura 1	Población de 60-79 años y de 80 años o más, en los años 2000, 2015, 2030 y 2050. Modificado de ONU (Organización de las Naciones Unidas) (2015).	**1**
Figura 2	Incremento de la población mundial con respecto al año 2000, por grupos de edad, entre los años 2000-2050. Modificado de ONU (Organización de las Naciones Unidas) (2015).	**2**
Figura 3	Desequilibrio entre la respuesta inmunológica tipo Th1 y tipo Th2 que tiene lugar durante el envejecimiento.	**7**
Figura 4	Relación mutualista entre el hospedador y la microbiota intestinal.	**10**
Figura 5	Algunas de las funciones de la microbiota intestinal en el hospedador.	**13**
Figura 6	Factores de virulencia de *C. difficile*.	**20**
Figura 7	Cambios en el enterocito mediados por las toxinas A y B de *C. difficile.* El dominio catalítico de la toxina aparece en rojo, el dominio proteasa cisteína en azul, el dominio de translocación en amarillo y el dominio de unión en verde. Modificado de Awad y cols. (2014).	**24**
Figura 8	Principales consecuencias del efecto citotóxico de las toxinas de *C. difficile* sobre los enterocitos.	**25**
Figura 9	Fotografía de microscopia electrónica de *B. longum*.	**32**
Figura 10	Efectos beneficiosos atribuidos a cepas probióticas específicas y su evidencia científica. Modificado de Gueimonde y cols., 2008.	**37**

Figura	Leyenda	Página
Figura 11	Efectos beneficiosos atribuidos a sustratos prebióticos específicos y su evidencia científica.	**40**
Figura 12	Criterios de selección de probióticos y prebióticos utilizados en este trabajo para la modulación de la microbiota intestinal de ancianos. Modificado de Salazar y cols. (2013).	**105**
Figura 13	Cepas potencialmente probióticas, sustratos prebióticos y combinaciones de ambos seleccionados por presentar un perfil de modulación de la microbiota intestinal en ancianos adecuado (Artículos 1 y 2).	**106**
Figura 14	Equipo RTCA (Real time cell analyser) xCELLIgence (ACEA Bioscience Inc., San Diego, CA) localizado en el IPLA-CSIC.	**111**
Figura 15	Escrutinio de potenciales probióticos para contrarrestar el efecto citotóxico de *C. difficile* LMG21717 sobre la línea intestinal HT29 mediante técnicas de monitorización en tiempo real (Artículo 4).	**114**
Figura 16	Escrutinio de potenciales simbióticos *in vitro* para la reducción del crecimiento de *C. difficile* LMG21717 y su citotoxicidad sobre la línea intestinal HT29 (Artículo 5).	**117**
Figura 17	Esquema de los principales resultados de esta Tesis Doctoral.	**119**



RESUMEN

Durante la segunda mitad del siglo pasado, la natalidad y la mortalidad de la población mundial han sufrido un descenso considerable y se espera que sus niveles continúen disminuyendo en la primera mitad del siglo en curso. Esta transición demográfica ha provocado un progresivo envejecimiento de la población. Al mismo tiempo que se está incrementando el número de personas de edad avanzada, se está produciendo un auge en la prevalencia de las enfermedades asociadas a la vejez, entre ellas las gastrointestinales. Por lo tanto, el incremento de las comorbilidades asociadas a la senectud, como consecuencia del aumento de este grupo de población, ha dado lugar a una elevación del gasto sanitario en los países desarrollados.

El tracto intestinal alberga la mayor y más compleja comunidad microbiana del cuerpo humano, denominada microbiota intestinal. Ésta lleva a cabo funciones esenciales para la salud del hospedador, ya que aporta energía, nutrientes y protección frente a patógenos. El envejecimiento se ha relacionado con cambios en la composición de esta microbiota que incluyen, en general, una reducida diversidad bacteriana, una disminución de los niveles de microorganismos potencialmente beneficiosos y un incremento del número de las bacterias potencialmente patógenas. Estos cambios en su composición incrementan la susceptibilidad a sufrir patologías gastrointestinales, como las infecciones asociadas a *Clostridium difficile* (IACD). Este microorganismo provoca enfermedad, generalmente, en personas de edad avanzada hospitalizadas que están siendo tratadas con antibióticos. El tratamiento estándar de estas infecciones consiste en la administración de dos antibióticos (metronizadol y vancomicina); sin embargo, el alto porcentaje de recurrencia de la IACD hace que se estén estudiando nuevas alternativas y estrategias adyuvantes para su prevención y tratamiento.

Una vía para mejorar la calidad de vida de las personas de edad avanzada y la reducción de los gastos sanitarios, sería el desarrollo de alimentos funcionales específicamente dirigidos a la modulación de la microbiota intestinal de este grupo de población. Es importante destacar que dichos productos, diseñados para cubrir las necesidades de este grupo poblacional, no están actualmente disponibles en el mercado. Diferentes estudios de intervención en personas de edad avanzada han demostrado el efecto modulador sobre la microbiota intestinal que tiene el consumo

de alimentos funcionales probióticos, prebióticos o simbióticos (combinación de ambos). Sin embargo, para la selección de cepas y/o sustratos, en la mayoría de los casos, no se han tenido en cuenta las necesidades específicas de este grupo de población.

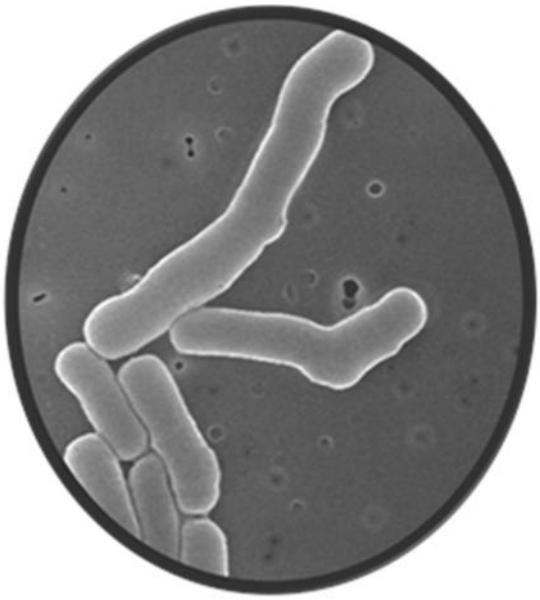
En este contexto, los objetivos que nos planteamos en la presente Tesis Doctoral fueron: 1) modular la microbiota intestinal de personas de edad avanzada mediante cepas potencialmente probióticas, sustratos prebióticos y/o combinaciones de ambos con características adecuadas, y 2) reducir el crecimiento y toxicidad de *C. difficile* mediante cepas potencialmente probióticas, sustratos prebióticos y/o simbióticos.

El primer capítulo se centra en la selección de probióticos, prebióticos y simbióticos eficaces para la modulación de la microbiota intestinal de personas de edad avanzada. Previamente, a partir de las alteraciones específicas identificadas en la microbiota intestinal de ancianos de nuestra población, definimos unos objetivos concretos para tratar de corregir las alteraciones detectadas. Se empleó un modelo *in vitro* de cultivos fecales (con inóculos fecales de ancianos de nuestra población) que, a partir de 16 cepas de bifidobacterias y 4 sustratos prebióticos, nos permitió seleccionar los que presentaron mejor capacidad para modular la microbiota intestinal de esta población. Las cepas y el sustrato más adecuados para, de forma independiente, incrementar ciertos grupos bacterianos beneficiosos, así como para aumentar la producción de ácidos grasos de cadena corta en esta población objetivo, fueron *Bifidobacterium longum* IPLA20021 e IPLA20022 y el fructooligosacárido de cadena corta (scFOS, “short-chain fructooligosaccharide”) Actilight. Posteriormente, llevamos a cabo cultivos discontinuos a pH libre para estudiar si 6 cepas seleccionadas eran capaces de fermentar diversos sustratos prebióticos (scFOS e inulina). Los resultados obtenidos de estos cultivos nos permitieron identificar a los scFOS como los sustratos prebióticos más adecuados para su combinación con cepas de *Bifidobacterium animalis* y/o *B. longum* con el objetivo de desarrollar alimentos funcionales simbióticos.

El segundo capítulo de esta Tesis tiene como objetivo la búsqueda de probióticos, prebióticos y simbióticos para contrarrestar el efecto del patógeno *C. difficile*. Para ello, desarrollamos un modelo biológico *in vitro* basado en la monitorización continua del comportamiento de líneas celulares intestinales, mediante medidas de impedancia, a través del equipo RTCA ("real time cell analyser"); esta tecnología permite estudiar la cinética de citotoxicidad de sobrenadantes procedentes de un cultivo de *C. difficile* toxigénico sobre líneas celulares intestinales. Una vez desarrollado este modelo, lo utilizamos para evaluar el potencial probiótico de 20 cepas de bifidobacterias y lactobacilos para reducir los niveles de toxina en el sobrenadante de *C. difficile* LMG21717 (Toxinotipo 0, Ribotipo 001) y, por tanto, contrarrestar su efecto citotóxico sobre la línea intestinal humana HT29. Las cepas de *B. longum* y *Bifidobacterium breve* estudiadas fueron las que mostraron una mayor capacidad protectora sobre la línea HT29 destacando, por su mayor eficacia, la cepa *B. longum* IPLA20022. Finalmente, determinamos el potencial de 4 cepas del género *Bifidobacterium* (tres seleccionadas en base a su capacidad para reducir la toxicidad de los sobrenadantes de un cultivo de *C. difficile* y una cepa ampliamente usada como probiótico) para reducir el crecimiento y la citotoxicidad de *C. difficile* LMG21717 en presencia de sustratos prebióticos seleccionados (scFOS e inulina) por su perfil adecuado para modular la microbiota intestinal en ancianos. Con este propósito, realizamos cocultivos de bifidobacteria-patógeno en presencia de los diferentes sustratos prebióticos como fuentes de carbono. En los cocultivos de *C. difficile* con *B. longum* IPLA20022 o *B. breve* IPLA20006 en presencia de Synergy 1 y Actilight se redujo significativamente el crecimiento del patógeno, así como la toxicidad de los sobrenadantes procedentes del cocultivo. Por tanto, las cepas *B. longum* IPLA20022 y *B. breve* IPLA20006, junto con estos scFOS, se mostraron como las combinaciones simbióticas con mayor potencial para inhibir el crecimiento y reducir la toxicidad de *C. difficile*.

En conjunto, estos trabajos nos han permitido identificar cepas y prebióticos concretos con gran potencial para su aplicación en el desarrollo de productos funcionales dirigidos a la tercera edad, destacando *B. longum* IPLA20022 y los scFOS

Synergy 1 y Actilight al mostrarse eficaces para lograr los dos objetivos planteados en esta Tesis doctoral.



1-INTRODUCCIÓN

1.1. Problemática del envejecimiento de la población

Desde mediados del siglo XX, la población mundial ha experimentado un gran envejecimiento, y se estima que esta tendencia se intensificará durante el siglo XXI (Figura 1). La intensidad y la profundidad de este envejecimiento varían considerablemente entre países y regiones, iniciándose en las más desarrolladas y, actualmente, teniendo lugar también en los países en vías en desarrollo (ONU, 2013).

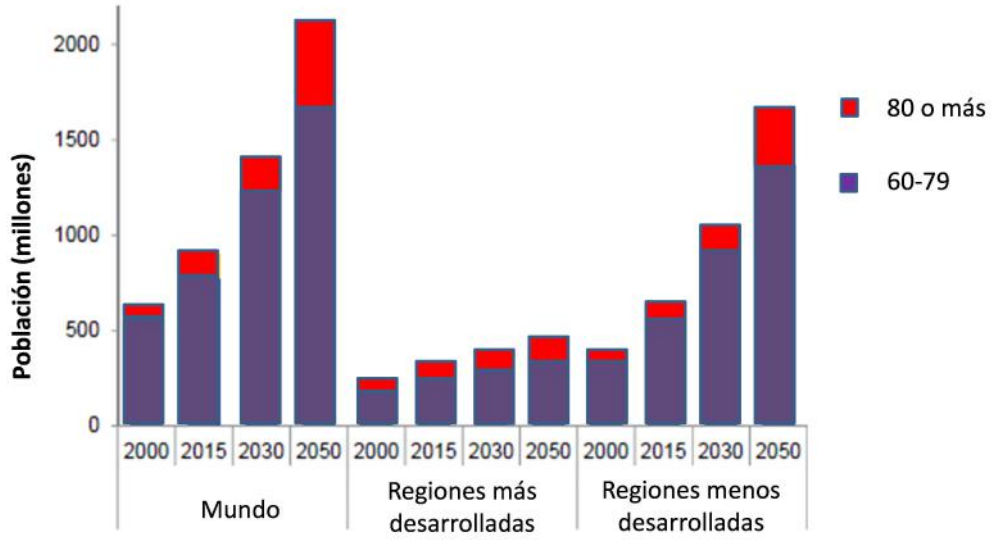


Figura 1. Población de 60-79 años y de 80 años o más, en los años 2000, 2015, 2030 y 2050. Modificado de ONU (Organización de las Naciones Unidas) (2015).

Este proceso implica un incremento del número de personas de edad avanzada (personas de 60 años o más), y resulta de una transición demográfica, proceso por el cual una reducción en la mortalidad va seguida de una reducción en la fertilidad. Las consecuencias son una proporción reducida de niños y una gran proporción de ancianos (ONU, 2013). En el año 2000, el número de personas mayores de 60 años en el mundo era de 607 millones, mientras que en 2015 alcanzó los 901 millones, lo que supone un incremento del 48%. Para el año 2030, se estima que el número de personas de 60 años o más crezca hasta los 1400 millones y para el 2050, se piensa

que puede alcanzar los 2100 millones. Además, se ha observado que el número de personas de 80 años o más está aumentando más rápidamente que el número de personas mayores de 60 años. A nivel mundial, en el año 2000, el número de personas con una edad igual o superior a 80 años era de 71 millones y en el 2015 de 125 millones, lo que supone un incremento del 77%. Para el 2030, se ha estimado que el número de personas de este grupo de edad puede alcanzar los 202 millones y para el 2050, puede llegar a ser de 434 millones (ONU, 2015).

El envejecimiento no sólo está determinado por la tasa de crecimiento de la población anciana, sino también por las tasas de crecimiento de otros grupos de edad. A nivel mundial, el número de personas de edad avanzada está creciendo más rápidamente que el número de personas de otros grupos (Figura 2). Como se ha indicado, para el año 2050, el número de personas de más de 60 años será el triple que

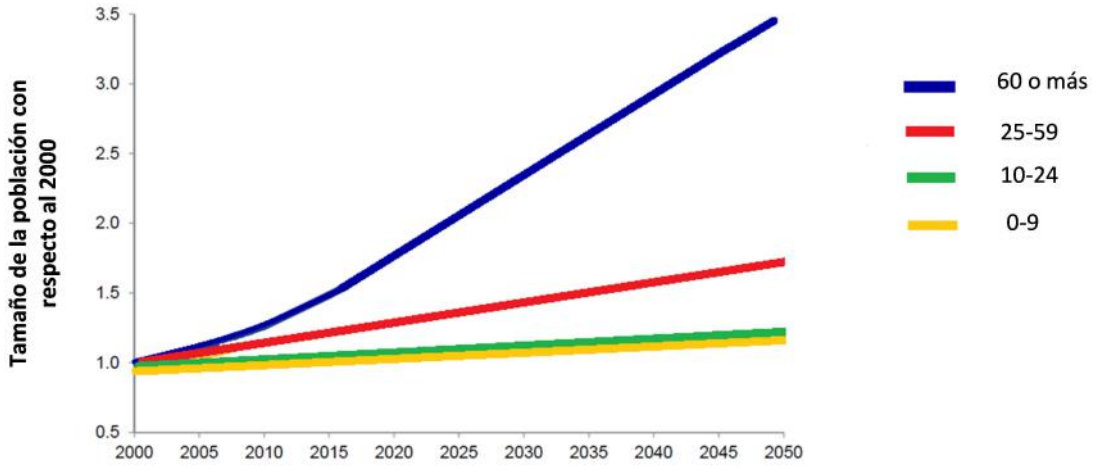


Figura 2. Incremento de la población mundial con respecto al año 2000, por grupos de edad, entre los años 2000-2050. Modificado de ONU (2015).

en el año 2000. Por el contrario, el número de individuos del grupo comprendido entre los 10-24 años de edad se predice que va a variar poco; ya que en el año 2050, se estima que sólo haya aumentado en un 11% con respecto al 2000. El número total de adultos de 25-59 años está creciendo más rápidamente que el número de niños, pero

no más que la población adulta de 60 años o más. En 2015, hubo un 29% más de personas con una edad comprendida entre los 29 y 59 años que las existentes en el año 2000, y en 2050, se estima que habrá un 62% más que en el año 2000 (ONU, 2015).

El principal factor involucrado en el envejecimiento de la población ha sido el descenso continuado, durante varias décadas, de la fertilidad a nivel mundial; la tasa de fertilidad ha pasado de 5,0 niños por mujer en 1950-1955 a 2,5 niños por mujer en 2010-2015 (ONU, 2015). Otro factor importante implicado en este envejecimiento es el incremento de la esperanza de vida. Este incremento se ha observado en todas la regiones del mundo. A nivel mundial, entre 1950-1955 la esperanza de vida era de 46,8 años, sin embargo, entre 2010-2015 se ha estimado en 70,5 años (ONU, 2015). El incremento en la esperanza de vida se asocia principalmente a la mejora en la calidad de vida y fundamentalmente a los avances en la ciencia y en la medicina que han tenido lugar en las últimas décadas (Woodmansey, 2007); factores que han producido una disminución de la mortalidad infantil y de otros grupos de edad, lo que favorece la posibilidad de que más personas puedan llegar a la vejez.

Este envejecimiento de la población ha conducido a un aumento de la prevalencia de las enfermedades relacionadas con la edad, como infecciones, cáncer, enfermedades neurodegenerativas y cardiovasculares (Christensen y cols., 2009). Por tanto, el incremento de este grupo de población tiene importantes consecuencias económicas, entre ellas, un mayor gasto sanitario, ya que aumenta la demanda de atención sanitaria y de servicios y tecnologías para prevenir y tratar las enfermedades crónicas asociadas a la vejez. Se ha descrito que actualmente los ancianos utilizan un alto porcentaje de los servicios médicos de un país, una parte importante de este porcentaje se debe a la aparición de enfermedades relacionadas con el tracto gastrointestinal (Sinoff, 2011). Una estrategia para incrementar la calidad de vida de las personas de edad avanzada, con la consecuente reducción del gasto sanitario, sería el desarrollo de alimentos funcionales específicamente dirigidos a este grupo de población (Arboleya y cols., 2012).

1.2. Cambios fisiológicos y nutricionales asociados al envejecimiento

1.2.1. Alteraciones del sistema digestivo

El sistema digestivo está constituido por regiones anatómica y funcionalmente distintas. Estas regiones, gracias a los distintos tipos de células que las constituyen, llevan a cabo funciones esenciales para la vida (secreción, digestión, absorción, excreción y defensa). Además, está asociado con otros sistemas orgánicos, como el circulatorio y el nervioso, así como a órganos glandulares (hígado, páncreas, vesícula biliar y glándulas salivares), que van a desempeñar papeles esenciales en las funciones intestinales. Por tanto, los cambios durante el envejecimiento en las células que integran el sistema digestivo pueden tener un fuerte impacto en las funciones de éste. En estudios llevados a cabo en humanos se ha observado una reducción del número de células intersticiales de Cajal (Gómez-Pinilla y cols., 2011), una reducción del número de neuronas del plexo mientérico (Saffrey, 2013) y un incremento de las mutaciones en el ADN mitocondrial de las células madre del epitelio intestinal (Taylor y cols., 2003). Estas mutaciones en las células madre pueden dar lugar a defectos en la cadena respiratoria, los cuales han sido observados (Greaves y cols., 2011), y se han relacionado con una reducción de la proliferación y un incremento de la apoptosis celular (Nooteboom y cols., 2010).

En la boca y orofaringe, los procesos de masticación y deglución se ven dificultados por la pérdida de dientes y por la disminución de masa muscular y de la fuerza motriz de la lengua (Woodmansey, 2007; Grassi y cols., 2011). También se ha observado un incremento del umbral del sabor (como consecuencia de la disminución del número de papilas gustativas en la lengua), lo que hace que los alimentos sean insípidos y poco apetecibles. Todos estos cambios pueden conducir a que los ancianos tengan una dieta nutricionalmente desequilibrada (Woodmansey, 2007).

En el esófago, con la edad, se ha observado que se produce: incompleta relajación del esfínter esofágico superior, disminución del peristaltismo esofágico, eliminación incompleta del contenido, dilatación esofágica y disminución de la presión del esfínter esofágico inferior (Soergel y cols., 1964; Ferriolli y cols., 1996). Estos

cambios favorecen la aparición de disfagia y de reflujo gastroesofágico (Grassi y cols., 2011).

El vaciamiento gástrico y las secreciones gástricas, por lo general, son normales en ancianos (Kekii y cols., 1982; Madsen, 1992; Katelaris y cols., 1993; Feldman y cols., 1996), mientras que la barrera de mucus y bicarbonato y la proliferación celular en la pared gástrica están disminuidas (Kawano y cols., 1991; Grassi y cols., 2011). Se han descrito casos de peristaltismo reducido (Huang y cols., 1995) y de reducción del vaciamiento del contenido gástrico (Kao y cols., 1994; Brogna y cols., 1999). Ambos cambios pueden incrementar la incidencia y prevalencia del reflujo gastroesofágico y de la dispepsia funcional (Grassi y cols., 2011). Los individuos que sufren gastritis atrófica presentan la secreción basal y estimulada de HCl reducida (Grassi y cols., 2011) y los que sufren infección por *Helicobacter pylori* presentan la secreción de pepsina reducida (Pilotto y Salles, 2002). La gastritis atrófica, además de reducir la secreción de ácido, tiene otras consecuencias: favorece la proliferación bacteriana, disminuye la absorción de micronutrientes como la vitamina B12, provoca anemia macrocítica (Van Asselt y cols., 1996) y favorece la producción de especies reactivas de oxígeno que aumentan el riesgo de carcinogénesis (Pignatelli y cols., 2001; Lenaz y cols., 2002). La disminución de la barrera protectora de mucus y bicarbonato se produce como consecuencia de la baja concentración de prostaglandinas, especialmente la prostaglandina E2, la cual influye en la secreción de ambos compuestos (Newton, 2004). Por otro lado, la disminución de la actividad proliferativa en las células de la mucosa gástrica parece ser debida a una baja expresión de factores de crecimiento (Fligiel y cols., 1994; Relan y cols., 1995). Por tanto, la disminución de las secreciones gástricas y la alteración de la barrera de mucus y bicarbonato pueden favorecer el desarrollo de una úlcera gástrica (Grassi y cols., 2011).

La mayoría de los estudios indican que no se produce cambio en la motilidad (Husebye y Engedal, 1992; Kagaya y cols., 1997; Brogna y cols., 1999) y en la estructura (Höhn y cols., 1978; Corazza y cols., 1986) del intestino delgado con el envejecimiento; sin embargo, los resultados obtenidos en cuanto a la absorción de nutrientes varían. En un estudio se observó que la absorción de la grasa en individuos sanos de edad

avanzada era normal (Arora y cols., 1989), mientras que en otro se observó que era más lenta (Holt y Balint, 1993).

La disminución de la motilidad en el colon da lugar a la retención fecal y a estreñimiento. El incremento del tiempo de retención fecal se ha asociado con el incremento de la actividad de las bacterias proteolíticas en el intestino y, como consecuencia, con un incremento de los niveles de amoniaco y fenol (Woodmansey, 2007).

A nivel ano-rectal, los cambios relacionados con la edad incluyen: adelgazamiento del esfínter externo y engrosamiento del interno, reducción de la presión en el canal anal en reposo (McHugh y Diamant, 1987; Rasmussen y cols., 1992) y reducida presión umbral de relajación de ambos esfínteres. Todos estos cambios aumentan la probabilidad de incontinencia fecal y de gas en ancianos (Grassi y cols., 2011).

A menudo, la secreción pancreática exocrina está disminuida (Laugier y cols., 1991), así como la secreción de secretina (hormona implicada en la secreción del páncreas exocrino) (Stevens y cols., 2008). Los cambios relacionados con el envejecimiento en la secreción exocrina del páncreas incluyen la disminución de las tasas de flujo y la disminución de la producción de bicarbonato y enzimas (Laugier y cols., 1991). En cuanto a la bilis, se ha observado una disminución del contenido en sales biliares de la misma. Hay estudios que indican que la hiposecreción de bilis y lipasas afecta a la absorción de carbohidratos y grasas, mientras que en otros no se observan cambios (Grassi y cols., 2011).

1.2.2. Inmunosenescencia

El término inmunosenescencia hace referencia a los cambios relacionados con el envejecimiento en el sistema inmunológico (tanto a nivel celular como humoral). Sus principales cambios incluyen: reducción de la respuesta humoral después de infecciones o de vacunaciones y disminución tanto de la eficacia de activación de las poblaciones de células T y B como de la generación de nuevas células T y B vírgenes y de la citotoxicidad de las células asesinas naturales (NK: “natural killer”) (Ibrahin y

cols., 2010). También se ha observado un incremento en los niveles de citoquinas pro-inflamatorias, como el factor de necrosis tumoral-α, interleucina-12 o la interleucina-8 (Salazar y cols., 2013), lo que da lugar a un desequilibrio entre la respuesta inmunológica tipo Th1 y la tipo Th2 del individuo (Figura 3), que desencadenan una inflamación sistémica crónica de bajo grado (Baylis y cols., 2013). Todos estos cambios provocan un incremento de la frecuencia y severidad de las enfermedades infecciosas, desórdenes inflamatorios crónicos, enfermedades autoinmunes, de la incidencia del cáncer y una disminución de la eficacia de las vacunaciones (Haq y McElhaney, 2014).

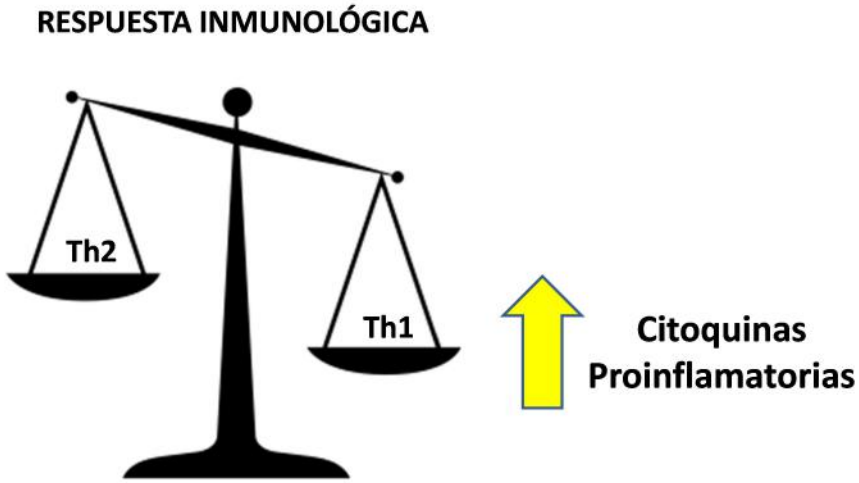


Figura 3: Desequilibrio entre la respuesta inmunológica tipo Th1 y tipo Th2 que tiene lugar durante el envejecimiento.

1.2.3. Alteraciones en la función cognitiva

El envejecimiento también afecta al cerebro (tanto a nivel celular como a nivel funcional), provocando la disminución de las funciones sensoriales, motoras y cognitivas (Salthouse, 2009; Schaffer y cols., 2012). Normalmente, la alteración de la función cognitiva se produce como resultado de la interacción entre los cambios relacionados con el envejecimiento y las enfermedades características de personas de edad avanzada. Según el grado de afectación, las manifestaciones clínicas de la disminución de la función cognitiva pueden ir desde una cognición intacta a un deterioro leve y finalmente demencia (Caracciolo y cols., 2014). En el deterioro

cognitivo leve (estado de alteración intermedio) se ven afectadas una o más áreas cognitivas, sin embargo se conserva la independencia funcional (Petersen, 2004), mientras que la demencia (estado más avanzado de alteración cognitiva) se caracteriza por el deterioro progresivo de múltiples dominios cognitivos, por tanto, es lo suficientemente grave como para interferir con el funcionamiento diario (Wimo y Prince, 2010; Caracciolo y cols., 2012). Las causas más comunes de demencia en personas de edad avanzada son el Alzheimer (Blennow y cols., 2006), alteraciones vasculares en el cerebro (consecuencia de una enfermedad cerebrovascular o patología cardiovascular) (Roman, 2003) o ambas (Langa y cols., 2004). Hay otros factores, actualmente en estudio, que se han relacionado con alteraciones en la función cognitiva como son los relacionados con el estilo de vida (particularmente la dieta) (Solfrizzi y cols., 2008) y enfermedades como hipertensión (Qiu y cols., 2005), hipercolesterolemia (Anstey y cols., 2008), obesidad o diabetes mellitus (Luchsinger y Gustafson, 2009). También se ha sugerido que los cambios en la microbiota intestinal relacionados con la edad pueden alterar la función cognitiva en ancianos (Caracciolo y cols., 2014).

1.2.4. Alteraciones nutricionales

El estado nutricional de las personas de edad avanzada es un factor determinante de la calidad de vida y de la mortalidad, sin embargo, la malnutrición es un problema frecuente en este grupo de población (Brownie, 2006). Las deficiencias nutricionales en ancianos son debidas a cambios físicos y fisiológicos asociados al envejecimiento (cambios en la composición del cuerpo, en el tracto gastrointestinal, en la función sensorial, en la regulación de líquidos y de electrolitos y enfermedades crónicas), así como a otros factores como medicaciones, hospitalizaciones y otros determinantes psicológicos y/o sociales (Hickson, 2005; Brownie, 2006). Esta malnutrición afecta a su salud, ya que provoca debilidad en el sistema inmunológico (incrementa el riesgo de infecciones), debilidad muscular (incrementa el riesgo de caídas y de fracturas) y dificulta la cicatrización de las heridas (Amarya y cols., 2015). Aunque, no hay una definición ampliamente aceptada de malnutrición en personas de

edad avanzada, si hay unos indicadores comunes como la pérdida de peso involuntaria, índice de masa corporal alterado, deficiencias en vitaminas específicas o disminución de la ingesta de alimentos (Wells y Dumbrell, 2006).

1.3. Cambios en la microbiota intestinal durante el envejecimiento

Los cambios asociados con el avance de la edad descritos anteriormente afectan inevitablemente a la composición del colectivo de microorganismos del intestino o microbiota intestinal (Biagi y cols., 2010). Las alteraciones en la composición y función de la microbiota (disbiosis) hacen que las personas de edad avanzada sean más propensas a enfermar e incrementan su susceptibilidad a infecciones, como la causada por *Clostridium difficile* (Claesson y cols., 2012; Biagi y cols., 2013).

1.3.1. Microbiota intestinal: composición y función

El tracto intestinal alberga la mayor y más compleja comunidad microbiana del cuerpo humano (Figura 4), denominada microbiota intestinal (Montalto y cols., 2009). Esta comunidad está compuesta principalmente por bacterias pero también por eucariotas (en su mayoría levaduras), arqueas metanogénicas (*Methanobrevibacter smithii*) y virus (incluidos bacteriófagos) (Tojo y cols., 2014). El microbioma intestinal humano (colección de genomas de la microbiota intestinal) contiene más de 5 millones de genes, esta cantidad supera en dos órdenes de magnitud el potencial genético humano. Cada individuo tiene un microbioma único, ya que hay muchos factores que influyen en su composición: el genotipo y fisiología del hospedador, los sucesos acaecidos durante la colonización microbiana del intestino durante la primeras etapas de vida y factores medioambientales (como dieta y fármacos) (Zoetendal y cols., 2001). Los productos de los genes del microbioma intervienen en rutas metabólicas y bioquímicas que complementan la fisiología del hospedador (Sommer y Bäckhed, 2013). Por tanto, la relación que existe entre la microbiota y el hospedador es mutualista (Figura 4), ya que la primera lleva a cabo funciones esenciales para la fisiología y salud humana mientras que el hospedador le proporciona un ambiente rico

en nutrientes y unas condiciones adecuadas para su supervivencia (Guinane y Cotter, 2013; Sommer y Bäckhed, 2013). La distribución de esta microbiota a lo largo del intestino es desigual, con bajas concentraciones de bacterias en el intestino delgado (10^3-10^7 células/g) y más altas en el intestino grueso (superiores a 10^{12} células/g) (Montalto y cols., 2009; Bull y cols., 2014). Su composición varía también a lo largo de las distintas regiones del intestino. En muestras obtenidas en biopsias se ha observado que el intestino delgado está enriquecido con ciertos miembros del filo *Firmicutes*, mientras que el intestino grueso tiene una mayor abundancia de miembros del filo *Bacteroidetes*. Además, se ha descrito que la microbiota del lumen intestinal difiere de la microbiota situada cerca o unida al epitelio (Bull y Plummer, 2014).

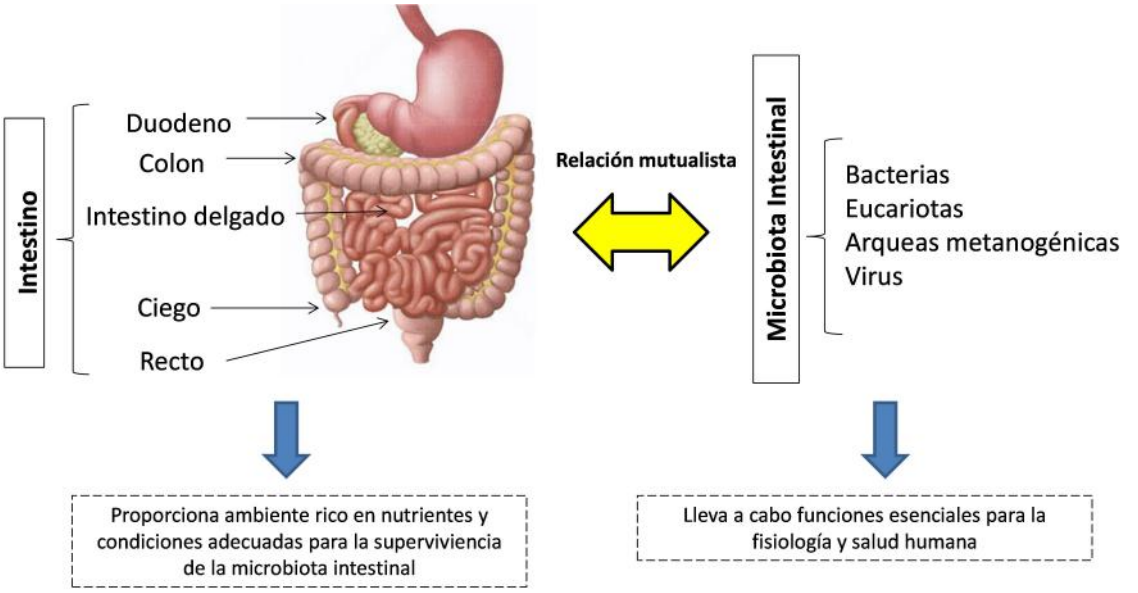


Figura 4: Relación mutualista entre el hospedador y la microbiota intestinal.

Tradicionalmente, se ha considerado que la colonización del tracto gastrointestinal comienza inmediatamente después del nacimiento y que, por tanto, el ambiente intrauterino y el intestino del feto se mantienen estériles hasta el parto. Sin embargo, estudios recientes han demostrado la presencia de bacterias en el ambiente

intrauterino, placenta, fluido amniótico, sangre del cordón umbilical y meconio (Salazar y cols., 2014; Rodríguez y cols., 2015a). La colonización microbiana del neonato va a estar influida por muchos factores (como el tipo de parto, edad gestacional, tipo de alimentación o el uso de antibióticos) (Matamoros y cols., 2013; Salazar y cols., 2014; Rodríguez y cols., 2015a). La microbiota intestinal alcanza una población similar a la de un adulto aproximadamente a los 3 años de vida (Montalto y cols., 2009; Rodríguez y cols., 2015a). En la edad adulta, la composición se mantiene relativamente estable, aunque factores externos tales como la dieta, tratamientos médicos o enfermedades, provocan variaciones (Montalto y cols., 2009; Bäckhed y cols., 2012; Salazar y cols., 2014; Voreades y cols., 2014; Rodríguez y cols., 2015a). Muchos autores han pretendido describir la composición de la microbiota intestinal en sujetos sanos; sin embargo, esta tarea ha sido muy difícil debido a la gran variabilidad que existe entre individuos y a las diferentes metodologías empleadas para este propósito (Rodríguez y cols., 2015a). Actualmente, se sabe que los filos predominantes en el intestino grueso son *Firmicutes* y *Bacteroidetes* y en menor proporción *Actinobacteria* (Turnbaugh y cols., 2009; Claesson y cols., 2011). Además, se ha observado que cuando la microbiota del intestino grueso ha alcanzado una composición microbiana estable, ésta se mantiene durante largos periodos de tiempo (Faith y cols., 2013). Por otro lado, el intestino delgado principalmente contiene especies del género *Streptococcus* y un número variable de bacterias de los géneros *Clostridium* y *Veillonella* (Zoetendal y cols., 2012; Leimena y cols., 2013); además, su composición sufre variaciones temporales, ya que se ha observado que el perfil de la mañana difiere significativamente del de la tarde (Booijink y cols., 2010). Se ha tratado de identificar una porción del microbioma intestinal común (genes altamente conservados) entre individuos adultos, responsable de la funcionalidad de la microbiota intestinal y de su papel en la salud humana; sin embargo, varios factores han dificultado la identificación de este microbioma conservado entre individuos (Qin y cols., 2010; Human Microbiome Consortium, 2012; Voreades y cols., 2014). También se ha sugerido clasificar a los individuos en tres grandes grupos de microbiota, llamados enterotipos, según la mayor abundancia relativa de uno de los siguientes géneros: *Bacteroides* (Enterotipo I)*, Prevotella* (Enterotipo 2) o *Ruminococcus* (Enterotipo 3)

(Arumugam y cols., 2011); sin embargo, esta clasificación en enterotipos ha sido muy debatida. Trabajos de intervención a largo plazo indican que los enterotipos son dependientes del tipo de dieta del individuo (Wu y cols., 2011), mientras que, otros estudios a corto plazo sugieren que estos enterotipos permanecen estables (David y cols., 2014; Roager y cols., 2014). Por el contrario, otros autores sugieren que el concepto de enterotipo no está claro y que debería de desarrollarse y emplearse una metodología estandarizada para definirlo (Huse y cols., 2012; Koren y cols., 2013; Voreades y cols., 2014).

La microbiota intestinal lleva a cabo funciones esenciales para el hospedador (Figura 5), entre ellas, el metabolismo de nutrientes procedentes de la dieta (Montalto y cols., 2009; Hsiao y cols., 2013; Bull y Plummer, 2014; Jandhyala y cols., 2015). La microbiota del colon es capaz de fermentar componentes de los alimentos (principalmente carbohidratos) que no pueden ser digeridos por el hospedador, dando lugar a ácidos grasos de cadena corta (AGCCs), alcoholes (como etanol) y gases (como CO_2 y H_2). La producción de AGCCs en el intestino está determinada por distintos factores (la microbiota presente en el colon, el sustrato utilizado como fuente de carbono y el tiempo de tránsito intestinal) (Hijova y Chmelarova, 2007; Byrne y cols., 2015; Ríos-Covián y cols., 2016a) y requiere que la microbiota intestinal trabaje como una comunidad, de tal manera que los productos finales procedentes del metabolismo de unos microorganismos son utilizados por otros; por ejemplo, las arqueas producen CH_4 a partir del CO_2 y H_2, mientras que microorganismos acetogénicos convierten el CO_2 en acetato (Den Besten y cols., 2013; Ríos-Covián y cols., 2016a). Los AGCCs colónicos más abundantes (90-95%) son acetato (2 átomos de carbono), propionato (3 átomos de carbono) y butirato (4 átomos de carbono), los cuales son producidos en una proporción molar de aproximadamente 3:1:1, respectivamente (Hijova y Chmelarova, 2007; Den Besten y cols., 2013; Byrne y cols., 2015). El filo *Bacteroidetes* principalmente produce acetato y propionato, mientras que el filo *Firmicutes* comprende los principales productores de butirato en el ambiente intestinal. El 95% de los AGCCs son rápidamente absorbidos por los colonocitos (por intercambio con bicarbonato) y el resto (5%) son secretados en heces (Hijova y Chmelarova, 2007; Den

Besten y cols., 2013). Por tanto, el pH del lumen intestinal (Figura 5) es el resultado de la producción microbiana de AGCCs y de la capacidad neutralizante del bicarbonato.

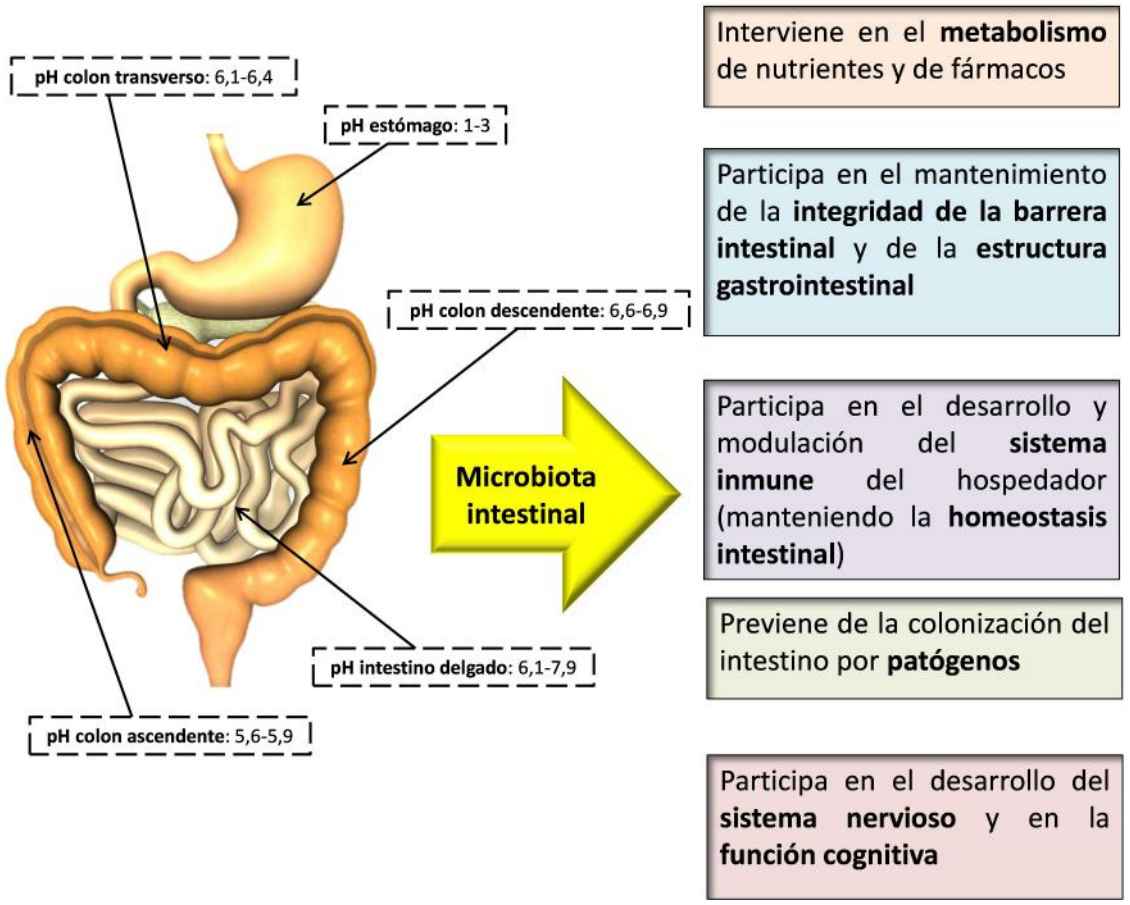


Figura 5. Algunas de las funciones de la microbiota intestinal en el hospedador.

Una gran concentración de AGCCs en el intestino grueso provoca una bajada de pH, que a su vez da lugar a un cambio de composición de la microbiota y previene el crecimiento excesivo de bacterias patógenas sensibles a pH ácidos como *Escherichia coli* (Hijova y Chmelarova, 2007; Den Besten y cols., 2013; Ríos-Covián y cols., 2016a). Una gran parte de estos AGCCs son usados como fuentes de energía; en humanos, por ejemplo, proporcionan el 10% de los requerimientos calóricos diarios. Desde los enterocitos, estos AGCCs pasan al torrente sanguíneo y a través de la sangre llegan a los órganos diana, donde actúan como sustratos o como señales moleculares (Den

Besten y cols., 2013). La mayor parte del acetato pasa al hígado, donde se utiliza como fuente de energía, como sustrato para la síntesis de colesterol y ácidos grasos de cadena larga y como co-sustrato para la síntesis de glutamina y glutamato. El resto del acetato es metabolizado en otros tejidos como el corazón, tejido adiposo, riñón y músculo (Hijova y Chmelarova, 2007; Den Besten y cols., 2013). La mayor parte del propionato pasa al hígado, donde actúa como precursor de la gluconeogénesis, y la mayoria del butirato es utilizado por los colonocitos como fuente de energía (Den Besten y cols., 2013; Ríos-Covián y cols., 2016a). El butirato parece que ayuda a mantener la integridad de la función barrera, debido a que incrementa la producción de mucina y mejora la integridad de las uniones estrechas entre las células (Ríos-Covián y cols., 2016a). También se ha descrito que el butirato protege contra los cánceres de colon y recto debido a que promueve la motilidad intestinal, reduce la inflamación, incrementa la irrigación de las vísceras, estimula la apoptosis y la inmunogenicidad de las células cancerígenas (Hijova y Chmelarova, 2007; Ríos-Covián y cols, 2016a). Estudios recientes en este campo sugieren que los AGCCs tienen efectos beneficiosos en la fisiología del hospedador regulando el metabolismo energético y el apetito: estimulan la oxidación de los ácidos grasos e inhiben la síntesis de los mismos y la lipólisis, reducen los niveles de glucosa en plasma e incrementan la glucosa disponible en los distintos órganos y también reducen los niveles de colesterol en plasma (Den Besten y cols., 2013; Byrne y cols., 2015; Kasubuchi y cols., 2015). Estudios llevados a cabo en animales indican que la reducción del apetito se lleva a cabo por dos vías: estimulación de la liberación de hormonas en el intestino (por butirato y propionato) (Lin y cols., 2012) o por interacción con el sistema nervioso (por acetato) (Frost y cols., 2014). Sin embargo, en otro estudio más reciente llevado a cabo con ratones, se observó como un aumento en la producción de acetato, debido a una microbiota alterada, provoca la activación del sistema nervioso parasimpático. Esta activación promueve el incremento de la secreción de la hormona ghrelina, hiperfagia y obesidad (Perry y cols., 2016).

1.3.2. Alteraciones asociadas al envejecimiento

Se ha observado que la microbiota intestinal de las personas de edad avanzada presenta diferente composición y función, y una mayor variabilidad entre individuos que la de individuos adultos más jóvenes (Claesson y cols., 2012). Esta microbiota en ancianos se caracteriza por una reducida diversidad de especies, una disminución de los microorganismos beneficiosos, variaciones en las especies dominantes, un incremento de las bacterias anaerobias facultativas y aerotolerantes y una disminución de la disponibilidad de AGCCs (Salazar y cols., 2014).

El efecto del envejecimiento, observado en distintos estudios sobre la abundancia de los filos dominantes *Firmicutes* y *Bacteroidetes* es polémico, ya que los resultados varían dependiendo de la nacionalidad de los sujetos implicados en tales trabajos (Biagi y cols., 2013). Un estudio finlandés y otro irlandés mostraron que la proporción *Firmicutes*/*Bacteroidetes* es más baja en ancianos que en adultos más jóvenes (Mäkiuvokko y cols., 2010; Claesson y cols., 2011). Sin embargo, otro estudio no encontró diferencias significativas entre las proporciones *Firmicutes*/*Bacteroidetes* de italianos centenarios, ancianos y adultos más jóvenes (Biagi y cols., 2010). Dentro del filo *Firmicutes*, se ha observado una disminución de los niveles del grupo *Clostridium* XIVa (grupo *Blautia coccoides*), en ancianos japoneses, italianos, finlandeses y españoles (Hayashi y cols., 2003; Mueller y cols., 2006; Mäkiuvokko y cols., 2010; Salazar y cols., 2013) y lo contrario se observó en ancianos alemanes (Mueller y cols., 2006). Dentro del grupo *Clostridium* IV, un efecto bien documentado del envejecimiento es la disminución de *Faecalibacterium prausnitzii* en ancianos italianos y españoles (Mueller y cols., 2006; Biagi y cols., 2010; Salazar y cols., 2013); sin embargo, hay estudios llevados a cabo con ancianos de otras localizaciones (Francia, Alemania, Suecia e Irlanda) que no confirman ese resultado (Mueller y cols., 2006; Claesson y cols., 2011). Los niveles del género *Lactobacillus* también se han visto afectados con la edad, ya que se ha observado un incremento en sus niveles en ancianos irlandeses, finlandeses y españoles (Tiihonen y cols., 2008; Mäkivuokko y cols., 2010; Claesson y cols., 2012; Salazar y cols., 2013) y lo contrario en ancianos escoceses (Hopkins y cols., 2001; Woodmansey y cols., 2004). La disminución de los

miembros del filo *Firmicutes* se ha asociado con ancianos hospitalizados o tratados con antibióticos o antiinflamatorios (Biagi y cols., 2013). El efecto de la edad sobre el filo *Bacteroidetes* es bastante confuso y parece estar también relacionado fuertemente con la nacionalidad (Biagi y cols., 2012): se observó un incremento en su abundancia en ancianos alemanes, australianos, finlandeses e irlandeses (Mueller y cols., 2006; Zwielehner y cols., 2009; Mäkivuokko y cols., 2010; Claesson y cols., 2011), mientras que se observó lo contrario en ancianos de otros países del norte de Europa, italianos y españoles (Mueller y cols., 2006; Rajilic-Stojanovic y cols., 2009; Salazar y cols., 2013). Estas diferencias podrían ser, al menos parcialmente, explicadas por las diferentes metodologías utilizadas, indicando la necesidad de metodologías estándar para el estudio de la microbiota.

Dentro del filo *Actinobacteria*, se ha detectado reducción de los niveles de bifidobacterias y alteración en la composición de las mismas en la microbiota de ancianos (Hopkins y cols., 2001; Woodmansey y cols., 2004; Salazar y cols., 2013).

1.4. *Clostridium difficile* como agente infeccioso en la tercera edad

C. difficile es considerado la causa más común de diarrea nosocomial en los países industrializados. Produce una gran mortalidad y morbilidad provocando además pérdidas económicas, sobre todo por el gran coste que supone la recurrencia de la enfermedad a la que da lugar, denominada infección asociada a *C. difficile* (IACD) (Rodríguez y cols., 2015b; Shields y cols., 2015). Generalmente este microorganismo provoca enfermedad en personas de edad avanzada hospitalizadas (mayores de 65 años) que están siendo tratadas con antimicrobianos de amplio espectro (Yuille y cols., 2015).

1.4.1. Fisiología y taxonomía

C. difficile es un bacilo gram-positivo, anaerobio estricto y formador de esporas (endoesporas ovales y subterminales); además, algunas cepas son móviles gracias a la presencia de flagelos peritricos (alrededor de toda la célula) (Baverud, 2002).

La esporulación permite a este patógeno sobrevivir en distinas superficies, alimentos y agua durante largos periodos de tiempo (Martin y cols., 2016), ya que la espora es resistente frente a condiciones adversas como calor, desecación, oxígeno y agentes químicos (Baverud, 2002), de tal manera que esta forma de resistencia favorece la transmisión del patógeno entre individuos (Martin y cols., 2016). Las esporas también son resistentes a los antibióticos, lo que les permite permanecer en el tracto gastrointestinal durante el tratamiento específico frente a la forma vegetativa, favoreciendo la recaida de la enfermedad (Heinlen y Ballard, 2011; Zanella y cols., 2014; Shields y cols., 2015). Un estudio ha demostrado la diseminación aérea de las esporas y se ha sugerido que este hecho podría explicar la dificultad de erradicación de este patógeno en los hospitales (Roberts y cols., 2008). Hasta este momento, el único compuesto que se considera eficaz contra las esporas es el hipoclorito de sodio. Su eficacia ha sido demostrada después de la implantación de un programa de limpieza con este compuesto en un centro de atención sanitaria, comprobándose que se produjo una reducción de la tasa de infección de este patógeno (Hacek y cols., 2010).

Otra característica a resaltar de *C. difficile* es que es capaz de sintetizar p-cresol (4-metilfenol) a partir de la tirosina, además de tolerar dicha sustancia. Varios estudios han demostrado que este compuesto fenólico es bacteriostático (capaz de inhibir el crecimiento de otras bacterias), lo cual le puede suponer una ventaja competitiva frente a otros integrantes de la microbiota intestinal permitiendo su proliferación en el intestino (Dawson y cols., 2011).

La temperatura óptima de crecimiento de *C. difficile in vitro* es de 30-37 ^{o}C; sin embargo, se ha observado que crece entre los 25 y los 45 ^{o}C (Baverud, 2002). Cuando este microorganismo se crece en placas de agar sangre se obtienen colonias de 2-5 mm de diámetro, circulares o rizoides, planas o ligeramente convexas, opacas, de color grisáceo y con una superficie brillante (Baverud, 2002).

Según la clasificación actual del Manual Bergey de Bacteriología Sistemática, *C. difficile* está incluido en el Filo *Firmicutes*, Clase *Clostridia*, Orden *Clostridiales* y Familia *Peptostreptococcaceae* (Ludwig y cols. 2009). Una revisión taxonómica más reciente de la Clase *Clostridia* basada en el gen del ARNr 16S y en las secuencias de proteínas ribosomales, confirmó que *C. difficile* pertenecía a la Familia *Peptostreptococcaceae* y

se propuso el cambio de nombre de este microorganismo de *Clostridium* a *Peptoclostridium* (Yutin y Galperin, 2013). Sin embargo, a pesar de que el nuevo nombre aparece en la clasificación taxonómica del NCBI ("National Center for Biotechnology Information"), en el ámbito clínico y en la comunidad científica se continua denominando *C. difficile* (Knight y cols., 2015).

1.4.2. *C. difficile* como patógeno

1.4.2.1. Epidemiología

C. difficile es un microorganismo ubicuo que puede estar presente en el intestino de individuos sanos. Se ha detectado en las heces de un 3% de adultos sanos y en un 16-35% de individuos hospitalizados. El incremento de ese porcentaje es proporcional tanto a la duración de la estancia en el hospital como a la duración de la exposición a antibióticos (Aslam y cols., 2005).

Los reservorios de *C. difficile* incluyen los pacientes colonizados asíntomáticos (incluidos los recién nacidos) o infectados, ambientes y superficies contaminadas, animales y alimentos (Martin y cols., 2016). La vía de transmisión de este patógeno entre personas es oral-fecal. El contacto con las esporas de este microorganismo en los sistemas de atención sanitaria se produce indirectamente, a través del contacto con ambientes y superficies contaminadas de hospitales o residencias geriátricas, y también directamente, a través del contacto con pacientes colonizados (asintomáticos o sintomáticos) y con personal sanitario (Asensio y Monge, 2012; Stanley y cols., 2013; Shields y cols., 2015; Martin y cols., 2016).

C. difficile es un patógeno oportunista que, cuando se produce una alteración de la microbiota intestinal, es capaz de dar lugar a una infección (Rodríguez y cols., 2015b). Hay factores de riesgo que favorecen que este microorganismo pueda desencadenar la enfermedad, siendo los más significativos para el desarrollo, la recurrencia y la mortalidad de IACD la exposición a antibióticos y la edad avanzada (Mizusawa y cols., 2015). Las personas de más de 65 años son el grupo más susceptible a esta enfermedad ya que, como se ha indicado en el apartado anterior, con el avance de la edad se producen cambios en la composición de la microbiota intestinal, se

presenta una respuesta inmune deficiente y se suelen padecer enfermedades crónicas como insuficiencia renal, lo que hace que se incremente el uso de fármacos, las intervenciones quirúrgicas y el tiempo de exposición a los sistemas de atención sanitaria (Keller y Surawicz, 2014; Mizusawa y cols., 2015; Rodríguez y cols., 2015b). Por otro lado, en general, el uso de antibióticos supone un riesgo de contraer IACD (sobre todo los que tienen un amplio espectro de actividad) ya que la resistencia de *C. difficile* a muchos antibióticos le permite sobrevivir mejor que otras especies comensales y, por tanto, la disminución de éstas crea condiciones favorables para que se produzca la colonización del patógeno (Stanley y cols., 2013; Rodríguez y cols., 2015b). Los antibióticos más comúnmente asociados con la IACD son la clindamicina, penicilinas, fluoroquinolonas y las cefalosporinas (Faris y cols., 2010; DePestel y Aronoff, 2013; Stanley y cols., 2013; Rodríguez y cols., 2015b).

La incidencia, severidad y mortalidad de la IACD ha aumentado significativamente en la última década. Este hecho se ha asociado con la aparición de una cepa de mayor virulencia denominada BI/NAP1/027 (cepa caracterizada como grupo BI por análisis por restricción de endonucleasas; como tipo NAP1 por electroforesis en gel de campo pulsado y finalmente 027 como el ribotipo correspondiente) (Zanella y cols., 2014; Mizusawa y cols., 2015). Se ha descrito que esta cepa es capaz de producir más cantidad de toxinas, tiene una esporulación más eficiente, es resistente a las fluoroquinolonas y produce una toxina adicional, la toxina binaria (Warny y cols., 2005; Leffler y Lamont, 2015; Shields y cols., 2015). Otras cepas pertenecientes a otros ribotipos se han visto implicadas en casos severos de la enfermedad (Stanley y cols., 2013; Gerding y Lessa, 2015) como es el caso del ribotipo 001, uno de los más frecuentemente encontrados en Europa (Martin y cols., 2016).

Aunque los individuos más afectados siguen siendo las personas de edad avanzada, se está produciendo un aumento de la incidencia de estas infecciones en poblaciones previamente no consideradas en riesgo, como niños o embarazadas (Rupnik y cols., 2009; Asensio y Monge, 2012; Stanley y cols., 2013).

1.4.2.2. Factores de virulencia

C. difficile presenta una gran variedad de factores de virulencia (Figura 6) (Sebaihia y cols., 2006; Carter y cols., 2012; Awad y cols., 2014; Martin y cols., 2016);

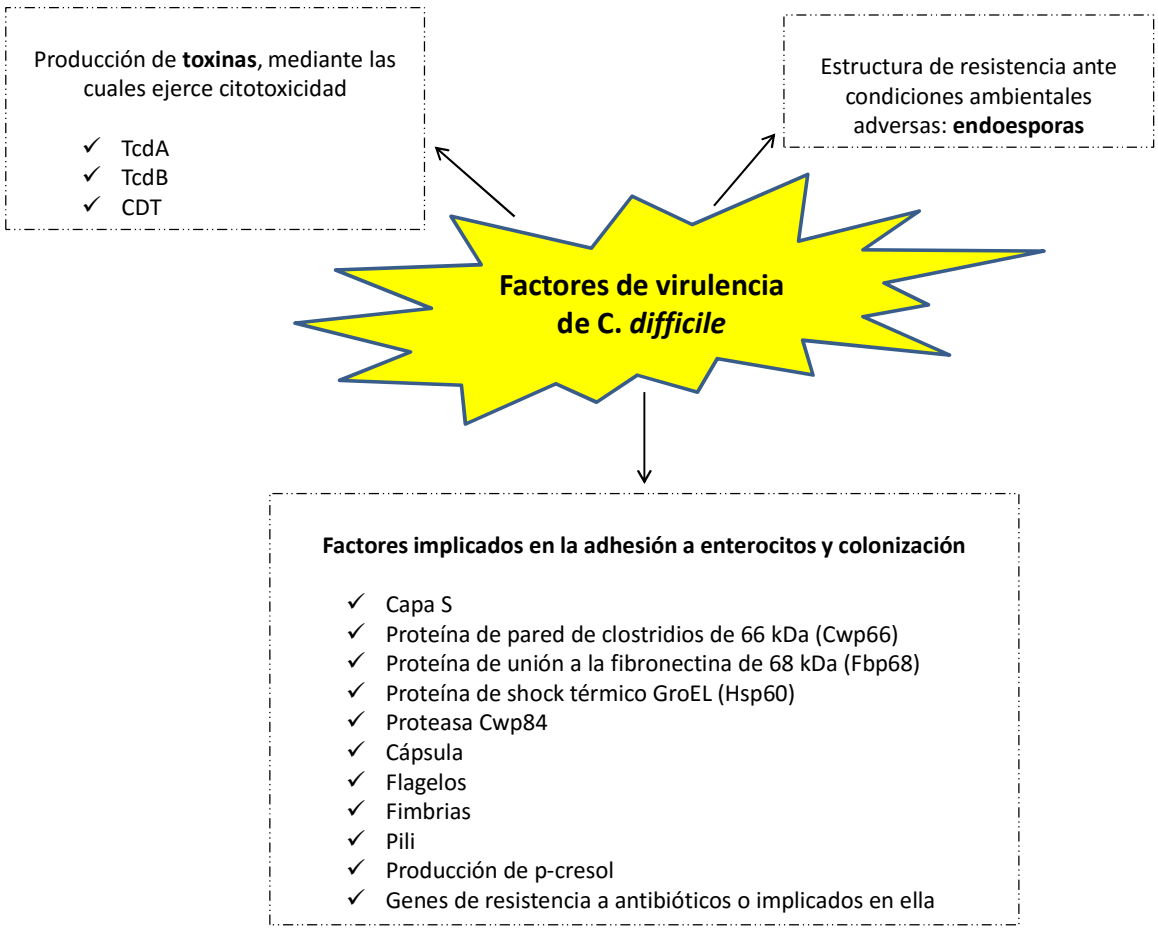


Figura 6. Factores de virulencia de *C. difficile*.

siendo los más importantes las exotoxinas, sobre todo la A (TcdA) y la B (TcdB), al ser las responsables del cuadro clínico de la enfermedad (Carter y cols., 2012; Stanley y cols., 2013; Rodríguez y cols., 2015b; Shields y cols., 2015). Se ha descrito que las toxinas son producidas tanto en la fase de crecimiento exponencial (Stanley y cols., 2013) como en la fase estacionaria (Yuille y cols., 2015). Hay cepas de este microorganismo que no producen toxinas (cepas no toxigénicas) y dentro de las cepas

productoras (cepas toxigénicas) no todas producen todos los tipos de toxinas (Geric y cols., 2004; Rupnik y cols., 2009; Shields y cols., 2015; Martin y cols., 2016).

Las toxinas A (300 kDa) y B (269,6 kDa) pertenecen al grupo de las grandes toxinas clostridiales (LCTs: "large clostridial toxins"), las cuales son monoglucosiltransferasas que inactivan a las GTPasas de la famila Rho a través de la transferencia covalente de un resto de glucosa. Estas toxinas están codificadas por genes localizados en una región del cromosoma de 19.6 kb denominada PaLoc (locus de patogenicidad) y están compuestas por cuatro dominios funcionales: dominio catalítico, dominio proteasa cisteína, dominio de translocación y dominio de unión (Carter y cols., 2012). El dominio catalítico es el que tiene la actividad glucosiltransferasa y se encuentra en el extremo N-terminal. El dominio proteasa cisteína es el requerido para el procesamiento y escisión de la toxina. El dominio de translocación se extiende por la parte central de la proteína e incluye una región hidrófoba, que puede estar involucrada en la formación de poros en la membrana del endosoma de la célula eucariota y en la liberación del dominio catalítico al citosol del enterocito. En la porción C-terminal de la proteína se encuentra el dominio de unión, implicado supuestamente en la unión a receptores de los enterocitos (Awad y cols., 2014). Se han realizado muchos estudios para determinar cuál de las dos toxinas es la más relevante para el desarrollo de la enfermedad. En principio se indicaba que la toxina A era la fundamental para el desarrollo de la infección; sin embargo, más recientemente se sugiere un incremento en la importancia de la toxina B como factor de virulencia (Awad y cols., 2014).

La toxina binaria (CDT: "*C. difficile* transferase") la sintetizan adicionalmente ciertas cepas de *C. difficile* (como el ribotipo 027). Pertenece al grupo de las toxinas binarias clostridiales, las cuales son ADP-ribosiltransferasas específicas de la actina, provocando la desorganización del citoesqueleto de la célula diana. Esta toxina está codificada por unos genes que están localizados en locus cromosómico CDTLoc, en una región del cromosoma de 6.2 kb. Su estructura la conforma una subunidad "a" (componente de la toxina que se introduce en la célula y ejerce su acción tóxica) y una subunidad "b", que se une al receptor celular (Awad y cols., 2014). El papel que tiene esta toxina en el desarrollo de la enfermedad no está claro. Se ha descrito que la

desorganización de la actina provoca la formación de microtúbulos, los cuales parecen incrementar la adherencia del patógeno al enterocito (Schwan y cols., 2009). Un estudio reciente con células Caco-2 ha sugerido que estos microtúbulos y un sensor dependiente de calcio (Stim 1) permiten el paso de vesículas con fibronectina y retículo endoplásmico desde la parte basolateral a la parte apical de la membrana del enterocito, dando lugar a un incremento en la capacidad del patógeno para adherirse a la célula hospedadora (Schwan y cols., 2014). Estudios en animales han demostrado que esta toxina por sí sola no es suficiente para desencadenar la sintomatología típica de la enfermedad (Awad y cols., 2014).

1.4.2.3. Patogenia

Las esporas o las células vegetativas de *C. difficile* son ingeridas por el hospedador; la mayoría de las células vegetativas mueren en el estómago mientras que las esporas, al ser resistentes al pH ácido existente, llegan al intestino delgado (Sunenshine y McDonald, 2006; Yuille y cols., 2015). Las esporas del intestino pueden proceder del exterior pero también estar presentes internamente, ya que este microorganismo forma parte de la microbiota intestinal. En el intestino delgado éstas pueden germinar si las condiciones son favorables, al ser expuestas a ácido cólico o derivados como ácido taurocólico y ciertos aminoácidos como L-glicina o L-histidina (Hernández-Rocha y cols, 2012), o permanecer allí en forma esporulada (Sunenshine y McDonald, 2006). Una vez que se ha producido la germinación el siguiente paso es la colonización del colon; sin embargo ésta sólo tiene lugar si se ha producido una alteración de la microbiota intestinal (disbiosis) por cualquier causa, ya que la microbiota de un individuo sano generalmente no permite la colonización por *C. difficile* (Poutanen y Simor, 2004; Rodríguez y cols., 2015b; Yuille y cols., 2015). Un análisis del microbioma intestinal, mediante secuenciación del ARNr 16S, demostró una reducción de la diversidad bacteriana y una alteración de las especies predominantes en pacientes con IACD, cuando se les comparaba con individuos sanos o colonizados asintomáticamente (Martin y cols., 2016).

Tras la colonización se produce la síntesis de las toxinas, lo cual depende de las cepas y de las condiciones medioambientales (Rupnik y cols., 2009). No todos los individuos colonizados por *C. difficile* desarrollan la sintomatología de la enfermedad, ya que su desarrollo depende de la virulencia de la cepa y de la respuesta inmune del individuo (Martin y cols., 2016). Hay estudios en los que se observa que pacientes colonizados con *C. difficile* con una alta producción de anticuerpos frente a las toxinas permanecen asintomáticos, mientras que pacientes con una respuesta inmune inadecuada tienen un mayor riesgo de diarrea asociado a este patógeno (Leffler y Lamont, 2015). Se ha descrito que los pacientes colonizados con cepas toxigénicas, pero sin presentar síntomas, son más comunes que los colonizados con síntomas de IACD, lo cual es un problema, ya que los colonizados asintomáticos actúan como reservorios y vectores de transmisión de *C. difficile* al diseminar las esporas a través de sus deposiciones (Morgan y cols., 2015).

El siguiente paso en el proceso de toxicidad es la unión de las toxinas por el dominio de unión (C-terminal) a uno o más receptores presentes en la superficie de la célula diana, que es el enterocito (Figura 7). La naturaleza exacta de estos receptores no se conoce, y además, depende de la especie estudiada: se ha descrito que la toxina A se une a α-Gal(1,3)β-Gal(1,4)βGlcNAc en animales y a la glicoproteína 96 en la línea celular de origen intestinal humano HT29. También se ha descrito que los receptores de la toxina A se sitúan en la parte apical de las células diana, mientras que los receptores de la toxina B se sitúan en la parte basolateral de la membrana de la célula susceptible (Rupnik y cols., 2009; Belyi y Aktories, 2010). La introducción de la toxina en la célula se produce por endocitosis a través de la vía clatrina-dinamina. Se piensa que se produce un cambio conformacional de la toxina debido a la acidificación del endosoma eucariota por medio de una H^+-ATPasa. Este cambio de conformación provoca que las toxinas expongan una región de su estructura (dominio de translocación), lo que permite que éstas puedan insertarse en la membrana del endosoma dando lugar a la formación de poros en la misma. En el caso de la toxina A, la formación de poros parece ser dependiente de la presencia del colesterol. Estos poros permiten que la toxina se pueda unir al inositol hexafosfato presente en el citoplasma de la célula, dando lugar a otro cambio conformacional de la toxina en el

dominio proteasa cisteína, que a su vez provoca la autoescisión de la misma liberando al citosol el dominio catalítico activo (Carter y cols., 2012; Awad y cols., 2014). El dominio catalítico de las toxinas va a llevar a cabo la glucosilación de las GTPasas de la

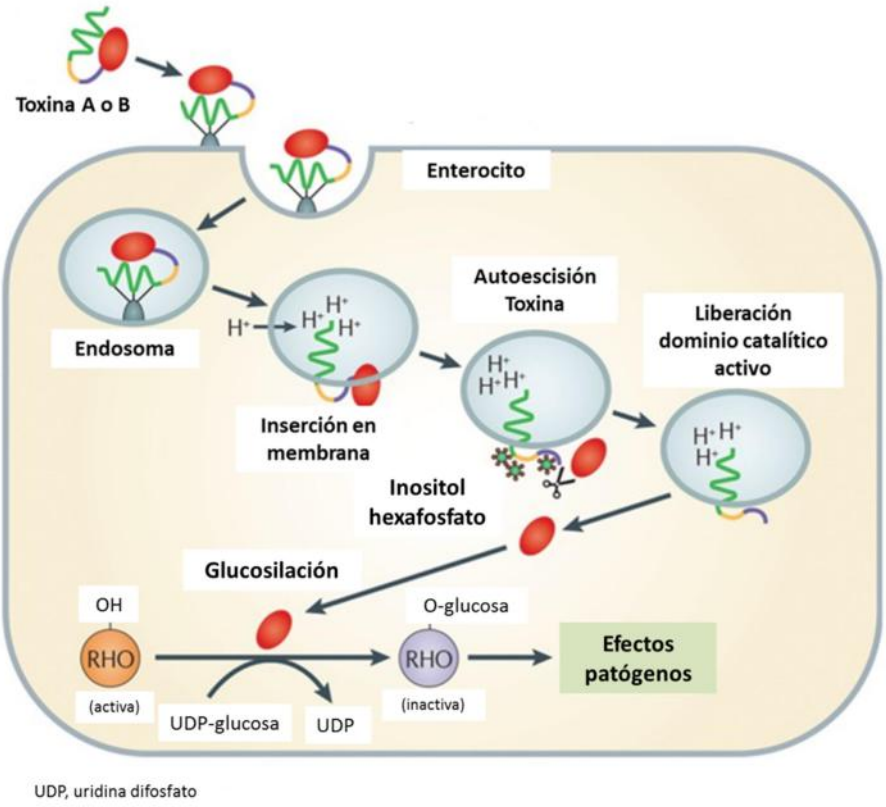


Figura 7. Cambios en el enterocito mediados por las toxinas A y B de *C. difficile.* El dominio catalítico de la toxina aparece en rojo, el dominio proteasa cisteína en azul, el dominio de translocación en amarillo y el dominio de unión en verde. Modificado de Awad y cols. (2014).

familia Rho (Rho, Rac, Ras, Ral y Cdc42), lo que provoca que estas proteínas tengan una conformación inactiva irreversible, afectando a importantes rutas celulares. La inactivación de estas proteínas por las toxinas provoca la pérdida de la integridad estructural de la célula diana como consecuencia de una disminución en la cantidad de F-actina, debido a que no tiene lugar la polimerización de las moléculas de actina G y, además, se promueve la despolimerización de los filamentos de F-actina. Esta pérdida de integridad estructural da lugar al redondeo de las células, que normalmente precede a la apoptosis celular mediada por la via caspasa-3 y la via caspasa-9, y a la

ruptura de las uniones estrechas entre las células vecinas (Figura 8) (Carter y cols., 2012; Awad y cols., 2014). En las células epiteliales en las que penetran las toxinas, se favorece la síntesis y liberación de citoquinas que atraen a neutrófilos y mastocitos al punto de infección y, por otro lado, se activan los nervios entéricos y las neuronas sensoriales de la lámina propia. Todo esto da lugar a la producción de numerosas citoquinas proinflamatorias y neuropéptidos que van a generar una gran respuesta inflamatoria con formación de pseudomembranas (colitis pseudomembranosa). La ruptura de las uniones estrechas entre los colonocitos muertos da lugar a un aumento de la permeabilidad intestinal y a la pérdida de la función barrera y, como consecuencia, al acúmulo de líquido que se manifiesta externamente con la diarrea (Carter y cols., 2012; Awad y cols., 2014).

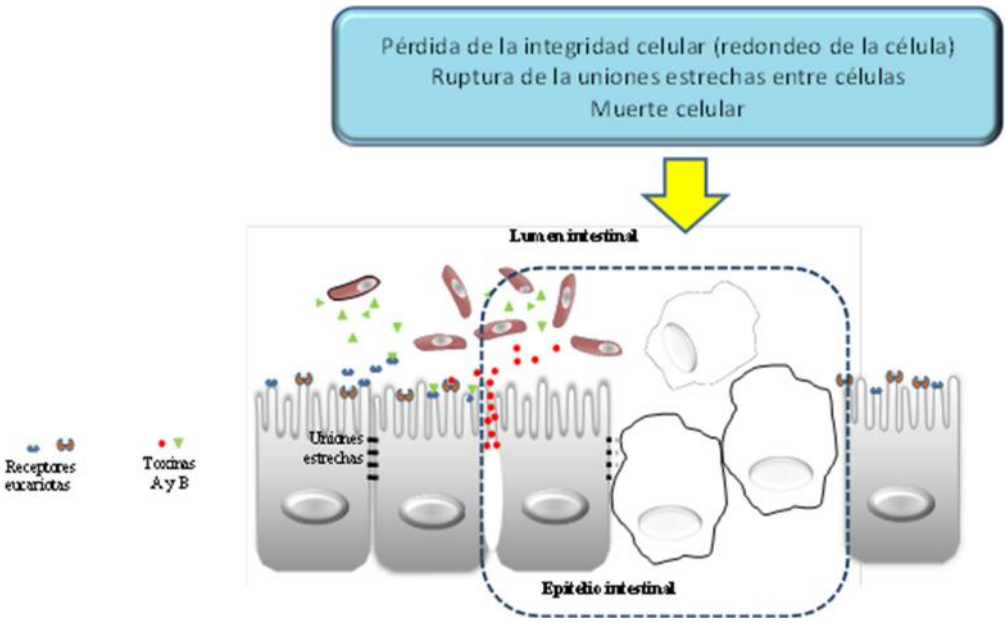


Figura 8. Principales consecuencias del efecto citotóxico de las toxinas de *C. difficile* sobre los enterocitos.

1.4.2.4. Cuadro clínico

El cuadro clínico de la IACD es variable; su severidad, riesgo de recurrencia y mortalidad no sólo dependen de la virulencia del patógeno, sino también de la respuesta inmune del hospedador (Bouza y cols., 2005; Martin y cols., 2016). Esta enfermedad afecta fundamentalmente al colon y raramente al intestino delgado (Rodríguez y cols., 2015b). Puede cursar con diarrea, colitis, colitis pseudomembranosa y megacolon tóxico, pudiendo llegar a producirse perforación intestinal, sepsis, shock y muerte (Keller y Surawicz, 2014; Martin y cols., 2016). También se puede presentar sintomatología general como dolor abdominal, fiebre y leucocitosis en las 48-72 horas post-infección (Rodríguez y cols., 2015b; Martin y cols., 2016).

1.4.3. Tratamientos terapéuticos actuales y emergentes

El tratamiento estándar de la IACD durante aproximadamente 30 años ha sido el uso de dos antibióticos: el metronidazol y la vancomicina. Se pensaba que la efectividad de ambos antibióticos era la misma; sin embargo, actualmente hay estudios que muestran que la vancomicina es más efectiva (Shields y cols., 2015). Debido a la disbiosis originada por estos antibióticos (ya que tienen un amplio espectro de actividad bactericida) y a la resistencia del patógeno a los mismos, el porcentaje de recurrencia de la enfermedad es muy alto (20-35%) (Zanella y cols., 2014; Mizusawa y cols., 2015; Rodríguez y cols., 2015b; Yuille y cols., 2015; Kociolek y Gerding, 2016). Otro antibiótico aprobado por la FDA ("Food and Drug Administration", de EE.UU.) para tratar IACD es la fidaxomicina, aunque tiene una actividad bactericida más reducida que el metronidazol y la vancomicina, sigue presentando altas tasas de recurrencia (superiores al 10%) (Kociolek y Gerding, 2016).

La recurrencia de la enfermedad puede ser debida a la germinación de las esporas o a las bacterias presentes en el intestino y que han resistido al tratamiento antibiótico (recaída); o bien a la adquisición de la cepa original o de una nueva cepa procedente del medio ambiente (reinfección) (Shields y cols., 2015). Debido a los altos porcentajes de recaída de la IACD, hay un creciente interés en el estudio de alternativas y estrategias adyuvantes para la prevención y tratamiento de la

enfermedad. Estas estrategias tienen como objetivos: i) reducir al máximo la disbiosis de la microbiota intestinal y/o restaurarla, ii) reducir la colonización del tracto intestinal por parte de cepas toxigénicas de *C. difficile,* iii) reducir la carga y actividad de las toxinas de *C. difficile* en el intestino y iv) reforzar la respuesta inmune del hospedador contra las toxinas de este patógeno (Musgrave y cols., 2011; Kociolek y Gerding, 2016).

Actualmente existen otros antibióticos en desarrollo clínico (como cadazolid), que tienen una actividad limitada contra varias especies bacterianas comensales entéricas y por esta razón podrían reducir el riesgo de recurrencia de la enfermedad (Kociolek y Gerding, 2016). Recientemente se ha descrito un fármaco (ebselen), no antibiótico, capaz de reducir la sintomatología de la enfermedad en ratones, lo cual se ha asociado con la inhibición del dominio proteasa cisteína de las toxinas A y B por parte de este fármaco (Bender y cols., 2015).

Una manera de prevenir la colonización del intestino con cepas toxigénicas de *C. difficile* puede lograrse con una restauración de la microbiota intestinal, bien a través del uso de probióticos, bien a través del transplante de microbiota fecal, o incluso a través de la colonización del intestino con cepas no toxigénicas de *C. difficile*. El uso de probióticos para prevenir y tratar la IACD es un área de investigación de interés creciente desde la pasada década, ya que son bien tolerados y tienen nulos efectos adversos. Hay evidencias de que ciertas mezclas de probióticos son efectivas para reducir el desarrollo de la IACD y sus tasas de recurrencia; sin embargo, los datos no son suficientes para recomendar su uso como terapia adyuvante para la prevención y tratamiento de la enfermedad (Musgrave y cols., 2011; Stanley y cols., 2013; Mizusawa y cols., 2015; Yuille y cols., 2015). Actualmente, existen estudios sobre probióticos con capacidad para disminuir el crecimiento de *C. difficile* o reducir la producción/actividad de sus toxinas (Castagliuolo y cols., 1996; Banerjee y cols., 2009; Trejo y cols., 2010; Carasi y cols., 2012; Kolling y cols., 2012; Bolla y cols., 2013; Yun y cols., 2014). Al igual que los probióticos, el objetivo del transplante de microbiota fecal es resolver la disbiosis por medio de la recuperación de la microbiota intestinal (Zanella y cols., 2014). El transplante de microbiota fecal consiste en la instilación de heces de un donante dentro del tracto gastrointestinal en pacientes con IACD

recurrente a través de un tubo nasoduodenal, enema, gastroscopia, colonoscopia o a través de un preparado fecal congelado en cápsula. Este método ha mostrado ser efectivo en casos severos de IACD y reduce el riesgo de recurrencia; sin embargo, se desconocen los efectos secundarios a largo plazo (Mizusawa y cols., 2015; Kociolek y Gerding, 2016). Por otro lado, la colonización con cepas no toxigénicas de *C. difficile* consiste en la administración oral de esporas de la cepa M3 no toxigénica de esta especie. Se ha descrito que esta cepa no toxigénica compite con las toxigénicas por los nutrientes y la adherencia en el intestino (Musgrave y cols., 2011). Se ha observado que en pacientes colonizados con la cepa no toxigénica el porcentaje de recurrencia de la enfermedad es bajo, pero esa colonización es transitoria (Kociolek y Gerding, 2016) y además, se ha sugerido que estas cepas pueden llegar a producir toxinas si se les transfiere por vía horizontal los genes del locus PaLoc (Martin y cols., 2016).

La inmunoterapia se ha convertido en un área de gran interés a partir de la aparición de estudios que sugieren que la respuesta humoral del individuo contra *C. difficile* protege frente al desarrollo de la IACD. Las terapias inmunológicas emergentes incluyen: el desarrollo de vacunas para proteger a largo plazo frente a la IACD, o la administración oral o intravenosa de anticuerpos monoclonales contra las toxinas de *C. difficile,* de tal manera que los anticuerpos se unen y neutralizan las mismas; sin embargo, la colonización del tracto gastrointestinal del patógeno no se ve afectada por estas estrategias (Musgrave y cols., 2011; Kociolek y Gerding, 2016). Por esta razón, existe gran interés en prevenir la colonización por *C. difficile* desarrollando vacunas contra proteínas de la superficie del patógeno. Hasta ahora sólo se ha logrado una modesta protección frente a la enfermedad en modelos animales (Kociolek y Gerding, 2016). Andersen y colaboradores han logrado reducir los síntomas IACD en hámster a través de la administración por vía oral de un lactobacilo modificado genéticamente capaz de producir anticuerpos frente a la toxina B (Andersen y cols., 2015).

Se han descrito quelantes de toxinas, como la colestiramina o el colestipol, que son capaces de formar un complejo con las toxinas, el cual no es absorbible en el intestino; sin embargo, éstos también pueden unirse a la vancomicina y al metronidazol pudiendo bloquear el efecto de los antibióticos. También se han señalado otros quelantes, como el tolevamer, el cual no parece unirse a los

antibióticos pero se considera que los beneficios que aporta no son suficientes como para considerarlo una opción viable de tratamiento (Musgrave y cols., 2011; Shields y cols., 2015).

1.5. Alimentos funcionales: probióticos y prebióticos

El concepto de alimento funcional nació en Japón. Las autoridades sanitarias japonesas, hacia 1980, se dieron cuenta de que para controlar los gastos sanitarios generados por el envejecimiento de la población, había que garantizar una mejor calidad de vida. Por esta razón, se planteó la necesidad de fomentar programas de desarrollo de productos alimentarios que pudieran ejercer un efecto positivo sobre la salud, reducir el riesgo de enfermedad y, como consecuencia, reducir los altos costes sanitarios (Siró y cols., 2008).

1.5.1. Definiciones

Un alimento puede ser considerado funcional "si se logra demostrar satisfactoriamente que posee un efecto beneficioso sobre una o más funciones específicas en el organismo, más allá de los efectos nutricionales habituales, de modo tal que mejore el estado general de salud y bienestar y/o reduzca el riesgo de enfermedad" (ILSI, 2008). Los alimentos funcionales pueden ser naturales o procesados. Los procesados son aquellos en cuya composición uno o varios componentes han sido añadido(s), o eliminado(s), o sustituido(s), o su naturaleza o su biodisponibilidad ha sido modificada, o cualquier combinación de estas posibilidades, mediante procedimientos tecnológicos diversos (enzimáticos, químicos, biotecnológicos, etc…) (ILSI, 2008).

Dentro de los alimentos funcionales procesados, están aquellos a los que se han añadido microorganismos probióticos. En concreto, los productos lácteos, al mejorar la supervivencia microbiana durante el tránsito gastrointestinal, se consideran el vehículo ideal para administrar probióticos en humanos debido al efecto tampón y protector de la matriz láctea (Kaur y cols., 2014). La OMS (Organización Mundial de la Salud) y la FAO (Organización de las Naciones Unidas para la Alimentación y la

Agricultura) definieron en el año 2001 a los probióticos como "microorganismos vivos que, cuando se administran en cantidades adecuadas, confieren un beneficio a la salud del consumidor" (FAO/OMS, 2006; Hill y cols., 2014). Este documento recoge que la seguridad y la eficacia de cada cepa probiótica tiene que ser demostrada, y que tiene que resistir las barreras biológicas presentes en el tracto gastrointestinal (pH ácido del estómago, enzimas digestivas y bilis) llegando viable al sitio de acción (Sánchez y cols., 2009). La WGO ("World Gastroenterology Organisation"), basándose en el documento de la FAO/OMS del 2002 (FAO/OMS, 2006), recoge una serie de criterios mínimos que debe cumplir un microorganismo probiótico incluido en alimentos (Guarner y cols., 2011): i) identificación correcta de la cepa (ya que los efectos saludables demostrados para una cepa microbiana específica no son extrapolables o atribuibles a otras cepas de la misma especie), ii) ser inocuo (carecer de factores de virulencia, de capacidad para producir metabolitos indeseables y de resistencia transmisible a antibióticos), iii) mantenerse vivo hasta el final de su vida útil (tiene que sobrevivir no sólo a la barreras biológicas presentes en el tracto gastrointestinal, sino también a las condiciones de procesado, almacenamiento y a las propias de los alimentos), iv) administrarse en dosis adecuadas y v) mostrarse eficaz en estudios de intervención en humanos (los estudios de laboratorio *in vitro* y en modelos animales no se consideran prueba suficiente de eficacia en salud humana).

El mercado de los alimentos prebióticos (alimentos a los que se han añadido sustratos prebióticos) también ha aumentado en los últimos años (Corzo y cols., 2015). En una de las últimas definiciones, Roberfroid y colaboradores indican que los prebióticos "son ingredientes que producen una estimulación selectiva del crecimiento y/o actividad(es) de uno o de un limitado número de géneros/especies de microorganismos en la microbiota intestinal confiriendo beneficios para la salud del hospedador" (Roberfroid y cols., 2010). Al igual que para los probióticos, se han establecido unos criterios que debe cumplir un compuesto para ser considerado prebiótico (Corzo y cols. 2015): i) ser resistente a la acidez gástrica y a la hidrólisis por enzimas digestivas, ii) no ser absorbido en el intestino delgado, iii) ser fermentado por un número limitado de bacterias beneficiosas del colon y iv) ser capaz de inducir efectos fisiológicos beneficiosos para la salud.

1.5.2. Microorganismos más comúnmente utilizados en alimentos funcionales

Como microorganismos probióticos para aplicación en humanos se utilizan, sobre todo, especies de los géneros *Lactobacillus* y *Bifidobacterium* aunque no exclusivamente (también se usan cepas de otras especies de bacterias y levaduras) (Sánchez y cols., 2009). Algunas especies de ambos géneros son consideradas seguras por la EFSA ("European Food Safety Authority") para su inclusión en alimentos para humanos y animales, según se establece en la lista de microorganismos QPS ("Qualified Presumption of Safety") (EFSA, 2015). Además, ambos géneros son miembros importantes de la microbiota intestinal del hombre y de animales (De Vrese y Schrezenmeir, 2008).

El género *Bifidobacterium* está incluido en el filo *Actinobacteria*, clase *Actinobacteria*, subclase *Actinobacteridae*, orden *Bifidobacteriales* y familia *Bifidobacteriaceae* (Lugli y cols., 2014). Hasta el momento se han descrito 39 especies y 9 subespecies de este género (Lugli y cols., 2014; Brandt y Barrangou, 2016). Estas bacterias se caracterizan por ser Gram-positivas, con alto contenido de guanina y citosina en su ADN cromosómico (entre 55 y 67%), no formadoras de esporas, no filamentosas e inmóviles. Se trata de bacilos polimórficos (Figura 9), ligeramente curvados o con ramificaciones en forma de Y o V (forma más común), y pueden organizarse individualmente o formando cadenas, agregados o en empalizada (Margolles y cols., 2011). Son bacterias catalasa negativas, con algunas excepciones como *Bifidobacterium indicum* y *Bifidobacterium asteroides* cuando crecen en presencia de aire (Felis y Dellaglio, 2007). Su temperatura óptima de crecimiento oscila entre 36-38 ^{o}C para las especies de origen humano y 41-43 ^{o}C para las especies de origen animal (Margolles y cols., 2011). Sin embargo, hay una especie, *Bifidobacterium thermacidophilum,* cuya temperatura óptima de crecimiento oscila entre 49 y 50 ^{o}C (Dong y cols., 2000) y la especie *Bifidobacterium psychraerophilum* es capaz de crecer a temperaturas tan bajas como de 4 ^{o}C (Simpson y cols., 2004). Las bifidobacterias tienen una escasa tolerancia a la acidez (a excepción de *Bifidobacterium animalis* subsp. *lactis*), siendo su pH óptimo de crecimiento entre 6,5 y 7,0 (González-Rodríguez y cols., 2013b). Son bacterias anaerobias estrictas, aunque algunas especies pueden

tolerar concentraciones moderadamente altas de oxígeno (como *B. animalis* o *B. psychraerophilum*) (Li y cols., 2010; Margolles y cols., 2011). Por ello, *B. animalis* subsp. *lactis* es la bifidobacteria más usada en productos lácteos fermentados, ya que tolera mejor el pH ácido y la presencia de oxígeno (Margolles y cols., 2011).

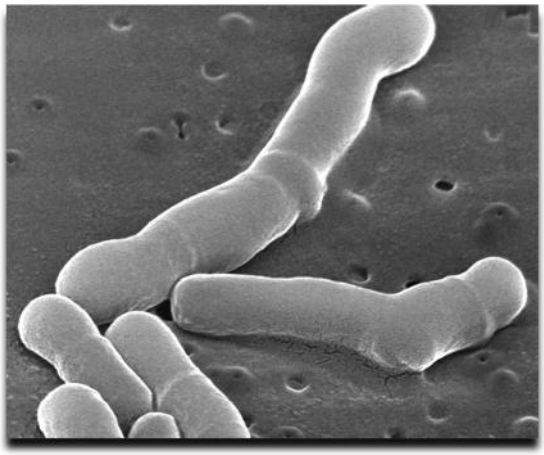


Figura 9: Fotografía de microscopia electrónica de *B. longum*.

El metabolismo de las bifidobacterias es fermentativo, a través de la utilización de las hexosas. Por la ruta bífida, o vía de la fructosa-6-fosfato fosfocetolasa, producen fundamentalmente ácido acético y láctico, así como pequeñas cantidades de etanol, ácido fórmico y otros compuestos. Para poder llevar a cabo esta ruta se necesita una enzima, la xilulosa-5-fosfato/fructosa-6-fosfato fosfocetolasa, que no está presente en otras bacterias intestinales (Sánchez y cols., 2010). Esta enzima cataliza la conversión de una molécula de xilulosa-5-fosfato en gliceraldehido-3-fosfato y acetil fosfato, o de una molécula de fructosa-6-fostato en eritrosa-4-fosfato y acetil fosfato. Teóricamente, por cada mol de glucosa consumido se forman 1,5 moles de ácido acético, 1 mol de ácido láctico y 2,5 moles de ATP (González-Rodríguez y cols., 2013a).

Por otro lado, el género *Lactobacillus* está incluido en el filo *Firmicutes*, clase *Bacilli*, orden *Lactobacillales* y familia *Lactobacillaceae* (Felis y Dellaglio, 2007). Se caracterizan por ser bacilos o coco-bacilos Gram-positivos, catalasa negativos, con un

bajo contenido en guanina y citosina en su ADN cromosómico (entre 32 y 54 %), no formadores de esporas, aerotolerantes o anaerobios y con requerimientos nutricionales complejos (como aminoácidos o vitaminas) (Kandlder y Weiss, 1986; Sánchez y Sanz, 2011). La temperatura de crecimiento varía de 2 a 53 ^{o}C, pero su temperatura óptima oscila entre 30 y 40 ^{o}C. Su pH óptimo se sitúa entre 5,5 y 6,2 aunque pueden crecer en un amplio intervalo, desde 3 a 8 (Salvetti y cols., 2012). El metabolismo puede ser homofermentativo obligado o heterofermentativo (obligado o facultativo). Las homofermentativas obligadas fermentan las hexosas casi exclusivamente (>85%) a ácido láctico por la vía Embden-Meyerhof-Parnas (EMP), pero no pueden metabolizar las pentosas y el gluconato por la carencia de la actividad fosfocetolasa. Las heterofermentativas facultativas degradan las hexosas por la vía EMP (poseen una fructosa difosfato aldolasa) pero también fermentan las pentosas (y a menudo el gluconato) por la vía pentosa fosfato (poseen una fosfocetolasa inducible). Por último, las heterofermentativas obligadas degradan exclusivamente las hexosas y pentosas por la vía pentosa fosfato ya que poseen una fosfocetolasa, pero no una fructosa difosfato aldolasa, y como resultado se obtiene una mezcla equimolar de lactato, etanol o ácido acético y CO_2 (Felis y Dellaglio, 2007; Sánchez y Sanz, 2011;). El balance energético a través de la ruta EMP es de 2 moles de ATP, mientras que por la ruta pentosa fosfato es de tan solo 1 mol (Madigan y cols., 1999).

1.5.3. Sustratos prebióticos más comúnmente utilizados en distintos alimentos funcionales.

Los carbohidratos prebióticos están disponibles de forma natural (en bajas concentraciones) en la leche materna y en ciertos vegetales pero, además, pueden ser sintetizados artificialmente (oligosacáridos sintéticos) (Toward y cols., 2012). En el mercado existen prebióticos con gran evidencia científica sobre sus propiedades beneficiosas en humanos (inulina, fructooligosacáridos (FOS), galactooligosacáridos (GOS), lactulosa y oligosacáridos de la leche humana (HMO: “human milk oligosaccharides”). También se están estudiando otros prebióticos (como xilooligosacáridos, lactosacarosa, isomaltooligosacáridos, oligosacáridos de soja y

glucooligosacáridos) cuyo grado de evidencia científica, en cuanto a sus beneficios para la salud humana, es menor. Además, hay otros en fase de estudio inicial como pectooligosacáridos, polidextrosa, exopolisacáridos bacterianos y polisacáridos de macroalgas (Corzo y cols. 2015).

La inulina y los FOS están presentes en la naturaleza en gran variedad de alimentos (como la achicoria), aunque los FOS también puede ser sintetizados (por síntesis enzimática a partir de la sacarosa o por degradación enzimática de la inulina) (Singh y cols., 2016). La estructura general, tanto de la inulina como de los FOS, está formada por varias unidades de fructosa unidas a una glucosa terminal (GF_n) mediante enlaces glucosídicos β-(2-1) o unidades de fructosa (FF_n) unidas entre sí por el mismo tipo de enlace. La inulina está formada por oligosacáridos y polisacáridos con una estructura mayoritaria GF_n y un grado de polimerización que varía de 2 a 65 unidades. Cuando los FOS son obtenidos por la hidrólisis de la inulina, se obtiene una mezcla de oligosacáridos (GF_n o FF_n) con un grado de polimerización de 2 a 7. Por otro lado, cuando los FOS son sintetizados enzimáticamente a partir de la sacarosa, se obtiene una mezcla de oligosacáridos GF_n en los que los enlaces glucosídicos pueden ser β-(2-1) o β-(2-6). Tanto la inulina como los FOS, son moléculas que no pueden ser hidrolizadas por enzimas digestivas humanas debido a la carencia de la actividad β-fructosidasa (Corzo y cols., 2015; Singh y cols., 2016).

Los GOS están presentes de forma natural en la leche y calostro humano, pero también pueden ser sintetizados a partir de la lactosa mediante la actividad galactosiltransferasa (β-galactosidasa o lactasa). Estas enzimas, bajo condiciones determinadas, catalizan la hidrólisis de la lactosa pero también la formación de un enlace β-glicosídico entre la galactosa, liberada en la hidrólisis, y la lactosa u otros carbohidratos presentes en la reacción. Estos oligosacáridos contienen de 2 a 10 moléculas de galactosa unidas a una glucosa terminal y se diferencian entre sí en la longitud de la cadena y el tipo de enlace (Villamiel y cols., 2014; Corzo y cols., 2015).

La lactulosa es un disacárido sintético (β-D-galactosa-(1-4)-D-fructosa) que puede obtenerse mediante isomerización química o enzimática de la lactosa o por síntesis enzimática con β-galactosidasas y lactosa y fructosa como sustratos. Altas concentraciones de lactulosa puede producir flatulencias y diarrea (se utiliza para

tratar el estreñimiento crónico), por esta razón hay que establecer las dosis adecuadas para poder ser usada como prebiótico (Meléndez y cols., 2011; Corzo y cols., 2015).

Los HMO son producidos en la glándula mamaria, se encuentran en el calostro (22-24 g/L) y en la leche humana (12-14 g/L). Estos oligosacáridos están constituidos por una molécula de lactosa en cuyo extremo reductor se unen diferentes carbohidratos (para ello es necesario la acción de diferentes glicosiltransferasas). Hay HMO neutros (compuestos de lactosa, galactosa y varias unidades de N-acetil-glucosamina y fucosa) y ácidos (compuestos de lactosa, galactosa y varias unidades de N-acetil-glucosamina, fucosa y una o más unidades de N-acetil-neuramínico). La presencia de fucosa y de N-acetil-neuramínico en posición términal hace que los HMO no puedan ser digeridos por las enzimas digestivas y tampoco por microorganismos intestinales que no contengan glicosil hidrolasas específicas. Los HMO con fucosa y N-acetil-neuramínico comparten estructuras con los glicanos del epitelio intestinal de los lactantes (receptores de patógenos), por tanto la presencia de estos oligosacáridos en la leche materna supone un mecanismo de defensa (Barile y Rastall, 2013; Corzo y cols., 2015).

1.5.4. Aplicaciones de los alimentos funcionales

1.5.4.1. Alimentos funcionales probióticos

Los mecanismos empleados por los probióticos para ejecer su efecto sobre el hospedador son diferentes en función de la cepa probiótica de la que se trate. Éstos pueden actuar a distintos niveles: en el lumen intestinal, en la mucosa y epitelio intestinal, así como en otros órganos (como el cerebro) (Martínez-Cuesta y cols., 2012). Pueden ejercer su efecto beneficioso modulando la composición de la microbiota intestinal, favoreciendo la presencia y diversidad de grupos microbianos beneficiosos dentro de esta comunidad microbiana, o inhibiendo a microorganismos patógenos (Dobson y cols., 2012). Esta inhibición de patógenos puede llevarse a cabo por distintos mecanismos: producción de sustancias inhibitorias como ácidos orgánicos o bacteriocinas, competición por sitios de unión al epitelio o por nutrientes, o bien por degradación de las toxinas o bloqueo de los receptores de las toxinas que producen

(Pandey y cols., 2015). Ciertos probióticos han mostrado ser eficaces en el mantenimiento de la integridad de la barrera intestinal, así como en la prevención y reparación de daños en la mucosa causados por distintos agentes (alérgenos de los alimentos, patógenos o citoquinas proinflamatorias). Los mecanismos de acción implicados en este efecto pueden ser: el incremento de la expresión y secreción de mucina por las células caliciformes, el incremento de la expresión y secreción de defensinas (péptidos antimicrobianos) al mucus por parte de las células epiteliales y la mejora de la estabilidad de las uniones estrechas entre los enterocitos (Ohland y MacNaughton, 2010). La pérdida de la función barrera está asociada a patologías como: enfermedad inflamatoria intestinal, infecciones intestinales, enfermedad celiaca y algunas enfermedades autoinmunes (Dobson y cols., 2012). El efecto inmunomodulador de los probióticos se debe a que son capaces de actuar sobre la inmunidad innata y la adquirida, de tal manera que pueden proteger al hospedador frente a infecciones y atenuar procesos de inflamación intestinal crónica como la enfermedad de Crohn (Dobson y cols., 2012). Las células del epitelio intestinal y del sistema inmunológico poseen receptores celulares capaces de discriminar entre la microbiota comensal y la patógena; ante la presencia de patógenos son capaces de inducir la síntesis y liberación de mediadores de la respuesta inmunológica innata y, por tanto, de inducir respuestas inmunológicas adaptativas adecuadas (Werner y Haller, 2007).

Son muchos los estudios que muestran, con mayor o menor evidencia científica, cómo se puede pueden prevenir determinadas enfermedades o reducir su sintomatología usando cepas probióticas (Figura 10).

La intolerancia a la lactosa se atribuye a la incapacidad para digerir la lactosa (debido a la baja o nula actividad de la enzima β-galactosidasa). Los síntomas incluyen: diarrea, distensión abdominal, dolor abdominal y flatulencias (Pandey y cols., 2015). Existe gran evidencia científica a cerca de la reducción de la sintomatología de la intolerancia a la lactosa por probióticos, incluyendo los microorganismos presentes en el yogurt (*Streptococcus thermophilus* y *Lactobacillus delbrueckii* subsp. *bulgaricus*) (Gueimonde y cols., 2008). Se ha demostrado que la combinación de *Lactobacillus casei* Shirota y *Bifidobacterium breve* Yakult sobrevive al tracto gastrointestinal y que

mejora los síntomas asociados a esta intolerancia; además, este efecto parece persistir durante al menos 3 meses una vez que cesa la administración de esta combinación probiótica (Almeida y cols., 2012).

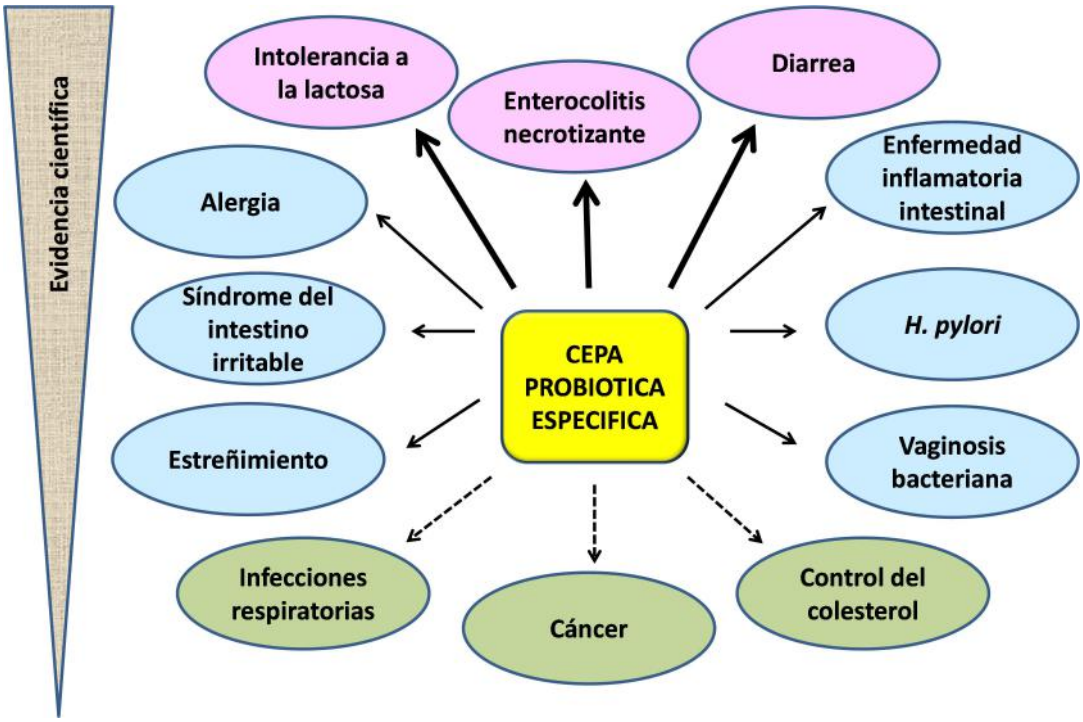


Figura 10. Efectos beneficiosos atribuidos a cepas probióticas específicas y su evidencia científica. Modificado de Gueimonde y cols., 2008.

Diversos estudios han confirmado el efecto de los probióticos en diferentes tipos de diarreas (Narayan y cols., 2010). Se ha descrito que *Lactobacillus rhamnosus* GG y *Saccharomyces boulardii* son eficaces en la reducción de la duración de la diarrea aguda en niños (principalmente debida a rotavirus) y para prevenir la diarrea asociada a antibióticos en niños y adultos (McFarland, 2006; Gueimonde y cols., 2008; Flonch y cols., 2015). Sin embargo, no hay evidencias clínicas concluyentes sobre la eficacia de los probióticos en la prevención y tratamiento de la diarrea asociada a *C. difficile* (Mizusawa y cols., 2015; Yuille y cols., 2015). En el caso de las diarreas del viajero (producidas por distintos agentes etiológicos) se ha observado un efecto beneficioso

de ciertos probióticos (cepas de lactobacilos, bifidobacterias, enterococos y estreptococos) en la prevención de este tipo de diarreas. Además, se ha descrito que *S. boulardii* es más efectivo en diarreas bacterianas y que *L. rhamnosus* GG resulta más eficaz frente a virus y diarreas idiopáticas (McFarland, 2007).

La enterocolitis necrotizante afecta fundamentalmente a niños prematuros. Una revisión sistemática y un meta-análisis reciente han mostrado el efecto preventivo de los probióticos, en general, en esta enterocolitis en neonatos prematuros (Aceti y cols., 2015).

El término enfermedad inflamatoria intestinal (EII) hace referencia a distintas patologías: colitis ulcerosa, enfermedad de Crohn y pouchitis. Estas EII se caracterizan por presentar periodos de recurrencia y de remisión de los síntomas, por su evolución crónica y por presentar daños en la función barrera de la mucosa intestinal (Pandey y cols., 2015). En la enfermedad de Crohn, ninguna de las cepas probióticas clínicamente probadas ha mostrado eficacia en el mantenimiento de la remisión, ni en la prevención de la recurrencia clínica de la enfermedad (Guarner y cols., 2011). En el caso de la colitis ulcerosa, la cepa *Escherichia coli* Nissle 1917 y la mezcla de probióticos VSL#3 (distintas cepas de *Streptococcus thermophilus*, *Lactobacillus* spp. y *Bifidobacterium* spp.) han sido eficaces en el mantenimiento de la remisión de los síntomas. En la pouchitis, la mezcla de probióticos VSL#3 ha resultado ser efectiva en la prevención de la recurrencia y en el mantenimiento de la remisión inducida por tratamiento antibiótico (Flonch y cols., 2015).

Los mayores beneficios de los probióticos en las alergias se han observado en la dermatitis atópica en niños; sin embargo, la variabilidad de los resultados obtenidos (incluso con la misma cepa) no permite recomendar, de forma rutinaria, el empleo de probióticos para prevenir la enfermedad (Martínez-Cuesta y cols., 2012).

En cuanto a la infección por *Helicobacter pylori,* se ha observado que ciertas cepas de lactobacilos y de bifidobacterias reducen los efectos colaterales de las terapias antibióticas; sin embargo, no hay datos suficientes que avalen la eficacia de los probióticos solos (sin antibiótico) en la erradicación del microorganismo (Guarner y cols., 2011).

El síndrome del intestino irritable es una enfermedad crónica que se caracteriza por recurrentes ataques de distensión y dolor abdominal, flatulencias y cambios en el ritmo intestinal (Pandey y cols., 2015). En una revisión sistemática de ensayos clínicos se ha concluido que los probióticos disminuyen los síntomas; sin embargo, no se ha confirmado que cepas son más efectivas (Moayyedi y cols., 2010). Se ha observado que la mezcla de probióticos VSL#3 reduce la flatulencia y la distensión abdominal (Chapman y cols., 2011) y que la cepa *L. rhamnosus* GG provoca una reducción moderada del dolor en niños (Horvath y cols., 2011).

El estreñimiento es uno de los desórdenes más comunes del intestino, afecta a una parte importante de la población de los países industrializados y se agrava con la edad. Existen pocos estudios sobre esta disfunción y a menudo son trabajos de baja calidad (Gueimonde y cols., 2008). Ensayos clínicos han mostrado que la cepa *B. animalis* subp. *lactis* mejora la función intestinal, particularmente, en subgrupos con un estreñimiento leve (Jungersen y cols., 2014).

Algunas cepas de *L. rhamnosus* y *Lactobacillus reuteri* han mostrado resultados prometedores en la prevención y tratamiento de la vaginosis bacteriana (De Preter y cols., 2007) y se ha observado que la administración de cepas de lactobacilos, tanto en niños como en adultos, puede disminuir el riesgo de aparición de síntomas asociados a infecciones del tracto respiratorio (Hao y cols., 2011).

En una revisión sistemática de estudios clínicos también se ha señalado el papel de los probióticos en la reducción de la concentración plasmática de las lipoproteínas de baja densidad (LDL) y del colesterol total (Guo y cols., 2011). Igualmente, existen algunas evidencias, *in vitro* y en modelos animales, que han descrito un efecto beneficioso potencial de los probióticos en la prevención de la carcinogénesis (Kaga y cols., 2013; Pandey y cols., 2015); sin embargo, se requieren estudios más sólidos para confirmar dicho efecto (Pandey y cols., 2015).

1.5.4.2. Alimentos funcionales prebióticos

Existen distintos estudios que indican que los prebióticos pueden tener efectos beneficiosos en la prevención y reducción de la sintomatología de determinadas enfermedades (Figura 11). Algunos autores sugieren que estos efectos podrían ser debidos a su capacidad para modular la microbiota intestinal, ya que estimulan el crecimiento de bacterias beneficiosas y controlan el crecimiento de microorganismos

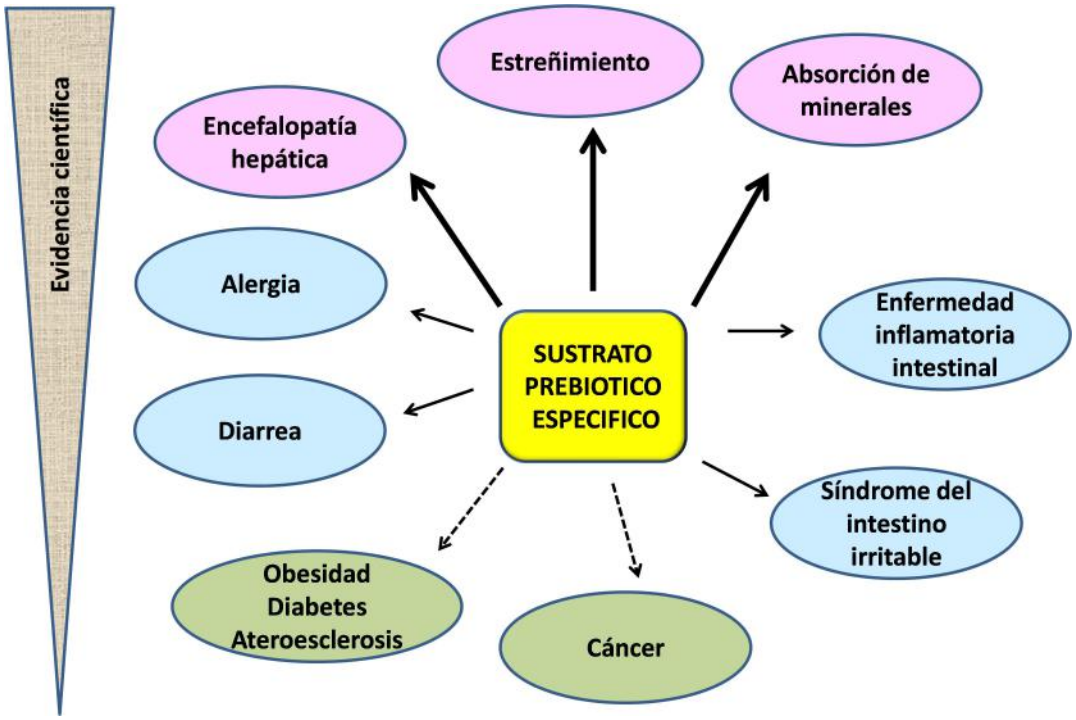


Figura 11. Efectos beneficiosos atribuidos a sustratos prebióticos específicos y su evidencia científica.

que pueden tener efectos perjudiciales (Guarner y cols., 2011). Hay prebióticos que pueden tener un efecto protector frente a patógenos, ya que actuan como análogos de los receptores, tanto de microorganimos patógenos como de las toxinas, presentes en las células epiteliales del colon, impidiendo que se unan a las dichas células (Corzo y cols., 2015). Los prebióticos parece que también pueden tener un efecto inmunomodulador, aunque no se conocen los mecanismos que median este efecto

(Corzo y cols., 2015; Pandey y cols., 2015). Se ha observado que los FOS y otros prebióticos incrementan el número de linfoncitos y/o leucocitos en el tejido linfoide asociado al intestino y en sangre periférica (Schley y Field, 2002). Además, los prebióticos tienen efecto sobre el metabolismo del hospedador derivado de los AGCCs (obtenidos tras su fermentación) que favorecen la absorción de minerales necesarios para el buen funcionamiento del organismo (Guarner y cols., 2011).

Los efectos beneficiosos de los prebióticos en la prevención o alivio del estreñimiento son bien conocidos (Nyman, 2002; Schumann, 2002), ya que reducen el tiempo de tránsito intestinal y aumentan el número de deposiciones (al producir un aumento del volumen del bolo fecal) (Pandey y cols., 2015).

Numerosos estudios muestran que la absorción de minerales, como el calcio, puede ser incrementada por el consumo de prebióticos, fundamentalmente fructanos (Abrams y cols., 2005; Whisner y cols., 2013); sin embargo, otros trabajos no han confirmado dicho efecto o han observado un aumento concomitante en la excreción de minerales, por lo que la existencia de un beneficio debido al aumento de la absorción no está claro (Gueimonde y cols., 2008).

La encefalopatía hepática, una complicación de la cirrosis hepática, es una enfermedad que afecta al sistema nervioso central debido al incremento de sustancias tóxicas, como el amoniaco, en sangre. Algunos prebióticos, como la lactulosa, se utilizan para prevenir y tratar esa encefalopatía (Shukla y cols., 2011).

Pese a que se han realizado diversos estudios, algunos con resultados positivos, se considera que aún existen pocas evidencias científicas sobre el efecto de la suplementación con prebióticos sobre las alergias en niños (Roberfroid y cols., 2010).

En cuanto al uso de prebióticos en la EII, hay algunos estudios que han indicado que podrían ser potencialmente útiles para aliviar la inflamación, si bien la evidencia a este respecto es insuficiente (Lindsay y cols., 2006; Peña, 2007; Langen y cols., 2009). De modo similar, aún hay pocos trabajos donde se haya observado que los prebióticos tengan un efecto protector en la diarrea del viajero, en la relacionada con el consumo de antibióticos (Corzo y cols., 2015) o en la diarrea asociada a *C. difficile* (Lewis y cols., 2005).

Algunos autores han descrito que los prebióticos podrían ser útiles para disminuir la inflamación asociada al síndrome del intestino irritable (Paineau y cols., 2008; Silk y cols., 2009; Pandey y cols., 2015); sin embargo, otros autores han sugerido que no está claro que los prebióticos sean adecuados para este tratamiento y la reducción de la sintomatología de la enfermedad, ya que los gases que se producen al ser fermentados podrían empeorar la sintomatología (Corzo y cols., 2015); pero estos efectos deben ser comprobados en estudios de más duración y tamaño muestral que los realizados hasta la fecha.

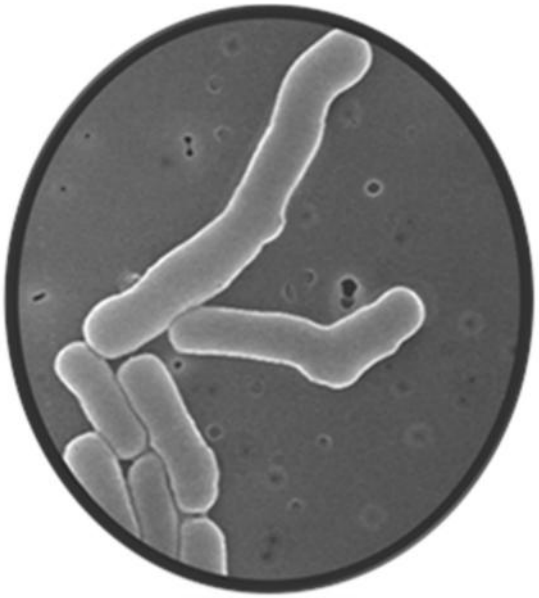
Los prebióticos podrían tener un efecto beneficioso en la prevención de enfermedades como diabetes, obesidad y ateroesclerosis, ya que se ha indicado que reducen los niveles en sangre de glucosa, colesterol, triglicéridos y fosfolípidos, así como la síntesis de triglicéridos y ácidos grasos en el hígado (Corzo y cols., 2015).

Con respecto a la prevención de la carcinogénesis, existen estudios que han descrito un efecto beneficioso potencial de los prebióticos; sin embargo, se requieren estudios más rigurosos para confirmar dicho efecto (Corzo y cols., 2015; Pandey y cols., 2015).

1.5.5. Probióticos y prebióticos en productos desarrollados para la tercera edad

En varios estudios clínicos llevados a cabo en ancianos se ha observado que el consumo de alimentos probióticos y prebióticos tiene un efecto modulador de la microbiota intestinal y del sistema inmunológico (Gill y cols., 2001; Bouhnik y cols., 2007; Fukushima y cols., 2007; Schiffrin y cols., 2007; Takeda y Okumura, 2007; Ibrahim y cols., 2010; Tiihonen y cols., 2010; Forssten y cols., 2011). Sin embargo, en estos trabajos no se han tenido en cuenta las necesidades específicas de esta población, en términos de modulación de la microbiota intestinal, a la hora de seleccionar el probiótico o el prebiótico a utilizar. El desarrollo de alimentos dirigidos a la modulación de la microbiota de personas de edad avanzada, actualmente inexistentes en el mercado, requiere la identificación de las alteraciones específicas presentes en la microbiota de este grupo de población para, a partir de éstas, definir unos objetivos concretos para la modulación de la misma. Estos objetivos tienen que

ser tenidos en cuenta para llevar a cabo una selección racional de microorganismos probióticos y/o sustratos prebióticos específicos para personas de edad avanzada (Arboleya y cols., 2012). Una vez seleccionados, éstos deberían ser utilizados para su inclusión en alimentos funcionales, de composición nutricional adecuada, formulados en función de las necesidades y hábitos específicos de las personas de la tercera edad.



2- OBJETIVOS

En prácticamente todos los países del mundo, a lo largo de las últimas décadas, está teniendo lugar un incremento tanto del número como de la proporción de personas de edad avanzada, es decir, se está produciendo un proceso de envejecimiento de la población. Al mismo tiempo, está aumentando la prevalencia de las enfermedades asociadas a la vejez, como los trastornos gastrointestinales, lo cual tiene importantes repercusiones económicas (incremento del gasto sanitario de los diferentes países) y sociales.

Este envejecimiento se ha asociado con cambios fisiológicos en el tracto gastrointestinal, en la alimentación, en el estilo de vida y en la funcionalidad del sistema inmunológico (inmunosenescencia). Estas variaciones, inevitablemente, afectan a la comunidad microbiana del intestino. La microbiota intestinal juega un papel importante en la salud del individuo y, por lo tanto, alteraciones en la composición y función de la misma pueden dar lugar a una mayor susceptibilidad a sufrir afecciones gastrointestinales, como la infección asociada a *C. difficile* (IACD). Esta infección afecta fundamentalmente a personas de edad avanzada y, además, es precisamente en este grupo poblacional donde tienen lugar infecciones de carácter más severo, mayores complicaciones médicas, mayor riesgo de recurrencia y de mortalidad.

Una estrategia para contrarrestar los cambios en la microbiota intestinal asociados a la vejez, que permitiría reducir los gastos sanitarios generados por el envejecimiento y mejorar la calidad de vida de esta población, sería el desarrollo de alimentos funcionales específicamente dirigidos a la modulación de la microbiota intestinal de personas de edad avanzada, los cuales no están disponibles en el mercado actualmente.

Distintos estudios de intervención han demostrado el efecto modulador de la microbiota intestinal y del sistema inmunológico que tiene el consumo de alimentos funcionales probióticos, prebióticos o simbióticos en ancianos. Sin embargo, en la mayoría de los casos, para la selección de cepas y/o sustratos no se ha tenido en cuenta las necesidades específicas de este grupo de población, en términos de modulación de la microbiota.

En este contexto, en la presente Tesis Doctoral nos planteamos identificar *in vitro* cepas potencialmente probióticas, sustratos prebióticos y combinaciones de ambos (simbióticos), adecuadas para el desarrollo de alimentos funcionales dirigidos a modular la microbiota intestinal de personas de edad avanzada, así como, para la reducción del crecimiento y de la toxicidad de *C. difficile*. Para ello establecimos dos objetivos principales:

OBJETIVO 1: Modular la microbiota intestinal de personas de edad avanzada mediante cepas potencialmente probióticas, sustratos prebióticos y combinaciones de ambos con características adecuadas.

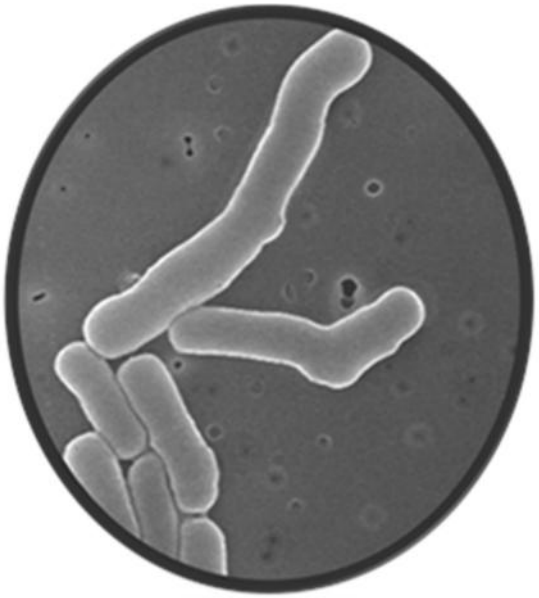
El trabajo realizado para llevar a cabo este objetivo se engloba en el primer capítulo de la Tesis:

CAPITULO 1: Selección de probióticos, prebióticos y simbióticos adecuados para la modulación de la microbiota intestinal de personas de edad avanzada.

OBJETIVO 2: Reducir el crecimiento y toxicidad de *C. difficile* mediante cepas potencialmente probióticas, sustratos prebióticos y combinaciones de ambos con características adecuadas.

El trabajo experimental llevado a cabo en este objetivo se recoge en el segundo capítulo de la Tesis:

CAPITULO 2: Selección de probióticos, prebióticos y simbióticos frente a *Clostridium difficile*.



3- TRABAJO EXPERIMENTAL

CAPÍTULO 1

Selección de probióticos, prebióticos y simbióticos para la modulación de la microbiota intestinal de personas de edad avanzada.

La primera parte de esta Tesis Doctoral se centró en identificar, *in vitro,* cepas potencialmente probióticas, sustratos prebióticos y combinaciones de ambos, adecuadas para el desarrollo futuro de alimentos funcionales dirigidos a modular la comunidad microbiana presente en el intestino de personas de edad avanzada.

El primer paso fue definir unos objetivos concretos para la modificación de los microorganismos intestinales a partir de alteraciones específicas previamente identificadas en la microbiota de ancianos de nuestra población. Estos objetivos fueron utilizados como criterios de selección para llevar a cabo una búsqueda *in vitro* de cepas potencialmente probióticas y de sustratos prebióticos, específicamente dirigidos a la modulación de la microbiota. El estudio se realizó utilizando un modelo de cultivos fecales *in vitro* en discontinuo, a pH libre, con heces de ancianos de nuestra población. Mediante PCR cuantitativa se determinaron los efectos de las cepas/prebióticos sobre los niveles de diversos grupos microbianos de la microbiota intestinal y, mediante cromatografía de gases, se evaluó su función cuantificando la concentración fecal de AGCCs.

El siguiente paso fue estudiar si las cepas elegidas, por su perfil adecuado para modificar la microbiota intestinal en ancianos, eran capaces de utilizar *in vitro* los sustratos prebióticos seleccionados con el mismo fin, para, posteriormente, determinar el potencial simbiótico de las diferentes combinaciones probiótico-prebiótico. En los distintos cultivos discontinuos a pH libre, se determinaron los niveles de las cepas por recuentos en placa; se midió el pH, y la concentración de ácidos orgánicos fue estimada por cromatografía líquida (HPLC).

Los resultados correspondientes a este capítulo 1 se presentan en los siguientes artículos:

- Artículo 1. **Valdés L.**, Salazar N., González S., Arboleya S., Ríos-Covian D., Genovés S., Ramón D., de los Reyes-Gavilán C.G., Ruas-Madiedo P. y Gueimonde M. (2016). Selection of potential probiotic bifidobacteria and prebiotics for elderly by using *in vitro* faecal batch cultures. European Food Research and Technology, en prensa ("online" doi: 10.1007/s00217-016-2732-y).

- Artículo 2. **Valdés-Varela L.**, Ruas-Madiedo P. y Gueimonde M. (2016). *In vitro* fermentation of different fructo-oligosaccharides by *Bifidobacterium* strains for the selection of synbiotic combinations. (Manuscrito enviado al International Journal of Food Microbiology).

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ORIGINAL PAPER

Selection of potential probiotic bifidobacteria and prebiotics for elderly by using in vitro faecal batch cultures

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Abstract The gut microbiota plays an important role in host health. The ageing process affects this microbial com-munity, and therefore, the use of functional foods to restore the microbiota of elderly constitutes an interesting strategy. To this end, probiotics and prebiotics targeted at correcting the specific microbiota alterations occurring during senes-cence are needed. We performed an in vitro selection of bifidobacterial strains and prebiotic substrates on the basis of their ability to counterbalance the specific microbiota aberrancies previously identified in the elderly population. Batch cultures of faeces from elderly were carried out add-ing different strains of *Bifidobacterium* or prebiotics. The effects of these strains/prebiotics upon gut microbiota were assessed by quantitative PCR and the concentrations of short chain fatty acids determined by gas chromatography-FID/MS. The target-specific selection process applied in this study allowed the preliminary selection of two *Bifi-dobacterium* strains and a prebiotic fructooligosaccharide on the basis of their specific properties for the modulation of the microbiota of elders. Overall, this study identifies potentially probiotic strains and prebiotic substrates for the development of functional foods specifically targeted to the senior population.

Keywords Probiotics · Microbiota · *Bifidobacterium* · Fructooligosaccharides · Inulin · Elder

Introduction

The rise on life expectancy and the resulting increase in the proportion of senior citizens represent a challenge for our society [1]. Extending the period of good health in this population group would increase quality of life, reducing the ageing-associated care costs. In this way, the develop-ment of functional foods specifically targeted to the elderly population may constitute a useful strategy of social and commercial interest [2].

Recent studies have underlined the important role of the intestinal microbiota in the maintenance of host's health. The ageing process is known to affect the compo-sition of this microbial community [3–6], although dif-ferences among individuals from different countries have been reported [7]. In a previous study, we observed that, when compared to middle-age adults, Spanish elderly har-bour significantly lower levels of *Bacteroides*, *Clostridium* XIVa (*Blautia coccoides* group) and *Faecalibacterium prausnitzii*, a trend towards reduced levels of bifidobac-teria and increased numbers of *Lactobacillus* and *Akker-mansia* [8]. Reduced levels of *Bacteroides* and *Clostrid-ium* XIVa in elderly have been repeatedly observed [3–5, 9–11]. Regarding lactobacilli, the information available is unclear; in some cases, a reduction in this microbial group with senescence has been found [12, 13] whilst in other cases an increase has been communicated [5, 10]. Several

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studies have also reported reduced levels of bifidobacte-ria in elderly people [12] although this reduction did not always reach statistical significance [3, 14].

As a consequence of these microbiota alterations, the faecal short chain fatty acids (SCFA) profile of elderly is also modified. A decrease in faecal total SCFA levels in samples from elderly has often been reported [4, 8] which has been related to a change from a predominantly sac-charolytic towards a predominantly putrefactive microbial metabolism [13]. Moreover, not only the concentration but also the ratios among different faecal SCFA have been found to be affected, with elderly displaying a higher faecal acetate/propionate ratio [8].

In this context, the use of probiotics and prebiotics could be an affordable strategy for microbiota modulation, and it represents a promising option for the development of spe-cific foods to promote a healthy ageing. Several human intervention studies have reported the modulation of the intestinal microbiota and the immune system in elderly by foods containing pro- and prebiotics [6, 15–21]. However, in most cases, the specific needs of the elder population in terms of microbiota modulation have not been considered for the selection of the better suited strains and compounds. Such an approach would require defining the specific tar-gets in the elderly microbiota for the rational selection of pro-/prebiotics for this population group [2]. Moreover, recent studies underline the high inter-individual variabil-ity existing in the response to probiotics [22–24] and the importance of a target-specific selection [25].

In this work, we consider previously identified alterations in the microbiota of elderly [8] as targets for microbiota modulation. These targets were used in the selection of prebiotic compounds and probiotic strains for the development of functional foods for elderly. In this context, the used selection criteria included the ability to increase the levels of *Bacteroides*, *B. coccoides*, *Bifidobacterium*, *Faecalibacterium* and SCFA and to decrease the acetate/propionate ratio. A batch faecal culture model was used for the initial screening and selection of the best suited prebiotics and *Bifidobacterium* strains for the modulation of the elderly gut microbiota.

Materials and methods

Strains and culture conditions

Sixteen *Bifidobacterium* strains (Table 1) from frozen stocks were reactivated in MRS broth (Difco, Becton Dickinson and Company, Le Pont de Claix, France) supplemented with 0.25 % (w/v) L-cysteine (MRSc; Sigma Chemical Co., St. Louis, MO) by overnight incubation at 37 °C in an anaerobic chamber MG500 (Don Whitley Scientific, West York-shire, UK) under 80 % (v/v) N_2, 10 % (v/v) CO_2 and 10 % (v/v) H_2 atmosphere. Bacterial inocula for the faecal culture experiments were prepared by grow-ing the strains overnight at 37 °C in MRSc under anaerobic conditions, collecting cells by centrifugation and suspend-ing them in the carbohydrate-free medium used for faecal cultures (see below). These sixteen strains were preselected from a collection, including over 50 bifidobacterial strains, on the basis of previous data obtained by our group on acid and bile tolerance and adhesion to human epithelial cells (unpublished data).

Faecal batch culture fermentations

Faecal samples were obtained from six healthy institutionalized elderly who had not taken antibiotics during the 6 months previous to the study and had not ingested probiotic/prebiotic products during the previous month. The Clinical Ethical Committee of Asturias approved the study, and a written informed consent was obtained from the faecal sample donors. Samples were collected and immediately introduced into anaerobic jars (Anaerocult A System, Merck, Darmstadt, Germany) for transportation to the laboratory within 1 h after collection. A 1/10 (w/v) dilution was made in pre-reduced PBS solution and homogenized in a Lab-Blender 400 stomacher (Seward Medical, London, UK) for 2 min. For the in vitro faecal batch cultures, the homogenized faecal mixtures were added (10 %, v/v) to a carbohydrate-free basal medium (CFBM) as previously described [26] and stabilized by keeping at 37 °C overnight under anaerobic conditions.

Six independent faecal batch fermentations, each of them corresponding to a faecal sample from a different donor, were carried out in CFBM. Three of these faecal cultures were used for the prebiotic study (donors of 79, 83 and 87 years of age), and the other three for the assessment of the *Bifidobacterium* strains (76, 80 and 90 years old). For testing prebiotics, overnight stabilized faecal mixtures in CFBM were added with 0.3 % (w/v) of different commercial prebiotic substrates (Table 1). One additional tube was kept without adding carbon source and used as a negative control. With regard to the assessment of the *Bifidobacterium* strains, the stabilized faecal mixtures in CFBM were supplemented with sterilized (by autoclaving) porcine mucin (1 g/l; Sigma) and starch (3 g/l; Sigma) and distributed into glass tubes. Tubes were then inoculated with the different bifidobacteria strains (Table 1) at a final level of 1×10^6 cfu/ml, and one tube was kept without adding bifidobacteria (negative control).

Faecal cultures were carried out in anaerobic chamber MG500 (Don Whitley Scientific) at 37 °C for 24 h. Samples were taken at time 0 and after 24 h of incubation. At each sampling point, one millilitre of each culture was

Table 1 Potentially probiotic bacterial strains and prebiotic substrates used in the faecal batch cultures

Species	Strain	Origin
Potentially probiotic strains		
Bifidobacterium animalis ssp. *lactis*	Bb12	Human faeces
	IPLA 20030	Commercial dairy culture
	IPLA 20031	Bile-adapted from IPLA 20030
	IPLA 20032	Derivative from IPLA 20031
	IPLA 20020	Human faeces
Bifidobacterium longum	IPLA 20001	Human milk
	IPLA 20002	Human milk
	IPLA 20003	Human milk
	IPLA 20021	Human faeces
	IPLA 20022	Human faeces
Bifidobacterium breve	IPLA 20004	Human milk
	IPLA 20005	Human milk
	IPLA 20006	Human milk
Bifidobacterium pseudocatenulatum	IPLA 20026	Human faeces
Bifidobacterium bifidum	IPLA 20015	Human faeces
	IPLA 20017	Human faeces

Commercial name	Composition	References
Prebiotic substrates		
FOS-P95 (Beneo-Orafti, Barcelona, Spain)	95 % FOS (DP: 2–10), 5 % glucose, fructose and sucrose	[37]
ScFOS Actilight 950P (Beghin Meiji, Marckolsheim, France)	95 % FOS (DP: 2–5), 5 % glucose, fructose and sucrose	[37]
Synergy 1(Beneo-Orafti, Barcelona, Spain)	92 % FOS/Inulin mixture, 8 % glucose, fructose and sucrose	[37]
Inulin (from dahlia tubers; Sigma-Aldrich)	FOS (Average DP: 36)	–

DP degree of polymerization

centrifuged (16,000×*g* for 10 min), and pellets and supernatants were collected and immediately frozen at −20 °C until their use.

pH measurement

Determination of pH in faecal culture supernatants was car-ried out by direct measurement with a pH meter Basic 20+ (Crison Instruments SA, Barcelona, Spain).

Quantification of intestinal microbial groups by qPCR

The DNA was extracted from pellets of faecal batch cultures using the QIAamp DNA stool kit (Qiagen, GmbH, Hilden, Germany), following the manufacturer's instructions. Purified DNA samples were stored at −20 °C until use.

The primers and conditions indicated in Table 2 were used for quantification of the different bacterial populations analysed by qPCR. All reactions were performed on MicroAmp optical plates sealed with MicroAmp optical caps (Applied Biosystems, Foster City, CA, USA) with a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems). One millilitre of template faecal DNA was used in the 25 µl of the PCR mixture. Thermal cycling consisted of an initial step of 95 °C 10 min, followed by 40 cycles of 95 °C 15 s and 1 min at the corresponding annealing temperature (Table 2). Standard curves were performed with pure cultures of appropriate strains (Table 2) grown overnight in GAM medium (Nissui Pharmaceutical Co, Tokyo, Japan) under anaerobic conditions. For *F. prausnitzii* DSMZ 17677, the microorganism was grown anaerobically in RCM formula (Oxoid) without agar. Samples were analysed in duplicate in at least two independent qPCR runs.

Analysis of SCFA by gas chromatography (GC)-FID/MS

Cell-free supernatants from faecal batch cultures, filtered through 0.2 µm filters, were used for the quantification of SCFA by GC. A chromatographic system composed of a 6890 N GC injection module (Agilent Technologies Inc., Palo Alto, Ca, USA) connected to a flame injection detector (FID) and a mass spectrometry (MS) 5973 N detector

Table 2 Primers and annealing temperatures used for quantification of intestinal microbial groups by qPCR

Target	Strain used for standard curves	Primer sequence (5′–3′)	Tm. (°C)	References
Blautia coccoides-Eubacterium rectale group	*Blautia coccoides* DSMZ935	F: CGGTACCTGACTAAGAAGC R: AGTTTYATTCTTGCGAACG	55	[32]
Bacteroides-Prevotella	*Bacteroides thetaiotaomicron* DSMZ2079	F: GAGAGGAAGGTCCCCCAC R: CGCKACTTGGCTGGTTCAG	60	[32]
Lactobacillus-Weissella group	*Lactobacillus gasseri* IPLA20126	F: AGCAGTAGGGAATCTTCCA R: CATGGAGTTCCACTGTCCTC	60	[32]
Faecalibacterium prausnitzii	*Faecalibacterium prausnitzii* DSMZ17677	F: GGAGGAAGAAGGTCTTCGG R: AATTCCGCCTACCTCTGCACT	60	[28]
Bifidobacterium	*Bifidobacterium longum* NCIMB8809	F: GATTCTGGCTCAGGATGAACGC R: CTGATAGGACGCGACCCCAT	60	[38]
Akkermansia	*Akkermansia muciniphila* CIP 107961	F: CAGCACGTGAAGGTGGGGAC R: CCTTGCGGTTGGCTTCAGAT	60	[38]

(Agilent) was used for quantification and identification of SCFA as described previously [27].

Statistical analyses

When appropriate, one-way analysis of variance (ANOVA) was performed using the IBM SPSS Statistics for Windows version 22.0 (IBM Corp, Armonk, NY, USA). Data were expressed as mean ± standard deviation (SD) of the three experiments. The conventional probability value (0.05) for significance was used in the interpretation of results.

Results

We used faecal cultures from elders for assessing the impact of the addition of different prebiotics or bifidobacterial strains upon some intestinal microbial groups and the faecal SCFA profile. In this sort of studies, a high inter-individual variability is often present; therefore, in order to facilitate the interpretation of the data, the results of the faecal cultures from each individual were calculated as increments with respect to the corresponding time zero value (24 h). Then, the results were compared with its negative control without bifidobacteria or prebiotic added and reported as fold change (FC) with regard to this negative control. The results obtained varied depending on both the compound/strain tested and the intestinal microbial group analysed. These differences, however, did not reach statistical significance, likely due to the high inter-individual variability in the intestinal microbiota composition. In spite of this, specific patterns were observed for some strains/compounds.

Prebiotic substrates

After 24 h of incubation, all prebiotic substrates pro-moted a significant decrease ($p < 0.05$) in the initial pH (7.11 ± 0.10) of the faecal cultures, with final pH ranging from 4.46 to 4.77 (no statistically significant differences among prebiotics) whilst such reduction was not observed for the control without carbon source added (final pH 6.97 ± 0.12).

The characteristics of the basal microbiota varied largely among donors. *Akkermansia* was not detected in the faeces from one donor (Donor 1), whereas in the other two donors the initial levels ($t = 0$) were clearly different (Table 3). For the other microbial groups analysed, the basal levels on the different donors were not as different among them as for *Akkermansia*, but still showed variability. The prebi-otics tested displayed limited effects upon most of the bacterial groups analysed, with the exception of the *Lac-tobacillus* group. The levels of *Akkermansia* in the faecal cultures from the two positive donors for this microorgan-ism slightly decreased with prebiotics as compared with the negative control culture (Table 3) (fold changes FCs rang-ing between −0.1 and −3.6, depending on the donor and the prebiotic). In contrast, a stimulation of the growth of *Lactobacillus* group, known lactic acid producers, with all the prebiotics tested was found (Table 3), which correlates well with the observed drop in pH. For the other micro-bial groups studied, the impact of the prebiotics was less pronounced.

Taking into account the specific selection criteria proposed, the most consistent increases in the levels of SCFA were promoted by Inulin and Actilight, whilst FOS-95 was the prebiotic inducing the highest decrease in the

Table 3 Initial bacterial levels (T = 0; Log cells/g) and increases/decreases with respect to T = 0 obtained for the different microbial populations analysed after 24 h of incubation (24 h; Log cells/g) with the different prebiotic substrates in three independent faecal batches

Donor	*Akkermansia*			*Bacteroides* group			*B. coccoides* group		
	1	2	3	1	2	3	1	2	3
T = 0 24 h (FC)	nd	7.79	6.44	10.13	10.38	9.76	8.90	9.88	8.7
Synergy 1	nd	−0.54 (−3.6)	0.59 (−0.3)	0.26 (1.7)	−0.27 (−2.1)	0.41 (−0.4)	0.69 (2.1)	−0.47 (−4.4)	0.2 (1.7)
FOS 95	nd	−0.24 (−2.1)	0.07 (−0.9)	0.15 (1.4)	0.36 (0.5)	0.15 (−0.8)	2.93 (5.7)	−0.62 (−5.4)	−0.27 (0.0)
Inulin	nd	−0.22 (−2.0)	0.25 (−0.7)	0.12 (1.3)	0.24 (0.0)	0.26 (−0.6)	0.46 (1.7)	−0.1 (−1.7)	3.35 (13.4)
Actilight	nd	−0.14 (−1.7)	0.80 (−0.1)	0.79 (3.2)	0.31 (0.3)	0.72 (0.1)	−3.90 (−5.3)	−0.31 (−3.2)	−0.35 (−0.3)

Donor	*Bifidobacterium*			*Lactobacillus* group			*F. prausnitzii*		
	1	2	3	1	2	3	1	2	3
T = 0 24 h (FC)	10.29	8.80	9.64	7.62	8.81	8.36	7.79	8.44	7.76
Synergy 1	0.08 (1.5)	−0.15 (−1.7)	0.80 (0.4)	2.51 (23.8)	0.26 (1.2)	2.28 (75.0)	0.07 (1.2)	−0.49 (−3.1)	0.38 (−0.2)
FOS 95	0.01 (1.1)	−0.02 (−1.1)	0.62 (0.1)	4.44 (41.4)	0.36 (2.0)	2.11 (69.3)	0.49 (2.4)	0.08 (1.7)	0.22 (−0.5)
Inulin	0.21 (2.2)	0.09 (−0.6)	0.56 (−0.1)	1.97 (18.9)	0.13 (0.1)	1.49 (48.7)	0.14 (1.4)	−0.19 (−0.6)	0.22 (−0.5)
Actilight	0.45 (3.6)	0.33 (0.6)	0.78 (0.3)	0.62 (6.6)	0.53 (3.4)	2.53 (83.3)	0.75 (3.1)	0.01 (1.1)	0.81 (0.7)

The fold changes (FC) with respect to the negative control without prebiotic added are indicated between brackets *Nd* non-detected

Table 4 Initial concentrations (mM) of acetate, propionate and butyrate, as well as the sum of the three of them (Total SCFA) and the acetate-to-propionate ratio, in the initial (T = 0) samples from the faecal cultures of the three donors

Donor	*Acetate*			*Propionate*			*Butyrate*		
	1	2	3	1	2	3	1	2	3
T = 0 24 h (FC)	18.67	18.29	16.16	3.25	3.10	2.51	2.27	2.32	2.31
Synergy 1	15.3 (1.2)	8.84 (2.0)	0.91 (−0.9)	1.09 (0.3)	3.81 (1.2)	0.70 (−0.4)	0.62 (−0.3)	1.78 (3.0)	0.53 (−0.4)
FOS 95	7.8 (0.1)	1.36 (−0,5)	−3.18 (−1.3)	0.61 (−0.3)	3.48 (1.0)	0.45 (−0.6)	0.64 (−0.2)	1.37 (2.1)	0.55 (−0.4)
Inulin	15.2 (1.2)	8.36 (1.8)	11.91 (0.0)	1.01 (0.2)	3.85 (1.2)	1.40 (0.1)	0.54 (−0.3)	1.68 (2.8)	1.12 (0.3)
Actilight	9.3 (0.4)	5.63 (0.9)	16.7 (0.4)	1.14 (0.4)	2.91 (0.7)	1.92 (0.6)	0.91 (0.1)	0.95 (1.2)	1.59 (0.8)

Donor	Total SCFA			*A/P* ratio		
	1	2	3	1	2	3
T = 0 24 h (FC)	24.19	23.7	20.98	5.75	5.97	6.44
Synergy 1	17.04 (1.0)	14.44 (1.8)	2.15 (−0.8)	2.06 (2.7)	−2.48 (−1.0)	−1.16 (−1.9)
FOS 95	9.02 (0.1)	6.21 (0.2)	−2.18 (−1.2)	0.99 (0.8)	−4.08 (−2.3)	−2.33 (−2.8)
Inulin	16.71 (1.0)	13.89 (1.7)	14.42 (0.1)	2.27 (3.1)	−1.72 (−0.4)	0.85 (−0.3)
Actilight	11.31 (0.3)	9.49 (0.8)	20.26 (0.4)	0.66 (0.2)	−1.67 (−0.4)	1.17 (−0.1)

Increases/decreases with regard to T = 0 obtained for the different SCFA analysed after 24 h of incubation (24 h, mM) with the different prebiotic substrates. The fold changes (FC) with respect to the negative control without prebiotic added are indicated between brackets

acetate-to-propionate ratio (Table 4). With regard to the microbiota modulation, none of the prebiotic substrates consistently increased the levels of *B. coccoides* group, whilst Actilight was the prebiotic showing better potential for increasing the levels of *Bacteroides*, *Faecalibacterium* and *Bifidobacterium*.

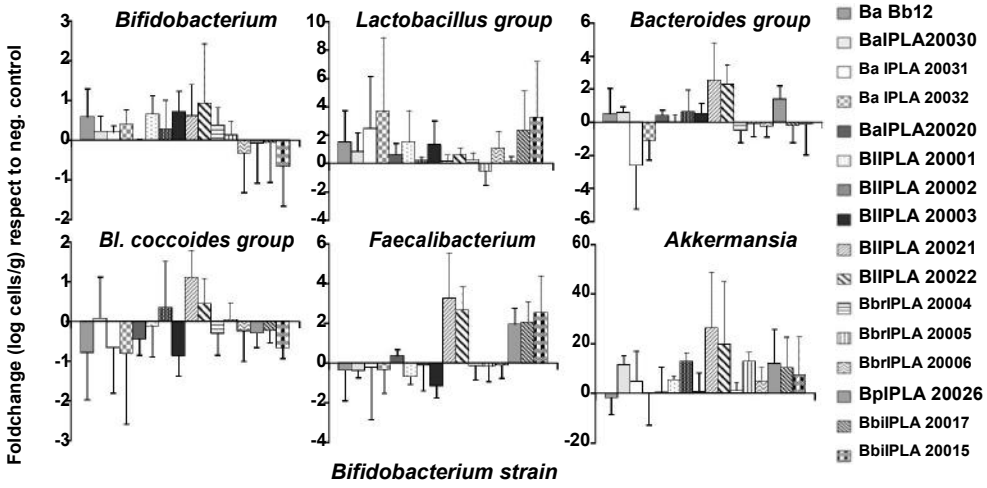

Fig. 1 Fold changes with regard to the negative control (without bifidobacteria added) obtained for the different microbial populations analysed by qPCR after 24 h of incubation of the faecal cultures with different bifidobacterial strains added. Results are presented as the mean ± SD of the results obtained in the faecal cultures of the three different donors. Ba, *Bifidobacterium animalis* subsp. *lactis*; Bl, *Bifidobacterium longum*, Bbr, *Bifidobacterium breve*; Bp, *Bifidobacterium pseudocatenulatum*; Bbi, *Bifidobacterium bifidum*

Potentially probiotic *Bifidobacterium* strains

The pH values dropped significantly ($p < 0.05$) in all faecal cultures (from initial pH of 7.00 ± 0.09 to values between 4.87 and 4.92 after 24 h incubation), and no statistically significant differences in the pH values were obtained between the cultures with the different strains or without bifidobacteria added (negative control).

Differences among donors in the basal levels of the microbial groups analysed were also observed. Initial lev-els of *Akkermansia* ranged between 7.39 (donor 2) and 9.25 (donor 3) Log cells/g, those of *Bifidobacterium* between 8.91 (donor 1) and 10.14 (donor 3) Log cells/g, whilst lev-els of *Lactobacillus* group varied from 7.34 (donor 2) to 9.98 (donor 3). The other bacterial groups analysed showed less inter-individual variability, with *Bacteroides* ranging between 10.17 and 10.39 Log cells/g, *B. coccoides* group between 9.31 and 9.69 and *F. prausnitzii* from 7.59 to 7.98 Log cells/g. In spite of this, clear differences among fae-cal cultures added with the different bifidobacteria strains became evident (Fig. 1, Supplementary File).

Most of the *Bifidobacterium* strains promoted the rise of bifidobacteria counts, with the exception of *B. breve* IPLA 20006, *B. pseudocatenulatum* IPLA 20026 and the two *B. bifidum* strains (IPLA 20015 and 20017) (Fig. 1). The *B. longum* strains IPLA 20022, 20021, 20003 and 20001 followed by *B. animalis* subsp. *lactis* Bb12 were the microorganisms causing the highest increases in bifidobacteria levels after 24 h of incubation. These increments ranged between 0.71 and 0.86 Log units with regard to time zero values (Supplementary File) representing FC between 0.47 and 0.85 with respect to the negative control (Fig. 1).

Similarly all the strains, with the exception of *B. breve* IPLA 20005, promoted variable increases in the counts of lactobacilli and all, but *B. animalis* subsp. *lactis* Bb12 and *B. animalis* subsp. *lactis* IPLA 20032 strains, in those of *Akkermansia* (Fig. 1). *B. longum* strains IPLA 20021 and IPLA 20022 were the microorganisms promoting the most pronounced increases in *Akkermansia*, which could be mainly attributed to the increment obtained with the faecal culture from donor 2 (almost 2 Log units, Supplementary File) who had low initial levels of this microorganism in faces.

For the other faecal microbial groups analysed, increases or decreases were observed depending on the bifidobacte-rial strain tested and the intestinal microbial groups ana-lysed. Only five of the strains tested were able to increase the levels of *B. coccoides* group, although at variable extent depending on the strain and the faecal donor. These included *B. animalis* subsp. *lactis* IPLA 20030 strain, a *B. breve* strain (IPLA 20005), and three *B. longum* strains (IPLA 20002, 20021 and 20022), the latter two microor-ganisms (*B. longum* IPLA 20021 and IPLA 20022) being the ones inducing the highest increases (Fig. 1). Similarly, these two last microorganisms and the strain *B. pseudo-catenulatum* IPLA 20026 promoted the largest rises in the counts of *Bacteroides*, with over 2 FC increase with respect to that found in the negative control without bifidobacteria added (Fig. 1). With regard to *F. prausnitzii*, six bifidobac-terial strains enhanced its growth; again *B. longum* strains IPLA 20021 and 20022 being the best performing ones. Thus, *B. longum* IPLA 20021 and 20022 were the strains better fulfilling the selection criteria established in terms of elderly microbiota modulation.

Regarding the effects of the *Bifidobacterium* strains tested upon the production of SCFA or the acetate-to-propionate ratio, these were scarce. Actually, FC values with respect to the negative control higher than 1 were rarely observed (data not shown).

Discussion

Previous reports indicate specific microbiota alterations related to the senescence process [3–5, 8]. In this study, we aim at selecting prebiotic compounds and bifidobacte-ria strains for the development of functional foods specifically designed to counterbalance those alterations. In spite of the high inter-individual variability, our results point out to differences among the prebiotic substrates and the bifidobacterial strains tested, in their ability to modulate the elderly intestinal microbiota in vitro. The prebiotic fructooligosaccharides tested (Synergy, FOS-95, Actilight and inulin), independently on their polymerization degree, were fermented to organic acids by the gut microbiota. In con-trast to prebiotics, and in spite of the differences observed among the different *Bifidobacterium* strains, no statistically significant differences were found in the final pH values among the different cultures with or without bifidobacte-ria added. This fact also correlated with the low variability detected in the total SCFA production following incubation with bifidobacteria.

With regard to the potential for microbiota modula-tion in the elderly population, previous qPCR-based stud-ies allowed us to define targets for the selection of specific prebiotics and probiotics for elderly [8]. These included the ability to increase *Bacteroides*, *B. coccoides*, *Bifi-dobacterium, Faecalibacterium* and total SCFA and to decrease acetate/propionate ratio. We identified Actilight as the best suited prebiotic for microbiota modulation in the elderly, being the compound promoting the most con-sistent increases in the levels of *Bacteroides*, *Faecalibac-terium* and *Bifidobacterium* in the three donors. Human intervention studies carried out with adults have reported increases in intestinal populations, such as those of bifido-bacteria and *F. prausinitzii*, following inulin consumption [28]. In our elderly-faecal culture model, Actilight was the prebiotic providing the most consistent results, with higher increases than those observed for inulin. Regarding the bifidobacterial strains, *B. longum* IPLA 20021 and IPLA 20022 showed the most promising microbiota modulatory profile. These strains display the most notable increases in the levels of *Bacteroides* and *F. prausnitzii* and behav-ing also well in terms of effects on *Bifidobacterium* and *B. coccoides* levels. We did not observe any remarkable differ-ences among prebiotics or strains on the profile of SCFA. This fact suggests a limited SCFA production ability by our faecal cultures, which can be due to the reduced SCFA pro-duction capability of the elderly microbiota [4, 8, 13].

Faecal culture models have been widely used in prebiotics research [29–32], and such approach has been recently employed by other authors for the selection of probiotics and prebiotics [33]. In a similar way, we were able to identify some *Bifidobacterium* strains with the ability to promote an increase in the levels of anaerobic microorganisms such as *Bacteroides*, *Bifidobacterium* or *Faecalibacterium* in faecal cultures from elderly. These microbial populations repeatedly reported as reduced at advanced age [3–5, 8] are known as mediators of anti-inflammatory effects and immune system regulators in the intestinal mucosa [34]. Similar faecal culture models have also been employed for the selection of strains with specific properties in terms of modulation of the microbiota in premature newborns [25]. However, it should be pointed out that promising in vitro data do not always translate into clear effects in clinical intervention trials. Actually, human probiotics have often shown only limited microbiota modulation ability [16, 28, 35]. This resilience of the gut microbiota seems to depend on the basal microbiota composition, which may determine the responder or non-responder phenotype towards probiotic intervention in elderly [36].

The fundamental role of the background microbiota in determining the microbiota modulatory capability of probiotics and prebiotics has been reported [22, 33], underlin-ing the importance of a specific target population selec-tion of strains and prebiotics. This highlights the need for strains/compounds with improved capability for microbiota modulation in elders, and it points out an opportunity for developing foods specifically designated for the elderly population. It should be noted, however, that the high inter-individual diversity makes such selection process very challenging. Moreover, the fact that qPCR does not differentiate between active and non-active microorganisms may preclude the detection of differences existing at functional but not at microbial composition level.

In spite of the above-mentioned limitations, the target-specific selection process applied in this study, based on the use of in vitro faecal cultures from elderly donors, seems to be appropriate for the preliminary screening and identifi-cation of prebiotic substrates and suitable probiotic strains for microbiota modulation in elderly. However, these fae-cal culture model results should be considered just part of an initial screening, requiring further in vivo confirmation before drawing firm conclusions.

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Compliance with ethical standards

Conflict of interest All authors disclose any conflict of interest.

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In vitro fermentation of different fructo-oligosaccharides by *Bifidobacterium* strains for the selection of synbiotic combinations

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Abstract

The use of selected probiotics, prebiotics and/or synbiotics, constitute an interesting dietary strategy for intestinal microbiota modulation in case of disbiosis. Species of the genus *Bifidobacterium* are among the most currently used probiotics for human consumption, since they have shown beneficial effects in the prevention and treatment of some disorders. Bifidobacteria are saccharolytic microorganisms, but their ability to use different carbohydrates varies among strains. In this study, we investigate the utilization of three prebiotic substrates (two different short-chain fructo-oligosaccharides [scFOS] and inulin) by strains of *Bifidobacterium,* in order to determine the synbiotic potential of the different probiotic/prebiotic combinations. Batch culture fermentations from six *Bifidobacterium* strains (*Bifidobacterium longum* IPLA20021, *B. longum* IPLA20022, *Bifidobacterium animalis* IPLA20031, *B. animalis* IPLA20032, *B. animalis* IPLA20020 and *B. animalis* Bb12) were carried out in the presence of inulin or scFOS (Synergy or Actilight), or glucose, as carbon source. Bifidobacteria levels were quantified by plate counting. The pH and production of organic acids in the different batch-culture fermentations were also determined. Our results showed that all the studied strains of *B. animalis* and *B. longum* were able to utilize scFOS but not inulin. The use of scFOS as carbon source affected the pattern of metabolite´s production, when compared with cultures carried out in glucose, particularly in the case of *B. longum* . The results indicated that the scFOS are well suited to be used in combination with *B. animalis* or *B. longum* strains for the development of synbiotic foods or food supplements.

Keywords: probiotic, *Bifidobacterium*, prebiotic, fructo-oligosaccharide, synbiotic

1. Introduction

Functional foods are defined as “food that may provide a health benefit beyond the traditional nutrients it contains” (Champagne et al., 2005). Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit to the host” (FAO/WHO 2006). Many studies have focused on using lactobacilli and bifidobacteria as probiotics (Duncan and Flint, 2013; Sims et al., 2014). Some strains of *Bifidobacterium*, a natural inhabitant of the gastrointestinal tract (Lugli et al., 2014), are among the most frequently commercialized probiotics for human consumption, dairy products being the most common vehicle

(Prasanna et al., 2014). These bacteria are able to breakdown complex carbohydrates and oligosaccharides releasing monosaccharides that are metabolized by the so-called fructose 6-phosphate phosphoketolase pathway or "bifid shunt"; the most abundant end metabolites of this pathway being acetate and lactate (González-Rodríguez et al., 2013a). On the other hand, prebiotics have been defined as "a selectively fermented ingredient that results in specific changes in the composition and /or activity of the gastrointestinal microbiota thus conferring benefit(s) upon host health" (Gibson et al., 2010). The most common prebiotics used in humans are fructo-oligosaccharides (FOS) of different polymerization degrees (Guerreiro et al., 2016), either obtained from vegetables by hydrolysis or enzymatically synthesized (Flores-Maltos et al., 2014). They consist of short and medium chains of fructose units, with a terminal glucose moiety, linked by β-(2-1) glycosidic bonds. These molecules cannot be hydrolyzed by the mammalian digestive system due to the lack of β-fructosidase activity, therefore remaining undigested and reach the colon where they can be fermented by bifidobacteria and other intestinal microorganisms (Sabater-Molina et al., 2009). A synbiotic is a mixture of probiotic(s) and prebiotic(s). In such combination the prebiotic(s) can favor the growth and improve the survival of the probiotic(s) as well as to benefit the autochthonous bacteria in the gastrointestinal tract (Sims et al., 2014). *In vivo* and in *vitro* studies have shown the positive effects of certain probiotic-prebiotic combinations in humans, in some cases suggesting higher effects than those of products containing only probiotics or prebiotics (Champagne et al., 2005). Thus, the use of probiotics, prebiotics or combinations of them could be an affordable strategy for intestinal microbiota modulation in the case of dysbiosis related with ageing or disease states. However, for identifying the best suitable combination a rational selection process, based on the metabolic properties of the probiotic, should be carried out.

In this study six strains from two *Bifidobacterium* species (*Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium longum*) were cultivated in batch cultures with three different prebiotics or glucose as carbon source. Our final aim was to evaluate the synbiotic potential of various prebiotic/ bifidobacteria combinations.

2. Material and Methods

2.1. Bacterial strains and culture conditions

Five strains of bifidobacteria from IPLA culture collection were used; three of them were originally isolated from breastfed infant's faeces (*B. longum* IPLA20021, *B. longum* IPLA20022 and *B. animalis* subsp. *lactis* IPLA20020) (Solís et al., 2010), whereas *B. animalis* subsp. *lactis* IPLA20031 and IPLA20032 were obtained after adaptation to increasing concentrations of bile salts from a parental strain isolated from a dairy product (Ruas-Madiedo et al., 2010). In addition the widely used commercial probiotic strain *B. animalis* subsp. *lactis* Bb12 was also included.

Strains were routinely grown in MRSC [MRS (Biokar Diagnostics, Beauvois, France) supplemented with 0.25% L-cysteine (Sigma-Chemical Co.,

St. Louis, MO, USA)] at 37ºC in the anaerobic chamber MG500 (Don Whitley Scientific, Yorkshire, UK) under 80% N_2, 10% CO_2 and 10% H_2 atmosphere. Overnight cultures (18 h) were used to prepare the bacterial inocula for the batch culture experiments, which were obtained after collecting cells by centrifugation and suspending them in the same volume of the medium without a carbon source described below.

2.2. Batch culture fermentations

Uncontrolled-pH batch cultures were performed in a carbohydrate-free basal medium (CFBM) previously described (Salazar et al., 2009a). This CFBM contained 2 g/L peptone water (Merck, Darmstadt, Germany), 2 g/L yeast extract (Difco, BD, Biosciences, San Diego, CA), 0.1 g/L sodium chloride (Merck), 0.04 g/L dipotassium phosphate (Merck), 0.04 g/L monopotassium phosphate (Merck), 0.01 g/L magnesium sulphate (Merck), 0.01 g/L hexahydrate calcium chloride (Merck), 2 g/L monosodium carbonate (Merck), 2.5 g/L L-cysteine-HCl (Sigma), 0.5 g/L bile salts (Oxoid Ltd., Basingstoke, Hampshire, UK), 2 mL Tween 80 (Sigma), 0.05 g/L haemin (Sigma) and 10 µL vitamin K1 (Sigma). CFBM were added with 0.3% (w/v) of three different commercial prebiotic substrates: Actilight 950P (Beghin Meiji, Marckolsheim, France: 95% FOS with degree of polymerization (DP) 2-5, 5% glucose, fructose and sucrose), Synergy 1 (Beneo-Orafti, Barcelona, Spain: 92% FOS/Inulin mixture with DP<10, 8% glucose, fructose and sucrose) and inulin (from dahlia tubers; Sigma-Aldrich: FOS average DP 36), or glucose (positive control). Each media was distributed into different tubes; moreover, one additional tube was kept without adding carbon source and used as a negative control. Then, tubes were inoculated with the different bifidobacteria at a 1% v/v. Fermentations were carried out in anaerobic chamber at 37ºC for 72 hours. Samples were collected at fixed incubation times (0, 6, 24 and 72 h) for analyses. All cultures were carried out in triplicate.

2.3. Determination of bacterial growth and pH measurement

At the defined sampling points, 0.1 mL of each culture was taken to prepare decimal dilutions in ringer solution (Merck) which were plated on MRSC agar. Plates were incubated in anaerobic chamber at 37ºC for 48 h and colonies were enumerated. Microbial counts were reported as Log CFU/mL.

Determination of pH in batch cultures was carried out by direct measurement with a pH meter Basic 20+ (Crison Instruments S. A., Barcelona, Spain).

2.4. Analysis of organic acids by HPLC

One mL of each culture collected at the defined sampling points was centrifuged (16000 x g for 10 min), to obtain supernatants which were immediately frozen at -20ºC until their use. These cell-free supernatants were filtered through 0.2 µm-pore-size filters. The quantification of organic acids was carried out in an HPLC chromatographic system composed of an Alliance 2690 module injector, a PDA 996 photodiode array detector, a 2414 differential refractometer detector and the Empower software (Waters, Milford, MA, USA).

Chromatographic conditions were those indicated previously by Salazar and co-workers (Salazar et al., 2009b).

2.5. Statistical analysis

Statistical analysis of the results was performed using the IBM SPSS Statistics for Windows version 22.0 (IBM Corp, Armonk, NY, USA). In each culture, an one-way analysis of variance (ANOVA) followed by SNK (Student-Newman-Keuls, $p<0.05$) was carried out to analyse different parameters by comparing the five carbon sources (FOS, glucose and negative control) within each incubation time.

3. Results and discussion

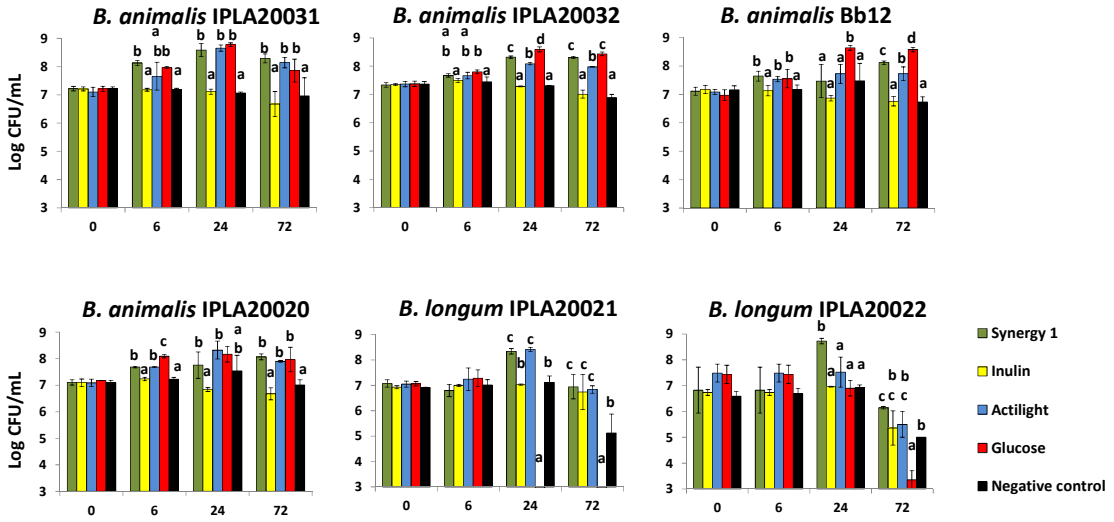
Several studies have reported beneficial effects of the intake of foods containing probiotics, prebiotics or synbiotics in different human populations (Champagne et al., 2005; Gourbeyre et al., 2011; Kaur et al., 2016; Saulnier et al., 2009; Wang, 2009). However, often there has not been a rational selection of the best suited probiotic strains and prebiotic substrates for their combination in a synbiotic product targeted to a specific population group. To this regard the strains and compounds tested in this study have been independently selected on the basis of their activity upon the modulation of the elderly gut microbiota (Valdés et al., submitted).

The saccharolytic ability of probiotics varies depending on the strains and species. *B. animalis* subsp. *lactis* HN019 and *Lactobacillus acidophilus* NCFM were reported to grow on a galacto-oligosaccharide (GOS), whilst *Lactobacillus rhamnosus* HN001 did not (Mäkeläinen et al., 2010). Another study confirmed that the first two strains were able to utilize FOS and inulin (Sims et al., 2014). In the specific case of *Bifidobacterium*, a study carried out with 55 strains found that different FOS (raftilose synergy, raftilose HP and raftilose P95) were fermented by most of the strains whilst only 8 of them grew when inulin was used as the carbon source (Rossi et al., 2005). Falony and co-workers (Falony et al., 2009) further demonstrated that many *Bifidobacterium* strains are not able to degrade inulin. Moreover, it was recently observed that although degradation of inulin-type fructans seems to be similar along human intestine, in general, the mucosa-associated strains isolated from biopsies preferred fructose and oligofructose, while the lumen-associated ones showed a higher degradation degree of inulin (Selak et al., 2016). These reports are in good agreement with our results, showing that the six bifidobacteria strains tested were able to grow or to be metabolically active in glucose and in scFOS (Synergy and Actilight) but not in inulin (Figure 1).

At stationary phase (24 and 72 hours of incubation), we observed that glucose, followed by Actilight and Synergy, promoted the highest growth and/or metabolic activity in all *B. animalis* and *B. longum* strain cultures (Figures 1 and 2). With regard to the *B. animalis* strains studied, when the data obtained were analyzed, we observed higher cell counts ($p<0.05$) in cultures with glucose, Synergy or Actilight, than in those with inulin or without carbon source added (Figure 1). The growth in the prebiotic substrates Actilight and Synergy, as well as in glucose, promoted a significant decrease of the pH values of the cultures (Figure 2). The pH value obtained with Actilight was

lower ($p<0.05$) than that obtained with synergy, however for both scFOS the pH values remained higher ($p<0.05$) than those obtained with glucose as carbon source. These results were

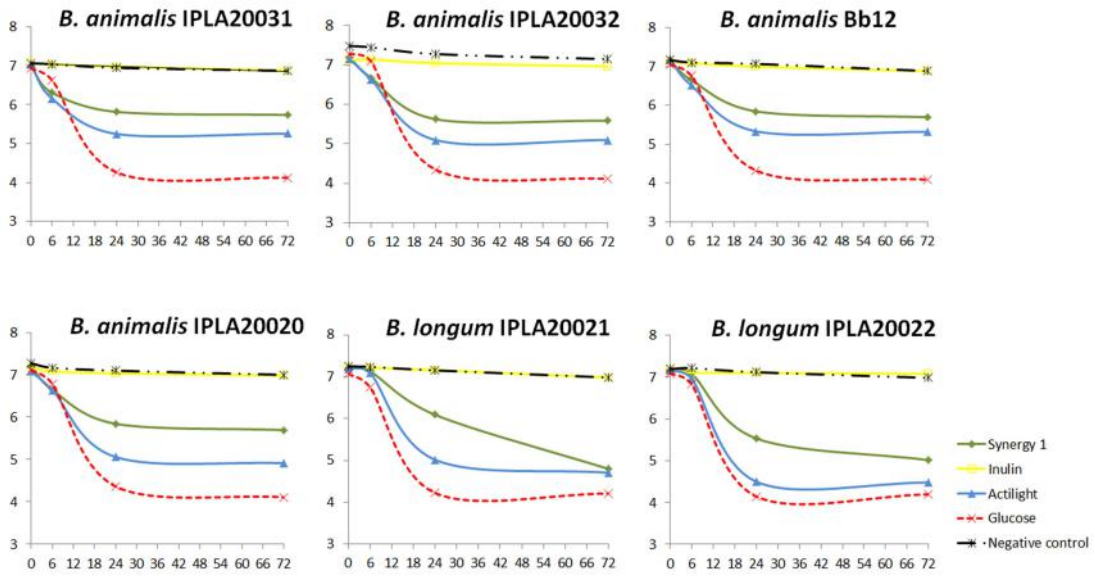
Fig 1. Microbial counts (mean ± SD) obtained at the different incubation times analysed (0, 6, 24 and 72 h) of six *Bifidobacterium* strains grown in batch cultures with different carbon sources. Within the same incubation time, bars that does not share a common letter showed statistically ($p<0.05$) differences among carbon sources.



further confirmed by the corresponding increase in the concentration of total organic acids (Figure 3, supplementary file 1). At long incubation times glucose promoted the highest production of organic acids ($p<0.05$), followed by Actilight and Synergy. In general, significant ($p<0.05$) differences in the production of total organic acids were observed between Actilight and Synergy, being higher in the former. As expected, significantly lower levels ($p<0.05$) of organic acids were observed in the cultures with inulin or without carbon source added. With regard to both *B. longum* strains, they showed highest cell counts ($p<0.05$) with both scFOS, but mainly with Synergy (Figure 1). Interestingly, we found that the cultures of both *B. longum* strains in glucose showed reduced cell counts at long incubation times; however, these bacteria were metabolically active during the first 24 hours of incubation as it was confirmed by continuous decrease in pH and the accumulation of organic acids (Figure 2 and 3). This is likely due to the decrease in the pH values promoted by the faster growth of *B. longum* with glucose as carbon source. It is known that bifidobacteria have a low tolerance to acid, perhaps with the exception of *B. animalis* and related species (González-Rodríguez et al., 2013b), and this low tolerance may explain the observed drop on viability. On the other hand, during exponential phase (after 6 hours of incubation),

Actilight and Synergy promoted higher decreases in the pH values than glucose (Figure 2, supplementary file 2), with a concomitantly higher ($p<0.05$) production of total organic acids in *B. animalis* at that sampling time (supplementary file 1), whilst the contrary was true for the *B. longum* strains (Figure 2, supplementary files 1 and 2). Thus, it may be that *B. animalis* has preference on some oligosaccharides present in scFOS over glucose. To this regard it has been previously reported that some *Bifidobacterium* species preferentially ferment disaccharides or oligosaccharides over monosaccharides (Amaretti et al., 2006, 2007; González-Rodríguez et al., 2013a; Gopal et al., 2001; Parche et al., 2006)

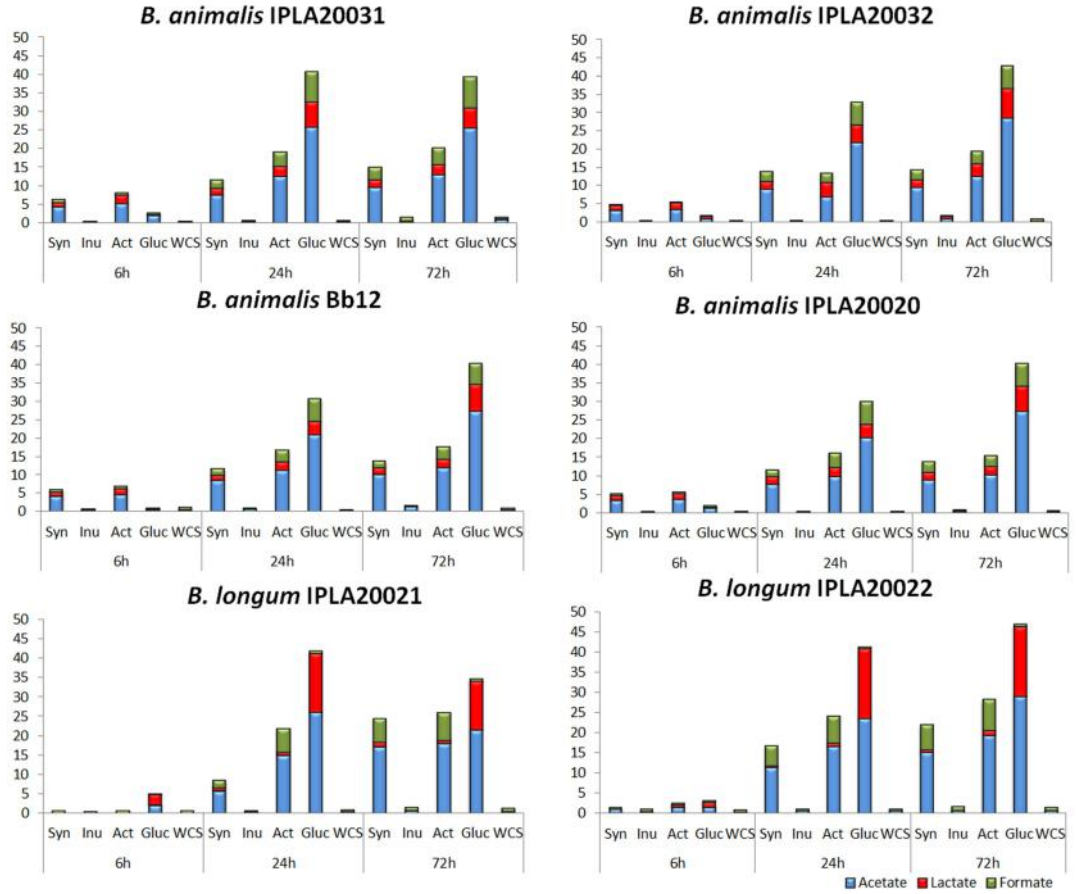
Fig 2. pH values of batch cultures supplemented with different prebiotics obtained at different incubations times (0, 6, 24 and 72 h) during the fermentations with the six *Bifidobacterium* strains. Within each incubation time, asterisks indicate statistically differences ($p<0.05$) among carbon sources. The coefficient of variation (SD/ mean) of this data range between 0 and 13.37%.



Bifidobacteria ferment carbohydrates through the so called bifid-shunt that lead to the production of lactic and acetic acids as main metabolic end products. Theoretically the bifid shunt produces 3 moles of acetic acid, 2 moles of lactic acid and 5 moles of ATP per 2 moles of glucose consumed (De Vuyst et al., 2013). However, several papers have shown that the patterns of organic acids produced, and therefore this ratio, vary depending on both the carbon source, the bifidobacteria strain and the growth phase (González-Rodríguez et al., 2013a; Rios-Covián et al., 2016), which is in good agreement with our results. Acetic acid was the most abundant organic acid formed, but lactic and formic acids were also produced at lower proportions (Figure

3). A previous study indicated that formic acid is produced at stationary phase by strains from the species *B. animalis* and *B. bifidum* (Rios-Covián et al., 2016) growing in glucose. However, we found that *B. longum* also formed formic acid at the stationary phase when grown on scFOS (Synergy or Actilight) (Figure 3). Changes in the

Fig 3. Concentration (mM) of the main organic acids (acetate, lactate and formate) in the batch cultures supplemented with different prebiotics at the different incubation times (6, 24 and 72 h). The SD of these means and the statistical analysis are showed in supplementary table 2.



direction of the bifid-shunt towards the formation of one or the other end-product, depending on the bifidobacterial species, the rate of substrate consumption, nutrient starvation or the exposure to environmental stressing conditions have been previously described (Rios-Covián et al., 2016). Moreover, it has been suggested that a metabolic shift toward the production of acetate at the expense of lactate allows the cells to gain extra ATP generation, whilst metabolic shift toward the production of lactic acid formation allows cells the increase the generation of NAD^+ at the expense of acetate (González-Rodríguez et al., 2013a). Therefore, bifidobacteria seem to be versatile microorganisms in directing their metabolic fluxes to one of the other branch of the bifid-shunt depending on the metabolic needs of the bacterium.

In general, large amounts of lactate and smaller amounts of acetate, formate and ethanol are produced when the energy source is consumed fast, whereas less lactate and more acetate, formate and ethanol are produced when the energy source is less easily fermentable (De Vuyst et al., 2014). Accordingly, in our *B. longum* cultures, at stationary phase, a metabolic shift towards more lactic acid production was observed with glucose (Figure 3), with A/L ratios close to theoretical ratio of the bifid shunt (Table 1). On the contrary a metabolic shift toward more acetic and formic acid production, at the expense of lactic acid production, was observed with Actilight and Synergy (Figure 3), their A/L ratio values being considerably higher than the theoretical ratio (Table 1). This

Table 1. Acetic to lactic and formic to lactic acid ratios of supernatants (mean ± standard deviation) from *Bifidobacteriu*m strains cultures with glucose and different prebiotics collected at different times of incubation (6, 24 and 72 hours). Letters indicate significant differences among carbon source from each strain (P<0.05).

			Ratios					
			Acetic/Lactic acid			Formic/Lactic acid		
Specie	Strain	Carbon source	6 h	24h	72h	6 h	24h	72h
B. animalis	IPLA20031	Synergy	3.93±0.35^{a}	4.92±1.74	4.49±0.75	0.58±0.06^{b}	1.52±0.73	1.51±0.32
		Actilight	2.31±0.36^{a}	4.80±0.46	5.02±0.48	0.29±0.09^{a}	1.46±0.19	1.82±0.38
		Glucose	7.74±1.53^{b}	3.72±0.58	5.20±1.90	1.89±0.22^{c}	1.15±0.09	1.71±0.78
	IPLA20032	Synergy	2.37±0.04^{b}	4.51±0.03^{b}	4.82±0.19^{b}	NC$^{\#}$	1.33±0.02^{b}	1.36±0.09^{b}
		Actilight	1.87±0.02^{a}	1.79±1.27^{a}	3.47±0.06^{a}	NC$^{\#}$	0.66±0.03^{a}	0.90±0.12^{a}
		Glucose	2.50±0.27^{b}	4.53±0.27^{b}	3.57±0.03^{a}	NC$^{\#}$	1.28±0.16^{b}	0.79±0.05^{a}
	Bb12	Synergy	3.23±0.49	5.86±0.27	5.65±1.37	0.55±0.09^{b}	1.28±0.22	1.07±0.48
		Actilight	2.57±0.24	5.07±0.56	5.42±0.78	0.34±0.01^{a}	1.38±0.17	1.59±0.51
		Glucose	2.41±1.41	5.53±0.18	3.83±0.50	NC$^{\#}$	1.59±0.11	0.81±0.18
	IPLA20020	Synergy	2.32±0.35^{a}	4.35±0.66	4.61±0.91	NC$^{\#}$	1.08±0.44	1.42±0.22
		Actilight	2.20±0.33^{a}	3.95±1.25	4.65±0.95	NC$^{\#}$	1.39±0.48	1.26±0.44
		Glucose	5.18±1.26^{b}	5.51±0.82	4.06±0.38	1.30±0.57	1.63±0.39	0.92±0.20
B. longum	IPLA20021	Synergy	NC$^{\#}$	7.14±0.43^{a}	13.30±3.03^{b}	NC$^{\#}$	2.16±0.18^{a}	4.57±1.04^{b}
		Actilight	NC$^{\#}$	18.74±4.98^{b}	18.51±3.52^{b}	NC$^{\#}$	7.47±2.40^{b}	7.35±1.46^{c}
		Glucose	0.96±0.76	1.72±0.03^{a}	1.69±0.08^{a}	NC$^{\#}$	0.04±0.01^{a}	0.05±0.01^{a}
	IPLA20022	Synergy	NC$^{\#}$	30.59±1.81^{c}	23.74±2.86^{c}	NC$^{\#}$	13.46±0.88^{c}	9.72±0.64^{c}
		Actilight	2.61±0.62	18.28±6.30^{b}	15.40±3.81^{b}	0.93±0.22^{b}	7.43±2.74^{b}	6.22±1.72^{b}
		Glucose	1.35±0.59	1.34±0.06^{a}	1.67±0.06^{a}	0.41±0.18^{a}	0.02±0.00^{a}	0.04±0.00^{a}

NC$^{\#}$, no calculated because concentrations of some organic acids were under quantification limit values.

metabolic shift toward more production of formic acid has been previously observed for other *Bifidobacterium* strains when grown with oligofructose as carbon source (Falony et al., 2009). A previous study, conducted with resting cells in glucose, observed a decrease in the molar proportion of acetic acid and an increase in lactic acid during the stationary phase in *Bifidobacterium* (Rios-Covián et al., 2016). However, we observed a metabolic shift toward more production of formic acid in *B. animalis* strains with glucose, Actilight and Synergy as carbon source (Figure 3), their A/L ratio values being higher than the theoretical ratio (Table 1). Thus, this result suggest that this species has an the capability to regenerate more NAD+ with could be one of the reason explaining its good adaptation to non-strictly anaerobic environments (Ruiz et al., 2011).

In conclusion, our results show that all *Bifidobacterium* strains tested

were not able to ferment inulin, and therefore do not point out at inulin as a good prebiotic for the development of a synbiotic product including our bifidobacterial strains. On the contrary, our results suggest the scFOS, may be well suited prebiotics for the combination with *B. animalis* and/or *B. longum* strains for the development of synbiotic functional foods. Moreover, the pattern of metabolite´s production varies depending on the carbon source in *B. longum* but not in *B. animalis*. This underlines the variability of the metabolic interactions between carbon sources and potential probiotic bifidobacteria and points out at the need of a careful assessment of the specific probiotic/prebiotic combinations used in synbiotic products.

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CAPITULO 2

Selección de probióticos, prebióticos y simbióticos frente a *Clostridium difficile*.

La segunda parte de esta Tesis Doctoral se centró en identificar cepas, sustratos y combinaciones de ambos con capacidad para reducir el crecimiento y toxicidad de *C. difficile*, que es la causa más importante de diarrea infecciosa de transmisión nosocomial en ancianos.

Para ello, como paso inicial, desarrollamos un modelo biológico *in vitro* basado en la monitorización continua en tiempo real de líneas celulares intestinales a través del RTCA ("real time cell analyser"), que permite estudiar la cinética de citotoxicidad de los sobrenadantes procedentes de un cultivo toxigénico de *C. difficile* sobre las líneas celulares.

A continuación, evaluamos el potencial probiótico de 20 cepas para contrarrestar, *in vitro*, el efecto citotóxico de *C. difficile* LMG21717 (Toxinotipo 0, Ribotipo 001) sobre la línea intestinal humana HT29. Con este objetivo incubamos los sobrenadantes toxigénicos de *C. difficile* con las diferentes bacterias a evaluar. Tras esta incubación, recogimos los nuevos sobrenadantes para cuantificar las toxinas A y B remanentes, por medio de pruebas ELISA, y para determinar el efecto tóxico residual de los sobrenadantes toxigénicos sobre la línea celular HT29, a través del modelo biológico mencionado anteriormente. También se analizó el comportamiento de la línea HT29 mediante técnicas de imagen; tomamos fotografías a tiempo real con un microscopio óptico invertido e imágenes a tiempo final con un microscopio confocal láser de barrido.

Por último, determinamos el potencial de 4 cepas del género *Bifidobacterium,* tres seleccionadas en base a su capacidad para reducir la toxicidad de los sobrenadantes toxigénicos de *C. difficile* anteriormente descritas y una cepa ampliamente usada como probiótico, para reducir el crecimiento y la toxicidad de *C. difficile* LMG21717 en presencia de varios sustratos prebióticos seleccionados. Con

este propósito, realizamos cocultivos de la cepa *C. difficile* LMG21717 con las cuatro bifidobacterias en presencia de cada uno de los diferentes sustratos prebióticos como fuente de carbono, y los resultados se compararon con los obtenidos en los correspondientes monocultivos. En los cocultivos y monocultivos llevados a cabo determinamos los niveles de las bifidobacterias y de *C. difficile*, por medio de q-PCR, así como el pH y la producción de AGCCs. Además, recogimos los sobrenadantes de los cultivos para evaluar su toxicidad usando el modelo biológico desarrollado.

Los resultados correspondientes a este capítulo 2 se presentan en los artículos siguientes:

- Artículo 3. **Valdés L.**, Gueimonde M. y Ruas-Madiedo P. (2015). Monitoring in real time the cytotoxic effect of *Clostridium difficile* upon the intestinal epithelial cell line HT29. Journal of Microbiological Methods, 119, 66-73.

- Artículo 4. **Valdés-Varela L.**, Alonso-Guervos M., Garcia-Suárez O., Gueimonde M. y Ruas-Madiedo P. (2016). Screening of bifidobacteria and lactobacilli able to antagonize the cytotoxic effect of *Clostridium difficile* upon intestinal epithelial HT29 monolayer. Frontiers in Microbiology, 7, 577.

- Artículo 5. **Valdés-Varela L.**, Hernández-Barranco A.M., Ruas-Madiedo P. y Gueimonde M. (2016). Effect of *Bifidobacterium* upon *Clostridium difficile* growth and toxicity when co-cultured in different prebiotic substrates. Frontiers in Microbiology, 7, 738.

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Monitoring in real time the cytotoxic effect of *Clostridium difficile* upon the intestinal epithelial cell line HT29

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ABSTRACT

The incidence and severity of *Clostridium difficile* infections (CDI) has been increased not only among hospitalized patients, but also in healthy individuals traditionally considered as low risk population. Current treatment of CDI involves the use of antibiotics to eliminate the pathogen, although recurrent relapses have also been reported. For this reason, the search of new antimicrobials is a very active area of research. The strategy to use inhibitors of toxin's activity has however been less explored in spite of being a promising option. In this regard, the lack of fast and reliable *in vitro* screening methods to search for novel anti-toxin drugs has hampered this approach. The aim of the current study was to develop a method to monitor in real time the cytotoxicity of *C. difficile* upon the human colonocyte-like HT29 line, since epithelial intestinal cells are the primary targets of the toxins. The label-free, impedance based RCTA (real time cell analyser) technology was used to follow overtime the behaviour of HT29 in response to *C. difficile* LMG21717 producing both A and B toxins. Results obtained showed that the selection of the medium to grow the pathogen had a great influence in obtaining toxigenic supernatants, given that some culture media avoided the release of the toxins. A cytotoxic dose- and time-dependent effect of the supernatant obtained from GAM medium upon HT29 and Caco2 cells was detected. The sigmoid-curve fit of data obtained with HT29 allowed the calculation of different toxicological parameters, such as EC50 and LOAEL values. Finally, the modification in the behaviour of HT29 reordered in the RTCA was correlated with the cell rounding effect, typically induced by these toxins, visualized by time-lapsed captures using an optical microscope. Therefore, this RTCA method developed to test cytotoxicity kinetics of *C. difficile* supernatants upon IEC could be a valuable in vitro model for the screening of new anti-CDI agents.

1. Introduction

Clostridium difficile is a Gram-positive, spore forming, anaerobic bacterium that inhabits the large intestine of healthy individuals. However, when the intestinal microbiota is disturbed (e.g. administration of oral antibiotics) this microorganism is able to overgrowth leading to different pathologies, such as *C. difficile* infections (CDI) in humans and animals (Rupnik et al., 2009). The main mechanism of virulence in *C. difficile* is related to the production of the two large protein toxins TcdA (308 kDa) and TcdB (260 kDa) and some variants are also able to produce the binary CDT toxin (Rupnik, 2008). Both A and B toxins have the same enzymatic activity, although TcdA acts mainly as enterotoxin whereas TcdB has a broad cytotoxic activity. Both toxins act as glycosyltransferases inactivating host cell GTPases, then causing disruption of the actin cytoskeleton and leading to colonocyte death via apoptosis; this produces a loss of intestinal epithelial barrier function by opening tight junctions between cells, which increased intestinal permeability and fluid accumulation, followed by the onset of diarrhoea (Jank and Aktories, 2008; Voth and Ballard, 2005). Toxins also induce the release of cytokines which lead to the activation of neutrophils, mast cells, enteric nerves and sensory neurons within the intestinal lamina propria. These, in turn, induce the release of neuropeptides and pro-inflammatory cytokines resulting in an inflammatory response and pseudomembrane formation (Shen, 2012; Sun and Hirota, 2015).

C. difficile is responsible for 20 to 30% of antibiotic-associated diarrhoea and is the most frequent in nosocomial diarrhoea (Abou-Chakra et al., 2014). The CDI manifestation ranges from asymptomatic carriage to clinical problems: from mild diarrhoea to more severe disease syndromes, including abdominal pain, fever and leucocytosis. Fulminant or severe complicated CDI is characterized by inflammatory lesions and the formation of pseudomembranes in the colon, toxic megacolon or bowel perforation, sepsis, shock and death (Faris et al., 2010). CDI have traditionally been assumed to be restricted to health-care settings. However, it is known that certain environments, animals and foods are predictable sources of *C. difficile*, although zoonotic and foodborne transmissions have not been confirmed yet (Rodriguez-Palacios et al., 2013). The main groups of risk are elders hospitalized and patients after hospitalization receiving antibiotics. However, CDI is increasing

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in younger populations, with no previous contact either with the hospital environment or with antibiotics, and in specific populations that were previously considered of low risk, such as children and pregnant women (Carter et al., 2012). In the recent years, the incidence and mortality of CDI has significantly increased due to the emergence in North America and Europe of strains with increased virulence, or hyper-virulent isolates, belonging to restriction endonuclease type BI, North American pulsed-field type 1 and PCR-ribotype 027. In addition, emerging strains belonging to PCR-ribotype 017 and 078, which are also associated with severe disease, have been isolated in parts of Asia and Europe (Bouillaut et al., 2013; Drudy et al., 2007; Kim et al., 2008). Hyper-virulent strains are characterized by significantly production of more A and B toxins and by their resistance to fluoroquinolones; they often produce more spores, in comparison to historical strains, and they synthesize the binary toxin CDT (*C. difficile* transferase) belonging to the family of binary ADP-ribosylating toxins (Gerding et al., 2014; Schwan et al., 2014).

Therapies against CDI comprise the use of antibiotics such as metronidazole or vancomycin, however in some cases this treatment does not prevent for the relapse of CDI. Emerging therapeutic options are currently under investigation for treatment of CDI (Mathur et al., 2014); in this regard, it is of pivotal relevance the use of fast, reliable, and accurate methods allowing the screening of new potential agents against *C. difficile* toxicity. This was the aim pursued in the current work, in which intestinal cellular lines were used as biological model to follow in real time the toxic effect of a *C. difficile* strain producing both A and B toxins.

2. Material and methods

2.1. C. difficile culture conditions and quantification of toxins

The type strain *C. difficile* LMG21717 (~ATCC®9689, Ribotype 001, genes *tcdA tcdB,tcdA+*, *tcdB+*, *cdtB-*) producing both TcdA and TcdB toxins (Toxinotype 0) was purchased from the "Belgian Coordinated Collections of Microorganisms" (BCCM, Gent, Belgium) and the non-toxigenic strain *C. difficile* ATCC®43601 (Ribotype 031, genes *tcdA-*, *tcdB-*, *cdtB-*) from the "American Type Culture Collection" (Manassas, VA, USA). Both strains were routinely grown (for 16 h) in Reinforced Clostridium Medium [RCM, Oxoid, Thermo Fisher Scientific Inc., Waltham, MA] in Hungate tubes. Incubations took place at 37 °C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) in a MG500 chamber (Don Whitley Scientific, West Yorkshire, UK). Several broth media (Table 1, supplementary Table S1) and incubation periods (24, 48, 72 and 120 h) were tested to select the best conditions for survival and toxigenic activity. The OD (600 nm), pH and counts, made in each corresponding medium supplemented with 2% agar, were measured to follow *C. difficile* activity. To determine cytotoxicity, bacterial cultures at the selected conditions were centrifuged (16,000 ×*g*, 10 min) to collect supernatants and pellets. Supernatants were directly tested, whereas pellets were washed ones with PBS buffer and resuspended (1/10 of the initial culture volume) in McCoy's medium which is defined below.

Table 1
Composition of culture media used to select the best conditions for growth and toxin production of *Clostridium difficile* LMG21717.

Medium	Composition	Brands[a]
RCM	(Supplementary table S1)	Oxoid
BHI	(Supplementary table S1)	Oxoid
GAM	(Supplementary table S1)	Nissui
RCM + BHI + FSB	1 vol RCM + 1 vol BHI + Foetal serum bovine (5%)	Oxoid, Sigma
BHI-C	BHI + L-cysteine (0.25%)	Oxoid, Sigma
BHI-Suppl.	BHI + L-cysteine (0.25%) + yeast extract (0.5%) + sodium thioglycolate (0.1%)	Oxoid, BD-Difco, Sigma
BHI-C-FSB	BHI + L-cysteine (0.25%) + Foetal serum bovine (5%)	Oxoid, Sigma
GAM-FSB	GAM + Foetal serum bovine (5%)	Nissui, Sigma

[a] Oxoid, Thermo Fisher Scientific Inc., Waltham, MA; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan; Sigma-Aldrich Co., St. Louis, MO; BD-Difco, Becton Dickinson Co., Franklin Lakes, NJ.

The concentration of A and B toxins produced by *C. difficile* LMG21717 supernatant was determined by independent ELISA tests (tgcBIOMICS GmbH, Bingen, Germany) following the manufacturer's instructions.

2.2. Cell-line cultures

The intestinal epithelial cell (IEC) lines HT29 (ECACC 91072201) and Caco2 (ECACC 86010202) were purchased from the "European Collection of Cell Cultures" (Salisbury, UK) and stored at IPLA under liquid N_2. Both cell lines were maintained under standard conditions using two specific media: supplemented McCoy's medium (or MM), for HT29, and supplemented Dulbecco's modified Eagle medium (or DMEM), for Caco2 (Hidalgo-Cantabrana et al., 2014). Both media were added, as well, with a mixture of antibiotics (50 μg/ml streptomycin-penicillin, 50 μg/ml gentamicin and 1.25 μg/ml amphotericin B). All media and reagents were purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO). For maintenance, the cell lines were incubated at 37 °C, 5% CO_2 atmosphere in a CO2-Series Shel-Lab incubator (Sheldon Manufacturing Inc., OR, USA) and were weekly trypsinized. A few consecutive passages were used: from p146 to p148, for HT29, and from p47 to p48, for Caco2. Cells were grown in 25 cm^2 bottles with vented (0.2 μm membrane) cap (Falcon®, Corning Inc. Life Science, Tewksbury, MA) and after 5 or 7 days of incubation these cultures were used to harvest cells for the real time experiments.

2.3. Monitoring cell-line behaviour in real-time

The RTCA (real time cell analyser) xCELLigence equipment (ACEA Bioscience Inc., San Diego, CA), which monitors three independent 16-well E-plates (each well having the same surface than 96-well standard plates), was used to test the behaviour of the IEC (Hidalgo-Cantabrana et al., 2014). The equipment was introduced in a Heracell-240 Incubator (Thermo Electron LDD GmbH, Langenselbold, Germany), set at 37 °C with 5% CO_2 atmosphere, and it was connected with a computer that controls and records the RTCA-curves. Initially, both IEC were titrated to determine the number of cells needed for further experiments. A cell suspension (1×10^6 cell/ml) was made in the corresponding medium for each IEC and, afterwards, serial 1/2 dilutions were prepared. Finally, 200 μl of the different cell suspensions were seed, in duplicated wells, in two independent 16-well E-plates (Fig. 1A). These micro-plates were hold in the equipment and incubated at 37 °C, 5% CO_2 to record the cell index (CI) every 15 min, for 50 h. The CI is an arbitrary unit that indicates variations of the impedance in gold-microelectrodes, placed in the bottom of the E-plates, as consequence of the IEC attachment and growth as well as due to morphological changes.

2.4. Monitoring the cytotoxic effect of C. difficile upon IEC

To analyse the cytotoxic effect of *C. difficile* LMG21717 both IEC were used in confluent (monolayer) state and the standard working parameters were defined accordingly to the titration results (Fig. 1B). An initial number of 2×10^5 cells (in 100 μl) were seed per well, thus allowing the formation of a monolayer around 14–15 h post-seeding. After 7–8 h of post-confluent state (around 22 h of total incubation) culture medium was removed; then different *C. difficile* samples (in 200 μl) were added and the monitoring continued (every 10 min) for additional 23 ± 1 h under the standard incubation conditions. The duration of a typical experiment was 44 h, which ends with the data analysis carried out with the RTCA software 1.2.1 (ACEA Bioscience). Data normalization

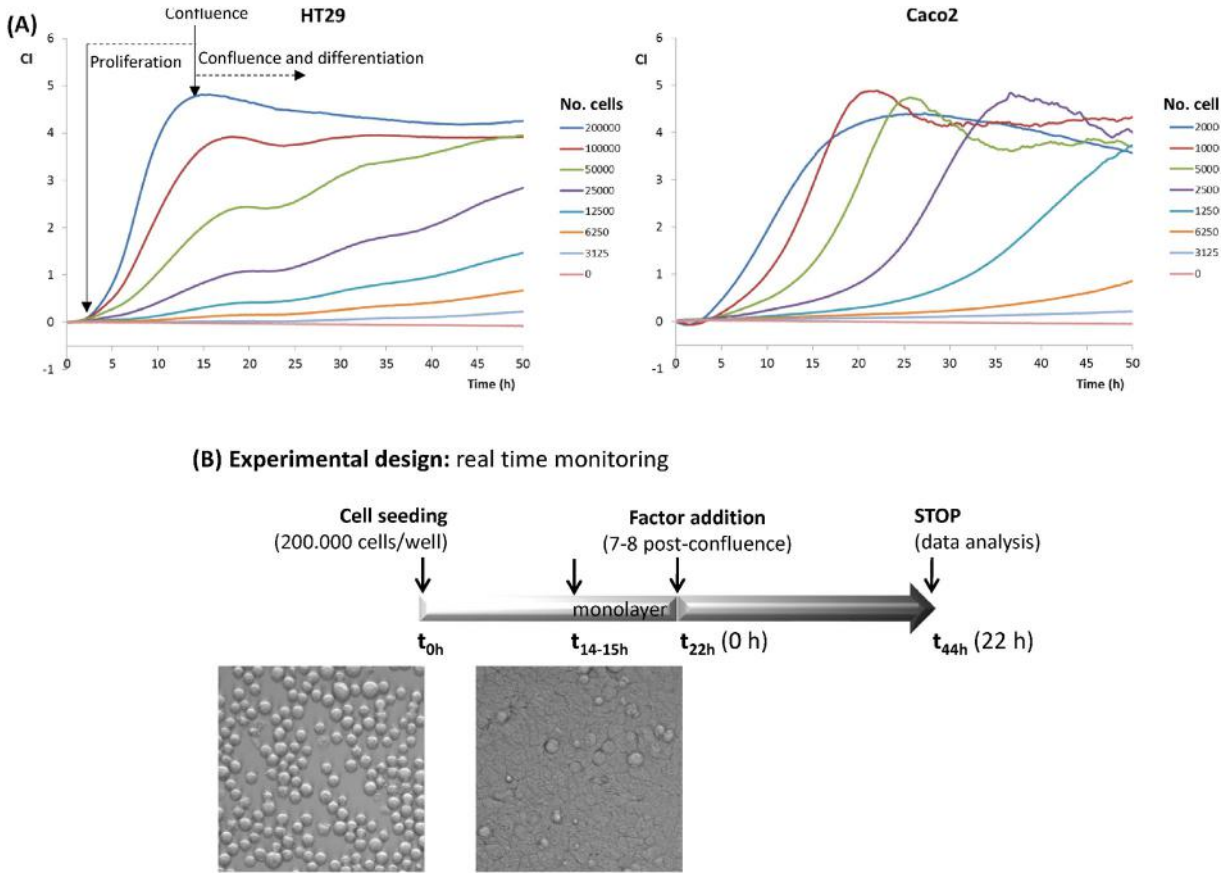

Fig. 1. Titration of HT29 and Caco2 cells dependent on the initial number of cells seeded (A). Experimental design used to test the toxic effect of *C. difficile* LMG21717 (B) and microscopic visualization of the HT29 cell morphology immediately after seeding (left microphotograph) and when the confluent monolayer state was reached (right microphotograph).

was performed as follows; first, the normalized-CI at a given time point was calculated by dividing the CI at this point by the CI at the normalization time point which, in our case, was the first time monitored immediately after *C. difficile* supernatant addition (time lapsed 10 min). Thus at the normalization time-point the normalized-CI is equal 1 for all wells. Afterwards, all data were referred to the "baseline CI" by subtracting the normalized-CI of each well from the normalized-CI baseline, which in our case was the control (culture medium without *C. difficile* supernatant addition); thus, the normalized-CI of the control is the "0 line" that is showed in the figures of the results section.

2.5. Image monitoring in real-time conditions

The compact, inverted, optical microscope LumaScope-600 Series (Etaluma, Carlsbad, CA) with a 40× objective was used to visualize, in time-lapsed capture images, the toxic effect of *C. difficile* samples under culturing conditions. For that purpose, the microscope was introduced in the Heracell-240 Incubator and was connected with an external computer that captures the images, every 10 min, by means of the LumaView600Cy 13.7.17.0 software (Etaluma). An optical quality (equivalent to coverslip no. 1.5), standard bottom μ-Slide 2-well plate (Ibidi GmbH, Martinsried, Germany) was used to monitor the formation of the HT29 monolayer after initially seeding 1 ml of 2×10^6 cells/ml suspension (Fig. 1C). Once that the confluent state was reached, and at the time defined with the RTCA-DP equipment (~22 h), MM supplemented with 2.5% *C. difficile* supernatant (collected from 48 h cultures in GAM) was added and the image capture continued for additional 24 h.

2.6. Statistical analysis

The statistical package IBM SPSS Statistics for Window Version 22.0 (IBM Corp., Armonk NY) was used to asses differences in response (normalized CI) of HT29 due to the supernatant dose by means of one-way ANOVA test. Afterwards, differences among doses were determined by the Duncan mean comparison test which allowed identifying the LOAEL (lowest observed adverse effect level) and NOAEL (non-observed adverse effect level) doses (Jeffery et al., 2004). Finally, the EC50 (concentration at which the half of the maximum adverse effect was detected) at a defined time point was calculated by the RTCA software 1.2.1 (ACEA Bioscience) from the normalized-CI vs. log concentration data fitted to a sigmoidal-curve.

3. Results

3.1. C. difficile LMG21717 cultures in GAM has the highest toxic effect upon HT29

The initial step to develop a method to follow in real-time the cytotoxic activity of the toxinotype 0 *C. difficile* strain upon IEC was obtaining a toxigenic supernatant. For that purpose, eight different media and four sampling points were screened. The strain LMG21717 was not able to survive, at any incubation time, in RCM, BHI-C, BHI-Suppl, or BHI-C-FSB after 48 h incubation (Table 2). In the remaining media, the highest counts ($>1 \times 10^7$ CFU/ml) were reached at this incubation period, whilst longer periods led to a reduction in the bacterial counts (data no shown). To test the toxigenic capability of *C. difficile*, the behaviour of HT29 in presence of 20% supernatants (Fig. 2A) or pellets (Fig. 2B)

Table 2
Cultures of *Clostridium difficile* LMG21717, after 48 h of incubation, made in different media used to select the best conditions for growth and toxigenic activity. The highest toxigenic activity was established at the lowest normalized-cell index (CI) value obtained from the intestinal cell line HT29 after 4 h of *C. difficile* factors addition.

	C. difficile			Normalized-CI HT29	
Media	pH	OD	CFU/ml	Supernatant	Pellet
RCM	5.62	1.26	$<10^5$	−0.0191	−0.0793
BHI	5.88	1.10	8.3×10^7	−0.0912	−0.6199
RCM + BHI + FSB	5.64	1.66	1.1×10^7	−0.3077	−0.7109
GAM	5.88	1.25	1.6×10^7	−0.7052	−0.5211
BHI-C	5.47	1.52	$<10^5$	−0.0566	−0.1958
BHI-Suppl.	5.51	1.72	$<10^5$	−0.1069	−0.2708
BHI-C-FSB	5.50	1.24	$<10^5$	−0.0739	−0.1721
GAM-FSB	5.84	1.64	1.4×10^7	−0.7267	−0.6820

collected from the cultures in different media and incubation periods, was monitored in real-time. The CI values obtained were normalized by the control sample (MM without *C. difficile* factors) and by the time at which the factors were added. The lowest normalized-CI value indicates the highest toxigenic capability of *C. difficile* factors upon HT29 since the reduction of this unit reflects the detachment of the cell line, from the gold-microelectrode in the E-plate, or modifications in the morphology of the cell-line monolayer. For practical purposes, we have arbitrary set the point of 4-h after factor addition, to obtain numeric values allowing comparisons among factors (Table 2). In agreement with the reduced growth, both supernatants and pellets collected from 48-h cultures in RCM, BHI-C, BHI-Suppl, or BHI-C-FSB showed the highest normalized CI values along all monitored period (Fig. 2A and B) or at the defined 4-h point (Table 2). Remarkably, the pellet harvested from BHI medium retained all toxigenic activity, since its corresponding supernatant showed a normalized-CI near cero. An intermediate pattern was denoted for *C. difficile* grown in RCM + BHI + FSB given that produced the most toxigenic pellet, but being able to release part of the toxins to the supernatant. Finally, the two media containing GAM produced the more toxic supernatants and also their corresponding pellets showed low normalized-CI values, i.e. high toxicity. In order to be able to choose one of these two GAM media, the normalized-CI after 4-h was calculated for both factors harvested from grown cultures after 72 and 120 h of *C. difficile* incubation. The supernatant collected from GAM, without supplementation, showed the highest toxigenic capability which remained stable during prolonged *C. difficile* culturing periods (Fig. 2C). Therefore, as conditions to obtain toxigenic supernatants for further experiments we have selected cultivation of *C. difficile* LMG21717 in GAM medium for 48 h. The concentration of toxin A and toxin B quantified under these conditions was 210 ± 51 ng/ml and 16 ± 8 ng/ml, respectively. The supernatant collected from these culture conditions was stored at −80 °C until its use, since we have detected that higher temperature (−20 °C) of storage reduced the activity of the supernatants (data no shown).

3.2. *C. difficile* LMG21717 toxic effect is dose dependent

Next, we aimed at determining whether the toxigenic effect was dependent on the amount of supernatant added and if was maintained during prolonged periods. To achieve this goal experiments upon HT29 monolayers were performed using a wide supernatant-percentage range. Immediately after the supernatant addition, it is noticeable the increase in the impedance (CI) signal, which it can be easily followed in the normalized-CI graphic (Fig. 3A). The maximum normalized-CI values are reached around 45 min after supernatant addition, the highest being at the highest doses (CI > 0.05 for concentrations above 2.5%). This behaviour is related with changes in the morphology and shape of the eukaryotic cells. Afterwards, the CI signal showed a continuous reduction

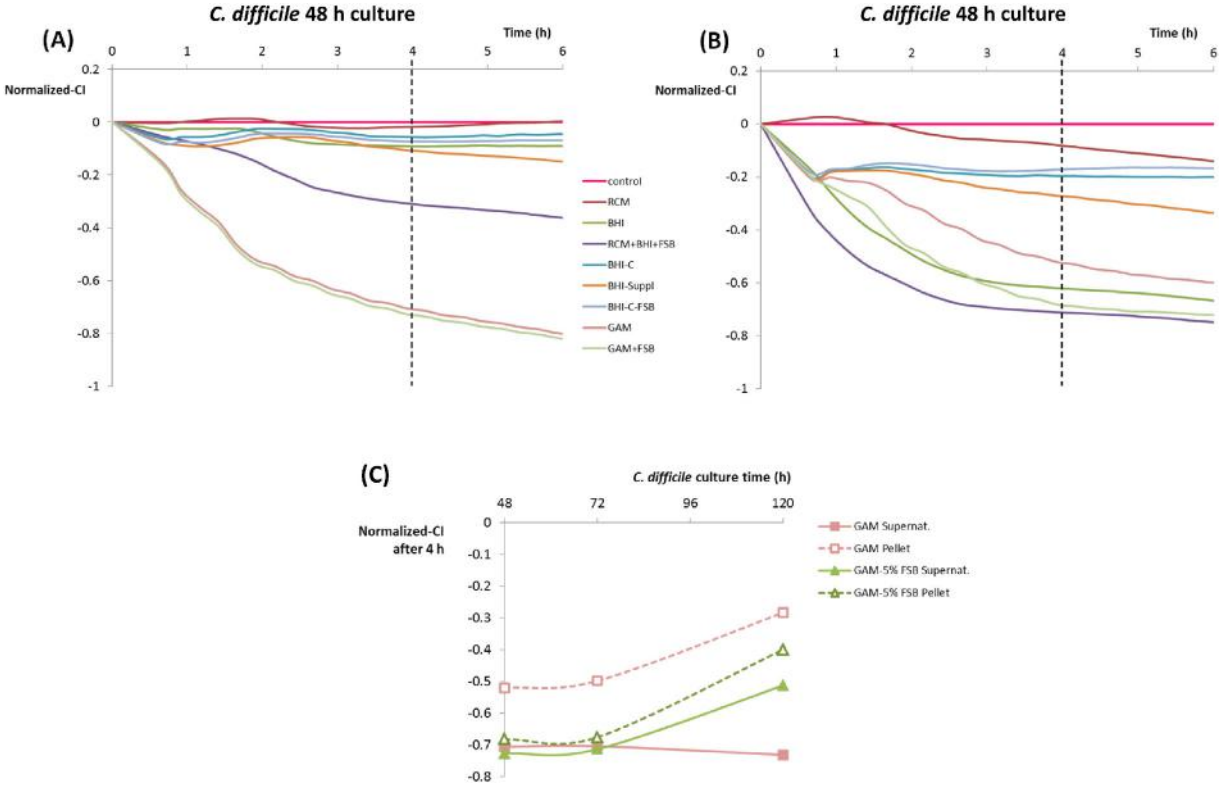

Fig. 2. Behaviour of HT29 monolayers in the presence of the toxigenic supernatants and pellets collected from *C. difficile* cultures grown for 48 h in different culture media. Normalized cell index (CI) obtained from E-plates to test McCoy's medium supplemented with 20% of supernatants from each medium (A) or with pellets resuspended (1/10 vol. of the initial 48-h culture) in McCoy's medium (B). The CI was normalized by the control (non-supplemented McCoy's medium) sample at the time of the supernatant or pellet addition. Normalized-CI values 4-h after addition of factors obtained from 48, 72 and 120 h of *C. difficile* cultures in media containing GAM (C).

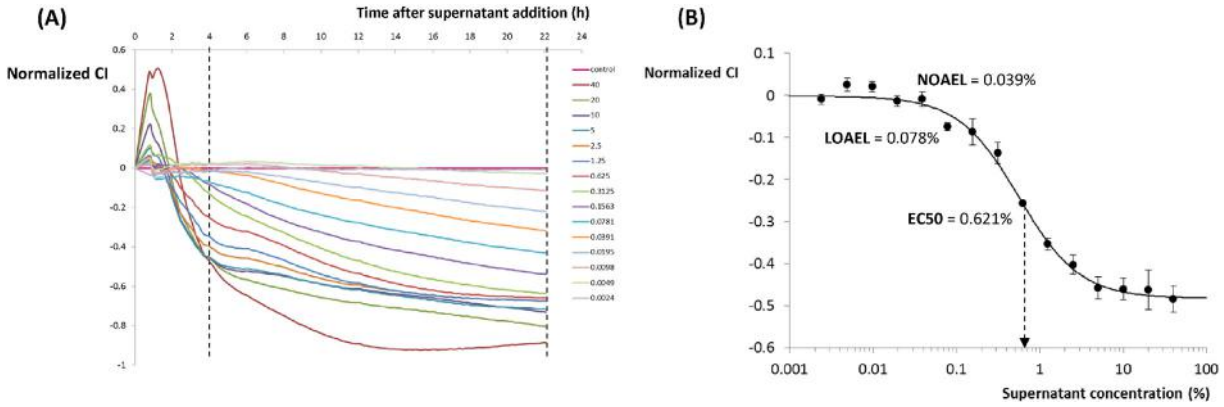

Fig. 3. Behaviour of HT29 monolayers in the presence of different percentages of the toxigenic supernatant collected from *C. difficile* cultures grown for 48 h in GAM. Mean values of data after normalization of the CI by the control sample (non-supplemented McCoy's medium) and by the time of the supernatant addition (A). Dose–response data (mean and SD) fitted to a sigmoidal trend line curve allowing the identification of the EC50 (concentration where supernatant produced 50% of the maximum toxic effect), LOAEL (lowest observed adverse effect level, $p < 0.05$) and NOAEL (non-observed adverse effect level) doses (B).

over time and after 2 h, the CI recorded for most concentrations tested started to show negative values. The normalized-CI drop was more drastic at higher supernatant concentrations, as indicated the higher slopes obtained in the normalized-CI vs. time curves. This behaviour suggests a loss in the monolayer integrity, which could be due to a disruption of the tight junctions between adjacent cells, to cellular death, or to a combination of both events. The sigmoid-curve fit ($R^2 = 0.9941$) of normalized-CI vs. log concentration (percentages) obtained 4-h after supernatants addition, clearly demonstrated the toxigenic dose-dependent effect of the *C. difficile* supernatant upon HT29 monolayers (Fig. 3B). Using this fit the LOAEL, i.e. the lowest concentration of *C. difficile* supernatants that produced a detectable toxic effect ($p < 0.05$), was 0.0781% which corresponded to 164 and 12.5 pg/ml of TcdA and TcdB, respectively. Therefore, the NOAEL, or the highest concentration of supernatant tested that causes no toxic effect, was 0.0391% (390.8 ppm). The concentration inducing half of the maximum effect (EC50 dose) was 0.6206% (1304 and 99 pg/ml for TcdA and TcdB, respectively). After longer periods of co-incubation (22-h) the different doses of *C. difficile* supernatants still had a stronger toxigenic effect upon HT29 cells, as the lower normalized-CI values reached indicates (Table 3). Finally, aiming to undoubtedly assign that the observed effect of LMG21717 supernatants upon HT29 was due to the production of these toxins, the same dose-effect experiment was performed with the non-toxin producer ATCC43601 strain. Data obtained (Supplementary Fig. 1 and Supplementary Table 2) showed that the normalized-CI values of the HT29 in presence of supernatants from this strain remained almost stable during the time course of the experiment (~22 h); thus non-toxigenic effect was detected with a no toxin producing strain.

In a step further, we want to assess whether results obtained with strain LMG21717 could be reproduced in another biological model. In this regard, Caco2 and HT29 cell lines have extensively being used in research to mimic the human intestinal epithelium. Under confluent and differentiated state both cell lines express characteristic of enterocytes, but Caco2 monolayers are composed exclusively of absorptive cells, whilst HT29 also includes mucus-secretory Goblet cells (Hilgendorf et al., 2000). Then in our study the IEC line Caco2 was confronted with the *C. difficile* toxigenic supernatant in percentages ranging from 10% to 0.16% (Fig. 4). Indeed, although we have observed a toxigenic effect of the *C. difficile* supernatant upon Caco2 monolayers, the normalized-CI values showed a different tendency with respect to that of HT29. After a short 4-h contact period, only concentrations of *C. difficile* supernatants higher than 1.25% were toxic; this cut-off percentage went down to 0.63% for prolonged co-incubation period (Table 3). Therefore, the intrinsic characteristics of each IEC accounted for the development of a model to address the toxigenic effect of this pathogen.

Table 3
Normalized-cell index (CI) values obtained from the intestinal cell lines HT29 and Caco2 after 4 h and 22 h of addition of different concentrations of *Clostridium difficile* LMG21717 toxigenic supernatants.

	Normalized-CI			
C. difficile	HT29		Caco2	
Supernatant	4 h	22 h	4 h	22 h
10%	−0.6253	−0.8468	−0.2741	−0.6662
5%	−0.5665	−0.8228	−0.2596	−0.6427
2.5%	−0.4935	−0.7956	−0.1151	−0.6367
1.25%	−0.4125	−0.7728	0.0191	−0.6135
0.63%	−0.3262	−0.7303	0.0576	−0.4668
0.31%	−0.2301	−0.6923	0.0048	0.0279
0.16%	−0.1604	−0.6198	−0.0756	0.1079
0 (control)	0	0	0	0

3.3. Visualization of *C. difficile* toxic effect

Image analysis was performed in order to correlate with the events that are recorded with the RTCA technology. HT29 monolayers were tested with 2.5% *C. difficile* supernatant and several images were captured in real time over time (Fig. 5, and Supplementary video). Initially, before the supernatant addition, HT29 cells displayed typical morphology of an intestinal monolayer with most adjacent cells well connected, indicating that tight junctions and cytoskeleton are intact. In a short period after supernatant addition, around 2 h, the cells into the monolayer become to acquire a spherical shape and tend to detach from the adjacent ones. This is in agreement with the way of action of the toxins which disarray actin cytoskeleton and finally induce cell death.

4. Discussion

Treatment for CDI involves the use of antibiotics to eliminate the pathogen or, more recently, the restoration of the intestinal microbiota to avoid relapse (van Nood et al., 2013) which is still a controversial issue limiting its widespread use. Novel, -alternative to antibiotics-, antimicrobial strategies towards *C. difficile* are currently under evaluation; for example, the use of other microbial-origin molecules such as bacteriocins (Gebhart et al., 2015) and bacteriophage endolysins (Dunne et al., 2014). Another less explored strategy is the use of drugs targeting *C. difficile* toxins which could act as co-adjuvants to palliate the acute effect induced by the pathogen (Tam et al., 2015). In this article, we report the optimization of a label-free, impedance-based RTCA method to follow the cytotoxicity kinetics of human colonocyte-like cells exposed to *C. difficile* supernatants, method that could be used for the screening of new toxin-activity inhibitors. Different label-free

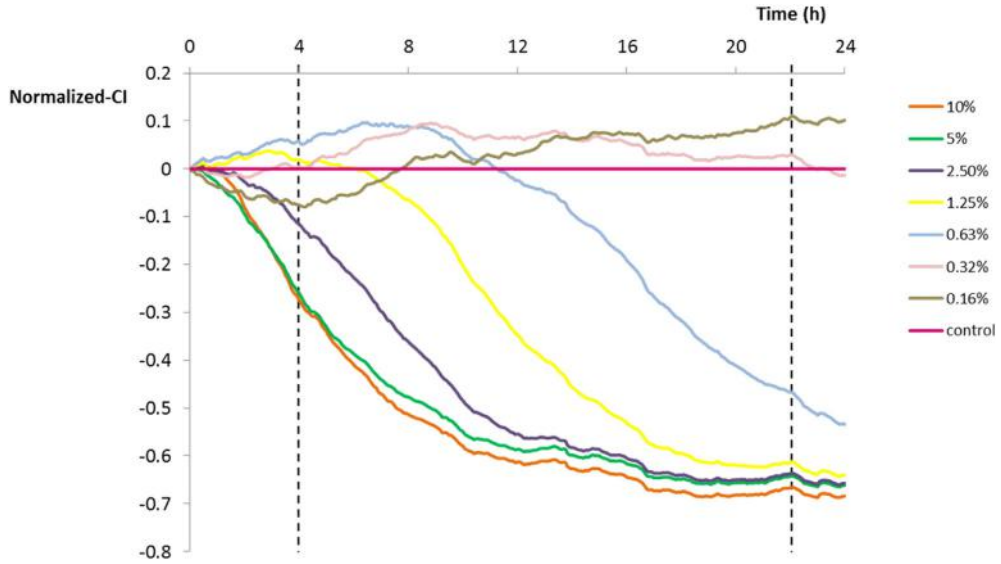

Fig. 4. Behaviour of Caco2 monolayers in the presence of different percentages of the toxigenic supernatant collected from *C. difficile* cultures grown for 48 h in GAM. The CI was normalized by the control (non-supplemented DMEM) sample at the time of the supernatant addition.

technologies, alternative to classic label-based endpoint methods, are currently been used in different research fields such as evaluation of new drugs (Xi et al., 2008) and cancer development studies (Limame et al. 2012), but also for assessment of the toxic effect of infectious bacteria upon host cells (Slanina et al., 2011; Ye et al., 2015). Specifically the RTCA technology, also known as RC-CES (real time-cell electronic sensing), was recently applied to develop methods allowing the clinical diagnosis of toxigenic *C. difficile* in different biological samples (Huang et al., 2014a). For this purpose cell lines from non-intestinal origin were used, such as HS27 cells (fibroblast) obtained from human skin (Huang et al., 2014b; Ryder et al., 2010), mRG1-1 cells genetically modified from CHO cells (epithelial morphology) which come from Hamster ovary (He et al., 2009; Steele et al., 2012) or Vero cells (epithelial morphology) obtained from a monkey kidney (Yu et al., 2015). However, given that the aim pursued in our work was to develop a method allowing the screening of new potential bio-actives against *C. difficile* toxicity acting within the human gut, we have choose as cellular model an intestinal epithelial line since enterocytes are the primary action targets of these toxins. Additionally, although the results obtained with HT29 cells in proliferative state were similar (data no shown), we worked in a confluent state (monolayer) because this better mimic the physiological conditions of an intestinal epithelium.

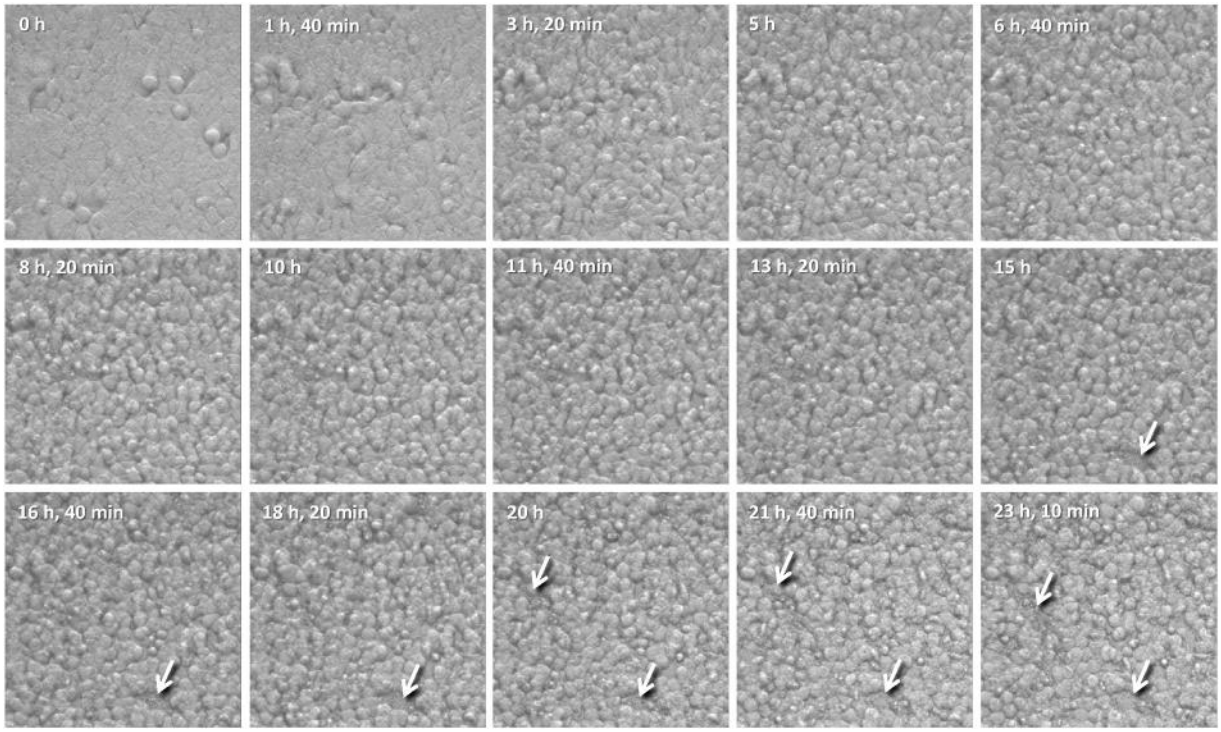

Fig. 5. Visualization over time (from 0 to 24 h, photo-capture interval of 1 h, 40 min) under inverted optical microscope (objective ×40) of the HT29 monolayer in the presence of 2.5% *C. difficile* toxigenic supernatant collected from 48 h cultures in GAM medium. Arrows indicate some areas of monolayer disaggregation. (See Supplementary video, made with a selection of 100 microphotographs captured at 10 min interval, from 0 to 24 h).

One of the remarkable findings observed in the context of our work was the fact that the culture media used to grow *C. difficile* under laboratory conditions had great influence in the release (secretion) of the toxins to the supernatant. Indeed, the non-selective BHI medium, classically used for growing this bacterium, retained almost all toxigenic activity attached to the bacterial pellet, whereas the two media based on GAM were those more effective releasing the toxins. It has been reported that culture conditions, i.e. micro- and macronutrient composition, have strong influence in both induction and repression of toxin production (Lei and Bochner, 2013). In fact it was proven that common rich media components, such as yeast or beef and pork peptones, are able to *in vitro* and *in vivo* inhibit the toxicity of *C. difficile* toxin A (Duncan et al., 2009). In addition, culture media also influence the composition of *C. difficile* protein secretome, including their toxins (Boetzkes et al., 2012). The exact mechanism of TcdA and TcdB secretion is unknown, but it seems that secretion signals or bacterial lysis are not involved. Other mechanisms of toxin release, such as the formation of a holin-like protein, are still controversial (Govind and Dupuy, 2012; Olling et al., 2012). Therefore, to *in vitro* evaluate the efficacy of any potential toxin's inhibitor, the laboratory *C. difficile* culture conditions must be carefully chosen. In this regard, our RTCA strategy allowed a fast, reliable and tailor-made method for the selection of culturing and toxin secretion conditions.

The data of cytotoxicity kinetics obtained from the dose–response curve showed an initial increase in the normalized-CI, which can be related with a modification in the morphology of the cells (Hidalgo-Cantabrana et al., 2014; Yu et al., 2006), followed by a drastic and continuous drop of this value during prolonged incubation times. Additionally, a strong dose–response behaviour was detected with a good fit ($R^2 > 0.99$) to a sigmoid-curve allowing the calculation of different toxicological parameters (Jeffery et al., 2004). The lowest concentration at which our model of *C. difficile* supernatants induced a significantly toxic effect upon HT29 cells, after a short exposition-period (4 h), was 164 and 12.5 pg/ml for toxin A and B, respectively. In this regard, it has been reported that TcdB is at least 100-fold more potent than TcdA upon other non-intestinal cell lines (Tam et al., 2015). Thus, our data confirm this ratio upon human colonocyte-like cells. As far as we could achieve after an exhaustive literature search, there are not reports about the toxicity range of *C. difficile* culture supernatants upon HT29, or enterocyte-like cells, allowing comparison with the results obtained in this study. Regarding other cellular models, Ryder et al. (2010) have also detected a dose- and time-dependent effect of purified toxins upon HS27 cells, being the detection limit for toxin B ~ 200 pg/ml with a detection time between 15 to 18 h. They also succeed with the detection of toxigenic *C. difficile* in stool samples collected from 300 CDI patients. This assay was improved latter when an immunomagnetic separation enrichment process was incorporated during stool preparation (Huang et al., 2014b). In parallel, He et al. (2009) reported in mRG1-1 cells detection limits about 10 pg/ml and 10 ng/ml for TcdB and TcdA, respectively, after prolonged (~20 h) incubation time; the range of the latter decreased to 1–10 pg/ml if the assay is carried out in the presence of the anti-*C. difficile* toxin A monoclonal antibody A1H3, since the mRG1-1 cells were engineered to express the murine Fc gamma receptor (FcγRI)-α-chain, thus increasing the sensitivity of the assay. The model develop by this group was also successfully used in the detection of toxins present in blood plasma of CDI animal models (Steele et al., 2012) and in two cases of human *C. difficile* toxaemia (Yu et al., 2015). These reports underlines that the detection limits are very dependent on the cellular model used, as well as on the preparation of complex biological samples.

Results obtained with our RTCA cytotoxic assay were confirmed with the images captured every 10-min with the time lapsed optical microscope. A time-dependent cell rounding of HT29 cells was observed, which it is a typical morphological change observed in different cell lines treated with *C. difficile* toxins (Steele et al., 2012; May et al. 2013). This loss of shape is due to modification in the cellular cytoskeleton induced by the toxins, which are able to depolymerize or disassemble F-actins (May et al., 2013). Another label-free, end-point independent technology to measure cellular events involves the use of a lens-free, video microscopy platform (Kesavan et al., 2014). The image-monitoring in real time records similar events than those measured by the impedance-based RTCA, such cell adhesion, spreading, division and death. Indeed, this alternative high-throughput imaging-based methodology was also used to identify new *C. difficile* TcdB inhibitors that protected human (non-enterocyte) cells, in this case CHO, Vero and IMR-90, from cell rounding (Tam et al., 2015).

Finally, is worth noting that the cellular model used to carry out cytotoxic assays, even if the cells are from the same tissue type, had an impact on the results obtained; we have previously observed similar findings in other types of studies using different IEC lines (Hidalgo-Cantabrana et al., 2014). In relation to the aim of the current work, this cell-dependent effect is especially relevant to test the efficacy of any potential anti-toxin bioactive since toxicological parameters, such as EC50 or LOAEL, can vary. Thus, although a broad screening can be performed with a single cell line, it would be advisable the use of more than one cellular type for the definition of the efficacy range of any novel candidate to inhibit *C. difficile* toxic activity.

5. Conclusion

In this work we have developed a valuable *in vitro* model to test cytotoxicity kinetics of *C. difficile* supernatants upon IEC, which could be eventually used in the screening of new anti-CDI agents. The model allows the preliminary selection of the appropriate culture conditions, as well as the choice of the accurate doses, to undertake the further toxicological assays of *C. difficile* toxins. However, it must be taken into consideration the variability of the results related to the model of eukaryotic cell used to analyse cytotoxicity. In addition, we consider that this model could be extended to the study of other microbial cyto-toxins.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2015.09.022.

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Screening of Bifidobacteria and Lactobacilli Able to Antagonize the Cytotoxic Effect of *Clostridium difficile* upon Intestinal Epithelial HT29 Monolayer

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Clostridium difficile is an opportunistic pathogen inhabiting the human gut, often being the aetiological agent of infections after a microbiota dysbiosis following, for example, an antibiotic treatment. *C. difficile* infections (CDI) constitute a growing health problem with increasing rates of morbidity and mortality at groups of risk, such as elderly and hospitalized patients, but also in populations traditionally considered low-risk. This could be related to the occurrence of virulent strains which, among other factors, have high-level of resistance to fluoroquinolones, more efficient sporulation and markedly high toxin production. Several novel intervention strategies against CDI are currently under study, such as the use of probiotics to counteract the growth and/or toxigenic activity of *C. difficile*. In this work, we have analyzed the capability of twenty *Bifidobacterium* and *Lactobacillus* strains, from human intestinal origin, to counteract the toxic effect of *C. difficile* LMG21717 upon the human intestinal epithelial cell line HT29. For this purpose, we incubated the bacteria together with toxigenic supernatants obtained from *C. difficile*. After this co-incubation new supernatants were collected in order to quantify the remnant A and B toxins, as well as to determine their residual toxic effect upon HT29 monolayers. To this end, the real time cell analyser (RTCA) model, recently developed in our group to monitor *C. difficile* toxic effect, was used. Results obtained showed that strains of *Bifidobacterium longum* and *B. breve* were able to reduce the toxic effect of the pathogen upon HT29, the RTCA normalized cell-index values being inversely correlated with the amount of remnant toxin in the supernatant. The strain *B. longum* IPLA20022 showed the highest ability to counteract the cytotoxic effect of *C. difficile* acting directly against the toxin, also having the highest capability for removing the toxins from the clostridial toxigenic supernatant. Image analysis showed that this strain prevents HT29 cell rounding; this was achieved by preserving the *F*-actin microstructure and tight-junctions between adjacent cells, thus keeping the typical epithelium-like morphology. Besides, preliminary evidence showed that the viability of *B. longum* IPLA20022 is needed to exert the protective effect and that secreted factors seems to have anti-toxin activity.

Keywords: probiotics, *Clostridium difficile*, toxins, RTCA, xCelligence, *Bifidobacterium*, *Lactobacillus*, microscopy

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INTRODUCTION

Clostridium difficile is a Gram-positive, spore-forming, motile and strict anaerobe rod that can be found in the gastrointestinal tract of humans and animals (Janezic et al., 2014). The current classification of the "Bergey's Manual of Systematic Bacteriology" includes *C. difficile* in the Phylum *Firmicutes*, Class *Clostridia*, Order *Clostridiales* and Family *Peptostreptococcaceae* (Ludwig et al., 2009). A recent taxonomic study, based on 16S rRNA and ribosomal protein sequences, ascertains that *C. difficile* belongs to this family and proposes that it should be renamed as *Peptoclostridium difficile* (Yutin and Galperin, 2013); this new name appears in the taxonomic classification and nomenclature catalog of NCBI[1], but still *C. difficile* remains as the name recognized by the clinical and scientific community.

C. difficile infection (CDI) is the main cause of diarrhea associated with antibiotic use or related to health-care environments (Leffler and Lamont, 2015) and increasing incidence is reported among populations previously considered as low risk, such as pregnant women and children (Carter et al., 2012). The ubiquity of this bacterium, in combination with its capability to form spores, makes hospital environments a good source for *C. difficile* acquisition, although zoonotic (Bauer and Kuijper, 2015) and food transmissions (Troiano et al., 2015) have been proposed as well. The incidence and severity of CDI has been growing since the beginning of this century due to the global occurrence of hypervirulent strains such as BI/NAP1/027 (group BI by restriction endonuclease analysis, North American pulse-field type NAP1 by pulse-field gel electrophoresis, and ribotype 027; Rupnik et al., 2009; Yakob et al., 2015). The antibiotics metronidazole and vancomycin are the current treatments for CDI, but this does not prevent the high rates of recurrence. Thus, new emerging therapeutic options, such as fecal microbiota transplantation (FMT), new antibiotics, bacteriocins, bacteriophages, and probiotics are under evaluation for the control of CDI (Martin et al., 2013; Dunne et al., 2014; Mathur et al., 2015). Indeed probiotics, which are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO-WHO, 2001; Hill et al., 2014), have been proposed as biotherapeutic agents to help microbiota restoration after a dysbiosis caused by antibiotics or infections (Reid et al., 2011).

The information encoded on the genomes of this species, excellently reviewed by Knight et al. (2015), reveals high plasticity and very low levels of conservation among strains. This genetic diversity is reflected in its physiological adaptation to different ecosystems and in the occurrence of different phenotypes. In addition, the presence of a wide variety of transposons and phages explain the lineage evolution of clinically relevant loci, such as the antimicrobial resistance genes and the PaLoc (pathogenicity locus), among others (Knight et al., 2015). The PaLoc harbors, together with three additional genes, *tcdA* and *tcdB* coding for toxin A and toxin B, respectively, which are the major *C. difficile* virulence factors (Monot et al., 2015). The modulating environmental signals regulating the expression of PaLoc is not totally understood and a recent report shows that toxin synthesis is regulated through quorum-sensing signaling (Darkoh et al., 2015). TcdA and TcdB are large toxins whose main mechanism of action is known, although host receptors and toxin-mediated responses still remain to be fully deciphered. They act as intracellular glycosyltransferases modifying the Ras superfamily of small GTPases thus inducing intracellular changes, including *F*-actin condensation, transcriptional activation and cell apoptosis of intestinal epithelial cells. This promotes the disruption of the tight junctions and barrier integrity, leading to an increase in the gut permeability and neutrophil infiltration. Downstream effects also include modifications in the chemokine and cytokine production patterns toward an inflammatory response and fluid accumulation, ending with the clinical manifestations of leukocytosis and diarrhea (Voth and Ballard, 2005; D'Auria et al., 2013; Carter et al., 2015; Leslie et al., 2015). Therefore, anti-toxin therapies to counteract the negative effects of these potent *C. difficile* virulence factors could be valuable tools to reduce the course of CDI (Tam et al., 2015).

In a previous study we developed a biological model, using the (human) intestinal epithelial cell line HT29, to follow in real time the effect of supernatants collected from *C. difficile* cultures of a TcdA+, TcdB+ (toxinotype 0) strain. This method is based on the continuous monitoring of the impedance signal, transmitted through gold microelectrodes placed in the bottom of microtiter plates, of HT29 monolayers (Valdés et al., 2015). Our aim in the present work is to search for lactobacilli and bifidobacteria probiotic candidates with anti-toxin capability able to protect HT29 cells from the cytotoxicity caused by toxigenic *C. difficile* supernatants.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The *Bifidobacterium* and *Lactobacillus* species used in this study are listed in **Table 1**. Most strains belonging to IPLA culture collection were isolated from infant feces and breast milk (Solís et al., 2010), whereas IPLA20031 and IPLA20032 were obtained after adaptation to increasing concentrations of bile salts from a parental strain isolated from a dairy product (Ruas-Madiedo et al., 2010). Strains were grown in MRSC [MRS (Biokar Diagnostics, Beauvois, France) supplemented with 0.25% L-cysteine (Sigma-Chemical Co., St. Louis, MO, USA)] at 37°C in the anaerobic chamber MG500 (Don Whitley Scientific, Yorkshire, UK) under 80% N_2, 10% CO_2 and 10% H_2 atmosphere. As standard procedure bacterial stocks, kept at −80°C in MRSC + 20% glycerol, were spread onto the surface of agar-MRSC and incubated for 3 days. A single colony was picked to inoculate MRSC broth which, after 24 h incubation, was used to inoculate (2%) 10 ml fresh MRSC broth. This culture was incubated overnight (18 h) to prepare the bacterial suspensions that will be described next.

[1]http://www.ncbi.nlm.nih.gov/taxonomy/?term=Peptoclostridium%20difficile

TABLE 1 | Strains included in this study and normalized cell index (CI) obtained at 4 and 22 h after addition of neutralized cell-free supernatants (NCFS) collected from incubations of each bifidobacteria or lactobacilli strain with 2.5% of toxigenic *Clostridium difficile* LGM21717 supernatant (Tox-S).

		Mean ± SD			
		Normalized-CI		Remnant toxin (ng/ml)	
NCFS	**Strain**	**After 4 h**	**After 22 h**	**TcdA**	**TcdB**
C. difficile Tox-S (2.5%)	LMG21717*	−0.64 ± 0.13	−0.93 ± 0.11	4.41 ± 0.01	0.48 ± 0.0
B. bifidum	LMG13195*	−0.62 ± 0.13	−0.97 ± 0.10	4.05 ± 0.33	0.15 ± 0.01
	IPLA20024	−0.43 ± 0.12	−0.92 ± 0.10	2.87 ± 0.02	0.11 ± 0.01
	IPLA20025	−0.64 ± 0.08	−1.03 ± 0.07	3.47 ± 0.09	0.14 ± 0.02
	IPLA20017	−0.71 ± 0.11	−1.04 ± 0.07	3.50 ± 0.12	0.42 ± 0.01
B. animalis	DSM15954† (Bb12)	−0.58 ± 0.09	−1.00 ± 0.13	3.71 ± 0.33	0.41 ± 0.04
	IPLA20031 (A1dOx)	−0.53 ± 0.05	−1.04 ± 0.07	4.60 ± 0.48	0.58 ± 0.26
	IPLA20032 (A1dOxR)	−0.64 ± 0.11	−1.00 ± 0.12	4.32 ± 0.3	0.42 ± 0.03
	IPLA20020	−0.62 ± 0.07	−1.05 ± 0.08	4.08 ± 0.42	0.42 ± 0.04
B. longum	IPLA20021	−0.16 ± 0.11	−0.60 ± 0.20	1.50 ± 0.14	0.29 ± 0.14
	IPLA20022	−0.06 ± 0.05	−0.06 ± 0.12	0.54 ± 0.18	0.26 ± 0.04
	IPLA20001	−0.09 ± 0.06	−0.33 ± 0.19	1.71 ± 0.05	0.26 ± 0.04
	IPLA20002	−0.07 ± 0.08	−0.57 ± 0.09	1.25 ± 0.16	0.22 ± 0.05
B. breve	IPLA20004	−0.00 ± 0.04	−0.24 ± 0.12	0.75 ± 0.09	0.21 ± 0.08
	IPLA20005	−0.03 ± 0.03	−0.25 ± 0.09	0.56 ± 0.29	0.15 ± 0.01
	IPLA20006	−0.12 ± 0.01	−0.66 ± 0.08	1.05 ± 0.37	0.12 ± 0.01
B. pseudocatenulatum	IPLA20026	−0.45 ± 0.06	−0.91 ± 0.13	3.31 ± 0.02	0.40 ± 0.12
L. crispatus	IPLA20120	−0.44 ± 0.06	−0.91 ± 0.00	4.09 ± 1.64	0.22 ± 0.09
L. gasseri	IPLA20121	−0.42 ± 0.04	−0.91 ± 0.03	3.68 ± 1.28	0.43 ± 0.16
L. paracasei	IPLA20124	−0.39 ± 0.03	−0.83 ± 0.03	4.57 ± 0.26	0.46 ± 0.03
L. rhamnosus	LMG18243* (GG)	−0.41 ± 0.03	−0.83 ± 0.03	3.35 ± 0.44	0.42 ± 0.04

**LMG: Belgian Coordinated Collections of Microorganisms" (BCCM, Gent, Belgium).*
†*DSM: German Collection of Microorganisms and Cell cultures (DSMZ, Braunschweig, Germany).*
The remnant toxins in these NCFS were quantified by ELISA tests. Data were obtained from two biological replicates each measured by duplicate.

The strain *C. difficile* LMG21717 (∼ATCC9689, Ribotype 001, genes *tcdA*+, *tcdB*+, *cdtB*-) producing both TcdA and TcdB toxins (Toxinotype 0) was purchased from the "Belgian Coordinated Collections of Microorganisms" (BCCM, Gent, Belgium). The strain was routinely grown in Reinforced Clostridium Medium (RCM, Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA) in Hungate tubes under anaerobic conditions at 37°C. Frozen stocks (−80°C in RCM + 20% glycerol) were directly activated in RCM broth incubated for 24 h and this culture was used to inoculate (2%) fresh medium that was cultivated for 13 h. This culture was used as inoculum to obtain the toxigenic supernatant.

Preparation of Toxigenic *C. difficile* Supernatant

Conditions to obtain toxigenic supernatant from *C. difficile* LMG21717 have previously been determined and published (Valdés et al., 2015). In short, 300 µl of RCM grown culture were used to inject into Hungates tubes containing 15 ml of Gifu Anaerobic Medium (GAM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). GAM cultures were incubated for 48 h and centrifuged (16,000 × *g*, 10 min) to obtain the *C. difficile*-free toxigenic supernatant (Tox-S), which was kept in several aliquots at −80°C.

Two independent ELISA tests (tgcBIOMICS GmbH, Bingen, Germany) were used to quantify the concentration of TcdA or TcdB in the toxigenic supernatant, as well as the remnant toxins in the neutralized bacterial-supernatants obtained after incubation of Tox-S with bifidobacteria and lactobacilli.

Incubation of Bifidobacteria or Lactobacilli with Toxigenic *C. difficile* Supernatant

The experimental design carried out in this study is schematized in **Supplementary Figure S1A**. Bifidobacteria and lactobacilli cultures grown for 18 h in MRSC were washed twice with PBS and resuspended at 10^9 cfu/ml in the HT29-cultivation medium (MM, described below) supplemented with 5% of Tox-S from *C. difficile* or without supplementation (controls). After incubation for 1 h under anaerobic conditions and mild stirring (∼300 rpm), the bacterial suspensions were centrifuged (16,000 × *g*, 10 min) to obtain bifidobacteria- or lactobacilli-free bacterial supernatants. Then, the pH was increased to 7.55 ± 0.05 with 1 and 0.1 N NaOH and the volume obtained was adjusted to twice the initial one with MM; this means that the maximum amount of remnant toxin that could be present was 2.5%. These neutralized cell-free supernatants (NCFS) were directly used to test their cytotoxicity upon HT29 monolayers as well as to quantify the remnant TcdA and TcdB toxins. This screening was performed with two biological replicates, each analyzed in duplicate, of each bacterial strain using HT29 monolayers of two consecutive passages (p147 and p148).

Incubation of Dead and Live *B. longum* IPLA20022 with Toxigenic *C. difficile* Supernatant

The strain *B. longum* IPLA20022 was selected in order to determine whether the capability to diminish the cytotoxic effect of *C. difficile* supernatant was dependent on bacterial viability. For that purpose UV-treated IPLA20022 suspensions were prepared from MRSC-grown cultures that were washed and resuspended in PBS at 10^9 cfu/ml. Then, the PBS suspension was poured into several petri dishes allowing a high surface spread and they were submitted to ultra violet radiation in a UV-chamber (15W, Selecta, Barcelona, Spain). Three UV cycles of 30 min were applied, homogenizing the PBS suspension in each interval, and the absence of viability was checked by plating serial dilutions of UV-treated IPLA20022 suspension in agar-MRSC (López et al., 2012). Incubation of this UV-treated suspension (dead IPLA20022) with toxigenic *C. difficile* supernatant was performed as previously described. A non UV-killed suspension (live IPLA20022) of the same culture was used as control. After incubation for 1 h, both suspensions were processed to obtain the respective NCFS (**Supplementary Figure S1A**). This experiment was carried out with three independent cultures (biological replicates) of strain IPLA20022 upon HT29 monolayers within the same passage (p149), each measured in duplicate.

Incubation of Supernatants from *B. longum* IPLA20022 with Toxigenic *C. difficile* Supernatant

To test the activity of putative secreted factors by *B. longum* IPLA20022 against *C. difficile* toxins, cell-free bifidobacterial supernatants obtained from three independent-culture replicates (each analyzed in duplicate) were incubated with 50% toxigenic (Tox-S) supernatant for 1 h under anaerobic conditions. Afterward, supernatants were neutralized (pH $\geq$ 7.5) and its cytotoxic activity tested upon HT29 monolayers (passage p149) at 2.5% in MM (**Supplementary Figure S1B**).

Intestinal Epithelial Cell Line HT29 and Culture Conditions

The intestinal cell line HT29 (ECACC 91072201), from human colon adenocarcinoma, was purchased from the "European Collection of Cell Cultures" (Salisbury, UK) and stored at IPLA under liquid N_2. McCoy's Medium (MM) supplemented with 10% foetal bovine serum (FBS), 3 mM L-glutamine and a mixture of antibiotics (50 μg/ml streptomycin-penicillin, 50 μg/ml gentamicin and 1.25 μg/ml amphotericin B) was used for HT29 cultivation. The pH value of supplemented MM was 7.48 ± 0.02. All media and reagents were purchased from Sigma–Aldrich. Maintenance of the cell line, between passages 145 to 149, was performed under standard conditions, at 37°C 5% CO_2 atmosphere, in a CO2-Series Shel-Lab incubator (Sheldon Manufacturing Inc., OR, USA).

Monitoring Behavior of HT29 in RTCA

The real time cell analyzer (RTCA-DP) xCelligence (ACEA Bioscience Inc., San Diego, CA, USA) used to monitor HT29 cells performance upon the different conditions tested, was introduced in a Heracell-240 Incubator (Thermo Electron LDD GmbH, Langenselbold, Germany) set at 37°C with 5% CO_2 atmosphere. This technology records variations in impedance due to the adhesion, growth and morphological changes of HT29 cells during interaction with gold-microelectrodes placed in the bottom of specific microtiter plates (E-plates). The impedance signal is converted in the arbitrary "cell index" (CI) unit which is recorded in the external computer allowing, as well, data analyses through the RTCA software 1.2.1 (ACEA Bioscience).

The method to monitor the damage caused by *C. difficile* toxins was previously described by Valdés et al. (2015). In short, 16-well E-plates were seeded with 2 × 10^5 HT29 cells (in 100 μl) and monitored (recording signal every 15 min) for 22 h to ensure the formation of a monolayer (confluent state). Afterward, the medium was removed and 200 μl of the different bacterial NCFS were added per well. Additionally, wells containing 200 μl of a control without bacteria but with Tox-S (added at 2.5% in MM, cytotoxic control) or 200 μl MM medium without bacteria or Tox-S added (non-cytotoxic control) were included in each experiment. The monitoring continued (every 10 min) for an additional 20–22 h under standard incubation conditions. CI values recorded were normalized by the time of the supernatant addition and by the control sample (MM) as previously described (Valdés et al., 2015). Samples of each bacterial supernatant were obtained from, at least, duplicated biological experiments (two independent Tox-S vs. bifidobacteria or lactobacilli incubations) and each NCFS was tested in duplicate (two independent wells within the same E-plate). Thus, four normalized-CI data were obtained per each bacterial strain tested.

Image Analysis of HT29 Behavior

Time-Lapsed Monitoring in Real Time

Several images were captured in real time using the compact, inverted, optical microscope (40× objective) LumaScope-600 Series (Etaluma, Carlsbad, CA, USA) which was placed inside the Heracell-240 incubator. Images were recordered in an external computer with the software LumaView600Cy 13.7.17.0 (Etaluma). To this end, 2-well μ-Slide (ibiTreat, 1.5 polymer coverslip, tissue culture treated, sterilized slides, Ibidi GmbH, Martinsried, Germany) were seeded with 2 × 10^6 HT29 cells/ml (1 ml) and placed on top of the microscope objective. Images were recorded every 15 min until the confluent state was reached (about 22 h); afterward, culture medium was removed and 1 ml of fresh medium containing 2.5% Tox-S or 1 ml of the NCFS collected after incubation of live *B. longum* IPLA20022 with Tox-S, was added in two independent μ-Slides. Image capture was performed for additional an 16 h.

End-Point CSLM Analysis

HT29 monolayers submitted to different treatments were analyzed by confocal scanning laser microscopy (CSLM) after an end-point incubation period of 20 h. For this, 8-well μ-Slide (ibiTreat, Ibidi GmbH) were seeded with 2 × 10^6 HT29 cells/ml (0.3 ml) and incubated for 22 h to reach confluent state. Afterward, supernatant was removed and wells (in duplicate) were filled with the same volume of fresh medium containing MM (control), 2.5% Tox-S, and NCFS from live or dead *B. longum* IPLA20022 incubated with Tox-S. Incubation continued for additional 20 h; then, supernatant of each well was

removed and HT29 monolayers fixed with 1 vol (0.3 ml) of cold (−20°C) acetone for 10 min. Samples were washed twice with PBS for 5 min under mild stirring and permeabilised with PBS containing 0.1% Triton 100x (Sigma) for 15 min. The nonspecific binding sites were blocked with FBS (25% in PBS) for 20 min and finally washed once with PBS. The Phalloidin-Alexa-Fluor-568 probe (Molecular Probes-Thermo Fisher, Life Technologies S.A., Madrid, Spain) toward *F*-actin was added in 0.3 ml of PBS (final concentration of 25 μl/ml) and samples were incubated overnight at 4°C in darkness. After washing twice with PBS, HT29 nucleus were stained with DAPI probe (Merck-Millipore Cor., Billerica, MA, USA) used at 1:1000 (final dilution in PBS) and incubated under the same conditions for, at least, 6 h. Finally, samples were washed and added to 0.3 ml of PBS previous visualization under microscope.

For the CSLM analysis the Leica TCS AOBS SP8 X confocal microscopy (Leica Microsystems GmbH, Heidelberg, Germany) located in the Scientific-Technical Services of Oviedo University, was used. DAPI and Alexa-Fluor-568 fluorochromes were excited at 405 nm by a blue–violet laser diode and at 578 nm by a white light laser, respectively. *Z*-stacks of HT29 samples were acquired using a 63x/1.4 oil objective applying a line average of 2 to reduce noise on the final images and a z-step of 1 micron. Details of a region were later acquired using a 2.50 optical zoom. Image-captures were recorded with the "Leica Application Suite X" software version 1.8.1.13759 (Leica).

Statistical Analysis

To assess differences in the response (normalized CI) of HT29 due to the anti-toxin activity of *B. longum* IPLA20022, one-way ANOVA followed by SNK (Student-Newman–Keuls, $p < 0.05$) mean comparison tests were performed. The statistical package IBM SPSS Statistics for Window Version 22.0 (IBM Corp., Armonk, NY, USA) was used to carry out these analyses. Legend of **Figure 4** describes the comparisons made in each type of experiment.

RESULTS

The method previously developed by our group to detect in real time the toxic effect of *C. difficile* upon intestinal cell lines was used to address the anti-toxin probiotic potential of twenty bifidobacteria and lactobacilli strains. As an initial step several parameters were optimized in order to establish conditions for the screening using as a biological model confluent-HT29 monolayers (data no shown). Finally, neutralized (pH $\geq$ 7.5) cell-free supernatants (NCFS), obtained after incubation (1 h, 37°C, anaerobiosis) of each strain (about 1×10^9 cfu/ml) with 5% *C. difficile* supernatant (Tox-S), were used for this study (**Supplementary Figure S1A**). The behavior of HT29 monolayers was monitored in real time recording the variations in the impedance signal (normalized-CI) over time due to the presence of the NCFS, the toxigenic control (2.5% Tox-S), or the culture media alone (MM; **Figure 1**). To understand the impedance graphs is worth noting that the lowest normalized-CI value indicates the highest toxigenic capability of *C. difficile* supernatant upon HT29; thus, in **Figure 1**, the red line (representing values obtained with 2.5% Tox-S) is the control for damage, whereas the pink line represents the non-toxigenic control (MM) used as a reference for normalization of all CI values being the reason to have "0 value." Regarding the effect of NCFS, those obtained after incubation of strains in MM medium without *C. difficile* toxins (dotted lines) showed normalized-CI values equal or higher to the control, therefore indicating the absence of any toxic effect induced by the putative probiotics. However, when the NCFS obtained from bacteria incubated with Tox-S were analyzed, HT29 monolayers behaved differently depending on the species considered (**Figure 1**). Graphics obtained clearly show that strains belonging to species *Bifidobacterium bifidum* and *B. animalis* subsp. *lactis* had no protective effect against *C. difficile* toxins since the normalized-CI lines obtained showed similar, or even lower, values than the toxigenic Tox-S control. By contrast, the normalized-CI obtained from the four lactobacilli tested, as well as the strains of *B. longum*, *B. breve,* and *B. pseudocatenulatum* were higher than those induced by *C. difficile* supernatant. In general, normalized-CI lines from *B. longum* and *B. breve* strains were the closest to the control, thus being the strains showing higher anti-toxin capability.

Normalized-CI obtained 4 h after NCFS addition (short term effect) or 22 h after (long term effect) were analyzed in more detail (**Table 1**). Results obtained in the short term showed that all strains belonging to *B. longum* and *B. breve*, as well as *L. gasseri* IPLA20121, *L. paracasei* IPLA20124 and *L. rhamnosus* GG, seemed to have higher values of normalized-CI than the toxigenic control. However, none of the lactobacilli were able to keep the protective effect upon HT29 for a prolonged period (22 h). The strain *C. difficile* LMG21717 used in this study produced about ten-times more TcdA than TcdB (**Table 1**) and the strains showing high protective effect were those that apparently were more effectively in reducing the concentration of TcdA, i.e., belonging to *B. longum* and *B. breve* species (**Table 1**). Indeed, the NCFS obtained from strain *B. longum* IPLA20022 that promoted the lowest damage after 22 h only had 12% of remnant TcdA. Of note is that NCFS from *B. bifidum* and *B. breve* seemed to have a good ability to reduce TcdB levels (remnant between 23 and 44%), although this fact was not correlated with higher protective effect in *B. bifidum* because this species seemed to be less effective against TcdA.

Time-lapsed microphotographs (**Figure 2**) showed that HT29 cells treated with 2.5% toxigenic *C. difficile* supernatant become spherical and the integrity of the monolayer was lost when incubation was prolonged (**Figure 2A**). However, monolayers added with NCFS from live *B. longum* IPLA20022 incubated with Tox-S remained more stable and only after a long incubation period (16 h) some cellular particles were released to the culture medium (**Figure 2B**). Furthermore, although the cytopathic mechanism of *C. difficile* toxins is well known, we performed immunohistochemistry CSLM analysis to confirm the cellular events under different treatments (**Figure 3**). Control HT29 monolayers (grown in MM for 20 h) showed a typical *F*-actin cytoskeleton in which the nucleus is imbibed, thus having an epithelial-like morphology with annexed cells well connected.

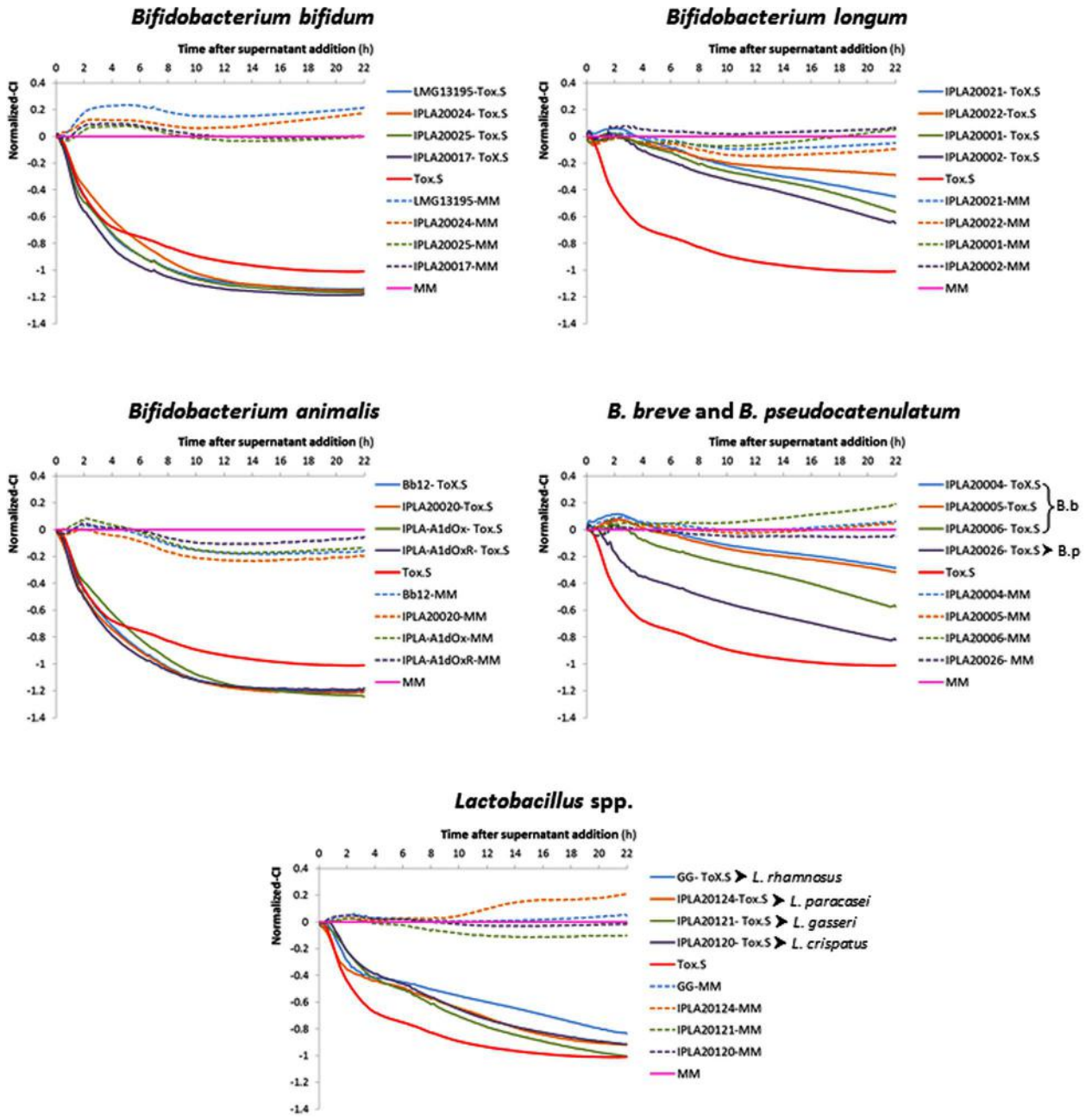

FIGURE 1 | Variation in the normalized cell index (Normalized-CI) of HT29 monolayers treated with different neutralized cell-free supernatants (NCFS; neutralized cell-free supernatants) obtained in a representative experiment after incubation of toxigenic *Clostridium difficile* supernatant (Tox-S) with different strains of *Bifidobacterium* and *Lactobacillus* species. The Tox-S was tested alone at 2.5% (red line). Normalization was performed with respect to the point of NCFS's addition and with respect to the control sample (culture medium MM without supernatant addition) which is the 0-reference control (pink line). The dotted lines represent results obtained with the NCFS obtained after incubation of the same strains in MM (without Tox-S). Representative SD values of these data are collected in **Table 1**.

However, monolayers treated for the same period with Tox-S supernatant lost the interconnection among *F*-actin filaments (**Figure 3B**) and the nucleus seems to be in the initial stages of apoptosis, i.e., the chromatin initiates the condensation showing more intense blue due to DAPI staining (**Figure 3C** and **Supplementary Figure S2**); therefore, HT29 cells become more spherical (non-epithelial morphology) and it seems that the tight junctions that maintain the monolayer integrity might have been disrupted (**Figure 3A**). The photographs obtained from HT29 monolayer treated for 20 h with the NCFS from live *B. longum* IPLA20022 were more similar to the control without toxin; the *F*-actin cytoskeleton still showed an interconnected structure and

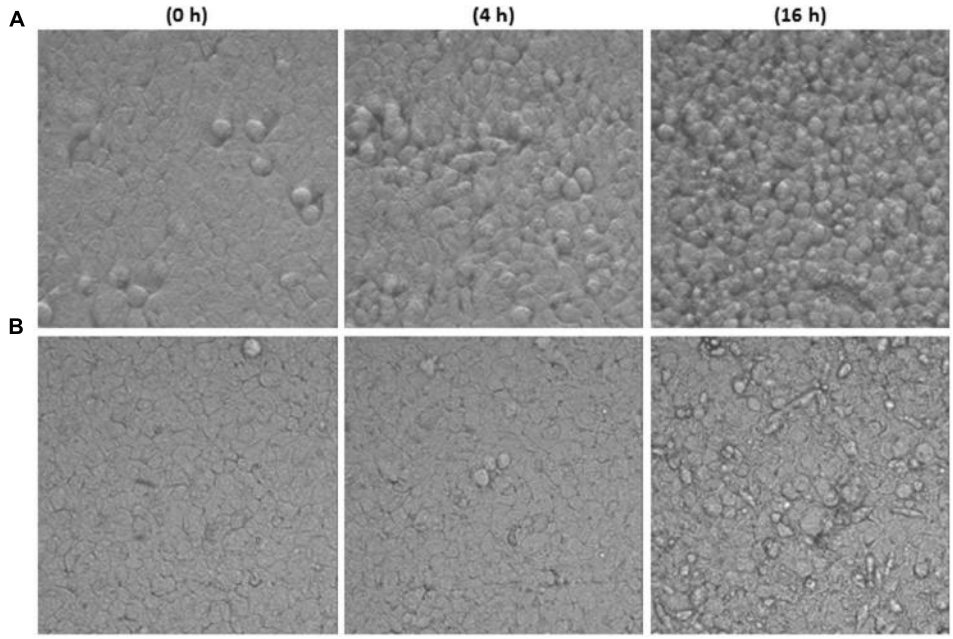

FIGURE 2 | Images of HT29 monolayers captured in real time (37°C, 5% CO_2) with the inverted optical microscope (objective 40×) at three incubation times (0, 4, and 16 h). Monolayer treated with toxigenic *C. difficile* supernatant (Tox-S, **A)** or with NCFS (neutralized cell-free supernatants) obtained after incubation of live *Bifidobacterium longum* IPLA20022 with Tox-S **(B)**.

the nucleus showed less intense DAPI staining comparable to that of the negative control than the toxigenic control. This structure, resembling that of intact epithelial monolayers, is in agreement with the presence of lower amounts of remnant toxin in the NCFS and higher normalized-CI due to the capability of this strain to counteract the effect of clostridial toxins.

In order to determine whether this strain retains its anti-toxin capability under non-viable conditions, *B. longum* IPLA20022 suspension was irradiated with UV light for 90 min. The RTCA monitoring clearly showed that this treatment modified the protective effect of the bifidobacteria upon HT29 since the normalized-CI of the dead strain followed the same tendency as the toxigenic control (**Figure 4A**). Indeed, the statistical analysis performed at 4 and 22 h after NCFS addition showed that live IPLA20022 had a significantly ($p < 0.05$) higher normalized-CI, i.e., higher protective capability, than the dead strain and the toxigenic control (**Figure 4A**). Consequently, the immunohistochemistry study confirmed that the morphology of HT29 treated for 20 h with the NCFS from dead *B. longum* IPLA20022 was more similar to that obtained with the toxigenic control (**Figure 3**). Indeed, besides the *F*-actin modification, some apoptotic bodies were evidenced in both toxigenic and dead-IPLA20022 samples (**Supplementary Figure S2**) suggesting that the UV treatment of this strain, which probably affected the structure and function of the cell envelope, abolished the anti-clostridial effect of *B. longum* IPLA20022. Finally, we have tested the activity against clostridial toxins of the supernatants obtained from overnight cultures of this bifidobacterial strain. Surprisingly, the normalized-CI values were similar to those of those obtained with (live) pellets and both of them were statistically ($p < 0.05$) higher than the toxigenic control (**Figure 4B**). This result suggests that *B. longum* IPLA20022 is able to secrete factors having activity against the toxins of *C. difficile*.

DISCUSSION

The search for novel approaches to treat or prevent CDI is a current "hot-topic" in which the scientific community is devoting much effort. Different approaches are under investigation, most of them toward restoring the dysbiotic intestinal microbiota following infection through FMT (Youngster et al., 2014; Satokari et al., 2015) or using a consortia of defined species (Lawley et al., 2012), but also toward the application of new antibiotics (Babakhani et al., 2013; Vickers et al., 2015) and drugs to treat infections (Oresic-Bender et al., 2015), as well as vaccinations with non-toxigenic *C. difficile* strains (Senoh et al., 2015) or anti-toxin antibodies (Yang et al., 2015). Probiotic bacteriotherapy is becoming an option for the prevention of *C. difficile* recurrent infection (Leffler and Lamont, 2015), and also for the attenuation of CDI symptoms. The choice of the appropriate probiotic against *C. difficile* is of pivotal relevance since, although some formulations seem to be promising (Auclair et al., 2015), not all of them are efficient (Allen et al., 2013).

Probiotic action against CDI is based on different bacterial antagonistic mechanisms, such as competition for adhesion to gut mucosa (Banerjee et al., 2009; Zivkovic et al., 2015)

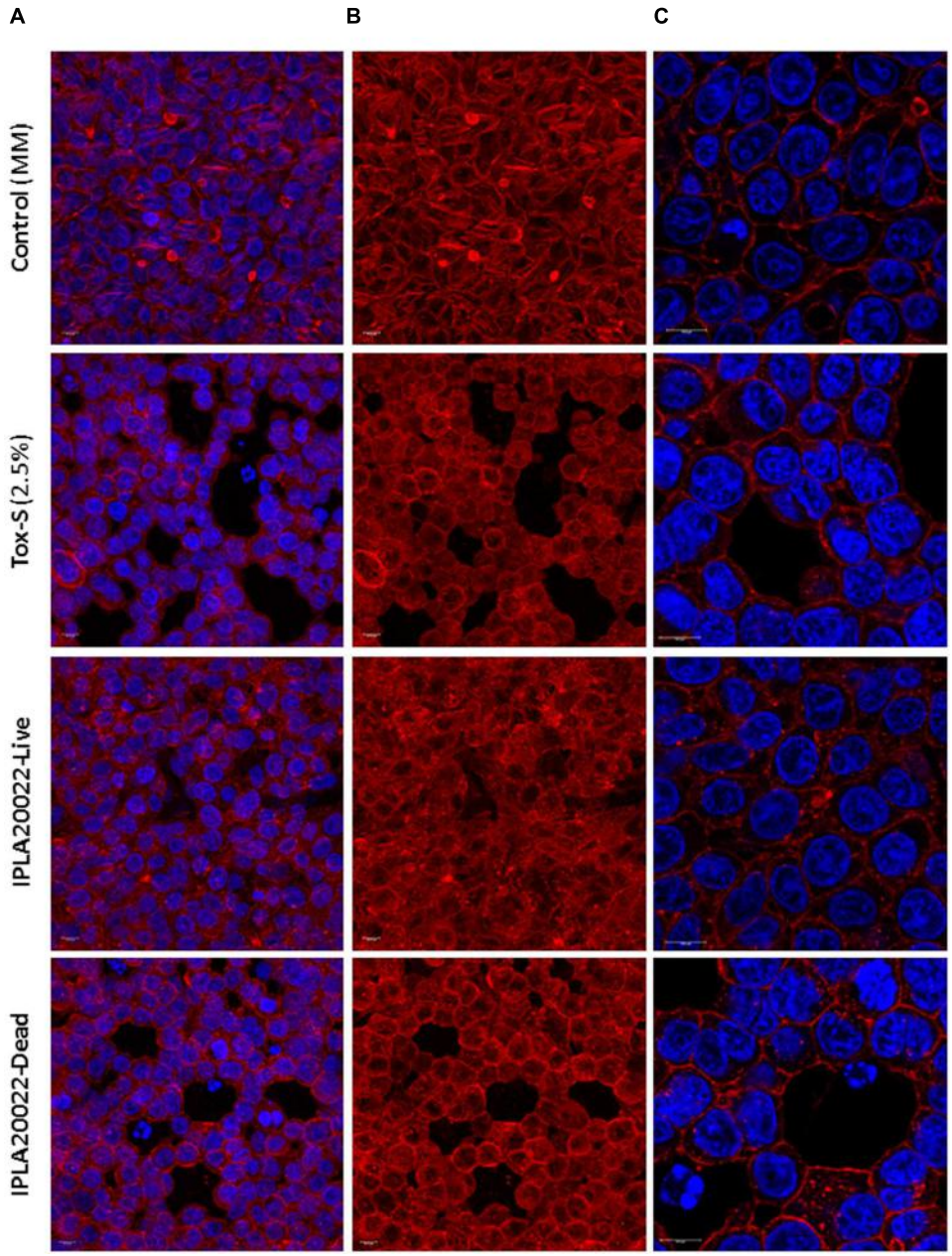

FIGURE 3 | Immunofluorescence images obtained by CSLM of HT29 after 20 h of incubation without toxigenic *C. difficile* supernatant Tox-S (control) and with Tox-S (damage control), and with NCFS (neutralized cell-free supernatants) obtained after incubation of live or dead *B. longum* IPLA20022 with Tox-S. (A) and **(B)** show a *Z*-projection (thickness about 13–15 μm) of 10 XY-slides and **(C)** shows a CSLM-zoom of a XY-region. **(A)** and **(C)** show the combination of DAPI-stained nucleus (blue, excited at 405 nm by a blue–violet laser diode) and *F*-actin stained with Phalloidin-Alexa-Fluor-568 probe (red, excited at 578 nm by a white light laser); *F*-actin is also shown as a single channel in **(B)**. Bars 10 μm.

and for colonization of the intestinal environment (Kondepudi et al., 2014), production of antimicrobial molecules (Schoster et al., 2013; Gebhart et al., 2015) or modulation of intestinal inflammation (Boonma et al., 2014). Another target for probiotic action is the reduction of toxicity caused by *C. difficile* (Trejo et al., 2013). In any case, if one of the active strains would be administered as a probiotic therapy to CDI patients, then the effect would only be present as long as the probiotic is consumed since stable colonization of probiotics in humans has not been shown yet.

In our study, we have explored the capability of twenty lactobacilli and bifidobacteria to counteract the effect of toxins (TcdA and TcdB) from *C. difficile* LMG21717 (equivalent to ATCC9689). The method used, based on impedance

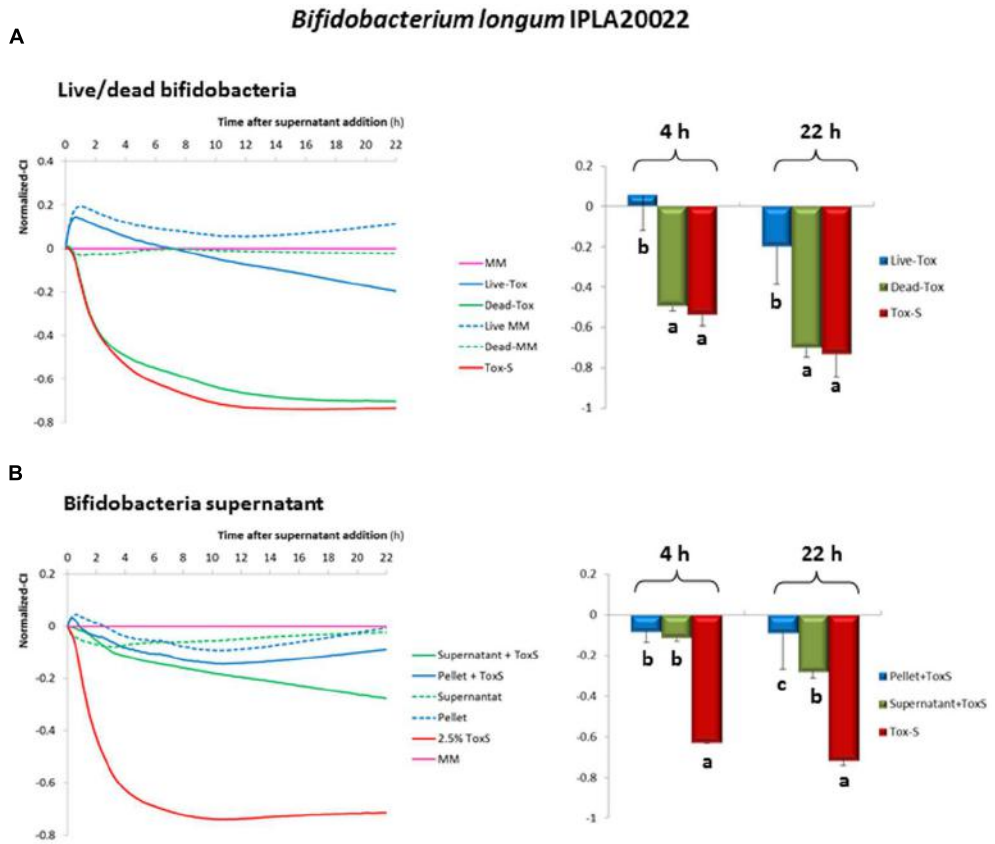

FIGURE 4 | Evolution of the normalized cell index (CI) of HT29 monolayers treated with NCFS (neutralized cell-free supernatants) obtained after incubation of toxigenic *C. difficile* supernatant (Tox-S) with live or dead *B. longum* IPLA20022 (A). Evolution of the normalized-CI of HT29 monolayers treated with 2.5% of neutralized supernatant obtained after incubation of toxigenic *C. difficile* supernatant (Tox-S) with *B. longum* IPLA20022 supernatant **(B)**. Normalization was performed with respect to the point of NCFS's addition and with respect to the control sample (culture medium MM without supernatant addition) which is the 0-reference line (pink line). The dotted lines represent results obtained with the NCFS obtained after incubation of the live or dead *B. longum* IPLA20022 or culture supernatant in MM (without Tox-S). Histograms located in the right of **(A)** and **(B)** sections, represented the mean and standard deviation of normalized-CI values obtained at 4 and 22 h from three biological replicates each measured in duplicate; within the same time, those means that do not share a common letter are statistically different ($p < 0.05$) according to one-way ANOVA and the mean comparison SNK (Student-Newman–Keuls) test.

measurement of HT29 monolayers (Valdés et al., 2015), allowed a quick search of the strains showing the highest anti-toxin ability which those were belonging to *B. longum* and *B. breve* species. This fact suggests that some species-specific characteristics could account for the observed effect, although differences were also detected among strains within the same species. As far as we know, there are few comparative studies among different probiotic species; Trejo et al. (2010) co-cultivated two *C. difficile* strains (including ATCC9689) with twenty five bifidobacteria or lactobacilli and they found that the capability to antagonize the toxic effect upon Vero line (monkey fibroblast-like kidney cells) was strain dependent, but they did not report a species-efficacy association. Nevertheless, the experimental procedure used in our screening for detecting anti-toxicity was based on the incubation of the probiotic strains with a toxigenic supernatant from *C. difficile*, previous to analyze the effect of NCFS upon the biological model HT29. Then, *a priori*, the putative mechanisms that could be behind the anti-toxin capability detected with our approach are the modification of the *C. difficile* toxin and/or its availability for acting on the epithelial cells.

Some authors have reported that probiotics are able to reduce the activity of *C. difficile* toxins. Banerjee et al. (2009) observed that *Lactobacillus delbrueckii* subsp. *bulgaricus* B-30892 releases bioactive components, of unknown nature, able to decrease the toxic effect of *C. difficile* ATCC9689 upon epithelial intestinal Caco2 cells. Similarly, *Lactococcus lactis* subsp. *lactis* CIDCA8221 secretes heat-sensitive products, higher than 10 kDa, that are not affected by treatment with proteases or protease-inhibitors, which were able to protect Vero cells from *C. difficile* toxins (Bolla et al., 2013). *Saccharomyces boulardii* releases an extracellular serin-protease that was able to breakdown the toxin A, as well as to inhibit its binding to the receptor in the brush border of ileal tissue (Castagliuolo et al., 1996). In our case, analysis of the bioactivity of the supernatant collected from strain IPLA20022 directly incubated with the toxigenic *C. difficile* supernatant showed similar effect on HT29

than that obtained with the bifidobacterial pellet. Then, it seems that this strain secreted molecules able to reduce the cytotoxic effect of clostrial toxins. As far as we could find, no exo-proteases have been described for bifidobacteria and only a few peptidases have been characterized (Janer et al., 2005; Seo et al., 2007). Additionally, other molecules inducing conformational changes in proteins that disrupt the active site of other proteins, which could putatively be involved in the inactivation of *C. difficile* toxins, have been described; these are serpins (serin protein inhibitors) found in the genome of *B. longum* (Schell et al., 2002) and *B. breve* (Turroni et al., 2010) and ion chelating agents such as the iron-chelating siderophores (Cronin et al., 2012; Vazquez-Gutierrez et al., 2015). Thus, further and extensive work will be needed in order to decipher the nature of the bifidobacterial secreted factors acting against *C. difficile* toxicity.

Regarding the adsorption as mechanism to reduce toxins activity, it has been demonstrated that the soluble S-layer protein from the surface of *L. kefir* strains diminish the damage of clostridial toxins upon Vero cells, suggesting a direct interaction between the S-layer and the toxins (Carasi et al., 2012). However, as far as we could know, this type of protein cover has not been described for bifidobacteria. Additionally, cellular extracts from *L. acidophilus* GP1B were able to interfere with quorum-sensing signals from *C. difficile* and down-regulated expression of some virulence genes; both, cellular extract and *L. acidophilus* strain, were efficient in increasing the survival rate of animals in a CDI murine model (Yun et al., 2014). The lactic acid synthesized by this lactobacilli strain also had an inhibitory effect on *C. difficile* growth. Similarly, Kolling et al. (2012) reported a bactericidal effect induced by the lactic acid synthesized by *Streptococcus thermophilus* LMD-9 and, furthermore, non-inhibitory levels (10 mM) decreased the *tcdA* expression and toxin-A release. *In vivo* (CDI mouse model) treatment with live *S. thermophilus* showed a significant inverse correlation between levels of luminal lactic acid and *C. difficile* abundance in the murine gut, thus reducing the disease activity indexes of experimentation animals (Kolling et al., 2012). In our experimental design, bifidobacteria were in contact for 1 h only with the toxigenic clostridial supernatant, but not with *C. difficile*, and the putative effect of the organic acids (lactate and/or acetate) produced in this short incubation period by lactobacilli or bifidobacteria was neutralized.

Based on the results describe in this article, the adsorption of toxins to the bifidobacterial surface as well as the presence of secreted molecules responsible for the anti-toxigenic effect observed, are both plausible mechanisms of action. Nevertheless bacterial viability, which may be also needed to keep a functional bifidobacterial envelope, is required in order to maintain the anti-clostridial activity. Finally, the highest anti-toxin capability of *B. longum* and *B. breve* strains (pointing to a species-dependent efficacy) suggests that some specific characteristics of these two phylogenetically close species (Lugli et al., 2014) could account for the anti-clostridial toxicity. Further experiments must be performed in order to understand the mechanism of action behind bifidobacterial anti-*C. difficile* toxicity. Another interesting observation that will deserve further attention is the (apparently) better capability of *B. bifidum*, and to a lower extent of *B. breve*, to specifically reduce TcdB levels.

CONCLUSION

In this work we have optimized a protocol to search for potential probiotics with anti-toxic activity against toxins synthesized by *C. difficile*. The impedance-based, RTCA xCelligence was a fast, reliable and efficient method for the screening of a large collection of bacteria allowing the selection of those strains with higher protection capability. In our case, strains from *B. breve* and *B. longum* showed the better performance, since they were able to reduce the levels of toxins from *C. difficile* supernatants. The best candidate to be used as probiotic to alleviate CDI was *B. longum* IPLA20022; this was the strain with the highest *in vitro* capability for reducing the levels of clostrial toxins, as well as for avoiding the cytopatic effect upon the intestinal epithelial cellular line HT29. Apart for elucidating the mechanism behind this anti-toxigenic capability, the next steps will be to study the efficacy of *B. longum* IPLA20022 in more complex *in vitro* and *in vivo* biological models before proposing its human application to treat CDI.

AUTHOR CONTRIBUTIONS

MG and PR-M contributed with the conception, experimental design and results interpretation of this study. LV-V carried out all experiments, OG-S advised the immunohistochemistry analysis and MA-G perform the CSLM analysis. PR-M was in charge of writing the drafted manuscript. All authors performed a critical revision of the manuscript and approved the final version.

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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.2016.00577

FIGURE S1 | Final conditions used to perform the screening of the anti-*Clostridium difficile* cytotoxic activity upon HT29 monolayers of twenty bifidobacteria and lactobacilli strains (A). Scheme of the flow-work followed to test the capability of *Bifidobacterium longum* IPLA20022 culture supernatant to act against toxigenic *C. difficile* supernatant **(B)** MRSC, MRS broth supplemented with 0.25% L-cysteine; MM, McCoy's Medium added with supplements and antibiotics described in material and methods section; NCFS, neutralized cell-free supernatant.

FIGURE S2 | Immunofluorescence images obtained by CSLM of HT29 after 20 h of incubation without toxigenic *C. difficile* supernatant Tox-S (control) and with Tox-S (damage control), and with NCFS (neutralized cell-free supernatants) obtained after incubation of live or dead *B. longum* IPLA20022 with Tox-S. Images show a CSLM-2.50 optical zoom of a XY-slide. Upper-part images show the combination of DAPI-stained nucleus (blue, excite at 405 nm by a blue–violet laser diode) and *F*-actin stained with Phalloidin-Alexa -Fluor-568 probe (red, excited at 578 nm by a white light laser) **(A)**. Bottom-part images show the same magnification view at the visible (transmitted light) channel **(B)**. Arrows indicates the apoptotic bodies. Bars 10 μm.

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Effect of *Bifidobacterium* upon *Clostridium difficile* Growth and Toxicity When Co-cultured in Different Prebiotic Substrates

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The intestinal overgrowth of *Clostridium difficile*, often after disturbance of the gut microbiota by antibiotic treatment, leads to *C. difficile* infection (CDI) which manifestation ranges from mild diarrhea to life-threatening conditions. The increasing CDI incidence, not only in compromised subjects but also in traditionally considered low-risk populations, together with the frequent relapses of the disease, has attracted the interest for prevention/therapeutic options. Among these, probiotics, prebiotics, or synbiotics constitute a promising approach. In this study we determined the potential of selected *Bifidobacterium* strains for the inhibition of *C. difficile* growth and toxicity in different carbon sources. We conducted co-cultures of the toxigenic strain *C. difficile* LMG21717 with four *Bifidobacterium* strains (*Bifidobacterium longum* IPLA20022, *Bifidobacterium breve* IPLA20006, *Bifidobacterium bifidum* IPLA20015, and *Bifidobacterium animalis* subsp. *lactis* Bb12) in the presence of various prebiotic substrates (Inulin, Synergy, and Actilight) or glucose, and compared the results with those obtained for the corresponding mono-cultures. *C. difficile* and bifidobacteria levels were quantified by qPCR; the pH and the production of short chain fatty acids was also determined. Moreover, supernatants of the cultures were collected to evaluate their toxicity using a recently developed model. Results showed that co-culture with *B. longum* IPLA20022 and *B. breve* IPLA20006 in the presence of short-chain fructooligosaccharides, but not of Inulin, as carbon source significantly reduced the growth of the pathogen. With the sole exception of *B. animalis* Bb12, whose growth was enhanced, the presence of *C. difficile* did not show major effects upon the growth of the bifidobacteria. In accordance with the growth data, *B. longum* and *B. breve* were the strains showing higher reduction in the toxicity of the co-culture supernatants.

Keywords: probiotics, prebiotics, inhibition, *Clostridium difficile*, *Bifidobacterium*, toxin, HT29, RTCA

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INTRODUCTION

Clostridium difficile is often present in the intestinal microbiota of both infants and adults, where it may be found in about 70 and 17% of the subjects, respectively (Ozaki et al., 2004; Jangi and Lamont, 2010). However, this microorganism is also the main causative agent of antibiotic associated diarrhea in nosocomial environments (Leffler and Lamont, 2015). The epidemiology of

C. difficile infection (CDI) is changing, with an increasing occurrence in populations traditionally considered of low-risk (Carter et al., 2012), likely due to the appearance of hipervirulent strains (Rupnik et al., 2009; Yakob et al., 2015). CDI is treated with antibiotics but a high rate of recurrence is present. In this context, new therapeutic alternatives for treating or preventing CDI are being continuously explored, among them the inhibition of *C. difficile* growth by the use of probiotics or prebiotics has been tested (Ambalam et al., 2015; Auclair et al., 2015; Forssten et al., 2015).

In general, probiotics and prebiotics have been proposed as biotherapeutic agents to prevent the dysbiosis caused by antibiotics or infections, and to help the microbiota restoration after it (Reid et al., 2011). The development of food products targeting at the inhibition of *C. difficile* constitutes an interesting approach in the context of the marketing of products bearing health claims. Reducing the intestinal levels of specific pathogens, such as *C. difficile*, has been considered by the European Food Safety Authority (EFSA) as a beneficial physiological effect [(EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2011)]. Therefore, such an effect would constitute an opportunity for the development of food products bearing a health claim in the area of gastrointestinal health.

To date, different probiotic strains and prebiotic substrates have been reported to increase colonization resistance against *C. difficile* (Hopkins and Macfarlane, 2003; Kondepudi et al., 2014; Auclair et al., 2015; Forssten et al., 2015). In addition to their microbiota-modulatory properties, probiotics have been found to protect against infections by other mechanisms, such as production of antimicrobial compounds or competition by adhesion sites or nutrients (Servin, 2004). The ability of certain probiotics, mainly bifidobacteria and lactobacilli, to inhibit *in vitro* the adhesion of *C. difficile* to intestinal epithelial cells or intestinal mucus is well established (Collado et al., 2005; Banerjee et al., 2009). Similarly, the ability to produce antimicrobials inhibiting the growth of *C. difficile in vitro* has been repeatedly reported (Lee et al., 2013; Schoster et al., 2013). However, other potential targets of probiotics and prebiotics on CDI, such as their impact on toxin production by the pathogen, and/or toxin activity, have been explored to a lesser extent and have not attracted attention until recently (Kondepudi et al., 2014; Yun et al., 2014; Andersen et al., 2015). Ambalam et al. (2015) recently reported the ability of cell-free supernatants from some *Lactobacillus* strains, and a probiotic mix, to inhibit the growth of *C. difficile* strains in variable way depending on the carbon source used. Moreover, the authors observed a reduction of toxin titers in those *C. difficile* cultures with inhibitory cell-free supernatants added. Moreover, we have demonstrated that incubation of toxigenic *C. difficile* cell-free culture supernatants with specific bifidobacterial strains reduces the cytotoxic effect upon human epithelial intestinal cells (Valdés et al., 2016). However, the influence of prebiotic substrates upon *C. difficile* growth and toxicity when co-cultured with bifidobacteria remains largely unknown.

In this context the aim of this study was to evaluate *in vitro* the potential of four bifidobacterial strains for inhibiting the growth of *C. difficile* when co-cultured with different prebiotics as carbon source. Moreover, the effect of the strains and prebiotics on the toxicity of the co-culture supernatants upon human intestinal epithelial cells (HT29) was also determined.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The widely used probiotic strain *Bifidobacterium animalis* subsp. *lactis* Bb12 and three strains of bifidobacteria from IPLA culture collection, two of them isolated from infant's feces (*Bifidobacterium longum* IPLA20022 and *Bifidobacterium bifidum* IPLA20015) (Solís et al., 2010) and the other one from breast-milk (*Bifidobacterium breve* IPLA20006) (Arboleya et al., 2011), were used. These last three strains were selected based on the good ability to reduce toxicity of *C. difficile* supernatants (Valdés et al., 2016). With regard to *C. difficile* we used the strain LMG21717, known to produce TcdA toxin and also, although at lower quantities, TcdB. This strain belongs to ribotype 001, which is one of the most common ones found in Europe (Martin et al., 2016). The *Bifidobacterium* strains were routinely grown in MRS (Biokar Diagnostics, Beauvois, France) supplemented with 0.25% L-cysteine (Sigma-Chemical Co., St. Louis, MO, USA) in an anaerobic chamber MG500 (Don Whitley Scientific, Yorkshire, UK) and *C. difficile* was grown in Reinforced Clostridial Medium (RCM, Oxoid, Thermo Fisher Scientific Inc., Waltham, MA) in Hungate tubes as previously described (Valdés et al., 2016). Overnight cultures (18 h) of the bifidobacterial strains and 13 h-old cultures of *C. difficile* were used to inoculate the batch culture fermentations.

For the batch mono- and co-culture fermentations a defined medium with the following composition was used: proteose peptone (10 g/L) (BD-Difco, New Jersey, EE.UU.), beef extract (10 g/L) (BD-Difco), yeast extract (5 g/L) (BD-Difco), polysorbate 80 (1 mL/L) (Sigma), ammonium citrate (2 g/L) (Sigma), sodium acetate (5 g/mL) (Sigma), magnesium sulfate (0.2 g/L) (Probus, Barcelona, Spain), manganese sulfate (0.056 g/L) (Panreac, Barcelona, Spain), and dipotassium phosphate (2 g/L) (Merck, New Jersey, EE.UU). Pairwise combinations of the *C. difficile* strain with the different *Bifidobacterium* strains, as well as the corresponding monocultures, were performed in the medium described above with a 2% (w/v) of different commercial prebiotic substrates added [Synergy 1 (Beneo-Orafti, Barcelona, Spain), Inulin (Sigma) and Actilight (Beghin Meiji and Tereos Syral, Marckolsheim, France)], glucose or without adding any carbon source (used as control). Each media was distributed into Hungate tubes which were inoculated with different *Bifidobacterium* strains at a final level of about 10^5 CFU/ml in case of *B. longum*/*B. breve* and 10^4 CFU/ml in case of *B. bifidum*/*B. animalis*, with *C. difficile* strain at final level of 10^6 CFU/ml or with both of them, in the case of the co-culture. The bifidobacteria were inoculated at a different level depending on the strain with the aim of allowing a balanced growth of both microorganisms (bifidobacteria and clostridia). The appropriate inoculum size was determined in previous experiments (data not shown).

Co-cultures, and the corresponding mono-cultures, in different carbon sources were carried out in triplicate under anaerobic conditions at 37°C for 24 h. Samples were taken at 0 and 24 h for bacterial growth assessment by quantitative PCR (qPCR), quantification of SCFA by Gas Chromatography (GC), pH measurements (pH meter Basic 20+, Crison Instruments S.A., Barcelona, Spain), and toxigenicity determination. One milliliter of each mono-culture or co-culture was centrifuged (16,000 × g for 10 min), and pellets and supernatants were collected. For toxigenic activity upon HT29 cells, the pH of 0.7 ml cell-free supernatant from each batch culture was adjusted to 7.55 ± 0.05 with 1 and 0.1 N NaOH. All supernatants and pellets were immediately frozen at −80°C until use.

Quantification of Bacterial Growth by qPCR

DNA was extracted from pellets of batch cultures using the GenElute Bacterial Genomic DNA Kit (Sigma) and kept at −80°C until analyzed. The levels of *C. difficile* and bifidobacteria in the cultures were determined as DNA copies per ml by qPCR using previously described primers and conditions (Arboleya et al., 2012). Reactions were performed on MicroAmp optical plates sealed with MicroAmp optical caps (Applied Biosystems, Foster City, CA, USA) with a 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). One microlitre of template DNA was used in the 25 mL PCR mixture. Standard curves were made with pure cultures of *B. longum* NCIMB8809 and *C. difficile* LMG 21717. In all cultures the levels of the microorganisms were above the corresponding detection limit of the technique (1×10^3 and 3×10^3 for bifidobacteria and *C. difficile*, respectively). Samples were analyzed by duplicate in at least two independent PCR runs.

Determination of the Production of Short Chain Fatty Acids by GC-MS

Cell-free supernatant (0.1 mL) from each batch culture was mixed with 1 ml methanol, 0.1 ml internal standard solution (2-ethylbutyric 1.05 mg/ml), and 0.1 ml 20% formic acid. This mixture was centrifuged and the supernatant obtained was used for quantification of SCFA by GC in a system composed of a 6890NGC injection module (Agilent Technologies Inc., Palo Alto, Ca, USA) connected to a flame injection detector (FID) and a mass spectrometry (MS) 5973N detector (Agilent) as described previously (Salazar et al., 2011).

Monitoring the Cytotoxic Effect of the Culture Supernatants upon Intestinal Epithelial Cells

The intestinal cell line HT29 (ECACC 91072201) was purchased from the "European Collection of Cell Cultures" (Salisbury, UK) and stored under liquid N_2. McCoy's Medium (MM) supplemented with 10% fetal serum bovine, 3 mM L-glutamine and a mixture of antibiotics (50 μg/ml streptomycin-penicillin, 50 μg/ml gentamicin, and 1.25 μg/ml amphotericin B) was used for HT29 cultivation. All media and reagents were purchased from Sigma-Aldrich. Maintenance of the cell line, between passages 145 and 149, was performed under standard conditions at 37°C 5% CO_2 atmosphere, in a CO_2-Series Shel-Lab incubator (Sheldon Manufacturing Inc., OR, USA). The experimental procedures were carried out with the cell passage 149.

We used an RTCA (real time cell analyser) xCelligence (ACEA Bioscience Inc., San Diego, CA) system, introduced in a Heracell-240 Incubator (Thermo Electron LDD GmbH, Langenselbold, Germany) set at 37°C with 5% CO_2 atmosphere, to monitor HT29 cells behavior. A method previously described, allowing the assessment of the damage caused by *C. difficile* supernatants, was used (Valdés et al., 2015). This method is based in the real-time monitoring of the cell index (CI). This CI is an arbitrary unit that measures the impedance, in gold-microelectrodes coating the surface of E-plates, which changes as consequence of the HT29 cells attachment and growth.

In short, 16-well E-plates were seed with 2×10^5 HT29 cells (in 100 μl), hold in the RTCA equipment, incubated for 22 h to ensure the formation of a monolayer (confluent state) and the CI was monitored (recording signal every 15 min). After this incubation the medium was removed from the wells and the methodology followed was slightly different depending on the experiment. To determine the effect of the carbon source on the toxicity of *C. difficile,* 200 μL of MM containing different concentrations (from 0.63 to 40%, v/v) of cell-free neutralized-supernatants from *C. difficile* mono-cultures were added to the wells. EC50 values (concentration at which half of the maximum damage was detected) for the cultures, in the different carbon sources tested, were then calculated as previously described (Valdés et al., 2015). To determine the effect of bifidobacteria on the toxigenic capability of *C. difficile* in the different carbon sources, 200 μL of MM containing a 5% (v/v) of the neutralized supernatant from each mono- and co-culture were added to the wells. Additionally, wells filled with 200 μl of MM (non-cytotoxic control) were included in each experiment. Then, monitoring continued (recording signal every 10 min) up to 20 h under standard incubation conditions. The data analyses were carried out through RTCA software 1.2.1 (ACEA Bioscience). The CI values were normalized as previously described (Valdés et al., 2015) by dividing the CI at every point by the CI at time zero (the time of the supernatant addition, thus making the CI equal to 1 at this time) and then referred to the normalized CI of the control sample (MM) (the normalized-CI of the control sample is then the "0 line" shown in figures).

Toxin A concentration in the supernatant of *C. difficile* mono-cultures in different carbon sources was determined by ELISA test (tgcBIOMICS GmbH, Bingen, Germany).

Statistical Analysis

To asses differences among carbon sources or between mono- and co-cultures, one-way ANOVAs followed by SNK (Student-Newman-Keuls, $p < 0.05$) mean comparison test were performed. The statistical package IBM SPSS Statistics for Window Version 22.0 (IBM Corp., Armonk NY) was used to carry out these analyses.

RESULTS AND DISCUSSION

Inhibition of *C. difficile* Growth When Co-cultured with *Bifidobacterium* Strains in Different Carbon Sources

There is a great scientific interest on the development of interventions for preventing or treating CDI, including vaccines (Senoh et al., 2015), antimicrobials (Gebhart et al., 2015; Vickers et al., 2015), anti-toxin antibodies (Yang et al., 2015), or genetically engineered bacteria producing them (Andersen et al., 2015), among others. Fecal transplants have demonstrated a high efficacy to treat recurrent CDI (Lee et al., 2016), underlining the importance of the gut microbiota in this disease. Probiotics and prebiotics constitute another interesting option although differences among strains and substrates seem to exist (Allen et al., 2013).

In our study the mono-culture of the *Bifidobacterium* strains (**Figure 1**) in different substrates (dark colored bars) showed that all the strains grew well in glucose. In agreement with previous reports (Rossi et al., 2005; Kondepudi et al., 2012), the strains showed the ability to grow in short-chain fructooligosaccharides (Synergy and Actilight) (scFOS) but they were not able to grow, or did it poorly, in Inulin (**Figure 1**). This observation was further supported by the production of bacterial metabolites (**Figure 2**, Supplementary File) and the pH (Supplementary Figure 1), which in the case of Inulin remained similar to those of the negative control without carbon source added (WCS). Interestingly, *B. longum* IPLA20022 showed a significantly higher growth ($p < 0.05$) in the prebiotics Synergy and Actilight than in glucose (**Figure 1**), whereas no statistically significant differences were observed for *B. breve* IPLA20006 or *B. bifidum* IPLA20015 between glucose and these two prebiotics. The mono-cultures of *B. animalis* Bb12 showed a significantly lower ($p < 0.05$) growth in all prebiotics than in glucose. This strain exhibited the lowest growth of all bifidobacteria in glucose, Synergy, and Actilight (**Figure 1**), which correlates with the limited drop in pH observed for this strain after 24 h of incubation (Supplementary Figure 1). With regard to the pathogen, *C. difficile* grew well in Synergy, not differing significantly from glucose, and to a lower extent in Actilight (**Figure 1**). Therefore, in spite of generally claimed high specific fermentation of prebiotic substrates, some intestinal pathogens may also be able to ferment and grow in some of them. This underlines the importance of a careful selection of the most appropriate strains, substrates, and combinations.

When co-cultured with *C. difficile* in the different carbon sources, the behavior of the bifidobacteria was, in general, similar to that observed in the mono-cultures. We observed increases in bifidobacterial counts in glucose, Synergy, and Actilight and poor grow in Inulin. Regarding *C. difficile*, it grew better in glucose, followed by Synergy, which is in agreement with the

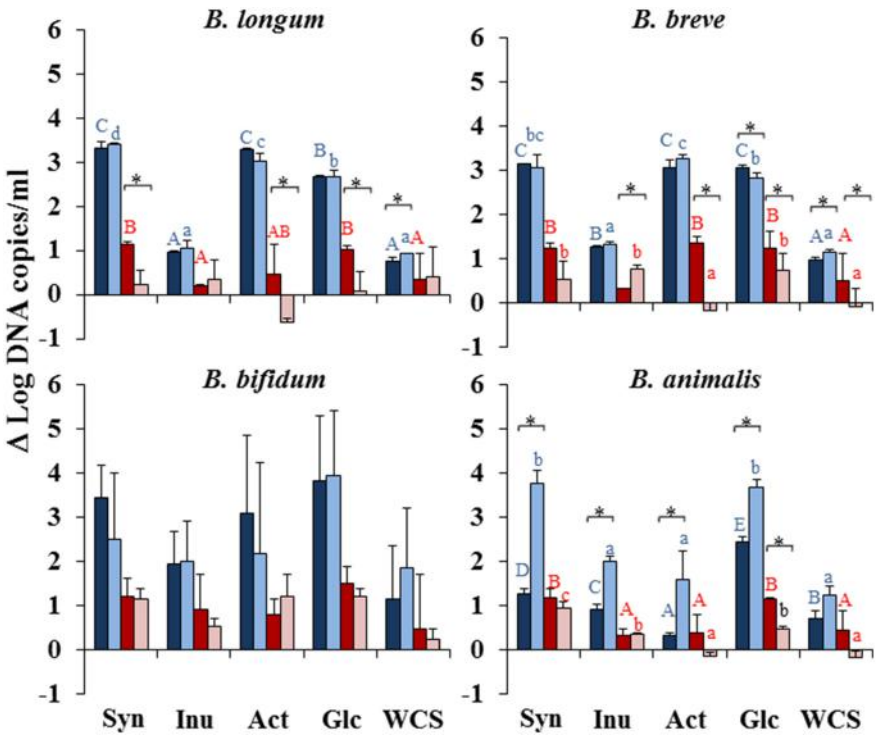

FIGURE 1 | Increments, with respect to time zero, on the levels (Log CFU/mL) of the strains when grown in mono-culture (*Bifidobacterium* dark-blue column and *C. difficile* dark-red) or co-culture (*Bifidobacterium* light-blue and *C. difficile* light-red column) in the prebiotics Synergy (Syn), Inulin (Inu), and Actilight (Act), in glucose (Glc) or without any carbon source added (WCS). Different capital letters above columns denote statistically significant differences ($p < 0.05$) among carbon sources in the mono-cultures of the corresponding bacterial strain, whereas different lowercase letters indicate differences in the co-cultures (either Bifidobacterium in blue letters or *C. difficile* in red letters). *Indicates statistically significant differences ($p < 0.05$) for the corresponding bacterial strain between mono- and co-culture within the same substrate.

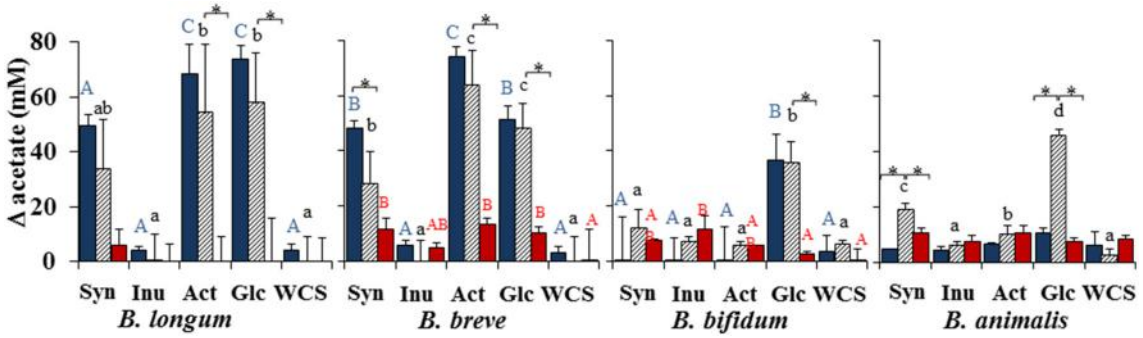

FIGURE 2 | Increments, with respect to time zero, in the concentration of acetate on the bacterial cultures when grown in mono-culture (*Bifidobacterium* blue-bars and *C. difficile* red-bars) or in co-culture (stripped bars) in the prebiotics Synergy (Syn), Inulin (Inu), and Actilight (Act), in glucose (Glc) or without any carbon source added (WCS). Different letters above columns denote statistically significant differences ($p < 0.05$) among carbon sources in the corresponding bacterial cultures, either mono-cultures (capital letter; red color for bifidobacteria and blue color for *C. difficile*) or co-cultures (lowercase letters). *Indicates statistically significant differences ($p < 0.05$) for the corresponding bacterial strain between mono- and co-culture.

mono-culture data, but the growth in Actilight was, in general, significantly ($p < 0.05$) worse in co-culture, the contrary being true for Inulin (**Figure 1**). This growth behavior of *C. difficile* in the different carbon sources was further confirmed by the metabolites production pattern (Supplementary File), showing in general a lower production of *C. difficile* metabolites, such as propionate or branched-SCFA, in co-culture with Actilight as carbon source than in the corresponding mono-culture, whilst the contrary was observed for Inulin.

When co- and mono-cultures were compared within the same carbon source, the growth of *C. difficile* was significantly reduced ($p < 0.05$) by *B. longum* IPLA20022, *B. breve* IPLA20006, or *B. animalis* Bb12 in glucose. The first two microorganisms also reduced *C. difficile* growth when co-cultured in Actilight and, in the case of *B. longum* also when Synergy was used as carbon source (**Figure 1**). On the contrary, no statistically significant differences between mono- and co-cultures were observed for *B. bifidum* in any carbon source. These results showed a good correlation with the pattern of production of *C. difficile* metabolites and the drop in pH (Supplementary File). This suggests the production of organic acids, with the concomitant reduction of the pH, as an important mechanism of inhibition (Tejero-Sariñena et al., 2012).

These results point out at *B. longum* IPLA20022 and *B. breve* IPLA20006, and the prebiotics Synergy and Actilight, as the most promising alternatives for inhibiting the growth of *C. difficile*. Moreover, they suggest that the pathogen inhibition is strain and substrate specific, which is in agreement with previous reports (Kondepudi et al., 2012; Tejero-Sariñena et al., 2013; Ambalam et al., 2015). Interestingly, the growth of *C. difficile* was significantly increased ($p < 0.05$) by *B. breve* in the presence of Inulin, indicating a potential risk of such combination and underlining the importance of a careful strain and substrate specific assessment.

Interestingly, effects of the co-culture with *C. difficile* on the growth of the bifidobacterial strains were also observed. Whilst in glucose the co-culture with the clostridia did not affect the growth of *B. longum*, it significantly ($p < 0.05$) reduced that of *B. breve* but increased that of *B. animalis*. Moreover, the growth of the latter microorganism was also increased by the presence of *C. difficile* in the three prebiotics tested, mainly Synergy (**Figure 1**) which was further confirmed by an enhanced production of acetate in the co-culture than in the corresponding monoculture (**Figure 2**) and a higher drop in pH (Supplementary Figure 1).

The Carbon Source Determines the Toxicity of *C. difficile* Supernatants

In addition to bacterial growth, inhibiting the toxicity caused by *C. difficile*, for example by reducing toxin production or toxic activity, represents another target in CDI (Trejo et al., 2010, 2013). The toxicity of *C. difficile* culture supernatants has been found to be dependent on the culture media used (Valdés et al., 2015), suggesting a potential role of the carbon source available. Therefore, it is important to know whether the availability of different prebiotics as carbon source may have an impact on the toxicity of *C. difficile*. To clarify this point we determined the toxicity of neutralized cell-free supernatants, obtained from *C. difficile* monocultures after 24 h of incubation in the different carbon sources, upon the human epithelial cell line HT29 by using a real-time monitoring system (RTCA). To this end the EC50 values, defined as the concentration of supernatant causing 50% of the maximum cell damage, were calculated (Valdés et al., 2015). Supernatants obtained from the mono-cultures carried out without any carbon source or with Actilight added were significantly ($p < 0.05$) more toxic than the others (**Figure 3**). They showed EC50 values below 2%, which means that a concentration of monoculture supernatant lower than 2% already produced half of the maximum cell damage. On the contrary, the supernatant of the mono-culture in glucose resulted significantly ($p < 0.05$) less toxic than all the others (EC50 value over 6%), followed by that on Synergy and the one carried out with Inulin as carbon source (**Figure 3**). The method used (Valdés et al., 2015) allowed us to determine that the *C. difficile* supernatants' toxicity was higher when no carbon source was added or when the available carbon source supported only a limited growth of

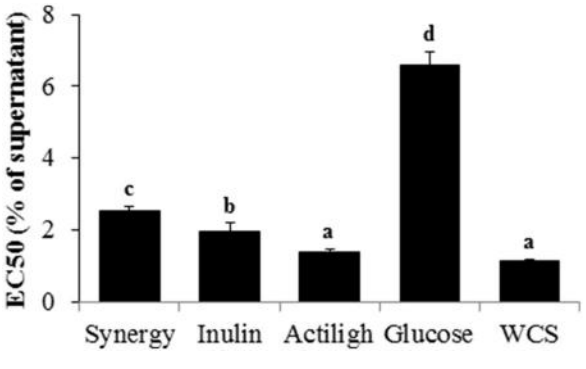

FIGURE 3 | Concentration (% v/v) of supernatants of *C. difficile* mono-cultures, in the different carbon sources tested, showing 50% of the maximum cell damage (EC50). To calculate EC50s the cell indexes obtained after 12 h of incubation of the HT29 cells with supernatants were used. Different letters above the columns denote statistically significant differences ($p < 0.05$).

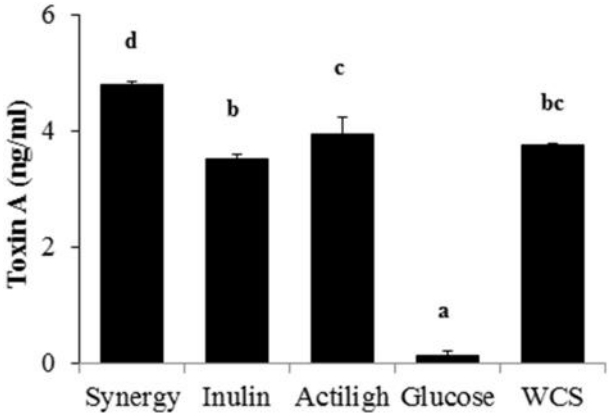

FIGURE 4 | Toxin A concentration in the different *C. difficile* supernatants obtained when the microorganism was growth in the different carbon sources. Different letters above the columns denote statistically significant differences ($p < 0.05$).

the pathogen, such as in the case of Actilight. On the contrary, the supernatant obtained when the pathogen was grown in glucose, in spite of the good growth of *C. difficile*, resulted less toxic. The availability of rapidly metabolizable sugars has been reported to inhibit toxin synthesis in *C. difficile* (Bouillaut et al., 2015). This inhibition is mediated through repression of *treR* (also known as *tdcR*), an alternative sigma factor responsible for the positive regulation of *toxA* and *toxB* genes (Mani et al., 2002). Our results seem to confirm the higher production of toxins by *C. difficile* under nutrient limitation or stress conditions in which readily fermentable sugars are not available. Moreover, in *C. difficile* a co-induction of metabolic pathways, such as that of butyrate production, and toxin production has been reported (Karlsson et al., 2000). In our study the *C. difficile* monoculture grown in glucose showed, in general, lowest butyrate production than those carried out with Synergy, Actilight or WCS added, which is in good agreement with the lower toxin production in glucose. However, the culture of the strain in Inulin, in spite of a lower production of butyrate than that in glucose, showed higher toxin concentrations, comparable to those found WCS or in the other prebiotics tested. These results indicate that, at least in some circumstances, toxin production by *C. difficile* is uncoupled from the production of metabolites such as butyrate.

In accordance with the above mentioned toxicity data, the concentration of *C. difficile* toxin A showed the lowest value in the supernatant from the culture in glucose (**Figure 4**). The supernatants obtained from cultures grown in Synergy, Actilight showed the highest toxin concentrations, whilst those from the growth on *C. difficile* in Inulin or WCS showed intermediate levels.

Co-culture with Bifidobacteria in Different Carbon Sources Reduces *C. difficile* Toxicity

The ability of certain bifidobacterial strains, such as *B. longum* IPLA20022, to remove toxins from *C. difficile* cell-free supernatants, then diminishing their cytotoxicity, has been recently reported (Valdés et al., 2016). Now we compared the toxicity of the *Clostridium-Bifidobacterium* co-culture supernatants with that of the pathogen monoculture. In general we observed a significant reduction on the toxicity of the supernatants in co-culture. However, differences depending on the strain and the carbon source used were also observed, confirming the high specificity of these interactions (Trejo et al., 2010). The toxicities obtained for the co-cultures in the different carbon sources were compared by using the normalized cell index (CI) obtained after 12 h of incubation of HT29 cells with a 5% of the culture supernatants. As it was the case for the monocultures, supernatants from co-cultures carried out on the different carbon sources showed differences among them ($p < 0.05$) (**Table 1**). Similarly to the mono-cultures, supernatants obtained in glucose showed the lowest toxicity whilst those in Inulin, or without any carbon source added, resulted the most toxic. When the supernatants of the co-cultures with the different bifidobacteria were compared with the *C. difficile* monoculture no statistically significant differences ($p > 0.05$) were obtained in media WCS added. However, in all the carbon sources tested, either glucose or prebiotics, statistically significant differences ($p < 0.05$) were observed depending on the bifidobacterial strain used (**Table 1**). Co-culture in Synergy or Actilight of *C. difficile* with *B. longum* IPLA20022 or *B. breve* IPLA20006 significantly ($p < 0.05$) inhibited the toxicity of the supernatant (i.e., higher normalized CI) when compared with the mono-culture of *C. difficile*. However, *B. bifidum* IPLA20015 only was able to reduce ($p < 0.05$) the toxicity of the pathogen with Actilight as carbon source whilst *B. animalis* Bb12 did not produce toxicity inhibition in any prebiotic. The four bifidobacteria tested were able to reduce ($p > 0.05$) the toxicity of the supernatant when co-cultured in glucose, in comparison to the *C. difficile* mono-culture, but none of them did it when the carbon source was Inulin. In the latter case, even, an increase in the toxicity was observed when the pathogen was co-incubated

TABLE 1 | Normalized cell index (mean ± sd) obtained after 12 h of incubation of HT29 cells with the supernatants (5%) of the *C. difficile* mono-culture or *C. difficile-Bifidobacterium* co-cultures grown in different prebiotics, glucose or without any carbon source added (WCS).

Culture	Normalized cell index				
	Carbon source				
	Synergy	Inulin	Actilight	Glucose	WCS
C. difficile	$-0.39 \pm 0.03^{a,1}$	$-0.30 \pm 0.03^{a,2}$	$-0.35 \pm 0.04^{a,1}$	$-0.23 \pm 0.01^{b,1}$	-0.34 ± 0.04^{a}
C. difficile–B. longum	$-0.06 \pm 0.04^{b,2}$	$-0.32 \pm 0.07^{a,2}$	$-0.13 \pm 0.05^{b,3}$	$0.01 \pm 0.02^{b,3}$	-0.43 ± 0.13^{a}
C. difficile–B. breve	$-0.02 \pm 0.02^{d,2}$	$-0.32 \pm 0.03^{b,2}$	$-0.07 \pm 0.01^{c,4}$	$0.00 \pm 0.01^{d,3}$	-0.37 ± 0.01^{a}
C. difficile–B. bifidum	$-0.40 \pm 0.08^{b,1}$	$-0.56 \pm 0.02^{a,1}$	$-0.24 \pm 0.01^{c,2}$	$0.00 \pm 0.01^{d,3}$	-0.34 ± 0.01^{b}
C. difficile–B. animalis	$-0.35 \pm 0.02^{a,1}$	$-0.31 \pm 0.03^{a,2}$	$-0.32 \pm 0.02^{a,1}$	$-0.03 \pm 0.00^{b,2}$	-0.34 ± 0.02^{a}

**Different superscripts letters within the same row indicate statistically significant differences ($p < 0.05$) among carbon sources, whereas different superscript numbers within the same column denote differences ($p < 0.05$) among cultures.*

with *B. bifidum* (**Table 1**), suggesting a potential risk for such combination.

Our results show that *B. longum* IPLA20022 and *B. breve* IPLA20006 reduced the toxicity of the co-cultures with sc-FOS as carbon source. Interestingly these two strains have previously shown the ability to remove *C. difficile* toxins from solution (Valdés et al., 2016). Although the putative mechanism behind toxin inactivation remains to be elucidated, it has been demonstrated that certain microorganisms produce compounds able to degrade *C. difficile* toxins or to reduce their toxicity (Castagliuolo et al., 1996; Banerjee et al., 2009; Carasi et al., 2012; Valdés et al., 2016). These mechanisms may be involved in the effect observed by us. However, given that in our case both microorganisms are co-incubated, the direct inhibition of the growth of the pathogen and/or an modulation of the expression of the toxin genes in *C. difficile* by the presence of bifidobacteria, similarly to that previously reported for *Lactobacillus acidophilus* (Yun et al., 2014), may also be involved. Previous studies pointed out a role of organic acids, such as lactic acid, in the inhibition of both growth and toxin production by *C. difficile* (Kolling et al., 2012; Yun et al., 2014). Therefore, the ability of bifidobacteria to produce acids, mainly acetic and lactic acids, and the pH drop caused by them may partially explain our observations. However, the role of other interactions cannot be overruled, especially since behaviors not explained by the acids, such as the increased toxicity of the co-culture *C. difficile-B. bifidum* in Inulin, were also observed.

CONCLUSION

Co-culture with *B. longum* IPLA20022 or *B. breve* IPLA20006 in the presence of scFOS, but not of Inulin, reduces significantly the growth of *C. difficile*. Moreover, co-culture with these two strains in Synergy or Actilight reduced the toxicity of the *C. difficile* supernatants. Therefore, *B. longum* IPLA20022 and *B. breve* IPLA20006, in combination with Synergy or Actilight, are the most promising strains and compounds for the development of probiotic, prebiotic, or synbiotic products targeting at the reduction of CDI. However, future *in vitro* studies aiming at other clinically relevant *C. difficile* strains, as well as *in vivo* evaluation of the efficacy of the products, would be needed before drawing firm conclusions.

AUTHOR CONTRIBUTIONS

MG and PR contributed with the conception, experimental design, and results interpretation of this study. LV carried out all experiments, AH performed chromatographic analyses. MG was in charge of writing the drafted manuscript. All authors performed a critical revision of the manuscript and approved the final version.

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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.2016.00738

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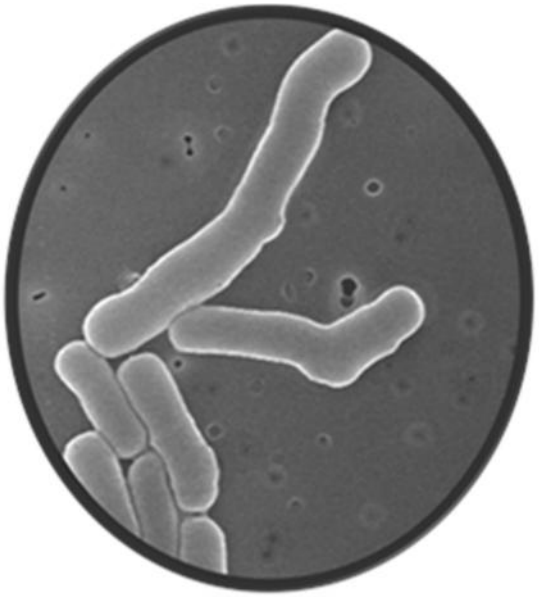
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



4-Discusión General

Los avances en ciencia y medicina, así como la mejora de las condiciones de vida, han conducido a un aumento de la esperanza de vida, y como consecuencia, a un incremento en la proporción de personas de edad avanzada (Woodmansey, 2007). Este envejecimiento de la población implica el concomitante aumento de la prevalencia de enfermedades asociadas a la vejez (Christensen y cols., 2009). Por lo tanto, el incremento de este grupo de población y las comorbilidades asociadas al envejecimiento suponen un gran gasto sanitario para los países desarrollados, en los que la población ya está envejecida y se prevé que envejezca notablemente más durante los próximos años (Sinoff, 2011). Esto pone de manifiesto la necesidad de desarrollar propuestas destinadas a la mejora de la calidad de vida y la salud de las personas de edad avanzada, con la consecuente disminución de los costes sanitarios asociados.

El envejecimiento se ha definido como "la regresión de la función fisiológica que acompaña al avance de la edad" (Biagi y cols., 2010); además, se ha relacionado con cambios en la nutrición (Amarya y cols., 2015), la fisiología del sistema digestivo (Grassi y cols., 2011), el sistema inmunológico (Ibrahim y cols., 2010) y en la composición de la microbiota intestinal (Claesson y cols., 2012; Biagi y cols., 2013; Salazar y cols., 2013). La microbiota intestinal juega un papel muy importante en la salud del hospedador, ya que aporta energía, nutrientes y protección frente a microorganismos patógenos (Woodmansey, 2007). Por lo tanto, las alteraciones en la composición de la microbiota intestinal que tienen lugar durante el envejecimiento pueden incrementar la susceptibilidad a sufrir infecciones y/o otras afecciones (Claesson y cols., 2012; Biagi y cols., 2013). Por esta razón el desarrollo de alimentos funcionales probióticos, prebióticos o simbióticos (combinación de probiótico(s)-prebiótico(s)) específicamente dirigidos a modular la microbiota intestinal de personas de edad avanzada, constituye una estrategia de gran interés. Cabe señalar que dichos productos, diseñados para cubrir las necesidades de este grupo de población específico, no están disponibles actualmente en el mercado.

En este sentido, el trabajo de investigación de esta Tesis Doctoral se ha centrado en la selección racional de probióticos, prebióticos y simbióticos capaces de modular de modo beneficioso la microbiota intestinal de personas de edad avanzada y

activos frente a *C. difficile*, un patógeno de especial relevancia en esta población. En los artículos que componen la sección de trabajo experimental se discuten en detalle los resultados obtenidos en cada uno de ellos; por ello, en esta discusión general se intentará dar una visión global de los hallazgos más destacados de esta Tesis Doctoral, situándolos en el contexto actual de la investigación en microbiota intestinal de personas de la tercera edad, probióticos, prebióticos y simbióticos.

Diversos estudios han indicado que los cambios observados en la composición de la microbiota intestinal asociados al envejecimiento incluyen una menor diversidad microbiana, una disminución de los niveles de microorganismos potencialmente beneficiosos, un incremento de los niveles de bacterias anaerobias facultativas y aerotolerantes, incluyendo bacterias potencialmente patógenas, la disminución de la concentración de AGCCs producidos por la actividad metabólica de la microbiota intestinal y la disminución de la proporción acético/propiónico (Salazar y cols., 2014). Se ha observado también que existen diferencias en la composición de la microbiota intestinal de ancianos en función de su localización geográfica (Mueller y cols., 2006; Biagi y cols., 2013), así como el papel fundamental que juega la composición de la microbiota intestinal basal en la capacidad de modulación de la misma por probióticos y prebióticos (Arboleya y cols., 2013; Likotrafiti y cols., 2014). Por tanto, el desarrollo de alimentos funcionales para personas de edad avanzada, específicamente dirigidos a modular la microbiota intestinal, requiere de una identificación previa de las alteraciones específicas presentes en el grupo de población a tratar; éste sería el paso previo a la selección de probióticos/prebióticos para la modulación de esta comunidad microbiana en la población anciana objetivo (Arboleya y cols., 2012). Varios estudios de intervención han mostrado efectos beneficiosos del consumo de probióticos, prebióticos o ambos, sobre la microbiota intestinal y el sistema inmune en ancianos (Gill y cols., 2001; Bouhnik y cols., 2007; Fukushima y cols., 2007; Schiffrin y cols. 2007; Takeda y Okumura, 2007; Ibrahim y cols., 2010; Tiihone y cols., 2010; Forssten y cols., 2011). Sin embargo, en la mayoría de los casos, para la selección de cepas y/o sustratos no se han tenido en cuenta las necesidades específicas de este grupo de población en términos de modulación de la microbiota, lo que podría explicar, al menos parcialmente, las limitaciones observadas respecto a los resultados positivos en

las cepas o sustratos disponibles actualmente. A diferencia de esos estudios, en el presente trabajo de Tesis Doctoral definimos "*a priori*" unos objetivos delimitados y concretos para la modulación de la microbiota intestinal a partir de las alteraciones específicas, identificadas previamente, en la microbiota de ancianos de nuestra población (Salazar y cols., 2013). Posteriormente, estos objetivos fueron utilizados como criterios de selección (Figura 12) para llevar a cabo una búsqueda *in vitro* de cepas potencialmente probióticas (16 cepas) y de sustratos prebióticos (4 sustratos), específicamente dirigidos a la modulación de la microbiota intestinal de ancianos (Artículo 1).

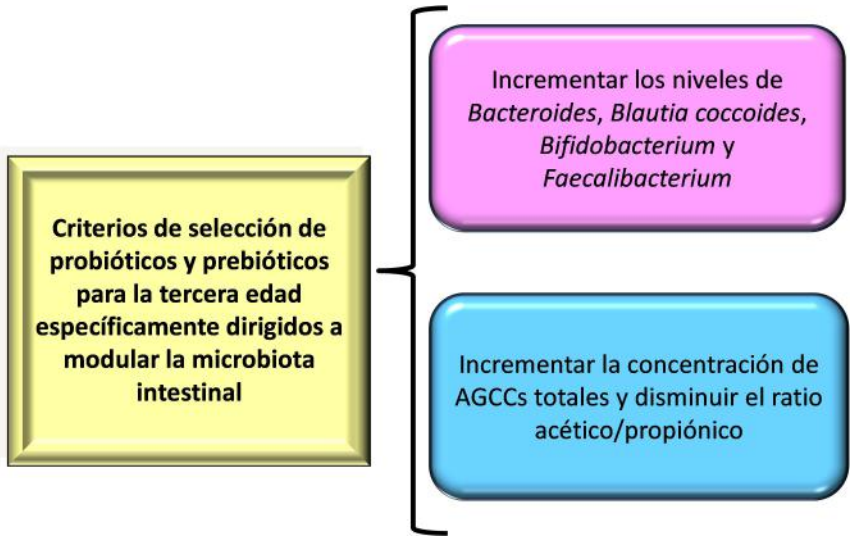


Figura 12. Criterios de selección de probióticos y prebióticos utilizados en este trabajo para la modulación de la microbiota intestinal de ancianos. Modificado de Salazar y cols. (2013).

El modelo de cultivos fecales utilizado en esta Tesis Doctoral (con inóculos fecales de ancianos) permitió seleccionar los sustratos prebióticos y las cepas en base a su actividad sobre la modulación de la microbiota intestinal de esta población (Figura 13). El Actilight resultó ser el sustrato más adecuado para tal fin; en este sentido otros autores también han identificado a los fructooligosacáridos de cadena corta ("short-chain fructooligosaccharides", scFOS) como sustratos adecuados para la modulación

de la microbiota intestinal humana (Gibson y cols., 2010). En cuando a las cepas de bifidobacterias, *B. longum* IPLA20021 e IPLA20022 resultaron ser las potencialmente más adecuadas para aumentar los niveles de los grupos microbianos establecidos en los criterios de selección (Artículo 1).

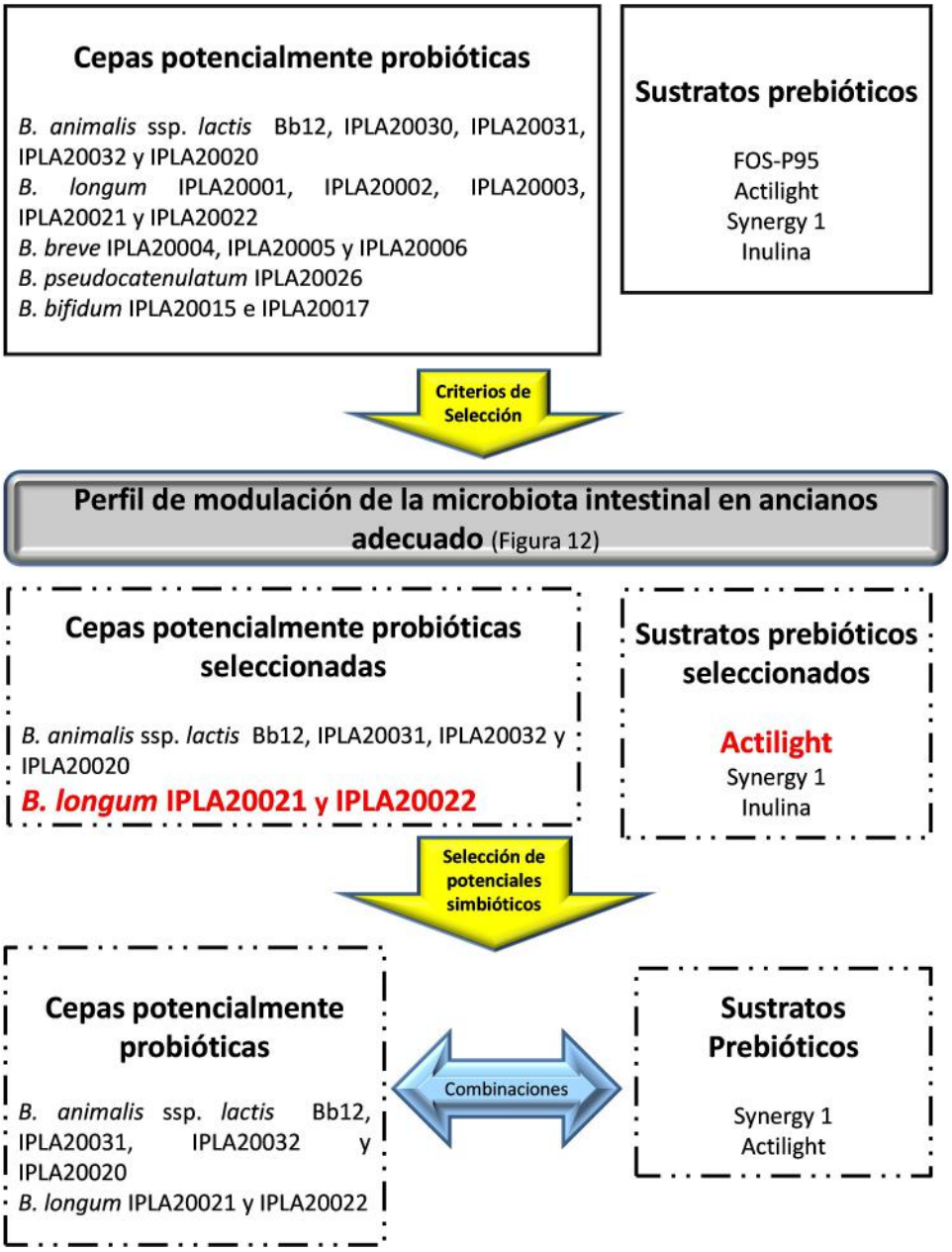


Figura 13. Cepas potencialmente probióticas, sustratos prebióticos y combinaciones de ambos seleccionados por presentar un perfil adecuado para la modulación de la microbiota intestinal en ancianos (Artículos 1 y 2).

Con frecuencia, los estudios de intervención en humanos en los que se administraron probióticos y/o prebióticos se han centrado en determinar sus efectos sobre grupos microbianos muy concretos, especialmente evaluando el incremento de los niveles en heces de *Bifidobacterium* y/o *Lactobacillus*. Sin embargo, los efectos sobre otros grupos microbianos, que también están alterados en las personas de edad avanzada, no han sido muy estudiados (Tiihonen y cols., 2010, Salazar y cols., 2013). Nuestros resultados nos permiten proponer cepas probióticas y prebióticos específicos con potencial para incrementar no sólo los niveles de *Bifidobacterium,* sino también los de otros microorganismos anaerobios, comensales o mutualistas, como *Bacteroides* o *Faecalibacterium* (Artículo 1). Estos microorganismos son, además, conocidos mediadores de efectos antiinflamatorios y reguladores del sistema inmune en la mucosa intestinal (Sokol y cols., 2009; Troy y Kasper, 2011).

En los productos simbióticos, los prebióticos pueden favorecer el crecimiento y mejorar la supervivencia de los probióticos, así como beneficiar a las bacterias autóctonas del tracto gastrointestinal (Sims y cols., 2014). Sin embargo, es bien conocido que la capacidad sacarolítica (Rossi y cols., 2005; Falony y cols., 2009; Mäkeläinen y cols., 2010; Sims y cols., 2014; Selak y cols., 2016), así como la preferencia por oligosacáridos o monosácaridos (Gopal y cols., 2001; Amaretti y cols., 2006; Parche y cols., 2006; Amaretti y cols., 2007; González-Rodríguez y cols., 2013a) de los probióticos depende de la cepa utilizada. Por esta razón, se decidió investigar si las cepas previamente seleccionadas eran capaces de utilizar *in vitro* los sustratos prebióticos seleccionados, para, posteriormente, determinar el potencial simbiótico de las diferentes combinaciones probiótico-prebiótico (Artículo 2, Figura 13). Varios estudios han mostrado que numerosas cepas del género *Bifidobacterium* no son capaces de degradar la inulina (Rossi y cols., 2005; Falony y cols., 2009; Selak y cols., 2016). Nuestros resultados parecen confirmar esta observación ya que ninguna de las 6 cepas de bifidobacterias estudiadas fue capaz de crecer en presencia de inulina. Por el contrario, las cepas si crecieron o fueron metabólicamente activas cuando se utilizaron scFOS como fuente de carbono. Estos resultados indican que Actilight y

Synergy 1 constituyen sustratos adecuados para su combinación con las cepas seleccionadas en un potencial producto simbiótico (Artículo 2).

Las bifidobacterias tienen un metabolismo fermentativo, degradando las hexosas a través de la ruta bífida, también conocida como vía de la fructosa-6-fosfato fosfocetolasa (Sánchez y cols., 2010). Como ya hemos indicado, teóricamente, a través de esta ruta se producen 1.5 moles de ácido acético, 1 mol de láctico y 2,5 moles de ATP por cada mol de glucosa consumido (González-Rodríguez y cols., 2013a); sin embargo, se han observado variaciones en el patrón de ácidos orgánicos producidos, y de sus proporciones, en función de la fuente de carbono y la cepa utilizada (González-Rodríguez y cols., 2013a; Ríos-Covián y cols., 2016b). Esto concuerda con nuestros resultados, ya que en el caso de las cepas de *B. longum* se observaron diferencias en el patrón de metabolitos producidos en función de la fuente de carbono disponible; sin embargo, esto no fue así en el caso de las cepas de *B. animalis* (Artículo 2). Estas observaciones ponen de manifiesto que las interacciones metabólicas entre la fuente de carbono y las cepas potencialmente probióticas son altamente variables. Por tanto, es necesario evaluar cuidadosamente cada una de las combinaciones probiótico-prebiótico usadas en los productos simbióticos. Además, se ha descrito que las diferencias en los metabolitos producidos por distintas cepas de *Bifidobacterium* pueden afectar a la normalización de las propiedades organolépticas de los productos que las contienen y pueden determinar la acción beneficiosa de los microorganismos ingeridos (Ríos-Covián y cols., 2016b). En este sentido, en el presente trabajo hemos seleccionado tanto cepas potencialmente probióticas como sustratos prebióticos en base a su capacidad para modular la microbiota intestinal de personas de edad avanzada. Además, hemos estudiado las características de cada una de las combinaciones probiótico-prebiótico de potencial interés, para el desarrollo futuro de productos simbióticos destinados a la tercera edad.

Como ya se ha indicado anteriormente, las alteraciones en la composición de la microbiota intestinal debidas al proceso del envejecimiento incrementan el riesgo de desarrollar enfermedades, como las infecciones causadas por bacterias toxigénicas. Un ejemplo de este caso, de especial relevancia en personas mayores, es la infección por

C. difficile. La diarrea asociada a este microorganismo es la mayor complicación nosocomial en ancianos hospitalizados (Biagi y cols., 2013). Su tratamiento estándar consiste en el uso de dos antibióticos (metronizadol y vancomicina) (Shields y cols., 2015); sin embargo, el alto porcentaje de recurrencia de la enfermedad (Zanella y cols., 2014; Mizusawa y cols., 2015; Rodríguez y cols., 2015b; Yuille y cols., 2015; Kociolek y Gerding, 2016) hace que se estén estudiando nuevas alternativas y estrategias adyuvantes para la prevención y tratamiento de estas infecciones. Entre estas estrategias, una que ha recibido un creciente interés en los últimos años consiste en el uso del probióticos, prebióticos o simbióticos (Kondepudi y cols., 2014; Ambalam y cols., 2015; Auclair y cols., 2015; Forssten y cols., 2015). El desarrollo de alimentos funcionales con estos microorganismos y sustratos dirigidos a inhibir a *C. difficile* resultaría de gran interés, ya que la EFSA considera la reducción de niveles de patógenos específicos en el intestino como un efecto fisiológico beneficioso (EFSA, NDA ["Panel on Dietetic Products, Nutrition and Allergies"], 2011). Por tanto, el desarrollo de productos funcionales con dicha capacidad constituiría una gran oportunidad para la comercialización de probióticos, prebióticos y/o simbióticos que en su etiquetado podrían portar una declaración de propiedades saludables en el área de salud gastrointestinal.

Distintos estudios han observado como algunos probióticos y prebióticos, debido a su capacidad para modular la microbiota intestinal, poseen la cualidad de incrementar la resistencia a la colonización por *C. difficile* (Hopkins y Macfarlane, 2003; Kondepudi y cols., 2014; Auclair y cols., 2015; Forssten y cols., 2015). Además, se ha mostrado que ciertas cepas probióticas pueden actuar contra *C. difficile* por otros mecanismos, como puede ser la competición por la adhesión a la mucosa intestinal (Collado y cols., 2005; Banerjee y cols., 2009; Zivkovic y cols., 2015), la producción de moléculas antimicrobianas (Lee y cols., 2013; Schoster y cols., 2013; Gebhart y cols., 2015) o la modulación de la inflamación intestinal (Boonma y cols., 2014). Otro potencial mecanismo, poco estudiado aún, es la reducción de la toxicidad causada por este microorganismo (Trejo y cols., 2013; Kondepudi y cols., 2014; Yun y cols., 2014; Andersen y cols., 2015). Algunos autores ya han mostrado como los probióticos pueden reducir la actividad de las toxinas de *C. diffícile,* las cuales son las responsables

del cuadro clínico de la enfermedad (Castagliuolo y cols., 1996; Banerjee y cols., 2009; Bolla y cols., 2013). En esta Tesis Doctoral nos propusimos explorar el efecto de cepas potencialmente probióticas y de sustratos prebióticos sobre la toxicidad de este microorganismo. Para ello, como paso inicial, desarrollamos un modelo capaz de determinar, en tiempo real, la toxicidad de *C. difficile* sobre los enterocitos. Las toxinas A y B de *C. difficile*, una vez en el interior del enterocito, provocan la inactivación irreversible de las GTPasas de la familia Rho, afectando a importantes vías celulares. El redondeo de las células afectadas, como consecuencia de la pérdida de la integridad estructural, precede a la muerte de las mismas y a la ruptura de las uniones estrechas entre ellas. Esta ruptura de las uniones estrechas entre los colonocitos da lugar al aumento de la permeabilidad intestinal y a la pérdida de la función barrera. Las toxinas también inducen la liberación de citoquinas proinflamatorias y neuropéptidos, los cuales dan lugar a una fuerte respuesta inflamatoria (Carter y cols., 2012; Awad y cols., 2014). Por tanto, un enfoque terapéutico de gran interés para el tratamiento de los síntomas asociados con la infección de *C. difficile* sería el desarrollo de agentes contra la actividad de las toxinas que eviten los efectos perjudiciales que se producen como consecuencia de la acción de las mismas (Tam y cols., 2015). Hasta ahora, la búsqueda de estos agentes se había visto obstaculizada por la carencia de un método de selección *in vitro* rápido y fiable. En el contexto de esta Tesis, hemos desarrollado un método biológico *in vitro* basado en la monitorización continua de la impedancia a través del RTCA (Figura 14), que permite estudiar la cinética de citotoxicidad de los sobrenadantes procedentes de un cultivo de *C. difficile* toxigénico sobre una línea celular intestinal. Este método puede ser utilizado para el escrutinio de nuevos agentes contra la actividad de las toxinas. Se utilizó la cepa de *C. difficile* LMG21717 por pertenecer al toxinotipo 0 (productor de las toxinas A y B) y a un ribotipo (001) muy frecuente en Europa (Martin y cols., 2016). El método desarrollado resultó ser rápido, eficaz y fiable para monitorizar la toxicidad, que resultó ser dosis-dependiente, de esta cepa sobre las líneas celulares HT29 y Caco2 (Artículo 3).

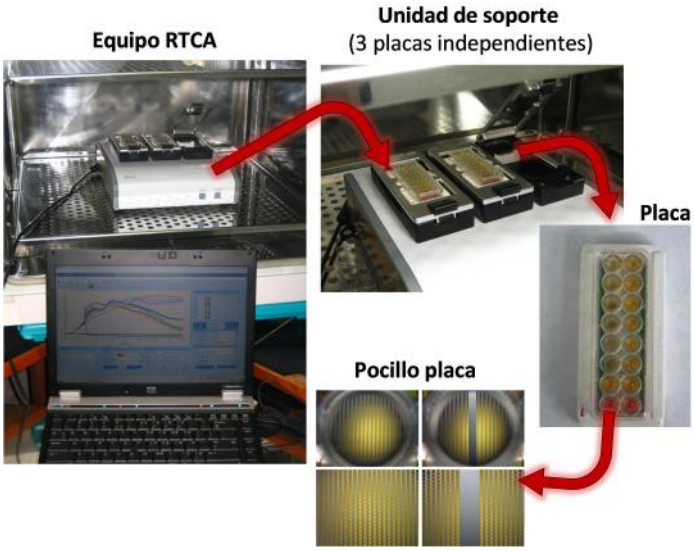


Figura 14. Equipo RTCA (Real time cell analyser) xCELLIgence (ACEA Bioscience Inc., San Diego, CA) localizado en el IPLA-CSIC.

Dentro de las tecnologías sin marcaje para ensayos celulares se encuentran las basadas en la impedancia, como el RTCA, cuya principal aplicación es la evaluación de nuevos fármacos (Xi y cols., 2008). El RTCA recoge variaciones en la impedancia debidas a la adhesión, crecimiento y cambios morfológicos de la línea celular detectados por unos microelectrodos de oro ubicados en el fondo de placas específicas. La señal de impedancia es convertida en una unidad arbitraria "cell index" (CI), que es recogida por un ordenador. Esta tecnología también ha sido usada para la detección de toxinas bacterianas, el análisis de la interacción entre bacteria y célula hospedadora, la cuantificación del efecto citopático inducido por virus y la detección de la neutralización de anticuerpos, entre otras aplicaciones (Huang y cols., 2014b). En cuanto a la detección de toxinas bacterianas, algunos investigadores han empleado el RTCA para la cuantificación del daño causado por las toxinas de *C. difficile* (He y cols., 2009; Ryder y cols., 2010). Además, este equipo se ha usado para desarrollar un método que permite la detección directa de las toxinas de *C. difficile* en muestras fecales (Huang y cols., 2014b). Sin embargo, para llevar a cabo estos estudios con las

toxinas de *C. difficile* se han utilizado líneas celulares de origen no intestinal, como la línea celular HS27 (fibroblastos obtenidos de piel humana) (Ryder y col., 2010; Huang y cols., 2014a) o las células mRG1-1 (células modificadas genéticamente que proceden de células CHO, con morfología epitelial que derivan del ovario de hámster) (He y cols., 2009; Steele y cols., 2012). En nuestro caso, teniendo en cuenta que el objetivo era desarrollar un método que, posteriormente, permitiera llevar a cabo un escrutinio de potenciales agentes contra la actividad de las toxinas de *C. difficile* capaces de actuar en el intestino humano, escogimos líneas celulares de este origen (HT29 y Caco2), ya que los enterocitos son la principal diana sobre las que van actuar estas toxinas. Además, decidimos trabajar con las células intestinales en fase de confluencia, ya que en esa fase se encuentran en un estado (monocapa) que es más semejante a las condiciones fisiológicas del epitelio intestinal.

Los resultados obtenidos a través del RTCA para monitorizar el efecto citotóxico de sobrenadantes neutralizados procedentes del cultivo de *C. difficile* LMG21717 sobre la HT29 mostraron una gran influencia del medio de cultivo utilizado para el crecimiento de *C. difficile*. Entre los medios empleados los hay que impidieron la liberación/secreción de las toxinas al sobrenadante, ya que en ciertos casos quedan adheridas al precipitado bacteriano (Artículo 3). Además, en estudios de otros autores se ha observado que las condiciones de cultivo (Lei y Bochner, 2013) o el medio de cultivo utilizado (Boetzkes y col., 2012) pueden inducir o reprimir la síntesis de toxinas por parte de *C. difficile*. También se ha demostrado que la levadura, así como el extracto de carne porcina y vacuna, tienen potencial para contrarrestar los efectos perjudiciales de la toxina A (Duncan y cols., 2009). En nuestro caso, de todos los medios usados, el sobrenadante obtenido de un cultivo de *C. difficile* con mayor efecto tóxico sobre las líneas celulares HT29 y Caco2 fue el recogido del cultivo con GAM ("Gifu Anaerobic Medium") a las 48 horas de incubación. El efecto citotóxico observado sobre ambas líneas celulares con este sobrenadante fue dependiente de la dosis, lo cual nos permitió calcular distintos parámetros toxicológicos: LOAEL ("Lowest Observed Adverse Effect Level"), NOAEL ("No Observed Adverse Effect Level") y EC50 ("Half Maximal Effective concentration") (Artículo 3). Al comparar nuestros resultados, sobre una línea celular de origen intestinal, con los resultados obtenidos en otros

estudios sobre líneas celulares de origen no intestinal (He y cols., 2009; Ryder y cols., 2010; Steele y cols., 2012; Huang y cols., 2014a) llegamos a la conclusión de que el límite de detección de la toxicidad de estas toxinas es variable y dependiente, tanto de la línea celular utilizada, como de la preparación de las muestras biológicas. En otros estudios con líneas celulares también se ha observado que el modelo celular utilizado (incluso usando células procedentes del mismo tejido, por ejemplo HT29 y Caco2) va a influir en los resultados obtenidos (Hidalgo-Cantabrana y cols., 2014). Esto concuerda con nuestros resultados, ya que utilizando la línea celular Caco2 de origen intestinal humano también observamos un efecto citotóxico dependiente de la dosis del sobrenadante procedente del cultivo de *C. difficile,* pero a dosis más altas; por tanto, los parámetros toxicológicos son diferentes a los obtenidos con la línea celular HT29 (Artículo 3). Por otro lado, los resultados obtenidos a través del RTCA fueron confirmados con imágenes capturadas en tiempo real por un microscopio óptico invertido. Las imágenes obtenidas nos permitieron observar el redondeo de las células HT29 inducido por las toxinas (Artículo 3). Este cambio morfológico ya había sido observado en otras líneas celulares de origen no intestinal al ser tratadas con las toxinas de *C. difficile* (Steele y col., 2012; May y col., 2013). Las imágenes obtenidas a tiempo real permiten detectar eventos celulares como la muerte celular; por ello, recientemente se ha desarrollado un método basado en imágenes para identificar moléculas que protegen a células de origen no intestinal (CHO-K1, Vero y IMR-90) del redondeo inducido por la toxina B (Tam y cols., 2015).

Una vez desarrollado el modelo para monitorizar a tiempo real el efecto citotóxico de *C. difficile* sobre líneas celulares intestinales, se aplicó para evaluar el potencial probiótico de 20 cepas, pertenecientes a los géneros *Bifidobacterium* y *Lactobacillus* (Figura 15), para contrarrestar *in vitro* el efecto citotóxico de *C. difficile* LMG21717 sobre la línea intestinal humana HT29, es decir, para analizar su capacidad antitoxigénica (Artículo 4). Para llevar a cabo este objetivo incubamos las bacterias con los sobrenadantes de *C. difficile* obtenidos en GAM. Tras esta incubación, recogimos los nuevos sobrenadantes para cuantificar las toxinas A y B remanentes y para determinar el efecto tóxico residual sobre la línea celular HT29, a través del modelo

biológico *in vitro* descrito anteriormente. Las cepas de *B. longum* y *B. breve* estudiadas (Figura 15) fueron las que mostraron una mayor capacidad protectora sobre la línea HT29 y fueron las más efectivas en reducir los niveles de toxinas en los sobrenadantes procedentes del cultivo de *C. difficile*. Esto sugiere que algunas características específicas de estas dos especies, las cuales están muy próximas filogenéticamente (Lugli y cols., 2014), podrían explicar el efecto antitoxigénico observado, aunque también se detectaron diferencias entre cepas de la misma especie. Dentro de las cepas con mejor capacidad antitoxigénica, *B. longum* IPLA20022 fue la que mostró una mayor reducción *in vitro* de los niveles de toxina, atenuando los efectos perjudiciales de las toxinas sobre la línea celular HT29 (Figura 15).

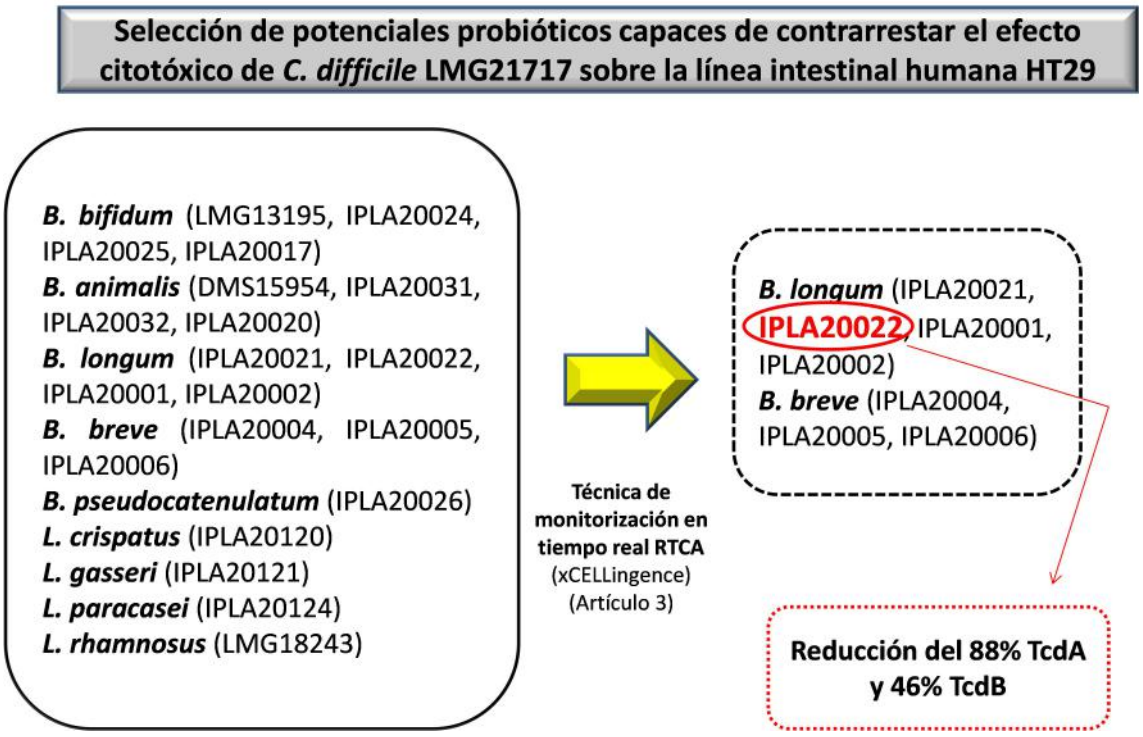


Figura 15. Escrutinio de potenciales probióticos para contrarrestar el efecto citotóxico de *C. difficile* LMG21717 sobre la línea intestinal HT29 mediante técnicas de monitorización en tiempo real (Artículo 4).

Por otra parte, nuestros resultados mostraron que es necesario que la cepa *B. longum* IPLA20022 esté viable para que pueda ejercer su efecto protector sobre la línea HT29 (Artículo 4). Además, los resultados obtenidos al examinar la actividad antitoxigenica de los sobrenadantes obtenidos de cultivos de *B. longum* IPLA20022 crecidos durante 18 horas fueron similares a los obtenidos con las propias células bacterianas. Esto sugiere que *B. longum* IPLA20022 es capaz de secretar al sobrenadante un(os) factor(es) con actividad contra las toxinas de *C. difficile*. Es conocido que la levadura *S. boulardii,* así como *L. delbrueckii* subsp*. bulgaricus* B-30892 y *Lactococcus lactis* subsp. *lactis* CIDCA8221, también son capaces de liberar agentes que reducen la toxicidad de *C. difficile* (Castagliuolo y cols., 1996; Banerjee y cols., 2009; Bolla y cols., 2013). Otro resultado a destacar, es que algunas cepas de *B. bifidum* y de *B. breve* se mostraron como las más efectivas para reducir los niveles de toxina B; sin embargo, este hecho no se correlacionó con un mayor efecto protector sobre la línea HT29, probablemente debido a que estas especies se mostraron menos efectivas contra la toxina A, que es la toxina mayoritaria de *C. difficile* LMG21717. El análisis del comportamiento de la línea HT29 a través de técnicas de imagen confirmó los datos obtenidos a través del RTCA; observándose que la cepa *B. longum* IPLA20022 previene el redondeo de las células, ya que mantienen su morfología epitelial. Esto es debido a que se conserva la microestructura de su citoesqueleto de F-actina y las uniones estrechas entre células, según se visualizó en imágenes de inmunofluorescencia obtenidas con microscopio confocal láser de barrido a un tiempo final determinado (Artículo 4).

Hasta la fecha, se conoce poco acerca de la influecia que tienen los sustratos prebióticos sobre el crecimiento y la toxicidad de *C. difficile* cuando se cocultiva con bifidobacterias. Por esta razón, decidimos determinar el potencial de distintas cepas del género *Bifidobacterium* (*B. animalis* Bb12, *B. longum* IPLA20022, *B. breve* IPLA20006, *B. bifidum* IPLA20015) para reducir el crecimiento y la toxicidad de *C. difficile* LMG21717 en presencia de varios sustratos prebióticos seleccionados (Synergy 1, Actilight e inulina) (Artículo 5). Con esta finalidad, realizamos cocultivos de la cepa *C. difficile* LMG21717 con las 4 cepas del género *Bifidobacterium* en presencia de los

diferentes sustratos prebióticos como fuentes de carbono, comparándose los resultados con los obtenidos en los correspondientes monocultivos. La cepa *B. animalis* Bb12 se incluyó en este estudio por ser ampliamente usada como probiótico, mientras que el resto fueron seleccionadas en base a su capacidad para reducir la toxicidad de los sobrenadantes de un cultivo de *C. difficile* (Artículo 4). En los cocultivos y monocultivos llevados a cabo, determinamos los niveles de las bifidobacterias y de *C. difficile* por medio de la q-PCR y también se siguió el pH y la producción de AGCCs. Además, recogimos los sobrenadantes neutralizados de los cultivos para evaluar su toxicidad usando el modelo biológico desarrollado por nosotros (Artículo 3). Algunos autores ya han sugerido que la capacidad para inhibir el crecimiento de *C. difficile* (Kondepudi y cols., 2012; Tejero-Sariñena y cols., 2013; Ambalam y cols., 2015) o para disminuir la toxicidad de sus sobrenadantes (Trejo y cols., 2010) es específica de cepa y de sustrato, lo cual hemos corroborado con nuestros resultados (Artículo 5). En los cocultivos con Synergy 1 y Actilight de *C. difficile* con *B. longum* IPLA20022 o *B. breve* IPLA20006 se redujo significativamente el crecimiento del patógeno y la toxicidad de los sobrenadantes procedentes del cocultivo (Figura 16); sin embargo, el crecimiento del patógeno aumentó en el cocultivo con *B. breve* IPLA20006 en presencia de la inulina, así como la toxicidad de los sobrenadantes procedentes del cocultivo con *B. bifidum* también se vio incrementada en presencia de inulina.

Por otro lado, nuestros resultados mostraron como *C. difficile* es capaz de crecer en monocultivo con Synergy 1, y en menor medida, en Actilight. Además, observamos que la toxicidad y la concentración de toxinas en el monocultivo del patógeno vienen determinadas por la fuente de carbono añadida (Artículo 5). Se ha descrito que la disponibilidad de azúcares rápidamente metabolizables inhibe la síntesis de toxina en *C. difficile* (Bouillaut y cols., 2015). Nuestros resultados parecen confirmar esta observación, ya que la toxicidad y la cantidad de toxina en los sobrenadantes procedentes del monocultivo fueron mayores en el realizado sin fuente de carbono añadida, o en aquel en el que la fuente de carbono disponible solo permitió un crecimiento limitado del patógeno, como fue el caso del Actilight. Por tanto, los resultados obtenidos de este trabajo demuestran la importancia de llevar a cabo una selección minuciosa de las cepas, sustratos y las combinaciones de ambos

más adecuadas para contrarrestar el efecto de *C. difficile*. De nuevo, las cepas *B. longum* IPLA20022 y *B. breve* IPLA20006, junto con los sustratos Synergy 1 y Actilight, se señalan como las combinaciones que presentan mayor potencial para la inhibición del crecimiento y la reducción de la toxicidad de *C. difficile* (Figura 16).

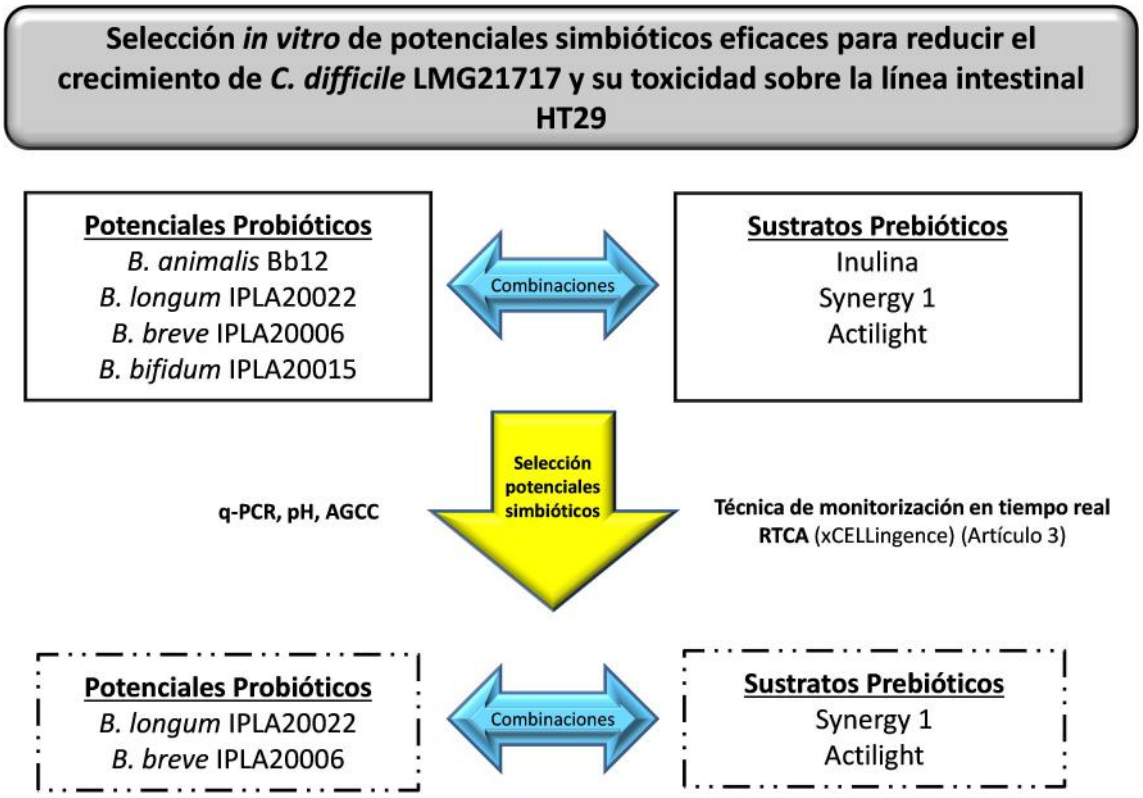


Figura 16. Escrutinio de potenciales simbióticos *in vitro* para la reducción del crecimiento de *C. difficile* LMG21717 y su citotoxicidad sobre la línea intestinal HT29 (Artículo 5).

En resumen (Figura 17), en los resultados de esta Tesis Doctoral mostrados en el capítulo 1 se han identificado *in vitro* varias cepas potencialmente probióticas, prebióticos y combinaciones simbióticas adecuadas para el desarrollo de alimentos funcionales dirigidos a modular la microbiota intestinal de personas de edad avanzada. Mientras que a partir de los obtenidos en el capítulo 2, se han seleccionado candidatos de los mismos agentes, eficaces para reducir *in vitro* el crecimiento y toxicidad de *C.*

difficile, patógeno muy común en ancianos hospitalizados. Además, se ha desarrollado un modelo biológico *in vitro* que ha posibilitado monitorizar a tiempo real el efecto citotóxico de sobrenadantes de un cultivo de *C. difficile,* el cual se podría hacer extensible a otros patógenos productores de enterotoxinas. Estos trabajos nos han permitido identificar cepas y prebióticos concretos, destacando *B. longum* IPLA20022 y los scFOS Actiligh y Synergy 1, con gran potencial para su aplicación en el desarrollo de productos funcionales dirigidos a la tercera edad, lo que constituía el objetivo último de este trabajo.

Además, los resultados que se presentan en esta memoria de Tesis Doctoral abren nuevas vías de trabajo, como confirmar los resultados obtenidos con las cepas potencialmente probióticas, sustratos prebióticos y simbióticos seleccionados en modelos biológicos más complejos y en estudios de intervención en humanos. Se identifica también una vía de estudio dirigida a dilucidar los mecanismos moleculares por los cuales las cepas seleccionadas, como *B. longum* IPLA20022, son capaces de reducir el efecto negativo de las toxinas producidas por *C. difficile*.

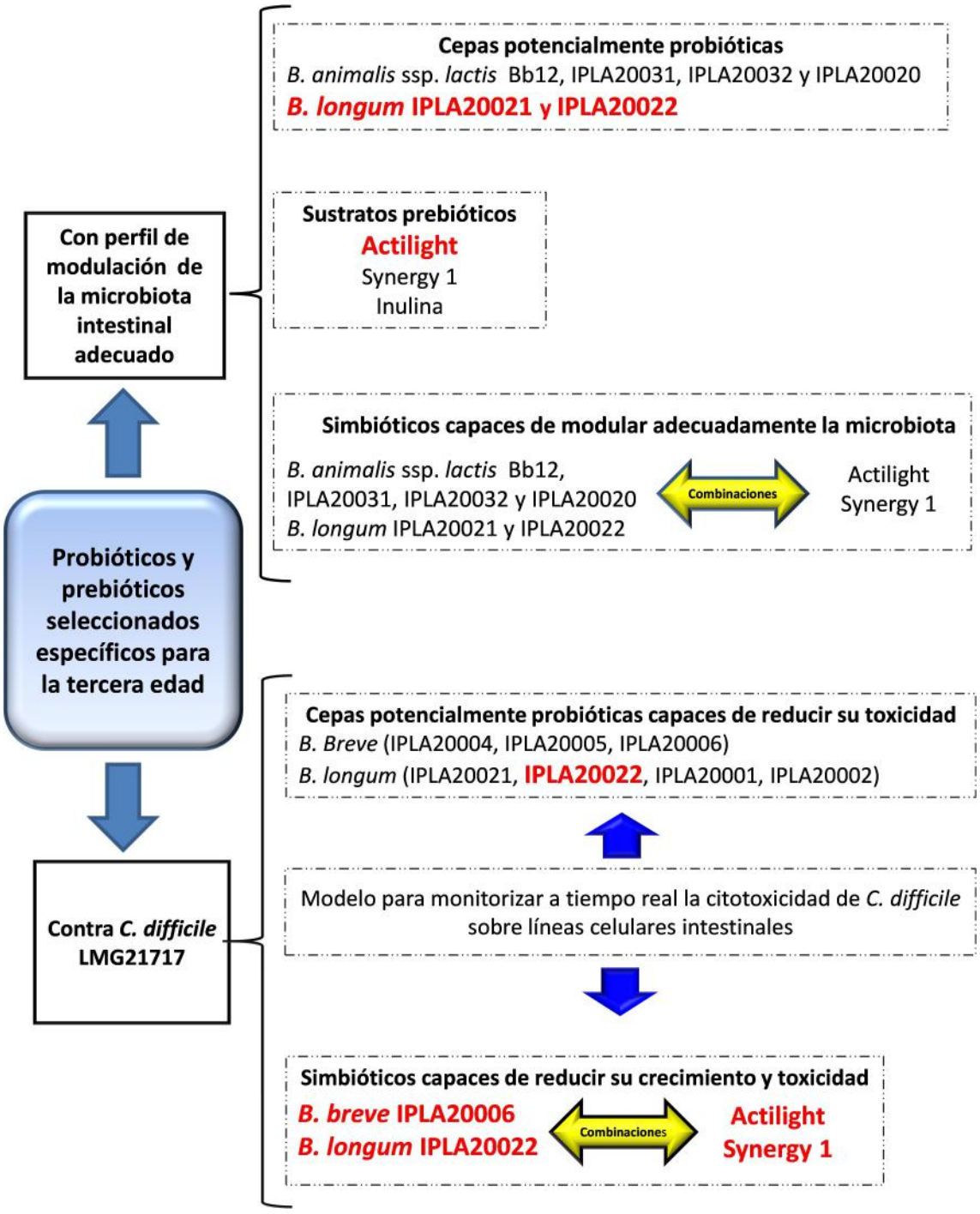
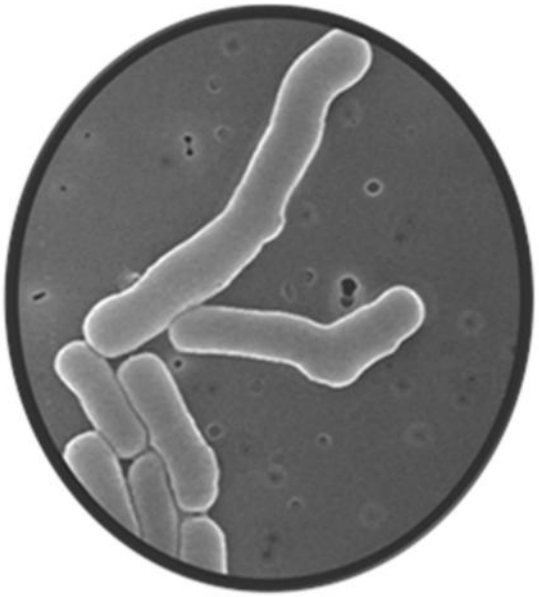


Figura 17. Esquema de los principales resultados de esta Tesis Doctoral.



5-Conclusiones

1. Mediante el uso de cultivos fecales, con inóculos procedentes de ancianos, y aplicando criterios de selección basados en alteraciones específicas de la microbiota intestinal, previamente identificadas en este grupo de población, hemos detectado sustratos prebióticos y cepas potencialmente probióticas con características adecuadas para modular la microbiota de personas de edad avanzada. Las cepas *B. longum* IPLA20021 e IPLA20022 y el fructooligosacárido de cadena corta Actilight resultaron ser los más adecuados para aumentar los niveles de bacterias anaerobias, como *Bacteroides*, *Bifidobacterium* y *Faecalibacterium*, aunque no se detectaron cambios significativos en el nivel de AGCCs.

2. Los cultivos discontinuos a pH libre de cepas seleccionadas, por su perfil adecuado para la modulación de la microbiota intestinal en ancianos, en presencia de los sustratos prebióticos seleccionados con el mismo fin mostraron, que ninguna de las bifidobacterias estudiadas fue capaz de crecer en presencia de inulina.

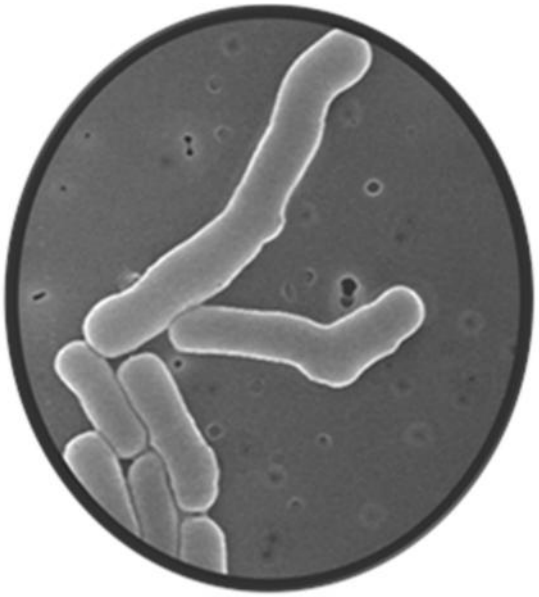
3. Nuestros resultados indicaron que los fructooligosacáridos de cadena corta constituyen sustratos adecuados para su combinación con las cepas de *B. animalis* y/o *B. longum*, con el objetivo de desarrollar productos simbióticos. Además, el empleo de estos prebióticos como fuente de carbono afectó al patrón de metabolitos producidos en el caso de las cepas de *B. longum*, no detectándose modificaciones en las cepas de *B. animalis*.

4. Hemos desarrollado un modelo *in vitro* basado en la monitorización continua del comportamiento de líneas celulares intestinales, a través de medidas de impedancia empleando la tecnología “Real Time Cell Analyzer”, que es eficaz para estudiar la cinética de citotoxicidad de los sobrenadantes procedentes de un cultivo toxigénico de *C. difficile*. Este método es capaz de detectar efectos citotóxicos dependientes de la dosis de sobrenadante toxigénico empleada y del tiempo de exposición sobre la línea celular.

5. El método desarrollado permitió evaluar el potencial probiótico de distintas cepas para contrarrestar *in vitro* el efecto citotóxico de *C. difficile* LMG21717, productor de toxinas A y B, sobre la línea intestinal HT29. Las cepas con mayor capacidad protectora sobre esta línea celular y las más efectivas en reducir los niveles de toxinas en los sobrenadantes procedentes del cultivo de *C. difficile* fueron las pertenecientes a las especies *B. longum* y *B. breve*, destacando por su mayor eficacia *B. longum* IPLA20022.

6. Los cocultivos de 4 cepas del género *Bifidobacterium* con *C. difficile* LMG21717 en presencia de varios sustratos prebióticos, mostraron que los fructooligosacáridos de cadena corta, Synergy 1 y Actilight, constituyen los sustratos adecuados para su combinación con las cepas *B. longum* IPLA20022 y *B. breve* IPLA20006, con el objetivo de desarrollar productos simbióticos dirigidos a reducir el crecimiento y la toxicidad de *C. difficile*.

7. El trabajo desarrollado en esta Tesis Doctoral nos ha permitido seleccionar cepas y prebióticos concretos con gran potencial para su aplicación en el desarrollo de productos funcionales dirigidos a personas de la tercera edad, destacando *B. longum* IPLA20022 y los fructooligosacáridos de cadena corta Synergy 1 y Actilight al mostrarse eficaces para lograr los dos objetivos planteados en esta Tesis.



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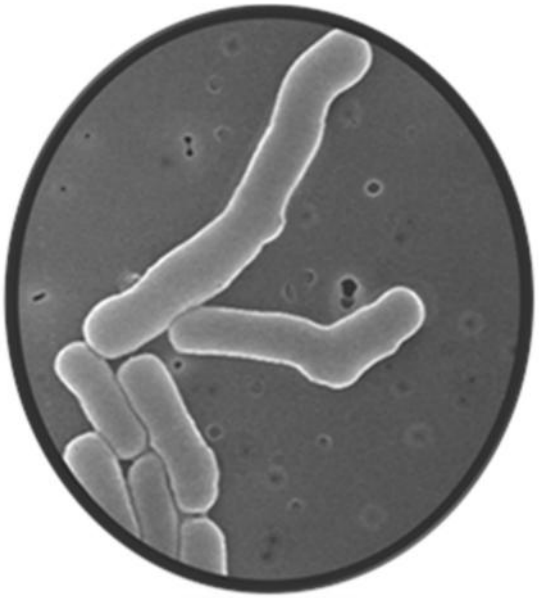


7-ANEXOS

INFORMACIÓN SUPLEMENTARIA DEL ARTÍCULO 1

Supplementary File. Initial bacterial levels (T=0; Log cells/g) and increases/decreases respect to T=0 obtained for the different microbial populations analyzed after 24 hours of incubation (Δ 24h; Log cells/g) with the different prebiotic substrates in three independent faecal batches.

MICROBIAL GROUPS (Log CFU/mL; MEAN ± SD)							
		Bifidobacterium			*Lactobacillus-Weisella group*		
Time (h)	**Treatment**	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
0 h	Synergy 1	10.46 ± 0.09	8.97 ± 0.02	9.62 ± 0.02	7.85 ± 0.05	8.43 ± 0.94	8.29 ± 0.15
	FOS 95	10.3 ± 0.04	8.72 ± 0.06	9.6 ± 0.04	6 ± 0	8.82 ± 0.1	8.39 ± 0.04
	Inulina	10.21 ± 0	9.02 ± 0.13	10.02 ± 0.05	7.53 ± 0.02	8.86 ± 0.07	8.76 ± 0.2
	Actilight	10.16 ± 0.12	8.71 ± 0.17	9.58 ± 0.11	8.1 ± 0.02	8.77 ± 0.17	8.18 ± 0.05
	Control negativo	10.37 ± 0.01	8.75 ± 0.05	9.63 ± 0.13	8.12 ± 0.4	9.08 ± 0.19	8.27 ± 0.24
Δ after 24	Synergy 1	0.08 ± 0.03	-0.15 ± 0.11	0.8 ± 0.07	2.51 ± 0	0.26 ± 0.93	2.28 ± 0.43
	FOS 95	0.01 ± 0.08	-0.02 ± 0.04	0.62 ± 0.01	4.44 ± 0.25	0.36 ± 0	2.11 ± 0.07
	Inulina	0.21 ± 0.05	0.09 ± 0.15	0.56 ± 0.07	1.97 ± 0.1	0.13 ± 0.15	1.49 ± 0.13
	Actilight	0.45 ± 0.18	0.33 ± 0.21	0.78 ± 0.23	0.62 ± 0.57	0.53 ± 0.1	2.53 ± 0.32
	Control negativo	-0.17 ± 0.08	0.21 ± 0.02	0.59 ± 0.19	-0.11 ± 0.39	0.12 ± 0.1	0.03 ± 0.26

MICROBIAL GROUPS (Log CFU/mL; MEAN ± SD)							
		Bacteroides-Prevotella group			*Blautia coccoides-Eubacterium rectale group*		
Time (h)	**Treatment**	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
0 h	Synergy 1	10.13 ± 0.03	10.38 ± 0.06	9.76 ± 0.04	8.9 ± 0.03	9.88 ± 0.01	8.7 ± 0.28
	FOS 95	9.99 ± 0.07	10.04 ± 0.05	9.85 ± 0.17	6.34 ± 0.49	10.19 ± 0.02	8.96 ± 0.06
	Inulina	9.99 ± 0.03	10.35 ± 0.04	9.97 ± 0.04	8.09 ± 0.39	10.43 ± 0.19	6 ± 0
	Actilight	9.78 ± 0.06	10.3 ± 0	9.69 ± 0.13	9.9 ± 0.07	9.88 ± 0.1	8.72 ± 0.01
	Control negativo	10.17 ± 0.01	10.35 ± 0.01	9.56 ± 0.02	9.59 ± 0.35	10.41 ± 0.05	8.93 ± 0.08
Δ after 24	Synergy 1	0.26 ± 0.02	-0.27 ± 0.05	0.41 ± 0.01	0.69 ± 0.38	-0.47 ± 0.22	0.2 ± 0.26
	FOS 95	0.15 ± 0.06	0.36 ± 0.09	0.15 ± 0.18	2.93 ± 0.7	-0.62 ± 0.05	-0.27 ± 0.21
	Inulina	0.12 ± 0.07	0.24 ± 0.12	0.26 ± 0.07	0.46 ± 0.4	-0.1 ± 0.18	3.35 ± 0.16
	Actilight	0.79 ± 0.11	0.31 ± 0.06	0.72 ± 0.16	-3.9 ± 0.07	-0.31 ± 0.15	-0.35 ± 0.22
	Control negativo	-0.36 ± 0.02	0.24 ± 0.02	0.68 ± 0.04	-0.62 ± 0.42	0.14 ± 0.11	-0.27 ± 0.06

MICROBIAL GROUPS (Log CFU/mL; MEAN ± SD)							
		Faecalibacterium prausnitzii			*Akkermansia*		
Time (h)	**Treatment**	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
0 h	Synergy 1	8.15 ± 0.02	8.55 ± 0.02	7.77 ± 0.08	ND	7.83 ± 0.04	6.3 ± 0.11
	FOS 95	7.62 ± 0.02	8.13 ± 0.05	7.81 ± 0.06	ND	7.6 ± 0.07	6.54 ± 0.12
	Inulina	7.81 ± 0.11	8.45 ± 0	8.02 ± 0.07	ND	7.85 ± 0.06	6.78 ± 0.02
	Actilight	7.68 ± 0.08	8.48 ± 0.01	7.69 ± 0.05	ND	7.75 ± 0.02	6.36 ± 0.04
	Control negativo	7.87 ± 0.13	8.53 ± 0.03	7.56 ± 0.01	ND	7.86 ± 0.02	6.33 ± 0.06
Δ after 24	Synergy 1	0.07 ± 0.01	-0.49 ± 0.01	0.38 ± 0.15	ND	-0.54 ± 0	0.59 ± 0.09
	FOS 95	0.49 ± 0.08	0.08 ± 0.16	0.22 ± 0.03	ND	-0.24 ± 0.04	0.07 ± 0.01
	Inulina	0.14 ± 0.1	-0.19 ± 0.14	0.22 ± 0.09	ND	-0.22 ± 0.01	0.25 ± 0.02
	Actilight	0.75 ± 0.09	0.01 ± 0.03	0.81 ± 0.07	ND	-0.14 ± 0.02	0.8 ± 0.05
	Control negativo	-0.35 ± 0.07	-0.12 ± 0.01	0.47 ± 0.02	ND	0.21 ± 0	0.87 ± 0.36

Supplementary File. Initial concentrations (mM) of acetate, propionate and butyrate, as well as the sum of the three of them (Total SCFA) and the acetate to propionate ratio, in the initial (T=0) samples from the fecal cultures of the three donors. Increases/decreases with regard to T=0 obtained for the different SCFA analyzed after 24 hours of incubation (Δ 24h, mM) with the different prebiotic substrates.

		Acetate Mm±SD			**Propionate** Mm±SD		
Time (h)	**Treatment**	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
0 h	Synergy 1	19.12 ± 0.11	16.73 ± 0.32	16.59 ± 0.06	3.3 ± 0.04	2.58 ± 0.05	2.52 ± 0.02
	FOS 95	21.12 ± 0.29	21.82 ± 0.16	19.45 ± 0.14	3.33 ± 0.03	2.81 ± 0.02	2.45 ± 0.01
	Inulina	17.84 ± 0.07	16.92 ± 0.04	15 ± 0.18	3.21 ± 0	3.18 ± 0.03	2.42 ± 0.01
	Actilight	17.73 ± 0.17	16.86 ± 0.03	14.71 ± 0.13	3.17 ± 0.04	3.12 ± 0.01	2.42 ± 0.02
	Control negativo	17.66 ± 0.16	19.04 ± 0.16	15.51 ± 0.09	3.2 ± 0.02	3.53 ± 0.03	2.67 ± 0
Δ after 24	Synergy 1	15.33 ± 0.31	8.84 ± 0.25	0.91 ± 0.08	1.09 ± 0.05	3.81 ± 0.04	0.7 ± 0.01
	FOS 95	7.77 ± 0.29	1.36 ± 0.22	-3.18 ± 0.2	0.61 ± 0.03	3.48 ± 0.04	0.45 ± 0.06
	Inulina	15.17 ± 0.19	8.36 ± 0.05	11.91 ± 0.23	1.01 ± 0.01	3.85 ± 0.01	1.4 ± 0.03
	Actilight	9.26 ± 0.09	5.63 ± 0.08	16.75 ± 0.1	1.14 ± 0.03	2.91 ± 0.05	1.92 ± 0
	Control negativo	6.82 ± 0.32	2.96 ± 0.04	12.09 ± 0.26	0.83 ± 0.04	1.75 ± 0.02	1.21 ± 0.01

		Butyrate Mm±SD			**Total SCFA** Mm		
Time (h)	**Treatment**	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
0 h	Synergy 1	2.3 ± 0.01	2.01 ± 0.04	2.25 ± 0.01	24.72 ± 0.13	21.33 ± 0.41	21.35 ± 0.06
	FOS 95	2.25 ± 0.01	2.18 ± 0.01	2.18 ± 0.01	26.7 ± 0.24	26.81 ± 0.19	24.08 ± 0.13
	Inulina	2.23 ± 0.01	2.32 ± 0.03	2.22 ± 0.01	23.28 ± 0.07	22.42 ± 0.1	19.65 ± 0.19
	Actilight	2.2 ± 0.03	2.26 ± 0.01	2.21 ± 0.01	23.1 ± 0.23	22.25 ± 0.04	19.34 ± 0.16
	Control negativo	2.29 ± 0.01	2.64 ± 0.02	2.53 ± 0.01	23.15 ± 0.19	25.22 ± 0.21	20.71 ± 0.1
Δ after 24	Synergy 1	0.62 ± 0.01	1.78 ± 0.04	0.53 ± 0.02	17.04 ± 0.36	14.44 ± 0.33	2.15 ± 0.1
	FOS 95	0.64 ± 0.01	1.37 ± 0.02	0.55 ± 0.02	9.02 ± 0.25	6.21 ± 0.28	-2.18 ± 0.28
	Inulina	0.54 ± 0.01	1.68 ± 0.02	1.12 ± 0.01	16.71 ± 0.19	13.89 ± 0.05	14.42 ± 0.25
	Actilight	0.91 ± 0.02	0.95 ± 0.01	1.59 ± 0.03	11.31 ± 0.14	9.49 ± 0.13	20.26 ± 0.12
	Control negativo	0.83 ± 0.02	0.44 ± 0.01	0.88 ± 0.02	8.48 ± 0.38	5.15 ± 0.04	14.18 ± 0.28

		A/P Ratio		
Time (h)	**Treatment**	Donor 1	Donor 2	Donor 3
0 h	Synergy 1	5.79 ± 0.06	6.48 ± 0.01	6.6 ± 0.06
	FOS 95	6.35 ± 0.15	7.77 ± 0.02	7.93 ± 0.09
	Inulina	5.56 ± 0.03	5.31 ± 0.04	6.19 ± 0.04
	Actilight	5.6 ± 0.05	5.4 ± 0.01	6.08 ± 0.04
	Control negativo	5.52 ± 0.02	5.39 ± 0.01	5.82 ± 0.03
Δ after 24	Synergy 1	2.06 ± 0.05	-2.48 ± 0.01	-1.16 ± 0.03
	FOS 95	0.99 ± 0.15	-4.08 ± 0.01	-2.33 ± 0.06
	Inulina	2.27 ± 0.06	-1.72 ± 0.04	0.85 ± 0.03
	Actilight	0.66 ± 0.02	-1.67 ± 0.02	1.17 ± 0.04
	Control negativo	0.55 ± 0.03	-1.23 ± 0.02	1.29 ± 0.04

Supplementary File. Initial bacterial levels (T=0; Log cells/g) and increases/decreases respect to T=0 obtained for the different microbial populations analyzed after 24 hours of incubation (Δ 24h; Log cells/g) with different bifidobacterial strains added in three independent faecal batches.

		MICROBIAL GROUPS (Log CFU/mL; MEAN ± SD)					
		Bifidobacterium			*Lactobacillus-Weisella group*		
Time (h)	**Treatment**	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
0	BB12	8.57 ± 0.06	9.23 ± 0.25	10.15 ± 0.01	8.43 ± 0.11	7.7 ± 0.07	9.97 ± 0.12
	IPLA20030	8.76 ± 0.04	8.96 ± 0.48	10.18 ± 0.02	8.8 ± 0.11	7.42 ± 0.26	10.26 ± 0.01
	IPLA20031	8.74 ± 0.11	9.13 ± 0.05	10.08 ± 0.07	8.81 ± 0.02	7.6 ± 0.13	9.84 ± 0.01
	IPLA20032	8.72 ± 0.03	9.25 ± 0.13	9.87 ± 0.05	8.75 ± 0	7.67 ± 0.14	9.67 ± 0.21
	IPLA20001	8.82 ± 0.02	8.97 ± 0.77	10.18 ± 0.07	8.6 ± 0.09	7.68 ± 0.05	9.95 ± 0.02
	IPLA20002	9.04 ± 0.14	8.64 ± 0.16	10.16 ± 0.05	8.77 ± 0.02	7.54 ± 0	10.14 ± 0.02
	IPLA20003	8.7 ± 0.03	8.84 ± 0.51	10.03 ± 0.02	8.52 ± 0.04	7.52 ± 0.04	9.99 ± 0.04
	IPLA20021	9.09 ± 0.12	8.51 ± 0.05	10.28 ± 0.02	8.85 ± 0.01	7.21 ± 0.1	10.06 ± 0.06
	IPLA20022	8.7 ± 0.11	9.59 ± 0.07	10.19 ± 0.02	8.55 ± 0.02	7.11 ± 0	10.23 ± 0.03
	IPLA20004	9.05 ± 0	9.26 ± 0.22	10.02 ± 0.07	8.77 ± 0.1	7.4 ± 0.19	9.87 ± 0.03
	IPLA20005	8.87 ± 0.1	9.49 ± 0.13	10.2 ± 0.03	8.85 ± 0.04	7.33 ± 0.17	10.12 ± 0.02
	IPLA006	9.23 ± 0.03	9.32 ± 0.26	10.16 ± 0.02	8.71 ± 0.07	7.34 ± 0.2	9.96 ± 0.02
	IPLA20026	8.74 ± 0.08	8.96 ± 0.12	10.2 ± 0.01	8.8 ± 0.07	7.2 ± 0.1	10 ± 0
	IPLA20017	9.21 ± 0.04	9.1 ± 0.16	10.08 ± 0.11	8.62 ± 0.1	7.48 ± 0.06	9.72 ± 0.01
	IPLA20015	9.76 ± 0.01	9.32 ± 0.06	10.26 ± 0.02	8.8 ± 0.07	7.26 ± 0.04	9.77 ± 0.03
	IPLA20020	8.84 ± 0.04	9.17 ± 0.14	10.05 ± 0.08	8.76 ± 0.11	7.35 ± 0.04	10.09 ± 0.05
	Control	8.74 ± 0.02	9.25 ± 0.02	10.24 ± 0.02	8.94 ± 0.1	7.25 ± 0.2	10.15 ± 0.09
Δ 24	BB12	0.95 ± 0.09	0.84 ± 0.27	0.34 ± 0.2	1.24 ± 0.13	0.48 ± 0	0.35 ± 0.14
	IPLA20030	0.53 ± 0.09	1.16 ± 0.56	0.22 ± 0.05	0.56 ± 0.01	0.86 ± 0.3	0.23 ± 0.12
	IPLA20031	0.34 ± 0.15	0.97 ± 0.05	0.46 ± 0.01	0.6 ± 0.01	0.65 ± 0.09	0.54 ± 0.04
	IPLA20032	0.32 ± 0.03	1.01 ± 0.13	0.66 ± 0.05	0.72 ± 0	0.72 ± 0.19	0.75 ± 0.32
	IPLA20001	0.81 ± 0.01	1.31 ± 0.91	0.33 ± 0.03	0.92 ± 0.05	0.72 ± 0.03	0.35 ± 0.09
	IPLA20002	0.19 ± 0.04	1.77 ± 0.13	0.32 ± 0.08	0.97 ± 0.05	0.98 ± 0	0.09 ± 0.14
	IPLA20003	0.81 ± 0.02	1.47 ± 0.53	0.32 ± 0.06	1.06 ± 0.07	0.94 ± 0.08	0.29 ± 0.07
	IPLA20021	0.71 ± 0.16	1.82 ± 0.13	0.15 ± 0.18	0.82 ± 0.07	1.32 ± 0.1	0.06 ± 0.11
	IPLA20022	1.5 ± 0.3	0.75 ± 0.09	0.21 ± 0.06	1.18 ± 0.07	1.26 ± 0.02	0.13 ± 0.01
	IPLA20004	0.73 ± 0.11	0.92 ± 0.23	0.3 ± 0.03	0.71 ± 0.11	0.9 ± 0.23	0.12 ± 0
	IPLA20005	0.59 ± 0.07	0.67 ± 0.13	0.29 ± 0.03	0.91 ± 0.02	0.84 ± 0.25	-0.03 ± 0.01
	IPLA006	0.02 ± 0.04	0.83 ± 0.31	0.29 ± 0.08	1.33 ± 0.01	0.97 ± 0.31	0.23 ± 0.06
	IPLA20026	0.2 ± 0.16	1.12 ± 0.04	0.24 ± 0.05	0.91 ± 0.16	1.22 ± 0.18	0.06 ± 0.01
	IPLA20017	0.21 ± 0.01	1.05 ± 0.2	0.28 ± 0.05	1.28 ± 0.08	1.12 ± 0.04	0.45 ± 0.08
	IPLA20015	-0.28 ± 0.01	0.89 ± 0.09	0.2 ± 0.03	1.18 ± 0	1.18 ± 0.04	0.61 ± 0.08
	IPLA20020	0.36 ± 0.06	0.73 ± 0.15	0.35 ± 0.05	1.25 ± 0.05	0.74 ± 0.08	0.17 ± 0
	Control	0.36 ± 0.1	0.75 ± 0.02	0.34 ± 0.17	1.23 ± 0.16	0.73 ± 0.25	0.06 ± 0.03

		MICROBIAL GROUPS (Log CFU/mL; MEAN ± SD)					
		Bacteroides-Prevotella group			*Blautia coccoides-Eubacterium rectale group*		
Time (h)	**Treatment**	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
0	BB12	9.87 ± 0.09	10.55 ± 0.08	10.31 ± 0.08	9.11 ± 0.03	9.96 ± 0.13	10.06 ± 0.03
	IPLA20030	10.17 ± 0.04	10.25 ± 0.01	10.48 ± 0.07	9.25 ± 0.18	9.64 ± 0.13	10.13 ± 0.01
	IPLA20031	10.29 ± 0.08	10.35 ± 0.16	9.93 ± 0.06	9.56 ± 0.08	9.82 ± 0.01	9.68 ± 0.03
	IPLA20032	10.12 ± 0.04	10.57 ± 0.2	10.2 ± 0.05	9.41 ± 0.16	10.14 ± 0.12	9.32 ± 0.04
	IPLA20001	10.04 ± 0.07	10.6 ± 0.05	10.36 ± 0.01	9.48 ± 0.03	9.87 ± 0.06	9.58 ± 0.01
	IPLA20002	10.21 ± 0.04	10.26 ± 0.14	10.54 ± 0.03	9.58 ± 0.08	9.42 ± 0.07	9.84 ± 0.05
	IPLA20003	10.09 ± 0.03	10.52 ± 0.03	10.52 ± 0.04	9.09 ± 0.34	9.83 ± 0.05	9.12 ± 0.23
	IPLA20021	10.24 ± 0.05	8.66 ± 0.12	10.57 ± 0.06	9.67 ± 0.04	8.19 ± 0.18	9.84 ± 0.21
	IPLA20022	10.08 ± 0.04	9.19 ± 0.02	10.6 ± 0.05	9.5 ± 0.1	8.69 ± 0.08	9.17 ± 0.18
	IPLA20004	10.23 ± 0.02	10.4 ± 0.02	10.31 ± 0.06	9.34 ± 0.04	9.33 ± 0.19	9.85 ± 0.05
	IPLA20005	10.23 ± 0.08	10.23 ± 0.01	10.55 ± 0.01	9.36 ± 0.01	9.57 ± 0.1	10.04 ± 0.03
	IPLA006	10.23 ± 0.02	10.37 ± 0.05	10.4 ± 0.01	9.3 ± 0.18	9.9 ± 0.09	9.87 ± 0.07
	IPLA20026	10.16 ± 0.01	10.02 ± 0.02	10.47 ± 0.03	8.95 ± 0.1	9.46 ± 0.04	9.9 ± 0.18
	IPLA20017	10.24 ± 0.01	10.2 ± 0.05	10.17 ± 0.06	9.09 ± 0.14	9.8 ± 0.11	9.57 ± 0.02
	IPLA20015	10.24 ± 0.05	9.96 ± 0.06	10.25 ± 0	8.99 ± 0.28	9.59 ± 0.05	9.13 ± 0.49
	IPLA20020	10.13 ± 0.08	10.4 ± 0	10.5 ± 0.07	9.37 ± 0.09	9.76 ± 0.09	9.78 ± 0.04
	Control	10.28 ± 0.03	10.37 ± 0.03	10.41 ± 0.08	9.3 ± 0.02	9.2 ± 0.13	9.8 ± 0.38
Δ 24	BB12	0.65 ± 0.04	0.35 ± 0.04	0.03 ± 0.1	-0.61 ± 0.18	-0.71 ± 0.28	-0.89 ± 0.25
	IPLA20030	0.18 ± 0.03	0.58 ± 0	-0.17 ± 0.08	-1.21 ± 0.07	-0.1 ± 0	-0.83 ± 0.25
	IPLA20031	-0.13 ± 0.05	0.48 ± 0.12	0.5 ± 0.01	-0.9 ± 0.22	-0.62 ± 0.73	-0.43 ± 0.44
	IPLA20032	0.07 ± 0	0.38 ± 0.12	0.17 ± 0.04	-1.3 ± 0.02	-1.1 ± 0.73	-0.15 ± 0.44
	IPLA20001	0.28 ± 0.07	0.22 ± 0.04	-0.07 ± 0.03	-0.93 ± 0.16	0.08 ± 0.05	-0.43 ± 0.15
	IPLA20002	-0.05 ± 0.08	0.64 ± 0.19	-0.29 ± 0.06	-1.42 ± 0.25	0.01 ± 0.22	-0.97 ± 0.45
	IPLA20003	0.23 ± 0.01	0.26 ± 0.02	-0.22 ± 0.05	-0.42 ± 0.23	-0.28 ± 0.09	-0.11 ± 0.13
	IPLA20021	0.11 ± 0.09	2.22 ± 0.1	-0.27 ± 0.02	-1.07 ± 0.04	1.3 ± 0.3	-1.12 ± 0.85
	IPLA20022	0.37 ± 0	1.61 ± 0.04	-0.24 ± 0.1	-0.74 ± 0.33	1 ± 0.01	-0.51 ± 0.52
	IPLA20004	-0.1 ± 0.1	0.44 ± 0.01	-0.06 ± 0.02	-0.55 ± 0.16	-0.06 ± 0.18	-1.05 ± 0.05
	IPLA20005	-0.04 ± 0.08	0.41 ± 0.06	-0.15 ± 0.01	-0.7 ± 0.03	0.19 ± 0.14	-1.23 ± 0.13
	IPLA006	0.11 ± 0.07	0.56 ± 0.12	0.01 ± 0	-0.74 ± 0.03	-0.18 ± 0.01	-1.1 ± 0.01
	IPLA20026	0.2 ± 0	0.89 ± 0.06	-0.29 ± 0.04	-0.07 ± 0.11	0.5 ± 0.03	-0.87 ± 0.13
	IPLA20017	0.12 ± 0	0.75 ± 0.07	0.06 ± 0.08	-0.57 ± 0.45	0.21 ± 0.24	-0.62 ± 0.14
	IPLA20015	0.26 ± 0.08	0.96 ± 0.03	0.17 ± 0.11	-0.2 ± 0.41	0.29 ± 0.04	0.08 ± 0.73
	IPLA20020	0.19 ± 0.12	0.4 ± 0	-0.17 ± 0.03	-0.48 ± 0.22	-0.03 ± 0.25	-0.7 ± 0.15
	Control	0.17 ± 0.05	0.32 ± 0.06	-0.09 ± 0.16	-0.46 ± 0.11	0.45 ± 0.38	-1.04 ± 0.34

MICROBIAL GROUPS (Log CFU/mL; MEAN ± SD)							
		Faecalibacterium prausnitzii			*Akkermansia*		
Time (h)	**Treatment**	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
0	BB12	7.5 ± 0.04	8.14 ± 0.06	7.95 ± 0	8.6 ± 0.05	7.7 ± 0.01	9.45 ± 0.03
	IPLA20030	7.72 ± 0.07	7.95 ± 0.1	8.05 ± 0	8.76 ± 0.04	7.53 ± 0.09	9.49 ± 0.06
	IPLA20031	7.69 ± 0.12	7.93 ± 0.14	7.7 ± 0	8.81 ± 0.03	7.55 ± 0.01	9.02 ± 0.02
	IPLA20032	7.6 ± 0.02	8.22 ± 0.08	7.71 ± 0	8.78 ± 0.05	7.75 ± 0.05	8.65 ± 0.01
	IPLA20001	7.52 ± 0.05	8.19 ± 0.14	7.89 ± 0	8.75 ± 0.06	7.81 ± 0.04	9.34 ± 0.03
	IPLA20002	7.72 ± 0	7.88 ± 0.19	8.19 ± 0.01	8.85 ± 0	7.44 ± 0.04	9.48 ± 0.02
	IPLA20003	7.58 ± 0.08	8.26 ± 0.07	8.09 ± 0.1	8.61 ± 0	7.71 ± 0.03	9.1 ± 0.08
	IPLA20021	7.77 ± 0	6.98 ± 0.06	8.29 ± 0.12	8.85 ± 0.06	6.18 ± 0.23	9.62 ± 0.1
	IPLA20022	7.6 ± 0.02	7.25 ± 0.09	8.29 ± 0.07	8.71 ± 0.05	6.35 ± 0.05	9.6 ± 0.09
	IPLA20004	7.71 ± 0.04	7.98 ± 0.05	7.9 ± 0.03	8.79 ± 0.02	7.61 ± 0.01	8.86 ± 0.17
	IPLA20005	7.49 ± 0.19	7.88 ± 0.07	8.1 ± 0	8.73 ± 0.08	7.33 ± 0.05	9.54 ± 0.05
	IPLA006	7.59 ± 0.06	8.19 ± 0.17	7.99 ± 0.03	8.71 ± 0.07	7.68 ± 0.08	9.45 ± 0.02
	IPLA20026	7.47 ± 0.08	7.61 ± 0.13	7.87 ± 0.01	8.63 ± 0.07	7.22 ± 0.03	9.11 ± 0.01
	IPLA20017	7.46 ± 0.01	7.83 ± 0.06	7.68 ± 0.04	8.66 ± 0.11	7.32 ± 0.09	8.91 ± 0.13
	IPLA20015	7.61 ± 0.25	7.59 ± 0.1	7.8 ± 0.03	8.78 ± 0.05	7.1 ± 0.05	9.14 ± 0.01
	IPLA20020	7.52 ± 0.03	7.95 ± 0.14	8.06 ± 0.01	8.78 ± 0.1	7.47 ± 0	9.22 ± 0.01
	Control	7.53 ± 0.05	8.06 ± 0.14	8.03 ± 0.02	8.79 ± 0.05	8.01 ± 0	9.41 ± 0.05
Δ 24	BB12	0.28 ± 0.03	0.06 ± 0.06	0.11 ± 0.08	0.22 ± 0.02	0.18 ± 0.05	-0.1 ± 0.02
	IPLA20030	-0.13 ± 0.15	0.21 ± 0.11	-0.04 ± 0.04	-0.25 ± 0.11	0.54 ± 0.12	-0.27 ± 0.06
	IPLA20031	-0.13 ± 0.11	-0.01 ± 0.17	0.55 ± 0.08	-0.35 ± 0.04	0.37 ± 0.02	0.5 ± 0.08
	IPLA20032	0.1 ± 0.03	-0.01 ± 0.01	0.37 ± 0.01	-0.13 ± 0.06	0.43 ± 0.05	0.73 ± 0.04
	IPLA20001	0.16 ± 0.05	-0.04 ± 0.02	0.2 ± 0.03	-0.09 ± 0.13	0.26 ± 0.13	-0.23 ± 0.08
	IPLA20002	-0.2 ± 0.09	0.3 ± 0.05	-0.04 ± 0.02	-0.26 ± 0.1	0.57 ± 0.04	-0.38 ± 0.01
	IPLA20003	0.26 ± 0.02	-0.06 ± 0.11	-0.09 ± 0.07	0.19 ± 0.12	0.27 ± 0.01	-0.22 ± 0.07
	IPLA20021	-0.34 ± 0.4	1.27 ± 0.05	-0.1 ± 0.06	-0.22 ± 0.06	1.83 ± 0.28	-0.39 ± 0.12
	IPLA20022	0.18 ± 0.07	0.87 ± 0.05	0.18 ± 0.05	0.08 ± 0.03	1.75 ± 0.08	-0.31 ± 0.15
	IPLA20004	-0.15 ± 0	0.2 ± 0.01	0.14 ± 0	-0.14 ± 0.07	0.06 ± 0.06	0.13 ± 0.13
	IPLA20005	0.17 ± 0.22	0.2 ± 0.03	-0.03 ± 0.07	-0.19 ± 0.03	0.58 ± 0.01	-0.5 ± 0.05
	IPLA006	0.11 ± 0.14	0.16 ± 0.08	0.12 ± 0.02	0.01 ± 0.05	0.42 ± 0.05	-0.16 ± 0.11
	IPLA20026	0.21 ± 0.05	0.71 ± 0	0.09 ± 0.06	-0.13 ± 0.01	1.01 ± 0.01	0.05 ± 0.07
	IPLA20017	0.19 ± 0.07	0.61 ± 0.05	0.39 ± 0.02	-0.1 ± 0.05	0.89 ± 0.1	0.04 ± 0.03
	IPLA20015	0.57 ± 0.27	0.64 ± 0.05	0.43 ± 0.05	0.08 ± 0.09	0.94 ± 0.11	0.08 ± 0.01
	IPLA20020	0.58 ± 0.07	0.19 ± 0.04	0.12 ± 0.11	0.19 ± 0.01	0.5 ± 0.05	0.13 ± 0.01
	Control	0.42 ± 0.04	0.09 ± 0.13	0.2 ± 0.02	-0.02 ± 0.02	0.03 ± 0.02	-0.04 ± 0.03

Supplementary File. Initial concentrations (mM) of acetate, propionate and butyrate, as well as the sum of the three of them (Total SCFA) and the acetate to propionate ratio, in the initial (T=0) samples from the fecal cultures of the three donors. Increases/decreases with regard to T=0 obtained for the different SCFA analyzed after 24 hours of incubation (Δ 24h, mM) with different bifidobacterial strains added.

		Acetate Mm±SD			**Propionate** Mm±SD		
Time (h)	**Treatment**	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
0 h	BB12	14.35 ± 0.82	15.69 ± 0.33	17.99 ± 0.25	2.32 ± 0.11	3.18 ± 0.06	3.01 ± 0.02
	IPLA20030	23.65 ± 1.51	18.75 ± 0.92	18.95 ± 0.48	3.91 ± 0.29	3.69 ± 0.02	2.96 ± 0.03
	IPLA20031	24.26 ± 0.29	14.98 ± 0.3	16.81 ± 0.65	3.92 ± 0.02	3.01 ± 0.04	2.69 ± 0.05
	IPLA20032	14.89 ± 0.2	18.06 ± 0.52	14.66 ± 0.14	4.31 ± 0.09	4.68 ± 0.11	3.63 ± 0.05
	IPLA20001	23.43 ± 0.53	15.72 ± 0.13	20.14 ± 0.31	3.84 ± 0.15	3.14 ± 0.04	3.12 ± 0.01
	IPLA20002	21.39 ± 0.25	15.96 ± 0.32	18.32 ± 0.18	3.53 ± 0.06	3.15 ± 0.02	2.92 ± 0.01
	IPLA20003	29.16 ± 0.7	17.82 ± 0.33	19.17 ± 0.54	4.47 ± 0.06	3.47 ± 0.05	3.02 ± 0.01
	IPLA20021	17.37 ± 0.4	17.14 ± 0.53	20.73 ± 0.65	3 ± 0.03	3.31 ± 0.02	3.69 ± 0.19
	IPLA20022	17.17 ± 0.31	17.38 ± 0.35	19.12 ± 1.16	2.91 ± 0.04	3.37 ± 0.08	3.22 ± 0.01
	IPLA20004	19.75 ± 0.33	17.77 ± 0.42	16.7 ± 0.03	3.09 ± 0.09	3.41 ± 0.06	2.95 ± 0.01
	IPLA20005	17.76 ± 0.48	16.82 ± 0.13	18.15 ± 0.52	2.87 ± 0.03	3.26 ± 0.03	3.05 ± 0.01
	IPLA006	17.78 ± 0.71	18.96 ± 0.16	16.82 ± 0.35	3.06 ± 0.09	3.53 ± 0.03	2.96 ± 0.02
	IPLA20026	17.85 ± 0.89	18.42 ± 0.19	17.13 ± 0.51	2.89 ± 0.05	3.48 ± 0.02	3 ± 0.05
	IPLA20017	20.42 ± 0.33	17.84 ± 0.47	17.12 ± 0.44	3.07 ± 0.02	3.39 ± 0.07	2.94 ± 0.03
	IPLA20015	16.91 ± 0.22	17.33 ± 0.22	18.66 ± 0.07	4.75 ± 0.06	4.45 ± 0.03	4.28 ± 0.06
	IPLA20020	20.31 ± 1.25	16.44 ± 0.11	19.31 ± 0.54	3.11 ± 0.03	3.15 ± 0.05	3.18 ± 0.03
	Control	16.04 ± 0.17	17.49 ± 0.45	17.62 ± 0.5	2.53 ± 0.01	3.23 ± 0.03	2.9 ± 0.05
Δ after 24 h	BB12	8.99 ± 1.47	10.47 ± 0.59	16.07 ± 0.32	2.62 ± 0.12	3.31 ± 0.15	1.49 ± 0.04
	IPLA20030	-2.28 ± 1.32	7.18 ± 1.07	23.62 ± 0.49	0.76 ± 0.29	2.91 ± 0.02	2.15 ± 0.03
	IPLA20031	-2.35 ± 0.38	14.06 ± 0.43	19.23 ± 1.33	0.92 ± 0.03	4.09 ± 0.07	2.01 ± 0.06
	IPLA20032	6.4 ± 0.48	7.4 ± 0.72	18.95 ± 1.12	0.15 ± 0.08	1.7 ± 0.12	0.87 ± 0.14
	IPLA20001	0.79 ± 0.69	9.08 ± 0.24	17.48 ± 0.64	0.76 ± 0.14	3.21 ± 0.06	1.68 ± 0.02
	IPLA20002	1.48 ± 0.46	10.09 ± 0.53	13.17 ± 0.21	0.95 ± 0.08	3.29 ± 0.04	1.16 ± 0.08
	IPLA20003	-5.72 ± 1.26	6.22 ± 0.55	11.42 ± 2.6	0.31 ± 0.05	2.57 ± 0.13	0.88 ± 0.13
	IPLA20021	6.68 ± 0.55	8.88 ± 0.67	8.59 ± 0.5	1.2 ± 0.05	3.22 ± 0.08	0.13 ± 0.22
	IPLA20022	9.01 ± 0.11	8.71 ± 0.27	17.04 ± 1.78	1.83 ± 0.03	3.15 ± 0.08	1.4 ± 0.08
	IPLA20004	5.58 ± 0.68	5.3 ± 0.46	22.27 ± 0.79	2.1 ± 0.04	2.43 ± 0.06	2.14 ± 0.04
	IPLA20005	2.65 ± 0.73	9.34 ± 3.18	9.51 ± 0.88	1.4 ± 0.06	3.28 ± 0.56	0.55 ± 0.04
	IPLA006	4.66 ± 0.7	8.32 ± 3.34	16.72 ± 1	1.62 ± 0.07	3.47 ± 0.58	1.28 ± 0.03
	IPLA20026	5.28 ± 0.96	5.51 ± 4.46	13.48 ± 0.74	1.94 ± 0.04	2.49 ± 0.78	1.02 ± 0.09
	IPLA20017	0.53 ± 0.37	8.58 ± 0.47	16.36 ± 0.55	1.4 ± 0.02	3.27 ± 0.08	1.25 ± 0.06
	IPLA20015	4.33 ± 0.41	9.04 ± 0.45	15.19 ± 0.39	0.05 ± 0.06	2.06 ± 0.03	0.17 ± 0.04
	IPLA20020	1.7 ± 1.3	12.17 ± 2.99	11.6 ± 0.68	1.45 ± 0.09	3.7 ± 0.53	0.95 ± 0.08
	Control	5.84 ± 0.04	15.83 ± 1.19	14.31 ± 0.5	2.46 ± 0.01	5.27 ± 0.03	1.33 ± 0.04

		Butyrate Mm±SD			Total SCFA Mm		
Time (h)	**Treatment**	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
0 h	BB12	1.45 ± 0.08	2.38 ± 0.04	2.54 ± 0.02	18.12 ± 1	21.26 ± 0.43	23.54 ± 0.26
	IPLA20030	2.57 ± 0.21	2.72 ± 0.01	2.5 ± 0.03	30.13 ± 2	25.16 ± 0.95	24.41 ± 0.54
	IPLA20031	2.56 ± 0.03	2.22 ± 0.02	2.26 ± 0.03	30.74 ± 0.27	20.22 ± 0.35	21.76 ± 0.73
	IPLA20032	2.47 ± 0.05	2.64 ± 0.12	2.4 ± 0.03	21.67 ± 0.29	25.38 ± 0.46	20.68 ± 0.18
	IPLA20001	2.53 ± 0.13	2.34 ± 0.03	2.65 ± 0.03	29.8 ± 0.79	21.2 ± 0.2	25.91 ± 0.31
	IPLA20002	2.33 ± 0.05	2.34 ± 0.01	2.49 ± 0.02	27.25 ± 0.3	21.45 ± 0.35	23.73 ± 0.17
	IPLA20003	2.94 ± 0.04	2.58 ± 0.03	2.54 ± 0.01	36.57 ± 0.74	23.88 ± 0.4	24.74 ± 0.55
	IPLA20021	1.96 ± 0.01	2.45 ± 0.02	2.94 ± 0.06	22.33 ± 0.44	22.9 ± 0.54	27.36 ± 0.5
	IPLA20022	1.9 ± 0.05	2.5 ± 0.05	2.75 ± 0.02	21.98 ± 0.39	23.25 ± 0.45	25.09 ± 1.13
	IPLA20004	1.98 ± 0.07	2.52 ± 0.04	2.55 ± 0.01	24.82 ± 0.47	23.7 ± 0.52	22.19 ± 0.05
	IPLA20005	1.85 ± 0.03	2.4 ± 0.01	2.6 ± 0.01	22.48 ± 0.52	22.48 ± 0.12	23.8 ± 0.52
	IPLA006	2 ± 0.08	2.61 ± 0.03	2.62 ± 0.01	22.84 ± 0.81	25.1 ± 0.2	22.39 ± 0.33
	IPLA20026	1.85 ± 0.02	2.57 ± 0.01	2.62 ± 0.04	22.59 ± 0.96	24.47 ± 0.2	22.74 ± 0.59
	IPLA20017	1.94 ± 0.01	2.49 ± 0.04	2.56 ± 0.02	25.42 ± 0.35	23.73 ± 0.57	22.62 ± 0.49
	IPLA20015	2.69 ± 0.05	2.76 ± 0.08	2.81 ± 0.03	24.35 ± 0.32	24.54 ± 0.28	25.75 ± 0.04
	IPLA20020	1.96 ± 0.01	2.31 ± 0.05	2.72 ± 0.01	25.37 ± 1.27	21.9 ± 0.16	25.21 ± 0.57
	Control	1.6 ± 0.01	2.35 ± 0.01	2.5 ± 0.04	20.18 ± 0.18	23.07 ± 0.48	23.03 ± 0.58
Δ after 24 h	BB12	2.11 ± 0.07	1.34 ± 0.08	1.14 ± 0.05	13.72 ± 1.66	15.12 ± 0.79	18.71 ± 0.35
	IPLA20030	0.85 ± 0.23	1.08 ± 0.02	1.49 ± 0.04	-0.66 ± 1.83	11.17 ± 1.09	27.26 ± 0.54
	IPLA20031	0.85 ± 0.02	1.8 ± 0.04	1.7 ± 0.06	-0.57 ± 0.39	19.95 ± 0.43	22.95 ± 1.43
	IPLA20032	0.37 ± 0.04	1.07 ± 0.11	1.34 ± 0.05	6.93 ± 0.55	10.17 ± 0.69	21.17 ± 1.28
	IPLA20001	0.53 ± 0.14	1.34 ± 0.04	1.29 ± 0.04	2.08 ± 0.87	13.64 ± 0.33	20.44 ± 0.62
	IPLA20002	0.46 ± 0.06	1.36 ± 0.04	0.85 ± 0.07	2.89 ± 0.55	14.74 ± 0.48	15.18 ± 0.26
	IPLA20003	0.42 ± 0.02	0.9 ± 0.05	0.64 ± 0.13	-4.99 ± 1.31	9.69 ± 0.72	12.95 ± 2.85
	IPLA20021	0.62 ± 0.06	1.31 ± 0.05	0.15 ± 0.08	8.5 ± 0.47	13.41 ± 0.78	8.87 ± 0.32
	IPLA20022	1.15 ± 0.05	1.36 ± 0.09	1.01 ± 0.07	11.98 ± 0.12	13.23 ± 0.4	19.46 ± 1.81
	IPLA20004	1.7 ± 0.03	0.95 ± 0.04	1.82 ± 0.01	9.39 ± 0.63	8.67 ± 0.54	26.23 ± 0.8
	IPLA20005	0.76 ± 0.03	1.42 ± 0.38	0.49 ± 0.03	4.81 ± 0.81	14.04 ± 4.12	10.55 ± 0.94
	IPLA006	0.81 ± 0.05	1.32 ± 0.27	0.89 ± 0.09	7.09 ± 0.82	13.1 ± 4.18	18.89 ± 1.1
	IPLA20026	1.13 ± 0.02	0.87 ± 0.36	0.72 ± 0.08	8.36 ± 1.02	8.88 ± 5.6	15.21 ± 0.91
	IPLA20017	0.77 ± 0.01	1.36 ± 0.01	0.81 ± 0.02	2.7 ± 0.39	13.21 ± 0.55	18.42 ± 0.58
	IPLA20015	0.13 ± 0.05	0.95 ± 0.05	0.91 ± 0.04	4.51 ± 0.51	12.06 ± 0.49	16.27 ± 0.48
	IPLA20020	0.75 ± 0.07	1.56 ± 0.26	0.7 ± 0.02	3.9 ± 1.41	17.43 ± 3.78	13.25 ± 0.73
	Control	1.4 ± 0.01	2.34 ± 0.03	1.24 ± 0.02	9.7 ± 0.02	23.44 ± 1.19	16.87 ± 0.53

		A/P Ratio		
Time (h)	**Treatment**	Donor 1	Donor 2	Donor 3
0 h	BB12	6.19 ± 0.1	4.93 ± 0.01	5.97 ± 0.06
	IPLA20030	6.06 ± 0.06	5.08 ± 0.23	6.4 ± 0.1
	IPLA20031	6.19 ± 0.09	4.98 ± 0.05	6.26 ± 0.13
	IPLA20032	3.45 ± 0.03	3.86 ± 0.1	4.04 ± 0.04
	IPLA20001	6.11 ± 0.14	5.01 ± 0.02	6.45 ± 0.09
	IPLA20002	6.06 ± 0.11	5.07 ± 0.08	6.27 ± 0.08
	IPLA20003	6.52 ± 0.14	5.13 ± 0.04	6.34 ± 0.16
	IPLA20021	5.79 ± 0.08	5.18 ± 0.16	5.64 ± 0.46
	IPLA20022	5.91 ± 0.06	5.15 ± 0.08	5.94 ± 0.37
	IPLA20004	6.39 ± 0.11	5.21 ± 0.04	5.67 ± 0.01
	IPLA20005	6.18 ± 0.14	5.16 ± 0.07	5.96 ± 0.19
	IPLA006	5.8 ± 0.21	5.37 ± 0.05	5.69 ± 0.15
	IPLA20026	6.16 ± 0.21	5.3 ± 0.05	5.72 ± 0.08
	IPLA20017	6.65 ± 0.09	5.26 ± 0.04	5.83 ± 0.09
	IPLA20015	3.56 ± 0.01	3.89 ± 0.04	4.36 ± 0.07
	IPLA20020	6.54 ± 0.36	5.22 ± 0.09	6.08 ± 0.14
	Control	6.34 ± 0.07	5.42 ± 0.11	6.07 ± 0.08
Δ after 24 h	BB12	-1.46 ± 0.22	-0.9 ± 0.04	1.59 ± 0.06
	IPLA20030	-1.48 ± 0.1	-1.15 ± 0.25	1.94 ± 0.12
	IPLA20031	-1.67 ± 0.09	-0.89 ± 0.07	1.42 ± 0.28
	IPLA20032	1.31 ± 0.05	0.13 ± 0.12	3.43 ± 0.12
	IPLA20001	-0.84 ± 0.14	-1.1 ± 0	1.39 ± 0.17
	IPLA20002	-0.95 ± 0.09	-1.03 ± 0.14	1.44 ± 0.17
	IPLA20003	-1.61 ± 0.23	-1.15 ± 0.02	1.48 ± 0.44
	IPLA20021	-0.07 ± 0.21	-1.2 ± 0.15	2.03 ± 0.47
	IPLA20022	-0.38 ± 0.06	-1.15 ± 0.08	1.89 ± 0.37
	IPLA20004	-1.52 ± 0.2	-1.26 ± 0.06	1.99 ± 0.14
	IPLA20005	-1.4 ± 0.17	-1.17 ± 0.17	1.73 ± 0.22
	IPLA006	-1.01 ± 0.16	-1.49 ± 0.17	2.22 ± 0.17
	IPLA20026	-1.39 ± 0.23	-1.31 ± 0.24	1.91 ± 0.08
	IPLA20017	-1.97 ± 0.1	-1.29 ± 0.05	2.16 ± 0.12
	IPLA20015	0.86 ± 0.04	0.16 ± 0.06	3.24 ± 0.05
	IPLA20020	-1.71 ± 0.32	-1.05 ± 0.13	1.42 ± 0.17
	Control	-1.96 ± 0.03	-1.5 ± 0.14	1.47 ± 0.14

INFORMACIÓN SUPLEMENTARIA DEL ARTÍCULO 2

Supplementary file S1. Concentration (mM) of the total organic acids (acetate, lactate and formate) (mean ± standard deviation) in the batch cultures supplemented with different prebiotics at the different incubation times (6, 24 and 72 h).

		Total organic acids (mM)			
		B. animalis IPLA20031		*B. animalis* IPLA20032	
Hours	**Carbon source**	**Mean±sd**	**p-valor**	**Mean±sd**	**p-valor**
0h	Synergy	0.337 ± 0.183	0.279	0.227 ± 0.009	0.279
	Inulin	0.254 ± 0.15		0.232 ± 0.010	
	Actilight	0.142 ± 0.124		0.181 ± 0.054	
	Glucose	0.201 ± 0.031		0.177 ± 0.047	
	Negative control	0.131 ± 0.026		0.281 ± 0.114	
6h	Synergy	6.419 ± 0.662 [c]	0	5.034 ± 0.421 [c]	0
	Inulin	0.459 ± 0.076 [a]		0.565 ± 0.008 [a]	
	Actilight	8.303 ± 0.262 [d]		5.617 ± 0.408 [d]	
	Glucose	2.975 ± 0.219 [b]		1.882 ± 0.150 [b]	
	Negative control	0.496 ± 0.152 [a]		0.683 ± 0.274 [a]	
24h	Synergy	11.778 ± 1.255 [b]	0	13.978 ± 0.261 [b]	0
	Inulin	0.720 ± 0.049 [a]		0.629 ± 0.030 [a]	
	Actilight	19.082 ± 0.218 [c]		13.535 ± 4.905 [b]	
	Glucose	40.601 ± 1.442 [d]		32.896 ± 0.092 [c]	
	Negative control	0.647 ± 0.097 [a]		0.627 ± 0.120 [a]	
72h	Synergy	15.018 ± 1.507 [b]	0	14.513 ± 0.269 [b]	0
	Inulin	1.736 ± 0.174 [a]		2.094 ± 1.312 [a]	
	Actilight	20.369 ± 0.302 [c]		19.423 ± 0.425 [c]	
	Glucose	39.166 ± 1.264 [d]		42.860 ± 0.452 [d]	
	Negative control	1.781 ± 1.233 [a]		1.085 ± 0.229 [a]	

Letters indicate differences significant diferences among carbon sources.

Total organic acids (mM)					
		B. animalis Bb12		*B. animalis* IPLA20020	
Hours	**Carbon source**	**Mean±sd**	**p-valor**	**Mean±sd**	**p-valor**
0h	Synergy	0.231 ± 0.005	0.119	0.174 ± 0.015	0.069
	Inulin	0.252 ± 0.001		0.267 ± 0.012	
	Actilight	0.599 ± 0.291		0.289 ± 0.069	
	Glucose	0.337 ± 0.154		0.219 ± 0.072	
	Negative control	0.385 ± 0.163		0.353 ± 0.113	
6h	Synergy	6.091 ± 0.341 [b]	0	5.300± 0.267 [c]	0
	Inulin	0.688 ± 0.031 [a]		0.582 ± 0.151 [a]	
	Actilight	6.939 ± 0.280 [c]		5.818 ± 0.342 [c]	
	Glucose	1.023 ± 0.171 [a]		2.231 ± 1.309 [b]	
	Negative control	1.119 ± 0.243 [a]		0.595 ± 0.044 [a]	
24h	Synergy	11.768 ± 0.407 [b]	0	11.803 ± 1.012 [b]	0
	Inulin	0.919 ± 0.007 [a]		0.603 ± 0.119 [a]	
	Actilight	16.677 ± 0.509 [c]		16.315 ± 3.309 [c]	
	Glucose	30.638 ± 1.250 [d]		29.907 ± 2.267 [d]	
	Negative control	0.436 ± 0.052 [a]		0.611 ± 0.115 [a]	
72h	Synergy	13.871 ± 0.476 [b]	0	13.96 ± 0.959 [b]	0
	Inulin	1.627 ± 0.283 [a]		0.909 ± 0.252 [a]	
	Actilight	17.644 ± 0.843 [c]		15.453 ± 1.806 [b]	
	Glucose	40.294 ± 1.216 [d]		40.215 ± 2.802 [c]	
	Negative control	0.894 ± 0.158 [a]		0.962 ± 0.414 [a]	

Letters indicate differences significant diferences among carbon sources.

Total organic acids (mM)					
		B. longum IPLA20021		*B. longum* IPLA20022	
Hours	**Carbon source**	**Mean±sd**	**p-valor**	**Mean±sd**	**p-valor**
0h	Synergy	0.680 ± 0.185 [a]	0.023	0.817 ± 0.068	0.25
	Inulin	0.975 ± 0.063 [b]		0.912 ± 0.158	
	Actilight	0.881 ± 0.120 [a,b]		0.822 ± 0.137	
	Glucose	0.657 ± 0.071 [a]		0.777 ± 0.017	
	Negative control	0.895 ± 0.085 [a,b]		0.971 ± 0.104	
6h	Synergy	0.827 ± 0.081 [a]	0	1.569 ± 0.059 [b]	0
	Inulin	0.761 ± 0.004 [a]		1.093 ± 0.146 [a,b]	
	Actilight	0.975 ± 0.208 [a]		2.648 ± 0.404 [c]	
	Glucose	5.317 ± 1.167 [b]		3.215 ± 0.458 [d]	
	Negative control	0.854 ± 0.062 [a]		0.905 ± 0.069 [a]	
24h	Synergy	8.645 ± 0.154 [a]	0	16.706 ± 0.372 [b]	0
	Inulin	0.973 ± 0.040 [a]		1.140 ± 0.011 [a]	
	Actilight	21.886 ± 5.559 [b]		24.172 ± 0.764 [c]	
	Glucose	41.924 ± 6.530 [c]		41.163 ± 0.812 [d]	
	Negative control	1.112 ± 0.178 [a]		1.094 ± 0.092 [a]	
72h	Synergy	24.442 ± 0.957 [b]	0	22.031 ± 1.163 [b]	0
	Inulin	1.821 ± 0.344 [a]		1.803 ± 0.076 [a]	
	Actilight	26.126 ± 1.307 [c]		28.225 ± 0.928 [c]	
	Glucose	34.706 ± 0.474 [d]		46.829 ± 0.760 [d]	
	Negative control	1.647 ± 0.373 [a]		1.299 ± 0.584 [a]	

Letters indicate differences significant diferences among carbon sources.

Supplementary file S2. Increase of Log CFU/mL and decrease of pH (mean ± standard deviation) in the samples from *Bifidobacteriu*m strains cultures with glucose and different prebiotics collected at 6 hours of incubation. Letters indicate significant differences among carbon source from each strain (P<0.05).

			Decrease of pH		Increase of Log CFU/mL	
Specie	**Strain**	**Carbon source**	**Mean±sd**	**p-valor**	**Mean±sd**	**p-valor**
B. animalis	IPLA20031	Synergy	-0.68 ± 0.47 ^{a,b}	0.019	0.91 ± 0.08 ^{b}	0.000
		Inulin	-0.03 ± 0.04 ^{b}		-0.03 ± 0.02 ^{a}	
		Actilight	-0.88 ± 0.44 ^{a}		0.55 ± 0.37 ^{b}	
		Glucose	-0.31 ± 0.20 ^{a,b}		0.75 ± 0.07 ^{b}	
		WCS	-0.02 ± 0.06 ^{b}		-0.02 ± 0.03 ^{a}	
	IPLA20032	Synergy	-0.52 ± 0.02 ^{a}	0.000	0.34 ± 0.02 ^{b}	0.000
		Inulin	-0.02 ± 0.06 ^{c}		0.15 ± 0.04 ^{a}	
		Actilight	-0.52 ± 0.05 ^{a}		0.3 ± 0.02 ^{b}	
		Glucose	-0.19 ± 0.02 ^{b}		0.43 ± 0.02 ^{c}	
		WCS	-0.04 ± 0.00 ^{c}		0.08 ± 0.08 ^{a}	
	Bb12	Synergy	-0.50 ± 0.37 ^{a}	0.005	0.53 ± 0.16 ^{b}	0.000
		Inulin	-0.00 ± 0.00 ^{b}		-0.05 ± 0.03 ^{a}	
		Actilight	-0.67 ± 0.16 ^{a}		0.45 ± 0.15 ^{b}	
		Glucose	-0.29 ± 0.01 ^{a,b}		0.58 ± 0.13 ^{b}	
		WCS	-0.06 ± 0.09 ^{b}		0.02 ± 0.03 ^{a}	
	IPLA20020	Synergy	-0.56 ± 0.11 ^{a}	0.000	0.58 ± 0.09 ^{b}	0.000
		Inulin	-0.09 ± 0.11 ^{c}		0.14 ± 0.07 ^{a}	
		Actilight	-0.44 ± 0.09 ^{a,b}		0.59 ± 0.15 ^{b}	
		Glucose	-0.33 ± 0.03 ^{b}		0.91 ± 0.06 ^{c}	
		WCS	-0.11 ± 0.11 ^{c}		0.13 ± 0.1 ^{a}	
B. longum	IPLA20021	Synergy	-0.07 ± 0.06 ^{b}	0.049	-0.27 ± 0.38	0.295
		Inulin	-0.02 ± 0.00 ^{b}		0.07 ± 0.02	
		Actilight	-0.10 ± 0.19 ^{b}		0.19 ± 0.32	
		Glucose	-0.32 ± 0.17 ^{a}		0.22 ± 0.33	
		WCS	-0.01 ± 0.03 ^{b}		0.09 ± 0.23	
	IPLA20022	Synergy	-0.08 ± 0.05 ^{b,c}	0.005	0.33 ± 0.81	0.089
		Inulin	-0.07 ± 0.04 ^{b,c}		0.28 ± 0.04	
		Actilight	-0.19 ± 0.06 ^{a,b}		0.98 ± 0.26	
		Glucose	-0.26 ± 0.13 ^{a}		0.91 ± 0.35	
		WCS	-0.02 ± 0.03 ^{c}		0.09 ± 0.15	

Letter indicate differences significant diferences among carbon sources.

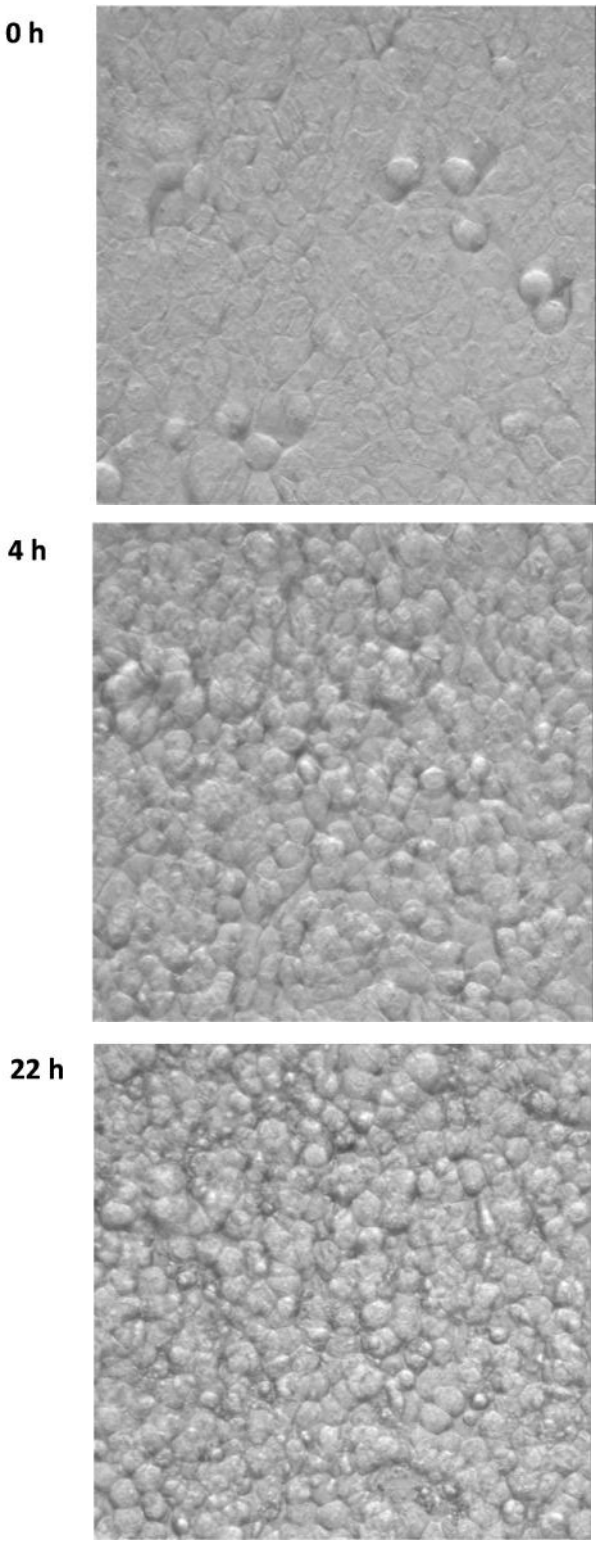
INFORMACIÓN SUPLEMENTARIA DEL ARTÍCULO 3

Supplementary Table S1. Composition of culture media used to select the best conditions for growth and toxin production of *Clostridium difficile* LMG21717.

RCM (CM0149, Oxoid[1])	(g/L)	**BHI** (CM1135, Oxoid[1])	(g/L)	**GAM** (05422, Nissui[2])	(g/L)
Peptone	10	Brain infusion solids	12.5	Peptone	10
"Lab-Lemco" powder	10	Beef heart infusion solids	5	Soya peptone	3
Yeast extract	3	Proteose peptone	10	Proteose peptone	10
Glucose	5	Glucose	2	Digested serum	13.5
Soluble starch	1	Sodium chloride	5	Yeast extract	5
Sodium chloride	5	Disodium phosphate	2.5	Meat extract	2.2
Sodium acetate	3			Liver extract	1.2
Cystein hydrochloride	0.5			Dextrose	3
Agar	0.5			Potasium dihydrogen phosphate	2.5
				Sodium chloride	3
				Soluble starch	5
				L-cysteine hydrochloride	0.3
				Sodium thioglycollate	0.3
pH 6.8 ± 0.2		pH 7.4 ± 0.2		pH 7.1 ± 0.2	

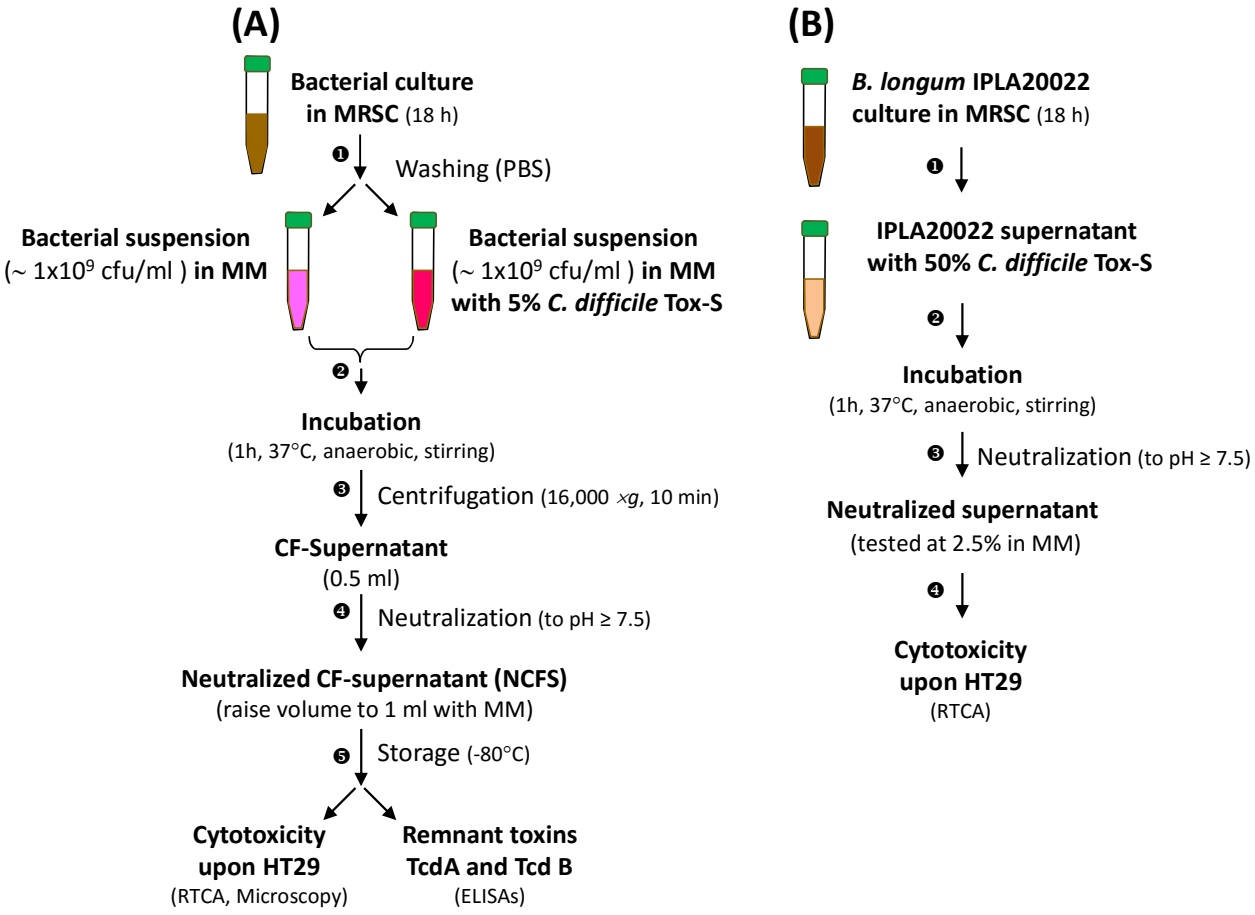
[1] Oxoid, Thermo Fisher Scientific Inc., Waltham, MA; [2] Nissui Pharmaceutical Co., Ltd., Tokyo, Japan.

Supplementary Figure S1. Visualization under inverted optical microscope (objective x60) of the HT29 monolayer in the presence of 2.5% *C. difficile* toxigenic supernatant collected from 48 h cultures in GAM medium. Microphotographs were captured immediately before the addition of the supernatant (0 h) and 4 h or 22 h after addition.

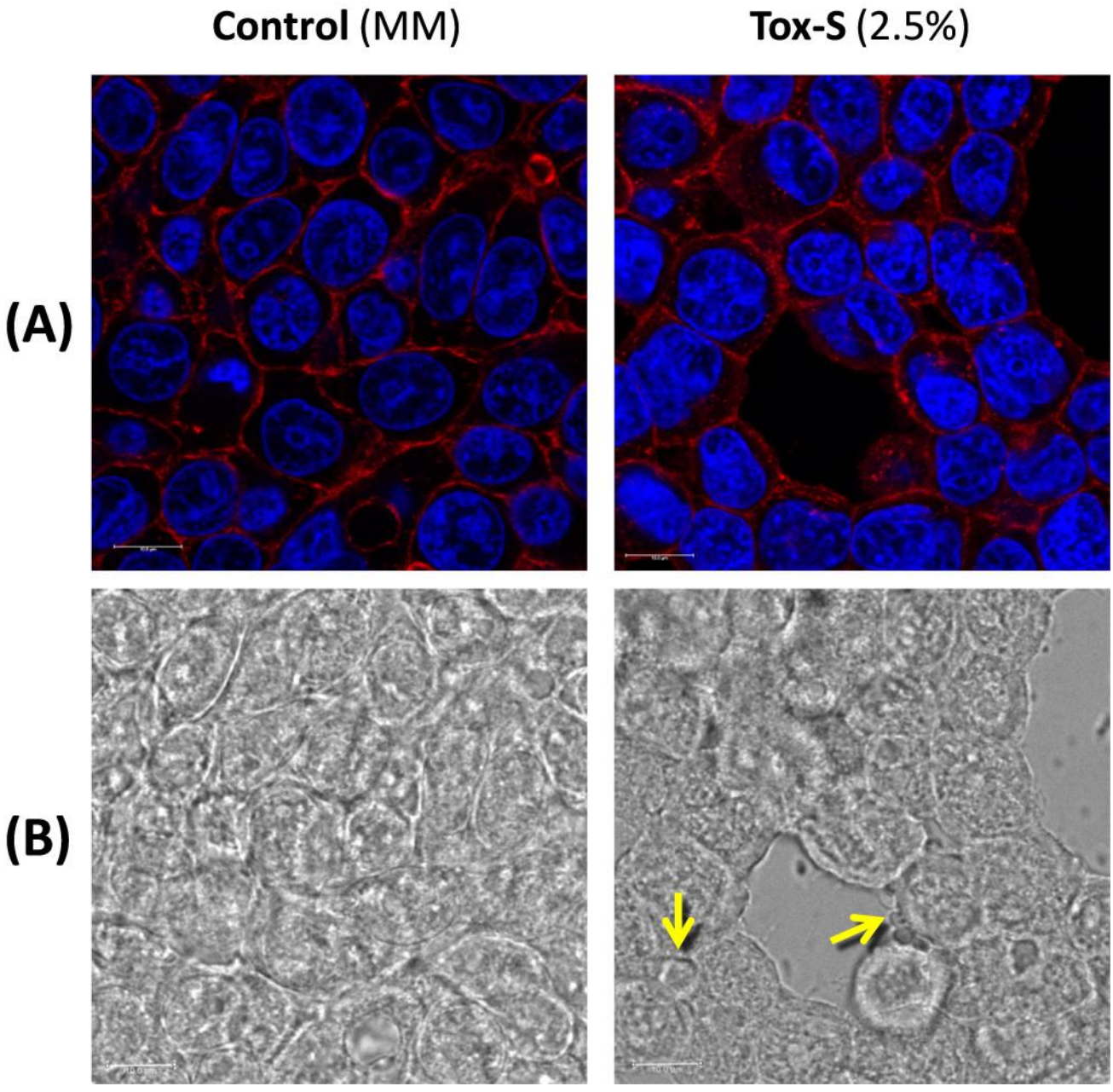


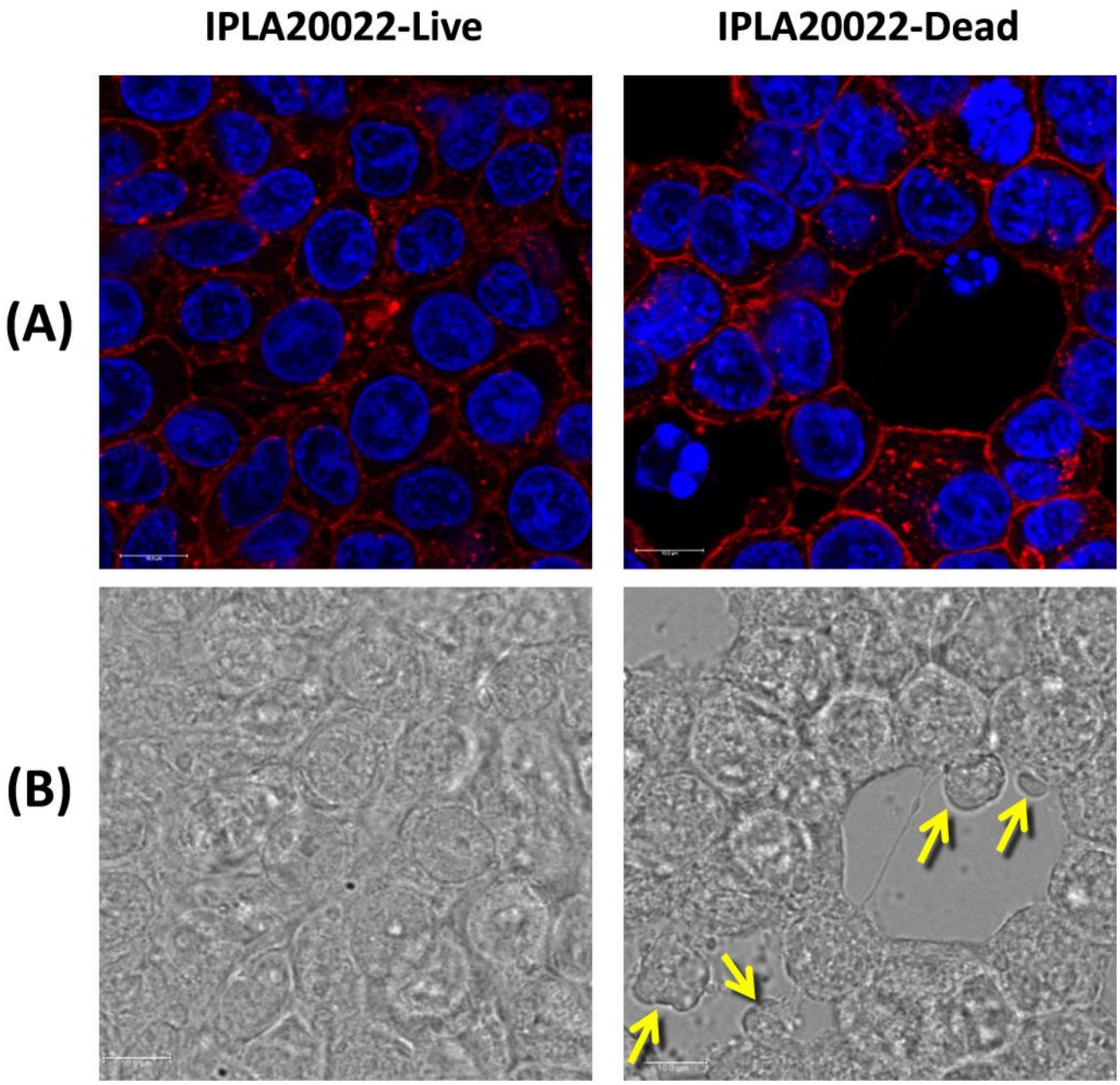
INFORMACIÓN SUPLEMENTARIA DEL ARTÍCULO 4:

Supplementary Figure S1. Final conditions used to perform the screening of the anti-*C. difficile* cytotoxic activity upon HT29 monolayers of twenty bifidobacteria and lactobacilli strains (**A**). Scheme of the flow-work followed to test the capability of *B. longum* IPLA20022 culture supernatant to act against toxigenic *C. difficile* supernatant (**B**) MRSC, MRS broth supplemented with 0.25% L-cysteine; MM, McCoy´s Medium added with supplements and antibiotics described in material and methods section; NCFS, neutralized cell-free supernatant.



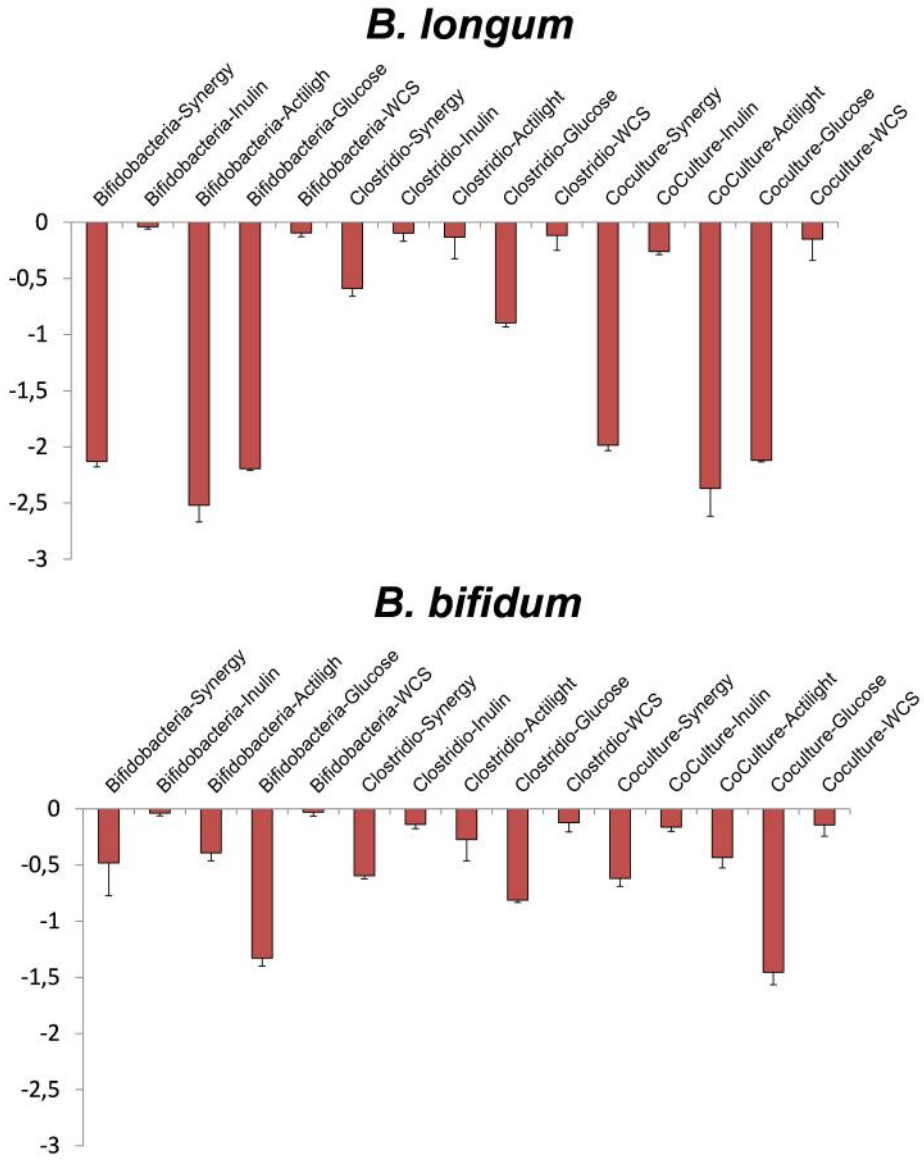
Supplementary Figure S2. Immunofluorescence images obtained by CSLM of HT29 after 20 h of incubation without toxigenic *C. difficile* supernatant Tox-S (control) and with Tox-S (damage control), and with NCFS (neutralized cell-free supernatants) obtained after incubation of live or dead *B. longum* IPLA20022 with Tox-S. Images show a CSLM-2.50 optical zoom of a XY-slide. Upper-part images show the combination of DAPI-stained nucleus (blue, excite at 405 nm by a blue-violet laser diode) and F-actin stained with Phalloidin-Alexa-Fluor-568 probe (red, excited at 578 nm by a white light laser) (**A**). Bottom-part images show the same magnification view at the visible (transmitted light) channel (**B**). Arrows indicates the apoptotic bodies. Bars 10 μm.

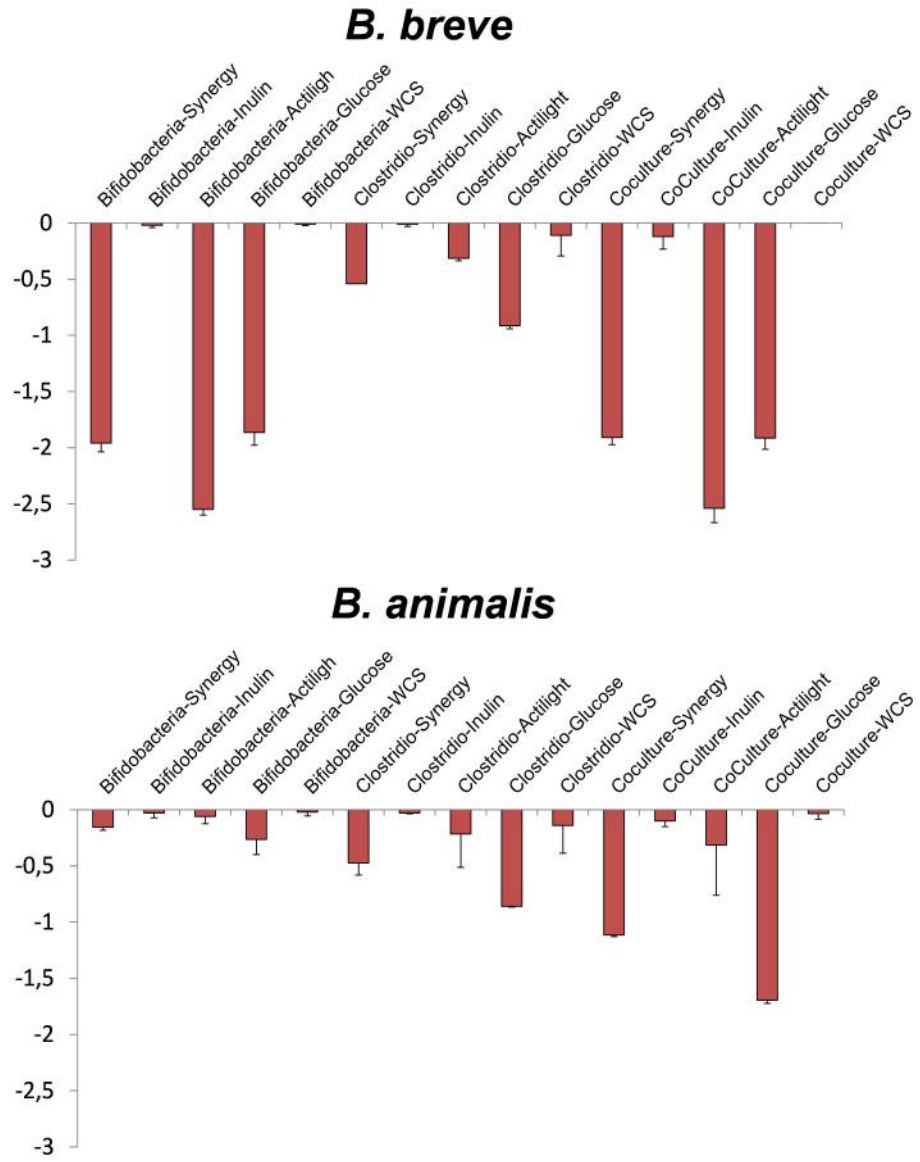



IPLA20022-Live
IPLA20022-Dead
(A)
(B)

INFORMACIÓN SUPLEMENTARIA DEL ARTÍCULO 5:

Figure Supplementary 1. Reduction of pH, with regard to time zero, obtained after 24 hours of incubation of the Bifidobacteria-*C. difficile* co-cultures, and their corresponding mono-cultures, in the different carbon sources.




B. breve
Bifidobacteria-Synergy
Bifidobacteria-Inulin
Bifidobacteria-Actiligh
Bifidobacteria-Glucose
Bifidobacteria-WCS
Clostridio-Synergy
Clostridio-Inulin
Clostridio-Actilight
Clostridio-Glucose
Clostridio-WCS
Coculture-Synergy
CoCulture-Inulin
CoCulture-Actilight
Coculture-Glucose
Coculture-WCS
0
-0,5
-1
-1,5
-2
-2,5
-3
B. animalis
Bifidobacteria-Synergy
Bifidobacteria-Inulin
Bifidobacteria-Actiligh
Bifidobacteria-Glucose
Bifidobacteria-WCS
Clostridio-Synergy
Clostridio-Inulin
Clostridio-Actilight
Clostridio-Glucose
Clostridio-WCS
Coculture-Synergy
CoCulture-Inulin
CoCulture-Actilight
Coculture-Glucose
Coculture-WCS
0
-0,5
-1
-1,5
-2
-2,5
-3

Supplementary File. Increments, with respect to time zero, in the concentration of butyrate on the bacterial cultures when grown in mono-culture or in co-culture in the prebiotics Synergy, Inulin and Actilight, in glucose or without any carbon source added (WCS).

Strains	Carbon source	Culture	Δ Butyrate bifidobacteria (mM) mean ± sd	p-value mono-culture vs co-culture	Δ Butyrate clostridia (mM) mean ± sd	p-value mono-culture vs co-culture
B. longum IPLA20022 & C. difficile LMG21717	Synergy	Mono-culture	0,009 ± 0,008	0.09	1,116 ± 0,640	0.33
		Co-culture	0,604 ± 0,473		0,605 ± 0,469	
	Inuline	Mono-culture	-0,008 ± 0,0071	0.16	0,559 ± 0,450	0.22
		Co-culture	3,378 ± 3,356		3,378 ± 3,358	
	Actilight	Mono-culture	-0,003 ± 0,007	***0.02***	4,004 ± 3,985	0.16
		Co-culture	0,0154 ± 0,006		0,0154 ± 0,006	
	Glucose	Mono-culture	-0,029 ± 0,038	***0.04***	1,570 ± 0,435	0.06
		Co-culture	0,679 ± 0,401		0,679 ± 0,401	
	WCS	Mono-culture	0,006 ± 0,009	0.15	3,215 ± 3,206	0.24
		Co-culture	0,604 ± 0,590		0,604 ± 0,590	
B. breve IPLA20006 & C. difficile LMG21717	Synergy	Mono-culture	-0,004 ± 0,007	***<0,01***	1,849 ± 0,023	***<0,01***
		Co-culture	0,657 ± 0,015		0,657 ± 0,015	
	Inuline	Mono-culture	-0,006 ± 0,002	0.09	0,664 ± 0,015	0.22
		Co-culture	1,955 ± 1,553		1,955 ± 1,553	
	Actilight	Mono-culture	-0,004 ± 0,001	***<0,01***	5,891 ± 1,343	***<0,01***
		Co-culture	0,006 ± 0,001		0,007 ± 0,001	
	Glucose	Mono-culture	0,021 ± 0,007	***<0,01***	1,986 ± 0,177	***<0,01***
		Co-culture	0,964 ± 0,319		0,965 ± 0,319	
	WCS	Mono-culture	-0,003 ± 0,002	0.80	3,558 ± 3,553	0.16
		Co-culture	-0,001 ± 0,015		0,001 ± 0,015	
B. animalis Bb12 & C. difficile LMG21717	Synergy	Mono-culture	-0,013 ± 0,011	***<0,01***	3,684 ± 2,654	0.10
		Co-culture	0,390 ± 0,093		0,390 ± 0,093	
	Inuline	Mono-culture	-0,001 ± 0,005	***<0,01***	0,557 ± 0,124	0.12
		Co-culture	0,415 ± 0,006		0,416 ± 0,006	
	Actilight	Mono-culture	-0,003 ± 0,006	0.76	3,768 ± 3,783	0.16
		Co-culture	-0,002 ± 0,001		-0,002 ± 0,001	
	Glucose	Mono-culture	-0,095 ± 0,083	***<0,01***	1,377 ± 0,066	***<0,01***
		Co-culture	0,935 ± 0,054		0,935 ± 0,054	
	WCS	Mono-culture	0,003 ± 0,002	***<0,01***	3,349 ± 3,343	0.16
		Co-culture	0,016 ± 0,001		0,016 ± 0,001	
B. bifidum IPLA 20015 & C. difficile LMG21717	Synergy	Mono-culture	0,062 ± 0,125	0.11	5,305 ± 4,729	0.82
		Co-culture	6,307 ± 5,198		6,307 ± 5,198	
	Inuline	Mono-culture	0,086 ± 0,094	0.06	5,639 ± 0,517	0.17
		Co-culture	4,592 ± 3,087		4,592 ± 3,087	
	Actilight	Mono-culture	-0,006 ± 0,009	0.16	7,879 ± 0,363	0.23
		Co-culture	4,355 ± 4,339		4,356 ± 4,339	
	Glucose	Mono-culture	0,004 ± 0,019	***<0,01***	5,506 ± 3,262	0.13
		Co-culture	1,877 ± 0,282		1,877 ± 0,282	
	WCS	Mono-culture	-0,010 ± 0,006	0.16	3,541 ± 3,467	0.95
		Co-culture	3,735 ± 3,721		3,736 ± 3,721	

Supplementary File. Increments, with respect to time zero, in the concentration of propionate on the bacterial cultures when grown in mono-culture or in co-culture in the prebiotics Synergy, Inulin and Actilight, in glucose or without any carbon source added (WCS).

Strains	Carbon source	Culture	Δ Propionate bifidobacteria (mM) mean ± sd	p-value mono-culture vs co-culture	Δ Propionate clostridia (mM) mean ± sd	p-value mono-culture vs co-culture
B. longum IPLA20022 & C. difficile LMG21717	Synergy	Mono-culture	-0,072 ± 0,057	0.12	0,437 ± 0,049	***<0,01***
		Co-culture	0,026 ± 0,065		0,026 ± 0,065	
	Inuline	Mono-culture	-0,032 ± 0,039	0.13	0,152 ± 0,038	0.38
		Co-culture	0,357 ± 0,356		0,357 ± 0,356	
	Actilight	Mono-culture	0,001 ± 0,055	0.36	0,045 ± 0,014	0.77
		Co-culture	0,060 ± 0,081		0,060 ± 0,081	
	Glucose	Mono-culture	-0,034 ± 0,013	***0.04***	0,033 ± 0,001	***<0,01***
		Co-culture	-0,012 ± 0,003		-0,012 ± 0,003	
	WCS	Mono-culture	0,003 ± 0,017	0.20	0,379 ± 0,374	0.57
		Co-culture	0,219 ± 0,246		0,219 ± 0,246	
B. breve IPLA20006 & C. difficile LMG21717	Synergy	Mono-culture	-0,007 ± 0,008	0.14	0,421 ± 0,010	***<0,01***
		Co-culture	0,030 ± 0,034		0,030 ± 0,034	
	Inuline	Mono-culture	0,003 ± 0,008	0.11	0,124 ± 0,000	0.25
		Co-culture	0,354 ± 0,298		0,354 ± 0,298	
	Actilight	Mono-culture	-0,013 ± 0,006	0.70	0,671 ± 0,003	***<0,01***
		Co-culture	-0,018 ± 0,019		-0,018 ± 0,019	
	Glucose	Mono-culture	-0,033 ± 0,005	0.37	0,100 ± 0,024	***<0,01***
		Co-culture	-0,018 ± 0,025		-0,018 ± 0,025	
	WCS	Mono-culture	-0,003 ± 0,001	0.05	0,341 ± 0,388	0.17
		Co-culture	-0,035 ± 0,020		-0,035 ± 0,020	
B. animalis Bb12 & C. difficile LMG21717	Synergy	Mono-culture	-0,117 ± 0,133	***0.03***	0,335 ± 0,081	***<0,01***
		Co-culture	0,148 ± 0,028		0,148 ± 0,028	
	Inuline	Mono-culture	-0,046 ± 0,050	***0.01***	0,110 ± 0,005	***<0,01***
		Co-culture	0,078 ± 0,007		0,078 ± 0,007	
	Actilight	Mono-culture	-0,024 ± 0,017	0.16	0,301 ± 0,285	0.14
		Co-culture	-0,004 ± 0,012		-0,004 ± 0,012	
	Glucose	Mono-culture	-0,010 ± 0,020	***<0,01***	0,045 ± 0,012	0.13
		Co-culture	0,058 ± 0,002		0,058 ± 0,002	
	WCS	Mono-culture	0,005 ± 0,008	0.16	0,371 ± 0,359	0.16
		Co-culture	0,013 ± 0,002		0,013 ± 0,002	
B. bifidum IPLA 20015 & C. difficile LMG21717	Synergy	Mono-culture	-0,024 ± 0,023	0.06	0,390 ± 0,335	0.69
		Co-culture	0,510 ± 0,347		0,510 ± 0,347	
	Inuline	Mono-culture	-0,003 ± 0,012	***0.02***	0,790 ± 0,112	0.09
		Co-culture	0,472 ± 0,217		0,472 ± 0,217	
	Actilight	Mono-culture	-0,234 ± 0,252	0.09	0,696 ± 0,049	0.12
		Co-culture	0,316 ± 0,335		0,316 ± 0,335	
	Glucose	Mono-culture	-0,075 ± 0,081	0.05	0,045 ± 0,078	0.85
		Co-culture	0,054 ± 0,010		0,054 ± 0,010	
	WCS	Mono-culture	-0,035 ± 0,059	0.12	0,315 ± 0,329	0.75
		Co-culture	0,416 ± 0,393		0,416 ± 0,393	

Supplementary File. Increments, with respect to time zero, in the concentration of caproate on the bacterial cultures when grown in mono-culture or in co-culture in the prebiotics Synergy, Inulin and Actilight, in glucose or without any carbon source added (WCS).

Strains	Carbon source	Culture	Δ Caproate bifidobacteria (mM) mean ± sd	p-value mono-culture vs co-culture	Δ Caproate clostridia (mM) mean ± sd	p-value mono-culture vs co-culture
B. longum IPLA20022 & C. difficile LMG21717	Synergy	Mono-culture	-0,004 ± 0,004	0.10	0,006 ± 0,006	0.64
		Co-culture	0,003 ± 0,005		0,003 ± 0,005	
	Inuline	Mono-culture	-0,005 ± 0,004	0.07	0,003 ± 0,005	0.63
		Co-culture	0,002 ± 0,003		0,002 ± 0,003	
	Actilight	Mono-culture	-0,003 ± 0,002	0.31	0,006 ± 0,003	0.17
		Co-culture	0,000 ± 0,005		0,000 ± 0,005	
	Glucose	Mono-culture	-0,005 ± 0,000	0.22	0,001 ± 0,006	0.76
		Co-culture	0,000 ± 0,006		0,000 ± 0,006	
	WCS	Mono-culture	-0,003 ± 0,003	0.34	0,003 ± 0,000	0.26
		Co-culture	0,000 ± 0,003		0,000 ± 0,003	
B. breve IPLA20006 & C. difficile LMG21717	Synergy	Mono-culture	-0,003 ± 0,000	0.16	0,002 ± 0,003	0.81
		Co-culture	0,001 ± 0,004		0,001 ± 0,004	
	Inuline	Mono-culture	-0,004 ± 0,002	0.07	0,000 ± 0,002	0.13
		Co-culture	0,013 ± 0,012		0,013 ± 0,012	
	Actilight	Mono-culture	-0,001 ± 0,001	0.30	0,007 ± 0,007	0.32
		Co-culture	0,002 ± 0,004		0,002 ± 0,004	
	Glucose	Mono-culture	-0,003 ± 0,001	***<0,01***	0,000 ± 0,006	0.39
		Co-culture	0,003 ± 0,001		0,003 ± 0,001	
	WCS	Mono-culture	0,000 ± 0,002	0.86	0,007 ± 0,003	0.13
		Co-culture	0,001 ± 0,004		0,001 ± 0,004	
B. animalis Bb12 & C. difficile LMG21717	Synergy	Mono-culture	-0,010 ± 0,009	0.21	0,004 ± 0,002	0.06
		Co-culture	-0,001 ± 0,003		-0,001 ± 0,003	
	Inuline	Mono-culture	-0,004 ± 0,002	0.17	0,000 ± 0,002	0.21
		Co-culture	-0,002 ± 0,001		-0,002 ± 0,001	
	Actilight	Mono-culture	-0,005 ± 0,000	0.20	-0,001 ± 0,002	0.80
		Co-culture	0,000 ± 0,006		0,000 ± 0,006	
	Glucose	Mono-culture	0,001 ± 0,008	0.92	-0,001 ± 0,000	0.09
		Co-culture	0,001 ± 0,002		0,001 ± 0,002	
	WCS	Mono-culture	-0,004 ± 0,002	0.81	0,001 ± 0,001	***0.04***
		Co-culture	-0,005 ± 0,003		-0,005 ± 0,003	
B. bifidum IPLA 20015 & C. difficile LMG21717	Synergy	Mono-culture	0,003 ± 0,002	0.92	0,002 ± 0,008	0.79
		Co-culture	0,004 ± 0,004		0,004 ± 0,004	
	Inuline	Mono-culture	0,004 ± 0,006	0.96	0,004 ± 0,001	0.88
		Co-culture	0,004 ± 0,004		0,004 ± 0,004	
	Actilight	Mono-culture	-0,004 ± 0,005	0.08	0,003 ± 0,001	0.43
		Co-culture	0,006 ± 0,006		0,006 ± 0,006	
	Glucose	Mono-culture	-0,005 ± 0,001	***0.01***	0,001 ± 0,001	0.80
		Co-culture	0,001 ± 0,002		0,001 ± 0,002	
	WCS	Mono-culture	0,000 ± 0,001	0.33	0,002 ± 0,005	0.86
		Co-culture	0,002 ± 0,004		0,002 ± 0,004	

Supplementary File. Increments, with respect to time zero, in the concentration of valerate on the bacterial cultures when grown in mono-culture or in co-culture in the prebiotics Synergy, Inulin and Actilight, in glucose or without any carbon source added (WCS).

Strains	Carbon source	Culture	Δ Valerate bifidobacteria (mM) mean ± sd	p-value mono-culture vs co-culture	Δ Valerate clostridia (mM) mean ± sd	p-value mono-culture vs co-culture
B. longum IPLA20022 & C. difficile LMG21717	Synergy	Mono-culture	0,000 ± 0,000	0.16	0,166 ± 0,023	0.08
		Co-culture	0,068 ± 0,068		0,068 ± 0,068	
	Inuline	Mono-culture	0,000 ± 0,000	0.16	0,149 ± 0,013	0.63
		Co-culture	0,114 ± 0,114		0,114 ± 0,114	
	Actilight	Mono-culture	0,000 ± 0,000	0.16	0,145 ± 0,145	0.32
		Co-culture	0,046 ± 0,046		0,046 ± 0,046	
	Glucose	Mono-culture	0,000 ± 0,000	1.00	0,072 ± 0,072	0.16
		Co-culture	0,000 ± 0,000		0,000 ± 0,000	
	WCS	Mono-culture	0,000 ± 0,000	0.16	0,108 ± 0,108	0.68
		Co-culture	0,074 ± 0,074		0,074 ± 0,074	
B. breve IPLA20006 & C. difficile LMG21717	Synergy	Mono-culture	0,000 ± 0,000	0.16	0,201 ± 0,006	***<0,01***
		Co-culture	0,046 ± 0,046		0,046 ± 0,046	
	Inuline	Mono-culture	0,000 ± 0,000	***<0,01***	0,158 ± 0,002	0.16
		Co-culture	0,209 ± 0,052		0,209 ± 0,052	
	Actilight	Mono-culture	0,000 ± 0,000	0.16	0,283 ± 0,032	***<0,01***
		Co-culture	0,065 ± 0,065		0,065 ± 0,065	
	Glucose	Mono-culture	0,000 ± 0,000	0.16	0,146 ± 0,003	0.10
		Co-culture	0,065 ± 0,065		0,065 ± 0,065	
	WCS	Mono-culture	0,000 ± 0,000	1.00	0,133 ± 0,087	0.06
		Co-culture	0,000 ± 0,000		0,000 ± 0,000	
B. animalis Bb12 & C. difficile LMG21717	Synergy	Mono-culture	-0,069 ± 0,069	0.07	0,238 ± 0,089	0.06
		Co-culture	0,068 ± 0,068		0,068 ± 0,068	
	Inuline	Mono-culture	-0,022 ± 0,022	***<0,01***	0,155 ± 0,003	0.65
		Co-culture	0,154 ± 0,004		0,154 ± 0,004	
	Actilight	Mono-culture	0,000 ± 0,000	1.00	0,140 ± 0,140	0.16
		Co-culture	0,000 ± 0,000		0,000 ± 0,000	
	Glucose	Mono-culture	0,000 ± 0,000	1.00	0,118 ± 0,026	***<0,01***
		Co-culture	0,000 ± 0,000		0,000 ± 0,000	
	WCS	Mono-culture	0,000 ± 0,000	1.00	0,113 ± 0,113	0.16
		Co-culture	0,000 ± 0,000		0,000 ± 0,000	
B. bifidum IPLA 20015 & C. difficile LMG21717	Synergy	Mono-culture	0,000 ± 0,000	***0.01***	0,229 ± 0,137	0.77
		Co-culture	0,259 ± 0,100		0,259 ± 0,100	
	Inuline	Mono-culture	0,000 ± 0,000	***<0,01***	0,276 ± 0,027	0.62
		Co-culture	0,250 ± 0,081		0,250 ± 0,081	
	Actilight	Mono-culture	0,000 ± 0,000	0.16	0,292 ± 0,059	0.13
		Co-culture	0,131 ± 0,131		0,131 ± 0,131	
	Glucose	Mono-culture	0,000 ± 0,000	0.16	0,244 ± 0,100	0.08
		Co-culture	0,076± 0,076		0,076 ± 0,076	
	WCS	Mono-culture	0,000 ± 0,000	0.16	0,162 ± 0,112	0.80
		Co-culture	0,135± 0,135		0,135 ± 0,135	

Supplementary File. Increments, with respect to time zero, in the concentration of iso-butyrate on the bacterial cultures when grown in mono-culture or in co-culture in the prebiotics Synergy, Inulin and Actilight, in glucose or without any carbon source added (WCS).

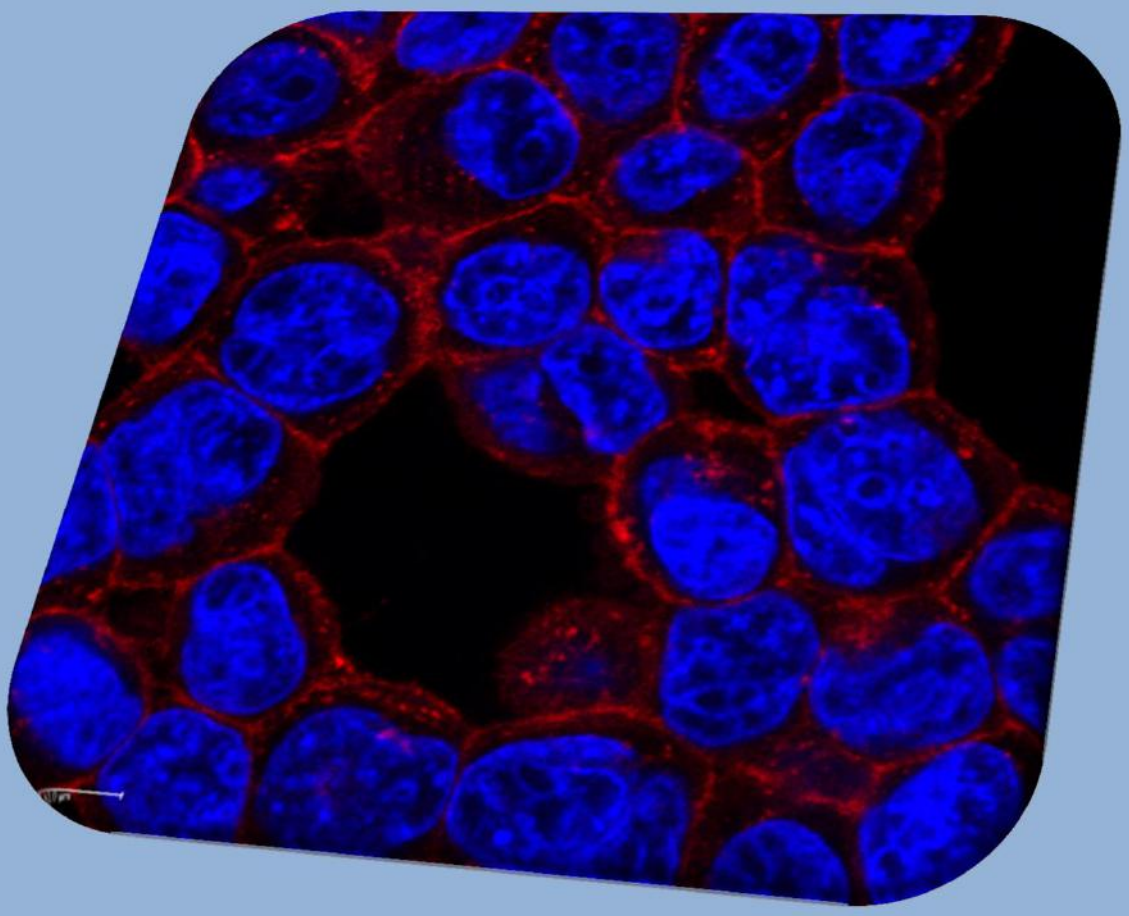
Strains	Carbon source	Culture	Δ Iso-Butyrate bifidobacteria (mM) mean ± sd	p-value mono-culture vs co-culture	Δ Iso-Butyrate clostridia (mM) mean ± sd	p-value mono-culture vs co-culture
B. longum IPLA20022 & C. difficile LMG21717	Synergy	Mono-culture	0,014 ± 0,0356	0.07	0,919 ± 0,384	***0.02***
		Co-culture	0,122 ± 0,068		0,122 ± 0,068	
	Inuline	Mono-culture	0,000 ± 0,001	0.16	0,245 ± 0,187	0.32
		Co-culture	0,755 ± 0,751		0,755 ± 0,750	
	Actilight	Mono-culture	0,004 ± 0,001	0.67	0,741 ± 0,737	0.16
		Co-culture	0,006 ± 0,008		0,006 ± 0,008	
	Glucose	Mono-culture	0,030 ± 0,024	0.75	0,108 ± 0,021	***0.01***
		Co-culture	0,036 ± 0,020		0,036 ± 0,020	
	WCS	Mono-culture	-0,003 ± 0,001	0.15	0,755 ± 0,754	0.98
		Co-culture	0,738 ± 0,728		0,738 ± 0,729	
B. breve IPLA20006 & C. difficile LMG21717	Synergy	Mono-culture	0,002 ± 0,001	***0.04***	1,272 ± 0,018	***<0,01***
		Co-culture	0,0589 ± 0,033		0,059 ± 0,032	
	Inuline	Mono-culture	-0,001 ± 0,004	0.09	0,272 ± 0,006	0.23
		Co-culture	0,796 ± 0,632		0,795 ± 0,632	
	Actilight	Mono-culture	0,001 ± 0,001	0.23	1,497 ± 0,032	***<0,01***
		Co-culture	0,003 ± 0,002		0,003 ± 0,002	
	Glucose	Mono-culture	-0,004 ± 0,005	***<0,01***	0,143 ± 0,013	***<0,01***
		Co-culture	0,031 ± 0,010		0,031 ± 0,010	
	WCS	Mono-culture	-0,001 ± 0,001	0.10	0,749 ± 0,739	0.15
		Co-culture	-0,006 ± 0,003		-0,006 ± 0,003	
B. animalis Bb12 & C. difficile LMG21717	Synergy	Mono-culture	0,076 ± 0,086	***0.04***	1,168 ± 0,226	***<0,01***
		Co-culture	0,222 ± 0,002		0,222 ± 0,002	
	Inuline	Mono-culture	0,002 ± 0,000	***<0,01***	0,228 ± 0,044	0.31
		Co-culture	0,191 ± 0,034		0,191 ± 0,034	
	Actilight	Mono-culture	-0,002 ± 0,002	0.63	0,713 ± 0,713	0.16
		Co-culture	-0,004 ± 0,003		-0,003 ± 0,004	
	Glucose	Mono-culture	-0,085 ± 0,080	0.06	0,076 ± 0,007	***<0,01***
		Co-culture	0,036 ± 0,011		0,036 ± 0,011	
	WCS	Mono-culture	0,000 ± 0,002	0.79	0,762 ± 0,770	0.16
		Co-culture	0,001 ± 0,006		0,001 ± 0,006	
B. bifidum IPLA 20015 & C. difficile LMG21717	Synergy	Mono-culture	0,065 ± 0,094	***0.02***	1,018 ± 0,603	0.65
		Co-culture	1,239 ± 0,503		1,239 ± 0,503	
	Inuline	Mono-culture	0,077 ± 0,077	***0.02***	1,620 ± 0,046	0.16
		Co-culture	1,115 ± 0,499		1,115 ± 0,499	
	Actilight	Mono-culture	0,000 ± 0,000	0.16	1,584 ± 0,009	0.16
		Co-culture	0,787 ± 0,790		0,787 ± 0,791	
	Glucose	Mono-culture	0,001 ± 0,005	***<0,01***	0,423 ± 0,331	0.16
		Co-culture	0,096 ± 0,023		0,096 ± 0,024	
	WCS	Mono-culture	-0,002 ± 0,002	0.16	0,804 ± 0,794	1.00
		Co-culture	0,803 ± 0,797		0,803 ± 0,797	

Supplementary File. Increments, with respect to time zero, in the concentration of iso-caproate on the bacterial cultures when grown in mono-culture or in co-culture in the prebiotics Synergy, Inulin and Actilight, in glucose or without any carbon source added (WCS).

Strains	Carbon source	Culture	Δ Iso-caproate bifidobacteria (mM) mean ± sd	p-value mono-culture vs co-culture	Δ Iso-Caproate clostridia (mM) mean ± sd	p-value mono-culture vs co-culture
B. longum IPLA20022 & C. difficile LMG21717	Synergy	Mono-culture	-0,017 ± 0,031	0.11	1,812 ± 0,018	0.06
		Co-culture	0,791 ± 0,675		0,791 ± 0,675	
	Inuline	Mono-culture	-0,005 ± 0,002	0.14	0,375 ± 0,242	0.36
		Co-culture	0,916 ± 0,881		0,916 ± 0,881	
	Actilight	Mono-culture	-0,004 ± 0,006	0.09	0,943 ± 0,878	0.15
		Co-culture	0,030 ± 0,026		0,030 ± 0,026	
	Glucose	Mono-culture	0,004 ± 0,007	***0.04***	1,509 ± 0,034	***0.04***
		Co-culture	0,753 ± 0,429		0,753 ± 0,429	
	WCS	Mono-culture	-0,003 ± 0,000	0.14	0,944 ± 0,869	0.95
		Co-culture	0,900 ± 0,837		0,900 ± 0,837	
B. breve IPLA20006 & C. difficile LMG21717	Synergy	Mono-culture	0,005 ± 0,001	***<0,01***	1,750 ± 0,028	***<0,01***
		Co-culture	0,945 ± 0,002		0,945 ± 0,002	
	Inuline	Mono-culture	-0,001 ± 0,002	0.09	0,393 ± 0,010	0.24
		Co-culture	0,996 ± 0,763		0,996 ± 0,763	
	Actilight	Mono-culture	-0,002 ± 0,000	0.18	1,826 ± 0,037	***<0,01***
		Co-culture	-0,015 ± 0,014		-0,015 ± 0,014	
	Glucose	Mono-culture	-0,001 ± 0,001	***<0,01***	1,517 ± 0,032	***<0,01***
		Co-culture	0,894 ± 0,231		0,894 ± 0,231	
	WCS	Mono-culture	-0,005 ± 0,003	0.82	0,916 ± 0,897	0.15
		Co-culture	-0,010± 0,040		-0,010 ± 0,040	
B. animalis Bb12 & C. difficile LMG21717	Synergy	Mono-culture	0,002 ± 0,008	***<0,01***	1,727 ± 0,100	***<0,01***
		Co-culture	1,320 ± 0,039		1,320 ± 0,039	
	Inuline	Mono-culture	-0,001 ± 0,005	***<0,01***	0,364 ± 0,083	0.64
		Co-culture	0,332 ± 0,072		0,332 ± 0,072	
	Actilight	Mono-culture	0,000 ± 0,005	0.18	0,848 ± 0,837	0.16
		Co-culture	0,014 ± 0,014		0,014 ± 0,014	
	Glucose	Mono-culture	-0,001 ± 0,000	***<0,01***	1,339 ± 0,018	***0.01***
		Co-culture	1,138 ± 0,075		1,138 ± 0,075	
	WCS	Mono-culture	0,002 ± 0,010	***<0,01***	0,916 ± 0,888	0.18
		Co-culture	0,081 ± 0,019		0,081 ± 0,019	
B. bifidum IPLA 20015 & C. difficile LMG21717	Synergy	Mono-culture	-0,052 ± 0,051	***<0,01***	1,685 ± 0,247	0.25
		Co-culture	1,924 ± 0,182		1,924 ± 0,182	
	Inuline	Mono-culture	-0,002 ± 0,008	***<0,01***	1,886 ± 0,073	0.17
		Co-culture	1,459 ± 0,443		1,459 ± 0,443	
	Actilight	Mono-culture	0,004 ± 0,006	0.14	1,941 ± 0,008	0.16
		Co-culture	0,996 ± 0,949		0,996 ± 0,949	
	Glucose	Mono-culture	0,002 ± 0,003	***<0,01***	1,591 ± 0,186	0.21
		Co-culture	1,429 ± 0,003		1,429 ± 0,003	
	WCS	Mono-culture	-0,003 ± 0,000	0.14	0,973 ± 0,887	0.99
		Co-culture	0,960 ± 0,893		0,960 ± 0,893	

Supplementary File. Increments, with respect to time zero, in the concentration of iso-valerate on the bacterial cultures when grown in mono-culture or in co-culture in the prebiotics Synergy, Inulin and Actilight, in glucose or without any carbon source added (WCS).

Strains	Carbon source	Culture	Δ Iso-valerate bifidobacteria (mM) mean ± sd	p-value mono-culture vs co-culture	Δ Iso-valerate clostridia (mM) mean ± sd	p-value mono-culture vs co-culture
B. longum IPLA20022 & C. difficile LMG21717	Synergy	Mono-culture	-0,025 ± 0,016	0.09	0,723 ± 0,157	***<0,01***
		Co-culture	0,078 ± 0,079		0,078 ± 0,079	
	Inuline	Mono-culture	-0,009 ± 0,013	0.19	0,216 ± 0,177	0.44
		Co-culture	0,506 ± 0,559		0,506 ± 0,559	
	Actilight	Mono-culture	-0,009 ± 0,016	0.44	0,477 ± 0,483	0.16
		Co-culture	0,000 ± 0,006		0,000 ± 0,006	
	Glucose	Mono-culture	-0,063 ± 0,012	0.21	0,114 ± 0,000	***<0,01***
		Co-culture	-0,039 ± 0,025		-0,039 ± 0,025	
	WCS	Mono-culture	-0,030 ± 0,015	0.15	0,547 ± 0,548	0.93
		Co-culture	0,506 ± 0,514		0,506 ± 0,514	
B. breve IPLA20006 & C. difficile LMG21717	Synergy	Mono-culture	0,000 ± 0,005	0.06	0,851 ± 0,038	***<0,01***
		Co-culture	0,048 ± 0,031		0,048 ± 0,031	
	Inuline	Mono-culture	0,003 ± 0,007	0.12	0,205 ± 0,023	0.28
		Co-culture	0,569 ± 0,500		0,569 ± 0,500	
	Actilight	Mono-culture	0,042 ± 0,055	0.16	0,996 ± 0,084	***<0,01***
		Co-culture	-0,015 ± 0,017		-0,015 ± 0,017	
	Glucose	Mono-culture	-0,035 ± 0,002	0.39	0,122 ± 0,044	***<0,01***
		Co-culture	-0,021 ± 0,024		-0,021 ± 0,024	
	WCS	Mono-culture	-0,003 ± 0,002	0.10	0,537 ± 0,519	0.14
		Co-culture	-0,013 ± 0,008		-0,013 ± 0,008	
B. animalis Bb12 & C. difficile LMG21717	Synergy	Mono-culture	0,001 ± 0,007	***<0,01***	0,744 ± 0,094	***<0,01***
		Co-culture	0,259 ± 0,021		0,259 ± 0,021	
	Inuline	Mono-culture	-0,070 ± 0,076	***0.01***	0,168 ± 0,032	0.17
		Co-culture	0,132 ± 0,018		0,132 ± 0,018	
	Actilight	Mono-culture	-0,006 ± 0,010	***0.04***	0,442 ± 0,431	0.16
		Co-culture	0,014 ± 0,005		0,014 ± 0,005	
	Glucose	Mono-culture	-0,105 ± 0,128	0.15	0,088 ± 0,000	***<0,01***
		Co-culture	0,026 ± 0,010		0,026 ± 0,010	
	WCS	Mono-culture	0,075 ± 0,072	0.16	0,545 ± 0,542	0.16
		Co-culture	0,003 ± 0,002		0,003 ± 0,002	
B. bifidum IPLA 20015 & C. difficile LMG21717	Synergy	Mono-culture	-0,025 ± 0,025	***<0,01***	0,754 ± 0,228	0.41
		Co-culture	0,906 ± 0,177		0,906 ± 0,177	
	Inuline	Mono-culture	0,030 ± 0,030	***<0,01***	1,276 ± 0,043	0.06
		Co-culture	0,951 ± 0,210		0,951 ± 0,210	
	Actilight	Mono-culture	0,001 ± 0,001	0.15	1,126 ± 0,032	0.12
		Co-culture	0,537 ± 0,521		0,537 ± 0,521	
	Glucose	Mono-culture	0,000 ± 0,182	0.33	0,333 ± 0,214	0.16
		Co-culture	0,119 ± 0,024		0,119 ± 0,024	
	WCS	Mono-culture	0,003 ± 0,146	0.17	0,591 ± 0,568	0.99
		Co-culture	0,600 ± 0,598		0,600 ± 0,598	



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