UNIVERSIDAD DE GRANADA

FACULTAD DE FARMACIA

DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR II

TESIS DOCTORAL

COLONIZACIÓN, SEGURIDAD Y TOLERANCIA DE *Lactobacillus paracasei* CNCM I-4034, *Lactobacillus rhamnosus* CNCM I-4036 Y *Bifidobacterium breve* CNCM I-4035 EN ADULTOS SANOS Y SUS EFECTOS SOBRE EL METABOLISMO Y SISTEMA INMUNITARIO EN RATAS ZUCKER

JULIO RAMÓN PLAZA DÍAZ

2014

Editor: Editorial de la Universidad de Granada
Autor: Julio Ramón Plaza Díaz
D.L.: GR 2090-2014
ISBN: 978-84-9083-123-6

UNIVERSIDAD DE GRANADA

FACULTAD DE FARMACIA

DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR II

TESIS DOCTORAL

Colonización, seguridad y tolerancia de *Lactobacillus paracasei* CNCM I-4034, *Lactobacillus rhamnosus* CNCM I-4036 y *Bifidobacterium breve* CNCM I-4035 en adultos sanos y sus efectos sobre el metabolismo y sistema inmunitario en ratas Zucker

Tesis Doctoral para optar al grado de Doctor por la Universidad de Granada presentada por:

Julio Ramón Plaza Díaz

Bajo la dirección de los doctores:

Ángel Gil Hernández

Carolina Gómez Llorente

Luis Fontana Gallego

Granada, 2014

El doctorando Julio Ramón Plaza Díaz y los directores de la tesis Ángel Gil Hernández, Carolina Gómez Llorente y Luis Fontana Gallego, garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada, 9 de Mayo de 2014

Director/es de la Tesis

Fdo.:

Ángel Gil Hernández

Doctorando

Fdo.:

Julio Ramón Plaza Díaz

Carolina Gómez Llorente

Luis Fontana Gallego

Ángel Gil Hernández, Catedrático del Departamento de Bioquímica y Biología Molecular II de la Universidad de Granada.

CERTIFICA

Que la Tesis Doctoral titulada "COLONIZACIÓN, SEGURIDAD Y TOLERANCIA DE *Lactobacillus paracasei* CNCM I-4034, *Lactobacillus rhamnosus* CNCM I-4036 Y *Bifidobacterium breve* CNCM I-4035 EN ADULTOS SANOS Y SUS EFECTOS SOBRE EL METABOLISMO Y SISTEMA INMUNITARIO EN RATAS ZUCKER", de la que es autor Don Julio Ramón Plaza Díaz, ha sido realizada bajo mi dirección y asesoramiento y reúne las condiciones y calidad científica deseadas para ser presentada por el interesado para optar al grado de Doctor.

Y para que conste y surta los efectos oportunos, firmo el presente certificado en Granada, a 9 de mayo de 2014.

Campus de Cartuja
18071 Granada
Tfno.34 958 243838
Fax.34 958 248960

Facultad de Farmacia
Departamento de Bioquímica y Biología Molecular II

Carolina Gómez Llorente, Contratada de investigación del Departamento de Bioquímica y Biología Molecular II de la Universidad de Granada.

CERTIFICA

Que la Tesis Doctoral titulada "COLONIZACIÓN, SEGURIDAD Y TOLERANCIA DE *Lactobacillus paracasei* CNCM I-4034, *Lactobacillus rhamnosus* CNCM I-4036 Y *Bifidobacterium breve* CNCM I-4035 EN ADULTOS SANOS Y SUS EFECTOS SOBRE EL METABOLISMO Y SISTEMA INMUNITARIO EN RATAS ZUCKER", de la que es autor Don Julio Ramón Plaza Díaz, ha sido realizada bajo mi dirección y asesoramiento y reúne las condiciones y calidad científica deseadas para ser presentada por el interesado para optar al grado de Doctor.

Y para que conste y surta los efectos oportunos, firmo el presente certificado en Granada, a 9 de mayo de 2014.

Campus de Cartuja
18071 Granada
Tfno.34 958 243838
Fax.34 958 248960

Facultad de Farmacia
Departamento de Bioquímica y Biología Molecular II

Luis Fontana Gallego, Profesor titular del Departamento de Bioquímica y Biología Molecular II de la Universidad de Granada.

CERTIFICA

Que la Tesis Doctoral titulada "COLONIZACIÓN, SEGURIDAD Y TOLERANCIA DE *Lactobacillus paracasei* CNCM I-4034, *Lactobacillus rhamnosus* CNCM I-4036 Y *Bifidobacterium breve* CNCM I-4035 EN ADULTOS SANOS Y SUS EFECTOS SOBRE EL METABOLISMO Y SISTEMA INMUNITARIO EN RATAS ZUCKER", de la que es autor Don Julio Ramón Plaza Díaz, ha sido realizada bajo mi dirección y asesoramiento y reúne las condiciones y calidad científica deseadas para ser presentada por el interesado para optar al grado de Doctor.

Y para que conste y surta los efectos oportunos, firmo el presente certificado en Granada, a 9 de mayo de 2014.

Campus de Cartuja
18071 Granada
Tfno.34 958 243838
Fax.34 958 248960

Facultad de Farmacia
Departamento de Bioquímica y Biología Molecular II

Alberto Vargas Morales, Catedrático y director del Departamento de Bioquímica y Biología Molecular II de la Universidad de Granada

CERTIFICA

Que la Tesis Doctoral titulada "COLONIZACIÓN, SEGURIDAD Y TOLERANCIA DE *Lactobacillus paracasei* CNCM I-4034, *Lactobacillus rhamnosus* CNCM I-4036 Y *Bifidobacterium breve* CNCM I-4035 EN ADULTOS SANOS Y SUS EFECTOS SOBRE EL METABOLISMO Y SISTEMA INMUNITARIO EN RATAS ZUCKER", de la que es autor Don Julio Ramón Plaza Díaz ha sido realizado en las instalaciones del Instituto de Nutrición y Tecnología de los Alimentos, Centro de Investigación Biomédica, Universidad de Granada, donde están ubicados los laboratorios del grupo de investigación CTS-461 Bioquímica Nutricional: Implicaciones terapéuticas, perteneciente a este Departamento.

Campus de Cartuja
18071 Granada
Tfno.34 958 243838
Fax.34 958 248960

Facultad de Farmacia
Departamento de Bioquímica y Biología Molecular II

Este estudio se ha financiado a través de dos contratos de investigación entre la Fundación General Universidad de Granada Empresa y la Empresa HERO ESPAÑA S.A.: Proyecto nº 3582, “Colonización, seguridad, tolerancia y efectos sobre el sistema inmunitario de tres cepas probióticas en adultos sanos”, y Proyecto nº 3545, “Evaluación de los efectos de tres cepas con actividad probiótica (*Lactobacillus paracasei* CNCM I-4034, *Lactobacillus rhamnosus* CNCM I-4036 y *Bifidobacterium breve* CNCM I-4035) sobre el sistema inmunitario y el metabolismo en un modelo de ratas obesas (Zucker *fa/fa*)".

AGRADECIMIENTOS

Creo personalmente que, cuando se termina una etapa en la vida, hay que agradecer a las personas que han hecho posible tal objetivo. Mi familia fue un pilar importante en la decisión de salir de Chile, conseguir con ello los sueños que tenía de joven y obtener en la actualidad, mucho más de lo que planteé en el principio, es gracias a ellos. Papá, mamá y hermanos, muchas gracias por confiar en mí; esto es para vosotros. También debo destacar más personas cercanas de mi familia, que cuando me veían siempre me daban ánimos; abuelos, tíos y amigos en general muchas gracias por vuestra confianza en mí. A mis dos abuelos que ya no están conmigo porque la vida nos separó, el tiempo disfrutado con cada uno de ustedes fue genial para mí, gracias tata Ramón y yaya Mati.

Agradecer infinitamente a la persona que confio en mí sin conocerme, que me dió la oportunidad de acercarme al mundo de la investigación y que es un ejemplo de vida en todo ámbito, Don Ángel Gil, gracias por ayudarme en todo y disponer de tiempo para solucionar cualquier problema.

A mis directores Carolina y Luis, muchas gracias por vuestras enseñanzas, tiempo y dedicación para conmigo. Carolina, agradezco los momentos en que me preguntaste cómo estaba y cuando te tomaste el tiempo de pasar alguna fiesta importante conmigo, invitándome a disfrutarla junto a tu familia, muchas gracias de verdad. Luis, te considero un gran amigo y un ejemplo de persona; gracias por abrirme las puertas de tu casa y por dejarme conocer a tu gran familia. Además, gracias por sumarte a la dirección de la tesis, junto a Carolina y Ángel habeís formado un equipo excelente.

A la gran pareja chilena que en el presente es un feliz matrimonio, muchas gracias Sergio y Carola por tan buenos momentos, por alegrarme y ayudarme en todo lo que podíais, os estoy agradecido de corazón.

A mis compañeros de trabajo, instituto y departamento, muchas gracias por vuestro tiempo para conmigo. Muchas gracias Laura, Vivi, Mari Cruz, Susana, Belén, Estefania, Carolina, Chiqui, MD, Mari Carmen, Miguel "Finut", Domingo, Óscar, Jesús, José Luis Periago, José Manuel, a la gente del departamento de Fisiología, a Paco Abadía, al personal de conserjería del CIBM y finalmente, a Mohammed y Ana; interactuamos muy poco, pero agradezco cada consejo. A mis compañeros de piso, Silvia muchas gracias por todo y Javier; que vaya todo bien.

A Fran, eres una gran persona y te doy las gracias por cada momento que compartí junto a ti. Gracias por entenderme y, por supuesto, muchas gracias por toda la ayuda brindada. Te considero personalmente uno de mis mejores amigos.

A María José, hemos interactuado poco (mucho menos de lo que me hubiera gustado), pero creo que eres una gran compañera. Espero sinceramente que podamos alcanzar juntos el objetivo planteado para tu trabajo.

Para terminar agradezco a Belén, mi pareja. Muchas gracias por apoyarme en cada momento, por tratar de entenderme cuando nadie lo hacía y por intentar cada día hacerme mejor persona, con tus consejos, preocupaciones y opiniones. Sinceras gracias también para tu madre y familia.

Julio Ramón Plaza Díaz, Granada 2014.

ABREVIATURAS

A

AGCC Ácidos grasos de cadena corta

ALC Ácido linoleico conjugado

ALT Alanina aminotransferasa

ARN Ácido ribonucleico

AST Aspartato aminotransferasa

ATCC Colección americana de cultivos tipo

C

CDM Células dendríticas mieloides

CEI Célula epiteliales intestinales

CNCM *Colléction Nationale de Cultures de Microorganismes*

CMP Células madres pluripotenciales

COX-2 Ciclooxigenasa 2

CU Colitis ulcerosa

D

DDBJ DNA Data Bank of Japan

E

EcN1917 *Escherichia coli* Nissle 1917

EFSA *European Food Safety Authority*

ELISA *Enzyme-Linked ImmunoSorbent Assay*

ENA *European Nucleotide Archive*

ENC Enterocolitis necrotizante

F

FAO/WHO *Food and Agriculture Organization of the United Nations*

FACS Clasificación de células activadas fluorescentemente

FISH *Fluorescence in situ hybridization*

FISH-CF *Fluorescence in situ hybridization coupled flow cytometry*

FITC *Fluorescein isothiocyanate isomer I*

G

GSRS *Gastrointestinal Symptom Rating Scale*

H

HDL *High density lipoprotein*

HOMA-IR *Homeostatic model assessment*

I

IBI Inflamación de la bolsa ileal

IFN Interferón

Ig Inmunoglobulina

IL Interleuquina

IMC Indice de masa corporal

L

LAB *Lactic acid bacteria*

LDL *Low density lipoprotein*

LPS Lipopolisacárido

M

MAPK *Mitogen-activated protein kinase*

MRS Medio de cultivo de Man, Rogosa y Sharpe

MRS-C Medio de cultivo de Man, Rogosa y Sharpe adicionado con cisteína

MUC Mucinas

MyD88 Proteína de respuesta primaria de diferenciación mieloide

N

NEFA *Non-esterified fatty acids*

NF-κβ *Nuclear factor kappa-beta*

NIH *National Institutes of Health*

NLR *NOD-like receptors*

P

PAM Proteínas antimicrobianas

PAMP Patrones moleculares asociados a patógenos

pb Pares de bases

PCR Reacción en cadena de la polimersa

PE Ficoeritrina

Per-CP Conjugado con peridina-clorofila

PQC Proteína quinasa C

PRR Receptores de reconocimiento de patrones antigénicos

Q

qPCR PCR cuantitativa

QRS *Qualified presumption of safety*

R

RANTES *Regulated on Activation, Normal T Cell Expressed and Secreted*

S

SII Síndrome de intestino irritable

SRI Síndrome de sistencia a la insulina

T

TG Triacilglicéridos

TGI Tracto gastrointestinal

TIR Receptor *toll*/interleuquina 1

TLR *Toll-like receptors*

TNF-α Factor de necrosis tumoral alfa

TSA Triptona-soja agar

T-RFLP *Terminal restriction fragment length polymorphism*

TRIF Adaptador que contiene el dominio TIR que induce interferón β

U

UCP-2 Proteína desacoplante de la fosforilación oxidativa 2

UFC Unidades formadoras de colonias

ÍNDICE

INDICE DE FIGURAS Y TABLAS

Esta Tesis Doctoral ha dado lugar a las siguientes publicaciones y comunicaciones a congresos:

a) **Publicaciones**

- Plaza-Diaz J, Gomez-Llorente C, Abadia F, Saez-Lara MJ, Muñoz-Quezada S, Campaña-Martin L, Romero F, Gil A, Fontana L. Effects of *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 on hepatic steatosis in Zucker rats. Plos One 2014, En prensa

- Plaza-Diaz J, Gomez-Llorente C, Campaña-Martin L, Matencio E, Ortuño I, Martínez-Silla R, Gomez-Gallego C, Periago MJ, Ros G, Chenoll E, Genovés S, Casinos B, Silva A, Corella D, Portolés O, Romero F, Ramón D, Perez de la Cruz A, Gil A, Fontana L. Safety and immunomodulatory effects of three probiotic strains isolated from the feces of breast-fed infants in healthy adults: SETOPROB study. Plos One 2013, 8(10):e78111.

b) **Comunicaciones a congresos**

- Plaza-Diaz J, Gomez-Llorente C, Campaña-Martin L, Bermudez-Brito M, Matencio E, Romero F, Pérez de la Cruz A, Gil A, Fontana L, and other members of the PROBIENSA study. Three novel probiotic strains isolated from feces of breast-fed infants modulate the immune system of healthy adults. Ann Nutr Metab 2013, 63(suppl 1): 1562.

- Plaza-Diaz J, Gomez-Llorente C, Abadía F, Sáez-Lara MJ, Muñoz-Quezada S, Campaña-Martín L, Bermúdez-Brito M, Jiménez-Valera M, Ruiz-Bravo A, Matencio E, Bernal-Cava MJ, Gil A, Fontana L. *Lactobacillus paracasei* CNCM I-4034 enhances the intestinal immune response in obese Zucker rats. Proc Nutr Soc 2013, 72 (OCE1):E61.

- Plaza-Diaz J, Gomez-Llorente C, Abadía F, Sáez-Lara MJ, Muñoz-Quezada S, Campaña-Martín L, Bermúdez-Brito M, Jiménez-Valera M, Ruiz-Bravo A, Matencio E, Bernal-Cava MJ, Gil A, Fontana L. *Lactobacillus rhamnosus* CNCM I-4036 estimula la producción de IgA intestinal y disminuye la concentración sérica de TNF-α en ratas Zucker obesas. Nutr Hosp 2012, 27(5):1690-1691.

RESUMEN

Nuestro grupo ha descrito el aislamiento y la caracterización de tres cepas probióticas (*Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 y *Lactobacillus rhamnosus* CNCM I-4036) a partir de las heces de niños recién nacidos y alimentados exclusivamente con leche materna. Estas cepas demostraron adherirse a la mucosa intestinal en estudios *in vitro*, tener la característica de ser sensibles al uso de antibióticos y resistir condiciones gástricas de pH elevado y presencia de sales biliares.

En el presente trabajo se ha realizado un estudio multicéntrico, aleatorizado, doble ciego y controlado por placebo con 100 individuos sanos de tres ciudades españolas, en donde se ha evaluado la tolerancia, seguridad, colonización intestinal y efectos inmunomoduladores de estas tres cepas probióticas. Los voluntarios se sometieron a un primer período de lavado de 15 días y seguidamente fueron divididos aleatoriamente en cinco grupos para recibir diariamente un placebo, una cápsula que contenía una de las tres cepas, o bien una mezcla de *Bifidobacterium breve* CNCM I-4035 y *Lactobacillus rhamnosus* CNCM I-4036 durante 30 días. Tras la intervención, hubo un segundo lavado de 15 días. Los voluntarios no consumieron ningún producto que contuviera alguna otra cepa probiótica, ni productos fermentados, durante la totalidad del estudio.

Los síntomas gastrointestinales, la frecuencia y la consistencia de las heces no se alteraron por la ingesta de ninguna cepa probiótica. No se observaron cambios relevantes en la sangre ni el suero de los voluntarios, ni eventos adversos en la totalidad del estudio. La administración de las cepas probióticas modificó ligeramente las poblaciones bacterianas de las heces de los voluntarios. La persistencia intestinal ocurrió en los voluntarios que recibieron la cepa *Lactobacillus rhamnosus* CNCM I-4036. La administración de *Bifidobacterium breve* CNCM I-4035 aumentó significativamente el contenido de IgA secretora en heces. Las concentraciones séricas de IL-4 e IL-10 aumentaron, mientras IL-12 disminuyó, en los voluntarios que recibieron probióticos. Estos resultados demuestran que el consumo de las tres cepas bacterianas es seguro y que ejercen efectos inmunomoduladores.

Por otro lado, se ha evaluado el efecto de estas cepas en la esteatosis hepática de ratas obesas, y el efecto inmunomodulador de dichas cepas. Se utilizaron ratas Zucker como modelo genético de obesidad. Las ratas Zucker recibieron una de las tres cepas, una mezcla de *L. paracasei* CNCM I-4034 y *B. breve* CNCM I-4035, o bien un placebo durante 30 días.

No hubo alteraciones en la histología intestinal de ninguno de los grupos experimentales. El contenido de triacilglicéridos hepáticos disminuyó significativamente en las ratas Zucker obesas que recibieron *L. rhamnosus* CNCM I-4036, *B. breve* CNCM I-4035 y la mezcla de *B. breve* CNCM I-4035 y *L. paracasei* CNCM I-4034. También, el área correspondiente a los lípidos neutros fue significativamente menor en los hígados de todos los grupos de ratas Zucker obesas que recibieron alguna cepa probiótica, comparado con las ratas obesas que recibieron placebo. Cabe destacar que las ratas Zucker obesas que recibieron placebo tenían mayor contenido de lipopolisacárido sérico comparado con las ratas Zucker obesas que recibieron probióticos. Por lo que se refiere a efectos inmunomoduladores, los valores séricos de TNF-α disminuyeron en las ratas Zucker obesas que recibieron *L. rhamnosus* CNCM I-4036, *B. breve* CNCM I-4035 y la mezcla de *B. breve* CNCM I-4035 y *L. paracasei* CNCM I-4034, mientras que las ratas que recibieron *L. paracasei* CNCM I-4034 presentaron menores valores séricos de IL-6. En conclusión, las cepas probióticas redujeron la esteatosis hepática, en parte debido a la disminución sérica de lipopolisacárido, y demostraron poseer cierto efecto antiinflamatorio en las ratas Zucker obesas.

OBJETIVOS

INTRODUCCIÓN

El nivel de salud de los individuos está condicionado primordialmente por factores genéticos y ambientales. De estos últimos, la variable externa más importante es la alimentación. Una alimentación apropiada debe satisfacer diariamente las necesidades nutricionales individuales e incorporar valores culturales, gastronómicos y de satisfacción personal. Estudios recientes en el ámbito de la nutrición humana demuestran que los desajustes alimentarios son la principal causa del desarrollo precoz de la mayor parte de las enfermedades crónicas o degenerativas en la sociedad actual. Por lo tanto, en nuestra mano está la posibilidad de incorporar hábitos de vida saludable, un mejor perfil alimentario y conseguir una disminución global de los factores de riesgo existentes en la población (Plaza-Díaz *et al.*, 2013).

En el tratamiento de algunas patologías digestivas se ha comenzado a introducir nuevas estrategias basadas en el uso de probióticos, "microorganismos vivos no patógenos que, ingeridos en cantidades suficientes, modulan o actúan sobre determinadas funciones del organismo, produciendo un efecto beneficioso, más allá del puramente nutricional, como contribuir al equilibrio de la microbiota intestinal del hospedador y potenciar el sistema inmunitario" (FAO, 2002). En general, éstos suelen ser bacterias lácticas del género *Bifidobacterium*, *Lactobacillus,* una mezcla de ellas u otros microorganismos que pueden ser empleados en adultos y niños.

Para que una bacteria pueda ser considerada "probiótica" es necesario que supere diferentes etapas: su aislamiento, identificación, caracterización y evaluación. La identificación de los microorganismos es el primer paso en la selección de potenciales probióticos. Las nuevas técnicas de biología molecular permiten discriminar, con diversos grados de éxito, bacterias estrechamente relacionadas. Métodos moleculares adicionales, tales como la electroforesis en gel de gradiente y la fluorescencia mediante hibridación *in situ* (por sus siglas en inglés *fluorescent in situ hybridization,* FISH), se emplean para identificar y caracterizar éstas nuevas bacterias.

Uno de los beneficios clínicos mejor demostrados de los probióticos es la prevención y el tratamiento de la diarrea aguda asociada a los antibióticos. No obstante, hay cada vez más evidencia de su beneficio en el tratamiento de alergias, enfermedades metabólicas e intestinales.

Existen varios mecanismos propuestos por los que los probióticos ejercen sus efectos beneficiosos, ya sea por la regulación de la permeabilidad intestinal, la mejora de la función barrera y el equilibrio entre citoquinas pro y anti-inflamatorias. En la actualidad, hay un gran número de estudios llevados a cabo *in vitro* y en animales para probar los efectos de los probióticos. Sin embargo, el método más fiable para evaluar los beneficios terapéuticos de cualquier cepa probiótica es el uso de ensayos aleatorizados en humanos controlados por placebo (Fontana *et al.*, 2013).

Recientemente, nuestro grupo ha identificado y caracterizado tres nuevas cepas probióticas, *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 y *Lactobacillus rhamnosus* CNCM I-4036 que se aislaron de heces de niños alimentados exclusivamente con leche materna (Muñoz-Quezada *et al.*, 2013). Tras una evaluación inicial de las tres cepas en modelos *in vitro,* el Instituto Pasteur (Vieites Fernández *et al.*, 2010) las calificó como bacterias nuevas. El **objetivo general** de este trabajo fue evaluar la seguridad, tolerancia y efectos de las 3 cepas probióticas sobre el sistema inmunitario tanto de voluntarios sanos como de ratas obesas Zucker (*fa*/*fa*).

Para ello los **objetivos específicos** fueron:

1. Evaluar la seguridad y tolerancia de las tres cepas probióticas en individuos sanos.
2. Investigar los efectos de las tres cepas sobre el sistema inmunitario de individuos sanos.
3. Determinar si la administración de las tres cepas probióticas modificó las poblaciones microbianas de los voluntarios sanos.
4. Evaluar el efecto de la ingesta de las cepas probióticas sobre valores antropométricos y el perfil lipídico en ratas obesas.
5. Determinar el efecto de la ingesta de las tres cepas probióticas sobre biomarcadores de riesgo cardiovascular e inflamación de ratas obesas.
6. Determinar el efecto de la ingesta de las tres cepas probióticas sobre la esteatosis hepática y su efecto sobre los niveles séricos de lipopolisacárido en ratas obesas.

El siguiente trabajo sigue el esquema que a continuación se detalla. En la parte de Antecedentes se exponen tres revisiones publicadas y un trabajo original:

- Fontana L, Bermudez-Brito M, Plaza-Diaz J, Muñoz-Quezada S, Gil A. Sources, isolation, characterisation and evaluation of probiotics. Br J Nutr 2013, 109(Suppl 2):S35-50.

- Bermudez-Brito M, Plaza-Diaz J, Muñoz-Quezada S, Gomez-Llorente C, Gil A. Probiotic mechanisms of action. Ann Nutr Metab 2012, 61(2):160-74.

- Bermudez-Brito M, Plaza-Diaz J, Fontana L, Muñoz-Quezada S, Gil A. In vitro cell and tissue models for studying host-microbe interactions: a review. Br J Nutr 2013, 109(Suppl 2):S27-34.

- Muñoz-Quezada S, Bermudez-Brito M, Chenoll E, Genovés S, Gomez-Llorente C, Plaza-Diaz J, Matencio E, Bernal MJ, Romero F, Ramón D, Gil A. Competitive inhibition of three novel bacteria isolated from faeces of breast milk-fed infants against selected enteropathogens. Br J Nutr 2013, 109(Suppl 2):S63-69.

En la parte de Resultados se incluyen dos publicaciones sobre los hallazgos principales de los proyectos de investigación realizados:

- Plaza-Diaz J, Gomez-Llorente C, Campaña-Martin L, Matencio E, Ortuño I, Martínez-Silla R, Gomez-Gallego C, Periago MJ, Ros G, Chenoll E, Genovés S, Casinos B, Silva A, Corella D, Portolés O, Romero F, Ramón D, Perez de la Cruz A, Gil A, Fontana L. Safety and immunomodulatory effects of three probiotic strains isolated from the feces of breast-fed infants in healthy adults: SETOPROB study. Plos One 2013, 8(10):e78111.

- Plaza-Diaz J, Gomez-Llorente C, Abadia-Molina F, Saez-Lara MJ, Campaña-Martin L, Muñoz-Quezada S, Romero F, Gil A, Fontana L. Effects of *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 on hepatic steatosis in Zucker rats. Plos ONE 2014, En prensa.

Finalmente, en un apartado de Anexos se recogen los trabajos publicados en relación al tema de probióticos y microbiota intestinal:

- Plaza-Diaz J, Gomez-Llorente C, Fontana L, Gil A. Modulation of immunity and inflammatory gene expression by probiotics. World J Gastroenterol 2014. En prensa.

- Gomez-Llorente C, Plaza-Diaz J, Aguilera M, Muñoz-Quezada S, Bermudez-Brito M, Peso-Echarri P, Martinez-Silla R, Vasallo-Morillas MI, Campaña-Martin L, Vives-Piñera I, Ballesta-Martinez MJ, Gil A. Three main factors define changes in fecal microbiota associated with feeding modality in infants. J Pediatr Gastroenterol Nutr 2013 57:461-466.

ANTECEDENTES

ANTECEDENTES

Las superficies cutáneas y mucosas del cuerpo humano están expuestas a ser colonizadas por microbios durante toda la vida del individuo (Hooper *et al.*, 2002). A medida que estos microbios van aumentando en número forman ecosistemas muy diversos y complejos. En particular, el tracto intestinal alberga la mayor comunidad, ya que es un ambiente rico en nutrientes, con una distribución estimada cercana a los 100 billones. De esta inmensa cantidad de microbios, la gran mayoría reside en el colon (Whitman *et al*., 1998). Por ello, el intestino humano constituye un hábitat microbiano sustancial de nuestra biosfera. Al nacer, el intestino de un recién nacido es estéril. Sin embargo, tras el nacimiento comienza a ser colonizado por bacterias de origen materno y del medio ambiente (Plaza-Díaz y Gil, 2013).

La diversidad microbiana en nuestro planeta es enorme, ya que existen más de 50 *Phyla* de bacterias y 13 de Archea (Rappe y Giovannoni, 2003; Rondón *et al*., 1999). No obstante, a pesar de toda la diversidad microbiana existente, el intestino posee exclusivamente solo dos *Phyla* mayoritarios de bacterias, *Bacteroidetes* y *Firmicutes*, y uno de *Archaea*, *Methanobrevibacter smithii* (Backhed *et al*., 2005; Eckburg *et al.*, 2005). Por esto, se presume que existen estrictos requisitos de la comunidad microbiana intestinal para el ingreso como miembro en este ambiente (Ley *et al.*, 2006).

La microbiota intestinal en adultos se estima formada por entre 1000 y 1150 especies bacterianas, sugiriendo algunos expertos que sólo 160 de estas especies constituyen el núcleo de la microbiota que está presente en la mayoría de los individuos. Aunque muchas de estas especies se encuentran en la mayoría de las personas, su abundancia relativa puede variar, existiendo una gran variabilidad individual (Plaza-Díaz y Gil, 2013).

La distribución de la microbiota en el intestino no es homogénea, el número de bacterias presentes en el intestino humano va desde 10 a 10^3 unidades formadoras de colonias (UFC) en el estómago y duodeno, aumentando de 10^{11} a 10^{12} UFC en el colon (O´Hara y Shanahan, 2006, Frank *et al.,* 2007). Existen, además, diferencias en la heterogeneidad longitudinal a través del tubo digestivo y en la variación latitudinal, debido a que el epitelio intestinal está separado del lumen

por una espesa y compleja capa de *mucus*, generando un tipo de hábitat diferente al que pueda existir en el lumen o en la superficie del epitelio intestinal, causando una clara diferencia de microbiota en cada uno de estos hábitats (Muñoz-Quezada, 2011).

Cada una de las funciones que posee la microbiota intestinal se afecta por factores microbiológicos, del hospedador, dietéticos y ambientales que explican la variabilidad interindividual, como cambios en la fisiología del hospedador, reactividad del sistema inmunitario y dieta, entre otros (Plaza-Díaz y Gil, 2013) (Figura 1).

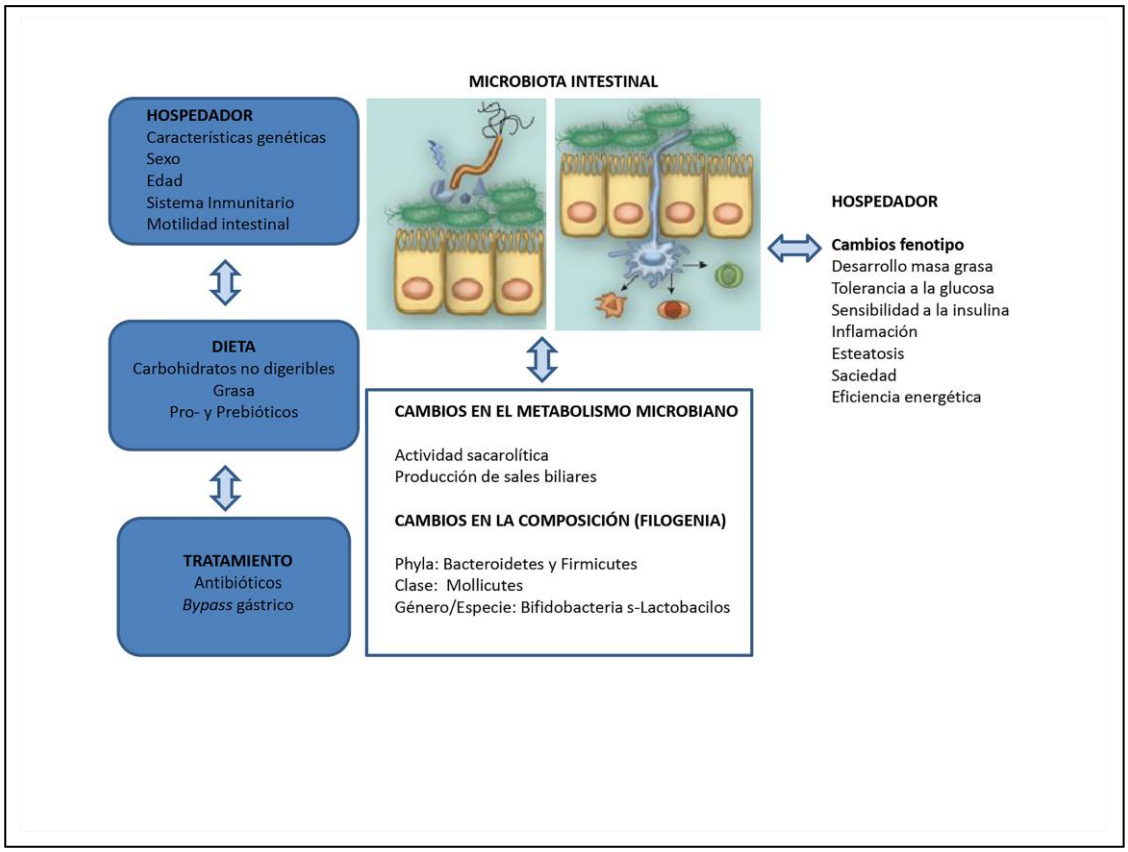


FIGURA 1. Relación entre microbiota intestinal y hospedador.

COMPOSICIÓN DE LA MICROBIOTA INTESTINAL

El intestino humano es el hábitat natural para una comunidad bacteriana amplia y dinámica, pero una parte sustancial de estas poblaciones permanece aún sin conocerse.

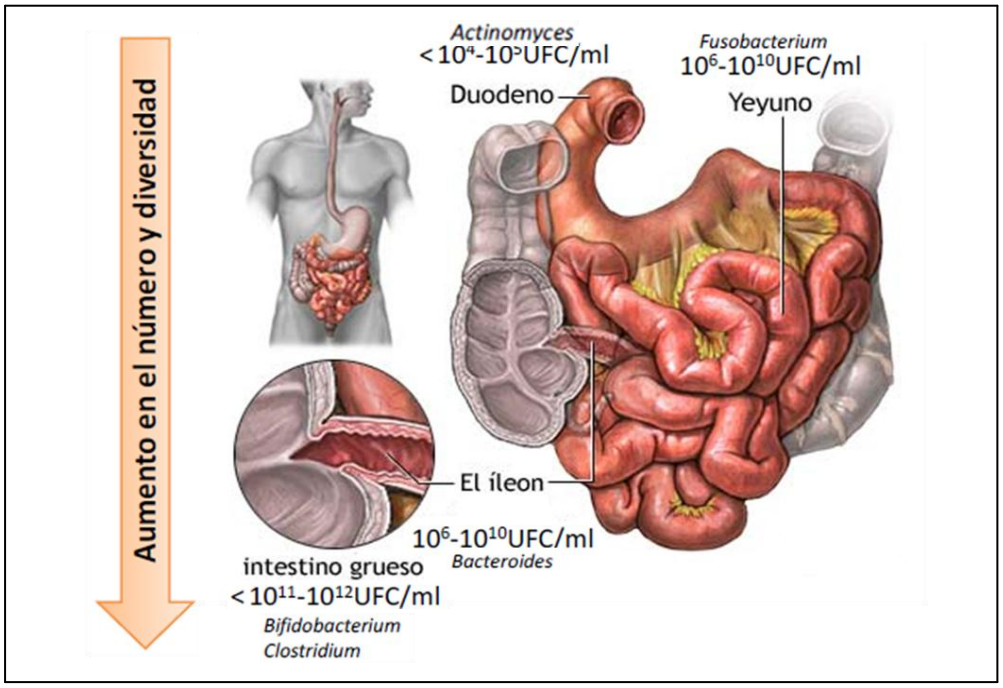


FIGURA 2. Variación en composición y número de la microbiota a lo largo del intestino humano.

En el intestino delgado, desde el duodeno en adelante, la composición de la microbiota es escasa y contiene usualmente menos de 10^5 UFC. En el intestino delgado proximal, se encuentran *Haemophilus, Actinomyces* y algunos anaerobios y algunos lactobacilos (Mackie *et al.*, 1999). En el yeyuno e íleon existe un incremento continuo en el número (>10^8UFC) y variedad de la microbiota apareciendo bifidobacterias, anaerobios facultativos (*Bacteroides* y *Fusobacterium*) y anaerobios estrictos que están presentes en número creciente a partir de la válvula ileocecal (Holzapfel *et al.*, 1998). La microbiota en la parte distal del intestino delgado es más densa (> 10^9 UFC) y se asemeja a la del ciego, con gran cantidad de bacterias anaerobias estrictas. (Mackie *et al.*, 1999; Kleessen *et al.*, 2000; Hooper *et al.*, 2002). En el intestino grueso, se encuentran comúnmente microorganismos anaerobios facultativos (*Streptococcus* y *Enterococcus*) (Kleessen *et al.*, 2000). Finalmente, en el ciego destacan eubacterias, bifidobacterias, *Clostridium* y cocos Gram positivos (Kleessen *et al.*, 2000; Macfarlane *et al.*, 2000, Eckburg *et al.*, 2005) (Figura 2).

La compleja comunidad bacteriana presente en el intestino humano no permanece constante a lo largo del tiempo sino que puede variar por diversos factores entre los cuales podemos encontrar las propias condiciones ambientales del tubo digestivo, la cantidad y variedad de las bacterias en las diferentes regiones del mismo, la cual es determinada por una gran diversidad de factores complejos intrínsecos y extrínsecos (Mackie *et al.*, 1999). Existe a su vez, una relación recíproca entre el hospedador y la microbiota que lo habita, ya que la microbiota puede tener un importante impacto sobre el organismo que lo aloja y estos efectos pueden ser beneficiosos o perjudiciales para la salud del individuo (Hooper *et al.*, 2002).

FUNCIONES DE LA MICROBIOTA INTESTINAL

Después de enumerar cada uno de los grupos de bacterias presentes en el intestino humano, podemos suponer que esta compleja comunidad bacteriana pueda tener algunas funciones en la mucosa intestinal del hospedador. Las bacterias forman una barrera de defensa natural que puede desarrollar actividades metabólicas que tienen por objetivo mantener la energía y mejorar la absorción de ciertos nutrientes, ejercer efectos tróficos importantes sobre la estructura y función inmunitaria del epitelio intestinal, así como proteger al hospedador frente a la colonización por otros microbios (Guarner y Magaleda, 2003; O´Hara y Shanahan, 2006) (Figura 3).

La influencia de las bacterias en la fisiología intestinal ha sido demostrada en estudios con animales libres de gérmenes y de colonización. En 1995, Usemaki *et al*. demostraron que la reconstitución de ratones libres de gérmenes con microbiota intestinal externa era suficiente para recuperar la función inmunitaria de la mucosa intestinal (Umesaki *et al.*, 1995). De hecho, la colonización de ratones libres de gérmenes con una sola especie, *Bacteroides thetaiotaomicron*, afectaba la expresión de varios genes que controlaban funciones como la absorción de nutrientes, el metabolismo, la angiogénesis, la función de barrera mucosa y el desarrollo del sistema nervioso entérico (Xu y Gordon, 2003).

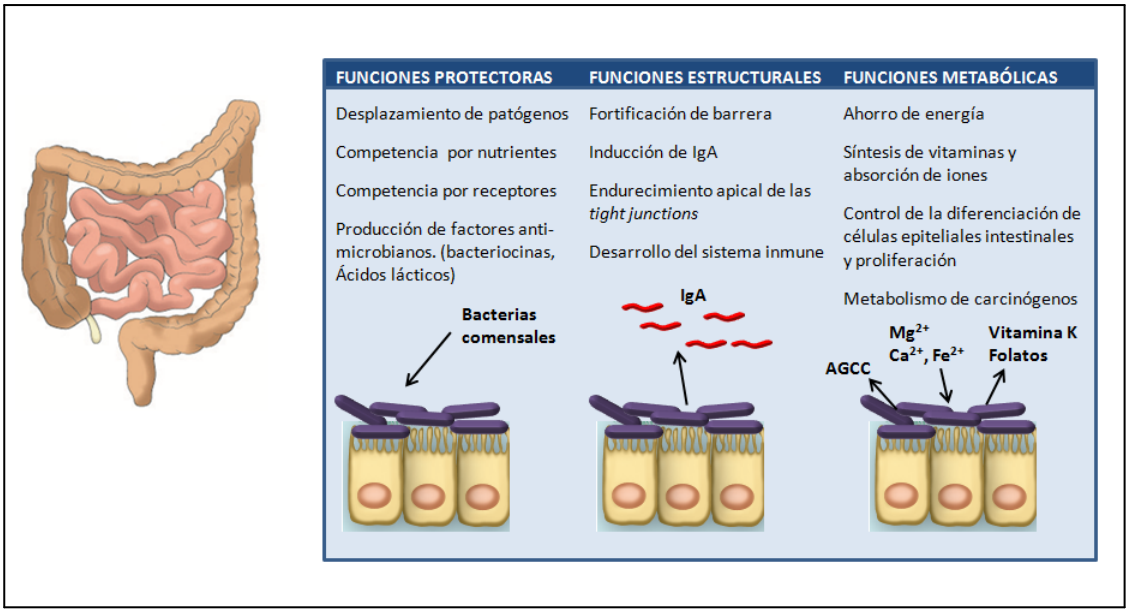


FIGURA 3. Funciones de la microbiota Intestinal. Adaptado de O'Hara AM and Shanahan F, 2006. AGCC: ácidos grasos de cadena corta.

Por otra parte, los fragmentos de las bacterias comensales influencian el normal desarrollo del sistema inmunitario de la mucosa (Mazmanian *et al.*, 2005; Rakoff-Nahoum *et al.*, 2004), además de afectar profundamente al desarrollo de los componentes humorales de dicho sistema (Weinstein y Cebra, 1991), modulando células T y T *helper* tipo 1 y 2 (Cebra, 1999; O' Hara y Shanahan, 2006). El microbioma, definido como la colección completa de microbios (bacterias, hongos, virus, etc.) que existen de forma natural dentro de un nicho biológico particular, tiene una actividad metabólica que es adaptable y a la vez renovable (Bocci, 1992). A través de la producción de ácidos grasos de cadena corta (AGCC), bacterias residentes influyen positivamente en la diferenciación de células del epitelio intestinal y en su proliferación, pudiendo mediar otros efectos metabólicos (Figura 3). Toda esta compleja actividad metabólica recupera valiosa energía y sustratos absorbibles para el hospedador, al mismo tiempo que entrega energía y nutrientes para el crecimiento y la proliferación bacteriana (O´Hara y Shanahan, 2006).

FUENTES, AISLAMIENTO, CARACTERIZACIÓN Y EVALUACIÓN DE BACTERIAS PROBIÓTICAS

En la búsqueda de microorganismos que poseen propiedades probióticas, es necesario centrarse en un procedimiento muy claramente definido. Este comienza con la investigación de diversas fuentes en las que se pueden encontrar tales bacterias, luego una etapa de caracterización en la que se evalúan la tolerancia a las condiciones gastrointestinales (tanto gástricas como intestinales), la capacidad de adherirse a la mucosa gastrointestinal y la competición frente a bacterias patógenas (Collins *et al.,* 1998, Ouwehand *et al.,* 2002, Muñoz-Quezada *et al.,* 2013). Finalmente, existe una etapa de evaluación que tiene como propósito investigar el efecto y/o beneficio en estudios experimentales y clínicos.

Las distintas etapas tienen como principios imprescindibles que las cepas halladas puedan:

- Haber demostrado efectos beneficiosos en el hospedador.
- Ser no patogénico, no tóxico y no presentar ningún efecto adverso significativo.
- Ser capaz de sobrevivir a condiciones gastrointestinales en estudios *in vitro* e *in vivo.*
- Estar presente en un producto en cantidad adecuada de células viables para proporcionar beneficios en la salud.
- Ser compatible con la matriz del producto, procesamiento y condiciones de almacenamiento para mantener las condiciones deseadas (Collado *et al.,* 2010).

Cada una de las etapas mencionadas es detallada a continuación.

FUENTES

Los lácteos y los productos derivados de los lácteos son una buena fuente de probióticos (Liong *et al.*, 2011). En este contexto, las LAB (bacterias del ácido láctico, por sus siglas en inglés *lactic acid bacteria*), bifidobacterias y otros microorganismos obtenidos a partir de leche fermentada han sido utilizados durante siglos. Un ejemplo de ello es la fermentación espontánea de la leche realizada durante varias generaciones en regiones de Mongolia y África por conferir efectos beneficiosos a quien las ingiriera (Yu *et al.*, 2011). En general, la gran parte de los

microorganismos aislados a partir de los productos fermentados pertenecen al género *Lactobacillus* (Vizoso-Pinto *et al.,* 2006; Lim e Im, 2009; Won *et al.,* 2011), pero existen excepciones como el caso de una cepa de *Weisella*, aislada de alimentos fermentados nigerianos y seleccionada posteriormente como probiótico (Ayeni *et al.,* 2011).

Dentro de las leches tradicionales fermentadas que contienen diversas especies bacterianas no es raro encontrarse con bacterias candidatas que podrían ser consideradas probióticos. Así, se aislaron 148 cepas de LAB de una leche tradicional de yak llamada "Kurut", que se fermenta de forma natural en regiones de China, siendo *L. delbrueckii* subsp. *bulgaricus y Streptococcus thermophilus* las poblaciones microbianas predominantes (Sun *et al.,* 2010). Hay que destacar además que levaduras y cepas de *Lactobacillus* con propiedades probióticas han sido aisladas también de granos de kéfir, leche Masai y *koumiss* (un fermentado de bebida de leche) (Lopitz-Otsoa *et al.,* 2006; Romanin *et al.,* 2010; Patrignani *et al.,* 2006; Ya *et al.,* 2008). Dentro de los productos lácteos fermentados, el queso es un producto lácteo con gran potencial como fuente de microorganismos probióticos para el intestino humano. Cepas de *L. plantarum* han sido aisladas de quesos italianos, argentinos y búlgaros (Zago *et al.,* 2011; Ugarte *et al.,* 2006; Georgieva *et al.,* 2008).

Una fuente importante de probióticos es la leche materna. Desde el descubrimiento de que la leche materna no es estéril, incluso cuando se extraía asépticamente, se planteó la posibilidad de que pudiera ser un inoculo bacteriano (West *et al.,* 1979). La presencia de bacterias en la leche materna había sido considerada siempre como consecuencia de una contaminación previa (piel). Sin embargo, los lactobacilos presentes en la leche humana son genotípicamente diferentes de los aislados de la piel (Martin *et al.,* 2009; O´Hara y Shanahan, 2006). Las cepas de LAB que se encuentran presentes en la leche materna se observan también en las heces de los lactantes alimentados con ella (Martin *et al.,* 2003). Por todo ello, la leche materna constituye una interesante fuente de LAB y bifidobacterias, tomando en consideración a estas cepas para incluirlas en fórmulas infantiles y alimentos destinados a infantes (Arboleya *et al.,* 2012).

Hay que agregar también que recién nacidos alimentados con leche materna tienen menos alergias e infecciones gastrointestinales que los que han recibido fórmulas, por lo que la microbiota

intestinal de los niños que reciben leche materna podría ser considerada "más sana" (Solis *et al.,* 2010).

La leche materna humana contiene una variada gama de bacterias, encontrándose de manera predominante estafilococos, estreptococos, micrococos, lactobacilos, enterococos, lactococos y bifidobacterias (O´Hara y Shanahan, 2006; Martin *et al.,* 2003; Solis *et al.,* 2010; Gueimonde *et al.,* 2007; Martin *et al.,* 2004; Pérez-Cano *et al.,* 2010) y su ingesta favorece el predominio de las bifidobacterias y lactobacilos en la microbiota intestinal infantil. Varios autores han señalado que los lactobacilos aislados de la leche materna son una alternativa eficiente para el tratamiento de la mastitis infecciosa durante la lactancia comparados con los antibióticos comúnmente prescritos (Arroyo *et al.,* 2010; Jiménez *et al.,* 2008). Por otra parte, se ha descrito que dos cepas de *Lactobacillus* aisladas de la leche materna humana mejoraban la respuesta inmunitaria a través de la activación de las células *natural killer* y la expansión de células T reguladoras (Pérez-Cano *et al.,* 2010).

Otra fuente importante de probióticos es el tracto gastrointestinal (TGI) humano. Más de 500 especies diferentes de bacterias residen en el intestino humano adulto. De hecho, muchas de las cepas de probióticos utilizadas en la actualidad han sido aislados a partir de esta fuente, tales como *L. gasseri* y *L. reuteri* (Ryan *et al.,* 2008). Además, se ha descrito que *L. fermentum*, aislado a partir de muestras de biopsia de mucosa de colon humano, posee actividad antimicrobiana frente a patógenos transmitidos por los alimentos. Cepas probióticas tales como *B. longum* (Srutková *et al,.* 2011) y *L. acidophilus* RY2 (Lin *et al.,* 2009), han sido aisladas de muestras de heces de adultos sanos y lactantes, respectivamente. En concordancia con la lactancia materna, varios estudios han descrito también el aislamiento de probióticos de las heces de estos lactantes (Martin *et al.,* 2006; Acharya y Shah, 2002; Muñoz-Quezada *et al.,* 2013). En la figura 4 se muestran los diferentes pasos necesarios para la caracterización de cepas probióticas (Varma *et al.,* 2010).

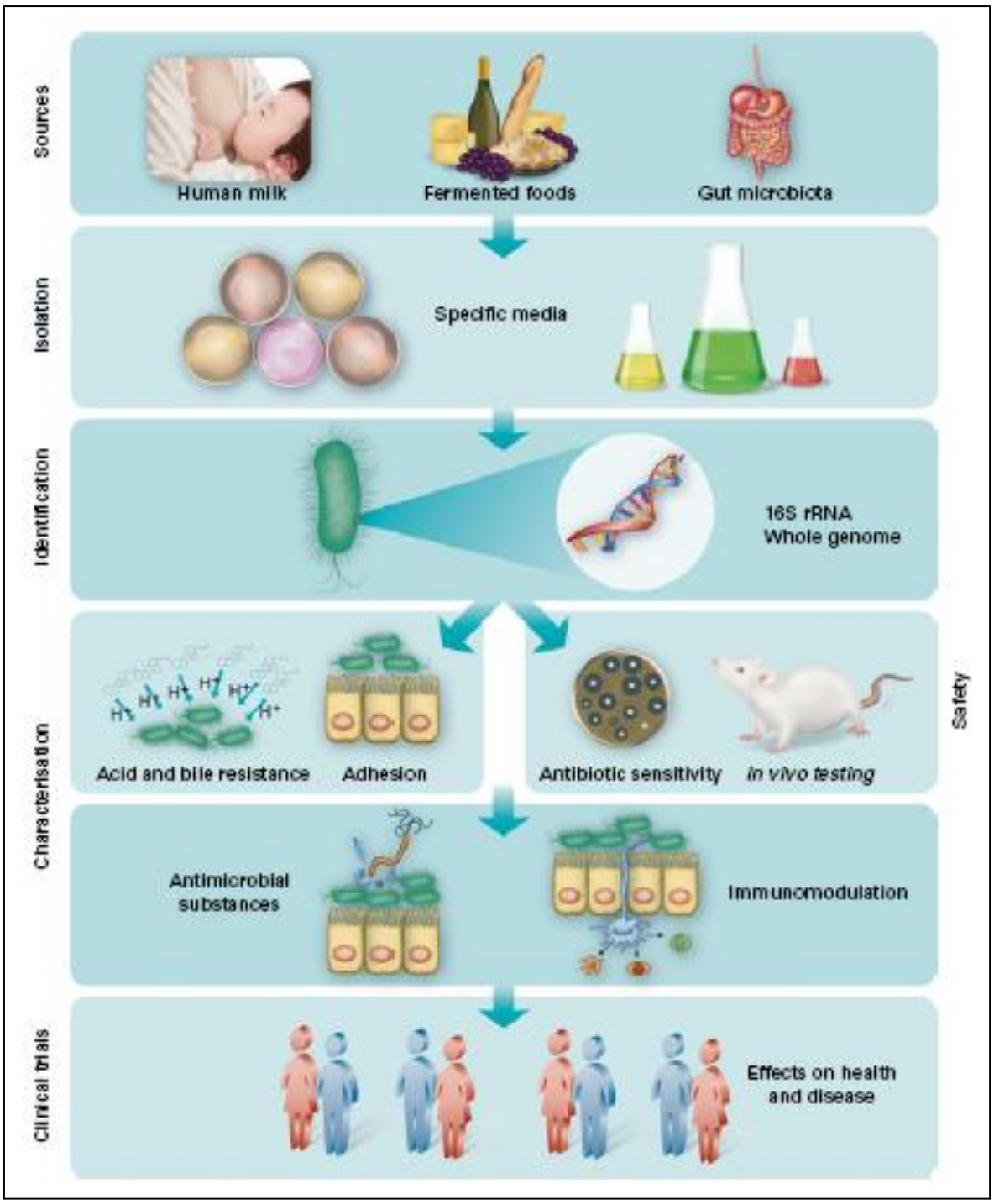


FIGURA 4. Diagrama de flujo que describe los diferentes pasos a seguir para que una cepa bacteriana pueda ser considerada como un nuevo probiótico. rRNA, ARN ribosomal. (Fontana *et al.,* 2013).

El aislamiento de probióticos no se limita necesariamente al TGI humano. El TGI de varias especies de animales, como cerdos, ratas e incluso aves de corral, es una buena fuente de probióticos (Petrof, 2009). Recientemente, se aisló *L. johnsonii* CRL 1647 del intestino de la abeja *Apis mellifera* L., demostrando un efecto beneficioso sobre las colonias de éstas abejas (Audisio y

Benítez-Ahrendts, 2011). También se han obtenido cepas probióticas del TGI de peces marinos y de agua dulce, tales como *Carassius auratus gibelio* (Chu *et al.,* 2011), la trucha arco iris (Pérez-Sánchez *et al.* 2011) y el camarón (Hill *et al.,* 2009).

Aunque la gran mayoría de las cepas probióticas se han obtenido a partir de productos lácteos, fermentados lácteos y del TGI, hay estudios que muestran que las cepas probióticas también se encuentran en sustratos fermentados no lácteos (Rivera-Espinoza y Gallardo-Navarro, 2010). Por ejemplo, en experimentos *in vitro* se ha demostrado que ciertas cepas bacterianas aisladas de la carne (*L. sakei*, *L. curvatus* y *Staphylococcus carnosus*) y de frutas (*L. paracasei* y *L. plantarum*), pueden expresar propiedades funcionales y metabólicas similares a las de las bacterias intestinales humanas (Haller *et al.,* 2001).

AISLAMIENTO

Hoy día se acepta que los enfoques basados en métodos de cultivo proporcionan una imagen incompleta de la diversidad microbiana. Los nichos ecológicos presentan una compleja interrelación entre las diferentes especies de microbios, que no se pueden imitar utilizando métodos de cultivo tradicionales. Enfoques moleculares que omiten el paso de cultivo se han vuelto populares como método para identificar la diversidad microbiana de diferentes fuentes. Estos métodos suministran información importante sobre los ecosistemas microbianos.

El primer paso en el estudio de un ecosistema es el aislamiento de sus miembros (Fontana *et al.,* 2013). En el aislamiento de bacterias probióticas es importante mantener la muestra en condiciones adecuadas antes de proceder a incubarla en medios selectivos. La gran mayoría de los probióticos son anaeróbicos o anaeróbicos facultativos. Por ello, la muestra recogida debe ser inmediatamente puesta en condiciones de anaerobiosis y procesada lo antes posible. Se recomienda que una vez obtenida, no se superen las 3 horas. Las muestran deben ser homogeneizadas rápidamente y luego diluidas para proceder a cultivarlas en medios selectivos (Muñoz-Quezada *et al.,* 2013).

Se han desarrollado varios medios de cultivo para el aislamiento selectivo de bifidobacterias y lactobacilos (Hartemink y Rombouts, 1999; Hartemink *et al.,* 1996; Beerens, 1990; Dave y Shah, 1995; Munoa y Pares, 1988; Nebra y Blanch, 1999; Silvi *et al.,* 1996; Rogosa *et al.,* 1951; Downes e Ito, 2001; MacFaddin, 1985). Rogosa *et al.* desarrollaron un medio selectivo para el aislamiento y recuento de lactobacilos y bifidobacterias, tanto orales como fecales, que contiene una base de agar Columbia suplementado con ácido propiónico. El pH ácido de este medio es fácilmente tolerado por lactobacilos y bifidobacterias, pero, además, inhibe el crecimiento de otros organismos presentes en las heces humanas, tales como especies de *Bacteroides* y *Eubacterium*. Las placas de agar son incubadas a 37 ^{o}C durante un rango de 48 a 72 horas en un ambiente anaeróbico para el crecimiento de bifidobacterias y otras especies. Otra alternativa es una atmósfera rica en CO_2 para favorecer el crecimiento de lactobacilos. Posteriormente, las colonias se aíslan y transfieren a un medio líquido o a una nueva placa de agar.

IDENTIFICACIÓN

La identificación de microbios aislados desde el TGI o de alimentos es la primera etapa en la selección de potenciales probióticos.

Para el caso de muchos ecosistemas, sólo un pequeño porcentaje de los microbios pueden crecer en los medios de cultivo utilizados hoy día (Amann *et al.,* 1995). La clasificación taxonómica podría ser definida como el proceso de catalogación de la biodiversidad basado en un enfoque con muchas fases (Vandamme *et al.,* 1996), que implica métodos genotípicos y fenotípicos. Históricamente, los métodos fenotípicos se han utilizado para identificar a las bacterias. La taxonomía durante muchas décadas se basó en el tipo de fermentación que se producía por las bacterias, determinado por cuál era el azúcar fermentado y los productos posteriormente generados. Por este motivo, los probióticos han sido principalmente clasificados como LAB.

Hoy en día, el análisis de la subunidad ribosomal 16S se ha convertido en el método de elección para su correcta identificación. Durante las últimas dos décadas, los microbiólogos han utilizado este fragmento conservado para la clasificación filogenética (Woese, 1987; Winker y Woese, 1991). También la relación entre los organismos se estima a través de la comparación de

sus secuencias en las bases de datos disponibles, DDBJ (*DNA Data Bank of Japan*), ENA (*European Nucleotide Archive*) y GenBank (*National Institutes of Health* (NIH) *genetic sequence database*).

El análisis de la subunidad 16S se ha sido combinado con otros métodos para identificar comunidades bacterianas del intestino y fuentes ecológicas. Dentro de estas técnicas podemos nombrar la reacción en cadena de la polimerasa (PCR), la electroforesis en gel de agarosa, FISH o la digestión con enzimas de restricción del fragmento amplificado correspondiente a la subunidad 16S (*terminal restriction fragment length polymorphism,* T-RFLP). Sin embargo, el fragmento obtenido es extremadamente pequeño, alrededor de 1500 pares de bases (pb) en comparación con el genoma bacteriano completo de 30.000 a 40.000 pb. Por lo tanto, es necesario obtener información complementaria para poder diferenciar cepas de una misma especie. La región intergénica (16S-23S) exhibe una gran variación que ha permitido diferenciar entre distintas especies de procariotas (Leblond-Bourget *et al.,* 1996). Finalmente, el análisis del genoma bacteriano es la herramienta más utilizada en la actualidad para identificar cualquier tipo de microorganismos.

CARACTERIZACIÓN

Los géneros *Lactobacillus* y *Bifidobacterium* son los más importantes cuando nos referimos al término "probiótico", en cuanto a especies documentadas. Cuando éstas son ingeridas en un número suficiente deben superar el TGI y persistir durante algún tiempo para poder ejercer sus efectos beneficiosos. En la parte introductoria del tema se definieron los principios que debían poseer las cepas candidatas para ser consideradas como probióticas. Para ello, las bacterias debían ser administradas vivas, aunque algunos autores han demostrado que los efectos beneficiosos también pueden existir cuando las cepas se administran muertas (de los Reyes Gavilán *et al.,* 2011).

La capacidad de tolerar un pH extremadamente ácido (1,5 - 3,0), las enzimas gástricas, las sales biliares y otras enzimas intestinales presentes en el TGI son las distintas barreras que deben superar las cepas administradas para llegar en cantidad suficiente para ejercer sus efectos (Masco *et al.,* 2007). Varios ensayos *in vitro* han sido diseñados para imitar estas condiciones a las que son sometidas las cepas que se consideran potenciales probióticos.

Resistencia a pH ácido y sales biliares: La tolerancia a un medio ácido es uno de los criterios generales para la selección de posibles cepas probióticas, con la idea de garantizar su viabilidad y funcionalidad (FAO, 2002). Sistemas *in vitro*, en los que se incluyen incubaciones controladas con jugos gástricos reales y/o simuladas (pH 2,0 a 4,0 y 70-180 min), se han utilizado preferentemente en la evaluación de nuevas cepas probióticas (Sanz, 2006). También se han desarrollado modelos complejos que simulan el tránsito gastrointestinal (Masco *et al.,* 2007; Mainville *et al.,* 2005). Por otra parte, se han realizado incubaciones de 1 a 4 h en medios enzimáticos y/o químicos en intervalos de pH de 1,5 a 3,0. Las sales biliares facilitan la digestión de compuestos lipofílicos, pero también se comportan como agentes antimicrobianos por influir en el establecimiento de la microbiota intestinal. Las concentraciones fisiológicas de sales biliares en la bilis humana van desde 0,3 a 0,5% (Dunne *et al.,* 2001; Zavaglia *et al.,* 1998). Los ensayos *in vitro* con sales biliares se llevan a cabo con bilis bovina de concentraciones entre 0,3 y 0,7% durante 60-180 min.

Los probióticos muestran resistencia variable tanto a los medios ácidos como a los que presentan sales biliares, siendo ésta característica dependiente de la cepa. Existen en la literatura controversias sobre la supervivencia de las bifidobacterias; hay estudios que han descrito que son muy sensibles a valores ácidos de pH. Algunas especies presentan tasas de supervivencia nula a pH 2,0 durante 90 min (Sanz, 2006; Charteris *et al.,* 1998), menos de 1% a pH 3,0 durante 2 h (Takahashi *et al.,* 2004) y un aumento de supervivencia cuando el pH varía desde 3,0 a 5,0 durante 3 h (Matsumoto *et al.,* 2004). En cambio, algunos autores destacan que los porcentajes más altos de tasas de supervivencia se han descrito para las bifidobacterias (Matto *et al.,* 2006; Chenoll *et al.,* 2011; Muñoz *et al.,* 2011; Li *et al.,* 2010). Para las cepas de *Lactobacillus* se han demostrado altas resistencias a pH ácidos. Un estudio en el que se evaluaron 20 cepas de *Lactobacillus* demostró una tasa de supervivencia que variaba de 2 a 100% con un pH de 3,0 durante 1 h. Sobre las sales biliares, las tasas de supervivencia de las bifidobacterias varía desde 1 a 70% en concentraciones de 0,3% de sales durante 90 min (Mainville *et al.,* 2005). Para el caso de los lactobacilos, se han probado dos cepas de *L. plantarum* que han tenido una supervivencia mayor al 50% con una concentración de sales biliares desde 0,3 a 1% durante 2 a 3 h (Bosch *et al.,* 2012).

Por los datos expuestos anteriormente, se observa que las bacterias desarrollan cierta adaptación cuando se les somete a condiciones de estrés, tales como medios ricos y/o pobres en nutrientes, distintos pH ácidos y porcentajes de sales biliares (Mills *et al.,* 2011).

Adherencia a células epiteliales intestinales: La adherencia a las células epiteliales intestinales y también al *mucus* es una característica importante de los probióticos para promover el tiempo de residencia en el intestino, la exclusión de algunos patógenos y las interacciones que pueden ocurrir con el hospedador y con el sistema inmunitario.

En los últimos 25 años, la línea celular Caco-2 ha sido la más usada para determinar la capacidad de adhesión de las cepas probióticas (Dicks y Botes, 2010). Las células Caco-2 forman una monocapa homogénea, que se asemeja a la de los enterocitos maduros humanos en el intestino delgado (Lenaerts *et al.,* 2007); además forman criptas, estructuras típicas de la monocapa epitelial (Huang *et al.,* 2009).

Existe otro tipo de línea celular de colon, la HT-29 que también muestra características típicas de diferenciación de enterocitos y se ha utilizado en ensayos de adhesión *in vitro* (Gopal *et al.,* 2001). Los resultados obtenidos en los estudios de adhesión en modelos *in vitro*, líneas celulares y/o su combinación son muy variados (Laparra y Sanz, 2009). De hecho, lactobacilos, bifidobacterias y bacterias patógenas exhiben diferencias en la adhesión al *mucus*, células Caco-2, Caco-2 más *mucus*, HT-29-MTX y Caco-2/HT-29-MTX. Así por ejemplo, para *L. rhamnosus* GG se han obtenido capacidades de adhesión en los sistemas antes mencionados de 10,21%, 5,17%, 3,19%, 0,84% y 0,85%, respectivamente. Muchos estudios *in vitro* evalúan la adhesión de potenciales bacterias probióticas y las interacciones con patógenos en la interfaz del epitelio intestinal, obteniendo resultados que dependen fundamentalmente de la técnica y cepa utilizada (Izquierdo *et al.,* 2008).

Las diferencias existentes entre las condiciones experimentales utilizadas para la tolerancia en medio ácido, (medios acidificados usando ácido clorhídrico o láctico, con y sin enzimas), resistencia a sales biliares (dosis y origen de las sales biliares) y adhesión (*mucus*, líneas celulares, células más *mucus*) generan un problema a la hora de comparar los diferentes estudios y resultados. Además, es importante destacar que cada una de las etapas descritas tiene siempre la

cualidad de ser cepa-específica, variando entre diferentes especies y géneros. Así, los estudios clínicos posteriores son la herramienta definitiva para establecer la verdadera funcionalidad de la cepa estudiada (Fontana *et al.,* 2013).

Actividad antimicrobiana: Los probióticos, por definición, cuando son administrados en cantidades adecuadas ejercen efectos beneficiosos para el hospedador. Uno de los más importantes es la actividad antimicrobiana frente a patógenos (Laparra y Sanz, 2009).

Las infecciones intestinales están mediadas por la adhesión de las bacterias patógenas a la superficie de las mucosas, lo que produce un cambio en la microbiota intestinal presente. Las bacterias probióticas pueden tener un rol protector a través de diversos mecanismos que incluyen la producción de sustancias antimicrobianas, la competencia con los propios patógenos por nutrientes, la adhesión a sitios de unión que impiden la posterior infección de los mismos y la estimulación del sistema inmunitario (Collado *et al.,* 2007).

Ferreira *et al.* evaluaron la capacidad de 7 cepas de *L. gasseri* para adherirse a la mucosa intestinal frente a los patógenos *Cronobacter sakazakii* (ATCC 29544) y *Clostridium difficile* (ATCC 1296). Estas cepas probióticas produjeron una disminución en la adhesión de estos patógenos (Ferreira *et al.,* 2011).

La capacidad de las cepas probióticas de inhibir el crecimiento de patógenos en medios de cultivo sólido y líquido y la posterior modulación mediante la producción de citoquinas y factores de crecimiento en líneas celulares, ha sido documentada usando modelos *in vitro* para la evaluación de sus efectos biológicos. Hay que añadir que también se han realizado estudios de actividad antimicrobiana de probióticos en modelos animales.

Finalmente, varias cepas de lactobacilos y bifidobacterias han demostrado la inhibición del crecimiento de *Escherichia coli* (Gopal *et al.,* 2001; Todoroki *et al.,* 2001; Chu *et al.,* 2005; Tsai *et al.,* 2008; Candela *et al.,* 2008), *Salmonella typhimurium*, *Shigella flexneri* (Jankowska *et al.,* 2008; Tien *et al.,* 2006; Cho *et al.,* 2009) y *Clostridium difficile* (Pillai y Nelson, 2008).

SEGURIDAD

Hasta el año 2002, fecha en que se creó la Autoridad Europea de Seguridad Alimentaria (EFSA por sus siglas en inglés, *European Food Safety Authority*), no existía ninguna guía formal que detallara la seguridad en productos alimentarios que contuvieran microbios. En 2007, el Comité Científico sobre Nutrición Animal propuso las normas "*qualified presumption of safety*" (QRS), sobre presunción de seguridad en productos alimentarios que tuvieran algún microbio. Las LAB se convirtieron en uno de los primeros grupos sometidos a estas normas, que se basan en cuatro etapas que deben cumplirse obligatoriamente:

- Definición de la taxonomía del microbio.
- Entrega de información referente a literatura científica, historia de uso, aplicaciones industriales y datos en intervenciones animales y humanas para proporcionar el *status* de presunción de seguridad.
- Exclusión de la patogenicidad.
- Definición de su uso final.

Los factores que se deben considerar para evaluar la seguridad de los probióticos incluyen: historia de aislamiento y clasificación taxonómica del probiótico potencial, controles de fabricación que eliminen la contaminación (incluida la contaminación cruzada entre lotes) de probióticos con microbios u otras sustancias, evaluación de la existencia de asociación de los probióticos con infección y toxicidad a nivel de cepa y determinación del estado fisiológico de la población consumidora del producto, con especial consideración en bebés recién nacidos y pacientes en estado crítico (dosis administrada y método de administración).

Si se superan todas estas cuestiones, los probióticos son considerados generalmente como "seguros", pero ésta hipótesis no puede ser asumida en términos absolutos, pues los análisis para asegurar la seguridad deben ser cada vez más específicos en alimentos y suplementos dietéticos que contengan alguna cepa y que sean administrados a la población general.

Una vez superadas las distintas etapas de aislamiento, identificación, caracterización y seguridad de la cepa se procede a la producción industrial. En ella hay dos aspectos fundamentales. En primer lugar, el microorganismo necesita ser cultivado en un medio adecuado que permita el crecimiento en grandes cantidades; y, en segundo lugar, se debe asegurar la viabilidad de las cepas durante la fabricación. Ambos aspectos son importantes y la producción se convierte en una fase clave para un posible probiótico que debe superar el crecimiento y los procesos a los que será sometido (Fontana *et al.,* 2013).

EVALUACIÓN

Estudios *in vitro*: Numerosos estudios han utilizado líneas celulares animales y humanas como modelos de intestino (Cencič y Langerholc, 2010), tales como células Caco-2, HT-29, IEC-6, IEC-18 y T84, por nombrar algunas. En la mayoría de estos modelos experimentales *in vitro*, las células epiteliales son cultivadas en una monocapa, no pudiendo reproducir las particularidades del epitelio funcional. Para superar este problema, los investigadores han tratado de entender los mecanismos que explican las interacciones dinámicas entre el epitelio intestinal, las bacterias y el sistema inmunitario y han concluido que la mejor manera de aunar todo esto es utilizando co-cultivos de células dendríticas y epiteliales de intestino con probióticos (Borchers *et al.,* 2009), así como modelos en 3 dimensiones (Cencič y Langerholc, 2010). Los modelos en 3 dimensiones se generan usando una línea celular epitelial intestinal de origen no-carcinogénico que se cultiva en una membrana microporosa, permitiendo la polarización de las células intestinales. Por debajo de la membrana microporosa (lado basolateral), las células epiteliales forman otra capa con células inmunitarias (macrófagos y células dendríticas), imitando el tejido linfoide de la mucosa. La microbiota intestinal es añadida a la parte apical de la membrana para estudiar sus efectos. Estos tres componentes (epitelios, células inmunitarias y microbiota) son los factores más importantes en el intestino; por tanto, estos modelos tratan de imitar la situación que realmente ocurre *in vivo*.

EVALUACIÓN CLÍNICA

Muchos estudios clínicos han tratado de evaluar una gran variedad de probióticos en diversas condiciones fisiológicas y patológicas. Sin embargo, muchos de estos estudios han sido

cuestionados debido al pequeño número de pacientes utilizados o a la falta de un grupo de control. De hecho, la EFSA emite dictámenes científicos sobre la validez de las alegaciones de salud relacionadas con cepas probióticas. Un alto porcentaje de las alegaciones son rechazadas por la EFSA porque la relación de causa y efecto no está claramente establecida entre el consumo del probiótico y los efectos beneficiosos que se suponía que tenía. El método más fiable para evaluar los beneficios terapéuticos de cualquier cepa probiótica es el uso de ensayos aleatorizados, controlados con placebo, los cuales se revisan a continuación dividiéndolos por estado fisiológico y patología.

Embarazo y lactancia: Asemi *et al.* evaluaron los efectos del consumo diario de un yogur que contenía cepas probióticas sobre parámetros inflamatorios en mujeres embarazadas. Las mujeres consumieron 200 g de yogur probiótico con *L. acidophilus* La5 y *B. animalis* BB12, ó 200 g de yogur convencional al día durante 9 semanas. El yogur probiótico disminuyó de manera significativa la expresión de la proteína C-reactiva, pero no tuvo ningún efecto sobre los niveles del factor de necrosis tumoral alfa (TNF-α) en las mujeres embarazadas. Además, tuvo lugar un aumento de los niveles de glutatión reductasa en las embarazadas sin afectar a ningún otro índice de estrés oxidativo (Asemi *et al.,* 2012). Dugoua *et al.* describieron que *Lactobacillus* y *Bifidobacterium* no presentaron ningún efecto sobre la incidencia de cesárea, peso al nacer y/o edad gestacional (Dugoua *et al.,* 2009).

Alergia: Vliagoftis *et al.* evaluaron la evidencia clínica para el uso de los probióticos como alternativa terapéutica en la rinitis alérgica y el asma. La revisión incluyó 12 estudios clínicos aleatorizados. Un total de 9 ensayos mostraron una mejora de la rinitis alérgica debido a la utilización de probióticos. Dentro de ellos, todos los ensayos relativos a rinitis alérgica persistente demostraron reducción en los síntomas y cantidad de medicación administrada comparado con el placebo. Por otra parte, en los ensayos donde se evaluaba la rinitis alérgica estacional, se observó mejoría clínica. Los 9 estudios en los que se describieron diversas mediciones inmunológicas de alergia no mostraron ningún efecto significativo debido al probiótico, al igual que los estudios sobre el efecto de la administración de probióticos en el tratamiento del asma. Tomados en conjunto, estos resultados sugieren que los probióticos podrían tener un efecto beneficioso en la rinitis

alérgica en dos aspectos: la reducción de algunos síntomas severos y de la medicación utilizada (Vliagoftis *et al.* 2008).

En el estudio en el que se analizó el efecto simbiótico, entre un probiótico y un prebiótico, en la prevención de la enfermedad atópica, Kuitunen *et al.* evaluaron a niños de 1.223 madres embarazadas con un alto riesgo de alergia (por lo menos uno de los padres con asma diagnosticado, rinitis alérgica o eczema atópico). Cada mujer recibió dos veces al día una combinación probiótica de *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* BB99 y *Propionibacterium freudenreichii* ssp *shermanii* JS o un placebo durante 4 semanas antes del parto. Sus recién nacidos recibieron los mismos probióticos, agregando también 0,8 g de un galacto-oligosacárido o placebo una vez al día desde el nacimiento hasta los 6 meses de edad. Después, se realizó un seguimiento de los niños hasta los 2 años de edad por si desarrollaban cualquier enfermedad alérgica. En las muestras de sangre y heces se evaluaron los valores hematológicos, calprotectina, α-1-antitripsina, TNF-α e IgA. A los 6 meses, el grupo de niños que recibió el probiótico mostró una disminución significativa de los valores de hemoglobina comparado con el grupo placebo. Además, se encontró una correlación negativa entre los valores de hemoglobina a los 6 meses de edad y la expresión de calprotectina fecal a los 3 meses de edad. Los valores hematológicos fueron similares en ambos grupos a los 2 años de edad (Kuitunen *et al.,* 2009).

Olivares *et al*. realizaron un estudio doble ciego, controlado por placebo en 44 niños alérgicos, distribuidos aleatoriamente en dos grupos: un grupo de yogur y un grupo de probiótico. Se recogieron muestras de sangre y heces. El consumo del producto probiótico indujo una disminución significativa en el nivel de IgE en el plasma y un aumento en las células reguladoras T CD4+/CD25+. La disminución de IgE sérica se acompañó de un aumento significativo de IgA en la mucosa. No se detectaron cambios en otras células implicadas en las reacciones alérgicas. El consumo del producto probiótico también produjo cambios significativos en la respuesta innata, con un aumento significativo de las células *natural killer* (Olivares *et al.* 2006).

No hay evidencias que sugieran que los probióticos puedan ser un tratamiento efectivo para el eczema en los niños, pues la administración del probiótico ha llevado a la producción de eventos adversos (infecciones e isquemia intestinal), sin mostrar ningún beneficio en comparación con el

placebo (Boyle *et al.,* 2009). Un meta-análisis de seis estudios clínicos de prevención y cuatro de tratamiento, doble ciegos, aleatorizados y controlados por placebo en niños entre 0 y 13 años de edad indicó que el uso de probióticos para la prevención de la dermatitis atópica pediátrica es favorable, pero no para el tratamiento (Lee *et al.* 2008). Un análisis adicional en el que se excluyó el único estudio con un protocolo postnatal reveló un menor riesgo relativo, que en términos de tratamiento no mostró ninguna diferencia estadística.

Enfermedades relacionadas con el intestino: Olivares *et al.* investigaron el efecto de un producto fermentado que contenía dos cepas probióticas, *L. gasseri* CECT5714 y *L. coryniformis* CECT5711, sobre varios parámetros sanguíneos y fecales relacionados con la función intestinal de voluntarios sanos. Un total de 30 voluntarios sanos fueron divididos aleatoriamente en dos grupos, uno tomó un yogur estándar y el otro las cepas antes mencionadas administradas vía oral. El grupo que recibió los probióticos no tuvo ningún evento adverso significativo y, además, las cepas administradas pudieron aislarse de las heces de los voluntarios. De hecho, la concentración en las heces de LAB aumentó en el grupo probiótico. Adicionalmente, la administración oral de los probióticos mejoró algunos parámetros intestinales, como la producción de AGCC, humedad fecal, frecuencia y volumen de las heces (Olivares *et al.,* 2006).

Diarrea infecciosa: Una revisión Cochrane sobre la eficacia de los probióticos en el tratamiento de la diarrea infecciosa, que incluyó tanto niños como adultos, evalúo 63 estudios con un total de 8014 participantes. No se atribuyeron eventos adversos relacionados a la administración de los probióticos y su uso disminuyó la duración de la diarrea. Sin embargo, el efecto fue muy variable en los estudios analizados. Los autores concluyeron que su uso es seguro y ejerce efectos beneficiosos en disminuir la duración de los episodios de diarrea y la frecuencia de las diarreas infecciosas agudas junto con una buena hidratación (Allen *et al.,* 2010).

Diarrea asociada al uso de antibióticos: Un meta-análisis realizado por Johnston *et al.* valoró los resultados obtenidos en los estudios clínicos, paralelos, aleatorizados y controlados por placebo realizados que investigaban sobre la diarrea asociada al uso de antibióticos en niños de 0 a 18 años (Johnston *et al.,* 2011). El tratamiento con probióticos fue comparado frente al tratamiento con placebo, profilaxis alternativa y/o no tratamiento y la incidencia de diarrea secundaria al uso de

antibióticos. Los estudios incluían el tratamiento con *Bacillus* spp., *Bifidobacterium* spp., *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc cremoris*, *Saccharomyces* spp. y *Streptococcus* spp., de manera individual y/o combinada. A pesar de la gran heterogeneidad de las cepas probióticas utilizadas, la dosis y la duración y la calidad de los estudios, la evidencia global sugiere un efecto protector de los probióticos en la prevención de las diarreas asociadas al uso de antibióticos.

Diarrea persistente: Los resultados científicos que sugieren que los probióticos puedan ser efectivos en tratar la diarrea persistente en niños son escasos. Bernaola Aponte *et al*. revisaron cuatro estudios clínicos aleatorizados comparando una cepa especifica de probióticos frente a placebo en niños con diarrea persistente. En cuatro estudios, con un total de 464 participantes, el tratamiento con probióticos redujo la duración de la diarrea persistente en dos de los estudios. De manera similar, la frecuencia de las deposiciones, disminuyó en el grupo de probióticos en dos de los estudios. En otro estudio, se informó de una disminución en los días de hospitalización, sin encontrarse eventos adversos (Bernaola Aponte *et al.,* 2010).

Enterocolitis necrotizante: Alfaleh *et al*. valoraron el empleo de probióticos en el tratamiento de la enterocolitis necrotizante (ENC) en 16 estudios aleatorizados y cuasi estudios clínicos en un meta-análisis que involucró a 2842 niños prematuros de menos de 37 semanas de gestación y/o menos de 2500 g de peso al nacer. Dentro de los estudios examinados existía una gran variabilidad en los criterios de inclusión (peso al nacer y edad gestacional), riesgo inicial de desarrollo de ENC para los grupos control, tiempo, dosis, formulación de probióticos y regímenes de alimentación. La administración enteral de probióticos redujo significativamente la incidencia de la ENC severa (etapa II o posterior) y la mortalidad. No hubo evidencia de una reducción significativa de sepsis nosocomial. Además, no se encontró evidencia de infección sistémica con el uso de probióticos en este estudio. Los autores concluyeron que la administración enteral con probióticos previene la ENC severa, aunque son necesarios más estudios para asegurar la eficacia del uso de probióticos en caso extremos como niños con bajo peso al nacer, donde es de suma importancia establecer claramente la eficiencia de la formulación y la dosis a utilizar. Braga *et al*. evaluaron el efecto de la administración de una combinación de *L. casei* y *B. breve* en niños prematuros con bajo peso al nacer que desarrollarían ENC. El uso de la combinación tuvo un efecto

beneficioso en la incidencia de ENC en la etapa II o posterior. Además, se asoció con una mejora en la motilidad intestinal en el tiempo necesario para alcanzar la alimentación enteral completa (Braga *et al.,* 2011).

Colitis ulcerosa: Se ha descrito que el tratamiento con probióticos es efectivo en la remisión de la colitis ulcerosa (CU) (Sang *et al.,* 2010). 13 estudios clínicos aleatorizados revisaron el tratamiento de la CU con probióticos. Comparado con el grupo placebo, se demostró que la tasa de remisión de la CU para el grupo de pacientes que recibieron probióticos fue 2,0% (95% IC 1,35-2,96). Durante el curso del tratamiento, los pacientes que recibieron probióticos durante al menos 12 meses tuvieron una tasa de remisión de CU de 1,36% (95% IC 1,07-1,73), la incidencia de CU en el grupo probióticos fue 0,69% (95% IC 1,01-2,47), comparados con el grupo placebo. Para el grupo de CU de gravedad media a moderada la tasa de recurrencia fue 0,25% (95% IC 0,12-0,51). Dentro de los grupos tratados con probióticos, el que recibió *B. bifidum* mostró una tasa de recurrencia de 0,25% (95% IC 0,12-0,50), comparados ambos con el grupo placebo.

La inflamación de la bolsa ileal (IBI) es la complicación más importante después de la anastomosis ileo-anal en pacientes con CU. Mimura *et al*. describieron que una dosis diaria de 6 g de la mezcla probiótica VSL#3 fue eficaz en la remisión de IBI en pacientes tratados durante 1 año. La remisión se mantuvo durante 1 año en el 85% de los pacientes del grupo de VSL#3, comparado con el 6% del grupo de placebo (Mimura *et al.*, 2004). En un trabajo más reciente, pacientes en remisión con IBI inducida por tratamiento con antibióticos fueron reclutados para recibir VSL#3 o placebo durante el mantenimiento de la remisión (Kühbacher *et al.,* 2006). Se obtuvieron biopsias antes y dos meses después del inicio de la administración de VSL#3 y del placebo. La terapia con VSL#3 aumentó el número total de células bacterianas intestinales, la riqueza y la diversidad de la microbiota bacteriana, especialmente la anaerobia, mientras que la microbiota de hongos fue reprimida. En contraste, los pacientes que recibieron el placebo recayeron más con una marcada reducción en la diversidad de la microbiota.

Enfermedad de Crohn: Doherty *et al*. revisaron en 2010 los estudios clínicos que comparaban el uso de antibióticos y/o probióticos con placebo en la prevención de la recurrencia clínica de la enfermedad de Crohn (Doherty *et al.,* 2010). Estudiaron un total de siete estudios que

incluían 2 estudios con antibióticos frente a placebo y 5 frente a probióticos y placebo. La administración de probióticos no se asoció con ninguna diferencia significativa en el riesgo de recurrencia de la enfermedad de Crohn cuando se comparó con el placebo.

Síndrome de intestino irritable: El síndrome de intestino irritable (SII) es una condición crónica que afecta de un 3 a 25% de la población y para la cual no existe disponible ningún tratamiento específico, tan solo sintomático. Cuando se descubrió que la microbiota intestinal normal se alteraba en el SII, se empezó a considerar el uso de probióticos en la mitigación de los síntomas. McFarland y Dublin revisaron 20 estudios clínicos que incluyeron un total de 1404 sujetos con SII. El uso de probióticos se asoció con una mejora en los síntomas globales de SII y un menor dolor abdominal comparados con el placebo (McFarland y Dublin, 2008). Gawrońska *et al*. investigaron la eficacia de *L. rhamnosus* GG para el tratamiento de la dispepsia funcional, SII y/o dolor abdominal en niños. Los autores encontraron que *L. rhamnosus* GG reducía la frecuencia de dolor en niños con SII (Gawrońska *et al.*, 2007). En contraste con los resultados anteriores, Bausserman y Michail encontraron que la administración de *L. rhamnosus* GG a 50 pacientes de entre 6 y 20 años con SII durante 6 semanas no disminuyó el dolor abdominal cuando se comparó con un placebo. No hubo diferencias significativas en otros síntomas gastrointestinales, exceptuando la percepción de la distensión abdominal (Bausserman y Michail, 2005).

Se ha observado que el tratamiento del SII con lisados de *Enterococcus faecalis* y *E. coli* ha sido efectivo y superior al placebo en la reducción de los síntomas típicos, tales como dolor abdominal y distensión (Enck *et al.,* 2008). En 297 pacientes con SII tratados durante 8 semanas con éstos lisados en un estudio clínico, doble ciego y aleatorizado, los pacientes que respondieron al tratamiento tuvieron al menos un 50% menos de síntomas globales y de dolor abdominal. La tasa de éxito en los voluntarios tratados con probióticos, en los síntomas globales fue de 102/149 (68,5%) comparado con el placebo de 56/148 (37,8%; $P< 0,001$). En la mejora del dolor abdominal el grupo probiótico produjo un 72,5% (108/149) y el placebo un 44,6% (66/148). El análisis de Kaplan-Meier reveló que el promedio de respuesta del grupo tratado con los lisados fue de 4 a 5 semanas frente a las más de 8 semanas del placebo.

Enfermedad hepática crónica: Los pacientes con enfermedad hepática crónica generalmente tienen una microbiota intestinal desequilibrada que afecta directamente al desarrollo y empeoramiento de la enfermedad. Liu *et al*. efectuaron un estudio clínico aleatorizado, controlado por placebo. Los pacientes en el grupo tratado una leche fermentada durante 14 días que contenía *Bacillus bifidus*, *L. acidophilus*, *L. bulgaricus* y *S. thermophilus.* Después de la intervención, el grupo con la leche fermentada tuvo un menor recuento de *E. coli* y menor desequilibrio en la microbiota intestinal que el grupo con placebo. Además, hubo una mejoría de los síntomas de la enfermedad, como ingesta de alimento, apetito, distensión abdominal y fluido ascítico (Liu *et al.,* 2010). Por otra parte, Aller *et al*. demostraron que la ingesta de un comprimido con 500 millones de bacterias de *L. bulgaricus* y *S. thermophilus* mejoraba los niveles de aminotransferasa hepática en pacientes con enfermedad de hígado graso no alcohólico (Aller *et al.,* 2011).

Pancreatitis aguda: Zhang *et al*. revisaron todos los estudios clínicos aleatorizados importantes que estudiaban el efecto de probióticos, prebióticos (ingrediente alimentarios no digerible, que mejoran la salud humana por la estimulación selectiva del crecimiento y/o actividad de las bacterias existentes en el colon) y la combinación de ambos (simbióticos) en pacientes con pancreatitis aguda. Se incluyeron un total de siete estudios clínicos aleatorizados con 559 pacientes. Ninguno de los tratamientos mostró influencia en la incidencia de infecciones postoperatorias, infección pancreática, fallo orgánico múltiple y síndrome de respuesta sistémica inflamatoria. Tampoco hubo diferencias significativas en la duración de la terapia con antibióticos y la mortalidad. Sin embargo, el tratamiento se asoció con una menor estancia hospitalaria (Zhang *et al.,* 2010).

Sharma *et al*. (2011) investigaron el papel de los probióticos en la permeabilidad del intestino y de la endotoxemia en pacientes con pancreatitis aguda, aunque dicho estudio no tuvo fuerza, ya que tuvo que terminar de manera prematura, por la gravedad de los pacientes incluidos. Los pacientes fueron asignados aleatoriamente para recibir un placebo o una mezcla de *L. acidophilus*, *B. longus*, *B. bifidum*, *B. infantalis* y 25 mg de fructo-oligosacáridos.

MECANISMOS DE ACCIÓN DE LOS PROBIÓTICOS

La defensa del hospedador a los microorganismos patógenos, mediada por la microbiota intestinal, requiere una fina interpretación del microambiente presente, ya que debe distinguir entre organismos comensales y patógenos ocasionales, y diferenciar las respuestas para ambos casos. Los epitelios de las mucosas, en particular el epitelio intestinal, representan la primera línea de defensa frente a la colonización por patógenos. Los enterocitos superficiales sirven como sensores aferentes en el microambiente luminal, secretando péptidos antibacterianos, IgA y quimioquinas que alertan y dirigen la respuesta inmunitaria al sitio de la infección. Las células M que recubren los folículos linfoides transportan los antígenos luminales a las células dendríticas subyacentes y a otras células presentadoras de antígenos. Finalmente, las células dendríticas intestinales juegan un rol esencial de sensores inmunitarios y pueden directamente monitorizar el contenido intestinal, ya sea por la entrada o la extensión de las dendritas entre los enterocitos superficiales sin alterar las uniones estrechas. Además, pueden ingerir y mantener vivas las bacterias comensales, y viajar a los ganglios linfáticos mesentéricos, donde se induce una respuesta inmunitaria local frente a las bacterias comensales.

La fina distinción entre bacterias patógenas y comensales está mediada por el sistema de receptores de reconocimiento de patrones antigénicos (PRR), que incluyen la familia de receptores *toll-like* (TLR) y los receptores NOD-*like* (NLR). La microbiota controla también la proliferación y diferenciación de las células epiteliales, y modula la maduración y la actividad de la respuesta inmunitaria innata y adaptativa. Un deterioro en la composición de la microbiota puede ser remediada por la utilización de prebióticos, actuando a través de diversos mecanismos, como por ejemplo la inducción de un sistema de equilibrio en la composición de la microbiota del intestino y/o un efecto directo a través de la activación o bloqueo de receptores celulares. Los prebióticos pueden mejorar el equilibrio inmunológico en los recién nacidos, generando una menor incidencia de infecciones tempranas en la vida, además de correlacionarse con una mejoría de la microbiota intestinal claramente establecida por un aumento de los niveles de bifidobacterias. El consumo de un probiótico, en combinación con un prebiótico adecuado (simbiótico), puede dar lugar a efectos sinérgicos, mejorando la supervivencia del probiótico, aportando un sustrato fácilmente disponible para la fermentación y aumento del contenido de bifidobacterias autóctonas. En definitiva, los

probióticos y prebióticos ofrecen una estrategia atractiva para reducir los cambios desfavorables del intestino manteniendo una microbiota intestinal más "sana", que puede ayudar a la función intestinal (Plaza-Díaz y Gil, 2013).

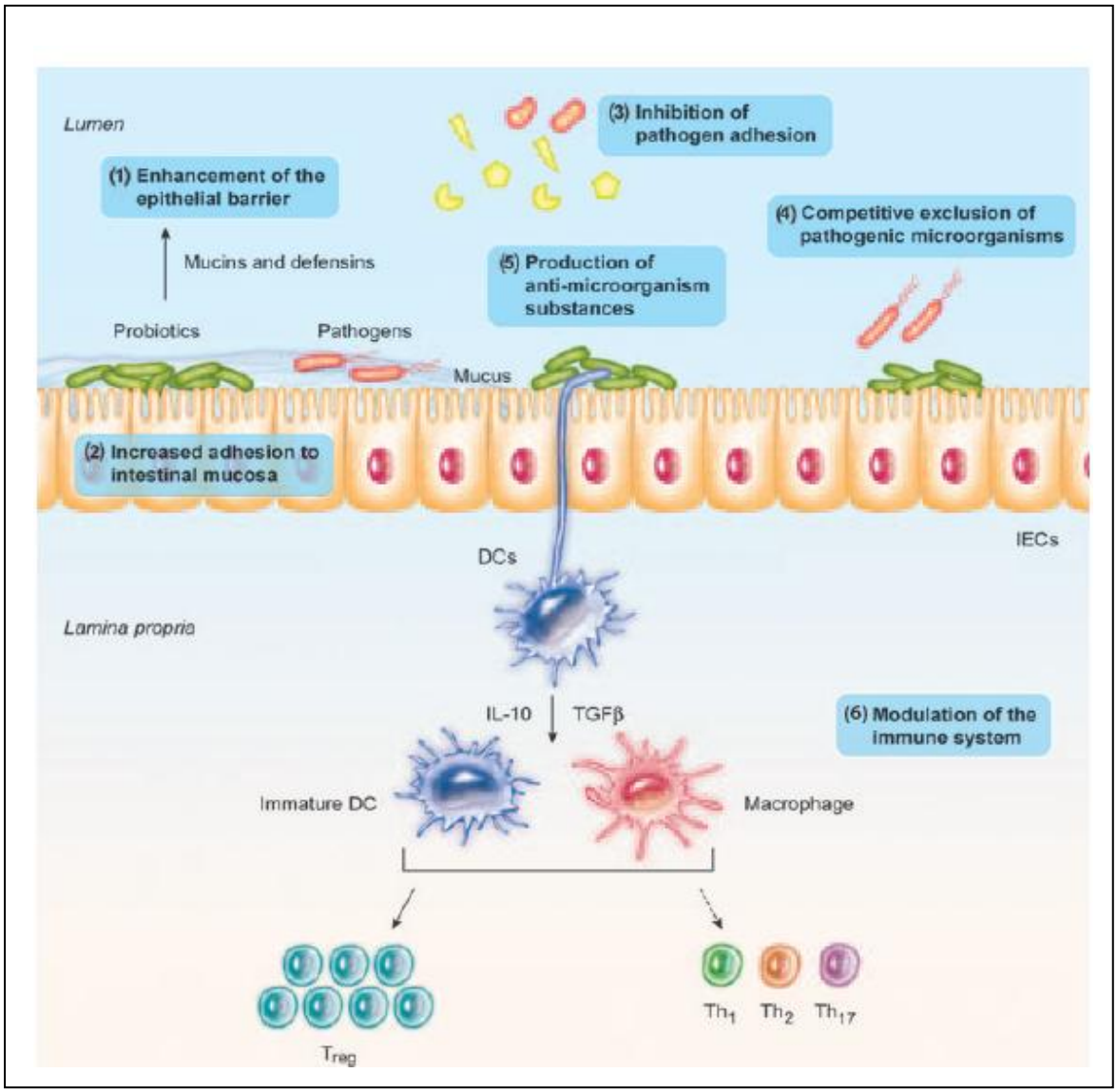


FIGURA 5. Mecanismos de acción de probióticos. (Bermudez-Brito *et al.,* 2012).

Los principales mecanismos de acción de los probióticos incluyen: la mejora de la barrera epitelial, el aumento de la adhesión a la mucosa intestinal, la inhibición concomitante del patógeno mediante la adhesión, la exclusión competitiva de los microorganismos patógenos, la producción de sustancias anti-microbianas y la modulación del sistema inmunitario (Figura 5).

MEJORA DE LA BARRERA EPITELIAL

El epitelio intestinal está en contacto permanente con el contenido luminal y con la microbiota (dinámica y variable). La barrera intestinal es un mecanismo de defensa importante utilizado para mantener la integridad epitelial y para proteger al organismo del medio ambiente. Las defensas de la barrera intestinal comprenden la capa mucosa, péptidos antimicrobianos, IgA secretora y el complejo de adhesión para la unión epitelial (Ohland y Macnaughton, 2010). Cuando la función de barrera se pierde, los antígenos bacterianos y derivados de los alimentos pueden llegar a la submucosa e inducir respuestas inflamatorias, resultando en la aparición de desórdenes intestinales como el SII (Hooper *et al.,* 2001; Hooper *et al.,* 2003; Sartor, 2006). El consumo de bacterias no patógenas puede contribuir a la función de barrera intestinal, siendo los probióticos las bacterias más estudiadas por su participación en el mantenimiento de esta barrera. Sin embargo, los mecanismos por los que los probióticos mejoran la barrera intestinal no se entienden completamente.

Varios estudios sugieren que el aumento de la expresión de genes implicados en la señalización de las uniones estrechas entre las células epiteliales es un posible mecanismo para reforzar la integridad de la barrera intestinal (Anderson *et al.,* 2010). Por ejemplo, los lactobacilos regulan varios genes que codifican proteínas de adhesión, tales como E-cadherina y β-catenina, en un modelo de barrera con células T84. Además, la incubación de células intestinales con lactobacilos influye diferencialmente en la fosforilación de proteínas de adhesión y en la abundancia de las isoformas de la proteína quinasa C (PQC), tales como PQCδ, que afecta positivamente la función de barrera del epitelio (Hummel *et al.,* 2012).

Datos recientes han indicado que los probióticos pueden iniciar la reparación de la función de barrera dañada. Así, por ejemplo, *Escherichia coli* Nissle 1917 (EcN1917) no sólo previene la disrupción de la barrera mucosa producida por *E. coli* enteropatógena, sino que restaura la integridad de la mucosa en células T84 y Caco-2. Este efecto está mediado por la mejora en la expresión y la redistribución de las proteínas de adhesión de las uniones estrechas (Zyrek *et al.,* 2007; Stetinova *et al.,* 2010). *Lactobacillus casei* DN-114001 y VSL#3 son capaces de mantener la función de barrera intestinal por mecanismos similares (Parassol *et al.,* 2005; Otte *et al.,* 2004).

En la prevención del daño epitelial inducido por citoquinas, característico en la enfermedad inflamatoria intestinal (Sartor, 2006), el uso de probióticos es eficaz, ya que contribuyen a la función de barrera de la mucosa. Dos péptidos secretados por *L. rhamnosus* GG, designadas p40 y p75, han demostrado recientemente prevenir la apoptosis celular inducida por citoquinas a través de la activación de la proteína quinasa B y por inhibición de la proteína quinasa pro-apoptótica p38 activada por mitógenos (Yan *et al.,* 2002; Yan *et al.,* 2007).

Las mucinas (MUC) son los principales constituyentes del moco epitelial y han sido durante mucho tiempo relacionadas con la salud y con el desarrollo de enfermedades. Los probióticos pueden promover la secreción mucosa, como un mecanismo que mejora la función de barrera y la exclusión de agentes patógenos. Varias especies de lactobacilos aumentan la expresión de mucinas en líneas celulares de intestino humano. Sin embargo, este efecto protector depende de la adhesión de los lactobacilos a la monocapa de células, algo que probablemente no se produce *in vivo* (Mack *et al.,* 2003; Mattar *et al.,* 2002). Por el contrario, hay datos que demuestran que un extracto celular de *L. acidophilus* es suficiente para aumentar la expresión de *MUC2* en células HT29 independientemente del proceso de fijación (Kim *et al.,* 2008). Estudios *in vivo*, menos consistentes ya que sólo se han realizado unos pocos, muestran resultados controvertidos. Ratones que recibieron diariamente VSL#3 durante 14 días no presentaron ningún cambio en la expresión de mucinas (Gaudier *et al.,* 2005). Por el contrario, ratas que recibieron VSL#3 en una dosis similar a la anterior durante 7 días tuvieron una expresión de *MUC2* 60 veces superior. La secreción de mucinas también aumentó (Caballero-Franco *et al.* 2007). Por consiguiente, la producción de moco puede incrementarse mediante el uso de probióticos *in vivo*, pero son necesarios más estudios para obtener conclusiones sólidas.

AUMENTO DE LA ADHESIÓN A LA MUCOSA INTESTINAL

La adhesión a la mucosa intestinal está considerada como un requisito fundamental para la colonización y para la interacción entre las cepas probióticas y el hospedador (Juntunen *et al., 2*001; Beachey *et al.,* 1981; Schiffrin *et al.,* 1997). La adhesión también es importante en la modulación del sistema inmunitario y en el antagonismo frente a patógenos (Schiffrin *et al.,* 1997; Perdigon *et al.,* 2002; Hirano *et al.,* 2003). Para que las LAB se unan a la superficie es necesaria la

interacción las células epiteliales intestinales (CEI) y el moco epitelial. Las CEI secretan mucinas que evitan la adhesión de bacterias patógenas (Collado *et al.,* 2005; González-Rodríguez *et al.,* 2012). Además, en las CEI existen lípidos, proteínas libres, inmunoglobulinas y sales (Neutra, 1987). La interacción específica existente entre las proteínas de superficie de las bacterias probióticas con las CEI puede ser la principal causa en la exclusión competitiva de los probióticos frente a distintos patógenos (Ouwehand *et al.,* 2002; Haller *et al.,* 2001; Van Tassell *et al.,* 2011).

El proceso de adhesión está mediado por proteínas, aunque hay otros factores implicados como el ácido lipoteicoico (Vélez *et al.,* 2007). El ejemplo más estudiado de adhesinas bacterianas es el de proteínas unidas al moco epitelial producidas por *L. reuteri* (Buck *et al.,* 2005; Hynönen *et al.,* 2002). Bajo ciertas circunstancias, las proteínas pueden desempeñar un papel facilitando la colonización del intestino humano a través de la degradación de la matriz extracelular de las células (Candela *et al.,* 2007; Candela *et al.,* 2009; Candela *et al.,* 2007; Guglielmetti *et al.,* 2008; Sánchez *et al.,* 2010). Probióticos tales como *L. plantarum* consiguen la inducción de las mucinas *MUC2* y *MUC3* para inhibir la adhesión de *E. coli* enteropatógena, proporcionando protección frente a la invasión de patógenos (Hirano *et al.,* 2003; Voltan *et al.,* 2007; Kim *et al.,* 2010).

Del mismo modo, Collado *et al.* (2006) evaluaron la adhesión de cepas de *B. longum* y *B. catenulatum* al moco intestinal humano y compararon los resultados obtenidos frente a cepas controles sensibles a los ácidos. Los resultados obtenidos mostraron que las cepas que tenían capacidad de resistencia al medio ácido poseían además una alta capacidad de adhesión a la mucosa intestinal. Esta característica en la adhesión no siempre es mejorada con la adquisición de la resistencia al medio ácido. En general, la inducción de resistencia a un medio ácido en bifidobacterias puede ser una estrategia de selección para cepas con mayor estabilidad y mejores propiedades probióticas (Collado *et al.,* 2006; Bermudez-Brito *et al.,* 2012).

Los probióticos pueden también modificar las mucinas intestinales para impedir la unión de patógenos (Kim *et al.,* 2010). El componente bacteriano encargado de la adhesión de cepas de *L. acidophilus* es una proteasa resistente asociada a la superficie bacteriana (Chauvière *et al.,* 1992; Coconier *et al.,* 1992; Greene y Klaenhammer, 1994). De manera singular, el componente

bacteriano también se degrada en un péptido antimicrobiano con propiedades frente a patógenos que son beneficiosas para el hospedador (Gopal *et al.,* 2001).

Las cepas probióticas además pueden inducir la liberación de defensinas por las células epiteliales. Estos péptidos/proteínas pequeños estabilizan la función de barrera del intestino (Furrie *et al.,* 2005). Los estudios realizados indican que en respuesta al ataque por bacterias patógenas, la primera línea de defensa química es el aumento en la producción de proteínas antimicrobianas (PAM), tales como α y β-defensinas, catelicidinas, lectinas tipo-C y ribonucleasas (Ayabe *et al.,* 2000; O'neil *et al.,* 1999; Takahashi *et al.,* 2001; Ogushi *et al.,* 2001; Ganz, 2003; Gallo y Hooper, 2012). Muchas PAM son enzimas que matan bacterias por un ataque enzimático en las estructuras de la pared celular. Las enzimas expresadas por las células de Paneth atacan las membranas bacterianas. La lisozima hidroliza el enlace glicosídico del peptidoglicano de la pared (Müller *et al.,* 2005) y la fosfolipasa A_2,y los fosfolípidos de la membrana (Koprivnjak *et al.,* 2002). Las defensinas constituyen una familia importante de los péptidos de membrana en los vertebrados. La interacción es no-específica y principalmente por unión a los grupos fosfolípidos aniónicos de la superficie de la membrana, a través de interacciones electrostáticas. Esta interacción crea poros en la membrana bacteriana que alteran la integridad y promueven la lisis de los microorganismos (Kagan *et al.,* 1990). Las catelicidinas son generalmente catiónicas, péptidos en hélice α que se unen a las membranas bacterianas por interacciones electrostáticas y, como las defensinas, inducen la disrupción de la membrana (Bals y Wilson, 2003).

En el proceso de adhesión microbiana de las LAB se incluyen fuerzas pasivas, interacciones electrostáticas, interacciones hidrofóbicas, fuerzas estéricas, ácidos lipoteicoicos y estructuras específicas, tales como apéndices externos cubiertos por lectinas. Una amplia variedad de moléculas que median la adhesión de bacterias patógenas han sido caracterizadas. Sin embargo, el conocimiento de qué factores median la adhesión de *Lactobacillus* es muy limitado (Abbot *et al.,* 2007; Westerlund y Korhonen, 1993; Sun *et al.,* 2012).

EXCLUSIÓN COMPETITIVA DE MICROORGANISMOS PATÓGENOS

Greenberg fue el primero en describir el término "exclusión competitiva", cuando se refirió al escenario en que especies de bacterias competían por sitios de unión frente a otras especies (Greenberg, 1969). Los mecanismos usados por las especies para la exclusión y/o la reducción en el crecimiento de otras especies es variable e incluye: creación de un microambiente hostil, disminución de los sitios de unión disponibles, producción y secreción de sustancias antimicrobianas, así como la disminución de los nutrientes esenciales para el crecimiento (Rolfe, 1991).

Las propiedades de adhesión específicas se deben a la interacción entre las proteínas de superficie y las mucinas. Lactobacilos y bifidobacterias inhiben un amplio rango de patógenos, en los que se pueden incluir *E. coli*, *Salmonella*, *Helicobacter pylori, Listeria monocytogenes* y *Rotavirus* (Chenoll *et al.,* 2011; Sgouras *et al.,* 2004; Todoriki *et al.,* 2001; Chu *et al.,* 2005; Tsai *et al.,* 2008; Muñoz *et al.,* 2011; Nakamura *et al.,* 2012). La exclusión se produce debido a diversos mecanismos y propiedades de los probióticos para inhibir la adhesión de patógenos, que incluyen la producción de PAM y la estimulación de CEI. La exclusión competitiva por bacterias intestinales se basa en la interacción bacteria-bacteria mediada por la competencia por los nutrientes disponibles y la adhesión a los sitios de la mucosa. Para obtener ventaja, las bacterias pueden modificar el ambiente para que sea menos adecuado para sus competidores. La producción de PAM es un ejemplo de la modificación del ambiente (Schiffrin *et al.,* 2002).

Algunos lactobacilos y bifidobacterias comparten características en la unión de hidratos de carbono con algunos enteropatógenos (Nesser *et al.,* 2000; Fujiwara *et al.,* 2001), lo que hace posible que las cepas compitan con patógenos específicos por los sitios de unión (Mukai *et al.,* 2002). En general, las cepas probióticas son capaces de inhibir la unión de bacterias patógenas por medio del impedimento estérico en los receptores de patógenos de los enterocitos (Coconier *et al.,* 1993). El efecto de las bacterias probióticas en la exclusión competitiva de patógenos se ha demostrado usando modelos de mucosa humana *in vitro* (Hirano *et al.,* 2003; Tuomola *et al.,* 1999), además de modelos a base de mucosas animales (Hirn *et al.,* 1992; Genovese *et al.,* 2000). Hirano *et al*. demostraron que *L. rhamnosus*, una cepa con mucha capacidad de adhesión, es apta para

inhibir la internalización de *E. coli* enterohemorrágica en una línea celular intestinal humana C2BBe1 (Hirano *et al.,* 2003).

PRODUCCIÓN DE SUSTANCIAS ANTIMICROBIANAS

Unos de los mecanismos propuestos por el cual los probióticos ejercen beneficios en la salud, es la formación de compuestos de bajo peso molecular, tales como ácidos orgánicos y PAM conocidos como bacteriocinas.

Los ácidos orgánicos son considerados el principal componente antimicrobiano capaz de inhibir la actividad de los patógenos; en particular los ácidos acético y láctico tienen un efecto inhibitorio frente a bacterias Gram negativo (Alakomi *et al.,* 2000; De Keersmaecker *et al.,* 2006; Makras *et al.,* 2006). La forma no disociada de los ácidos orgánicos entra en la bacteria y se disocia dentro del citoplasma, ocurriendo una disminución del pH o una acumulación intracelular de las formas ionizadas de los ácidos orgánicos que pueden conducir a la muerte del patógeno (Ouwehand, 1998; Russel y Diez-Gonzalez, 1998). Las bacterias intestinales producen una gran variedad de ácidos grasos que promueven la salud. De hecho, ciertas cepas de bifidobacterias intestinales y lactobacilos han demostrado producir ácido linoleico conjugado (ALC), potente agente anticancerígeno (O'Shea *et al.,* 2012; Macouzet *et al.,* 2009). Recientemente, se ha demostrado la capacidad de modular la composición de los ácidos grasos del hígado y del tejido adiposo del hospedador tras la administración oral de bifidobacterias y lactobacilos productoras de ALC en ratones (O'Shea *et al.,* 2012, Lee *et al*., 2007). Por último, las bacterias probióticas son capaces de producir derivados de las sales biliares, que tienen actividad antimicrobiana en comparación con las sales biliares sintetizados por el organismo. Sin embargo, queda por dilucidar cómo los probióticos se protegen a sí mismos de los distintos metabolitos con actividad microbiana que producen (Oelschlaeger, 2010).

Con respecto a la producción de bacteriocinas, hay que destacar que varios estudios han puesto de manifiesto que la producción de las mismas confiere a las cepas productoras una ventaja competitiva dentro de entornos microbianos complejos, como consecuencia de su actividad antimicrobiana asociada. La producción de bacteriocinas puede permitir el establecimiento del

microorganismo y aumentar la prevalencia de las cepas productoras, así como permitir la inhibición directa en la proliferación de patógenos dentro del TGI (O'Shea *et al.,* 2012).

Muchas LAB generan bacteriocinas con actividad sólo contra bacterias estrechamente relacionadas, pero existen algunas que incluso son activas frente a patógenos transmitidos por los alimentos (Nielsen *et al.,* 2010). Los mecanismos comunes de acción de las bacteriocinas incluyen la destrucción mediante la formación de poros y/o inhibición de la síntesis de la pared celular (Hassan *et al.,* 2012). Por ejemplo, la nisina forma un complejo con el último precursor de la pared celular, el lípido dos, inhibiendo de este modo la biosíntesis de la pared celular y posteriormente formando un poro en la membrana bacteriana (Bierbaum y Sahl, 2009).

Algunos compuestos antibacterianos específicos han sido descritos para cepas de bifidobacterias y, dentro de ellos, sólo una bacteriocina (bifidocina B), producida por *B. bifidum* NCFB 1454, es activa frente a bacterias Gram positivas (Makras *et al.,* 2006; Yildirim *et al.,* 1999). Liévin *et al*. describieron una gran actividad de eliminación de dos cepas de *Bifidobacterium* contra varias bacterias patógenas, como *Salmonella enterica* ser. *typhimurium* SL1344 y *E. coli* C1845. Dicha actividad, fue atribuida a la producción de una molécula lipofílica de bajo peso molecular (Liévin *et al.,* 2000; Gibson y Wang, 1994).

PROBIÓTICOS Y SISTEMA INMUNITARIO

Los TLRs son proteínas transmembrana expresadas en diversas células, tanto inmunitarias como no, entre las que se encuentran las células B, células *natural killer*, células dendríticas, macrófagos, fibroblastos, células epiteliales y células endoteliales. En los mamíferos, la familia de TLRs incluye 11 proteínas (TLR1-TLR11). En los seres humanos, TLR1, TLR2, TLR4, TLR5, TLR6 y TLR10 se encuentran en la membrana plasmática asociados principalmente a la respuesta de patrones moleculares asociados a patógenos (PAMP). Los receptores TLR3, TLR7, TLR8 y TLR9 se encuentran en la superficie de los endosomas, respondiendo a los PAMP de virus y bacterias (Figura 6) (Gómez-Llorente *et al.,* 2010).

La vía de señalización de los TLRs, a excepción del receptor TLR3, implica el reclutamiento de MyD88 (proteína de respuesta primaria de diferenciación mieloide), que activa las vías de MAPK (*mitogen-activated protein kinase*) y la del factor nuclear (NF)-kappa beta (κβ) (Lebeer *et al.,* 2010; Kawai y Akira, 2010; Wells, 2011). El receptor TLR3 utiliza el TRIF (adaptador que contiene el dominio TIR —receptor *toll*/interleuquina (IL)-1— que induce interferón β), lo que lleva a la expresión de interferón tipo I (Wells, 2011). La señalización mediada por TLRs ha demostrado controlar la maduración de las células dendríticas induciendo la sobreexpresión de varios marcadores de maduración, tales como CD80, CD83 y CD86, así como el receptor de quimioquinas CCR7.

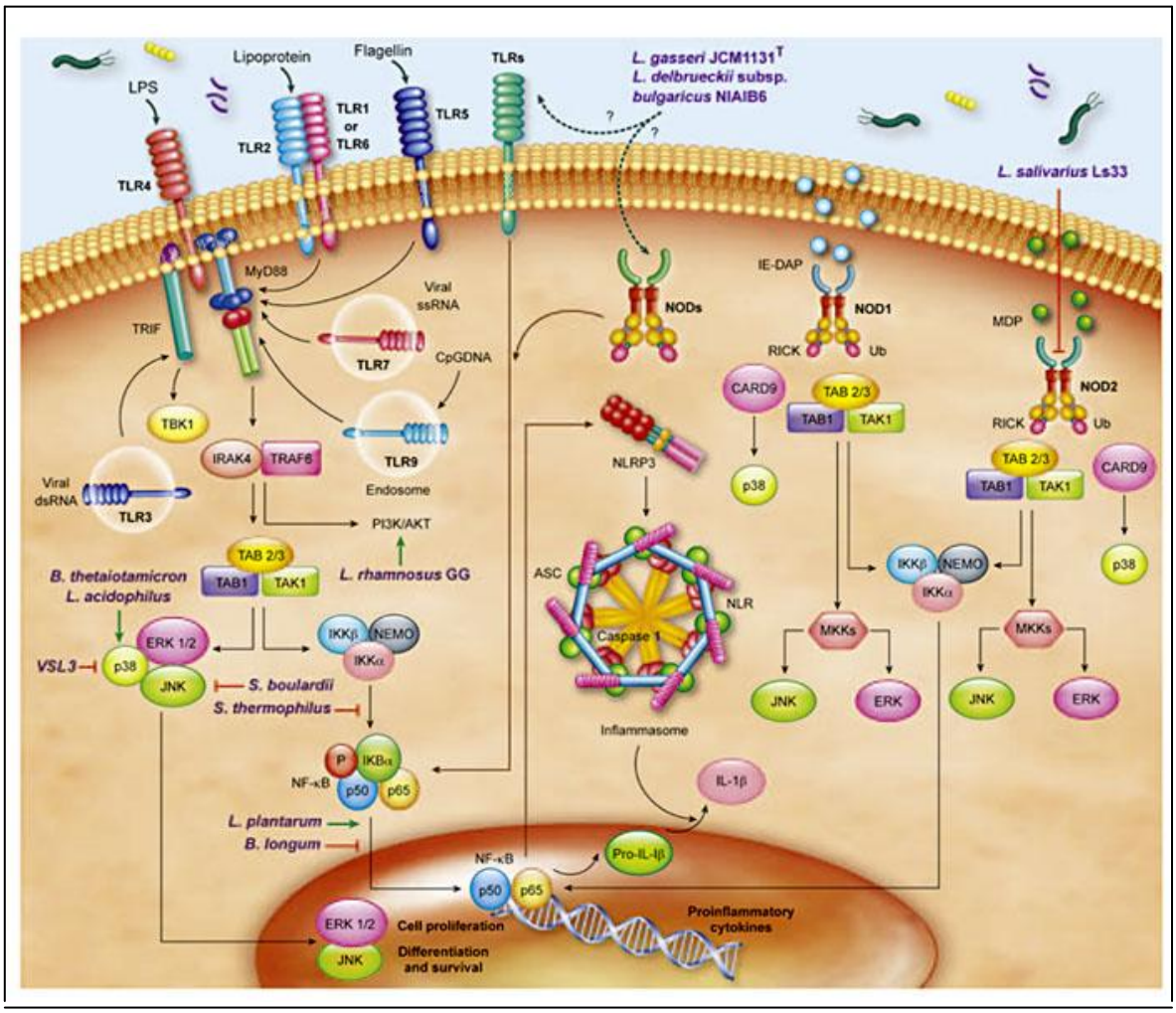


FIGURA 6. Interacción de los probióticos con el sistema inmunitario en el intestino (Bermudez-Brito *et al.,* 2012).

Por otra parte, los microorganismos comensales y los probióticos pueden crear un estado de tolerancia general mediado por la acción de TLRs en las células dendríticas. La señalización del receptor TLR9 es esencial en la intervención de la acción antiinflamatoria producida por los

probióticos. Sin embargo, otros estudios han implicado a otros TLRs, tales como TLR3 y TLR7, en la tolerancia inducida por bacterias comensales y probióticas (Gómez-Llorente *et al.*, 2010).

Después de la activación producida por los microorganismos comensales y probióticos, las células dendríticas inician una respuesta adecuada, como la diferenciación de células Th_0 a T_{reg}, que tiene un efecto inhibidor sobre las respuestas inflamatorias Th1, Th2 y Th17. Está bien establecido que los probióticos pueden suprimir la inflamación intestinal a través de la regulación a la baja de la expresión de TLRs, la secreción de metabolitos que pueden inhibir al TNF-α y la inhibición de la señalización de NF-κβ en los enterocitos (Gómez-Llorente *et al.,* 2011).

El receptor TLR2 reconoce al peptidoglicano, principal componente de las bacterias Gram positivas, entre las que se incluyen los géneros *Lactobacillus*. Varios estudios han demostrado que TLR2 es necesario para que algunas cepas de *Lactobacillus* puedan ejercer sus efectos inmunomoduladores. Vinderola *et al*. (2005) demostraron que *L. casei* CRL 431 interactúa con las CEI a través de TLR2, y que esta interacción induce un incremento en el número de receptores CD-206 y TLR2, principalmente en las células implicadas en la respuesta inmunitaria innata. Además, los componentes de la pared celular de los lactobacilos pueden potenciar la señal de unión de TLR2 en combinación con TLR6. Los anclajes de la membrana diacilados de lipoproteínas y ácidos lipoteicoicos se unen a TLR2 y TLR6, promoviendo así la dimerización y activación mediada por MyD88, de la vía canónica de NF-κβ (Wells, 2011). La estimulación de TLR2 aumenta la producción de citoquinas y su activación tiene un papel importante en la mejora de la resistencia transepitelial a las bacterias invasoras (Vizoso Pinto *et al.,* 2009).

En este sentido, Shida *et al*. mostraron que *L. casei* induce altos niveles de IL-12, tanto en macrófagos normales como en deficientes de TLR2, y que el peptidoglicano induce bajos niveles de IL-12 en macrófagos normales e incluso más bajos en los deficientes de TLR2. Por lo tanto, los autores sugirieron que el peptidoglicano intacto de los lactobacilos en realidad actúa vía TLR2 inhibiendo la producción de IL-12. Aunque, el reconocimiento por TLR2 es esencial, sólo del 12 al 48% de la producción de IL-12 en los macrófagos deficientes de TLR2 fue inhibida por el peptidoglicano, lo que sugiere que otros mecanismos independientes de TLR2 pueden estar implicados (Shida *et al.,* 2009).

Igualmente, se ha demostrado que cepas de *L. rhamnosus* GG y *L. plantarum* BFE 1685 aumentan la expresión de TLR2 en experimentos con células intestinales humanas. Más recientemente, *L. casei* CRL431 ha demostrado ejercer efectos similares en ratones sanos y ratones infectados con *S. enterica* serovar *typhimurium* (Abreu *et al.,* 2005; Castillo *et al.,* 2011). La administración de probióticos a ratones sanos produjo un aumento en la expresión de TLR2, TLR4 y TLR9 y mejora la secreción de TNF-α, IFN-γ e IL-10 en las placas de Peyer (Castillo *et al.,* 2011).

TLR2 también tiene un papel importante en el reconocimiento de las bifidobacterias. Hoarau *et al*. han descrito que el producto de fermentación por *B. breve* C50 puede inducir la maduración y producción de IL-10, así como prolongar la supervivencia de las células dendríticas vía TLR2 (Hoarau *et al.,* 2006). Del mismo modo, Zeuthen *et al*. señalaron que células dendríticas sin el gen *TLR2* producen más IL-2 y menos IL-10 en respuesta a bifidobacterias, concluyendo que los efectos inmuno-inhibitorios dependen de TLR2 (Zeuthen *et al.,* 2008).

Con respecto al receptor de TLR4 se ha observado que tiene un papel en la defensa del hospedador frente a las infecciones *in vivo* de *Salmonella.* En ratones sanos, *L. casei* CRL 431 activa este receptor y es capaz de utilizarlo como mecanismo de supervivencia frente a bacterias patógenas (Castillo *et al.,* 2011). La activación de TLR4 induce mediadores pro-inflamatorios, un aumento en la expresión de TLR2 y una reducción en su propia expresión, que cursa con un reclutamiento de células inflamatorias y con el inicio de una respuesta apropiada en el bazo (Castillo *et al.,* 2011; Weiss *et al.,* 2004; Totemeyer *et al.,* 2003).

Asimismo, cepas de *L. rhamnosus* GG, *L. delbrueckii* subsp. *bulgaricus* inactivadas por calor pueden disminuir la expresión de TLR4, de manera similar a la del lipopolisacárido (LPS), en células dendríticas derivadas de monocitos humanos tras 12 h de incubación. Éstas cepas probióticas pueden alterar la respuesta inmunitaria a nivel post-transcripcional por modificación de la expresión del microARN (Giahi *et al.,* 2012).

Otro TLR relevante es el TLR9, que reconoce ADN bacteriano CpG. Fragmentos de ADN no metilado que contiene CpG son liberados por probióticos *in vivo* teniendo un efecto anti-inflamatorio potencial, a través de la señalización de TLR9 en la superficie. Se sabe que las especies

de *Lactobacillus* difieren en su composición G+C. Por lo tanto, la capacidad de distintas especies de estimular TLR9 puede ser diferente (Wells, 2011; Hemmi *et al.,* 2000). La estimulación de TLR9 a través de las superficies apical y basolateral activa diferentes vías de señalización intracelular en células epiteliales polarizadas. Mientras el TLR9 basolateral activa la degradación de Iκβα y la activación de la vía de NF-κβ, el TLR9 apical induce una acumulación citoplasmática de Iκβ ubiquitinada e inhibe a NF-κβ (Lee *et al.,* 2006). Cepas de *B. breve* (NumRes 204), *L. rhamnosus* (NumRes 1) y *L. casei* (DN-114001) inducen distintos niveles de producción de citoquinas en células inmunitarias primarias de humanos y ratones. Se ha demostrado que la cepa de *B. breve* genera niveles mucho más bajos de IFN-γ que *L. rhamnosus* y *L. casei*. Por otra parte, la cepa de *B. breve* y lactobacilos estimulan citoquinas de manera independiente de TLR9 y disminuyen la inflamación por efectos inhibitorios de TLR2 (Plantiga *et al.,* 2011).

NLRs Y PROBIÓTICOS

Existe otra familia de receptores, los NLRs. Se encuentran localizados en el citoplasma y son importantes en los tejidos donde TLRs se expresan en niveles muy bajos. Los miembros más caracterizados son NOD1 y NOD2, pero actualmente hay más de 20 NLRs (Hakansson y Molin, 2011). NOD1 se expresa de forma ubicua. NOD2 se expresa en macrófagos, células de Paneth, células dendríticas, células intestinales, células de pulmón y células epiteliales orales, y en bajos niveles en células T. El receptor NOD1 puede detectar restos de peptidoglicano, asociado principalmente a la identificación de bacterias Gram positivas; y NOD2 puede detectar motivos dipeptídicos de ácido murámico, lo que permite el reconocimiento de un amplio rango de bacterias (Biswas *et al.,* 2012). Después del reconocimiento de su agonista, tanto NOD1 como NOD2 se oligomerizan para reclutar y activar la proteína adaptadora RICK, una proteín-quinasa que regula la apoptosis mediada por CD95, esencial en la activación de MAPK y NF-κβ, aumentando la transcripción y producción de mediadores inflamatorios, entre ellos citoquinas, quimioatractantes, ciclooxigenasa-2 (COX-2) y la enzima oxido nítrico sintasa inducible (Chen *et al.,* 2009).

Hay algunos estudios que demuestran el efecto de los probióticos en los NLRs. Fernandez *et al*. señalaron recientemente que la capacidad protectora de *L. salivarius* Ls33 se correlaciona con la producción local de IL-10, que fue neutralizada en ratones deficientes de NOD2. De hecho, estos

autores establecen que el efecto anti-inflamatorio de Ls33 está mediado por NOD2 (Fernandez *et al.,* 2011).

Otra importante vía activada por los NLRs implica la escisión de pro-IL-1β y pro-IL-18 en sus formas maduras y activas. Los NLRs participan en la formación del inflamasoma, lo que conduce a la activación de la caspasa-1. Existen tres inflamasomas principales: NLRP1, NLRP3 y NLRC4. El NLRP3 detecta LPS, muramildipéptidos, ARN bacteriano y viral (Chen *et al.,* 2009). Existen dos pasos fundamentales para la completa activación del inflamasoma NLRP3, uno necesario para inducir la transcripción del ARN mensajero de NLRP3 y otro en el que se reconozcan los PAMP para la completa expresión de NLRP3 (Bauernfeind *et al.,* 2009; Bauernfeind *et al.,* 2010).

Se ha sugerido que NLRP3 tiene un papel importante en la regulación de la inflamación intestinal en adultos, como en el caso de la enfermedad de Crohn (Hirota *et al.,* 2011). A su vez, una alteración en la expresión de NLRP3 resulta en una interrupción en la homeostasis inmunitaria asociada a enfermedades auto-inflamatorias en humanos (Anderson *et al.,* 2008). Debido a que el nivel de expresión de NLRP3 es bajo en las células inmunitarias, la inducción de la expresión de NLRP3 es un primer paso para la activación apropiada del NLRP3 con el fin de responder al estímulo producido por los PAMP (Bauernfeind *et al.,* 2009; Bauernfeind *et al.,* 2010; Meylan *et al.,* 2006; Martinon *et al.,* 2009).

MODELOS EXPERIMENTALES PARA EL ESTUDIO DE PROBIÓTICOS

La barrera entre el lumen intestinal y el tejido conectivo del hospedador está formada por las CEI. Sin embargo, estudios recientes han demostrado que también se encuentran involucrados en procesos inmunológicos que discriminan entre patógenos y bacterias comensales. Además las CEI secretan una amplia gama de PAM, mencionados anteriormente. Las CEI interactúan con células presentadoras de antígenos, siendo la mayoría de ellas células dendríticas y macrófagos asociados a tejido linfoide. Este tipo de células es capaz de polarizar células T *naive* y producir inmunotolerancia o una respuesta inflamatoria.

La interacción entre CEI, células dendríticas, macrófagos, bacterias comensales y/o patógenos estimula una secreción diferencial de citoquinas dependiendo de la situación presente. La presencia de bacterias comensales y probióticas hace que las CEI liberen IL-10; en cambio, la existencia de bacterias patógenas genera citoquinas pro-inflamatorias (IL-8 y TNF-α). Si pensamos en las situaciones descritas anteriormente (interacción entre las distintas células), el resultado global podría estar determinado por citoquinas secretadas por las CEI y células dendríticas, que estimularían a su vez a células inmaduras y macrófagos, aumentando los niveles de tales citoquinas en respuesta (Th1, Th2, Th17 o T_{reg}) a la situación del medio. Estudiar en su conjunto tal compleja red de células y citoquinas secretadas es difícil de imitar *in vitro.* Por ello, los modelos experimentales que se han desarrollado implican CEI y células dendríticas generadas a partir de monocitos o segmentos de intestino de rata o humano (Uematsu *et al.,* 2008; Ng *et al.,* 2011; Smits *et al.,* 2005), y células T aisladas de células mononucleares de sangre periférica (Smits *et al.,* 2005). Dichos modelos pueden incluir sólo un tipo de células, o bien más de uno (co-cultivo de varios tipos de células en un esfuerzo por imitar el tejido intestinal), o un cultivo de explantes intestinales (Jarry *et al.,* 2011). Usualmente, los modelos permiten determinar la diferencia de secreción de citoquinas, o distinguir entre diferentes porciones de células T y dendríticas, usando fenotipos de superficies celulares y/o factores de transcripción que son específicos para cada tipo celular (Figura 7).

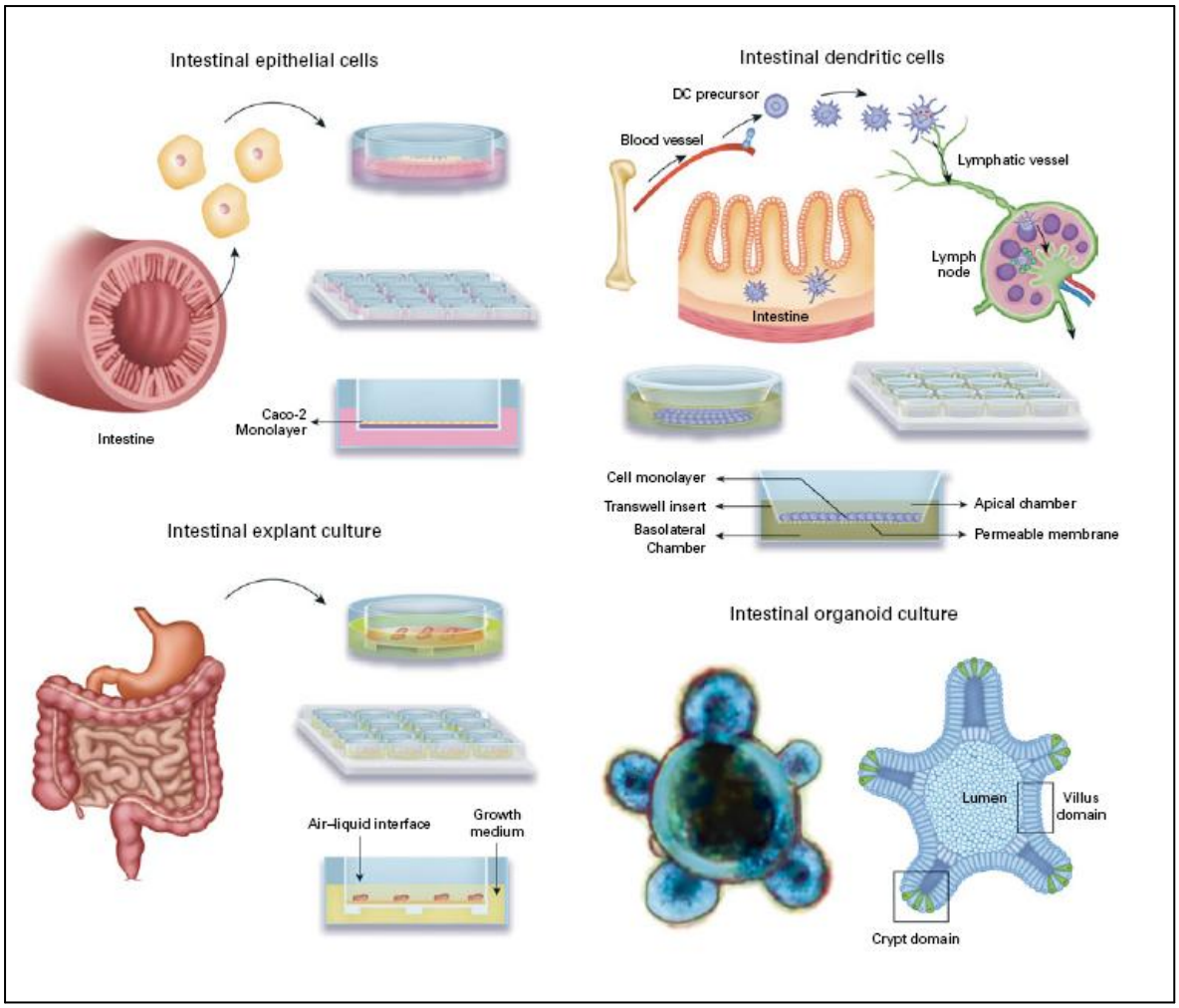


FIGURA 7. Modelos experimentales para el estudio de las interacciones entre el hospedador y los microbios (Bermudez-Brito *et al.,* 2013).

En apartados anteriores hemos descrito modelos celulares con CEI para el estudio de probióticos. Profundizaremos en este apartado en los modelos con células dendríticas, macrófagos, explantes, organoides y animales.

CÉLULAS DENDRÍTICAS

Las células dendríticas pueden diferenciarse al menos en cuatro líneas que abarcan las células de Langerhans, células dendríticas mieloides (CDM), células dendríticas linfoides y células dendríticas plasmocitoides (Wu y Liu, 2007). Estas células juegan un papel crítico en la organización de la respuesta inmunitaria adaptativa que se produce por tolerancia e inmunidad (Cools *et al.,* 2007; Quah y O'neill, 2005; Steinman *et al.,* 2003). Las células dendríticas inmaduras residen en

tejidos periféricos, tales como la mucosa intestinal, y monitorizan el microambiente presente en busca de PAMP. Posteriormente, liberan quimioquinas y citoquinas que amplifican la respuesta inmunitaria (Banchereau y Steinman, 1998; Sabatté *et al.,* 2007).

Los probióticos ejercen efectos estimulantes diferentes en células dendríticas *in vitro*, desarrollando niveles de producción muy variables de citoquinas, y funciones efectoras diferentes (Christensen *et al.,* 2002; Zeuthen *et al.,* 2008; Fink *et al.,* 2007). Algunas cepas modulan la producción de citoquinas en células dendríticas *in vitro* e inducen respuesta antiinflamatorias, mientras otras inducen una respuesta pro-inflamatoria (Evrard *et al.,* 2011). Este efecto dependiente de la cepa resulta de la interacción específica entre la bacteria y el PAMP. Braat. *et al.* (2004) propusieron que *L. rhamnosus* modula la función de las células dendríticas para inducir una nueva forma de respuesta de células T menos reactiva, un mecanismo por el que se podrían explicar los efectos beneficiosos observados por la administración de probióticos en algunas enfermedades clínicas. El análisis de células dendríticas inmaduras derivadas de médula ósea muestra que todas las cepas aumentan la expresión del marcador de superficie CD86, que es indicativo de la maduración de las células dendríticas. Sin embargo, las distintas cepas varían en la intensidad de la expresión inducida de CD86. Ninguna cepa indujo apreciablemente los niveles de IL-10 e IL-12 en células dendríticas inmaduras derivadas de médula ósea, mientras que la expresión de TNF-α fue inducida en particular por *L. paracasei* y *L. fermentum* (D'Arienzo *et al.,* 2009).

Mohamadzadeh *et al*. (2005) investigaron tres especies de *Lactobacillus* y encontraron que modularon el fenotipo y la función de CDM humanas. Las CDM expuestas a *Lactobacillus* aumentaban la expresión de antígenos de leucocitos, CD83, CD40, CD80 y CD86 y altos niveles de secreción de IL-12 e IL-18, pero no de IL-10. La IL-12 fue persistente en las CDM expuestas a las tres especies de *Lactobacillus* en presencia de LPS de *E. coli*, mientras la inducción de IL-10 por LPS estuvo inhibida. La activación de CDM con lactobacilos produjo un cambio de células T $CD4^+$ y $CD8^+$ a Th1 y polarización de Tc1, como evidenció la secreción de IFN-γ, pero no de IL-4 o IL-13.

L. reuteri y *L. casei,* pero no *L. plantarum*, dieron lugar a que las células dendríticas derivadas de monocitos desarrollaran células T_{reg}. Las células T_{reg} producen niveles elevados de IL-10 y son capaces de inhibir la proliferación de células Th. *Lactobacillus reuteri* y *L. casei* se unen a la

molécula de adhesión intercelular denominada DCSIGN. Se observó que anticuerpos que bloquean DCSIGN inhiben la inducción de las células T_{reg} causada por estas bacterias probióticas.

Weiss *et al*. (2011) demostraron que los lactobacilos pueden ser divididos en dos grupos de bacterias que tienen efectos contrarios y que las bifidobacterias exhiben efectos uniformes. En conclusión, las LAB inician "interacciones" por una vía de maduración de las células dendríticas. Éstas cepas de LAB pueden representar herramientas útiles para modular el equilibrio de citoquinas e impulsar potentes respuestas inmunitarias tipo-1 y/o prevenir la desregulación inmunitaria asociada con polarizaciones específicas de células T (Fink *et al.,* 2007).

MACRÓFAGOS

Los lactobacilos han demostrado activar monocitos y macrófagos, que juegan un papel significativo en la presentación de antígenos, la activación de la inmunidad por anticuerpos específicos y la estimulación de IgA. En particular, éstas células son esenciales en la respuesta inmunitaria de tipo 1 (con células efectoras citotóxicas) o respuestas de tipo 2 (caracterizadas por la producción de anticuerpos). La respuesta de tipo 2 se relaciona con la secreción de IL-4, IL-5, IL-9 e IL-13, que promueve la inducción de IgE y de respuestas alérgicas. Por incubación de suspensiones bacterianas con células THP-1 tipo macrófagos, Drago *et al.* (2010) analizaron cuatro cepas de *L. salivarius* (LDR0723, CRL1528, BNL1059 y RGS1746) por su habilidad en la modulación de las citoquinas pro y anti-inflamatorias. LDR0723 y CRL1528 produjeron un incremento persistente en la producción de IL-12 e IFN-γ y disminuyeron la liberación de IL-4 e IL-5. En cambio, BNL1059 y RGS1746 favorecieron la respuesta Th2, produciendo una disminución de la proporción Th1/Th2 con respecto a células no estimuladas. Ivec *et al.* (2007) mostraron que las bacterias probióticas, sin importar si fueran lactobacilos o bifidobacterias, tenían la habilidad de disminuir la infección viral mediante un estado antiviral en los macrófagos, a través de la producción de óxido nítrico y de citoquinas inflamatorias, tales como IL-6 e IFN-γ.

EXPLANTES DE TEJIDO Y PROBIÓTICOS

El número de estudios en los que se utilizan explantes y probióticos es muy limitado. La gran mayoría de ellos se centra en el estudio de enfermedades intestinales, particularmente de la enfermedad de Crohn. Utilizando un modelo de cultivo con mucosa intestinal y cepas bacterianas seleccionadas, Carol *et al.* (2006) observaron una disminución en la actividad de linfocitos T y de la secreción de TNF-α en la mucosa inflamada de pacientes con la enfermedad de Crohn. *L. casei* puede restaurar la homeostasis inmunitaria en la mucosa ileal inflamada de esos pacientes, favoreciendo la apoptosis de linfocitos T. Además, Carol *et al.* (2006) también señalaron que ciertos lactobacilos, tales como *L. casei* DN-11 401 y *L. bulgaricus* LB10, podueden disminuir la respuesta inflamatoria cuando se exponen a la mucosa inflamada en el modelo celular (Carol *et al.,* 2006; Borruel *et al.,* 2002; Borruel *et al.,* 2003). Estos autores concluyeron que los probióticos interactúan con células inmunocompetentes, a través de la interfaz de la mucosa y la modulación local en la producción de citoquinas pro-inflamatorias. Recientemente, Mencarelli *et al.* (2011) cultivaron explantes de grasa abdominal de cinco pacientes con enfermedad de Crohn y cinco pacientes con cáncer de colon con VSL#3. Estos autores encontraron que la exposición de los tejidos a VSL#3, condicionaba la liberación de leptina.

Dicho grupo trató también explantes de colon de ratón con proteínas purificadas de *L. rhamnosus* GG en ausencia o presencia de TNF-α (Mencarelli *et al.*, 2011). Dos proteínas purificadas denominadas p75 y p40 activaron la proteína quinasa B, inhibiendo la apoptosis de células epiteliales inducida por citoquinas y promoviendo el crecimiento celular en explantes celulares de colon de ratón. Adicionalmente, el daño epitelial del colon inducido por TNF-α se redujo de manera significativa. Estos hallazgos sugieren que los componentes bacterianos de los probióticos pueden ser útiles en la prevención de las enfermedades gastrointestinal mediadas por citoquinas (Yan y Polk, 2007).

ORGANOIDES

Intentos para conseguir la arquitectura del tejido intestinal *in vitro* incluyen la disgregación de subunidades del intestino de ratas adultas, denominados actualmente como "organoides", para

extraer células madre adultas y generar de manera espontánea tejido intestinal de cuerpos embrioides (Howell y Wells, 2011). Recientemente, se ha descrito que células madres pluripotenciales (CMP) humanas son capaces de diferenciarse directamente en tejido intestinal *in vitro* (Spence *et al.,* 2011).

Las CMP ofrecen oportunidades prometedoras para generar tejido intestinal que pueda aprovecharse en enfermedades intestinales (Howell y Wells, 2011). Por ejemplo, CMP humanas se han diferenciado en un cultivo en monocapa de hepatocitos de hígado y células endocrinas pancreáticas (Cai *et al.,* 2007; D'Amour *et al.,* 2005; Song *et al.,* 2009; Zhang *et al.,* 2009), con eficacia terapéutica en modelos animales de enfermedad hepática (Zhang *et al.,* 2009; Basma *et al.,* 2009; Touboul *et al.,* 2009) y diabetes (Kroon *et al.,* 2008). Varios autores han diferenciado CMP de ratones y humanas hasta conseguir tejido intestinal. Las estructuras en tres dimensiones resultantes consisten en epitelios tipo columna polarizados que siguen un modelo estructural de vellosidad intestinal y de cripta, con zonas de proliferación que expresan marcadores de células madres intestinales. Los epitelios contienen un número normal de células madre Lgr5 positivas, células de Paneth y del dominio que forman la cripta, y líneas celulares diferenciados (enterocitos y células enteroendocrinas) en el dominio de la vellosidad intestinal (Koo *et al.,* 2011). Éste tejido intestinal es funcional, pudiendo secretar mucinas (Spence *et al.,* 2011; McCracken *et al.,* 2011).

El modelo de los organoides se basa en el uso de factores de crecimiento y Matrigel®, es un sistema de cultivo bien establecido que mantiene características *in vivo*, tales como la cinética de auto-renovación (Sato *et al.,* 2011). Sin embargo, a pesar de tener muchas ventajas, este sistema tiene ciertas limitaciones. Por ejemplo, los organoides intestinales tienen muchos menos componentes que el intestino *in vivo y* dentro de ellos no podemos encontrar el sistema nervioso entérico y el sistema vascular, linfático e inmunitario. Mientras la mayoría de los tipos celulares son generados en proporciones similares a los hallados *in vivo*, la arquitectura de los organoides no es regular, variando las criptas de un organoide a otro (McCracken *et al.,* 2011). A pesar de las desventajas detalladas, el sistema posee una utilidad extraordinaria para entender y reproducir el desarrollo, homeostasis y enfermedad del intestino humano.

PROBIÓTICOS Y ANIMALES

Los efectos inmunomoduladores de los probióticos han sido demostrados en modelos experimentales de alergia, enfermedades autoinmunes y enfermedades inflamatorias intestinales (Borchers *et al.,* 2009). La administración de probióticos ha demostrado efectos protectores en la colitis espontánea e inducida químicamente, a través de la regulación negativa en la producción de citoquinas inflamatorias y/o la inducción de mecanismos de regulación de una manera específica para cada cepa. En modelos animales (de sensibilización a alérgenos, de asma y rinitis alérgica en ratón), la administración oral de probióticos causó una disminución en la producción de IgE a través de la modulación en la producción sistémica de citoquinas. Algunos probióticos han disminuido la hipersensibilidad de las vías respiratorias y la inflamación a través de la inducción de la mecanismos de regulación (Fontana *et al.,* 2013). Como hemos descrito, los probióticos pueden modular tanto la microbiota intestinal como el sistema inmunitario asociado a las mucosas y, por tanto, al sistema inmunitario sistémico. Se ha observado que la administración con *L. acidophilus* y *L. casei* aumenta la eficacia del *dahi* (yogur griego) en la supresión de la diabetes inducida por estreptotozina en ratas, previniendo la dislipidemia e inhibiendo la peroxidación lipídica y la formación de nitritos (Yadav *et al*., 2008). En ratones *ob/ob*, obesos y diabéticos, la administración de la mezcla probiótica VSL#3 disminuyó los niveles de lípidos hepáticos y la actividad de la alanina-aminotransferasa, lo que indica un efecto protector frente a la esteatohepatitis asociada a la obesidad (Li *et al.,* 2003). Estos beneficios se asociaron a una normalización de la β-oxidación y a la reducción de la actividad del factor proinflamatorio NF-κB y de la expresión de la proteína desacoplante-2 (UCP-2). Asimismo, en el modelo de obesidad, resistencia insulínica y esteatosis hepática provocada por una dieta elevada en grasa también se ha demostrado la eficacia de VSL#3, siendo el efecto mediado por las células NKT (Ma *et al.,* 2008).

En el presente estudio, hemos utilizado ratas con una mutación del receptor de la leptina *Lepr*^{fa} descrito en 1961 en un stock de ratas de Lois y Theodroe Zucker, estableciéndose como modelo genético de obesidad en 1991. Dichas ratas reciben el nombre de ratas Zucker y se trata de un modelo de obesidad muy bien caracterizado, pues presenta las complicaciones metabólicas típicas del obeso humano: resistencia insulínica, dislipidemia y diabetes tipo 2.

British Journal of Nutrition (2013), **109**, S63–S69
doi:10.1017/S0007114512005600

Competitive inhibition of three novel bacteria isolated from faeces of breast milk-fed infants against selected enteropathogens

Sergio Muñoz-Quezada[1], Miriam Bermudez-Brito[1], Empar Chenoll[2], Salvador Genovés[2], Carolina Gomez-Llorente[1], Julio Plaza-Diaz[1], Esther Matencio[3], María José Bernal[3], Fernando Romero[3], Daniel Ramón[2] and Angel Gil[1]*

[1]*Department of Biochemistry and Molecular Biology II, Institute of Nutrition and Food Technology "José Mataix", Biomedical Research Centre, University of Granada, Avenida del Conocimiento s/n, 18100 Armilla, Granada, Spain*
[2]*Department of Food Biotechnology, Biópolis S.L., Parc Científic Universitat de València, C/Catedrático Agustín Escardino 9, Edificio 2, 46980 Paterna, Valencia, Spain*
[3]*Hero Global Technology Centre For Infant Nutrition, Hero Group, Avenida Murcia 1, 30820-Alcantarilla, Murcia, Spain*

Abstract

Numerous *in vitro* and *in vivo* studies conducted using different probiotic micro-organisms have demonstrated their ability to interfere with the growth and virulence of a variety of enteropathogens. The reported beneficial effects of the use of probiotics to complement antibiotic therapy or prevent diarrhoea or gastrointestinal infection in infants have increased in recent years. In the present study, we demonstrated the capacity of supernatants obtained from three novel probiotics (*Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036) isolated from the faeces of breastfed infants to inhibit the growth of enterotoxigenic and enteropathogenic (EPEC) bacteria, such as *Escherichia coli*, *Salmonella* and *Shigella*. To assess their potential antimicrobial activity, the 17 and 24 h cell-free supernatants broth concentrates (10×) having 1, 2 or 4% of the three probiotics were incubated with EPEC bacteria strains. After 17 h of co-culture, the supernatants were able to inhibit the growth of *E. coli*, *Salmonella* and *Shigella* up to 40, 55 and 81%, respectively. However, the inhibitory capacity of some supernatants was maintained or completely lost when the supernatants (pH 3·0) were neutralised (pH 6·5). Overall, these results demonstrated that *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036 produce compounds that exhibited strain-specific inhibition of enterobacteria and have the potential to be used as probiotics in functional foods.

Key words: **Bacterial competition; Bifidobacterium breve; enteropathogens; Lactobacillus paracasei; Lactobacillus rhamnosus; probiotics.**

Probiotics are defined as living micro-organisms that confer a health benefit to the host when administered in adequate amounts[1]. One of the most frequent health claims for probiotics concerns the putative reduction and prevention of infectious disease in the gastrointestinal tract. The regular intake of probiotic micro-organisms has been demonstrated to prevent several infectious diseases, allergic disorders, diarrhoea and inflammatory diseases, such as inflammatory bowel disease[2].

Bacteria are present in the food, water and environment, promoting gastrointestinal tract susceptibility to different types of infection, which produce a variety of illnesses in human subjects worldwide. *Escherichia coli* strains possess variable mechanisms of pathogenesis, while enterotoxigenic (ETEC) strains produce enterotoxin and enteropathogenic (EPEC) strains that adhere to epithelial cells; the latter type of infection remains a formidable cause of diarrhoeal illnesses. Zoonotic pathogens and those transmitted through faecal–oral contact, such as *Salmonella* and *Shigella*, respectively, constitute an important public health problem, especially in developing countries with substandard hygiene and unsafe water supplies. Antibiotics represent the first line of treatment, but the use of antibiotics causes an imbalance in the complex ecosystem of the human gastrointestinal tract. Thus, therapeutic alternatives to prevent or complement antibiotic therapy are currently being assessed. In this respect, the use of probiotics is a promising tool to prevent EPEC infections.

Several studies have demonstrated antimicrobial activity by lactobacilli and bifidobacteria against gastrointestinal microbial pathogens[3]. The mechanism underlying these effects of probiotics are largely unknown but are likely to be multifactorial, including the reduction of luminal pH, competition for nutritional sources, inhibition of adhesion to

Abbreviations: EPEC, enteropathogenic; ETEC, enterotoxigenic.

* **Corresponding author:** A. Gil, fax +34 958 819132, email agil@ugr.es

British Journal of Nutrition

epithelial cells, stimulation of the host immune system and the production of organic acids, bacteriocin or bacteriocin-like substances[4].

Some probiotics produce metabolites that inhibit the growth of bacteria and fungi[5,6] and have been used to prevent intestinal pathogenic infections, such as those caused by *Salmonella*, *Shigella*, *E. coli*, *Listeria* and *Helicobacter pylori*[7–11]. Due to their reported health benefits and the large number of novel probiotic strains, they have become attractive candidates for incorporation into functional foods and food products.

Among all probiotic bacteria, those isolated from the faeces of exclusively breastfed infants are of special interest due to lactic acid bacteria predominance, and these strains also seem to provide protection against enteric and systemic disorders caused by bacterial pathogens[12,13].

Recently, we reported the selection and characterisation of three probiotic strains (*Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036) isolated from the faeces of breastfed infants[11]. We demonstrated that these strains exhibited probiotic potential, survival in gastrointestinal conditions and adhesion to intestinal cells, and they were also innocuous to human health. Their safety status has been confirmed by sensitivity to antibiotics, toxicology, the absence of undesirable metabolites and acute ingestion studies in immunocompetent and immunosuppressed mice. Furthermore, antimicrobial activity against human rotavirus and *Listeria monocytogenes* has also been described for these probiotics.

The aim of the present study was to demonstrate the ability of *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036 to inhibit the growth of *E. coli* ETEC, *E. coli* EPEC, *Salmonella* and *Shigella*.

Materials and methods

Lactic acid bacteria strains from exclusively breastfed infants

Three novel strains of lactic acid bacteria were isolated from the faeces of breastfed infants, and strains that demonstrated adhesion to intestinal epithelial cells and resistance to gastrointestinal conditions were identified and denominated as *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036, according to a previously described protocol[11]. Briefly, twelve healthy, exclusively breastfed infants, aged 1 month, were selected for the study at the Clinic Hospital of the University of Granada. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethical Committee of the University of Granada. Written informed consent was obtained from the parents after a careful explanation of the nature of the study.

Isolation of supernatants from lactic acid bacteria strains

To obtain probiotic bacteria supernatants with 1× or 10× concentrations, the bacterial strains were grown anaerobically for 17 or 24 h at 37°C in Man, Rogosa and Sharpe (lactobacilli) or Man, Rogosa and Sharpe plus cysteine (bifidobacteria) media, respectively.

The supernatants were obtained by centrifugation at 12 000 ***g*** for 10 min and were then neutralised to pH 6·5 using NaOH (1 M). To obtain the 10× supernatant, the neutralised 1× supernatant was concentrated by freeze-drying. The supernatants were sterilised by filtration through 0·22 μm pore-sized filters and stored at − 20°C until further use.

Escherichia coli *ETEC*, Escherichia coli *EPEC*, Salmonella *and* Shigella *strains and growth conditions*

E. coli ETEC, *E. coli* EPEC, *Salmonella typhimurium*, *Salmonella typhi* and *Shigella sonnei* strains were obtained from the Spanish Type Culture Collection (CECT). We chose the following strains:

E. coli ETEC: CECT 501 and CECT 515; *E. coli* EPEC: CECT 727 and CECT 729; *S. typhimurium* CECT 443 and CECT 4594; *S. typhi*: CECT 725; and *S. sonnei*: CECT 457, CECT 4887^T and CECT 413.

The *E. coli* and *Salmonella* strains were grown in tryptone soy broth and incubated aerobically for 24 h at 37°C. The *Shigella* strains were grown in nutrient broth and incubated aerobically for 24 h at 37°C.

Activity of lactic acid bacteria supernatants against pathogens

These assays were performed in polystyrene ninety-six-well (volume, 200 μl/well) plates (Maxisorp). Tryptone soy (*E. coli* and *Salmonella*) or nutrient (*Shigella*) broth was inoculated with a 5% (v/v) concentrated microbial cell solution and grown overnight (*E. coli* and *Salmonella*). The supernatants, with or without neutralisation, were added to a final concentration (v/v) of 1 and 4% (*E. coli*, *Salmonella* and *Shigella*) or just neutralised to 2 and 4% (*Shigella*). The ability of each strain to inhibit the pathogenic strains was evaluated by monitoring bacterial growth at 37°C in tryptone soy or nutrient medium in ninety-six-well plates according to the methods of Chenoll *et al.*[10]. Bacterial growth was analysed at 620 nm using a Multiskan microplate reader (Thermo Fisher Scientific). In each case, the percentage of resistance was calculated by comparing the final optical densities at 620 nm obtained with different concentrations of supernatants with those of the corresponding control samples.

Statistical analysis

Results are expressed as mean and standard deviation. The differences between the mean values for the different treatments with lactic acid bacteria supernatants were analysed using one-way ANOVA. The least significant difference test was used for *a posteriori t* paired comparison of the mean values. The statistical analysis was performed using Statgraphics plus (version 5.1) software (Manugistics).

Results

Pathogen inhibition assays

The *L. paracasei* CNCM I-4034 supernatants were specific in their effects against the pathogenic strains. The not neutralised

17 h supernatant when used at 1 and 4% concentrations inhibited the growth of *S. typhi* CECT 725 by 23 and 41%, respectively. However, when it was neutralised, the inhibitory capacity was lost. The not neutralised 24 h supernatant used at 1 and 4% concentrations inhibited the growth of *S. typhi* CECT 725 by 25 and 23%, respectively; when this supernatant was neutralised, growth was inhibited by 24 and 23%, respectively (Fig. 1(c)). These results suggest that compounds of different nature might be present in the supernatant.

When both not neutralised supernatants were used at 4% concentration, the 17 h supernatant inhibited the growth of *S. typhimurium* CECT 443 and the 24 h supernatant inhibited the growth of *S. typhimurium* CECT 4594 by 29 and 39%, respectively (Fig. 1(a) and (b)). The neutralised supernatants inhibited the growth of *S. sonnei* CECT 413 by 81% (17 h) and 82·10% (24 h) when utilised at 4% concentration and by 32·5% (17 h) when utilised at 2% concentration (Fig. 4(a)). Similar results were observed against *S. sonnei* CECT 4887^T, as the supernatants used at 4% concentration showed 9·2% (17 h) and 20·5% (24 h) inhibition, and those used at 2% concentration showed 9·9% (24 h) inhibition (Fig. 4(b)). Only the 17 h supernatant was effective against *S. sonnei* CECT 457, as growth was inhibited by 6·3 and 25·9% when utilised at 2 and 4% concentrations, respectively (Fig. 4(c)).

When used at 1 and 4% concentrations, the not neutralised 17 h *B. breve* CNCM I-4035 supernatant was able to inhibit the growth of *S. typhi* CECT 725 by 37 and 46%, respectively, and by 25 and 29%, respectively, when the supernatants were neutralised. Similar results were obtained with the 24 h supernatants at 1 and 4% concentrations, as these showed 34 and 48% inhibition, respectively, when the supernatants were not neutralised and 34 and 15% inhibition, respectively, when the supernatants were neutralised (Fig. 2(c)). The supernatants did not inhibit the growth of *E. coli* EPEC CECT 727 and *E. coli* EPEC CECT 729 (Fig. 2(a) and (b)). However, the neutralised supernatants did inhibit the growth of *S. sonnei* CECT 457 by 19·4% (24 h) when utilised at 4% concentration and by 25·8% (24 h) when utilised at 2% concentration (Fig. 4(f)). The neutralised supernatants were not able to inhibit *S. sonnei* CECT 4887^T and *S. sonnei* CECT 413 (Fig. 4(e) and (d), respectively).

The *L. rhamnosus* CNCM I-4036 not neutralised 17 and 24 h supernatants inhibited the growth of *S. typhi* CECT 725 by 55 and 29%, respectively, but this inhibition was completely lost

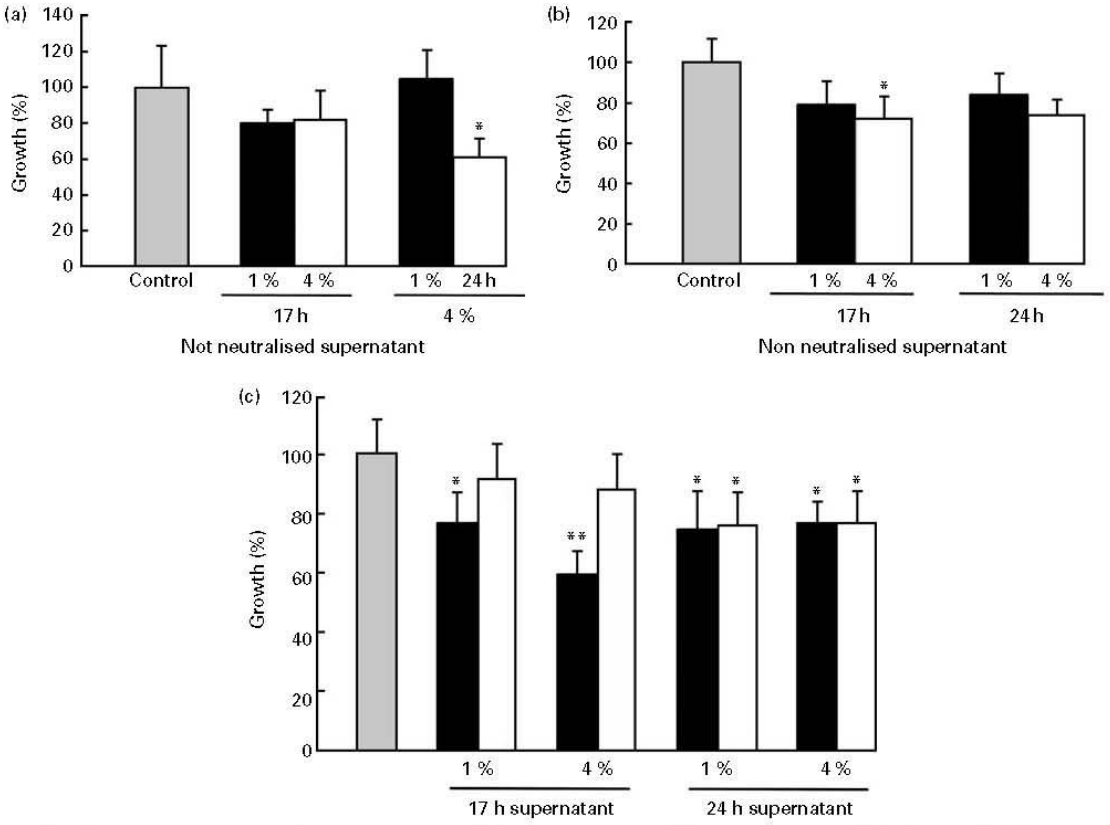


Fig. 1. Effect of 17 and 24 h supernatants of *Lactobacillus paracasei* CNCM I-4034 on growth of *Salmonella typhimurium* (a) CECT 4594, (b) CECT 443 and (c) *Salmonella typhi* CECT 725. Values were significantly different: * $P<0·05$; ** $P<0·01$. □, Control; ■, not neutralised; □, neutralised.

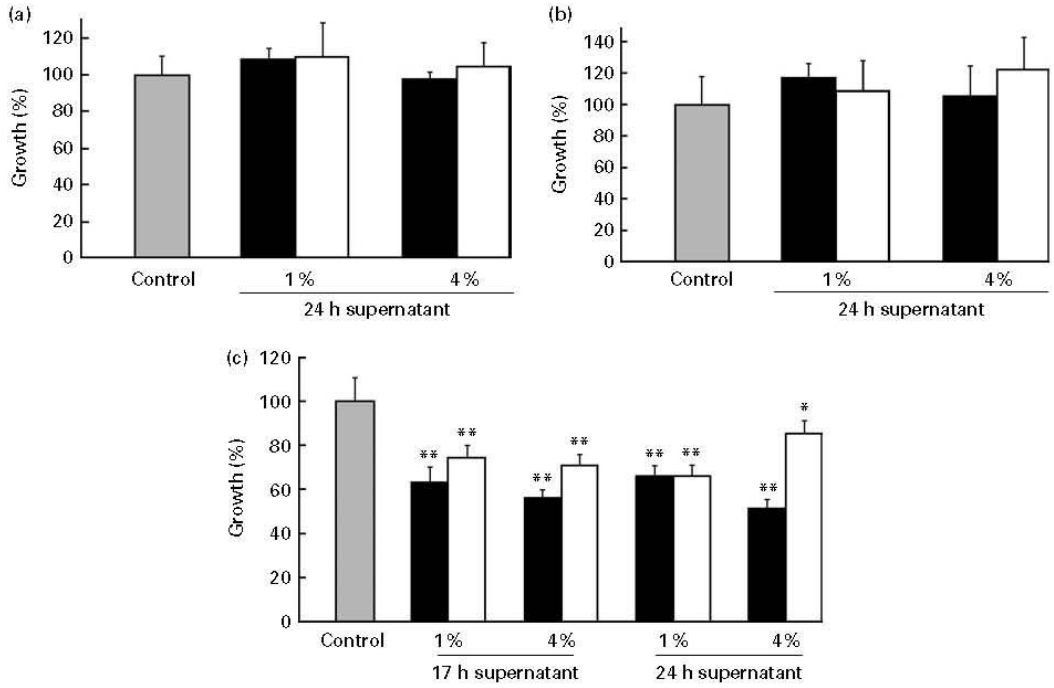


Fig. 2. Effect of supernatant of *Bifidobacterium breve* CNCM I-4035 on growth of *Escherichia coli* enteropathogenic (a) CECT 729, (b) CECT 727 and (c) *Salmonella typhi* CECT 725. Values were significantly different: * $P<0{\cdot}05$; ** $P<0{\cdot}01$. □, Control; ■, not neutralised; □, neutralised.

when the supernatants were neutralised (Fig. 3(a)). Similar results were observed against *E. coli* ETEC CECT 501, with 40 and 25% inhibition, and these 17 and 24 h supernatants also inhibited the growth of *E. coli* ETEC CECT 515 by 29 and 31%, respectively (Fig. 3(b) and (d)). The growth of *E. coli* EPEC CECT 729 was only affected by the 17 h supernatants used at 4% concentration (27% inhibition, Fig. 3(c)). The neutralised supernatants did inhibit the growth of *S. sonnei* CECT 413 by 81% (17 h) and 82% (24 h) when utilised at 4% concentration, and 16·3% (24 h) inhibition was observed when utilised at 2% concentration (Fig. 4(g)). The supernatants were slightly effective against *S. sonnei* CECT 4887^T, with minor inhibitory percentages of 29·1% (24 h) when utilised at 4% concentration (Fig. 4(h)), and against *S. sonnei* CECT 457, showing 33·8% (24 h) inhibition when the supernatant was utilised at 4% concentration and 16% (24 h) inhibition when utilised at 2% concentration (Fig. 4(i)).

Discussion

Probiotics display important characteristics that benefit human health. Although knowledge of the potential mechanisms underlying the effects of probiotics against enteropathogens is largely unknown, these mechanisms are likely multifactorial. Important mechanisms that have been shown to underlie these antagonistic effects include the reduction of luminal pH, competition for adhesion sites and nutritional sources, secretion of antimicrobial substances, toxin inactivation and immune stimulation[3,14]. Several previous reports have indicated that lactic acids, organic acids, bacteriocins, proteases, peroxides and exopolysaccharides exert antibacterial and antifungal effects[3,5,15]. In addition, three novel probiotics (*L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036) were previously demonstrated to resist low pH and high bile salt concentrations and to adhere to the colon[11]. Moreover, a safety evaluation of these probiotics, including their antibiotic resistance patterns, an assessment of certain metabolic activities (D-lactate production, bile salt deconjugation and amine biogen production) and *in vivo* acute ingestion profiles (immunosuppressed and immunocompetent mice), was also performed. Taken together, these previous results demonstrated that these probiotics were able to inhibit some strains of *L. monocytogenes* and the infection of human cells with rotavirus *in vitro*[11].

The results of the present study demonstrated that supernatants from cultures of the three novel probiotics *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036 inhibited the growth of EPEC bacteria in a strain-specific manner. Tsai *et al.*[6] showed a similar effect of three *Lactobacillus* strains against *E. coli* ETEC during a 20 h incubation, and similar results have also been obtained using organic acids[16,17]. For *Bifidobacterium* strains, the production of different compounds with inhibitory capacity against enteropathogen strains has also been described[18–20].

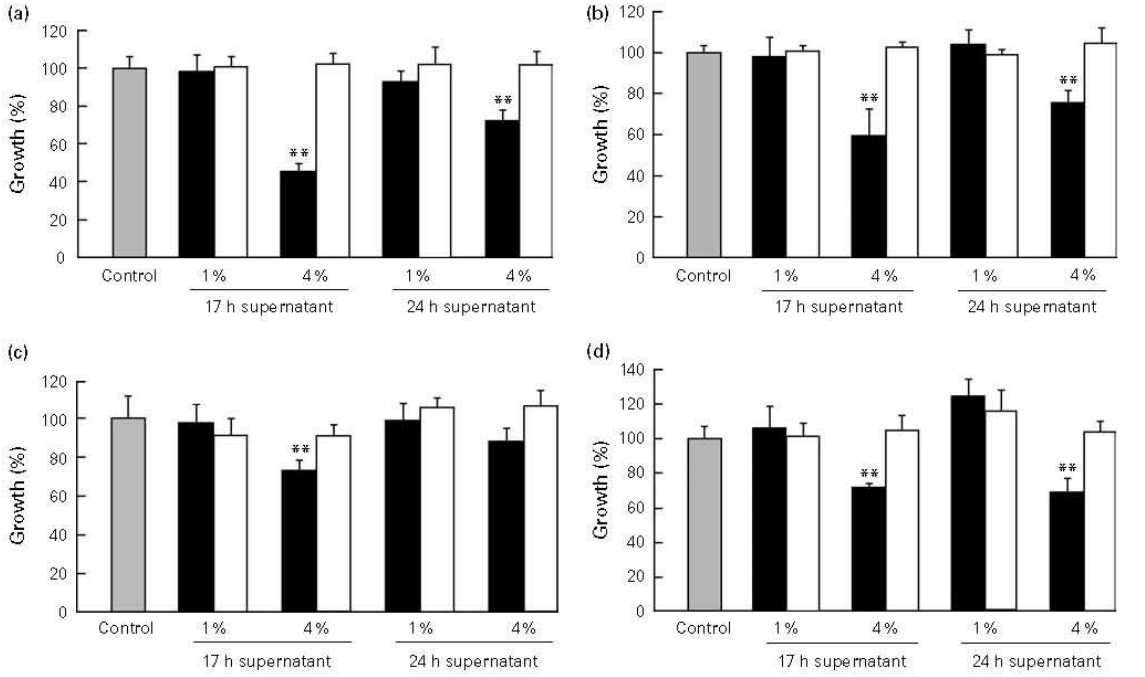


Fig. 3. Effect of supernatant of *Lactobacillus rhamnosus* CNCM I-4036 on growth of (a) *Salmonella typhi* CECT 725, (b) *Escherichia coli* ETEC CECT 501, (c) *E. coli* EPEC CECT 729 and *E. coli* ETEC CECT 515. ** Value was significantly different ($P<0.01$). □, Control; ■, not neutralised; □, neutralised.

The *L. paracasei* CNCM I-4034 17 h supernatants showed a differential effect against *S. typhi* CECT 725, as inhibition was observed when the supernatants were not neutralised, but these effects were lost upon neutralisation. A similar finding demonstrated that when both *Lactobacillus* supernatants were used against *S. typhimurium* CECT 4594, *S. typhimurium* CECT 443 and *E. coli* EPEC CECT 729, only the supernatant that was not neutralised was able to inhibit the growth of EPEC bacteria when utilised at 4% concentration. These results are consistent with those described in the study by Mauch *et al.*[15], who found that the activity of the compounds produced by *Lactobacillus brevis* PS1 was higher at low pH values, i.e. pH 5, and that this effect was partially diminished after proteolytic treatment, indicating the production of organic acid and proteinaceous compounds[5]. In another study, the total loss of antimicrobial activity at pH 7 suggested that organic acids were involved[21]. At low pH values, organic acids exist in non-dissociated forms, which facilitate their penetration into the hydrophobic cell membranes of bacteria. Several reports have also shown that the major groups of inhibitory compounds produced by the probiotics include lactic acid, volatile acids and bacteriocins[5,15,21–23].

The *B. breve* CNCM I-4035 supernatants showed a strain-specific effect on *S. typhi* CECT 725, as growth was inhibited in all cases. The nature of compounds produced by bifidobacteria differs from organic acid[18]; these substances, which are present in the supernatant, are highly effective against *S. typhimurium* and *E. coli*, and this effect has been attributed to the production of low-molecular-weight lipophilic molecules[19].

Some authors have attributed the production of organic acid to antimicrobial mechanisms[24,25]. In addition, Fukuda *et al.*[20] found that the acetate produced by bifidobacteria improves intestinal defence against *E. coli* EHEC.

L. rhamnosus CNCM I-4036 inhibited the growth of enteropathogens by 10–50% when supernatants that were not neutralised were used; however, these effects were diminished upon neutralisation. De Keersmaecker *et al.*[26] proposed that the production of lactic acid may be responsible for the antimicrobial effect of *L. rhamnosus* against *S. typhimurium*. However, we cannot disregard the possibility that this inhibitory effect was due to the added production of organic acids and bacteriocins that remain active in an acidic pH.

The extent of the culture incubation may also serve to concentrate the inhibitory substances, which suggests that the concentration of the supernatant after fewer hours of culture may not be high enough to inhibit the growth of enteropathogens. For example, *L. paracasei* CNCM I-4034 24 h supernatants exhibited an inhibitory effect against *S. typhi* CECT 725, which was not affected by neutralisation; however, a different result was observed with the 17 h supernatants.

The antimicrobial activity of probiotics against a wide range of pathogenic micro-organisms has been observed. *S. sonnei* is an enteroinvasive pathogen that induces the inflammatory destruction of the intestinal epithelium, leading to acute recto-colitis and lethal complications[27]. Some authors have demonstrated the inhibition of *S. sonnei* using *Lactobacillus* supernatants that were not neutralised[21,28,29]. Zhang *et al.*[28] described the inhibition of *S. sonnei* using five different

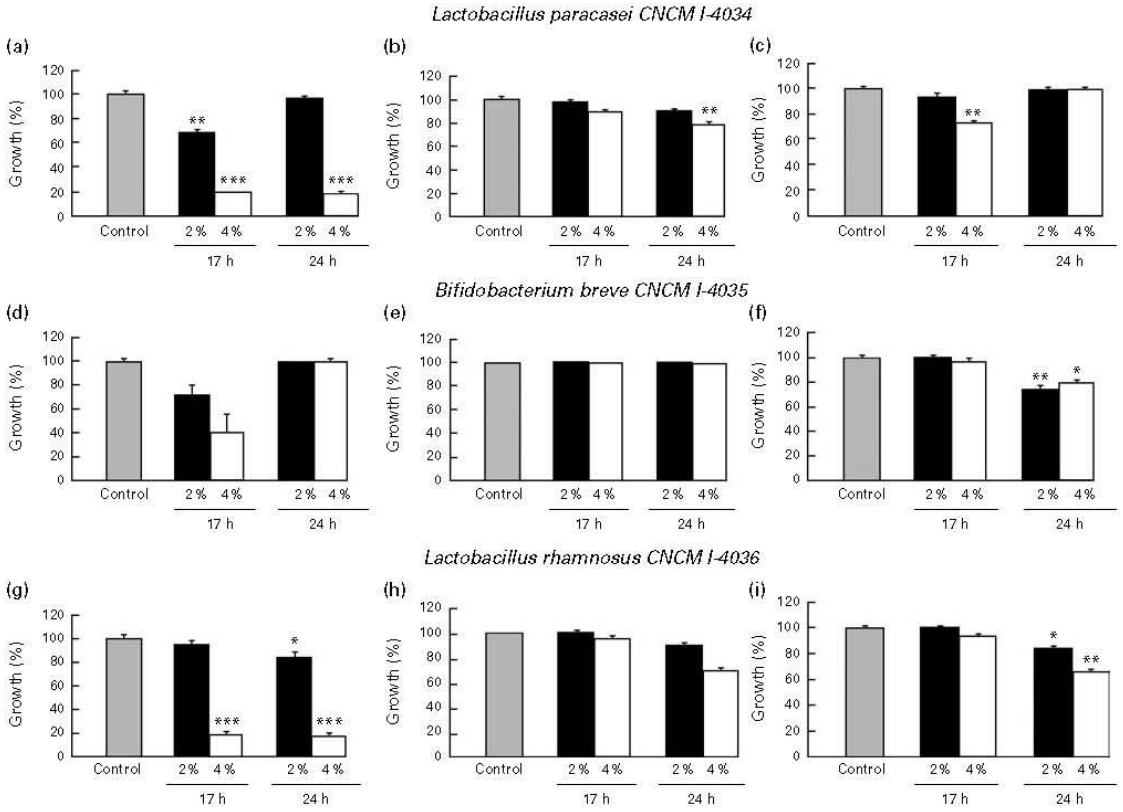


Fig. 4. Effect of 17 and 24 h neutralised supernatants of *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 on growth of *Shigella sonnei* (a, d, g) CECT 413, (b, e, h) CECT 4887^T and (c, f, i) CECT 457. Values were significantly different: * $P<0.05$, ** $P<0.01$; *** $P<0.001$. □, Control; ■, 2%; □, 4%.

supernatants from *Lactobacillus* strains, although this capacity was completely lost when the supernatants were neutralised. These results suggest that the action of organic acids is important and that bacteriocins are not involved in the antimicrobial activity of probiotics. However, different results were obtained with the strains tested in the present study, as the highest percentages of growth inhibition with *L. paracasei* CNCM I-4034 and *L. rhamnosus* CNCM I-4036 against *S. sonnei* CECT 413 (>80%) were obtained with neutralised supernatants, which indicates that substances other than organic acids were involved. *B. breve* CNCM I-4035 did not inhibit *S. sonnei* CECT 4887^T and *S. sonnei* CECT 413. Low percentages of inhibition (< 25 %) were observed against *S. sonnei* CECT 457, and other studies have demonstrated similar results for bifidobacteria strains[30].

A mixture of organic acids and proteinaceous compounds may affect the growth of EPEC bacteria in a strain-specific manner. Furthermore, due to the reduction in pH, this antimicrobial activity may be attributed to the presence of non-dissociated forms of acids. However, neutralised supernatants were shown to both inhibit and stimulate the growth of enteropathogens in a strain-specific manner, although the role of additional substances is possible. Taken together, the present results demonstrated that the inhibitory effects of probiotic supernatants were highly strain specific, and similar results have been previously described by other authors[31,32].

In conclusion, in the present study, we showed that supernatants harvested at different culture times from three novel probiotic strains can inhibit the growth of selected strains of *E. coli*, *Salmonella* and *Shigella*. These results suggest that these probiotic strains may produce substances that are either permissive or harmful to enteropathogens, depending on the neutralisation, concentration and culture time of the supernatants. These compounds could be organic acid and/or bacteriocins and are strain specific. Reactome analysis are ongoing to determine the nature of these substances.

Acknowledgements

S. M.-Q. and E. C. carried out the majority of the experiments and were responsible to write the first draft of the manuscript. S. M.-Q. and M. B.-B. were involved in the experiments with *E. coli* and *Salmonella* and E. C. with those of *S. sonnei*. C. G.-L. and S. G. helped in the studies of bacteria competition. J. P.-D. was involved in the evaluation and preparation of the manuscript. E. M., M. J. B. and F. R. participated in the

process of strain characterisation. D. R. and A. G. were the scientist leaders of the present work and contributed to the design and supervision of the experiments and results; they also were involved in writing the manuscript. This work was supported by the HERO Group trough its company HERO Spain S.A. (contract no. 3143 signed with the Fundación General Universidad de Granada Empresa contract and a private contract signed with the Spanish Biotechnology Company Biópolis S.L.). Hero Spain S.A. in turn was funded by the CDTI, Spanish Ministry of Health. C. G.-L. is a recipient of a postdoctoral fellowship from Plan Propio of the University of Granada. E. M., M. J. B. and F. R. are members of the Department of Research & Development, Hero Institute for Infant Nutrition. This Institute forms part of the food company HERO with headquarters in Switzerland. E. C., S. G. and D. R. are members of Biópolis S.L. (Spain), a spin-off of the High Scientific Research Council (Consejo Superior de Investigaciones Cientificas), Ministry of Education, Spain. No other authors declare conflict of interest.

References

1. FAO/WHO (2002) *Guidelines for the Evaluation of Probiotics in Food. Working Group Report.* London, Ontario: Food and Health Agricultural Organisation of the United Nations – World Health Organisation.
2. Minocha A (2009) Probiotics for preventive health. *Nutr Clin Pract* **24**, 227–241.
3. Servin AL (2004) Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiol Rev* **28**, 405–440.
4. Collado MC, Gueimonde M, Salminem S (2010) In: *Bioactive foods in promoting health, probiotics and prebiotics,* 1st ed., pp. 353–370 [Watson RR and Preedy VR, editors]. London, UK: Academic Press, Elsevier.
5. Wang H, Yan Y, Wang J, *et al.* (2012) Production and characterization of antifungal compounds produced by *Lactobacillus plantarum* IMAU10014. *PLoS One* **7**, e29452.
6. Tsai CC, Lin PP & Hsieh YM (2008) Three *Lactobacillus* strains from healthy infant stool inhibit enterotoxigenic *Escherichia coli* grown *in vitro*. *Anaerobe* **14**, 61–67.
7. Tsai CC, Hsih HY, Chiu HH, *et al.* (2005) Antagonistic activity against *Salmonella* infection *in vitro* and *in vivo* for two *Lactobacillus* strains from swine and poultry. *Int J Food Microbiol* **102**, 185–189.
8. de LeBlancade M, Castillo NA & Perdigon G (2010) Anti-infective mechanisms induced by a probiotic *Lactobacillus* strain against *Salmonella enterica* serovar *Typhimurium* infection. *Int J Food Microbiol* **138**, 223–231.
9. Dobson A, Cotter PD, Ross RP, *et al.* (2012) Bacteriocin production: a probiotic trait? *Appl Environ Microbiol* **78**, 1–6.
10. Chenoll E, Casinos B, Bataller E, *et al.* (2011) Novel probiotic *Bifidobacterium bifidum* CECT 7366 strain active against the pathogenic bacterium *Helicobacter pylori*. *Appl Environ Microbiol* **77**, 1335–1343.
11. Muñoz-Quezada S, Chenoll E, Vieites JM, *et al.* (2012) Isolation, identification and characterization of three novel probiotic strains (*Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036) from the faeces of exclusively breast-fed infants. *Br J Nutr.* (doi:10.1017/S0007114512005211).
12. Gribble KD (2011) Mechanisms behind breastmilk's protection against, and artificial baby milk's facilitation of, diarrhoeal illness. *Breastfeed Rev* **19**, 19–26.
13. Le Huërou-Luron I, Blat S & Boudry G (2010) Breast- *v.* formula-feeding: impacts on the digestive tract and immediate and long-term health effects. *Nutr Res Rev* **23**, 23–36.
14. Fooks LJ, Fuller R & Gibson GR (1999) Prebiotics, probiotics and human gut microecology. *Int Dairy J* **9**, 53–61.
15. Mauch A, Dal Bello F, Coffey A, *et al.* (2010) The use of *Lactobacillus brevis* PS1 to *in vitro* inhibit the outgrowth of *Fusarium culmorum* and other common *Fusarium* species found on barley. *Int J Food Microbiol* **141**, 116–121.
16. Bernet-Camard MF, Liévin V, Brassart D, *et al.* (1997) The human *Lactobacillus acidophilus* strain LA1 secretes a non-bacteriocin antibacterial substance(s) active *in vitro* and *in vivo*. *Appl Environ Microbiol* **63**, 2747–2753.
17. Coconnier MH, Liévin V, Bernet-Camard MF, *et al.* (1997) Antibacterial effect of the adhering human *Lactobacillus acidophilus* strain LB. *Antimicrob Agents Chemother* **41**, 1046–1052.
18. Collado MC, Hernández M & Sanz Y (2005) Production of bacteriocin-like inhibitory compounds by human fecal *Bifidobacterium* strains. *J Food Prot* **68**, 1034–1040.
19. Liévin V, Peiffer I, Hudault S, *et al.* (2000) *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. *Gut* **47**, 646–652.
20. Fukuda S, Toh H, Hase K, *et al.* (2011) Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* **469**, 543–547.
21. Lin WH, Yu B, Jang SH, *et al.* (2007) Different probiotic properties for *Lactobacillus fermentum* strains isolated from swine and poultry. *Anaerobe* **13**, 107–113.
22. Millette M, Luquet FM & Lacroix M (2007) *In vitro* growth control of selected pathogens by *Lactobacillus acidophilus*- and *Lactobacillus casei*-fermented milk. *Lett Appl Microbiol* **44**, 314–319.
23. Lee H, Yoon H, Ji Y, *et al.* (2011) Functional properties of *Lactobacillus* strains isolated from kimchi. *Int J Food Microbiol* **145**, 155–161.
24. Makras L & de Vuyst L (2006) The *in vitro* inhibition of Gram-negative pathogenic bacteria by bifidobacteria is caused by the production of organic acids. *Int Dairy J* **16**, 1049–1057.
25. Morita H, Hashimoto H, Hosoda M, *et al.* (2002) Intestinal *Bifidobacterium* species induce varying cytokine production. *J Allergy Clin Immunol* **109**, 1035–1036.
26. De Keersmaecker SC, Verhoeven TL, Desair J, *et al.* (2006) Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. *FEMS Microbiol Lett* **259**, 89–96.
27. Niyogi SK (2005) Shigellosis. *J Microbiol* **43**, 133–143.
28. Zhang Y, Zhang L, Du M, *et al.* (2011) Antimicrobial activity against *Shigella sonnei* and probiotic properties of wild lactobacilli from fermented food. *Microbiol Res* **167**, 27–31.
29. Jara S, Sánchez M, Vera R, *et al.* (2011) The inhibitory activity of *Lactobacillus* spp. isolated from breast milk on gastrointestinal pathogenic bacteria of nosocomial origin. *Anaerobe* **17**, 474–477.
30. Hütt P, Shchepetova J, Lõivukene K, *et al.* (2006) Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. *J Appl Microbiol* **100**, 1324–1332.
31. Gueimonde M, Jalonen L, He F, *et al.* (2006) Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli. *Food Res Int* **39**, 467–471.
32. Salminen S, Nybom S, Meriluoto J, *et al.* (2010) Interaction of probiotics and pathogens – benefits to human health? *Curr Opin Biotechnol* **21**, 157–167.

British Journal of Nutrition

MATERIALES Y MÉTODOS

ESTUDIO EN HUMANOS

DECLARACIÓN DE PRINCIPIOS ÉTICOS

Todos los pacientes que participaron en el estudio firmaron un formulario de consentimiento informado. El estudio siguió las normas establecidas en la declaración de Helsinki y fue aprobado por los comités de evaluación ética de las universidades de Granada, Murcia y Valencia.

SUJETOS DE ESTUDIO

Los criterios de inclusión fueron: hombre o mujer, edad entre 18 y 50 años, defecaciones normales, parámetros sanguíneos dentro del rango normal o que no se consideraran clínicamente significativos, IMC entre 18,0 y 29,9 kg/m^2.

Los criterios de exclusión fueron: embarazo y lactancia, parámetros sanguíneos fuera del rango normal considerado como clínicamente significativo, una historia de enfermedad metabólica o gastrointestinal, alergias a alimentos, uso reciente de antibióticos o medicamentos laxantes, diarrea, estreñimiento, diabetes mellitus, tabaquismo y presión arterial mayor a 140/90 mmHg.

PROBIÓTICOS

Se utilizaron las cepas probióticas *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 y *Lactobacillus rhamnosus* CNCM I-4036 descritas anteriormente por nuestro grupo (Muñoz-Quezada *et al.,* 2013). Estas cepas se sometieron a ensayos de actividad enzimática y utilización de carbohidratos y fueron depositadas en la *Collection Nationale de Cultures de Microorganismes* (CNCM) del Instituto Pasteur (Muñoz-Quezada *et al.,* 2013).

DISEÑO EXPERIMENTAL

Se ha realizado un estudio multicéntrico, aleatorizado, doble ciego y controlado por placebo, registrándose en www.clinicaltrials.gov como NCT01479543. La asignación al azar fue simple y no sujeta a ningún tipo de restricción, tales como bloqueo o tamaño de bloque. Ciento tres voluntarios sanos fueron reclutados en tres ciudades de España (Granada, Murcia y Valencia). A cada ciudad se le asignaron 35 códigos tomados de una tabla de aleatorización. Seis códigos fueron asignados a cada tratamiento en cada ciudad. Se utilizó una secuencia de asignación al azar (Ríos, 1967). Los sobres que contenían los códigos fueron asignados a cada participante del estudio. Finalmente, los voluntarios fueron reclutados, asignados aleatoriamente y de forma ciega por Carolina Gómez-Llorente (Granada), Gaspar Ros (Murcia) y Dolores Corella (Valencia). En la figura 8 se representa un diagrama de flujo del diseño experimental del estudio.

Los voluntarios se sometieron a un período de lavado de 15 días (t_1). Tras este periodo se dividieron de forma aleatoria y ciega en 5 grupos que recibieron diariamente un placebo, una cápsula que contenía $9x10^9$ UFC de una de las 3 cepas, o una cápsula que contenía $9x10^9$ UFC de una mezcla de *B. breve* CNCM I-4035 y *L. rhamnosus* CNCM I-4036, durante 30 días (t_2). El placebo contenía 67% de leche de vaca en polvo, 32,5% de sacarosa, y 0,56% de vitamina C. El período de intervención de 30 días fue seguido por un segundo lavado de otros 15 días (t_3). Los voluntarios no consumieron ningún producto fermentado durante todo el estudio.

Los voluntarios fueron reclutados entre julio y octubre de 2011. El primer lavado fue en octubre de 2011. La intervención terminó en noviembre de 2011, y el segundo lavado en diciembre de 2011. Todas las determinaciones acabaron en diciembre de 2012. Las muestras de sangre fueron tomadas en el instante t_1 y t_2. La sangre se centrifugó para separar el suero de las células. Las muestras de heces fueron tomadas en los tiempos t_1, t_2 y t_3. Los datos de referencia de todos los voluntarios aparecen en Plaza Díaz *et al.*, 2013 (Anexos).

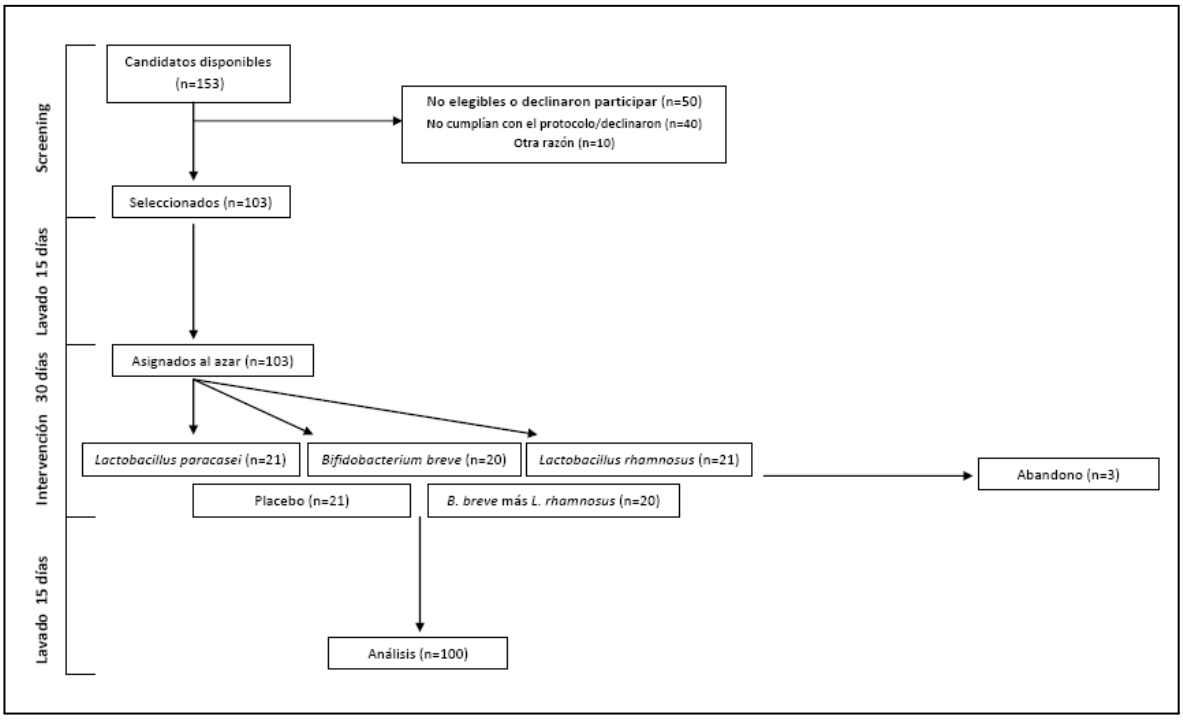


FIGURA 8. Diagrama de flujo del estudio NCT01479543 (Plaza-Díaz *et al.,* 2013).

Las variables principales del estudio fueron seguridad, tolerancia y persistencia. Las variables secundarias fueron las poblaciones de bacterias evaluadas en heces, efectos inmunomoduladores (citoquinas y producción de IgA secretora), análisis microbiológicos y poblaciones de linfocitos. El cálculo del tamaño muestral se realizó a partir de la varianza de una de las variables principales (persistencia), recuento de la cepa probiótica (log cepa UFC/g) en las heces de los voluntarios, con una diferencia del 25% comparado con el grupo placebo (Wind *et al.,* 2010). Se asumió un error de tipo 1 con una valor de α=0,05 y una potencia del 90 % (β=0,1). El número mínimo calculado de sujetos por grupo fue de 19.

El número inicial de voluntarios por grupo fue el siguiente: placebo n=21; *L. paracasei* CNCM I-4034 n=21; *B. breve* CNCM I-4035 n=20; *L. rhamnosus* CNCM I-4036 n=21; mezcla de *B. breve* CNCM I-4035 y *L. rhamnosus* CNCM I-4036 n=20. Un sujeto de cada uno de los grupos de placebo, *L. paracasei* CNCM I-4034 y *L. rhamnosus* CNCM I-4036, abandonó el estudio de manera voluntaria.

Las determinaciones que se describen a continuación se llevaron a cabo en todos los voluntarios (20 por grupo) con la excepción de la resistencia frente a los antibióticos, que se realizó en Valencia con 3 voluntarios por grupo (n=15).

COLECCIÓN Y PREPARACIÓN DE LAS MUESTRAS DE HECES

Las muestras de heces fueron recogidas por cada voluntario en recipientes de plástico estéril que contenía un indicador de anaerobiosis. El recipiente fue introducido en una bolsa plástica en condiciones anaeróbicas y sellada inmediatamente por una pinza plástica. Las muestras fueron analizadas en un tiempo máximo de 4 h.

PARÁMETROS DE TOLERANCIA Y SEGURIDAD GASTROINTESTINAL

La tolerancia gastrointestinal se determinó mediante el cuestionario de calificación de síntomas gastrointestinales GSRS, por sus siglas en inglés (Svedlund *et al.,* 1988), la consistencia de las heces (Whelan *et al.,* 2004), la recopilación diaria de síntomas gastrointestinales (náuseas, vómitos, diarrea, eructos, distensión abdominal y flatulencia) (Van Aerde *et al.,* 2004) y la frecuencia en la defecación. Los valores del GSRS y la consistencia de las heces fueron medidas por los investigadores a las 4 y 6 semanas. El cumplimiento en la toma del producto se registró en un diario. La intolerancia fue definida con una puntuación de los síntomas GSRS de 2 o superior (moderada o grave). Los parámetros de seguridad fueron el número y el tipo de eventos adversos registrados a lo largo de todo el estudio y los cambios en los parámetros sanguíneos entre el inicio y el final del período de administración de las cepas. Los parámetros sanguíneos se midieron en los laboratorios de Análisis Clínicos del Hospital Virgen de las Nieves (Granada), Hospital Clínico (Valencia) y el laboratorio MegaLab (Murcia).

ANÁLISIS DE FLUORESCENCIA DE HIBRIDACIÓN IN SITU ACOPLADO A CITOMETRÍA DE FLUJO (FISH-CF)

Un gramo de heces se homogeneizó con 9 ml de tampón fosfato salino (PBS) y posteriormente 0,2 ml de la suspensión se mezcló con 0,6 ml de paraformaldehído al 4% en PBS. Se fijó durante toda la noche a 4 ^{o}C. Los grupos bacterianos se evaluaron mediante FISH-CF tal como han descrito Fallani *et al*. (2006 y 2010) y Gomez-Llorente *et al*. (2013) La composición de la microbiota se analizó con un panel de 10 sondas específicas para grupos y especies unidas covalentemente a Cy5 en el extremo 5' (Langendijk *et al.,* 1995; Manz *et al.,* 1996; Sghir *et al.,* 2000; Franks *et al.,* 1998; Harmsen *et al.,* 1999; Harmsen *et al.,* 2000; Lay *et al.,* 2005; Fallani *et al.,* 2006; Fallani *et al.,* 2010, Gomez-Llorente *et al.,* 2013).

La hibridación se realizó en una placa de 96 pocillos durante la noche a 35 ^{o}C. La solución de hibridación contenía 4 ng/µl de cada sonda, en 150 µl por pocillos. Las células se lavaron para eliminar el exceso de sonda mediante la incubación de las muestras a 37 ^{o}C durante 20 minutos en una solución de lavado (64 mmol/L de NaCl; 20 mmol/L de Tris-HCl; pH 8,0; 5 mmol/L de EDTA pH 8,0 y 0,01% dodecil sulfato sódico, pH 7,2). Finalmente, se volvió a suspender la muestra en PBS. Las muestras se examinaron en un clitómetro de flujo FACSCanto II (Becton Dickinson, NJ, Estados Unidos), en el Centro de Instrumentación Científica de la Universidad de Granada.

ANÁLISIS MICROBIOLÓGICOS

Se llevaron a cabo los siguientes análisis microbiológicos de las muestras de heces: recuento en placa mediante diluciones seriadas en agar Wilkins-Chalgren (Panreac Química, Barcelona, España) para determinar el número total de bacterias anaeróbicas; cuantificación del número de lactobacilos por determinación en medio agar MRS (Oxoid, Basingstoke, Reino Unido); y finalmente, se cuantificó el número de bifidobacterias con el agar de Beerens (Oxoid, Basingstoke, Reino Unido). La cepa *L. rhamnosus* CNCM I-4036 se determinó empleando un medio MRS específico modificado, en el que se sustituyó la glucosa por ramnosa. Sólo *L. rhamnosus* y muy pocas especies de *Lactobacillus* del TGI humano son capaces de crecer en este medio (Wind *et al.,* 2010; Scardovi, 1986).

ANÁLISIS DE RESISTENCIA ANTIBIÓTICA

La sensibilidad de las cepas probióticas frente a ampicilina y tetraciclina fue determinada en las muestras de heces de los voluntarios mediante recuento en placas con diluciones seriadas en agar MRS (Oxoid, Basingstoke, Reino Unido), suplementado con un 0,05% (p/v) de cisteína (Sigma-Aldrich) (medio MRS-C) y TSA agar (TSA, Oxoid) con y sin ampicilina (2 y 4 µg/ml, Sigma Aldrich, St. Louis, MO), o tetraciclina (4 y 8 µg/ml, Sigma Aldrich). Las placas fueron incubadas durante un rango de 48 a 72 horas a 37 ^{o}C en atmósfera anaeróbica, generada utilizando el sistema AnaeroGen® (AnaeroGen®, Oxoid, Basingstoke, Reino Unido), para el medio MRS y MRS-C y aeróbicamente a 30 ^{o}C en el caso de las placas de TSA.

AISLAMIENTO DE Lactobacillus rhamnosus

Para cada voluntario, se seleccionaron de 5 a 10 colonias del agar MRS modificado (sustitución de glucosa por ramnosa, que favorece el crecimiento de *L. rhamnosus*), para inocularlas individualmente en medio MRS tradicional (Oxoid, Basingstoke, Reino Unido) durante 2 días a 37 ^{o}C bajo condiciones anaeróbicas De estos cultivos se aisló el ADN de *L. rhamnosus* CNCM I-4036 empleando el kit mini DNA QIAamp (QIAGEN, Barcelona, España) para posteriormente identificar la cepa mediante PCR cuantitativa (qPCR) con oligonucleótidos específicos.

REACCIÓN EN CADENA DE LA POLIMERA (PCR) CUANTITATIVA (qPCR)

La qPCR fue utilizada para identificar *Lactobacillus*, *Bifidobacterium* spp., *Bacteroides* y *Clostridium difficile* en heces y confirmar la persistencia intestinal de *L. rhamnosus* CNCM I-4036. Para aislar el ADN de las heces de los voluntarios se utilizó el kit Mini DNA Stool QIAamp (QIAGEN, Barcelona, España). Para el caso de *L. rhamnosus*, el protocolo de aislamiento se ha descrito anteriormente. La tabla 1 muestra los cebadores utilizados en cada caso. La qPCR fue realizada por triplicado en un equipo Eppendorf Mastercycle EP Gradient. Los cebadores específicos fueron comprados en Sigma-Aldrich (Barcelona, España). La qPCR se llevó a cabo, usando Power SYBR Green Master Mix (Applied Biosystems, Barcelona, España). El programa empleado fue: un paso inicial de activación/desnaturalización de 95 ^{o}C durante 5 minutos seguido de 30 a 40 ciclos de 15

segundos a 95 ^{o}C, 30 a 40 segundos de anillamiento en un rango de temperatura de 55 a 68 ^{o}C, y un paso final de extensión de 33 a 45 segundos a 72 ^{o}C. Se realizó una cuantificación absoluta empleando una curva estándar. Para el caso de *L. rhamnosus* la qPCR fue realizada en triplicado en un equipo StepOne Real-Time PCR (ABI). Los cebadores fueron diseñados en Thermo Fisher (Thermo Fisher Scientific, Waltham, MA). La qPCR fue realizada con Power SYBR Green Master Mix (ABI). La Taq polimerasa fue activada a 95 ^{o}C durante 10 minutos. Los parámetros para la desnaturalización fueron 95 ^{o}C durante 15 segundos, y 64 ^{o}C durante 50 segundos para el anillamiento (30 ciclos). Las colonias se identificaron como *L. rhamnosus* CNCM I-4036 cuando la amplificación fue positiva.

TABLA 1. Cebadores utilizados en análisis microbiológicos mediante PCR (Plaza-Díaz *et al.,* 2013).

Microbial target	Strain used for standard curve	**Sequence 5´→ 3¨**
Bifidobacterium spp.	*B. longum* CECT 4503	CTCCTGGAAACGGGTGG (Forward)
		GGTGTTCTTCCCGATATCTACA (Reverse)
B. longum	B longum CECT 4503	TTCCAGTTGATCGCATGGTCT (Forward)
		GGCTACCCGTCGAAGCCACG (Reverse)
B. breve	*B breve* CECT 4839	CCGGATGCTCCATCACAC (Forward)
		ACAAAGTGCCTTGCTCCCT (Reverse)
B. dentium	*B dentium* CECT 687	ATCCCGGGGGTTCGCCT (Forward)
		GAAGGGCTTGCTCCCGA (Reverse)
B. bifidum	*B bifidum* DSM 20456	CCACATGATCGCATGTGATTG (Forward)
		CCGAAGGCTTGCTCCCAAA (Reverse)
B. catenulatum	*B. catenulatum* DSM 16992	GCCGGATGCTCCGACTCCT (Forward)
		ACCCGAAGGCTTGCTCCCGAT (Reverse)
B. adolescentes	*B adolescentes* CECT 5781	CTCCAGTTGGATGCATGTC (Forward)
		CGAAGGCTTGCTCCCAGT (Reverse)
Lactobacillus	*L. paracasei* CNCM-I-4034	TGGATGCCTTGGCACTAGGA (Forward)
		AAATCTCCGGATCAAAGCTTAC (Reverse)
Bacteroides fragilis	*Bacteroides fragilis* DSM 2151	GAGAGGAAGGTCCCCCAC (Forward)
		CGCTACTTGGCTGGTTCAG (Reverse)
Clostridium difficile	*Clostridium difficile* CECT 531	TTGAGCGATTTACTTCGGTAAAGA (Forward)
		TGTACTGGCTCACCTTTGATATTCA (Reverse)
L. rhamnosus CNCM I-4036	*L. rhamnosus* CNCM I-4036	TGGCACTCACTGCAATTCGT (Forward)
		GATGCTTTGGCGTTGGTGTA (Reverse)

DETERMINACIÓN DEL CONTENIDO DE INMUNOGLOBULINA A SECRETORA (IgA) EN HECES

La IgA secretora se analizó en las heces de los voluntarios mediante un ensayo por inmunoabsorción ligado a enzimas (ELISA) de Inmundiagnostik AG (Bensheim, Alemania), siguiendo las indicaciones del fabricante.

PREPARACIÓN Y RECOLECCIÓN DE LAS MUESTRAS DE SANGRE

Las muestras de sangre fueron recolectadas en tubos Vacutainer® con ácido etilendiaminotetraacético (Becton Dickinson, NJ, Estados Unidos). Una alícuota de sangre fue usada para las determinaciones hematológicas. Una segunda alícuota fue centrifugada durante 10 minutos a 1000 g y 4 ^{o}C para separar el suero de las células. El suero se guardó a -80 ^{o}C para el análisis de citoquinas.

DETERMINACIÓN DE DIFERENTES POBLACIONES DE LINFOCITOS MEDIANTE CLASIFICACIÓN DE CELULAS ACTIVADAS FLUORESCENTEMENTE (FACS)

Estos análisis fueron realizados en un tiempo no superior a 24 h tras la toma de las muestras de sangre, para evitar la lisis celular. Los anticuerpos anti-CD14 conjugado con peridina-clorofila (PerCP)-Cy®, anti-CD4 conjugado con ficoeritrina (PE), anti-CD4 conjugado con fluoresceína (FITC), anti-CD25 conjugado con PE, anti-CD127 conjugado con AlexaFluor®, anti-CD19 conjugado con PE y anti-CD8 conjugado con PerCP, todos ellos frente a la proteína humana, se compraron a Becton Dickinson (San Diego, California, Estados Unidos) para ser utilizados en el análisis mediante citometría de flujo. Los anticuerpos monoclonales se incubaron con 200 µl de las muestras de sangre completa durante 15 minutos, en ausencia de luz. Los eritrocitos se lisaron con la solución hipotónica con Pharm LyseTM (BD Biosciences, San Diego, CA), limpiando las muestras de acuerdo a las especificaciones del fabricante. La citometría de flujo fue realizada usando el citómetro de flujo FACS Calibur® y Cell Quest (Becton Dickinson). Para cada anticuerpo se utilizaron $2x10^4$ linfocitos.

CUANTIFICACIÓN DE CITOQUINAS EN SUERO

Las citoquinas IL-4, IL-6, IL-10, IL-12, TNF-α y TGF-β fueron medidas utilizando la tecnología MILLIplex^{TM} (Merck-Millipore, MA, Estados Unidos) en un equipo Luminex 200, de acuerdo a las indicaciones del fabricante. Las citoquinas fueron determinadas en la Universidad de Granada.

ANÁLISIS ESTADÍSTICO

Todos los valores están expresados como media ± error típico de la media, a menos que se indique lo contrario. El análisis estadístico de los síntomas gastrointestinales fue realizado con el test *U* de Mann-Whitney. Para las comparaciones de los tiempos de cada uno de los parámetros que se distribuían normalmente, se utilizó un modelo lineal general de medidas repetidas. Para las variables en las que se encontró una diferencia significativa (diferencia entre tiempos específicos) se empleó el test *t* de Student para muestras pareadas, mientras que se recurrió al test de Wilcoxon para las variables que no tenían una distribución normal ($P<0,05$). Todos los análisis fueron realizados con el paquete estadístico de IBM, SPSS Statistics 20 (Somers, NY, Estados Unidos).

ESTUDIO EN RATAS ZUCKER

DECLARACIÓN DE PRINCIPIOS ÉTICOS

El estudio se llevó a cabo de acuerdo a las recomendaciones de las guías para la investigación animal de la Universidad de Granada (España). Todos los animales recibieron cuidado humanitario. El protocolo fue aprobado por el Comité de Ética de Experimentación Animal de la Universidad de Granada (Permiso número: 2011-377).

DISEÑO EXPERIMENTAL

Cuarenta y ocho ratas obesas Zucker-Lepr$^{fa/fa}$ y 16 ratas Zucker-lean$^{+/fa}$ machos que pesaban entre 168 y 180 g fueron compradas al Laboratorio Charles River (Ciudad, País). Las ratas fueron alojadas en jaulas metabólicas con un ciclo de luz/oscuridad de 12 h y acceso libre al agua y a la comida. Tras 5 días de adaptación, 8 ratas Zucker-lean$^{+/fa}$ y 8 ratas Zucker-Lepr$^{fa/fa}$ fueron sacrificadas como referencia (tiempo basal). Las restantes 40 ratas obesas Zucker-Lepr$^{fa/fa}$ fueron divididas aleatoriamente para recibir un placebo, 10^{10} UFC de una de las tres cepas probióticas o una mezcla de *L. paracasei* CNCM I-4034 y *B.* CNCM I-4035 por vía oral, durante 30 días. Un grupo adicional de 8 ratas Zucker-lean$^{+/fa}$ recibieron placebo durante 30 días. El placebo contenía 67% de leche de vaca en polvo, 32,5% de sacarosa y 0,56% de vitamina C. El objetivo de este estudio fue examinar las diferencias entre las ratas obesas tratadas con probióticos frente al placebo.

Tras la intervención los animales fueron anestesiados y sedados con una mezcla de ketamina y xilazina. La sangre fue extraída de la aorta y centrifugada durante 10 minutos a 1000 g a 4 ^{o}C para separar el suero de las células. Las muestras de íleon, colon e hígado se congelaron en nitrógeno líquido y se mantuvieron a -80 ^{o}C, o bien se fijaron con paraformaldehido al 4% para su posterior análisis.

HISTOLOGÍA INTESTINAL

Las muestras de íleon y colon se fijaron con 4 % de paraformaldehido durante 4 h a temperatura ambiente y se incluyeron posteriormente en parafina. Tres piezas de cada íleon y colon se incluyeron en el mismo bloque de parafina. Se obtuvieron secciones de 5 µm de espesor y se tiñeron con hematoxilina-eosina para su examen microscópico. Dos ratas por grupo y 8 secciones por cada rata se tiñeron y examinaron.

DETERMINACIÓN DEL CONTENIDO DE TRIACILGLICEROLES HEPÁTICOS

El contenido hepático de triacilgliceroles se determinó utilizando un kit comercial, siguiendo las instrucciones del fabricante (Spinreact, Gerona, España). Los valores de triacilglicerol fueron normalizados con respecto al peso del hígado.

TINCIÓN DE OIL RED O

Las muestras de hígado fueron fijadas con 4% de paraformaldehido, criopreservadas en PBS-sacarosa al 30%, enfriadas en un baño de nitrógeno líquido-isopentano e incluidas en el compuesto OCT^{TM}. Tres piezas de hígado de cada animal fueron fijadas e incluidas en el mismo bloque. Secciones de criostato de 7 µm de espesor se obtuvieron para luego ser teñidas con la solución de Oil Red O al 3% en 60% de isopropanol. De cuatro a ocho secciones por cada bloque se tiñeron para tomar las correspondientes imágenes. El porcentaje del área de cada imagen que correspondía al contenido de lípidos se calculó utilizando el software ImageJ (National Institutes of Health, Estados Unidos). Dos ratas por cada grupo fueron utilizadas para este estudio.

BIOQUÍMICA SÉRICA

Las concentraciones de glucosa, insulina, fosfolípidos, triacilglicerol, colesterol HDL, colesterol LDL y ácidos grasos no esterificados (NEFA), así como las actividades de AST (Aspartato aminotransferasa) y ALT (Alanina aminotransferasa) se determinaron en el suero de las ratas

utilizando kits comerciales (Spinreact, Gerona, España). Además, se calculó la resistencia a la insulina mediante el valor de HOMA-IR (homeostasys model assessment-insulin resistance).

CONCENTRACIÓN DE LIPOPOLISACÁRIDO EN SUERO

La concentración de LPS sérico se midió con un ensayo de inmunoabsorbancia de la compañía Cloud-Clone Corp., Houston, USA, siguiendo las indicaciones descritas por el fabricante.

CUANTIFICACIÓN DE CITOQUINAS Y ADIPOQUINAS EN EL SUERO

Las concentraciones séricas de leptina, adiponectina, TNF-α e IL-6 se midieron utilizando la tecnología MILLIplex^{TM} (Merck-Millipore, MA, Estados Unidos) en un equipo Luminex 200, de acuerdo a las indicaciones del fabricante.

ANALISIS ESTADÍSTICO

Todos los valores están expresados como media ± error típico de la media, a menos que se indique lo contrario. Los análisis estadísticos entre las ratas obesas Zucker-Lepr$^{fa/fa}$ y las ratas Zucker-lean$^{+/fa}$ se realizaron mediante la prueba de *t* de Student entre el grupo de referencia (tiempo basal) y al final de la intervención. Las diferencias significativas entre las ratas obesas que recibieron placebo y cualquier grupo de ratas obesas que recibieron alguna cepa específica después de la intervención se analizaron usando el test de ANOVA de un factor, corregido mediante una prueba *posthoc* (test de Bonferroni, $P< 0,05$). Todos los análisis fueron realizados con el paquete estadístico de IBM, SPSS Statistics 20 (Somers, NY, Estados Unidos).

RESULTADOS

ESTUDIO EN HUMANOS

Para ver detalladamente los resultados que se exponen aquí de forma muy resumida, véase en la sección de anexos el artículo Plaza Díaz *et al.*, 2013.

La frecuencia y la consistencia de las heces no se alteraron por la ingesta de ninguna cepa probiótica. No se observaron cambios en los parámetros séricos ni sanguíneos de los voluntarios. Tampoco se produjeron efectos adversos de tipo gastrointestinal en los voluntarios que recibieron probióticos durante todo el estudio.

La administración de las cepas probióticas modificó algunas de las poblaciones bacterianas evaluadas de las heces de los voluntarios. La persistencia intestinal pudo ser corroborada en las heces de los voluntarios que recibieron *L. rhamnosus* CNCM I-4036. La administración de *B. breve* CNCM I-4035 aumentó significativamente el contenido de IgA secretora en heces.

Las concentraciones de IL-4 e IL-10 sérica aumentaron, mientras IL-12 disminuyó, en los voluntarios que recibieron alguna de las tres cepas probióticas.

ESTUDIO EN RATAS ZUCKER

Para ver de forma detallada los resultados que se exponen aquí de forma muy resumida, véase en la sección de anexos el artículo Plaza Díaz *et al.*, 2014.

El contenido de triacilgliceroles hepáticos disminuyó en las ratas Zucker obesas que recibieron *L. rhamnosus* CNCM I-4036, *B. breve* CNCM I-4035 o la mezcla de *B. breve* CNCM I-4035 y *L. paracasei* CNCM I-4034. Además, el área correspondiente a los lípidos neutros fue significativamente menor en cortes histológicos de hígados de las ratas Zucker obesas que recibieron alguna cepa probiótica, comparado con las ratas obesas que recibieron placebo.

Es de destacar que se encontraron valores de LPS significativamente menores en el suero de las ratas Zucker obesas que recibieron probióticos en comparación con ratas obesas que recibieron

placebo. Los valores séricos de TNF-α disminuyeron en las ratas Zucker obesas que recibieron *L. rhamnosus* CNCM I-4036, *B. breve* CNCM I-4035 o la mezcla de *B. breve* CNCM I-4035 y *L. paracasei* CNCM I-4034, mientras que las ratas que recibieron *L. paracasei* CNCM I-4034 presentaron menores valores séricos de IL-6.

DISCUSIÓN

Los probióticos se definen como “microorganismos vivos que ingeridos en cantidades adecuadas resultan beneficiosos para la salud” (FAO/WHO, 2001), aunque bacterias muertas o componentes bacterianos también pueden exhibir propiedades probióticas (Plaza-Díaz *et al.*, 2014). Muchos de ellos, cuando se ingieren de forma habitual, modifican la ecología intestinal o son al menos transeúntes de la microbiota del colon. Los probióticos, como hemos descrito con anterioridad, son principalmente bacterias pertenecientes a los géneros *Lactobacillus y Bifidobacterium*, aunque también hay algunas levaduras. Los efectos beneficiosos que ejercen pueden producirse a través de variados mecanismos de acción: competitividad con patógenos intestinales por la adhesión al epitelio intestinal y nutrientes luminales, estimulación de la función de barrera del epitelio intestinal, producción y secreción de sustancias antimicrobianas, y una gran variedad de acciones sobre el sistema inmunitario, que aún en la actualidad no comprendemos detalladamente (Muñoz-Quezada, 2011).

Los probióticos podrían considerarse ingredientes de alimentos funcionales, que se definen como aquellos que con independencia de aportar nutrientes han demostrado científicamente que afectan beneficiosamente a una o varias funciones del organismo, de manera que proporcionan un mejor estado de salud y bienestar. En la actualidad existen productos que incluyen probióticos en sus formulaciones, tal es el caso de numerosos productos lácteos fermentados e incluso de chicles con *Lactobacillus reuteri* que aumentan la IgA salival (Ericson *et al.*, 2013). Aunque los probióticos están siendo extensa e intensamente estudiados, aún se desconocen todos los efectos que producen, así como el mecanismo por el que actúan.

En el presente estudio se evaluaron los efectos de la administración de tres cepas probióticas (*Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 y *Lactobacillus rhamnosus* CNCM I-4036) en individuos sanos y en un modelo de obesidad ampliamente caracterizado (ratas Zucker obesas). En el caso de los individuos sanos, se valoró la seguridad, tolerancia, persistencia y efectos sobre el sistema inmunitario de las cepas probióticas; en el caso de las ratas Zucker obesas se investigaron los cambios en los valores antropométricos, en el perfil de lípidos, de marcadores relacionados con el riesgo cardiovascular y la obesidad, y su efecto sobre la esteatosis hepática.

ESTUDIO EN HUMANOS

Los síntomas gastrointestinales (recogidos con las tablas de síntomas diarios y en el cuestionario de calificación de síntomas gastrointestinales), así como la frecuencia y consistencia de las deposiciones no se alteraron en los individuos sanos que ingirieron alguna de las cepas evaluadas, cuando se comparó cada variable con el grupo que recibió el placebo durante 30 días. Igualmente, no se observaron cambios adversos durante o después de la intervención. Estudios similares que evaluaron la seguridad de la administración de cepas probióticas en individuos sanos también han demostrado no producir eventos adversos gastrointestinales graves, ni cambios hematológicos significativos (Wind *et al.*, 2010; Ulsemer *et al.*, 2012; Zhang *et al.*, 2013).

Este primer punto merece ser destacado, ya que se cumple el primer objetivo del trabajo que se refiere a la seguridad y tolerancia de las cepas probióticas en individuos sanos. Además, estos resultados complementan los datos obtenidos con anterioridad en el estudio de la resistencia frente a antibióticos de las cepas (Plaza-Diaz *et al.*, 2013, Anexos), que demostraron que todas fueron sensibles a tetraciclina y ampicilina, lo que permite prevenir el desarrollo de posibles infecciones (Wind *et al.*, 2010).

La seguridad y la tolerancia son puntos importantes a la hora de establecer posibles utilidades futuras de las cepas. La oportunidad de evaluar las cepas probióticas en otros tipos de estudios, ya no con pacientes sanos, sino con alguna patología y/o diferentes grupos etarios que permitan conocer y verificar los efectos observados, es un paso que debe ser considerado en el futuro.

Con respecto a la persistencia, *L. rhamnosus* CNCM I-4036 fue la única cepa que pudo ser identificada al finalizar la intervención en las muestras de heces de los voluntarios que la tomaron. Estos resultados no necesariamente implican una colonización exitosa, sino más bien una persistencia de la cepa en el período de tiempo evaluado. La detección en heces de *L. rhamnosus* CNCM I-4036 tras un período de tiempo mayor (dos semanas después de finalizar la intervención) podría ayudar a determinar si la cepa pudo de hecho colonizar el TGI. Cabe destacar que el uso de nuevas tecnologías, como la secuenciación masiva, podrían ser útiles para clarificar este asunto. La

persistencia en las heces de los voluntarios que recibieron las restantes cepas, *L. paracasei* CNCM I-4034 y *B. breve* CNCM I-4035 no pudo ser llevada a cabo debido a la falta de medios de cultivo específicos y selectivos.

Es importante destacar que, en nuestro estudio, la administración de las cepas probióticas modificó las poblaciones bacterianas en las heces de los voluntarios, de acuerdo con los datos obtenidos mediante PCR a tiempo real y FISH. Las poblaciones bacterianas modificadas por las cepas bacterianas fueron: *Clostridium coccoides*, *Lactobacillus*, *Clostridium leptum*, *Streptococcus* y *Clostridium perfringens* y *Clostridium difficile*. Algunas de estas modificaciones fueron transitorias, cuando se evaluaron dos semanas después de la intervención. Al observar las poblaciones bacterianas en las heces, se aprecia que los cambios producidos fueron pequeños, pero hay que recordar que los individuos seleccionados en el estudio eran sanos y no presentaron ninguna patología durante y después del estudio.

Uno de los hallazgos más relevantes del trabajo fue el aumento de la población de *Clostridium difficile* que se produjo en las heces de los voluntarios que recibían *L. rhamnosus* CNCM I-4036 al evaluar las dos semanas siguientes de finalizado el estudio, suponemos que la cepa *L. rhamnosus* CNCM I-4036 pudo haber evitado el aumento de *Clostridium difficile* durante la intervención y el hecho de finalizar la administración produjo tal aumento. Este hecho pudo ser debido a que los voluntarios experimentasen un desplazamiento de *C. difficile* durante los 30 días de intervención con *L. rhamnosus* CNCM I-4036. Varios estudios han demostrado una disminución en la adhesión de *C. difficile* a la mucosa intestinal causada por probióticos (Gorbach *et al.* 1987; Lawrence *et al.*, 2005; Na y Kelly, 2011). El recuento de bifidobacterias también disminuyó en el grupo tratado con *L. rhamnosus* CNCM I-4036 cuando se compararon las dos semanas después de la intervención y el primer lavado. La disminución del número total de de bifidobacterias en el grupo de voluntarios tratado con *B. breve* CNCM I-4035 sugiere que la cepa administrada no alcanzó un estado viable en un número significativo en el colon, o bien no proliferó en el mismo (Guarner y Magaleda, 2003).

Sorprendentemente, ciertas poblaciones bacterianas cambiaron en las heces de los voluntarios que recibieron el placebo. Este efecto pudo deberse a su composición (leche de vaca

y/o sacarosa). El hecho de que los linfocitos T reguladores estuvieran aumentados en el grupo placebo también es intrigante. Estos dos datos, junto con la disminución de la IL-4 en el suero del grupo placebo, pueden indicar que el tamaño de la muestra, a pesar de haber sido calculado *a priori*, no fue el adecuado para las tres variables mencionadas.

Otro importante hallazgo de este trabajo fue que la administración de *B. breve* CNCM I-4035 resultó en un aumento significativo en el contenido de IgA secretora en heces, tras los 30 días de intervención. Dicho incremento de IgA secretora retornó a los valores iniciales dos semanas despés de finalizar la intervención, lo que apunta a un claro efecto debido a los probióticos. Este resultado confirma hallazgos previos de nuestro grupo (Muñoz-Quezada *et al.*, 2013), que describió que *B. breve* CNCM I-4035 aumenta las concentraciones de IgA en heces y en plasma de ratones (Muñoz-Quezada *et al.*, 2013). La modificación producida en la IgA secretora tiene un claro e importante efecto en el sistema inmunitario, pues la IgA secretora funciona como una primera línea de defensa en la protección del epitelio intestinal frente a toxinas entéricas y microorganismos patógenos (Mantis *et al.*, 2011).

En la actualidad, se ha sugerido que la seguridad de los probióticos debe ser evaluada por los cambios producidos en parámetros del sistema inmunitario, pues hay una evidencia clara de que estos microorganismos, especialmente los pertencientes a los géneros *Lactobacillus* y *Bifidobacterium*, pueden tener ciertas propiedades inmunomoduladoras (Ashraf y Shah, 2014). El principal hallazgo de nuestro análisis de citoquinas fue que *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 y *L. rhamnosus* CNCM I-4036 exhibieron efectos inmunomoduladores en los pacientes que recibieron las cepas cuando se compararon los tiempos finales (t_2) e iniclales (t_1) de la intervención. Así, los niveles de algunas moléculas antiinflamatorias (IL-4 e IL-10) aumentaron en el suero de los voluntarios que tomaron *L. rhamnosus* CNCM I-4036, mientras el índice proinflamatorio TNF-α/IL-10 disminuyó en el mismo grupo de voluntarios. Del mismo modo, la IL-12 (proinflamatoria) disminuyó en voluntarios que recibieron *B. breve* CNCM I-4035, y el índice antiinflamatorio (IL-10/IL-12) aumentó en el grupo que recibió *L. paracasei* CNCM I-4034.

Estos resultados confirman que estas tres cepas presentan efectos inmunoduladores tal y como ha sido descrito con anterioridad por nuestro grupo en experiementos *in vitro* (Bermudez-

Brito *et al.* 2012; Bermudez-Brito *et al.* 2013; Bermudez-Brito *et al.* 2014). *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 y *L. rhamnosus* CNCM I-4036 inhiben la producción de citoquinas y quimioquinas proinflamatorias en células dendríticas expuestas a bacterias patógenas, tales efectos pueden ser mediados por la expresión de TLR 1, 5 y 9 (Bermudez-Brito *et al.* 2012; Bermudez-Brito *et al.* 2013; Bermudez-Brito *et al.* 2014). Sin embargo, es necesario determinar por qué ocurrieron estos cambios en el perfil de las citoquinas llevando a cabo otro tipo de estudios que permitan entender el mecanismo de acción de las cepas analizadas.

ESTUDIO EN LAS RATAS ZUCKER

El síndrome metabólico o de resistencia a la insulina (SRI), término este último que se prefiere hoy día, fue originalmente definido como la concomitancia de dislipidemias, hipertensión, resistencia a la insulina y obesidad (Reaven 1989, Reaven 2004). El SRI a menudo precede a la aparición de diabetes mellitus tipo 2, e incrementa el riesgo de enfermedad cardiovascular (Lakka *et al.*, 2002; Kahn *et al.*, 2005), suponiendo por ello un gran problema de salud pública. Dentro de los modelos animales, la rata Zucker posee varias de las características del SRI, por lo que es uno de los modelos genéticos más utilizados para estudiar este síndrome (Kahn *et al.*, 2005). En el caso de nuestro estudio experimental, las ratas Zucker-Lepr$^{fa/fa}$ desarrollaron obesidad, hiperglucemia, resistencia a la insulina, hipercolesterolemia y presentaron concentraciones elevadas de ácidos grasos libres y triacilglicéridos en el suero tras 30 días de intervención en el grupo alimentado con placebo, en comparación con las ratas Zucker-lean$^{+/fa}$ que también recibieron el placebo. Además, las ratas Zucker-Lepr$^{fa/fa}$ mostraron esteatosis hepática y niveles elevados de AST y ALT en suero, indicando que el componente hepático del SRI también estaba presente en este modelo.

Al igual que ocurrió en nuestro estudio con voluntarios sanos (Plaza-Diaz *et al.*, 2013), la administración de las tres cepas probióticas a las ratas Zucker fue segura, pues la histología intestinal no mostró alteraciones ni diferencias entre las ratas que recibieron el placebo y las que recibieron alguna de las distintas cepas durante los 30 días de intervención. Además, la administración de las cepas probióticas no afectó a los parámetros bioquímicos, la resistencia a la insulina ni tampoco al perfil de adipoquinas séricas. Estos hallazgos coinciden con los resultados obtenidos en el estudio de intervención realizado en voluntarios sanos (Plaza-Diaz *et al.*, 2013).

El principal resultado de esta parte del estudio fue la reducción de la esteatosis hepática observada en las ratas que recibieron alguna cepa probiótica. Las ratas Zucker-Lepr$^{fa/fa}$ que recibieron *L. rhamnosus* CNCM I-4036, *B. breve* CNCM I-4035 o la mezcla de *B. breve* CNCM I-4035 y *L. paracasei* CNCM I-4034 presentaron un menor contenido de triacilgliceroles (TG) hepáticos que las ratas que fueron alimentadas con el placebo durante 30 días. Además, dado que la administración de *L. paracasei* CNCM I-4034 no disminuyó la cantidad de TG en el hígado, la disminución de los TG hepáticos por la mezcla de los dos probióticos puede atribuirse a la cepa *B. breve* CNCM I-4035. Esta disminución en el contenido de TG hepáticos no puede ser atribuida a una disminución en la concentración de insulina en suero porque la insulinemia fue similar en las ratas Zucker-Lepr$^{fa/fa}$ que recibieron placebo y las cepas. La tinción con Oil red O de los lípidos hepáticos neutros confirmó estos hallazgos.

Resultados similares han sido descritos por otros autores. La administración de probióticos produjo una disminución de TG y colesterol hepáticos en ratones y ratas alimentados con una dieta alta en grasa. (Cani *et al.,* 2009; Paik *et al.*, 2005; Xie *et al.*, 2011; Awaisheh *et al*., 2013; Yoo *et al.*, 2013). Sin embargo, ésta es la primera vez que se describe el efecto de probióticos en la disminución del contenido hepático de TG en un modelo genético de obesidad. Existe gran evidencia que señala que componentes bacterianos pueden afectar el metabolismo del hígado y, por lo tanto, causar enfermedades sistémicas (Delzenne y Cani, 2011, Nolan 1979). Se ha propuesto que los niveles en suero de LPS aumentan en obesidad y esteatosis, produciendo una endotoxemia metabólica capaz de modular las citoquinas proinflamatorias, así como la glucosa y el metabolismo lipídico en el hígado o en el tejido adiposo (Cani y Delzenne 2009; Lanthier *et al.*, 2009; Neyrinck *et al.*, 2009). La endotoxemia tiene un papel importante en la inducción de la inflamación hepática en la esteatohepatitis no alcohólica y en la enfermedad de hígado graso no alcohólico en los seres humanos (Adachi *et al.*, 1995; Verdam *et al.*, 2010; Harte *et al.* 2010; Thuy *et al.*, 2010). Cani *et al.* han demostrado alteraciones de la función de barrera intestinal en modelos genéticos de obesidad (Cani *et al.*, 2009). En conjunto, estos estudios sugieren una relación directa entre la microbiota intestinal, la barrera intestinal y alteraciones hepáticas. Este efecto reductor de la esteatosis que observamos en nuestro estudio puede estar mediado, al menos en parte, por la disminución de LPS en el suero observada en las ratas obesas que recibieron alguna cepa probiótica. En general, nuestros resultados apoyan la evidencia actual de que la microbiota

intestinal puede afectar al metabolismo hepático (Delzenne y Cani, 2011; Nolan, 1979; Kahn *et al.*, 2005).

Asimismo, se han encontrado claros efectos antiinflamatorios mediados por los probióticos en este estudio: 1) La disminución de los niveles séricos de TNF-α en las ratas Zucker-Lepr$^{fa/fa}$ obesas que recibieron *L. rhamnosus* CNCM I-4036, *B. breve* CNCM I-4035 o la mezcla de *B. breve* CNCM I-4035 y *L. paracasei* CNCM I-4034 comparados con las ratas Zucker-Lepr$^{fa/fa}$ obesas que sólo tomaron placebo; y 2) La disminución en la concentración sérica de IL-6 en las ratas obesas que recibieron *L. paracasei* CNCM I-4034 comparados con las ratas obesas que tomaron placebo. Dos estudios previos de nuestro grupo describen los efectos de *L. paracasei* CNCM I-4034 y *B. breve* CNCM I-4035 en la producción de citoquinas y quimioquinas utilizando cultivos de células dendríticas humanas expuestas a *Salmonella typhi* (Bermudez-Brito *et al.* 2012; Bermudez-Brito *et al.* 2013). *Lactobacillus paracasei* CNCM I-4034 disminuyó la producción de citoquinas y quimioquinas proinflamatorias en estas células (Bermudez-Brito *et al.* 2012), mientras *B. breve* CNCM I-4035 fue un potente inductor de factores proinflamatorios como TNF-α, IL-8 y RANTES (*Regulated on Activation, Normal T Cell Expressed and Secreted*) y también de factores anti-inflamatorios como IL-10 (Bermudez-Brito *et al.* 2013). En el presente estudio la administración de *L. paracasei* CNCM I-4034 disminuyó las concentraciones séricas de citoquinas proinflamatorias como IL-6.

Otros autores han descrito efectos antiinflamatorios tras la administración de probióticos. Estos probióticos han demostrado suprimir significativamente la activación en la señalización de NF-κB mediada por una dieta alta en grasa (Ma *et al.*, 2008). Además, la administración de lactobacilos a ratas con enfermedad hepática inducida por alcohol redujo los niveles de endotoxina plasmática y una mejora en la puntuación de la patología hepática (Nanji *et al.*, 1994). Una mezcla de bifidobacterias, lactobacilos y *Streptococcus thermophilus* disminuyó la inflamación hepática en ratones obesos genéticamente (Li *et al.*, 2003) y en ratas jóvenes a las que se les indujo obesidad mediante una dieta alta en grasa (Esposito *et al.*, 2009).

CONCLUSIONES

Las conclusiones del estudio en humanos son las siguientes:

1) Se ha evaluado la seguridad y la tolerancia de tres cepas probióticas (*Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 y *Lactobacillus rhamnosus* CNCM I-4036) en un estudio multicentrico, aleatorizado, doble ciego y controlado por placebo en voluntarios sanos. Ninguna de las cepas produjo trastornos gastrointestinales de ningún tipo, ni alteraciones en la frecuencia o en la consistencia de las deposiciones de los voluntarios. Tampoco se observaron cambios relevantes en los parámetros hematológicos y séricos.

2) Las cepas probióticas estudiadas presentaron efectos inmunomoduladores. La administración de *Lactobacillus rhamnosus* CNCM I-4036 disminuyó las concentraciones séricas de IL-12 (citoquina proinflamatoria) y aumentó las de IL-4 e IL-10 (citoquinas antiinflamatorias). *Bifidobacterium breve* CNCM I-4035 disminuyó los niveles séricos de IL-12 y finalmente, *Lactobacillus paracasei* CNCM I-4034 aumentó el índice antiinflamatorio IL-10/IL-12.

3) La administración de *Bifidobacterium breve* CNCM I-4035 aumentó el contenido de IgA secretora, primera línea de defensa en la protección del epitelio intestinal frente a toxinas entéricas y microorganismos patógenos, en las heces de los voluntarios.

4) La cepa *Lactobacillus rhamnosus* CNCM I-4036 fue la única cuya persistencia pudo demostrarse en las heces de los voluntarios tras 30 días de intervención.

Las conclusiones del estudio con ratas obesas son las siguientes:

5) Las ratas Zucker-Lepr$^{fa/fa}$ desarrollaron obesidad, hiperglucemia, resistencia a la insulina e hipercolesterolemia, concentraciones elevadas en suero de ácidos grasos libres y triacilgliceroles así como esteatosis hepática tras de 30 días de intervención con el placebo, en comparación con las ratas Zucker lean$^{+/fa}$.

6) La esteatosis hepática presente en las ratas Zucker-Lepr$^{fa/fa}$ disminuyó significativamente en las ratas que recibieron cepas probióticas. Este efecto estuvo mediado, en parte, por la disminución en suero de la concentración de LPS.

7) Los valores de TNF-α disminuyeron en el suero de las ratas Zucker-Lepr$^{fa/fa}$ que recibieron *L. rhamnosus* CNCM I-4036, *B. breve* CNCM I-4035 o la mezcla de *B. breve* CNCM I-4035 y *L. paracasei* CNCM I-4034; en cambio, las ratas que recibieron *L. paracasei* CNCM I-4034 presentaron menores valores séricos de IL-6. Estos resultados apuntan a un claro efecto antiinflamatorio de las cepas probióticas.

Conclusión general

Los resultados obtenidos en esta Tesis indican que las cepas probióticas *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 y *Lactobacillus rhamnosus* CNCM I-4036, son seguras y bien toleradas por individuos sanos. Presentan efectos inmunomoduladores tanto en el estudio realizado en las ratas Zucker-Lepr$^{fa/fa}$como en el estudio clínico, afectando la homeostasis intestinal, gracias a la liberación de citoquinas antiinflamatorias como la IL-10 y la producción de IgA. Finalmente, nuestros resultados sugieren la necesidad de continuar la investigación con las cepas probióticas para probar los efectos encontrados en otro tipo de estudios, que tal vez puedan incluir voluntarios con patologías de tipo inflamatorio.

BIBLIOGRAFÍA

Abbot EL, Smith WD, Siou GP, et al. (2007) Pili mediate specific adhesion of *Streptococcus pyogenes* to human tonsil and skin. Cell Microbiol 9: 1822–1833.

Abreu MT, Fukata M, Arditi M (2005) TLR signaling in the gut in health and diseases. J Immunol 174: 4453–4460.

Acharya MR and Shah RK (2002) Selection of human isolates of Bifidobacteria for their use as probiotics. Appl Biochem Biotechnol 102-103: 81-98.

Adachi Y, Moore LE, Bradford BU, et al. (1995) Antibiotics prevent liver injury in rats following long-term exposure to ethanol. Gastroenterology 108: 218-224.

Alakomi HL, Skytta E, Saarela M, et al. (2000) Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Appl Environ Microbiol 66: 2001–2005.

Alfaleh K, Anabrees J, Bassler D, et al. (2011) Probiotics for prevention of necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev 16: CD005496.

Allen SJ, Martinez EG, Gregorio GV, et al. (2010) Probiotics for treating acute infectious diarrhoea. Cochrane Database Syst Rev 10: CD003048.

Aller R, De Luis DA, Izaola O, et al. (2011) Effect of a probiotic on liver aminotransferases in nonalcoholic fatty liver disease patients: a double blind randomized clinical trial. Eur Rev Med Pharmacol Sci 15: 1090-1095.

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual cells without cultivation. Microbiol Rev 59: 143-169.

Anderson JP, Mueller JL, Misaghi A, et al. (2008) Initial description of the human NLRP3 promoter. Gene Immun 9: 721–726.

Anderson RC, Cookson AL, McNabb WC, et al. (2010) *Lactobacillus plantarum* MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. BMC Microbiol 10: 316.

Arboleya S, Binetti A, Salazar N, et al. (2012) Establishment and development of intestinal microbiota in preterm neonates. FEMS Microbiol Ecol 79: 763-772.

Arroyo R, Martín V, Maldonado A, et al. (2010) Treatment of infectious mastitis during lactation: antibiotics versus oral administration of Lactobacilli isolated from breast milk. Clin Infect Dis 15: 1551-1558.

Asemi Z, Jazayeri S, Najafi M, et al. (2012) Effect of daily consumption of probiotic yoghurt on oxidative stress in pregnant women: a randomized controlled clinical trial. Ann Nutr Metab 60: 62-68.

Ashraf R and Shah NP (2014) Immune system stimulation by probiotic microorganisms. Crit Rev Food Sci Nut. 54:938-56.

Audisio MC and Benítez-Ahrendts MR (2011) *Lactobacillus johnsonii* CRL1647, isolated from Apis mellifera L. bee-gut, exhibited a beneficial effect on honeybee colonies. Benef Microbes 2: 29-34.

Awaisheh SS, Khalifeh MS, Al-Ruwaili MA, et al. (2013) Effect of supplementation of probiotics and phytosterols alone or in combination on serum and hepatic lipid profiles and thyroid hormones of hypercholesterolemic rats. J Dairy Sci 96: 9-15.

Ayabe T, Satchell DP, Wilson CL, et al. (2000) Secretion of microbicidal alphadefensins by intestinal Paneth cells in response to bacteria. Nat Immunol 1: 113–118.

Ayeni FA, Sánchez B, Adeniyi BA, et al. (2011) Evaluation of the functional potential of *Weissella* and *Lactobacillus* isolates obtained from Nigerian traditional fermented foods and cow's intestine. Int J Food Microbiol 147: 97-104.

Backhed F, Ley RE, Sonnenburg JL, et al. (2005) Host-bacterial mutualism in the human intestine. Science 307:1915-1920.

Bals R and Wilson JM (2003) Cathelicidins – a family of multifunctional antimicrobial peptides. Cell Mol Life Sci 60: 711–720.

Banchereau J and Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392: 245–252.

Basma H, Soto-Gutiérrez A, Yannam GR, et al. (2009) Differentiation and Transplantation of Human Embryonic Stem Cell-Derived Hepatocytes. Gastroenterology 136: 990-999.

Bauernfeind F, Ablasser A, Bartok E, et al. (2010) Inflammasomes: current understanding and open questions. Cell Mol Life Sci 68: 765–783.

Bauernfeind FG, Horvath G, Stutz A, et al. (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol 183: 787–791.

Bausserman M and Michail S (2005) The use of *Lactobacillus* GG in irritable bowel syndrome in children: a double-blind randomized control trial. J Pediatr 147: 197-201.

Beachey EH (1981) Bacterial adherence: adhesinreceptor interactions mediating the attachment of bacteria to mucosal surfaces. J Infect Dis 143: 325–345.

Beerens H (1990) An elective and selective isolation medium for *Bifidobacterium* spp. Lett Appl Microbiol 11: 155-157.

Bermudez-Brito M, Muñoz-Quezada S, Gomez-Llorente C, et al. (2012) Human intestinal dendritic cells decrease cytokine release against *Salmonella* infection in the presence of *Lactobacillus paracasei* upon TLR activation. Plos One 7: e43197.

Bermudez-Brito M, Muñoz-Quezada S, Gomez-Llorente C, et al. (2013) Cell-free culture supernatant of *Bifidobacterium breve* CNCM I-4035 decreases pro-inflammatory cytokines in human dendritic cells challenged with *Salmonella typhi* through TLR activation. Plos One 8: e59370.

Bermudez-Brito M, Muñoz-Quezada S, Gomez-Llorente C, et al. (2014) *Lactobacillus rhamnosus* and its cell-free culture supernatant differentially modulate inflammatory biomarkers in *Escherichia coli*-challenged human dendritic cells. Br J Nutr 111:1727-37.

Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S (2012) Probiotic mechanisms of action. Ann Nutr Metab 61:160-74.

Bernaola Aponte G, Bada Mancilla CA, Carreazo Pariasca NY, et al. (2010) Probiotics for treating persistent diarrhoea in children. Cochrane Database Syst Rev 10: CD007401.

Bierbaum G and Sahl (2009) Lantibiotics: mode of action, biosynthesis and bioengineering. Curr Pharm Biotechnol 10: 2–18.

Biswas A, Petnicki-Ocwieja T, Kobayashi KS (2012) Nod2: a key regulator linking microbiota to intestinal mucosal immunity. J Mol Med (Berl) 90: 15–24.

Bocci V (1992) The neglected organ: bacterial flora has a crucial immunostimulatory role. Perspect Biol Med 35:251-60.

Borchers AT, Selmi C, Meyers FJ, et al. (2009) Probiotics and immunity. J Gastroenterol 44: 26-46.

Borruel N, Carol M, Casellas F, et al. (2002) Increased mucosal tumour necrosis factor alpha production in Crohn's disease can be downregulated ex vivo by probiotic bacteria. Gut 51: 659-664.

Borruel N, Casellas F, Antolín M, et al. (2003) Effects of nonpathogenic bacteria on cytokine secretion by human intestinal mucosa. Am J Gastroenterol 98: 865-870.

Bosch M, Rodriguez M, Garcia F, et al. (2012) Probiotic properties of *Lactobacillus plantarum* CECT 7315 and CECT 7316 isolated from faeces of healthy children. Lett Appl Microbiol 54: 240-246.

Boyle RJ, Bath-Hextall FJ, Leonardi-Bee J, et al. (2009) Probiotics for treating eczema. A systematic review. Clin Exp Allergy 39: 1117-1127.

Braat H, van den Brande J, van Tol E, et al. (2004) *Lactobacillus rhamnosus* induces peripheral hyporesponsiveness in stimulated CD4+ T cells via modulation of dendritic cell function. Am J Clin Nutr 80: 1618-1625.

Braga TD, da Silva GAP, de Lira PIC, et al. (2011) Efficacy of *Bifidobacterium breve* and *Lactobacillus casei* oral supplementation on necrotizing enterocolitis in very-low-birth-weight preterm infants: a double-blind, randomized, controlled trial. Am J Clin Nutr 93: 81-86.

Buck BL, Altermann E, Svingerud T, et al. (2005) Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCNCFM. Appl Environ Microbiol 71:8344–8351.

Caballero-Franco C, Keller K, De Simone C, et al. (2007) The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol 292:G315–G322.

Cai J, Zhao Y, Liu Y, et al. (2007) Directed differentiation of human embryonic stem cells into functional hepatic cells. Hepatology 45: 1229–1239.

Candela M, Bergmann S, Vici M, et al. (2007) Binding of human plasminogen to *Bifidobacterium*. J Bacteriol 189: 5929–5936.

Candela M, Biagi E, Centanni M, et al. (2009) Bifidobacterial enolase, a cell surface receptor for human plasminogen involved in the interaction with the host. Microbiology 155:3294–3303.

Candela M, Perna F, Carnevali P et al. (2008) Interaction of probiotic *Lactobacillus* and *Bifidobacterium* strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8 production. Int J Food Microbiol 31: 286-292.

Cani PD and Delzenne NM (2009) Interplay between obesity and associated metabolic disorders: new insights into the gut microbiota. Curr Opin Pharmacol 9: 737-743.

Cani PD and Delzenne NM (2009) The role of the gut microbiota in energy metabolism and metabolic disease. Curr Opin Pharmacol 15: 1546-1558.

Cani PD, Possemiers S, Van de WT, et al. (2009) Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 58: 1091-1103.

Carol M, Borruel N, Antolin M, et al. (2006) Modulation of apoptosis in intestinal lymphocytes by a probiotic bacteria in Crohn's disease. J Leukoc Biol 79: 917-922.

Castillo NA, Perdigón G, De Moreno de Le Blanc A (2011) Oral administration of a probiotic *Lactobacillus* modulates cytokine production and TLR expression improving the immune response against *Salmonella enterica* serovar *typhimurium* infection in mice. BMC Microbiol 11: 177–189.

Cebra JJ (1999) Influences of microbiota on intestinal immune system development. Am J Clin Nutr 69:1046S-1051S.

Cencič A and Langerholc T (2010) Functional cell models of the gut and their applications in food microbiology – A review. Int J Food Microbiol 141: S4-S14.

Charteris WP, Kelly PM, Morelli L, et al. (1998) Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J Appl Microbiol 84: 759-768.

Chauvière G, Coconnier MH, Kerneis S, et al. (1992) Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. J Gen Microbiol 138: 1689–1696.

Chen G, Shaw MH, Kim YG, et al. (2009) NOD-like receptors: role in innate immunity and inflammatory disease. Annu Rev Pathol 4: 365–398.

Chenoll E, Casinos B, Bataller E, et al. (2011) Novel probiotic *Bifidobacterium bifidum* CECT 7366 strain active against the pathogenic bacterium *Helicobacter pylori*. Appl Environ Microbiol 77: 1335–1343.

Cho IL, Lee NK, Hahm YT (2009) Characterization of *Lactobacillus* spp. Isolated from the feces of breast-feeding piglets. J Biosci Bioeng 108: 194-198.

Christensen HR, Frøkiær H, Pestka JJ (2002) Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol 168: 171–178.

Chu H, Kang S, Ha S, et al. (2005) *Lactobacillus acidophilus* expressing recombinant K99 adhesive fimbriae has an inhibitory effect on adhesion of enterotoxigenic *Escherichia coli*. Microbiol Immunol 49: 941-948.

Chu W, Lu F, Zhu W, et al. (2011) Isolation and characterization of new potential probiotic bacteria based on quorum-sensing system. J Appl Microbiol 110: 202-208.

Coconnier MH, Bernet MF, Chauviere G, et al. (1993) Adhering heat-killed human *Lactobacillus acidophilus*, strain LB, inhibits the process of pathogenicity of diarrhoeagenic bacteria in cultured human intestinal cells. J Diarrhoeal Dis Res 11: 235–242.

Coconnier MH, Klaenhammer TR, Kerneis S, et al. (1992) Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus secreting cell lines in culture. Appl Environ Microbiol 58: 2034–2039.

Collado MC, Gueimonde M, Hernández M, et al. (2005) Adhesion of selected *Bifidobacterium* strains to human intestinal mucus and the role of adhesion in enteropathogen exclusion. J Food Prot 68:2672–2678.

Collado MC, Gueimonde M, Salminen S (2010) Probiotics in adhesion of pathogens: mechanisms of action; in Watson RR, Preedy VR (eds): Bioactive Foods in Promoting Health,Chennai, Academic Press, Elsevier, vol 23, pp 353–370.

Collado MC, Gueimonde M, Sanz Y, et al. (2006) Adhesion properties and competitive pathogen exclusion ability of bifidobacteria with acquired acid resistance. J Food Prot 69: 1675–1679.

Collado MC, Meriluoto J, Salminen S (2007) Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. Lett Appl Microbiol 45: 454-460.

Collins JK, Thornton G, Sullivan GO (1998) Selection of probiotic strains for human application. Int Dairy J 8: 487–490.

Cools N, Ponsaerts P, Van Tendeloo VF, et al. (2007) Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. J Leukoc Biol 82: 1365–1374.

D'Amour KA, Agulnick AD, Eliazer S, et al. (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol 23: 1534–1541.

D'Arienzo R, Maurano F, Lavermicocca P, et al. (2009) Modulation of the immune response by probiotic strains in a mouse model of gluten sensitivity. Cytokine 48: 254-259.

Dave RI and Shah NP (1995) Evaluation of media for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, and bifidobacteria. J Dairy Sci 79: 1529-1536.

de Keersmaecker SC, Verhoeven TL, Desair J, et al. (2006) Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. FEMS Microbiol Lett 259: 89–96.

de los Reyes-Gavilán CG, Suárez A, Fernández-García M et al. (2011) Adhesion of bile-adapted *Bifidobacterium* strains to the HT29-MTX cell line is modified after sequential gastrointestinal challenge simulated in vitro using human gastric and duodenal juices. Res Microbiol 162: 514-519.

Delzenne NM and Cani PD (2011) Interaction between obesity and the gut microbiota: relevance in nutrition. Annu Rev Nutr 31: 15-31.

Dicks LM and Botes M (2010) Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. Benef Microbes 1: 11-29.

DNA data Bank of Japan DDBJ (2014) http://www.ddbj.nig.ac.jp/v.

Doherty GA, Bennett GC, Cheifetz AS, et al. (2010) Meta-analysis: targeting the intestinal microbiota in prophylaxis for post-operative Crohn's disease. Aliment Pharmacol Ther 31: 802–809.

Downes FP and Ito K (2001) Compendium of methods for the microbiological examination of foods, 4th ed., pp 601-648. American Public Health Association (APHA). Washington, D.C. USA.

Drago L, Nicola L, Iemoli E, et al. (2010) Strain-dependent release of cytokines modulated by *Lactobacillus salivarius* human isolates in an in vitro model. BMC Res Notes 3: 44.

Dugoua JJ, Machado M, Zhu X, et al. (2009) Safety in pregnancy: a systematic review and meta-analysis of RCT of *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces* spp. J Obstet Gynaecol Can 31: 542-552.

Dunne C, O´Mahony L, Murphy L et al. (2001) In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. Am J Clin Nutr 73: 386-392.

Eckburg PB, Bik EM, Bernstein CN, et al. (2005) Diversity of the human intestinal microbial flora. Science 308:1635–1638.

Enck P, Zimmermann K, Menke G, et al. (2008) A mixture of *Escherichia coli* (DSM 17252) and *Enterococcus faecalis* (DSM 16440) for treatment of the irritable bowel syndrome – A randomized controlled trial with primary care physicians. Neurogastroenterol Motil 20: 1103–1109.

Ericson D1, Hamberg K, Bratthall G, et al. (2013) Salivary IgA response to probiotic bacteria and mutans streptococci after the use of chewing gum containing *Lactobacillus reuteri*. Pathog Dis 68: 82-7.

Esposito E, Iacono A, Bianco G, et al. (2009) Probiotics reduce the inflammatory response induced by a high-fat diet in the liver of young rats. J Nutr 139: 905-911.

European Bioinformatics Institute (2014) European Nucleotide Archive (ENA) http://www.ebi.ac.uk/ena/

Evrard B, Coudeyras S, Dosgilbert A, et al. (2011) Dose-dependent immunomodulation of human dendritic cells by the probiotic *Lactobacillus rhamnosus* Lcr35. PlosOne 18: e18735.

Fallani M, Rigottier-Gois L, Aguilera M, et al. (2006) *Clostridium difficile* and *Clostridium perfringens* species detected in infant faecal microbiota using 16S rRNA targeted probes. J Microbiol Methods 67: 150-16.

Fallani M, Young D, Scott J, et al. (2010) Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. J Pediatr Gastroenterol Nutr 51: 77-84.

FAO/WHO (2001) Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. www.fao.org

FAO/WHO (2002) Guidelines for the evaluation of probiotics in food. Food and Health Agricultural Organisation of the United Nations — World Health Organisation. Working group report. London, Ontario, Canada. www.fao.org

Fernandez M, Valenti V, Rockel C, et al. (2011) Anti-inflammatory capacity of selected lactobacilli in experimental colitis is driven by NOD2-mediated recognition of a specific peptidoglycan-derived muropeptide. Gut 60: 1050–1059.

Ferreira CL, Grześkowiak Ł, Collado MC, et al. (2011) In vitro evaluation of *Lactobacillus gasseri* strains of infant origin on adhesion and aggregation of specific pathogens. J Food Prot 74: 1482-1487.

Fink LN, Zeuthen LH, Ferlazzo G, et al. (2007) Human antigen-presenting cells respond differently to gut-derived probiotic bacteria but mediate similar strain-dependent NK and T cell activation. FEMS Immunol Med Microbiol 51: 535-546.

Fontana L, Bermudez-Brito M, Plaza-Diaz J, et al. (2013) Sources, isolation, characterisation and evaluation of probiotics. Br J Nutr 109 Suppl 2:S35-50.

Frank DN, St Amand AL, Feldman RA, et al. (2007) Molecularphylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci USA 104:13780–13785.

Franks AH, Harmsen HJM, Raagns GC, et al. (1998) Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group specific 16S rRNA-targeted oligonucleotide probes. Appl Environ Microbiol 64: 3336-3345.

Fujiwara S, Hashiba H, Hirota T, et al. (2001) Inhibition of the binding of enterotoxigenic *Escherichia coli* Pb176 to human intestinal epithelial cell line HCT-8 by an extracellular protein fraction containing BIF of *Bifidobacterium longum* SBT2928: suggestive evidence of blocking of the binding receptor gangliotetraosylceramide on the cell surface. Int J Food Microbiol 67: 97–106.

Furrie E, Macfarlane S, Kennedy A, et al. (2005) Synbiotic therapy (*Bifidobacterium longum*/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. Gut 54: 242–249.

Gallo RL and Hooper LV (2012) Epithelial antimicrobial defence of the skin and intestine. Nat Rev Immunol 12: 503–516.

Ganz T (2003) Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol 3:710–720.

Gaudier E, Michel C, Segain JP, et al. (2005) The VSL#3 probiotic mixture modifies microflora but does not heal chronic dextran-sodium sulfate-induced colitis or reinforce the mucus barrier in mice. J Nutr 135: 2753–2761.

Gawrońska A, Dziechciarz P, Horvath A, et al. (2007) A randomized double-blind placebo-controlled trial of *Lactobacillus* GG for abdominal pain disorders in children. Aliment Pharmacol Ther 25: 177-1784.

Genovese KJ, Anderson RC, Harvey RB, et al. (2000) Competitive exclusion treatment reduces the mortality and fecal shedding associated with enterotoxigenic *Escherichia coli* infection in nursery-raised neonatal pigs. Can J Vet Res 64: 204–207.

Georgieva RN, Iliev IN, Chipeva VA, et al. (2008) Identification and in vitro characterization of *Lactobacillus plantarum* strains from artisanal Bulgarian white brined cheeses. J Basic Microbiol 48: 234-244.

Giahi L, Aumueller E, Elmadfa I, et al. (2012) Regulation of TLR4, p38 MAPkinase, IκB and miRNAs by inactivated strains of lactobacilli in human dendritic cells. Benef Microbes 4: 91–98.

Gibson GR and Wang X (1994) Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J Appl Bacteriol 77:412–420.

Gómez-Llorente C, Muñoz S, Gil A (2010) Role of Toll-like receptors in the development of immunotolerance mediated by probiotics. Proc Nutr Soc 69: 381–389.

Gomez-Llorente C, Plaza-Diaz J, Aguilera M, et al. (2013) Three main factors define changes in fecal microbiota associated with feeding modality in infants. J Pediatr Gastroenterol Nutr 57: 461-466.

González-Rodríguez I, Sánchez B, Ruiz L, et al. (2012) Role of extracellular transaldolase from *Bifidobacterium bifidum* in mucin adhesion and aggregation. Appl Environ Microbiol 78: 3992–3998.

Gopal PK, Prasad J, Smart J, et al. (2001) In vitro adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. Int J Food Microbiol 67: 207-216.

Gorbach SL, Chang TW, Goldin B (1987) Successful treatment of relapsing *Clostridium difficile* colitis with *Lactobacillus* GG. Lancet 2: 1519.

Greenberg B (1969) *Salmonella* suppression by known populations of bacteria in flies. J Bacteriol 99: 629–635.

Greene JD and Klaenhammer TR (1994) Factors involved in adherence of lactobacilli to human Caco-2 cells. Appl Environ Microbiol 60: 4487–4494.

Guarner F and Malagelada JR (2003) Gut flora in health and disease. Lancet 361: 512-519.

Gueimonde M, Laitinen K, Salminen S, et al. (2007) Breast milk: a source of bifidobacteria for infant gut development and maturation. Neonatology 92: 64-66.

Guglielmetti S, Tamagnini I, Mora D, et al. (2008) Implication of an outer surface lipoprotein in adhesion of *Bifidobacterium bifidum* to Caco-2 cells. Appl Environ Microbiol 74: 4695–4702.

Hakansson A and Molin G (2011) Gut microbiota and inflammation. Nutrients 3: 637–682.

Haller D, Colbus H, Ganzle MG, et al. (2001) Metabolic and functional properties of lactic acid bacteria in the gastro-intestinal ecosystem: a comparative in vitro study between bacteria of intestinal and fermented food origin. Syst Appl Microbiol 24: 218–226.

Harmsen HJ, Wildeboer-Veloo AC, et al. (2000) Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr 30: 61-67.

Harmsen HJM, Elfferich P, Schut F, et al. (1999) A 16S rRNA-targeted probe for detection of *Lactobacilli* and *Enterococci* in faecal samples by fluorescent *in situ* hybridization. Microb Ecol Health Dis 11: 3-12.

Harte AL, da Silva NF, Creely SJ, et al. (2010) Elevated endotoxin levels in nonalcoholic fatty liver disease. J Inflamm (Lond.) 7: 15.

Hartemink R and Rombouts FM (1999) Comparison of media for the detection of bifidobacteria, lactobacilli and total anaerobes from faecal samples. J Microbiol Meth 36: 181-192.

Hartemink R, Kok BJ, Weenk GH, et al. (1996) Raffinose-*Bifidobacterium* (RB) agar, a new selective medium for bifidobacteria. J Microbiol Meth 27: 33-43.

Hassan M, Kjos M, Nes IF, et al. (2012) Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. J Appl Microbiol 113: 723-736.

Hemmi H, Takeuchi O, Kawai T, et al. (2000) Toll-like receptor recognizes bacterial DNA. Nature 408: 740–745.

Hill JE, Baiano JC, Barnes AC (2009) Isolation of a novel strain of *Bacillus pumilus* from penaeid shrimp that is inhibitory against marine pathogens. J Fish Dis 32: 1007-1016.

Hirano J, Yoshida T, Sugiyama T, et al. (2003) The effect of *Lactobacillus rhamnosus* on enterohemorrhagic *Escherichia coli* infection of human intestinal cells in vitro. Microbiol Immunol 47: 405–409.

Hirn J, Nurmi E, Johansson T, et al. (1992) Long-term experience with competitive exclusion and salmonellas in Finland. Int J Food Microbiol 15: 281–285.

Hirota SA, Ng J, Lueng A, et al. (2011) NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis. Inflamm Bowel Dis 17: 1359–1372.

Hoarau C, Lagaraine C, Martin I, et al. (2006) Supernatant of *Bifidobacterium breve* induces dendritic cell maturation, activation, and survival through a Toll-like receptor pathway. J Allergy Clin Immunol 117: 696–702.

Holzapfel WH, Haberer P, Snel J, et al. (1998) Overview of gut flora and probiotics. Int J Food Microbiol 41: 85-101.

Hooper LV, Midtvedt T, Gordon JI (2002) How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr 22: 283-307.

Hooper LV, Stappenbeck TS, Hong CV, et al. (2003) Angiogenins: a new class of microbicidal proteins involved in innate immunity. Nat Immunol 4: 269–273.

Hooper LV, Wong MH, Thelin A, et al. (2001) Molecular analysis of commensal host-microbial relationships in the intestine. Science 291: 881-884.

Howell JC and Wells JM (2011) Generating intestinal tissue from stem cells: potential for research and therapy. Regen Med 6: 743-755.

Huang SH, He L, Zhou Y, et al. (2009) *Lactobacillus rhamnosus* GG suppresses meningitic *E. coli* K1 penetration across human intestinal epithelial cells in vitro and protects neonatal rats against experimental hematogenous meningitis. Int J Microbiol 2009: 647862.

Hummel S, Veltman K, Cichon C, et al. (2012) Differential targeting of the E-cadherin/β-catenin complex by Gram-positive probiotic lactobacilli improves epithelial barrier function. Appl Environ Microbiol 78: 1140–1147.

Hynönen U, Westerlund-Wikström B, Palva A, et al. (2002) Identification by flagellum display of an epithelial cell and fibronectin binding function in the SlpA surface protein of *Lactobacillus brevis*. J Bacteriol 184: 3360–3367.

Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA (2007). http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178667590178.htm

Ivec M, Botić T, Koren S, et al. (2007) Interactions of macrophages with probiotic bacteria lead to increased antiviral response against vesicular stomatitis virus. Antiviral Res 75: 266-274.

Izquierdo E, Medina M, Ennahar S, et al. (2008) Resistance to simulated gastrointestinal conditions and adhesion to mucus as probiotic criteria for *Bifidobacterium longum* strains. Curr Microbiol 56: 613–618.

Jankowska A, Laubitz D, Antushevich H, et al. (2008) Competition of *Lactobacillus paracasei* with *Salmonella enterica* for adhesion to Caco-2 Cells. J Biomed Biotechnol 2008: 357964.

Jarry A, Bossard C, Sarrabayrouse G, et al. (2011) Loss of interleukin-10 or transforming growth factor β signaling in the human colon initiates a T-helper 1 response via distinct pathways. Gastroenterology 141: 1887-1896.

Jiménez E, Fernández L, Maldonado A, et al. (2008) Oral administration of Lactobacillus strains isolated from breast milk as an alternative for the treatment of infectious mastitis during lactation. Appl Environ Microbiol 74: 4650-4655.

Johnston BC, Goldenberg JZ, Vandvik PO, et al. (2011) Probiotics for the prevention of pediatric antibiotic-associated diarrhea. Cochrane Database Syst Rev 9: CD004827.

Juntunen M, Kirjavainen PV, Ouwehand AC, et al. (2001) Adherence of probiotic bacteria to human intestinal mucus in healthy infants and during rotavirus infection. Clin Diag Lab Immunol 8: 293–296.

Kagan BL, Selsted ME, Ganz T, et al. (1990) Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. Proc Natl Acad Sci USA 87: 210–214.

Kahn R, Buse J, Ferrannini E, et al. (2005) The metabolic syndrome: time for a critical appraisal: joint statement from the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 28: 2289-2304.

Kawai T and Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11: 373–384.

Kim Y, Kim SH, Whang KY, et al. (2008) Inhibition of *Escherichia coli* O157:H7 attachment by interactions between lactic acid bacteria and intestinal epithelial cells. J Microbiol Biotechnol 18: 1278–1285.

Kim YS, Ho SB (2010) Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep 12: 319–330.

Kleessen B, Bezirtzoglou E, Mättö J (2000) Culture based knowledge on biodiversity development and stability of human gastrointestinal microflora. Microb Ecol Health Dis 12: 53-63.

Koo BK, Stange DE, Sato T, et al. (2011) Controlled gene expression in primary Lgr5 organoid cultures. Nat Methods 9: 81-83.

Koprivnjak T, Peschel A, Gelb MH, et al. (2002) Role of charge properties of bacterial envelope in bactericidal action of human group IIA phospholipase A2 against *Staphylococcus aureus*. J Biol Chem 277: 47636–47644.

Kroon E, Martinson LA, Kadoya K, et al. (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose responsive insulin-secreting cells in vivo. Nat Biotechnol 26: 443-452.

Kühbacher T, Ott SJ, Helwig U, et al. (2006) Bacterial and fungal microbiota in relation to probiotic therapy (VSL#3) in pouchitis. Gut 55: 833–841.

Kuitunen M, Kukkonen K, Savilahti EJ (2009) Pro- and prebiotic supplementation induces a transient reduction in hemoglobin concentration in infants. J Pediatr Gastroenterol Nutr 49: 626-630.

Lakka HM, Laaksonen DE, Lakka TA, et al. (2002) The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. JAMA 288: 2709-2716.

Langendijk PS, Schut F, Jansen GL, et al. (1995) Quantitative fluorescent in situ hybridization of *Bifidobacterium* spp. with genus specific 16S ribosomal-RNA targeted probes and its application in fecal samples. Appl Environ Microbiol 61: 3069-3075.

Lanthier N, Molendi-Coste O, Horsmans Y, et al. (2009) Kupffer cell activation is a causal factor for hepatic insulin resistance. Am J Physiol Gastrointest Liver Physiol 298: 107-116.

Laparra JM and Sanz Y (2009) Comparison of in vitro models to study bacterial adhesion to the intestinal epithelium. Lett Appl Microbiol 49: 695-701.

Lawrence SJ, Korzenik JR, Mundy LM (2005) Probiotics for recurrent *Clostridium difficile* disease. J Med Microbiol 54: 905–906.

Lay C, Sutren M, Rochet V, et al. (2005) Design and validation of 16S rRNA probes to enumerate members of the *Clostridium leptum* subgroup in human faecal microbiota. Environ Microbiol 7: 933-946.

Lebeer S, Vanderleyden J, De Keersmaecker CJ (2010) Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol 8: 171–184.

Leblond-Bourget N, Philippe H, Mangin I, et al. (1996) 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter-and intraspecific *Bifidobacterium* phylogeny. Int J Syst Bacteriol 46: 102-11.

Lee J, Mo JH, Katura K, et al. (2006) Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. Nat Cell Biol 8: 1327–1336.

Lee J, Seto D, Bielory L (2008) Meta-analysis of clinical trials of probiotics for prevention and treatment of pediatric atopic dermatitis J Allergy Clin Immunol 121: 116-121.

Lee K, Paek K, Lee HY, et al. (2007) Antiobesity effect of trans-10,cis-12-conjugated linoleic acid-producing *Lactobacillus plantarum* PL62 on diet-induced obese mice. J Appl Microbiol 103: 1140–1146.

Lenaerts K, Bouwman FG, Lamers WH, et al. (2007) Comparative proteomic analysis of cell lines and scrapings of the human intestinal epithelium. BMC Genomics 8: 91.

Ley RE, Peterson DA, Gordon JI (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124:837–848.

Li Q, Chen Q, Ruan H, et al. (2010) Isolation and characterisation of an oxygen, acid and bile resistant *Bifidobacterium animalis* subsp. *lactis* Qq08. J Sci Food Agric 90: 1340-1346.

Li Z, Yang S, Lin H, et al. (2003) Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. Hepatology 37: 343-350.

Liévin V, Peiffer I, Hudault S, et al. (2000) *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut 47: 646–652.

Lim SM and Im DS (2009) Screening and characterization of probiotic lactic acid bacteria isolated from Korean fermented foods. J Microbiol Biotechnol 19: 178-186.

Lin PP, Hsieh YM, Tsai CC (2009) Antagonistic activity of *Lactobacillus acidophilus* RY2 isolated from healthy infancy feces on the growth and adhesion characteristics of enteroaggregative *Escherichia coli*. Anaerobe 15: 122-126.

Liong MT (2011) Probiotics: Biology, Genetics and Health aspects. Microbiol Monographs.

Liu JE, Zhang Y, Zhang J, et al. (2010) Probiotic yoghurt effects on intestinal flora of patients with chronic liver disease. Nurs Res 59: 426-432.

Lopitz-Otsoa F, Rementeria A, Elguezabal N, et al. (2006) Kefir: a symbiotic yeasts-bacteria community with alleged healthy capabilities. Rev Iberoam Micol 23: 67-74.

Ma X, Hua J, Li Z (2008) Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. J Hepatol 49: 821-30.

MacFaddin JD (1985) Media for isolation-cultivation-identification- maintenance of medical bacteria, vol. 1, pp. 275-284. Williams & Wilkins. Baltimore, MD.

MacFarlane S, Hopkins MJ, Macfarlane GT (2000) Bacterial growth and metabolism on sufaces in the large intestine. Microb Ecol Health Dis 2(Suppl): 64-72.

Mack DR, Ahrne S, Hyde L, et al. (2003) Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells in vitro. Gut 52: 827-33.

Mackie RI, Sghir A, Gaskins HR (1999) Developmental microbial ecology of neonatal gastrointestinal tract. Am J Clin Nutr 69: 1035-45.

Macouzet M, Lee BH, Robert N (2009) Production of conjugated linoleic acid by probiotic *Lactobacillus acidophilus* La-5. J Appl Microbiol 106: 1886–1891.

Mainville I, Arcand Y, Farnworth ER (2005) A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. Int J Food Microbiol 99: 287-296.

Makelainen H, Tahvonen R, Salminen S, et al. (2003) In vivo safety assessment of two *Bifidobacterium longum* strains. Microbiol Immunol 47: 911–914.

Makras L, Triantafyllou V, Fayol-Messaoudi D, et al. (2006) Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar *typhimurium* reveals a role for lactic acid and other inhibitory compounds. Res Microbiol 157: 241–247.

Mantis NJ, Rol N, Corthésy B (2011) Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol 4: 603-611.

Manz W, Amann R, Ludwig W, et al. (1996) Application of a suite of 16S rRNA specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. Microbiology (Reading, Engl.) 142: 1097-1106.

Martin R, Jiménez E, Heilig H, et al. (2009) Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR. Appl Environ Microbiol 75: 965-969.

Martín R, Jiménez E, Olivares M, et al. (2006) *Lactobacillus salivarius* CECT 5713, a potential probiotic strain isolated from infant feces and breast milk of a mother-child pair. Int J Food Microbiol 112: 35-43.

Martin R, Langa S, Reviriego C, et al. (2003) Human milk is a source of lactic acid bacteria for the infant gut. J. Pediatr 143: 754-758.

Martin R, Langa S, Reviriego C, et al. (2004) The commensal microflora of human milk: new perspectives for food bacterio therapy and probiotics. Trends Food Sci. Technol 15: 121-127.

Martinon F, Mayor A, Tschopp J (2009) The inflammasomes: guardians of the body. Annu Rev Immunol 27: 229–265.

Masco L, Crockaert C, van Hoorde K, et al. (2007) In vitro assessment of the gastrointestinal transit tolerance of taxonomic reference strains from human origin and probiotic product isolated of *Bifidobacterium*. J Dairy Sci 90: 3572-3578.

Matsumoto M, Ohishi H, Benno Y (2004) +-ATPase activity in *Bifidobacterium* with special reference to acid tolerance. Int J Food Microbiol 93: 109-113.

Mattar AF, Teitelbaum DH, Drongowski RA, et al. (2002) Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model. Pediatr Surg Int 18: 586-90.

Matto J, Alakomi HL, Vaari A et al. (2006) Influence of processing conditions on *Bifidobacterium animalis* subsp, *lactis* functionality with a special focus on acid tolerance and factors affecting it. Int Dairy J 16, 1029–1037.

Mazmanian SK, Liu CH, Tzianabos AO, et al. (2005) An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 122: 107-118.

McCracken KW, Howell JC, Wells JM, et al. (2011) Generating human intestinal tissue from pluripotent stem cells in vitro. Nat Protoc 6: 1920-1928.

McFarland LV and Dublin S (2008) Meta-analysis of probiotics for the treatment of irritable bowel syndrome. World J Gastroenterol 14: 2650-2661.

Mencarelli A, Distrutti E, Renga B, et al. (2011) Probiotics modulate intestinal expression of nuclear receptor and provide counter-regulatory signals to inflammation-driven adipose tissue activation. PLoS One 6: e22978.

Meylan E, Tschopp J, Karin M (2006) Intracellular pattern recognition receptors in the host response. Nature 442: 39–44.

Mills S, Stanton C, Fitzgerald GF, et al. (2011) Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. Microb Cell Fact 10: S19.

Mimura T, Rizzello F, Helwig U, et al. (2004) Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. Gut 53: 108–114.

Mohamadzadeh M, Olson S, Kalina WV, et al. (2005) Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. Proc Natl Acad Sci USA 102: 2880-2885.

Mukai T, Asasaka T, Sato E, et al. (2002) Inhibition of binding of *Helicobacter pylori* to the glycolipid receptors by probiotic *Lactobacillus reuteri*. FEMS Immunol Med Microbiol 32: 105–110.

Müller CA, Autenrieth IB, Peschel A (2005) Innate defenses of the intestinal epithelial barrier. Cell Mol Life Sci 62: 1297–1307.

Munoa FJ and Pares R (1988) Selective medium for isolation and enumeration of *Bifidobacterium* species. Appl Env Microbiol 54: 1715-1718.

Muñoz JA, Chenoll E, Casinos B, et al. (2011) Novel Probiotic *Bifidobacterium longum* subsp*. infantis* CECT 7210 strain active against rotavirus infections. Appl Environ Microbiol 77: 8775-8783.

Muñoz-Quezada S (2011) Tesis doctoral Universidad de Granada. Aislamiento, identificación y caracterización de *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 y *Lactobacillus rhamnosus* CNCM I-4036, obtenidos a partir de heces de niños alimentados exclusivamente con leche materna.

Muñoz-Quezada S, Chenoll E, Vieites JM, et al. (2013) Isolation, identification and characterisation of three novel probiotic strains (*Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036) from the faeces of exclusively breast-fed infants. Br J Nutr 109: S51-62.

Na X and Kelly C (2011) Probiotics in *Clostridium difficile* Infection. J Clin Gastroenterol 45: S154-158.

Nakamura S, Kuda T, An C, et al. (2012) Inhibitory effects of *Leuconostoc mesenteroides* 1RM3 isolated from narezushi, a fermented fish with rice, on *Listeria monocytogenes* infection to Caco-2 cells and A/J mice. Anaerobe 18: 19–24.

Nanji AA, Khettry U, Sadrzadeh SM (1994) *Lactobacillus* feeding reduces endotoxemia and severity of experimental alcoholic liver (disease). Proc Soc Exp Biol Med 205: 243-247.

Nebra Y and Blanch AR (1999) A new selective medium for *Bifidobacterium* spp. Appl Env Microbiol 65: 5173-5176.

Nesser JR, Granato D, Rouvet M, et al. (2000) *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. Glycobiology 10: 1193–1199.

Neutra MR and Forstner JF (1987) Gastrointestinal mucus: synthesis, secretion and function; in Johnson LR (ed): Physiology of the Gastrointestinal Tract, ed 2. New York, Raven.

Neyrinck AM, Cani PD, Dewulf EM, et al. (2009) Critical role of Kupffer cells in the management of diet-induced diabetes and obesity. Biochem Biophys Res Commun 385: 351-356.

Ng SC, Benjamin JL, McCarthy NE, et al. (2011) Relationship between human intestinal dendritic cells, gut microbiota, and disease activity in Crohn's disease. Inflamm Bowel Dis 17: 2027-2037.

Nielsen DS, Cho GS, Hanak A, et al. (2010) The effect of bacteriocin-producing *Lactobacillus plantarum* strains on the intracellular pH of sessile and planktonic *Listeria monocytogenes* single cells. Int J Food Microbiol 141: S53–S59.

Nolan JP (1979) The contribution of gut-derived endotoxins to liver injury. Yale J Biol Med 52: 127-133.

O´hara AM and Shanahan F (2006) The gut flora as a forgotten organ. EMBO Rep 7: 688-693.

O'neil DA, Porter EM, Elewaut D, et al. (1999) Expression and regulation of the human betadefensins hBD-1 and hBD-2 in intestinal epithelium. J Immunol 163: 6718–6724.

O'Shea EF, Cotter PD, Stanton C, et al. (2012) Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanisms: bacteriocins and conjugated linoleic acid. Int J Food Microbiol 152: 189–205.

Oelschlaeger TA (2010) Mechanisms of probiotic actions – a review. Int J Med Microbiol 300: 57–62.

Ogushi K, Wada A, Niidome T, et al. (2001) *Salmonella enteritidis* FliC (flagella filament protein) induces human betadefensin-2 mRNA production by Caco-2 cells. J Biol Chem 276: 30521–30526.

Ohland CL and Macnaughton WK (2010) Probiotic bacteria and intestinal epithelial barrier function. Am J Physiol Gastrointest Liver Physiol 298: G807–G819.

Olivares M, Díaz-Ropero MO, Gómez N et al. (2006) Oral administration of two probiotic strains, *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711, enhances the intestinal function of healthy adults. Int J Food Microbiol 107: 104 –111.

Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of microorganisms resistant to antibiotics of human clinical and veterinary importance (2002). http://ec.europa.eu/food/fs/sc/scan/out108_en.pdf

Otte JM and Podolsky DK (2004) Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. Am J Physiol Gastrointest Liver Physiol 286: G613–G626.

Ouwehand AC (1998) Antimicrobial components from lactic acid bacteria; in Salminen S, von Wright A (eds): Lactic Acid Bacteria: Microbiology and Functional Aspects. New York, Dekker, pp 139–159.

Ouwehand AC, Salminen S, Isolauri E (2002) Probiotics: an overview of beneficial effects. Antonie van Leeuwenhoek 82: 279–289.

Ouwehand AC, Salminen S, Tolkko S, et al. (2002) Resected human colonic tissue: new model for characterizing adhesion of lactic acid bacteria. Clin Diag Lab Immunol 9: 184–186.

Paik HD, Park JS, Park E (2005) Effects of Bacillus polyfermenticus SCD on lipid and antioxidant metabolisms in rats fed a high-fat and high-cholesterol diet. Biol Pharm Bull 28: 1270-1274.

Parassol N, Freitas M, Thoreux K, et al. (2005) *Lactobacillus casei* DN-114001 inhibits the increase in paracellular permeability of enteropathogenic *Escherichia coli*-infected T84cells. Res Microbiol 156: 256–262.

Patrignani F, Lanciotti R, Mathara JM, et al. (2006) Potential of functional strains, isolated from traditional Maasai milk, as starters for the production of fermented milks. Int J Food Microbiol 107: 1-11.

Perdigon G, Maldonado Galdeano C, et al. (2002) Interaction of lactic acid bacteria with the gut immune system. Eur J Clin Nutr 56: S21–S26.

Perez-Cano FJ, Dong K, Yaqoob P (2010) In vitro immunomodulatory activity of *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713: two probiotic strains isolated from human breast milk. Immunobiology 215: 996-1004.

Pérez-Sánchez T, Balcázar JL, García Y, et al. (2011) Identification and characterization of lactic acid bacteria isolated from rainbow trout, *Oncorhynchus mykiss* (Walbaum), with inhibitory activity against *Lactococcus garvieae*. J Fish Dis 34: 499-507.

Petrof EO (2009) Probiotics and Gastrointestinal Disease: Clinical Evidence and Basic Science. Antiinflamm Antiallergy Agents Med Chem 8: 260-269.

Pillai A and Nelson R (2008) Probiotics for treatment of *Clostridium difficile*-associated colitis in adults. Cochrane Database Syst Rev 23: CD004611.

Plantiga TS, van Maren WWC, van Bergenhenegouwen J, et al. (2011) Differential Toll-like receptor recognition and induction of cytokine profile by *Bifidobacterium breve* and *Lactobacillus* strains of probiotics. Clin Vaccine Immunol 18: 621–628.

Plaza-DIaz J, Gil A (2013) Fibra y microbiota intestinal. I Jornada Universitaria UCM-CLAS sobre fibra dietética. pp. 12-21. ISBN: 978-84-695-6335-9.

Plaza-Diaz J, Gomez-Llorente C, Campaña-Martin L, et al. (2013) Safety and immunomodulatory effects of three probiotic strains isolated from the feces of breast-fed infants in healthy adults: SETOPROB study. Plos One 8:e78111.

Plaza-Diaz J, Gomez-Llorente C, Abadía Molina F, et al. (2014) Effects of *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 on Hepatic Steatosis in Zucker Rats. Plos One, En prensa

Plaza-Díaz J, Martínez Augustín O, Gil A (2013) Foods as sources of mono and disaccharides: biochemical and metabolic aspects. Nutr Hosp 28: 5-16.

Quah BJ and O'Neill HC (2005) Maturation of function in dendritic cells for tolerance and immunity. J Cell Mol Med 9: 643–654.

Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, et al. (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 118:229-41.

Rappé MS and Giovannoni SJ (2003) The uncultured microbial majority. Annu Rev Microbiol 57:369-94.

Reaven GM (1988) Role of insulin resistance in human disease. Diabetes 37: 1595-1607.

Reaven GM (2004) The metabolic syndrome or the insulin resistance syndrome? Different names, different concepts, and different goals. Endocrinol Metab Clin North Am 33: 283-303.

Rivera-Espinoza Y and Gallardo-Navarro Y (2010) Non-dairy probiotic products. Food microbiology 27: 1-11.

Rogosa M, Mitchell JA, Wiseman RF (1951) A selective medium for the isolation and enumeration of oral and fecal lactobacilli. J Bacteriol 62: 132-133

Rolfe RD (1991) Population dynamics of the intestinal tract; in Blankenship LC (ed): Colonization Control of Human Bacterial Enteropathogens in Poultry. San Diego, Academic Press, pp 59–75.

Romanin D, Serradell M, González Maciel D, et al. (2010) Down-regulation of intestinal epithelial innate response by probiotic yeasts isolated from kefir. Int J Food Microbiol 140: 102-108.

Rondon MR, Goodman RM, Handelsman J (1999) The Earth's bounty: assessing and accessing soil microbial diversity. Trends Biotechnol 17: 403-409.

Russell JB and Diez-Gonzalez F (1998) The effects of fermentation acids on bacterial growth. Adv Microb Physiol 39: 205–234.

Ryan KA, Jayaraman T, Daly P, et al. (2008) Isolation of lactobacilli with probiotic properties from the human stomach. Lett Appl Microbiol 47: 269-274.

Sabatté J, Maggini J, Nahmod K, et al. (2007) Interplay of pathogens, cytokines and other stress signals in the regulation of dendritic cell function. Cytokine Growth Factor Rev 18: 5–17.

Sánchez B, Urdaci MC, Margolles A (2010) Extracellular proteins secreted by probiotic bacteria as mediators of effects that promote mucosa-bacteria interactions. Microbiology 156: 3232–3242.

Sang LX, Chang B, Zhang WL, et al. (2010) Remission induction and maintenance effect of probiotics on ulcerative colitis: A meta-analysis. World J Gastroenterol 16: 1908-1915.

Sanz Y (2006) Ecological and functional implications of the acid adaptation ability of *Bifidobacterium*: A way of selecting improved probiotic strains. Int dairy J 17: 1284-1289.

Sartor RB (2006) Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol 3: 390–407.

Sato T, Stange DE, Ferrante M, et al. (2011) Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology 141: 1762-172.

Scardovi V (1986) Genus *Bifidobacterium*. In: Sneath PHA, Mair NS and Sharpe ME, editors. Bergey's Manual of Systematic Bacteriology. pp. 1418–1434.

Schiffrin EJ, Blum S (2002) Interactions between the microbiota and the intestinal mucosa. Eur J Clin Nutr 56: S60–S64.

Schiffrin EJ, Brassart D, Servin AL, et al. (1997) Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. Am J Clin Nutr 66: 515S–520S.

Sghir A, Gramet G, Suau A, et al. (2000) Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl Environ Microbiol 66: 2263-2266.

Sgouras D, Maragkoudakis P, Petraki K, et al. (2004) In vitro and in vivo inhibition of *Helicobacter pylori* by *Lactobacillus casei* strain Shirota. Appl Environ Microbiol 70: 518–526.

Sharma B, Srivastava S, Singh N, et al. (2011) Role of probiotics on gut permeability and endotoxemia in patients with acute pancreatitis. J Clin Gastroenterol 45: 442–448.

Shida K, Kiyoshima-Shibata J, Nagaoka M, et al. (2009) Peptidoglycan from lactobacilli inhibits interleukin-12 production by macrophages induced by *Lactobacillus casei* through Toll-like receptor 2-dependent and independent mechanisms. Inmmunology 128: e858–e869.

Silvi S, Rumney CJ, Rowland IR (1996) An assessment of three selective media for bifidobacteria in faeces. J Appl Bacteriol 81: 561-564.

Smits HH, Engering A, van der Kleij D, et al. (2005) Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J Allergy Clin Immunol 115: 1260-1267.

Solis G, Reyes-Gavilan CG, De los Fernandez N, et al. (2010) Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breastmilk and the infant gut. Anaerobe 16: 307-310.

Song Z, Cai J, Liu Y, et al. (2009) Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. Cell research 19: 1233–1242.

Spence JR, Mayhew CN, Rankin SA, et al. (2011) Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature 470: 105-109.

Srůtková D, Spanova A, Spano M, et al. (2011) Efficiency of PCR-based methods in discriminating *Bifidobacterium longum* ssp. *longum* and *Bifidobacterium longum* ssp. *infantis* strains of human origin. J Microbiol Methods 87: 10-16.

Steinman RM, Hawiger D, Nussenzweig MC (2003) Tolerogenic dendritic cells. Annu Rev Immunol 21: 685–711.

Stetinova V, Smetanova L, Kvetina J, et al. (2010) Caco-2 cell monolayer integrity and effect of probiotic *Escherichia coli* Nissle 1917 components. Neuro Endocrinol Lett 31: 51–56.

Sun Z, Kong J, Hu S, et al. (2012) Characterization of a S-layer protein from *Lactobacillus crispatus* K313 and the domains responsible for binding to cell wall and adherence to collagen. Appl Microbiol Biotechnol 97: 1941-1952.

Sun Z, Liu W, Gao W, et al. (2010) Identification and characterization of the dominant lactic acid bacteria from kurut: the naturally fermented yak milk in Qinghai, China. J Gen Appl Microbiol 56: 1-10.

Svedlund J, Sjodin I, Dotevall G (1988) GSRS – a clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. Dig Dis Sci 33: 129–134.

Takahashi A, Wada A, Ogushi K, et al. (2001) Production of betadefensin-2 by human colonic epithelial cells induced by Salmonella enteritidis flagella filament structural protein. FEBS Lett 508: 484–488.

Takahashi N, Xiao JZ, Miyaji K, et al. (2004) Selection of acid tolerant bifidobacteria and evidence for a low-pH-inducible acid tolerance response in *Bifidobacterium longum*. J Dairy Res 71: 340–345.

Thuy S, Ladurner R, Volynets V, et al. (2008) Nonalcoholic fatty liver disease in humans is associated with increased plasma endotoxin and plasminogen activator inhibitor 1 concentrations and with fructose intake. J Nutr 138: 1452-1455.

Tien MT, Girardin SE, Regnault B, et al. (2006) Anti-inflammatory effect of *Lactobacillus casei* on *Shigella*-infected human intestinal epithelial cells. J Immunol 176: 1228-1237.

Todoriki K, Mukai T, Sato S, et al. (2001) Inhibition of adhesion of food-borne pathogens o Caco-2 cells by *Lactobacillus* strains. J Appl Microbiol 91: 154-159.

Totemeyer S, Foster N, Kaiser P, et al. (2003) Toll-like receptor expression in C3H/HeJ and C3H/HeJ mice during *Salmonella enterica* serovar *typhimurium* infection. Infect Immun 71: 6653–6657.

Touboul T, Hannan NR, Corbineau S, et al. (2009) Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. Hepatology 51: 1754-1765.

Tsai CC, Lin PP, Hsieh YM (2008) Three *Lactobacillus* strains from healthy infant stool inhibit enterotoxigenic *Escherichia coli* grown in vitro. Anaerobe 14: 61-67.

Tuomola EM, Ouwehand AC, Salminen S (1999) The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. FEMS Immunol Med Microbiol 26: 137–142.

U.S. Department of Health & Human Services (2011) National Institutes of Health (NIH) National Center for Biotechnology Information (NCBI) GenBank. http://www.ncbi.nlm.nih.gov/genbank/

Uematsu S, Fujimoto K, Jang MH, et al. (2008) Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. Nat Immunol 9: 769-776.

Ugarte MB, Guglielmotti D, Giraffa G, et al. (2006) Nonstarter lactobacilli isolated from soft and semihard Argentinean cheeses: genetic characterization and resistance to biological barriers. J Food Prot 69: 2983-2991.

Ulsemer P, Toutounian K, Kressel G, et al. (2012) Safety and tolerance of *Bacteroides xylanisolvens* DSM 23964 in healthy adults. Benef Microbes 3: 99-111.

Umesaki Y, Okada Y, Matsumoto S, et al. (1995) Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. Microbiol Immunol 39: 555-562.

Van Aerde J, Alarcon P, Lam W (2003) Tolerance and safety of energy-dense enteral formulae for young children. Int Pediatr 18: 95–99.

Van Tassell ML, Miller MJ (2011) *Lactobacillus* adhesion to mucus. Nutrients 3: 613–636.

Vandamme P, Pot B, Gillis M, et al. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematic. Microbiol Rev 60: 407-438.

Varma P, Dinesh KR, Menon KK, et al. (2010) *Lactobacillus fermentum* isolated from human colonic mucosal biopsy inhibits the growth and adhesion of enteric and foodborne pathogens. J Food Sci 75: M546-M551.

Vélez MP, De Keersmaecker SC, Vanderleyden J (2007) Adherence factors of *Lactobacillus* in the human gastrointestinal tract. FEMS Microbiol Lett 276: 140–148.

Verdam FJ, Rensen SS, Driessen A, et al. (2010) Novel evidence for chronic exposure to endotoxin in human nonalcoholic steatohepatitis. J Clin Gastroenterol 5: 149-152.

Vieites Fernández JM, Muñoz Quezada S, Llamas Company I, et al. (2010) PCT AX090006WO.

Vinderola G, Matar C, Perdigón G (2005) Role of the epithelial cells in the immune effects mediated by Gram-positive probiotic bacteria. Involvement of Toll-like receptors. Clin Diagn Lab Immunol 12: 1075–1084.

Vizoso Pinto MG, Franz CM, Schillinger U, et al. (2006) *Lactobacillus* spp. with in vitro probiotic properties from human faeces and traditional fermented products. Int J Food Microbiol 109: 205-214.

Vizoso Pinto MG, Rodriguez Gómez M, Seifert S, et al. (2009) Lactobacilli stimulate the innate immune response and modulate the TLR expression of HT29 intestinal epithelial cells in vitro. Int J Food Microbiol 31: 86-93.

Vliagoftis H, Kouranos VD, Betsi GI, et al. (2008) Probiotics for the treatment of allergic rhinitis and asthma: systematic review of RCT. Ann Allergy Asthma Immunol 101: 570-579.

Voltan S, Castagliuolo I, Elli M, et al. (2007) Aggregating phenotype in *Lactobacillus crispatus* determines intestinal colonization and TLR2 and TLR4 modulation in murine colonic mucosa. Clin Vaccine Immunol 14: 1138–1148.

Weinstein PD and Cebra JJ (1991) The preference for switching to IgA expression by Peyer's patch germinal center B cells is likely due to the intrinsic influence of their microenvironment. J Immunol 147: 4126-35.

Weiss DS, Raupach B, Takeda K, et al. (2004) Toll-like receptors are temporally involved in host defense. J Immunol 172: 4463-4469.

Weiss G, Christensen HR, Zeuthen LH, et al. (2011) Lactobacilli and bifidobacteria induce differential interferon-β profiles in dendritic cells. Cytokine 56: 520-530.

Wells JM (2011) Immunomodulatory mechanisms of lactabacilli. Microb Cell Fact 10: S17.

West PA, Hewitt JH, Murphy OM (1979) Influence of methods of collection and storage on the bacteriology of human milk. J Appl Bacteriol 46: 269-277.

Westerlund B and Korhonen TK (1993) Bacterial proteins binding to the mammalian extracellular matrix. Mol Microbiol 9: 687–694.

Whelan K, Judd PA, Taylor MA (2004) Assessment of fecal output in patients receiving enteral tube feeding: validation of a novel chart. Eur J Clin Nutr 58: 1030–1037.

Whitman WB, Coleman DC, Wiebe WJ (1998) Prokaryotes: the unseen majority. Proc Natl Acad Sci USA 95: 6578-83.

Wind RD, Tolboom H, Klare I, et al. (2010) Tolerance and safety of the potentially probiotic strain *Lactobacillus rhamnosus* PRSF-L477: a randomised, double-blind placebo-controlled trial in healthy volunteers. Br J Nutr 104: 1806-1816.

Winker S and Woese CR (1991) A definition of the domains Archaea, Bacteria and Eucarya in terms of small subunit ribosomal RNA characteristics. Syst Appl Microbiol 14: 305-310.

Woese CR (1987) Bacterial evolution. Microbiol Rev 51: 221–271.

Won TJ, Kim B, Lim YT, et al. (2011) Oral administration of *Lactobacillus* strains from Kimchi inhibits atopic dermatitis in NC/Nga mice. J Appl Microbiol 110: 1195-1202.

Wu L and Liu YJ (2007) Development of dendritic-cell lineages. Immunity 26: 741–750.

Xie N, Cui Y, Yin YN, et al. (2011) Effects of two *Lactobacillus* strains on lipid metabolism and intestinal microflora in rats fed a high-cholesterol diet. BMC Complement Altern Med 11: 53.

Xu J and Gordon JI (2003) Inaugural article: honor thy symbionts. Proc Natl Acad Sci USA 100: 10452–10459.

Ya T, Zhang Q, Chu F, et al. (2008) Immunological evaluation of *Lactobacillus casei* Zhang: a newly isolated strain from koumiss in Inner Mongolia, China. BMC Immunol 9: 68.

Yadav H, Jain S, Sinha PR (2008) Oral administration of dahi containing probiotic *Lactobacillus acidophilus* and *Lactobacillus casei* delayed the progression of streptozotocin-induced diabetes in rats. J Dairy Res 75: 189-195.

Yan F and Polk DB (2011) Probiotics and immune health. Curr Opin Gastroenterol 27: 496-501.

Yan F, Cao H, Cover TL, et al. (2007) Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. Gastroenterology 132: 562–575.

Yan F, Polk DB (2002) Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. J Biol Chem 277: 50959–50965.

Yildirim Z, Winters DK, Johnson MG (1999) Purification, amino acid sequence and mode of action of bifidocin B produced by *Bifidobacterium bifidum* NCFB 1454. J Appl Microbiol 86: 45–54.

Yoo SR, Kim YJ, Park DY, et al. (2013) Probiotics *L. plantarum* and *L. curvatus* in combination alter hepatic lipid metabolism and suppress diet-induced obesity. Obesity (Silver Spring) 21: 2571-2578.

Yu J, Wang WH, Menghe BL, et al. (2011) Diversity of lactic acid bacteria associated with traditional fermented dairy products in Mongolia. J Dairy Sci 94: 3229-3241.

Zago M, Fornasari ME, Carminati D, et al. (2011) Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. Food Microbiol 28: 1033-1040.

Zavaglia AG, Kociubinsky G, Pérez P, et al. (1998). Isolation and characterization of *Bifidobacterium* strains for probiotic formulation. J Food Protection 61: 865-873.

Zeuthen LH, Fink LN, Frøkiær H (2008) Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-β. Immunology 123: 197–208.

Zeuthen LH, Fink LN, Frokiaer H (2008) Toll-like receptor 2 and nucleotide-binding oligomerization domain-2 play divergent roles in the recognition of gut derived lactobacilli and bifidobacteria in dendritic cells. Inmmunology 124: 489–502.

Zhang H, Sun J, Liu X, et al. (2013) *Lactobacillus paracasei* subsp. *paracasei* LC01 positively modulates intestinal microflora in healthy young adults. J Microbiol 51: 777-782.

Zhang D, Jiang W, Liu M, et al. (2009) Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. Cell Res 19: 429-38.

Zhang MM, Cheng JQ, Lu YR, et al. (2010) Use of pre-, pro- and synbiotics in patients with acute pancreatitis: A meta-analysis. World J Gastroenterol 16: 3970–3978.

Zyrek AA, Cichon C, Helms S, et al. (2007) Molecular mechanisms underlying the probiotic effects of *Escherichia coli* Nissle 1917 involve ZO-2 and PKC redistribution resulting in tight junction and epithelial barrier repair. Cell Microbiol 9: 804–816.

.

.

ANEXOS

British Journal of Nutrition (2013), **109**, S35–S50 doi:10.1017/S0007114512004011

Sources, isolation, characterisation and evaluation of probiotics

Luis Fontana, Miriam Bermudez-Brito, Julio Plaza-Diaz, Sergio Muñoz-Quezada and Angel Gil*

Department of Biochemistry & Molecular Biology II, School of Pharmacy and Institute of Nutrition & Food Technology "José Mataix", Biomedical Research Centre, University of Granada, Granada, Spain

Abstract

Probiotics are live microorganisms that, when ingested in adequate amounts, provide health benefits to the host. The strains most frequently used as probiotics include lactic acid bacteria and bifidobacteria, which are isolated from traditional fermented products and the gut, faeces and breast milk of human subjects. The identification of microorganisms is the first step in the selection of potential probiotics. The present techniques, including genetic fingerprinting, gene sequencing, oligonucleotide probes and specific primer selection, discriminate closely related bacteria with varying degrees of success. Additional molecular methods, such as denaturing gradient gel electrophoresis/temperature gradient gel electrophoresis and fluorescence *in situ* hybridisation, are employed to identify and characterise probiotics. The ability to examine fully sequenced genomes has accelerated the application of genetic approaches to elucidate the functional roles of probiotics. One of the best-demonstrated clinical benefits of probiotics is the prevention and treatment of acute and antibiotic-associated diarrhoea; however, there is mounting evidence for a potential role for probiotics in the treatment of allergies and intestinal, liver and metabolic diseases. These positive effects are generally attributed to the ability of probiotics to regulate intestinal permeability, normalise host intestinal microbiota, improve gut immune barrier function and equilibrate the balance between pro-inflammatory and anti-inflammatory cytokines. However, the positive effects of probiotics are not always substantiated by findings from properly conducted clinical trials. Notably, even when the results from randomised, placebo-controlled trials support the beneficial effects of a particular probiotic for a specific indication, the benefits are generally not translatable to other probiotic formulations.

Key words: **Bifidobacteria: Lactic acid bacteria: Lactobacilli: Probiotics: Diseases**

Currently, there is an increasing interest in and demand for probiotics, after a long history of safe use in fermented dairy products and an increased recognition of the beneficial effects of probiotics to human gut health[1]. According to the FAO of the UN and the WHO[2], probiotics are 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host'. In particular, strains belonging to *Bifidobacterium* and *Lactobacillus*, the predominant and subdominant groups of the gastrointestinal microbiota, respectively[3], are the most widely used probiotic bacteria and are included in many functional foods and dietary supplements[4–6]. The yeast *Saccharomyces boulardii* has also been shown to have health benefits[7].

For probiotics to be successful, they must possess certain characteristics. The criteria for the selection of probiotics include tolerance to gastrointestinal conditions (gastric acid and bile), ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens[8,9]. Traditionally, it has been proposed that a useful probiotic must fulfil the following criteria:

(1) Have a demonstrated beneficial effect on the host.
(2) Be non-pathogenic, non-toxic and free of significant adverse side effects.
(3) Be able to survive through the gastrointestinal tract (GIT; *in vitro* and *in vivo*).
(4) Be present in the product in an adequate number of viable cells to confer the health benefit.
(5) Be compatible with product matrix, processing and storage conditions to maintain the desired properties, and labelled accurately[10].

The results of evidence-based analyses from human studies and animal models have shown the potential clinical effectiveness of probiotics on many diseases[11]. In fact, probiotics have been reported to suppress diarrhoea[12], alleviate lactose intolerance[13] and post-operative complications[14], exhibit antimicrobial[15] and anti-colorectal cancer activities[16,17], reduce irritable bowel symptoms[18] and prevent inflammatory bowel disease[19]. However, generalisations concerning the potential health benefits of probiotics should be not made

Abbreviations: AR, allergic rhinitis; IBS, irritable bowel syndrome; LAB, lactic acid bacteria; NEC, necrotising enterocolitis; RCT, randomised controlled trial; UTI, urinary tract infections.

* **Corresponding author:** Professor A. Gil, fax +34 958 819132, email agil@ugr.es

British Journal of Nutrition

because probiotic effects tend to be strain specific; thus, the health benefit attributed to one strain is not necessarily applicable to another strain, even within one species[20].

The mechanisms underlying the beneficial effects of probiotics are largely unknown but are likely to be multi-factorial. However, several important mechanisms underlying the antagonistic effects of probiotics on various microorganisms include modification of the gut microbiota, competitive adherence to the mucosa and the epithelium, strengthening of the gut epithelial barrier and modulation of the immune system to convey an advantage to the host.

The aim of the present work was to review the sources, isolation methodology, characterisation and evaluation of probiotic strains. The various steps needed to characterise a bacterial strain as a novel probiotic are depicted in Fig. 1.

In the present study, we sought to conduct a literature review of the sources, isolation and characterisation and evaluation of probiotic strains. The present review summarises a total of 1500 works, published to the date from PubMed database (February 2012), and intends to provide an historical context and the state of this field. For this aim, the following search combinations were used: probiotics and sources; lactobacillus and isolation; bifidobacteria and isolation; probiotics and breast milk; probiotics and origin probiotics and fermented foods; isolation and characterisation and probiotics; probiotics and evaluation; and probiotics and randomised controlled trial (RCT).

Sources

Dairy and dairy-related products are a good source of probiotics[1]. Within this context, lactic acid bacteria (LAB), bifidobacteria and other microorganisms obtained from fermented milks have been used for centuries. Spontaneous milk fermentation has a long history in different regions of Mongolia or Africa, and the use of beneficial microorganisms in fermented dairy products has been practised for many generations[21].

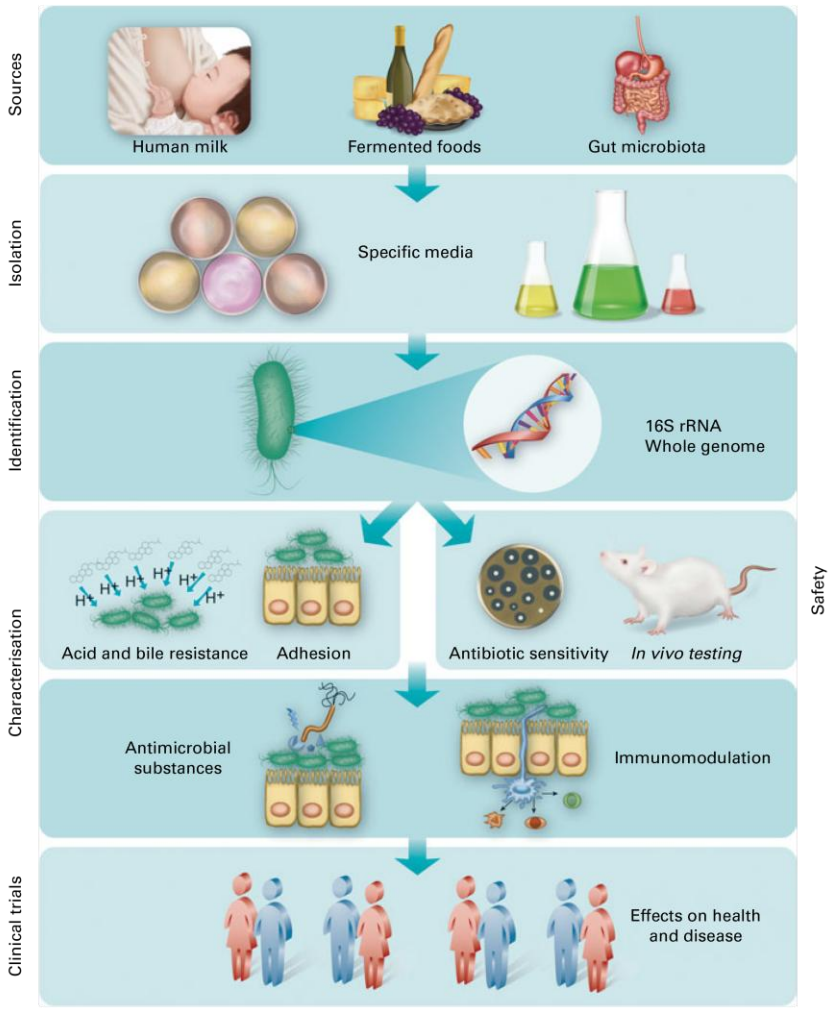


Fig. 1. Flow chart describing the various steps to be followed in order for a bacterial strain to qualify as a novel probiotic. rRNA, ribosomal RNA.

British Journal of Nutrition

These traditional fermented milks contain complex compositions of LAB species and therefore provide a useful source of probiotic strains. Thus, it is not surprising that in a recent work, 148 LAB strains were isolated from Kurut, a traditional naturally fermented yak milk from China in which *L. delbrueckii* subsp *bulgaricus* and *Streptococcus thermophilus* are the predominant microbial populations[22]. In addition, yeasts and lactobacillus strains with probiotic properties have been isolated from kefir grains, Masai milk and Koumiss, a fermented milk drink; these microorganisms are able to influence immune responses[23–26].

Recent studies were conducted to evaluate traditional fermented products as potential natural sources of probiotic bacteria. Generally, most of the microorganisms isolated from fermented products belong to the *Lactobacillus* genus[27–29]. Interestingly, in a recent work, a *Weisella* strain was isolated from Nigerian fermented foods and selected for its probiotic potential[30].

Cheese is a dairy product with potential for the delivery of probiotic microorganisms into the human intestine. *L. plantarum* strains have been isolated from Italian, Argentinean and Bulgarian cheeses[31–33].

Interestingly, it was observed that breast milk is not sterile, even when collected aseptically, which raises the possibility that breast milk harbours a natural bacterial inoculum[34]. The bacteria in breast milk have long been considered to be a consequence of skin or faecal contamination. Although the lactobacilli present in human milk are genotypically different from those isolated from the skin[35,36], and the LAB strains present in breast milk were also observed in the faeces of the corresponding infants[37], it has only recently become accepted that breast milk constitutes an interesting source of probiotic LAB and bifidobacteria for inclusion in infant formulas and foods targeted to both pre-term and full-term infants[38]. In addition, it has been reported that breast-fed infants have fewer allergies and gastrointestinal infections than formula-fed infants; therefore, the intestinal microbiota in the breast-fed infant might be considered to be ideally healthy[39]. Human breast milk comprises several predominant bacteria, such as staphylococci, streptococci, micrococci, lactobacilli, enterococci, lactococci and bifidobacteria[36,37,39–42], and its intake favours the predominance of bifidobacteria and lactobacilli in the infant intestinal microbiota. Several authors have reported that lactobacilli isolated from breast milk are an efficient alternative to the use of commonly prescribed antibiotics for the treatment of infectious mastitis during lactation[43,44]. Moreover, it was reported that two *Lactobacillus* strains isolated from human breast milk enhanced natural and acquired immune responses through the activation of the natural killer and T-cell subsets and the expansion of regulatory T cells[42].

Another source of probiotics is the human GIT. More than 500 different bacterial species reside in the adult human gut. In fact, many of the probiotic strains used today have been isolated from this source, such as *L. gasseri* and *L. reuteri*[45]. In addition, it has been reported that *L. fermentum*, isolated from human colonic mucosal biopsy samples, possesses antimicrobial activities against food-borne pathogens[46]. A common misconception is that probiotics must always colonise the intestinal tract to exert their effects. In fact, certain probiotics (e.g. *B. longum* and *Bacteroides thetaiotaomicron*) reside in the human intestinal microbiota, but others (e.g. *L. casei* and *B. animalis*) do not[47]. Most of the probiotic strains, such as *B. longum*[48] and *L. acidophilus* RY2[49], were isolated from the faecal samples of healthy adults and infants, respectively. Notably, in concordance with breast milk, several studies have reported the isolation of probiotics from breast-fed infant faeces[50,51].

The isolation of probiotics is not limited to the human tract. The guts of several animal species, including pigs, rats and even poultry, are good sources of probiotics[52]. Recently, *L. johnsonii* CRL 1647, isolated from the *Apis mellifera* L. bee gut, was shown to exhibit a beneficial effect on honeybee colonies[53]. Additionally, probiotic strains have been obtained from the intestinal tracts of marine and freshwater fish, such as *Carassius auratus gibelio*[54], rainbow trout[55] or shrimp[56].

Other studies show that probiotic strains are also found in non-dairy fermented substrates[57]. *In vitro* experiments have demonstrated that certain bacterial strains, isolated from meat (*L. sakei*, *L. curvatus* and *Staphylococcus carnosus*) and fruits (*L. paracasei* and *L. plantarum*), can display functional and metabolic properties similar to those of human intestinal bacteria[58]. In addition, a recent work described the isolation of a *Lactobacillus* strain from brines of naturally fermented Aloreña green table olives[59]. Moreover, *L. buchneri* P2, isolated from pickled juice, demonstrated probiotic properties, such as cholesterol reduction, acid and bile tolerance and antimicrobial activity[60].

Isolation, identification, characterisation and safety

In microbial ecology, it is generally accepted that cultivation-based approaches provide an incomplete picture of microbial diversity. Ecological niches present a complex interrelation between the different species of microbes that cannot be mimicked using traditional culture methods. Molecular approaches that bypass the cultivation step have become popular as a means of identifying the microbial diversity of different sources. These methods have provided important information concerning microbial ecosystems, including the sources of probiotics. The first important step in studying an ecosystem is the isolation of its members. The identification of the microbes, especially in probiotic bacteria, is not valuable when we want to determine *in vivo* functions associated with beneficial effects on human health.

Isolation

The first step in the isolation of probiotic bacteria is to maintain the sample in adequate conditions before incubation in selective media. Most probiotics are anaerobic or facultatively anaerobic; therefore, the samples should be immediately placed under anaerobic conditions and processed as soon as possible (within 3 h). The samples should be homogenised quickly and diluted and cultured in selective media (Sergio Muñoz-Quezada, Empar Chenoll, Jose Maria Vieites, Salvador Genoves, Jose Maldonado, Miriam Bermudez-Brito, Carolina Gomez-Llorente, Esther Matencio, Maria Jose Bernal, Fernando Romero, Antonio Suárez, Daniel Ramon, Angel Gil, unpublished results).

Several media have been devised for the elective or selective isolation of bifidobacteria and lactobacilli[61–70]. Rogosa *et al.*[68] developed a selective medium for the isolation and enumeration of oral and faecal lactobacilli and *Bifidobacterium* that contains a Columbia agar base supplemented with propionic acid. The low pH of this medium, which is tolerated by lactobacilli and bifidobacteria, inhibits the growth of other predominating organisms in human faeces, such as *Bacteroides* and *Eubacterium* species.

The plates are incubated at 37°C for 48–72 h in an anaerobic atmosphere for the growth of bifidobacteria and other anaerobic species or in a CO_2-rich atmosphere for the growth of lactobacilli. Subsequently, the colonies are isolated and transferred to broth or a new agar plate.

Identification

The identification of microbes in the GIT or food sources is the first step in the selection of potential probiotics. For many ecosystems, only a small percentage of microbes can be grown in culture[71]. The taxonomic classification might be defined as the process of cataloguing biodiversity based on a polyphasic approach[72], which involves genotypic and phenotypic methods. Historically, phenotypic methods have been used to identify bacteria. The taxonomy for many decades heavily relied on the type of sugar fermentation and the fermentation products generated. Thus, the probiotics have been primarily classified as LAB. Today, 16S RNA gene analysis has become the method of choice. For the past two decades, microbiologists have used this conserved fragment for phylogenetic classification[73,74], and the relatedness among organisms is estimated through the comparison of their sequences in available databases (DDBJ, ENA, GenBank)[75–77]. The 16S RNA gene analysis has been combined with other methods to identify bacterial communities of the gut or ecological sources. The amplified 16S DNA can be coupled with PAGE using temperature (temperature gradient gel electrophoresis) or chemical denaturation (denaturing gradient gel electrophoresis)[78], hybridised using fluorescent oligonucleotide probes that target specific 16S (fluorescence *in situ* hybridisation)[79,80] or digested with restriction enzymes (Terminal restriction fragment length polymorphism (T-RFLP)).

However, the 16S DNA fragment is extremely small (1500 bp) compared with the bacterial genome (30 000–40 000 bp). Complementary information is typically necessary due to insufficient base sequence diversity to differentiate strains of a given species. The 16S to 23S intergenic spacer region exhibits a great deal of sequence and length variation[81]. The variation in this region has been used for differentiating species of prokaryotes. Undoubtedly, the analysis of the bacterial genome is the most useful tool to identify and characterise the processes underlying speciation and evolution in prokaryotes[82]. However, genome sequencing remains a laborious and relatively expensive technique.

Characterisation

The species of the genera *Lactobacillus* and *Bifidobacterium* are among the most important taxa of probiotics. When ingested, sufficient numbers of metabolically active bacteria must overcome the GIT barrier and transitorily persist in the GIT to exert their beneficial effects. This characteristic is important, although certain authors have shown beneficial effects of dead probiotics[83]. The capacity to tolerate an extremely low pH (1·5–3·0), gastric enzymes, bile salts and other intestinal enzymes, are the challenges for arriving alive in the GIT[84]. Various *in vitro* assays have been designed to mimic these stress conditions.

Resistance to low pH and biliary salts. Acid tolerance is one of the general criteria that is considered during the selection of potential probiotic strains to guarantee their viability and functionality[85]. *In vitro* systems, including controlled incubations in real or simulated gastric juices (pH 2·0–4·0; 70–180 min[86]), have been preferentially used in the evaluation of new probiotic strains. Complex models that simulate gastrointestinal transit have been developed[84,87]. Moreover, 1–4 h incubations in chemical and/or enzymatic media at a pH range of 1·5–3·0 have also been performed.

The biliary salts facilitate the digestion of lipophilic compounds, but also behave as an antimicrobial agent by directly influencing the establishment of the intestinal microbiota. The relevant physiological concentrations of human bile range from 0·3 to 0·5%[88,89]. *In vitro* assays are conducted in 0·3–0·7% bovine bile (Oxgall) for 60–180 min. Probiotics show highly variable resistance to acid and bile salts, and this characteristic is both species and strain dependent. Several studies have reported that bifidobacteria are highly sensitive to low pH values. Certain species exhibit survival rates of 0% at pH 2·0 for 90 min[86,90], less than 1% at pH 3·0 for 2 h[91] or increased percentages at pH 3·0–5·0 for 3 h[92]. The highest survival rates have been described for certain bifidobacteria[93–96]. Several *Lactobacillus* strains have shown a high resistance to low pH. A study involving twenty *Lactobacillus* strains reported survival rates of 2–100% at pH 3·0 for 1 h. Certain bifidobacteria demonstrated a survival rate of 1–70% in 0·3% Oxgall for 90 min[87]. A total of two *L. plantarum* strains showed greater than 50% survival at pH 2·0 and 3·0 and 1·0% survival in 73–180% bile salt[97].

Bacteria develop an adaptive response under moderate stress conditions, such as nutrient-rich or nutrient-poor media, pH and salt content[98]. Surprisingly, the modulation of protein complexes, transduction of signals or induction of genes[99] might be used to modify food features[100].

Adherence to intestinal epithelial cells. The adherence to intestinal epithelial cells and/or mucus is an important characteristic of probiotics to promote the gut residence time, pathogen exclusion and host and immune system interactions. Over the past 25 years, the Caco-2 cell line has been used extensively to determine adhesion capacity[101]. Caco-2 cells form a homogeneous monolayer that resembles that of human mature enterocytes in the small intestine[102]; they also form crypts, which are typical structures of the epithelial monolayer[103]. The colonic cell line HT-29 also displays typical characteristics of enterocyte differentiation and has been used for *in vitro* adhesion assays[104]. Adhesion to the intestinal mucosa is based on the immobilisation of mucin bound to the surface of microwell plates[105,106] in several commercially

available *in vitro* assays, whereas other useful *in vitro* models utilise cell lines developed to simulate a mucus-secreting environment (HT-29-MTX)[107,108]. The results of *in vitro* adhesion models, cell lines or their combination are highly variable[109]. In fact, lactobacilli, bifidobacteria and pathogens show differences in adhesion to mucus, Caco-2, Caco-2 plus mucus, HT-29 MTX and Caco-2/HT29MTX. For *L. rhamnosus* GG, the reported capacities for adhesion in those systems are 10·21, 5·17, 3·19, 0·84 and 0·85 %, respectively. Several *in vitro* studies have evaluated the adhesion of potential probiotic bacteria and their interactions with pathogens at the intestinal epithelial interface, and the results were dependent on the technique and strains used[105].

Differences in the experimental conditions for assays of acid tolerance (medium acidified using HCl or lactic acid, with or without enzymes), bile resistance (bile origin and dose) and adhesion (mucus, cell lines, cells plus mucus) make it extremely difficult to compare their results. Remarkably, these characteristics are strain-specific traits that can be extremely variable within the species or genus. Therefore, the use of *in vitro* models is necessary to select the most promising strains. Thus, human clinical trials are the definitive tool to establish probiotic functionality.

Antimicrobial activity. When administered in adequate amounts, probiotics confer health benefits to the host[85]. An important beneficial effect is antimicrobial activity against pathogens[109]. Probiotics might act through a variety of mechanisms, including the production of antimicrobial substances, competition with pathogens for nutrients and adhesion sites and stimulation of the immune system[110].

Intestinal infections are mediated by the adhesion of pathogenic bacteria to mucosal surfaces and disruption of the intestinal microbiota. The probiotic bacteria might play protective roles through adhesion and colonisation of the mucosal surfaces, effectively competing with pathogens for binding sites and nutrients or/and immune stimulation[111,112]. Ferreira *et al.*[113] evaluated the ability of seven newly isolated strains of *L. gasseri* to adhere to intestinal mucosa and to auto-aggregate and co-aggregate with the model pathogens *Cronobacter sakazakii* (ATCC 29 544) and *Clostridium difficile* (ATCC 1296). All of the viable and non-viable bacterial strains used alone or in combination were able to auto-aggregate. The co-aggregation with *C. sakazakii* or *C. difficile* was higher ($P<0·05$) in the non-viable bacterial strains.

The ability of probiotic strains to inhibit the growth of pathogens in broth and agar plates and to modulate the production of cytokines and growth factors in cell lines has been well documented using *in vitro* models in the evaluation of their biological effects. In addition, mice and other animal models are also useful to study the antimicrobial activity of probiotics. The antimicrobial effects of novel probiotics have been tested against *Listeria monocytogenes* and *Helicobacter pylori in vitro*, and against human rotavirus using *in vivo* infection models[94,95]. Several strains of lactobacilli and bifidobacteria successfully inhibited the growth of *Escherichia coli*[104,114–117], *Salmonella typhimurium*, *Shigella flexneri*[118–120] and *C. difficile*[121]. Moreover, an *L. plantarum* strain produced compounds with antifungal activity[122].

Notably, in these studies, single strains were tested and the antimicrobial activities in most cases were due to the mixed host immune modulation and anti-infective activity of probiotics.

Although, in clinical studies, the use of probiotics is promising for the management of diarrhoea, *H. pylori* infection, atopic disease, necrotising enterocolitis (NEC) and inflammatory bowel diseases, there are uncertainties concerning the relative significance of probiotics, and the results from meta-analyses to determine the beneficial effects of probiotics[123] are contradictory. Moreover, individual or mixtures of probiotic strains and the required dosages need to be determined, and additional clinical trials should be conducted to improve the available information.

Safety

Detailed reviews and opinions of present practices in the safety assessment of probiotics for human subjects have been published[124,125]. The European Food Safety Authority was established in 2002 to address the increasingly important and complex scientific and technical issues concerning food and feed safety in the European Union (regulation no. 178/2002), but no formal safety testing guidelines for food-associated microbes have been established. The Scientific Committee on Animal Nutrition proposed the 'qualified presumption of safety'[126] as an approach to safety evaluation, which involves four steps: (1) defining the taxonomy of the microbe; (2) collecting sufficient information providing the basis for qualified presumption of safety status, including scientific literature, history of use, industrial applications and ecological and human intervention data; (3) excluding pathogenicity and (4) defining the end use. If there are no safety concerns for a certain taxonomic group, or if any safety concerns have been allayed (qualification), then qualified presumption of safety status may be granted[127].

The LAB will be among the first groups to be evaluated. The introduction of this system appears to be favourably received and is considered to be more flexible than the Generally Recognised As Safe system used in the United States because it considers new emerging safety risks, such as the acquisition of antibiotic resistance and virulence determinants.

A variety of factors are considered in the assessment of the safety of probiotics, which include the following: (1) recording the isolation history and taxonomic classification of candidate probiotics, (2) manufacturing controls that eliminate contamination (including cross-contamination between batches) of probiotics with microbes or other substances, (3) assessing the association of probiotics with infectivity or toxicity at the strain level and (4) determining the physiological status of the consuming population, with special consideration for use in vulnerable populations, including newborn infants and the critically ill (dose administered and method of administration). When considering all of these factors, probiotics are generally considered to be 'safe', but this assumption cannot be made broadly, and such an assessment is specific to the many conditions indicated earlier. To market probiotics as

British Journal of Nutrition

British Journal of Nutrition

foods or dietary supplements, the safety of each particular strain for the general population[128] needs to be determined.

Industrial production of probiotics

The next step after a probiotic strain has been isolated, identified and characterised, and its safety has been approved, is scale-up production. Industrial production relies on two aspects. First, the microorganism needs to be cultured in adequate medium to allow growth in large quantities. Second, probiotic viability during manufacturing has to be secured. Both aspects are important, and scale-up production may become a bottleneck for an initially promising microorganism. Thus, certain strains might not grow properly, stand freeze- or spray-drying processes, or addition of preservatives to maintain viability throughout the shelf-life of the manufactured product.

Evaluation of probiotics

Preclinical evaluation

There is substantial evidence from *in vitro* and animal studies that known and potential probiotics exhibit strain-specific immunomodulatory effects.

In vitro studies. A large inventory of animal and human cell lines is available as models of the gut[129], such as Caco-2, HT-29, IEC-6, IEC-18 and T84, to name a few. In most of the *in vitro* experimental models, the epithelial cells are cultivated as monolayers in which the establishment of a functional epithelial feature is not achieved.

To overcome this problem, researchers have attempted to reconcile the mechanisms underlying the complex and dynamic interactions between the intestinal epithelium and bacteria on the luminal side, and the epithelium and cells of the immune system on the basolateral side, using co-culture experiments with probiotics, dendritic cells, intestinal epithelial cells[130] and 3D models[129]. The 3D models are generated using an intestinal epithelial cell line of non-carcinogenic origin, which is cultured on a microporous membrane, enabling the polarisation of the cells. Below the microporous membrane (basolateral side), the epithelial cells are underlaid with immune cells (macrophages, dendritic cells), mimicking mucosal lymphoid tissue. Intestinal microbiota are added to the apical side of the membrane to study the effects of the microbiota. These three components (epithelia, immune cells and microbiota) are the most important factors in the gut; therefore, these models closely mimic the *in vivo* situation.

Animal studies. The immunomodulatory effects of probiotics have been demonstrated in experimental models of allergy, autoimmunity and inflammatory bowel disease[130]. Probiotic supplementation has exhibited protective effects during spontaneous and chemically induced colitis by down-regulating the production of inflammatory cytokines or inducing regulatory mechanisms in a strain-specific manner. In animal models of allergen sensitisation and murine models of asthma and allergic rhinitis (AR), orally administered probiotics have demonstrated a strain-dependent decrease in IgE production by modulating systemic cytokine production. Certain probiotics have been shown to decrease airway hyperresponsiveness and inflammation through the induction of regulatory mechanisms.

Clinical evaluation

Many clinical studies have attempted to evaluate a great variety of probiotics under diverse physiological conditions and pathologies. However, many of these studies are flawed due to the small number of patients used or the lack of a control group. In fact, the European Food Safety Authority delivers scientific opinions on the substantiation of health claims related to probiotic strains. A high percentage of these claims is rejected by the European Food Safety Authority because a cause and effect relationship is not clearly established between the consumption of the probiotic and the beneficial effect it is supposed to have (mostly due to the small number, or even lack, of human intervention studies demonstrating such effects). The most reliable method of assessing the therapeutic benefits of any probiotic strain is the use of randomised, placebo-controlled trials, which are reviewed later and appear in Table 1.

Pregnancy and lactation. Asemi *et al.*[131] assessed the effects of the daily consumption of probiotic yoghurt on inflammatory factors in pregnant women. The subjects consumed 200 g of probiotic yoghurt containing *L. acidophilus* La5 and *B. animalis* BB12 or 200 g of conventional yoghurt daily for 9 weeks. The consumption of the probiotic yoghurt significantly decreased the expression of C-reactive protein, but had no effect on TNF-α in these subjects. In addition, the consumption of probiotic yoghurt among pregnant women resulted in increased levels of erythrocyte glutathione reductase but did not affect other indices of oxidative stress.[132]

Dugoua *et al.*[133] reported that *Lactobacillus* and *Bifidobacterium* had no effect on the incidence of Caesarean section, birth weight or gestational age.

As mentioned in the 'Sources' section of the present review, lactobacilli isolated from breast milk are an efficient alternative to the use of commonly prescribed antibiotics for the treatment of infectious mastitis during lactation[43,44]

Allergy. Vliagoftis *et al.*[134] evaluated the clinical evidence for the use of probiotics as a therapeutic modality for AR and asthma. The review included twelve RCT. A total of nine trials that evaluated clinical outcomes in AR showed an improvement due to the use of probiotics. All of the trials concerning perennial AR showed reduced symptom scoring and medication use with the administration of probiotics compared with the placebo. Moreover, in the five trials concerning seasonal AR, an improvement in the clinical outcomes was shown. The nine studies that reported various immunologic measurements of allergy showed no significant probiotic effects. The trials concerning the effect of probiotic administration on the treatment of asthma showed no positive effects. Taken together, these results suggest that probiotics might

British Journal of Nutrition

Table 1. Summary of selected human studies evaluating probiotic strains

Study	Probiotics	Main outcomes
Pregnancy and lactation		
Asemi *et al.*[131]	*Lactobacillus acidophilus* La5 *Bifidobacterium animalis* BB12	↓ C-reactive protein
Asemi *et al.*[132]	*L. acidophilus* La5 *B. animalis* BB12	↑ Erythrocyte glutathione levels
Dugoua *et al.*[133]	*Lactobacillus* *Bifidobacterium*	No effects on birth weight, gestational age or incidence of C-section
Arroyo *et al.*[43] Jiménez *et al.*[44]	Lactobacilli from breast milk	↓ Mastitis during lactation
Allergy		
Vliagoftis *et al.*[134]	*B. longum* *L. acidophilus* *Bacillus clausii* *L. paracasei* *L. casei* *L. rhamnosus*	↓ Symptom severity of allergic rhinitis and medication use
Kuitunen *et al.*[135]	*L. rhamnosus* GG *L. rhamnosus* LC705 *B. breve* Bb99 *Propionibacterium freudenreichii ssp shermanii* JS	↓ Hb in infants Negative correlation between Hb values at 6 months and faecal calprotectin at age 3 months
Martínez-Cañavate *et al.*[136]	*L. gasseri* CECT5714 *L. coryniformis* CECT5711	↓ Plasma Ig E, ↑ mucosal Ig A ↑ CD4 + /CD25 + T cells ↑ Natural killer cells
Boyle *et al.*[138]	*L. rhamnosus* GG	No benefit in the treatment of eczema in children Risk of adverse effects
Lee *et al.*[139]	*L. rhamnosus* GG	Effective in prevention but not treatment of pediatric atopic dermatitis
Intestinal-related diseases		
Olivares *et al.*[137]	*L. gasseri* CECT5714 *L. coryniformis* CECT5711	Improvement in intestinal habits
Allen *et al.*[140]	*L. casei* strain GG *Saccharomyces boulardii* *Enterococcus* LAB SF68	↓ Duration and ↓ stool frequency in acute infectious diarrhoea
Johnston *et al.*[141]	*Bacillus* spp. *Bifidobacterium* spp. *Lactobacillus* spp. *Lactococcus* spp. *Leuconostoc cremoris* *Saccharomyces* spp. *Streptococcus* spp.	Protective effect in preventing antibiotic-associated diarrhoea
Bernaola Aponte *et al.*[142]	Lactobacilli Bifidobacteria Lactococci *Saccharomyces*, etc	↓ Duration and ↓ stool frequency in persistent diarrhoea
Alfaleh *et al.*[143]	Mainly lactobacilli	↓ Incidence and mortality in necrotising enterocolitis
Braga *et al.*[144]	*L. casei* *B. breve*	Benefit on the occurrence of necrotising enterocolitis Improvement in intestinal motility
Sang *et al.*[145]	*B. bifidum*	↑ Remission rate and ↓ recurrence rate of ulcerative colitis
Mimura *et al.*[146]	VSL#3	Effective in maintaining antibiotic-induced remission in patients with pouchitis for 1 year
Kühbacher *et al.*[147]	VSL#3	↑ Total number of intestinal bacteria in pouchitis ↑ Richness and diversity of the bacterial microbiota, especially the anaerobic microbiota Repression in fungal microbiota
Doherty *et al.*[148]	VSL#3 *Lactobacillus rhamnosus* GG *L. johnsonii* LA1	No effect

Table 1. *Continued*

Study	Probiotics	Main outcomes
McFarland & Dublin[149]	Mainly lactobacilli and bifidobacteria	Improvement in IBD symptoms ↓ Abdominal pain
Gawrońska *et al.*[150]	*L. rhamnosus* GG	↓ Frequency but not the severity of pain in children with IBD
Bausserman & Michail[151]	*L. rhamnosus* GG	↓ Incidence of abdominal distention in children with IBD
Enck *et al.*[152]	*Enterococcus faecalis* *Escherichia coli*	↓ Typical symptoms of IBD
Chronic liver disease		
Liu *et al.*[153]	*Bacillus bifidus* *L. acidophilus* *L. bulgaricus* *S. thermophilus*	↓ *E. coli* count, ↓ intestinal flora imbalance Improved symptoms and signs (debilitation, food intake, appetite, abdominal distension and ascitic fluid)
Aller *et al.*[154]	*L. bulgaricus* *S. thermophilus*	Improved liver aminotransferases in NAFLD patients
Acute pancreatitis		
Zhang *et al.*[155]	*L. plantarum* 299 *Pediococcus pentosaceus* *Leuconostoc mesenteroides* *L. paracasei* *L. plantarum*	↓ Length of hospital stay
Sharma *et al.*[156]	*L. acidophilus* *B. longus* *B. bifidum* *B. infantalis*	No effects
Type 2 diabetes		
Ejtahed *et al.*[157]	*L. acidophilus* La5 *B. lactis* Bb12	↓ Total cholesterol, LDL-C and atherogenic indices
AIDS		
Hummelen *et al.*[158]	*L. rhamnosus* GR-1 *L. reuteri* RC-14	No impact on the immune function of HIV-infected women who were naïve to anti-retroviral treatment
Trois *et al.*[159]	*B. bifidum* *S. thermophilus*	Preservation of the immune function of HIV-infected children
Anukam *et al.*[160]	*L. rhamnosus* GR-1	Preservation of the immune function of women naïve to anti-retrovirals
Urinary tract infections		
Stapleton *et al.*[161]	*L. crispatus*	↑ Vaginal colonisation, ↓ recurrent urinary tract infections
Respiratory infections		
Hao *et al.*[162]	*L. casei* DN-114 001	↓ Episodes of acute upper respiratory infections ↓ Antibiotic use
Siempos *et al.*[163]	Lactobacilli *P. pentosaceus* *Leuconostoc mesenteroides*	↓ Incidence of ventilator-associated pneumonia
Spondyloarthritis		
Jenks *et al.*[164]	*S. salivarius* *B. lactis* *L. acidophilus*	No benefit over placebo

↓, Decrease; ↑, increase; LAB, lactic acid bacteria; IBD, inflammatory bowel disease; NAFLD, non-alcoholic fatty liver disease.

have a beneficial effect in AR by reducing symptom severity and medication use.

In a study examining the effect of pre- and probiotics on the prevention of atopic disease, Kuitunen *et al.*[135] conducted a randomised study of 1223 eligible mothers carrying a child with a high risk for allergy (at least one parent with doctor-diagnosed asthma, AR or atopic eczema). Each subject received twice daily a probiotic combination of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bb99 and *Propionibacterium freudenreichii ssp shermanii* JS or placebo 4 weeks before delivery. Their infants received the same probiotics and 0·8 g of a galacto-oligosaccharide or placebo once daily from birth until 6 months of age. The children were observed until 2 years of age for the development of any allergic disease. Blood samples were obtained from ninety-eight infants at 6 months and 658 children at 2 years of age to measure

British Journal of Nutrition

the haematologic values. Faecal samples were collected at 3 and 6 months of age to measure immunologic development by the expression of calprotectin, α-1-antitrypsin, TNF-α and IgA. At 6 months of age, the infants in the probiotic group had significantly lower Hb values than the placebo group. A significant negative correlation emerged between the Hb values at 6 months of age and the expression of faecal calprotectin at 3 months of age. The hematologic values in both groups were similar at 2 years of age.

Martínez-Cañavate *et al.*[136] evaluated the immunological effects of two probiotic strains, *L. gasseri* CECT5714 and *L. coryniformis* CECT5711, in children suffering with allergies. Olivares *et al.*[137] previously described a double-blinded, randomised, placebo-controlled comparative study with forty-four allergic children, who were randomly distributed into two groups: a yoghurt group and a probiotic group. In the present study, intestinal and immunological parameters were measured in faecal and blood samples. The consumption of the probiotic product induced a significant decrease in the level of IgE in the plasma and an increase in CD4 + / CD25 + T regulatory cells. The decrease in IgE was accompanied by a significant increase in mucosal IgA. Changes in other effector cells potentially involved in allergic reactions, such as eosinophiles, basophiles or other IgE + cells, were not detected. The consumption of the probiotic product also induced significant changes in the innate response, as a significant increase in natural killer cells was detected.

No evidence suggests that probiotics are an effective treatment for eczema in children; probiotic treatment carries a small risk of adverse events (infections and bowel ischaemia) and does not show any benefit in comparison with the placebo[138]. A meta-analysis of six prevention and four treatment double-blind, randomised, placebo-controlled clinical trials in children with an age ranging from 0 to 13 years indicated that present evidence favours the use of probiotics for the prevention but not the treatment of paediatric atopic dermatitis[139]. There was a 61 % risk reduction associated with the use of prenatal and/or postnatal probiotics for paediatric atopic dermatitis prevention. An additional analysis, which excluded the single study using a postnatal protocol, revealed a lower relative risk ratio. This result suggests that a prenatal component might be clinically important for maximising the prophylactic potential of probiotics. In terms of treatment, the summary effect size derived for both intergroup and intragroup differences failed to show any statistical significance.

Intestinal-related diseases

Intestinal function. Olivares *et al.*[137] investigated the effect of a fermented product containing two probiotic strains, *L. gasseri* CECT5714 and *L. coryniformis* CECT5711, on several blood and faecal parameters related to intestinal function in the host. A total of thirty healthy volunteers were randomly distributed into two groups, one receiving a standard yoghurt and the other a similar dairy fermented product in which the *L. delbreuckii* subsp. *bulgaricus* yoghurt strain had been replaced by a combination of the probiotic strains *L. gasseri* CECT5714 and *L. coryniformis* CECT5711. The volunteers that received the probiotics reported no adverse effects, and the strains could be isolated from their faeces at a relatively high level. In fact, the concentration of faecal LAB significantly increased in the probiotic group. Additionally, the oral administration of the probiotics led to an improvement in several parameters, such as the production of SCFA, faecal moisture and frequency and volume of the stools. As a result, the volunteers assigned to the probiotic group perceived a clear improvement in their intestinal habits[137].

Infectious diarrhoea. A Cochrane review on the efficacy of probiotics for treating infectious diarrhoea, including both adults and children, evaluated sixty-three studies with a total of 8014 participants. No adverse events were attributed to probiotic intervention. The use of probiotics reduced the duration of diarrhoea, although the size of the effect varied considerably between studies. The average of the effect was significant for the mean duration of diarrhoea (lasting ≥ 4 d) and stool frequency on day 2. The authors concluded that, when used alongside rehydration therapy, probiotics appear to be safe and have clear beneficial effects in shortening the duration and reducing stool frequency in acute infectious diarrhoea[140].

Antibiotic-associated diarrhoea. A 2011 Cochrane review meta-analysis evaluated the results of sixteen randomised, parallel, placebo-controlled trials that investigated antibiotic-associated diarrhoea in children (0–18 years of age) receiving antibiotics[141]. Treatment with probiotics was compared with treatment with placebo, active alternative prophylaxis or no treatment, and the incidence of diarrhoea secondary to antibiotic use was measured. The trials included treatment with *Bacillus* spp., *Bifidobacterium* spp., *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc cremoris*, *Saccharomyces* spp. or *Streptococcus* spp., individually or in combination. Despite the heterogeneity in probiotic strain, dose and duration, and the quality of the study, the overall evidence suggests a protective effect of probiotics in preventing antibiotic-associated diarrhoea.

Persistent diarrhoea. The evidence suggesting that probiotics might be effective in treating persistent diarrhoea in children is scarce. Bernaola Aponte *et al.*[142] reviewed RCT comparing a specified probiotic agent with placebo or no probiotic in children with persistent diarrhoea. In all, four trials, with a total of 464 participants, were included in this meta-analysis. Treatment with probiotics reduced the duration of persistent diarrhoea in two trials. Similarly, the stool frequency was reduced with the use of probiotics in two trials. One trial reported a shorter hospital stay, which was significant, but the numbers were small. No adverse events were reported.

Necrotising enterocolitis. Alfaleh *et al.*[143] performed a meta-analysis with sixteen randomised or quasi-RCT that involved 2842 preterm infants of < 37 weeks gestational age and/or weighing < 2500 g at birth. These trials were highly variable with regard to enrolment criteria (i.e. birth weight and gestational age), baseline risk of NEC in the control groups, timing, dosing, probiotics formulations and feeding regimens. The data regarding extremely low birth weight infants could not be extrapolated. Enteral probiotic supplementation significantly reduced the incidence of severe NEC (stage II or more) and mortality. There was no evidence

British Journal of Nutrition

of a significant reduction of nosocomial sepsis. Moreover, there was no evidence of systemic infection with the use of probiotics in these trials. The authors concluded that enteral supplementation with probiotics prevents severe NEC, although more studies are needed to assess the efficacy of probiotic use in extremely low birth weight infants and assess the most effective formulation and dose to be utilised.

Braga *et al.*[144] evaluated the effect of a combined supplementation of *L. casei* and *B. breve* in preterm infants with low birth weight on the occurrence of NEC as a primary outcome. The use of probiotics had a beneficial effect on the occurrence of NEC at stage ≥2 using Bell's criteria and was associated with an improvement in intestinal motility based on the time required to reach full enteral feeding.

Ulcerative colitis. Probiotic treatment is effective in maintaining remission in ulcerative colitis[145]. A total of thirteen RCT were reviewed. Compared with the non-probiotics group, the remission rate for ulcerative colitis patients who received probiotics was 1·35 (95 % CI 0·98, 1·85). Compared with the placebo group, the remission rate of ulcerative colitis patients that received probiotics was 2·00 (95 % CI 1·35, 2·96). During the course of treatment, patients who received probiotics for less than 12 months showed a remission rate of 1·36 (95 % CI 1·07, 1·73) compared with the group treated with non-probiotics. Compared with the non-probiotics group, the recurrence rate of ulcerative colitis in patients that received probiotics was 0·69 (95 % CI 1·01, 2·47). The recurrence rate was 0·25 (95 % CI 0·12, 0·51) in the mild-to-moderate group that received probiotics compared with the group that did not receive probiotics. The group that received *B. bifidum* treatment had a recurrence rate of 0·25 (95 % CI 0·12, 0·50) compared with the non-probiotics group.

Pouchitis is a major complication after ileal pouch anal anastomosis in patients with ulcerative colitis. Mimura *et al.*[146] showed that a single daily high dose (6 g) of probiotic VSL#3 was effective in maintaining antibiotic-induced remission in patients with pouchitis for 1 year. The remission was maintained for 1 year in 85 % of patients in the VSL#3 group compared with 6 % of patients in the placebo group. In a more recent paper, patients with pouchitis in remission that had been induced by antibiotic therapy were recruited to receive either the VSL#3 probiotic compound or placebo for the maintenance of remission[147]. Biopsies were obtained before and 2 months after the initiation of VSL#3 or placebo treatment. Therapy with VSL#3 increased the total number of intestinal bacterial cells and the richness and diversity of the bacterial microbiota, especially the anaerobic microbiota, whereas the fungal flora was repressed. In contrast, patients who relapsed while receiving placebo showed a reduced diversity of the mucosal microbiota.

Crohn's disease. Doherty *et al.*[148] recently reviewed trials comparing antibiotics or probiotics with placebo in the prevention of endoscopic or clinical recurrence of Crohn's disease following surgical resection. A total of seven studies were identified as suitable for inclusion (two comparing antibiotics with the placebo and five comparing probiotics with the placebo). Probiotic administration was not associated with any significant difference in the risk of recurrence compared with the placebo.

Irritable bowel syndrome. Irritable bowel syndrome (IBS) is a chronic condition affecting 3–25 % of the population for which no curative treatment is available. Accordingly, therapy is aimed at reducing symptoms. Because an alteration of the normal intestinal microbiota has been observed in IBS, probiotics were considered to be useful in reducing symptoms. McFarland & Dublin[149] reviewed twenty trials that included a total of 1404 subjects. Probiotic use was associated with improvements in global IBS symptoms compared with the placebo. Probiotics were also associated with less abdominal pain.

Gawrońska *et al.*[150] investigated the efficacy of *L. rhamnosus* GG for treating functional dyspepsia, IBS or functional abdominal pain in children. These authors found that *L. rhamnosus* GG reduced the frequency but not the severity of pain in children with IBS.

In contrast to these findings, the administration of *L. rhamnosus* GG to fifty children (6–20 years) with IBS for 6 weeks was not superior to the placebo in relieving abdominal pain. There was no difference in the other gastrointestinal symptoms, except for a lower incidence of perceived abdominal distension[151].

Treatment of IBS with the bacterial lysate of *Enterococcus faecalis* and *E. coli* was effective and superior to the placebo in reducing the typical symptoms of IBS in patients treated by general practitioners[152]. In all, 297 patients with IBS were treated for 8 weeks with this bacterial lysate or a placebo, in a double-blinded, randomised fashion. The responders had at least a 50 % decrease in the global symptom score, and the abdominal pain score was ≥1 visit during treatment. The responder rate in global symptom score to the probiotics was 102/149 (68·5 %) compared with the placebo rate of 56/148 (37·8 %; $P<0·001$), the improvement in abdominal pain score was 108/149 (72·5 %) and 66/148 (44·6 %), respectively ($P=0·001$). The number-needed-to-treat was 3·27 for global symptom score and 3·59 for abdominal pain score. The Kaplan–Meier analysis revealed an average response time of 4–5 weeks for active treatment and more than 8 weeks for treatment with the placebo ($P<0·0001$).

Chronic liver disease. Patients with chronic liver disease generally have an intestinal microbiota imbalance that is related to the development and worsening of the disease. Liu *et al.*[153] conducted a randomised, placebo-controlled trial, pre-test/post-test controlled group design. Patients were randomised to an experimental group (forty-one patients) or a control group (forty patients). Patients in the experimental group were given probiotic yoghurt containing *Bacillus bifidus*, *L. acidophilus*, *L. bulgaricus* and *S. thermophilus*. The subjects in the control group had meals only and were not provided with the probiotic yoghurt. After intervention, the experimental group had a lower *E. coli* count and a reduced intestinal microbiota imbalance. A comparison of the experimental and control groups after the intervention showed that the former had improved symptoms and signs, including a significant improvement in debilitation, food intake, appetite, abdominal distension and ascitic fluid.

British Journal of Nutrition

Aller *et al.*[154] showed that the ingestion of a tablet of 500 million *L. bulgaricus* and *S. thermophilus* improved liver aminotransferase levels in patients with non-alcoholic fatty liver disease.

Acute pancreatitis. Zhang *et al.*[155] reviewed all relevant RCT that studied the effects of pre-, pro- or synbiotics in patients with acute pancreatitis. A total of seven randomised studies with 559 patients were included. Pre-, pro- or synbiotic treatment showed no influence on the incidence of postoperative infections, pancreatic infection, multiple organ failure and systemic inflammatory response syndrome. There were also no significant differences in the length of antibiotic therapy and mortality. However, pre-, pro- or synbiotic treatment was associated with a reduced length of hospital stay.

Sharma *et al.*[156] investigated the role of probiotics on gut permeability and endotoxaemia in patients with acute pancreatitis. Patients were randomised to receive either a placebo or a mixture of *L. acidophilus*, *B. longus*, *B. bifidum*, *B. infantalis* and 25 mg of fructo-oligosaccharide. No significant trend was identified concerning the effect of probiotics on gut permeability or endotoxaemia in acute pancreatitis. However, the study was underpowered owing to premature study termination.

Type 2 diabetes. Ejtahed *et al.*[157] investigated the administration of probiotics in type 2 diabetic patients, who were randomised to receive either 300 g of probiotic yoghurt containing *L. acidophilus* La5 and *B. lactis* Bb12 or 300 g of conventional yoghurt for 6 weeks. Probiotic consumption caused significant decreases in total cholesterol, LDL-C and the atherogenic indices total cholesterol:HDL-C ratio and LDL-C:HDL-C ratio compared with the controls.

AIDS. HIV-infected women who were naïve to antiretroviral treatment were randomised to receive oral capsules containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 or placebo twice daily for 25 weeks. The CD4 count and immune markers (IgG, IgE, IFNγ and IL-10) were measured at baseline and during follow-up. Probiotics had no impact on the immune function in the present study[158].

Other trials have shown a preservation of the immune function with the use of probiotics among non-responsive children or those treated with the anti-retrovirals *B. bifidum* and *S. termophilus* in Brazil[159] and among women naïve to anti-retrovirals who were treated with *L. rhamnosus* GR-1 in Nigeria[160].

Urinary tract infections. Urinary tract infections (UTI) are common among women and frequently recur. The depletion of vaginal lactobacilli is associated with UTI risk, which suggests that repletion might be beneficial. Stapleton *et al.*[161] conducted a double-blind placebo-controlled trial of a *L. crispatus* intravaginal suppository probiotic for the prevention of recurrent UTI in premenopausal women. Recurrent UTI occurred in 15 % of women receiving probiotics compared with 27 % of women receiving placebo (relative risk, 0·5; 95 % CI 0·2, 1·2). High-level vaginal colonisation with *L. crispatus* throughout follow-up was associated with a significant reduction in recurrent UTI only in the group that received probiotics.

Respiratory infections. Hao *et al.*[162] performed a meta-analysis that included ten RCT comparing probiotics with placebo to prevent acute upper respiratory tract infections. Probiotics were more effective than the placebo in reducing the number of participants experiencing episodes of acute upper respiratory tract infections, the rate ratio of episodes of acute upper respiratory tract infections and reducing antibiotic use.

A meta-analysis of five RCT showed that the administration of probiotics is associated with lower incidence of ventilator-associated pneumonia compared with the placebo[163].

Spondyloarthritis. Jenks *et al.*[164] studied the effect of an orally administered probiotic on disease activity, fatigue, quality of life and intestinal symptoms in patients with active spondyloarthritis. In the present randomised placebo-controlled trial, the probiotic combination did not demonstrate significant benefit over the placebo.

Conclusions and future directions

Lactobacilli and bifidobacteria are the genera most frequently used as probiotics. Traditional fermented products and the breast milk, GIT and faeces of human subjects are the primary sources of these microorganisms. Probiotics are isolated by culture in selective media. Currently, the identification of probiotic strains is facilitated by the sequencing of their *16S RNA* genes. Prior to their evaluation, probiotics must be characterised using the following criteria: (1) the capacity to resist extremely low pH, gastric and intestinal enzymes and bile salts, (2) the capacity to adhere to intestinal epithelial cells, (3) antimicrobial activity and (4) safety. The evaluation of probiotics can be conducted at the preclinical (cell and animal models) and clinical levels. Among the latter, the most reliable studies to assess the therapeutic benefits of any probiotic strain are randomised, placebo-controlled trials.

Probiotics have been shown to promote a variety of biological effects in a number of physiological conditions and pathologies, including allergy, intestinal and liver diseases, urinary and upper respiratory infections, AIDS and metabolic diseases. These effects are strain specific and are primarily mediated through changes in the faecal microbiota and immune modulation. RCT concerning the appropriate clinical evaluation of probiotics, with an adequate and statistically sufficient number of subjects related to the main outcome variables, should be performed in a variety of diseases. In addition, multi-centre and replicate studies are necessary to evaluate the actual role of probiotics in the amelioration of symptoms for many diseases. The number of studies concerning the mechanism of probiotics in cell and animal models is scarce. Apparently, many probiotics are able to modulate both the innate and adaptive immune responses; however, the molecular basis of these effects remains unknown.

Acknowledgements

The authors declare that they have no conflict of interest. Part of the research currently in progress in our laboratory is funded by the company Hero España, S. A. through the

grants no. 3143 and 3545 managed by the Fundación General Empresa-Universidad de Granada. The author contributions are as follows: M. B.-B. wrote the abstract, the introduction and the section 'Sources'. J. P.-D. and S. M.-Q. wrote the section 'Isolation, identification, characterisation and safety' and made Fig. 1. L. F. and A. G. wrote the section 'Evaluation' and Table 1.

References

1. Liong MT (editor) (2011) Probiotics: biology, genetics and health aspects. Berlin: Springer-Verlag.
2. FAO/WHO (2001) Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf
3. Guarner F & Malagelada JR (2003) Gut flora in health and disease. *Lancet* **361**, 512–519.
4. Gourbeyre P, Denery S & Bodinier M (2011) Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. *J Leukoc Biol* **89**, 685–695.
5. Macpherson AJ & Harris NL (2004) Interactions between comensal intestinal bacteria and the immune system. *Nat Rev Immunol* **4**, 478–485.
6. Frick JS, Schenk K, Quitadamo M, *et al.* (2007) *Lactobacillus fermentum* attenuates the proinflammatory effect of *Yersinia enterocolitica* on human epithelial cells. *Inflamm Bowel Dis* **13**, 83–90.
7. McFarland (2006) Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of *Clostridium difficile* disease. *Am J Gastroenterol* **101**, 812–822.
8. Collins JK, Thornton G & Sullivan GO (1998) Selection of probiotic strains for human application. *Int Dairy J* **8**, 487–490.
9. Ouwehand AC, Salminen S & Isolauri E (2002) Probiotics: an overview of beneficial effects. *Antonie van Leeuwenhoek* **82**, 279–289.
10. Collado MC, Gueimonde M & Salminen S (2010) Probiotics in adhesion of pathogens: mechanisms of action. *Bioactive Foods Promot Health* **23**, 353–370.
11. Yan F & Polk DB (2011) Probiotics and immune health. *Curr Opin Gastroenterol* **27**, 496–501.
12. Lye HS, Kuan CY, Ewe JA, *et al.* (2009) The improvement of hypertension by probiotics: effects on cholesterol, diabetes, renin, and phytoestrogens. *Int J Mol Sci* **10**, 3755–3775.
13. Pelletier X, Laure-Boussuge S & Donazzolo Y (2001) Hydrogen excretion upon ingestion of dairy products in lactose-intolerant male subjects: importance of the live flora. *Eur J Clin Nutr* **55**, 509–512.
14. Woodard GA, Encarnacion B, Downey JR, *et al.* (2009) Probiotics improve outcomes after Roux-en-Y gastric bypass surgery: a prospective randomized trial. *J Gastrointest Surg* **13**, 1198–1204.
15. Karska-Wysocki B, Bazo M & Smoragiewicz W (2010) Antibacterial activity of *Lactobacillus acidophilus* and *Lactobacillus casei* against methicillin-resistant *Staphylococcus aureus* (MRSA). *Microbiol Res* **165**, 674–686.
16. Liong MT (2008) Safety of probiotics: translocation and infection. *Nutr Rev* **66**, 192–202.
17. Rafter J, Bennett M, Caderni G, *et al.* (2007) Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. *Am J Clin Nutr* **85**, 488–496.
18. Moayyedi P, Ford AC, Talley NJ, *et al.* (2010) The efficacy of probiotics in the treatment of irritable bowel syndrome: a systematic review. *Gut* **59**, 325–332.
19. Golowczyc MA, Mobili P, Garrote GL, *et al.* (2007) Protective action of *Lactobacillus kefir* carrying S-layer protein against *Salmonella enterica* serovar *enteritidis*. *Int J Food Microbiol* **118**, 264–273.
20. Williams NT (2010) Probiotics. *Am J Health Syst Pharm* **67**, 449–458.
21. Yu J, Wang WH, Menghe BL, *et al.* (2011) Diversity of lactic acid bacteria associated with traditional fermented dairy products in Mongolia. *J Dairy Sci* **94**, 3229–3241.
22. Sun Z, Liu W, Gao W, *et al.* (2010) Identification and characterization of the dominant lactic acid bacteria from kurut: the naturally fermented yak milk in Qinghai, China. *J Gen Appl Microbiol* **56**, 1–10.
23. Lopitz-Otsoa F, Rementeria A, Elguezabal N, *et al.* (2006) Kefir: a symbiotic yeasts-bacteria community with alleged healthy capabilities. *Rev Iberoam Micol* **23**, 67–74.
24. Romanin D, Serradell M, González Maciel D, *et al.* (2010) Down-regulation of intestinal epithelial innate response by probiotic yeasts isolated from kefir. *Int J Food Microbiol* **140**, 102–108.
25. Patrignani F, Lanciotti R, Mathara JM, *et al.* (2006) Potential of functional strains, isolated from traditional Maasai milk, as starters for the production of fermented milks. *Int J Food Microbiol* **107**, 1–11.
26. Ya T, Zhang Q, Chu F, *et al.* (2008) Immunological evaluation of *Lactobacillus casei* Zhang: a newly isolated strain from koumiss in Inner Mongolia, China. *BMC Immunol* **9**, 68.
27. Vizoso Pinto MG, Franz CM, Schillinger U, *et al.* (2006) *Lactobacillus* spp. with *in vitro* probiotic properties from human faeces and traditional fermented products. *Int J Food Microbiol* **109**, 205–214.
28. Lim SM & Im DS (2009) Screening and characterization of probiotic lactic acid bacteria isolated from Korean fermented foods. *J Microbiol Biotechnol* **19**, 178–186.
29. Won TJ, Kim B, Lim YT, *et al.* (2011) Oral administration of *Lactobacillus* strains from Kimchi inhibits atopic dermatitis in NC/Nga mice. *J Appl Microbiol* **110**, 1195–1202.
30. Ayeni FA, Sánchez B, Adeniyi BA, *et al.* (2011) Evaluation of the functional potential of Weissella and *Lactobacillus* isolates obtained from Nigerian traditional fermented foods and cow's intestine. *Int J Food Microbiol* **147**, 97–104.
31. Zago M, Fornasari ME, Carminati D, *et al.* (2011) Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiol* **28**, 1033–1040.
32. Ugarte MB, Guglielmotti D, Giraffa G, *et al.* (2006) Nonstarter lactobacilli isolated from soft and semihard Argentinean cheeses: genetic characterization and resistance to biological barriers. *J Food Prot* **69**, 2983–2991.
33. Georgieva RN, Iliev IN, Chipeva VA, *et al.* (2008) Identification and *in vitro* characterization of *Lactobacillus plantarum* strains from artisanal Bulgarian white brined cheeses. *J Basic Microbiol* **48**, 234–244.
34. West PA, Hewitt JH & Murphy OM (1979) Influence of methods of collection and storage on the bacteriology of human milk. *J Appl Bacteriol* **46**, 269–277.
35. Martin R, Jiménez E, Heilig H, *et al.* (2009) Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR. *Appl Environ Microbiol* **75**, 965–969.

36. O'hara AM & Shanahan F (2006) The gut flora as a forgotten organ. *EMBO Rep* **7**, 688–693.
37. Martin R, Langa S, Reviriego C, *et al.* (2003) Human milk is a source of lactic acid bacteria for the infant gut. *J Pediatr* **143**, 754–758.
38. Arboleya S, Binetti A, Salazar N, *et al.* (2012) Establishment and development of intestinal microbiota in preterm neonates. *FEMS Microbiol Ecol* **79**, 763–772.
39. Solis G, Reyes-Gavilan CG, De los Fernandez N, *et al.* (2010) Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breastmilk and the infant gut. *Anaerobe* **16**, 307–310.
40. Gueimonde M, Laitinen K, Salminen S, *et al.* (2007) Breast milk: a source of bifidobacteria for infant gut development and maturation. *Neonatology* **92**, 64–66.
41. Martin R, Langa S, Reviriego C, *et al.* (2004) The commensal microflora of human milk: new perspectives for food bacterio therapy and probiotics. *Trends Food Sci Technol* **15**, 121–127.
42. Perez-Cano FJ, Dong K & Yaqoob P (2010) *In vitro* immunomodulatory activity of *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713: two probiotic strains isolated from human breast milk. *Immunobiology* **215**, 996–1004.
43. Arroyo R, Martín V, Maldonado A, *et al.* (2010) Treatment of infectious mastitis during lactation: antibiotics versus oral administration of lactobacilli isolated from breast milk. *Clin Infect Dis* **15**, 1551–1558.
44. Jiménez E, Fernández L, Maldonado A, *et al.* (2008) Oral administration of *Lactobacillus* strains isolated from breast milk as an alternative for the treatment of infectious mastitis during lactation. *Appl Environ Microbiol* **74**, 4650–4655.
45. Ryan KA, Jayaraman T, Daly P, *et al.* (2008) Isolation of lactobacilli with probiotic properties from the human stomach. *Lett Appl Microbiol* **47**, 269–274.
46. Varma P, Dinesh KR, Menon KK, *et al.* (2010) *Lactobacillus fermentum* isolated from human colonic mucosal biopsy inhibits the growth and adhesion of enteric and foodborne pathogens. *J Food Sci* **75**, M546–M551.
47. Ohland CL & Macnaughton WK (2010) Probiotic bacteria and intestinal epithelial barrier function. *Am J Physiol Gastrointest Liver Physiol* **298**, G807–G819.
48. Srůtková D, Spanova A, Spano M, *et al.* (2011) Efficiency of PCR-based methods in discriminating *Bifidobacterium longum* ssp. *longum* and *Bifidobacterium longum* ssp. *infantis* strains of human origin. *J Microbiol Methods* **87**, 10–16.
49. Lin PP, Hsieh YM & Tsai CC (2009) Antagonistic activity of *Lactobacillus acidophilus* RY2 isolated from healthy infancy feces on the growth and adhesion characteristics of enteroaggregative *Escherichia coli*. *Anaerobe* **15**, 122–126.
50. Martín R, Jiménez E, Olivares M, *et al.* (2006) *Lactobacillus salivarius* CECT 5713, a potential probiotic strain isolated from infant feces and breast milk of a mother-child pair. *Int J Food Microbiol* **112**, 35–43.
51. Acharya MR & Shah RK (2002) Selection of human isolates of bifidobacteria for their use as probiotics. *Appl Biochem Biotechnol* **102–103**, 81–98.
52. Petrof EO (2009) Probiotics and gastrointestinal disease: clinical evidence and basic science. *Antiinflamm Antiallergy Agents Med Chem* **8**, 260–269.
53. Audisio MC & Benítez-Ahrendts MR (2011) *Lactobacillus johnsonii* CRL1647, isolated from *Apis mellifera* L. bee-gut, exhibited a beneficial effect on honeybee colonies. *Benef Microbes* **2**, 29–34.
54. Chu W, Lu F, Zhu W, *et al.* (2011) Isolation and characterization of new potential probiotic bacteria based on quorum-sensing system. *J Appl Microbiol* **110**, 202–208.
55. Pérez-Sánchez T, Balcázar JL, García Y, *et al.* (2011) Identification and characterization of lactic acid bacteria isolated from rainbow trout, *Oncorhynchus mykiss* (Walbaum), with inhibitory activity against *Lactococcus garvieae*. *J Fish Dis* **34**, 499–507.
56. Hill JE, Baiano JC & Barnes AC (2009) Isolation of a novel strain of *Bacillus pumilus* from penaeid shrimp that is inhibitory against marine pathogens. *J Fish Dis* **32**, 1007–1016.
57. Rivera-Espinoza Y & Gallardo-Navarro Y (2010) Non-dairy probiotic products. *Food Microbiol* **27**, 1–11.
58. Haller D, Colbus H, Ganzle MG, *et al.* (2001) Metabolic and functional properties of lactic acid bacteria in the gastrointestinal ecosystem: a comparative *in vitro* study between bacteria of intestinal and fermented food origin. *Syst Appl Microbiol* **24**, 218–226.
59. Abriouel H, Benomar N, Pulido RP, *et al.* (2011) Annotated genome sequence of *Lactobacillus pentosus* MP-10, which has probiotic potential, from naturally fermented Aloreña green table olives. *J Bacteriol* **193**, 4559–4560.
60. Zeng XQ, Pan DD & Guo YX (2010) The probiotic properties of *Lactobacillus buchneri* P2. *J Appl Microbiol* **108**, 2059–2066.
61. Hartemink R & Rombouts FM (1999) Comparison of media for the detection of bifidobacteria, lactobacilli and total anaerobes from faecal samples. *J Microbiol Meth* **36**, 181–192.
62. Hartemink R, Kok BJ, Weenk GH, *et al.* (1996) Raffinose-Bifidobacterium (RB) agar, a new selective medium for bifidobacteria. *J Microbiol Meth* **27**, 33–43.
63. Beerens H (1990) An elective and selective isolation medium for *Bifidobacterium* spp. *Lett Appl Microbiol* **11**, 155–157.
64. Dave RI & Shah NP (1995) Evaluation of media for selective enumeration of *Streptococcus thermophilus, Lactobacillus delbrueckii* ssp. *bulgaricus, Lactobacillus acidophilus*, and bifidobacteria. *J Dairy Sci* **79**, 1529–1536.
65. Munoa FJ & Pares R (1988) Selective medium for isolation and enumeration of *Bifidobacterium* species. *Appl Environ Microbiol* **54**, 1715–1718.
66. Nebra Y & Blanch AR (1999) A new selective medium for *Bifidobacterium* spp. *Appl Environ Microbiol* **65**, 5173–5176.
67. Silvi S, Rumney CJ & Rowland IR (1996) An assessment of three selective media for bifidobacteria in faeces. *J Appl Bacteriol* **81**, 561–564.
68. Rogosa M, Mitchell JA & Wiseman RF (1951) A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *J Bacteriol* **62**, 132–133.
69. Downes FP & Ito K (2001) *Compendium of Methods for the Microbiological Examination of Foods*, 4th ed. pp. 601–648. Washington, DC: American Public Health Association.
70. MacFaddin JD (1985) Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria. vol. 1, pp. 275–284. Baltimore, MD: Williams & Wilkins.
71. Amann RI, Ludwig W & Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual cells without cultivation. *Microbiol Rev* **59**, 143–169.
72. Vandamme P, Pot B, Gillis M, *et al.* (1996) Polyphasic taxonomy, a consensus approach to bacterial systematic. *Microbiol Rev* **60**, 407–438.
73. Woese CR (1987) Bacterial evolution. *Microbiol Rev* **51**, 221–271.

British Journal of Nutrition

74. Winker S & Woese CR (1991) A definition of the domains Archaea, Bacteria and Eucarya in terms of small subunit ribosomal RNA characteristics. *Syst Appl Microbiol* **14**, 305–310.
75. DDBJ: DNA Data Bank of Japan (2012) http://www.ddbj.nig.ac.jp/intro-e.html (accessed February 2012).
76. European Bioinformatics Institute (EBI) (2012) Databases at the EBI. http://www.ebi.ac.uk/Databases/
77. National Institutes of Health (NIH) National Center for Biotechnology Information (NCBI) (2012) GenBank. http://www.ncbi.nlm.nih.gov
78. Muyzer G & Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek* **73**, 127–141.
79. Langendijk PS, Schut F, Jansen GJ, *et al.* (1995) Quantitative fluorescence *in situ* hybridization of *Bifidobacterium* spp. With genus-specific 16S rRNA targeted probes and its application in fecal samples. *Appl Environ Microbiol* **61**, 3069–3075.
80. Fallani M, Young D, Scott J, *et al.* (2010) Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *J Pediatr Gastroenterol Nutr* **51**, 77–84.
81. Leblond-Bourget N, Philippe H, Mangin I, *et al.* (1996) 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter-and intraspecific *Bifidobacterium* phylogeny. *Int J Syst Bacteriol* **46**, 102–111.
82. Fehér T, Burland V & Pósfai G (2012) In the fast lane: large-scale bacterial genome engineering. *J Biotechnol* **160**, 72–79.
83. de los Reyes-Gavilán CG, Suárez A, Fernández-García M, *et al.* (2011) Adhesion of bile-adapted *Bifidobacterium* strains to the HT29-MTX cell line is modified after sequential gastrointestinal challenge simulated *in vitro* using human gastric and duodenal juices. *Res Microbiol* **162**, 514–519.
84. Masco L, Crockaert C, van Hoorde K, *et al.* (2007) *In vitro* assessment of the gastrointestinal transit tolerance of taxonomic reference strains from human origin and probiotic product isolated of *Bifidobacterium*. *J Dairy Sci* **90**, 3572–3578.
85. FAO/WHO (2002) Guidelines for the evaluation of probiotics in food. Food and Health Agricultural Organisation of the United Nations – World Health Organisation. Working group report. London, Ontario. http://www.fao.org.
86. Sanz Y (2006) Ecological and functional implications of the acid adaptation ability of *Bifidobacterium*: a way of selecting improved probiotic strains. *Int dairy J* **17**, 1284–1289.
87. Mainville I, Arcand Y & Farnworth ER (2005) A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. *Int J Food Microbiol* **99**, 287–296.
88. Dunne C, O'Mahony L, Murphy L, *et al.* (2001) *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *Am J Clin Nutr* **73**, 386–392.
89. Zavaglia AG, Kociubinsky G, Pérez P, *et al.* (1998) Isolation and characterization of *Bifidobacterium* strains for probiotic formulation. *J Food Prot* **61**, 865–873.
90. Charteris WP, Kelly PM, Morelli L, *et al.* (1998) Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *J Appl Microbiol* **84**, 759–768.
91. Takahashi N, Xiao JZ, Miyaji K, *et al.* (2004) Selection of acid tolerant bifidobacteria and evidence for a low-pH-inducible acid tolerance response in *Bifidobacterium longum*. *J Dairy Res* **71**, 340–345.
92. Matsumoto M, Ohishi H & Benno Y (2004) H + -ATPase activity in *Bifidobacterium* with special reference to acid tolerance. *Int J Food Microbiol* **93**, 109–113.
93. Matto J, Alakomi HL, Vaari A, *et al.* (2006) Influence of processing conditions on *Bifidobacterium animalis* subsp., *lactis* functionality with a special focus on acid tolerance and factors affecting it. *Int Dairy J* **16**, 1029–1037.
94. Chenoll E, Casinos B, Bataller E, *et al.* (2011) Novel probiotic *Bifidobacterium bifidum* CECT 7366 strain active against the pathogenic bacterium *Helicobacter pylori*. *Appl Environ Microbiol* **77**, 1335–1343.
95. Muñoz JA, Chenoll E, Casinos B, *et al.* (2011) Novel probiotic *Bifidobacterium longum* subsp. *infantis* CECT 7210 strain active against rotavirus infections. *Appl Environ Microbiol* **77**, 8775–8783.
96. Li Q, Chen Q, Ruan H, *et al.* (2010) Isolation and characterisation of an oxygen, acid and bile resistant *Bifidobacterium animalis* subsp. *lactis* Qq08. *J Sci Food Agric* **90**, 1340–1346.
97. Bosch M, Rodriguez M, Garcia F, *et al.* (2012) Probiotic properties of *Lactobacillus plantarum* CECT 7315 and CECT 7316 isolated from faeces of healthy children. *Lett Appl Microbiol* **54**, 240–246.
98. Mills S, Stanton C, Fitzgerald GF, *et al.* (2011) Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. *Microb Cell Fact* **10**, Suppl. 1, S19.
99. Marles-Wright J & Lewis R (2007) Stress response of bacteria. *Curr Opin Struct Biol* **17**, 755–760.
100. Rizzello CG, Cassone A, Di Cagno R, *et al.* (2008) Synthesis of angiotensin I-converting enzyme (ACE)-inhibitory peptides and g-aminobutyric acid (GABA) during sourdough fermentation by selected lactic acid bacteria. *J Agric Food Chem* **56**, 6936–6943.
101. Dicks LM & Botes M (2010) Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. *Benef Microbes* **1**, 11–29.
102. Lenaerts K, Bouwman FG, Lamers WH, *et al.* (2007) Comparative proteomic analysis of cell lines and scrapings of the human intestinal epithelium. *BMC Genomics* **8**, 91.
103. Huang SH, He L, Zhou Y, *et al.* (2009) *Lactobacillus rhamnosus* GG suppresses meningitic *E. coli* K1 penetration across human intestinal epithelial cells *in vitro* and protects neonatal rats against experimental hematogenous meningitis. *Int J Microbiol* **2009**, 647862.
104. Gopal PK, Prasad J, Smart J, *et al.* (2001) *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. *Int J Food Microbiol* **67**, 207–216.
105. Izquierdo E, Medina M, Ennahar S, *et al.* (2008) Resistance to simulated gastrointestinal conditions and adhesion to mucus as probiotic criteria for *Bifidobacterium longum* strains. *Curr Microbiol* **56**, 613–618.
106. Tuomola EM, Ouwehand AC & Salminen SJ (1999) The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. *FEMS Immunol Med Microbiol* **26**, 137–142.
107. Lesuffleur T, Barbat A, Dussaulx E, *et al.* (1990) Growth adaption to methotrexate of HT-29 human colon carcinoma cell is associated with their ability to differentiate into columnar absorptive and mucus secreting cells. *Cancer Res* **50**, 6334–6343.
108. Leteurtre E, Gouyer V, Rousseau K, *et al.* (2004) Differential mucin expression in colon carcinoma HT-29 clones with

variable resistance to 5-fluorouracil and methotrexate. *Biol Cell* **96**, 145–151.
109. Laparra JM & Sanz Y (2009) Comparison of *in vitro* models to study bacterial adhesion to the intestinal epithelium. *Lett Appl Microbiol* **49**, 695–701.
110. Collado MC, Meriluoto J & Salminen S (2007) Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. *Lett Appl Microbiol* **45**, 454–460.
111. Salminen S, Bouley C, Boutron-Ruault MC, *et al.* (1998) Functional food science and gastrointestinal physiology and function. *Br J Nutr* **80**, 147–171.
112. Sambuy Y, De Angelis I, Ranaldi G, *et al.* (2005) The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol* **21**, 1–26.
113. Ferreira CL, Grześkowiak Ł, Collado MC, *et al.* (2011) *In vitro* evaluation of *Lactobacillus gasseri* strains of infant origin on adhesion and aggregation of specific pathogens. *J Food Prot* **74**, 1482–1487.
114. Todoriki K, Mukai T, Sato S, *et al.* (2001) Inhibition of adhesion of food-borne pathogens o Caco-2 cells by *Lactobacillus* strains. *J Appl Microbiol* **91**, 154–159.
115. Chu H, Kang S, Ha S, *et al.* (2005) *Lactobacillus acidophilus* expressing recombinant K99 adhesive fimbriae has an inhibitory effect on adhesion of enterotoxigenic *Escherichia coli*. *Microbiol Immunol* **49**, 941–948.
116. Tsai CC, Lin PP & Hsieh YM (2008) Three *Lactobacillus* strains from healthy infant stool inhibit enterotoxigenic *Escherichia coli* grown *in vitro*. *Anaerobe* **14**, 61–67.
117. Candela M, Perna F, Carnevali P, *et al.* (2008) Interaction of probiotic *Lactobacillus* and *Bifidobacterium* strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8 production. *Int J Food Microbiol* **31**, 286–292.
118. Jankowska A, Laubitz D, Antushevich H, *et al.* (2008) Competition of *Lactobacillus paracasei* with *Salmonella enterica* for adhesion to Caco-2 cells. *J Biomed Biotechnol* **2008**, 357964.
119. Tien MT, Girardin SE, Regnault B, *et al.* (2006) Anti-inflammatory effect of *Lactobacillus casei* on *Shigella*-infected human intestinal epithelial cells. *J Immunol* **176**, 1228–1237.
120. Cho IL, Lee NK & Hahm YT (2009) Characterization of *Lactobacillus* spp. isolated from the feces of breast-feeding piglets. *J Biosci Bioeng* **108**, 194–198.
121. Pillai A & Nelson R (2008) Probiotics for treatment of *Clostridium difficile*-associated colitis in adults. *The Cochrane Database of Systematic Reviews* issue 1, CD004611.
122. Wang H, Yan Y, Wang J, *et al.* (2012) Production and characterization of antifungal compounds produced by *Lactobacillus plantarum* IMAU10014. *PLoS One* **7**, e29452.
123. Naidoo K, Gordon M, Fagbemi AO, *et al.* (2011) Probiotics for maintenance of remission in ulcerative colitis. *The Cochrane Database of Systematic Reviews* issue 12, CD007443.
124. Von Wright A (2005) Regulating the safety of probiotics – the European approach. *Curr Pharm Des* **11**, 17–23.
125. Wassenaar TM & Klein G (2008) Safety aspects and implications of regulation of probiotic bacteria in food and food supplements. *J Food Prot* **71**, 1734–1741.
126. Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA (2007). http://www.efsa.europa.eu/en/efsajournal/pub/587.htm
127. Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of micro-organisms resistant to antibiotics of human clinical and veterinary importance (2002). http://ec.europa.eu/food/fs/sc/scan/out64_en.pdf (accessed February 2012).
128. Sanders ME, Akkermans LM, Haller D, *et al.* (2010) Safety assessment of probiotics for human use. *Gut Microbes* **1**, 164–185.
129. Cencič A & Langerholc T (2010) Functional cell models of the gut and their applications in food microbiology. *Int J Food Microbiol* **141**, Suppl. 1, S4–S14.
130. Borchers AT, Selmi C, Meyers FJ, *et al.* (2009) Probiotics and immunity. *J Gastroenterol* **44**, 26–46.
131. Asemi Z, Jazayeri S, Najafi M, *et al.* (2011) Effects of daily consumption of probiotic yoghurt on inflammatory factors in pregnant women: a randomized controlled trial. *Pak J Biol Sci* **14**, 476–482.
132. Asemi Z, Jazayeri S, Najafi M, *et al.* (2012) Effect of daily consumption of probiotic yoghurt on oxidative stress in pregnant women: a randomized controlled clinical trial. *Ann Nutr Metab* **60**, 62–68.
133. Dugoua JJ, Machado M, Zhu X, *et al.* (2009) Safety in pregnancy: a systematic review and meta-analysis of RCT of *Lactobacillus, Bifidobacterium*, and *Saccharomyces* spp. *J Obstet Gynaecol Can* **31**, 542–552.
134. Vliagoftis H, Kouranos VD, Betsi GI, *et al.* (2008) Probiotics for the treatment of allergic rhinitis and asthma: systematic review of RCT. *Ann Allergy Asthma Immunol* **101**, 570–579.
135. Kuitunen M, Kukkonen K & Savilahti EJ (2009) Pro- and prebiotic supplementation induces a transient reduction in hemoglobin concentration in infants. *J Pediatr Gastroenterol Nutr* **49**, 626–630.
136. Martínez-Cañavate A, Sierra S, Lara-Villoslada F, *et al.* (2009) A probiotic dairy product containing *L. gasseri* CECT5714 and *L. coryniformis* CECT5711 induces immunological changes in children suffering from allergy. *Pediatr Allergy Immunol* **20**, 592–600.
137. Olivares M, Díaz-Ropero MO, Gómez N, *et al.* (2006) Oral administration of two probiotic strains, *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711, enhances the intestinal function of healthy adults. *Int J Food Microbiol* **107**, 104–111.
138. Boyle RJ, Bath-Hextall FJ, Leonardi-Bee J, *et al.* (2009) Probiotics for treating eczema. A systematic review. *Clin Exp Allergy* **39**, 1117–1127.
139. Lee J, Seto D & Bielory L (2008) Meta-analysis of clinical trials of probiotics for prevention and treatment of pediatric atopic dermatitis. *J Allergy Clin Immunol* **121**, 116–121.
140. Allen SJ, Martinez EG, Gregorio GV, *et al.* (2010) Probiotics for treating acute infectious diarrhoea. *The Cochrane Database of Systematic Reviews* issue 11, CD003048.
141. Johnston BC, Goldenberg JZ, Vandvik PO, *et al.* (2011) Probiotics for the prevention of pediatric antibiotic-associated diarrhea. *The Cochrane Database of Systematic Reviews* issue 11, CD004827.
142. Bernaola Aponte G, Bada Mancilla CA, Carreazo Pariasca NY, *et al.* (2010) Probiotics for treating persistent diarrhoea in children. *The Cochrane Database of Systematic Reviews* issue 11, CD007401.
143. Alfaleh K, Anabrees J, Bassler D, *et al.* (2011) Probiotics for prevention of necrotizing enterocolitis in preterm infants. *The Cochrane Database of Systematic Reviews* issue 3, CD005496.
144. Braga TD, da Silva GAP, de Lira PIC, *et al.* (2011) Efficacy of *Bifidobacterium breve* and *Lactobacillus casei* oral supplementation on necrotizing enterocolitis in

British Journal of Nutrition

very-low-birth-weight preterm infants: a double-blind, randomized, controlled trial. *Am J Clin Nutr* **93**, 81–86.
145. Sang LX, Chang B, Zhang WL, *et al.* (2010) Remission induction and maintenance effect of probiotics on ulcerative colitis: a meta-analysis. *World J Gastroenterol* **16**, 1908–1915.
146. Mimura T, Rizzello F, Helwig U, *et al.* (2004) Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut* **53**, 108–114.
147. Kühbacher T, Ott SJ, Helwig U, *et al.* (2006) Bacterial and fungal microbiota in relation to probiotic therapy (VSL#3) in pouchitis. *Gut* **55**, 833–841.
148. Doherty GA, Bennett GC, Cheifetz AS, *et al.* (2010) Meta-analysis: targeting the intestinal microbiota in prophylaxis for post-operative Crohn's disease. *Aliment Pharmacol Ther* **31**, 802–809.
149. McFarland LV & Dublin S (2008) Meta-analysis of probiotics for the treatment of irritable bowel syndrome. *World J Gastroenterol* **14**, 2650–2661.
150. Gawrońska A, Dziechciarz P, Horvath A, *et al.* (2007) A randomized double-blind placebo-controlled trial of *Lactobacillus* GG for abdominal pain disorders in children. *Aliment Pharmacol Ther* **25**, 177–1784.
151. Bausserman M & Michail S (2005) The use of *Lactobacillus* GG in irritable bowel syndrome in children: a double-blind randomized control trial. *J Pediatr* **147**, 197–201.
152. Enck P, Zimmermann K, Menke G, *et al.* (2008) A mixture of *Escherichia coli* (DSM 17252) and *Enterococcus faecalis* (DSM 16440) for treatment of the irritable bowel syndrome – a randomized controlled trial with primary care physicians. *Neurogastroenterol Motil* **20**, 1103–1109.
153. Liu JE, Zhang Y, Zhang J, *et al.* (2010) Probiotic yoghurt effects on intestinal flora of patients with chronic liver disease. *Nurs Res* **59**, 426–432.
154. Aller R, De Luis DA, Izaola O, *et al.* (2011) Effect of a probiotic on liver aminotransferases in nonalcoholic fatty liver disease patients: a double blind randomized clinical trial. *Eur Rev Med Pharmacol Sci* **15**, 1090–1095.
155. Zhang MM, Cheng JQ, Lu YR, *et al.* (2010) Use of pre-, pro- and synbiotics in patients with acute pancreatitis: a meta-analysis. *World J Gastroenterol* **16**, 3970–3978.
156. Sharma B, Srivastava S, Singh N, *et al.* (2011) Role of probiotics on gut permeability and endotoxemia in patients with acute pancreatitis. *J Clin Gastroenterol* **45**, 442–448.
157. Ejtahed HS, Mohtadi-Nia J, Homayouni-Rad A, *et al.* (2011) Effect of probiotic yoghurt containing *Lactobacillus acidophilus* and *Bifidobacterium lactis* on lipid profile in individuals with type 2 diabetes mellitus. *J Dairy Sci* **94**, 3288–3294.
158. Hummelen R, Changalucha J, Butamanya NL, *et al.* (2011) Effect of 25 weeks probiotic supplementation on immune function of HIV patients. *Gut Microbes* **2**, 80–85.
159. Trois L, Cardoso EM & Miura E (2008) Use of probiotics in HIV-infected children: a randomized double-blind controlled study. *J Trop Pediatr* **54**, 19–24.
160. Anukam KC, Osazuwa EO, Osadolor HB, *et al.* (2008) Yoghurt containing probiotic *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 helps resolve moderate diarrhea and increases CD4 count in HIV/AIDS patients. *J Clin Gastroenterol* **42**, 239–243.
161. Stapleton AE, Au-Yeung M, Hooton TM, *et al.* (2011) Randomized, placebo-controlled phase 2 trial of a *Lactobacillus crispatus* probiotic given intravaginally for prevention of recurrent urinary tract infection. *Clin Infect Dis* **52**, 1212–1217.
162. Hao Q, Lu Z, Dong BR, *et al.* (2011) Probiotics for preventing acute upper respiratory tract infections. *The Cochrane Database of Systematic Reviews* issue 9, CD006895.
163. Siempos Nt II, aidou TK & Falagas ME (2010) Impact of the administration of probiotics on the incidence of ventilator-associated pneumonia: a meta-analysis of RCT. *Crit Care Med* **38**, 954–962.
164. Jenks K, Stebbings S, Burton J, *et al.* (2010) Probiotic therapy for the treatment of spondyloarthritis: a randomized controlled trial. *J Rheumatol* **37**, 2118–2125.

British Journal of Nutrition

Systematic Review

Annals of Nutrition & Metabolism

Ann Nutr Metab 2012;61:160–174
DOI: 10.1159/000342079

Received: July 19, 2012
Accepted: July 20, 2012
Published online: October 2, 2012

Probiotic Mechanisms of Action

Miriam Bermudez-Brito Julio Plaza-Díaz Sergio Muñoz-Quezada
Carolina Gómez-Llorente Angel Gil

Department of Biochemistry and Molecular Biology II, Institute of Nutrition and Food Technology 'José Mataix', Biomedical Research Center, University of Granada, Armilla, Spain

Key Words
Antimicrobial responses · Bifidobacteria · Lactic acid bacteria · Lactobacilli · Probiotic mechanism of action · Probiotics

Abstract
Probiotics are live microorganisms that provide health benefits to the host when ingested in adequate amounts. The strains most frequently used as probiotics include lactic acid bacteria and bifidobacteria. Probiotics have demonstrated significant potential as therapeutic options for a variety of diseases, but the mechanisms responsible for these effects have not been fully elucidated yet. Several important mechanisms underlying the antagonistic effects of probiotics on various microorganisms include the following: modification of the gut microbiota, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier and modulation of the immune system to convey an advantage to the host. Accumulating evidence demonstrates that probiotics communicate with the host by pattern recognition receptors, such as toll-like receptors and nucleotide-binding oligomerization domain-containing protein-like receptors, which modulate key signaling pathways, such as nuclear factor-κB and mitogen-activated protein kinase, to enhance or suppress activation and influence downstream pathways. This recognition is crucial for eliciting measured antimicrobial responses with minimal inflammatory tissue damage. A clear understanding of these mechanisms will allow for appropriate probiotic strain selection for specific applications and may uncover novel probiotic functions. The goal of this systematic review was to explore probiotic modes of action focusing on how gut microbes influence the host.

Introduction

According to the Food and Agriculture Organization of the United Nations and the World Health Organization [1], probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts. In particular, strains belonging to *Bifidobacterium* and *Lactobacillus*, which are the predominant and subdominant groups of the gastrointestinal microbiota, respectively [2], are the most widely used probiotic bacteria and are included in many functional foods and dietary supplements [3–5]. *Saccharomyces boulardii* yeast has also been shown to have health benefits [6]. After a long history of safe use of probiotics in fermented dairy products and an increased recognition of their beneficial effects on human health [7], the food industry has become increasingly interested in these types of microorganisms. Often the criteria for the selection of probiotics include the tolerance to gastrointestinal conditions (gas-

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0250–6807/12/0612–0160$38.00/0

Accessible online at:
www.karger.com/anm

Prof. Angel Gil
Institute of Nutrition and Food Technology 'José Mataix' (INyTA)
Biomedical Research Center, University of Granada
Avenida del Conocimiento s/n, ES–18100 Armilla (Spain)
E-Mail agil@ugr.es

tric acid and bile), ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens [8, 9]. The mechanisms underlying the beneficial effects of probiotics are largely unknown but are likely to be multifactorial. Several mechanisms related to the antagonistic effects of probiotics on various microorganisms include the following mechanisms: secretion of antimicrobial substances, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier and modulation of the immune system [10].

The results of evidence-based analyses from human studies and animal models have shown the clinical potential of probiotics against many diseases [11]. Probiotics have been reported to suppress diarrhea [12], alleviate lactose intolerance [13] and postoperative complications [14], exhibit antimicrobial [15] and anti-colorectal cancer activities [16, 17], reduce irritable bowel symptoms [18] and prevent inflammatory bowel disease [19]. However, generalizations concerning the potential health benefits of probiotics should not be made because probiotic effects tend to be strain specific. Thus, the health benefit attributed to one strain is not necessarily applicable to another strain even within one species [20].

In the present study, we sought to conduct a systematic review on the mechanisms of action of probiotic strains. Using the following equation: 'epithelial barrier' [All Fields] OR 'antimicrobial substances'[All Fields] OR 'bacteriocins'[All Fields] OR 'BIF'[All Fields] OR 'adhesion'[All Fields] OR 'competitive exclusion'[All Fields] OR 'defensins'[All Fields] OR 'mucins'[All Fields] OR 'bacterial adhesins' [All Fields] OR 'antifungals'[All Fields] OR 'intestinal microbiota'[All Fields] OR 'fatty acids'[All Fields] OR 'mechanisms'[All Fields] OR 'TLR2'[All Fields] OR 'TLR4'[All Fields] OR 'TLR9'[All Fields] OR 'toll-like receptor'[All Fields] OR 'NOD1'[All Fields] OR 'NOD2' [All Fields] OR 'inflammasome'[All Fields] OR 'NLRP3' [All Fields] AND 'probiotics'[MeSH], we have selected 165 relevant articles of 1,731 articles published until June 25, 2012, from the PubMed and SCOPUS databases.

Mechanisms of Action of Probiotics

Major probiotic mechanisms of action include enhancement of the epithelial barrier, increased adhesion to intestinal mucosa, and concomitant inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms, production of anti-microorganism substances and modulation of the immune system (fig. 1).

Enhancement of the Epithelial Barrier

The intestinal epithelium is in permanent contact with luminal contents and the variable, dynamic enteric flora. The intestinal barrier is a major defense mechanism used to maintain epithelial integrity and to protect the organism from the environment. Defenses of the intestinal barrier consist of the mucous layer, antimicrobial peptides, secretory IgA and the epithelial junction adhesion complex [21]. Once this barrier function is disrupted, bacterial and food antigens can reach the submucosa and can induce inflammatory responses, which may result in intestinal disorders, such as inflammatory bowel disease [22–24]. Consumption of non-pathogenic bacteria can contribute to intestinal barrier function, and probiotic bacteria have been extensively studied for their involvement in the maintenance of this barrier. However, the mechanisms by which probiotics enhance intestinal barrier function are not fully understood.

Several studies have indicated that enhancing the expression of genes involved in tight junction signaling is a possible mechanism to reinforce intestinal barrier integrity [25]. For instance, lactobacilli modulate the regulation of several genes encoding adherence junction proteins, such as E-cadherin and β-catenin, in a T84 cell barrier model. Moreover, incubation of intestinal cells with lactobacilli differentially influences the phosphorylation of adherence junction proteins and the abundance of protein kinase C (PKC) isoforms, such as PKCδ, thereby positively modulating epithelial barrier function [26].

Recent data have indicated that probiotics may initiate repair of the barrier function after damage. *Escherichia coli* Nissle 1917 (EcN1917) not only prevents the disruption of the mucosal barrier by enteropathogenic *E. coli*, but it even restores mucosal integrity in T84 and Caco-2 cells. This effect is mediated by the enhanced expression and redistribution of tight junction proteins of the zonula occludens (ZO-2) and PKC resulting in the reconstruction of the tight junction complex [27, 28]. Similarly, *Lactobacillus casei* DN-114001 [29] and VSL3 (a mixture of pre- and probiotics) [30] are capable of sustaining the intestinal barrier function by similar mechanisms. A recent paper has reported that VSL3 protects the epithelial barrier and increases tight junction protein expression in vivo and in vitro by activating the p38 and extracellular regulated kinase signaling pathways [31].

A link between altered levels of pro-inflammatory cytokines and intestinal permeability has been described in a number of intestinal diseases [32]. Using probiotics, the prevention of cytokine-induced epithelial damage, which is characteristic of inflammatory bowel disease [24], may

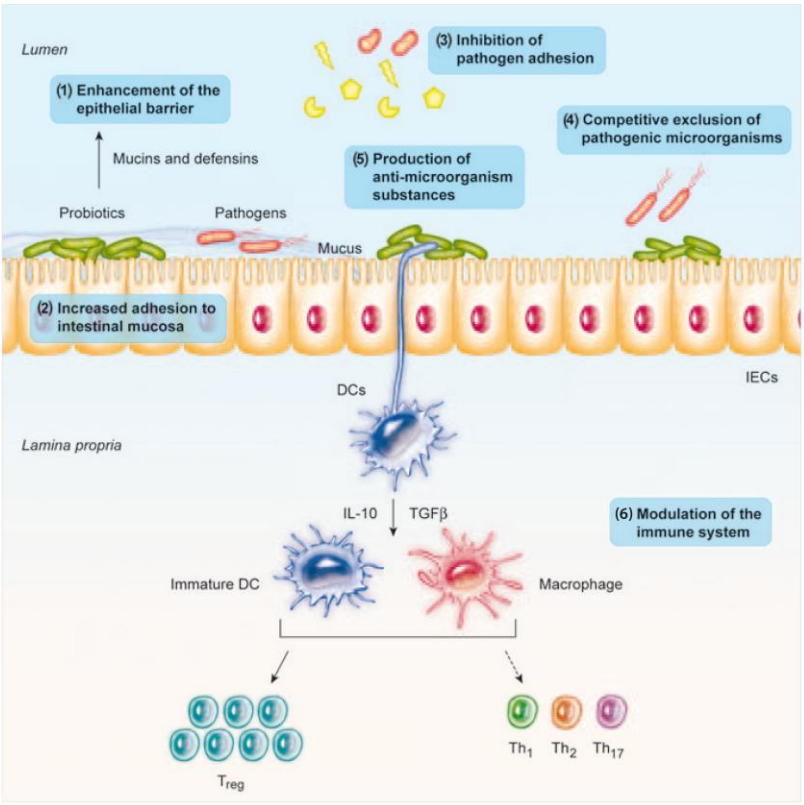


Fig. 1. Major mechanisms of action of probiotics.

also contribute to the reinforcement of the mucosal barrier. Two isolated and purified peptides secreted by *Lactobacillus rhamnosus* GG (LGG), which are designated p40 and p75, have recently been demonstrated to prevent cytokine-induced cell apoptosis by activating the anti-apoptotic protein kinase B (PKB/Akt) in a phosphatidyl inositol-3′-kinase-dependent pathway and by inhibiting the pro-apoptotic p38/mitogen-activated protein kinase (MAPK) [33, 34]. The evidence that p40 and p75 are responsible for the observed effects is derived from the observation that the anti-apoptotic function is abolished when p40- and p75-specific antibodies are added in vitro to murine and human epithelial cells or to colon explants derived from mice [34]. Other low-molecular-weight (LMW) peptides secreted from LGG induce expression of heat shock proteins and activate MAPKs [35].

Mucin glycoproteins (mucins) are major macromolecular constituents of epithelial mucus and have long been implicated in health and disease. Probiotics may promote mucous secretion as one mechanism to improve barrier function and the exclusion of pathogens. Several *Lactobacillus* species increase mucin expression in human intestinal cell lines. However, this protective effect is dependent on *Lactobacillus* adhesion to the cell monolayer, which likely does not occur in vivo [36, 37]. Conversely, another group has shown that *Lactobacillus acidophilus* A4 cell extract is sufficient to increase *MUC2* expression in HT29 cells independent of attachment [38]. Additionally, VSL3, which contains some *Lactobacillus* species, increases the expression of *MUC2*, *MUC3* and *MUC5AC* in HT29 cells [30]. In vivo studies are less consistent because only a few have been performed. Mice given VSL3 daily for 14 days do not exhibit altered mucin expression or mucous layer thickness [39]. Conversely, rats given VSL3 at a similar daily dose for 7 days have a 60-fold increase in *MUC2* expression and a concomitant increase in mucin secretion [40]. Therefore, mucous production may be increased by probiotics in vivo, but further studies are needed to make a conclusive statement.

Increased Adhesion to Intestinal Mucosa

Adhesion to intestinal mucosa is regarded as a prerequisite for colonization and is important for the interaction between probiotic strains and the host [41–43]. Adhesion of probiotics to the intestinal mucosa is also important for modulation of the immune system [43, 44] and antagonism against pathogens [45].

Thus, adhesion has been one of the main selection criteria for new probiotic strains [41, 46–48] and has been related to certain beneficial effects of probiotics [49]. Lactic acid bacteria (LABs) display various surface determinants that are involved in their interaction with intestinal epithelial cells (IECs) and mucus. IECs secrete mucin, which is a complex glycoprotein mixture that is the principal component of mucous, thereby preventing the adhesion of pathogenic bacteria [47, 50]. Additionally, lipids, free proteins, immunoglobulins and salts are present in mucous gel [51]. This specific interaction has indicated a possible association between the surface proteins of probiotic bacteria and the competitive exclusion of pathogens from the mucus [52–54]. As mentioned above, several *Lactobacillus* proteins have been shown to promote mucous adhesion [54], and bacteria display surface adhesins that mediate attachment to the mucous layer [55]. This process is mainly mediated by proteins, although saccharide moieties and lipoteichoic acids have also been implicated [56]. The most studied example of mucus-targeting bacterial adhesins is MUB (mucus-binding protein) produced by *Lactobacillus reuteri* [55, 57]. The proteins playing a role in the mucous adhesion phenotype of lactobacilli are mainly secreted and surface-associated proteins, which are either anchored to the membrane through a lipid moiety or embedded in the cell wall [58–61]. The involvement of surface proteins in the interaction with human plasminogen or enterocytes has been reported in *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium bifidum*, respectively. Under certain circumstances, these proteins may play a role in facilitating the colonization of the human gut through degradation of the extracellular matrix of cells or by facilitating close contact with the epithelium [62–66]. MapA (mucous adhesion-promoting protein) has been reported to mediate the binding of *L. reuteri* and *L. fermentum* to mucus [52]. Probiotics, such as *L. plantarum*, have been reported to induce MUC2 and MUC3 mucins and to inhibit the adherence of enteropathogenic *E. coli*. These observations indicate that enhanced mucous layers and glycocalyx overlying the intestinal epithelium as well as the occupation of microbial binding sites by *Lactobacillus* spp. provide protection against invasion by pathogens [45, 67, 68].

Collado et al. [69] evaluated the adhesion of *Bifidobacterium longum* and *Bifidobacterium catenulatum* strains to human intestinal mucus and compared the results to those of control experiments that were run with the original acid-sensitive strains. They reported that in half of the 4 studied cases, the acid-resistant derivative shows a greater ability to adhere to human intestinal mucus than the original strain. The ability of bifidobacteria to inhibit pathogen adhesion to mucus is not generally improved by the acquisition of acid resistance. Overall, the induction of acid resistance in bifidobacteria may be a strategy for selecting strains with enhanced stability and improved surface properties that favor their potential functionality as probiotics against specific pathogens.

The mixture of probiotics and VSL3 has been reported to increase the synthesis of cell surface mucins and to modulate mucin gene expression in a manner dependent on the adhesion of bacterial cells to the intestinal epithelium [40].

Probiotics also cause qualitative alterations in intestinal mucins that prevent pathogen binding [68]. The bacterial component involved in the adhesion of the LB and BG2FO4 *L. acidophilus* strains is protease resistant and is associated with the bacterial surface [70–72]. Interestingly, the bacterial component is also degraded into an antimicrobial peptide, which lends anti-pathogenic properties to the host and provides an example of how large surface proteins may exhibit evolutionarily beneficial pleiotropic effects [73].

Probiotic strains can also induce the release of defensins from epithelial cells. These small peptides/proteins are active against bacteria, fungi and viruses. Moreover, these small peptides/proteins stabilize the gut barrier function [74]. Observations have indicated that in response to attack by pathogenic bacteria, the host engages its first line of chemical defense by increasing the production of antimicrobial proteins (AMPs), such as α- and β-defensins, cathelicidins, C-type lectins and ribonucleases [75–80]. Many AMPs are enzymes that kill bacteria by carrying out an enzymatic attack on cell wall structures and/or non-enzymatic disruption of the bacterial membrane. Enzymes expressed by Paneth cells attack the bacterial membranes. Lysozyme hydrolyzes the glycosidic linkage of wall peptidoglycan [81] and phospholipase A_2 bacterial membrane phospholipids [82]. Defensins comprise a major family of membrane-disrupting peptides in vertebrates. The interaction is non-specific and mainly by binding to anionic phospholipid groups of the membrane surface through electrostatic interactions. This interaction creates defensin pores in the bacterial mem-

brane that disrupt membrane integrity and promote lysis of microorganisms [83]. Cathelicidins are usually cationic, α-helical peptides that bind to bacterial membranes through electrostatic interactions and, like the defensins, induce membrane disruption [84].

The microbial adhesion process of LAB also includes passive forces, electrostatic interactions, hydrophobic interactions, steric forces, lipoteichoic acids and specific structures, such as external appendages covered by lectins. A wide variety of molecules mediating the adhesion of pathogenic bacteria has been characterized. However, the understanding of the factors that mediate adhesion for *Lactobacillus* is extremely limited [85–87]. Further studies are needed for the identification and analysis of the functional significance of various components of mucous layers as well as the complex interactions of mucous layers, microbiota (including probiotics) and epithelial cells with underlying innate and adaptive immune systems [68].

Competitive Exclusion of Pathogenic Microorganisms

In a report addressing the total exclusion of *Salmonella typhimurium* from maggots of blowflies published in 1969, Greenberg [88] first used the 'competitive exclusion' term for the scenario in which one species of bacteria more vigorously competes for receptor sites in the intestinal tract than another species. The mechanisms used by one species of bacteria to exclude or reduce the growth of another species are varied, including the following mechanisms: creation of a hostile microecology, elimination of available bacterial receptor sites, production and secretion of antimicrobial substances and selective metabolites, and competitive depletion of essential nutrients [89].

Specific adhesiveness properties due to the interaction between surface proteins and mucins may inhibit the colonization of pathogenic bacteria and are a result of antagonistic activity by some strains of probiotics against adhesion of gastrointestinal pathogens [90]. Lactobacilli and bifidobacteria have been shown to inhibit a broad range of pathogens, including *E. coli*, *Salmonella*, *Helicobacter pylori, Listeria monocytogenes* and *Rotavirus* [91–97]. Exclusion is the result of different mechanisms and properties of probiotics to inhibit pathogen adhesion, including the production of substances and the stimulation of IECs. Competitive exclusion by intestinal bacteria is based on a bacterium-to-bacterium interaction mediated by competition for available nutrients and for mucosal adhesion sites. To gain a competitive advantage, bacteria can also modify their environment to make it less suitable for their competitors. The production of antimicrobial substances, such as lactic and acetic acid, is one example of this type of environmental modification [98]. Some lactobacilli and bifidobacteria share carbohydrate-binding specificities with some enteropathogens [99, 100], which makes it possible for the strains to compete with specific pathogens for the receptor sites on host cells [101]. In general, probiotic strains are able to inhibit the attachment of pathogenic bacteria by means of steric hindrance at enterocyte pathogen receptors [102].

The effect of probiotic bacteria on the competitive exclusion of pathogens has been demonstrated using human mucosal material in vitro [45, 103] as well as chicken [104] and pig mucosal material in vivo [105]. Hirano et al. [45] showed that *L. rhamnosus,* a strongly adhering strain, is capable of inhibiting the internalization of EHEC (enterohemorrhagic *E. coli*) in a human intestinal cell line.

Production of Antimicrobial Substances

One of the proposed mechanisms involved in the health benefits afforded by probiotics includes the formation of LMW compounds (<1,000 Da), such as organic acids, and the production of antibacterial substances termed bacteriocins (>1,000 Da).

Organic acids, in particular acetic acid and lactic acid, have a strong inhibitory effect against Gram-negative bacteria, and they have been considered the main antimicrobial compounds responsible for the inhibitory activity of probiotics against pathogens [106–108]. The undissociated form of the organic acid enters the bacterial cell and dissociates inside its cytoplasm. The eventual lowering of the intracellular pH or the intracellular accumulation of the ionized form of the organic acid can lead to the death of the pathogen [109, 110].

Many LAB produce antibacterial peptides, including bacteriocins and small AMPs. Bacteriocins produced by Gram-positive bacteria (usually LAB, including lactacin B from *L. acidophilus*, plantaricin from *L. plantarum* and nisin from *Lactococcus lactis*) have a narrow activity spectrum and act only against closely related bacteria, but some bacteriocins are also active against food-borne pathogens [111]. The common mechanisms of bacteriocin-mediated killing include the destruction of target cells by pore formation and/or inhibition of cell wall synthesis [112]. For example, nisin forms a complex with the ultimate cell wall precursor, lipid II, thereby inhibiting cell wall biosynthesis of mainly spore-forming bacilli. Subsequently, the complex aggregates and incorporates peptides to form a pore in the bacterial membrane [113]. Several studies have revealed that bacteriocin production

confers producing strains with a competitive advantage within complex microbial environments as a consequence of their associated antimicrobial activity. Bacteriocin production may enable the establishment and increase the prevalence of producing strains as well as enable the direct inhibition of pathogen growth within the gastrointestinal tract [114].

Some specific antibacterial compounds have been described for several *Bifidobacterium* strains, and a unique bacteriocin, bifidocin B, which is produced by *B. bifidum* NCFB 1454 and is active towards Gram-positive bacteria, has been described as well [108, 115]. Liévin et al. [116] described a strong killing activity of two *Bifidobacterium* strains against several pathogenic bacteria, including *Salmonella enterica* ser. *typhimurium* SL1344 and *E. coli* C1845. This activity has been attributed to the production of a potential LMW lipophilic molecule [117]. In addition, an LMW protein termed BIF, which is produced by *B. longum* BL1928, is the only compound characterized thus far that is active against Gram-negative bacteria [100, 118, 119]. This protein has no direct inhibitory or killing effect, but it inhibits the binding of *E. coli* to human epithelial cell lines.

Intestinal bacteria also produce a diverse array of health-promoting fatty acids. Indeed, certain strains of intestinal bifidobacteria and lactobacilli have been shown to produce conjugated linoleic acid (CLA), a potent anticarcinogenic agent [114, 120]. An anti-obesity effect of CLA-producing *L. plantarum* has been observed in diet-induced obesity in mice [121]. Recently, the ability to modulate the fatty acid composition of the liver and adipose tissue of the host upon oral administration of CLA-producing bifidobacteria and lactobacilli has been demonstrated in a murine model [114].

Finally, probiotic bacteria are able to produce so-called de-conjugated bile acids, which are derivatives of bile salts. De-conjugated bile acids show a stronger antimicrobial activity compared to that of the bile salts synthesized by the host organism. It remains to be elucidated how probiotics protect themselves from their own bactericidal metabolites or if they are resistant to de-conjugated bile acids at all [122].

It is well known that some strains of probiotics produce metabolites that inhibit the growth of fungi and other species of bacteria [123, 124]. Some researchers have reported that *Lactobacillus* can produce antifungal substances, such as benzoic acid, methylhydantoin, mevalonolactone [125, 126] and short-chain fatty acids [127]. Magnusson and Schnürer [128] discovered that *Lactobacillus coryniformis* can produce proteinaceous compounds exhibiting antifungal properties, and Rouse et al. [129] characterized the antifungal peptides produced by LAB. These reports showed that the antifungal culture has the ability to prevent the growth of molds found in apple spoilage. Dal Bello et al. [130] reported the identification and chemical characterization of four antifungal substances produced by *L. plantarum* FST 1.7, including lactic acid, phenyllactic acid and two cyclic dipeptides [cyclo(L-Leu-L-Pro) and cyclo(L-Phe-L-Pro)]. A study described the antifungal culture as having the ability to retard growth of *Fusarium culmorum* and *Fusarium graminearum* found on breads. Another such study has reported the production of the antifungal cyclic dipeptides, cyclo (L-Phe-L-Pro) and cyclo(L-Phe-traps-4-OH-L-Pro), by LAB, which inhibit the growth of food- and feed-borne filamentous fungi and yeasts in a dual-culture agar plate assay [131].

Probiotics and the Immune System

It is well known that probiotic bacteria can exert an immunomodulatory effect. These bacteria have the ability to interact with epithelial and dendritic cells (DCs) and with monocytes/macrophages and lymphocytes. The immune system can be divided between the innate and adaptive systems. The adaptive immune response depends on B and T lymphocytes, which are specific for particular antigens. In contrast, the innate immune system responds to common structures called pathogen-associated molecular patterns (PAMPs) shared by the vast majority of pathogens [132]. The primary response to pathogens is triggered by pattern recognition receptors (PPRs), which bind PAMPs. The best-studied PPRs are toll-like receptors (TLRs). In addition, extracellular C-type lectin receptors (CLRs) and intracellular nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors (NLRs) are known to transmit signals upon interaction with bacteria [133].

It is well established that the host cells that interact most extensively with probiotics are IECs. In addition, probiotics can encounter DCs, which have an important role in innate and adaptive immunity. Both IECs and DCs can interact with and respond to gut microorganisms through their PPRs [132, 133]. Figure 2 shows a summary of how probiotics may interact and modulate the immune system

TLRs and Probiotics

TLRs are transmembrane proteins expressed on various immune and non-immune cells, such as B cells, natural killer cells, DCs, macrophages, fibroblasts, epithelial

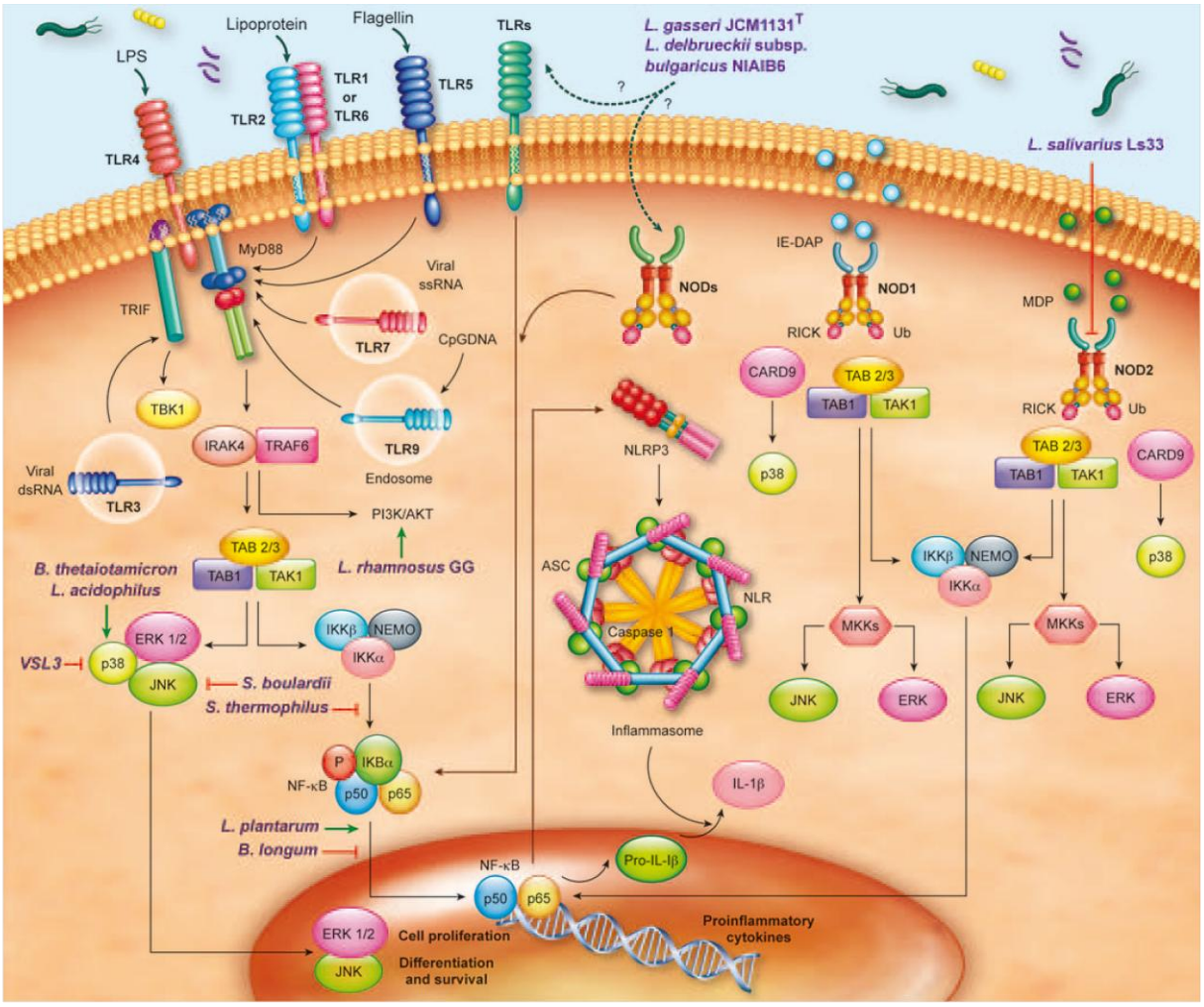


Fig. 2. Interaction of probiotics with the gut-associated immune system. ASC = Apoptosis-associated speck-like protein containing a CARD; *B. thetaiotamicron* = *Bacteroides thetaiotamicron*; CARD9 = caspase recruitment domain-containing protein 9; ERK = extracellular regulated kinase; IE-DAP = D-gamma-glutamyl-meso-DAP; IKK = IκB kinase; IRAK4 = IL-1 receptor-associated kinase 4; JNK = Jun N-terminal kinase; MDP = muramyl dipeptide; MKK = mitogen-activated kinase kinase; NEMO = NF-κB essential modulator; TAB1/2/3 = TAK binding proteins; TAK1 = ubiquitin-dependent kinase of MKK and IKK; TBK1 = serine/threonine-protein kinase 1; TRAF6 = TNF receptor-associated factor 6; Ub = ubiquitin.

cells and endothelial cells. In mammals, the TLR family includes eleven proteins (TLR1–TLR11). However, there is a stop codon in the human TLR11 gene that results in a lack of production of human TLR11. Activation of TLRs occurs after binding of the ligand to extracellular leucine-rich repeats. In humans, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are outer membrane associated and primarily respond to bacterial surface-associated PAMPs. TLR3, TLR7, TLR8 and TLR9 are found on the surface of endosomes where they respond primarily to nucleic acid-based PAMPs from viruses and bacteria [132]. Dimerization of TLRs and the highly conserved toll-interleukin-1 (IL-1) receptor (TIR) domains leads to the recruitment of adaptor molecules, such as myeloid differentiation primary response protein (MyD88), TIR domain-containing adaptor protein and TIR domain-containing adapter-

inducing interferon (IFN)-β (TRIF), to initiate signaling activation. The TLR signaling pathway, except for TLR3, involves the recruitment of MyD88, which activates the MAPK and nuclear factor (NF)-κB signaling pathways [133–135]. TLR3 utilizes the adaptor protein TRIF, leading to the expression of type 1 IFNs [135]. Furthermore, TLR-mediated signaling has been shown to control DC maturation inducing the upregulation of various maturation markers, such as CD80, CD83 and CD86, as well as the CCR7 chemokine receptor. Moreover, commensal and probiotic microorganisms can create an overall tolerant state mediated by the action of TLRs on DCs. It is clear that TLR9 signaling is essential to mediate the anti-inflammatory effect of probiotics. However, different studies have implicated other TLRs, such as TLR3 and TLR7, in the tolerance induced by commensal and probiotic bacteria. After activation by commensal and probiotic microorganisms, DCs initiate an appropriate response, such as the differentiation of Th_0 to T_{reg}, which has an inhibitory effect on Th_1, Th_2 and Th_{17} inflammatory responses.

It is well established that probiotics can suppress intestinal inflammation via the downregulation of TLR expression, secretion of metabolites that may inhibit TNF-α from entering blood mononuclear cells and inhibition of NF-κB signaling in enterocytes [132].

In this regard, cell wall components of lactobacilli can potentially signal through binding TLR2 in combination with TLR6. The diacylated membrane anchors of lipoproteins and lipoteichoic acids bind to TLR2 and TLR6, thereby promoting dimerization and MyD88-mediated activation of the canonical pathway of NF-κB [135]. Stimulation of TLR2 increases the production of cytokines, and TLR2 activation has an important role in enhancing transepithelial resistance to invading bacteria [136].

TLR2 recognizes peptidoglycan, which is the main component of Gram-positive bacteria, including the *Lactobacillus* genus. Several studies have demonstrated that TLR2 is required for some *Lactobacillus* strains to exert their immunomodulatory effects. Vinderola et al. [137] demonstrated that *L. casei* CRL 431 interacts with epithelial cells through TLR2 and that the interaction between *L. casei* and gut-associated immune cells induces an increase in the number of CD-206 and TLR2 receptors, mainly in the cells involved in the innate immune response.

In addition, Shida et al. [138] showed that *L. casei* induces a high level of IL-12 production in both wild-type and TLR2-deficient macrophages, and that peptidoglycan induces low levels of IL-12 production in wild-type macrophages and even lower levels in TLR2-deficient macrophages. They also suggested that the intact peptidoglycan of lactobacilli actually signals via TLR2 to inhibit IL-12 production. Although the recognition by TLR2 is essential, 12–48% of IL-12 production in TLR2-deficient macrophages is inhibited by peptidoglycan, thus suggesting that other TLR2-independent mechanisms may also be involved. Furthermore, it has been demonstrated that *Lactobacillus* strains, such as *L. rhamnosus* GG (LGG) and *L. plantarum* BFE 1685, enhance TLR2 in vitro in experiments using human intestinal cells, and more recently, *L. casei* CRL 431 has been shown to exert a similar effect on healthy mice and mice infected with *S. enterica* serovar *typhimurium* [139, 140]. For instance, probiotic administration to healthy mice increases expression of TLR2, TLR4 and TLR9, and it improves the secretion of TNF-α, IFN-γ and IL-10 in Peyer's patches [140].

Similarly, when porcine IECs encounter *Lactobacillus jensenii* TL2937, TLR2 may act synergistically and cooperatively with one or more PRRs, which may result in a coordinated sum of signals that induce the upregulation of several negative regulators of TLRs, including A20, Bcl-3 and MKP-1 [141].

TLR2 also has an important role in the recognition of bifidobacteria. Hoarau et al. [142] reported that a fermentation product from *Bifidobacterium breve* C50 can induce maturation, high IL-10 production and prolonged survival of DCs via the TLR2 pathway.

Similarly, Zeuthen et al. [143] showed that TLR2–/– DCs produce more IL-2 and less IL-10 in response to bifidobacteria, and they concluded that the immuno-inhibitory effect of bifidobacteria is dependent on TLR2.

Recently, Kailova et al. [144] reported that oral administration of *B. bifidum* OLB 6378 to rats with necrotizing enterocolitis (NEC) stimulates TLR2 expression in the ileal epithelium, enhances epithelial expression of COX-2 and increases intestinal production of prostaglandin E_2. Indeed, pretreatment of IEC-6 cells with the probiotic strain stimulates TLR2 and COX-2 expression and blocks cytokine-induced apoptosis. However, there is no evidence of a clear link between TLR2 activation and the upregulation of COX-2.

In contrast, it has been shown that the *L. reuteri* strains DSM 17938 and ATCC PTA 4659 have a beneficial effect on preventing NEC in rats. In response to the probiotic, mRNA expression of IL-6, and expression levels of TNF-α, TLR4 and NF-κB are significantly downregulated, and mRNA levels of IL-10 are significantly upregulated. Moreover, *L. reuteri* treatment leads to de-

creases in intestinal protein levels of TLR4, IL-1β and TNF-α in newborn rats with NEC. Furthermore, *L. reuteri* significantly increases survival rate, reduces both the incidence and severity of NEC and decreases pro-inflammatory cytokine levels in parallel with inhibition of TLR4 signaling via the NF-κB pathway.

Moreover, TLR4 has a significant role in the host defense against *Salmonella* infection in vivo. In healthy mice, *L. casei* CRL 431 activates this receptor and can be used as a surveillance mechanism against pathogenic bacteria [140]. Activation of TLR4 leads to the induction of pro-inflammatory mediators, an increase in TLR2 expression, and a reduction in its own expression, which leads to the recruitment of inflammatory cells and the initiation of the appropriate responses in the spleen. Collectively, these events allow for the control of bacterial replication [140, 146, 147].

Similarly, heat-inactivated LGG and *Lactobacillus delbrueckii* subsp. *bulgaricus* can decrease TLR4 expression similar to lipopolysaccharide (LPS) after 12 h in human monocyte-derived DCs. Moreover, LGG downregulates p38 expression, and *L. delbrueckii* subsp. *bulgaricus* reduces inhibitor protein κB (IκB) expression. In addition, these probiotic strains can modify the immune response at the post-transcriptional level by modifying miRNA expression [148].

Another relevant TLR is TLR9, which recognizes bacterial CpG DNA and synthetic unmethylated CpG oligonucleotide mimics (CpG-ODN). Unmethylated DNA fragments containing CpG motifs that are released from probiotics in vivo have the potential to mediate anti-inflammatory effects through TLR9 signaling at the epithelial surface. It is known that *Lactobacillus* species differ in their C+G composition. Thus, the ability of different species to stimulate TLR9 is likely to be different [135, 149]. TLR9 activation through apical and basolateral surfaces activates different intracellular signaling pathways in polarized epithelial cells. Whereas basolateral TLR9 triggers IκBα degradation and NF-κB pathway activation, apical TLR9 induces cytoplasmic accumulation of ubiquitinated IκB and inhibition of NF-κB activation [150].

Using polarized HT29 and T84 cell monolayers, Ghadimi et al. [151] showed that binding of natural commensal-origin DNA to the apical TLR9 initiates an intracellular signaling cascade in a specific manner that is associated with the attenuation of TNF-α-induced NF-κB activation and NF-κB-mediated IL-8 expression. When LGG DNA was apically applied, they showed a detracted TNF-α-induced NF-κB activation by reduced IκBα degradation and p38 MAPK phosphorylation, thereby indicating that intracellular chemical signals may coordinately regulate multiple properties of TLR9 expression that are relevant in multicellular functional responses of TLR9 to bacterial DNA. They also showed that TLR9 silencing abolishes the inhibitory effect of natural commensal-origin DNA on TNF-α-induced IL-8 secretion.

Similarly, *B. breve* (NumRes 204), *L. rhamnosus* (NumRes 1) and *L. casei* (DN-114 001) strains induce different cytokine production levels by human and mouse primary immune cells. It has been demonstrated that the *B. breve* strain induces lower levels of the pro-inflammatory cytokine IFN-γ than *L. rhamnosus* and *L. casei*. Moreover, *B. breve* and lactobacilli induce cytokines in a TLR9-dependent manner, and the lower inflammatory profile of *B. breve* is due to inhibitory effects of TLR2 [152].

In addition, it has been shown that purified genomic DNA from *L. plantarum* (p-gDNA) does not substantially stimulate pro-inflammatory cytokines. However, p-gDNA inhibits LPS-induced TNF-α production by THP-1 cells. Furthermore, p-gDNA reduces the expression of TLR2, TLR4 and TLR9, which induces the activation of NF-κB through the LPS signaling pathway, leading to the upregulation of inflammatory cytokines [153, 154]. Pretreatment of p-gDNA inhibited the phosphorylation of MAPKs and NF-κB, and also inhibited LPS-induced TNF-α production in subsequent LPS stimulation. In this regard, *L. plantarum* genomic DNA-mediated inhibition of signaling and TNF-α was accompanied by the suppression of TLR2, TLR4 and TLR9, as well as the induction of IL-1 receptor-associated kinase M (a negative regulator of TLR) [154].

NLRs and Probiotics

As mentioned before, there is another family of membrane-bound receptors: NLRs. They are located in the cytoplasm and are important in tissues where TLRs are expressed at low levels. The most thoroughly characterized members are NOD1 and NOD2, but currently more than 20 different NLRs have been identified [155]. Unlike NOD1, which is ubiquitously expressed, the expression of NOD2 is restricted to DCs, macrophages, Paneth cells, intestinal cells, lung cells and oral epithelial cells, and it is expressed at low levels in T cells. NOD1 can sense peptidoglycan moieties containing meso-diaminopimelic acid, which are associated with Gram-negative bacteria, but NOD2 senses muramyl dipeptide motifs, which can be found in a wide range of bacteria [156]. Upon recogni-

tion of their agonist, both NOD1 and NOD2 self-oligomerize to recruit and activate the adaptor protein RICK, a protein kinase that regulates CD95-mediated apoptosis, which is essential for the activation of NF-κB and MAPKs, resulting in the upregulation of transcription and production of inflammatory mediators (e.g. cytokines, chemoattractants, COX-2 and inducible nitric oxide synthase) [157].

There are a few studies showing the effect of probiotics on NLR. However, Fernandez et al. [158] recently demonstrated that the protective capacity of *L. salivarius* Ls33 correlates with local IL-10 production, which is abolished in NOD2-deficient mice. Indeed, these authors showed that the anti-inflammatory effect of Ls33 is mediated via NOD2.

Another important pathway activated by NLRs involves apoptosis-associated speck-like protein with caspase recruitment to activated caspase 1, an adaptor protein which is necessary for the cleavage of pro-IL-1β and pro-IL-18 into their mature and biologically active forms. NLRs participate in the formation of inflammasomes, which leads to the activation of caspase-1. There are three principal inflammasomes named after the NLR involved as follows: NOD-like receptor family, pyrin domain containing protein (NLRP) 1, NLRP3 and NLRC4. NLRP3 detects LPS, muramyl dipeptide, bacterial RNA and viral RNA [157].

The following two steps are required for the complete activation of the NLRP3 inflammasome: a priming step to induce transcription of NLRP3 mRNA and a sequential step to recognize various PAMPs and danger-associated molecular patterns by fully expressed NLRP3 itself [159, 160]. With regard to probiotic mechanisms associated with NLRP3, Tohno et al. [161] found that *L. delbrueckii* subsp. *bulgaricus* NIAI B6 and *L. gasseri* JCM1131^{T} are able to enhance NLRP3 expression in the GALT of adult and newborn swine. Their results suggested that immunobiotic *Lactobacillus* strains directly promote NLRP3 expression via TLR and NOD-mediated signaling, resulting in the induction of appropriate NLRP3 activation in porcine GALT. Furthermore, their results indicated that NLRP3 expression is upregulated by TLR2, TLR9, NOD1 and NOD2 agonists in adult and newborn porcine GALT. It has been suggested that NLRP3 has an important role in the regulation of human intestinal inflammation, such as in Crohn's disease [162], and that dysregulated NLRP3 expression results in the disruption of immune homeostasis associated with autoinflammatory disease in humans [163]. Because the potential expression level of NLRP3 is low in immune cells, induction of cellular NLRP3 expression itself is a first step to evoke the appropriate activation of the NLRP3-mediating signaling pathway in order to respond to danger-associated molecular patterns and PAMP stimuli [159, 160, 164, 165].

Conclusions

Probiotics have considerable potential for preventive or therapeutic applications in various gastrointestinal disorders. However, it is important to note that many probiotic health claims have not yet been substantiated by experimental evidence. In addition, the efficacy demonstrated for one given bacterial strain cannot necessarily be transferred to other probiotic organisms. Moreover, the mechanisms underlying probiotic action have not yet been fully elucidated.

This study reviewed the mechanisms of action of probiotics. Several important mechanisms underlying the antagonistic effects of probiotics on various microorganisms include the following: modification of the gut microbiota, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier and modulation of the immune system to convey an advantage to the host. The recent characterization of the host families of pattern-recognition molecules, such as TLR and NOD-like receptors, as well as modulating key signaling pathways, such as NF-κB and MAPK, with respect to their ability to enhance or suppress activation and influence downstream pathways will shed light onto the complex interplay of host-microbe interactions. Stimulation of these receptors by commensal bacteria has a crucial role to elicit measured antimicrobial responses with minimal inflammatory tissue damage.

Future Perspectives

In the present review, we provided an overview of the mechanisms of action of probiotics. It must be noted that many reported mechanisms of probiotic action are the results of in vitro experiments. Considerable effort has been invested in the development of methods enabling the in-depth analysis of the molecular mechanisms of probiotics. The complex and dynamic interactions that exist between the intestinal epithelium and bacteria on the luminal side as well as between the epithelium and the underlying immune system on the basolateral side must be reconciled in co-culture experiments with probiotics,

DCs and IECs as well as in 3D models. Other models include tissue explants, bioreactors and organoids. In vitro models have improved our current knowledge regarding specific probiotic modes of action. However, a number of limitations have to be taken into account. For example, results obtained with different IECs have to be carefully interpreted because not all cell lines share the same characteristics. It should also be noted that culture conditions may influence the expression of certain molecular characteristics.

The molecular elucidation of probiotic action in vivo will help to identify true probiotics and to select the most suitable ones for the prevention and/or treatment of particular diseases. It is important to note that results obtained in animal models cannot be directly transferred to humans. The physiology of animals differs considerably from that of humans, but this disadvantage is outweighed by the possibility of using animals with virtually identical genetic backgrounds, such as human microbiota-associated animals.

The quest for a better understanding of how probiotics operate has catalyzed an enormous interest in the molecular processes underlying host-microbe interactions. Gaining insight into the mechanisms of probiotic action may not only help to improve the credibility of the probiotic concept but also to foster the development of novel strategies for the treatment or prevention of gastrointestinal and autoimmune diseases.

References

1 FAO/WHO: Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. 2001. www.fao.org.

2 Guarner F, Malagelada JR: Gut flora in health and disease. Lancet 2003;361:512–519.

3 Gourbeyre P, Denery S, Bodinier M: Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. J Leukoc Biol 2011;89:685–695.

4 Macpherson AJ, Harris NL: Interactions between comensal intestinal bacteria and the immune system. Nat Rev Immunol 2004;4:478–485.

5 Frick JS, Schenk K, Quitadamo M, et al: *Lactobacillus fermentum* attenuates the proinflammatory effect of *Yersinia enterocolitica* on human epithelial cells. Inflamm Bowel Dis 2007;13:83–90.

6 McFarland LV: Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of *Clostridium difficile* disease. Am J Gastroenterol 2006;101:812–822.

7 Liong MT: Probiotics: Biology, Genetics and Health Aspects. Microbiology Monographs. Heidelberg, Springer, 2011.

8 Collins JK, Thornton G, Sullivan GO: Selection of probiotic strains for human application. Int Dairy J 1998;8:487–490.

9 Ouwehand AC, Salminen S, Isolauri E: Probiotics: an overview of beneficial effects. Antonie van Leeuwenhoek 2002;82:279–289.

10 Collado MC, Gueimonde M, Salminen S: Probiotics in adhesion of pathogens: mechanisms of action; in Watson RR, Preedy VR (eds): Bioactive Foods in Promoting Health, Chennai, Academic Press, Elsevier, 2010, vol 23, pp 353–370.

11 Yan F, Polk DB: Probiotics and immune health. Curr Opin Gastroenterol 2011;27:496–501.

12 Lye HS, Kuan CY, Ewe JA, et al: The improvement of hypertension by probiotics: effects on cholesterol, diabetes, renin, and phytoestrogens. Int J Mol Sci 2009;10:3755–3775.

13 Pelletier X, Laure-Boussuge S, Donazzolo Y: Hydrogen excretion upon ingestion of dairy products in lactose-intolerant male subjects: importance of the live flora. Eur J Clin Nutr 2001;55:509–512.

14 Woodard GA, Encarnacion B, Downey JR, et al: Probiotics improve outcomes after Roux-en-Y gastric bypass surgery: a prospective randomized trial. J Gastrointest Surg 2009;13:1198–1204.

15 Karska-Wysocki B, Bazo M, Smoragiewicz W: Antibacterial activity of *Lactobacillus acidophilus* and *Lactobacillus casei* against methicillin-resistant *Staphylococcus aureus* (MRSA). Microbiol Res 2010;165:674–686.

16 Liong MT: Safety of probiotics: translocation and infection. Nutr Rev 2008;66:192–202.

17 Rafter J, Bennett M, Caderni G, et al: Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. Am J Clin Nutr 2007;85:488–496.

18 Moayyedi P, Ford AC, Talley NJ, et al: The efficacy of probiotics in the treatment of irritable bowel syndrome: a systematic review. Gut 2010;59:325–332.

19 Golowczyc MA, Mobili P, Garrote GL, et al: Protective action of Lactobacillus kefir carrying S-layer protein against *Salmonella enterica* serovar *enteritidis*. Int J Food Microbiol 2007;118:264–273.

20 Williams NT: Probiotics. Am J Health System Pharm 2010;67:449–458.

21 Ohland CL, Macnaughton WK: Probiotic bacteria and intestinal epithelial barrier function. Am J Physiol Gastrointest Liver Physiol 2010;298:G807–G819.

22 Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI: Molecular analysis of commensal host-microbial relationships in the intestine. Science 2001;291:881–884.

23 Hooper LV, Stappenbeck TS, Hong CV, Gordon JI: Angiogenins: a new class of microbicidal proteins involved in innate immunity. Nat Immunol 2003;4:269–273.

24 Sartor RB: Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol 2006;3:390–407.

25 Anderson RC, Cookson AL, McNabb WC, Park Z, McCann MJ, Kelly WJ, Roy NC: Lactobacillus plantarum MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. BMC Microbiol 2010;10:316.

26 Hummel S, Veltman K, Cichon C, Sonnenborn U, Schmidt MA: Differential targeting of the E-cadherin/β-catenin complex by Gram-positive probiotic lactobacilli improves epithelial barrier function. Appl Environ Microbiol 2012;78:1140–1147.

27 Zyrek AA, Cichon C, Helms S, Enders C, Sonnenborn U, Schmidt MA: Molecular mechanisms underlying the probiotic effects of *Escherichia coli* Nissle 1917 involve ZO-2 and PKC redistribution resulting in tight junction and epithelial barrier repair. Cell Microbiol 2007;9:804–816.

28 Stetinova V, Smetanova L, Kvetina J, Svoboda Z, Zidek Z, Tlaskalova-Hogenova H: Caco-2 cell monolayer integrity and effect of probiotic *Escherichia coli* Nissle 1917 components. Neuro Endocrinol Lett 2010;31:51–56.

29 Parassol N, Freitas M, Thoreux K, Dalmasso G, Bourdet-Sicard R, Rampal P: Lactobacillus casei DN-114001 inhibits the increase in paracellular permeability of enteropathogenic *Escherichia coli*-infected T84cells. Res Microbiol 2005;156:256–262.

30 Otte JM, Podolsky DK: Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. Am J Physiol Gastrointest Liver Physiol 2004;286:G613–G626.

31 Dai C, Zhao DH, Jiang M: VSL#3 probiotics regulate the intestinal epithelial barrier in vivo and in vitro via the p38 and ERK signaling pathways. Int J Mol Med 2012;29:202–208.

32 Bruewer M, Samarin S, Nusrat A: Inflammatory bowel disease and the apical junctional complex. Ann NY Acad Sci 2006;1072:242–252.

33 Yan F, Polk DB: Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. J Biol Chem 2002;277:50959–50965.

34 Yan F, Cao H, Cover TL, Whitehead R, Washington MK, Polk DB: Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. Gastroenterology 2007;132:562–575.

35 Tao Y, Drabik KA, Waypa TS, Musch MW, Alverdy JC, Schneewind O, Chang EB, Petrof EO: Soluble factors from *Lactobacillus* GG activate MAPKs and induce cytoprotective heat shock proteins in intestinal epithelial cells. Am J Physiol Cell Physiol 2006;290:C1018–C1030, erratum in 2006;291:C194.

36 Mack DR, Ahrne S, Hyde L, Wei S, Hollingsworth MA: Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells in vitro. Gut 2003;52:827–833.

37 Mattar AF, Teitelbaum DH, Drongowski RA, Yongyi F, Harmon CM, Coran AG: Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model. Pediatr Surg Int 2002;18:586–590.

38 Kim Y, Kim SH, Whang KY, Kim YJ, Oh S: Inhibition of *Escherichia coli* O157:H7 attachment by interactions between lactic acid bacteria and intestinal epithelial cells. J Microbiol Biotechnol 2008;18:1278–1285.

39 Gaudier E, Michel C, Segain JP, Cherbut C, Hoebler C: The VSL# 3 probiotic mixture modifies microflora but does not heal chronic dextran-sodium sulfate-induced colitis or reinforce the mucus barrier in mice. J Nutr 2005;135:2753–2761.

40 Caballero-Franco C, Keller K, De Simone C, Chadee K: The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol 2007;292:G315–G322.

41 Juntunen M, Kirjavainen PV, Ouwehand AC, Salminen SJ, Isolauri E: Adherence of probiotic bacteria to human intestinal mucus in healthy infants and during rotavirus infection. Clin Diag Lab Immunol 2001;8:293–296.

42 Beachey EH: Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J Infect Dis 1981;143:325–345.

43 Schiffrin EJ, Brassart D, Servin AL, Rochat F, Donnet-Hughes A: Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. Am J Clin Nutr 1997;66:515S–520S.

44 Perdigon G, Maldonado Galdeano C, Valdez JC, Medici M: Interaction of lactic acid bacteria with the gut immune system. Eur J Clin Nutr 2002;56:S21–S26.

45 Hirano J, Yoshida T, Sugiyama T, Koide N, Mori I, Yokochi T: The effect of *Lactobacillus rhamnosus* on enterohemorrhagic *Escherichia coli* infection of human intestinal cells in vitro. Microbiol Immunol 2003;47:405–409.

46 Salminen S, Bouley C, Boutron-Ruault MC, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau MC, Roberfroid M, Rowland I: Functional food science and gastrointestinal physiology and function. Br J Nutr 1998;80:S147–S171.

47 Collado MC, Gueimonde M, Hernández M, Sanz Y, Salminen S: Adhesion of selected *Bifidobacterium* strains to human intestinal mucus and the role of adhesion in enteropathogen exclusion. J Food Prot 2005;68:2672–2678.

48 Crociani J, Grill JP, Huppert M, Ballongue J: Adhesion of different bifidobacterias strains to human enterocyte-like Caco-2 cells and comparison with in vivo study. Lett Appl Microbiol 1995;21:146–148.

49 Castagliuolo I, Galeazzi F, Ferrari S, Elli M, Brun P, Cavaggioni A, Tormen D, Sturniolo GC, Morelli L, Palù G: Beneficial effect of auto-aggregating *Lactobacillus crispatus* on experimentally induced colitis in mice. FEMS Immunol Med Microbiol 2005;43:197–204.

50 González-Rodríguez I, Sánchez B, Ruiz L, Turroni F, Ventura M, Ruas-Madiedo P, Gueimonde M, Margolles A: Role of extracellular transaldolase from *Bifidobacterium bifidum* in mucin adhesion and aggregation. Appl Environ Microbiol 2012;78:3992–3998.

51 Neutra MR, Forstner JF: Gastrointestinal mucus: synthesis, secretion and function; in Johnson LR (ed): Physiology of the Gastrointestinal Tract, ed 2. New York, Raven, 1987.

52 Ouwehand AC, Salminen S, Tolkko S, Roberts P, Ovaska J, Salminen E: Resected human colonic tissue: new model for characterizing adhesion of lactic acid bacteria. Clin Diag Lab Immunol 2002;9:184–186.

53 Haller D, Colbus H, Ganzle MG, Scherenbacher P, Bode C, Hammes WP: Metabolic and functional properties of lactic acid bacteria in the gastro-intestinal ecosystem: a comparative in vitro study between bacteria of intestinal and fermented food origin. Syst Appl Microbiol 2001;24:218–226.

54 Van Tassell ML, Miller MJ: *Lactobacillus* adhesion to mucus. Nutrients 2011;3:613–636.

55 Buck BL, Altermann E, Svingerud T, Klaenhammer TR: Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCNCFM. Appl Environ Microbiol 2005;71:8344–8351.

56 Vélez MP, De Keersmaecker SC, Vanderleyden J: Adherence factors of *Lactobacillus* in the human gastrointestinal tract. FEMS Microbiol Lett 2007;276:140–148.

57 Hynönen U, Westerlund-Wikström B, Palva A, Korhonen TK: Identification by flagellum display of an epithelial cell and fibronectin-binding function in the SlpA surface protein of *Lactobacillus brevis*. J Bacteriol 2002;184:3360–3367.

58 Goh YJ, Klaenhammer TR: Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol 2010;76:5005–5012.

59 Sánchez B, González-Tejedo C, Ruas-Madiedo P, Urdaci MC, Margolles A: *Lactobacillus plantarum* extracellular chitin-binding protein and its role in the interaction between chitin, Caco-2 cells, and mucin. Appl Environ Microbiol 2011;77:1123–1126.

60 von Ossowski I, Reunanen J, Satokari R, Vesterlund S, Kankainen M, Huhtinen H, Tynkkynen S, Salminen S, de Vos WM, Palva A: Mucosal adhesion properties of the probiotic *Lactobacillus rhamnosus* GG SpaCBA and SpaFED pilin subunits. Appl Environ Microbiol 2010;6:2049–2057.

61 von Ossowski I, Satokari R, Reunanen J, Lebeer S, De Keersmaecker SC, Vanderleyden J, de Vos WM, Palva A: Functional characterization of a mucus-specific LPXTG surface adhesin from probiotic *Lactobacillus rhamnosus* GG. Appl Environ Microbiol 2011;77:4465–4472.

62 Candela M, Bergmann S, Vici M, Vitali B, Turroni S, Eikmanns BJ, Hammerschmidt S, Brigidi P: Binding of human plasminogen to *Bifidobacterium*. J Bacteriol 2007;189:5929–5936.

63 Candela M, Biagi E, Centanni M, Turroni S, Vici M, Musiani F, Vitali B, Bergmann S, Hammerschmidt S, Brigidi P: Bifidobacterial enolase, a cell surface receptor for human plasminogen involved in the interaction with the host. Microbiology 2009;155:3294–3303.

64 Candela M, Centanni M, Fiori J, Biagi E, Turroni S, Orrico C, Bergmann S, Hammerschmidt S, Brigidi P: DnaK from *Bifidobacterium animalis* subsp. *lactis* is a surface-exposed human plasminogen receptor upregulated in response to bile salts. Microbiology 2010;156:1609–1618.

65 Guglielmetti S, Tamagnini I, Mora D, Minuzzo M, Scarafoni A, Arioli S, Hellman J, Karp M, Parini C: Implication of an outer surface lipoprotein in adhesion of *Bifidobacterium bifidum* to Caco-2 cells. Appl Environ Microbiol 2008;74:4695–4702.

66 Sánchez B, Urdaci MC, Margolles A: Extracellular proteins secreted by probiotic bacteria as mediators of effects that promote mucosa-bacteria interactions. Microbiology 2010;156:3232–3242.

67 Voltan S, Castagliuolo I, Elli M, Longo S, Brun P, D'Incà R, Porzionato A, Macchi V, Palù G, Sturniolo GC, Morelli L, Martines D: Aggregating phenotype in *Lactobacillus crispatus* determines intestinal colonization and TLR2 and TLR4 modulation in murine colonic mucosa. Clin Vaccine Immunol 2007; 14:1138–1148.

68 Kim YS, Ho SB: Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep 2010; 12:319–330.

69 Collado MC, Gueimonde M, Sanz Y, Salminen S: Adhesion properties and competitive pathogen exclusion ability of bifidobacteria with acquired acid resistance. J Food Prot 2006;69:1675–1679.

70 Chauvière G, Coconnier MH, Kerneis S, Fourniat J, Servin AL: Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. J Gen Microbiol 1992;138:1689–1696.

71 Coconnier MH, Klaenhammer TR, Kerneis S, Bernet MF, Servin AL: Protein-mediated adhesion *of Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. Appl Environ Microbiol 1992;58:2034–2039.

72 Greene JD, Klaenhammer TR: Factors involved in adherence of lactobacilli to human Caco-2 cells. Appl Environ Microbiol 1994; 60:4487–4494.

73 Gopal PK, Prasad J, Smart J, Gill HS: In vitro adherence properties of *Lactobacillus rhamnosus* DR 20 and *Bifidobacterium lactis* DR 10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. Int J Food Microbiol 2001;67:207–216.

74 Furrie E, Macfarlane S, Kennedy A, Cummings JH, Walsh SV, O'neil DA, Macfarlane GT: Synbiotic therapy (*Bifidobacterium longum*/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. Gut 2005;54:242–249.

75 Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ: Secretion of microbicidal alphadefensins by intestinal Paneth cells in response to bacteria. Nat Immunol 2000;1:113–118.

76 O'neil DA, Porter EM, Elewaut D, Anderson GM, Eckmann L, Ganz T, Kagnoff MF: Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. J Immunol 1999;163:6718–6724.

77 Takahashi A, Wada A, Ogushi K, Maeda K, Kawahara T, Mawatari K, Kurazono H, Moss J, Hirayama T, Nakaya Y: Production of beta-defensin-2 by human colonic epithelial cells induced by *Salmonella enteritidis* flagella filament structural protein. FEBS Lett 2001; 508:484–488.

78 Ogushi K, Wada A, Niidome T, Mori N, Oishi K, Nagatake T, Takahashi A, Asakura H, Makino S, Hojo H, Nakahara Y, Ohsaki M, Hatakeyama T, Aoyagi H, Kurazono H, Moss J, Hirayama T: *Salmonella enteritidis* FliC (flagella filament protein) induces human betadefensin-2 mRNA production by Caco-2 cells. J Biol Chem 2001;276:30521–30526.

79 Ganz T: Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol 2003;3: 710–720.

80 Gallo RL, Hooper LV: Epithelial antimicrobial defence of the skin and intestine. Nat Rev Immunol 2012;12:503–516.

81 Müller CA, Autenrieth IB, Peschel A: Innate defenses of the intestinal epithelial barrier. Cell Mol Life Sci 2005;62:1297–1307.

82 Koprivnjak T, Peschel A, Gelb MH, Liang NS, Weiss JP: Role of charge properties of bacterial envelope in bactericidal action of human group IIA phospholipase A2 against *Staphylococcus aureus*. J Biol Chem 2002; 277:47636–47644.

83 Kagan BL, Selsted ME, Ganz T, Lehrer RI: Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. Proc Natl Acad Sci USA 1990;87:210–214.

84 Bals R, Wilson JM: Cathelicidins – a family of multifunctional antimicrobial peptides. Cell Mol Life Sci 2003;60:711–720.

85 Abbot EL, Smith WD, Siou GP, Chiriboga C, Smith RJ, Wilson JA, Hirst BH, Kehoe MA: Pili mediate specific adhesion of *Streptococcus pyogenes* to human tonsil and skin. Cell Microbiol 2007;9:1822–1833.

86 Westerlund B, Korhonen TK: Bacterial proteins binding to the mammalian extracellular matrix. Mol Microbiol 1993;9:687–694.

87 Sun Z, Kong J, Hu S, Kong W, Lu W, Liu W: Characterization of a S-layer protein from *Lactobacillus crispatus* K313 and the domains responsible for binding to cell wall and adherence to collagen. Appl Microbiol Biotechnol 2012, DOI: 10.1007/s00253-012-4044-x.

88 Greenberg B: Salmonella suppression by known populations of bacteria in flies. J Bacteriol 1969;99:629–635.

89 Rolfe RD: Population dynamics of the intestinal tract; in Blankenship LC (ed): Colonization Control of Human Bacterial Enteropathogens in Poultry. San Diego, Academic Press, 1991, pp 59–75.

90 Servin AL: Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol Rev 2004;28: 405–440.

91 Chenoll E, Casinos B, Bataller E, Astals P, Echevarría J, Iglesias JR, Balbarie P, Ramón D, Genovés S: Novel probiotic *Bifidobacterium bifidum* CECT 7366 strain active against the pathogenic bacterium *Helicobacter pylori*. Appl Environ Microbiol 2011;77:1335–1343.

92 Sgouras D, Maragkoudakis P, Petraki K, Martinez-Gonzalez B, Eriotou E, Michopoulos S, Kalantzopoulos G, Tsakalidou E, Mentis A: In vitro and in vivo inhibition of *Helicobacter pylori* by *Lactobacillus casei* strain Shirota. Appl Environ Microbiol 2004;70:518–526.

93 Todoriki K, Mukai T, Sato S, Toba T: Inhibition of adhesion of food-borne pathogens to Caco-2 cells by *Lactobacillus* strains. J Appl Microbiol 2001;91:154–159.

94 Chu H, Kang S, Ha S, Cho K, Park SM, Han KH, Kang SK, Lee H, Han SH, Yun CH, Choi Y: *Lactobacillus acidophilus* expressing recombinant K99 adhesive fimbriae has an inhibitory effect on adhesion of enterotoxigenic *Escherichia coli*. Microbiol Immunol 2005; 49:941–948.

95 Tsai CC, Lin PP, Hsieh YM: Three *Lactobacillus* strains from healthy infant stool inhibit enterotoxigenic *Escherichia coli* grown in vitro. Anaerobe 2008;14:61–67.

96 Muñoz JA, Chenoll E, Casinos B, Bataller E, Ramón D, Genovés S, Montava R, Ribes JM, Buesa J, Fàbrega J, Rivero M: Novel probiotic *Bifidobacterium longum* subsp. *infantis* CECT 7210 strain active against rotavirus infections. Appl Environ Microbiol 2011;77: 8775–8783.

97 Nakamura S, Kuda T, An C, Kanno T, Takahashi H, Kimura B: Inhibitory effects of *Leuconostoc mesenteroides* 1RM3 isolated from narezushi, a fermented fish with rice, on *Listeria monocytogenes* infection to Caco-2 cells and A/J mice. Anaerobe 2012;18:19–24.

98 Schiffrin EJ, Blum S: Interactions between the microbiota and the intestinal mucosa. Eur J Clin Nutr 2002;56:S60–S64.

99 Nesser JR, Granato D, Rouvet M, Servin A, Teneberg S, Karlsson KA: *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. Glycobiology 2000;10:1193–1199.

100 Fujiwara S, Hashiba H, Hirota T, Forstner JF: Inhibition of the binding of enterotoxigenic *Escherichia coli* Pb176 to human intestinal epithelial cell line HCT-8 by an extracellular protein fraction containing BIF of *Bifidobacterium longum* SBT2928: suggestive evidence of blocking of the binding receptor gangliotetraosylceramide on the cell surface. Int J Food Microbiol 2001;67:97–106.

101 Mukai T, Asasaka T, Sato E, Mori K, Matsumoto M, Ohori H: Inhibition of binding of *Helicobacter pylori* to the glycolipid receptors by probiotic *Lactobacillus reuteri*. FEMS Immunol Med Microbiol 2002;32:105–110.

102 Coconnier MH, Bernet MF, Chauviere G, Servin AL: Adhering heat-killed human *Lactobacillus acidophilus*, strain LB, inhibits the process of pathogenicity of diarrhoeagenic bacteria in cultured human intestinal cells. J Diarrhoeal Dis Res 1993;11:235–242.

103 Tuomola EM, Ouwehand AC, Salminen S: The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. FEMS Immunol Med Microbiol 1999;26:137–142.

104 Hirn J, Nurmi E, Johansson T, Nuotio L: Long-term experience with competitive exclusion and salmonellas in Finland. Int J Food Microbiol 1992;15:281–285.

105 Genovese KJ, Anderson RC, Harvey RB, Nisbet DJ: Competitive exclusion treatment reduces the mortality and fecal shedding associated with enterotoxigenic *Escherichia coli* infection in nursery-raised neonatal pigs. Can J Vet Res 2000;64:204–207.

106 Alakomi HL, Skytta E, Saarela M, Mattila-Sandholm T, Latva-Kala K, Helander IM: Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Appl Environ Microbiol 2000;66:2001–2005.

107 De Keersmaecker SC, Verhoeven TL, Desair J, Marchal K, Vanderleyden J, Nagy I: Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. FEMS Microbiol Lett 2006;259:89–96.

108 Makras L, Triantafyllou V, Fayol-Messaoudi D, Adriany T, Zoumpopoulou G, Tsakalidou E, Servin A, DeVuyst L: Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar *typhimurium* reveals a role for lactic acid and other inhibitory compounds. Res Microbiol 2006;157:241–247.

109 Ouwehand AC: Antimicrobial components from lactic acid bacteria; in Salminen S, von Wright A (eds): Lactic Acid Bacteria: Microbiology and Functional Aspects. New York, Dekker, 1998, pp 139–159.

110 Russell JB, Diez-Gonzalez F: The effects of fermentation acids on bacterial growth. Adv Microb Physiol 1998;39:205–234.

111 Nielsen DS, Cho GS, Hanak A, Huch M, Franz CM, Arneborg N: The effect of bacteriocin-producing *Lactobacillus plantarum strains* on the intracellular pH of sessile and planktonic Listeria monocytogenes single cells. Int J Food Microbiol 2010;141:S53–S59.

112 Hassan M, Kjos M, Nes IF, Diep DB, Lotfipour F: Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. J Appl Microbiol. 2012, DOI: 10.1111/j.1365-2672.2012.05338.

113 Bierbaum G, Sahl: Lantibiotics: mode of action, biosynthesis and bioengineering. Curr Pharm Biotechnol 2009;10:2–18.

114 O'Shea EF, Cotter PD, Stanton C, Ross RP, Hill C: Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanisms: bacteriocins and conjugated linoleic acid. Int J Food Microbiol 2012;152:189–205.

115 Yildirim Z, Winters DK, Johnson MG: Purification, amino acid sequence and mode of action of bifidocin B produced by *Bifidobacterium bifidum* NCFB 1454. J Appl Microbiol 1999;86:45–54.

116 Liévin V, Peiffer I, Hudault S, Rochat F, Brassart D, Neeser JR, et al: *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut 2000;47:646–652.

117 Gibson GR, Wang X: Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J Appl Bacteriol 1994;77:412–420.

118 Fujiwara S, Hashiba H, Hirota T, Forstner JF: Proteinaceous factor(s) in culture supernatant fluids of bifidobacteria which prevents the binding of enterotoxigenic *Escherichia coli t*o gangliotetraosylceramide. Appl Environ Microbiol 1997;63:506–512.

119 Fujiwara S, Hashiba H, Hirota T, Forstner JF: Purification and characterization of a novel protein produced by *Bifidobacterium longum* SBT2928 that inhibits the binding of enterotoxigenic Escherichia coli Pb176 (CFA/II) to gangliotetraosylceramide. J Appl Microbiol 1999;86:615–621.

120 Macouzet M, Lee BH, Robert N: Production of conjugated linoleic acid by probiotic *Lactobacillus acidophilus* La-5. J Appl Microbiol 2009;106:1886–1891.

121 Lee K, Paek K, Lee HY, Park JH, Lee Y: Antiobesity effect of trans-10,cis-12-conjugated linoleic acid-producing *Lactobacillus plantarum* PL62 on diet-induced obese mice. J Appl Microbiol 2007;103:1140–1146.

122 Oelschlaeger TA: Mechanisms of probiotic actions – a review. Int J Med Microbiol 2010;300:57–62.

123 Coloretti F, Carri S, Armaforte E, Chiavari C, Grazia L, Zambonelli C: Antifungal activity of lactobacilli isolated from salami. FEMS Microbiol Lett 2007;271:245–250.

124 Lindgren SE, Dobrogosz WJ: Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol Rev 1990;7:149–163.

125 Prema P, Smila D, Palavesam A, Immanuel G: Production and characterization of an antifungal compound (3-phenyllactic acid) produced by *Lactobacillus plantarum* strain. Food Bioprocess Technol 2008;3:379–386.

126 Niku-Paavola ML, Laitila A, Mattila-Sandholm T, Haikara A: New types of antimicrobial compounds produced by *Lactobacillus plantarum*. J Appl Microbiol 1999;86:29–35.

127 Sjogren J, Magnusson J, Broberg A, Schnurer J, Kenne L: Antifungal 3-hydroxy fatty acids from *Lactobacillus plantarum* MiLAB 14. Appl Environ Microbiol 2003;69:7554–7557.

128 Magnusson J, Schnürer J: *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. Appl Environ Microbiol 2001;67:1–5.

129 Rouse S, Harnett D, Vaughan A, van Sinderen D: Lactic acid bacteria with potential to eliminate fungal spoilage in foods. J Appl Microbiol 2008;104:915–923.

130 Dal Bello F, Clarke CI, Ryan LAM, Ulmer H, Schober TJ, Ström K, Sjögren J, van Sinderen D, Schnürer J, Arendt EK: Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. J Cereal Sci 2007;45:309–318.

131 Strom K, Sjogren J, Broberg A, Schnurer J: *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Appl Environ Microbiol 2002;68:4322–4327.

132 Gómez-Llorente C, Muñoz S, Gil A: Role of Toll-like receptors in the development of immunotolerance mediated by probiotics. Proc Nutr Soc 2010;69:381–389.

133 Lebeer S, Vanderleyden J, De Keersmaecker CJ: Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol 2010;8:171–184.

134 Kawai T, Akira S: The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010;11:373–384.

135 Wells JM: Immunomodulatory mechanisms of lactabacilli. Microb Cell Fact 2011;10(suppl 1):S17.

136 Vizoso Pinto MG, Rodríguez Gómez M, Seifert S, Waltz B, Holzapfel WH, Franz CM: Lactobacilli stimultates the innate immune response and modulate TLR expression of HT29 intestinal epithelial cells in vitro. Int J Food Microbiol 2009;133:86–93.

137 Vinderola G, Matar C, Perdigón G: Role of the epithelial cells in the immune effects mediated by Gram-positive probiotic bacteria. Involvement of Toll-like receptors. Clin Diagn Lab Immunol 2005;12:1075–1084.

138 Shida K, Kiyoshima-Shibata J, Nagaoka M, Nanno M: Peptidoglycan from lactobacilli inhibits interleukin-12 production by macrophages induced by *Lactobacillus casei* through Toll-like receptor 2-dependent and independent mechanisms. Inmmunology 2009;128:e858–e869.

139 Abreu MT, Fukata M, Arditi M: TLR signaling in the gut in health and diseases. J Immunol 2005;174:4453–4460.

140 Castillo NA, Perdigón G, De Moreno de Le Blanc A: Oral administration of a probiotic *Lactobacillus* modulates cytokine production and TLR expression improving the immune response against *Salmonella enterica* serovar *typhimurium* infection in mice. BMC Microbiol 2011;11:177–189.

141 Shimazu T, Villena J, Tohno M, Fujie H, Hosoya S, Shimosato T, Aso H, Suda Y, Kawai Y, Saito T, Makino S, Ikegami S, Itoh H, Kitazawa H: Immunobiotic *Lactobacillus jensenii* elicits anti-inflammatory activity in porcine intestinal epithelial cells by modulating negative regulators of the Toll-like receptor signaling pathway. Infect Inmmun 2012;80:276–288.

142 Hoarau C, Lagaraine C, Martin I, Velge-Roussel F, Lecranchu Y: Supernatant of Bifidobacterium breve induces dendritic cell maturation, activation, and survival through a Toll-like receptor pathway. J Allergy Clin Immunol 2006;117:696–702.

143 Zeuthen LH, Fink LN, Frokiaer H: Toll-like receptor 2 and nucleotide-binding oligomerization domain-2 play divergent roles in the recognition of gut derived lactobacilli and bifidobacteria in dendritic cells. Inmmunology 2008;124:489–502.

144 Kailova L, Mount Patrick SK, Arganbright KM, Halpern M, Kinouchi T, Dvorak B: *Bifidobacterium bifidum* reduces apoptosis in the intestinal epithelium in necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 2010;299:G1118–G1127.

145 Liu Y, Fatheree NY, Mangalat N, Rhoads JM: *Lactobacillus reuteri* strains reduce incidence and severity of experimental necrotizing enterocolitis via modulation of TLR4 and NF-κB signaling in the intestine. Am J Physiol Gastrointest Liver Physiol 2012;302:G608–G617.

146 Weiss DS, Raupach B, Takeda K, Akira S, Zychlinsky A: Toll-like receptors are temporally involved in host defense. J Immunol 2004;172:4463–4469.

147 Totemeyer S, Foster N, Kaiser P, Maskell DJ, Bryant CE: Toll-like receptor expression in C3H/HeJ and C3H/HeJ mice during Salmonella enterica serovar typhimurium infection. Infect Immun 2003;71:6653–6657.

148 Giahi L, Aumueller E, Elmadfa I, Haslberger AG: Regulation of TLR4, p38 MAPkinase, IκB and miRNAs by inactivated strains of lactobacilli in human dendritic cells. Benef Microbes 2012;4:91–98.

149 Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, et al: Toll-like receptor recognizes bacterial DNA. Nature 2000;408:740–745.

150 Lee J, Mo JH, Katura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S, et al: Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. Nat Cell Biol 2006;8:1327–1336.

151 Ghadimi D, de Vrese M, Heller KJ, Schrezenmeir J: Effect of natural commensal-origin DNA on Toll-like receptor 9 (TRL9) signaling cascade, chemokine IL-8 expression, and barrier integrity of polarized intestinal epitelial cells. Inflamm Bowel Dis 2010;16:410–427.

152 Plantiga TS, van Maren WWC, van Bergenhenegouwen J, Hameetman M, Nierkens S, Jacobs C, de Jong DJ, Joosten LAB, van't Land B, Garssen J, Adema GJ, Netea MG: Differential Toll-like receptor recognition and induction of cytokine profile by *Bifidobacterium breve* and *Lactobacillus* strains of probiotics. Clin Vaccine Immunol 2011;18:621–628.

153 Verstrepen L, Bekaert T, Chau TL, Tavernier J, Chariot A, Beyaert R: TLR4, IL-1R and TNF-R signaling to NF-kappaB: variation on a common theme. Cell Mol Life Sci 2008;65:2964–2978.

154 Kim CH, Kim HG, Kim JY, Kim NR, Jung BJ, Jeong JH, Chung DK: Probiotic genomic DNA reduces the production of pro-inflammatory cytokine tumor necrosis factor-alpha. FEMS Microbiol Lett 2012;328:13–19.

155 Hakansson A, Molin G: Gut microbiota and inflammation. Nutrients 2011;3:637–682.

156 Biswas A, Petnicki-Ocwieja T, Kobayashi KS: Nod2: a key regulator linking microbiota to intestinal mucosal immunity. J Mol Med (Berl) 2012;90:15–24.

157 Chen G, Shaw MH, Kim YG, Nuñez G: NOD-like receptors: role in innate immunity and inflammatory disease. Annu Rev Pathol 2009;4:365–398.

158 Fernandez M, Valenti V, Rockel C, Hermann C, Pot B, Boneca IG, Grangette C: Anti-inflammatory capacity of selected lactobacilli in experimental colitis is driven by NOD2-mediated recognition of a specific peptidoglycan-derived muropeptide. Gut 2011;60:1050–1059.

159 Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemeri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E: Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol 2009;183:787–791.

160 Bauernfeind F, Ablasser A, Bartok E, Kim S, Schmid-Burgk J, Cavlar T, Hornung V: Inflammasomes: current understanding and open questions. Cell Mol Life Sci 2010;68:765–783.

161 Tohno M, Shimasato T, Aso H, Kitazawa H: Immunobiotic *Lactobacillus* strains augment NLRP3 expression in newborn and adult porcine gut-associated lymphoid tissues. Vet Immunol Immunopathol 2011;144:410–416.

162 Hirota SA, Ng J, Lueng A, Khajah M, Parhar K, Li Y, Lam V, Potentier MS, Ng K, Bawa M, McCafferty DM, Rioux KP, Ghosh S, Xaier RJ, Colgan SP, Tschopp J, Muruve D, Macdonald JA, Beck PL: NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis. Inflamm Bowel Dis 2011;17:1359–1372.

163 Anderson JP, Mueller JL, Misaghi A, Anderson S, Sivagnanam M, Kolodner RD, Hoffman HM: Initial description of the human NLRP3 promoter. Gene Immun 2008;9:721–726.

164 Meylan E, Tschopp J, Karin M: Intracellular pattern recognition receptors in the host response. Nature 2006;442:39–44.

165 Martinon F, Mayor A, Tschopp J: The inflammasomes: guardians of the body. Annu Rev Immunol 2009;27:229–265.

British Journal of Nutrition (2013), **109**, S27–S34
doi:10.1017/S0007114512004023

In vitro cell and tissue models for studying host–microbe interactions: a review

Miriam Bermudez-Brito, Julio Plaza-Díaz, Luis Fontana, Sergio Muñoz-Quezada and Angel Gil*

Department of Biochemistry & Molecular Biology II, School of Pharmacy and Institute of Nutrition & Food Technology "José Mataix", Biomedical Research Centre, University of Granada, Granada, Spain

Abstract

Ideally, cell models should resemble the *in vivo* conditions; however, in most *in vitro* experimental models, epithelial cells are cultivated as monolayers, in which the establishment of functional epithelial features is not achieved. To overcome this problem, co-culture experiments with probiotics, dendritic cells and intestinal epithelial cells and three-dimensional models attempt to reconcile the complex and dynamic interactions that exist *in vivo* between the intestinal epithelium and bacteria on the luminal side and between the epithelium and the underlying immune system on the basolateral side. Additional models include tissue explants, bioreactors and organoids. The present review details the *in vitro* models used to study host–microbe interactions and explores the new tools that may help in understanding the molecular mechanisms of these interactions.

Key words: **Probiotics: Cell models: Tissue explants: Bioreactors: Organoids**

Over the course of evolution, lactobacilli, other lactic acid bacteria (LAB) and bifidobacteria have been abundant colonisers of the human small intestinal mucosa and coexist in mutualistic relationships with the host. Some members of these groups exert additional probiotic properties that provide health benefits to the host via the regulation of immune system and other physiological functions[1,2].

The immune system can be divided into two systems: innate and adaptive. The adaptive immune response depends on B- and T-lymphocytes, which are specific for particular antigens. By contrast, the innate immune system responds to common structures, called pathogen-associated molecular patterns, which are shared by the vast majority of pathogens. The primary response to pathogens is triggered by the pattern recognition receptors that bind pathogen-associated molecular patterns; pattern recognition receptors comprise Toll-like receptors (TLR), nucleotide-binding oligomerisation domains, adhesion molecules and lectins[3].

The use of probiotics is considered to be a potentially important strategy for modulating infectious and inflammatory responses in the gastrointestinal tract of the host. The effect of these probiotics is diverse and includes the modulation of the gut immune system through the interaction with gut epithelial cells and immune cells. These interactions primarily involve gut-associated dendritic cells (DC), which have the capability to respond to microbial signals through TLR signalling[4–6].

For a micro-organism to qualify as a probiotic, it is essential to scientifically demonstrate that it is beneficial to the health of the host. Before testing probiotics in human subjects, a *sine qua non* condition is to conduct studies in cell and animal models. *In vitro* and animal studies may provide valuable information, such as the mechanism through which a probiotic acts, but these types of studies alone are not proof of the benefit of a putative probiotic to human health.

Intestinal epithelial cells (IEC) are a barrier between the intestinal lumen and host connective tissue. However, recent studies have demonstrated that IEC are involved in the immunological process of the discrimination between pathogenic and commensal bacteria. IEC also secrete a broad range of antimicrobial peptides, including defensins, cathelicidins and calprotectins. The IEC interact with subepithelial professional antigen-presenting cells that can sample antigens and micro-organisms and are mostly populations of DC and macrophage-associated lymphoid tissues. These subepithelial cells are able to polarise naïve T cells and produce an immunotolerance or an inflammatory response. The interaction of IEC, DC and macrophages with commensal or pathogenic bacteria stimulates the differential secretion of cytokines. Thymic stromal lymphopoietin and IL-10 are secreted by IEC

Abbreviations: 3D, three-dimensional; DC, dendritic cells; DCSIGN, dendritic cells-specific intercellular adhesion molecule 3-grabbing non-integrin; IEC, intestinal epithelial cells; IFN, interferon; LAB, lactic acid bacteria; MDC, myeloid dendritic cells; PSC, pluripotent stem cells; Th, T helper; TLR, Toll-like receptors; T_{reg}, regulatory T cells.

* **Corresponding author:** Professor Á. Gil, fax +34 958 819132, email agil@ugr.es

in the presence of commensal or probiotic bacteria, whereas the pro-inflammatory cytokines IL-8 and TNF-α are secreted when pathogenic bacteria are present[7]. DC can sample antigens directly from the intestinal lumen by forming tight-junction-like structures with IEC[8]; alternatively, DC can be stimulated by the cytokines secreted from IEC. An environment containing cytokines secreted by IEC and DC stimulates immature DC and macrophages to produce a further increase in the level of cytokines. The subepithelial naïve T cells are stimulated for immunotolerance or in response to a profile of inflammatory cytokines and promote the generation of Th1, Th2, Th17 or regulatory T cells (T_{reg}). Because this complex net of secreted cells and cytokines is not easy to mimic *in vitro*, different models have been developed that principally involve IEC and DC generated from monocytes or segments of intestine (rat, human)[9–11] and T cells isolated from peripheral blood mononuclear cells[11]. Such models may include only one type of cell, a co-culture of various cell types or, in an effort to mimic the intestinal tissue, a culture of explants from the intestines[12]. Often, the models determine the different cytokines secreted or distinguish between different subsets of DC or T cells using cell surface phenotypes or transcription factors that are specific for each cell type. Here, we review the cell and tissue models that are currently used or could potentially be used to ascertain the mechanism through which probiotics act (Fig. 1).

In vitro models

Although human clinical trials are the definitive tool for establishing probiotic functionality, the use of *in vitro* models is necessary to select the most promising strains for these trials. Several *in vitro* studies evaluate the adhesion ability of potential probiotic bacteria and their interactions with pathogens at the intestinal epithelial interface[13–15]. The main goals of these studies are to understand the immunomodulatory effects of different bacterial strains on *in vitro* cell models and to evaluate whether the strain-dependent characteristics of commensal bacteria make them appropriate strains for the prevention and treatment of diseases.

A wide variety of cells are used as *in vitro* models for probiotic evaluation. Available models include both normal and

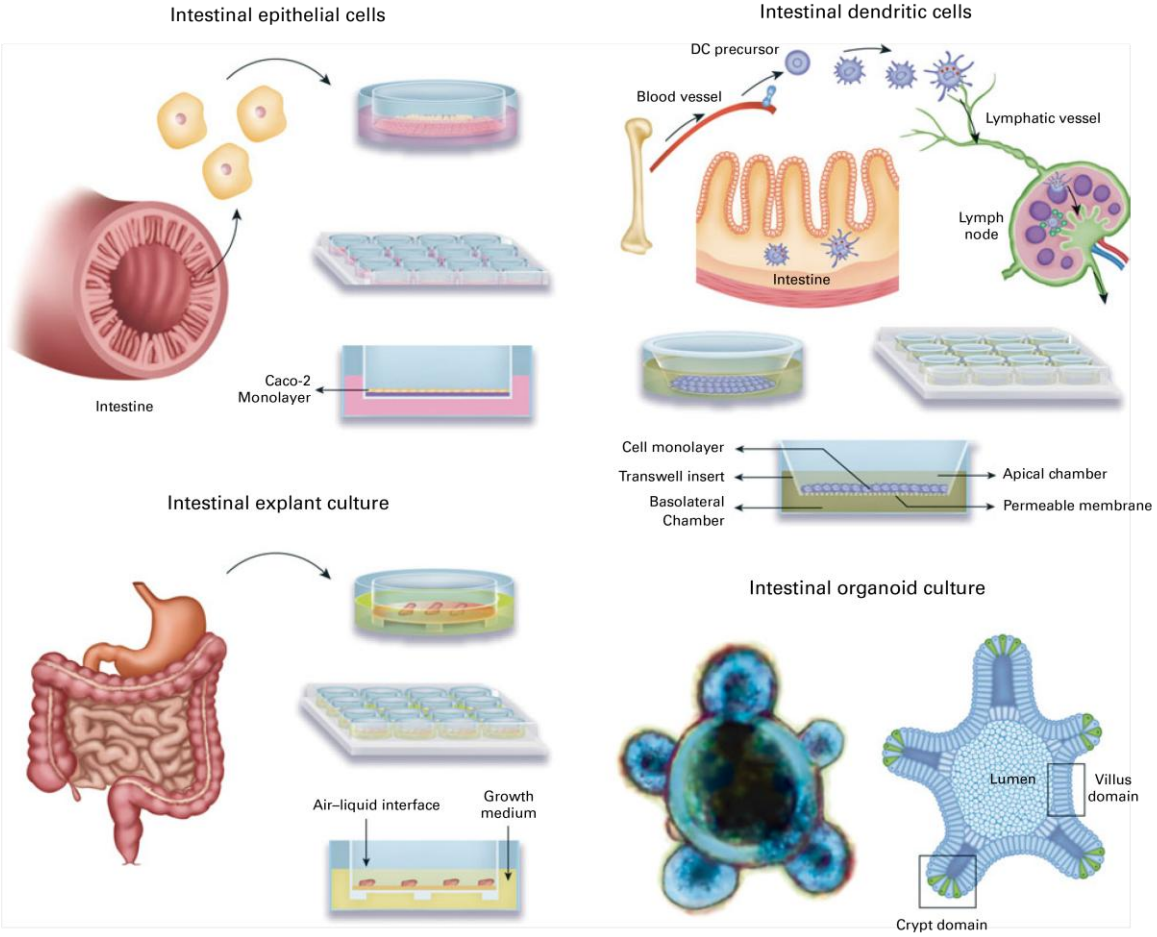


Fig. 1. Available *in vitro* models for studying host–microbe interactions and the mechanisms of action of probiotics. DC, dendritic cells.

British Journal of Nutrition

carcinogenic cells of different origins (intestine and blood), species (human, rat, pig, calf, goat, sheep and chicken) and types (epithelial and monocyte/macrophage).

Intestinal epithelial cells

Three of the most widely used commercially available human cell lines are Caco-2, T84 and HT-29[16,17], all of which were isolated from colon adenocarcinomas, express the features of enterocytes and are useful for attachment and mechanistic studies. In the differentiated state, these cell lines mimic the typical characteristics of the human small intestinal epithelium, including a well-developed brush border with such associated enzymes as alkaline phosphatase and sucrose isomaltase[18]. The HT29-MTX is a cell line obtained from HT29 cells adapted to methotrexate[19], which differentiate into goblet cells and secrete mucin, although of gastric immunoreactivity[20,21]. Nevertheless, these three cell models are different from the small intestine in several aspects, and their phenotypes are dependent on the duration of the culture period[22,23]. Enterocytes and goblet cells represent the two major cell phenotypes in the intestinal epithelium, and IEC-6 and IEC-18 are the most widely used among the rodent cell lines[24,25]. Both are commercially available and are derived from normal (non-carcinogenic) rat small intestine. Other available cell lines have been extensively reviewed by Cencič & Langerholc[26].

The models of IEC are focused on studying the receptors of the innate immune system (TLR and nucleotide oligomerization domain (NOD)) and the pathways that result in the secretion of cytokines. Ma *et al.*[27] showed that live *Lactobacillus reuteri* cells were able to reduce TNF-α-induced IL (IL-8) levels in Caco-2 cells. In addition, Vizoso Pinto *et al.*[28] showed that TLR9 and TLR2 were up-regulated when HT29 cells were incubated with lactobacilli but not when incubated only with *Salmonella typhimurium*. Using polarised HT29 and T84 cell monolayers, Ghadimi *et al.*[29] demonstrated that apically applied DNA from *Lactobacillus rhamnosus* GG (a human commensal and probiotic bacteria) attenuated TNF-α-enhanced NF-κB activity by reducing the degradation of the inhibitor subunit α of NF-κB (IκBα) and p38 subunit mitogen-activated protein kinase phosphorylation. *In vitro* studies have suggested that through the secretion of such immunoregulatory molecules as IL-8, TNF-α, TSLP, transforming growth factor-β and PGE_2, IEC limit pro-inflammatory cytokine production in DC. Thus, the secretion of immunoregulatory molecules by IEC is important for the maintenance of intestinal immune homeostasis[30,31].

Dendritic cells

DC comprise a complex, heterogeneous group of multifunctional antigen-presenting cells that comprise a critical arm of the immune system[32–35]. DC differentiate into at least four lines: Langerhans cells, myeloid DC (MDC), lymphoid DC and plasmacytoid DC[36]. These cells play critical roles in the orchestration of the adaptive immune response by inducing both tolerance and immunity[37–39]. The present paradigm is that this dual role results from the division of the total DC population into a network of DC subsets having distinct functions[36,40].

Immature DC reside in peripheral tissues, such as the gut mucosa, where they sense the microenvironment via pattern recognition receptors, including TLR and C-type lectin receptors, which recognise pathogen-associated molecular patterns[41]. Immature DC also release chemokines and cytokines to amplify the immune response[35]. Therefore, the regulatory role of DC is of particular importance at such mucosal surfaces as the intestine, where the immune system exists in intimate association with commensal bacteria, including LAB[42]. Probiotics exert differential stimulatory effects on DC *in vitro*, giving rise to varying production levels of different cytokines and, accordingly, different effector functions[43–45].

The response of the immune system to probiotics remains controversial. Some strains modulate the cytokine production by DC *in vitro* and induce a regulatory response, whereas others induce a pro-inflammatory response[46]. These strain-dependent effects are thought to be linked to specific interactions between bacteria and pattern recognition receptors.

Braat *et al.*[47] proposes that *L. rhamnosus* modulates DC function to induce a novel form of T-cell hyporesponsiveness, a mechanism that might be an explanation for the observed beneficial effects of probiotic treatment in clinical diseases.

The analysis of immature bone marrow-derived DC showed that all strains up-regulated the surface expression of B7-2 (CD86), which is indicative of DC maturation. However, the different strains up-regulated CD86 with varying intensities. No strain induced appreciable levels of IL-10 or IL-12 in immature bone marrow-derived DC, whereas TNF-α expression was elicited by *Lactobacillus paracasei* and *Lactobacillus fermentum*[48] in particular.

Although efficiently taken up by DC *in vitro*, selected LAB strains induced only a partial maturation of DC[43,49]. The transfer of probiotic-treated DC conferred protection against 2,4,6-trinitrobenzenesulfonic acid solution-induced colitis, and the preventive effect required Myeloid differentiation primary (MyD88)-, TLR2- and NOD2-dependent signalling and also the induction of CD4+ CD25+ regulatory cells in an IL-10-independent pathway[49].

Mohamadzadeh *et al.*[50] investigated three species of *Lactobacillus* and found that they modulated the phenotype and function of human MDC. *Lactobacillus*-exposed MDC up-regulated human leukocyte antigen-DR (HLA-DR), CD83, CD40, CD80 and CD86 and secreted high levels of IL-12 and IL-18 but not IL-10. IL-12 was sustained in the MDC exposed to all three of the *Lactobacillus* species in the presence of lipopolysaccharide from *Escherichia coli*, whereas lipopolysaccharide-induced IL-10 was greatly inhibited. The MDC activated with lactobacilli clearly skewed the $CD4^+$ and $CD8^+$ T cells to T helper (Th) 1 and T cell 1 (Tc1) polarisation, as evidenced by the secretion of interferon (IFN)-γ but not IL-4 or IL-13.

L. reuteri and *Lactobacillus casei*, but not *Lactobacillus plantarum*, prime monocyte-derived DC to drive the development of T_{reg} cells. The T_{reg} cells then produce increased levels of IL-10 and are capable of inhibiting the proliferation of

British Journal of Nutrition

bystander T cells in an IL-10-dependent fashion. Strikingly, both *L. reuteri* and *L. casei*, but not *L. plantarum*, bind the C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DCSIGN). Blocking antibodies against DCSIGN inhibited the induction of the T_{reg} cells by these probiotic bacteria, stressing that binding of DCSIGN can actively prime DC to induce T_{reg} cells[11].

All bifidobacteria and certain lactobacilli strains are low IL-12 and TNF-α inducers, and the IL-10 and IL-6 levels showed less variation than and no correlation with IL-12 and TNF-α. The DC matured by strong IL-12-inducing strains also produced high levels of IFN-β. When combining two strains, the low IL-12 inducers inhibited both this IFN-β production and the IL-12 and Th1-skewing chemokines. Weiss *et al.*[51] demonstrate that lactobacilli can be divided into two groups of bacteria that have contrasting effects; conversely, all bifidobacteria exhibit uniform effects.

In conclusion, LAB potently initiate 'interactions' via DC maturation. Hence, these LAB strains may represent useful tools for modulating the cytokine balance and promoting potent type-1 immune responses or preventing the immune deregulation associated with specific T-cell polarisation[45].

Macrophages

Lactobacilli have been shown to activate monocytes and macrophages, which play pivotal roles in antigen processing and presentation, the activation of antigen-specific immunity and the stimulation of IgA immunity. In particular, these cells are essential in the deviation of the immune response to the so-called type 1 response with cytotoxic effector cells or towards the type 2 response that is characterised by antibody production. The type 2 response is related to the secretion of IL-4, IL-5, IL-9 and IL-13, which promote the induction of IgE and allergic responses. By incubating bacterial suspensions with THP-1 macrophage-like cells, Drago *et al.*[52] analysed four strains of *Lactobacillus salivarius* for their ability to modulate the release of pro- and anti-inflammatory cytokines. LDR0723 and CRL1528 led to a sustained increase in the production of IL-12 and IFN-γ and a decrease in the release of IL-4 and IL-5; by contrast, BNL1059 and RGS1746 favoured a Th2 response, leading to a decrease in the Th1:Th2 ratio with respect to unstimulated cells.

Ivec *et al.*[53] showed that probiotic bacteria, either from *Lactobacillus* sp. or bifidobacteria, have the ability to decrease viral infection by establishing the antiviral state in macrophages through the production of NO and inflammatory cytokines, such as IL-6 and IFN-γ.

Three-dimensional cell models and probiotics

Much of the present knowledge about how microbial pathogens cause infection is based on studying experimental infections of standard cell monolayers grown as flat two-dimensional cultures on impermeable glass or plastic surfaces. Whereas these models continue to contribute to the present understanding of infectious diseases, they are greatly limited because they are unable to model the complexity of intact three-dimensional (3D) tissue[54,55]. In addition, cells grown as standard two-dimensional monolayers are also unable to respond to chemical and molecular gradients in three dimensions (at the apical, basal and lateral cell surfaces), resulting in many departures from the *in vivo* behaviour[56].

There are a variety of methods that have been used to enhance the differentiation of cultured cells, including permeable inserts, transplanted human cells grown as xenografts in animals and explanted human biopsies[57]. Together, with animal experiments, organotypical cell cultures are important models for analysing the cellular interactions of the mucosal epithelium and pathogenic mechanisms in the gastrointestinal tract[58]. Although these advanced *in vitro* models have provided important insights into microbial pathogenesis, they suffer from several limitations, including short lifetimes, labour-intensive design, experimental variability, availability and limited numbers of cells[57,59].

Three-dimensional cell cultures and probiotics

Epithelial cells cultured in a 3D matrix self-assemble into polarised monolayers that separate central apical lumens from a basal environment containing extracellular matrix; therefore, these 3D epithelial culture systems allow key events in the life cycle of IEC, such as proliferation, differentiation, apoptosis and migration, to be controlled in concert by organising principles that are determined by the spatial context of the cells. 3D culture systems mimic essential aspects of the *in vivo* organisation of epithelial cells of various origins. Compared with cell monolayers, the 3D culture of human intestinal cell lines (small intestine and colon) enhanced many characteristics associated with fully differentiated functional intestinal epithelia *in vivo*, including a distinct apical and basolateral polarity, the increased expression and better organisation of the tight junctions, extracellular matrix and brush border proteins and the highly localised expression of mucins. All of these important physiological features of *in vivo* intestinal epithelium were either absent or not expressed or distributed at physiologically relevant levels in monolayer cultures of the same cells[60].

Recently, Mappley *et al.*[61] reported a study using a 3D culture model and probiotics. In *in vitro* adherence and invasion assays using HT29-16E 3D cells, the adherence and invasion of *Brachyspira pilosicoli* B2904 into epithelial cells were significantly reduced by the presence of the cell-free supernatants of two *Lactobacillus* strains, *L. reuteri* LM1 and *L. salivarius* LM2.

Tissue explants and probiotics

A limited number of studies used explants and probiotics, and all of these studies focused on intestinal diseases, particularly Crohn's disease.

Using an organ-culture model with intestinal mucosa explants and selected bacterial strains, Carol *et al.*[62] reported a decrease of activated T lymphocytes and TNF-α secretion by the inflamed mucosa of patients with Crohn's disease. By favouring the apoptosis of T lymphocytes, *L. casei* may restore

British Journal of Nutrition

immune homeostasis in the inflamed ileal mucosa of these patients. In addition, Carol *et al.*[62] also demonstrated that some lactobacilli, such as *L. casei* DN-11 401 and *L. bulgaricus* LB10, may down-regulate inflammatory responses when exposed to inflamed mucosa in organ culture[63,64]. These authors conclude that probiotics interact with immunocompetent cells through the mucosal interface and locally modulate the production of pro-inflammatory cytokines. It is important to note that, although this model is useful for studying whole tissue responses in Crohn's disease, it does not permit the investigation of signals between mucosal cells because mucosa explants include a great variety of cells in their natural disposition for cell-to-cell communication.

More recently, Mencarelli *et al.*[65] cultured abdominal fat explants from five Crohn's disease patients and five patients with colon cancer (as controls) using VSL#3 (VSL Pharmaceuticals, Fort Lauderdale FL) medium and found that the exposure of these tissues to the VSL#3 conditioned medium abrogates leptin release. Thus, probiotics seem to correct inflammation-driven metabolic dysfunction.

Lastly, another group treated mouse colon epithelial cells and cultured colon explants with purified *L. rhamnosus* GG proteins in the absence or presence of TNF-α. Two novel purified proteins p75 and p40 activated protein kinase B (Akt), inhibited cytokine-induced epithelial cell apoptosis and promoted cell growth in human and mouse colon epithelial cells and cultured mouse colon explants. Furthermore, TNF-induced colon epithelial damage was significantly reduced. These findings suggest that probiotic bacterial components may be useful for preventing cytokine-mediated gastrointestinal diseases[66].

Future models

3D culture advanced *in vitro* models have provided important insights into microbial pathogenesis. However, they have several limitations, including short lifetimes, labour-intensive design, experimental variability, availability and limited numbers of cells. The development of novel relevant *in vitro* models of human intestinal epithelium, such as bioreactors and organoids, provides a viable starting point for future efforts aimed at bioengineering human intestine. 3D culture in bioreactors represents an easy, reproducible and high-throughput platform that provides a large number of differentiated cells. Another promising line is human pluripotent stem cells (PSC) that offer a unique and promising means to generate intestinal tissue, resulting in 3D intestinal 'organoids' formed villus-like structures and crypt-like proliferative zones. This intestinal tissue is functional, as it can secrete mucins into luminal structures.

Bioreactors

Tissue engineering represents a biology-driven approach by which bioartificial tissues are engineered through the combination of material technology and biotechnology. Bioreactors constitute and maintain physiological tissue conditions at desired levels, enhance mass transport rates and expose cultured cells to specific stimuli. It has been shown that bioreactor technologies providing appropriate biochemical and physiological regulatory signals guide cell and tissue differentiation and influence the tissue-specific function of bioartificial 3D tissues.

BioVaSc. BioVaSc is generated from a decellularised porcine small bowel segment with preserved tubular structures of the capillary network within the collagen matrix. BioVaSc is a prerequisite technique for the generation of bioartificial tissues endowed with a functional artificial vascular network. The technology has been performed in artificial human liver, intestine, trachea and skin models. These various human tissue models are a new technology that is an alternative to animal experiments for pharmacokinetic (drug penetration, distribution and metabolisation) and pharmacodynamic studies[67], and also to study probiotic interactions with the host.

Rotating-wall vessel bioreactor. Another alternative model utilises rotating-wall vessel technology to engineer biologically meaningful 3D models of human large intestinal epithelia and can be used in conjunction with the established models. Many reports have described the fact that cells cultured in an rotating-wall vessel bioreactor can assume physiologically relevant phenotypes that have not been possible with other models. In addition, 3D culture in rotating-wall vessel bioreactors represents an easy, reproducible and high-throughput platform that provides a large number of differentiated cells.

Optimally, the design of cell culture models should mimic both the 3D organisation and differentiated function of an organ, while allowing for experimental analysis in a high-throughput platform. Originally designed by National Aeronautics and Space Administration (NASA) engineers, the rotating-wall vessel technology is an optimised suspension culture design for growing 3D cells that maintain many of the specialised features of *in vivo* tissues[68,69].

To date, several works have shown the use of 3D cell culture systems in infection studies with the following pathogens: *S. typhimurium* (small intestine and colon models)[59,70], *Pseudomonas aeruginosa* (lung model)[71,72], human cytomegalovirus (placental model)[73] and Hepatitis C virus (hepatocyte model)[74]. Additional studies with other 3D models and infectious agents are ongoing and include bacteria, viruses and parasites that are difficult or impossible to culture using conventional methods.

Organoids

Intestinal resection and malformations in adult and paediatric patients result in devastating consequences. Unfortunately, allogeneic transplantation of intestinal tissue into patients has not been met with the same measure of success as the transplantation of other organs. Attempts to engineer intestinal tissue *in vitro* include the disaggregation of adult rat intestine into subunits called organoids, harvesting native adult stem cells from mouse intestine and spontaneous generation of intestinal tissue from embryoid bodies[75]. Recently, by utilising principles gained from the study of developmental biology, human PSC have been demonstrated to be capable of directed differentiation into intestinal tissue *in vitro*[76].

British Journal of Nutrition

PSC offer a unique and promising means to generate intestinal tissue for the purposes of modelling intestinal disease, understanding embryonic development and providing a source of material for therapeutic transplantation[75]. For example, human PSC have been differentiated into monolayer cultures of liver hepatocytes and pancreatic endocrine cells[77–80] that have therapeutic efficacy in animal models of liver disease[80–82] and diabetes[83], respectively.

Several authors have differentiated PSC from mice and human subjects into intestinal tissue. The resulting 3D intestinal 'organoids' consisted of a polarised, columnar epithelia that were patterned into villus-like structures and crypt-like proliferative zones that expressed intestinal stem cell markers. The epithelia contained the normal number of Lgr5-positive stem cells, Paneth cells and transit-amplifying cells in the crypt domain and the three differentiated cell lineages (enterocytes, goblet and enteroendocrine cells) of the villus domain[84]. This intestinal tissue is functional, as it can secrete mucins into luminal structures[76,85]. Furthermore, as based on defined growth factors and Matrigel, this well-established culture system retains critical *in vivo* characteristics, such as lineage composition and self-renewal kinetics[86].

However, despite offering such great potential, this system is not without its limitations. For example, the intestinal organoids lack several components of the intestine *in vivo*, such as the enteric nervous system and the vascular, lymphatic and immune systems. Additionally, whereas all of the major epithelial cell types are generated in proportions similar to those found *in vivo*, and there is evidence of crypt-like domains housing stem cells, the 3D architecture is not as regular as that seen *in vivo*, and the villus-like structures are variable from one organoid to the next[85]. Regardless of these drawbacks, this system has extraordinary experimental utility for understanding and modelling human intestinal development, homeostasis and disease. Moreover, this system provides a viable starting point for future efforts aimed at bioengineering human intestine.

Finally, Sato *et al.*[86] developed a technology that can be used to study infected, inflammatory or neoplastic tissues from the human gastrointestinal tract. Encouraged by the establishment of murine small intestinal cultures, these researchers adapted that culture condition to mouse and human colonic epithelia. However, long-term adult human IEC culture has remained difficult. Although there have been some long-term culture models, these techniques and cell lines have not gained wide acceptance, possibly as a result of the inherent technical difficulties in extracting and maintaining viable cells. These tools might have applications in regenerative biology through *ex vivo* expansion of the intestinal epithelia.

Conclusions

In most *in vitro* experimental models, epithelial cells are cultivated as monolayers, in which the establishment of functional epithelial features is not achieved. Compared with cell monolayers, the 3D culture of human intestinal cell lines enhanced many characteristics associated with fully differentiated functional intestinal epithelia *in vivo*. However, despite providing important insights, a number of limitations have to be taken into account, including short lifetimes, labour-intensive design, experimental variability, availability and limited numbers of cells.

Considerable effort has been invested in the development of new tools to study host–microbe interactions. Recent studies based on the ability to generate human intestinal tissues may help in understanding the molecular mechanisms of host–microbe interactions. Bioreactors and organoids provide a viable starting point for future efforts aimed at bioengineering human intestine. However, to date, these new approaches have also limitations that must be considered. For example, in organoids, the 3D architecture is not as regular as that seen *in vivo* and the villus-like structures are variable from one organoid to the next.

Acknowledgements

The authors declare that they have no conflict of interest. Part of the research currently in progress in our laboratory is funded by the company Hero España, S.A. through the grants no. 3143 and 3545 managed by the Fundación General Empresa-Universidad de Granada. The author contributions were as follows: L. F. and M. B.-B. wrote the abstract; J. P.-D., S. M.-Q. and L. F. wrote the introduction and the section '*In vitro* models'. M. B.-B. and A. G. wrote the sections 'Three dimensional models and probiotics', 'Future models' and 'Conclusions'. J. P.-D. and S. M.-Q. made the figure.

References

1. Konstantinov SR, Smidt H, de Vos WM, *et al.* (2008) S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. *Proc Natl Acad Sci U S A* **105**, 19474–19479.
2. MacDonald TT & Monteleone G (2005) Immunity, inflammation, and allergy in the gut. *Science* **307**, 1920–1925.
3. McCole DF & Barret KE (2007) Varied role of the gut epithelium in mucosal homeostasis. *Curr Opin Gastroenterol* **23**, 647–654.
4. Pamer EG (2007) Immune responses to commensal and environmental microbes. *Nat Immunol* **11**, 1173–1178.
5. Coombes JL & Powrie F (2008) Dendritic cells in intestinal immune regulation. *Nat Rev Immunol* **8**, 435–446.
6. Goriely S, Neurath MF & Goldman M (2008) How microorganisms tip the balance between interleukin-12 family members. *Nat Rev Immunol* **8**, 81–86.
7. Wallace TD, Bradley S, Buckley ND, *et al.* (2003) Interactions of lactic acid bacteria with human intestinal epithelial cells: effects on cytokine production. *J Food Prot* **66**, 466–472.
8. Rescigno M, Urbano M, Valzasina B, *et al.* (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Immunol* **2**, 361–367.
9. Uematsu S, Fujimoto K, Jang MH, *et al.* (2008) Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat Immunol* **9**, 769–776.
10. Ng SC, Benjamin JL, McCarthy NE, *et al.* (2011) Relationship between human intestinal dendritic cells, gut microbiota,

and disease activity in Crohn's disease. *Inflamm Bowel Dis* **17**, 2027–2037.
11. Smits HH, Engering A, van der Kleij D, *et al.* (2005) Selective probiotic bacteria induce IL-10-producing regulatory T cells *in vitro* by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *J Allergy Clin Immunol* **115**, 1260–1267.
12. Jarry A, Bossard C, Sarrabayrouse G, *et al.* (2011) Loss of interleukin-10 or transforming growth factor β signaling in the human colon initiates a T-helper 1 response via distinct pathways. *Gastroenterology* **141**, 1887–1896.
13. Sanz Y, Nadal I & Sánchez E (2007) Probiotics as drugs against human gastrointestinal infections. *Recent Pat Anti-infect Drug Discov* **2**, 148–156.
14. Izquierdo E, Medina M, Ennahar S, *et al.* (2008) Resistance to simulated gastrointestinal conditions and adhesion to mucus as probiotic criteria for *Bifidobacterium longum* strains. *Curr Microbiol* **56**, 613–618.
15. Sánchez E, Nadal I, Donat E, *et al.* (2008) Reduced diversity and increased virulence-gene carriage in intestinal enterobacteria of coeliac children. *BMC Gastroenterol* **8**, 50.
16. Fogh J & Trempe G (1975) New human tumor cell lines. In *Human Tumor Cells In Vitro*, pp. 115–140 [J Fogh, editor]. New York, NY: Plenum.
17. Fogh J, Fogh JM & Orfeo T (1977) One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* **59**, 221–226.
18. Lenaerts K, Bouwman FG, Lamers WH, *et al.* (2007) Comparative proteomic analysis of cell lines and scrapings of the human intestinal epithelium. *BMC Genomics* **8**, 91.
19. Lesuffleur T, Barbat A, Dussaulx E, *et al.* (1990) Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells. *Cancer Res* **50**, 6334–6343.
20. Lesuffleur T, Porchet N, Aubert JP, *et al.* (1993) Differential expression of the human mucin genes MUC1 to MUC5 in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations. *J Cell Sci* **106**, 771–783.
21. Leteurtre E, Gouyer V, Rousseau K, *et al.* (2004) Differential mucin expression in colon carcinoma HT-29 clones with variable resistance to 5-fluorouracil and methotrexate. *Biol Cell* **96**, 145–151.
22. Engle MJ, Goetz GS & Alpers DH (1998) Caco-2 cells express a combination of colonocyte and enterocyte phenotypes. *J Cell Physiol* **174**, 362–369.
23. Mehran M, Levy E, Bendayan M, *et al.* (1997) Lipid, apolipoprotein, and lipoprotein synthesis and secretion during cellular differentiation in Caco-2 cells. *In Vitro Cell Dev Biol Anim* **33**, 118–128.
24. Quaroni A, Isselbacher KJ & Ruoslahti E (1978) Fibronectin synthesis by epithelial crypt cells of rat small intestine. *Proc Natl Acad Sci U S A* **75**, 5548–5552.
25. Quaroni A & Isselbacher KJ (1981) Cytotoxic effects and metabolism of benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene in duodenal and ileal epithelial cell cultures. *J Natl Cancer Inst* **67**, 1353–1362.
26. Cencič A & Langerholc T (2010) Functional cell models of the gut and their applications in food microbiology – a review. *Int J Food Microbiol* **141**, S4–S14.
27. Ma D, Forsythe P & Bienenstock J (2004) Live *Lactobacillus reuteri* is essential for the inhibitory effect on tumor necrosis factor alpha-induced interleukin (IL)-8 expression. *Infect Immunol* **72**, 5308–5314.
28. Vizoso Pinto MG, Rodriguez Gómez M, Seifert S, *et al.* (2009) Lactobacilli stimulate the innate immune response and modulate the TLR expression of HT29 intestinal epithelial cells *in vitro*. *Int J Food Microbiol* **31**, 86–93.
29. Ghadimi D, De Vrese M, Heller KJ, *et al.* (2010) Effect of natural commensal-origin DNA on toll-like receptor 9 (TLR9) signalling cascade, chemokine IL-8 expression, and barrier integrity of polarized intestinal epithelial cells. *Inflamm Bowel Dis* **16**, 410–427.
30. Wang J & Xing F (2008) Human TSLP-educated DCs. *Cell Mol Immunol* **5**, 99–106.
31. Dignass AU & Podolsky DK (1993) Cytokine modulation of intestinal epithelial cell restitution: central role of transforming growth factor β. *Gastroenterology* **105**, 1323–1332.
32. Steinman RM & Banchereau J (2007) Taking dendritic cells into medicine. *Nature* **449**, 419–426.
33. Banchereau J (2002) The long arm of the immune system. *Sci Am* **287**, 52–59.
34. Banchereau J, Briere F, Caux C, *et al.* (2000) Immunobiology of dendritic cells. *Annu Rev Immunol* **18**, 767–811.
35. Banchereau J & Steinman RM (1998) Dendritic cells and the control of immunity. *Nature* **392**, 245–252.
36. Wu L & Liu YJ (2007) Development of dendritic-cell lineages. *Immunity* **26**, 741–750.
37. Cools N, Ponsaerts P, Van Tendeloo VF, *et al.* (2007) Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. *J Leukoc Biol* **82**, 1365–1374.
38. Quah BJ & O'Neill HC (2005) Maturation of function in dendritic cells for tolerance and immunity. *J Cell Mol Med* **9**, 643–654.
39. Steinman RM, Hawiger D & Nussenzweig MC (2003) Tolerogenic dendritic cells. *Annu Rev Immunol* **21**, 685–711.
40. Shortman K & Naik SH (2007) Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* **7**, 19–30.
41. Sabatté J, Maggini J, Nahmod K, *et al.* (2007) Interplay of pathogens, cytokines and other stress signals in the regulation of dendritic cell function. *Cytokine Growth Factor Rev* **18**, 5–17.
42. Stagg AJ, Hart AL, Knight SC, *et al.* (2004) Microbial–gut interactions in health and disease. Interactions between dendritic cells and bacteria in the regulation of intestinal immunity. *Best Pract Res Clin Gastroenterol* **18**, 255–270.
43. Christensen HR, Frøkiær H & Pestka JJ (2002) Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *J Immunol* **168**, 171–178.
44. Zeuthen LH, Fink LN & Frøkiær H (2008) Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-β. *Immunology* **123**, 197–208.
45. Fink LN, Zeuthen LH, Ferlazzo G, *et al.* (2007) Human antigen-presenting cells respond differently to gut-derived probiotic bacteria but mediate similar strain-dependent NK and T cell activation. *FEMS Immunol Med Microbiol* **51**, 535–546.
46. Evrard B, Coudeyras S, Dosgilbert A, *et al.* (2011) Dose-dependent immunomodulation of human dendritic cells by the probiotic *Lactobacillus rhamnosus* Lcr35. *PLoS One* **18**, e18735.
47. Braat H, van den Brande J, van Tol E, *et al.* (2004) *Lactobacillus rhamnosus* induces peripheral hyporesponsiveness in stimulated $CD4^+$ T cells via modulation of dendritic cell function. *Am J Clin Nutr* **80**, 1618–1625.

48. D'Arienzo R, Maurano F, Lavermicocca P, *et al.* (2009) Modulation of the immune response by probiotic strains in a mouse model of gluten sensitivity. *Cytokine* **48**, 254–259.
49. Foligne B, Zoumpopoulou G, Dewulf J, *et al.* (2007) A key role of dendritic cells in probiotic functionality. *PLoS One* **2**, e313.
50. Mohamadzadeh M, Olson S, Kalina WV, *et al.* (2005) Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. *Proc Natl Acad Sci U S A* **102**, 2880–2885.
51. Weiss G, Christensen HR, Zeuthen LH, *et al.* (2011) Lactobacilli and bifidobacteria induce differential interferon-β profiles in dendritic cells. *Cytokine* **56**, 520–530.
52. Drago L, Nicola L, Iemoli E, *et al.* (2010) Strain-dependent release of cytokines modulated by *Lactobacillus salivarius* human isolates in an *in vitro* model. *BMC Res Notes* **3**, 44.
53. Ivec M, Botić T, Koren S, *et al.* (2007) Interactions of macrophages with probiotic bacteria lead to increased antiviral response against vesicular stomatitis virus. *Antiviral Res* **75**, 266–274.
54. Abbott A (2003) Biology's new dimension. *Nature* **424**, 870–872.
55. Schemeichel KL & Bissell MJ (2003) Modeling tissue-specific signaling and organ function in three dimensions. *J Cell Sci* **116**, 2377–2388.
56. Zhang S (2004) Beyond the Petri dish. *Nat Biotechnol* **22**, 151–152.
57. Nickerson CA, Richter EG & Ott CM (2007) Studying host–pathogen interactions in 3-D: organotypic models for infectious disease and drug development. *J Neuroimmune Pharmacol* **2**, 26–31.
58. Bareiss PM, Metzger M, Sohn K, *et al.* (2008) Organotypical tissue cultures from adult murine colon as an *in vitro* model of intestinal mucosa. *Histochem Cell Biol* **129**, 795–804.
59. Höner zu Bentrup K, Ramamurthy R, Ott CM, *et al.* (2006) Three-dimensional organotypic models of human colonic epithelium to study the early stages of enteric salmonellosis. *Microbes Infect* **8**, 1813–1825.
60. Juuti-Uusitalo K, Klunder LJ, Sjollema KA, *et al.* (2011) Differential effects of TNF (TNFSF2) and IFN-γ on intestinal epithelial cell morphogenesis and barrier function in three-dimensional culture. *PLoS One* **6**, e22967.
61. Mappley LJ, Tchórzewska MA, Cooley WA, *et al.* (2011) Lactobacilli antagonize the growth, motility, and adherence of *Brachyspira pilosicoli*: a potential intervention against avian intestinal spirochetosis. *Appl Environ Microbiol* **77**, 5402–5411.
62. Carol M, Borruel N, Antolin M, *et al.* (2006) Modulation of apoptosis in intestinal lymphocytes by a probiotic bacteria in Crohn's disease. *J Leukoc Biol* **79**, 917–922.
63. Borruel N, Carol M, Casellas F, *et al.* (2002) Increased mucosal tumour necrosis factor alpha production in Crohn's disease can be downregulated *ex vivo* by probiotic bacteria. *Gut* **51**, 659–664.
64. Borruel N, Casellas F, Antolín M, *et al.* (2003) Effects of nonpathogenic bacteria on cytokine secretion by human intestinal mucosa. *Am J Gastroenterol* **98**, 865–870.
65. Mencarelli A, Distrutti E, Renga B, *et al.* (2011) Probiotics modulate intestinal expression of nuclear receptor and provide counter-regulatory signals to inflammation-driven adipose tissue activation. *PLoS One* **6**, e22978.
66. Yan F, Cao H, Cover TL, *et al.* (2007) Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. *Gastroenterology* **132**, 562–575.
67. Schanz J, Pusch J, Hansmann J, *et al.* (2010) Vascularised human tissue models: a new approach for the refinement of biomedical research. *J Biotechnol* **148**, 56–63.
68. Unsworth BR & Lelkes PI (1998) Growing tissues in microgravity. *Nat Med* **4**, 901–907.
69. Nickerson CA, Ott CM, Wilson JW, *et al.* (2004) Microbial responses to microgravity and other low-shear environments. *Microbiol Mol Biol Rev* **68**, 345–361.
70. Nickerson CA, Goodwin TJ, Terlonge J, *et al.* (2001) Three-dimensional tissue assemblies: novel models for the study of *Salmonella pathogenesis*. *Infect Immun* **69**, 7106–7120.
71. Carterson AJ, Höner zu Bentrup K, Ott CM, *et al.* (2005) A549 lung epithelial cells grown as three-dimensional aggregates: alternative tissue culture model for *Pseudomonas aeruginosa* pathogenesis. *Infect Immun* **73**, 1129–1140.
72. Crabbé A, Sarker SF, Van Houdt R, *et al.* (2011) Alveolar epithelium protects macrophages from quorum sensing-induced cytotoxicity in a three-dimensional co-culture model. *Cell Microbiol* **13**, 469–481.
73. LaMarca HL, Ott CM, Höner Zu Bentrup K, *et al.* (2005) Three-dimensional growth of extravillous cytotrophoblasts promotes differentiation and invasion. *Placenta* **26**, 709–720.
74. Sainz B Jr, TenCate V & Uprichard SL (2009) Three-dimensional Huh7 cell culture system for the study of Hepatitis C virus infection. *Virol J* **6**, 103.
75. Howell JC & Wells JM (2011) Generating intestinal tissue from stem cells: potential for research and therapy. *Regen Med* **6**, 743–755.
76. Spence JR, Mayhew CN, Rankin SA, *et al.* (2011) Directed differentiation of human pluripotent stem cells into intestinal tissue *in vitro*. *Nature* **470**, 105–109.
77. Cai J, Zhao Y, Liu Y, *et al.* (2007) Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology* **45**, 1229–1239.
78. D'Amour KA, Agulnick AD, Eliazer S, *et al.* (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol* **23**, 1534–1541.
79. Song Z, Cai J, Liu Y, *et al.* (2009) Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. *Cell Res* **19**, 1233–1242.
80. Zhang D, Jiang W, Liu M, *et al.* (2009) Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. *Cell Res* **19**, 429–438.
81. Basma H, Soto-Gutiérrez A, Yannam GR, *et al.* (2009) Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. *Gastroenterology* **136**, 990–999.
82. Touboul T, Hannan NR, Corbineau S, *et al.* (2009) Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. *Hepatology* **51**, 1754–1765.
83. Kroon E, Martinson LA, Kadoya K, *et al.* (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose responsive insulin-secreting cells *in vivo*. *Nat Biotechnol* **26**, 443–452.
84. Koo BK, Stange DE, Sato T, *et al.* (2011) Controlled gene expression in primary Lgr5 organoid cultures. *Nat Methods* **9**, 81–83.
85. McCracken KW, Howell JC, Wells JM, *et al.* (2011) Generating human intestinal tissue from pluripotent stem cells *in vitro*. *Nat Protoc* **6**, 1920–1928.
86. Sato T, Stange DE, Ferrante M, *et al.* (2011) Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762–1172.

British Journal of Nutrition

OPEN ACCESS Freely available online

PLOS ONE

Safety and Immunomodulatory Effects of Three Probiotic Strains Isolated from the Feces of Breast-Fed Infants in Healthy Adults: SETOPROB Study

Julio Plaza-Diaz[1,2], Carolina Gomez-Llorente[1,2], Laura Campaña-Martin[2], Esther Matencio[3], Inmaculada Ortuño[3], Rosario Martínez-Silla[3], Carlos Gomez-Gallego[4], Maria Jesús Periago[4], Gaspar Ros[4], Empar Chenoll[5], Salvador Genovés[5], Beatriz Casinos[5], Ángela Silva[5], Dolores Corella[6,7], Olga Portolés[6,7], Fernando Romero[3], Daniel Ramón[5], Antonio Perez de la Cruz[8], Angel Gil[1,2], Luis Fontana[1,2*]

1 Department of Biochemistry & Molecular Biology II, School of Pharmacy, University of Granada, Granada, Spain, **2** Institute of Nutrition & Food Technology "José Mataix", Biomedical Research Center, University of Granada, Granada, Spain, **3** Hero Global Technology Center, Hero Spain, S.A., Alcantarilla, Murcia, Spain, **4** Department of Human Nutrition and Food Science, Faculty of Veterinary Sciences, University of Murcia, Murcia, Spain, **5** Department of Food Biotechnology, Biopolis s.l., Parc Científic Universitat de Valencia, Paterna, Valencia, Spain, **6** Department of Preventive Medicine and Public Health, School of Medicine, University of Valencia, Valencia, Spain, **7** CIBER Fisiopatologia de la Obesidad y Nutricion, Instituto de Salud Carlos III, Madrid, Spain, **8** Unit of Nutrition and Dietetics, Virgen de las Nieves Hospital, Granada, Spain

Abstract

We previously described the isolation and characterization of three probiotic strains from the feces of exclusively breast-fed newborn infants: *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036. These strains were shown to adhere to intestinal mucus *in vitro*, to be sensitive to antibiotics and to resist biliary salts and low pH. In the present study, a multicenter, randomized, double-blind, placebo-controlled trial with 100 healthy volunteers in three Spanish cities was carried out to evaluate the tolerance, safety, gut colonization and immunomodulatory effects of these three probiotics. Volunteers underwent a 15-day washout period, after which they were randomly divided into 5 groups that received daily a placebo, a capsule containing one of the 3 strains or a capsule containing a mixture of two strains for 30 days. The intervention was followed by another 15-day washout period. Patients did not consume fermented milk for the entire duration of the study. Gastrointestinal symptoms, defecation frequency and stool consistency were not altered by probiotic intake. No relevant changes in blood and serum, as well as no adverse events occurred during or after treatment. Probiotic administration slightly modified bacterial populations in the volunteers' feces. Intestinal persistence occurred in volunteers who received *L. rhamnosus* CNCM I-4036. Administration of *B. breve* CNCM I-4035 resulted in a significant increase in fecal secretory IgA content. IL-4 and IL-10 increased, whereas IL-12 decreased in the serum of volunteers treated with any of the three strains. These results demonstrate that the consumption of these three bacterial strains was safe and exerted varying degrees of immunomodulatory effects.

Trial Registration: ClinicalTrials.gov NCT01479543

Citation: Plaza-Diaz J, Gomez-Llorente C, Campaña-Martin L, Matencio E, Ortuño I, et al. (2013) Safety and Immunomodulatory Effects of Three Probiotic Strains Isolated from the Feces of Breast-Fed Infants in Healthy Adults: SETOPROB Study. PLoS ONE 8(10): e78111. doi:10.1371/journal.pone.0078111

Editor: Ernesto T. A. Marques, University of Pittsburgh, United States of America

Received June 26, 2013; **Accepted** September 5, 2013; **Published** October 28, 2013

Funding: Part of the research currently in progress in the authors' laboratory is funded by the company Hero Spain, S. A. through the grant #3582 managed by the Fundacion General Empresa-Universidad de Granada. No additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: Part of the research currently in progress in the authors' laboratory is funded by the company Hero Spain, S. A. through the grant #3582 managed by the Fundacion General Empresa-Universidad de Granada. EM, IO, RMS and FR are employed by Hero Global Technology Center, Hero Spain, S.A. This center is part of the food company HERO, headquartered in Switzerland. EC, SG, BC, AS and DR are employed by Biopolis S.L., a spin-off of the Cover Letter National Spanish Research Council (Consejo Superior de Investigaciones Científicas), Ministry of Education, Spain. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

* E-mail: fontana@ugr.es

Introduction

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) define probiotics as live microorganisms that confer a health benefit to the host when administered in adequate amounts [1]. Strains belonging to *Bifidobacterium* and *Lactobacillus*, the predominant and subdominant groups of the gastrointestinal microbiota, respectively [2,3], are the most widely used probiotic bacteria and are included in many functional foods and dietary supplements [4-6].

The FAO/WHO [1] and the European Union (EU)-funded Product Safety Enforcement Forum of Europe (EU-PROSAFE) project [7] have attempted to create consensus guidelines for probiotic safety evaluation. These groups have recommended that i) the genus and species of the microorganism must first be definitively determined by phenotypic and genotypic techniques, ii) the strains must be deposited in an internationally recognized culture collection, and iii) the safety of the bacterial strain must be evaluated through acute ingestion studies in murine models and the estimation of potential side effects in human studies.

For probiotics to be successful, they must possess certain characteristics. The criteria for the selection of probiotics include tolerance to gastrointestinal conditions (gastric acid and bile), ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens [8,9].

We have previously described the isolation of three lactic acid bacteria (LAB) strains from the feces of exclusively breast-fed newborn infants. These strains were selected based on their probiotic properties, such as adhesion to intestinal mucus, sensitivity to antibiotics and resistance to biliary salts and low pH. We identified these strains as *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 [10]. In addition, their safety has been assessed by acute ingestion in immunocompetent and immunosuppressed BALB/c mouse models. The three strains inhibited *Listeria monocytogenes*, the etiological agent of meningitis, and human rotavirus infections *in vitro* [10].

The immunomodulatory effects of probiotics have been demonstrated in experimental models of allergy, autoimmunity and inflammatory bowel disease [2]. In the present study, a multicentric, randomized, double-blind placebo-controlled trial with healthy volunteers was undertaken to investigate the tolerance, safety and colonization of the aforementioned probiotic strains, following the FAO/WHO guidelines [1]. Additionally, we have evaluated their potential immunomodulatory effects by quantitating cytokines and secretory IgA in volunteers' serum and feces, respectively.

Materials and Methods

Ethical statement

All patients enrolled in this study signed an informed consent form. The study followed the guidelines laid down in the Declaration of Helsinki and was approved by the ethics review committees of the University of Granada, Murcia and Valencia.

Probiotics

The probiotic strains *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 have been described elsewhere [10]. These strains were assayed for enzymatic activity and carbohydrate utilization, and they were deposited in the Collection Nationale de Cultures de Microorganismes (CNCM) of the Institute Pasteur [10].

Experimental design

This study was a multicenter, randomized, double-blind, placebo-controlled trial. The trial was registered at www.clinicaltrials.gov as NCT01479543. Randomization was simple and not subjected to any kind of restriction such as blocking or block size. One hundred and three healthy volunteers were enrolled in three different cities in Spain (Granada, Murcia and Valencia). We used a random allocation sequence [11]. Briefly, each city was assigned 35 codes taken from a randomization table. Six codes were assigned to each treatment in each city. Envelopes containing the codes and matching those in the randomization table were assigned to each participant. Volunteers were enrolled and assigned by Gomez-Llorente C (Granada), Ros G (Murcia) and Corella D (Valencia). This was a double blind study. A flow chart of the study design is depicted in Figure 1. The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1.

Volunteers underwent a 15-day washout period (t_1), after which they were randomly and blindly divided into 5 groups that received daily either a placebo, a capsule containing $9x10^9$ CFUs of one of the 3 strains, or a capsule containing $9x10^9$ CFUs of a mixture of *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036, for 30 days (t_2).

The placebo contained 67% cow's milk powder, 32.5% sucrose, and 0.56% vitamin C. The 30-day intervention period was followed by a second washout of another 15 days (t_3) (Figure 1). Patients did not consume any fermented milk for the entire duration of the study. Blood samples were taken at t_1 and t_2. Blood was centrifuged to separate serum from cells. Fecal samples were taken at t_1, t_2 and t_3. Baseline data appear in Table 1.

Volunteers were recruited between July and October 2011. The first washout was in October 2011. Intervention ended in November 2011, and the second washout in December 2011. All determinations were finished by December 2012.

Primary outcome variables were safety, tolerance and persistence. Secondary outcome variables were bacterial populations, immunomodulatory effects (cytokine and secretory IgA production), microbiological analyses, and lymphocyte populations. Calculation of sample size was done based on the variance in the main outcome variable persistence, i.e., probiotic strain count (log strain CFU/g) in feces and a difference of 25% compared with the placebo (12). A type 1 error of α=0.05 and a power of 90%, (β=0.1) were assumed. The calculated minimum number of subjects per group was 19. The initial number of volunteers per group was as follows: placebo, n=21; *L. paracasei* CNCM I-4034, n=21; *B. breve* CNCM I-4035, n=20; *L. rhamnosus* CNCM I-4036, n=21;

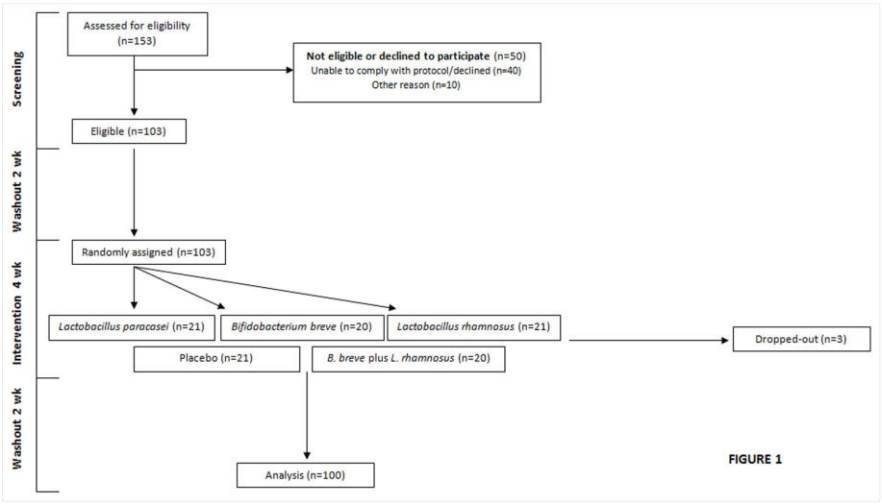


Figure 1. CONSORT flow diagram of the subjects in the SETOPROB study (NCT01479543).
doi: 10.1371/journal.pone.0078111.g001

mixture of *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036, n=20. One subject each of groups placebo, *L. paracasei* CNCM I-4034 and *L. rhamnosus* CNCM I-4036 voluntarily dropped out of the study. No changes in the estimated sample size or its precision occurred as all selected volunteers received intended treatment and were analyzed.

The *inclusion criteria* were as follows: healthy male or female, age 18–50 years, normal defecation pattern, blood parameters within the normal range or not considered clinically significant if outside of the normal range, BMI 18.0–29.9 kg/m^2 and written informed consent. The *exclusion criteria* were pregnancy or breast-feeding, blood parameters outside of the normal range and considered clinically significant, a history of metabolic or gastrointestinal disease, food allergies, recent use of antibiotics or laxative drugs, diarrhea, constipation, diabetes mellitus, smoking and blood pressure > 140/90 mmHg.

Determinations described below were carried out in all volunteers (20 per group) with the exception of antibiotic resistance, which was done in Valencia (3 volunteers/group, n=15).

Collection and preparation of fecal samples

Fecal samples were collected from each volunteer in plastic pots lined with a sterile plastic bag in anaerobic conditions and submitted immediately by courier to the laboratory. Samples were analyzed within a maximum of 4 h.

Gastrointestinal tolerance and safety parameters

Gastrointestinal tolerance was determined using the gastrointestinal symptom rating scale (GSRS) [13], the King's Stool Chart for stool consistency [14], daily recorded gastrointestinal symptoms (nausea, vomiting, diarrhea, burping, abdominal distension and flatulence) [15] and defecation frequency. Baseline GSRS and stool consistency were measured by the investigator and at 4 and 6 weeks. Product compliance was recorded daily in a diary. Intolerance was defined as a symptom score of 2 or higher (moderate or severe) on the GSRS. Safety parameters were the number and type of adverse events recorded throughout the entire study and changes from baseline blood parameters determined at the end of the supplementation period. The measurement of blood parameters was performed in the Clinical Analysis Laboratory of the Virgen de las Nieves Hospital (Granada), Clinical Hospital (Valencia) and Megalab laboratory (Murcia).

Table 1. Baseline characteristics of the study groups.

	Probiotic groups	Placebo group
	(n=80)	(n=20)
Sex (male/female)	37/43	9/11
Age (years)	28.7 ± 0.7	28.5 ± 1.7
Height (m)	1.71 ± 0.1	1.7 ± 0.1
Weight (kg)	68.4 ± 1.4	67 ± 2.5
BMI (kg/m^2)	23.1 ± 0.4	22.8 ± 0.5
Heart rate (beats/min)	72.0 ± 1.5	71.2 ± 3.0
Blood pressure (mm/Hg)		
Systolic	116.8 ± 1.8	117.6 ± 3.1
Diastolic	71.5 ± 1.2	71.6 ± 2.3

Values are means ± SEM unless otherwise indicated. There were no significant differences between groups.

doi: 10.1371/journal.pone.0078111.t001

Fluorescence *in situ* hybridization-flow cytometry analysis (FISH-FC)

Fecal samples were processed as previously described [16,17]. One gram of feces was homogenized in 9 mL of PBS (phosphate-buffered saline), and then 0.2 mL of the suspension was mixed with 0.6 mL of 4% paraformaldehyde (PFA) in PBS and fixed overnight at 4°C. Fecal bacterial populations were assessed by FISH-CF analysis as described

by Fallani et al. and Gomez-Llorente et al. [16,17]. A panel of 10 group- and species-specific probes covalently linked with Cy5 at their 5' end was used to assess the microbiota composition [18-25] (Table S1).

Hybridization was performed in a 96-well microtiter plate overnight at 35°C in hybridization solution containing 4 ng/µL of the appropriate probes, and then 150 µL of hybridization solution was added to each well. Cells were pelleted and washed to remove any nonspecific probe binding by incubating the bacterial cells at 37°C for 20 min in wash solution (64 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0, 5 mmol/L EDTA pH 8.0, 0.01% sodium dodecyl sulfate, pH 7.2). Finally, the cells were pelleted and resuspended in PBS. Samples were analyzed in a FACSCanto II flow cytometer (Becton Dickinson, NJ, USA).

Microbiological analysis

Fecal samples were analyzed by plating appropriate dilutions onto Wilkins-Chalgren agar (Panreac Quimica, Barcelona, Spain) to determine the total number of anaerobic bacteria, de Man-Rogosa-Sharpe (MRS) agar (Oxoid, Basingstoke, United Kingdom) to determine the number of *Lactobacilli*, and Beerens agar (Oxoid, Basingstoke, United Kingdom) to determine the number of bifidobacteria. The *Lactobacillus rhamnosus* CNCM I-4036 count was determined on modified MRS medium in which glucose was substituted with rhamnose. Only *L. rhamnosus* and a few other rare *Lactobacillus* species in the human gastrointestinal tract are able to grow on this medium [12,26].

Antibiotic resistance analysis

The sensitivity of probiotic strains to ampicillin and tetracycline was analyzed in volunteers' fecal samples by plating appropriate dilutions onto MRS agar (Oxoid, Basingstoke, United Kingdom) supplemented with 0.05% (wt/vol) cysteine (Sigma-Aldrich) (MRS-C medium) and trypticase soy agar (TSA, Oxoid) with or without ampicillin (2 and 4 µg/mL; Sigma-Aldrich, St. Louis, MO) or tetracycline (4 and 8 µg/mL; Sigma-Aldrich). The plates were incubated for 48-72 h at 37°C in an anaerobic atmosphere, which was generated using an AnaeroGen® system, for MRS and MRS-C, and aerobically at 30°C in the case of TSA plates.

Isolation procedure

From each patient, 5 to 10 random colonies grown on modified MRS agar were individually inoculated into MRS broth medium (Oxoid, Basingstoke, United Kingdom) for 2 days, at 37°C under anaerobic conditions (Anaerogen® Oxoid, Basingstoke, United Kingdom). DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN, Barcelona, Spain) and used for identification by quantitative real-time PCR (qPCR) with specific primers (see Table S2) for this probiotic strain.

Real-time polymerase chain reaction (PCR)

Real-time PCR was used i) to identify *Lactobacillus*, *Bifidobacterium* spp., *Bacteroides* and *Clostridium difficile* in feces and ii) to confirm intestinal persistence by *Lactobacillus rhamnosus*. For the former (i), DNA was isolated from volunteers' feces with the QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain). For the latter (ii), DNA was isolated from bacterial cultures (see sections Microbiological determinations and Re-isolation procedure). The primer sequences appear in Table S2.

PCR was performed in triplicate in an Eppendorf Mastercycle EP Gradient. The primer sequences are shown in Table S2 and were purchased from Sigma-Aldrich (Barcelona, Spain). PCR was carried out using Power SYBR Green Master Mix (Applied Biosystems, Barcelona, Spain). The PCR program was as follows: an initial activation/denaturation step at 95°C for 5 min followed by 30-40 cycles of 15 sec at 95°C, 30-40 sec for annealing at 55-68°C and a final extension step for 33-45 s at 72°C. Quantitation was performed using a standard curve.

In the case of strain-specific reactions, PCR was performed in triplicate in the StepOne Real-Time PCR System (ABI). Primers were purchased from Thermo Fisher (Thermo Fisher Scientific, Waltham, MA). PCR was carried out using Power SYBR Green Master Mix (ABI). Taq polymerase was activated at 95°C for 10 min. The cycling parameters were denaturation at 95°C for 15 sec and extension at 64°C for 50 sec (for 30 cycles). Colonies were identified as *L. rhamnosus* CNCM I-4036 when amplification appeared.

Determination of the fecal content of secretory IgA

Secretory IgA was analyzed in feces by enzyme-linked immunosorbant assay (Immundiagnostik AG, Bensheim, Germany) according to the manufacturer's instructions.

Preparation and collection of blood samples

Blood samples were collected into BD Vacutainer® tubes (Becton Dickinson, NJ, USA). An aliquot of the blood was used for hematological determination. A second aliquot was centrifuged for 10 min at 1000 x g and 4°C to separate serum from cells. Serum was collected for cytokine analysis.

Determination of differences in the lymphocyte population by fluorescence-activated cell sorting (FACS)

These analyses were performed at the University of Murcia in the 24-hour period after blood collection to avoid cell lysis. PerCP-Cy-conjugated anti-human CD14, PE-conjugated anti-CD4, FITC-conjugated anti-CD4, PE-Cy7 conjugated anti-CD25, AlexaFluor®-conjugated anti-CD127, PE-conjugated anti-CD19 and PerCP-conjugated anti-CD8 antibodies purchased from Becton Dickinson (San Diego, California, USA) were used to perform multicolor flow cytometric analysis.

The monoclonal antibodies were incubated with 200 µL of the whole blood samples obtained from volunteers for 15 min protected from light. Erythrocytes were removed by hypotonic lysis using Pharm Lyse™ (BD Biosciences, San Diego, CA), and samples were cleaned according to the manufacturer's instructions. Flow cytometry was performed using a fluorescence activated cell sorter (FACS) Calibur® flow cytometer (Becton Dickinson) and Cell Quest (BD). For each antibody panel analysis, $2x10^4$ lymphocytes were gated.

Table 2. Gastrointestinal symptom score according to the Gastrointestinal Symptom Rating Scale (GSRS).

	GSRS symptom score											
	Probiotic groups (n=80)						Placebo group (n=20)					
Symptom	t_1		t_2		t_3		t_1		t_2		t_3	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Abdominal pain (q1)	0		0	0-1	0		0		0		0	
Heartburn (q2)	0		0	0-1	0	0-1	0	0-1	0	0-1	0	
Acid regurgitation (q3)	0	0-1	0	0-1	0	0-1	0		0	0-1	0	0-1
Sucking sensations in the epigastrium (q4)	0		0	0-1	0	0-1	0	0-1	0	0-1	0	0-1
Nausea and vomiting (q5)	0	0-1	0	0-1	0	0-1	0	0-1	0		0	
Borborygmus (q6)	0		0	0-1	0		0		0		0	
Abdominal distension (q7)	0	0-1	0	0-1	0	0-1	0	0-1	0	0-2	0	0-1
Eructation (q8)	0	0-1	0	0-1	0	0-1	0	0-1	0	0-2	0	0-1
Loose stools (q12)	0	0-2	0	0-2	0	0-1	0	0-1	0	0-2	0	0-1
Hard stools (q13)	0	0-2	0	0-2	0	0-1	0	0-1	0	0-2	0	0-1
Urgent need for defecation (q14)	0	0-1	0	0-1	0		0	0-1	0	0-1	0	0-1
Sensation of incomplete evacuation (q15)	0		0		0		0		0		0	
Dyspeptic syndrome (q1-5)	0	0-0.4	0	0-0.8	0	0-0.8	0	0-0.2	0	0.6	0	0.4
Indigestion syndrome (q6-8)	0	0-0.67	0	0-1	0	0-0.67	0	0-0.8	0	0-1	0	0-0.8
Bowel dysfunction syndrome (q12-15)	0	0-1	0	0-1	0	0-0.67	0	0-1	0	0-1	0	0-1

Values are the median and range. q, question number of questionnaire. 0 = absent; 1 = mild; 2 = moderate; 3 = severe. t_1, first washout; t_2, intervention; t_3 , second washout.

doi: 10.1371/journal.pone.0078111.t002

Cytokine quantification in serum

IL-4, IL-6, IL-10, IL-12(p70), TNF-α, and TGF-β were measured using MILLIplex™ immunoassays (Merck-Millipore, MA, USA) on the Luminex 200 system according to the manufacturer's instructions.

Statistical analysis

All results are expressed as the mean ± SEM unless otherwise indicated. Statistical analyses of gastrointestinal symptom scores were performed using the Mann–Whitney *U* test for equivalence. Time comparisons for normally distributed parameters were tested for statistical significance by a lineal model of variance for repeated measures. For those variables found significantly different, specific time differences were tested using the paired t test while the paired Wilcoxon test was used for non-normally distributed parameters. All analyses were performed using the statistical package IBM SPSS (Statistical Package for the Social Sciences) Statistics 20 (Somers, NY, USA).

Results

Subjects

Of the 103 patients enrolled in the study, 3 dropped out during the intervention period (Figure 1). Baseline features of the volunteers appear in Table 1. The average age was 28 years in the placebo and probiotic groups. There was no significant difference between volunteers who received placebo and those fed probiotics regarding height, weight, body mass index, heart rate or blood pressure at baseline.

Tolerance and safety

Symptom scores as measured by the GSRS questionnaire are described in Table 2. All symptom scores were less than 2, and there was no significant difference between the control group and the probiotic-treated group. The median score of the daily recorded gastrointestinal symptoms of acid regurgitation, nausea, vomiting, abdominal distension, and eructation did not change during the probiotic supplementation (intervention) and subsequent follow-up period. Additionally, the stool consistency and defecation frequency did not change during the supplementation period and the subsequent follow-up period in the probiotic and placebo groups (Table S3)

Therefore, no serious adverse events occurred during the supplementation period in any of the groups based on the GSRS questionnaire, which shows that the differences between the probiotic and placebo groups were not significant for any of the reported symptoms.

Likewise, no difference between placebo and probiotic groups occurred in any of the hematological (hemoglobin, hematocrit, mean corpuscular volume, and leucocyte count) and biochemical (cholesterol, glucose, AST, ALT, γ-GT, and creatinine) parameters (Table 3). There was an initial significant difference in γ-GT between placebo and probiotics (17.6 ± 1.2 vs. 14.0 ± 0.9 probiotics vs. placebo at t_1. *P*=0.021); however, such difference in γ-GT remained after the intervention (17.0 ± 1.1 vs. 13.4 ± 0.8 probiotics vs. placebo at t_2. *P*=0.011).

All three probiotic strains were found to be sensitive to ampicillin and tetracycline. In addition, antibiotic sensitivity was similar among the strains at both t_1 and at t_2 (Table S4).

Table 3. Volunteers' hematological and biochemical data.

Parameter	Probiotic groups (n=80)		Placebo group (n=20)	
	t_1	t_2	t_1	t_2
Hemoglobin	14.2 ± 0.2	14.1 ± 0.2	14.0 ± 0.3	13.9 ± 0.3
Hematocrit	42.7 ± 0.8	41.4 ± 0.5	41.7 ± 0.9	41.2 ± 1
Mean Corpuscular Volume	88.2 ± 0.5	87.7 ± 0.5	86.5 ± 1.2	86.0 ± 1.1
Leucocytes	6.2 ± 0.1	6.0 ± 0.2	6.4 ± 0.3	6.2 ± 0.4
Total cholesterol	182.4 ± 3.8	182.3 ± 4	180.0 ± 4.6	183.0 ± 7.5
HDL-cholesterol	65.6 ± 1.9	65.0 ± 2	59.0 ± 3	58.0 ± 3.7
LDL-cholesterol	103.1 ± 3.2	104.8 ± 3.6	109.0 ± 3.4	112.0 ± 6.1
Glucose	83.9 ± 0.9	83.4 ± 0.4	85.0 ± 1.8	86.4 ± 1.8
Aspartate transaminase	23.7 ± 1.5	22.3 ± 1.0	25.4 ± 2.1	23.9 ± 1.5
Alanine transaminase	19.1 ± 1.3	21.5 ± 1.2	18.8 ± 1.9	21.1 ± 1.7
γ-glutamyl transferase	17.6 ± 1.2*	17.0 ± 1.1*	14.0 ± 0.9	13.4 ± 0.8
Creatinine	0.8 ± 0.01	0.8 ± 0.02	0.8 ± 0.03	0.8 ± 0.03

Values are means ± SEM. Hemoglobin (g/dL), hematocrit (%), mean corpuscular volume (fL), leukocytes (x10^3/μL), total cholesterol (mg/dL), HDL-cholesterol (mg/dL), LDL-cholesterol (mg/dL), glucose (mg/dL), aspartate transaminase (U/L), alanine transaminase (U/L), γ-glutamyl transferase (U/L), creatinine (mg/dL). *P<0.05 probiotic groups vs. placebo; t_1, first washout; t_2, intervention.

doi: 10.1371/journal.pone.0078111.t003

Overall, these results indicate that the three probiotic strains were safe and well tolerated by healthy subjects.

Fecal bacterial populations

FISH and real-time PCR were used to investigate whether fecal bacterial populations changed due to the various treatments (Table 4 and Figure 2). The *Bifidobacterium* genus and the *Atopobium* cluster significantly decreased, whereas the *Bacteroides* group increased in the feces of the volunteers who received the placebo. These changes did not occur in any of the groups fed probiotic strains (Table 4).

A transient increase in the *Clostridium coccoides* population occurred at the end of the intervention (t_2) in those patients fed *B. breve*. However, the percentage of live *C. coccoides* after the second washout returned to initial values (t_1). The *C. coccoides* population significantly increased with *L. paracasei* administration after the second washout (Table 4).

The *Clostridium leptum* population increased in both the placebo and *L. rhamnosus*-treated groups. However, the increase was continuous in the placebo group, whereas the initial increase in *C. leptum* slightly but significantly dropped with *L. rhamnosus* treatment (Table 4).

Increases in *Lactobacillus* and *Streptococcus* groups were observed in patients fed a mixture of *B. breve* and *L. rhamnosus*. Percentages of live *C. difficile* and *C. perfringens* also increased with administration of the probiotic mixture, but in this case, both *Clostridium* spp. remained elevated after the second washout (t_2) (Table 4).

Real-time PCR analysis confirmed the increase in *Bacteroides* and the decrease in *Bifidobacterium* spp. observed by FISH-FC in the placebo group (Figure 2C and Figure 2E). *Bacteroides* also increased upon treatment with the mixture of *B. breve* and *L. rhamnosus* (Figure 2C). Interestingly, *C.*

Table 4. Bacterial populations in healthy volunteers' fecal samples by FISH-CF analysis.

Targeted group	Capsule	Time (t)		
		t_1	t_2	t_3
	Placebo	9.6 ± 2.3^{ab}	8.7 ± 1.7^{a}	5.8 ± 0.8^{b}
	L. rhamnosus	12.2 ± 2.4	8.3 ± 1.3	9.0 ± 1.3
Bif164	*B. breve*	6.3 ± 1.3	6.0 ± 1.3	6.8 ± 1.5
	B. breve plus *L. rhamnosus*	8.5 ± 1.8	12.0 ± 3.1	7.2 ± 1.7
	L. paracasei	7.3 ± 1.4	7.3 ± 1.3	8.3 ± 1.5
	Placebo	0.2 ± 0.07	0.7 ± 0.3	0.6 ± 0.2
	L. rhamnosus	1.0 ± 0.3	0.2 ± 0.1	0.3 ± 0.1
Erec482	*B. breve*	0.2 ± 0.1^{a}	0.5 ± 0.2^{b}	0.2 ± 0.01^{ac}
	B. breve plus *L. rhamnosus*	0.4 ± 0.1	1.0 ± 0.4	0.3 ± 0.2
	L. paracasei	0.3 ± 0.2^{ab}	0.3 ± 0.2^{a}	1.1 ± 0.3^{b}
	Placebo	37.7 ± 3.6^{ab}	39.0 ± 2.4^{a}	44.6 ± 2.7^{b}
	L. rhamnosus	42.2 ± 3.1^{a}	49.3 ± 3.1^{b}	43.0 ± 2.9^{ab}
Clep886	*B. breve*	44.9 ± 2.6	45.7 ± 3.5	45.5 ± 2.1
	B. breve plus *L. rhamnosus*	52.5 ± 3.4	45.1 ± 2.4	47.0 ± 3.7
	L. paracasei	46.3 ± 2.4	48.7 ± 3.7	48.7 ± 2.7
	Placebo	12.2 ± 2.1^{a}	7.5 ± 1.4^{b}	6.7 ± 1.2^{bc}
	L. rhamnosus	7.9 ± 1.4	7.0 ± 0.9	5.0 ± 0.7
Ato291	*B. breve*	6.3 ± 1.3	5.4 ± 0.9	5.9 ± 1.0
	B. breve plus *L. rhamnosus*	7.9 ± 2.1	6.1 ± 1.7	6.8 ± 1.5
	L. paracasei	4.2 ± 0.8	5.2 ± 0.8	6.2 ± 0.9
	Placebo	16.6 ± 3.2^{a}	20.7 ± 2.7^{ab}	20.1 ± 2.3^{b}
	L. rhamnosus	14.6 ± 2.0	15.5 ± 2.1	15.7 ± 2.0
Bac303	*B. breve*	20.4 ± 2.6	20.6 ± 2.6	21.7 ± 2.4
	B. breve plus *L. rhamnosus*	15.8 ± 2.2	13.7 ± 2.1	18.9 ± 2.8
	L. paracasei	17.6 ± 2.0	19.4 ± 2.7	14.1 ± 1.9
	Placebo	0.9 ± 0.2	0.9 ± 0.4	1.4 ± 0.4
	L. rhamnosus	1.8 ± 0.5	1.1 ± 0.3	1.3 ± 0.3
Enter1432	*B. breve*	1.3 ± 0.4	1.0 ± 0.3	1.3 ± 0.3
	B. breve plus *L. rhamnosus*	1.2 ± 0.4	1.1 ± 0.4	1.2 ± 0.5
	L. paracasei	0.6 ± 0.2	1.3 ± 0.4	1.7 ± 0.5
	Placebo	3.0 ± 0.5	1.8 ± 0.5	2.5 ± 0.4
	L. rhamnosus	2.6 ± 0.5	3.1 ± 0.5	3.6 ± 0.7
Lab158	*B. breve*	2.9 ± 0.6	2.5 ± 0.5	2.8 ± 0.6
	B. breve plus *L. rhamnosus*	1.8 ± 0.6^{a}	3.2 ± 0.8^{b}	2.6 ± 0.5^{ab}
	L. paracasei	2.2 ± 0.4	3.2 ± 0.6	2.9 ± 0.5
	Placebo	5.5 ± 0.8	4.8 ± 0.7	5.5 ± 0.7
	L. rhamnosus	5.3 ± 0.8	4.7 ± 0.8	5.7 ± 0.7
Strc493	*B. breve*	5.3 ± 0.7	5.1 ± 0.6	6.1 ± 0.8
	B. breve plus *L. rhamnosus*	3.7 ± 0.8^{a}	5.2 ± 0.7^{b}	5.0 ± 0.7^{ab}
	L. paracasei	3.8 ± 0.6	5.4 ± 0.8	6.2 ± 0.9
	Placebo	6.0 ± 1.0	5.4 ± 0.8	6.4 ± 0.8
	L. rhamnosus	6.8 ± 0.6	7.3 ± 1.2	7.8 ± 1.0
Cdif198 plus Cperf191	*B. breve*	7.3 ± 1.1	6.8 ± 0.9	7.3 ± 0.7

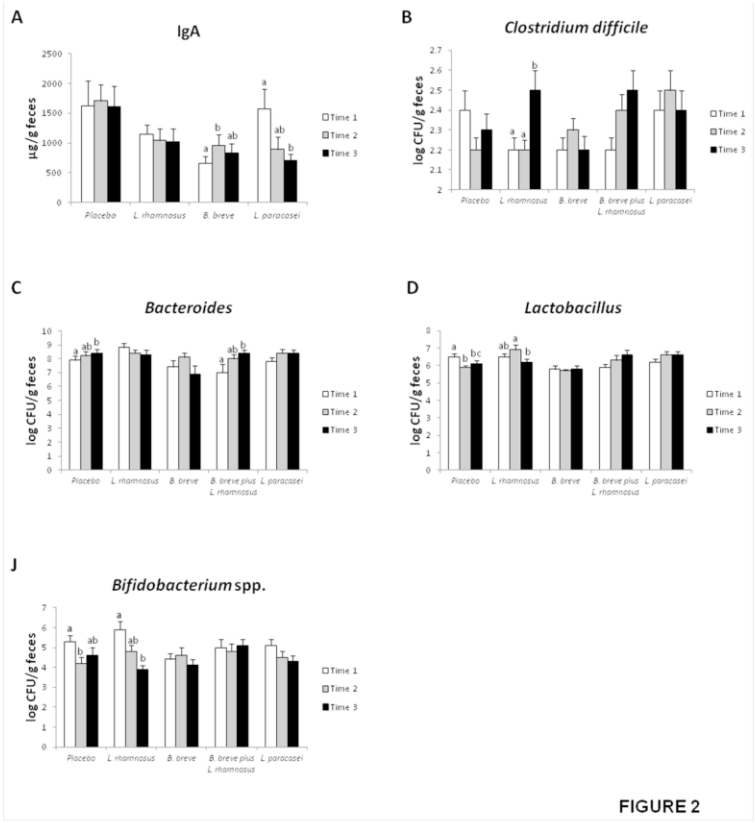


Figure 2. Secretory IgA content (A) and populations of *Clostridium difficile* (B), *Bacteroides* (C), *Lactobacillus* (D), and *Bifidobacterium* spp. (E) in the feces of healthy adults fed one daily probiotic capsule or placebo for 4 weeks as log CFU/g feces. Values are means ± SEM, n=20 per group. Labeled means without a common letter differ, $P<0.05$. Time 1, first washout; Time 2, intervention; Time 3, second washout.

doi: 10.1371/journal.pone.0078111.g002

Table 4 (continued).

Targeted group	Capsule		Time (t)	
	B. breve plus *L. rhamnosus*	5.5 ± 0.9[a]	7.3 ± 1.1[ab]	9.2 ± 0.6[b]
	L. paracasei	6.2 ± 0.9	6.9 ± 1.0	7.2 ± 0.9

Values are means ± SEM, in percentages of living bacteria. n=20 per group. Labeled means without a common letter differ. $P<0.05$. t_1, first washout; t_2, intervention; t_3, second washout.

doi: 10.1371/journal.pone.0078111.t004

difficile increased in volunteers fed *L. rhamnosus* immediately after treatment with this strain ceased (t_2) (Figure 2B).

Lactobacillus significantly decreased in the placebo group after 30 days of intervention and remained low after the second washout. *L. rhamnosus* feeding also resulted in a decrease in *Lactobacillus* at t_2 (Figure 2D).

As for *Bifidobacterium* spp., this population significantly decreased in those volunteers who received a daily capsule of *L. rhamnosus* when treatment with the probiotic ceased (Figure 2E).

Altogether, these results indicate that both probiotic and placebo administration modified bacterial populations in the volunteers' feces.

Fecal strain persistence

A total of 75 colonies from patients fed *L. rhamnosus* CNCM I-4036 were picked from dishes containing MRS modified medium (glucose substituted with rhamnose) and were subsequently grown under anaerobic conditions. DNA analysis by real-time PCR with specific primers for *L. rhamnosus* CNCM I-4036 revealed that 86% of the colonies were positive for this species. This result suggests that at least *L. rhamnosus*, for which there is available specific and selective culture medium, colonized the intestine of volunteers fed this strain for 30 days.

Fecal secretory IgA content

Secretory IgA content was measured in the stools of the various groups of healthy volunteers (Figure 2A). *B. breve* administration resulted in a significant increase in the fecal

secretory IgA content after the 30-day intervention (t_2), but this increase returned to initial values after the second washout (t_3). The secretory IgA content did not change in the feces of volunteers who received, *L. rhamnosus*, *L. paracasei* or the *L. rhamnosus/B. breve* mixture.

White blood cell (WBC) subsets

The effects of probiotic administration on various WBC subsets were analyzed by flow cytometry (Table 5). The most relevant findings were as follows: i) the significant increases in the percentage of CD4+ T lymphocytes and CD4+/CD8+ ratio in the blood of volunteers who received *L. paracasei* and ii) the increase in the percentage of regulatory T lymphocytes observed in the placebo, *L. rhamnosus* and *B. breve* groups.

Cytokine concentrations in volunteers' serum

Serum IL-4, IL-10 and IL-12 concentrations, as well as the IL-10/IL-12 and TNF-α/IL-10 ratios appear in Figure 3. All patient groups showed similar values of IL-6, TNF-α and TGF-β (not shown).

Whereas the anti-inflammatory cytokine IL-4 decreased in the group fed the placebo for 30 days, the serum concentration of this cytokine remained unchanged in the groups that received *B. breve*, *L. paracasei* or the combination of both. In contrast, IL-4 increased in those volunteers fed *L. rhamnosus* (Figure 3A). The latter probiotic strain also increased the concentration of another anti-inflammatory cytokine, IL-10 (Figure 3B).

Volunteers fed the placebo, *L. rhamnosus* or *B. breve* exhibited significantly lower values of the pro-inflammatory cytokine IL-12 at the end of the intervention (t_2) compared with baseline (t_1) (Figure 3C). The IL-10/IL-12 ratio, an anti-inflammatory index, significantly increased in patients who received *L. rhamnosus* and *L. paracasei* (Figure 3D). In contrast, *L. rhamnosus* treatment decreased the TNF-α/IL-10 ratio, a pro-inflammatory index (Figure 3E).

Altogether, these findings point to a clear immunomodulatory effect of the three probiotic strains, with *L. rhamnosus* exerting the most robust effect.

Table 5. Analysis of immune system populations in volunteers' blood.

Subset population	Capsule	Time (t)	
		t_1	t_2
	Placebo	69.2 ± 1.9	71.4 ± 1.4
	L. rhamnosus	70.8 ± 2.5	72.2 ± 1.4
CD3+(T cells)	*B. breve*	69.6 ± 3.0	70.1 ± 1.8
	B. breve plus *L. rhamnosus*	74.0 ± 2.5	73.3 ± 2.2
	L. paracasei	70.5 ± 1.9	72.2 ± 1.2
	Placebo	9.6 ± 0.7	9.8 ± 0.8
	L. rhamnosus	10.1 ± 0.5	10.1 ± 0.5
CD19+(B cells)	*B. breve*	9.8 ± 0.7	9.5 ± 0.9
	B. breve plus *L. rhamnosus*	8.6 ± 0.8	9.6 ± 0.6
	L. paracasei	8.7 ± 0.5	8.8 ± 0.7
	Placebo	45.5 ± 1.9	47.6 ± 1.2
	L. rhamnosus	46.0 ± 2.1	45.7 ± 1.6
CD3+ CD4+ (T helper cells)	*B. breve*	45.4 ± 3.0	47.0 ± 2.3
	B. breve plus *L. rhamnosus*	49.5 ± 2.0	50.3 ± 2.0
	L. paracasei	43.6 ± 1.7	47.8 ± 1.5*
	Placebo	21.3 ± 1.6	22.5 ± 1.1
	L. rhamnosus	15.8 ± 2.0	20.3 ± 0.7
CD3+ CD8+ (T cytolytic cells)	*B. breve*	20.1 ± 1.6	20.4 ± 0.9
	B. breve plus *L. rhamnosus*	20.4 ± 1.8	19.8 ± 1.4
	L. paracasei	23.4 ± 1.3	22.4 ± 1.1
	Placebo	2.0 ± 0.2	2.2 ± 0.1
	L. rhamnosus	2.1 ± 0.2	2.3 ± 0.1
CD4+/CD8+ cells	*B. breve*	2.1 ± 0.2	2.4 ± 0.2
	B. breve plus *L. rhamnosus*	2.6 ± 0.2	2.7 ± 0.2
	L. paracasei	1.8 ± 0.1	2.1 ± 0.1*
	Placebo	4.1 ± 0.2	4.9 ± 0.2*
	L. rhamnosus	4.0 ± 0.3	4.9 ± 0.3*
CD3+ CD4+ CD25+ CD127- (T regulatory cells)	*B. breve*	3.8 ± 0.3	5.0 ± 0.6*
	B. breve plus *L. rhamnosus*	4.5 ± 0.4	5.7 ± 0.8
	L. paracasei	4.6 ± 0.2	5.1 ± 0.6
	Placebo	3.1 ± 0.4	4.1 ± 0.5
	L. rhamnosus	3.5 ± 0.4	3.6 ± 0.5
CD14+	*B. breve*	4.6 ± 0.6	5.3 ± 0.7
	B. breve plus *L. rhamnosus*	3.6 ± 0.6	3.9 ± 0.4
	L. paracasei	2.8 ± 0.5	3.9 ± 0.6

Results are mean ± SEM, as percentage of total accounted cells. n=20 per group.

*P<0.05. t_1, first washout; t_2, intervention.

doi: 10.1371/journal.pone.0078111.t005

Discussion

In this study, the safety, tolerance, persistence and effects on the immune system of the probiotic strains *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 [10] were investigated in 100 healthy volunteers. We found that the recorded gastrointestinal symptoms (GSRS and daily recorded symptoms), defecation frequency and stool consistency were not altered by probiotic intake in healthy volunteers. Moreover, no relevant changes in blood and serum parameters, and no adverse events occurred during and after treatment. All three probiotic strains were sensitive to ampicillin and tetracycline.

Probiotic administration modified bacterial populations in the volunteers' feces as evidenced by real-time PCR and fluorescence *in situ* hybridization. Some of the alterations were transient, whereas others were stable. The most relevant finding regarding bacterial populations was the increase in *Clostridium difficile* that took place in feces when *L. rhamnosus* CNCM I-4036 administration ceased (t_3, Figure 2B), which points to a clear beneficial effect by this probiotic strain. Volunteers may have experienced a displacement of *C. difficile* by *L. rhamnosus* CNCM I-4036 during the intervention of 30 days. Many studies have shown a decrease in *C. difficile* adhesion to intestinal mucosa by probiotics [27-29].

The fact that total bifidobacteria counts were reduced in the group treated with *B. breve* CNCM I-4035 suggests that the administered strain either did not reach the colon in a viable state in significant numbers or did not proliferate in the colon.

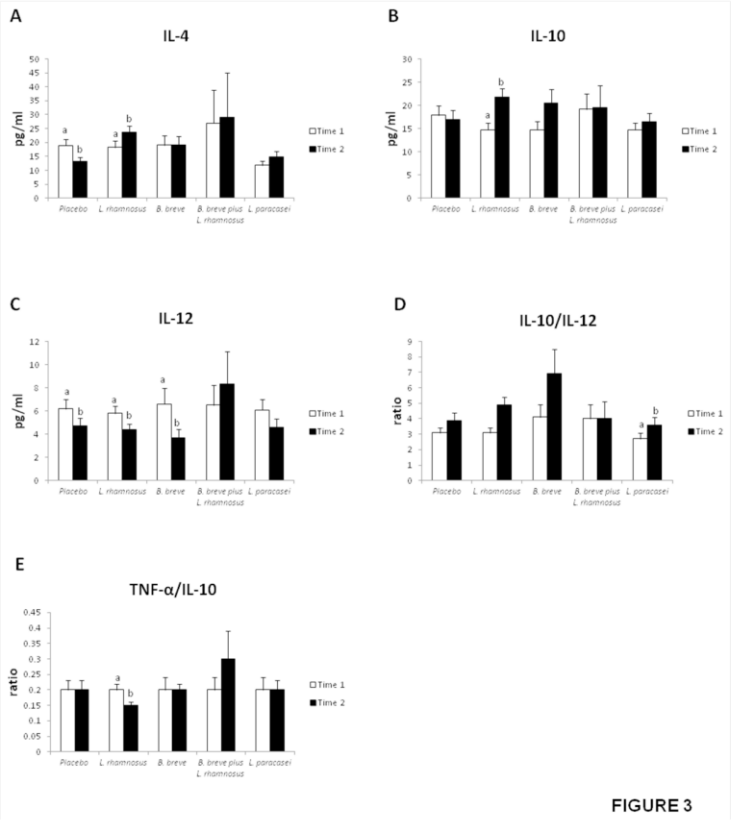


Figure 3. Serum IL-4 (A), IL-10 (B), and IL-12 (C) concentrations and IL-10/TNF-α (D), and IL-10/IL-12 ratios (E) in healthy adults fed one daily capsule of probiotics or placebo for 4 weeks. Values are means ± SEM, n=20 per group. Labeled means without a common letter differ, *P*<0.05. Time 1, first washout; Time 2, intervention.

doi: 10.1371/journal.pone.0078111.g003

Bifidobacteria counts also decreased by *L. rhamnosus* CNCM I-4036 feeding when t_3 is compared with t_1 (Figure 2E).

Strikingly, certain bacterial populations changed in the feces of volunteers in the placebo group. This effect caused by the placebo might be due to the cow's milk and/or sucrose included in its composition. The fact that regulatory T lymphocytes were increased in the placebo group is also intriguing. The same is true for the observation that IL-4 was decreased in the placebo group. This may indicate that the sample size, while calculated prior to start of the trial for the main outcome, was too low for these two variables.

Interestingly, *L. rhamnosus* CNCM I-4036 was identified after the intervention (t_2) in fecal samples of volunteers that received this bacterial strain. This finding does not necessarily imply successful colonization but rather persistence of the strain at this time period. Detection of *L. rhamnosus* CNCM I-4036 for a much longer period would be needed to determine whether the strain does in fact colonize the gastrointestinal tract. Also, high-throughput sequencing techniques would be helpful. Persistence in feces by the two other assayed strains, *L. paracasei* CNCM I-4034 and *B. breve* CNCM I-4035, could not be proven due to the lack of specific and selective culture media.

Another interesting finding of this work was that the *B. breve* CNCM I-4035 administration resulted in a significant increase in secretory IgA content after the 30-day intervention. After the second washout, this increase returned to initial values, which points to a clear effect due to the probiotic. This result confirms previous results from our group [10]. We have reported that *B. breve* CNCM I-4035 led to a higher IgA concentration in both feces and plasma of mice [10]. Modification of secretory IgA has a clear and important effect on the immune system. Secretory IgA serves as the first line of defense in protecting the intestinal epithelium from enteric toxins and pathogenic microorganisms (30). Secretory IgA promotes the clearance of antigens and pathogenic microorganisms from the intestinal lumen by blocking their access to epithelial receptors, entrapping them in mucus, and facilitating their removal by peristaltic and mucociliary activities [30].

It has been suggested that the safety of probiotics should be further evaluated by the detection of undesirable changes in immune parameters [31] because of growing evidence that probiotics, especially lactobacilli and bifidobacteria, have

immunomodulatory properties. The main finding of our cytokine analysis was that *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036 exerted immunomodulatory effects. Increased levels of anti-inflammatory molecules (IL-4, IL-10, IL-10/IL-12) and decreased levels of the pro-inflammatory index (TNF-α/IL-10) were found in the serum of volunteers fed *L. rhamnosus* CNCM I-4036. IL-12 also decreased in volunteers that received *B. breve* CNCM I-4035, whereas the anti-inflammatory index (IL-10/IL-12) increased in the group fed *L. paracasei* CNCM I-4034. Immunomodulation by these three probiotic strains has been reported in *in vitro* experiments by Bermudez-Brito et al. [32,33]. These authors showed that *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036 inhibited the production of pro-inflammatory cytokines and chemokines by human intestinal dendritic cells challenged with pathogenic bacteria and that such an effect seems to be mediated through a decreased expression of toll-like receptor (TLR)-1, TLR-5 and TLR-9 [32,33].

In summary, our results demonstrate that the intake of the three bacterial strains was safe and exerted a varying degree of immunomodulatory effects. In particular, *L. rhamnosus* CNCM I-4036 colonized the intestine, and *B. breve* CNCM I-4035 enhanced production of intestinal secretory IgA. Our findings also confirm previous results obtained in mice. Overall, these results warrant further studies and open the possibility of undertaking similar trials in patients affected by intestinal pathologies.

Supporting Information

Checklist S1. CONSORT Checklist.
(DOC)

Protocol S1. Trial Protocol.
(DOC)

Table S1. Probes used in fluorescence in situ hybridization (FISH).
(DOCX)

Table S2. Primer sequences used in real-time PCR.
(DOCX)

Table S3. Frequency and consistency of the feces.
(DOCX)

Table S4. Percentage of antimicrobial resistance populations.
(DOCX)

Author Contributions

Conceived and designed the experiments: AG LF CGL FR DC GR EM. Performed the experiments: JPD LCM CGL IO RMS CGG MJP EC SG BC AS OP. Analyzed the data: JPD CGL LCM EM IO RMS CGG MJP GR EC SG BC AS DC OP FR DR APC AG LF. Contributed reagents/materials/analysis tools: N/A. Wrote the manuscript: JPD CGL AG LF. N/A.

References

1. World Health Organization and Food & Agriculture Organization (2002) Guidelines for the Evaluation of Probiotics in Food. Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. London, Ontario, Canada. Retrieved onpublished at whilst December year 1111 from ftp://ftp.fao.org/es/esn/food/wgreport2.pdf.
2. Fontana L, Bermudez-Brito M, Plaza-Diaz J, Muñoz-Quezada S, Gil A (2013) Sources, isolation, characterisation and evaluation of probiotics. Br J Nutr 109: S35-S50. doi:10.1017/S0007114512004011. PubMed: 23360880.
3. Guarner F, Malagelada JR (2003) Gut flora in health and disease. Lancet. 361: 512-519. doi:10.1016/S0140-6736(03)12489-0. PubMed: 12583961.
4. Gourbeyre P, Denery S, Bodinier M (2011) Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. J Leukoc Biol 89: 685-695. doi:10.1189/jlb.1109753. PubMed: 21233408.
5. Macpherson AJ, Harris NL (2004) Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol 4: 478-485. doi:10.1038/nri1373. PubMed: 15173836.
6. Frick JS, Schenk K, Quitadamo M, Kahl F, Köberle M et al. (2007) *Lactobacillus fermentum* attenuates the proinflammatory effect of *Yersinia enterocolitica* on human epithelial cells. Inflamm Bowel Dis 13: 83-90. doi:10.1002/ibd.20009. PubMed: 17206643.
7. Vankerckhoven V, Huys G, Vancanneyt M, Vael C, Klare I et al. (2008) Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE Project. Trends Food Sci Technol 19: 102–114. doi:10.1016/j.tifs.2007.07.013.
8. Collins JK, Thornton G, Sullivan GO (1998) Selection of probiotic strains for human application. Int Dairy J 8: 487–490. doi:10.1016/S0958-6946(98)00073-9.
9. Ouwehand AC, Salminen S, Isolauri E (2002) Probiotics: an overview of beneficial effects. Antonie Van Leeuwenhoek 82: 279–289. doi:10.1023/A:1020620607611. PubMed: 12369194.
10. Muñoz-Quezada S, Chenoll E, Vieites JM, Genovés S, Maldonado J et al. (2013) Isolation, identification and characterisation of three novel probiotic strains (*Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036) from the faeces of exclusively breast-fed infants. Br J Nutr 109: S51-S62. doi:10.1017/S0007114512005211. PubMed: 23360881.
11. Rios S (1967) Métodos estadísticos. Madrid, Spain: Ediciones del Castillo.
12. Wind RD, Tolboom H, Klare I, Huys G, Knol J (2010) Tolerance and safety of the potentially probiotic strain *Lactobacillus rhamnosus* PRSF-L477: a randomised, double-blind placebo-controlled trial in healthy volunteers. Br J Nutr 104: 1806-1816. doi:10.1017/S0007114510002746. PubMed: 20691131.
13. Svedlund J, Sjödin I, Dotevall G (1988) GSRS – a clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. Dig Dis Sci 33: 129–134. doi:10.1007/BF01535722. PubMed: 3123181.
14. Whelan K, Judd PA, Taylor MA (2004) Assessment of fecal output in patients receiving enteral tube feeding: validation of a novel chart. Eur J Clin Nutr 58: 1030–1037. doi:10.1038/sj.ejcn.1601927. PubMed: 15220945.
15. Van Aerde J, Alarcon P, Lam W (2003) Tolerance and safety of energy-dense enteral formulae for young children. Int Pediatr 18: 95–99.
16. Fallani M, Young D, Scott J, Norin E, Amarri S et al. (2010) Intestinal microbiota of 6-weeks-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. J Pediatr

Gastroenterol Nutr 51: 77-84. doi:10.1097/MPG.0b013e3181d1b11e. PubMed: 20479681.
17. Gomez-Llorente C, Plaza-Diaz J, Aguilera M, Muñoz-Quezada S, Bermudez-Brito et al. (2013) Three main factors define changes in fecal microbiota associated with feeding modality in infants. J Pediatr Gastroenterol Nutr (In press).
18. Langendijk PS, Schut F, Jansen GL, Raangs GC, Kamphuis GR et al. (1995) Quantitative fluorescent in situ hybridization of *Bifidobacterium* spp. with genus specific 16S ribosomal-RNA targeted probes and its application in fecal samples. Appl Environ Microbiol 61: 3069-3075. PubMed: 7487040.
19. Manz W, Amann R, Ludwig W, Vancanneyt M, Schleifer KH (1996) Application of a suite of 16S rRNA specific oligonucleotide probes designed to investigate bacteria of the phylum Cytophaga-flavobacter-bacteroides in the natural environment. Microbiology (Reading, Engl.) 142: 1097-1106
20. Sghir A, Gramet G, Suau A, Rochet V, Pochart P et al. (2000) Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl Environ Microbiol 66: 2263-2266. doi:10.1128/AEM.66.5.2263-2266.2000. PubMed: 10788414.
21. Franks AH, Harmsen HJM, Raangs GC, Jansen GJ, Schut F et al. (1998) Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group specific 16S rRNA-targeted oligonucleotide probes. Appl Environ Microbiol 64: 3336-3345. PubMed: 9726880.
22. Harmsen HJM, Elfferich P, Schut F, Welling GW (1999) A 16S rRNA-targeted probe for detection of *Lactobacilli* and *Enterococci* in faecal samples by fluorescent *in situ* hybridization. Microb Ecol Health Dis 11: 3-12. doi:10.1080/089106099435862.
23. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N et al. (2000) Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr 30: 61-67. doi: 10.1097/00005176-200001001-00010. PubMed: 10630441.
24. Lay C, Sutren M, Rochet V, Saunier K, Doré J et al. (2005) Design and validation of 16S rRNA probes to enumerate members of the *Clostridium leptum* subgroup in human faecal microbiota. Environ Microbiol 7: 933-946. doi:10.1111/j.1462-2920.2005.00763.x. PubMed: 15946290.
25. Fallani M, Rigottier-Gois L, Aguilera M, Bridonneau C, Collignon A et al. (2006) *Clostridium difficile* and *Clostridium perfringens* species detected in infant faecal microbiota using 16S rRNA targeted probes. J Microbiol Methods 67: 150-116. doi:10.1016/j.mimet.2006.03.010. PubMed: 16647148.
26. Scardovi V (1986) Genus *Bifidobacterium*. In: PHA SneathNS MairME Sharpe. Bergey's Manual of Systematic. J Bacteriol: 1418–1434.
27. Gorbach SL, Chang TW, Goldin B (1987) Successful treatment of relapsing *Clostridium difficile* colitis with *Lactobacillus* GG. Lancet. 2: 1519. PubMed: 2892070.
28. Lawrence SJ, Korzenik JR, Mundy LM (2005) Probiotics for recurrent *Clostridium difficile* disease. J Med Microbiol 54: 905–906. doi:10.1099/jmm.0.46096-0. PubMed: 16091446.
29. Na X, Kelly C (2011) Probiotics in *clostridium difficile* Infection. J Clin Gastroenterol 45: S154-S158. doi:10.1097/MCG.0b013e31822ec787. PubMed: 21992956.
30. Mantis NJ, Rol N, Corthésy B (2011) Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol 4: 603-611. doi:10.1038/mi.2011.41. PubMed: 21975936.
31. Mäkeläinen H, Tahvonen R, Salminen S, Ouwehand AC (2003) *In vivo* safety assessment of two *Bifidobacterium longum* strains. Microbiol Immunol 47: 911–914. PubMed: 14695440.
32. Bermudez-Brito M, Muñoz-Quezada S, Gomez-Llorente C, Matencio E, Bernal MJ et al. (2012) Human intestinal dendritic cells decrease cytokine release against *Salmonella* infection in the presence of *Lactobacillus paracasei* upon TLR activation. PLOS ONE 7: e43197. doi:10.1371/journal.pone.0043197. PubMed: 22905233.
33. Bermudez-Brito M, Muñoz-Quezada S, Gomez-Llorente C, Matencio E, Bernal MJ et al. (2013) Cell-Free culture supernatant of *Bifidobacterium breve* CNCM I-4035 decreases pro-Inflammatory cytokines in human dendritic cells challenged with *Salmonella typhi* through TLR activation. PLOS ONE 8: e59370. doi:10.1371/journal.pone.0059370. PubMed: 23555025.

View Letter

Close

Date: Apr 23 2014 8:03AM
To: "Luis Fontana" fontana@ugr.es
From: "PLOS ONE" plosone@plos.org
Subject: PLOS ONE Decision: Accept [PONE-D-14-11447]

PONE-D-14-11447
Effects of Lactobacillus paracasei CNCM I-4034, Bifidobacterium breve CNCM I-4035 and Lactobacillus rhamnosus CNCM I-4036 on Hepatic Steatosis in Zucker Rats
PLOS ONE

Dear Dr. Fontana,

I am pleased to inform you that your revised manuscript has been deemed suitable for publication in PLOS ONE.

Your manuscript will now be passed on to our Production staff, who will check your files for correct formatting and completeness. During this process, you may be contacted to make necessary alterations to your manuscript, though not all manuscripts require this.

Please check the accepted PDF of your manuscript very closely. THERE IS NO AUTHOR PROOFING. You should consider the accepted PDF or any corrected files you upload during the production process as equivalent to a production proof. If you would like to make any corrections to your manuscript, please email our Production team (one_production@plos.org) as soon as possible with your request. The text you supply will be faithfully represented in your published manuscript exactly as you supply it. This is your last opportunity to correct any errors that are present in your manuscript files.

Now that your manuscript has been accepted, please log into Editorial Manager at http://www.editorialmanager.com/pone, click the "Update My Information" link at the top of the page, and update your user information to ensure an efficient production and billing process. If you have any questions about billing, please contact authorbilling@plos.org.

If you or your institution will be preparing press materials for this manuscript, you must inform our press team in advance. We no longer routinely supply publication dates to authors; if you need to know your paper's publication date for media purposes, you must coordinate with our press team. Your manuscript will remain under a strict press embargo until the publication date and time. For more information please contact onepress@plos.org.

Please contact one_production@plos.org if you have any other questions or concerns. Thank you for submitting your work to PLOS ONE.

With kind regards,

Jose Luis Balcazar, Ph.D.
Academic Editor
PLOS ONE

Additional Editor Comments (optional):

All reviewers' comments have been addressed satisfactorily. So, I feel this MS is suitable for publication.

Journal requirements:

When submitting your revision, we need you to address these additional requirements.

1. Thank you for stating the following financial disclosure [Part of the research currently in progress

in our laboratory is funded by the company Hero Spain, S. A. through the grant #3545 managed by the Fundacion General Empresa-Universidad de Granada. No additional external funding received for this study.].

Please respond by return e-mail so that we can complete your Financial Disclosure by including the following statement, if pertinent: "The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript." If this statement is not correct you must amend it as needed.

We will amend your financial disclosure and competing interests on your behalf.

2. We noticed that you did not deposit your data to a publicly available database, or include it in the submission form. All PLOS journals now require raw data underlying the findings described in the manuscript to be freely available to other researchers, either in a public repository, or in the manuscript itself.

As your paper has been accepted for publication, please respond by return email stating exactly where your data is deposited, and provide accession numbers or DOIs. For more information may refer to: http://www.plosone.org/static/policies#sharing

[Note: HTML markup is below. Please do not edit.]

Reviewers' comments:

Reviewer's Responses to Questions

Comments to the Author

1. Is the manuscript technically sound, and do the data support the conclusions?

The manuscript must describe a technically sound piece of scientific research with data that supports the conclusions. Experiments must have been conducted rigorously, with appropriate controls, replication, and sample sizes. The conclusions must be drawn appropriately based on the data presented.

Reviewer #1: Yes

---

2. Has the statistical analysis been performed appropriately and rigorously?

Reviewer #1: Yes

---

3. Does the manuscript adhere to the PLOS Data Policy?

Authors must follow the PLOS Data policy, which requires authors to make all data underlying the findings described in their manuscript fully available without restriction. Please refer to the author's Data Availability Statement in the manuscript. All data and related metadata must be deposited in an appropriate public repository, unless already provided as part of the submitted article or supporting information. If there are restrictions on the ability of authors to publicly share data—e.g. privacy or use of data from a third party— these reasons must be specified.

Reviewer #1: Yes

---

4. Is the manuscript presented in an intelligible fashion and written in standard English?

PLOS ONE does not copyedit accepted manuscripts, so the language in submitted articles must be clear, correct, and unambiguous. Any typographical or grammatical errors should be corrected at revision, so please note any specific errors here.

Reviewer #1: Yes

---

5. Review Comments to the Author

Please use the space provided to explain your answers to the questions above. You may also include additional comments for the author, including concerns about dual publication, research ethics, or publication ethics. (Please upload your review as an attachment if it exceeds 20,000 characters)

Reviewer #1: the authors have answered all my questions satisfactorily including the extra experiment comprising liver histology.

---

6. If you would like your identity to be revealed to the authors, please include your name here (optional).

Your name and review will not be published with the manuscript.

Reviewer #1: (No Response)

Close

PLOS ONE

Effects of Lactobacillus paracasei CNCM I-4034, Bifidobacterium breve CNCM I-4035 and Lactobacillus rhamnosus CNCM I-4036 on Hepatic Steatosis in Zucker Rats

--Manuscript Draft--

Manuscript Number:	PONE-D-14-11447
Article Type:	Research Article
Full Title:	Effects of Lactobacillus paracasei CNCM I-4034, Bifidobacterium breve CNCM I-4035 and Lactobacillus rhamnosus CNCM I-4036 on Hepatic Steatosis in Zucker Rats
Short Title:	Reduction in liver steatosis by three probiotic strains
Corresponding Author:	Luis Fontana University of Granada Granada, SPAIN
Keywords:	Probiotic bacteria, LPS, steatosis, inflammation, liver
Abstract:	We have previously described the safety and immunomodulatory effects of Lactobacillus paracasei CNCM I-4034, Bifidobacterium breve CNCM I-4035 and Lactobacillus rhamnosus CNCM I-4036 in healthy volunteers. The scope of this work was to evaluate the effects of these probiotic strains on the hepatic steatosis of obese rats. We used the Zucker rat as a genetic model of obesity. Zucker-Leprfa/fa rats received one of three probiotic strains, a mixture of L. paracasei CNCM I-4034 and B. breve CNCM I-4035, or a placebo for 30 days. An additional group of Zucker-lean+/fa rats received a placebo for 30 days. No alterations in intestinal histology, in the epithelial, lamina propria, muscular layers of the ileal or colonic mucosa, or the submucosae, were observed in any of the experimental groups. Triacylglycerol content decreased in the liver of Zucker-Leprfa/fa rats that were fed L. rhamnosus, B. breve, or the mixture of B. breve and L. paracasei. Likewise, the area corresponding to neutral lipids was significantly smaller in the liver of all four groups of Zucker-Leprfa/fa rats that received probiotics than in rats fed the placebo. Zucker-Leprfa/fa rats exhibited significantly greater serum LPS levels than Zucker-lean+/fa rats upon administration of placebo for 30 days. In contrast, all four groups of obese Zucker-Leprfa/fa rats that received LAB strains exhibited serum LPS concentrations similar to those of Zucker-lean+/fa rats. Serum TNF-α levels decreased in the Zucker-Leprfa/fa rats that received B. breve, L. rhamnosus, or the mixture, whereas L. paracasei feeding decreased IL-6 levels in the serum of Zucker-Leprfa/fa rats. In conclusion, the probiotic strains reduced hepatic steatosis in part by lowering serum LPS, and had an anti-inflammatory effect in obese Zucker rats.
Order of Authors:	Julio Plaza-Diaz
	Carolina Gomez-Llorente
	Francisco Abadia-Molina
	Maria Jose Saez-Lara
	Laura Campaña-Martin
	Sergio Muñoz-Quezada
	Fernando Romero
	Angel Gil
	Luis Fontana
Suggested Reviewers:	Ricardo Uauy Institute of Nutrition and Food Technology, University of Chile, Chile druauy@gmail.com
	Francisco Guarner University Hospital Vall d'Hebron fguarner@vhebron.net

Effects of *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 on Hepatic Steatosis in Zucker Rats

Julio Plaza-Diaz,[1,2] Carolina Gomez-Llorente,[1,2] Francisco Abadía-Molina,[3] Maria Jose Saez-Lara,[4] Laura Campaña-Martin,[2] Sergio Muñoz-Quezada,[1,2,$] Fernando Romero,[5] Angel Gil,[1,2] Luis Fontana[1,2,#]

[1]Dept. Biochemistry & Molecular Biology II, School of Pharmacy, University of Granada, Spain.
[2]Institute of Nutrition & Food Technology "José Mataix", Biomedical Research Center, University of Granada, Spain.
[3]Dept. Cell Biology, School of Sciences, University of Granada, Spain.
[4]Dept. Biochemistry & Molecular Biology I, School of Sciences, University of Granada, Spain.
[5]Hero Global Technology Center, Hero Spain, S.A., Alcantarilla, Murcia, Spain.

[$]**Current address**: National Agency for Medicines (ANAMED), Public Health Institute, Santiago, Chile.

24 #**Corresponding author**:
25 Luis Fontana, Ph. D.
26 Dept. Biochemistry and Molecular Biology II, School of Pharmacy
27 Campus de Cartuja s/n, 18071 Granada, Spain
28 Phone: 34958242335
29 Fax: 34958248960
30 E-mail: fontana@ugr.es
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48

ABSTRACT

We have previously described the safety and immunomodulatory effects of *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 in healthy volunteers. The scope of this work was to evaluate the effects of these probiotic strains on the hepatic steatosis of obese rats. We used the Zucker rat as a genetic model of obesity. Zucker-Lepr$^{fa/fa}$ rats received one of three probiotic strains, a mixture of *L. paracasei* CNCM I-4034 and *B. breve* CNCM I-4035, or a placebo for 30 days. An additional group of Zucker-lean$^{+/fa}$ rats received a placebo for 30 days. No alterations in intestinal histology, in the epithelial, lamina propria, muscular layers of the ileal or colonic mucosa, or the submucosae, were observed in any of the experimental groups. Triacylglycerol content decreased in the liver of Zucker-Lepr$^{fa/fa}$ rats that were fed *L. rhamnosus*, *B. breve*, or the mixture of *B. breve* and *L. paracasei*. Likewise, the area corresponding to neutral lipids was significantly smaller in the liver of all four groups of Zucker-Lepr$^{fa/fa}$ rats that received probiotics than in rats fed the placebo. Zucker-Lepr$^{fa/fa}$ rats exhibited significantly greater serum LPS levels than Zucker-lean$^{+/fa}$ rats upon administration of placebo for 30 days. In contrast, all four groups of obese Zucker-Lepr$^{fa/fa}$ rats that received LAB strains exhibited serum LPS concentrations similar to those of Zucker-lean$^{+/fa}$ rats. Serum TNF-α levels decreased in the Zucker-Lepr$^{fa/fa}$ rats that received *B. breve*, *L. rhamnosus,* or the mixture, whereas *L. paracasei* feeding decreased IL-6 levels in the serum of Zucker-Lepr$^{fa/fa}$ rats. In conclusion, the probiotic strains reduced hepatic

73 steatosis in part by lowering serum LPS, and had an anti-inflammatory effect in
74 obese Zucker rats.
75

INTRODUCTION

Obesity has reached pandemic levels and is becoming a serious health problem worldwide. In developing countries, the prevalence of obesity has tripled over the last 20 years owing to the adoption of a Western lifestyle (i.e., low physical activity levels, sedentariness, and excessive eating) [1, 2].

The increasing prevalence of type 2 diabetes (T2D), cardiovascular disease, and certain types of cancer is linked to obesity. Thus, approximately 90% of T2D cases are attributable to excess body weight, and 200 million people worldwide are estimated to have glucose intolerance and insulin resistance syndrome (IRS), a figure that is expected to rise to 420 million by the year 2025 [3].

Recent evidence indicates that the intestinal microbiota plays a crucial role in body weight and fat mass [4-6], and, accordingly, suggests an association between the gut microbiota and T2D [7-9]. Probiotics are live microorganisms that confer a health benefit on the host when administered in adequate amounts [10], although dead bacteria and bacterial molecular components may also exhibit probiotic properties. Strains belonging to *Bifidobacterium* and *Lactobacillus* are the most widely used probiotic bacteria and are included in many functional foods and dietary supplements [11-13]. Probiotics can modulate the gut microbiota and the mucosal immune system [14-16].

For probiotics to be successful, they must exhibit certain characteristics: i.e., tolerance to gastrointestinal conditions (gastric acid and bile), ability to adhere to the gastrointestinal mucosa, and competitive exclusion of pathogens [17, 18]. We have previously described the isolation of three lactic acid bacterial (LAB) strains from the feces of exclusively breast-fed newborn infants. These strains were selected based on their probiotic properties, such as adhesion to intestinal mucus, sensitivity to antibiotics and resistance to biliary salts and low pH. We identified these strains as *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035, and *Lactobacillus rhamnosus* CNCM I-4036 [19].

Recently, we demonstrated the tolerance and safety of these three strains in a multi-centre, randomized, double-blind, placebo-controlled trial with healthy volunteers [20]. Oral administration of these LAB strains modified the bacterial populations in the feces of the volunteers, and all three strains exerted varying degrees of immunomodulatory effects [20]. Thus, administration of *B. breve* CNCM I-4035 resulted in a significant increase in fecal secretory IgA content. In addition, IL-4 and IL-10 was increased, whereas IL-12 was decreased, in the serum of volunteers treated with any of the three strains.

A large body of evidence has highlighted the concept that putative intestinal bacteria–derived compounds may affect liver metabolism and, therefore, cause systemic diseases [6, 21, 22]. Serum LPS levels have been proposed to increase upon obesity and steatosis, leading to a metabolic endotoxemia capable of modulating proinflammatory cytokines, as well as glucose and lipid

metabolism in the liver or in the adipose tissue [23-26]. Endotoxemia is considered a major risk for inducing liver inflammation in nonalcoholic steatohepatitis (NASH) and nonalcoholic fatty liver disease (NAFLD) in humans [27-30]. NASH and NAFLD have been shown to be associated with increased gut permeability in humans [31,32]. Cani et al. have demonstrated the alteration of gut-barrier function in genetic models of obesity [33]. Overall, these studies strongly suggest a direct link between the gut microbiota, the gut barrier, and hepatic changes.

In the present study, we used the Zucker rat as a genetic model of obesity to test the probiotic properties of our three LAB strains. We chose the Zucker rat model because it has been exhaustively characterized and exhibits symptoms of IRS that are usually found in obese humans, including hyperglycemia, glucose intolerance, hyperinsulinemia, insulin resistance, hyperlipidemia, and hepatic steatosis [34-38]. We focused on evaluating the effects of the LAB strains on hepatic steatosis in lean and obese Zucker rats.

MATERIALS AND METHODS

Ethical Statement. This study was carried out in strict accordance with the recommendations in the guidelines for animal research of the University of Granada (Spain). All animals received humane care. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Granada (Permit Number. CEEA: 2011-377).

Microorganisms. The LAB strains *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035, and *Lactobacillus rhamnosus* CNCM I-4036 have been characterized and are described elsewhere [19]. These strains were assayed for enzymatic activity and carbohydrate utilization, and they were deposited in the Collection Nationale de Cultures de Microorganismes (CNCM) of the Institute Pasteur [19].

Experimental design. Forty-eight Zucker-Lepr$^{fa/fa}$ and 16 Zucker-lean$^{+/fa}$ male rats weighing 168-180 g were purchased from Harlan Laboratories (Charles River, Barcelona, Spain). The rats were housed in metabolic cages with a 12-h light-dark cycle and had free access to water and food. After 5 days of adaptation, 8 Zucker-lean$^{+/fa}$ and 8 Zucker-Lepr$^{fa/fa}$ rats were euthanized as a reference (baseline). The remaining 40 Zucker-Lepr$^{fa/fa}$ rats were then randomly assigned to receive 10^{10} CFUs of one of the three probiotic strains, a mixture of *Lactobacillus paracasei* CNCM I-4034 and *Bifidobacterium breve* CNCM I-4035, or a placebo by oral administration each day for 30 days. An additional group of 8 Zucker-lean$^{+/fa}$ rats received placebo for 30 days. The placebo contained 67% cow's milk powder, 32.5% sucrose, and 0.56% vitamin C. The goal of this study was to examine the differences between obese rats treated with probiotics and placebo.

After the intervention, the animals were anesthetized and sedated with ketamine and xylazine. Blood was drawn from the aorta and centrifuged for 10 min at 1000 x g and 4°C to separate the serum from cells. Samples of intestinal mucosa and liver were also taken.

175

176 **Intestinal histology.** Ileum and colon samples were fixed with 4%
177 paraformaldehyde for 4 h at room temperature and embedded in paraffin. Three
178 pieces of each ileum and colon were respectively embedded in the same
179 paraffin block. Five-μm-thick sections were obtained and routinely stained with
180 haematoxylin-eosin for their microscopic examination. Two rats per group and 8
181 sections per rat were stained and examined.

182

183 **Hepatic triacylglycerol (TG) assay.** Hepatic TG content was determined using
184 a commercial kit according to the manufacturer's instructions (SpinReact,
185 Gerona, Spain). The TG values were normalized to liver weight.

186

187 **Oil red O staining.** Liver samples were fixed with 4% paraformaldehyde,
188 cryopreserved in 30% phosphate-buffered saline (PBS)-sucrose, frozen in an
189 isopentane liquid nitrogen bath, and embedded in OCT^{TM} compound. Three
190 pieces of liver from each animal were fixed and embedded in the same block.
191 Seven μm-thick cryostat sections were obtained and stained with a solution of
192 0.3% Oil Red O in 60% isopropanol. Four to 8 sections per block were stained,
193 micrographs were taken and the percentage of the micrograph area
194 corresponding to the lipid staining was calculated using ImageJ software
195 (National Institutes of Health, USA). Two rats per group were used for this
196 study.

197

198 **Serum biochemistry.** Concentrations of glucose, insulin, phospholipids,
199 triacylglycerols, HDL-cholesterol, LDL-cholesterol, and non-esterified fatty acids

200 (NEFA), as well as the activities of AST and ALT were determined in the serum
201 of the rats using commercial kits. Relative insulin sensitivity was determined by
202 the homeostasis model assessment of insulin resistance (HOMA-IR) as
203 described [39].
204
205 **Serum lipopolysaccharide (LPS) concentration.** Serum LPS was measured
206 with an enzyme-linked immunosorbent assay kit from Cloud-Clone Corp.,
207 Houston, USA, following the manufacturer's directions.
208
209 **Adipokine and cytokine quantification in serum.** Serum concentrations of
210 leptin, adiponectin, TNF-α, and IL-6 were measured using MILLIplex^{TM}
211 immunoassays (Merck-Millipore, MA, USA) and the Luminex 200 system
212 according to the manufacturer's instructions.
213
214 **Statistical analysis.** All results are expressed as the mean ± SEM unless
215 otherwise indicated. Statistical analyses between Zucker-Lepr$^{fa/fa}$ and Zucker-
216 lean$^{+/fa}$ male rats were performed using the t test at the baseline and after the
217 intervention (placebo groups). Significant differences between obese rats that
218 received placebo and any group of obese rats that received a specific strain
219 after intervention were analyzed using one-factor ANOVA, which was corrected
220 by an *a posteriori* Bonferroni test (P<0.05). All analyses were performed using
221 the statistical package IBM SPSS (Statistical Package for the Social Sciences)
222 Statistics 20 (Somers, NY).
223
224

RESULTS

Zucker-Lepr$^{fa/fa}$ rats exhibited severe signs of insulin resistance syndrome (IRS) at the end of the intervention period. Although the body weights of Zucker-Lepr$^{fa/fa}$ (n=8) and Zucker-lean$^{+/fa}$ rats (n=8) were initially similar (179.9 g ± 2.2 g vs. 168.9 g ± 4.9 g, respectively, P>0.3), the Zucker-Lepr$^{fa/fa}$ rats were clearly obese after 30 days of feeding with the placebo (Zucker-Lepr$^{fa/fa}$ (n=8) 294.4 g ± 5.7 g vs. Zucker-lean$^{+/fa}$ (n=8) 241.5 g ± 5.6 g, P<0.001). No adverse events occurred during or after treatment.

We measured parameters related to carbohydrate and lipid metabolism, as well as hepatic function in the serum of the rats (n=8 per group, Table 1). At baseline, the glucose, insulin, HOMA-IR, phospholipid, TG, total cholesterol, and HDL cholesterol concentrations were significantly greater in the Zucker-Lepr$^{fa/fa}$ rats than in the Zucker-lean$^{+/fa}$ rats (P<0.05). With the exceptions of phospholipids and HDL cholesterol, all of these parameters were worse in obese rats that were fed the placebo for 30 days compared to the lean controls (P<0.05) (Table 1).

The LDL cholesterol and NEFA concentrations were similar in both groups of rats at baseline but were significantly higher in Zucker-Lepr$^{fa/fa}$ rats that were fed the placebo for 30 days compared to Zucker-lean$^{+/fa}$ rats (P<0.05) (Table 1). At baseline, the AST and ALT activities differed between the Zucker-Lepr$^{fa/fa}$ rats and the Zucker-lean$^{+/fa}$ rats and remained significantly elevated in the Zucker-Lepr$^{fa/fa}$ rats after the intervention (t=30) with the placebo (P<0.05) (Table 1).

The concentrations of TG, total cholesterol and NEFA, as well as the ALT activity, of Zucker-Lepr$^{fa/fa}$ rats worsened after 30 days of intervention (P<0.05).

The TG content was also measured in the livers of the rats (Figure 1). Hepatic TG content was similar in Zucker-Lepr$^{fa/fa}$ rats and Zucker-lean$^{+/fa}$ rats at baseline. However, the liver TG content was 2.5-fold greater in the Zucker-Lepr$^{fa/fa}$ rats at the end of the intervention with the placebo (P<0.05) (Figure 1). Together, these results indicated that Zucker-Lepr$^{fa/fa}$ rats showed clear signs of IRS.

LAB strains did not exert any effect, beneficial or detrimental, on intestinal histology. No alterations in intestinal histology, of the epithelial, lamina propria, or muscular layers of the ileal or colonic mucosa, or in the submucosae, were observed in any of the experimental groups (Figure 2). The remaining intestinal layers also appeared normal in all of the experimental groups. These results suggested that the probiotics did not alter the morphology of this organ, reinforcing the safety of all three LAB strains.

Steatosis was decreased in Zucker-Lepr$^{fa/fa}$ rats that were fed LAB strains. To investigate whether the bacterial strains affected hepatic steatosis, we measured the TG content in the liver of rats fed these strains (Figure 1). Strikingly, the TG content was significantly lower in the liver of Zucker-Lepr$^{fa/fa}$ rats that were fed *L. rhamnosus* CNCM I-4036, *B. breve* CNCM I-4035, or the mixture of *B. breve* CNCM I-4035 and *L. paracasei* CNCM I-4034 for 30 days

274 (P<0.05) than in the liver of Zucker-Lepr$^{fa/fa}$ rats that were fed the placebo
275 (Figure 1).
276 These results were confirmed by Oil red O staining of liver sections (Figure 3).
277 All four groups of Zucker-Lepr$^{fa/fa}$ rats that received probiotic bacteria exhibited
278 significantly lower percentages of neutral lipids in the liver compared with
279 Zucker-Lepr$^{fa/fa}$ rats fed the placebo (Figure 3).
280
281 **LAB strains did not affect serum biochemistry.** No significant differences in
282 HOMA-IR values or any of the biochemical parameters that were analyzed in
283 the serum were found among the various groups of Zucker-Lepr$^{fa/fa}$ rats that
284 received LAB strains after an intervention of 30 days (Table 1), suggesting that
285 the bacterial strains did not affect serum markers of IRS.
286
287 **LAB strains modified the profile of serum cytokines but not serum**
288 **adipokines.** We determined the serum concentrations of leptin, adiponectin,
289 TNF-α, and IL-6. At baseline, the concentrations of leptin and adiponectin
290 (Figure 4) were significantly greater in Zucker-Lepr$^{fa/fa}$ rats than in Zucker-
291 lean$^{+/fa}$ rats (P<0.05). These results further supported the above serum
292 biochemistry measurements that indicated that Zucker-Lepr$^{fa/fa}$ rats suffered
293 from IRS. At the end of the intervention with the placebo, leptin concentration
294 remained higher in the obese rats than in the lean controls (P<0.05) (Figure
295 4A). Probiotics exerted no effect on the levels of any of the adipokines that were
296 analyzed (Figure 4).
297

298 At baseline, the serum TNF-α and IL-6 concentrations were similar in Zucker-
299 Lepr$^{fa/fa}$ and Zucker-lean$^{+/fa}$ rats (Figure 5), but the TNF-α concentration was
300 significantly increased (Figure 5A) in obese rats after 30 days of intervention
301 with the placebo (P<0.05). Intervention with *L. rhamnosus* CNCM I-4036, *B.*
302 *breve* CNCM I-4035, or the mixture of *L. paracasei* CNCM I-4034 and *B. breve*
303 CNCM I-4035 decreased serum TNF-α concentrations in Zucker-Lepr$^{fa/fa}$ rats
304 (P<0.05) (Figure 5A). The serum IL-6 levels decreased upon *L. paracasei*
305 CNCM I-4034 administration (P<0.05) (Figure 5B).
306
307 **Administration of LAB strains to obese rats decreased serum LPS**
308 **concentrations.** To shed light on the potential mechanism of action of the
309 probiotic strains we measured LPS concentrations in serum samples. These
310 results appear in Figure 6. Zucker-Lepr$^{fa/fa}$ rats exhibited significantly greater
311 LPS levels than Zucker-lean$^{+/fa}$ rats upon administration of placebo for 30 days.
312 In contrast, all four groups of obese Zucker-Lepr$^{fa/fa}$ rats that received LAB
313 strains exhibited serum LPS concentrations similar to those of Zucker-lean$^{+/fa}$
314 rats. These results matched those obtained for liver TG content (Figure 1) and
315 clearly demonstrate the impact of probiotic administration on serum LPS.
316
317 **DISCUSSION**
318
319 Metabolic syndrome, which is better termed insulin resistance syndrome (IRS),
320 was originally defined as concomitant hyperlipidemia, hypertension, insulin
321 resistance and obesity [40, 41]. IRS often precedes the onset of type 2 diabetes
322 and increases the risk of cardiovascular disease [42, 43]; therefore, IRS has

become a major public health concern. The Zucker rat shows many of the features of IRS; therefore, it is one of the most commonly used genetic models of this syndrome [43]. Under our experimental conditions, Zucker-Lepr$^{fa/fa}$ rats exhibited obesity, hyperglycemia, insulin resistance, hypercholesterolemia, hypertriglyceridemia, and elevated serum free fatty acid concentrations after 30 days of intervention with the placebo in contrast to Zucker-lean$^{+/fa}$ rats. In addition, Zucker-Lepr$^{fa/fa}$ rats had hepatic steatosis, as well as elevated serum AST and ALT activities, indicating that the liver component of IRS was also present in this model.

As we have previously described in human subjects [20], the administration of our three LAB strains was safe, as determined by intestinal histology, which showed no difference between LAB strain-fed rats and placebo-fed rats.

We took advantage of the Zucker rat model to investigate the effects of three probiotic strains on IRS features and inflammation. Administration of the probiotic strains did not affect serum biochemical parameters, insulin resistance, or the adipokine profile. These findings were in accordance with results recently reported in human volunteers who were fed these same three LAB strains [20].

The main finding of this study was the reduction in liver steatosis observed in obese rats fed probiotics. Zucker-Lepr$^{fa/fa}$ rats that received *L. rhamnosus* CNCM I-4036, *B. breve* CNCM I-4035, or a mixture of *B. breve* CNCM I-4035 and *L. paracasei* CNCM I-4034 had a liver TG content lower than rats fed the

348 placebo. This drop in liver TG content may not be attributable to a decrease in
349 serum insulin concentration because insulinemia was similar in Zucker-Lepr$^{fa/fa}$
350 rats fed placebo and those fed LAB strains. Additionally, given that the
351 administration of *L. paracasei* CNCM I-4034 alone did not lower liver TG
352 content in the obese rats, the effect observed by mixing the two probiotics might
353 be attributable to *B. breve* CNCM I-4035. Oil red O staining of liver neutral lipids
354 confirmed this finding in probiotic-fed obese rats.
355
356 The administration of probiotics has been reported to lower the hepatic TG and
357 cholesterol content in mice and rats with high fat diet-induced obesity [33, 44-
358 47]. To our knowledge, however, this is the first study describing the effect of
359 probiotics on lowering the liver TG content in genetically obese Zucker rats.
360 This anti-steatotic effect seemed to be mediated, at least in part, by the lowering
361 of serum LPS observed in the probiotic-fed groups of obese rats. Overall our
362 results support the current evidence that intestinal bacteria may affect liver
363 metabolism [6, 21, 43].
364
365 Clear anti-inflammatory effects of probiotics were found in this study: i) the
366 lower serum TNF-α concentrations found in Zucker-Lepr$^{fa/fa}$ rats that received *L.*
367 *rhamnosus* CNCM I-4036, *B. breve* CNCM I-4035, or a mixture of *B. breve*
368 CNCM I-4035 and *L. paracasei* CNCM I-4034 than in Zucker-Lepr$^{fa/fa}$ rats fed
369 the placebo; and ii) the lower serum IL-6 concentrations in obese rats fed *L.*
370 *paracasei* CNCM I-4034 than in obese rats fed the placebo. Two previous
371 studies by our group have reported the effects of *L. paracasei* CNCM I-4034
372 and *B. breve* CNCM I-4035 on the production of cytokines and chemokines by

373 cultured human intestinal dendritic cells challenged with *Salmonella typhi* [48,
374 49]. *L. paracasei* CNCM I-4034 decreased the amounts of proinflammatory
375 cytokines and chemokines in these cells [48], whereas *B. breve* CNCM I-4035
376 was a potent inducer of pro-inflammatory factors (TNF-α, IL-8 and RANTES
377 (Regulated on Activation, Normal T Cell Expressed and Secreted) and anti-
378 inflammatory factors (IL-10) [49]. In the present study, the administration of *L.*
379 *paracasei* CNCM I-4034 decreased the concentrations of proinflammatory
380 cytokines such as IL-6.
381
382 Other authors have described the anti-inflammatory effects of probiotic
383 administration. Probiotics have been reported to significantly suppress the high-
384 fat-diet-induced activation of nuclear factor κ-B signaling that is involved in the
385 development of high-fat-diet-induced insulin resistance [50]. In addition, the
386 administration of lactobacilli to rats developing alcohol-induced metabolic
387 endotoxemia and liver disease reduced plasma endotoxin levels and the liver
388 pathology score [51]. A mixture of bifidobacteria, lactobacilli, and *Streptococcus*
389 *thermophilus* has been shown to decrease liver inflammation in genetically
390 obese mice [52] and high-fat-diet-induced hepatic inflammation in young rats
391 [53].
392
393 Taken together, the results suggested that our probiotic strains ameliorated
394 hepatic steatosis through a decrease in serum LPS and diminished the serum
395 profile of proinflammatory cytokines of obese Zucker rats. These findings, along
396 with those previously obtained by our group using these probiotic strains in *in*

397 *vivo* and human studies, warrant further study to investigate the potential use of
398 these bacterial strains as coadjuvants in the treatment of human disease.
399

400 **REFERENCES**

401

402 1. Haslam DW, James WPT (2005) Obesity Lancet 366: 1197-1209.

403 2. Hossain P, Katar B, El Vahas M (2007) Obesity and diabetes in the
404 developing world. A growing challenge. N Engl J Med 356: 213-215.

405 3. Wang Y, Lobstein T (2006) Worldwide trends in childhood overweight
406 and obesity. Int J Pediatr Obes 1: 11-25.

407 4. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, et al.
408 (2006) An obesity-associated gut microbiome with increased capacity for
409 energy harvest. Nature 444: 1027-1031.

410 5. Knaapen M, Kootte RS, Zoetendal EG, de Vos WM, Dallingha-Thie GM,
411 et al. (2013) Obesity, nonalcoholic fatty liver disease, and
412 atherothrombosis: a role for the intestinal microbiota?. Clin Microbiol
413 Infect 19: 331-337.

414 6. Delzenne NM, Cani PD (2011) Interaction between obesity and the gut
415 microbiota: relevance in nutrition. Annu Rev Nutr 31: 15-31.

416 7. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, et al.
417 (2005) Obesity alters gut microbial ecology. Proc Natl Acad Sci USA 102:
418 11070-11075.

419 8. Ley RE, Turnbaugh PJ, Klein S, Gordon JI (2006) Microbial ecology:
420 human gut microbes associated with obesity. Nature 444: 1022-1023.

421 9. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI (2005)
422 Host-bacterial mutualism in the human intestine. Science 307: 1915-
423 1920.

424 10. World Health Organization and Food & Agriculture Organization (2002)
425 Guidelines for the Evaluation of Probiotics in Food. Report of a joint
426 FAO/WHO working group on drafting guidelines for the evaluation of
427 probiotics in food. London, Ontario, Canada.
428 ftp://ftp.fao.org/es/esn/food/wgreport2.pdf

429 11. Gourbeyre P, Denery S, Bodinier M (2011) Probiotics, prebiotics, and
430 synbiotics: impact on the gut immune system and allergic reactions. J
431 Leukoc Biol 89: 685-695.

432 12. Macpherson AJ, Harris NL (2004) Interactions between commensal
433 intestinal bacteria and the immune system. Nat Rev Immunol 4: 478-485.

434 13. Frick JS, Schenk K, Quitadamo M, Kahl F, Köberle M, et al. (2007)
435 *Lactobacillus fermentum* attenuates the proinflammatory effect of
436 *Yersinia enterocolitica* on human epithelial cells. Inflamm Bowel Dis 13:
437 83-90.

438 14. Yadav H, Jain S, Sinha PR (2008) Oral administration of dahi containing
439 probiotic *Lactobacillus acidophilus* and *Lactobacillus casei* delayed the
440 progression of streptozotocin-induced diabetes in rats. J Dairy Res 75:
441 189-195.

442 15. Power SE, O'Toole PW, Stanton C, Ross RP (2014) Intestinal microbiota,
443 diet and health. Br J Nutr 111: 387-402.

16. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, et al. (2013) The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 341: 569-573.

17. Collins JK, Thornton G, Sullivan GO (1998) Selection of probiotic strains for human application. Int Dairy J 8: 487-490.

18. Ouwehand AC, Salminen S, Isolauri E (2002) Probiotics: an overview of beneficial effects. Antonie van Leeuwenhoek 82: 279-289.

19. Muñoz-Quezada S, Chenoll E, Vieites JM, Genovés S, Maldonado J, et al. (2013) Isolation, identification and characterisation of three novel probiotic strains (*Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036) from the faeces of exclusively breast-fed infants. Br J Nutr 109: S51-S62.

20. Plaza-Diaz J, Gomez-Llorente C, Campaña-Martin L, Matencio E, Ortuño I, et al. (2013) Safety and immunomodulatory effects of three probiotic strains isolated from the feces of breast-fed infants in healthy adults: Setoprob study. PLoS ONE 8: e78111.

21. Nolan JP (1979) The contribution of gut-derived endotoxins to liver injury. Yale J Biol Med 52: 127-133.

22. Nolan JP, Leibowitz AI (1978) The contribution of gut-derived endotoxins to liver injury. Gastroenterology 75: 765-766.

23. Cani PD, Delzenne NM (2009) Interplay between obesity and associated metabolic disorders: new insights into the gut microbiota. Curr Opin Pharmacol 9: 737-743.

467 24. Cani PD, Delzenne NM (2009) The role of the gut microbiota in energy
468 metabolism and metabolic disease. Curr Opin Pharmacol 15: 1546-1558.

469 25. Lanthier N, Molendi-Coste O, Horsmans Y, van RN, Cani PD, et al.
470 (2009) Kupffer cell activation is a causal factor for hepatic insulin
471 resistance. Am J Physiol Gastrointest Liver Physiol 298: 107-116.

472 26. Neyrinck AM, Cani PD, Dewulf EM, De BF, Bindels LB, et al. (2009)
473 Critical role of Kupffer cells in the management of diet-induced diabetes
474 and obesity. Biochem Biophys Res Commun 385: 351-356.

475 27. Adachi Y, Moore LE, Bradford BU, Gao W, Thurman RG (1995)
476 Antibiotics prevent liver injury in rats following long-term exposure to
477 ethanol. Gastroenterology 108: 218-224.

478 28. Verdam FJ, Rensen SS, Driessen A, Greve JW, Buurman WA (2010)
479 Novel evidence for chronic exposure to endotoxin in human nonalcoholic
480 steatohepatitis. J Clin Gastroenterol 5: 149-152.

481 29. Harte AL, da Silva NF, Creely SJ, McGee KC, Billyard T, et al. (2010)
482 Elevated endotoxin levels in nonalcoholic fatty liver disease. J Inflamm
483 (Lond.) 7: 15.

484 30. Thuy S, Ladurner R, Volynets V, Wagner S, Strahl S, et al. (2008)
485 Nonalcoholic fatty liver disease in humans is associated with increased
486 plasma endotoxin and plasminogen activator inhibitor 1 concentrations
487 and with fructose intake. J Nutr 138: 1452-1455.

488 31. Farhadi A, Gundlapalli S, Shaikh M, Frantzides C, Harrell L, et al. (2008)
489 Susceptibility to gut leakiness: a possible mechanism for endotoxaemia
490 in nonalcoholic steatohepatitis. Liver Int 28: 1026-1033.

491 32. Miele L, Valenza V, La TG, Montalto M, Cammarota G, et al. (2009)
492 Increased intestinal permeability and tight junction alterations in
493 nonalcoholic fatty liver disease. Hepatology 49: 1877-1887.

494 33. Cani PD, Possemiers S, Van de WT, Guiot Y, Everard A, et al. (2009)
495 Changes in gut microbiota control inflammation in obese mice through a
496 mechanism involving GLP-2-driven improvement of gut permeability. Gut
497 58: 1091-1103.

498 34. Serkova NJ, Jackman M, Brown JL, Liu T, Hirose R, et al. (2006)
499 Metabolic profiling of livers and blood from obese Zucker rats. J Hepatol
500 44: 956-962.

501 35. Obeid OA, Powell-Tuck J, Emery PW (2000) The postprandial rates of
502 glycogen and lipid synthesis of lean and obese female Zucker rats. Int J
503 Obes Relat Metab Disord 24: 508-513.

504 36. Erdös B, Snipes JA, Miller AW, Busija DW (2004) Cerebrovascular
505 dysfunction in Zucker obese rats is mediated by oxidative stress and
506 protein kinase C. Diabetes 53: 1352-1359.

507 37. Dokken BB, Henriksen EJ (2006) Chronic selective glucogen synthase
508 kinase-3 inhibition enhances glucose disposal and muscle insulin action
509 in prediabetic obese Zucker rats. Am J Physiol Endocrinol Metab 291:
510 207-213.

511 38. D'Angelo G, Mintz JD, Tidwell JE, Schreihofer AM, Pollock DM, et al.
512 (2006) Exaggerated cardiovascular stress responses and impaired β-
513 adrenergic-mediated pressor recovery in obese Zucker rats.
514 Hypertension 48: 1109-1115.

515 39. Singh R, Wang Y, Xiang Y, Tanaka KE, Gaarde WA, Czaja MJ.
516 Differential effects of JNK1 and JNK2 inhibition on murine steatohepatitis
517 and insulin resistance. Hepatology 2009; 49: 87-96.

518 40. Reaven GM (1988) Role of insulin resistance in human disease.
519 Diabetes 37: 1595-1607.

520 41. Reaven GM (2004) The metabolic syndrome or the insulin resistance
521 syndrome? Different names, different concepts, and different goals.
522 Endocrinol Metab Clin North Am 33: 283-303.

523 42. Lakka HM, Laaksonen DE, Lakka TA, Niskanen LK, Kumpusalo E, et al.
524 (2002) The metabolic syndrome and total and cardiovascular disease
525 mortality in middle-aged men. JAMA 288: 2709-2716.

526 43. Kahn R, Buse J, Ferrannini E, Stern M (2005) The metabolic syndrome:
527 time for a critical appraisal: joint statement from the American Diabetes
528 Association and the European Association for the Study of Diabetes.
529 Diabetes Care 28: 2289-2304.

530 44. Paik HD, Park JS, Park E (2005) Effects of *Bacillus polyfermenticus* SCD
531 on lipid and antioxidant metabolisms in rats fed a high-fat and high-
532 cholesterol diet. Biol Pharm Bull 28: 1270-1274.

533 45. Xie N, Cui Y, Yin YN, Zhao X, Yang JW, et al. (2011) Effects of two
534 *Lactobacillus* strains on lipid metabolism and intestinal microflora in rats
535 fed a high-cholesterol diet. BMC Complement Altern Med 11: 53.

536 46. Awaisheh SS, Khalifeh MS, Al-Ruwaili MA, Khalil OM, Al-Ameri OH, et al.
537 (2013) Effect of supplementation of probiotics and phytosterols alone or
538 in combination on serum and hepatic lipid profiles and thyroid hormones
539 of hypercholesterolemic rats. J Dairy Sci 96: 9-15.

540 47. Yoo SR, Kim YJ, Park DY, Jung UJ, Jeon SM, et al. (2013) Probiotics *L.*
541 *plantarum* and *L. curvatus* in combination alter hepatic lipid metabolism
542 and suppress diet-induced obesity. Obesity (Silver Spring) 21: 2571-
543 2578.

544 48. Bermudez-Brito M, Muñoz-Quezada S, Gomez-Llorente C, Matencio E,
545 Bernal MJ, et al. (2012) Human intestinal dendritic cells decrease
546 cytokine release against *Salmonella* infection in the presence of
547 *Lactobacillus paracasei* upon TLR activation. PLoS ONE 7: e43197.

548 49. Bermudez-Brito M, Muñoz-Quezada S, Gomez-Llorente C, Matencio E,
549 Bernal MJ, et al. (2013) Cell-Free culture supernatant of *Bifidobacterium*
550 *breve* CNCM I-4035 decreases pro-Inflammatory cytokines in human
551 dendritic cells challenged with *Salmonella typhi* through TLR activation.
552 PLoS ONE 8: e59370.

553 50. Ma X, Hua J, Li Z (2008) Probiotics improve high fat diet-induced hepatic
554 steatosis and insulin resistance by increasing hepatic NKT cells. J
555 Hepatol 49: 821-830.

556 51. Nanji AA, Khettry U, Sadrzadeh SM (1994) *Lactobacillus* feeding reduces
557 endotoxemia and severity of experimental alcoholic liver (disease). Proc
558 Soc Exp Biol Med 205: 243-247.

559 52. Li Z, Yang S, Lin H, Huang J, Watkins PA, et al. (2003) Probiotics and
560 antibodies to TNF inhibit inflammatory activity and improve nonalcoholic
561 fatty liver disease. Hepatology 37: 343-350.

562 53. Esposito E, Iacono A, Bianco G, Autore G, Cuzzocrea S, et al. (2009)
563 Probiotics reduce the inflammatory response induced by a high-fat diet in
564 the liver of young rats. J Nutr 139: 905-911.

565
566
567
568
569
570
571
572
573
574
575
576
577
578

579 **FIGURE LEGENDS**

580

581 **Figure 1.** Liver triacylglycerol content of Zucker-lean$^{+/fa}$ and Zucker-Lepr$^{fa/fa}$ rats
582 that were fed either a placebo or LAB strains for 30 days. Values are the means
583 ± SEM, n=8 per group. $^{\dagger}P<0.05$ (ZL + placebo vs. ZO + placebo), and $^{*}P<0.05$
584 (ZO + placebo vs. ZO + LAB strains). ZL, Zucker-lean$^{+/fa}$ rats; ZO, Zucker-
585 Lepr$^{fa/fa}$ rats.

586

587 **Figure 2.** Haematoxylin-eosin stained, 5-μm-thick sections of ileal (top panels,
588 A-D) and colonic (bottom panels, E-H) mucosa of Zucker-lean$^{+/fa}$ and Zucker-
589 Lepr$^{fa/fa}$ rats that were fed either a placebo or LAB strains for 30 days. Two rats
590 per group were used for this staining. Three pieces of tissue from each animal
591 were fixed and embedded in the same paraffin block. Four to 8 sections per
592 block were cut, stained and analyzed. Representative micrographs from various
593 groups are shown. A and E: Zucker-lean$^{+/fa}$ rats at baseline; B and F: Zucker-
594 Lepr$^{fa/fa}$ rats + placebo; C and G: Zucker-Lepr$^{fa/fa}$ rats + *L. rhamnosus*; and D
595 and H: Zucker-Lepr$^{fa/fa}$ rats + LAB mixture.

596

597 **Figure 3.** (A) Representative micrographs of 7-μm-thick liver sections stained
598 with 0.3% Oil red O in 60% isopropanol of Zucker-lean$^{+/fa}$ and Zucker-Lepr$^{fa/fa}$
599 rats that were fed either a placebo or LAB strains for 30 days. (B) Percentage of
600 the micrograph area corresponding to the lipid staining of liver sections
601 described in panel A was calculated. Values are the means ± SEM, n=2 per
602 group. $^{\dagger}P<0.05$ (ZL + placebo vs. ZO + placebo), and $^{*}P<0.05$ (ZO + placebo
603 vs. ZO + LAB strains). ZL, Zucker-lean$^{+/fa}$ rats; ZO, Zucker-Lepr$^{fa/fa}$ rats.

604

605 **Figure 4.** Serum leptin (A) and adiponectin (B) concentrations of Zucker-lean$^{+/fa}$
606 and Zucker-Lepr$^{fa/fa}$ rats that were fed either a placebo or LAB strains for 30
607 days. Values are the means ± SEM, n=8 per group. $^{\#}P<0.05$ (ZL baseline vs.
608 ZO baseline), and $^{\dagger}P<0.05$ (ZL + placebo vs. ZO + placebo). ZL, Zucker-lean$^{+/fa}$
609 rats; ZO, Zucker-Lepr$^{fa/fa}$ rats.

610

611 **Figure 5.** Concentrations of serum TNF-α (A) and IL-6 (B) of Zucker-lean$^{+/fa}$ and
612 Zucker-Lepr$^{fa/fa}$ rats that were fed either a placebo or LAB strains for 30 days.
613 Values are the means ± SEM, n=8 per group. $^{\dagger}P<0.05$ (ZL + placebo vs. ZO +
614 placebo), and $^{*}P<0.05$ (ZO + placebo vs. ZO + probiotic strains). ZL, Zucker-
615 lean$^{+/fa}$ rats; ZO, Zucker-Lepr$^{fa/fa}$ rats.

616

617 **Figure 6.** LPS concentration in serum of Zucker-lean$^{+/fa}$ and Zucker-Lepr$^{fa/fa}$ rats
618 that were fed either a placebo or LAB strains for 30 days. Values are the means
619 ± SEM, n=8 per group. $^{\dagger}P<0.05$ (ZL + placebo vs. ZO + placebo), and $^{*}P<0.05$
620 (ZO + placebo vs. ZO + LAB strains). ZL, Zucker-lean$^{+/fa}$ rats; ZO, Zucker-
621 Lepr$^{fa/fa}$ rats.

Table 1. Serum biochemical parameters of Zucker-lean$^{+/fa}$ and Zucker-Lepr$^{fa/fa}$ rats fed either a placebo or LAB strains

	Baseline		Placebo		Intervention with LAB strains			
	ZL	ZO	ZL	ZO	*L. rhamnosus*	*L. paracasei*	*B. breve*	Mixture
Glucose (mg/dL)	165.2 ± 11.1	257.4 ± 38.7$^{\#}$	191.1 ± 4.9	290.1 ± 33.2†	267.2 ± 14.7	243.2 ± 38.9	229.7 ± 19.6	272.7 ± 15.7
Insulin (µg/L)	0.8 ± 0.1	3.1 ± 1.1$^{\#}$	1.1 ± 0.1	3.4 ± 0.5†	3.8 ± 0.6	3.9 ± 1.0	3.5 ± 0.4	2.6 ± 0.4
HOMA-IR	3.1 ± 0.5	8.0 ± 1.1$^{\#}$	4.2 ± 0.2	18.2 ± 3.9†	13.8 ± 2.3	9.7 ± 2.3	13.2 ± 1.6	11.6 ± 2.1
Phospholipids (mg/dL)	185.4 ± 7.6	271.9 ± 20.8$^{\#}$	144.4 ± 7.8	242.9 ± 43.2†	283.7 ± 14.7	239.9 ± 49.2	318.2 ± 16.2	323.9 ± 23.1
Triacylglycerols (mg/dL)	54.3 ± 4.5	152.7 ± 23.2$^{\#}$	46.2 ± 1.8	256.4 ± 25.1$^{\dagger *}$	242.5 ± 26.9	364 ± 12.1	269.1 ± 51.3	297.7 ± 46.9
Total Cholesterol (mg/dL)	123.6 ± 6.5	143.5 ± 4.7$^{\#}$	99.6 ± 3.7	174.5 ± 13.1$^{\dagger *}$	191 ± 10.9	208.8 ± 22.8	211.3 ± 5.4	191 ± 10.9
HDL Cholesterol (mg/dL)	32.1 ± 0.9	40.7 ± 2.3$^{\#}$	18.9 ± 2.1	23.8 ± 7.1	39.7 ± 5.5	28.4 ± 8	33.7 ± 3.4	37.3 ± 1.8
LDL Cholesterol (mg/dL)	80.5 ± 5.3	77.2 ± 8.3	70.3 ± 3.3	99.3 ± 11.9†	93.9 ± 14.2	126.6 ± 20	108.8 ± 7.9	111.6 ± 12.3
NEFA (mmol/L)	0.3 ± 0.03	0.3 ± 0.01	0.3 ± 0.04	0.5 ± 0.05$^{\dagger *}$	0.4 ± 0.04	0.6 ± 0.1	0.6 ± 0.08	0.6 ± 0.08
AST (U/L)	110 ± 13.5	216.1 ± 19.9$^{\#}$	97.2 ± 10.8	356 ± 69.3†	309.7 ± 52.6	378.7 ± 61.6	424.3 ± 70.9	363.6 ± 58.5
ALT (U/L)	39.4 ± 2.5	130.8 ± 19.9$^{\#}$	35.8 ± 6.1	275 ± 52.6$^{\dagger *}$	235 ± 31.4	316.8 ± 46.3	347.9 ± 60.3	296 ± 61.9

Values are the means ± SEM, n=8 per group. $^{\#}P$<0.05 (ZL baseline vs. ZO baseline), $^{\dagger}P$<0.05 (ZL + placebo vs. ZO + placebo), $^{*}P$<0.05 (ZO baseline vs. ZO + placebo). ALT, alanine aminotransferase; AST, aspartate aminotransferase; NEFA, non-esterified fatty acids. HOMA-IR, homeostasis model assessment of insulin resistance. ZL, Zucker-lean$^{+/fa}$ rats; ZO, Zucker-Lepr$^{fa/fa}$ rats.

SUPPORTING INFORMATION LEGENDS

ARRIVE CHECKLIST S1. ARRIVE checklist of information included in this article.

Figure 1
Click here to download Figure: Figure 1.pptx

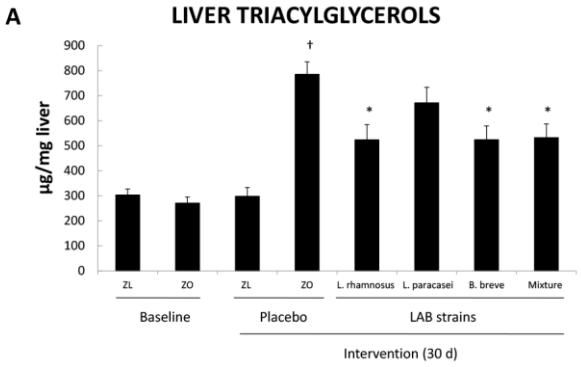


FIGURE 1

Figure 2
Click here to download Figure: Figure 2.pptx

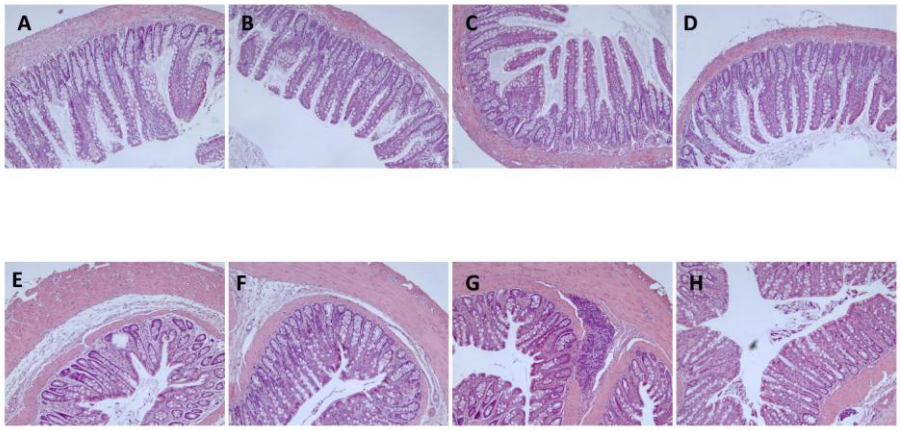


FIGURE 2

Figure 3A
Click here to download Figure: Figure3A.ppt

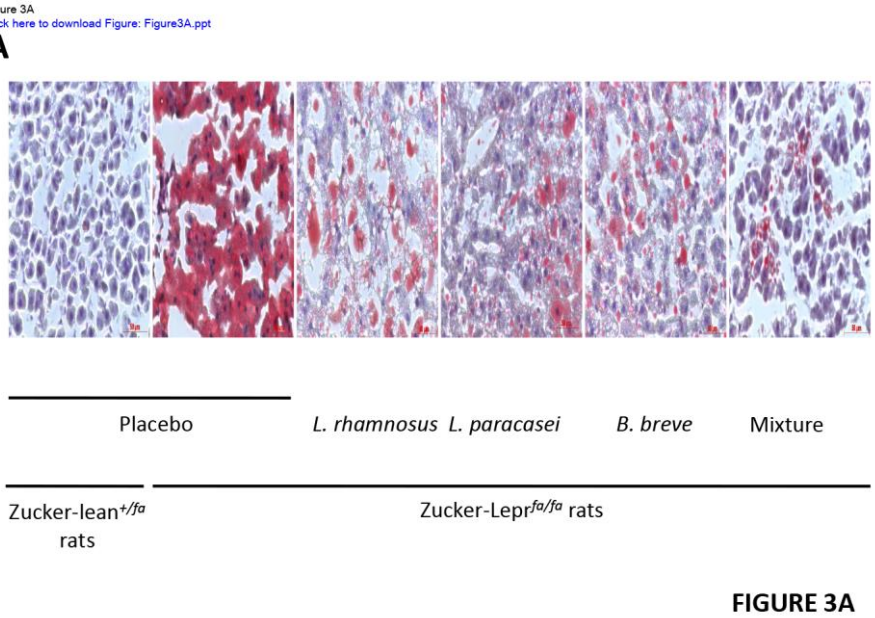


FIGURE 3A

Figure 3B
Click here to download Figure: Figure 3B.ppt

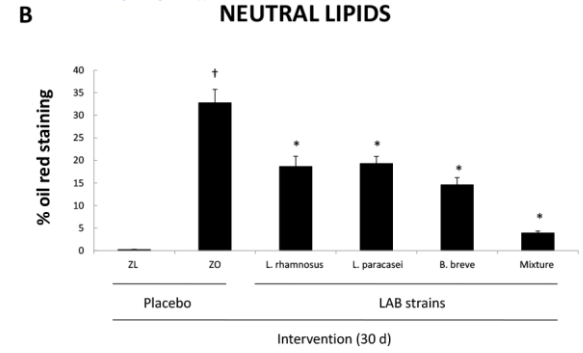
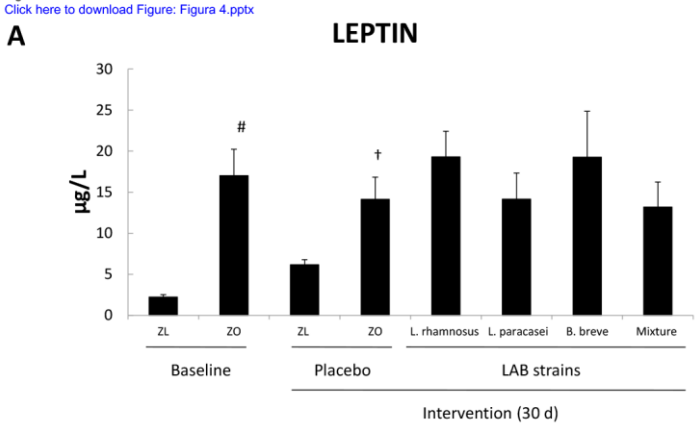


FIGURE 3B

Figure 4
Click here to download Figure: Figura 4.pptx



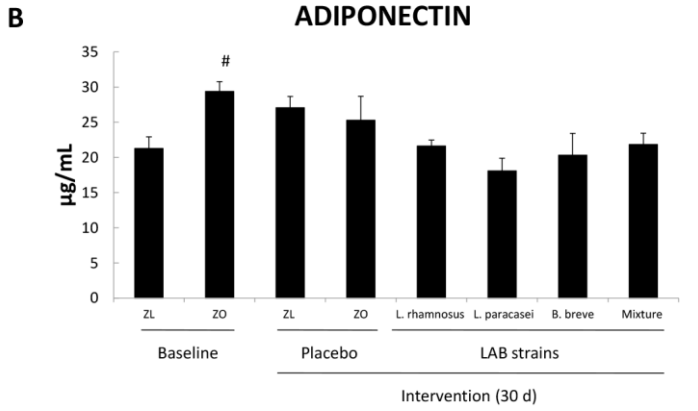
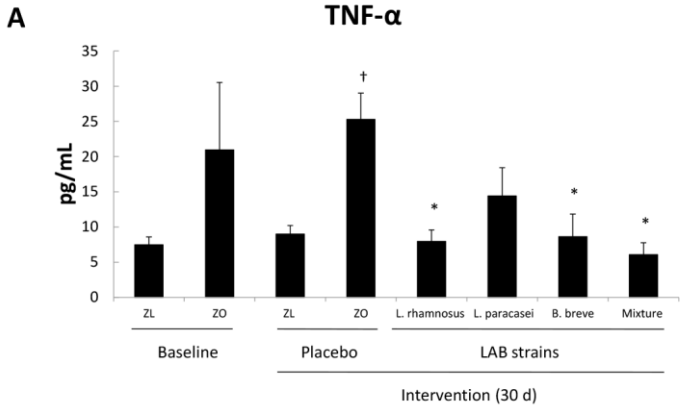


FIGURE 4

Figure 5
Click here to download Figure: Figura 5.pptx



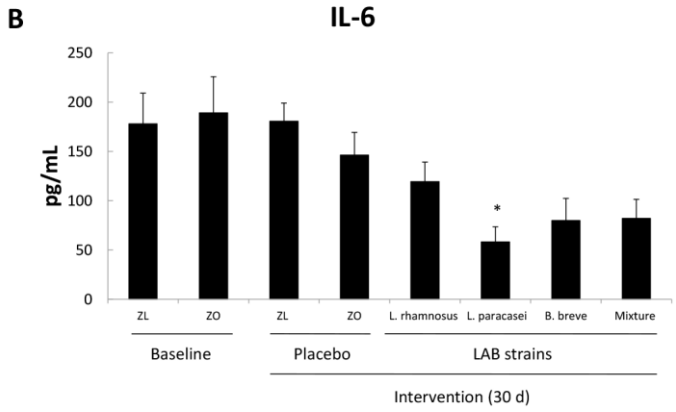


FIGURE 5

Figure 6
Click here to download Figure: Figure 6.pptx

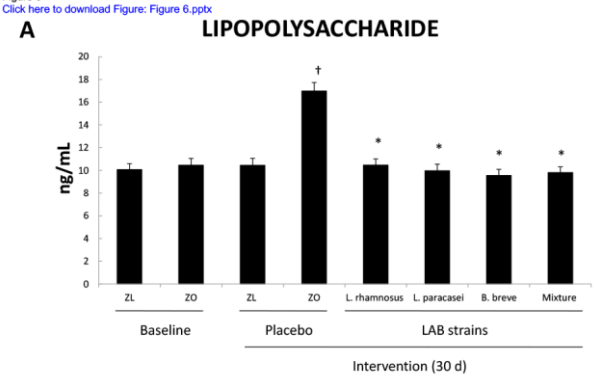


FIGURE 6

Name of journal: **World Journal of Gastroenterology**

ESPS Manuscript NO: 9823

Columns: **Topic Highlight**

Modulation of immunity and inflammatory gene expression by probiotics

Plaza-Diaz J *et al*. Modulation of immunity and inflammatory gene expression by probiotics

Julio Plaza-Diaz, Carolina Gomez-Llorente, Luis Fontana, Angel Gil

Julio Plaza-Diaz, Carolina Gomez-Llorente, Luis Fontana, Angel Gil, Department of Biochemistry and Molecular Biology II, School of Pharmacy and Institute of Nutrition and Food Technology "Jose Mataix", Biomedical Research Center, University of Granada, 18100 Armilla, Granada, Spain.

Author contributions: Each author wrote a different part of the manuscript.

Correspondence to: Prof. Angel Gil, PhD, Institute of Nutrition and Food Technology "Jose Mataix", Biomedical Research Center, University of Granada, Avenida del Conocimiento s/n, 18100 Armilla, Granada, Spain. Telephone: +34 958 241000 Ext. 20307 Fax: +34 958 819132. E-mail: agil@ugr.es

Received: February 28, 2014 **Revised:**

Accepted:

Published online:

Abstract

The potential for the positive manipulation of the gut microbiome through the introduction of beneficial microbes, as also known as probiotics, is currently an active area of investigation. The FAO/WHO define probiotics as live microorganisms that confer a health benefit to the host when administered in adequate amounts. However, dead bacteria and bacterial molecular components may also exhibit probiotic properties. The results of clinical studies have demonstrated the clinical potential of probiotics in many pathologies, such as allergic diseases, diarrhea, inflammatory bowel disease and viral infection. Several mechanisms have been proposed to explain the beneficial effects of probiotics, most of which involve gene expression regulation in specific tissues, particularly the intestine and liver. Therefore, the modulation of gene expression mediated by probiotics is an important issue that warrants further investigation. In the present paper, we performed a systematic review of the probiotic-mediated modulation of gene expression that is associated with the immune system and inflammation. Between January 1990 to February 2014, PubMed was searched for articles that were published in English using the MeSH terms “probiotics" and "gene expression" combined with “intestines", "liver", "enterocytes", "antigen-presenting cells", "dendritic cells", "immune system", and "inflammation". Two hundred and five original articles matching these criteria were initially selected, although only those articles that included specific gene expression results (77) were later considered for this review and separated into three major topics: the regulation of immunity and inflammatory gene expression in the gut, in inflammatory diseases of the gut and in the liver. Particular strains of bifidobacteria, lactobacilli, *Escherichia coli*, *Propionibacterium*, *Bacillus* and *Saccharomyces* influence the gene expression of mucins, Toll-like receptors, caspases, nuclear factor-κB, and interleukins and lead mainly to an anti-inflammatory response in cultured enterocytes. In addition, the interaction of commensal bacteria and probiotics with the surface of antigen-presenting cells *in vitro* results in the downregulation of pro-inflammatory genes that are linked to inflammatory signaling pathways, whereas other anti-inflammatory genes are upregulated.

The effects of probiotics have been extensively investigated in animal models ranging from fish to mice, rats and piglets. These bacteria induce a tolerogenic and hyporesponsive immune response in which many genes that are related to the immune system, in particular those genes expressing anti-inflammatory cytokines, are upregulated. By contrast, information related to gene expression in human intestinal cells mediated by the action of probiotics is scarce. There is a need for further clinical studies that evaluate the mechanism of action of probiotics both in healthy humans and in

patients with chronic diseases. These types of clinical studies are necessary for addressing the influence of these microorganisms in gene expression for different pathways, particularly those that are associated with the immune response, and to better understand the role that probiotics might have in the prevention and treatment of disease.

Keywords: Gene expression; immunity; immunotolerance; inflammation; microbiota; probiotics.

Core tip: Probiotics, which include live microorganisms as well as dead bacteria and bacterial molecular components, confer a health benefit to the host when administered in adequate amounts. Most of the published research articles that are devoted to probiotics evaluate the effects of probiotics on the prevention and treatment of diseases. However, only a few of these articles address the mechanism of action of these microorganisms. This paper reviews the mechanisms of action that have been proposed to explain the beneficial effects of probiotics, most of which involve gene expression regulation in specific tissues, particularly the intestine and liver. Several strains of lactic acid bacteria, *Escherichia coli*, *Propionibacterium*, *Bacillus* and *Saccharomyces* influence the gene expression in gut and liver cells, leading mainly to anti-inflammatory responses and to the enhancement of immunotolerance to foreign antigens.

Plaza Díaz J, Gomez-Llorente C, Fontana L, Gil A.

World J Gastroenterol 2014;

Available from:

DOI:

INTRODUCTION

The potential for the positive manipulation of the gut microbiome through the introduction of beneficial microbes, as also known as probiotics, is currently an active area of investigation[1,2]. Probiotics are generally recognized as live microorganisms that confer a health benefit to the host when administered in adequate amounts[3], although dead bacteria and bacterial molecular components may also exhibit probiotic properties[4]. In particular, strains belonging to *Bifidobacterium* and *Lactobacillus* are the most widely used probiotic bacteria[5] and exert health-promoting properties, including, for example, the maintenance of the gut barrier function and the local and systemic modulation of the host immune system[6,7].

Clinical studies have demonstrated the clinical potential of probiotics against many diseases[5], such as allergic pathologies (including atopic eczema[8] and rhinitis[9]), diarrhea[10], inflammatory bowel disease (IBD)[11] and viral infection[7]. However, generalizations concerning the potential health benefits of probiotics should not be made because probiotic effects tend to be strain-specific[12,13].

Several important mechanisms underlying the beneficial effects of probiotics include the modification of the gut microbiota, the competitive adherence to the mucosa and epithelium, the strengthening of the gut epithelial barrier and the regulation of the immune system and inflammation[5,13]. Most of these mechanisms involve gene expression regulation in specific tissues, particularly the intestine and liver.

In this sense, the probiotic-mediated modulation of gene expression is an important issue that needs to be addressed. The expression of mucin genes (*MUC)* can be affected by probiotics. Likewise, toll-like receptor (*TLR*) and nucleotide-binding oligomerization domain (*NOD*)-receptor genes as well as pro-inflammatory transcription factors, cytokines, and apoptosis- related enzyme genes can also be affected by commensal bacteria.

In the present paper, we performed a systematic review of the probiotic-mediated modulation of gene expression that is associated with the immune system and inflammation. Between 1990 to February 2014, PubMed was searched for articles that were published in English using the MeSH terms “probiotics" and "gene expression" combined with “intestines", "liver", "enterocytes", "antigen-presenting cells", "dendritic cells", "immune system", and "inflammation". Two hundred and five original articles matching these criteria were initially selected, although only those articles that included specific gene expression results (77) were later considered for the review and

separated into three major topics: the regulation of immunity and inflammatory gene expression in the gut, in inflammatory diseases of the gut and in the liver.

REGULATION OF IMMUNITY AND INFLAMMATORY GENE EXPRESSION IN THE GUT BY PROBIOTICS

The intestinal epithelium is constantly exposed to high levels of food and bacterial antigens. Under normal physiological conditions, the intestinal epithelial monolayer facilitates a controlled and selective flux of components between the lumen and the underlining mucosa[14]. The intestine and the gut-associated lymphoid tissue (GALT) are essential components of the immune defense, protecting the body from foreign antigens and pathogens while tolerating commensal bacteria and dietary antigens. The balance between tolerance and immunity in the intestine is, in part, dictated by antigen-presenting cell (APCs) populations in the gut. The dysregulation of this balance can contribute to the pathogenesis of numerous inflammatory conditions[15]. The inflammatory response in the intestinal tract is abrogated or avoided by the complex and well-regulated tolerance-inducing mechanisms in the GALT.

Several cells that are capable of antigen presentation exist in the GALT, including enterocytes and other intestinal epithelial cells (IEC), such as M cells, dendritic cells (DCs), macrophages, and T and B cells[16]. Microbes activate DCs directly via the DCs' pattern recognition receptors (PRR) or indirectly by capturing the apoptotic/necrotic products of other cells that are dying in response to microbial exposure[16]. PRRs are comprised of TLRs, NOD-like receptors (NLRs), adhesion molecules and lectins[13].

Commensal bacteria and probiotics can interact with these cells, thereby exerting immunomodulatory effects. Below we review the probiotic modulation of the genes that are involved in inflammation and immunity in intestinal cultured cells, as well as in animals and humans.

Intestinal cultured cells

Although most studies regarding probiotics have reported anti-inflammatory effects, certain probiotic strains have been shown to exert pro-inflammatory effects. The effects of selected probiotics on the gene expression in intestinal cells, namely HT-29, T84, Caco-2, APCs (e.g., RAW264.7 macrophages) and DCs, are reviewed below.

Enterocytes

Otte and Podolsky (2004) provided insight into the molecular mechanisms by which probiotic bacteria interact with the intestinal surface. The effects of *Escherichia coli* Nissle 1917 (EcN), the probiotic mixture VSL#3, bacterial cell lysates, and conditioned media on monolayer resistance, interleukin (IL)-8 secretion, mucin gene expression, and tight junction proteins were evaluated by these authors in T84 and HT-29 cells. The EcN as well as debris and cell extracts induced pro-inflammatory IL-8 secretion from the IEC, whereas no such effect was observed with VSL#3. A soluble factor that was released from VSL#3 increased monolayer resistance, prevented the pathogen-induced decrease in monolayer resistance, and stabilized tight junctions. VSL#3 induced the expression of mucins in intraepithelial cells, and these organisms as well as EcN diminished *Salmonella dublin*-induced cell death[17].

Similarly, Mack *et al.* (1999) showed that *Lactobacillus plantarum* 299v and *Lactobacillus rhamnosus* GG (LGG) quantitatively inhibited the adherence of an attaching and effacing pathogenic *E. coli* to HT-29 intestinal epithelial cells but did not inhibit the adherence to non-intestinal HEp-2 cells. Media enriched with MUC2 and MUC3 mucins were added exogenously to binding assays and were shown to inhibit the enteropathogen adherence to HEp-2 cells. The incubation of *L. plantarum* 299v with HT-29 cells increased the *MUC2* and *MUC3* mRNA expression levels. These authors proposed that probiotic agents, which can bind to epithelial cells *in vitro* and colonize the intestinal tract *in vivo*, induce epithelial cells to secrete mucins that diminish enteric pathogens that are bound to mucosal epithelial cells[18].

In another study using trans-epithelial electrical resistance (TEER) across Caco-2 cell layers, Anderson *et al.* (2010) described the effect of *Lactobacillus plantarum* MB452 on tight junction integrity. *L. plantarum* MB452 caused a dose-dependent TEER increase across Caco-2 cell monolayers compared to a control medium. Nineteen tight junction-related genes had altered expression levels in response to *L. plantarum* MB452. *Lactobacillus plantarum* MB452 also caused changes in tubulin and proteasome gene expression that may be linked to the intestinal barrier function[19].

Audy *et al.* (2013) investigated the differential gene expression of potential probiotics, LPS, and enteropathogenic bacteria on human intestinal epithelial cells using a custom-designed expression microarray evaluating 17 specific host-response pathways. The main outcome was the differential

regulation of the central mitogen-activated protein kinases (MAPK) signaling pathway in response to these probiotics, validated later with quantitative real-time PCR (qPCR)[20].

Different strains of bifidobacteria were tested for their effects on HT-29 in *in vitro* models of the non-inflamed and inflamed intestinal epithelium. None of the tested bifidobacteria induced the activation of nuclear factor (NF)-kappa beta (κB), indicating that bifidobacteria themselves do not induce inflammatory events. However, six out of eight tested bifidobacteria inhibited the LPS-induced NF-κB activation in a dose- and strain-dependent manner. By contrast, the NF-κB activation in response to challenge with tumor necrosis factor-alpha (TNF-α) was not affected by any of the tested bifidobacteria, indicating that the inhibitory effect of bifidobacteria is specific for LPS-induced inflammation in IECs. As shown with two of the six inhibition-positive bifidobacteria, the LPS-induced inhibition of NF-κB activation was accompanied by a dose-dependent decrease in IL-8 secretion and by lower mRNA levels for *IL-8*, *TNF-a*, cyclooxygenase 2 (*COX-2*), and intercellular adhesion molecule 1 (*ICAM-1*)[21].

Ruiz *et al.* (2005) characterized the molecular mechanisms for the initial interaction of probiotic *Bifidobacterium lactis* strain BB12 with native and IEC lines. *B. lactis*-monoassociated Fisher F344 rats transiently induced the phosphorylation/activation of the NF-κB transcriptionally active subunit RelA and the MAPK p38 in native IECs 5 days after the initial bacterial colonization. Additionally, *IL-6* gene expression significantly increased after 5 days. The adenoviral delivery of the mutant IKK-beta and the inhibition of the p38 MAPK pathway significantly blocked the *B. lactis*-induced *IL-6* gene expression in IECs, suggesting that *B. lactis* triggers NF-κB and MAPK signaling to induce gene expression in the intestinal epithelium[22]. Likewise, the inhibition of IL-8 secretion by intestinal epithelial INT-407 cells that were incubated with *B. lactis* HN019 has been reported by Liu *et al.* (2010)[23].

Three species of *Bifidobacterium* and *Enterococcus faecalis* differentially modulate the *in vitro* production of cytokines from LPS-stimulated RAW264.7 macrophages. The three species of *Bifidobacterium* significantly inhibited the phosphorylation of IκB-alpha that had been previously induced by LPS and modulated the *IL12p40*, *IL-1β*, and *TNF-a* mRNA levels. The mRNA levels of suppressor of cytokine signaling (*SOCS*)1 or *SOCS3* increased in response to exposure to *Bifidobacterium* species combined with LPS. Conversely, *E. faecalis* combined with LPS induced significantly lower levels of *SOCS* mRNA than those in those cells that were induced by *Bifidobacterium* species combined with LPS[24].

Imaoka *et al.* (2008) co-cultured peripheral blood mononuclear cells (PBMNC) that were isolated from ulcerative colitis (UC) patients or HT-29 cells with heat-killed probiotic bacteria or the culture supernatant of *Bifidobacterium breve* strain Yakult (BbrY) or *Bifidobacterium bifidum* strain Yakult (BbiY) to estimate the amount of secreted IL-10 or IL-8. Both strains of the probiotic bifidobacteria induced IL-10 production in the peripheral blood mononuclear cells (PBMNC), although BbrY was more effective than was BbiY. The inhibitory effect of the conditioned medium (CM) that was derived from BbiY was greater than that of the CM that was derived from BbrY. The DNAs of the two strains had a comparable inhibitory activity against the secretion of IL-8. The conditioned medium of BbiY induced a repression of the *IL-8* gene with a higher expression of *IκB-zeta* mRNA 4 h after the culture of HT-29 cells compared to that in the absence of CM[25].

Boesten *et al.* (2011) determined the genome-wide transcriptional response of HT-29 cells to TNF-α following exposure to *Bifidobacterium breve* strains M-16V, NR246 and UCC2003. Approximately 54% of the TNF-α induced genes were solely suppressed by the presence of *B. breve* M-16V. These genes included apoptosis-related cysteine protease caspase 7 (*CASP7*), interferon regulatory factor 3 (*IRF3*), amyloid beta (A4) precursor protein-binding family A member 1 (*APBA1*), NADPH oxidase (*NOX5*), and leukemia inhibitory factor receptor (*LIFR*). The extracellular IL-8 concentration did not change, indicating that *B. breve* M-16V only partially modulates the TNF-α pathway[26].

Anti-inflammatory effects by *Lactococcus lactis* subsp. *cremoris* FC have been shown by Nishitani *et al.* (2009) in both *in vivo* and *in vitro* experimental models. *L. lactis* subsp. *cremoris* FC showed preventive and therapeutic effects with the amelioration of colon length and histological score and an attenuation of pro-inflammatory cytokine mRNA expression in inflamed colon tissue. In an *in vitro* gut inflammation model consisting of a co-culture of intestinal epithelial cells (Caco-2) and macrophages (RAW264.7), treatment with the probiotic downregulated the pro-inflammatory *IL-8* mRNA expression in Caco-2 cells and inhibited the nuclear translocation of NF-κB in RAW264.7 cells[27].

Likewise, O'Flaherty and Klaenhammer (2012) demonstrated that the exposure time to *L. acidophilus* impacted the immune-related gene expression profiles of IECs. In this study, a 1-h rather than a 4- or 8-h exposure time resulted in the maximal differential expression of immune-related genes and genes that are targeted by the NF-κB complex. After an initial exposure to *L. acidophilus*, the expression of the immune-related genes returned to baseline levels[28].

Oksaharju *et al.* (2011) examined the effects of LGG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS, *Bifidobacterium animalis* spp. *lactis* Bb12 and their combination on human mast cell gene expression. The LGG and *L. rhamnosus* Lc705 suppressed genes that encoded the allergy-related high-affinity IgE receptor subunits α and γ (*FCER1A* and *FCER1G*, respectively) and the histamine H4 receptor. The LGG, *L. rhamnosus* Lc705 and the combination of the four probiotics had the strongest effect on the expression of genes involved in mast cell immune system regulation and on several genes that encoded proteins with a pro-inflammatory impact, such as *IL-8* and *TNF-a*, whereas genes that encoded proteins with anti-inflammatory functions, such as *IL-10*, were upregulated[29].

Paszti-Gere *et al*. (2012) investigated the immunomodulatory effect of the culture supernatant of five bacterial strains in a non-transformed cell line that was derived from porcine jejunal epithelial IPEC-J2 cells that had been previously subjected to oxidative stress with hydrogen peroxide. *Lactobacillus plantarum* 2142 had significantly decreased the *IL-8* and *TNF-a* mRNA levels with the concomitant upregulation of *HSP70* gene expression. However, *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Bacillus amyloliquefaciens* CECT 5940 had the opposite effect, increasing the gene expression of either *IL-8*, *TNF-a* or both. No effects were observed with *Enterococcus faecium* CECT 4515 or *Lactobacillus casei* Shirota[30].

Zanello *et al.* (2011) reported that the yeast *Saccharomyces cerevisiae* (strain CNCM I-3856) modulates transcript and protein expression in the inflammation, recruitment and activation of immune cells in differentiated porcine intestinal epithelial (PIE) IPEC-1 cells and demonstrated that viable *S. cerevisiae* inhibits the enterotoxigenic *Escherichia coli* (ETEC)-induced expression of pro-inflammatory transcripts (IL-6, IL-8, CCL20, CXCL2, and CXCL10) and proteins (IL-6, IL-8). This inhibition was associated with a decrease in ERK1/2 and p38 MAPK phosphorylation, an agglutination of ETEC by *S. cerevisiae* and an increase in the anti-inflammatory *PPAR-γ* nuclear receptor mRNA level[31].

Latvala *et al.* (2011) investigated which non-pathogenic bacteria could stimulate the expression of *SOCS3*, which controls the expression of pro-inflammatory cytokine genes in human primary macrophages. *Lactobacillus* and *Streptococcus* species induced *SOCS3* mRNA expression directly in the absence of protein synthesis and indirectly via bacteria-induced IL-10 production. The MAPK p38 signaling pathway played a key role in the bacteria-induced *SOCS3* gene expression[32].

The gene expression profiles of Caco-2 cells that were treated with EcN were analyzed via a microarray analysis by Ukena *et al.* (2005). The results revealed 126 genes that were specifically regulated after treatment with EcN. A second human intestinal cell line as well as pieces of small intestine from BALB/c mice were used to confirm the regulatory data of selected genes by qPCR. Among others, the expression of genes encoding the pro-inflammatory molecules monocyte chemo-attractant protein-1 (*MCP-1*), macrophage inflammatory protein-2 alpha (*MIP-2alpha*) and macrophage inflammatory protein-2 beta (*MIP-2beta*) increased up to 10-fold. Elevated levels of *MCP-1* and *MIP-2alpha* mRNA were confirmed using LoVo cells. *MCP-1* gene expression was also upregulated in mouse intestinal tissue[33].

Wang *et al.* (2013) analyzed the immunomodulatory effects of *Lactobacillus casei* Zhang (LcZ) in RAW264.7 macrophages. The immunostimulating effects of live LcZ were significantly attenuated in heat-killed LcZ. The live LcZ promoted *TLR2* mRNA transcription, whereas the heat-killed LcZ enhanced transcription of *TLR2*, *TLR3*, *TLR4*, and *TLR9*[34].

The effects of *Lactobacillus plantarum* genomic DNA on the LPS-induced MAPK activation, NF-κB activation, and the expressions of TNF-α, IL-1 receptor-associated kinase M, and the pattern recognition receptor were studied in human monocyte-like cells. *L. plantarum* genomic DNA inhibited this signaling pathway and TNF-α production accompanied by the suppression of *TLR2*, *TLR4*, and *TLR9* and the induction of IL-1 receptor-associated kinase M, a negative regulator of TLR[35].

Cammarota *et al.* (2009) analyzed the probiotic potential of *L. plantarum* DSMZ 12028 *in vitro* using the pathogen *E. coli* K4 and a certified probiotic, *L. paracasei* F19, as controls in Caco-2 and HT-29 cells. Real-time PCR was used to monitor the expression of TLRs and cytokines in a monocytic cell line (THP-1) following bacterial exposure. *L. plantarum* downregulated *TLR* mRNA levels with the exception of *TLR2*, while *L. paracasei* F19 and *E. coli* K4 significantly upregulated *TLR2* and 4, respectively[36].

Ghadimi *et al.* (2010) tested the effects of DNA from LGG and *Bifidobacterium longum* on the TLR9 signaling cascade and the barrier integrity of polarized HT-29 and T84 cells that had been previously treated with TNF-α. The HT-29 and T84 cells enhanced expression of TLR9 in a specific manner, which was subsequently associated with the attenuation of TNF-α-induced NF-κB activation and NF-κB mediated *IL-8* expression. *TLR9* silencing abolished this inhibitory effect. Apically applied LGG DNA attenuated the TNF-α enhanced NF-κB activity by reducing the IκBa

degradation and p38 phosphorylation. Likewise, LGG DNA diminished the TNF-α-induced membrane integrity reduction[14].

Eleven different probiotic strains with immunoregulatory capabilities used a common mechanism to induce tolerance in PIE cells. Immunoregulatory strains interacted with TLR2, upregulated the expression of *ubiquitin-editing enzyme A20* in PIE cells, and beneficially modulated the subsequent TLR4 activation by reducing the activation of MAPK and NF-κB pathways and the production of pro-inflammatory cytokines[37].

Gao *et al.* (2012) analyzed the myeloid differentiation primary response protein 88 (*MyD88*) expression using small interfering RNA in HT-29 cells. The knockdown of *MyD88* did not affect *Clostridium butyricum*-induced elevated levels of NF-κB, IL-8, IL-6, and TNF-α, suggesting a MyD88-independent route for TLR signal transduction in human epithelial cells. However, a significant reduction in the levels of NF-κB, *IL-8*, *IL-6*, and *TNF-a* was evident in the absence of *TLR2* expression, indicating the need for TLR2 in *C. butyricum* recognition[38].

In addition, the modulation of *TLR-4* gene expression by *Bacillus mesentericus* TO-A, *Clostridium butyricum* TO-A, and *Streptococcus faecalis* T-110 in human colonic epithelial cells HT-29 was investigated by Isono *et al.* (2007). Culture supernatants or heat-killed bacteria were added to HT-29 cells. Treatment with *C. butyricum* TO-A culture supernatant downregulated the *TLR4* mRNA and protein levels but only in the presence of butyrate. This effect seems to be mediated by the transcription factor PU.1[39].

Bacillus species, non-pathogen spore-forming microorganisms, are being used as probiotics owing to evidence indicating that these species are important for the development of a robust gut-associated lymphoid system[40]. Huang *et al.* (2008) tested the ability of six *Bacillus* strains (*B. subtillis* PY79, HU58 and HU68, *B. licheniformis* HU14 and HU53, and *B. flexus* HU37), from the human gut to induce the pro-inflammatory cytokines TNF-α, IL-6 and IL-1α in cultured RAW264.7 macrophages. The highest levels of induction were with TNF-α followed by IL-6, for which all of the strains induced expression. IL-1α was only induced by *B. subtillis* PY79 and HU68 and *B. licheniformis* HU14. The authors also determined the expression of *TLR-2* and *TLR-4* in RAW264.7 macrophages that were co-incubated with either spores or vegetative cells of *B. subtillis* PY79, demonstrating a clear temporal increase in the expression of both *TLR-2* and *TLR-4* over time. A closer inspection of TLR-2 and TLR-2 induction indicated that for vegetative cells, there was a

progressive increase in the expression during the 6-h period of evaluation. However, for induction by spores, there was a significant increase in the expression only between h 4 and 6[40].

In summary, specific strains of bifidobacteria, lactobacilli, EcN, *Propionibacterium*, *Bacillus* and *Saccharomyces* influence the gene expression of *mucins*, *TLRs*, *caspases*, *NF-κB*, and *interleukins*, leading mainly to an anti-inflammatory response. Notwithstanding, specific responses are dependent on particular strains and intestinal cell types.

Dendritic cells

Dendritic cells (DCs) are potent APCs that induce a primary immune response against microbial infection and other stimuli[41]. Upon activation, DCs upregulate co-stimulatory molecules and migrate to secondary lymphoid organs where they activate antigen-specific T cells. The types of cytokines and other factors that are secreted by DCs and other innate immune cells program the differentiation of naïve Th0 into Th1, Th2 or Th17 effector cells or Treg cells[16]. Understanding the direct interaction between commensal bacteria and DCs is particularly important in determining how the immune system of the gut is able to distinguish these bacteria from pathogens and elicit a tolerogenic response[42].

In this sense, the mixture probiotic VSL#3 induces the release of significant levels of IL-10 in DC culture supernatants if added over a period of 3 days. IL-10 is a critical Th2 cytokine that suppresses IL-12 production and therefore other cytokines, such as interferon gamma (IFN-γ) and TNF-α[43]. Drakes *et al*. (2004), using DCs that were generated from mice bone marrow showed that VSL#3 induces the release of higher amounts of IL-10 and more modest levels of IL-12[41]. This study highlighted the fact that the presence of probiotic bacteria during the development of DCs influences the outcome of the immune response[41]. Changes in the DC cell surface phenotypes may result in altered DC function or cytokine production[44]. To determine the action of probiotic VSL#3 on the DC surface phenotypes, Drakes *et al.* (2004) added the probiotic mixture during the DC generation phase, observing that the addition of 10^3 and 10^5 organisms/ml did not alter the immature phenotype of DC; however, higher concentrations (10^7 organisms/ml) upregulated the DC co-stimulatory molecule expression of CD80, CD86, CD40, and major histocompatibility complex class (MHC) class II I-A^d[41].

In agreement with the aforementioned study, Mastrangeli *et al.* (2009) demonstrated that the effects of VSL#3 on BALB/c mice bone marrow (BM) DC maturation were time- and dose-dependent and peaked after 18 h of co-culture at the 10^7-organisms/ml dose. Live and sonicated VSL#3 induced a

significant upregulation of CD83, CD86 and MHC class II. Moreover, both of the VSL#3 preparations were as effective as the LPS control in inducing DC maturation. In addition, live VSL#3 at the same dose induced a significant production by BMDCs of high levels of IL-12p70 and IL-10, significantly higher than those obtained after LPS stimulation. However, sonicated VSL#3 induced lower levels of IL-12 and IL-10, which were in any case significantly higher than those that were induced by LPS and higher than the control (medium only) levels[45].

In addition, D'Arienzo *et al.* (2009), using BMDCs that were generated from a DQ8 tg mouse, a well characterized model of food antigen sensitivity, demonstrated that incubation with 10^8 colony-forming units (CFU)/ml of *L. paracasei* IMPC2.1, *L. plantarum* ITM21B, *L. fermentum* DRL38 and *B. lactis* NCCC2818 two days before cell harvesting in the presence/absence of LPS stimulated CD86 expression, with *L. plantarum* and *L. paracasei* inducing higher expression levels. However, no strains modulated the expression of CD11c or CD80 or further enhanced the LPS-induced CD86 expression. These data clearly indicate that probiotic exposure alters the immature phenotype of DCs. Regarding the cytokine analysis, immature BMDCs revealed no significant differences for IL-12, whereas in mature BMDCs (LPS-treated), *L. plantarum* and *L. fermentum* enhanced IL-12 production. In addition, the IL-10 levels were low in both un-stimulated and LPS-induced DCs. Moreover, the *L. fermentum, L plantarum* and *B. lactis* strains induced a significant increase in TNF-α in immature BMDCs, while *L. fermentum* and *B. lactis* induced a significant increase in mature BMDCs[46].

Furthermore, the incubation of murine BMDCs with heat-killed *L. lactis* subsp. *cremoris* FC and *L. lactis* subsp. *cremoris* ATCC 19257 (as a control strain) enhanced the production of IL-10, IL-12, IL-6 and TNF-α in a dose-dependent manner[47]. Compared to *L. lactis* subsp. *cremoris* ATCC 19257, *L. lactis* subsp. *cremoris*, FC induced higher levels of IL-12 production, whereas treatment with *L. lactis* subsp. *cremoris* ATCC 19257 induced higher levels of IL-6 production. Moreover, the production of IL-10, IL-12, IL-6 and TNF-α that was induced by *L. lactis* subsp. *cremoris* FC was almost completely depleted in the culture supernatants of BMDCs that were derived from MYD88$^{-/-}$ mice. This result suggests that *L. lactis* subsp. *cremoris* FC activates DCs and induces cytokine production through a MyD88-dependent pathway[47].

Certain *Lactobacillus* spp. posses strong IL-12- inducing properties whose production depends on the upregulation of type I IFNs that are mainly involved in the immune response against viral infection[48]. In this sense, Weiss *et al.* (2010) investigated whether *L. acidophilus* NCFM could induce anti-viral defense gene expression in immature murine DCs. A genome-wide microarray analysis

revealed that the induction of virus-related genes was most prominent for the *RSAD2* gene (radical S-adenosyl methionine domain-containing 2). The genes encoding IFN-induced T cell-specific GTPase (*TGTP2),* IFN-stimulated gene 15 (*ISG15*), IFN-regulatory factor (*IRF-7*) and *TLR-3*, all of which are involved in the viral immune defense that is induced by IFN-β, were similar among the highest significantly upregulated genes. The upregulation of viral response genes seems to be caused by a rapid, strong and transient upregulation of *IFN-β,* which in turn stimulates the transcription of a number of other genes that are involved in viral defense. Moreover, the upregulation of *IFN-β* in DCs was much stronger upon stimulation with *L. acidophilus* NCFM compared with that of cells that were stimulated with Poly I:C, EcN and *B. bifidum* Z9. In addition, *IFN-β* expression was markedly reduced in TLR-2$^{-/-}$ DCs, dependent on endocytosis, the major cause of the induction of *IL-12* and *TLR-3* in DCs that were stimulated with *L. acidophilus* NCFM. These results reveal that certain lactobacilli trigger the expression of viral defense genes in DCs in a TLR-2-dependent manner[48].

The stimulation of chicken bone marrow dendritic cells (chi-BMDCs) with LPS, *Saccharomyces boulardii* (Sb), *Bacillus subtillis* B10 (Bs), and a co-culture of Sb+Bs and phosphate-buffered saline (PBS) as a control group revealed that the treatment groups modulate the phenotype and biological functions of chi-BMDCs. The gene expression levels of *MHC-II*, *CD40*, *CD80* and *CD86* were upregulated in the stimulated groups. Furthermore, the cell surface receptors *TLR-1*, 2, 4 and 15 showed significant upregulation at the mRNA levels. In addition, the levels of the associated factors *MyD88*, *TRAF6*, *TAB1* and *NF-κB* mRNA increased in all of the treatment groups compared to those of the control group. However, the NF-κB response was significantly higher in the LPS treatment. Regarding the cytokine production levels, the probiotics improved the production of IL-1β, IL-17, IL-4, transforming growth factor beta (TGF-β) and IL-10, whereas IL-8 and IFN-γ were downregulated[49].

In an interesting study, Latvala S *et al.* (2008) stimulated human monocyte-derived DCs (moDCs) with nine probiotic bacteria (two well-characterized probiotics, *L. rhamnosus* GG and *B. animals* Bb12, and seven potentially probiotic bacteria). These authors showed that *S. thermophilus* THS efficiently induced TNF-α, IL-6 and IL-12. *B. animalis* Bb12 and *B. breve* Bb99 were potent inducers of *TNF-a, IL-1β, IL-6, IL-10, IL-12*, and *IFN-γ*. However, *B. longum* strain 1/10 was not as efficient as *B. animalis* Bb12 and *B. breve* Bb99 in inducing cytokine production. *L lactis* subsp. *cremoris* ARH74 and *L. helveticus* 1129 were as efficient as bifidobacteria. By contrast, LGG and LC705 as well as *L. mesenteroides* subsp. *cremoris* PIA2 were poor inducers of cytokine production in moDCs[50]. The

cytokine responses were directly associated with the bacterial dose; a 40:1 bacteria:host cell ratio showed the highest cytokine production levels. In addition, all of the studied bacteria induced CCL20 production in a dose-dependent manner, whereas none of the aforementioned bacteria were able to induce CCL19 production[50]. According to the gene expression levels, *L. mesenteroides* subsp. *cremoris* PIA2, LGG and LC705 were weak inducers of moDC cytokine responses. By contrast, bifidobacteria, *S. thermophilus* THS, *L. lactis* ARH74 and *L. helveticus* 1129 induced the production of the pro-inflammatory cytokines and chemokines TNF-α, IL-6, IL-12, CCL20 and CXCL10. In addition, *S. thermophilus* THS and *B. breve* Bb99 stimulated the highest upregulation of human leukocyte antigen (HLA) class II (ratio 10:1) in the moDCs, whereas a ratio of 40:1 for *L. lactis* subsp. *cremoris* ARH74 was required to maximize HLA class II induction. MoDCs that were stimulated with probiotic bacteria matured equally well as cells that were stimulated with pathogenic *S. pyogenes,* a known inducer of moDC maturation[50].

Furthermore, human myeloid DCs that were isolated from PBMCs and treated with a ratio of 10:1 for *B. infantis* or *L. salivarius* for 48 h stimulated a significant increase in IL-10 and TNF-α secretion compared to that of the untreated DCs[51].

In an elegant study, Evrard *et al.* (2011) investigated the effects of *Lactobacillus rhamnosus* 35 (Lcr35) on human PBMNC, using a multiplicity of infection (MOI) ranging from 100 to 0.01. These authors' flow cytometry data indicated the Lcr35-induced semi-maturation of DCs with the upregulation of the expression of HLA-DR, CD86 and CD83 as well as the upregulation of CCR7. The Lcr35-induced phenotype was intermediate between that of immature DCs and that of fully mature LPS-induced DCs, a so-called semi-mature DCs phenotype. In addition, a gene array analysis showed great dose-dependent variations. At a MOI of 10, 823 genes were overexpressed (with a 3-fold change threshold), and 859 were downregulated. Most of these genes were involved in four main biological processes: immune and inflammatory responses, antigen processing and presentation via MHC, intracellular signaling and signal transduction. At an MOI of 0.01, the expression of 58 genes was upregulated, while that of 138 genes was downregulated. In addition, at an MOI of 10, these authors observed that the expression of genes directing a Th1 (*IL12A, IL12B* and *TNF-a*) or a Th17 (*IL1B, IL6, IL23A, Il12B* and *TGFB1)* profile was strongly upregulated[52]. A comparison of these results with the Torri's model[53] of the molecular signature of inflammation indicated that at an MOI of 10, Lcr35 exhibited a pro-inflammatory DC phenotype in response to 76% of the genes, neither a pro- nor an anti-inflammatory DC phenotype in response to 11% and an anti-inflammatory DC phenotype in response to 13%. A qRT-PCR analysis revealed that at an MOI of 10,

the transcription of the *CCL20, IL1B, IL12B* and *TNF-a* genes increased by approximately 100-, 300-, 400- and 200-fold, respectively. In addition, the expression of *IL-23* and *PYSG2* increased, although less strikingly, and the *CCR7, FCAR,* and *IL-8* genes were upregulated[52]. Regarding cytokine production, a strong dose-dependent increase of IL-12p40, TNF-α and, to a lesser extent, IL-10 was induced by Lcr35 compared to the untreated immature DCs[52].

Most of the studies on probiotic activity have been performed in human moDCs or murine DCs, which are different from human gut DCs[54]. Recently, our research group[55] co-incubated intestinal-like human DCs from cord blood CD34+ progenitor cells[56] with *B. breve* CNCM I-4035 or its cell-free supernatant (CFS), *S. typhi* or a combination of these treatments for 4 h. These treatments upregulated *TLR-9* gene transcription. In addition, CFS was a more powerful inducer of *TLR-9* expression than were the probiotic bacteria in the presence of *S. typhi.* In addition, both of the treatments induced Toll-interacting protein (*TOLLIP*) gene expression. Furthermore, CFS decreased the pro-inflammatory cytokines and chemokines in DCs that were challenged with *S. typhi*. By contrast, *B. breve* CNCM I-4035 was a potent inducer of the pro-inflammatory cytokines TNF-α, IL-8 and RANTES (regulated upon activation normal T cell expressed, and presumably secreted) as well as of anti-inflammatory cytokines, including IL-10. CFS restored the TGF-β levels in the presence of *S. typhi*. These results indicate that *B. breve* CNCM I-4035 affects the intestinal immune response, whereas its supernatant exerts anti-inflammatory effects that are mediated by DCs[55]. Likewise, *Lactobacillus paracasei* and its CFS also decreased the pro-inflammatory cytokines and chemokines in human intestinal DCs that were challenged with *Salmonella.* CFS was as effective as the bacteria in reducing pro-inflammatory cytokine expression. These treatments strongly induced the transcription of the *TLR-9* gene. In addition, an upregulation of the *CASP8* and *TOLLIP* genes was also observed. *L. paracasei* CNCM I-4034 was a potent inducer of TGF-β2 secretion, whereas the supernatant enhanced the innate immunity through the activation of TLR signaling[7]. Giahi *et al.* (2012) investigated the effect of heat-inactivated LGG and *Lactobacillus delbrueckii* subsp. *bulgaricus* on the expression of *TLR4* and signaling factors, such as *p38 MAPK* and *IκB*, at the transcription level in human monocyte-derived DCs. LGG significantly downregulated the expression of *p38*, while the *IκB* expression was significantly reduced in the *Lactobacillus delbrueckii* subsp. *bulgaricus*-treated DCs[4].

In summary, the interactions of commensal bacteria and probiotics with the surface of APCs, mainly through TLR, in most studies result in the downregulation of pro-inflammatory genes that are linked to inflammatory signaling pathways, whereas other anti-inflammatory genes are

upregulated. The probiotic-mediated increase in TGF-β and IL-10 expression can help to explain the immunotolerance process that is mediated by these microorganisms.

Animal studies

Matsumoto *et al*. (2011) supplemented the diet of 10-month-old Crj:CD-1 female mice with *B. animalis* subsp. *lactis* LKM512 for 11 months. The colonic mucosal function was better in LKM512 mice, with increased mucus secretion and better maintenance of tight junctions. *B. animalis* subsp. *lactis* LKM512 also downregulated the expression of aging-associated and inflammation-associated genes. The gene expression levels in 21-month-old *B. animalis* subsp. *lactis* LKM512-treated mice resembled those in 10-month-old untreated (younger) mice[57].

Ohtsuka *et al.* (2012) examined the immunomodulatory effects of *Bifidobacterium breve* M-16V during early infancy in rat pups during the newborn or weaning period. The numbers of upregulated and downregulated genes were greater during the weaning period than during the newborn period, and these were greatest in the colon, with fewer genes altered in the small intestine and the fewest in the spleen. The expression of inflammation-related genes, including lipoprotein lipase (*LPL*), glutathione peroxidase 2 (*GPX2*), and lipopolysaccharide-binding protein (*LBP*), was significantly reduced in the colon during the newborn period. In weaning rat pups, the expression of CD3d, a cell surface receptor-linked signaling molecule, was significantly enhanced in the colon; however, the expression of co-stimulatory molecules was not enhanced[58].

Trevisi *et al.* (2008) investigated the potential synergic action of one prebiotic with increasing dietary doses of a probiotic strain of *Bifidobacterium animalis* on the translocation of bifidobacteria and on TLR gene expression in different organs of weaned piglets. The linear effect of the dose of *B. animalis* on the expression of the TLR2-encoding gene in the lymph nodes was observed when fructo-oligosaccharides were added to the diet. Tumor necrosis factor-alpha-encoding gene expression was positively correlated with the *TLR4*- and *TLR2*-encoding genes[59].

The effect of live *Lactobacillus plantarum* 299v (Lp299v), *Lactobacillus rhamnosus* R0011 (LrR0011), and *Bifidobacterium bifidum* R0071 (BbR0071) were analyzed in rats. After killing the rats via CO_2 suffocation, the *MUC2*, *MUC3*, neuronal apoptosis inhibitor protein (*NAIP*), human inhibitor of apoptosis protein 1/cellular inhibitor of apoptosis 2 (*HIAP1/cIAP2*), and human inhibitor of apoptosis protein 2/cellular inhibitor of apoptosis 1 (*HIAP2/cIAP1*) mRNA and protein levels were analyzed via qPCR and immunohistochemistry. Live Lp299v, BbR0071, and LrR0011 increased MUC3 protein and mRNA expression in the jejunum and ileum. A heat-killed non-adherent

derivative of Lp299v failed to induce MUC3 expression. Lp299v did induce the expression of HIAP2/cIAP1 and NAIP expression. MUC3 mucin expression was elevated for 5 days after the oral administration of Lp299v; however, this effect was not sustained despite ongoing daily ingestion of a probiotic[60].

Three groups of rats orally received LGG, *Bifidobacterium animalis* MB5, or PBS for 28 days. Each group was divided into two subgroups of tolerized or immunized rats receiving ovalbumin (OVA; 7 mg) or PBS on days 7, 9, and 11. All of the rats were immunized with OVA (300 mg) on days 14 and 21. In the tolerized rats, the OVA-induced proliferative response of mesenteric lymph nodes (MLN) and spleen cells did not differ from those of the control, indicating that the two probiotics maintained the tolerance. *Lactobacillus rhamnosus* GG and *B. animalis* MB5 in the immunized rats reduced the OVA-induced proliferative response in the MLN but not in the spleen, whereas the proliferative response to anti-CD3 and concanavalin A of the MLN and spleen cells as well as the delayed-type hypersensitivity reaction were not affected by probiotic treatment, indicating that the OVA-specific hyporesponsiveness is restricted to intestinal immunity. This hyporesponsiveness was associated with CD4+CD25+Foxp3+T cell expansion, increased IL-10 and TGF-β after LGG, and increased apoptosis after *B. animalis* MB5 in MLN[61].

The effects of *L. acidophilus*, inulin, or both (synbiotic) on pathogen-induced inflammatory responses, NF-κB, and Smad 7 signaling were evaluated in a murine model to parallel infantile enteric disease. Newborn mice were inoculated bi-weekly for 4 weeks with *L. acidophilus*, inulin, or synbiotic and challenged with *Citrobacter rodentium* (Cr) at 5 weeks. The results showed that the host defense against Cr infection correlated with enhanced colonic *IL-10* and *TGF-β* expression and the inhibition of NF-κB in synbiotic-treated mice, whereas mice that were pretreated with synbiotic, *L. acidophilus*, or inulin had an attenuation of Cr-induced Smad 7 expression[62].

Deng *et al.* (2013) evaluated the ability of the co-administration of *Bacillus subtillis* RJGP16 and *Lactobacillus salivarius* B1 to stimulate local immune responses. Thirty two newborn piglets were divided into four groups and were orally administered with different combinations of probiotics (none; RJGP16; B1; RJGP16 and B1) at the ages of 0, 7 and 11 days. These authors analyzed the parameters of the mucosal immunity of piglets one week after weaning. The results showed that the expressions of *IL-6* in the duodenum and ileum and of porcine beta-defensins (*pBD*)-*2* in the duodenum significantly increased with the co-administration of RJGP16 and B1. Additionally, the expression and release of TLR-2 and the number of IgA-producing cells increased[63].

In addition, the cytokine gene expression in the spleen and in Peyer's patches of mice that received dahi supplemented with *L. casei* was analyzed. The mRNA levels of *IFN-γ* in both the spleen and in the Peyer's patches were significantly increased in the probiotic dahi group after 14 and 28 days compared with those of the control and dahi groups. The abundance of *IL-2* mRNA also significantly increased in the Peyer's patches of probiotic-fed animals[64].

The effects of lactic acid bacteria on the control of lactococcosis and the impact of probiotics on the expression of immune-related genes were investigated in the head kidney and intestine of rainbow trout. *Lactobacillus plantarum*, *Lactococcus lactis* and *Leuconostoc mesenteroides* were administered orally for 36 days. Twenty-one days after the start of the feeding period, the fish were challenged with *Lactococcus garvieae*. Only the fish that were fed the diet containing *L. plantarum* showed significantly improved protection against *L. garvieae* compared to that of the control. Subsequently, qPCR was used to measure the mRNA levels of *IL-1β*, *IL-8*, *IL-10* and *TNF-a* in the head kidney and of *IL-8*, *TLR5* and *IgT* in the intestine of the control and *L. plantarum* groups. The expression of *IL-1β, IL-10* and *TNF-a* was significantly upregulated by *L. plantarum*. Moreover, the mRNA levels of *IL-10*, *IL-8* and *IgT* were significantly higher in the *L. plantarum* group after *L. garvieae* infection, suggesting that *L. plantarum* can stimulate the immune response of rainbow trout. These findings demonstrate that direct probiotic-host interactions with the intestine are not always necessary to induce host stimulatory responses that ultimately enhance disease resistance[65].

Pirarat et al. (2011) investigated the modulation of immunity in Nile tilapia by LGG and found higher levels of *TNF-a* and *IL-1* gene expression. As described before for intestinal cultured cells, probiotic bacteria influence the immune response and inflammation by controlling TLR, NF-κB and cytokine gene expression in animal models[66].

In summary, the effects of probiotics have been extensively investigated in animal models ranging from fish to mice, rats and piglets. These bacteria induce a tolerogenic and hyporesponsiveness immune response in which many genes that are related to the immune system, in particular those expressing anti-inflammatory cytokines, are upregulated.

Human studies

Compared to intestinal cultured cells and animal models, there are only a few studies in humans evaluating the effects of probiotic bacteria on the expression of genes that are involved in immunity and inflammation.

Van Baarlen *et al.* (2011) obtained transcriptomes in an intervention study after a double-blind placebo-controlled cross-over study to investigate the *in vivo* mucosal responses of healthy adults to probiotics. In the mucosa of the proximal small intestine of healthy volunteers, probiotic strains from the species *Lactobacillus acidophilus*, *L. casei*, and *L. rhamnosus* each induced differential gene-regulatory networks and pathways in the human mucosa. Comprehensive analyses revealed that these transcriptional networks regulate major basal mucosal processes and uncovered remarkable similarity to the profiles that were obtained in response to specific bioactive molecules and drugs[67].

Lammers *et al.* (2005) analyzed the expression of *IL-1β*, *IL-6*, *IFN-γ*, *TNF-a*, *IL-12*, *IL-10*, *TGF-β* and *IL-8* in endoscopic samples. The data showed that patients who were treated with probiotics had significantly lower mucosal mRNA expression levels of *IL-1β*, *IL-8*, and *IFN-γ* compared with those of the placebo-treated patients[68].

Di Caro *et al.* (2005) evaluated the gene expression pattern that was induced by *Bacillus clausii* in the intestinal mucosa of healthy individuals. Six male patients who were affected by mild esophagitis were treated for one month with esomeprazole and were randomly selected to receive or not *B. clausii* (groups I and II, respectively). Duodenal biopsies were taken pre- and post-treatment to identify the modification of gene expression. After *B. clausii* administration, a total of 158 and 265 genes were upregulated and downregulated, respectively. *Bacillus clausii* mainly affected the expression of genes that are involved in the immune response and inflammation, apoptosis and cell growth, cell differentiation, cell-cell signaling, cell adhesion, signal transcription and transduction[69].

Information regarding gene expression in human intestinal cells that are mediated by the action of probiotics is very scarce (table 1 summarizes the principal results). Hence, new studies should consider this aspect to ascertain the mechanism of action of specific strains in the modulation of the immune response and inflammation, mainly in chronic disorders of the gut.

REGULATION OF GENE EXPRESSION BY PROBIOTICS IN INFLAMMATORY DISEASES OF THE GUT

The intestinal microbiota play essential roles in nutrient absorption and metabolism, immune stimulation, satiety and pain. An altered composition of intestinal microbiota has been reported in IBD patients[70]. IBD is linked to post-inflammatory and stress-correlated factors that cause changes in the perception of visceral events.

Probiotic bacteria may be effective in treating IBD symptoms[71]. The effects of *Bifidobacterium breve* (DSMZ 20213) and LGG on the expression of IL-17 and IL-23, which play an important role in IBD, and on the epigenetic machinery were evaluated in a 3D co-culture model that was composed of human intestinal HT-29/B6 or T84 cells and PBMCs. The cells were treated with LPS in the presence or absence of bacteria for 48 h, and the expression of *IL-17*, *IL-23*, and CD40 at the mRNA and protein levels was assessed using qPCR. The NF-κB activity was assessed by NF-κB-dependent luciferase reporter gene assays. *B. breve* and LGG diminished the LPS-induced expression of *IL-17*, *IL-23*, and CD40 as well as histone acetylation and slightly enhanced DNA methylation. These effects were paralleled by a decrease in the nuclear translocation of NF-κB, as demonstrated by a decrease in the expression of MyD88, IRAK-1, IκBα, the nuclear NF-κB p50/p65 subunits, p-p38 MAPK and p-MEK1 and the NF-κB-dependent luciferase reporter gene activity in LPS-stimulated cells[72].

To mimic the IBD response to Gram-negative bacteria, Grimoud *et al.* (2010) used HT-29 cells that were sensitized to the inflammatory response to LPS by IFN-γ, which increased the expression of *TLR4*, the LPS biosensor, and were then treated by probiotics, prebiotics and synbiotics. Only three probiotic strains induced a proliferation decrease but with a lack of reproducibility. Binary or ternary probiotic associations, complemented or not by prebiotics, significantly decreased proliferation, especially with a synbiotic association of *Bifidobacterium breve*, *Lactococcus lactis* and *L. oligoalternan*[73].

Angiogenesis is an integral process of inflammatory responses in IBD and is required for mucosal remodeling during restitution. Chen *et al.* (2013) indicated that *Saccharomyces boulardii* modulates angiogenesis to limit intestinal inflammation and promote mucosal tissue repair by regulating vascular endothelial growth factor (VEGF) receptor signaling using an adenovirus expressing VEGF-A(164) in the ears of adult nude mice[74].

IBD increases the risk of colorectal cancer. Bassaganya-Riera *et al.* (2012) studied the cellular and molecular mechanisms underlying the efficacy of probiotic bacteria in mouse models of inflammation-driven colorectal cancer. Immune cell subsets in the MLN, spleen and colonic lamina propria lymphocytes (LPL) were phenotypically and functionally characterized. The mice were treated with conjugated linoleic acid (CLA) or VSL#3 and recovered faster from the acute inflammatory phase of disease and had lower disease severity in the chronic, tumor-bearing phase of disease. VSL#3 increased the mRNA expression of *TNF-a*, angiostatin and *PPAR-γ*, whereas CLA decreased *COX-2* levels. Moreover, the VSL#3-treated mice had increased IL-17 expression in the

MLN CD4+ T cells and an accumulation of Treg LPL and memory CD4+ T cells[75]. Finally, IBD in a rat model with male neonatal maternal separation (NMS) was reported and treated orally with placebo or VSL#3 from days 3 to 60, while normal, not-separated rats were used as controls. A microarray analysis demonstrated that NMS induced a robust change in the expression of subsets of genes (*CCL2, NOS3, THP1, NTRK1, CCR2, BDRKRB1, IL-10, TNFRSF1B, TRPV4, CNR1* and *OPRL1*) that are involved in pain transmission and inflammation. TPH1, tryptophan hydroxylase 1, a validated target gene in IBD treatment, was markedly upregulated by NMS; this effect was reversed by VSL#3 intervention[71].

Ulcerative colitis

Garrido-Mesa *et al.* (2011) tested the association of minocycline and EcN in a mouse model of reactivated colitis. The mice were assigned to different groups: non-colitic and dextran sodium sulfate (DSS) control groups (without treatment), and minocycline, EcN, and minocycline plus EcN treated groups. Colitis was induced by adding DSS to the drinking water (3%) for 5 days; 2 weeks later, the colitis was reactivated by subsequent exposure to DSS. The inflammatory status was evaluated daily by a disease activity index (DAI), and the colonic damage was assessed histologically and biochemically by the mRNA relative expression of different mediators. Minocycline and EcN exerted an intestinal anti-inflammatory effect and attenuated the reactivation of the colitis, as shown by the reduced DAI values; these effects were greater when both of the treatments were combined. These effects were evidenced histologically and biochemically by the reduced expression of *TNFa, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS* and *MMP-9* together with an increased *MUC-3* and *ZO-1* expression[76]. In the same model, Claes *et al.* (2010) utilized a *dltD* mutant of the model probiotic LGG in its lipoteichoic acid molecules. The mice received either PBS, LGG wild-type or the *dltD* mutant via drinking water. The macroscopic parameters, histological abnormalities, and cytokine and TLR expression levels were analyzed to assess the disease activity. The mice that were treated with the *dltD* mutant showed an improvement of some of the colitic parameters compared to the LGG wild-type-treated mice in both experimental models. In addition, treatment with the *dltD* mutant correlated with a significant downregulation of *TRL-2* expression and of downstream pro-inflammatory cytokine expression in the colitic mice[77].

Lactobacillus rhamnosus OLL2838 was employed in the DSS model. The barrier function was restored by the administration of live and heat-killed OLL2838 to the DSS-treated animals, and an increased expression of ZO-1 (4.8-fold) and myosin light-chain kinase (3.1-fold) was found in IECs that were isolated from mice of the heat-killed OLL2838 group[78].

The efficacy of probiotics in the recurrent trinitrobenzenesulfonic (TNBS)-induced colitis model in BALB/c mice has been tested. A microarray analysis revealed differences in expression of genes that are related to inflammation and immune processes between untreated mice and those that were treated with the probiotics *Lactobacillus plantarum* NCIMB8826 or VSL#3. The effects of probiotics on colonic gene expression were most profound during active inflammation, in particular on gene clusters that are related to mast cells and antimicrobial peptides[79].

Amit-Romach *et al.* (2010) evaluated and compared the effects of two probiotic regimens, LGG and a mixture of *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacterium lactis* in both normal and TNBS acid colitis-induced rats. Colonic tissues were used for mRNA analysis via qPCR. The administration of both of the probiotic regimens reduced the expression of the pro-inflammatory cytokines *TNF-a* and *IL-6* and increased the expression of *MUC2* compared with the that of the colitis group[80]. Using the same model, Duary *et al.* (2012) examined the effects of *Lactobacillus plantarum* Lp91 on the gene expression of cytokines and other molecules. *L. plantarum* Lp91 downregulated *TNF-a* and *COX-2* in mice with colitis. Interleukin-10 was significantly upregulated in colitis and non-colitis mice that were treated with *L. plantarum* Lp91, while other anti-inflammatory markers, i.e., *COX-1, IL-4* and *IL-6,* were significantly upregulated in the colitis mice that were treated with *L. plantarum* Lp91. The MUC2 gene was also significantly up regulated in the non-colitis group[81].

The antioxidant potential of *Lactobacillus rhamnosus* CNCM I-3690 using the nematode *Caenorhabditis elegans* as host was investigated. The transcriptomic analysis of *C. elegans* that were fed this strain showed that an increased lifespan is correlated with the differential expression of the DAF-16/insulin-like pathway, which is highly conserved in humans. In addition, this *Lactobacillus* strain reduced inflammation in a murine model of colitis[82].

Finally, the synbiotic (*Bifidobacterium longum* and inulin-oligofructose) was tested in UC patients. The treatment was administered for a period of one month in a double blind, randomized, controlled trial using 18 patients with active UC. The sigmoidoscopy scores were reduced in the test group (start 4.5, end 3.1) compared with those of the placebo group (start 2.6, end 3.2). The mRNA levels for *human beta defensins 2, 3*, and *4*, which are strongly upregulated in active UC, were significantly reduced in the test group after treatment. *Tumor necrosis factor alpha* and *IL-1a*, which are inflammatory cytokines that drive inflammation and induce defensin expression, were also significantly reduced after treatment[83].

Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) afflicts extremely low-birth-weight neonates, and probiotics reduce its incidence and severity. Nitric oxide (NO) is involved in the pathogenesis of NEC, and caveolin-1 regulates NO signaling. D'Souza *et al.* (2010) evaluated the importance of NO in formula-fed neonatal rats that were supplemented with "Florastar Kids" and/or galacto-oligosaccharides and fructo-oligosaccharides. Samples from the terminal ileum were analyzed for total NO metabolites, growth factors, and gene expression of *caveolin-1*, *NOS isoforms*, and antioxidants. The data showed that formula feeding with and without supplementation resulted in significant growth restriction. *Caveolin-1, endothelial NOS*, and *neuronal NOS* were simultaneously downregulated with formula feeding, while the inducible NOS was upregulated. Superoxide dismutase and glutathione peroxidase were upregulated with supplementation[84]. Moreover, Lin *et al.* (2008) evaluated probiotics in the incidence of NEC. *Lactobacillus rhamnosus* GG reduced the chemically induced intestinal epithelial apoptosis, demonstrating that LGG upregulates a battery of genes with known and likely cytoprotective effects[85].

Other inflammatory disorders

There are many inflammation-based intestinal diseases. However, probiotics has been tested in only a few of these diseases.

The gastroprotective potential of *Bifidobacterium bifidum* BF-1 in a rat model of acid-ethanol-induced acute gastric injury was investigated to elucidate its potential compared with *Streptococcus thermophilus* YIT 2021. Living *B. bifidum* BF-1 and *S. thermophilus* YIT 2021 or vehicle was orally administered to rats, and acid-ethanol gastric injury was induced 2 h later. Mucin 5ac (muc5ac) gene expression in gastric corpus samples and gastric mucin production in stomach samples from the *B. bifidum* BF-1 group, but not the *S. thermophilus* YIT 2021 group, were significantly higher than those in the respective samples from the vehicle group[86].

Mirpuri *et al.* (2012) evaluated the enteral administration of LGG in mice with intestinal injury due to the administration of platelet-activating factor (PAF) and LPS. The probiotic strain downregulated the expression of *TNF-a* and *MIP-2* but failed to alter *IL-10* mRNA and protein expression. LGG did however induce the mRNA expression of the *IL-10R2* subunit of the IL-10 receptor. IL-10 receptor activation has been associated with the signal transducer and activator of transcription (STAT) 3-dependent induction of members of the SOCS family. In 2-week-old mice, LGG also induced STAT3 phosphorylation, increased the colonic expression of SOCS-3, and

attenuated the colonic production of MIP-2 and TNF-α. These LGG-dependent changes in phosphoSTAT3, SOCS3, MIP-2 and TNF-α were inhibited by the antibody-mediated blockade of the IL-10 receptor. Thus LGG decreased the baseline pro-inflammatory cytokine expression in the developing colon via the upregulation of IL-10 receptor-mediated signaling, most likely due to the combined induction of phospho-STAT3 and SOCS3[87]. The principal findings concerning gene expression in inflammatory diseases of the gut mediated by probiotics appear in table 2.

Although studies evaluating the mechanism of action of probiotics in IBD are heterogeneous because of the different methodological approaches, basically probiotics lead to the downregulation of a number of pro-inflammatory genes and the upregulation of others, e.g., mucin genes, which can help explain the beneficial effects of probiotics in decreasing the activity of these gut diseases.

REGULATION OF IMMUNITY AND INFLAMMATION GENE EXPRESSION IN THE LIVER BY PROBIOTICS

A large body of evidence has highlighted the concept that putative intestinal bacteria–derived compounds may affect liver metabolism and, therefore, cause systemic diseases[88,89]. Serum LPS levels have been proposed to increase upon obesity and steatosis, leading to a metabolic endotoxemia that can modulate pro-inflammatory cytokines as well as glucose and lipid metabolism in the liver or adipose tissue[90-93]. Endotoxemia is considered a major risk for inducing liver inflammation in nonalcoholic steatohepatitis (NASH) and nonalcoholic fatty liver disease (NAFLD) in humans[94-97]. NASH and NAFLD are associated with increased gut permeability in humans[98,99]. Cani *et al.* (2009) demonstrated the alteration of gut-barrier function in genetic models of obesity. Overall, these studies strongly suggest a direct link between the gut microbiota, the gut barrier, and hepatic changes[100].

Few papers have been published regarding the probiotic-mediated modulation of genes that are involved in immunity and inflammation in the liver. These few papers are reviewed below and are organized by pathology.

Sepsis

Bu *et al.* (2006) described a bacteria-free, lysozyme-modified probiotic product that was obtained by treating the probiotic bacteria, *Lactobacillus* spp., with lysozyme (LzMPC), which might be beneficial for the treatment of sepsis owing to the potent immunomodulatory effects of lysozyme on macrophages. The oral administration of LzMPC effectively protected rats against lethality from

polymicrobial sepsis that was induced by cecal ligation and puncture. LzMPC was engulfed by macrophages in the liver after crossing the intestinal barrier. The LzMPC-induced protection was associated with an increase in the bacterial clearance in the liver. Surgical stress or cecal ligation and puncture caused a decrease in the cathelicidin-related peptide (*CRAMP*) expression in the liver, whereas the enteral administration of LzMPC restored *CRAMP* gene expression in these animals. In addition, macrophages from LzMPC-treated rats had an enhanced capacity of cytokine production in response to LPS or LzMPC stimulation[101].

Inflammation

Mair *et al.* (2010) evaluated the 4-week administration of a probiotic mixture (*Enterococcus faecium*, *Lactobacillus salivarius*, *L. reuteri* and *Bifidobacterium thermophilum*) on cell turnover, growth and inflammatory marker gene expression (*caspase-3*; cyclin-dependent kinase-4, *CDK-4*; insulin-like growth factor I, *IGF-I*; *NF-κB; TNF-a*; and *TGF-β*) in piglets' intestines and liver. The gene expression of *CDK-4* and *TGF-β* was upregulated in the jejunum and the mesenteric lymph nodes, respectively, in the probiotic group. In addition, the probiotic group exhibited an upregulation in cell turnover marker genes in the colon and blood. No significant differences were observed in gene expression in the liver tissue[102].

The administration of *L. ingluviei* to mice promotes alterations in the intestinal microbiota, weight gain increase, and accelerated metabolism as well as liver enlargement and inflammation. Angelakis *et al.* (2012) studied the mRNA expression of genes that are involved in lipogenesis and inflammation in the liver of BALB/c mice gavaged for different periods of time with this probiotic strain. The mRNA expression of fatty acyl synthase (*FAS*), sterol regulatory element binding protein 1 (*SREBP-1*), cytochrome P450 2E1, 3-phosphoinositide-dependent protein kinase-1 (*PDPK1*), acyl-Coenzyme A dehydrogenase-11 (*Acad11*), ATP-binding cassette sub family member G (*ABCG2*), and DEAD box polypeptide 25 (*DDX25*) was significantly higher in the probiotic-fed mice compared with that of the control mice. This result was accompanied by a low-grade inflammatory state in the liver, suggested by a significantly increased mRNA expression of liver *TNF-a* in the mice that received probiotics[103].

Experimental liver disease

D'Argenio *et al.* (2013) specifically examined the effects of a synbiotic formulation on an experimental model of CCl4–induced liver fibrosis in rats. The synbiotic product was a mixture of a probiotic strain (*L. paracasei* B21060) with L-glutamine, arabinogalactan and xylo-oligosaccharides as

prebiotics. The serum ALT and AST activities as well as liver histology and collagen deposition improved in fibrotic mice with the synbiotic mixture compared with those of the placebo group. The serum levels of the pro-inflammatory cytokine TNF-α were significantly increased in rats with liver fibrosis compared with those of normal rats, whereas the synbiotic treatment normalized the plasma levels of TNF-α and significantly enhanced the anti-inflammatory cytokine IL-10. In the liver, *TNF-a*, *TGF-β*, *TLR4*, *TLR2*, *iNOS* and *aSMA* mRNA levels were upregulated in rats with CCl4-induced liver fibrosis and downregulated by the synbiotic treatment. Moreover, the *IL-10* and *eNOS* mRNA levels increased in the fibrotic rats that received synbiotics[104].

Aflatoxins are naturally occurring toxins that are produced by *Aspergillus flavus* and *Aspergillus parasiticus*, which are species of fungi. Aflatoxin exposure produces an acute hepatic necrosis that results in cirrhosis or hepatocarcinoma. Because aflatoxin-producing members of *Aspergillus* are common and widespread in nature, these compounds pose serious hazards to human and animal health, and chemoprevention strategies aimed at reducing their toxicity in animal diets are needed. Zuo *et al.* (2013) investigated one such strategy that was based on the administration of a mixture of three aflatoxin-degrading probiotic strains (*Lactobacillus casei*, *Bacillus subtillis*, and *Pichia anomala*) along with the aflatoxin-degrading enzyme from *Aspergillus oryzae* to Arbor Acres broilers that were fed an aflatoxin-supplemented diet. The administration of this mixture to chickens that were fed the aflatoxin-supplemented diet resulted in the restorations of i) the antioxidant enzymatic defense in the serum and liver and ii) the hepatic expression of an array of genes that are involved in apoptosis, cell growth, immunity and metabolism[105]. Table 3 summarizes the reported investigation related to gene expression in the liver by probiotics.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Probiotics exert their actions through interaction with intestinal cells, which in turn modify the expression of many genes that are mainly related to the gut-associated immune system. Although the specific actions are dependent on the particular bacteria and strains, probiotics mainly induce a tolerogenic response to external antigens by interacting with TLR and down-regulating the expression of NF-κB and pro-inflammatory cytokines. There is a need for further clinical studies that evaluate the mechanism of action of probiotics both in healthy humans and patients with chronic diseases. These types of clinical studies are necessary for addressing the influence of these microorganisms in gene expression for different pathways, particularly those that are associated with the immune response, and to better understand the role that probiotics might have in the prevention and treatment of disease.

REFERENCES

1 **Guarner F**. Prebiotics, probiotics and helminths: the 'natural' solution?. *Dig Dis* 2009; **27**: 412-417 [PMID: 19786773 DOI: 10.1159/000228582]

2 **Shanahan F**. Therapeutic implications of manipulating and mining the microbiota. *J Physiol* 2009; **578**: 4175-4179 [PMID: 19505978 DOI: 10.1113/jphysiol.2009.174649]

3 **FAO/WHO**. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. 2001

4 **Giahi L**, Aumueller E, Elmadfa I, Haslberger AG. Regulation of TLR4, p38 MAP kinase, IκB and miRNAs by inactivated strains of lactobacilli in human dendritic cells. *Benef Microbes* 2012; **3**: 91-98 [PMID: 22476320 DOI: 10.3920/BM2011.0052]

5 **Fontana L**, Bermudez-Brito M, Plaza-Diaz J, Muñoz-Quezada S, Gil A. Sources, isolation, characterisation and evaluation of probiotics. *Br J Nutr* 2013; **109**: S35-S50 [PMID: 23360880 DOI: 10.1017/S0007114512004011]

6 **Collado MC**, Isolauri E, Salminen S, Sanz Y. The impact of probiotic on gut health. *Curr Drug Metab* 2009; **10**: 68-78 [PMID: 19149514 DOI: 10.2174/138920009787048437]

7 **Bermudez-Brito M**, Muñoz-Quezada S, Gomez-Llorente C, Matencio E, Bernal MJ, Romero F, Gil A. Human intestinal dendritic cells decrease cytokine release against *Salmonella* infection in the presence of *Lactobacillus paracasei* upon TLR activation. *PloS One* 2012; **7**: e43197 [PMID: 22905233 DOI: 10.1371/journal.pone.0043197]

8 **Bongaerts GP**, Severijnen RS. Preventive and curative effects of probiotics in atopic patients. *Med Hypotheses* 2005; **64**: 1089–1092 [PMID: 15823690 DOI: 10.1016/j.mehy.2004.10.018]

9 **Ouwehand A**, Nermes M, Collado MC, Rautonen N, Salminen S, Isolauri E. Specific probiotics alleviate allergic rhinitis during the birch pollen season. *World J Gastroenterol* 2009; **15**: 3261–3268 [PMID: 19598302 DOI: 10.3748/wjg.15.3261]

10 **Huang JS**, Bousvaros A, Lee JW, Diaz A, Davidson EJ. Efficacy of probiotic use in acute diarrhea in children: a meta-analysis. *Dig Dis Sci* 2002; **47**: 2625–2634 [PMID: 12452406 DOI: 10.1023/A:1020501202369]

11 **Borchers AT**, Selmi C, Meyers FJ, Keen CL, Gershwin ME. Probiotics and immunity. *J Gastroenterol* 2009; **44**: 26–46 [PMID: 19159071 DOI: 10.1007/s00535-008-2296-0]

12 **Williams NT**. Probiotics. *Am J Health Syst Pharm* 2010; **67**: 449-458 [PMID: 20208051 DOI: 10.2146/ajhp090168]

13 **Bermudez-Brito M**, Plaza-Diaz J, Muñoz-Quezada M, Gomez-Llorente C, Gil A. Probiotics mechanisms of action. *Ann Nutr Metab* 2012; **61**: 160-174 [PMID: 23037511 DOI: 10.1159/000342079]

14 **Ghadimi D**, Vrese Md, Heller KJ, Schrezenmeir J. Effect of natural commensal-origin DNA on toll-like receptor 9 (TLR9) signaling cascade, chemokine IL-8 expression, and barrier integritiy of

polarized intestinal epithelial cells. *Inflamm Bowel Dis* 2010; **16**: 410-427 [PMID: 19714766 DOI: 10.1002/ibd.21057]

15 **Artis D**. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat Rev* 2008; **8**: 411–420 [PMID: 18469830 DOI: 10.1038/nri2316]

16 **Gómez-Llorente C**, Muñoz S, Gil A. Role of Toll-like receptors in the development of immunotolerance mediated by probiotics. *Proc Nutr Soc* 2010; **69**:381-389 [PMID: 20416121 DOI: 10.1017/S0029665110001527]

17 **Otte JM**, Podolsky DK. Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. *Am J Physiol Gastroenterol Liver Physiol* 2004; **286**: G613-G626 [PMID: 15010363 DOI: 10.1152/ajpgi.00341.2003]

18 **Mack DR**, Michail S, Wei S, McDougall L, Hollingsworth MA. Probiotics inhibit enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression. *Am J Physiol* 1999; **276**: G941-950 [PMID: 10198338]

19 **Anderson RC**, Cookson AL, McNabb WC, Park Z, McCann MJ, Kelly WJ, Roy NC. *Lactobacillus plantarum* MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. *BMC Microbiol* 2010; **10**: 316 [PMID: 21143932 DOI: 10.1186/1471-2180-10-316]

20 **Audy J**, Mathieu O, Belvis J, Tompkins TA. Transcriptomic response of immune signalling pathways in intestinal epithelial cells exposed to lipopolysaccharides, Gram-negative bacteria or potentially probiotic microbes. *Benef Microbes* 2012; **3**: 273-86 [PMID: 23234729 DOI: 10.3920/BM2012.0027]

21 **Riedel CU**, Foata F, Philippe D, Adolfsson O, Eikmanns BJ, Blum S. Anti-inflammatory effects of bifidobacteria by inhibition of LPS-induced NF-kappaB activation. *World J Gastroenterol* 2006; **12**: 3729-3735 [PMID: 16773690 DOI: 10.3748/wjg.v12.i23.3729]

22 **Ruiz PA**, Hoffmann M, Szcesny S, Blaut M, Haller D. Innate mechanisms for *Bifidobacterium lactis* to activate transient pro-inflammatory host responses in intestinal epithelial cells after the colonization of germ-free rats. *Immunology* 2005; **115**:441-50 [PMID: 16011513 DOI: 10.1111/j.1365-2567.2005.02176.x]

23 **Liu C**, Zhang ZY, Dong K, Guo XK. Adhesion and immunomodulatory effects of *Bifidobacterium lactis* HN019 on intestinal epithelial cells INT-407. *World J Gastroenterol* 2010; **16**: 2283-90 [PMID: 20458767 DOI: 10.3748/wjg.v16.i18.2283]

24 **Okada Y**, Tsuzuki Y, Hokari R, Komoto S, Kurihara C, Kawaguchi A, Nagao S, Miura S. Anti-inflammatory effects of the genus *Bifidobacterium* on macrophages by modification of phospho-I kappaB and SOCS gene expression. *Int J Exp Pathol* 2009; **90**: 131-140 [PMID: 19335551 DOI: 10.1111/j.1365-2613.2008.00632.x]

25 **Imaoka A**, Shima T, Kato K, Mizuno S, Uehara T, Matsumoto S, Setoyama H, Hara T, Umesaki Y. Anti-inflammatory activity of probiotic *Bifidobacterium*: enhancement of IL-10 production in

peripheral blood mononuclear cells from ulcerative colitis patients and inhibition of IL-8 secretion in HT-29 cells. *World J Gastroenterol* 2008; **14**: 2511-2516 [PMID: 18442197 DOI: 10.3748/wjg.14.2511]

26 **Boesten RJ**, Schuren FH, Willemsen LE, Vriesema A, Knol J, De Vos WM. *Bifidobacterium breve* - HT-29 cell line interaction: modulation of TNF-α induced gene expression. *Benef Microbes* 2011; **2**: 115-28 [PMID: 21831793 DOI: 10.3920/BM2011.0005]

27 **Nishitani Y**, Tanoue T, Yamada K, Ishida T, Yoshida M, Azuma T, Mizuno M. *Lactococcus lactis* subsp. *cremoris* FC alleviates symptoms of colitis induced by dextran sulfate sodium in mice. *Int Immunopharmacol* 2009; **9**: 1444-1451 [PMID: 19733697 DOI: 10.1016/j.intimp.2009.08.018]

28 **O'Flaherty S**, Klaenhammer TR. Influence of exposure time on gene expression by human intestinal epithelial cells exposed to *Lactobacillus acidophilus*. *Appl Environ Microbiol* 2012; **78**: 5028-5032 [PMID: 22562992 DOI: 10.1128/AEM.00504-12]

29 **Oksaharju A**, Kankainen M, Kekkonen RA, Lindstedt KA, Kovanen PT, Korpela R, Miettinen M. Probiotic *Lactobacillus rhamnosus* downregulates FCER1 and HRH4 expression in human mast cells. *World J Gastroenterol* 2011; **17**: 750-759 [PMID: 21390145 DOI: 10.3748/wjg.v17.i6.750]

30 **Paszti-Gere E**, Szeker K, Csibrik-Nemeth E, Csizinszky R, Marosi A, Palocz O, Farkas O, Galfi P. Metabolites of *Lactobacillus plantarum* 2142 prevent oxidative stress-induced overexpression of proinflammatory cytokines in IPEC-J2 cell line. *Inflammation* 2012; **35**: 1487-1499 [PMID: 22476971 DOI: 10.1007/s10753-012-9462-5]

31 **Zanello G**, Berri M, Dupont J, Sizaret PY, D'Inca R, Salmon H, Meurens F. *Saccharomyces cerevisiae* modulates immune gene expressions and inhibits ETEC-mediated ERK1/2 and p38 signaling pathways in intestinal epithelial cells. *PLoS One* 2011; **6**: e18573 [PMID: 21483702 DOI: 10.1371/journal.pone.0018573]

32 **Latvala S**, Miettinen M, Kekkonen RA, Korpela R, Julkunen I. *Lactobacillus rhamnosus* GG and *Streptococcus thermophilus* induce suppressor of cytokine signalling 3 (SOCS3) gene expression directly and indirectly via interleukin-10 in human primary macrophages. *Clin Exp Immunol* 2011; **165**: 94-103 [PMID: 21545585 DOI: 10.1111/j.1365-2249.2011.04408.x]

33 **Ukena SN**, Westendorf AM, Hansen W, Rohde M, Geffers R, Coldewey S, Suerbaum S, Buer J, Gunzer F. The host response to the probiotic *Escherichia coli* strain Nissle 1917: specific upregulation of the proinflammatory chemokine MCP-1. *BMC Med Genet* 2005; **6**: 43 [PMID: 16351713 DOI: 10.1186/1471-2350-6-43]

34 **Wang Y**, Xie J, Wang N, Li Y, Sun X, Zhang Y, Zhang H. *Lactobacillus casei* Zhang modulate cytokine and toll-like receptor expression and beneficially regulate poly I:C-induced immune responses in RAW264.7 macrophages. *Microbiol Immunol* 2013; **57**: 54-62 [PMID: 23350674 DOI: 10.1111/j.1348-0421.516.x]

35 **Kim CH**, Kim HG, Kim JY, Kim NR, Jung BJ, Jeong JH, Chung DK. Probiotic genomic DNA reduces the production of pro-inflammatory cytokine tumor necrosis factor-alpha. *FEMS Microbiol Lett* 2012; **328**: 13-19 [PMID: 22126103 DOI: 10.1111/j.1574-6968.2011.02470.x]

36 **Cammarota M**, De Rosa M, Stellavato A, Lamberti M, Marzaioli I, Giuliano M. *In vitro* evaluation of *Lactobacillus plantarum* DSMZ 12028 as a probiotic: emphasis on innate immunity. *Int J Food Microbiol* 2009; **135**: 90-98 [PMID: 19748696 DOI: 10.1016/j.ijfoodmicro.2009.08.022]

37 **Tomosada Y**, Villena J, Murata K, Chiba E, Shimazu T, Aso H, Iwabuchi N, Xiao JZ, Saito T, Kitazawa H. Immunoregulatory effect of bifidobacteria strains in porcine intestinal epithelial cells through modulation of ubiquitin-editing enzyme A20 expression. *PLoS One* 2013; **8**: e59259 [PMID: 23555642 DOI: 10.1371/journal.pone.0059259]

38 **Gao Q**, Qi L, Wu T, Wang J. *Clostridium butyricum* activates TLR2-mediated MyD88-independent signaling pathway in HT-29 cells. *Mol Cell Biochem* 2012; **361**: 31-37 [PMID: 21956671 DOI: 10.1007/s11010-011-1084-y]

39 **Isono A**, Katsuno T, Sato T, Nakagawa T, Kato Y, Sato N, Seo G, Suzuki Y, Saito Y. *Clostridium butyricum* TO-A culture supernatant downregulates TLR4 in human colonic epithelial cells. *Dig Dis Sci* 2007; **52**: 2963-2971 [PMID: 17404865 DOI: 10.1007/s10620-006-9593-3]

40 **Huang JM**, La Ragione RM, Nunez A, Cutting SM. Immunostimulatory activity of *Bacillus* spores. *FEMS Immunol Med Microbiol* 2008; **53**: 195-203 [PMID: 18430003 DOI: 10.1111/j.1574-695X.2008.00415.x]

41 **Drakes M**, Blanchard T, Czinn S. Bacterial probiotic modulation of dendritic cells. *Infect Immun* 2004; **72**: 3299-3309. [PMID: 15155633 DOI: 10.1128/IAI.72.6.3299-3309.2004]

42 **Rizzello V**, Bonaccorsi I, Dongarrà ML, Fink LN, Ferlazzo G. Role of natural killer and dendritic cell crosstalk in immunomodulation by commensal bacteria probiotics. *J Biomed Biotechnol* 2011; **2011**: 47307 [PMDI: 21660136 DOI: 10.1155/2011/473097]

43 **Cavani A**, Nasorri F, Prezzi C, Sebastiani S, Albanesi C, Girolomoni G. Human CD4+T lymphocytes with remarkable regulatory functions on dendritic cells and nickel-specific Th1 immune responses. *J Investig Dermatol* 2000; **114**: 295-302 [PMID: 10651989 DOI: 10.1046/j.1523-1747.2000.00881.x]

44 **De Jong EC**, Vieira PL, Kalinski P, Schuitemaker JH, Tanaka Y, Wierenga EA, Yazdanbakhs M, Kapsenberg ML. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells *in vitro* with diverse th cell-polarizing signals. *J Immunol* 2002; **168**: 1704-1709 [PMID: 11823500]

45 **Mastrangeli G**, Corinti S, Butteroni C, Afferni C, Bonura A, Boirivant M, Colombo P, Di Felice G. Effects of live and inactivated VSL#3 probiotic preparations in the modulation of *in vitro* and *in vivo* allergen-induced Th2 responses. *Int Arch Allergy Immunol* 2009; **150**: 133-143 [PMID: 19439979 DOI: 10.1159/000218116]

46 **D'Arienzo R**, Maurano F, Lavermicocca P, Ricca E, Rossi M. Modulation of the immune response by probiotic strains in a mouse model of gluten sensitivity. *Cytokine* 2009; **48**: 254-259 [PMID: 19736022 DOI: 10.1016/j.cyto.2009.08.003]

47 **Kosaka A**, Yan H, Ohashi S, Gotoh Y, Sato A, Tsutsui H, Kaisho T, Toda T, Tsutsui H, Kaisho T, Toda T, Tsuji N. *Lactoccocus lactis* subsp. cremoris FC triggers IFN-γ production from NK and T cells via IL-

12 and IL-18. *Int Immunopharmacol* 2012; **14**: 729-733 [PMID: 23102661 DOI: 10.1016/j.intimp.2012.10.007]

48 **Weiss** G, Rasmussen S, Zeurhen LH, Nielsen BN, Jespersen L, Frøkiaer H. *Lactobacillus acidophilus* induces virus immune defence genes in murine dendritic cells by a Toll-like receptor-2-dependent mechanism. *Immunology* 2010; **131**: 268-281 [PMID: 20545783 DOI: 10.1111/j.1365-2567.2010.03301.x]

49 **Rajput IR**, Hussain A, Li YJ, Zhang X, Xu X, Long MY, You DY, Li WF. *Saccharomyces boulardii* and *Bacillus subtilis* B10 modulate TLRs mediated signaling to induce immunity by chicken BMDCs. *J Cell Biochem* 2014; **115**: 189-198 [PMID: 24038094 DOI: 10.1002/jcb.24650]

50 **Latvala S**, Pietilä TE, Veckman V, Kekkonen RA, Tynkkynen S, Korpela R, Julkunen I. Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells. *World J Gastroenterol* 2008; **28**: 5570-5583 [PMID: 18810777 DOI: 10.3748/wjg.14.5570]

51 **O'Hara AM**, O'regan P, Fanning A, O'Mahony C, MacSharry J, Lyons A, Bienenstock J, O'Mahony, Shannhan F. Functional modulation of human intestinal epithelial cell responses by *Bifidobacterium infantis* and *Lactobacillus salivarius*. *Immunology* 2006; **118**: 202-215 [PMID: 16771855 DOI: 10.1111/j.1365-2567.2006.02358.x]

52 **Evrard B**, Coudeyras S, Dosgilbert A, Charbonnel N, Alamé J, Tridon A, Forestier C. Dose-dependent immunomodulation of human dendritic cells by the probiotic *Lactobacillus rhamnosus* Lcr35. *PLoS One* 2011; **6**: e18735 [PMID: 21533162 DOI: 10.1371/journal.pone.0018735]

53 **Torri A**, Beretta O, Ranghetti A, Granucci F, Ricciardi-Castagnoli P, Foti M. Gene expression profiles identify inflammatory signatures in dendritic cells. *PLoS One* 2010; **5**: e9404 [PMID: 20195376 DOI: 10.1371/journal.pone.0009404]

54 **Tsilingiri K**, Barbosa T, Penna G, Caprioli F, Sonzogni A, Viale G, Rescigno M. Probiotic and postbiotic activity in health and disease: comparison on a novel polarized ex-vivo organ culture model. *Gut* 2012; **61**: 1007-1005 [PMID: 22301383 DOI: 10.1136/gutjnl-2011-300971]

55 **Bermudez-Brito M**, Muñoz-Quezada S, Gomez-Llorente C, Matencio E, Bernal MJ, Romero F, Gil A. Cell-free culture supernatant of *Bifidobacterium breve* CNCM I-4035 decreases pro-inflammatory cytokines in human dendritic cells challenged with *Salmonella typhi* through TLR activation. *PLoS One* 2013; **8**: e59370 [PMID: 23555025 DOI: 10.1371/journal.pone.0059370]

56 **Ayehunie S**, Snell M, Chirld M, Klausner M. A plasmacytoid dendritic cell (CD123+/CD11c-) based assay system to predict contact allegenicity of chemicals. *Toxicology* 2009; **264**: 1-9 [PMID: 19665512 DOI: 10.1016/j.tox.2009.07.021]

57 **Matsumoto M**, Kurihara S, Kibe R, Ashida H, Benno Y. Longevity in mice is promoted by probiotic-induced suppression of colonic senescence dependent on upregulation of gut bacterial polyamine production. *PLoS One* 2011; **6**: e23652 [PMID: 21858192 DOI: 10.1371/journal.pone.0023652]

58 **Ohtsuka Y**, Ikegami T, Izumi H, Namura M, Ikeda T, Ikuse T, Baba Y, Kudo T, Suzuki R, Shimizu T. Effects of *Bifidobacterium breve* on inflammatory gene expression in neonatal and weaning rat intestine. *Pediatr Res* 2012; **71**: 46-53 [PMID: 22289850 DOI: 10.1038/pr.2011.11]

59 **Trevisi P**, De Filippi S, Minieri L, Mazzoni M, Modesto M, Biavati B, Bosi P. Effect of fructo-oligosaccharides and different doses of *Bifidobacterium animalis* in a weaning diet on bacterial translocation and Toll-like receptor gene expression in pigs. *Nutrition* 2008; **24**: 1023-1029 [PMID: 18562167 DOI: 10.1016/j.nut.2008.04.008]

60 **Dykstra NS**, Hyde L, Adawi D, Kulik D, Ahrne S, Molin G, Jeppsson B, Mackenzie A, Mack DR. Pulse probiotic administration induces repeated small intestinal Muc3 expression in rats. *Pediatr Res* 2011; **69**: 206-11 [PMID: 21135754 DOI: 10.1203/PDR.0b013e3182096ff0]

61 **Finamore A**, Roselli M, Britti MS, Merendino N, Mengheri E. *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* MB5 induce intestinal but not systemic antigen-specific hyporesponsiveness in ovalbumin-immunized rats. *J Nutr* 2012; **142**: 375-381 [PMID: 22223570 DOI: 10.3945/jn.111.148924]

62 **Foye OT**, Huang IF, Chiou CC, Walker WA, Shi HN. Early administration of probiotic *Lactobacillus acidophilus* and/or prebiotic inulin attenuates pathogen-mediated intestinal inflammation and Smad 7 cell signaling. *FEMS Immunol Med Microbiol* 2012; **65**: 467-480 [PMID: 22524476 DOI: 10.1111/j.1574-695X.2012.00978.x]

63 **Deng J**, Li Y, Zhang J, Yang Q. Co-administration of *Bacillus subtilis* RJGP16 and *Lactobacillus salivarius* B1 strongly enhances the intestinal mucosal immunity of piglets. *Res Vet Sci* 2013; **94**: 62-8 [PMID: 22901748 DOI: 10.1016/j.rvsc.2012.07.025]

64 **Jain S**, Yadav H, Sinha PR, Marotta F. Modulation of cytokine gene expression in spleen and Peyer's patches by feeding dahi containing probiotic *Lactobacillus casei* in mice. *J Dig Dis* 2009; **10**: 49-54 [PMID: 19236547 DOI: 10.1111/j.1751-2980.2008.00362.x]

65 **Pérez-Sánchez T**, Balcázar JL, Merrifield DL, Carnevali O, Gioacchini G, de Blas I, Ruiz-Zarzuela I. Expression of immune-related genes in rainbow trout (*Oncorhynchus mykiss*) induced by probiotic bacteria during *Lactococcus garvieae* infection. *Fish Shellfish Immunol* 2011; **31**: 196-201 [PMID: 21620974 DOI: 10.1016/j.fsi.2011.05.005]

66 **Pirarat N**, Pinpimai K, Endo M, Katagiri T, Ponpornpisit A, Chansue N, Maita M. Modulation of intestinal morphology and immunity in nile tilapia (*Oreochromis niloticus*) by *Lactobacillus rhamnosus* GG. *Res Vet Sci* 2011; **91**: e92-7 [PMID: 21536310 DOI: 10.1016/j.rvsc.2011.02.014]

67 **van Baarlen P**, Troost F, van der Meer C, Hooiveld G, Boekschoten M, Brummer RJ, Kleerebezem M. Human mucosal *in vivo* transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways. *Proc Natl Acad Sci USA* 2011; **108**: 4562-4569 [PMID: 20823239 DOI: 10.1073/pnas.1000079107]

68 **Lammers KM**, Vergopoulos A, Babel N, Gionchetti P, Rizzello F, Morselli C, Caramelli E, Fiorentino M, d'Errico A, Volk HD, Campieri M. Probiotic therapy in the prevention of pouchitis onset:

decreased interleukin-1beta, interleukin-8, and interferon-gamma gene expression. *Inflamm Bowel Dis* 2005; **11**: 447-54 [PMID: 15867584 DOI: 10.1097/01.mpa.0000160302.40931.7b]

69 **Di Caro S**, Tao H, Grillo A, Franceschi F, Elia C, Zocco MA, Gasbarrini G, Sepulveda AR, Gasbarrini A. *Bacillus clausii* effect on gene expression pattern in small bowel mucosa using DNA microarray analysis. *Eur J Gastroenterol Hepatol* 2005; **17**: 951-60 [PMID: 16093873]

70 **Kassinen A**, Krogius-Kurikka L, Mäkivuokko H, Rinttilä T, Paulin L, Corander J, Malinen E, Apajalahti J, Palva A. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology* 2007; **133**: 24-33 [PMID: 17631127 DOI: 10.1053/j.gastro.2007.04.005]

71 **Distrutti E**, Cipriani S, Mencarelli A, Renga B, Fiorucci S. Probiotics VSL#3 protect against development of visceral pain in murine model of irritable bowel syndrome. *PLoS One* 2013; **8**: e63893 [PMID: 23691109 DOI: 10.1371/journal.pone.0063893]

72 **Ghadimi D**, Helwig U, Schrezenmeir J, Heller KJ, de Vrese M. Epigenetic imprinting by commensal probiotics inhibits the IL-23/IL-17 axis in an *in vitro* model of the intestinal mucosal immune system. *J Leukoc Biol* 2012; **92**: 895-911 [PMID: 22730546 DOI: 10.1189/jlb.0611286]

73 **Grimoud J**, Durand H, de Souza S, Monsan P, Ouarné F, Theodorou V, Roques C. *In vitro* screening of probiotics and synbiotics according to anti-inflammatory and anti-proliferative effects. *Int J Food Microbiol* 2010; **144**: 42-50 [PMID: 20951454 DOI: 10.1016/j.ijfoodmicro.2010.09.007]

74 **Chen X**, Yang G, Song JH, Xu H, Li D, Goldsmith J, Zeng H, Parsons-Wingerter PA, Reinecker HC, Kelly CP. Probiotic yeast inhibits VEGFR signaling and angiogenesis in intestinal inflammation. *PLoS One* 2013; **8**: e64227 [PMID: 23675530 DOI: 10.1371/journal.pone.0064227]

75 **Bassaganya-Riera J**, Viladomiu M, Pedragosa M, De Simone C, Hontecillas R. Immunoregulatory mechanisms underlying prevention of colitis-associated colorectal cancer by probiotic bacteria. *PLoS One* 2012; **7**: e34676 [PMID: 22511958 DOI: 10.1371/journal.pone.0034676]

76 **Garrido-Mesa N**, Utrilla P, Comalada M, Zorrilla P, Garrido-Mesa J, Zarzuelo A, Rodríguez-Cabezas ME, Gálvez J. The association of minocycline and the probiotic *Escherichia coli* Nissle 1917 results in an additive beneficial effect in a DSS model of reactivated colitis in mice. *Biochem Pharmacol* 2011; **82**: 1891-1900 [PMID: 21930116 DOI: 10.1016/j.bcp.2011.09.004]

77 **Claes IJ**, Lebeer S, Shen C, Verhoeven TL, Dilissen E, De Hertogh G, Bullens DM, Ceuppens JL, Van Assche G, Vermeire S, Rutgeerts P, Vanderleyden J, De Keersmaecker SC. Impact of lipoteichoic acid modification on the performance of the probiotic *Lactobacillus rhamnosus* GG in experimental colitis. *Clin Exp Immunol* 2010; **162**: 306-14 [PMID: 20731672 DOI: 10.1111/j.1365-2249.2010.04228.x]

78 **Miyauchi E**, Morita H, Tanabe S. *Lactobacillus rhamnosus* alleviates intestinal barrier dysfunction in part by increasing expression of zonula occludens-1 and myosin light-chain kinase *in vivo*. *J Dairy Sci* 2009; **92**: 2400-2408 [PMID: 19447972 DOI: 10.3168/jds.2008-1698]

79 **Mariman R**, Kremer B, van Erk M, Lagerweij T, Koning F, Nagelkerken L. Gene expression profiling identifies mechanisms of protection to recurrent trinitrobenzene sulfonic acid colitis mediated by probiotics. *Inflamm Bowel Dis* 2012; **18**:1424-1433 [PMID: 22162025 DOI: 10.1002/ibd.22849]

80 **Amit-Romach E**, Uni Z, Reifen R. Multistep mechanism of probiotic bacterium, the effect on innate immune system. *Mol Nutr Food Res* 2010; **54**: 277-284 [PMID: 19998380 DOI: 10.1002/mnfr.200800591]

81 **Duary RK**, Bhausaheb MA, Batish VK, Grover S. Anti-inflammatory and immunomodulatory efficacy of indigenous probiotic *Lactobacillus plantarum* Lp91 in colitis mouse model. *Mol Biol Rep* 2012; **39**: 4765-4775 [PMID: 21947851 DOI: 10.1007/s11033-011-1269-1]

82 **Grompone G**, Martorell P, Llopis S, González N, Genovés S, Mulet AP, Fernández-Calero T, Tiscornia I, Bollati-Fogolín M, Chambaud I, Foligné B, Montserrat A, Ramón D. Anti-inflammatory *Lactobacillus rhamnosus* CNCM I-3690 strain protects against oxidative stress and increases lifespan in *Caenorhabditis elegans*. *PLoS One* 2012; **7**: e52493 [PMID: 23300685 DOI: 10.1371/journal.pone.0052493]

83 **Furrie E**, Macfarlane S, Kennedy A, Cummings JH, Walsh SV, O'neil DA, Macfarlane GT. Synbiotic therapy (*Bifidobacterium longum*/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut* 2005; **54**: 242-249 [PMID: 15647189 DOI: 10.1136/gut.2004.044834]

84 **D'Souza A**, Fordjour L, Ahmad A, Cai C, Kumar D, Valencia G, Aranda JV, Beharry KD. Effects of probiotics, prebiotics, and synbiotics on messenger RNA expression of caveolin-1, NOS, and genes regulating oxidative stress in the terminal ileum of formula-fed neonatal rats. *Pediatr Res* 2010; **67**: 526-531 [PMID: 20101198 DOI: 10.1203/PDR.0b013e3181d4ff2b]

85 **Lin PW**, Nasr TR, Berardinelli AJ, Kumar A, Neish AS. The probiotic *Lactobacillus* GG may augment intestinal host defense by regulating apoptosis and promoting cytoprotective responses in the developing murine gut. *Pediatr Res* 2008; **64**: 511-516 [PMID: 18552706 DOI: 10.1203/PDR.0b013e3181827c0f]

86 **Gomi A**, Harima-Mizusawa N, Shibahara-Sone H, Kano M, Miyazaki K, Ishikawa F. Effect of *Bifidobacterium bifidum* BF-1 on gastric protection and mucin production in an acute gastric injury rat model. *J Dairy Sci* 2013; **96**: 832-837 [PMID: 23200466 DOI: 10.3168/jds.2012-5950]

87 **Mirpuri J**, Sotnikov I, Myers L, Denning TL, Yarovinsky F, Parkos CA, Denning PW, Louis NA. *Lactobacillus rhamnosus* (LGG) regulates IL-10 signaling in the developing murine colon through upregulation of the IL-10R2 receptor subunit. *PLoS One* 2012; **7**: e51955 [PMID: 23272193 DOI: 10.1371/journal.pone.0051955]

88 **Delzenne NM**, Cani PD. Interaction between obesity and the gut microbiota: relevance in nutrition. *Annu Rev Nutr 2011*; **31**: 15-31 [PMID: 21568707 DOI: 10.1146/annurev-nutr-072610-145146]

89 **Nolan JP**. The contribution of gut-derived endotoxins to liver injury. *Yale J Biol Med* 1979; **52**: 127-133 [PMID: 377823]

90 **Cani PD**, Delzenne NM. Interplay between obesity and associated metabolic disorders: new insights into the gut microbiota. *Curr Opin Pharmacol* 2009; **9**: 737-743 [PMID: 19628432 DOI: 10.1016/j.coph.2009.06.016]

91 **Cani PD**, Delzenne NM. The role of the gut microbiota in energy metabolism and metabolic disease. *Curr Opin Pharmacol* 2009; **15**: 1546-1558 [PMID: 19442172 DOI: 10.2174/138161209788168164]

92 **Lanthier N**, Molendi-Coste O, Horsmans Y, van RN, Cani PD, Leclercq IA. Kupffer cell activation is a causal factor for hepatic insulin resistance. *Am J Physiol Gastrointest Liver Physiol* 2009; **298**: 107-116 [PMID: 19875703 DOI: 10.1152/ajpgi.00391.2009]

93 **Neyrinck AM**, Cani PD, Dewulf EM, De BF, Bindels LB, Delzenne NM. Critical role of Kupffer cells in the management of diet-induced diabetes and obesity. *Biochem Biophys Res Commun* 2009; **385**: 351-356 [PMID: 19463788 DOI: 10.1016/j.bbrc.2009.05.070]

94 **Adachi Y**, Moore LE, Bradford BU, Gao W, Thurman RG. Antibiotics prevent liver injury in rats following long-term exposure to ethanol. *Gastroenterology* 1995; **108**: 218-224 [PMID: 7806045 DOI: 10.1016/0016-5085(95)90027-6]

95 **Verdam FJ**, Rensen SS, Driessen A, Greve JW, Buurman WA. Novel evidence for chronic exposure to endotoxin in human nonalcoholic steatohepatitis. *J Clin Gastroenterol* 2010; **5**: 149-152 [PMID: 20661154 DOI: 10.1097/MCG.0b013e3181e12c24]

96 **Harte AL**, da Silva NF, Creely SJ, McGee KC, Billyard T, Youssef-Elabd EM, Tripathi G, Ashour E, Abdalla MS, Sharada HM, Amin AI, Burt AD, Kumar S, Day CP, McTernan PG. Elevated endotoxin levels in nonalcoholic fatty liver disease. *J Inflamm* 2010; **7**: 15 [PMID: 20353583 DOI: 10.1186/1476-9255-7-15]

97 **Thuy S**, Ladurner R, Volynets V, Wagner S, Strahl S, Königsrainer A, Maier KP, Bischoff SC, Bergheim I. Nonalcoholic fatty liver disease in humans is associated with increased plasma endotoxin and plasminogen activator inhibitor 1 concentrations and with fructose intake. *J Nutr* 2008; **138**: 1452-1455 [PMID: 18641190]

98 **Farhadi A**, Gundlapalli S, Shaikh M, Frantzides C, Harrell L, Kwasny MM, Keshavarzian A. Susceptibility to gut leakiness: a possible mechanism for endotoxaemia in nonalcoholic steatohepatitis. *Liver Int* 2008; **28**: 1026-1033 [PMID: 18397235 DOI: 10.1111/j.1478-3231.2008.01723.x]

99 **Miele L**, Valenza V, La TG, Montalto M, Cammarota G, Ricci R, Mascianà R, Forgione A, Gabrieli ML, Perotti G, Vecchio FM, Rapaccini G, Gasbarrini G, Day CP, Grieco A. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology* 2009; **49**: 1877-1887 [PMID: 19291785 DOI: 10.1002/hep.22848]

100 **Cani PD**, Possemiers S, Van de WT, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck A, Lambert DM, Muccioli GG, Delzenne NM. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut* 2009; **58**: 1091-1103 [PMID: 19240062 DOI: 10.1136/gut.2008.165886]

101 **Bu HF**, Wang X, Zhu YQ, Williams RY, Hsueh W, Zheng X, Rozenfeld RA, Zuo XL, Tan XD. Lysozyme-modified probiotic components protect rats against polymicrobial sepsis: role of macrophages and cathelicidin-related innate immunity. *J Immunol* 2006; **177**: 8767-876 [PMID: 17142779]

102 **Mair C**, Plitzner C, Pfaffl MW, Schedle K, Meyer HH, Windisch W. Inulin and probiotics in newly weaned piglets: effects on intestinal morphology, mRNA expression levels of inflammatory marker genes and haematology. *Arch Anim Nutr* 2010; **64**: 304-321 [PMID: 20722301 DOI: 10.1080/1745039X.2010.492137]

103 **Angelakis E**, Bastelica D, Ben Amara A, El Filali A, Dutour A, Mege JL, Alessi MC, Raoult D. An evaluation of the effects of *Lactobacillus ingluviei* on body weight, the intestinal microbiome and metabolism in mice. *Microb Pathog* 2012; **52**: 61-68 [PMID: 22020311 DOI: 10.1016/j.micpath.2011.10.004]

104 **D'Argenio G**, Cariello R, Tuccillo C, Mazzone G, Federico A, Funaro A, De Magistris L, Grossi E, Callegari ML, Chirico M, Caporaso N, Romano M, Morelli L, Loguercio C. Symbiotic formulation in experimentally induced liver fibrosis in rats: intestinal microbiota as a key point to treat liver damage?. *Liver Int* 2013; **33**: 687-697 [PMID: 23448378 DOI: 10.1111/liv.12117]

105 **Zuo RY**, Chang J, Yin QQ, Wang P, Yang YR, Wang X, Wang GQ, Zheng QH. Effect of the combined probiotics with aflatoxin B1-degrading enzyme on aflatoxin detoxification, broiler production performance and hepatic enzyme gene expression. *Food Chem Toxicol* 2013; **59**: 470-475 [PMID: 23831311 DOI: 10.1016/j.fct.2013.06.044]

P-Reviewers: S-Editor: Qi Y **L-Editor: E-Editor:**

Table 1 Regulation of immunity and inflammatory gene expression in the gut by probiotics

Study	Probiotic strain	Genes involved
Intestinal cultured cells		
Enterocytes		
Ghadimi *et al.*[14]	DNA from *L. rhamnosus* GG and B. longum	*TLR-9* and *IL-8*
Otte and Podolsky[17]	*E. coli* Nissle 1917 and VSL#3	Mucins genes
Mack *et al.*[18]	*L. plantarum* 299v and *L. rhamnosus* GG	*MUC2* and *MUC3*
Anderson *et al.*[19]	*L. plantarum* MB452	Tight junction-related genes
Audy *et al.*[20]	Lactobacilli and bifidobacteria strains	MAPK signaling pathway
Riedel *et al.*[21]	Bifidobacteria strains	NF-κB activation, *IL-8*, *TNF-a*, *COX-2*, and *ICAM-1*
Ruiz *et al.*[22]	*B. lactis* BB12	NF-κB, MAPK signaling, and *IL-6*
Liu *et al.*[23]	*B. lactis* HN019	*IL-8*
Okada *et al.*[24]	Bifidobacteria	*IL12p40*, *IL-1β*, *TNF-a*, and *SOCS1*
Imaoka *et al.*[25]	*B. breve* strain Yakult and *B. bifidum* strain Yakult	*IL-8* and *IκB-zeta*
Boesten *et al.*[26]	*B. breve* strains M-16V, NR246 and UCC2003	*CASP7*, *IRF3*, *A4*, *APBA1*, *NOX5*, and *LIFR*
Nishitani *et al.*[27]	*L. lactis* subsp. *cremoris* FC	*IL-8*
O'Flaherty and Klaenhammer[28]	*L. acidophilus*	NF-κB signaling

1

Oksaharju *et al.*[29]	Bifidobacteria, lactobacilli, and *P. freudenreichii*	*FCER1A*, *FCER1G*, *IL-8*, *TNF-a*, and *IL-10*
Paszti-Gere *et al.*[30]	*L. plantarum* 2142 and bifidobacteria	*IL-8* and *TNF-a*
Zanello *et al.*[31]	*Saccharomyces cerevisiae* CNCM I-3856	*PPAR-γ*
Latvala *et al.*[32]	*Lactobacillus* and *Streptococcus* species	*SOCS3*
Ukena *et al.*[33]	*E. coli* Nissle 1917	*MCP-1*, *MIP-2alpha* and *MIP-2beta*
Wang *et al.*[34]	*L. casei* Zhang	*TLR2*, *TLR3*, *TLR4*, and *TLR9*
Kim *et al.*[35]	*L. plantarum* genomic DNA	*TLR2*, *TLR4*, and *TLR9*
Cammarota *et al.*[36]	*L. plantarum* DSMZ 12028	*TLR2* and *TLR4*
Tomosada *et al.*[37]	Eleven different probiotic strains	MAPK and NF-κB pathways
Gao *et al.*[38]	*C. butyricum*	*IL-8*, *IL-6*, and *TNF-a*
Isono *et al.*[39]	*C. butyricum* TO-A	*TLR-4*
Huang *et al.*[40]	*Bacillus* species	*TLR-2* and *TLR-4*
Dendritic cells		
Bermudez-Brito *et al.*[7]	*L. paracasei* CNCM I-4034	*TLR9*, *CASP8*, and *TOLLIP*
Weiss *et al.*[48]	*L. acidophilus*	Genes encoding *IFN*, *TLR-3*, and *IL-12*
Rajput *et al.*[49]	*S. boulardii* and *B. subtillis* B10	*MyD88*, *NF-κB*, *TLR-1*, *2*, *4*, and *15*
Latvala *et al.*[50]	Bifidobacteria, lactobacilli, and *S. thermophilus* THS	*TNF-a*, *IL-1β*, *IL-6*, *IL-10*, *IL-12*, and *IFN-γ*
Evrard *et al.*[52]	*L. rhamnosus* 35	*IL12*, *TNF-a*, *IL1B*, *IL6*, *TGFB1*, *IL-23*, and *IL-8*
Bermudez-Brito *et al.*[55]	*B. breve* CNCM I-4035	*TLR-9* and *TOLLIP*
Ayehunie *et al.*[56]	*L. delbrueckii* subsp. *bulgaricus*	*TLR-4*, *p38*, and *IκB*

2

Animal studies		
Matsumoto *et al.*[57]	*B. animalis* subsp. *lactis* LKM512	Aging-associated and inflammation-associated genes
Ohtsuka *et al.* [58]	*B. breve* M-16V	*LBP*
Trevisi *et al.* [59]	*B. animalis*	*TLR-2* and *TLR-4*
Dykstra *et al.* [60]	Bifidobacteria and lactobacilli	*MUC2, MUC3, NAIP, HIAP1/cIAP2,* and HIAP2/cIAP1
Foye *et al.* [62]	*L. acidophilus*	*IL-10* and *TGF-β*
Deng *et al.* [63]	*B. subtillis* RJGP16 and *L. salivarius* B1	*IL-6* and *pBD-2*
Jain *et al.* [64]	*L. casei*	*IFN-γ* and *IL-2*
Pérez-Sánchez *et al.* [65]	*L. plantarum, L. lactis,* and *L. mesenteroides*	*IL-1β, IL-8, IL-10, TNF-a, IL-8, TLR5,* and *IgT*
Pirarat *et al.* [66]	*L. rhamnosus* GG	*TNF-a* and *IL-1*
Human studies		
van Baarlen *et al.*[67]	*L. acidophilus, L. casei,* and *L. rhamnosus*	Gene-regulatory networks and pathways in human mucosa
Lammers *et al.*[68]	VSL#3	*IL-1β, IL-6, IFN-γ, TNF-a, IL-12, IL-10, TGF-β,* and *IL-8*
Di Caro *et al.*[69]	*B. clausii*	Genes involved in the immune response and inflammation

3

Abbreviations: A4, amyloid beta; APBA1, precursor protein-binding family A member 1; CASP7, cysteine protease caspase 7; COX2, cyclooxygenase 2; FCER1A, allergy-related high-affinity IgE receptor subunits α; FCER1G, allergy-related high-affinity IgE receptor subunits γ; HIAP1/cIAP2, human inhibitor of apoptosis protein 1/cellular inhibitor of apoptosis 2; HIAP2/cIAP1, human inhibitor of apoptosis protein 2/cellular inhibitor of apoptosis 1; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon gamma; IL, interleukin; IRF3, interferon regulatory factor 3; LBP, lipopolysaccharide-binding protein; LIFR, leukemia inhibitory factor receptor; MAPK, mitogen-activated protein kinases; MCP-1, monocyte chemo-attractant protein-1, MIP-2α, macrophage inflammatory protein-2 alpha; MIP-2β, macrophage inflammatory protein-2 beta; MUC, mucins; MyD88, myeloid differentiation primary response protein 88; NAIP, neuronal apoptosis inhibitor protein; NF- κB, nuclear factor-kappa beta; NOX5, NADPH oxidase; PPAR-γ, peroxisome proliferator-activated receptor gamma; SOCS, suppressor of cytokine signaling; p-BD2, porcine beta-defensins 2; TGF-β, transforming growth factor beta; TLR, toll-like receptor; TNF- α, tumor necrosis factor-alpha; TOLLIP, Toll-interacting protein; VSL#3, mixture of *Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium breve, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus plantarum,* and *Streptococcus salivarius* subsp. *thermophilus.*

4

TABLE 2. Regulation of gene expression by probiotics in inflammatory diseases of the gut

Study	Probiotic strain	Genes involved
Inflammatory bowel disease		
Disfrutti *et al.*[71]	VSL#3	*IL-10, TNFRSF1B*
Ghadimi *et al.*[72]	*B. breve* (DSMZ 20213) *and L. rhamnosus* GG	*IL-17* and *IL-23*
Grimoud *et al.*[73]	*B. breve* and *L. lactis*	*TLR-4*
Chen *et al.*[74]	*Saccharomyces boulardii*	*VEGF*
Bassaganya-Riera *et al.*[75]	VSL#3	*TNF-a*, *COX-2*, and *PPAR-γ*
Ulcerative colitis		
Garrido-Mesa *et al.*[76]	*E. coli* Nissle 1917	*TNF-a, IL-1β, IL2, MIP-2, MCP-1, ICAM-1, MUC3*, and *ZO-1*
Claes *et al.* [77]	*L. rhamnosus* GG wild type and mutant	*TLR-2*
Miyauchi *et al.* [78]	*L. rhamnosus OLL2838*	*ZO-1*
Mariman *et al.* [79]	*L. plantarum* NCIMB8826 and VSL#3	Inflammation and immune genes
Amit-Romach *et al.* [80]	*L. rhamnosus* GG and a mixture of probiotics	*MUC2, IL-6*, and *TNF-a*
Duary *et al.* [81]	*L. plantarum* Lp91	*IL-4, IL-6,COX-1, COX-2*, and *TNF-a*
Grompone *et al.* [82]	*L. rhamnosus* CNCM I-3690	DAF-16/insulin-like pathway
Furrie *et al.* [83]	*B. longum*	*human beta defensins 2, 3*, and *4, TNF-a*, and *IL-1a*

5

Necrotizing enterocolitis		
D'Souza *et al.*[84]	*S. boulardii*	*Caveolin-1* and *NOS-isoforms*
Lin *et al.*[85]	*L. rhamnosus* GG	Genes with cytoprotective effects
Other inflammatory disorders		
Gomi *et al.*[86]	*B. bifidum BF-1*	*MUC5*
Mirpuri *et al.*[87]	*L. rhamnosus* GG	*IL-10,MIP-2*, and *TNF-a*

Abbreviations: COX1, cyclooxygenase 1; COX2, cyclooxygenase 2; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; MCP-1, monocyte chemo-attractant protein-1, MIP-2, macrophage inflammatory protein-2 alpha; MUC, mucins; NOS isoforms, constitutional neuronal isoform (nNOS), the inducible isoform (iNOS), and the endothelial isoform (eNOS); PPAR-γ, peroxisome proliferator-activated receptor gamma; TLR, toll-like receptor; TNF- α, tumor necrosis factor-alpha; TNFRSF1B, tumor necrosis factor receptor superfamily, member 1b; TOLLIP, Toll-interacting protein; VEGF, vascular endothelial growth factor; VSL#3, mixture of *Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus plantarum*, and *Streptococcus salivarius* subsp. *thermophilus*; ZO, zona occludens.

6

TABLE 3. Regulation of immunity and inflammation gene expression in the liver by probiotics

Study	Probiotic strain	Genes involved
Sepsis		
Bu *et al.*[101]	*Lactobacillus* spp.	*CRAMP*
Inflammation		
Mair *et al.*[102]	*E. faecium, L. salivarius, L. reuteri and B. thermophilum*	*CDK-4* and *TGF-β*
Angelakis *et al.* [103]	*L. ingluviei*	*TNF-a*
Experimental liver disease		
D'Argenio *et al.*[104]	*L. paracasei* B21060	*TNF-a, TGF-β, IL-10, TLR4, TLR2, iNOS, eNOS, and aSMA*
Zuo *et al.*[105]	*L. casei, B. subtillis, and Pichia anomala*	Genes involved in immunity

Abbreviations: CDK-4, cyclin-dependent kinase-4; CRAMP, cathelicidin-related peptide; IL, interleukin; TGF-β, transforming growth factor beta; NOS isoforms, inducible isoform (iNOS), and endothelial isoform (eNOS); TLR, toll-like receptor; TNF- α, tumor necrosis factor-alpha.

ORIGINAL ARTICLE: HEPATOLOGY AND NUTRITION

Three Main Factors Define Changes in Fecal Microbiota Associated With Feeding Modality in Infants

*Carolina Gomez-Llorente, *Julio Plaza-Diaz, †Margarita Aguilera, *Sergio Muñoz-Quezada, *Miriam Bermudez-Brito, ‡Patricia Peso-Echarri, §Rosario Martinez-Silla, §M. Isabel Vasallo-Morillas, *Laura Campaña-Martin, ||Inmaculada Vives-Piñera, ||Maria J. Ballesta-Martinez, and *Angel Gil

ABSTRACT

Objectives: There are many differences in the fecal infant microbiota associated with various feeding methods. The aim of this study was to examine the major differences in the fecal microbiota of breast-fed (BF) and formula-fed (FF) infants and to describe the principal bacterial components that would explain the variability in the predominant bacterial families and genus clusters.

Methods: Fecal samples from 58 infants, 31 of whom were exclusively BF and 27 of whom were exclusively FF with a standard formula in agreement with the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition recommendations, were analyzed by fluorescent in situ hybridization combined with flow cytometry. Principal component analysis was used to maximize the information gained for the predominant bacterial families and genus clusters using a minimal number of bacterial groups.

Results: The predominant detected group was *Bifidobacterium*, followed by Enterobacteriaceae and *Bacteroides* in both BF and FF infants. The *Lactobacillus* group was the only independent variable associated with FF infants. We also found that 3 principal components were sufficient to describe the association between the bacterial group, genus, and species studied in BF and FF infants; however, these components differed between BF and FF infants. For the former, the 3 factors found were *Bifidobacterium*/Enterobacteriaceae, *Lactobacillus*/*Bacteroides*, and *Clostridium coccoides*/*Atopobium*; for the latter, *Bifidobacterium*/Enterobacteriaceae, *Bacteroides* and *C coccoides* were observed.

Conclusions: There is a clear clustering of components of infant microbiota based on the feeding method.

Key Words: feeding methods, fluorescent in situ hybridization, infant fecal microbiota

(*JPGN* 2013;57: 461–466)

Microbiota plays an important role in human health and nutrition by preventing colonization of the gut by potential pathogenic microorganisms and preserving the health of the host through interactions with the immune system (1,2). The development of microbiota occurs primarily during infancy, and microbial dysbiosis has been linked to several disorders such as inflammatory bowel disease (3,4), irritable bowel syndrome (5), stomach cancer (6), mucosa-associated lymphoid tissue lymphoma (7), obesity (8,9), and necrotizing enterocolitis (10).

Bacterial colonization of the gastrointestinal tract is influenced by many factors such as infant diet (breast milk vs infant formula), mode of birth, perinatal antibiotics, mucosal maturation, age, country of birth, and geographical origin (1,11). Given the importance of the intestinal microbiota and its effect on infant health status, there is a considerable interest in determining the microbiota composition. It is well known that breast-fed (BF) infants differ widely in their microbiota compared to formula-fed (FF) infants (12,13). FF infants develop a complex fecal microbiota with higher levels of the facultative anaerobes *Bacteroides* and *Clostridium* than BF infants. The predominance of *Bifidobacterium* is also common in FF infants, although in lower number and frequency than BF infants of the same age (12,13); however, recent metagenomics studies have raised questions about the actual levels of *Bifidobacterium* (14,15).

The aim of this study was to determine the fecal microbiota in both BF and FF infants using fluorescent in situ hybridization combined with flow cytometry (FISH-FC), which allows the precise evaluation of living bacteria. In addition, the present work was undertaken to show that the main components of fecal microbiota explain the variability in BF and FF infants as a result of variables that include different bacterial groups, genus, and species as detected by FISH-FC.

Received December 19, 2012; accepted May 23, 2013.

From the *Department of Biochemistry and Molecular Biology II, the †Department of Microbiology, Institute of Nutrition and Food Technology ''Jose Mataix'' Biomedical Research Center, University of Granada, Armilla, Granada, the ‡Department of Food Science and Nutrition, Veterinary Faculty, University of Murcia, the §Hero Global Technology Center for Infant Nutrition, Hero Group, Alcantarilla, and the ||Pediatric Service, Virgen de la Arrixaca Hospital, Murcia, Spain.

Address correspondence and reprint requests to Prof Angel Gil, Instituto de Nutricion y Tecnologia de los Alimentos ''Jose Mataix'' (INyTA), Centro de Investigacion Biomedica (CIBM), Universidad de Granada, Avda. del Conocimiento s/n, 18100 Armilla, Granada, Spain (e-mail: agil@ugr.es).

This study was supported by the Spanish Plan Nacional de I+D+I through the projects Consolider Ingenio 2010 Programme (Ref. FUN-C-FOOD CSD2007–0623), AGL-2007–63504 and the Fundacion Empresa Universidad de Granada (FEUGR) contract no. 3318 with HERO Spain S.A. The present work is part of a project titled ''Evaluation of the Bifidogenic Effect of a Modified Starting Infant Milk Formula Versus a Standard One and Human Milk.''

C.G.L. is a recipient of a postdoctoral fellowship from Plan Propio of the University of Granada. R.M-S. and M.I.V-M., who participated in the clinical trial, are members of the Department of Research & Development at the Hero Institute for Infant Nutrition. This institute forms part of the food company HERO with headquarters in Switzerland.

The authors report no conflicts of interest.

DOI: 10.1097/MPG.0b013e31829d519a

TABLE 1. Baseline demographic data of infants involved in the study

	Sex		Gestation age, wk		Birth weight, kg	
	Boys	Girls	Mean	SD	Mean	SD
Breast-fed infants, n = 31	17	11	39.3	1.2	3.3	0.4
Formula-fed infants, n = 27	10	17	39.2	1.4	3.1	0.4

SD = Standard deviation.

METHODS

Subjects

Fifty-eight healthy infants born at the Neonatology Unit of University Hospital Virgen de la Arrixaca (Murcia, Spain) were selected: 31 were exclusively BF and 27 were exclusively FF. Based on the variance of the main outcome variable, that is, *Bifidobacterium* percentage in feces as determined by FISH-FC for Spanish infants (1), and assuming a type 1 error of $\alpha = 0.05$ and a power of 80% ($\beta = 0.2$), the minimum number of subjects per group was 30. Initially, we recruited 32 BF infants and 28 FF infants, but the number of subjects decreased to 31 and 27, respectively, because of fecal samples that were not fixed properly. The infant formulas used were in accordance with the recommendations of the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. The inclusion criteria were as follows: birth at full term (40 ± 2 weeks' gestation), normal birth weight (≥ 2.5 kg), and vaginal delivery. None of the infants received antibiotics during the study period. Table 1 shows the demographic characteristics of the infants involved in the study.

This study was conducted according to the guidelines of the Declaration of Helsinki, and all procedures involving human subjects were approved by the ethical committees on clinical research from Hospital Virgen de la Arrixaca. All parents gave their written informed consent to participate in the study.

Sample Collection

Fecal samples were collected from each infant at 12 weeks of age. Freshly soiled diapers were placed under anaerobic conditions at 4°C for a maximum of 4 hours before processing for cell fixation (2). Aliquots of 1 g of feces samples were added to 9 mL of phosphate-buffered saline (PBS). The suspension was mixed to complete homogeneity, and 0.2 mL of the suspension was added to 0.6 mL of 4% paraformaldehyde in PBS. After an overnight incubation at 4°C, the suspensions fixed in paraformaldehyde were stored at −80°C (1).

FISH-FC Analysis

Fecal bacteria populations were assessed by FISH-FC analysis as described by Fallani et al (1). In brief, 400 μL of the fixed suspension was mixed with 600 μL of PBS. Before hybridization, the cells were pelleted and resuspended in a volume of 1 mL. After washing in Tris-ethylenediaminetetraacetic acid buffer, the pellets were resuspended in Tris-ethylenediaminetetraacetic acid buffer containing 1 mg/mL of lysozyme and incubated for 10 minutes at room temperature. The cells were then washed in PBS and equilibrated in the hybridization solution. A 50-μL aliquot of this suspension was used for FISH-FC with control- and group-specific probes (ThermoFisher, Hesse, Germany). The EUB 338 probe was used as a positive control for hybridization, and the NON 338 probe was used as a negative control (1,16,17). These oligonucleotide probes were covalently linked at their 5′ end with either 6-FAM or Cy5 (ThermoFisher). Similar to Fallani et al (1), a panel of 10 group- and species-specific probes covalently linked with Cy5 at their 5′ end was used to assess the microbiota composition (Table 2) (18–25).

Hybridization was performed in a 96-well microtiter plate overnight at 35°C in the hybridization solution containing 4 ng/μL of the appropriate probes. Following hybridization, 150 μL of hybridization solution was added to each well, and cells were pelleted and washed to remove any nonspecific binding of the probe by incubating the bacterial cells at 37°C for 20 minutes in the washing solution. Finally, the cells were pelleted and resuspended in PBS. The samples were analyzed in a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ) through the instrumental scientific service of the University of Granada as described by Fallani et al (1). Enumeration of different bacterial families, genus, clusters, or species was performed by FISH-FC

TABLE 2. Panel of probes used for in situ determination of infant gut microbiota

Probes	Sequence from 5′ to 3′ end	Targeted groups	Ref
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* genus	(18)
Bac303	CCAATGTGGGGACCTT	*Bacteroides* group	(19)
Enter1432	CTTTTGCAACCCACT	Enterobacteriaceae	(20)
Str493	GTTAGCCGTCCCTTTCTGG	*Streptococcus* group	(21)
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus group*	(22)
Ato291	GGTCGGTCTCTCAACCC	*Atopobium* cluster	(23)
Erec482	GCTTCTTAGTCARGTACCG	*Clostridium coccoides* group	(21)
Clep866	GGTGGATWACTTATTGTG	*Clostridium leptum* group	(24)
Cpef191	GCTCCTTTGGTTGAATGATG	*Clostridium perfringens*	(25)
Cdif198	TCCATCCTGTACTGGCTCACC	*Clostridium difficile*	(25)

combining a specific probe labeled with Cy5 together with the EUB 338 6-FAM probes in the same tube.

Statistical Analysis

Data are expressed as the mean and standard error of the mean for the proportions of cells that were hybridized with each of the 10 oligonucleotide probes relative to the total bacteria (1). Paired *t* tests were used to compare any differences in variables (bacterial groups) detected in the feces of BF or FF infants.

Principal component analysis was used to maximize the information gained for the predominant bacterial families and gene clusters using a minimal number of bacterial groups. This mathematical model calculates new variables (principal components) that account for the variability in the data and enables the study of covariances or correlations between variables (bacterial groups). The combination of bacterial groups with the greatest amount of variability is the first principal component. The subsequent components (second and third principal components) describe the maximum amount of remaining variability (26,27). The data from each bacterial group were transformed into the arc cos square root of the percentage of that particular bacterial group. Extraction of the initial set of uncorrelated components was accomplished with the principal factor method and then the orthogonal rotation of components was used to facilitate interpretation. Factor loading was used to interpret the factor structure. Loadings are equivalent to Pearson correlation coefficients, and a higher loading indicates a stronger relation between a factor and an observed variable (27). Strong loading was defined as a value ≥ 0.6, and marginal loading as a value from 0.2 to 0.4. To evaluate the bacterial variables that were independently related to feeding modality (BF vs FF), we performed a binary logistic regression. All of the analyses were performed using the statistical package SPSS (SPSS Inc, Chicago, IL).

RESULTS

Fecal samples from healthy infants who had been exclusively BF or FF were collected at 12 weeks of age. The microbiota was analyzed by FISH-FC. In general, the predominant group detected was *Bifidobacterium*, followed by Enterobacteriaceae and *Bacteroides*. BF infants presented significantly lower proportions of *Atopobium* cluster ($P = 0.0001$), *Lactobacillus* group ($P = 0.004$), *C leptum* group ($P = 0.015$), and *Streptococcus* group ($P = 0.048$) compared with those of FF infants (Table 3). No significant differences were observed for the rest of the bacterial groups.

Regarding the principal component analysis results, 3 principal components were sufficient to explain the correlations among bacterial groups in both BF and FF infants. The 3 components explained 65.4% and 64% of the total variance in BF and FF infants, respectively. Those components were different for the BF and FF infants: *Bifidobacterium*/Enterobacteriaceae, *Lactobacillus*/*Bacteroides*, and *C coccoides*/*Atopobium* for BF infants (Table 4) and *Bifidobacterium*/*Enterobacteriaceae*, *Bacteroides*, and *C coccoides* for the FF infants (Table 5). The components were named according to the main group associated with BF or FF infant microbiota.

In BF infants, 29.9% of the variance was explained by the *Bifidobacterium*/Enterobacteriaceae component, 18.7% by the *Lactobacillus*/*Bacteroides* component, and 16.8% by the *C coccoides*/*Atopobium* component (Table 4). Similarly, in FF infants, the *Bifidobacterium*/Enterobacteriaceae component explained 29.2% of the variance, and 19.9% and 14.9% were explained by the *Bacteroides* and *C coccoides* components, respectively (Table 5).

Based on a binary logistic regression, we found that the *Lactobacillus* group was the only independent variable associated with fecal microbiota in FF infants (odds ratio 7.43, 95% confidence interval 2.00–27.64; $P = 0.003$).

DISCUSSION

In this study, the evaluation of the fecal microbiota using FISH-FC showed that the *Bifidobacterium* genus was the predominant group detected followed by Enterobacteriaceae and *Bacteroides* in both BF and FF infants. These results are in agreement with older data based on classic culture methods (13) and with those obtained recently by Turroni et al (28) and Yatsunenko et al (29), which revealed a predominance of bifidobacteria in the infant gut based on pyrosequencing data. Indeed, our data do not support the results of Palmer et al (14), who reported a low level of bifidobacteria in infant microbiota using 16S rRNA gene hybridization microarrays.

It is well known that bifidobacteria can be underrepresented when using genomic techniques for determination because DNA isolation can be affected by their thick cell walls (30). The paucity of bifidobacteria recently described can also be because of inefficient PCR analysis (28,31). Therefore, in the present study, we used FISH analysis, which does not require cell lysis, to detect bacteria with known DNA sequences with a sensitivity of $\geq 10^3$/g of feces (13). In fact, Fallani et al (1) used FISH-FC to show that the

TABLE 3. Comparison of the proportion of bacterial groups detected in fecal samples of breast-fed or formula-fed infants by fluorescent in situ hybridization and flow cytometry with a panel of 10 oligonucleotide probes

	Breast-fed infant, n = 31		Formula-fed infant, n = 27	
Targeted groups	Mean	SEM	Mean	SEM
Bifidobacterium genus (Bif164)	58.2	5.7	48.4	3.9
Enterobacteriaceae (Enter1432)	22.0	5.4	12.9	2.5
Streptococcus group (Strc493)	0.8	0.5	2.1*	0.4
Clostridium perfringens (Cpef191) + *Clostridium difficile* (Cdif198)	4.3	0.7	6.3	1.0
Clostridium leptum group (Clep1156)	0.5	0.2	2.4*	0.7
Lactobacillus group (Lab158)	0.5	0.1	2.3*	0.6
Bacteroides group (Bac303)	12.6	2.8	17.9	2.6
Atopobium cluster (Ato291)	0.3	0.1	7.7**	1.8
Clostridium coccoides group (Erec482)	0.9	0.9	0.1	0.0

SEM = standard error of the mean.
* Significant difference at $P < 0.05$.
** Significant difference at $P < 0.001$.

TABLE 4. Principal component analysis of breast-fed infants according to bacterial groups, genus, and species, as detected by fluorescent in situ hybridization and flow cytometry

	Component		
Targeted groups	*Bifidobacterium*/ Enterobacteriaceae	*Lactobacillus*/ *Bacteroides*	*Clostridium coccoides*/*Atopobium*
Bifidobacterium genus (Bif164)	−0.9	0.2	—
Enterobacteriaceae (Enter1432)	0.9	—	—
Streptococcus group (Strc493)	0.6	0.5	—
Clostridium perfringens (Cpef191) + *Clostridium difficile* (Cdif198)	0.5	0.3	−0.2
Clostridium leptum group (Clep1156)	0.2	—	—
Lactobacillus group (Lab158)	—	0.8	−0.3
Bacteroides group (Bac303)	—	−0.6	−0.2
Atopobium cluster (Ato291)	—	0.6	0.8
Clostridium coccoides group (Erec482)	—	—	0.9
Percent			
Variance	29.9	18.7	16.8
Cumulative proportion of variance	29.9	48.6	65.4

Results are expressed in loading factors. Factor loading is the product-moment correlation between an observed variable and an underlying factor. Strong loading was defined as a value ≥0.6 and marginal loading as a value from 0.2 to 0.4.

intestinal microbiota of 6-week-old BF infants had significantly higher proportions of *Bifidobacterium* genus and lower proportions of *Bacteroides, C coccoides*, and the *Lactobacillus* group compared with that of FF infants. Similarly, we found lower proportions of the *Lactobacillus* group in the BF infants; however, we also found significant differences in the proportions of the *Atopobium* cluster, *C leptum*, and the *Streptococcus* group between BF and FF infants, with a higher difference for the *Atopobium* cluster. Moreover, other studies have also shown that fecal samples of FF infants harbor higher numbers of the *Atopobium* cluster compared with BF infants (32,33). In addition, the number of clostridia found in BF infants is lower and has been considered as the only bacterial group that can be predictive for FF infants (34). Indeed, Fallani et al described *C coccoides* as a potential indicator group for FF infants (1). Thus, we found a higher proportion of *C leptum* in FF infants; however, in our study, the only independent bacterial group associated with FF infants was the *Lactobacillus* group.

Based on our study, the low frequencies of the *Atopobium* cluster, *C leptum, Lactobacillus* group, and the *Streptococcus* group in feces may explain the lower incidence of diseases in BF infants. Studies based on smaller infant groups identified bifidobacteria associated with protection from allergy development (35,36), whereas *Clostridium* spp, including *C difficile*, were associated with increased risk (35–37); however, a large study found no association between colonization by any particular bacterial group and development of atopic eczema or specific IgE to food antigens (38). Our group has previously shown that infants with cow's-milk protein allergy exhibit significantly higher numbers of the *C coccoides* group and the *Atopobium* cluster in their gut microbiota (39).

TABLE 5. Principal component analysis of formula-fed infants according to bacterial groups, genus, and species, as detected by fluorescent in situ hybridization and flow cytometry

	Component		
Targeted groups	*Bifidobacterium*/ Enterobacteriaceae	*Bacteroides*	*Clostridium coccoides*
Bifidobacterium genus (Bif164)	−0.8	−0.5	—
Enterobacteriaceae (Enter1432)	0.8	−0.2	−0.3
Clostridium leptum group (Clep1156)	0.7	—	—
Lactobacillus group (Lab158)	0.5	−0.5	0.3
Streptococcus group (Strc493)	0.5	—	0.6
Clostridium coccoides group (Erec482)	−0.3	—	0.7
Clostridium perfringens (Cpef191) + *Clostridium difficile* (Cdif198)	0.3	−0.4	0.6
Bacteroides group (Bac303)	—	0.8	—
Atopobium cluster (Ato291)	—	0.6	—
Percent			
Variance	29.2	19.9	14.9
Cumulative proportion of variance	29.2	49.1	64

Results are expressed in loading factors. Factor loading is the product-moment correlation between an observed variable and an underlying factor. Strong loading was defined as a value ≥0.6 and marginal loading as a value from 0.2 to 0.4.

Another main finding of this study was the identification of 3 main components that explain the major part of the variability of an infant's intestinal microbiota. Microbiota of BF and FF infants at the age of 12 weeks can be primarily described by the *Bifidobacterium*/Enterobacteriaceae component. According to this component, a BF infant harboring high levels of *Bifidobacterium* genus will also have low levels of the Enterobacteriaceae and *Streptococcus* group. In this regard, Tsuji et al (40) have reported that *Bifidobacterium* population levels were negatively correlated with those of Enterobacteriaceae in healthy Japanese infants. Although in FF infants the first component is also named *Bifidobacterium*/Enterobacteriaceae, high levels of *Bifidobacterium* genus have indicated low levels of Enterobacteriaceae and *C leptum.*

As mentioned before, *Bifidobacterium* have been associated with protection against allergy. Indeed, low levels of *Bifidobacterium* have been associated with allergic status (41–43), but other studies have found no association (44,45). In addition, a reduction of *Bifidobacterium* diversity has also been associated with allergy (46); however, the literature shows numerous discrepancies related to the link between bifidobacterial species and allergy (47). Accordingly, Waligora-Dupriet et al (47) showed that the diversity in *Bifidobacterium* colonization was not related to allergic status in both allergic and nonallergic French infants. Moreover, they showed that the link between *Bifidobacterium* colonization and allergy-based diseases is complex and cannot be restricted to the role attributed to *Bifidobacterium* species (47). Hence, we hypothesized that the high levels of *Bifidobacterium* genus and the lower levels of the Enterobacteriaceae and *Streptococcus* group in BF infants, compared with those found in FF infants, may contribute to the lower prevalence of allergy in the former.

The second component shows that harboring low levels of *Bacteroides* indicates higher levels of the *Lactobacillus* group and *Atopobium* cluster in BF infants. Recently, Jost et al (48) reported that neonates, exclusively BF, harboring high levels of *Bifidobacterium* show lower levels of *Bacteroides* and vice versa. According to our results, BF infants with low levels of *Bacteroides* also have high levels of *Bifidobacterium* genus, but with a marginal loading factor (0.2). Differences between the 2 studies may exist because they studied only 7 children. In FF infants, the *Bacteroides* component shows that harboring high levels of the *Bacteroides* group also indicates the presence of high levels of *Atopobium* cluster. Likewise, in FF infants, there was a weak negative correlation between *Bifidobacterium* genus and *Bacteroides.*

Clostridia are generally recognized to be more prevalent in FF than in BF infants (13). The last component, *C coccoides/Atopobium*, reveals the positive association of *Atopobium* cluster and *C coccoides* in BF infants. Nevertheless, in FF infants, the *C coccoides* component indicated that higher proportions of the C *coccoides* group are associated with higher proportions of *C perfringens* plus *C difficile*, and the *Streptococcus* group.

Our study has some limitations. Although FISH-FC technology allows the detection of living bacteria with good sensitivity, we only studied 10 bacterial groups, genus, and species, whereas many other species are known to be present in human microbiota. Approximately 150 common species have recently been identified in human feces associated with 3 enterotypes (49,50).

In conclusion, we found a differential clustering of bacterial components for the infant gut microbiota based on the feeding method. Dysregulation of these bacterial clusters may be associated with disease. Further studies are clearly needed in this field to identify the main strains for each cluster and to determine the association of the clusters to specific diseases.

REFERENCES

1. Fallani M, Young D, Scott J, et al. Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *J Pediatr Gastroenterol Nutr* 2010;51:77–84.
2. Kirjavainen PV, Arvola T, Salminen SJ, et al. Aberrant composition of gut microbiota of allergic infants: a target of bifidobacterial therapy at weaning? *Gut* 2002;51:51–5.
3. Tamboli CP, Neut C, Desreumaux P, et al. Dysbiosis in inflammatory bowel disease. *Gut* 2004;53:1–4.
4. Sokol H, Pigneur B, Watterlot I, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci USA* 2008;105:16731–6.
5. Kassinen A, Krogius-Kurikka L, Mäkivuokko H, et al. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology* 2007;133:24–33.
6. Parsonnet J, Friedman GD, Vandersteen DP, et al. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med* 1991;325:1127–31.
7. Lecuit M, Abachin E, Martin A, et al. Immunoproliferative small intestinal disease associated with Campylobacter jejuni. *N Engl J Med* 2004;350:239–48.
8. Turnbaugh PJ, Ley RE, Mahowald MA, et al. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006;444:1027–31.
9. Delzenne NM, Neyrinck AM, Bäckhed F, et al. Targeting gut microbiota in obesity: effects of prebiotics and probiotics. *Nat Rev Endocrinol* 2011;7:639–46.
10. De la Cochetiere MF, Piloquet H, des Robert C, et al. Early intestinal bacterial colonization and necrotizing enterocolitis in premature infants: the putative role of Clostridium. *Pediatr Res* 2004;56:366–70.
11. Peso P, Martínez C, Ros G, et al. Assessment of intestinal microbiota of full-term breast-fed infants from two geographical locations. *Early Hum Dev* 2011;87:511–3.
12. Mountzouris KC, McCartney AL, Gibson GR. Intestinal microflora of human infants and current trends for its nutritional modulation. *Br J Nutr* 2002;87:405–20.
13. Adlerberth I, Wold AE. Establishment of the gut microbiota in Western infants. *Acta Paediatr* 2009;98:229–38.
14. Palmer C, Bik EM, DiGiulio DB, et al. Development of the human infant intestinal microbiota. *Plos Biol* 2007;5:e177.
15. Koenig JE, Spor A, Scalfone N, et al. Succession of microbial consortia in the developing infant gut microbiome. *Proc Natl Acad Sci USA* 2011;109 (suppl 1):4578–85.
16. Amann RI, Krumholz L, Stahl DA. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol* 1990;172:762–70.
17. Wallner G, Amann R, Beisker W. Optimizing fluorescent in situ hybridization with ribosomal-RNA-targeted oligonucleotide probes for flow cytometry identification of microorganisms. *Cytometry* 1993;14:136–43.
18. Langendijk PS, Schut F, Jansen GL, et al. Quantitative fluorescent in situ hybridization of *Bifidobacterium* spp. with genus specific 16S ribosomal-RNA targeted probes and its application in fecal samples. *Appl Environ Microbiol* 1995;61:3069–75.
19. Manz W, Amann R, Ludwig W, et al. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology* 1996;142:1097–106.
20. Sghir A, Gramet G, Suau A, et al. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. *Appl Environ Microbiol* 2000;66:2263–6.
21. Franks AH, Harmsen HJM, Raagns GC, et al. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group specific 16S rRNA-targeted oligonucleotide probes. *Appl Envioron Microbiol* 1998;64:3336–45.
22. Harmsen HJM, Elfferich P, Schut F, et al. A 16S rRNA-targeted probe for detection of *Lactobacilli* and *Enterococci* in faecal samples by fluorescent in situ hybridization. *Microb Ecol Health Dis* 1999;11:3–12.

23. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, et al. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr* 2000;30:61–7.
24. Lay C, Sutren M, Rochet V, et al. Design and validation of 16S rRNA probes to enumerate members of the *Clostridium leptum* subgroup in human faecal microbiota. *Environ Microbiol* 2005;7:933–46.
25. Fallani M, Rigottier-Gois L, Aguilera M, et al. *Clostridium difficile* and *Clostridium perfringens* species detected in infant faecal microbiota using 16S rRNA targeted probes. *J Microbiol Methods* 2006;67:150–6.
26. Marques RC, Bernardi JV, Dórea JG, et al. Principal component analysis and discrimination of variables associated with pre- and post-natal exposure to mercury. *Int J Hyg Enviorn Health* 2008;211:606–14.
27. Weiss R, Dziura J, Burgert TS, et al. Obesity and the metabolic syndrome in children and adolescents. *N Engl J Med* 2004;350: 2362–2374.
28. Turroni F, Peano C, Pass DA, et al. Diversity of bifidobacteria within the infant gut microbiota. *PloS One* 2012;7:e36957.
29. Yatsunenko T, Rey FE, Manary MJ, et al. Human gut microbiome viewed across age and geography. *Nature* 2012;486:222–8.
30. De Boer R, Peters R, Gierveld S, et al. Improved detection of microbial DNA after bead-beating before DNA isolation. *J Microbiol Methods* 2010;80:209–11.
31. Sim K, Cox MJ, Wopereis H, et al. Improved detection of bifidobacteria with optimized 16S rRNA-gene based pyrosequencing. *PloS One* 2012;7:e32543.
32. Bezirtzoglou E, Tsiotsias A, Welling GW. Microbiota profile in feces of breast-and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe* 2011;17:478–82.
33. Harmsen HJM, Wildeboer-Veloo ACM, Grijpstra J, et al. Development of 16S rRNA-based probes for the Coriobacterium group and the Atopobium cluster and their application for enumeration of Coriobacteriaceae in human feces from volunteers of different age groups. *Appl Environ Microbiol* 2000;66:4523–7.
34. Tannock GW. The acquisition of the normal microflora of the gastrointestinal tract. In: Gibson SAW, ed. *Human Health: The Contribution of Microorganisms.* London: Springer-Verlag; 1994:1–16.
35. Björkstén B, Sepp E, Julge K, et al. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol* 2001;108:516–20.
36. Sepp E, Julge K, Mikelsaar M, et al. Intestinal microbiota and immunoglobulin E responses in 5-year-old Estonian children. *Clin Exp Allergy* 2005;33:1141–6.
37. Penders J, Thijs C, Van den Brandt PA, et al. Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. *Gut* 2007;56:661–7.
38. Adlerberth I, Stracahn DP, Matricardi PM, et al. Gut microbiota and development of atopic eczema in 3 European birth cohorts. *J Allergy Clin Immunol* 2007;120:343–50.
39. Thompson-Chagoyan OC, Fallani M, Maldonado J, et al. Faecal microbiota and short-chain fatty acid levels in faeces from infants with cow's milk protein allergy. *Int Arch Allergy Immunol* 2011;156:325–32.
40. Tsuji H, Oozeer R, Matsuda K, et al. Molecular monitoring of the development of intestinal microbiota in Japanese infants. *Benef Microbes* 2012;1:113–25.
41. Watanabe S, Narisawa Y, Arase S, et al. Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. *J Allergy Clin Immunol* 2003;111:587–91.
42. Mah KW, Bjorksten B, Lee BW, et al. Distinct pattern of commensal gut microbiota in toddlers with eczema. *Int Arch Allergy Immunol* 2006; 140:157–63.
43. Sepp E, Julge K, Mikelsaar M, et al. Intestinal microbiota and immunoglobulin E responses in 5-year-old Estonian children. *Clin Exp Allergy* 2005;35:1141–6.
44. Penders J, Stobbering EE, Thijs C, et al. Molecular fingerprints of the intestinal microbiota of infants in whom atopic eczema was or was not developing. *Clin Exp Allergy* 2005;35:741–5.
45. Songjinda P, Nakayama J, Tateyama A, et al. Differences in developing intestinal microbiota between allergic and non-allergic infants: a pilot study in Japan. *Biosci Biotechnol Biochem* 2007;71:2338–42.
46. Stsepetova J, Sepp E, Julge K, et al. Molecularly assessed shifts of *Bifidobacterium* ssp. and less diverse microbial communities are characteristic of 5-year-old allergic children. *FEMS Immunol Med Microbiol* 2007;51:260–9.
47. Waligora-Dupriet AJ, Campeotto F, Romero K, et al. Diversity of gut *Bifidobacterium* species is not altered between allergic and non-allergic French infants. *Anaerobe* 2011;17:91–6.
48. Jost T, Lacroix C, Braegger CP, et al. New insights in gut microbiota establishment in healthy breast fed neonates. *PloS One* 2012;7:e44595.
49. Arumugam M, Raes J, Pelletier E, et al. Enterotypes of the human gut microbiome. *Nature* 2011;473:174–80.
50. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010;464:59–65.

TABLA 2. Resistencia antibiótica

Culture media	Capsule	Time (t)	
		t_1	t_2
TSA+Ampicillin (2 µg/mL)	Placebo	1.45 ± 0.99	3.02 ± 0.43
	L. rhamnosus	4.11 ± 0.85	4.08 ± 0.04
	B. breve	0.50 ± 0.13	0.34 ± 0.18
	B. breve plus *L. rhamnosus*	3.36 ± 0.89	3.07 ± 0.33
	L. paracasei	5.79 ± 0.51	6.07 ± 0.55
TSA+Ampicillin (4 µg/mL)	Placebo	0.15 ± 0.08	0.13 ± 0.01
	L. rhamnosus	0.05 ± 0.02	0.01 ± 0.01
	B. breve	0.06 ± 0.01	0.02 ± 0.02
	B. breve plus *L. rhamnosus*	0.22 ± 0.12	0.22 ± 0.01
	L. paracasei	0.54 ± 0.14	0.37 ± 0.09
MRS+Ampicillin (2 µg/mL)	Placebo	8.30 ± 2.60	7.96 ± 1.67
	L. rhamnosus	5.32 ± 3.81	3.65 ± 0.83
	B. breve	2.24 ± 1.42	1.36 ± 1.13
	B. breve plus *L. rhamnosus*	5.12 ± 1.90	6.64 ± 2.85
	L. paracasei	5.67 ± 1.07	4.62 ± 1.89
MRS+Ampicillin (4 µg/mL)	Placebo	4.65 ± 3.27	5.05 ± 1.10
	L. rhamnosus	3.34 ± 1.63	2.55 ± 0.71
	B. breve	2.30 ± 1.67	2.29 ± 0.60
	B. breve plus *L. rhamnosus*	5.45± 2.21	4.28 ± 1.56
	L. paracasei	5.87 ± 0.47	4.26 ± 1.82
MRSC+Ampicillin (2 µg/mL)	Placebo	0.050 ± 0.030	0.019 ± 0.017
	L. rhamnosus	0.012 ± 0.010	0.012 ± 0.001
	B. breve	0.002 ± 0.003	0.002 ± 0.003

	B. breve plus *L. rhamnosus*	0.018 ± 0.012	0.014 ± 0.002
	L. paracasei	0.009 ± 0.002	0.004 ± 0.003
MRSC+Ampicillin (4 µg/mL)	Placebo	0.043 ± 0.033	0.033 ± 0.028
	L. rhamnosus	0.009 ± 0.001	0.010 ± 0.001
	B. breve	0.002 ± 0.003	0.001 ± 0.001
	B. breve plus *L. rhamnosus*	0.010 ± 0.006	0.013 ± 0.002
	L. paracasei	0.005 ± 0.001	0.003 ± 0.001
TSA+Tetracycline (4 µg/mL)	Placebo	0.28 ± 0.08	0.16 ± 0.05
	L. rhamnosus	0.15 ± 0.07	0.19 ± 0.02
	B. breve	0.02 ± 0.01	0.01 ± 0.01
	B. breve plus *L. rhamnosus*	0.09 ± 0.04	0.03 ± 0.01
	L. paracasei	0.16 ± 0.05	0.20 ± 0.02
TSA+Tetracycline (8 µg/mL)	Placebo	0.19 ± 0.07	0.24 ± 0.04
	L. rhamnosus	0.07 ± 0.01	0.05 ± 0.01
	B. breve	0.02 ± 0.01	0.01 ± 0.01
	B. breve plus *L. rhamnosus*	0.08 ± 0.02	0.04 ± 0.01
	L. paracasei	0.09 ± 0.02	0.12 ± 0.01
MRS+Tetracycline (4 µg/mL)	Placebo	1.95 ± 0.68	2.94 ± 0.72
	L. rhamnosus	2.81 ± 2.12	1.57 ± 0.91
	B. breve	4.01 ± 2.37	2.51 ± 1.81
	B. breve plus *L. rhamnosus*	8.79 ± 3.14	6.31 ± 1.49
	L. paracasei	2.79 ± 1.70	3.27 ± 1.18
MRS+Tetracycline (8 µg/mL)	Placebo	1.83 ± 0.99	0.85 ± 0.25
	L. rhamnosus	2.66 ± 2.38	0.93 ± 1.10
	B. breve	1.97 ± 1.72	1.79 ± 0.25
	B. breve plus *L. rhamnosus*	7.89 ± 2.36	6.45 ± 1.51

	L. paracasei	2.15 ± 1.12	2.21 ± 0.74
MRSC+Tetracycline (4 µg/mL)	Placebo	0.06 ± 0.04	0.03 ± 0.02
	L. rhamnosus	0.05 ± 0.02	0.01 ± 0.01
	B. breve	0.002 ± 0.001	0.001 ± 0.001
	B. breve plus *L. rhamnosus*	0.024 ± 0.0.22	0.014 ± 0.000
	L. paracasei	0.006 ± 0.001	0.003 ± 0.002
MRSC+Tetracycline (8 µg/mL)	Placebo	0.053 ± 0.029	0.027 ± 0.015
	L. rhamnosus	0.015 ± 0.013	0.012 ± 0.002
	B. breve	0.002 ± 0.004	0.002 ± 0.002
	B. breve plus *L. rhamnosus*	0.018 ± 0.013	0.014 ± 0.002
	L. paracasei	0.001 ± 0.001	0.001 ± 0.001

Values are means ± SEM, in percentages of resistant bacteria. n=3 per group. Means within a group without a common letter differ significantly. $P<0.05$. t_1, first washout; t_2, intervention. MRS, Man-Rogosa-Sharpe; MRSC, Man-Rogosa-Sharpe with cysteine; TSA, Tryptone soy agar.