TESIS DOCTORAL

EL SUEÑO DE ELIE METCHNIKOFF: EVALUACIÓN FUNCIONAL DE NUEVAS CEPAS DE PROBIÓTICOS

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EL SUEÑO DE ELIE METCHNIKOFF: EVALUACIÓN FUNCIONAL DE NUEVAS CEPAS DE PROBIÓTICOS

Memoria que presenta la bioquímica Mª Paz Díaz-Ropero Medina para aspirar al grado de Doctor en Bioquímica por la Universidad de Granada.

Lda. Mª Paz Díaz-Ropero Medina

Los directores de esta Tesis Doctoral, Dr. Jordi Xaus Pey, Doctor en Biología por la Universidad de Barcelona, del Departamento de Biomedicina de Puleva Biotech, S.A. (Granada) y de la Dra. Mónica Olivares Martín, Doctora en Farmacia por la Universidad de Granada, del Departamento de Biomedicina de Puleva Biotech, S.A. (Granada)

CERTIFICAN que los trabajos que se exponen en esta memoria de Tesis Doctoral: "El sueño de Elie Metchnikoff: Evaluación funcional de nuevas cepas de probióticos" han sido realizados en el Departamento de Biomedicina de Puleva Biotech S.A., bajo la tutela del Dr. Fermín Sánchez de Medina, del departamento de Bioquímica y Biología Molecular de la Facultad de Farmacia de Granada, en el Departamento de Farmacología de la Facultad de Farmacia de Granada y en el Departamento de Nutrición, Bromatología y Tecnología de los alimentos de la Universidad Complutense de Madrid, correspondiendo fielmente a los resultados obtenidos. La presente memoria ha sido revisada por nosotros, encontrándola conforme para ser presentada y aspirar al Grado de Doctor en Bioquímica por el tribunal que en su día se designe.

Dr. Jordi Xaus Pey

Dra. Mónica Olivares Martín

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Antonio Muñoz

"Nunca olvides tus ilusiones"

D-R Escribano

A mi madre y a Jesús

A mi Pacezurra

A mi padre

Índice

Abreviaturas

AGCC: Ácidos grasos de cadena corta

BAL: Bacterias ácido lácticas

CE: Comisión Europea

CECT: Colección Española de Cultivos Tipo

CEE: Comunidad Económica Europea

DE: Desviación estándar

DO: Densidad óptica

EDTA: Etilen-diamino tetraacetic acid (ácido etilen-diamino tetraacético)

EFSA: European Food Safety Authority (Agencia Europea de Seguridad Alimentaria)

FAO: Food and Agriculture Organization (Organización de las Naciones Unidas para la agricultura y la alimentación)

GALT: Gut-Associated Lymphoid Tissue (Tejido linfoide asociado a la mucosa intestinal)

GRAS: Generally Recognized As Safe (Reconocido generalmente como seguro)

IBD: Inflammatory bowel disease (Enfermedad inflamatoria intestinal)

LGG: Lactobacillus rhamnosus GG

LPS: Lipopolisácarido

MPO: Mieloperoxidasa

MRS: Medio de cultivo de Man, Ragosa, Sharpe

NK: Natural Killer

OMS: Organización Mundial de la Salud

PCR: Polymerase Chain Reaction (reacción en cadena de la polimerasa)

RAPD: Randomly Amplified Polymorphic DNA (Amplificación al azar de DNA polimórfico)

rDNA: DNA ribosómico

RT-PCR: Reverse Transcription-polymersase Chain Reaction (reacción en cadena de la polimerasa de transcripción reversa)

SIDA: Síndrome de Inmunodeficiencia Adquirida

TGI: Tracto gastrointestinal

TLR: Toll-Like receptors (Receptores Tipo Toll)

TNBS: Ácido trinitro-benceno sulfónico

TNO: Nederlandse Organisatie voor Toegepast-Natuurwetenschappelijk onderzoek (Netherlands Organisation for Applied Scientific Research) Leiden (Holanda)

TSB: Tryptic soy broth culture medium

ufc: Unidades formadoras de colonias

WHO: World Health Organization (Organización mundial de la salud)

16S rDNA: Gen que codifica la fracción 16S del rRNA

16S rRNA: Fracción 16S (sububidad menor) del rRNA

Introducción

1. La microbiota del TGI

1.1. Composición y distribución

El aparato digestivo humano está formado por cinco partes bien diferenciadas: la cavidad oro-faríngea, el esófago, el estómago, el intestino delgado y el intestino grueso. Las tres últimas partes conforman el tracto gastrointestinal (TGI), con funciones de digestión, absorción, secreción y de barrera. Además, cada vez se reconoce más su importancia como órgano endocrino y constituye el mayor órgano del sistema inmunitario humano.

La mucosa del TGI humano está colonizada por una comunidad microbiana extremadamente compleja. De hecho, se estima que contiene aproximadamente 10^{14} células procariotas, cifra diez veces mayor que la suma de todas las células eucariotas del cuerpo humano.

La diversidad taxonómica de las bacterias intestinales ha sido objeto de numerosas investigaciones durante las últimas décadas. En los años 70 y 80, el estudio de la microbiota intestinal dependía de la continua mejora en los procedimientos de enriquecimiento y en los sistemas para la generación de ambientes anaerobios. Los aislados se identificaban y caracterizaban mediante la combinación de diversos ensayos fenotípicos. En un estudio pionero, Moore y Holdeman (1974) analizaron la microbiota fecal de 20 personas y, a pesar de que únicamente pudieron identificar 113 especies, estimaron que el número real podría ser superior a 400.

Efectivamente, los estudios subsiguientes revelaron la gran diversidad bacteriana existente en el TGI, y confirmaron la existencia de más de 400 especies. Los grupos dominantes identificados en esos estudios iniciales pertenecían a los géneros *Bacteroides*, *Bifidobacterium*, *Clostridium, Eubacterium, Fusobacterium, Lactobacillus, Peptostreptococcus, Ruminococcus* y *Streptococcus* (que incluía entonces el género *Enterococcus*). El empleo de técnicas de cultivo, las únicas disponibles hasta hace pocos años, parecía sugerir que entre el 85 y el 99% de los microorganismos del TGI pertenecía a un máximo de unas 40 especies (Drasar y Barrow, 1985; Moore y Holdeman, 1974). Recientemente, el interés por el estudio de

la microbiota asociada a distintas mucosas se ha visto reforzado con la aparición de técnicas molecures que facilitan la caracterización y el seguimiento de una enorme variedad de grupos microbianos, incluyendo poblaciones no cultivables. Con estas nuevas herramientas se pueden llegar a conocer incluso los componentes individuales de los diversos grupos y su estado fisiológico de actividad (Vaughan y col., 2000; 2002; 2005; Zoetendal y col., 2004). Mediante el análisis del rDNA 16S amplificadas directamente de heces humanas y su comparación con secuencias cultivables, se ha estimado que sólo un 30% de las especies detectadas se corresponden con especies previamente aisladas e identificadas con las técnicas clásicas de cultivo (Amann y col., 1995; Hayashi y col., 2002; Suau y col., 1999; Wilson y Blitchington, 1996).

La microbiota aumenta en cantidad y complejidad a medida que avanzamos por el TGI (figura 1.1). Así, en individuos sanos, la marcada acidez del ambiente estomacal (pH ~ 3) únicamente permite el desarrollo de estreptococos y lactobacilos (10^3 -10^4 ufc/g) y de algunas levaduras (Macy y col., 1978; Rastall, 2004; Tannock, 1995). Además, *Helicobacter pylori* también coloniza la mucosa gástrica de un porcentaje considerable de individuos, si bien se necesita la concurrencia de diversos factores para que cause manifestaciones clínicas (Lee y col., 1993). En el intestino delgado, los principales factores limitantes para el establecimiento de los microorganismos son los movimientos peristálticos y la secreción de jugos pancreáticos y biliares. Aquí, los niveles aumentan progresivamente, desde 10^4-10^5 ufc/g en el duodeno, donde nuevamente son mayoritarios los lactobacilos y los estreptococos (Rastall, 2004; Simon y Gorbach, 1984), hasta más de 10^8 ufc/g en la región distal del íleon. Las especies cultivables más numerosas en esta región son las bifidobacterias, enterobacterias, bacteroides y fusobacterias (Croucher y col., 1983; Nord y Kager, 1984).

En el intestino grueso, el pH está más próximo a la neutralidad, la velocidad de tránsito es mucho más lenta y las secreciones biliar y pancreática están mucho más diluidas. Por ello, no es de extrañar que el mayor número de bacterias en el TGI humano resida precisamente en este segmento, donde constituye entre el 35 y el 55% del volumen del contenido sólido (Stephen y Cummings, 1980). Además, existe un ambiente muy reductor y desprovisto de oxígeno por lo que la mayoría de las poblaciones son anaerobias estrictas y constituyen lo que se denomina la microbiota dominante, caracterizada por concentraciones del orden de 10^9-10^{12} ufc/g. Dentro de esta microbiota, el género *Bacteroides* es uno de los más abundantes (Tannock,

1995). También son dominantes otros microorganismos Gram-positivos no esporulados pertenecientes a los géneros *Eubacterium*, *Bifidobacterium*, *Peptostreptococcus* y *Ruminococcus* (Conway, 1995). Los bacilos Gram-positivos esporulados están representados esencialmente por los clostridios. En concentraciones inferiores aparecen poblaciones de bacterias anaerobias facultativas como enterobacterias, enterococos, lactobacilos y estreptococos, que constituyen la microbiota subdominante, con tasas comprendidas entre 10^5 y 10^8 ufc/g (Hagiage, 1994; Holzapfel y col., 1998) y que, a pesar de su menor número, pueden resultar esenciales para la homeostasis microbiana en el intestino grueso. Algunas levaduras también se encuentran formando parte de esta microbiota, aunque en concentraciones relativamente bajas (10^2-10^4 ufc/ml) (Rastall, 2004; Satokari y col., 2001; Tannock, 1995).

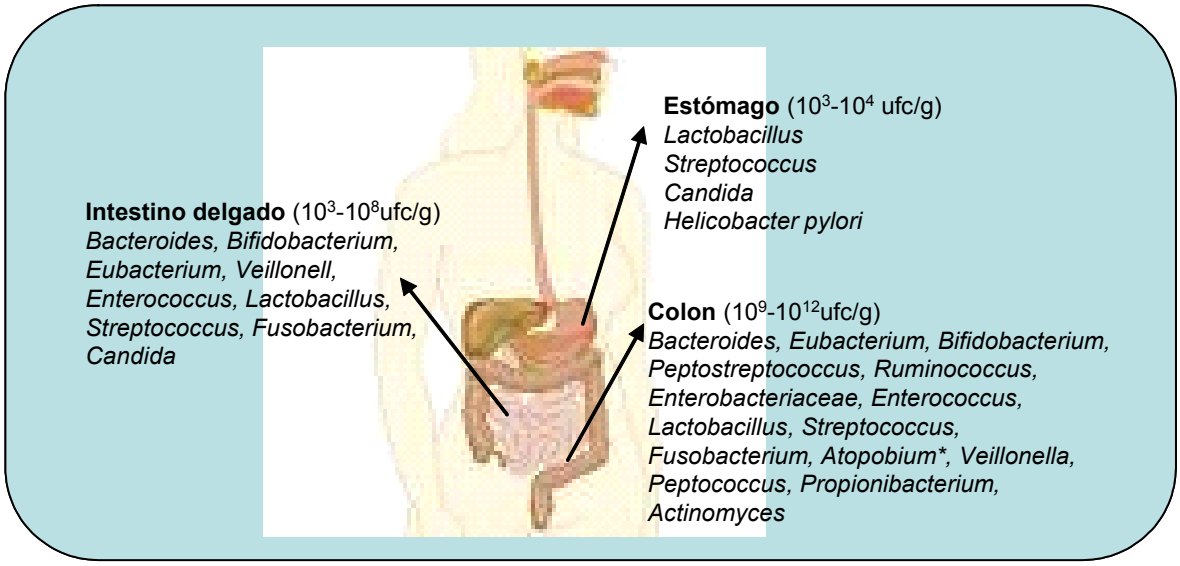

Figura 1.1: Niveles bacterianos en diferentes secciones del tracto gastrointestinal (*incluye *Atopobium*, *Coriobacterium*, *Eggherthella* y *Collinsella*).

1.2. Adquisición y evolución de la microbiota intestinal humana

La microbiota del TGI humano constituye un equilibrio dinámico que puede variar entre los grupos humanos, e incluso individualmente, debido a factores dependientes del hospedador como, por ejemplo, la base genética, la edad, el estado de salud, la dieta, el estrés o la administración de medicamentos y otras sustancias xenobióticas (Mitsuoka, 1992; Tannock, 1983; Toivanen y col., 2001;

Zoetendal y col., 2001). También, juegan un papel muy importante los factores ambientales y las interacciones que se establecen entre los distintos microorganismos (Finegold y col., 1983; Kleesen y col., 2000; Swift y col., 2000).

Desde los estudios clásicos de Tissier (1900) sobre la adquisición de la microbiota intestinal infantil, se ha aceptado la idea de que los fetos son estériles *in utero* y que la colonización bacteriana del intestino se inicia durante el tránsito por el canal del parto, por contaminación a partir de la microbiota vaginal e intestinal de la madre (Isolauri y col., 2001; Mackie y col., 1999; Tannock, 1995), a pesar de la ausencia de evidencias científicas. Según dicha hipótesis, la composición inicial de la microbiota intestinal estaría determinada fundamentalmente por el tipo de nacimiento y la alimentación del recién nacido (Adlerberth y col., 1991; Bettelheim y col., 1974; Brook y col., 1979; Grönlund y col., 1999; Harmsen y col., 2000a; MacGregor y Tunnessen, 1973; Mackie y col., 1999) e incluso por el contacto íntimo que se establece entre madre e hijo (Tannock, 1994). Por su parte, la principal fuente de bacterias para los niños nacidos por cesárea sería el ambiente hospitalario, incluyendo el instrumental, los equipos y la presencia de otros neonatos y del personal médico (Lennox-King y col., 1976a; 1976b).

Sin embargo, estudios muy recientes en los cuales nuestro grupo de investigación ha participado muy activamente, han demostrado la existencia de bacterias en muestras de líquido amniótico y sangre de cordón umbilical obtenidos de madres/neonatos sanos, en el meconio de niños nacidos tanto por parto como por cesárea, en el líquido amniótico de ratonas gestantes y en el intestino de sus correspondientes fetos (Bearfield y col., 2002; Buduneli y col., 2005; Jiménez y col., 2005; Martín y col., 2003; 2004). Paralelamente, otros autores han cuestionado la importancia del tránsito a través de la vagina, que tendría, en el mejor de los casos, un papel menor en la colonización bacteriana del neonato (Matsumiya y col., 2002). En este sentido, Tannock y col. (1990) observaron que la microbiota vaginal de la madre no se encuentra representada en la microbiota fecal de los recién nacidos. Más recientemente, la aplicación de técnicas moleculares al estudio de la posible transmisión vertical de lactobacilos presentes en la vagina de la madre reveló que únicamente una cuarta parte de los neonatos adquirían lactobacilos maternos en el momento del nacimiento y que, incluso en tales casos, esos lactobacilos no llegaban a colonizar persistentemente el intestino del neonato ya que eran rápidamente reemplazados por otros lactobacilos (Matsumiya y col., 2002).

Precisamente, en estos últimos años también se ha puesto de manifiesto que la leche materna es una fuente constante de bacterias comensales para el intestino del lactante, encontrándose en concentraciones relativamente elevadas (>10^4 ufc/ml) y siendo particularmente rica en estreptococos, estafilococos y bacterias lácticas (Beasley y Saris, 2004; Heikkilä y Saris, 2003; Martín y col., 2003; Ng y col., 2004; Perez y col., 2007).

Los estudios citados anteriormente sugieren que, existe un flujo de bacterias lácticas desde el intestino de las mujeres sanas hacia el de los fetos y neonatos. Inicialmente, existiría un tránsito cuantitativamente pequeño de ciertas especies bacterianas a través de la placenta que permitiría su transferencia al intestino prenatal; posteriormente, se iniciaría una segunda fase en la que el intestino del niño recibiría cantidades mucho más elevadas de bacterias a través de la leche materna.

En las primeras semanas de vida, bacterias anaerobias facultativas (estreptococos, enterococos, estafilococos, lactobacilos, enterobacterias) colonizan el TGI del neonato. Parece significativo que se trate precisamente de los grupos bacterianos más representativos de la microbiota de la leche materna. Estas bacterias crearían un ambiente reductor favorable para la colonización de bacterias anaerobias (bifidobacterias, bacteroides y clostridios). Obviamente, la alimentación juega un papel determinante en la evolución de la microbiota intestinal. Así, en los niños alimentados con leche materna se suele observar una menor presencia de enterobacterias, estreptococos, bacteroides y clostridios, en beneficio de una mayor cantidad de bifidobacterias, al revés de lo que sucede en los alimentados con fórmulas (Favier y col., 2002; Fuller, 1991; Martín y col., 2000). En contraste, Penders y col. (2005) no detectaron diferencias cuantitativas entre las bifidobacterias presentes en heces de lactantes y en las de niños alimentados con fórmulas infantiles. Por otra parte, se ha descrito una mayor concentración de bacterias del grupo *Coriobacterium* (*Coriobacterium* y *Collinsella*) en heces de niños alimentados con fórmulas infantiles (Harmsen y col., 2000b).

Cuando se inicia la fase de destete, se van introduciendo progresivamente alimentos sólidos y, paralelamente, se va reduciendo la ingesta de leche materna hasta su completa sustitución. Estas circunstancias producen grandes cambios en la composición de la microbiota intestinal infantil, de tal manera que, en poco tiempo, desaparecen las diferencias entre la microbiota de los lactantes y la de los alimentados con fórmulas (Mackie y col., 1999; Stark y Lee, 1982). En general, se

estima que los grupos microbianos dominantes en la microbiota intestinal de los niños de 2 años son similares a los de los adultos, aunque todavía existen diferencias en cuanto a las especies presentes (Favier y col., 2002). La evolución de la microbiota intestinal con la edad se muestra en la figura 1.2.

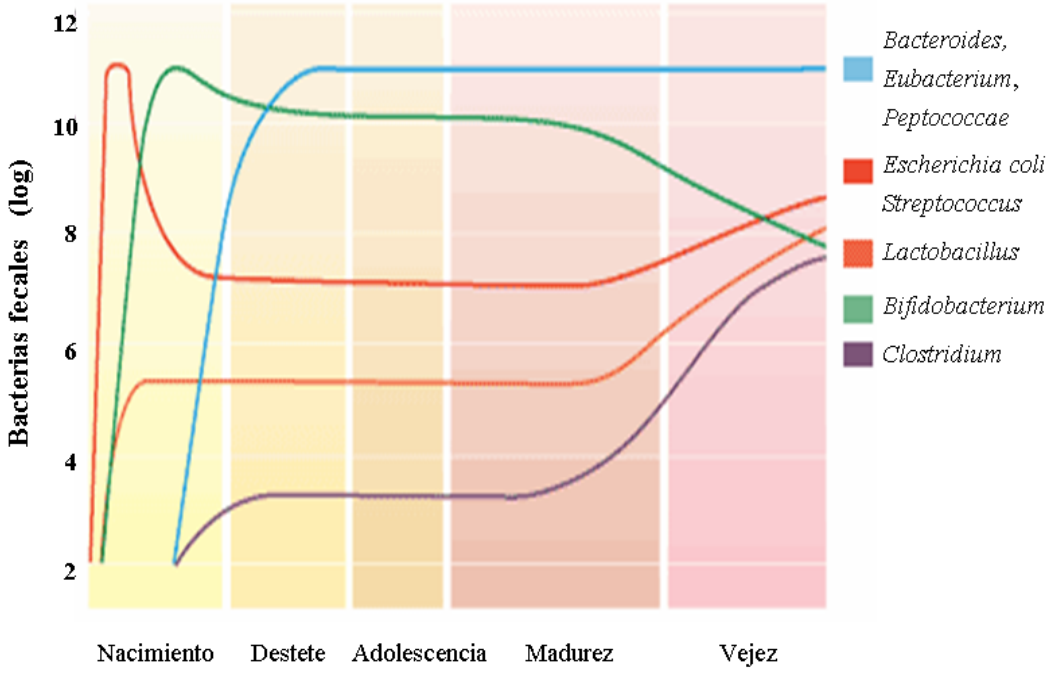

Figura 1.2: Evolución de la microbiota intestinal con la edad. Fuente: Blum y col. (2005).

En los últimos años, los estudios realizados aplicando técnicas moleculares sugieren que el 80% de las bacterias de la microbiota fecal de individuos adultos sanos pertenecen a 4 grupos filogenéticos: (1) *Bacteroides-Porphyromonas-Prevotella*, (2) *Clostridium coccoides-Eubacterium rectale*, (3) *Fusobacterium prausnitzii-Clostridium leptum*, y (4) *Bifidobacterium* (Doré, 2004). El número de clostridios aumenta a lo largo de la vida mientras que el de bifidobacterias disminuye. En ancianos es más frecuente el aislamiento de *C. difficile*, mohos y enterobacterias que en individuos jóvenes (Gorbach y col., 1967; Noack-Loebel y col., 1983). Aunque la asociación entre el tipo de dieta y los distintos grupos microbianos aún no está clara, la microbiota de comunidades occidentales (caracterizadas por una ingesta alta en grasa y proteínas de origen animal y un bajo contenido en fibra) parece contener mayores niveles de bacteroides y clostridios, y

menores de bacterias lácticas, en comparación con la de comunidades orientales (Benno y col., 1986; Hayashi y col., 2002). En cualquier caso, la microbiota intestinal parece ser específica de cada individuo y las poblaciones dominantes suelen permanecer estables a lo largo del tiempo (Franks y col., 1998; Moore y Moore, 1995; Simon y Gorbach, 1984; Zoetendal, y col., 1998; 2001).

La mayoría de los estudios sobre la microbiota del TGI se han realizado a partir de muestras de heces y, sólo ocasionalmente, se han analizado muestras de mucosa obtenidas mediante endoscopia o intervenciones quirúrgicas (Alander y col., 1997; 1999; Gibson y col., 1991; Zinkevich y Beech, 2000); incluso en estos casos, los resultados son difícilmente extrapolables a lo que puede suceder en individuos sanos, aunque parece claro que existen diferencias importantes entre la comunidad bacteriana asociada a la mucosa del colon y la presente en las heces (Ouwehand y col., 2004; Zoetendal y col., 2002).

1.3. Funciones de la microbiota intestinal humana

En los últimos años, se ha incrementado notablemente el interés por el estudio del complejo ecosistema microbiano del TGI humano (Tannock, 1999). Esto se debe a que las poblaciones microbianas ejercen una gran influencia sobre muchas características bioquímicas, fisiológicas e inmunológicas del hospedador en el que residen (Gill, 1998; Parodi, 1999; Salminen y col., 1998a), existiendo una relación cada vez más clara entre la microbiota intestinal y la salud (Guarner y Malagelada, 2003; Hart y col., 2002; Noverr y Huffnagle, 2004; Parodi, 1999).

La introducción de las técnicas moleculares ha revelado que aún no se conocen con precisión todos los microorganismos que integran las poblaciones intestinales, ni la importancia y el papel biológico con los que cada tipo contribuye a la salud del hospedador. Tampoco se conoce la importancia y el papel fisiológico de las poblaciones minoritarias que, sin duda, pueden ser determinantes en ciertas regiones del TGI o en situaciones específicas del hospedador (Cummings, 1997).

En general, la microbiota intestinal ejerce tres funciones básicas: metabólicas, interviniendo en la asimilación de nutrientes de la dieta; protectoras, contribuyendo al efecto barrera y al desplazamiento de microorganismos patógenos; y tróficas, interviniendo en el desarrollo y la proliferación celular (figura 1.3).

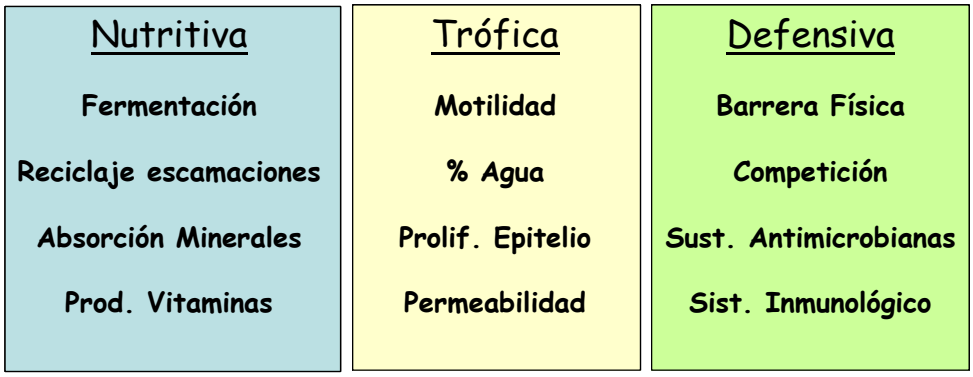

Figura 1.3: Funciones de la microbiota intestinal.

1.3.1. Funciones metabólicas

La fermentación de carbohidratos es la principal fuente de energía en el colon y se efectúa mayoritariamente en el colon proximal. Los diversos compuestos no digeribles, principalmente carbohidratos (25-60 g/día), que llegan a esta localización procedentes de la dieta constituyen los principales sustratos para la proliferación microbiana (Macfarlane y Cummings, 1991). La mayoría de estos carbohidratos son de origen vegetal (almidón resistente, celulosa, hemicelulosas, pectinas, inulina, oligosacáridos que escapan de la digestión, azúcares y alcoholes no absorbidos) (Cummings y col., 1987; 1996). Los productos metabólicos más importantes de este proceso fermentativo son los ácidos grasos de cadena corta (principalmente acetato, butirato y propionato), que tienen gran importancia en la fisiología del TGI. La producción de estos compuestos en el colon es de unos 200 mmol/día, de los cuales se excretan en heces entre 7 y 20 mmol/día. Además, también se producen gases (dióxido de carbono, metano e hidrógeno), etanol y ácido láctico.

Los ácidos grasos de cadena corta actúan directa e indirectamente sobre las células del epitelio intestinal y participan en el control de varios procesos metabólicos (Cummings y col., 2001). El butirato es metabolizado rápidamente por los enterocitos del colon, sirviéndoles como fuente de energía. Además, esta molécula es capaz de estimular la diferenciación celular y de disminuir el riesgo de cáncer de colon mediante la inducción de apoptosis de células tumorales (Avivi-Green y col., 2000; Buommino y col., 2000; Chai y col., 2000; Rowland, 1995). Por su parte, el acetato y el propionato llegan intactos al hígado a través de la vena

porta. El acetato se incorpora a los procesos de colesterogénesis y lipogénesis en el hígado, mientras que el propionato actúa como inhibidor competitivo del transportador de acetato hacia el interior de los hepatocitos. Este fenómeno parece contribuir a la disminución de la lipogénesis y colesterogénesis hepática (Delzenne y Williams, 2002). La producción fermentativa de altas concentraciones de propionato, explicaría la disminución de los niveles séricos y hepáticos de colesterol en animales de experimentación previamente alimentados con ciertos carbohidratos (Delzenne y Kok, 2001). Finalmente, la producción de acetato y propionato también interviene en la regulación del metabolismo hepático de la glucosa, reduciendo la glucemia postpandrial y la respuesta insulínica (Brighenti y col., 1995; Venter y col., 1990).

Los compuestos proteicos disponibles en el colon (5-20 g/día) incluyen proteínas no digeridas procedentes de la dieta, proteínas que forman parte del mucus, de bacterias lisadas o de los enterocitos muertos que se liberan al lumen intestinal como resultado de una regeneración (Quigley y Kelly, 1995). Los péptidos y proteínas se degradan mediante un proceso putrefactivo en el colon distal, originando ácidos grasos de cadena corta y también ácidos grasos de cadena ramificada, como el isobutirato, el metilbutirato o el isovalerato. Paralelamente, la degradación bacteriana de las proteínas puede generar algunos metabolitos que, en ciertas circunstancias, pueden ser perjudiciales para la salud, como amoniaco, aminas, indoles y fenoles (Macfarlane y col., 1986; Smith y Macfarlane, 1996).

En general, las grasas son digeridas casi totalmente, por lo que las cantidades que alcanzan el colon suelen ser pequeñas (3-4 g/día) y son metabolizadas parcialmente por la microbiota (Priebe y col., 2002). Por otra parte, las sales biliares secretadas al intestino delgado con la bilis durante la digestión, alcanzan el íleon distal donde son mayoritariamente reabsorbidas, volviendo al hígado por la circulación portal en el proceso que se conoce como "circulación enterohepática de las sales biliares" (Hofmann, 1984). Sin embargo, una fracción minoritaria aunque significativa de sales biliares no es absorbida y alcanza el intestino grueso, eliminándose con las heces. En el colon, las sales biliares pueden sufrir principalmente dos tipos de modificaciones por acción de la microbiota intestinal: una desconjugación de las formas conjugadas formando ácidos biliares primarios por acción de hidrolasas y, una deshidroxilación de los ácidos biliares primarios en la posición 7α dando lugar a los correspondientes ácidos biliares secundarios. Las hidrolasas de sales biliares (BSH, del inglés *Bile Salt Hydrolase*), responsables de la desconjugación, están presentes en muchas bacterias del TGI,

incluyendo lactobacilos (Lundeen y Savage, 1990) y bifidobacterias (Grill y col., 1995; Kim y col., 2004). Por el contrario, la hidrólisis del grupo hidroxilo en posición 7α del anillo esteroide se ha descrito básicamente en los géneros *Clostridium* y *Eubacterium* (Wells y Hylemon, 2000). Una excesiva desconjugación puede conducir a una malabsorción de grasas y vitaminas liposolubles, mientras que los ácidos biliares secundarios formados por deshidroxilación parecen estar implicados en la formación de cálculos biliares. Además, una elevada concentración de ácidos biliares secundarios en el intestino grueso incrementa el riesgo de padecer cáncer de colon (Nagengast y col., 1995).

La microbiota del colon también juega un papel importante en la síntesis de vitaminas como la biotina, la riboflavina, el ácido pantoténico, la piridoxina, la cianocobalamina o la vitamina K, un proceso en el que participan tanto bacterias Gram-negativas como Gram-positivas (Burgess y col., 2004; Conly y col., 1994; Hill, 1997; Quesada-Chanto y col., 1994; Sybesma y col., 2003). Además, favorecen la absorción de calcio, hierro y magnesio, gracias a la acción de los ácidos grasos de cadena corta (Miyazawa y col., 1996; Roberfroid y col., 1995; Younes y col., 2001).

1.3.2. Función protectora frente a infecciones

Quizás una de las funciones más relevantes de las bacterias residentes en el intestino es la regulación y establecimiento del ecosistema tanto intestinal como del resto de las mucosas, evitando la colonización por microorganismos exógenos, previniendo la proliferación de patógenos y oportunistas, constituyendo una primera barrera de defensa frente a infecciones.

Los mecanismos implicados en el efecto protector de la microbiota intestinal son muy variados, entre ellos, existe el fenómeno de competición no solo por el espacio sino también por los nutrientes disponibles entre los microorganismos ya instaurados y los patógenos. La adherencia de las bacterias al epitelio y mucus intestinal es una propiedad que depende de cada cepa, existiendo mecanismos de adhesión inespecíficos (gobernados por interacciones electrostáticas o hidrofóbicas) y específicos (interacciones ligando-receptor mediante polisacáridos exocelulares, proteínas y/o ácidos lipoteicoicos) (Charteris y col., 1998a). De este modo, las bacterias del TGI que poseen dicha capacidad formarían una película sobre el epitelio intestinal que impediría el contacto directo con microorganismos potencialmente patógenos (Bernet y col., 1994), fenómeno conocido como

"exclusión competitiva". Algunas bacterias del TGI también pueden inhibir la adhesión de ciertos patógenos a los enterocitos mediante el aumento de la producción de mucinas ileocolónicas, como MUC2 y MUC3 (Mack y col., 2003).

Otros mecanismos importantes en el efecto protector de la microbiota intestinal son la competencia por los nutrientes disponibles en el nicho ecológico (Hooper y col., 1999) y la producción de sustancias antimicrobianas. En este sentido, se ha descrito que, entre la microbiota fecal, existen bacterias que producen sustancias potencialmente antimicrobianas, como ácidos orgánicos, diacetilo, etanol, reuterina, reutericiclina, peróxido de hidrógeno o bacteriocinas (de Vuyst y col., 2004). La producción *in vitro* de sustancias antimicrobianas ha sido una propiedad particularmente investigada entre las bacterias lácticas, aunque prácticamente no existen estudios sobre su síntesis *in vivo* en el TGI.

Este fenómeno de competición se puede hacer patente: primero, con el uso de antibióticos de amplio espectro que minimizan de forma importante la microbiota, provocando infecciones por patógenos que en otras circunstancias quedarían aplacadas por la propia microbiota comensal; así, infecciones por *Clostridium dificile, E.coli, Pseudomonas* o *Candida* son relativamente comunes tras determinados tratamientos con antibióticos (Midvedt T, 1985) y segundo, en los modelos de animales *germ-free* (libres de gérmenes), donde un bajo número de bacterias patógenas, que en un individuo normal sería neutralizado sin problemas, provoca la infección e incluso la muerte (Baba y col., 1991; Taguchi y col., 2002).

1.3.3. Inmunomodulación

La mucosa intestinal constituye la mayor interfase entre el sistema inmunitario y el medio externo y, por lo tanto, no es de extrañar que la microbiota intestinal ejerza una influencia determinante en el desarrollo y maduración del tejido linfoide asociado a la mucosa intestinal (GALT, del inglés *Gut-Associated Lymphoid Tissue*). De hecho, el GALT representa el mayor órgano del sistema inmunitario humano (10^6 linfocitos/g), conteniendo entre el 80 y el 85% de las células inmunocompetentes de un individuo adulto sano (Brandtzaeg y col., 1989). Por otra parte, aproximadamente el 60% de las inmunoglobulinas producidas diariamente se secretan en el TGI (Salminen y col., 1998b). La característica más distintiva de las respuestas inmunitarias que se producen en las mucosas, es la producción de inmunoglobulinas A secretoras (Underdown y Schiff, 1986) que son secretadas por

las células plasmáticas y transportadas a través de la capa de células epiteliales hasta la luz intestinal, donde reaccionan con antígenos específicos; de este modo, impiden la interacción física de los agentes nocivos con la superficie de la mucosa y previenen las reacciones inflamatorias, que podrían dañar su integridad.

La respuesta inmunitaria adaptativa se desarrolla en los dos tipos de compartimentos presentes en el GALT (figura 1.4): (a) los tejidos linfoides organizados en forma de agregados, como los folículos asociados a las placas de Peyer y a los ganglios linfáticos mesentéricos; y (b) los tejidos linfoides difusos distribuidos en la lámina propia de la mucosa o en el epitelio intestinal (linfocitos intraepiteliales) (MacDonald, 2003; Mowat y Viney, 1997). El intestino delgado contiene alrededor de 250 placas de Peyer (agregados de 5 ó más folículos) y miles de folículos solitarios, un número que aumenta en el colon. Cada folículo se asocia a unas células especializadas, las células M, encargadas de transportar los antígenos para que contacten con las células inmunitarias (Blum y col., 2005). Una vez activados por el contacto con los antígenos, los linfocitos T y B proliferan en forma de clones antígeno-específicos y migran, primero a través de la linfa y posteriormente por el torrente sanguíneo a diferentes localizaciones extraintestinales, como el tracto respiratorio, el genitourinario o las glándulas secretoras, pudiendo regresar de nuevo a la lámina propia y al epitelio intestinal (Salvini y col., 2004).

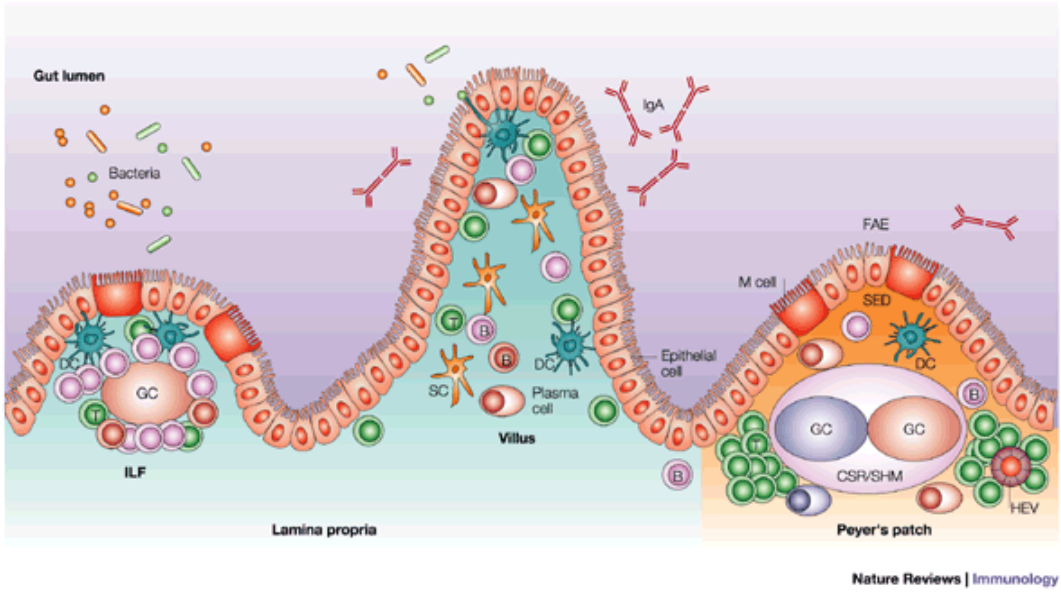

Figura 1.4: Organización GALT (Fagarasan y Honjo., 2003).

El establecimiento de la microbiota intestinal determina el desarrollo del sistema inmunitario (Grönlund y col., 2000), actuando como un regulador esencial en las respuestas inmunitarias (Noverr y Huffnagle, 2004). Los estudios realizados en animales neonatos y en animales axénicos (criados en condiciones de asepsia total) demuestran que, la interacción entre el epitelio y la microbiota es esencial para el desarrollo normal del sistema inmunitario, tanto humoral como celular. Se ha visto que los niños alérgicos exhiben una flora alterada en su composición con altos niveles de clostridios y bajos de bifidobacterias, contrastando con niños no alérgicos, colonizados con una importante cantidad de *B.bifidum,* típico de lactantes alimentados con leche materna (Kalliomäki y col, 2003). La colonización del intestino de mamíferos con nuevas especies bacterianas provoca un aumento en el número de células plasmáticas y en la producción de anticuerpos a lo largo del TGI (Butler y col., 2000; Helgeland y col., 1996; Umesaki y col., 1993) y, además, estimula la producción de células B antígeno-específicas en las placas de Peyer (Cebra y col., 1998). Por otra parte, en ausencia de microbiota intestinal, los animales presentan un sistema inmunitario intestinal infradesarrollado (Butler y col., 2000; Falk y col., 1998). Las continuas interacciones entre este sistema y la microbiota intestinal son las responsables del estado de "inflamación controlada" existente en el TGI, necesario para la generación rápida de una respuesta inflamatoria frente a patógenos (Neish y col., 2000). Este estado, debe ser compatible con la ausencia de respuestas inmunitarias (o respuestas de poca intensidad) frente a los antígenos que forman parte de los alimentos y de las bacterias no patógenas ya que, un estado de hipersensibilidad frente a la microbiota intestinal puede conducir a enfermedades inflamatorias crónicas, como la enfermedad de Crohn o la colitis ulcerosa (Lupp y Finlay, 2005; Shanahan, 2001).

Debido a la importancia y al gran número de funciones que ejerce la microbiota intestinal, los beneficios que se pueden derivar de la modificación de dicha microbiota han generado, desde hace décadas, un gran interés por el desarrollo de probióticos y prebióticos.

2. Probióticos

En los últimos años, la progresiva comprensión de las estrechas relaciones entre nutrición y salud ha permitido conocer el papel de ciertos alimentos (o de algunos de sus componentes) en la mejora de la salud y/o en la reducción del riesgo de enfermedad de los consumidores, más allá de los efectos atribuibles a su valor estrictamente nutritivo. De hecho, la salud se encuentra entre las tres primeras razones por las que los europeos eligen sus alimentos (Lappalainen y col., 1998), lo que, sin duda, ha favorecido el desarrollo de alimentos con un valor añadido para el consumidor, conocidos, en general, como "alimentos funcionales".

En Europa, el término "alimento funcional" no está claramente delimitado por una definición legal. No obstante, Goldberg (1994) ha propuesto una definición ampliamente aceptada hasta la fecha. Para este autor sería, cualquier alimento que tenga un impacto positivo, y diferenciado de su valor nutritivo, sobre la salud de un individuo. Se trataría de un alimento natural, o desarrollado a partir de ingredientes naturales, que se consumiría como parte de la dieta y que desempeñaría una función concreta en procesos tales como la mejora de los mecanismos biológicos de defensa frente a agentes nocivos, la prevención de enfermedades o el retraso del envejecimiento. En este contexto, los alimentos que contienen microorganismos probióticos representan el prototipo de alimento funcional, con un volumen de producción de más de 1 millón de Tm anuales, que se traducen en más de 120.000 millones de euros en ventas sólo en Europa (Buss, 2004).

2.1. Reseña histórica y definición

La modulación de la microbiota intestinal para mejorar la salud se ha efectuado empíricamente desde tiempos ancestrales, existiendo noticias del empleo de leche fermentada para el tratamiento de infecciones gastrointestinales ya en el año 76 a.c. (Stanton y col., 2005). A principios del siglo XX, se empezó a sugerir que la Humanidad no sólo había hecho uso inadvertido de una multitud de microorganismos para la elaboración y/o conservación de una parte sustancial de su suministro de alimentos, sino que además existían algunas bacterias que ejercían ciertos efectos beneficiosos para la salud de los hospedadores (Beijerinck, 1901;

Cahn, 1901; Moro, 1900). En 1907, Elie Metchnikoff publicó un libro que ejerció una gran influencia en la comunidad científica: *Prolongation of Life* (Metchnikoff, 1907). En él, Metchnikoff postulaba que las bacterias que intervenían en la fermentación del yogur contribuían al mantenimiento de la salud mediante la supresión de las "fermentaciones de tipo putrefactivo" de la microbiota intestinal y que ésta era la causa de la longevidad de los campesinos búlgaros, grandes consumidores de yogur.

Un año antes, Cohendy había administrado leche fermentada por *Lactobacillus bulgaricus* (actualmente *Lb. delbrueckii* subsp. *bulgaricus*) a pacientes con alteraciones en sus "fermentaciones intestinales", observando una notable mejoría tras 8-12 días de tratamiento (Cohendy, 1906a; 1906b). Paralelamente, Tissier (1906) no sólo había descubierto la existencia de bifidobacterias en el tracto intestinal de lactantes alimentados exclusivamente con leche materna, sino que había demostrado los beneficios clínicos derivados de la modulación de la microbiota intestinal de niños con infecciones intestinales. En 1909, Isaac Carasso fundó su primer establecimiento de yogures (Danone) en Barcelona, contribuyendo decisivamente al prestigio de un producto que durante varias décadas sólo se podía adquirir en farmacias y que se empleaba para prevenir o aliviar trastornos tan diversos como diarrea, estreñimiento, dispepsia, colitis mucosa, colitis ulcerativa crónica, disbiosis por antibioterapia, cistitis o dermatitis (Shortt, 1999a). Desde entonces, se han descrito y comercializado numerosas bacterias con propiedades probióticas.

Posiblemente, el término "probiótico" fue empleado por primera vez por Vergio (1954), cuando comparaba los efectos adversos ("*antibiotika*") que los antibióticos ejercían sobre la microbiota intestinal con las acciones beneficiosas ("*probiotika*") ejercidas por otros factores que no pudo determinar. Una década más tarde, Lilly y Stillwell (1965) se referían a los probióticos como aquellas sustancias secretadas por un microorganismo que estimula el crecimiento de otro, en contraposición al término "antibiótico", entendido como cualquier compuesto químico utilizado para eliminar o inhibir el crecimiento de organismos infecciosos. Fuller (1989) redefinió probióticos como "cualquier suplemento alimenticio vivo que beneficia al huésped mediante la mejora de su equilibrio microbiano intestinal". Más recientemente, la OMS los ha definido como "organismos vivos que ingeridos a dosis definidas ejercen efectos beneficiosos para la salud". Esta última definición es más amplia y tiene en cuenta los resultados de recientes investigaciones que demuestran

la existencia de efectos probióticos que no se restringen al ámbito intestinal (http://www.who.int/foodsafety). Recientemente, se ha propuesto que las bacterias inactivadas o alguno de sus componentes celulares también pueden ejercer ciertos efectos beneficiosos, aunque no al nivel de las células vivas (Isolauri y col., 2002; Ouwehand y Salminen, 1998).

Actualmente, existe una gran variedad de bacterias potencialmente probióticas entre las que podemos encontrar numerosas especies de lactobacilos y bifidobacterias, así como de otros géneros de bacterias lácticas y otros microorganismos (tabla 2.1).

Tabla 2.1: Principales especies microbianas utilizadas como probióticos

Lactobacilos	Bifidobacterias	Otras bacterias lácticas	No bacterias lácticas
Lb.johnsonii	*B.bifidum*	*E.faecium*	*P.freudenreichii*
Lb.casei	*B.breve*	*Str.thermophilus*	*Escherichia coli*
Lb.delbrueckii	*B.longum*		*Bacillus cereus*
Lb.rhamnosus	*B.lactis*		*S.cerevisae*
Lb.reuteri			
Lb.gasseri			
Lb.plantarum			

Fuente: Collins y col (1998), Fooks y col. (1999). *E.: Enterococcus; P.: Propionibacterium; S.: Saccharomyces; Str.: Streptococcus.*

Las bacterias lácticas y las bifidobacterias ocupan el lugar más destacado entre los microorganismos empleados con fines probióticos pero también se utilizan con este fin bacterias que pertenecen a otros géneros, como *Escherichia coli* y *Bacillus cereus*, y levaduras, principalmente *Saccharomyces cerevisiae* (Shortt, 1998; Vaughan y col., 2002) (tabla 2.1). Dentro de las bacterias lácticas, se incluyen los géneros *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus, Enterococcus,*

Streptococcus, Vagococcus, Weissela, Oenococcus, Atopobium, Alloicoccus, Aerococcus, Tetragenoccus y Carnobacterium (Holzapfel y Wood, 1995; Schleifer y Ludwig, 1995). Son bacilos o cocos Gram-positivos, generalmente catalasa negativos, no esporulados, inmóviles, y productores de ácido láctico como principal producto final de su metabolismo. El género *Bifidobacterium* no está relacionado filogenéticamente con las bacterias lácticas pero comparte con ellas diversas propiedades fisiológicas, bioquímicas y ecológicas (Aguirre y Collins, 1993).

Aunque todavía no existe una normativa sobre los requisitos que deben cumplir los probióticos que se incorporan a los alimentos, la FAO/WHO (2002) ha hecho unas recomendaciones acerca de los criterios de evaluación de éstos, incluyendo su identificación intraespecífica, pruebas *in vitro* e *in vivo* para constatar sus propiedades y efectividad, y guías sobre el etiquetado. En general, se considera que la concentración de una cepa probiótica en un producto debe ser de 10^8 ufc/ml o superior (Shortt, 1999a). En Japón, la *Fermented Milks and Lactic Acid Bacteria Beverages Association* exige que la concentración de bacterias sea como mínimo de 10^7 ufc/ml al final de la vida útil del producto (Stanton y col., 2005).

Los productos probióticos comercializados actualmente se pueden dividir en tres tipos :

a) los alimentos fermentados convencionales a los que se les adicionan probióticos y que se consumen, principalmente, con fines nutritivos (yogures, leche, quesos, etc.).

b) las leches cultivadas y fermentadas, utilizadas básicamente como, vehículos de bacterias probióticas (actimel, leche acidófila, etc.).

c) los suplementos dietéticos o preparaciones farmacéuticas liofilizadas (ultralevura, infloran, etc) (Sanz y col., 2003).

2.2. Criterios de selección y efectos beneficiosos de bacterias probióticas

El establecimiento de criterios de selección y controles de calidad para productos probióticos, se considera una prioridad debido a la rápida incorporación de estos productos al mercado y su distribución en el ámbito internacional.

Sin embargo, la selección de nuevas cepas con fines probióticos no resulta un trabajo fácil ya que, aunque se considera que los microorganismos probióticos ejercen efectos beneficiosos para el hospedador que los consume, aún se desconocen los mecanismos implicados. Además, no existe una uniformidad de criterios para la selección de estas bacterias y tampoco se puede extrapolar el efecto probiótico de una cepa a las restantes cepas de la misma especie. En este sentido, se han sugerido numerosos criterios de selección (Charteris y col., 1998a; Collins y col., 1998; FAO/WHO, 2002; Holzapfel y col., 1998; Mattila-Sandholm y col., 1999; Ouwehand y col., 1999). De todos ellos, hay seis sobre los que existe un acuerdo prácticamente general. No obstante, incluso algunos de estos criterios son objeto de controversia:

1. Habitante normal del hospedador diana
2. Ausencia de patogenicidad
3. Resistencia al tránsito por el aparato digestivo
4. Capacidad para resistir los procesos tecnológicos
5. Viabilidad durante toda la vida útil del producto
6. Evidencia científica de efecto(s) beneficioso(s) específico(s) en estudios clínicos correctamente diseñados.

2.2.1. Origen y seguridad

Tradicionalmente, se ha recomendado que las cepas probióticas sean aisladas de muestras de la misma especie a la que se vayan a aplicar, basándose en la creencia de que las cepas de origen humano se implantarían o colonizarían el epitelio gastrointestinal humano con mayor facilidad que las aisladas de otras fuentes (Holzapfel y col., 1998; Ouwehand y col., 1999; Shortt, 1999b). Sin embargo, no todas las cepas destinadas a uso humano que se encuentran actualmente en el mercado son de origen humano (Dunne y col., 2001; Mattila-Sandholm y col., 1999)

y, además, resulta extraordinariamente difícil, sino imposible, conocer el origen último de una cepa ya que cualquiera que esté presente en el intestino humano podría proceder inicialmente de un alimento determinado e, incluso, del animal o vegetal que proporcionó la materia prima para elaborar el alimento. Por otra parte, existen varios estudios que demuestran que la capacidad de cepas probióticas para adherirse al epitelio del hospedador es independiente de su origen (Johansson y col., 1993; Kirjavainen y col., 1998; Rinkinen y col., 2000, 2003). Por ello, un reciente informe de la FAO/WHO (2002) establece que la selección de una cepa potencialmente probiótica se debe basar, independientemente de su origen, en dos criterios fundamentales: su capacidad para llegar viable y en número suficiente a su lugar de acción, y su efectividad real.

La importancia de demostrar la seguridad de nuevos probióticos antes de su introducción en el mercado está fuera de cualquier duda, considerándose un criterio crítico para la selección de cepas probióticas (tabla 2.2). Diversos autores (O´Brien y col., 1999; Salminen y col., 1998a) han propuesto una serie de pruebas para evaluar la seguridad de las cepas bacterianas que se aplican, o se deseen aplicar, como probióticos. En los productos probióticos, es importante que la clasificación taxonómica de las bacterias que los integra sea exacta, ya que se trata de un aspecto que puede tener connotaciones para la seguridad y legalidad de los mismos (O´Brien y col., 1999).

Además del conocimiento de su origen y una correcta caracterización taxonómica al nivel de género, especie y cepa, hay que conocer aquellas propiedades intrínsecas relevantes desde el punto de vista de la seguridad, como posibles factores de virulencia, potencial invasivo, resistencia a antibióticos y facilidad para intercambiar material genético mediante procesos conjugativos. Además, las bacterias lácticas probióticas pueden tener en algunos casos actividades metabólicas que pudieran suponer un peligro, aunque sea potencial, para los consumidores en general o para ciertos colectivos en particular. Entre las actividades que tradicionalmente se han considerado merecedoras de una cuidadosa evaluación se encuentran las siguientes: (1) producción de D-láctico; (2) producción de aminas biógenas; (3) desconjugación de sales biliares (7a-deshidroxilasa); (4) producción de enzimas que degradan la mucina; (5) actividades azoreductasa y/o nitrorreductasa; (6) actividades ß-glucuronidasa y/o ß-glucosidasa. Cabe señalar que las reservas que existen en cuanto a la utilización de cepas productoras del isómero D(-) del ácido láctico derivan del *Codex* de 1976 (Comisión

del *Codex Alimentarius,* 1997), mientras que para el comité de expertos consultados por la FAO/WHO en 2001 la configuración de esta molécula no supone un factor de riesgo.

Tabla 2.2. Criterios para la evaluación de la seguridad de las cepas probióticas

Propiedad	**Prueba**	
	In vitro	***In vivo***
Actividad metabólica	Producción aminas biógenas Desconjugación de las sales biliares (actividad 7a-hidrolasa) Producción de ácido D-láctico Degradación de la mucina Actividad colil-glicina hidrolasa Actividad azoreductasa Actividad ß-glucuronidasa Actividad ß-glucosidasa	
Infectividad	Agregación de plaquetas Adhesión Hemólisis Agregación de eritrocitos	Datos epidemiológicos Ratones irradiados
Transferencia genética	Transferencia de plásmidos	Transferencia genética entre la microbiota intestinal en ratones
Función inmunitaria	Ensayos de fagocitosis	Ensayos en ratas inmunodeficientes

Los probióticos han sido usados desde hace siglos para conservar y producir alimentos. Este largo historial de consumo asegura su inocuidad. Entre 1961 y 1998 se realizaron, por lo menos, 143 estudios clínicos con probióticos en más de 7,500 sujetos; en ninguno de los casos se ha registrado un problema de salud. La mayor parte de infecciones humanas por bacterias lácticas han sido causadas por cepas de origen endógeno, es decir, procedentes de la propia microbiota del paciente y solo en contados casos se ha asociado al consumo de probióticos comerciales. Sin embargo, todos los casos estaban asignados a pacientes con patologías subyacentes (Aguirre y Collins., 1993).

Con los datos obtenidos en las investigaciones y estudios epidemiológicos realizados en la última década, resulta evidente que los probióticos empleados actualmente son seguros en las condiciones de uso autorizadas. Se debe poner especial énfasis en el hecho de que se han administrado bacterias lácticas probióticas a pacientes con enfermedad de Crohn, con diferentes tipos de gastroenteritis o con SIDA, y a enfermos de unidades oncológicas sujetos a radioterapia y quimioterapia, sin registrarse ni un solo caso de infección por el

probiótico administrado. Bien al contrario, los resultados obtenidos han sido prometedores. Tampoco se han descrito efectos adversos derivados de la administración de bacterias lácticas probióticas a mujeres gestantes, niños sanos, niños prematuros, niños alérgicos o ancianos (O'Brien y col., 1999).

2.2.2. Resistencia a las condiciones del TGI

Un determinante esencial en la elección de microorganismos probióticos es, su capacidad para sobrevivir y persistir en las condiciones ambientales existentes durante el tránsito por el aparato digestivo y de colonizar el TGI. Por lo tanto, la resistencia a la acidez gástrica y a las sales biliares se encuentra entre uno de los criterios de selección.

Para ello, se recurre a métodos *in vitro* como la acidificación y/o la adición de sales biliares al medio de cultivo, el empleo de secreciones gastrointestinales obtenidas de individuos sanos (Charteris y col., 1998b; Dunne y col., 2001; Fernández y col., 2003; Zárate y col., 2000), o el uso de modelos dinámicos más sofisticados que simulan las condiciones gastrointestinales *in vivo*, como el desarrollado por el TNO en Holanda (Marteau y col., 1997). Este modelo, proporciona datos con un mayor valor predictivo sobre la supervivencia de bacterias probióticas en el TGI que los obtenidos con las técnicas mencionadas anteriormente. Sin embargo, en algunas ocasiones los resultados obtenidos con estos simuladores han sido contradictorios con los obtenidos *in vivo* (Mattila-Sandholm y col., 1999). En cualquier caso, la capacidad de los probióticos para sobrevivir a las condiciones gastrointestinales es una característica específica de cepa (Charteris y col, 1998a; 1998b; Xanthopoulos y col., 2000; Zárate y col., 2000; Zavaglia y col., 1998).

Un factor importante para la supervivencia de bacterias probióticas en el TGI es el substrato o matriz con el que se vehiculan y, de hecho, los resultados obtenidos en medios de cultivo no son, en absoluto, extrapolables a lo que sucede cuando las cepas se vehiculan con alimentos (Charteris y col., 1998b). Por ejemplo, Saxelin y col. (1993; 1995) demostraron que la supervivencia de *Lb. rhamnosus* GG difiere si se ingiere mediante comprimidos, cápsulas gelatinosas, leches fermentadas o bebidas a base de soja.

2.2.3. Adhesión y colonización del epitelio intestinal

La concentración de probióticos viables que se estima que debe llegar al intestino para obtener un efecto beneficioso es de aproximadamente >= 10^6 ufc/ml en el intestino delgado y >= 10^8 ufc/g en el colon (Marteau y Shanahan, 2003). Sin embargo, harían falta más estudios que ofrezcan datos concluyentes sobre la relación dosis-respuesta. Una vez que los microorganismos probióticos alcanzan el intestino, son capaces de permanecer allí durante un corto período de tiempo en estado no proliferativo. Sin embargo, para establecerse como habitante permanente del TGI, deben adherirse a las células epiteliales intestinales o a la capa de mucus. La adhesión a la mucosa intestinal es el primer paso en la colonización, y muchos autores lo consideran un prerrequisito para ejercer efectos beneficiosos en el hospedador, como la exclusión competitiva de bacterias enteropatógenas (Bernet y col., 1993; 1994; Mack y col., 1999) o la inmunomodulación (Isolauri y col., 1999; Link-Amster y col., 1994; Schiffrin y col., 1995). Por el contrario, Colombel y col. (1987) y Lennoir-Winjkoop y Hopkins (2003) no sólo consideran que no es imprescindible que las bacterias probióticas colonicen el epitelio intestinal para que se ejerza el efecto beneficioso, sino que sugieren que ciertos efectos beneficiosos podrían llegar a perderse si las bacterias tuvieran esta propiedad.

El estudio de la adherencia de las bacterias *in vivo* supone una dificultad, por eso se ha propiciado el desarrollo de métodos *in vitro* que permiten estimar la capacidad de una cepa para colonizar el TGI. Los modelos más empleados se basan en líneas celulares humanas derivadas de adenocarcinoma de colon, como la HT-29 y la Caco-2 (Bernet y col., 1993; Elo y col., 1991; Tuomola y Salminen, 1998). Las células de la línea Caco-2 se diferencian y polarizan espontáneamente cuando confluyen, mostrando una membrana apical funcional con las microvellosidades completamente desarrolladas; por ello, se suelen utilizar para el estudio *in vitro* de la organización y función de células intestinales humanas. La principal característica de la línea celular HT29-MTX es que secreta mucus y, de hecho, se desarrolló para simular el ambiente mucoso del intestino humano (Lessufleur y col., 1990).

Numerosos trabajos han mostrado la utilidad de ambas líneas para el estudio de la adherencia de lactobacilos (Bernet y col., 1994; Coconnier y col., 1992; Hudault y col., 1997, Tuomola y Salminen, 1998) y bifidobacterias (Dunne y col., 2001; Thornton, 1996). Por otra parte, se ha observado una buena correlación entre la

capacidad adhesiva que manifiestan las cepas probióticas utilizando estos modelos y los resultados obtenidos *in vivo*.

2.2.4. Nuevos criterios de selección

Como ya se ha mencionado, el establecimiento de los criterios de selección y controles de calidad para productos probióticos se considera una prioridad, por ello, además de los comentados anteriormente, se establecen otra serie de criterios a la hora de seleccionar un probiótico que nos aseguren una buena elección; entre ellos destacamos algunos como son: fermentación de hidratos de carbono (API CH50), actividades enzimáticas, resistencia a antibióticos o en base a criterios algo más funcionales como pueden ser, producción de vitaminas, de mucinas, inducción de la expresión de citocinas. En la página 35 de esta Tesis, se muestra una tabla con una lista de posibles criterios llevados a cabo en nuestro laboratorio para la selección de nuevos probióticos (tabla 4.1).

2.2.5. Aspectos tecnológicos

Los probióticos además de cumplir todos los requisitos anteriormente mencionados presentan un elevado interés industrial y, por lo tanto, deben ser capaces de soportar las condiciones de producción industrial, mantener sus propiedades funcionales durante el proceso productivo y asegurar gran parte de su viabilidad durante el almacenamiento, en muchas ocasiones en refrigeración o congelación. Por otra parte, no deben afectar negativamente a las propiedades organolépticas de los alimentos finales (Ouwehand y col., 2001; Tuomola y col., 2001).

2.2.6. Efectos beneficiosos de los probióticos

El uso de probióticos se ha asociado con un gran número de efectos beneficiosos como la mejora de la intolerancia a la lactosa, la modulación del sistema inmunitario, la reducción de la hipercolesterolemia y la hipertensión, y la protección frente a enfermedades infecciosas, inflamatorias, alérgicas y tumorales (Amores y col., 2004; Gill, 2003). Sin embargo, no se debe asumir, bajo ningún concepto, que todas las bacterias lácticas y bifidobacterias posean propiedades beneficiosas. De igual manera, cuando se adscribe un efecto beneficioso a una cepa, tampoco se puede extrapolar esa propiedad a las restantes cepas de la misma

especie. Incluso la adscripción de un efecto beneficioso a una cepa depende de las condiciones de su empleo y, muy particularmente, de la dosis.

De los posibles efectos beneficiosos de los probióticos hablaremos a lo largo de los siguientes apartados de la Introducción de esta memoria.

3. Probióticos y enfermedad

Naidu y col. (1999) realizaron una exhaustiva revisión crítica de los efectos de los probióticos, incluyendo datos de estudios *in vitro* e *in vivo*. Según estos autores, desde 1961 a 1998 se efectuaron al menos 143 ensayos clínicos humanos con probióticos y los resultados indican que, efectivamente, existen ciertas cepas capaces de proporcionar efectos beneficiosos para la salud. A pesar de que, es necesario profundizar en el desarrollo de marcadores biológicos validados que permitan la comprobación interlaboratorial de los efectos probióticos que se produzcan *in vivo* (Diplock y col., 1999; Yoon y col., 1999), un informe científico financiado por la Unión Europea concluyó que los efectos beneficiosos para la salud humana atribuidos a ciertas cepas de lactobacilos y bifidobacterias, podían considerarse científicamente probados (Salminen y col., 1998b). El principal criterio seguido por la Comisión encargada de elaborar el informe fue que, el efecto probiótico fuese patente e inequívoco en al menos dos estudios humanos correctamente diseñados e independientes.

De hecho, los probióticos están siendo rigurosamente evaluados en diversos hospitales y centros médicos como adjuntos en el tratamiento y/o prevención de diversas enfermedades, y los resultados son prometedores.

Así, está ampliamente demostrado que la digestión de la lactosa mejora con la ingestión de yogurt y productos lácteos fermentados en sustitución de la leche (de Vrese y col., 2001; Marteau y col., 1990; Martini y col., 1991; Savaiano y col., 1984; Suárez y col., 1995). Esta mejora no es sin embargo igual para todas las cepas, encontrándose diferencias en los efectos e incluso algunos casos en los que no se ha podido detectar una mejora en la sintomatología (Saltzman y col., 1999).

El efecto beneficioso de las bacterias probióticas sobre las diarreas es, sin duda, uno de los efectos más demostrados. Los datos obtenidos hasta el momento

indican que ciertos probióticos mejoran las diarreas agudas en niños. El gran número de estudios realizados (más de 100 estudios publicados) ha permitido incluso la realización de ensayos tipo meta-análisis que indican la efectividad del tratamiento con determinadas cepas probióticas en los casos de diarrea (Szajewska y Mrukowicz, 2001; van Niel y col., 2002) así como, la reducción en la recurrencia de diarreas crónicas causadas por *C.difficile* (Plummer y col., 2004; Wullt y col., 2003). Algunos estudios preliminares también señalan el papel de ciertas cepas probióticas en el control de la infección por *H.pylori* que, aunque no erradican al patógeno, sí inhiben su crecimiento y reducen significativamente la inflamación gastroduodenal (Cruchet y col., 2003; Linsalata y col., 2004; Wang y col., 2004a). Además, leches fermentadas con probióticos reducen la colonización de la mucosa nasal por bacterias potencialmente patógenas tales como *Staphylococcus aureus*, *Streptococcus pneumoniae*, estreptococos ß-hemolíticos y *Haemophilus influenzae* (Gluck y Gebbers, 2003; Hatakka y col., 2001). Con estos resultados podemos observar como, el tratamiento con probióticos no sólo actúa sobre enfermedades que afectan al tracto gastrointestinal sino también al aparato respiratorio, para confirmar estos efectos hacen falta más estudios y así saber cuáles son los mecanismos implicados (Tubelius y col., 2005., Turchet y col., 2003). También, se ha demostrado un efecto beneficioso de ciertas cepas probióticas a nivel de infecciones vaginales en el que probablemente estén implicados los mismos mecanismos (Reid y col.,2001a; 2001b; Reid, 2002).

El efecto observado a nivel de protección frente a infecciones, es un fenómeno complejo multifactorial que involucra diferentes mecanismos como la producción de sustancias antibacterianas, la competición por la adhesión a la mucosa, competición por nutrientes y el efecto sobre la respuesta inmunológica.

Actualmente, muchos de los estudios que se están realizando para dilucidar los mecanismos responsables de los efectos probióticos se están centrando en las propiedades inmunomoduladoras de las cepas. Resulta cada vez más evidente que, muchas actividades probióticas están mediadas por procesos de regulación inmunitaria, como el aumento de la secreción de IgA (Schiffrin y col., 1995) y, especialmente, mediante el mantenimiento de un equilibrio entre citoquinas pro- y antiinflamatorias (Isolauri y col., 2001), lo que abre nuevas expectativas para el empleo de bacterias probióticas como agentes terapéuticos (Marteau y col., 2002). Estas propiedades inmunomoduladoras, son la base de la utilización de los probióticos en patologías derivadas de desequilibrios de la respuesta inmunitaria

tales como la alergia (Hattori y col., 2003; Kalliomäki y col., 2001a; 2001b; 2003; Viljanen y col., 2005; Wang y col., 2004b) o enfermedades inflamatorias crónicas como la enfermedad de Crohn, la colitis ulcerosa o la artritis reumatoide (Gosselink y col., 2004; Hatakka y col., 2003; Niedzielin y col., 2001). Pero las propiedades inmunomoduladoras no sólo pueden ayudar en situaciones patológicas, sino también, pueden ser un componente más en la protección frente a infecciones en individuos sanos o en condiciones fisiológicas de mayor susceptibilidad, como pueden ser la inmunosenescencia (Gill y col., 2001). Finalmente, se está valorando el uso de probióticos como coadyuvantes e incluso vehículo en procesos de vacunación (de Vresse et al 2005a; Wells y Mercenier, 2008).

Por último, se está estudiando el papel de los probióticos como adyuvantes en el tratamiento y/o prevención de ciertos tipos de cáncer (Kato y col., 1981; Van´t Veer y col., 1989), de la hipertensión (Minervini y col., 2003; Seppo y col., 2003), de la encefalopatía hepática (Bongaerts y col., 2005), o de la depresión (Logan y Katzman, 2005). Los datos epidemiológicos han demostrado que el consumo de leches fermentadas con ciertas bacterias lácticas, está asociado a una menor incidencia de úlceras gastroduodenales en Europa (Elmstahl y col., 1999) y de cáncer de vejiga en Japón (Shortt, 1999a). En la tabla 3.1, se resumen los principales efectos beneficiosos científicamente probados que se atribuyen a las principales bacterias probióticas comerciales.

Muchos de los mecanismos implicados en los efectos beneficiosos del hospedador podrán ser aclarados en un futuro próximo gracias al desarrollo de la genómica, la proteómica y la metabolómica. Recientemente, la comparación de los genomas de *Lb. plantarum* y *Lb. johnsonii* (Kleerebezem y col., 2003; Pridmore y col., 2004) ha puesto de manifiesto diferencias en el tamaño, organización y contenido de genes entre dos especies muy afines; también ha revelado la presencia de un reducido número de genes comunes que pueden estar relacionados con la adaptación al ambiente del TGI (Boekhorst y col., 2004) y la existencia de genes que confieren propiedades probióticas específicas (Bron, 2004; Granato y col., 2004). El estudio *in vivo* de estos genes, junto con otros análisis post-genómicos, contribuirán a esclarecer los mecanismos de acción de las bacterias probióticas (de Vos y col., 2004; Saxelin y col., 2005).

Tabla 3.1: Efectos beneficiosos de bacterias probióticas probados en estudios humanos

Efecto beneficioso	Probiótico	Referencia
Prevención de diarreas asociadas a tratamiento antibiótico	*L.rhamnosus* GG	Siitonen y col. (1990) Vanderhoof y col. (1999) Armuzzi y col. (2001)
	L.acidophilus y *B.longum*	Orrhage y col. (1994)
	L.acidophilus y L.bulgaricus	Gotz y col. (1979) Tankanow y col. (1990)
	L.sporogens	La Rosa y col. (2003)
	B.longum	Colombel y col. (1987)
	E.faecium SF68	Wunderlich y col. (1989)
	B.clausii	Nista y col. (2004)
	C.butyricum	Seki y col. (2003)
	S.boulardii	MacFarland y col. (1995)
	L.acidophilus y *B.bifidum*	Lewis y col. (1998) Madden y col. (2005)
Tratamiento de infecciones causadas por *Helicobacter pylori*	*L.johnsonii* La1	Michetti y col. (1999) Felley y col. (2001)
	L.acidophilus LB	Coconnier y col. (1998) Canducci y col. (2000)
	L.salivarius	Aiba y col. (1998)
	L.casei Shirota	Cats y col. (2003)
	L.reuteri	Imase y col. (2005)
	B.lactis Bb12	Wang y col. (2004)
	LGG Plus[a]	Myllyluoma y col.(2005)
Reducción de la duración de diarreas causadas por rotavirus	*L.rhamnosus* GG	Guarino y col. (1997) Guandalini y col. (2000)
	L.reuteri	Shornikova y col. (1997)
	B.lactis y *S.thermophilus*	Saavedra y col. (1994)
Control de infecciones del tracto urinario y/o vaginosis	*L.rhamnosus* GR-1y *L.fermentum* RC-14	Reid, (2001); (2002) Cadieux y col. (2002)
	L.acidophilus	Vanderhoof y col. (1998)
	B.lactis	Kalliomäki y col. (2001)
	L.rhamnosus GG	Pessi y col. (2000) Murch (2001) Kalliomäki y col. (2001); (2003)
Estimulación del sistema inmunitario	*L.rhamnosus* GG	Pelto y col. (1998) Pohjavuori y col. (2004)
Tratamiento de enfermedades inflamatorias intestinales	*E.coli Nissle* 1917	Rembacken y col. (1999)
	S.boulardii	Guslandi y col. (2000) Gionchetti y col. (2000); (2003)
	VSL#3[b]	Campieri y col. (2000) Kajander y col. (2005)
Reducción de otros problemas gastrointestinales	*L.plantarum* 299v	Nobaeck y col. (2000) Sen y col. (2002) Niedzielin y col. (2001)
	B.infantis y *L.salivarius*	O´Mahony y col. (2005)
	L.plantarum LP01 y *B.breve*	Saggioro y col. (2004)
	S.boulardii	Marteau y col. (2001)
	VSL#3	Kim y col. (2003)
Cáncer de colon	*L.acidophilus*	Gorbach (2000) Spanhaak y col. (1998) Ling y col. (1994) Goldin y Gorbach (1980)
Reducción de atopias y alergias	*L.rhamnosus GG*	Kalliomäki y col. (2001); (2003)
	L.paracasei-33	Wang y col. (2004)

[a] LGG Plus: *L.rhamnosus* GG, *L.rhamnosus* LC705, *P.freudenreichii, B.breve*

[b] VSL#3: *S.thermophilus, B.breve, B.longum, B.infantis, L.acidophilus, L.plantarum, L.casei, L.bugaricus*

4. Hereditum

Debido a que cada cepa específica de probióticos posee sus propias características y propiedades, la búsqueda de nuevos probióticos específicos es una de las principales preocupaciones y objetivos de los investigadores y de las empresas de alimentación funcional.

Como estrategia para esa búsqueda de nuevas cepas se inició el estudio de la leche materna como fuente de bacterias con valor probiótico.

4.1. Microbiota de la leche humana como fuente de nuevas cepas probióticas

La leche materna constituye el mejor alimento para los niños durante los primeros meses de vida. En primer lugar, satisface todos sus requerimientos nutritivos durante esta etapa de rápido crecimiento. En segundo lugar, diversos estudios han demostrado que la leche de la madre confiere al recién nacido una notable protección frente a enfermedades infecciosas (López-Alarcón y col., 1997; Wright y col., 1998a;1998b). Este efecto puede ser debido a la acción combinada de algunos componentes de la leche, como inmunoglobulinas, linfocitos, macrófagos y diversas sustancias con propiedades antimicrobianas (Saavedra, 2002). Además, la leche materna también contiene sustancias prebióticas que estimulan de forma selectiva el crecimiento de bacterias deseables en el intestino infantil (Dai y Walker, 1999; Drasar y Roberts, 1990).

Ya en los años 70, se demostró que la leche humana recién recolectada poseía actividad antimicrobiana (Hanson y Winberg, 1972; Mata y Wyatt, 1971) y que su pasteurización provoca la pérdida parcial o total de dicha actividad (Evans y col., 1978; Ford y col., 1977). Gracias a estas observaciones, las unidades de cuidados intensivos neonatales empezaron a administrar leche materna no tratada térmicamente en lugar de fórmulas infantiles, particularmente en el caso de niños prematuros ya que son especialmente sensibles a las infecciones (Siimes y Hallman, 1979; Williamson y col., 1978). De hecho, la alimentación de prematuros con leche materna durante las primeras 24 horas de vida disminuye la incidencia de enterocolitis necrotizante, mejora la tolerancia a otros alimentos y reduce los casos en los que es necesaria la nutrición parenteral (Schanler, 2000).

4.2. Origen de las bacterias aisladas de leche materna

La idea de que los fetos son estériles *in utero* y que la colonización bacteriana del intestino neonatal se inicia durante el tránsito por el canal del parto, por contaminación a partir de la microbiota vaginal e intestinal de la madre, es algo que tradicionalmente se ha aceptado (Isolauri y col., 2001; Mackie y col., 1999; Tannock, 1995), a pesar de la ausencia de evidencias científicas concluyentes.

Ciertas especies de estreptococos y estafilococos se pueden aislar frecuentemente a partir de la leche humana fresca (Dudgeon y Jewesbury, 1924; Eidelman y Szilagyi, 1979; Heikkilä y Saris, 2003; West y col., 1979). Se sugirió que los estafilococos coagulasa-negativos, con *S. epidermidis* como especie predominante, procederían de la piel materna y contaminarían la leche durante las tomas (West y col., 1979). En contraste, los estreptococos del grupo viridans, como *St. salivarius*, no suelen encontrarse en la piel, pero son comunes en la cavidad oral de los niños. El hecho de que la concentración de estreptococos en leches recolectadas antes de que un niño empiece a mamar sea muy baja (Plueckhahn y Banks, 1964) llevó a pensar que estos estreptococos son transferidos desde la boca del niño al pecho de la madre y, desde esa localización, a la leche (Heikkilä y Saris, 2003; West y col., 1979). Tradicionalmente se consideró que la cavidad oral, junto con el resto del tracto digestivo, se coloniza tras el nacimiento con bacterias procedentes de la microbiota vaginal y/o intestinal de la madre (Mackie y col., 1999), mientras que las bacterias que colonizan al TGI de niños nacidos por cesárea procederían del ambiente que lo rodea y de la microbiota fecal de la madre (Lennox-King y col., 1976a; 1976b; Schultz y col., 2004).

Heikkilä y Saris (2003) obtuvieron aislados de *Lb. crispatus* en leche humana y especularon que podría tener un origen vaginal, ya que se trata de una de las especies del género *Lactobacillus* predominantes en la vagina de las mujeres sanas (Song y col., 1999; Zhou y col., 2004). El niño se habría contaminado con esta especie durante el parto y, a su vez, habría contaminado la piel de la madre. No obstante, estos autores sólo pudieron aislar esta especie a partir de la leche de una de las madres que participaron en el estudio. Sin embargo, Matsumiya y col., (2002) demostraron que sólo una cuarta parte de los niños resultan colonizados por lactobacilos procedentes de la vagina de la madre durante el parto, y que además,

estos lactobacilos son rápidamente reemplazados por los procedentes de la leche o de otras fuentes.

Sin embargo, el aislamiento de especies bacterianas de líquido amniótico, sangre del cordón umbilical y meconio de neonatos sanos que todavía no habían ingerido leche, incluyendo algunos nacidos por cesárea (Hitti y col., 1997; Jiménez y col., 2005; Tannock y col., 1990), parece desmentir la versión tradicional. Además, se han aislado especies de bifidobacterias en muestras de meconio (Tannock y col., 1990) y una cepa de lactobacilos en sangre del cordón umbilical de un recién nacido sano (Salminen y col., 2004). En este último caso, la cepa se descartó para futuros estudios por el mero hecho de tener un origen atípico y difícilmente explicable.

Ante la falta de estudios rigurosos que demostraran o descartaran la presencia de bacterias en la leche materna, en el año 2003 Martín y col., publicaron los resultados de un estudio realizado con 8 mujeres y sus hijos recién nacidos, que demostraban la presencia de bacterias en la leche y su papel en la colonización del intestino del niño. De esta manera, se observó que ciertas cepas de *L.gasseri* y *Enterococcus faecium* se podían aislar simultáneamente de las muestras de leche, areola mamaria, cavidad oral infantil y heces proporcionadas por una misma pareja madre-hijo (figura 4.1). En contraste, ninguna de las bacterias lácticas compartió perfiles de RAPD con aislados de otros orígenes. Estos resultados revelaron que, la leche materna es una fuente importante de bacterias lácticas para el intestino neonatal y que las que están presentes en la leche materna, puede tener un origen endógeno (el intestino materno). Adicionalmente, la administración por vía oral de una cepa de *E.faecium* marcada genéticamente a ratonas gestantes, ha permitido observar que existe una transferencia de la cepa al intestino fetal (bajo nivel de transferencia) (Fernández y col., 2004). Posiblemente, el intestino fetal se coloniza con concentraciones relativamente bajas de bacterias lácticas. Tras el nacimiento, y una vez iniciada la lactancia, se iniciará una segunda fase en la que el intestino recibiría cantidades mucho más elevadas de estas bacterias que ejercerían efectos beneficiosos para el niño.

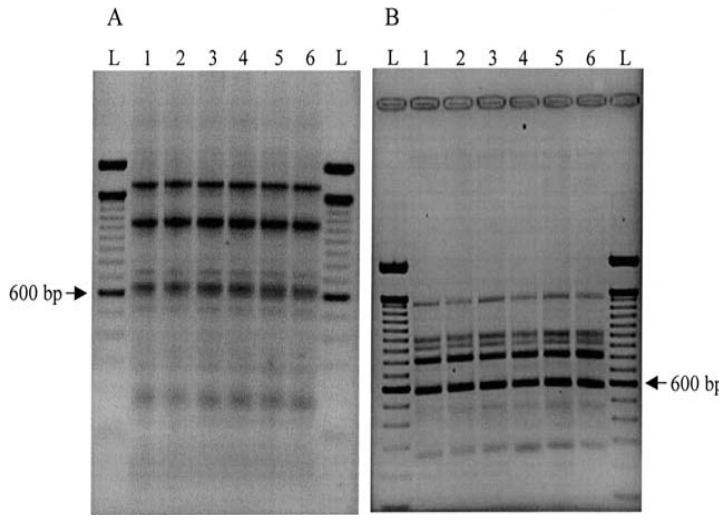

Figura 4.1: RAPD-PCR obtenidos (A) con el primer OPL5 para *L.gasseri* aislado de leche materna (líneas 1 y 2), areola mamaria (líneas 3 y 4), heces de niño (línea 5) y cavidad oral infantil (línea 6); (B) con la pareja de primers E1-E2 para *E.faecium* aislado de leche materna (líneas 1 y 2), areola mamaria (líneas 3 y 4), heces de niño (línea 5) y cavidad oral infantil (línea 6).L represena el patrón estándar de 100 bp (Invitrogen, Paisley, UK).

El aislamiento de cepas de *Lb. rhamnosus* con un perfil de RAPD idéntico al de la cepa comercial GG y de aislados de *Lactococcus lactis* productores de nisina a partir de la leche de mujeres finlandesas, resulta particularmente interesante (Beasley y Saris, 2004; Heikkilä y Saris, 2003). *Lb. rhamnosus* GG, es una cepa probiótica consumida frecuentemente en Finlandia a través de productos lácteos, mientras que las cepas de *L. lactis* productoras de nisina, se aíslan habitualmente de alimentos fermentados. Estas observaciones sugieren que, al menos algunas de las bacterias lácticas presentes en el intestino de la madre, pueden alcanzar la glándula mamaria a través de una vía endógena (la ruta entero-mamaria) y que, por lo tanto, la capacidad de algunas bacterias intestinales para extenderse a otros tejidos u órganos de hospedadores humanos sanos ha sido tradicionalmente infravalorada.

Las hipótesis de trabajo llevadas a cabo por el grupo del Dr. Juan Miguel Rodríguez de la Universidad Complutense de Madrid apuntan que, este mecanismo podría explicar cómo determinadas bacterias de la madre alcanzan la glándula mamaria. Serían las propias células dendríticas las que transportarían a determinadas bacterias intestinales, a las que han ayudado a translocarse, a través del torrente linfático y circulatorio hacia las diversas mucosas del organismo y a la

glándula mamaria, dichas bacterias (originalmente intestinales) pueden iniciar la colonización de la glándula mamaria y ser secretadas a la leche materna durante la lactancia, determinando así su transferencia al neonato y la colonización inicial de éste a través de la lactancia. Perez y col (2007) observaron que, la translocación bacteriana es un hecho fisiológico que se ve incrementado durante el embarazo y la lactancia y que serían las células del sistema inmune los responsables de dicha translocación.

Recientemente, se han obtenido evidencias clínicas que demuestran que ciertos lactobacilos probióticos administrados oralmente a mujeres con infecciones vaginales, eran capaces de llegar a la mucosa vaginal por vía endógena y ejercer un efecto antiinfeccioso (Reid y col., 2001a). Paradójicamente, la translocación bacteriana, que generalmente se ha considerado como un criterio negativo para la selección de cepas probióticas, podría jugar un papel importante en los efectos probióticos extraintestinales asociados a ciertas cepas.

Dado los efectos beneficiosos que las bacterias ácido lácticas presentes en la leche podrían estar aportando, la leche materna nos pareció una fuente excelente para la selección y el estudio de nuevas cepas probióticas.

4.3. Criterios de selección de Hereditum

A partir de una colección de 5000 aislados procedentes de leche materna y de fuentes relacionadas (Martín y col., 2003; Martín y col., 2004), se inició un proceso de selección con el fin de identificar nuevas cepas de lactobacilos con un elevado potencial probiótico.

Para ello, se aplicaron una serie de criterios siendo los primeros los de seguridad, descartando así desde el principio, todas aquellas cepas que tuvieran una dudosa seguridad. De esta manera, se seleccionaron cepas bacterianas Gram positivas, catalasa y oxidasa negativas. También, se tuvieron en cuenta en las primeras fases del proceso los aspectos tecnológicos, pues aunque una cepa tenga muy buenas propiedades probióticas, si su aplicación no es viable tecnológicamente debería eliminarse del proceso. Es en estas fases, dónde se descartaron la mayor cantidad de aislados.

Tabla 4.1: Criterios de selección de Hereditum

A. Criterios de seguridad
Taxonómicos: Bioquímicos (API CH50, SDS-protein) y genéricos (16S, RAPD, AFLP, TGGE)
Producción de aminas biogénicas: Tiamina, histamina, cadaverina y putrescina
Actividades enzimáticas: Degradación de mucinas, proteolísis, desconj.sales biliares, glucuronidasa
Resistencia a antibióticos: MIC, factores de virulencia
Otros: Infectividad, producción de feromonas
B. Criterios técnicos
Resistencia a la digestión: Ácido, bilis modelo global
Capacidad fermentativa: Azúcares simples, oligosacáridos y fibras
Colonización intestinal: Adhesión (Caco, HT-29), estudios de competición
C. Criterios funcionales
Producción de metabolitos: AGCC, vitaminas, glutation, PUFAs, oligosacáridos
Sustancias antimicrobianas: Bacteriocinas, reuterina, H_2O_2, estudios de competición
Parámetros inmunitarios: Activación linfocitaria, expresión de citocinas, fagocitosis
Otros: Producción de mucinas, reactividad sanguínea, supervivencia en leche materna
D. Criterios tecnológicos
Crecimiento: En condiciones limitantes, biomasa
Supervivencia: Al calor, contenido en sales, presión osmótica

El siguiente paso fue, la identificación de la especie, ya que sólo se seleccionaron aquellas especies que fuesen comúnmente usadas por su potencial probiótico y que fuesen consideradas GRAS (*Generally Recognized as Safe).* Los siguientes criterios que se aplicaron fueron aquellos que valorasen el potencial probiótico de las cepas. En esta fase, se comenzó midiendo las propiedades que asegurasen que la cepa llegase viva al intestino, descartándose aquellas en las que su baja resistencia a las condiciones de digestión o baja capacidad de adhesión a la mucosa intestinal pudiera indicar su ineficacia para alcanzar el intestino en un estado viable. Al final de esta fase sólo continuaron en el proceso aproximadamente el 1% de las cepas que lo iniciaron.

El último paso en el proceso de selección fue, la valoración de la funcionalidad de una cepa. Para ello, se valoraron las funcionalidades más demostradas en probióticos, como eran la actividad antimicrobiana y la capacidad inmunomoduladora, aunque también se incluyeron criterios de selección que pudieran suponer un beneficio para la salud, como por ejemplo la producción de vitaminas, moléculas antioxidantes como el glutation, oligosacáridos, etc.

Una vez finalizado el proceso de selección, para poder comparar las cepas entre ellas, se estableció un sistema de puntuación determinando la capacidad probiótica global de cada una de las cepas. En el proceso se incluyeron también las principales cepas comerciales con el fin de seleccionar cepas que como mínimo tuvieran un potencial probiótico similar o superior a las ya presentes en el mercado (figura 4.2)

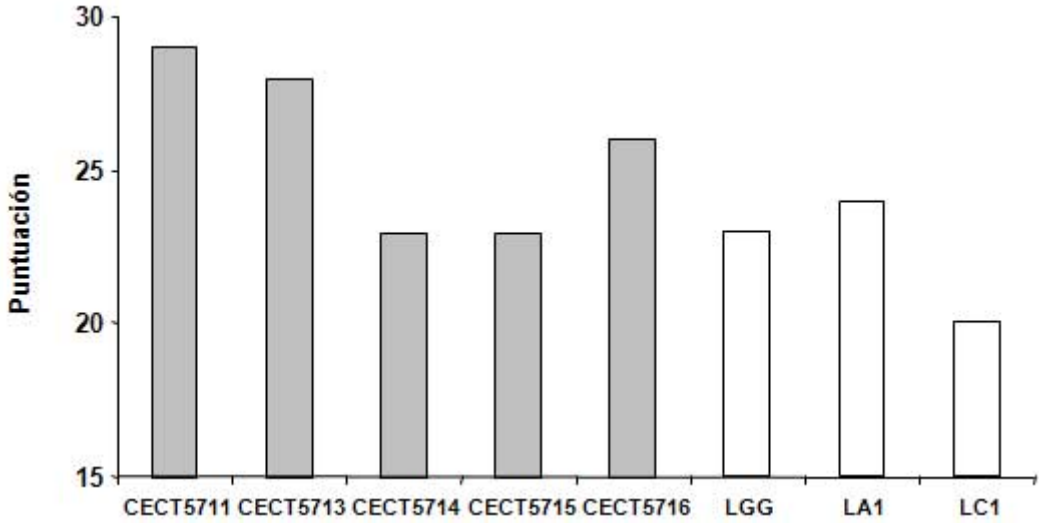

Figura 4.2: Capacidad probiótica de las cepas Hereditum comparada con las principales cepas probióticas comerciales (*L.rhamnosus* LGG de Valio, *L.johnsonii* La1 de Nestlé y *L.casei inmunitas* de Danone) y cuantificadas arbitrariamente..

Las cepas que se pre-seleccionaron fueron posteriormente evaluadas por su capacidad de ser trasferidas a la leche materna, y finalmente, las cinco cepas de lactobacilos que mostraron mejores resultados pasaron a formar parte de las cinco cepas **Hereditum** patentadas por Puleva Biotech (Xaus y col., 2002) (figura 4.3).

L.coryniformis CECT5711
L.salivarius CECT5713
L.gasseri CECT5714
L.gasseri CECT5715
L.fermentum CECT5716

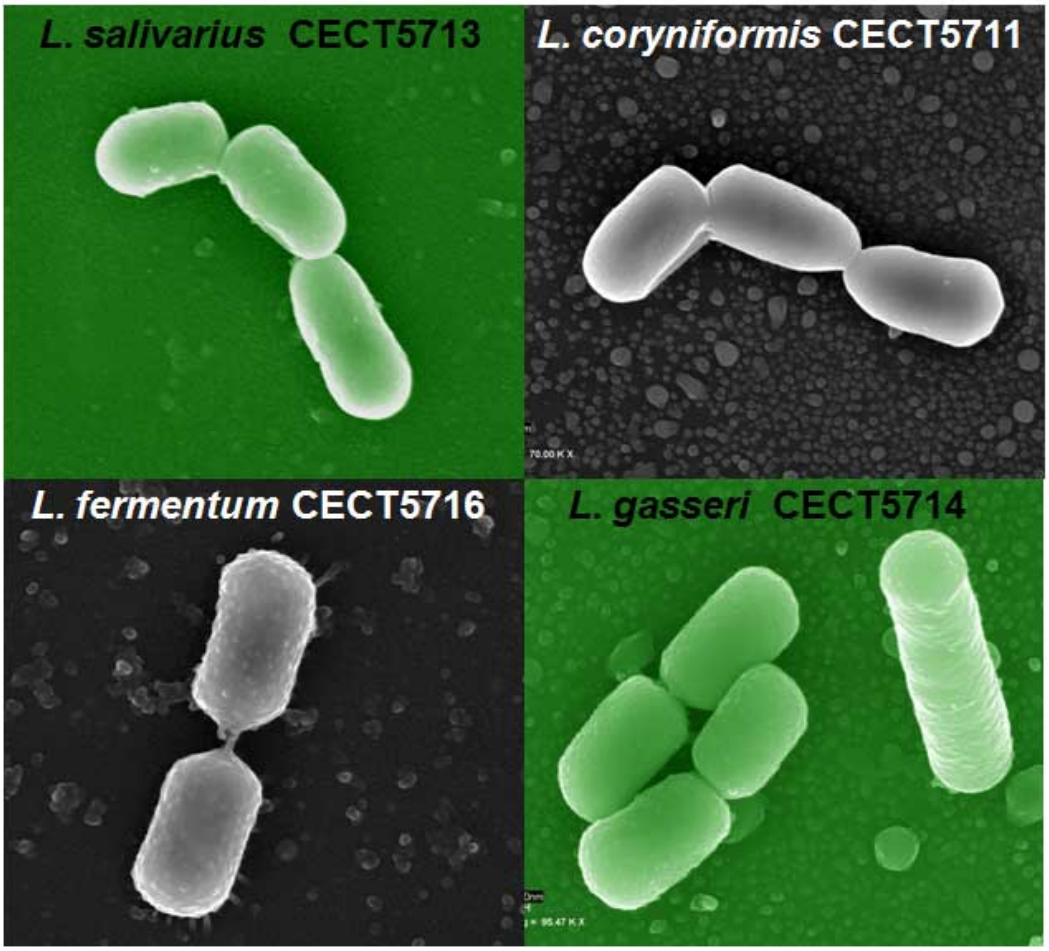

Figura 4.3: Cepas probióticas Hereditum

Con estas cinco cepas se inició una extensa tarea de investigación destinada a poder caracterizarlas, incluyendo un estudio de su tolerancia y de la ausencia de efectos perjudiciales, de su potencial probiótico y, por supuesto, de su capacidad para inducir efectos beneficiosos en la salud del consumidor.

4.4. Propiedades probióticas de Hereditum

Todos los lactobacilos de la leche humana seleccionados en esta Tesis para su análisis, mostraron una elevada supervivencia a las condiciones existentes durante el tránsito por el tracto gastrointestinal, y particularmente a un pH bajo y a la presencia de bilis (Martín y col.,2005a;2005b; Martín y col., 2006). Esta propiedad parece ser un prerrequisito crítico para una bacteria probiótica. El modelo elegido (Marteau y col., 1997) permitió una simulación aceptable de los factores que más influyen en la supervivencia de una bacteria. De las cinco cepas Hereditum, la que presentó una mayor supervivencia fue *L.fermentum* CECT5716 (70%) (Martín y col., 2005a), seguida de *L.salivarius* CECT5713 (55%) (Martín y col., 2006), *L.coryniformis* CECT5711 (50%) (Martín y col., 2005b) y las dos *L.gasseri* CECT5714 y CECT5715, con un 30% de supervivencia (Martín y col., 2005a). Estos resultados obtenidos, no difieren significativamente a los obtenidos con otras bacterias ácido lácticas comerciales en un modelo gastrointestinal dinámico y computerizado (Marteau y col., 1997). Las condiciones ambientales que se encuentran en el tracto gastrointestinal de los neonatos, generalmente, no son tan restrictivas como las presentes en adultos, por ello, la supervivencia de los lactobacilos en neonatos probablemente sea mayor.

Otra propiedad probiótica importante por su repercusión en la protección frente a infecciones, es la producción de sustancias antimicrobianas, como ácidos orgánicos, bacteriocinas, peróxido de hidrógeno o reuterina ya que pueden impedir la presencia y/o el crecimiento de patógenos en las mucosas. De hecho, tanto los lactobacilos aislados en esta Tesis, como otras bacterias lácticas aisladas por otros autores a partir de la leche materna (Beasley y Saris., 2004), mostraron un gran potencial para producir algunas de estas sustancias inhibitorias. Aunque no se pudo detectar la producción de bacteriocinas en los sobrenadantes de las cepas estudiadas en este trabajo, existen cepas de *L.gasseri* y *L.salivarius* de origen fecal con capacidad para producir bacteriocinas (Flynn y col.,2002; Toba y col., 1991). Beasley y Saris (2004), describieron que aproximadamente un 30% de las muestras

de leche de mujeres finlandesas contenían cepas de *L.lactis* productoras de la bacteriocina nisina; anteriormente, se había propuesto que tales cepas tenían un potencial como agente bioterapéutico para prevenir infecciones neonatales y mastitis causadas por *S.aureus* (Heikkilä y Saris., 2003).

De las cepas Hereditum, *L.coryniformis* CECT5711 se ha descrito como la primera cepa no perteneciente a la especie *L.reuteri,* que produce reuterina como sustancia antimicrobiana (junto con la producción de la vitamina cobalamina en el proceso) (Martín y col., 2005b) con un patrón que fue muy similar al obtenido por Talarico y col. (1988) para la producción de reuterina por *L.reuteri* 1063. Los rumiantes contienen microorganismos en su rumen que sintetizan cobalamina; pero, entre los microorganismos que constituyen la microbiota intestinal de los humanos tal capacidad es escasa, como resultado, los humanos deben absorber esta coenzima a partir de los alimentos (Roth y col., 1996).

Por lo que respecta a los ácidos orgánicos, todas las cepas Hereditum fueron capaces de producir ácido láctico en caldo MRS mientras que únicamente *L.fermentum* CECT5716 fue capaz de producir ácido acético en concentraciones relativamente elevadas (Martín y col., 2005a). Otras cepas de *L.fermentum* también han demostrado un gran potencial para producir ácidos acético y láctico (Annuk, 2002). Curiosamente, la cantidad de ácido láctico que produjeron las cepas evaluadas cuando crecieron en leche de vaca fue muy inferior a la que se observó en el medio de cultivo MRS (tabla 4.2). Este hecho explicaría porqué la leche materna no fermenta en el interior de la glándula mamaria a pesar de la presencia de bacterias potencialmente acidificantes. En cualquier caso, la capacidad de producir ácidos orgánicos puede resultar muy útil una vez que estas bacterias son transferidas al intestino del lactante ya que, en este ecosistema, pueden contribuir a mantener un pH ácido deseable. El ácido láctico, además de reducir el pH ambiental, funciona como un permeabilizador de la membrana externa de bacterias Gram-negativas y, por lo tanto, puede potenciar el efecto de otras sustancias antimicrobianas (Alalomi y col., 2000). En este sentido, Strus y col. (2004) observaron que el efecto bactericida del sobrenadante de bacterias productoras de peróxido de hidrógeno frente a *E.coli* y *Candida albicans,* era mayor que el que provocaba una cantidad equivalente de peróxido de hidrógeno puro.

Tabla 4.2: Concentración de L- y D-láctico ((mg/mL) ± DE) y pH de los sobrenadantes obtenidos de MRS y leche UHT de lactobacilos (n=4). Los valores iniciales de pH del MRS y de la leche UHT fueron 6,2 y 6,5 respectivamente. ND= no detectado

	MRS			Leche UHT		
Cepa	**pH**	**L-Láctico**	**D-Láctico**	**pH**	**L-Láctico**	**D-Láctico**
L.coryniformis CECT5711	4,04	4,34 ± 0,88	3,05 ± 0,55	5,7	0,18 ± 0,02	0,22 ± 0,03
L.salivarius CECT5713	3,93	10,26 ± 0,62	ND	5,75	0,19 ± 0,02	ND
L.gasseri CECT5714	4,03	3,16 ± 0,15	3,83 ± 0,74	6,16	ND	ND
L.gasseri CECT5715	3,96	2,73 ± 0,21	2,88 ± 1,22	6,2	ND	ND
L.fermentum CECT5716	4,24	4,83 ± 1,09	3,80 ± 0,12	5,9	ND	ND
L.rhamnosus GG	3,98	6,40 ± 1,14	0,45 ± 0,05	6,22	ND	ND
L.johnsonii La1	3,97	3,94 ± 0,47	7,95 ± 0,62	6,03	ND	ND
L.casei inmunitas	4,01	7,29 ± 0,87	0,61 ± 0,14	5,1	0,62 ± 0,16	ND

Por otra parte, la producción de peróxido de hidrógeno por *L.salivarius* CECT5713 y las dos cepas de *L.gasseri* CECT5714 y CECT5715, fue moderada, estando dentro de los valores obtenidos previamente en otras cepas presentes en neonatos pertenecientes a estas especies (Martín y col., 2005;2006; Song y col., 1999). La presencia de lactobacilos productores de peróxido de hidrógeno en leche materna también resulta muy interesante ya que se ha descrito que, en la mucosa vaginal, este metabolito representa uno de los principales mecanismos de defensa frente a patógenos (Ocaña y col., 1999; Redondo-López y col., 1990); es más, la ausencia de lactobacilos con esta propiedad está correlacionada con una elevada tasa de infecciones vaginales (Eschenbach y col., 1989; Hawes y col., 1996).

La producción del isómero D del ácido láctico es objeto de gran controversia en el campo de la nutrición infantil, aunque se trata de una polémica más asentada en el terreno de lo comercial que en el estrictamente científico. El Real Decreto 72/1998 incorporó al Ordenamiento Jurídico español la Directiva 91/321/CEE de la Unión Europea, que regula el empleo de bacterias tanto en fórmulas infantiles y de continuación dirigidas a niños sanos como en alimentos infantiles con fines médicos específicos, indica que se pueden utilizar bacterias no patógenas productoras del isómero L(+) del ácido láctico para la producción de leches acidificadas. Esta directiva recoge una recomendación del CODEX de 1976 basada en tres estudios realizados con niños (Droese y Stolley, 1962; 1965; Jacobs y Christian, 1957). Estos estudios se realizaron con ácido D-láctico puro en unas condiciones que no son en

absoluto extrapolables al efecto que ejercerían cepas productoras de D- o DL-lactato. Ante las escasas evidencias científicas que avalan las recomendaciones del CODEX, los expertos de la Unión Europea han recomendado que se estudien los casos de forma individualizada, teniendo en cuenta los datos disponibles sobre la seguridad y la eficacia de cada cepa (http://europa.eu.int/comn/food/fs/sc/outcome_en.htlm).

De hecho, el principal problema asociado con el D-lactato (acidosis) se presenta en niños con síndrome de intestino corto y, curiosamente, se ha demostrado que la administración de ciertas bacterias lácticas productoras de D-lactato a niños con este síndrome conduce a una remisión de la sintomatología más rápida que cuando se administran cepas probióticas que exclusivamente producen L-lactato (Vanderhoof y col., 1998). Adicionalmente, el ácido D-láctico sirve de sustrato para las bacterias productoras de butirato (Tsukahara y col., 2002), por lo que su presencia en el intestino estaría contribuyendo a la producción de un metabolito relacionado con importantes actividades anti-neoplásicas (DÁrgenio y Mazzacca, 2003). La capacidad de los lactobacilos para producir los isomeros D(-), L(+) del ácido láctico o la mezcla de ambos, es una característica propia de cada especie (Hammes y col., 1992).

Entre las bacterias analizadas en esta Tesis, sólo *L.salivarius* CECT5713 produjo L(+)-láctico (Martín y col., 2006), el resto dio lugar a una mezcla racémica de D(-) y L(+)- láctico. La proporción de ambos enantiómeros fue de, aproximadamente, 1:1 (D:L). Lapierre y col. (1999) encontraron para la cepa *L.johnsonii* La1 una relación 2:1(Martín y col., 2005a), cepa de la que cabe destacar que se encuentra en leches fermentadas que se consumen en todo el mundo sin que, hasta la fecha, se hayan registrado efectos negativos asociados a su consumo por la población infantil. Lo mismo sucede en el caso de *L.delbrueckii* Subs. *bulgaricus,* una de las bacterias que componen el cultivo iniciador del yogur y que produce exclusivamente el isómero D del ácido láctico. Finalmente, otras bacterias presentes en leche materna, como *Leuconostoc* sp. y *L.reuteri* (Hammes y col., 1992; Kandler y col., 1980; Karvonen y col., 1998), también producen D(-)-lactato y diversos estudios han demostrado la eficacia y seguridad de una cepa de *L.reuteri* aislada de leche humana en niños menores de un año (Shornikova y col., 1997a; 1997b; Weizman y col., 2003; 2005).

Las aminas biógenas son compuestos orgánicos alcalinos que se producen en diferentes tipos de alimentos, incluidos los productos lácteos, debido a la actividad amino-descarboxilasa de algunos microorganismos presentes en los alimentos. A pesar de que las aminas biógenas (histamina, tiramina, putrescina, cadaverina,…) son necesarias para ciertas funciones fisiológicas, el consumo de alimentos con altas concentraciones de estos compuestos puede derivar en graves problemas toxicológicos. La histamina y la tiramina tienen propiedades vasoactivas y psicoactivas, mientras que la putrescina y la cadaverina potencian los efectos de las anteriores y , además, son precursores de nitrosaminas carcinogénicas en alimentos (Bover-Cid y Holzapfel, 1999). Dado que algunos lactobacilos tienen capacidad para producir estas sustancias (Coton y col., 1998; de las Rivas y col., 2005) resulta conveniente evaluar esta propiedad en cualquier cepa que pretenda utilizarse con fines probióticos, especialmente en aquellas que se deseen vehicular en alimentos fermentados. Al contrario de lo que ocurre con el ácido D-láctico, la producción de aminas biógenas depende de cada cepa (Coton y col., 1998) y no es una característica propia de especie. No se pudo detectar la producción de aminas biógenas en los lactobacilos Hereditum (Martín y col., 2005a;2005b; Martín y col., 2006).

Los lactobacilos juegan un papel muy importante en las barreras microbiológicas primarias que se forman en las mucosas con el fin de prevenir infecciones. Para que puedan ejercer esta acción de manera eficaz, las bacterias deben poseer una propiedad particularmente relevante: capacidad de adhesión al epitelio intestinal. Esta propiedad representa un prerrequisito para la colonización intestinal por parte de una cepa probiótica y, adicionalmente, propicia los fenómenos de inmunomodulación y de exclusión competitiva de bacterias patógenas (Fernández y col., 2003; Mack y col., 1999; Schiffrin y col., 1995). Las líneas celulares HT-29 y Caco-2 han sido ampliamente empleadas para evaluar la capacidad de adhesión de bacterias lácticas y bifidobacterias (Bernet y col., 1994; Coconnier y col., 1993; Dunne y col., 2001; Elo y col., 1991; Forestier y col., 2001; Tuomola y Salminen, 1998) ya que, una vez diferenciadas, muestran características propias de los enterocitos maduros, como la polarización y la presencia de microvellosidades funcionales e hidrolasas en su extremo apical (Pinto y col., 1983). Todas las cepas Hereditum evaluadas en esta Tesis, mostraron un gran potencial de adhesión a cultivos de las dos líneas celulares mencionadas anteriormente, mostrando un comportamiento similar al de algunas cepas comerciales (tabla 4.3)

(Martín y col., 2005a;2005b; Martín y col., 2006). Previamente, se ha descrito la capacidad de colonización intestinal de diversas cepas de *L.fermentum* (Reid y col., 2001a), así como la gran adherencia de diversas cepas de *L.gasseri* de origen humano a las células Caco-2 (Fernández y col., 2003; Martín y col., 2005a).

Tabla 4.3: Capacidad de adhesión de lactobacilos a células HT-29 y Caco-2

Cepa	HT-29	Caco-2
L.coryniformis CECT5711	940,1 ± 690,7	388,00 ± 221,78
L.salivarius CECT5713	682,6 ± 354,6	242,5 ± 127,1
L.gasseri CECT5714	873,1 ± 452,5	249,9 ± 166,9
L.gasseri CECT5715	633,8 ± 535,21	325,2 ± 178,2
L.fermentum CECT5716	905,65 ± 596,6	277,01 ± 185,4
L.rhamnosus GG	920,0 ± 450,5	381,05 ± 208,6
L.johnsonii La1	644,0 ± 462,5	253,23 ± 161,3
L.casei inmunitas	144,7 ± 110,9	15,9 ± 10,43

Los modelos basados en líneas celulares Caco-2 y HT-29 resultan muy útiles dentro de los esquemas de preselección de bacterias probióticas y, de hecho, diversos estudios han demostrado una buena correlación entre la capacidad de adhesión *in vitro* y efectos probióticos *in vivo.* No obstante, ciertos autores cuestionan su valor predictivo debido, principalmente, a su incapacidad para sintetizar el mucus que recubre al epitelio intestinal (Bezkorovainy, 2001; Vaughan y col., 2002).

Aunque las propiedades probióticas de las cepas Hereditum ensayadas *in vitro* auguraban la efectividad de estas cepas *in vivo,* son necesarios estudios preclínicos en animales y finalmente los ensayos clínicos en humanos para poder determinar la utilidad de estas cepas para su uso en el mantenimiento o mejora del estado de salud.

Objetivos

Un gran número de evidencias científicas demuestran que, las bacterias lácticas ejercen numerosos efectos beneficiosos para la salud tales como la mejora de la intolerancia a la lactosa, la prevención y atenuación de infecciones intestinales, alergias y enfermedades inflamatorias intestinales, efectos inmunomoduladores, etc (Gill y Guarner, 2004). Estos estudios, han demostrado la singularidad de cada cepa, indicando la necesidad de estudiar las características propias de cada una de forma que, su selección para una determinada aplicación esté basada en sus propiedades particulares y en las condiciones de salud del consumidor al que vaya dirigido su consumo.

Actualmente, la mayor parte de las cepas probióticas que se utilizan comercialmente han sido inicialmente aisladas de heces humanas ya sean de adultos o de niños. Sin embargo, recientemente se ha demostrado que la leche materna no es estéril, sino más bien constituye una fuente excelente y continúa de bacterias que serán transferidas al bebé a través de la lactancia para iniciar su propia microbiota intestinal. Entre las bacterias comúnmente aisladas de la leche materna se encuentran representantes de los géneros estafilococos, estreptococos, lactococos, enterococos y lactobacilos (Heikkilä y col., 2003; Martín y col., 2003). La presencia de bacterias lácticas en la leche materna con efectos probióticos podría explicar, al menos en parte, la menor incidencia de infecciones o alergias en niños amamantados en comparación con los alimentados con fórmulas infantiles.

Por tanto, la leche materna es una fuente excelente de bacterias para buscar nuevas cepas con un destacado potencial probiótico que puedan ser aplicadas en humanos. Tras un proceso de selección que contempló aspectos de seguridad, funcionalidad y tecnológicos, 4 cepas a las que se denominó Hereditum®, fueron seleccionadas:

L. coryniformis CECT5711
L. salivarius CECT5713
L. gasseri CECT5714
L. fermentum CECT5716

El objetivo general de esta Tesis es la evaluación preclínica y clínica del efecto probiótico de las cepas Hereditum® con el fin de demostrar su eficacia y, determinar las posibles aplicaciones tanto en nutrición infantil como en la de adultos, así como, en el campo de la prevención y tratamiento de ciertas enfermedades.

Con este fin se plantearon los siguientes objetivos que se han desarrollado en la presente Tesis Doctoral:

1. Demostrar la tolerancia y seguridad de las cepas Hereditum® en modelos animales, utilizando dosis superiores a las dosis habitualmente consumidas por humanos, con el fin de evaluar su seguridad en humanos.

2. Validación preclínica de las cepas Hereditum® en animales de experimentación que, demuestren la eficacia de los probióticos *in vivo* y permitan caracterizar las propiedades probióticas de cada cepa en particular con el fin de definir las aplicaciones más adecuadas para cada una de ellas.

3. Validación clínica de las cepas Hereditum®. Demostrar la funcionalidad de cada cepa en la población a la que se vaya a dirigir su consumo ya sea en individuos sanos o enfermos.

Resultados

1. Evaluación de la seguridad de la cepa probiótica *L.salivarius* CECT5713 aislada de leche materna

RESUMEN

OBJETIVO

Aunque, gracias a su largo historial de consumo los lactobacilos son cepas consideradas como seguras, es cierto que hay algunos estudios que han identificado a bacterias ácido lácticas asociadas a patologías clínicas tales como, bacteremia y ocasionalmente endocarditis y abscesos. No obstante, en todos los casos la infección estaba asociada a patologías subyacentes. En los últimos años, los criterios de seguridad se han considerado críticos en la selección de nuevas cepas para su uso en alimentación, por ello, se han sugerido una serie de criterios de selección tales como, que la cepa sea habitante normal del hospedador diana y la ausencia de patogenicidad entre otros. Todos estos aspectos han sido contemplados en el proceso de selección de las cepas Hereditum.

Lactobacillus salivarius posee estatus QPS (Qualified Presumption of Safety) de la Agencia Europea de Seguridad Alimentaria (EFSA). El estatus QPS de la EFSA se otorga a especies de microorganismos para las que existe un amplio historial de uso seguro en alimentación y cuyo consumo no representa ningún riesgo para la salud, basado en la identidad de la especie, el conocimiento científico de la misma, los datos de patogenicidad y los usos finales. En este sentido, las cepas de microorganismos con estatus QPS no necesitan estudios adicionales de evaluación de seguridad.

Sin embargo, con el fin de avalar su seguridad se ha realizado un estudio de tolerancia oral del consumo de la cepa probiótica *L.salivarius* CECT5713 aislada de leche humana en un modelo murino.

RESULTADOS

Dosis entre 500 y 10000 veces mayores (por Kg de peso corporal) a las dosis que normalmente son consumidas por humanos, fueron administradas diariamente a ratones Balb/C durante 4 semanas. Se analizaron diversos parámetros bioquímicos y clínicos, así como, translocación bacteriana. Para excluir la toxicidad en el caso de una potencial translocación, se simuló un caso de translocación mediante la

adiministración intraperitoneal de $5x10^8$ ufc/ratón de la cepa probiótica a un grupo de ratones, evaluándose los efectos nocivos en diferentes tejidos.

La administración oral de *L.salivarius* CECT5713, no tuvo efectos adversos en los ratones incluso a la dosis superior. No se observaron signos de bacteremia ni de translocación asociada al consumo oral de la bacteria. La administración intraperitoneal no produjo muerte ni enfermedad de los ratones.

CONCLUSIÓN

Por tanto, *L.salivarius* CECT5713 es totalmente segura en animales sugiriendo la seguridad de su uso en humanos.

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Safety Assessment of the Human Milk-Isolated Probiotic *Lactobacillus salivarius* CECT5713

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ABSTRACT

The potential probiotic bacteria *Lactobacillus salivarius* CECT5713 has recently been isolated from human milk and characterized. The objective of the present study was to evaluate the oral toxicity of this potential probiotic bacteria in mice. With this aim, 50 Balb/C mice were divided in 5 groups (n = 10). Three of these groups were treated orally with different doses of *L. salivarius* CECT5713: 5×10^8, 2×10^9, or 10^{10} cfu/mouse per d for 28 d. One additional group was administered the vehicle alone and was used as a control. The last group were injected intraperitoneally with 10^8 cfu/mouse in a single dose and killed 2 (n = 5) and 5 (n = 5) d after intraperitoneal injection. Food intake, body weight, bacterial translocation, serum α-amyloid protein, and different biochemical parameters were analyzed. Oral administration of *L. salivarius* CECT5713 to mice had no adverse effects on mouse body weight or food intake. No bacteremia was shown and there was no treatment-associated bacterial translocation to the liver or spleen. Intraperitoneal administration caused a significant bacterial translocation to the liver and spleen, but not to the blood. However, this translocation was not related to illness or death at either d 2 or d 5, although an increase in plasma serum α-amyloid protein was observed at d 2. These results suggest that the strain *L. salivarius* CECT5713 is nonpathogenic for mice, even in doses 10,000 times higher (expressed per kilograms of body weight) than those normally consumed by humans. Thus, this strain is likely to be safe for human consumption.
Key words: probiotic, translocation, safety, mice

INTRODUCTION

Although the use of lactic acid bacteria (**LAB**) in fermented food dates back many centuries (Ballongue, 1998), in the last 2 decades there has been an increasing interest in the use of these bacteria for nutritional and even medical applications, taking advantage of their capacity to modulate intestinal microbiota. Thus, the health-promoting effects of probiotic bacteria, most of them included in the LAB group, are well documented both in animal models and in clinical trials. These include anti-infection properties (Isolauri et al., 1991), beneficial effects in intestinal inflammation (Perán et al., 2005), immunomodulatory activity (Olivares et al., 2006), and efficacy in the prevention of allergic diseases (Furrie, 2005). The health benefits described for probiotics make them good candidates for functional foods, and a high number of new bacterial strains are being identified and incorporated into food and pharmaceutical products.

In the selection of new probiotic strains, safety criteria are applied, such as a long history of use in humans and the absence of pathogenic mechanisms (Collins et al., 1998). However, some reports have identified LAB as being associated with clinical pathological conditions such as bacteremia (Bayer et al., 1978) and occasionally endocarditis and abscess (Aguirre and Collins, 1993), although these studies are rare and it is unlikely that LAB were the causative agent in these cases (Gasser, 1994). These reports have resulted in concern about the safety of these probiotic bacteria, particularly those strains that are being introduced into the human food chain. Thus, safety assessments are recommended for probiotic strains that are aimed at being incorporated into food products (Conway, 1996).

Recently, a new potential probiotic strain has been isolated and characterized in our laboratory, *Lactobacillus salivarius* CECT5713. This strain has been isolated from infant feces and breast milk of a mother-child pair (Marín et al., 2006). Concerning its probiotic characteristics, *L. salivarius* CECT5713 has been shown to survive in gastrointestinal tract conditions, to adhere to intestinal cells, and to have antimicrobial activity (Marín et al., 2006). Because its probiotic potential suggests that this strain could be of interest for human consumption, we decided to perform an oral safety assessment to preclude deleterious effects. With this aim, mice were administered doses 500 to 10,000

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times higher (per kilogram of BW) than those normally consumed by humans, and oral toxicity, biochemical and clinical parameters, and bacterial translocation were analyzed. To preclude toxicity even in the case of potential translocation, a group of mice were injected intraperitoneally with the bacteria and the harmful effects of the translocation of bacteria to different tissues were evaluated.

MATERIALS AND METHODS

Animals

Fifty male Balb/C mice, aged 6 to 8 wk, bred at the Universidad de Granada facilities (Granada, Spain) were housed in stainless-steel cages with a 12-h light/dark cycle (0800 to 2000 h) in a controlled atmosphere (temperature 22 ± 2°C, humidity 55 ± 2%). The animals were fed a purified diet ad libitum (Harlan, Barcelona, Spain) and had free access to water during the experimental protocol. The protocol was carried out according to the guidelines of the Helsinki declaration and was approved by the Ethics Committee of Animal Experiment **[Au: Ethics Committee on Animal Experimentation (or Experiments)?]** of the Universidad de Granada.

Experimental Design

After 7 d of acclimation, mice were randomly assigned into 1 of 5 experimental groups (n = 10 per group) as follows: 3 groups were orally administered with either 5×10^8, 2×10^9, or 10^{10} cfu/mouse per d dissolved in skim milk for 28 d (0.1 mL/mouse per d). One additional group received the vehicle alone and was used as a control. Intragastric feeding was performed by means of a poly(vinyl chloride) tube feeding needle purchased from Vygon (Ecoue, France). The last group received intraperitoneal administration of a single dose of 5×10^8 cfu/mouse dissolved in saline solution (0.1 mL/mouse) and animals were killed at d 2 (n = 5) and d 5 (n = 5) after intraperitoneal injection. Throughout the experiment, the activity, behavior, and general health of the animals were observed daily. Body weight and food intake were recorded once a week. Mice were killed by intraperitoneal administration of sodium pentothal (50 mg/kg), and blood was collected by cardiac puncture in EDTA-containing tubes in sterile conditions. The liver and spleen were removed in sterile conditions and weighed. The thymus, kidney, and heart were also removed from the intraperitoneally injected mice and weighed.

Bacterial Translocation

Bacterial translocation was analyzed in the blood, liver, and spleen. A 50-μL quantity of blood was cultured in de Man, Rogosa, Sharpe (**MRS**) agar medium and brain heart infusion (**BHI**) agar and incubated at 37°C for 48 h anaerobically for MRS and aerobically for BHI. Tissue samples were homogenized in buffered peptone water (1 g/mL) and 100 μL of the resulting homogenates were cultured in MRS and BHI agar as previously mentioned. After 48 h of incubation, colony-forming units were counted and the results were expressed as incidence of translocation (number of mice in which colony-forming units were detected/total number of mice). Positive growth on agar plates was defined by the presence of any microorganism (even a single colony).

Identification of Bacteria by PCR

For detection of *L. salivarius* CECT5713, PCR was performed directly from MRS agar colonies using the following specific primers: 5′-GAT CGC TAT TTT TTT ATT AGG TAT C- 3′ and 5′-TGG CTA ACT TGT TTT TTT ACT TC-3′.

Serum α-Amyloid Protein

With the aim of analyzing plasma markers of sepsis in mice injected intraperitoneally with the bacteria, plasma serum α-amyloid (**SAA**) concentration was measured with an ELISA kit (Biosource, Camarillo, CA). A 100-μL quantity of plasma or standard was added to each well of a microtiter plate previously coated with a monoclonal antibody specific for SAA. Immediately, 50 μL of a peroxidase-conjugated antimouse SAA was also added to each well. After 1 h of incubation at 37°C, 100 μL of tetramethylbenzidine was added to wells and the reaction was stopped by addition of 100 μL of H_2SO_4 (2 *M*). Finally, optical density was measured at 450 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Hercules CA).

Total Liver Glutathione Concentration

A 100-mg quantity of liver from each mouse was homogenized in a 7.5% TCA solution by means of an UltraTurrax homogenizer (Heidolph, Barcelona, Spain) and homogenates were centrifuged at 3,000 × *g* for 10 min at 4°C. Total glutathione (**GSH**) concentration was measured in the supernatants using a colorimetric commercial kit (OxisResearch, Portland, OR). Briefly, 40 μL of the homogenates or the standards was added to each well of a microtiter plate, together with 40 μL of a reducing agent [tris(2-carboxyethyl)phosphine in

Table 1. Body and tissues weights of mice treated orally with different doses of *Lactobacillus salivarius* CECT5713 for 28 d[1]

Item	Dose			
	Control	5×10^8 cfu/mouse per d	2×10^9 cfu/mouse per d	10^{10} cfu/mouse per d
Initial weight, g	18.30 ± 0.93	18.50 ± 0.65	18.37 ± 0.73	18.36 ± 0.67
Final weight, g	20.32 ± 1.41	20.46 ± 1.10	20.41 ± 1.10	20.24 ± 1.24
Weight increase, %	11.05 ± 1.32	10.58 ± 2.53	11.12 ± 2.10	10.23 ± 1.67
Food intake, g/mice per d	2.93 ± 0.35	2.85 ± 0.40	2.73 ± 0.38	2.93 ± 0.43
Liver weight, mg	1,041 ± 33	1,053 ± 110	1,029 ± 115	1,009 ± 96
Spleen weight, mg	94.7 ± 11.6	96.6 ± 13	95.9 ± 23	89.9 ± 17

[1]Data are means (n = 10) ± SD.

HCl], 40 μL of a chromogen (1-methyl-3-chloro-7-trifluoromethylquinolinium methylsulfate in HCl), and 40 μL of color developer (NaOH). After 30 min of incubation at room temperature and in the dark, optical density was measured at 415 nm using a microplate spectrophotometer (Bio-Rad Laboratories).

Malondialdehyde Plasma Concentration

Malondialdehyde (**MDA**) plasma concentration was measured by separation with HPLC, by using a previously described method based on thiobarbituric acid reaction and reverse-phase separation with fluorescence detection (Fukunaga et al., 1998).

Evaluation of the General Health Status of Mice

To evaluate possible changes in physiological functions, because of the oral treatment with bacteria, glucose concentration and glutamic-oxalacetic transaminase (**GOT**) activity were measured in plasma samples by means of a commercial kit (Stangest Biomedical IND, Tarragona, Spain).

Blood cell counts (white and red cells) were also analyzed with an automatic hematology counter (Minos, ABX, Montpellier, France) on fresh blood, to evaluate possible modifications due to infection caused by oral administration of the bacteria.

Statistical Analysis

All results are expressed as the mean ± standard deviation. Differences between means were tested for statistical significance using one-way ANOVA and post hoc least significance tests. Differences between proportions (bacterial translocation incidence) were analyzed with the chi-squared test. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC Inc., Rockville, MD), with statistical significance set at $P < 0.05$.

RESULTS

Oral Administration of Different Doses of L. salivarius CECT5713

During the experimental protocol, no noticeable activity or behavioral changes were observed in the mice and no illness or death occurred. There was no difference in aspect between animals in the treatment and control groups.

Oral administration of *L. salivarius* CECT5713 had no adverse effects on mouse food intake; there was no difference in food intake between the control and treatment groups throughout the experiment (Table 1). As shown in Table 1, no significant difference was observed in BW gain between the control group and the groups treated orally.

Concerning tissue weights, there were no significant differences in liver and spleen weights between control mice and those receiving oral administration of *L. salivarius* CECT5713 (Table 1). Previous studies in our laboratory showed that the higher dose of the potential probiotic (10^{10} cfu/d) did not cause changes ($P > 0.1$) in the weight of other tissues, such as the thymus, kidney, heart, or colon weight/length ratio in control mice compared with treated mice (10^{10} cfu/d). In agreement with the absence of clinical symptoms in mice, plasma GOT activity and glucose concentration did not change, even in the group receiving the highest dose of *L. salivarius* CECT5713 (10^{10} cfu/mice per d; Table 2).

The incidence of translocation of bacteria from the gut to different tissues is shown in Table 3. No bacteremia was observed in any of the experimental groups. There was also no statistically significant difference in the incidence of translocation to the liver or spleen between the control and treated groups at any of the tested doses. In addition, colonies found on agar plates were checked by conventional PCR using specific primers, and none of the colonies corresponded to the administered strain *L. salivarius* CECT5713 (data not shown).

Table 2. Biochemical and hematological data in control and *Lactobacillus salivarius* CECT5713-treated mice (10^{10} cfu/mouse per d)[1] **[Au: Please verify red blood cell values in col. 3]**

Item	Control	Dose, 10^{10} cfu/mouse per d
Leukocytes, cells/mL	3,987 ± 1,663	3,987 ± 1,664
Red blood cells, cells/mL	$6.4 \times 10^6 \pm 3.1 \times 10^5$	$7.4 \times 10^6 \pm 1.2 \times 10^6$
Glutamic-oxalacetic transaminase, IU **[Au: Ok as IU?]**	184.3 ± 80.6	199.8 ± 118.0
Glucose, g/L	125.0 ± 14.3	136.4 ± 11.0

[1]Data are means (n = 10) ± SD.

To detect signs of infection due to treatment, the oxidative status of mice was analyzed. With this aim, liver GSH concentration and plasma MDA concentration were determined. As shown in Table 4, oral administration of *L. salivarius* CECT5713 caused a decrease ($P < 0.05$) in the liver content of GSH. This decrease was not dose dependent, because there was no difference between the groups receiving different doses of the potential probiotic. In contrast, plasma concentration of MDA did not show changes, either between the control and treatment groups or between the groups receiving different doses of the potential probiotic. Intraperitoneal Administration of L. salivarius CECT5713

As shown in Table 5, intraperitoneal administration caused a significant translocation of *L. salivarius* CECT5713 to the liver and spleen. Although the presence of bacteria in the liver was similar 2 and 5 d after intraperitoneal injection, in the spleen the incidence of bacterial translocation was lower at d 5, even though the difference was statistically significant only for bacteria grown in MRS. In contrast, no bacteremia was shown in any of the mice injected intraperitoneally with *L. salivarius* CECT5713. The presence of bacteria in the liver and spleen was accompanied by a significant increase in liver weight at d 2 and in spleen weight at d 2 and 5 (Table 6), whereas the weight of the other analyzed tissues (thymus, heart, and kidney) did not change (data not shown).

In spite of bacterial translocation to the liver and spleen and the increase in these tissue weights, no noticeable behavioral changes were observed and there was no illness or death related to *L. salivarius* CECT5713 administration. In addition, there were no significant changes in mouse BW throughout the 5 d after intraperitoneal injection (Table 6).

Intraperitoneal injection of *L. salivarius* CECT5713 did not statistically modify the plasma concentration of MDA, a marker of oxidative stress, because the plasma MDA concentration was similar to that of the control mice. In contrast, a decrease in the hepatic GSH content was observed at d 2 after injection, although at d 5 the hepatic content of GSH was similar to that of the control mice (Table 6). Plasma concentration of SAA, an acute-phase protein reported to be increased in sepsis (Dofferhoff et al., 1992), was significantly increased in mice 2 d after injection, but it returned to basal values at d 5 (Table 6). Concerning the hematology results, intraperitoneal injection did not statistically **[Au: significantly?]** modify the number of red blood cells and leukocytes.

Table 3. Incidence of bacterial translocation to the blood, liver, and spleen in mice treated with different doses of *Lactobacillus salivarius* CECT5713[1]

	Dose			
Item[2]	Control	5×10^8 cfu/mouse per d	2×10^9 cfu/mouse per d	10^{10} cfu/mouse per d
Blood				
MRS	0/10	0/10	0/10	0/10
BHI	0/10	0/10	0/10	0/10
Liver				
MRS	3/10 (32–234)	2/10 (6–60)	2/10 (49–345)	0/10
BHI	5/10 (50–800)	5/10 (60–900)	3/10 (20–265)	2/10 (35–190)
Spleen				
MRS	1/10 (32)	0/10	1/10 (43)	2/10 (25–85)
BHI	1/10 (130)	0/10	1/10 (105)	0/10

[1]Numbers in parentheses represent the range of bacteria expressed as colony-forming units per organ.
[2]MRS = de Man, Rogosa, Sharpe agar medium; BHI = brain heart infusion agar.

Table 4. Liver glutathione (GSH) content and plasma concentration of malondialdehyde (MDA) in mice treated with different doses (cfu/mouse per d) of *Lactobacillus salivarius* CECT5713[1]

	Dose			
Item	Control	5×10^8 cfu/mouse per d	2×10^9 cfu/mouse per d	10^{10} cfu/mouse per d
Liver GSH, μmol/g	9.11 ± 1.38	7.41 ± 1.01*	7.72 ± 0.95*	7.71 ± 0.73*
Plasma MDA, μ*M*	4.57 ± 1.20	4.89 ± 1.03	4.18 ± 1.03	3.99 ± 0.40

[1]Data are means (n = 10) ± SD.

*$P < 0.05$ vs. the control.

DISCUSSION

During the last several years, there has been an increasing interest in the manipulation of intestinal microbiota with probiotics, which has resulted in the isolation of new LAB strains to be included in food and pharmaceutical products. For this reason, the safety of these probiotics has been the subject of active discussion. Although there have been no general guidelines on this issue up to now, acute oral toxicity has been proposed as a fundamental test for the assessment of probiotic safety (Stine and Brown, 1996) and has been applied previously in safety assessment studies (Donohue et al., 1993; Zhou et al., 2000).

Oral toxicity assessment showed that mice treated orally with huge doses of the potential probiotic strain *L. salivarius* CECT5713 were healthy and survived daily administration after 28 d. No adverse effects were observed on BW and food intake, indicating that the potential probiotic does not exhibit gross oral toxicity, and no deleterious effects were shown on the health status, growth, or development of the animals. In addition, no dose-dependent effect was observed in any of the parameters measured, thus suggesting the absence of a treatment-related deleterious effect.

Table 5. Incidence of translocation in mice injected intraperitoneally with 5×10^8 cfu/mouse of *Lactobacillus salivarius* CECT5713 at d 2 and 5 after injection[1]

Item[2]	Control	Day 2	Day 5
Blood			
MRS	0/10	0/5	0/10
BHI	0/10	0/5	0/10
Liver			
MRS	3/10 (50–230)	5/5* (90–1,800)	4/5* (98–1,116)
BHI	5/10 (90–900)	5/5* (312–1,025)	4/5* (50–1,130)
Spleen			
MRS	1/10 (55)	5/5* (185–1,024)	1/4[#] (24)
BHI	1/10 (98)	5/5* (193–880)	2/5*[#] (25–45)

[1]Numbers in brackets represent colony-forming units per organ.

[2]MRS = de Man, Rogosa, Sharpe agar medium; BHI = brain heart infusion agar.

*$P < 0.05$ vs. control; [#]$P < 0.05$ vs. d 2.

Bacterial translocation is a recommended indicator of probiotic toxicity, because it is the first step in the pathogenesis process for many opportunistic strains indigenous to the lumen (Steffen and Berg, 1983). In spite of the high doses of bacteria administered to mice, there was no bacteremia in any of the treated groups. We obtained bacterial cells from the liver and spleen (see Table 3), but in no case was *L. salivarius* CECT5713 found, and the incidence was similar in mice receiving the potential probiotic compared with control mice, thus suggesting that translocation was not associated with treatment. These data suggest that high doses of the test potential probiotic administered orally to mice do not cause bacterial translocation to the blood, spleen, or liver. The presence of some bacteria in the liver and spleen was previously described in healthy mice (Ma et al., 1990). According to our results, there were no signals of sepsis in mice because the plasma concentration of MDA, an oxidative stress marker (Gil et al., 2006), was similar in the control and treatment groups. Regarding the hepatic content of GSH, an increase in this parameter has been reported to be related to the acute phase of sepsis (Malmezat et al., 1998), but to our knowledge a decrease has not been reported to be clinically significant in sepsis. Although the decrease observed in the hepatic content of GSH in our experimental model was not related to any symptoms of infection, further investigation is needed to elucidate the biological relevance of this decrease.

In agreement with the absence of any symptoms of infection in mice treated orally with the bacteria, the blood markers of physiological functions (GOT and glucose), as well as the red blood cell and leukocyte counts, did not show any statistically significant difference compared with those of the control mice.

Although the data pointed to the fact that *L. salivarius* CECT5713 was not able to translocate from the gut to other tissues, we decided to induce this translocation by intraperitoneal administration of *L. salivarius* CECT5713 with the aim of precluding adverse effects even in this case. The injection of *L. salivarius* CECT5713 caused a significant initial translocation of

Table 6. Different clinical and biochemical data at d 2 and 5 after intraperitoneal injection (10^8 cfu/mouse) of *Lactobacillus salivarius* CECT5713[1]

Item	Control	Day 2	Day 5
Initial BW **[Au: units?]**	18.69 ± 0.91	19.19 ± 1.27	18.94 ± 1.75
Final BW **[Au: units?]**	19.01 ± 0.85	19.63 ± 1.45	19.82 ± 1.46
Liver weight, mg	1,041 ± 33	1,116 ± 70*	1,086 ± 13*
Spleen weight, mg	94.7 ± 11.6	120.1 ± 14.6*	158.6 ± 16.4*
Plasma malondialdehyde, m*M*	4.57 ± 1.20	3.63 ± 0.32	5.68 ± 2.50
Liver glutathione, μmol/g of tissue	9.11 ± 1.38	7.09 ± 0.64*	8.54 ± 0.46
Plasma serum α-amyloid, pg/mL	31.7 ± 17.1	388.8 ± 83.9*	49.23 ± 82.7
Red blood cells, cells/mL	$6.2 \times 10^6 \pm 3.0 \times 10^5$	$5.8 \times 10^6 \pm 2.5 \times 10^5$	$6.5 \times 10^6 \pm 2.8 \times 10^5$
Leukocytes, cells/mL	3,987 ± 1,664	4,152 ± 1,782	3,853 ± 1,663

[1]Values are mean (n = 10 for control; n = 5 for d 2 and 5) ± SD.

*$P < 0.05$ vs. control.

the bacteria to the liver and spleen, which produced a significant increase in liver and spleen weights. This increase was probably caused by a transient inflammatory process that could be maintained over time, even in the absence of bacteria. This could explain why the spleen weights did not return to normal values at d 5 in spite of the fact that translocation of the bacteria decreased. In contrast, bacterial translocation did not induce behavioral changes, and there was no illness or death in the injected mice. Intraperitoneal administration of the same dose of other pathogenic microorganisms, such as different strains of *Escherichia coli,* has been reported to be lethal for mice at d 2 after inoculation (Gras et al., 2006). In the case of *L. salivarius* CECT5713 administration, an immediate response was observed at d 2, mainly shown by the increase in the acute-phase protein SAA, but at d 5 this protein returned to basal values, thus suggesting a transitory and slight inflammatory response. Although more studies will be needed to elucidate the significance of the decrease in hepatic GSH content, our results did not show any signs of illness related to this decrease.

These results, suggesting the safety of *L. salivarius* CECT5713 in the case of translocation, are in agreement with previously published results. Perán et al. (2005) evaluated the effect of *L. salivarius* CECT5713 in a rat model of intestinal inflammation, a pathology related to increased intestinal permeability. Even in these intestinal conditions, oral treatment with *L. salivarius* CECT5713, far from being deleterious, showed a beneficial effect, improving clinical manifestation of the inflammation.

In summary, feeding mice with the new LAB strain *L. salivarius* CECT5713 at high doses for 28 d had no deleterious effects on mice. Even in the case of translocation, toxicity of the bacteria seemed to be negligible. These data, together with other important safety properties of *L. salivarius* CECT5713, such as its human milk origin and the absence of D-lactic acid production (Marín et al., 2006), a critical parameter for its use in infant nutrition, allow us to conclude that this strain is likely to be safe for human consumption.

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2. Potencial antimicrobiano de cuatro cepas de *Lactobacillus* aisladas de leche materna

RESUMEN

OBJETIVO

Las diarreas y enfermedades infecciosas causadas por microorganismos patógenos continúan siendo la mayor causa de morbilidad y mortalidad en el mundo. La microbiota intestinal ejerce un papel importante en el efecto barrera de la mucosa intestinal frente a patógenos, inhibiendo su crecimiento e invasión mediante la producción de sustancias antimicrobianas, competición por los mismos sustratos y/o por adhesión a mucinas. Además, las interacciones que tienen lugar en el tracto gastrointestinal entre microbiota, células epiteliales y sistema inmunitario refuerzan la defensa frente a patógenos. Actualmente, hay una gran evidencia de que algunos probióticos ejercen un efecto positivo en la prevención y/o tratamiento de enfermedades infecciosas. Por ello, el objetivo de este estudio fue analizar las propiedades antibacterianas que presentaban las cepas Hereditum.

RESULTADOS

Los resultados obtenidos *in vitro* mediante el uso de ensayos de difusión en agar, de adhesión competitiva y de expresión de mucinas así como, estudios *in vivo* utilizando un modelo de infección en ratones, mostraron que todas las cepas estudiadas presentan propiedades anti-microbianas frente a bacterias patógenas y sugieren que, los lactobacilos de leche materna podrían contribuir a la protección frente a infecciones demostrada en los recién nacidos. Sin embargo, el potencial anti-microbiano es distinto entre las diversas cepas, siendo la cepa *L. salivarius* CECT5713 la que mostró no sólo las mejores actividades anti-microbianas *in vitro*, sino que también mostró, la mayor protección frente a una infección por *Salmonella* en el modelo *in vivo* de ratón.

CONCLUSIÓN

Las cepas probióticas Hereditum y particularmente *L.salivarius* CECT5713, presentan una potente actividad antibacteriana y podrían contribuir a una protección anti-infectiva en recién nacidos, siendo candidatas excelentes para el desarrollo de productos probióticos infantiles.

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ORIGINAL ARTICLE

Antimicrobial potential of four *Lactobacillus* strains isolated from breast milk

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Abstract

Aims: The antimicrobial potential of four lactobacilli (*Lactobacillus salivarius* CECT5713, *Lactobacillus gasseri* CECT5714, *L. gasseri* CECT5715 and *Lactobacillus fermentum* CECT5716), isolated from fresh human breast milk, was evaluated in this study and compared with *Lactobacillus coryniformis* CECT5711, a reuterin-producing strain isolated from an artisan goat's cheese.

Methods and Results: Agar diffusion tests, competitive adhesion assays and mucin expression assays were carried out in order to value the antibacterial properties of the lactobacilli strains. The antibacterial capability of the strains was tested *in vivo* by using a murine infection model with *Salmonella choleraesuis*. The results revealed that all the strains studied, displayed antibacterial properties against pathogenic bacteria. However, the antibacterial potential varied among the lactobacilli tested and, in fact, *L. salivarius* CECT5713 showed not only the best *in vitro* antibacterial activity, but also the highest protective effect against a *Salmonella* strain in the murine infection model.

Conclusion: The four breast-milk lactobacilli, and particularly *L. salivarius* CECT5713, possess potent antibacterial activities that result in a higher protection against *S. choleraesuis* CECT4155 in a mouse infection model.

Significance and Impact of the Study: These results suggest that lactobacilli from breast milk could contribute to an anti-infective protection in neonates and would be excellent candidates for the development of infant probiotic products.

Introduction

The human intestinal mucosa constitutes the main interface with the external environment and, as a consequence, is the main entrance for foreign substances and micro-organisms. Therefore, organisms have developed potent and complex defence mechanisms that involve physical, chemical and biological barriers created by the epithelium, the immune system and the microbiota of the gastrointestinal tract (GIT). The GIT microbiota forms a very complex and diverse ecosystem and contains up to 1×10^{14} bacteria (Berg 1996).

The activities of the gut bacteria depend on a variety of factors, such as availability of nutrients, redox potential, pH, or their distribution along the GIT (Cummings and Mcfarlane 1991). The normal microbiota constitutes the first line of defence against pathogenic micro-organisms and, in spite of the underlying mechanisms being far from total elucidation, it seems that the production of a physiologically restrictive environment, production of antimicrobials, competition for the same substrates, and/or for adhesion to mucin, may play an important role in this protective effect (Fons *et al.* 2000). Moreover, the interactions that occur in the GIT between microbiota, epithelial cells and immune system reinforce the host defence system against pathogens (Adlerberth 1999; Servin 2004). In fact, there is increasing evidence that some probiotic strains exert a positive effect in the prevention

and/or treatment of a variety of infectious and inflammatory GI diseases (Saavedra *et al.* 1994; Guarino *et al.* 1997; Salminen *et al.* 1998; Huang *et al.* 2002).

Maturation of both the GIT and the immune system is a dynamic process that starts during the perinatal period and, therefore, it is not strange that their immaturity in neonates leads to an increase in infection risk (Insoft *et al.* 1996; van Elburg *et al.* 2003). The anti-infectious properties have been attributed to breast milk, because in breast-fed infants, the rate of infectious diseases is significantly lower than in formula-fed infants (Fallot *et al.* 1980; Victoria *et al.* 1987). The breast milk components, such as maternal immunoglobulins, lactoferrin, and oligosaccharides seem to be involved in this activity (Xanthou 1998; do Nascimento and Issler 2003). In addition, it has recently been observed that breast milk contains different species of lactobacilli, lactococci, and other lactic acid bacteria with probiotic potential (Heikkilä and Saris 2003; Martín *et al.* 2003, 2005a). The aim of this study was to analyse the antibacterial properties of four *Lactobacillus* strains isolated from breast milk: *Lactobacillus gasseri* CECT5714, *L. gasseri* CECT5715 and *Lactobacillus fermentum* CECT5716 (Martín *et al.* 2005a), and *Lactobacillus salivarius* CECT5713 (unpublished results) and to compare them with those of *Lactobacillus coryniformis* CECT5711, a reuterin-producing strain isolated from goat's cheese (Martín *et al.* 2005b).

Materials and methods

Micro-organisms

All the lactobacilli included in this study, *L. gasseri* CECT5714, *L. gasseri* CECT5715, *L. fermentum* CECT5716, *L. salivarius* CECT5713, and *L. coryniformis* CECT5711 were grown in MRS medium (Oxoid, Basingstoke, UK) at 37°C under anaerobic conditions generated by using the Anaerogen system (Oxoid).

The pathogenic strains *Salmonella choleraesuis* CECT4155, CECT409 and CECT443, *Escherichia coli* CECT439 and *E. coli* O157:H7 serovar CECT4076, *Staphylococus aureus* CECT4013 and CECT976, and *Listeria monocytogenes* Scott A, and the spoilage strain *Clostridium tyrobutyricum* CECT4011 were grown in tryptic soy broth (TSB; Biolife, Milano, Italy) at 37°C in aerobic conditions for 24–48 h.

Agar diffusion assays

The *Lactobacillus* strains were grown until early stationary phase ($A_{620}\sim1$). The cultures were centrifuged at 12 000 ***g*** for 10 min at 4°C, and the supernatants and a sample of noninoculated MRS-broth were tenfold concentrated in a Speed Vac System (Hucoa-Erlös, Madrid, Spain). Finally, the supernatants were filter-sterilized through 0·22-μm-pore-size filters (Millipore, Bedford, USA). Aliquots (50 μl) of the supernatants were placed in 7-mm wells previously cut in brain heart infusion (BHI) agar plates seeded ($\sim10^5$ CFU ml^{-1}) with the pathogenic bacteria. The plates were kept at 4°C for 2 h, and then incubated under the optimal growth conditions for each pathogenic bacterial strain. The inhibition halos were measured from the edge of the wells. To test if the inhibitory effect of the lactobacilli supernatants was exclusively due to its acidic pH, aliquots of noninoculated MRS broth adjusted to low pH values with lactic acid were included in the agar diffusion assays.

Broth inhibitory assays

To test the antibacterial activity of the lactobacilli in a broth assay format, 100 μl of an *S. choleraesuis* CECT4155 TSB culture was added to tubes containing the culture supernatants (5 ml) of the respective lactobacilli previously adjusted to pH 7·2 and supplemented up to the appropriate concentration of the TSB. Subsequently, the cultures were incubated at 37°C in aerobic conditions, and after 0, 6 and 24 h, the aliquots were collected, serially diluted and plated on tryptic soy agar (TSA) to determine bacterial counts.

Adhesion to mucin and co-aggregation to *S. choleraesuis* CECT4155

The adhesion of bacteria was determined according to the method described by Cohen and Laux (1995) with some modifications. In brief, 100 μl of a solution (1 mg ml^{-1}) of porcine mucin (Sigma, St Louis, MO, USA) in HEPES-buffered Hanks salt solution (HH) was immobilized in polystyrene microtitre plates (Maxisorp; Nunc, Roskilde, Denmark) after overnight incubation at 4°C. The wells were washed twice with 250 μl of HH. Simultaneously, bacteria were grown overnight at 37°C in MRS broth and the bacterial pellets from 1-ml fractions were obtained by centrifugation and washed with HH. Subsequently, 10 μl of 10 mmol l^{-1} carboxifluorescein (Sigma) was added to the pellets and the bacterial suspensions were incubated for 20 min at 37°C. Afterwards, the bacterial cells were washed thrice with HH and, finally, resuspended in 1 ml of HH. Following this, a suspension of 50 μl of the fluorescent-labelled bacteria ($\sim5\times10^7$ CFU) was added to each well. After incubation for 1 h at 37°C, the plates were washed twice with 250 μl of HH to remove unattached cells, and incubated for 1 h at 60°C in the presence of 50 μl of 1% sodium dodecyl sulphate (SDS)-NaOH (0·1 mol l^{-1}) to release and lyse the bound micro-organisms.

Fluorescence was measured in a fluorescence microplate reader (Tecan Austria GMBH, Salzburg, Austria). Adhesion was assessed as the percentage of the fluorescence retained in the wells after the washing steps when compared with that present in the labelled bacterial aliquots originally added to the wells.

Co-aggregation experiments between the lactobacilli and *S. choleraesuis* CECT4155 were performed according to McIntire *et al.* (1978). Micro-organism suspensions were adjusted to an A_{620} of ~2. Aliquots of 200 μl of each micro-organism (lactobacilli or *Salmonella*) or a mix of 100 μl of the lactobacilli cultures and 100 μl of the *Salmonella* culture were added to 96-well plates. The co-cultures were incubated at room temperature for 3 h, and then, the plates were centrifuged at approximately 7 ***g*** for 2 min. Subsequently, a fraction of the clear supernatants (100 μl) was discharged to facilitate the readings at A_{620}. The co-aggregation percentage was calculated as follows:

$$\frac{\frac{A620\text{lactobacilli}+A620\text{Salmonella}}{2} - A620(\text{lactobacilli}+\text{Salmonella})}{\frac{A620\text{lactobacilli}+A620\text{Salmonella}}{2}} \times 100$$

In vitro interference assays

Interference experiments were performed with fluorescent *S. choleraesuis* CECT4155. The pathogen was labelled with carboxifluorescein using the labelling protocol for lactobacilli described earlier. The plates with adhered porcine mucin were also prepared as described for the lactobacilli adhesion assay. For exclusion tests, lactobacilli (~5×10^7 CFU) were incubated in the mucin-containing wells for 30 min; then, the fluorescent-labelled *S. choleraesuis* cells (~5×10^7 CFU) were added and the incubation was continued for 1 h. For competition tests, the lactobacilli and the fluorescent pathogen were inoculated simultaneously in the mucin-containing wells and the co-cultures were incubated for 1 h. In the case of displacement tests, fluorescent *Salmonella* cells were added to the wells and incubated for 30 min before the addition of the lactobacilli and the additional 1-h incubation. For preincubation tests, each of the lactobacilli was premixed with the fluorescent *Salmonella* (v/v) and incubated for 1 h. Subsequently, the mix (5×10^7 of each micro-organism) was added to the mucin wells and the co-cultures were incubated for 1 h. In all the assays, the inhibition of *Salmonella* adhesion was calculated as the percentage of fluorescence recovered from the wells in the absence of *Lactobacillus* with respect to that found in the lactobacilli-containing wells after the incubation of the co-cultures.

Analysis of mucin gene expression by RT-PCR

The quantity of 1×10^5 of differentiated HT-29 cells (ATCC number HTB-38), 3 weeks postconfluence, were incubated in complete medium (Dulbecco's Modified Eagle's medium plus 10% fetal bovine serum, 1% of glutamine, 1% essential amino acids) with 5×10^7 CFU of each *Lactobacillus* strain for 2 h at 37°C in a humidified 5% CO_2 atmosphere. Afterwards, the cells were washed with sterile phosphate buffer saline to eliminate nonadhered bacteria and incubated for 20 h at 37°C in a humidified 5% CO_2 atmosphere in complete culture medium. Total mRNA was isolated from HT-29 cells by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. RT-PCR and polymerase chain reactions (PCR) were performed as previously described by Soler *et al.* (2001). The primers used in the PCR were purchased from MWG-Biotech AG (Ebersberg, Germany) and included: β-actin-forward (5′-TGG-AATCCTGTGGCATCC-3′), β-actin-reverse (5′-AACGC-AGCTCAGTAACAGTCC-3′), MUC-2-forward (5′-CTGC-ACCAAGACCGTCTTCATG-3′), MUC-2-reverse (5′-GC-AAGGACTGAACAAAGACTCAGAC-3′), MUC-5AC-forward (5′-TGATCATCCAGCAGCAGGGCT-3′), MUC-5AC-reverse (5′-CCGAGCTCAGAGGACATATGGG-3′), MUC-5B-forward (5′-CTGCGAGACCGAGGTCAACATC-3′), MUC-5B-reverse (5′-TGGGCAGCAGGAGCACG-GAG-3′).

Once the PCR was completed, 20 μl of each reaction mixture was used for electrophoresis on 2% agarose gels, and the DNA bands were visualized with ethidium bromide staining.

Animals

Female Balb/c mice (6-weeks old) were purchased from the Granada University breeding colony (Granada, Spain), and housed under a temperature (22°C) and light-controlled (12 h) cycle. Animals ($n = 10$ per group) were maintained on a regular mouse chow diet under specific pathogen-free conditions. Guidelines for the care and use of animals were followed as described (Institute of Laboratory Animal Resource Commission of Life Sciences 1996) and approved by the ethical committee of the Granada University. The global experiment was conducted thrice with similar results.

Infection of mice

Mice were infected with a single 0·2-ml dose (10^6 CFU) of *S. choleraesuis* CECT4155 through a gastric probe. During the 2 weeks preceeding the *Salmonella* inoculation, and also during the weeks after inoculation, mice received 4×10^8 CFU of the lactobacilli in 0·2 ml of skimmed milk every 2 days. Mice of the control group received 0·2 ml of non-lactobacilli-containing skimmed milk. Mice were weighed regularly and the mortality recorded.

Statistical analysis

Statistical analysis was performed with a microcomputer version of the Statistical Package for Social Science (SPSS) (version 12·0, Chicago, USA). The probability of survival was plotted with a Kaplan–Meier curve. The statistical significance was defined when $P < 0{\cdot}05$.

Results

In vitro assays

In this study, different *in vitro* assays were carried out to characterize the antimicrobial potential of the *Lactobacillus* strains studied. First, the ability of their culture supernatants to inhibit the growth of some pathogenic bacteria was examined by an agar diffusion assay. The pH values of the respective culture supernatants were between 3·86 and 4·40. As for some strains, such as *L. gasseri* CECT5715, the inhibition halos were difficult to detect with nonconcentrated supernatants, the supernatants of all the *Lactobacillus* strains were concentrated tenfold before being used (Table 1). The concentrated supernatants of *L. coryniformis* CECT5711, *L. salivarius* CECT5713, and *L. gasseri* CECT5714 inhibited all the indicator strains included in this work, while those of *L. gasseri* CECT5715 and *L. fermentum* CECT5716 failed to inhibit *E. coli* O157:H7 CECT4076 and *C. tyrobutyricum* NZ8; in addition, the *L. gasseri* CECT5715 supernatant did not inhibit the *Listeria* strain (Table 1). In contrast, when the pH of the supernatants was adjusted to 7, no inhibition zones could be detected. Interestingly, when noninoculated MRS broth with the pH adjusted to 3·9–4·4 using HCl, lactic acid, or acetic acid (similar to that found in the lactobacilli supernatants) was tested, the inhibition zones were up to 50% smaller than those obtained with the lactobacilli supernatants.

The viability of the enteropathogenic strain *S. choleraesuis* CECT4155 in broth inoculated with the lactobacilli supernatants was also studied. After 6 h of incubation, this pathogen grew only slightly in the presence of the *L. salivarius* CECT5713 and *L. fermentum* CECT5716 supernatants. After 24 h, no pathogen growth was detected in cultures incubated with *L. gasseri* CECT5714 and *L. gasseri* CECT5715 supernatants or even a decrease, up to 20%, in the case of cultures incubated with the supernatants obtained from the other lactobacilli (Fig. 1).

All the *Lactobacillus* strains were able to adhere to porcine mucin. *Lactobacillus coryniformis* CECT5711 showed the highest adhesion ability while *L. gasseri* CECT5714 and CECT5715 displayed the lowest adhesion values (Fig. 2a). The ability of the lactobacilli strains to co-aggregate with *S. choleraesuis* CECT4155 was also investigated. *Lactobacilus salivarius* CECT5713 and *L. coryniformis* CECT5711 showed potent co-aggregation capabilities in contrast to those of *L. gasseri* CECT5714 and CECT5715 and *L. fermentum* CECT57146, which were low or not detectable (Fig. 2b).

The effect of the *Lactobacillus* strains on the attachment of *S. choleraesuis* CECT4155 to hog mucin was investigated under conditions of exclusion (lactobacilli added before *Salmonella*), competition (lactobacilli and pathogen added simultaneously), displacement (pathogen added before lactobacilli), and preincubation (both strains preincubated together before adhesion analysis). For all the lactobacilli, the inhibition ability was similar when they were added to the mucin layers before or simultaneously

Table 1. Agar diffusion assays

	Salmonella choleraesuis			*Escherichia. coli*		*Staphylococcus aureus*		*Listeria*	*Clostridium*	
	4155	409	443	433	4076	976	4013	Scott A	NZ8	Σ (mm)
CECT5711	11	19	16	23	12	20	18	20	15	154
CECT5713	15	15	21	15	12	18	18	18	14	145
CECT5714	7	20	15	22	12	21	20	10	18	145
CECT5715	6	11	19	22	0	8	8	0	0	74
CECT5716	12	15	19	12	0	12	18	17	0	105

Results show mm of inhibition halo for 10X concentrated supernatants of the probiotic strains.
Σ is the summatory of the size halo for the nine pathogen strain tested.

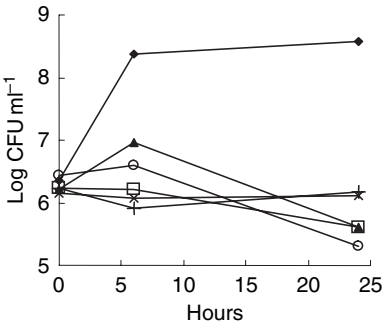

Figure 1 Broth inhibitory assay. *Salmonella choleraesuis* CECT4155 was incubated in presence of *Lactobacillus* supernatant and after 0, 6 and 24 h of incubation, the *Salmonella* growth was measured as tryptic soy agar (TSA) counts (CFU ml^{-1}). ◆, Control; □, CECT5711; ▲, CECT5713; ×, CECT5714; |, CECT5715; ○, CECT5716.

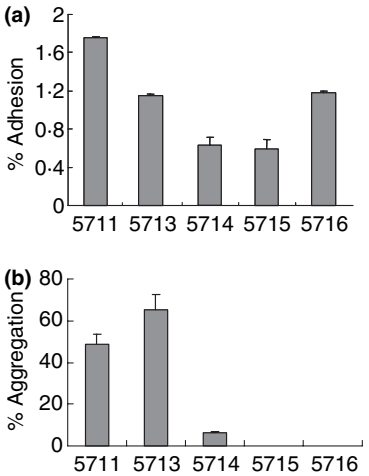

Figure 2 (a) Adhesion of the *Lactobacillus* strains to hog mucin. Carboxifluorescein- labelled bacteria were incubated for 1 h with hog mucin. The percentage of adhered bacteria was calculated from the relation between fluorescence of bacteria before adhesion and fluorescence of adhered bacteria. (b) Co-aggregation experiments between *Lactobacillus strains* and *Salmonella choleraesuis* CECT4155. The results are expressed as the percentage of aggregation after incubation for 3 h.

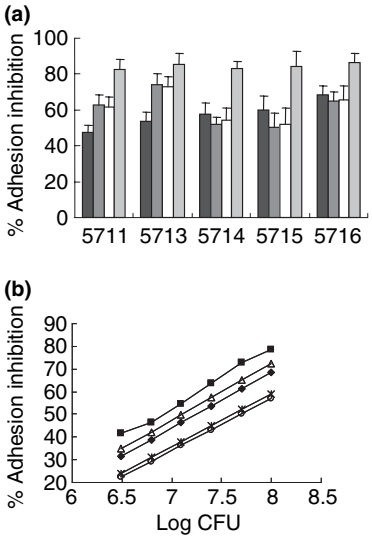

Figure 3 (a) Effect of *Lactobacillus* strains on the adhesion of fluorescent *Salmonella choleraesuis* on hog mucin under conditions of exclusion (▩), competition (□), displacement (■), and preincubation (▨). Results are expressed as the percentage of fluorescence recovered from the wells in absence of lactobacilli with respect to that found in the lactobacilli-containing wells after incubation. (b) Effect of the lactobacilli dose on the adhesion of fluorescent *S. choleraesuis* on hog mucin under competition conditions ◆, 5711; ■, 5713; ✕, 5714; ○, 5715; △, 5716.

to the pathogen, *L. salivarius* CECT5713 being the most effective strain (Fig. 3a). Similar results were obtained when the two *L. gasseri* strains and the *L. fermentum* strain were added after the inoculation of the mucin-containing wells with the *Salmonella* strain (Fig. 3a). However, in this particular assay, the efficacy of *L. salivarius* CECT5713 and *L. coryniformis* CECT5711 decreased with respect to competition or exclusion conditions (Fig. 3a). Finally, the highest inhibitory effect was observed when the respective lactobacilli were incubated together with *Salmonella* for 1 h before the adhesion assay. In this case, the effect was very similar among all the lactobacilli strains evaluated in this study (Fig. 3a). In a competition assay it was observed that this effect was dose-dependent (Fig. 3b).

The effect of the five *Lactobacillus* strains on mucin gene expression in HT-29 cells was analysed by RT-PCR. All the strains studied increased the expression of MUC-2 in these enterocyte-like cells although such effect was particularly strong in the case of *L. coryniformis* CECT5711 and *L. fermentum* CECT5716 (Fig. 4). No effect was observed in the expression of MUC-5AC. Finally, *L. gasseri* CECT5714 and *L. salivarius* CECT5713 also increased the expression of the MUC-5B gene (Fig. 4).

In vivo murine infection model

The protective effect of the *Lactobacillus* strains against infection of *S. choleraesuis* CECT4155 was studied in Balb/c mice. For this purpose, 4×10^8 CFU of the *Lactobacillus* strains were orally given every 2 days during the 2 weeks preceeding the infection and also during the 2 weeks after the *Salmonella* inoculation. The first death was observed 3 days after infection in the group that did not receive the lactobacilli treatment (control group), while the first death in the *Lactobacillus*-treated group occurred 2 days later (Fig. 5). On day 6, 50% of the mice in the control group

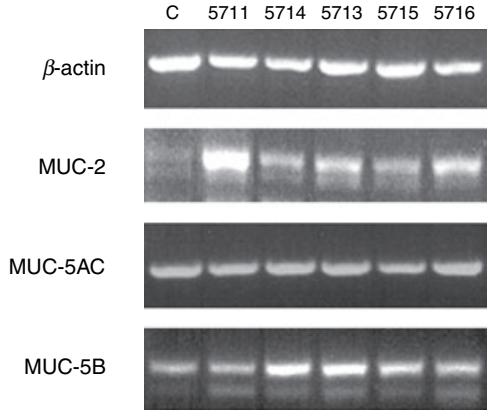

Figure 4 Effect of *Lactobacillus* strains on mucin gene expression in HT-29 cells. Gene expression was analysed by RT-PCR using β-actin as a control.

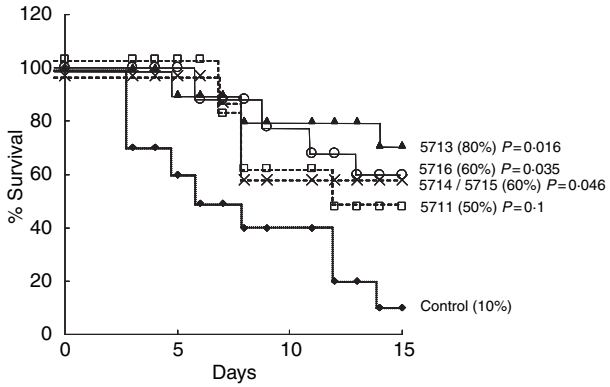

Figure 5 Mortality due to *Salmonella choleraesuis* CECT4155 infection in mice treated and nontreated with *Lactobacillus* strains is shown. Treated mice received 4×10^8 CFU of lactobacilli strain every 2 days during the 2 weeks preceeding the infection and the 2 weeks after infection. *Lactobacillus* strains: *Lactobacillus salivarius* CECT5713, *Lactobacillus fermentum* CECT5716, *Lactobacillus gasseri* CECT5714, *L. gasseri* CECT5715, and *Lactobacillus coryniformis* CECT5711 are named with the corresponding number of the CECT code. ◆, Control; □, 5711; ▲, 5713; ×, 5714/5715; ○ 5716.

had died, but those in the groups receiving the lactobacilli treatment maintained a survival rate of 90–100%. Two weeks after infection, the survival in the control group (10%) was significantly lower than that observed in the groups receiving the *Lactobacillus* strains (50–80%, depending on the lactobacilli strain) ($P < 0.05$) (Fig. 5). Thus, all the lactobacilli strains tested were able to exert an antagonistic effect against *Salmonella* infection *in vivo*.

Discussion

In the recent years, it has become evident that human milk is a major factor in the initiation and development of neonatal gut microbiota, because breast milk is a continuous source of commensal bacteria to the infant gut, several weeks after birth (Matsumiya *et al.* 2002; Heikkilä and Saris 2003; Martín *et al.* 2003). It is estimated that an infant consuming about 800 ml of milk will ingest between 1×10^5 and 1×10^7 of bacteria and, therefore, it is not surprising that the bacterial composition of the infant faecal microbiota reflects that of breast milk (Heikkilä and Saris 2003). This breast milk-induced microbiota and other components of breast milk have been attributed to the lower risk of infectious disease in breastfed infants (Xanthou 1998; Bourlioux *et al.* 2003; do Nascimento and Issler 2003).

The lactic acid bacteria that are present in fresh breast milk include different species of lactobacilli and, recently it has been observed that three of the breast milk strains included in this study (*L. fermentum* CECT5716, *L. gasseri* CECT5714, and *L. gasseri* CECT5715) display a series of properties, including antimicrobial activity against infant-related pathogens (Martín *et al.* 2005a). Since breastfeeding can be a significant source of lactobacilli to the infant gut, affecting the overall composition of the neonate gut microbiota and exerting biological functions that may include the inhibition of potential pathogenic bacteria, the antimicrobial potential of four lactobacilli isolated from human breast milk (the three cited previously and *L. salivarius* CECT5713) was further evaluated in this study and compared with that of *L. coryniformis* CECT5711, a strain isolated from goat's cheese producing reuterin, a wide-spectrum antimicrobial substance (Martín *et al.* 2005b).

The mechanism by which these species exert their beneficial effects remain largely to be determined but mechanisms, such as production of antimicrobial substances, competition for nutrients and competition for epithelium adhesion are involved (Bourlioux *et al.* 2003; Servin 2004).

Probiotic bacteria produce a variety of substances with antibacterial properties including organic acids, hydrogen peroxide, bacteriocins that affect not only the bacterial viability but may also affect bacterial metabolism or toxin production (Vandenberh 1993; Rolfe 2000). Therefore, the production of exocellular antimicrobial substances was assayed by an agar diffusion assay using different pathogenic bacteria as indicator. All the culture supernatants obtained from the different lactobacilli showed activity against all or most of the indicator bacteria. However, such activity disappeared when the pH of the supernatants was adjusted to 7. Therefore, the production of organic acids seems to be involved in the antimicrobial activity of these strains. In fact, it has been previously observed that *L. fermentum* CECT5716, *L. gasseri* CECT5714, and *L. gasseri* CECT5715 produce high

amounts of lactic acid, but not bacteriocins or reuterin, when grown in MRS broth (Martín *et al.* 2005a). In addition to lowering the environmental pH, lactic acid also functions as a permeabilizer of the outer membrane of Gram-negative bacteria and, therefore, may facilitate the effects of other antimicrobial substances (Alakomi *et al.* 2000). Although *L. gasseri* CECT5714 and *L. gasseri* CECT5715 are able to produce hydrogen peroxide (Martín *et al.* 2005a) and *L. coryniformis* CECT5711 produces reuterin (Martín *et al.* 2005b), the antimicrobial activities associated with these molecules could not be detected in the agar diffusion assays. On one hand, MRS is a medium unsuitable for hydrogen peroxide detection (Rodríguez *et al.* 1997), while reuterin production requires the addition of glycerol to the broth medium (Talarico *et al.* 1988). The high supernatant concentration needed to observe an inhibitory effect by agar diffusion assay suggests that, *in vivo*, this antimicrobial effect would affect near pathogenic bacteria on the epithelial surface only if high amounts of antimicrobial substances are produced.

Therefore, the antibacterial activity of these *Lactobacillus* strains seems to be a consequence of the activity of different factors with different potency and action spectrum. In fact, in broth medium, the two *L. gasseri* strains (CECT5714 and CECT5715) showed a bacteriostatic effect on *S. choleraesuis*, while *L. coryniformis* CECT5711, *L. fermentum* CECT5716, and *L. salivarius* CECT5713 showed a bactericide effect on this pathogen.

Once a pathogen reaches the intestinal mucosa, its adhesion to the components of the host's extracellular matrix seems to be a prerequisite for bacterial colonization and invasion of the subepithelial tissues, because it ensures that the pathogen will not be rapidly eliminated from the gut (Westerlund and Korhonen 1993). The ability of some *Lactobacillus* strains to co-aggregate with intestinal pathogens in intestinal lumen might prevent pathogens from reaching the intestinal mucosa. It has also been described that some *Lactobacillus* strains can strongly adhere to the gut mucosa, and as a consequence, they interfere with the adhesion of pathogenic bacteria to intestinal cells (Servin 2004). In this work, the five lactobacilli analysed were able to interfere with the adhesion of an enteropathogenic *Salmonella* strain to hog mucin. Under competition and exclusion conditions, *L. salivarius* CECT5713 showed the highest effect against the pathogen adhesion probably because of its strong ability to adhere to mucin and to co-aggregate with the *Salmonella* cells. In contrast, the two *L. gasseri* strains, CECT5714 and CECT5715, were less efficient inhibiting the pathogen adhesion through an exclusion or a competition mechanism. These strains showed a lower potential to adhere to mucin and also a lower ability to co-aggretate with the *Salmonella* cells. On the other hand, the preincubation of the lactobacilli strains with the *Salmonella* strain led to an important decrease in the adhesion of the pathogen to mucin, which suggests that some of the metabolites produced by the *Lactobacillus* strains could have affected inherent adhesion properties of this pathogenic strain. Similar results have been previously reported for *Lactobacillus acidophilus* LA1 (Bernet-Camard *et al.* 1997).

Another mechanism used by probiotic bacteria to inhibit the infection of pathogenic micro-organisms is to increase the expression of intestinal mucins (Mack *et al.* 1999). We report here that all the lactobacilli tested induced the expression of MUC-2 in HT29 cells, while *L. gasseri* CECT5714 and *L. salivarius* CECT5713 also induced the expression of MUC-5B. In conclusion, the antibacterial activity of the *Lactobacilli* strains tested is a multifactorial process involving different mechanisms of interference in pathogen adhesion.

The oral administration of the five *Lactobacillus* strains to mice inoculated with *S. choleraesuis* CECT4155 had a beneficial effect on the survival of the animals (50–80%) in contrast to the group that did not receive any of the lactobacilli (10%). The survival percentage among the animals of the different lactobacilli-treated groups depended on the strain, *L. salivarius* CECT5713 being the one that provided the highest survival rate. In conclusion, the four breast milk lactobacilli, and particularly *L. salivarius* CECT5713, are endowed with a potent antibacterial activity that together with their breast milk origin makes them excellent candidates for the development of infant probiotic products.

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3. Dos cepas de *Lactobacillus* aisladas de leche materna modulan la respuesta inmunitaria de forma diferente

RESUMEN

OBJETIVO

Numerosos estudios en animales y en humanos evidencian la capacidad de los lactobacilos para modular tanto la respuesta inmunitaria natural como la adquirida. Así, se han observado efectos como por ejemplo, aumento de la actividad fagocítica de leucocitos por administración a humanos adultos sanos con cepas específicas de lactobacilos, o el aumento en la actividad Natural Killer. Con respecto a la respuesta inmunitaria específica, numerosos estudios en humanos muestran que la ingesta de cepas de lactobacilos específicas es capaz de elevar la respuesta inmunitaria humoral frente a infecciones naturales. Los mecanismos por los cuales los lactobacilos modulan la respuesta inmunitaria no son totalmente conocidos, siendo evidente que la manera en la cual la bacteria impacta en el sistema inmunitario depende no solo de la especie bacteriana implicada sino también de la cepa individual de la bacteria, de su viabilidad o de la dosis de consumo y del estado de salud en el que se encuentre el hospedador. El objetivo de este estudio fue caracterizar mediante ensayos *in vitro* y en animales de experimentación, la capacidad para modular la respuesta inmunitaria de dos cepas Hereditum (*L.salivarius* CECT5713 y *L.fermentum* CECT5716).

RESULTADOS

El efecto de ambas cepas probióticas sobre macrófagos derivados de médula ósea de ratones fue contrapuesto. Mientras que, *L.fermentum* CECT5716 indujo en macrófagos la producción de citocinas pro-inflamatorias, *L.salivarius* CECT5713 se caracterizó por la inducción de la citocina inmunoreguladora IL-10. Sin embargo, en el caso de macrófagos sobre-estimulados por LPS, ambas cepas redujeron la respuesta inflamatoria, siendo el efecto de *L.salivarius* CECT5713 más eficiente debido a la producción de altas cantidades de citocina IL-10. Los ensayos en ratones mostraron resultados similares a los que se llevaron a cabo *in vitro*. Así, el consumo de *L.fermentum* incrementó la producción de citocinas de tipo Th1 por células esplenocíticas e incrementó la concentración de IgA en heces, mientras que, el consumo de *L.salivarius* CECT5713 indujo la producción de IL-10.

CONCLUSIÓN

Los resultados de este estudio mostraron que las dos cepas de lactobacilos aisladas de leche materna pueden ejercer efectos diferentes e incluso opuestos en la respuesta inmunitaria demostrando la especificidad de cada cepa. De esta manera, en condiciones normales el efecto de *L.fermentum* CECT5716 es inmunoestimulante con respecto al efecto anti-inflamatorio que presenta *L.salivarius* CECT5713. Sin embargo, en condiciones de inflamación ambas cepas presentan un carácter inmunomodulador que se ha demostrado en los estudios posteriores en el modelo de colitis en ratas.

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ORIGINAL ARTICLE

Two *Lactobacillus* strains, isolated from breast milk, differently modulate the immune response

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Abstract

Aims: The ability of two different *Lactobacillus* strains (*Lactobacillus salivarius* CECT5713 and *Lactobacillus fermentum* CECT5716), isolated from human breast milk, to modulate the immune response was examined.

Methods and Results: In rodent bone-marrow-derived macrophages (BMDM), the presence of *Lact. fermentum* CECT5716 induced pro-inflammatory cytokines, in contrast to the activation of IL-10 induced by *Lact. salivarius* CECT5713. Although both strains reduced the lipopolysaccharide (LPS)-induced inflammatory response in BMDM, the effect of *Lact. salivarius* CECT5713 was more efficient, probably because of the production of higher amounts of IL-10 cytokine. *In vivo* assays in mice showed similar results; the consumption of *Lact. fermentum* CECT5716 enhanced the production of Th1 cytokines by spleen cells and increased the IgA concentration in faeces. However, the consumption of *Lact. salivarius* CECT5713 induced IL-10 production by spleen cells.

Conclusion: Therefore, in general, the effect of *Lact. fermentum* CECT5716 is immunostimulatory in contrast to the anti-inflammatory effect of *Lact. salivarius* CECT5713.

Significance and Impact of the Study: The results of this study show that two *Lactobacillus* strains isolated from breast milk can exert different and even opposing effects on immune response demonstrating the specificity of each strain.

Introduction

The normal gastrointestinal flora is in close and continuous contact with epithelial and immune cells, and the resulting stimulation is essential for immune system function. In fact, the type of bacteria colonizing the intestine of newborns and the timing may determine the immunomodulation of the naïve system, as intestinal colonization acts as an important antigenic stimulus for the maturation of the immune response. It is involved in the induction and maintaining of the oral tolerance (Grönlund *et al.* 2000; Isolauri *et al.* 2001). Lactobacilli are a major component of the gastrointestinal flora and have long been used in the preparation of fermented foods. Evidence from clinical and animal studies has supported the idea that lactobacilli can modulate aspects of both natural and acquired immune responses in the host (Gill 1998; Salminen *et al.* 2000; Cross 2002a,b). For example, enhanced phagocytic activity of peripheral blood leukocytes have been reported in healthy human adults administered with specific *Lactobacillus* strains (Schiffrin *et al.* 1995; Arunachalam *et al.* 2000). In addition, significant improvements in natural killer (NK) cells activity and in the percentage of NK cells in the peripheral blood of human volunteers consuming fermented products containing probiotic strains have been detected (Gill *et al.* 2001). Regarding specific immune response, numerous human studies have shown that intake of specific *Lactobacillus* strains are able to enhance humoral immune response to natural infections (Kaila *et al.* 1992, 1995;

Majamaa *et al.* 1995) and to vaccination (Link-Amster *et al.* 1994; de Vrese *et al.* 2005). Mechanisms by which lactobacilli modulate immune response are not totally known, however, it is evident that the manner in which bacteria impact on the immune system varies depending not only on the bacterial species involved but also on the individual strain of bacteria, state of viability or level of consumption and on the health status of the host (Donnet-Hughes *et al.* 1999; Gill and Rutherfurd 2001; Karlsson *et al.* 2002; Ibnou-Zekri *et al.* 2003). Antigen-presenting cells, such as monocytes, macrophages and dendritic cells, are responsible for detecting microbes and presenting their antigenic structures to T cells, thus eliciting acquired immune responses. In addition, monocytes and macrophages kill micro-organisms by phagocytosis and produce pro-inflammatory cytokines that mediate the adaptative response. Therefore, a change in profile of macrophage-derived cytokines may polarize the T-cell response to Th1, Th2 or T regulatory cells (Christensen *et al.* 2002). It is also interesting to note that probiotic bacteria have been shown to modulate phagocytosis differently in healthy and allergic subjects: in healthy persons there was an immunostimulatory effect, whereas in allergic persons, downregulation of the inflammatory response has been detected (Pelto *et al.* 1998; Isolauri *et al.* 2001).

The establishment of the gut microbiota is a complex process influenced by microbial and host interactions and by external and internal factors. Extrinsic factors include the bacterial load of the environment, the composition of the maternal microbiota, diet, the mode of delivery and medication (Fanaro *et al.* 2003). In this process, human milk plays an important role because it is a major factor in the initiation and development of neonatal gut microbiota as it constitutes a continuous source of micro-organisms to the infant gut during several weeks after birth. Bacteria commonly isolated from this substrate include staphylococci, streptococci, micrococci, lactobacilli and enterococci (Martín *et al.* 2003, 2004). Two *Lactobacillus* strains (*Lactobacillus salivarius* CECT5713 and *Lactobacillus fermentum* CECT5716) were isolated from breast milk of two different healthy women, and numerous *in vitro* assays showed the probiotic potential of both strains (Martín *et al.* 2005, 2006). In this study, different *in vitro* and *in vivo* assays were performed to examine the ability of the two *Lactobacillus* strains isolated from breast milk to modulate the immune response.

Materials and methods

Micro-organisms

The *Lactobacillus* strains *Lact. fermentum* CECT5716 and *Lact. salivarius* CECT5713 property of Puleva Biotech SA (Granada, Spain) were grown in de Man, Rogosa and Sharpe medium (Oxoid, Basingstoke, UK) at 37°C under anaerobic conditions generated by the AnaeroGen system (Oxoid).

Induction of cytokines released by bone-marrow-derived macrophages

For this purpose, rodent bone-marrow-derived macrophages (BMDM), obtained as described previously (Xaus *et al.* 1999), were incubated with 10^6 CFU ml^{-1} of *Lact. salivarius* CECT5713 or *Lact. fermentum* CECT5716 in the presence or absence of 100 ng ml^{-1} of LPS for 2 h. Then, cells were washed with culture media to eliminate nonattached bacteria and cultured with new media, with or without LPS, for 12–14 h to allow the expression of the indicated cytokines.

Animals and treatment

Female BALB/c mice (6 weeks old) were purchased from the Granada University breeding colony (Granada, Spain) and housed under a temperature (22°C) and light-controlled (12 h) cycle. Animals ($n = 8$ per group) were maintained on a regular mouse chow diet under specific pathogen-free conditions. Guidelines for the care and use of animals were followed as described (Institute of Laboratory Animal Resource Commission of Life Sciences, 1996) and approved by the ethical committee of the Granada University. The experiment was conducted three times with similar results. Mice received 4×10^8 CFU of the lactobacilli in 0·2 ml of skimmed milk by oral gavage every 2 days during 4 weeks. Mice of the control group received 0·2 ml of non-lactobacilli-containing skimmed milk.

Phagocytic activity

In vitro phagocytic activity was determined by flow cytometry in whole blood samples after the uptake of fluoresceinated *Escherichia coli* (Gill *et al.* 2000). Basically, 50 μl of heparin-treated whole blood was incubated for 10 min at 37°C with 10 μl (10^8 CFU) of fluoresceinated bacteria. Erythrocytes were lysed with 100 μl of 4% formaldehyde and 1 ml of cool water. Samples were centrifuged to 2200 ***g*** for 5 min and suspended in 0·5 ml of 4% (w/v) formaldehyde in PBS. Samples were analysed by flow cytometry and the results were expressed as the percentage of blood leukocytes showing phagocytic activity.

Culture of spleen cells

Spleens were aseptically removed from all mice and lymphocytes cultured as described previously (Lara-Villos-

lada *et al.* 2005). Spleen-derived lymphocytes were cultured in six-well plates (10^6 cells per well) in 5 ml of medium and incubated for 48 h. The supernatants were collected after 48 h of incubation.

Intestinal content samples

Content from caecum and distal sections of intestine from each mouse was collected and homogenized in saline solution (50 mg ml^{-1}). Samples were centrifuged (1500 ***g*** for 15 min) and supernatants were used for IgA measurement.

Immunoglobulin and cytokine measurements

Concentrations of IgA, IgE and IgG in serum and IgA in supernatants from intestinal content were determined by ELISA quantitation kits (BETHYL, Montgomery, TX, USA). TNF-α, IL-12, IL-1β and IL-10 concentrations in supernatants from macrophages and lymphocytes were measured by ELISA quantitation kits (CytoSets, BIOSOURCE, Camarillo, CA, USA), following manufacturer instructions.

Statistical analysis

Statistical analysis was performed with a microcomputer version of the Statistical Package for Social Science (SPSS) (version 12.0, Chicago, IL, USA). Statistical significance ($P < 0{\cdot}05$) was calculated using Student's *t*-test.

Results

Cytokine production by murine bone-marrow-derived macrophages after incubation with the *Lactobacillus* strains

Lactobacillus strains were co-incubated with BMDM or LPS-stimulated BMDM to study cytokine production after bacterial stimulation (Fig. 1). Protein concentrations of IL-10, IL-12, IL-1β and TNF-α were measured in the supernatants. The two strains induced the production of all these cytokines in nonstimulated BMDM (Fig. 1a) but the highest response was observed in TNF-α production. The increase of IL-10 induced by *Lact. fermentum* CECT5716 in BMDM was not as big as that of TNF-α, and subsequently, the ratio TNF-α/IL-10 increased, suggesting a predominance of the inflammatory response. In contrast, *Lact. salivarius* CECT5713 induced more IL-10 production when compared with that of *Lact. fermentum* CECT5716, resulting in a decrease in the IL-12/IL-10 ratio, suggesting a Th2/Th3 predominant response. Curiously, the presence of the *Lactobacillus* strains decreased the production of

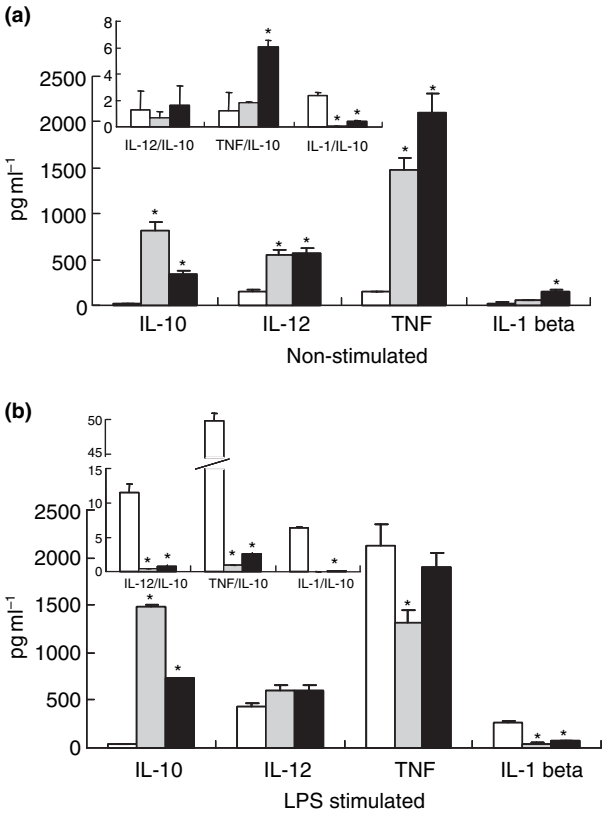

Figure 1 Cytokine production by bone-marrow-derived macrophages (BMDM) incubated with *Lactobacillus salivarius* CECT5713 (grey bars), *Lactobacillus fermentum* CECT5716 (black bars) or in the absence of bacteria (white bars). (a) nonstimulated BMDM; (b) LPS (10 ng ml^{-1}) stimulated BMDM. Results are expressed as cytokine concentration (pg ml^{-1}) ± SD. To elucidate the kind of immune response, triggered IL-12/IL-10, TNF/IL-10 and IL-1β/IL-10 ratio are represented. *Statistically significant difference ($P < 0{\cdot}01$) with respect to control group.

LPS-induced TNF-α and IL-1β without modifying the IL-12 response. In this sense, *Lact. salivarius* CECT5713 was more effective, reducing by 40% of the TNF-α production (Fig. 1b). On the contrary, IL-10 production was enhanced in the cultures where *lactobacilli* were added, resulting in the reduction of TNF/IL-10, IL-1/IL-10 and IL-12/IL-10 ratio in LPS-stimulated macrophages (Fig. 1b).

Effect of orally administered *Lactobacillus* strains on murine immune response

The *Lactobacillus* content in faeces and colonic contents were analysed at the end of the treatment and no significant differences were detected between groups consuming the probiotic strains and the control group, although the presence of the administered strains was only detectable by PCR in samples of the animals of the treated groups (data not shown).

Fresh blood was collected from each mouse after 4 weeks of treatment. The phagocytic activity was tested against a fluoresceinated *E. coli* bacteria. The consumption of both *Lactobacillus* strains increased the phagocytic activity of circulating blood leukocytes, although the effect of *Lact. fermentum* CECT5716 was significatively higher (Fig. 2).

Intestinal content from caecum and distal colonic sections was collected from each mouse and IgA concentration was measured (Table 1). A significant increase in the concentration of this immunoglobulin was observed in the case of the mice orally administered with *Lact. fermentum* CECT5716, but plasma IgA concentration did not change. A trend to decrease the IgE concentration in plasma was also observed when *Lact. fermentum* CECT5716 were administered, while IgG production was not affected by the administration of any of the probiotic strains (Table 1).

The absence of bacterial translocation to the spleen after the 4 weeks of treatment with the probiotic strains was evaluated, and no bacterial translocation in the spleen was detected because of probiotic consumption (data not shown). To investigate the systemic immune response triggered by the administered bacteria, the production of cytokines by spleen cells from the treated mice was analysed (Table 2). While both lactobacilli strains were equally efficient in the induction of IL-12 production in murine lymphocytes *in vitro*, the oral administration of *Lact. fermentum* CECT5716 and *Lact. salivarius* CECT5713 preferentially induced IL-12 and IL-10 production, respectively. Therefore, the ratio IL-12/IL-10 was affected towards Th1 immune response (*Lact. fermentum* CECT5716) or to attenuate Th1 immune response (*Lact. salivarius* CECT5713).

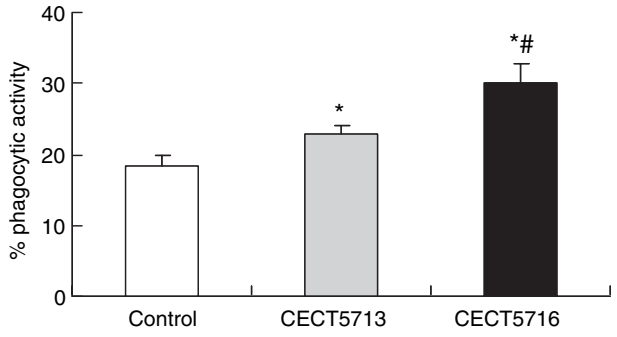

Figure 2 Phagocytic activity of circulating blood leukocytes. Results are expressed as the mean ±SEM of the percentage of leukocyte cells containing fluoresceinated *Escherichia coli* after *in vitro* incubation of the bacteria with fresh blood. White bars, control group; grey bars, group consuming *Lactobacillus salivarius* CECT5713 and black bars, *Lactobacillus fermentum* CECT5716. *Statistically significant difference ($P < 0{\cdot}01$) with respect to week 0. #Significant difference ($P < 0{\cdot}01$) between control and treatment groups.

Table 2 Cytokine production by spleen cells

	Control	CECT5713	CECT5716
IL-2 (pg ml^{-1})	71·4 ± 17·5	142·9 ± 14·9*	133·9 ± 28·8*
IL-4 (pg ml^{-1})	109·5 ± 19·2	137·3 ± 15·6	135·3 ± 20·8
IL-12 (pg ml^{-1})	40·0 ± 3·3	38·6 ± 4·5	62·2 ± 3·5*†
IL-10 (pg ml^{-1})	87·6 ± 5·7	167·5 ± 8·7*	91·8 ± 17·0*
IL-12/IL-10	0·45 ± 0·02	0·24 ± 0·02*	0·80 ± 0·20†‡

Data are presented as mean ± SEM ($n = 8$).
*Statistically significant difference with respect to control, $P < 0{\cdot}05$.
†Statistically significant difference between probiotic-treated groups, $P < 0{\cdot}05$.
‡$P = 0{\cdot}07$ CECT5713 and CECT5713 groups with respect to control.

Discussion

It is widely accepted that the immunomodulatory effects of lactobacilli are heterogeneous and strain dependent. Monocytes and macrophages recognize conserved molecular patterns of bacterial components through Toll-like receptors. These receptors signal through pathways that lead to activation of a variety of transcription factors, which triggers the production of cytokines instruments in the development of T-cell differentiation into Th1, Th2 or T regulatory cells (D'Andrea *et al.* 1993; Trinchieri 1993; Medzhitov and Janeway 1997; Takeuchi *et al.* 1999). Therefore, a change in the profile of macrophage-

Table 1 Mucosal and plasma immunoglobulin concentrations

	Control	CECT5713	CECT5716
Caecum IgA (mg g^{-1})	87·2 ± 8·7	96·0 ± 5·5	107·9 ± 3·6*†
Distal colon IgA (mg g^{-1})	123·3 ± 3·2	116·5 ± 6·6	141·1 ± 13·5*†
Plasma IgA (ng ml^{-1})	928·2 ± 107·6	930·9 ± 131·3	832·6 ± 124·8
Plasma IgG (mg ml^{-1})	74·0 ± 6·8	77·7 ± 7·6	67·9 ± 4·4
Plasma IgE (ng ml^{-1})	413·9 ± 65·9	411·9 ± 77·2	285·6 ± 74·3‡

Data are presented as mean ± SEM ($n = 8$).
*Statistically significant difference with respect to control, $P < 0{\cdot}05$.
†Statistically significant difference between probiotic-treated groups, $P < 0{\cdot}05$.
‡$P = 0{\cdot}1$ CECT5716 group with respect to control and CECT5713 groups.

derived cytokines could determine the immune response. The co-incubation of the *Lact. salivarius* CECT5713 or *Lact. fermentum* CECT5716 with murine bone-marrow-derived macrophages stimulated the production of cytokines, but the effect of each strain was different. The cytokine pattern induced by *Lact. fermentum* CECT5716 would favour a Th1 response, meanwhile the higher production of IL-10 induced by *Lact. salivarius* CECT5713 could have the opposite effect. Surprisingly, when macrophages were LPS stimulated, the induction of the inflammatory cytokine TNF-α was lower in the presence of both *Lactobacillus* strains, but specially in the case of *Lact. salivarius* CECT5713. The ability of some *Lactobacillus* strains to modulate the immune response after inflammatory stimulus has recently been reported (Borruel *et al.* 2003; Karlsson *et al.* 2004). Moreover, distinct regulatory effects have been detected in healthy subjects and in patients with inflammatory diseases such as inflammatory bowel disease in which IL-12 and TNF-α are involved (Isolauri *et al.* 2001). In fact, previous assays have demonstrated that the administration of the probiotic *Lact. salivarius* CECT5713 and *Lact. fermentum* CECT5716 facilitates the recovery of the inflamed tissue in a trinitrobenzene-sulfonic-acid-colitis-induced rat model, an effect associated with amelioration of the production of some of the mediators involved in the inflammatory response in the intestine, such as TNF-α and NO (Peran *et al.* 2005, 2006). The mechanisms by which bacteria induce different effects, depending on the health status of the host, are not totally known, but bacteria are likely to be recognized by a complex combination of receptors, which on acting together decide the outcome of the response.

The immunomodulatory properties of these *Lactobacillus* strains were corroborated by the *in vivo* assays in mice. The consumption of these strains stimulated both innate and specific immune responses. Regarding the innate response, the consumption of *Lact. fermentum* CECT5716 and *Lact. salivarius* CECT5713 induced an increase in phagocytic activity of blood leukocytes, being the effect of *Lact. fermentum* CECT5716 particularly higher than that of *Lact. salivarius* CECT5713. However, the effect on specific immune response differed more notably between both *Lactobacillus* strains. In the case of *Lact. fermentum* CECT5716, a significant increase in faecal IgA and in Th1 cytokine production by spleen cells of the treated mice was observed. The enhancement in antibody response and inflammatory-related response might improve the immune response against pathogenic agents and also might be beneficial in cases of deficient immune response such as immunosenescence. In fact, it has been shown that the oral administration of this strain increased significantly the survival of the mice in a murine infection model with *Salmonella* (Olivares *et al.* 2006). The immunostimulant activity of *Lact. fermentum* CECT5716 has also been shown in an anti-influenza vaccination protocol in humans, where the consumption of this strain before and after vaccination enhanced the immunological response of the vaccine by increasing Th1 response and virus-neutralizing antibodies (M. Olivares, M.P. Díaz-Ropero, S. Sierra, F. Lara-Villoslada, J. Fonollá, M. Navas, J.M. Rodríguez, J. Xaus, manuscript submitted).

The consumption of *Lact. salivarius* CECT5713, according to the *in vitro* assays, induced a significant increase in IL-10 production. IL-10 cytokine downregulates the production of Th1 cytokines (D'Andrea *et al.* 1993) and induces the development of regulatory T cells (Groux *et al.* 1996). The results mentioned above about the capability of this strain to decrease the inflammatory response in a model of rat colitis confirm the anti-inflammatory activity of this strain and demonstrate its efficacy *in vivo*. *In vitro* assays with murine BMDM and the *in vivo* assays in healthy mice showed that the IL-10 production is increased by *Lact. salivarius* CECT5713, without affecting IL-12 production. Therefore, the capacity to respond against pathogens would remain intact. In fact, it has been previously reported that the oral administration of this strain also protects mice against *Salmonella* infection in a mice model (Olivares *et al.* 2006).

Therefore, in general, *Lact. fermentum* CECT5716 acts as an immunostimulant agent in contrast with the anti-inflammatory/regulatory effect of *Lact. salivarius* CECT5713. These results confirm the strain-specific effect of each probiotic and the evidence that the selection of a strain for a determined application should be based on the knowledge of the particular capabilities of that strain and the health condition of the host. We show in this study that two *Lactobacillus* strains isolated from breast milk can exert different and even opposing effects on immune response. In a context such as the maturation of the immune system in neonates, which involves the induction of Th1/Th2/Th3 response, the bacterial colonization of the gut by a variety of bacterial strains exerting different responses might play a key role in the fine-tuning of the immune response.

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4. Efectos preventivos del probiótico *Lactobacillus salivarius* ssp. *salivarius* en el modelo de colitis experimental por TNBS en rata

RESUMEN

OBJETIVO

En los últimos años, numerosos estudios realizados con diversas cepas probióticas han mostrado un cierto efecto beneficioso en la Enfermedad Inflamatoria Intestinal. Esta enfermedad, está caracterizada por un proceso inflamatorio agudo en el que citocinas proinflamatorias como el TNF-alpha han mostrado un papel relevante. La capacidad inmunomoduladora de algunas cepas probióticas es probablemente uno de los mecanismos a través de los cuales las bacterias probióticas ejercen un efecto positivo en esta patología.

Lactobacillus salivarius CECT5713, ha mostrado una potente actividad inmunomoduladora con una elevada inducción de IL-10, citocina fundamental en la regulación de los procesos inflamatorios. Además, *L. salivarius* CECT5713 es capaz de adherirse a la mucosa intestinal e inhibir el crecimiento de bacterias potencialmente patógenas. El objetivo del presente estudio fue, ensayar el efecto preventivo de esta cepa en el modelo de colitis experimental por TNBS en ratas.

RESULTADOS

El tratamiento con *Lactobacillus salivarius* CECT5713 durante las dos semanas previas a la inducción de la enfermedad en las ratas y durante el curso de la enfermedad, mejoró la respuesta inflamatoria en comparación con las ratas colíticas no tratadas. Se observó un descenso significativo del grado de necrosis y/o inflamación (2,3 ± 0,4 cm vs. 3,4 ± 0,3 cm, $p<0.01$) y del cociente peso/longitud (143.3 ± 11.8 mg/cm vs. 209.7 ± 17.0 mg/cm, $p<0.01$), aumentados en las ratas colíticas como consecuencia de la administración del TNBS. El análisis histológico mostró una recuperación muy evidente del daño colónico, presentando una menor infiltración de neutrófilos en comparación con las ratas colíticas no tratadas. La reducción de la infiltración leucocitaria también se puso de manifiesto por una disminución en la actividad colónica de MPO (105,3 ± 26,0 U/g vs. 180,6 ± 21,9 U/g, $p<0.05$). El tratamiento con esta cepa de *Lactobacillus salivarius* también logró restaurar de manera significativa los niveles colónicos de glutation (1252 ± 42 nmol/g vs. 1087 ± 51 nmol/g, $p<0.05$), que disminuye a causa del estrés oxidativo provocado por el proceso inflamatorio. Además, el tratamiento probiótico redujo

también de manera significativa los niveles de TNF-a colónico (509,4 ± 68,2 pg/g vs. 782,9 ± 60,1 pg/g, $p<0.01$) y la expresión colónica de la iNOS en comparación con los animales colíticos no tratados. Finalmente, los animales tratados mostraron un mayor recuento de *Lactobacillus* en el contenido colónico, sin existir diferencias en el recuento de *Bifidobacterium*.

CONCLUSIÓN

El tratamiento con la cepa probiótica *Lactobacillus salivarius* CECT5713, facilita la recuperación del tejido inflamado en el modelo de colitis experimental por TNBS en rata, efecto asociado a la inhibición en la producción de algunos de los mediadores implicados en la respuesta inflamatoria intestinal, tales como citocinas, incluyendo el TNF-a, y la NO. Posteriores estudios en humanos deberán determinar si el carácter inmunomodulador que la cepa ha demostrado en este modelo experimental de colitis, puede tener efecto beneficioso en el tratamiento de la Enfermedad Inflamatoria Intestinal.

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• *BRIEF REPORTS* •

Preventative effects of a probiotic, *Lactobacillus salivarius ssp. salivarius*, in the TNBS model of rat colitis

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Abstract

AIM: To investigate the intestinal anti-inflammatory effect and mechanism of a probiotic *Lactobacillus salivarius ssp. salivarius* CECT5713 in the TNBS model of rat colitis.

METHODS: Female Wistar rats (180-200 g) were used in this study. A group of rats were administered orally the probiotic *L. salivarius ssp. salivarius* (5×10^8 CFU suspended in 0.5 mL of skimmed milk) daily for 3 wk. Two additional groups were used for reference, a non-colitic and a control colitic without probiotic treatment, which received orally the vehicle used to administer the probiotic. Two weeks after starting the experiment, the rats were rendered colitic by intracolonic administration of 10 mg of TNBS dissolved in 0.25 mL of 500 mL/L ethanol. One week after colitis induction, all animals were killed and colonic damage evaluated both histologically and biochemically. The biochemical studies performed in colonic homogenates include determination of myeloperoxidase (MPO) activity, glutathione (GSH) content, leukotriene B_4 (LTB_4) and tumor necrosis factor α (TNF-α) levels, as well as inducible nitric oxide synthase (iNOS) expression. In addition, the luminal contents obtained from colonic samples were used for microbiological studies in order to determine Lactobacilli and Bifidobacteria counts.

RESULTS: Treatment of colitic rats with *L. salivarius ssp. salivarius* resulted in amelioration of the inflammatory response in colitic rats when compared with the corresponding control group without probiotic treatment. This anti-inflammatory effect was evidenced macroscopically by a significant reduction in the extent of colonic necrosis and/or inflammation induced by the administration of TNBS/ethanol (2.3±0.4 cm *vs* 3.4±0.3 cm in control group, $P<0.01$) and histologically by improvement of the colonic architecture associated with a reduction in the neutrophil infiltrate in comparison with non-treated colitic rats. The latter was confirmed biochemically by a significant reduction of colonic MPO activity (105.3±26.0 U/g *vs* 180.6±21.9 U/g, $P<0.05$), a marker of neutrophil infiltration. The beneficial effect was associated with an increase of the colonic GSH content (1 252±42 nmol/g *vs* 1 087±51 nmol/g, $P<0.05$), which is depleted in colitic rats as a consequence of the oxidative stress induced by the inflammatory process. In addition, the treatment of colitic rats with *L. salivarius* resulted in a significant reduction of colonic TNF-α levels (509.4±68.2 pg/g *vs* 782.9±60.1 pg/g, $P<0.01$) and in a lower colonic iNOS expression, when compared to TNBS control animals without probiotic administration. Finally, treated colitic rats showed higher counts of Lactobacilli species in colonic contents than control colitic rats, whereas no differences were observed in Bifidobacteria counts.

CONCLUSION: Administration of the probiotic *L. salivarius ssp. salivarius* CECT5713 facilitates the recovery of the inflamed tissue in the TNBS model of rat colitis, an effect associated with amelioration of the production of some of the mediators involved in the inflammatory response in the intestine, such as cytokines, including TNF-α and NO. This beneficial effect could be ascribed to its effect on the altered immune response that occurs in this inflammatory condition.

Key words: *Lactobacillus salivarius ssp. salivarius*; TNBS rat colitis; Probiotic; Tumor necrosis factor α; Nitric oxide

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disease of

the digestive tract, and usually refers to two related conditions, namely ulcerative colitis and Crohn's disease, characterized by chronic and spontaneously relapsing inflammation. Although the etiology of IBD remains unknown, there is increasing experimental evidence to support a role for luminal bacteria in the initiation and progression of these intestinal conditions; probably related to an imbalance in the intestinal microflora, relative predominance of aggressive bacteria and insufficient amount of protective species[1,2]. This could justify the remission achieved in intestinal inflammation after treatment with antibiotics such as metronidazole or ciprofloxacin[3], or the fact that germ-free animals may fail to develop experimental intestinal inflammation[4]. In consequence, a possible therapeutic approach in IBD therapy is the administration to these patients of probiotic microorganisms, defined as viable nutritional agents conferring benefits to the health of human host. In fact, it has been reported that administration of a mixture of *Bifidobacterium* and *Lactobacillus*[5] or of non-pathogenic viable *Escherichia coli*[6] prolongs remission in ulcerative colitis. Moreover, there are reports on successful induction and maintenance of remission of chronic pouchitis after oral bacteriotherapy[7,8]. However, treatments of Crohn's disease with probiotic preparations reported conflicting results[9-12].

Different mechanisms have been proposed to participate in the therapeutic effects exerted by probiotic microorganisms. First, probiotic microorganisms may exert their action through a modulation of the intestinal bowel flora, which may result from competitive metabolic interactions with potential pathogens, production of anti-microbial peptides, or inhibition of epithelial adherence and translocation by pathogens[5,13]; second, probiotics have been proposed to modulate the host defenses by influencing the intestinal immune system[14,15]; and third, these microorganisms have been reported to positively affect the intestinal barrier function[16,17]. However, the detailed mechanisms by which these bacteria mediate their effects are not fully understood.

Although the results obtained after probiotic treatment in both human IBD and experimental colitis are promising, new studies are required in order to further understand this new concept for the therapy of IBD, even if we consider the fact that many studies have shown that not all bacterial species have equal activities in reducing intestinal inflammation[18,19]. Hence, the selection of new probiotic strains for the treatment of IBD can be based on their ability to regulate the immune response of the intestinal mucosa. This can be the case of *Lactobacilli* strains, which were able to downregulate the production of tumor necrosis factor α (TNF-α). In fact, previous *ex vivo* experiments have reported the ability of *L. casei* and of *L. bulgaricus* to downregulate TNF-α production in colonic explants from patients with Crohn's disease[20], thus supporting their future development for IBD therapy. This may be of special relevance since several studies have attributed a key role in the pathogenesis of IBD to this proinflammatory cytokine, as evidenced by the increased production of TNF-α in the intestinal mucosa from IBD patients[21,22] as well as by a number of clinical studies using anti-TNF-α mAb therapy that have clearly shown a beneficial effect in these patients[23].

The aim of the present study was to test the preventative effects of a *L. salivarius ssp. salivarius* strain in the trinitrobenzenesulfonic acid (TNBS) model of rat colitis, a well-established model of intestinal inflammation with some resemblance to human IBD[24]. The selection of this lactobacilli strain was based on previous *in vitro* studies that showed its ability to adhere to human intestinal cells, to inhibit pathogenic bacterial growth (unpublished results) and to reduce the production of inflammatory cytokines by immune cells. Special attention was paid to its effects on the production of some of the mediators involved in the inflammatory response, such as TNFα, leukotriene B_4 (LTB_4) and nitric oxide (NO). In addition, the correlation among the intestinal anti-inflammatory effect of *L. salivarius ssp. salivarius* and modifications on colonic flora induced by this probiotic was also studied.

MATERIALS AND METHODS

This study was carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the National Institute of Health.

Reagents

All chemicals were obtained from Sigma Chemical (Madrid, Spain), unless otherwise stated. Glutathione (GSH) reductase was provided by Boehringer Mannheim (Barcelona, Spain).

In vitro modulation of cytokine production by bacteria

Puleva Biotech's lactic acid bacteria collection was screened for *Lactobacilli* bacteria with the ability to reduce the production of inflammatory cytokines by activated macrophages. For this purpose, rodent bone marrow-derived macrophages, obtained as previously described[25], were stimulated with 100 ng/mL LPS in the presence or absence of 10^6 CFU/mL of each bacteria for 2 h. Then, cells were washed with culture media to eliminate non-attached bacteria, and cultured with new media for 12 h. TNF-α, IL-12, and IL-10 production was evaluated by ELISA in cell supernatants (CytoSets™, Biosource International, Nivelles, Belgium) following manufacturer's instructions.

Preparation and administration of the probiotic

L. salivarius ssp. salivarius CECT5713 was provided by Puleva Biotech (Granada, Spain) and it was normally grown in MRS media at 37 ℃ in anaerobic conditions using the AnaeroGen system (Oxoid, Basingstoke, UK). For probiotic treatment, bacteria was suspended in skimmed milk (10^9 CFU/mL) and stored at -80 ℃ until usage.

Experimental design

Female Wistar rats (180-200 g) were obtained from the Laboratory Animal Service of the University of Granada (Granada, Spain) and maintained in standard conditions. The rats were randomly assigned to three groups (n = 10); two of them (non-colitic and control groups) received no probiotic treatment and the other (treated group) received orally the probiotic (5×10^8 CFU suspended in 0.5 mL of skimmed milk) daily for 3 wk. Both non-colitic and control groups received orally the vehicle used to administer the probiotic (0.5 mL daily). Two weeks after starting the

experiment, the rats were fasted overnight and those from the control and treated groups were rendered colitic by the method originally described by Morris *et al*.[26]. Briefly, they were anesthetized with halothane and given 10 mg of TNBS dissolved in 0.25 mL of 500 mL/L ethanol by means of a Teflon cannula inserted 8 cm through the anus. Rats from the non-colitic group were administered intracolonically 0.25 mL of PBS instead of TNBS. All rats were killed with an overdose of halothane 1 wk after induction of colitis.

Assessment of colonic damage

The body weight, water and food intake were recorded daily throughout the experiment. Once the rats were killed, the colon was removed aseptically and placed on an ice-cold plate, longitudinally opened and luminal contents were collected for the microbiological studies (see below). Afterwards, the colonic segment was cleaned of fat and mesentery, blotted on filter paper; each specimen was weighed and its length measured under a constant load (2 g). The colon was scored for macroscopically visible damage on a 0-10 scale by two observers unaware of the treatment, according to the criteria described by Bell *et al*.[27] (Table 1), which takes into account the extent as well as the severity of colonic damage. Representative whole gut specimens were taken from a region of the inflamed colon corresponding to the adjacent segment to the gross macroscopic damage and were fixed in 4% buffered formaldehyde. Cross-sections were selected and embedded in paraffin. Equivalent colonic segments were also obtained from the non-colitic group. Full-thickness sections of 5 μm were obtained at different levels and stained with hematoxylin and eosin. The histological damage was evaluated by two pathologist observers (AN and AC), who were blinded to the experimental groups, according to the criteria described previously by Stucchi *et al*.[28] (Table 2). The colon was subsequently divided into four segments for biochemical determinations. Two fragments were frozen at -80 ℃ for myeloperoxidase (MPO) activity and inducible nitric oxide synthase (iNOS) expression, and another sample was weighed and frozen in 1 mL of 50 g/L trichloroacetic acid for total GSH content determinations. The remaining sample was immediately processed for the measurement of TNF-α and LTB_4 levels. All biochemical measurements were completed within 1 wk from the time of sample collection and were performed in duplicate.

MPO activity was measured according to the technique described by Krawisz *et al*.[29]; the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 μmoL hydrogen peroxide/min at 25 ℃. Total GSH content was quantified with the recycling assay described by Anderson[30], and the results were expressed as nanomole per gram of wet tissue. Colonic samples for TNF-α and LTB_4 determinations were immediately weighed, minced on an ice-cold plate and suspended in a tube with 10 mmol/L sodium phosphate buffer (pH 7.4) (1∶5 w/v). The tubes were placed in a shaking water bath (37 ℃) for 20 min and centrifuged at 9 000 r/min for 30 s at 4 ℃; the supernatants were frozen at -80 ℃ until assay. TNF-α was quantified by ELISA (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the results were expressed as picogram per gram of wet tissue. LTB_4 was determined by enzyme immunoassay (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the results expressed as nanogram per gram of wet tissue.

iNOS expression was analyzed by Western blotting as previously described[31]. Control of protein loading and transfer was conducted by detection of the β-actin levels.

Table 1 Criteria for assessment of macroscopic colonic damage

Score	Criteria
0	No damage
1	Hyperemia, no ulcers
2	Linear ulcer with no significant inflammation
3	Linear ulcer with inflammation at one site
4	Two or more sites of ulceration / inflammation
5	Two or more major sites of ulceration and inflammation or one site of ulceration / inflammation extending >1 cm along the length of the colon
6-10	If damage covers >2 cm along the length of the colon, the score is increased by one for each additional centimeter of involvement

Table 2 Criteria for assessment of microscopic colonic damage

Mucosal epithelium	
	Ulceration: none (0); mild surface (1); moderate (2); extensive-full thickness (3)
Crypts	
	Mitotic activity: lower third (0); mild mid-third (1); moderate mid-third (2); upper third (3)
	Mucus depletion: none (0); mild (1); moderate (2); severe (3)
Lamina propria	
	Mononuclear infiltrate: none (0); mild (1); moderate (2); severe (3)
	Granulocyte infiltrate: none (0); mild (1); moderate (2); severe (3)
	Vascularity: none (0); mild (1); moderate (2); severe (3)
Submucosal	
	Mononuclear infiltrate: none (0); mild (1); moderate (2); severe (3)
	Granulocyte infiltrate: none (0); mild (1); moderate (2); severe (3)
	Edema: none (0); mild (1); moderate (2); severe (3)

Maximum score: 27. Modified from Stucchi *et al*.[28].

Microbiological studies

Luminal content samples were weighed, homogenized, and serially diluted in sterile peptone water. Serial 10-fold dilutions of homogenates were plated on specific media for *Lactobacillus* (MRS media, Oxoid) or *Bifidobacterium* (MRS media supplemented with 0.5 mg/L dicloxacillin, 1 g/L LiCl and 0.5 g/L L-cysteine hydrochloride) and incubated under anaerobic conditions in an anaerobic chamber for 24-48 h at 37 ℃. Coliforms and enterobacteria were also determined by using specific Count Plates Petrifilm (3M, St. Paul, MN). After incubation, the final count of colonies was reported as $\log_{10}$ colony forming units per gram of material.

Statistical analysis

All results are expressed as mean±SE. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and post hoc least significance tests. Non-parametric data (score) are expressed as the median (range) and were analyzed using the Mann-Whitney *U*-test. Differences between proportions were analyzed with the χ^2 test. All statistical analyses were carried

out with the Statgraphics 5.0 software package (STSC, MD), with statistical significance set at $P<0.05$.

RESULTS

More than 30 lactic acid bacterial strains with the ability to adhere to human intestinal cell lines and to inhibit pathogenic bacterial growth *in vitro* belonging to the own Puleva Biotech collection were screened for their ability to modulate the production of inflammatory cytokines in LPS-stimulated macrophages. The results obtained were highly diverse including bacteria with the ability to enhance or to reduce inflammatory cytokine production (TNF-α and IL-12) modifying or not the expression of the anti-inflammatory cytokine IL-10 (data not shown).

Among all the screened bacteria, *L. salivarius ssp. salivarius* (CECT5713) showed the best TNF-α/IL-10 and IL-12/IL-10 ratio (Figure 1), since it was able not only to reduce the LPS-induced TNF-α and IL-12 production, but also to increase the levels of IL-10. For these reasons, we decided to use this strain to test its ability to prevent the inflammatory response in the *in vivo* assay of experimental colitis.

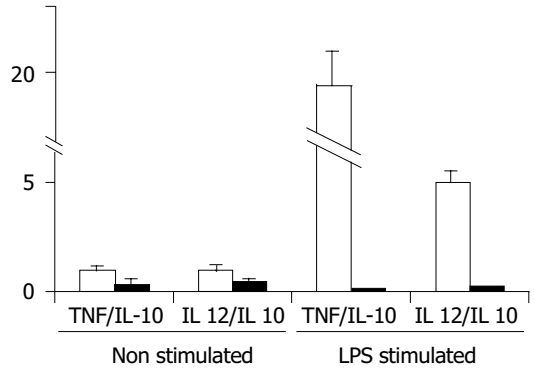

Figure 1 Production of inflammatory cytokines by bone marrow-derived macrophages (BMDM). TNF-α, IL-12, and IL-10 production was analyzed by ELISA in the supernatants of BMDM stimulated or not with LPS (100 ng/mL) and incubated with 10^6 CFU/mL of *L. salivarius ssp. salivarius* (CECT5713) (black bars) or in absence of bacteria (gray bars). The results are the mean of three assays±SE of the ratio between the proinflammatory cytokines (TNF-α and IL-12) and IL-10.

L. salivarius ssp. salivarius administration for 2 wk did not induce any symptoms of diarrhea or affected weight evolution. However, once the colitis was induced, the probiotic-treated rats showed an overall lower impact of TNBS-induced colonic damage compared to the TNBS control group. The anti-inflammatory effect was evidenced macroscopically by a significantly lower colonic damage score than that of control rats ($P<0.05$), with a significant reduction in the extent of colonic necrosis and/or inflammation induced by the administration of TNBS/ethanol (Table 3). This anti-inflammatory effect was also associated with a significant reduction in the colonic weight/length ratio between both colitic groups, an index of colonic edema, which increased significantly as a consequence of the inflammatory process (Table 3). The histological studies confirmed the intestinal anti-inflammatory effect exerted by *L. salivarius* (Figure 2). Histological assessment of colonic samples from the TNBS control group revealed severe transmural disruption of the normal architecture of the colon, extensive ulceration and inflammation involving all the intestinal layers of the colon, giving a score value of 18.9±1.1 (mean±SE). Colonic samples were characterized by severe edema, interstitial microhemorrhages and diffuse leukocyte infiltration, mainly composed of neutrophils in the mucosa layer and, to a lesser extent, lymphocytes in the submucosa. Most of the rats showed epithelial ulceration of the mucosa affecting over 75% of the surface. The inflammatory process was associated with crypt hyperplasia and dilation, and moderate goblet cell depletion. However, histological analysis of the colonic specimens from rats treated with the probiotic revealed a more pronounced recovery in the intestinal architecture than controls, with a score of 11.2±2.4 (mean±SE) ($P<0.01$ *vs* TNBS control group). Thus, most of the samples (7 of 10) showed almost complete restoration of the epithelial cell layer, in contrast to the extensive ulceration observed in non-treated animals; in fact, the zones with ulceration were surrounded by tissue in process of re-epithelization. Moreover, the transmural involvement of the lesions was reduced. The goblet cell depletion was less severe and thus they appeared replenished with their mucin content, and no dilated crypts were observed. The improvement in colonic histology was accompanied by a reduction in the inflammatory infiltrate, which was

Figure 2 Histological sections of colonic mucosa from colitic rats 1 wk after TNBS instillation stained with hematoxylin and eosin. **A**: Non-colitic group showing the normal histology of the rat colon (original magnification ×20). **B**: TNBS control group showing complete destruction of the mucosa, which has been substituted by inflammatory granulation tissue. There is evident edema and intense diffuse transmural inflammatory infiltrate (original magnification ×100). **C**: *L. salivarius ssp. salivarius* treated group showing amelioration of the inflammatory process and 'restoration' of the mucosal tissue with presence of mucin replenished goblet cells (original magnification ×100).

Table 3 Effects of *L. salivarius ssp. salivarius* (5×10^8 CFU/rat·day) treatment on macroscopic damage score, extent of the inflammatory lesion along the colon and changes in colon weight in TNBS experimental colitis in rats

Group (n = 10)	Damage score (0-10)	Extent of damage (cm)	Colon weight (mg/cm)
Non-colitic	0	0	63.3±2.5
TNBS control	6.5 (5-8)	3.4±0.3	209.7±17.0
TNBS probiotic	5 (3-7)[a]	2.3±0.4[b]	143.3±11.8[b]

Damage score for each rat was assigned according to the criteria described in Table 1 and data are expressed as median (range). Extent of damage and colon weight data are expressed as mean±SE. [a]$P<0.05$, [b]$P<0.01$ *vs* TNBS control. All colitic groups differ significantly from non-colitic group ($P<0.01$, not shown).

slight to moderate with a patchy distribution, although neutrophils were the predominant cell type.

The lower leukocyte infiltration was also assessed biochemically by the reduction in colonic MPO activity, a marker of neutrophil infiltration that was enhanced in the TNBS control group (Table 4). In addition, probiotic-treated colitic rats showed a significant increase in colonic GSH content, which is depleted in colitic rats as a consequence of the colonic oxidative stress induced by the inflammatory process, as previously reported in this model of experimental colitis[32] (Table 4). Finally, the colonic inflammation induced by TNBS was characterized by increased levels of colonic TNF-α and LTB_4 (Table 4) as well as by higher colonic iNOS expression (Figure 3) in comparison with non-colitic animals. Treatment of colitic rats with *L. salivarius* resulted in a significant reduction of colonic TNF-α levels (Table 4), that did not show any statistical differences with normal rats. No significant modification was observed on colonic LTB_4 levels. Finally, lower colonic iNOS expression was also seen in colitic animals that received the bacteria suspension when compared to TNBS control animals (Figure 3).

Effects of L. salivarius administration on colonic bacterial profile

TNBS colitis resulted in a significant reduction in fecal lactobacilli count in comparison with normal rats ($P<0.05$). Probiotic-treated colitic rats showed higher counts of Lactobacilli species in colonic contents than control colitic rats, without showing statistical differences with both non-colitic and colitic control groups (Figure 4). No statistical differences were observed in Bifidobacteria counts among three groups ($P>0.1$, Figure 4) nor in the amount of other fecal potential pathogenic bacteria such as enterobacteria or coliform bacteria (data not shown).

DISCUSSION

The results obtained in the present study reveal the efficacy of probiotic therapy with a *L. salivarius ssp. salivarius* strain in intestinal inflammation, incorporating a new microorganism to the probiotics that have been reported to attenuate the development of colonic injury in experimental and human IBD[33]. Thus, oral administration of the probiotic facilitated recovery from TNBS-induced colonic damage, as it was evidenced histologically, with a significant reduction in the extent and severity of inflamed tissue. This beneficial effect was also stated biochemically by a decrease in colonic MPO activity, a marker of neutrophil infiltration that has been previously described to be upregulated in experimental colitis[29], and is widely used to detect and follow intestinal inflammatory processes. In consequence, a reduction in the activity of this enzyme can be interpreted as a manifestation of the anti-inflammatory activity of a given compound[34]. The ability of the probiotic to reduce granulocyte infiltration, showed by MPO activity reduction, was confirmed histologically since the level of leukocyte infiltrate in the colonic mucosa was lower in treated colitic animals than in

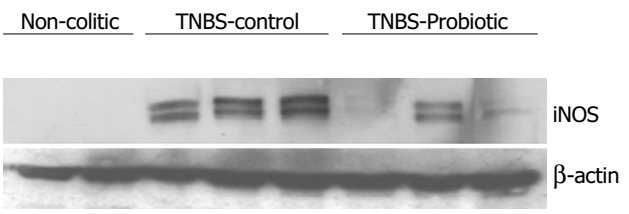

Figure 3 Effects of *L. salivarius ssp. salivarius* treatment (5×10^8 CFU/rat·day) on colonic nitric oxide synthase (NOS) expression in TNBS experimental colitis in rats.

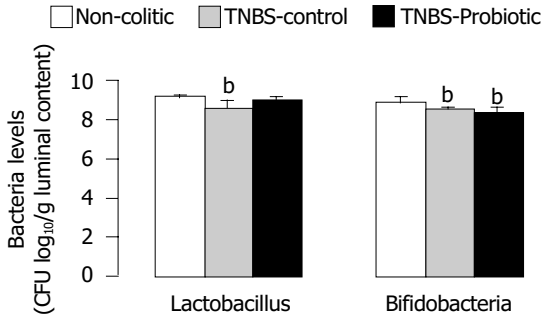

Figure 4 Effects of *L. salivarius ssp. salivarius* (5×10^8 CFU/rat·day) treatment on bacteria levels (Lactobacillus and Bifidobacteria) in TNBS experimental colitis in rats. [b]$P<0.01$ *vs* non-colitic group.

Table 4 Myeloperoxidase (MPO) activity, total GSH content, TNF-α and LTB_4 levels in colon specimens from non-colitic rats, TNBS control colitic rats and TNBS colitic rats treated with *L. salivarius ssp. salivarius* (5×10^8 CFU/rat·day)

Group (n = 10)	MPO activity (units MPO/g)	GSH (nmol/g)	LTB_4(ng/g)	TNF-α (pg/g)
Non-colitic	23.4±7.2	1 540±41	2.9±0.4	441.5±39.1
TNBS control	180.6±21.9[d]	1 087±51[d]	6.5±0.9[d]	782.9±60.1[d]
TNBS probiotic	105.3±26.0[a,d]	1 252±42[a,d]	6.9±0.8[d]	509.4±68.2[b]

Data are expressed as mean±SEM. [a]$P<0.05$, [b]$P<0.01$ *vs* TNBS control group; [d]$P<0.01$ *vs* non-colitic group.

the corresponding TNBS control groups. The inhibitory effect on the infiltration of inflammatory cells into the colonic mucosa might account for the beneficial effect of this probiotic against tissue injury, because margination and extravasation of circulating granulocytes contribute markedly to the colonic injury in this model of IBD[35]. These results are in agreement with other studies that describe the attenuation exerted by several probiotics in leukocyte–endothelial cell adhesion in this experimental model of rat colitis[36]. This effect can justify the inhibition of the synthesis and/or release of different mediators that participate in the inflammatory process, such as NO, since probiotic treatment of colitic rats was associated with a reduction in colonic iNOS expression. Moreover, this can also explain the improvement in the colonic oxidative stress in colitic rats after probiotic treatment, as evidenced by a partial restoration of the GSH depletion that took place as a consequence of the TNBS colonic damage.

During the last decade, it has become increasingly evident that chronic colonic inflammation, both in human IBD and in experimental colitis, is associated with enhanced NO production, mainly via iNOS activity[37-39], as well as with increased release of reactive oxygen metabolites, including superoxide[40-42]. The simultaneous overproduction of NO and superoxide can yield the highly toxic radical peroxynitrite in the inflamed intestine[43], which have been demonstrated to produce widespread colonic injury[44]. It is important to note that neutrophils are thought to be important source of both NO[45,46] and reactive oxygen metabolites[47]. Considering the above, the effect exerted by *L. salivarius ssp. salivarius* in decreasing the neutrophil infiltration that occurs in response to TNBS may preserve the colonic mucosa from oxidative insult. In fact, beneficial effects have previously been reported either after NOS inhibition[37,38] or by antioxidant therapy[31,42] in different experimental models of intestinal inflammation.

Probiotic treatment could attenuate neutrophil infiltration via inhibition of different mediators with chemotactic activity. The results obtained in the present study revealed that probiotic treatment did not significantly modify colonic LTB_4 levels, an eicosanoid with chemotactic activity involved in the pathogenesis of IBD[1]. In consequence, the inhibitory effect of leukocyte infiltration exerted by the probiotic should be related to the downregulation of other pro-inflammatory mediators, given the ability of this lactobacilli strain to modulate the immune response as demonstrated by the *in vitro* studies. In fact, the intestinal anti-inflammatory activity exerted by *L. salivarius ssp. salivarius* was also characterized by downregulation of colonic TNF-α. This may be relevant since TNF-α acts as a potent chemoattractant, thus contributing to the recruitment of neutrophil in the inflamed colonic mucosa and initiating the inflammatory pathogenic cascade that definitively perpetuates colonic inflammation[48]. The important role attributed to TNF-α in intestinal inflammation is strongly supported by the fact that different drugs capable of interfering with the activity of this mediator are being developed for IBD therapy[23]. The ability of probiotic bacteria to downregulate TNF-α production has been reported previously for other lactobacilli strains such as *L. casei* and of *L. bulgaricus* when they were cultured with inflamed mucosa from patients with Crohn's disease[20]. This effect was attributed to the existence of a cross talk between bacteria and mucosal cells, being able to downregulate the degree of activation of intestinal immune cells[20]. This has also been demonstrated in the present study for *L. salivarius ssp. salivarius* since it was able to modify the cytokine profile in macrophages, reducing the amount of inflammatory cytokines (TNF-α and IL-12) while increasing the amount of the anti-inflammatory cytokine IL-10. The high diversity of immuno-modulatory action of probiotics observed in the screening of the bacteria are in concordance with previous works showing both the ability of some lactic bacteria to promote TNF-α production[49] while others such as *L. rhamnosus* GG (LGG) reduced it[50]. LGG, a probiotic that also reduces the ratio TNF-α/IL-10, has been reported to exert intestinal anti-inflammatory effects both in human[12] and in experimental intestinal inflammation[51]. This effect of some probiotics on the immune response may be of special relevance because it would promote a possible shift from a T_H1-mediated immune response toward a T_H2/T_H3 profile, similarly to that proposed to occur with Lactobacillus GG[15]. It is important to note that replacing the bacteria responsible for the constant antigenic drive leading to T_H1 cellular activation with probiotic species that preferentially induce protective immune responses may alter the normal course of these relapsing intestinal conditions. In addition, probiotics like *Bifidobacterium longum* or *L. bulgaricus* have been shown to inhibit the IL-8 secretion in intestinal epithelia when stimulated by the proinflammatory cytokine TNF-α, thus reducing the activity of other proinflammatory cytokines with chemotactic activity[52].

However, the participation of the modification in the immune response in the intestinal anti-inflammatory effect exerted by this probiotic does not exclude mechanisms proposed for other probiotics, mainly due to a role in preventing the imbalance in the intestinal microflora, given the relative predominance of aggressive bacteria and insufficient amount of protective species that has been reported in these intestinal conditions[1,2]. Previous studies have suggested that in TNBS-induced colitis, specific strains from colonic microflora invades the colonic wall after disruption of the epithelium and the presence of bacteria within the wall participates in the transmural inflammation[53]. In fact, the present study reveals that the colonic damage induced by TNBS was associated with a significant reduction of lactobacilli count in the colonic lumen, which was counteracted after the probiotic treatment, since probiotic-treated rats showed no statistical differences from non-colitic rats in the lactobacilli content.

In conclusion, administration of the probiotic *L. salivarius ssp. salivarius* CECT5713 facilitates the recovery of the inflamed tissue in the TNBS model of rat colitis, an effect associated with amelioration of the production of some of the mediators involved in the inflammatory response of the intestine, such as cytokines, including TNF-α, and NO. This beneficial effect could be ascribed to its effect on the altered immune response characteristic of this inflammatory condition, which would attenuate the exacerbated immune response evoked by the colonic instillation of the hapten TNBS in the rats.

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5. La administración oral de dos cepas probióticas, *L.gasseri* CECT5714 y *L.coryniformis* CECT5711, mejora la función intestinal de adultos sanos

RESUMEN

OBJETIVO

La colonización, junto con la modificación de parámetros gastro-intestinales implicados en la función intestinal, son algunos de los principales efectos beneficiosos que se reclaman para los probióticos. Sin embargo, a pesar de que la gente sana es la principal consumidora de estos nuevos alimentos funcionales, el número de estudios clínicos analizando los efectos gastrointestinales de los probióticos en personas sanas, es realmente escaso. El objetivo de este estudio es el de evaluar los posibles efectos beneficiosos sobre la función intestinal de un producto fermentado que contenía las cepas probióticas, *Lactobacillus gasseri* CECT5714 y *Lactobacillus coryniformis* CECT5711.

RESULTADOS

Se realizó un estudio clínico aleatorio, doble-ciego y comparado con un placebo, que involucró a 30 adultos sanos, con el propósito de investigar los efectos en diversos parámetros relacionados con la función intestinal. Los voluntarios fueron asignados aleatoriamente en dos grupos, uno que tomó diariamente y durante 4 semanas un yogurt estándar y otro que tomó un producto fermentado similar en el cual el *Lactobacillus delbreuckii* subsp. *bulgaricus* del yogurt, fue sustituido por una combinación de las cepas probióticas *L. gasseri* CECT5714 y *L. coryniformis* CECT5711. No se detectó ningún efecto adverso relacionado con el consumo del producto fermentado y las cepas consumidas pudieron ser aisladas en las heces de los voluntarios en un porcentaje relativamente alto. De hecho, la concentración fecal de bacterias ácido lácticas aumentó significativamente en el grupo probiótico. Además, la administración oral de las cepas probióticas supuso una mejora de parámetros como la producción de ácidos grasos de cadena corta, el contenido de agua, volumen y frecuencia de defecación. Como resultado de ello, los voluntarios del grupo probiótico percibieron una clara mejoría de sus hábitos intestinales. Este estudio reveló que, el consumo de probióticos puede ejercer un efecto positivo en voluntarios sanos adultos.

CONCLUSIÓN

Por tanto, en este trabajo se demuestra que la administración oral de dos nuevas cepas probióticas, *L.coryniformis* CECT5711 y *L.gasseri* CECT5714, es bien tolerada y que ejerce efectos beneficiosos sobre la función intestinal de adultos sanos.

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Oral administration of two probiotic strains, *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711, enhances the intestinal function of healthy adults

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Abstract

Modifications in gastrointestinal parameters, intestinal colonization and tolerance are some of the main goals claimed for probiotics. However, although healthy people are the common target for these new functional food products, the number of clinical trials analysing the effects of probiotics in gastrointestinal parameters of healthy subjects is very scarce. A randomized, double blind, placebo-controlled human clinical trial involving 30 healthy adults was performed to investigate the effect of a fermented product containing two probiotic strains, *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711, on several blood and fecal parameters, most of them related to the host intestinal function. The volunteers were randomly distributed into two groups, one receiving a standard yogurt and the other a similar dairy fermented product in which the *Lactobacillus delbreuckii* subsp. *bulgaricus* yogurt strain had been replaced by a combination of the probiotic strains *L. gasseri* CECT5714 and *L. coryniformis* CECT5711. The volunteers that received the probiotic strains reported no adverse effects and the strains could be isolated from their feces at a relatively high level. In fact, the concentration of fecal lactic acid bacteria significantly increased in the probiotic group. Additionally, the oral administration of the probiotic strains led to an improvement of parameters such as the production of short chain fatty acids, the fecal moisture and the frequency and volume of the stools. As a result, the volunteers assigned to the probiotic group perceived a clear improvement in their intestinal habits. The study revealed that probiotics may exert a positive effect on healthy adults.

Keywords: *Lactobacillus gasseri*; *Lactobacillus coryniformis*; Probiotics; Gut microbiota; Human clinical trial

1. Introduction

The intestinal microbiota is composed of a wide diversity of bacteria that perform important functions for the host (Salminen et al., 1995). Its acquisition is a gradual process involving bacterial strains from maternal and environmental sources. The microbiota of breast-fed infants includes a relatively narrow spectrum of Gram-positive bacteria but, after weaning, it becomes more diverse and complex (Roderick et al., 1999; Martín et al., 2004). Recently, the use of probiotic strains (particularly lactobacilli and bifidobacteria) has been promoted as a means to balance the gut microbiota and, in fact, their potential preventive and therapeutical effects have received renewed research and industrial interest (Salminen et al., 1998; Ouwehand et al., 1999; Saavedra, 2001).

Recently, several well-designed clinical trials have been carried to determine the effects of specific probiotic strains on patients suffering gastrointestinal disorders, atopy, cancer and other conditions (Guandalini et al., 2000; Felley et al., 2001; Kalliomaki et al., 2003); however, few studies have dealt with

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the potential effects of probiotics on healthy adults despite, in such circumstances, probiotic bacteria could help people to preserve their health status (Ling et al., 1994; Schiffrin et al., 1995; Spanhaak et al., 1998). In addition, it should be taken into account that, at present, healthy people actually constitute the main target of the probiotic market.

In a previous work, we described that breast milk of healthy women is an important source of lactic acid bacteria to the infant gut and that *Lactobacillus gasseri* is among the main species found in this biologic fluid (Martín et al., 2003). More recently, we showed that *L. gasseri* CECT5714, one of the breast milk isolates selected for further studies, had a variety of in vitro probiotic properties (Martín et al., 2005). In parallel, we isolated *Lactobacillus coryniformis* CECT5711 from an artisan goat's milk cheese and its characterization revealed that it displayed a variety of potential probiotic properties such as production of reuterin and cobalamin, adhesion to enterocytes, resistance to conditions simulating those of the digestive tract or immunomodulation (Martín et al., 2005b). Further studies revealed that, from a technological point of view, the combination of these two strains was particularly suited for the production of fermented dairy products (unpublished data). In this context, we present the results of a human clinical trial performed to know if the daily administration of a fermented milk product containing both strains had any effect on a variety of intestinal-related parameters of healthy adults when compared with a similar group consuming a yogurt elaborated with a conventional starter (*Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*).

2. Material and methods

2.1. Design of the trial

A total of 30 healthy adult human volunteers (15 females and 15 males) with ages ranging from 23 to 43 years old were included in the study. Exclusion criteria were lactose intolerance, recent antibiotic treatment, frequent gastrointestinal disorders or metabolic diseases. The study was carried out according to the Helsinki declaration and informed written consent was obtained from all the subjects. The study consisted of three phases: a pre-treatment period (2 weeks), a treatment period (4 weeks) and, finally, a wash-out period (2 weeks). The volunteers were asked to exclude from their diet any kind of fermented food or drink with the exception of the dairy product tested. Only one participant had to abandon the study at the end of the pre-treatment period due to an oral bacterial infection independent of the study design that required antibiotherapy.

Volunteers were randomly distributed into two groups. Those belonging to the "yogurt" group used as a control received, on a daily basis, 200 ml of a yogurt elaborated with a standard yogurt starter provided by Puleva Food (Granada, Spain) containing 10^8 cfu of *S. thermophilus* and 4×10^9 cfu of *L. bulgaricus.* Those of the "probiotic" group ingested, with the same frequency, 200 ml of a similar dairy product containing the same concentration of the starter *S. thermophilus* but in which the *L. bulgaricus* starter strain had been replaced by approximately 2×10^9 cfu of each of the probiotic strains *L. coryniformis* CECT5711 and *L. gasseri* CECT5714.

2.2. Collection and analysis of the blood samples

Blood samples were taken from each volunteer immediately before and after the treatment period using EDTA-containing vacutainers (S-Monovette, Sarstedt, Germany). The blood biochemistry and the hemogram were performed in a SYMEX F-800 system at Laboratorios Algar SL (Granada, Spain). Total cholesterol, HDL-cholesterol and triacylglycerides plasma concentrations were measured using enzyme-spectrophotometry kits (BioSystems, Barcelona, Spain). The concentration of HDL-cholesterol was calculated using the Friedewald formula (LDL = total cholesterol − DL − TG/5).

2.3. Concentration of lactic acid bacteria and RAPD detection of the probiotic strains in the fecal samples

Once a week, fecal samples were collected and homogenized in a peptone-saline solution. To estimate the concentration of lactic acid bacteria, appropriate dilutions were spread in quadruplicate onto plates of MRS agar (Oxoid, Basingstoke, UK). The cultures were incubated in anaerobiosis at 37 °C for 24 h.

From the fecal samples of each volunteer, 50 colonies grown in MRS agar were randomly selected and checked for two properties: resistance to ciprofloxacin (a characteristic of *L. gasseri* CECT5714) and β-galactosidase activity (a characteristic of *L. coryniformis* CECT5711). For this purpose, the colonies were subcultured in MRS plates supplemented with ciprofloxacin (20 μg/ml). Parallel, their β-galactosidase activity was measured following the method described by Pardee et al. (1959). The colonies that displayed one of both the properties tested were submitted to the RAPD technique using the RAPD Analysis Primer Set (Amersham Biosciences, Piscataway, NJ) following the instructions of the manufacturer. The RAPD profiles were compared with those corresponding to *L. gasseri* CECT5714 and *L. coryniformis* CECT5711. The genomic DNA of the selected isolates was obtained using the DNeasy™ Tissue Kit (Qiagen GmbH, Hilden, Germany).

2.4. Quantification of short chain fatty acids and other parameters in the fecal samples

The concentration of short chain fatty acids (SCFAs) in the fecal samples was quantified according to the method described by Rodriguez-Cabezas et al. (2002). Briefly, fecal samples were homogenized with 150 mM $NaHCO_3$ (pH 7.8) (1 : 5, wt/v) in an argon atmosphere. Samples were incubated for 24 h at 37 °C and stored at −80 °C until the extraction. To extract the SCFAs, 50 μL of 100 mM 2-methylvaleric acid (internal standard), 10 μL of sulfuric acid and 0.3 ml of ethyl acetate were added to 1 ml of the homogenate. The mix was centrifuged at 10,000 ×*g* for 5 min at 4 °C. The supernatants were dehydrated with sodium sulphate (anhydrous) and centrifugated 10,000 ×*g* for 5 min at 4 °C. Later, the sample

(0.5 ml) was splitless inoculated into a gas chromatograph (mod. CP-3800, Varian, Lake Forest, CA) equipped with an ID (CPWAX 52CB 60 m × 0.25 mm), and connected to a FID detector (Varian). Helium was used as the carrier and the make-up gas, with a flow rate of 1.5 ml/min. The injection temperature was 250 °C. Acetate, propionate and butyrate concentrations were automatically calculated from the areas of the resulting peaks using the Star Chromatography WorkStation program (version 5.5), which was connected on-line to the FID detector.

The supernatants obtained after the homogenization of the fecal samples were used to measure the fecal pH and the ammonium concentration in a pH-meter (mod. GLP21-21, Crison, Barcelona, Spain) equipped with electrodes for pH and ammonium (mV) measurements. The water content of the feces was calculated by difference between fresh and dried samples. Fecal enzymatic activities were determined using the APIzym strip system (BioMerieux, Lyon, France).

2.5. Cytotoxicity of fecal supernatants

A cell lysis assay (Xaus et al., 2001) using the human tumor adenocarcinoma cell line (HT-29) was performed to determine the cytotoxicity of the supernatants obtained after the homogenization of the fecal samples. The HT-29 cell line was cultured in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD) with fetal bovine serum (10%), L-glutamine (2 mmol/L), penicillin (100,000 U/L) and streptomycin (100 mg/L). Then 50,000 cells were placed in each well of a 96-multiwell plate. Cells were cultured for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. Then, the fecal supernatants were filtered through 0.45 μm filters (Millipore, Bedford, MA), diluted using the medium cited above and added (50 μg/ml) to the cells. The cell cultures were incubated for 24 h. Wells with no supernatants added were included in each plate and used as negative control and each assay was performed by quadruplicate. After incubation, wells were washed twice in PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. After 3 washes by immersion in twice-distilled water, the cells were stained with a 0.1% crystal violet for 20 min. After 2 additional washes, the plates were dried at 37 °C and developed by exposition to 0.1 M HCl for 5 min; then, the absorbance of each well was measured at 630 nm.

2.6. Bowel habit

The participants were asked to annotate the frequency and volume of stools and to compare their bowel function with that existing before the treatment by using a scale ranging from 0 to 10, being 5 the individual status before the study. They were also asked to report any symptom of gastrointestinal discomfort and, in such case, to evaluate the severity of the symptoms by using a scale ranging from 0 (no symptoms) to 5 (severe symptoms). The final score values resulted from the addition of the daily recorded scores obtained during the first two weeks before treatment (week 0) or two week periods during treatment and after treatment.

2.7. Statistical analysis

The data were analysed using SPSS software (version 12.0, Chicago, USA). Data are expressed as means ± s.e.m. P values < 0.05 were considered significant.

For the Gaussian variables, the longitudinal effect of each yogurt within each group at the different time points of the study was analysed by one-way repeated measures ANOVA followed by paired t test (within-group comparison). Two-way repeated measures ANOVA was used to analyse statistical differences produced by the consumption of each yogurt followed by independent t test to assess in which time points the groups differed.

For the non-Gaussian variables, Wilcoxon, Mann–Whitney U, Moses extreme reactions, Kolmogorov–Smirnov Z, Wald–Wolfowitz runs comparisons were performed to assess differences within- and between-groups, respectively.

3. Results

3.1. Clinical status and blood analysis

Throughout the entire study, none of the volunteers reported any adverse effect associated with the consumption of the dairy fermented products nor diet restriction. Clinical examination by the medical staff of Puleva Biotech revealed that the health condition of all the participants was excellent during the study.

The blood analysis performed before and after the treatment period showed that all the parameters of clinical relevance were within their respective ranges of normality although, after the treatment, the values for red cells and hematocrit had experienced a significant increase in both groups, being higher in the case of the probiotic group (red cells count from 4482.2 ± 103.03 to $4847.6 \pm 110.55 \times 10^3 \times dL$ for control group and from 4805.3 ± 140.99 to $5167.8 \pm 148.53 \times 10^3 \times dL$ for probiotic group and in the case of hematocrit from $42.5 \pm 1.12\%$ to $45.07 \pm 1.18\%$ for control group and from $44.06 \pm 0.86\%$ to $47.32 \pm 1.08\%$ for probiotic group). In both groups, the values of mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were lower at the end of the treatment period but the concentration of hemoglobin did not change significantly (data not shown).

Plasma concentrations of cholesterol and triacylglycerides were also measured. No significant changes were found in total, HDL- and LDL-cholesterol after the treatment period. The concentration of triacylglycerides decreased in the probiotic group (from 71.95 ± 10.04 to 65.2 ± 4.87 mg/dL), in contrast with that of the yogurt group (from 76.88 ± 7.9 to 87.65 ± 11.9 mg/dL) although such differences were not statistically significant ($P = 0.086$).

3.2. Concentration of lactic acid bacteria and RAPD detection of the probiotic strains in the fecal samples

During the treatment, the consumption of the probiotic strains led to a significant increase in the number of fecal lactic

acid bacteria (from 6.97±0.158 to 7.59±0.213 cfu/g of feces). In contrast, the fecal MRS counts of the yogurt group decreased along the treatment period (from 6.82±0.235 to 6.48±0.232 cfu/g of feces) and were significantly lower than those obtained from the respective samples of the probiotic group (Fig. 1). However, the MRS counts decreased in the probiotic group after the wash-out period (6.52±0.32 log cfu/g of feces) to a level not significantly different to that found in the yogurt group (6.30±0.30 log cfu/g of feces).

To determine if the administration of the probiotic lactobacilli was playing a role in the increase of the MRS counts observed in the probiotic group, the RAPD technique was applied among the ciprofloxacin-resistant or β-galactosidase-positive colonies tested. Both lactobacilli strains could be isolated and detected from fecal samples of the probiotic group but not from those of the yogurt group (Fig. 2), accounting for 10–15% of the colonies tested for each of the strains (data not shown).

3.3. Quantification of short chain fatty acids and other biochemical parameters in the fecal samples

The ability of the fecal microbiota to produce SCFAs such as butyric acid, propionic acid and acetic acid was measured. Production of butyrate was higher in the probiotic group than in the control group during the treatment (Fig. 3). Similarly, production of propionic and acetic acid was higher in the probiotic group during the treatment period although differences were only significant at two weeks (353.77±56.92 versus 681.34±85.57 mg/L propionate ($P=0.004$) and 655.88±109.21 versus 1284.45±200.50 mg/L acetate ($P=0.001$), in all cases control versus probiotic group). At the end of the wash-out period the production of butyrate in probiotic group was still higher than in control group (221.46±48.28 mg/L in control group versus 474.41±86.21 mg/L in probiotic group ($P=0.019$)) meanwhile no differences between groups were observed.

At the end of the treatment period, the values for other biochemical parameters related to microbiota metabolism, such as the fecal pH or the fecal ammonium concentration were similar in both groups (data not shown).

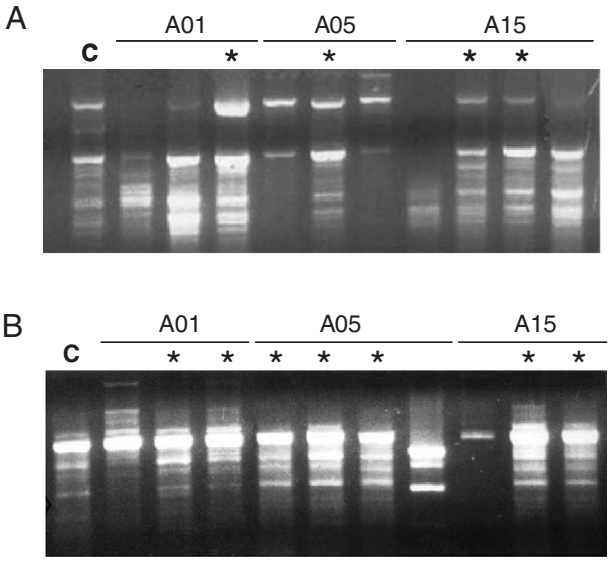

Fig. 2. RAPD profiles obtained with primer 2 (RAPD analysis primer set, Amersham) from bacterial DNA. A: lane C, pattern of *L. gasseri* CECT5714 (lane C); lanes A01, A05 and A015, ciprofloxacin-resistant colonies isolated from feces of the homonym hosts of the probiotic group (*RAPD profile identical to that of *L. gasseri* CECT5714). B: lane C, pattern of *L. coryniformis* CECT5711; lanes A01, A05 and A015, β-galactosidase-positive colonies isolated from feces of the homonym hosts of the probiotic group (*RAPD profile identical to that of *L. coryniformis* CECT5711).

Nineteen enzymatic activities were estimated in the feces of volunteers (Table 1). In general, the pattern of enzymatic activities displayed by both groups was very stable throughout the study. However, a significant increase ($P<0.05$) in the napthol-AS-BI-phosphohydrolase activity (a feature of the probiotic lactobacilli) was observed in the feces belonging to the probiotic group, and the increase was still significant after the wash-out period (Table 1). In addition, the leucine arylamidase activity (also characteristic of the probiotic strains) tended to increase ($P=0.08$) in the probiotic group but, in contrast, it decreased significantly in the yogurt group. Differences in β-glucuronidase activity were also found between the yogurt group and the probiotic group at the end of treatment but the differences did not reach a statistical significance ($P=0.10$).

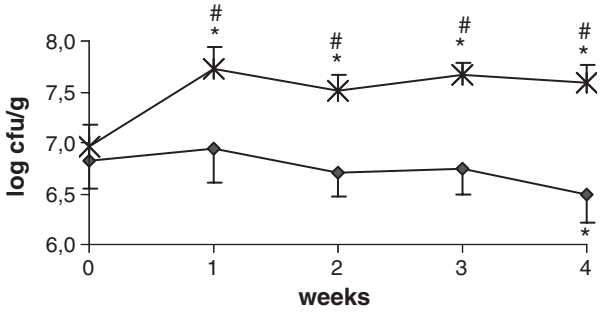

Fig. 1. MRS counts (log cfu/g) in fecal samples obtained before the treatment (week 0) and, then, weekly during the treatment period. Probiotic group (X) and control group (♦). *Statistically significant difference ($P<0.05$) with respect to week 0. #Significant difference ($P<0.05$) between control and treatment groups.

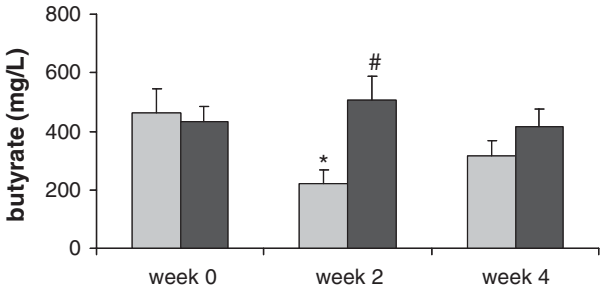

Fig. 3. SCFAs concentrations. Butyrate concentrations after 24 h of fermentation of the fecal samples are represented as means (±s.e.m.) in mg/L at the different time points. Probiotic group (black bars) and control group (grey bars). ##, #Significant difference (##$P<0.01$, #$P<0.05$) between control and treatment groups.

Table 1
Enzymatic activities in fecal samples

	Yogurt group		Probiotic group	
	Week 0	Week 4	Week 0	Week 4
Alkaline phosphatase	4.86±0.09	4.93±0.07	4.60±0.16	4.87±0.09
Esterase (C4)	3.00±0.18	3.40±0.14	2.80±0.17	3.13±0.18
Esterase lipase (C8)	1.73±0.18	2.00±0.40	1.67±0.12	1.87±0.19
Leucine arylamidase	3.21±0.49	2.86±0.44*	2.20±0.44	2.93±0.49
Valine arylamidase	0.93±0.38	0.50±0.31	0.33±0.15	0.73±0.28
Acid phosphatase	3.29±0.19	3.29±0.22	3.07±0.18	3.27±0.21
Napthol-AS-BI-phosphohydrolase	3.29±0.34	3.14±0.36	2.67±0.33	3.27±0.28*
α-galactosidase	3.14±0.35	3.71±0.29	3.33±0.30	3.20±0.42
β-galactosidase	3.79±0.28	4.29±0.22	3.60±0.21	3.93±0.28
β-glucuronidase	2.62±0.37	3.31±0.31	2.80±0.27	2.73±0.37
α-glucosidase	3.86±0.21	4.21±0.21	3.60±0.24	3.93±0.25
B-glucosidase	3.07±0.25	3.43±0.37	2.80±0.34	3.33±0.30
N-acetyl-β-glucosaminidase	3.79±0.23	3.93±0.26	3.60±0.23	3.87±0.27
α-fucosidase	0.86±0.25	0.86±0.17	0.86±0.14	0.87±0.25

Results are expressed as the mean±s.e.m. of values obtained at the end of the colorimetric reaction.
* Statistically significant difference ($P<0.05$) with respect to week 0.

3.4. Cytotoxicity of fecal supernatants

The enzymatic activity of the so-called beneficial bacteria has been extensively related to the reduction of toxic metabolites in the luminal content (De Boever et al., 2000; Wollowski et al., 2001). The analysis of the cytotoxicity of the supernatants obtained after homogenization of the fecal samples provided by the participants revealed a reduction of this parameter in the probiotic group in contrast to the increase observed in the yogurt group (Fig. 4); however, such differences had no statistical significance.

3.5. Bowel habit

Parameters related to the bowel habits of the participants were also measured to study the potential impact of the probiotic strains on their bowel function. The water content of the fecal samples increased significantly at the end of the treatment in the probiotic group and this effect was still observed after the wash-out period; in contrast, the values of the yogurt group samples tended to decrease and, in this case, the values for both groups were significantly different (Fig. 5A). In addition, the stool frequency was significantly higher in the probiotic group at the end of the consumption period and even after the wash-out period, whereas the stool frequency of the yogurt group participants decreased significantly (Fig. 5B). Parallel, the volunteers consuming the probiotic product reported a significant increase in the volume of their stools, a fact that was not detected among the people ascribed to the yogurt group (Fig. 5C). Finally, the participants belonging to the probiotic group preferred the intestinal habits acquired during the study, in contrast with the opinion of those belonging to the yogurt group (Fig. 5D). The level of gastrointestinal discomfort was very low in both groups and did not change significantly along the treatment period. In fact, the volunteers of both groups perceived that the consumption of the fermented milk products had a mild positive impact on their bowel habit. However, while the value obtained for this parameter did not change significantly in the control group, a slight but statistically significant ($P=0.015$) increase was observed in the probiotic group (from 5.7±0.15 at week 1 to 6.0±0.19 at the end of the treatment).

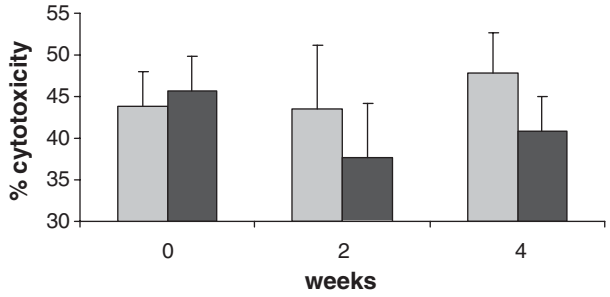

Fig. 4. Cytotoxicity (percentage of dead HT-29 cells) of the fecal supernatants obtained from the probiotic (black bars) and the yogurt (grey bars) groups.

4. Discussion

The consumption of the fermented products tested in this study was well tolerated by all the participants since none of them reported any health disturbance and the hematological values were within a normal range throughout the study. The tolerance of the probiotic product in healthy people was expected since no adverse effects have been reported in previous studies in which the participants had an impaired intestinal function, including pre-term neonates (Marini et al., 2003; Saggioro, 2004; Salminen et al., 2004).

An increase in the concentration of lactic acid bacteria in the feces of the hosts was observed after the consumption of the fermented product containing *L. gasseri* CECT5714 and *L. coryniformis* CECT5711; additionally, both strains could be isolated from such samples and specifically detected by RAPD profiling. The fact that the MRS counts returned to the basal level two weeks after the cessation of the probiotic treatment suggests that the presence of both strains in the gut of the hosts is transient. It has been previously reported that once the adult

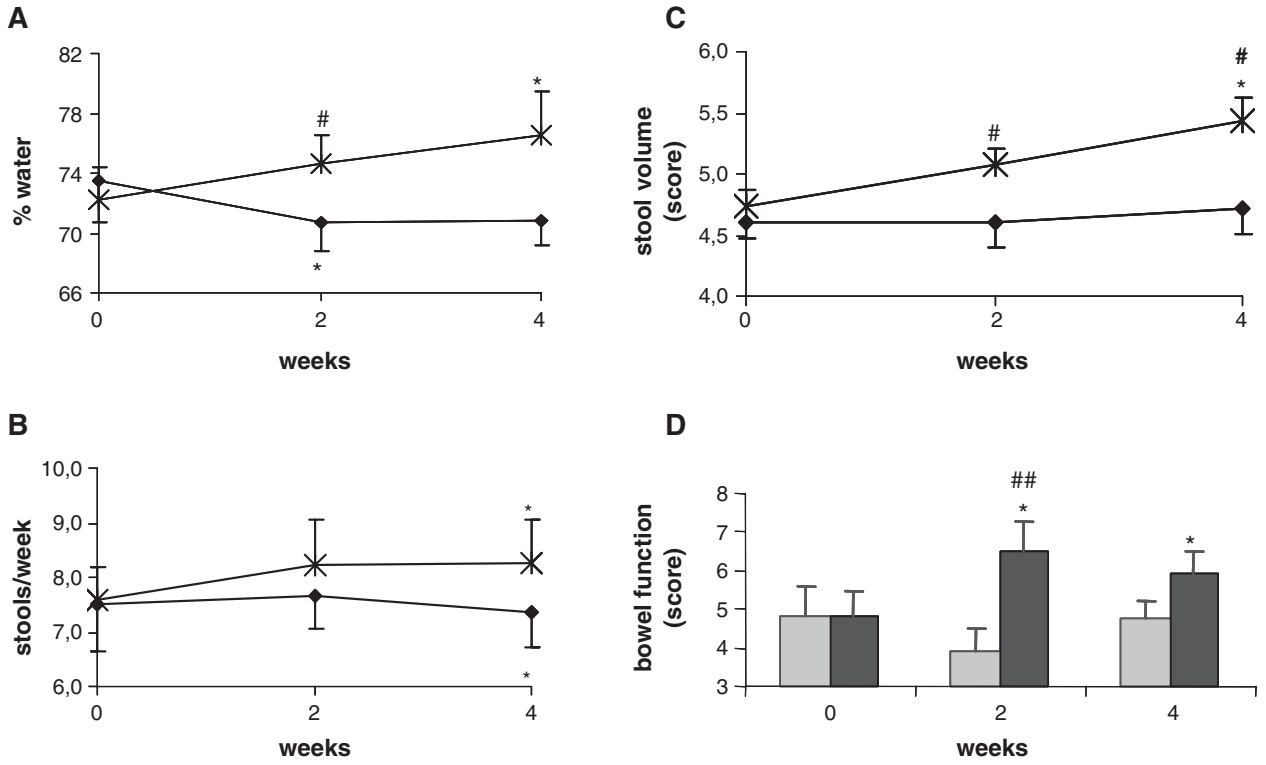

Fig. 5. Bowel habit-related parameters. A, Water content in feces is represented as the mean (±s.e.m.) of the percentage of water in fecal samples for probiotic group (X) and yogurt group (♦). B, Stool frequency is represented as the means (±s.e.m.) of the number of stool at week for probiotic group (X) and yogurt group (♦). C, Mean (±s.e.m.) of the valuation by volunteers of the stool volume is represented samples for probiotic group (X) and yogurt group (♦). D, Evolution of the intestinal habit is represented as the mean (±s.e.m.) of the valuation by volunteers of the defecation function for probiotic group (black bars) and yogurt group (grey bars). 0=worse, 5=no change, 10=best. *Statistically significant difference ($P<0.05$) with respect to week 0. $^{\#\#}$, $^{\#}$Significant difference ($^{\#\#}P<0.01$, $^{\#}P<0.05$) between control and treatment groups.

gut microbiota is established, the colonization with new strains is usually difficult and transient and sustained oral doses are required for their middle- and long-term maintenance (Roderick et al., 1999).

Moreover, the increase of the napthol-AS-BI-phosphohydrolase activity and the maintenance of the leucine arylamidase one in the feces of the probiotic group also indicated that *L. gasseri* CECT5714 and *L. coryniformis* CECT5711 were functional in the gut of the hosts since such activities are characteristic of the cited strains and their pattern was different in the feces of the yogurt group.

The fecal concentration of SCFAs, a parameter related to the fermentation of some carbohydrates by lactic acid bacteria and other gut bacteria, was significantly higher in the probiotic group when compared to the yogurt group. SCFAs are the main energy source for colonocytes and contribute to several gut functions including carbohydrate and lipid metabolism, control of the colonic pH, maintenance of the integrity of the colonic mucosa, intestinal motility or absorption (Yajima, 1985; Robertfroid et al., 1995; Mortensen and Clausen, 1996). The fact that, in this study, the parameters related to the bowel habit (water content of the feces, stool volume and frequency) obtained significantly better values in the probiotic group may be a consequence of the higher SCFAs concentrations in the feces of the participants belonging to this group.

Interestingly, it has been also observed that an increase in the fecal bulk dilutes carcinogens, mutagens and tumor promoters and results in a lower risk of colon cancer (Weisburger et al., 1993), while a faster transit can reduce exposure of the gut mucosa to the potential cancer-inducing agents that may be found among the intestinal content (Cummings et al., 1992). In addition, the presence of butyrate is usually linked to anti-neoplastic activities such as increased apoptosis, lower proliferation rate or down regulation of angiogenesis (D'Argenio and Mazzacca, 2003); therefore, the maintenance of butyrate concentrations in the colon may be considered as a potential health-promoting factor because of its potential role in the prevention of colon cancer. In this context, the cytotoxicity of the fecal water of the probiotic group feces was lower than that of the respective samples of the yogurt group. The reduction of toxic metabolites in the luminal content by the enzymatic activity of the so-called "beneficial bacteria" has been described previously (De Boever et al., 2000; Wollowski et al., 2001).

Few studies have been focused on the effects of probiotics on the intestinal function of healthy people and the observed effects depend on the strain used (Guerin-Danan et al., 1998; Spanhaak et al., 1998; Johansson et al., 1998; Ouwehand et al., 2002; Valeur et al., 2004). In this work we demonstrate that the oral administration of two new probiotic strains, *L.*

coryniformis CECT5711 and *L. gasseri* CECT5714, is well tolerated and exerts a beneficial effect on the bowel function of healthy adults. Studies are in progress to elucidate the effects of these strains on immunological parameters of healthy adult hosts.

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6. El consumo de dos nuevas cepas probióticas, *L.gasseri* CECT5714 y *L.coryniformis* CECT5711, estimula el sistema inmunitario de voluntarios sanos

RESUMEN

OBJETIVO

A principios de la década pasada se señaló la influencia que los probióticos tenían sobre la respuesta inmune, mostrando su capacidad inmunomoduladora tanto a nivel de respuesta innata como de respuesta específica. Sin embargo, existen diferencias en los efectos inmunomoduladores de las diferentes cepas probióticas que, dependen no sólo de las características propias de la cepa, sino también del entorno bacteriano en que se encuentre y del estado de salud del consumidor. Los estudios más recientes, tratan de conocer los mecanismos implicados y obtener un modelo en el que se pueda observar la relación causa-efecto entre la ingestión de una determinada cepa probiótica y la modulación del sistema inmunitario. El propósito de este estudio es, evaluar el potencial inmunomodulador de un producto fermentado que contiene las cepas probióticas Hereditum, *L.gasseri* CECT5714 y *L.coryniformis* CECT5711 en voluntarios sanos.

RESULTADOS

Se realizó un estudio clínico aleatorio, doble-ciego y comparado con un placebo, que involucró a 30 adultos sanos, con el propósito de investigar los efectos en diversos parámetros sanguíneos y fecales relacionados con la función del sistema inmune. Los voluntarios fueron asignados aleatoriamente en dos grupos, uno que diariamente y durante 4 semanas, tomó un yogurt estándar y otro que tomó un producto fermentado similar en el cual el *Lactobacillus delbreuckii* subsp. *bulgaricus* del yogurt, fue substituido por una combinación de las cepas probióticas *L. gasseri* CECT5714 y *L. coryniformis* CECT5711. El consumo tanto del nuevo producto fermentado como del yogurt incrementó la proporción de células fagocíticas, incluyendo monocitos y neutrófilos, así como su actividad fagocítica. Sin embargo, el producto fermentado que contenía la combinación de las cepas, *L.gasseri* CECT5714 y *L.coryniformis* CECT5711, también indujo un incremento en la proporción de células natural killer (NK) y en la concentración de IgA. Los efectos fueron mayores a las dos semanas después del tratamiento que a las 4 semanas, lo cuál sugiere una regulación en el sistema inmunitario.

CONCLUSIÓN

El consumo de un producto fermentado con las cepas probióticas *L.gasseri* CECT5714 y *L.coryniformis* CECT5711, mejoró parámetros inmunológicos relacionados tanto con la respuesta innata como con la específica que podrían incidir en una mejor o más eficaz defensa frente a infecciones.

RESEARCH ARTICLE

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The consumption of two new probiotic strains, *Lactobacillus gasseri* CECT 5714 and *Lactobacillus coryniformis* CECT 5711, boosts the immune system of healthy humans

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Summary. Orally ingested probiotic bacteria are able to modulate the immune system. However, differences exist in the immunomodulatory effects of different probiotic strains. Moreover, different regulatory effects, which depend on the health status of the consumer, have been identified. This work describes a randomized, double-blind, placebo-controlled human clinical trial to investigate the immune effects on healthy people of a fermented product containing two new probiotic strains, *Lactobacillus gasseri* CECT 5714 and *Lactobacillus coryniformis* CECT 5711, which was compared with another fermented product, a standard yogurt. Consumption of either the new product or yogurt increased the proportion of phagocytic cells, including monocytes and neutrophils, as well as their phagocytic activity. However, combination of the product containing the strains *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 also induced an increase in the proportion of natural killer (NK) cells and in IgA concentrations. The effects were higher after two weeks of treatment than after 4 weeks, which suggests regulation of the immune system. In addition, the new product enhanced immunity in the participants to a greater extent than did the control standard yogurt. [**Int Microbiol** 2006; 9(1):47-52]

Key words: *Lactobacillus gasseri* · *Lactobacillus coryniformis* · probiotics · human clinical trial · immune response

Introduction

Endogenous microbiota has a modulatory effect on the mucosal and systemic immune response. In fact, the type of bacteria that colonize the intestine in newborns determines immunomodulation of the naive immune system [9]. The usual components of the gut microbiota, such as lactobacilli, modulate the immune system, causing, for instance, host resistance to infections, atopy, and gut inflammation [13,26,30]. While the immune mechanisms of such bacteria, called probiotics, have not been totally defined, several clinical trials have studied the effects of probiotics consumption on human health. Most of these trials evaluated the effect of probiotics on patients suffering from diarrhea, *Helicobacter pylori* infections, intestinal inflammation, or atopy [4,10,14], and the results varied greatly. However, few data are available regarding the immune effects of probiotics in healthy people, the main target of such products currently on the market.

In a previous study, we reported that breast milk of healthy women is a major source of lactic acid bacteria to the infant gut, and that *Lactobacillus gasseri* is among the predominant

species [18]. The microbiota of newborns could be responsible for some of the health benefits observed in breast-fed compared to formula-fed infants [3,5,34]. A breast-milk strain of *L. gasseri* (CECT 5714) was selected based on its particular in vitro probiotic properties [19]. We also selected *L. coryniformis* CECT 5711 from an artisan goat's milk cheese; this strain exhibits peculiar probiotic properties, including the production of reuterin and cobalamin [20]. Also, in a randomized, double-blind, placebo-controlled human clinical trial, we investigated the effects of a fermented product containing the probiotic strains *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 on the intestinal function of healthy adults and compared them with the effects of a standard probiotic with a long consumption history and demonstrated beneficial effects [1,21,25,29]. An increase in the concentration of fecal lactic acid bacteria and a beneficial effect on the bowel function of healthy adults volunteers consuming the new probiotic strain was observed. In this work, we describe the effects of the new probiotic strains on the immune system of healthy adults in that clinical trial.

Material and methods

Design of the trial. Thirty healthy adult human volunteers (15 females and 15 males) ranging in age from 23 to 43 years old were included in the study. Exclusion criteria and the protocol followed was described elsewhere [25].

Collection and analysis of fecal samples. After an overnight fast lasting at least 10 h, blood samples were withdrawn using EDTA-containing vacutainers (S-Monovette, Sarstedt, Germany) from the volunteers just before and after the treatment period. The proportion of neutrophils was analyzed using a Symex F-800 counter (Coulter Electronic, Luton, London, UK). Major leukocyte subset phenotypes were counted in EDTA-treated whole-blood samples by flow cytometry on a FACScalibur (Becton Dickinson, Oxford, UK) system and using the following fluorochrome-conjugated monoclonal antibodies (Becton Dickinson): anti-CD3+, -CD19+, -CD4+, -CD8+, -CD45RO+, -CD56+, -CD25+, -CD14+. The results were expressed as the percentage of positively staining mononuclear cells. Fecal samples were collected weekly, placed into pre-weighed bottles, and then homogenized in a peptone-saline solution (100 mg/ml) within 12 h.

Phagocytic activity. In vitro phagocytic activity was determined by flow cytometry of whole-blood samples after the uptake of fluoresceinated *Escherichia coli* [7]. One hundred ml of heparin-treated whole blood was incubated at 37ºC for 10 min with 10 ml (10^8 colony-forming units, cfu) of fluoresceinated bacteria. Erythrocytes were lysed with 100 ml of 4% formaldehyde and 1 ml of cool water. Samples were centrifuged at 2200 ×*g* for 5 min, suspended in 0.5 ml of 4% (w/v) formaldehyde in PBS, and analyzed by flow cytometry. The results were expressed as the percentages of monocytes and granulocytes showing phagocytic activity.

Total immunoglobulin and cytokines measurements. IgA, IgG, and IgE concentrations in serum and total IgA concentration in feces were measured by ELISA (Bethyl, Montgomery, TX, USA). Cytokine concentrations in sera were measured by ELISA (CytoSets, Biosource, Camarillo, CA, USA).

Statistical analysis. Data were analyzed using SPSS software (version 12.0, Chicago, IL, USA) as described in [25] for the Gaussian variables.

Results

Clinical observations. None of the volunteers voluntarily left the study. Only one had to leave at the end of the pre-test period, due to the use of antibiotics for treatment of an oral bacterial infection not related to this study.

Leukocyte subsets and proportions. Flow cytometric analysis of the proportion of monocytes (CD14+ cells) showed an increase after 2 weeks of consumption of either of the fermented products, but the differences were statistically significant only in the case of the group receiving the new probiotic product. At the end of the treatment, the proportions of monocytes were only slightly higher than the initial values (Table 1). After 2 weeks of treatment, there was also a significant increase in the neutrophil proportion in both groups, but at the end of treatment it was maintained only in the new probiotic group. The proportion of total lymphocytes and cells staining positively for CD3+ (T lymphocytes), CD8+ (cytotoxic T lymphocytes), CD4+ (T-helper lymphocytes), CD19+ (B lymphocytes), CD3+CD45RO+ (memory T lymphocytes), CD4+CD25+ (suppressor T lymphocytes), and CD56+ (natural killer, NK, cells) were in the ranges of those for hematologically normal Caucasian adults (Table 1). However, a significant decrease, more evident at the end of treatment, was detected in the proportions of lymphocyte and of CD3+ and CD19+ cells in both groups. Despite that decrease, we observed that these values had increased during the two-week pretreatment period, probably due to the absence of fermented product in the diet before the treatment, and were normalized after consumption of both fermented products (data not shown). The most outstanding result was the significant increase in the proportion of NK cells after 2 and 4 weeks of treatment in the group that had ingested the new probiotic strains (Fig. 1). In contrast, NK proportions in the control group decreased at the end of treatment and were significantly lower than in the group receiving the new probiotic preparation (Fig. 1). Subjects receiving the latter were ranked and stratified depending on their preintervention levels of NK cells into two groups: a group with high preintervention values (above the mean +10% of the preintervention proportion of NK cells) and a group with medium/low preintervention values (below the mean +10% of the preintervention proportion of NK cells). To study whether the effect of treatment was related to the initial proportion of NK cells, the relative increases in NK-cell proportions were calculated. In the group receiving the new probiotic, changes in NK-cell proportions occurred in volunteers who initially had normal/low values, with percentage increases of 209.49 ± 8.92 and 133.29 ± 12.83, respectively, after 2 and 4 weeks of treatment. By contrast, there was no increase in the proportion of NK cells in subjects who had high initial values.

Table 1. Percentage of white blood cell subsets. Results are expressed as mean ± s.e.m.

	Control group			Probiotic group		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
Monocytes (%)	07.29 ± 0.58	09.03 ± 1.26	08.11 ± 0.94	07.02 ± 0.53	11.00 ± 1.40*	08.45 ± 1.62
Neutrophils (%)	50.55 ± 1.75	57.48 ± 2.81*	54.28 ± 2.06	50.90 ± 1.98	57.34 ± 2.37**	58.66 ± 1.37**
Lymphocytes(%)	45.81 ± 0.93	37.49 ± 2.42*	42.50 ± 2.19	43.97 ± 1.81	37.13 ± 2.61**	38.13 ± 1.33*
T lymphocytes (%)	67.65 ± 2.33	64.95 ± 2.09	61.15 ± 2.87	62.90 ± 2.36	56.48 ± 2.68*	56.49 ± 2.64**
T helper (%)	37.56 ± 1.80	33.14 ± 1.89	36.68 ± 2.72	39.25 ± 1.43	36.75 ± 1.12	39.31 ± 1.62
T cytotoxic (%)	25.21 ± 1.73	25.45 ± 2.02	26.32 ± 2.41	23.74 ± 1.08	22.24 ± 1.40	23.33 ± 0.97
T suppressor (%)	17.52 ± 1.44	19.41 ± 2.09	16.15 ± 0.96	18.42 ± 1.36	18.54 ± 1.81	17.75 ± 1.36
T memory (%)	39.32 ± 2.33	39.28 ± 4.10	36.49 ± 2.82	35.10 ± 2.01	37.31 ± 2.69	32.36 ± 1.91*
B lymphocytes (%)	11.38 ± 1.62	11.01 ± 1.42	09.24 ± 1.70**	09.48 ± 0.72	08.74 ± 0.84	08.23 ± 0.71*

Statistically significant difference with respect to week 0: * $p < 0.05$, ** $p < 0.01$.

Phagocytic activity. The percentages of both mononuclear and polymorphonuclear cells showing phagocytic activity in vitro increased significantly during the period in which the two fermented products were consumed (Fig. 2). Although the increase was higher in the group that had ingested the new probiotic preparation, the differences between the groups were not statistically significant.

Effects on cytokine expression pattern and immunoglobulin production. The cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-12, IL-10 and IL-4 were measured in serum. In the group consuming the new probiotic product, IL-10 and IL-4 increased significantly after 2 weeks of treatment. However, at the end of the treatment period, the IL-4 levels no longer differed from those at the beginning of treatment, while IL-10 levels were higher, but not significantly (Table 2). A significant increase in IgA concentrations was measured in the blood of volunteers who

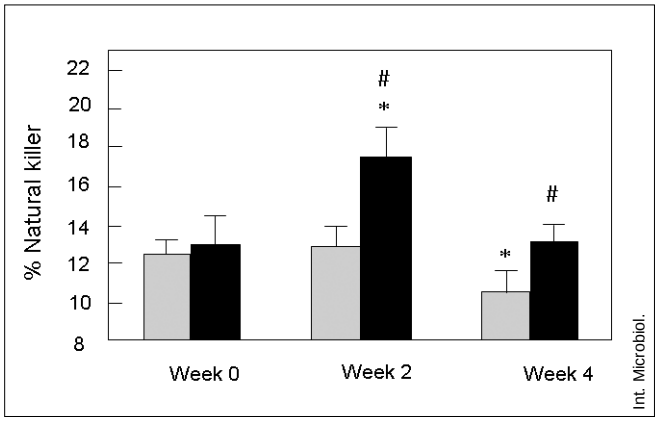

Fig. 1. Mean (±s.e.m.) of the percentage of lymphocytes staining positively for CD56+ (natural killer cells) in the blood of volunteers who received the new probiotic product (black bars) and in the control group (gray bars). * Statistically significant difference (* $p < 0.05$) respect to week 0. # Significant difference ($p < 0.05$) between control and treatment group.

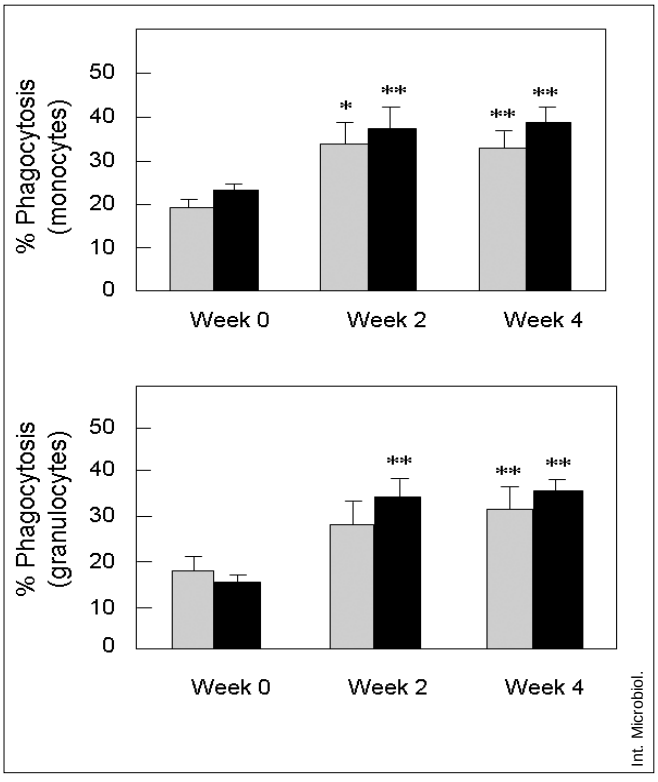

Fig. 2. Monocytes and granulocytes were differentiated by size and complexity by flow cytometer. Phagocytic activity of monocytes (**A**) and granulocytes (**B**) before and after 2 and 4 weeks of treatment is expressed as the mean (± s.e.m.) of the percentage of leukocytes containing fluoresceinated *Escherichia coli* after in vitro incubation of the bacteria with fresh blood. Black bars, probiotic group; gray bars, control group. *, ** Statistically significant difference (* $p < 0.05$, ** $p < 0.01$) respect to week 0.

Table 2. Cytokine concentrations in blood. Results are expressed as pg (mean ± s.e.m.) per ml of serum

	Control group			Probiotic group		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
TNF-α	38.49 ± 13.25	36.14 ± 13.14	37.49 ± 15.69	38.37 ± 9.24	46.57 ± 11.16	35.74 ± 11.54
IL-10	56.38 ± 27.03	60.71 ± 28.69	72.24 ± 35.68	64.27 ± 31.75	84.62 ± 39.29*	81.80 ± 54.25
IL-12	21.41 ± 5.57	23.17 ± 5.90	33.06 ± 9.40	21.43 ± 5.25	23.06 ± 5.78	22.03 ± 6.44
IL-4	30.96 ± 9.39	43.79 ± 17.52	47.27 ± 15.79	28.26 ± 9.93	37.96 ± 10.22*	25.7 ± 15.36

TNF, Tumor necrosis factor; IL, interleukin. Statistically significant difference with respect to week 0: * $p < 0.05$.

were given the new probiotic product (Table 3). In feces, there was a slight increase in IgA levels, but it was not significant. Serum IgG levels remained stable in both groups throughout the study. In addition, after 2 weeks there was a significant decrease in the serum IgE levels of volunteers in the new-probiotic group and in the control group; however, at the end of treatment the decreases were not significant (Table 2).

Discussion

Modulation of the immune response is one of the most acclaimed health benefits attributed to probiotic strains [6]. Previous studies in humans reported the effects of oral administration of probiotic strains on innate and specific immunity [2,31,32,37]. Although studies of the mechanisms by which probiotics exert this immunomodulatory effect are still in progress, recent data have shown differences in the immunomodulatory effects of different probiotic strains. Moreover, different regulatory effects have been detected in healthy subjects and in patients with inflammatory diseases [12]. These results suggest that the specific immunomodulatory properties of probiotic bacteria should be specifically characterized on target populations.

Our results showed that the consumption of either the new probiotic product or a standard yogurt preparation resulted in an increase in phagocytic cells, including monocytes and neutrophils, and enhanced phagocytic activity. These results are consistent with those of previous studies on other probiotic strains [31,32]. Although the new probiotic strains seemed to be more effective, consumption of the standard yogurt produced similar results. In fact, the effect of *L. delbrueckii* subsp. *bulgaricus* on monocytes and macrophages function was previously reported [29].

Monocytes and macrophages, together with dendritic cells, play a crucial role in the innate immune response against microbial antigens, which in turn leads to activation of the adaptive immune system [15]. These cells recognize conserved molecular patterns of bacterial components through toll-like receptors (TLR), leading to activation of a variety of transcription factors, which triggers cytokine production [36]. The ability of these cells to recognize conserved structures present in large groups of bacteria enables them to effectively detect a wide range of pathogens, and this response is strengthened even when the increase in the phagocytic function is induced by non-pathogen bacteria, such as those in probiotic preparations. NK cells are also involved in the innate response and play a major role in recognizing and killing both virus-infected cells and tumor cells. In our study, the percentage of NK cells increased in the new-probiotic group but decreased in the control group. Moreover, the increase in the proportion of NK cells was restricted to volunteers in the new-probiotic group that initially had normal/low numbers of NK cells, while there was no change in subjects with initially higher NK-cell counts. In elderly people, in individuals with poor lifestyle habits, such as

Table 3. Immunoglobulin concentrations in serum. Results are expressed as mean ± s.e.m.

	Control group			Probiotic group		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
IgA(mg/g feces)	200.94 ± 37.92	225.13 ± 73.20	214.66 ± 42.49	181.21 ± 37.33	214.95 ± 45.11	248.36 ± 55.91
IgA(mg/dl serum)	139.39 ± 16.98	151.89 ± 30.32	126.51 ± 23.23	137.08 ± 20.37	158.12 ± 19.51	159.29 ± 23.00*
IgG(mg/dl serum)	1045.5 ± 169.0	1317.1 ± 154.4	1141.0 ± 118.8	1144.1 ± 203.6	1303.2 ± 277.5	1114.5 ± 169.6
IgE(mg/dl serum)	190.40 ± 39.26	161.48 ± 35.25*	195.33 ± 52.95	192.58 ± 48.07	147.64 ± 39.61**	169.53 ± 39.21

Statistically significant difference with respect to week 0: * $p < 0.05$, ** $p < 0.01$.

smoking, or in those with mental stress, a decrease in the proportions of NK cells has been reported [11,24,33]. Since probiotics seem to offer benefits to people whose health could be improved, they could also be effective in people with typically low levels of NK function. The positive effect of probiotics on NK cells has been previously reported for other lactobacillus strains [8,23], and their stimulation of the immune system and antitumor effects in animal models of cancer have been reported [17,35]. The lack of an increase in the proportion of NK cells in the control group may have been due to a lower adherence of the starter strain, *L. delbrueckii* subsp. *bulgaricus*, to both the gut mucosa and Peyer's patches, which would have made contact with immune cells difficult [27]. In fact, we did not detect an increase in fecal lactic acid bacteria in the control group [25]. Therefore, the consumption of the new probiotic product might have increased the innate immune response. Innate immunity is activated very quickly after infection when acquired immunity has not yet developed. Thus the improvement of innate immunity would strengthen the response against possible infections or cell transformations. The effects were in generally higher after 2 weeks of treatment than after 4 weeks, suggesting that they involved regulation of the immune system. The effect of treatment on the response capability against stimuli occurring weeks after the end of the treatment finished is currently being investigated.

The level in serum of the main cytokines involved in the regulation of the immune response were also analyzed in order to determine whether the acquired immune response was also affected. The increase in IL-10 and IL-4 concentrations suggests a role for a Th2 response. However, the serum IgE concentration was lower in the new-probiotic group than in controls, which suggests that other cytokines should be involved in IgE switching. IgE is involved in the allergy processes, so a decrease in the levels of this immunoglobulin could be beneficial for allergic subjects. In fact, the beneficial effect of a strain of *L. rhamnosus* in the prevention of atopy has been reported [14,15].

The increase in Th2 cytokines, including IL-4, could also affect IgA switching. In fact, a significant increase in the total IgA concentration in serum was detected, but only in the group that received the new probiotic product. IgA is the main immunoglobulin involved in mucosal defense. Thus, an increase in its concentration enhances its protection against pathogens. It has been reported that increases in IgA are related to the anti-infectious properties of probiotics in the treatment of diarrheal disease [37,38].

Although both probiotic products—the new preparation and the control yogurt—induced immune effects, consumption of the former, containing two lactobacilli strains, *L. gasseri* (CECT 5714) and *L. coryniformis* (CECT 5711), enhanced immunity in healthy people to a greater extent than the standard yogurt consumption.

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El consumo de dos nuevas cepas probióticas, *Lactobacillus gasseri* CECT 5714 y *Lactobacillus coryniformis* CECT 5711, estimula el sistema inmunitario de individuos sanos

Resumen. La ingestión oral de bacterias probióticas puede modular el sistema inmunitario. Sin embargo, existen diferencias en los efectos inmunomoduladores de diferentes cepas probióticas. Además, se han identificado distintos efectos reguladores, que dependen del estado de salud del consumidor. Este trabajo describe un ensayo clínico aleatorizado, con ocultación doble (*double blind*) y con control de placebo llevado a cabo en humanos para estudiar los efectos inmunitarios del consumo de un producto fermentado que contiene dos nuevas cepas probióticas, *Lactobacillus gasseri* CECT 5714 y *Lactobacillus coryniformis* CECT 5711, comparados con los efectos producidos por otro producto fermentado, un yogurt clásico. El consumo del nuevo producto o del yogurt clásico aumentó la proporción de células fagocitarias, como monocitos y neutrófilos, así como su actividad fagocitaria. Sin embargo, la combinación del producto que contenía las cepas *L. gasseri* CECT 5714 y *L. coryniformis* CECT 5711 indujo además un aumento en la proporción de linfocitos citolíticos natuales (células NK) y en las concentraciones de IgA. Estos efectos fueron más acusados después de dos semanas de tratamiento que después de 4 semanas, lo que sugiere una regulación del sistema inmunitario. Además, el nuevo producto reforzó la inmunidad en las personas que participaron en el ensayo más que el yogur clásico usado como control. [**Int Microbiol** 2006; 9(1):47-52]

Palabras clave: *Lactobacillus gasseri* · *Lactobacillus coryniformis* · probióticos · ensayo clínico en humanos · respuesta inmunitaria

O consumo de duas novas cepas probióticas, *Lactobacillus gasseri* CECT 5714 e *Lactobacillus coryniformis* CECT 5711, estimula o sistema imunitário de indivíduos sãos

Resumo. A ingestão oral de bactérias probióticas pode modular o sistema imunitário. No entanto, existem diferenças nos efeitos imunomoduladores de diferentes cepas probióticas. Além disto, foram detectados diferentes efeitos reguladores dependentes do estado de saúde do consumidor. Este trabalho descreve um ensaio clínico aleatorizado, com ocultação dupla (*double blind*) e com controle de placebo levado a cabo em humanos para estudar os efeitos imunitários do consumo de um produto fermentado que contém duas novas cepas probióticas, *Lactobacillus gasseri* CECT 5714 e *Lactobacillus coryniformis* CECT 5711, comparados com os efeitos produzidos por outro produto fermentado, um iogurte clássico. O consumo tanto do novo produto como do iogurte clássico aumentou a proporção de células fagocitárias, como monócitos e neutrófilos, assim como sua atividade fagocitária. No entanto, a combinação do produto que continha as cepas *L. gasseri* CECT 5714 e *L. coryniformis* CECT 5711 induziu também um aumento na proporção de linfócitos citolíticos natuales (células NK) e nas concentrações de IgA. Estes efeitos foram mais aguçados após duas semanas de tratamento do que após 4 semanas, o que sugere uma regulação do sistema imunitário. Além disto, o novo produto reforçou de forma mais acentuada a imunidade das pessoas que participaram do ensaio do que o iogurte clássico usado como controle. [**Int Microbiol** 2006; 9(1):47-52]

Palavras chave: *Lactobacillus gasseri* · *Lactobacillus coryniformis* · probióticos · ensaio clínico em humanos · resposta imunitária

7. La restricción de productos fermentados en la dieta causa una caída en la respuesta inmunitaria innata. Las bacterias ácido lácticas pueden contrarrestar los efectos inmunológicos de esta restricción

RESUMEN

OBJETIVO

La microbiota intestinal se encuentra modulada por factores extrínsecos tales como la microbiota materna, la carga bacteriana del ambiente, la dieta y la medicación, influyendo su estabilidad sobre la maduración y la función del sistema inmunitario. En este trabajo, describimos los efectos inmunitarios y sobre la microbiota que supone un periodo de restricción en la dieta de alimentos fermentados en voluntarios sanos y, cómo se contrarrestan estos efectos con la ingesta de un producto fermentado con dos cepas probióticas, *L.gasseri* CECT5714 y *L.coryniformis* CECT571.

RESULTADOS

Se realizó un estudio clínico aleatorio, doble-ciego y comparado con un placebo, que involucró a 30 adultos sanos, con el propósito de investigar los efectos, en diversos parámetros sanguíneos y fecales relacionados con la función intestinal y del sistema inmunitario. El estudio constó de dos fases. Una primera fase de dos semanas de duración, en la cual todos los voluntarios mantuvieron una dieta restringida en el consumo de alimentos fermentados. Y una segunda fase, en la cual los voluntarios fueron asignados aleatoriamente en dos grupos, uno que tomó un yogurt estándar y otro que tomó un producto fermentado similar en el cual el *Lactobacillus delbreuckii* subsp. *bulgaricus* del yogurt fue substituido por una combinación de las cepas probióticas, *L. gasseri* CECT5714 y *L. coryniformis* CECT5711.

Tras dos semanas de restricción de dieta se observó una disminución significativa en los recuentos de lactobacilos, aerobios totales y en la concentración de ácidos grasos de cadena corta así como una disminución en la actividad fagocítica de los leucocitos. Por lo tanto, la restricción de productos fermentados podría inducir a una disminución en la respuesta inmunitaria innata que, afectaría a la capacidad de respuesta frente a infecciones.

La introducción en la dieta de ambos productos fermentados contrarrestó en gran medida los efectos negativos observados en el periodo de restricción, tanto a

nivel intestinal como del sistema inmunitario, siendo el efecto más potente en el caso del producto probiótico.

CONCLUSIÓN

La eliminación de productos fermentados en la dieta conlleva, una disminución en la respuesta inmunitaria que podría afectar a la capacidad de respuesta frente a infecciones. La administración de un producto fermentado con, *L. gasseri* CECT5714 y *L. coryniformis* CECT5711, es capaz de contrarrestar dicho efecto por lo que se muestran como una alternativa eficaz en los casos de dietas pobres en alimentos fermentados.

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Dietary deprivation of fermented foods causes a fall in innate immune response. Lactic acid bacteria can counteract the immunological effect of this deprivation

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Extrinsic factors such as maternal microbiota, bacterial load of the environment, diet and medication modulate the intestinal microbiota. Maturation and function of the immune system is influenced by established gut microbiota. In this work we describe the immunological effects of the dietary deprivation of fermented foods of healthy volunteers. Significant decreases in faecal lactobacillus and total aerobes counts and concentration of short chain fatty acids were observed following deprivation of fermented food of the normal diet. Moreover, a decrease in phagocytic activity in leukocytes was observed after two weeks of restricted diet. Therefore, the dietary deprivation of fermented foods could induce a decrease in innate immune response that might affect the capacity to respond against infections. The ingestion of a probiotic product containing the strains *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711 or a standard yogurt containing a conventional starter *Lactobacillus delbrueckii* sp. *bulgaricus* counteracted the fall in the immune response, although the probiotic product was more effective than the standard yogurt.

Keywords: Fermented food, probiotics, immune response.

The microbiota of the human intestine influences health and well-being. The list of beneficial functions attributed to intestinal bacteria is large and includes defence against pathogen infections, nutrient processing, the fine tuning and maturation of immune responses, etc. (Erickson & Hubbard, 2000; Isolauri et al. 2001; Fanaro et al. 2003; Grönlund et al. 2000). The establishment of the gut microbiota begins after birth and is a complex process influenced by microbial – host interactions, and by external and internal factors. Human milk is a major factor in the initiation and development of neonatal gut microbiota because it constitutes a continuous source of microorganisms to the infant gut during the first weeks after birth (Martín et al. 2003). Moreover, it also contains prebiotic substances, which selectively stimulate the growth of bacteria (Drasar & Roberts, 1990; Dai & Walker, 1999). After weaning, a bacterial community resembling the adult flora become established (at 2 years of age). Essential extrinsic modulating factors of microbiota include the bacterial load of the environment, the composition of the maternal microbiota, diet and medication (Fanaro et al. 2003).

Fermentation of foods has been in use for thousands of years for the preservation and improvement of a range of foods, unfortunately the consumption of fermented food is decreasing in the western diet. However, although this kind of food constitutes an important bacterial source, no studies have been carried out in order to investigate the effect of this lack in western diet. In recent years, the use of probiotic strains (particularly lactobacilli and bifidobacteria) has been encouraged as a mean to balance the gut microbiota and, in fact, their potential preventive and therapeutic effects have received a renewed research and industrial interest (Salminen et al. 1998; Ouwehand et al. 1999; Saavedra, 2001).

In this article we analysed the effect on microbiota and immune system during fermented food deprivation of healthy volunteers and the ability of a fermented product containing two probiotic strains, *Lactobacillus gasseri* CECT5714 isolated from breast milk (Martín et al. 2005a) and *Lactobacillus coryniformis* CECT5711 isolated from an

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artisan goat-cheese (Martín et al. 2005b) to counteract the effects of the restricted diet.

Material and Methods

Design of the trial

A total of 30 healthy adult human volunteers (15 females and 15 males) with an age ranging from 23 to 43 years were included in the study. Volunteers included in the study were regular consumers of fermented foods, consuming fermented milk products (yogurt and/or cheese) at least in an amount of 5 portions per week and other kinds of fermented food at least 3 portions per week. Exclusion criteria were lactose intolerance, recent antibiotic treatment, frequent gastrointestinal disorders or metabolic diseases. The study was carried out according to the Helsinki declaration and the protocol was approved by the ethical committee of Puleva Biotech SA and informed written consent was obtained from all the subjects. The study consisted of two phases: a restricted diet period (2 weeks from day –14 to day 0) followed by a period of restricted diet supplemented with a specific fermented product (2 weeks from day 0 to day +14). The volunteers were asked to exclude from their diet any kind of fermented food or drink, such as fermented milk and dairy products including cheese, fermented meat, and fermented beverages like wine, beer or vinegar, and also any other kind of fermented food product such as cured olives. The only exception was bread. Volunteers were allowed to consume only the specific fermented products supplied as the object of this study and then only during the period corresponding to the second phase, while maintaining all the other diet restrictions. Volunteer's diet was controlled by daily surveys.

After two weeks of restricted diet volunteers were randomly distributed into two groups. Those belonging to the 'yogurt' group used as a control received, on a daily basis, 200 ml of a yogurt elaborated with a standard yogurt starter provided by Puleva Food (Granada, Spain) containing 10^8 cfu *Streptococcus thermophilus* and 4×10^9 cfu *Lactobacillus delbruekii* sp. *bulgaricus*. Those of the 'probiotic' group ingested, with the same frequency, 200 ml of a similar dairy product containing the same concentration of the starter *Str. thermophilus* but in which the *Lb. bulgaricus* starter strain had been replaced by approximately 2×10^9 cfu of each of the probiotic strains *Lb. coryniformis* CECT5711 and *Lb. gasseri* CECT5714.

Collection of faecal samples and counts of fecal bacterial groups

Once a week, fresh faecal samples were collected and individually homogenized in a peptone-saline solution (100 mg/ml). To estimate the concentration of bacterial groups, appropriate dilutions were spread in quadruplicate onto plates of MRS agar for lactic acid bacteria, MRS agar supplemented with 0·5 mg dicloxacilin/l, 1 g LiCl/l and 0·5 g L-cysteine hydrochloride/l for bifidobacterium and Reinforced Clostridial Agar containing 20 μg polymixin/ml for Clostridium. All media were obtained from Oxoid (Basingstoke, UK) whereas antibiotics and other supplements were obtained from Sigma Chemical Co. (St Louis, MO). Culture plates were incubated in absence of oxygen at 37 °C for 24 to 48 h. Similarly, 1 ml of suitable dilution was spread onto specific Count Plates Petrifilm (3 M St Paul, MN) for total aerobes and for Enterobacteriaceae. Plates were incubated at 37 °C for 24 h. After the incubation, the specific colonies grown on the selective culture media were counted and the number of viable microorganism per gram of faeces (cfu/g) was calculated. The mean and standard error per group were calculated from the log values of the cfu/g.

Quantification of short chain fatty acids in the faecal samples

The concentration of short chain fatty acids (SCFA) in the faecal samples was quantified similarly to the method described by Rodríguez-Cabezas et al. (2002). Faecal samples were homogenized with 150 mM-$NaHCO_3$ (pH 7·8) (1 : 5, wt/v) in an argon atmosphere. Samples were incubated for 24 h at 37 °C and stored at –80 °C until extraction. To extract the SCFA, 50 μl 100 mM-2-methylvaleric acid (internal standard), 10 μl sulphuric acid and 0·3 ml ethyl acetate were added to 1 ml of the homogenate. The mix was centrifuged at $10\,000 \times \boldsymbol{g}$ for 5 min at 4 °C. The supernatants were dehydrated with sodium sulphate (anhydrous) and re-centrifuged. Then, the sample (0·5 ml) was injected (splitless) into a gas chromatograph (CP-3800, Varian, Lake Forest, CA) equipped with an ID (CPWAX 52CB 60 m × 0·25 mm), and connected to a FID detector (Varian). Helium was used as the carrier and the make-up gas, with a flow rate of 1·5 ml/min. The injection temperature was 250 °C. Acetate, propionate and butyrate concentrations were automatically calculated from the areas of the resulting peaks using the Star Chromatography WorkStation program (version 5.5), which was connected on-line to the FID detector.

Collection of blood samples

After an overnight fast lasting at least 10 h, blood samples were taken from the volunteers at the beginning of the trial (Day –14); before probiotic supplementation (Day 0) and at the end of the trial (Day +14) just using EDTA-containing vacutainers (S-Monovette, Sarstedt, Gemany). Samples were individually analysed. An automatic cell counter system, SYMEX F-800 (Symex, Nashua, NH), was used to analyse neutrophils proportion. Major leukocyte subset phenotypes were counted in EDTA-treated whole-blood samples *via* flow cytometry on a FACScalibur (Becton Dickinson, Oxford, United Kingdom) by using

Table 1. Effect of deprivation on bacterial groups in faeces. Log numbers of bacteria per gram of faeces are expressed

Values are means ± SEM for $n=30$

	Day −14	Day 0
Lactobacilli	8·13 ± 0·18	6·89 ± 0·14*
Bifidobacterium	8·99 ± 0·12	8·70 ± 0·13
Enterobacteriaceae	6·90 ± 0·18	6·96 ± 0·18
Clostridium	9·22 ± 0·15	9·20 ± 0·11
Total aerobes	7·41 ± 0·16	6·00 ± 0·19*

* Statistically significant difference ($P<0·01$) respect to Day −14

the following fluorochrome-conjugated monoclonal antibodies (Becton Dickinson): anti-CD3+, -CD19+, -CD4+, -CD8+, -CD45RO+, -CD56+, -CD25+, -CD14+. The results were expressed as the percentage of mononuclear cells that stained positively.

Phagocytic activity

In vitro phagocytic activity was determined by flow cytometry in whole-blood samples after the uptake of fluoresceinated *Escherichia coli* (Gill et al. 2000). Basically 100 µl heparin treated whole-blood were incubated for 10 min at 37 °C with 10 ml (10^8 cfu) fluoresceinated bacteria. Erythrocytes were lysed with 100 µl 40 g formaldehyde/l and 1 ml cool water. Samples were centrifuged to 2200 × ***g*** for 5 min and suspended in 0·5 ml 40 g formaldehyde/l PBS. Samples were analysed by flow cytometry and the results were expressed as the percentage of monocytes and granulocytes showing phagocytic activity.

Total immunoglobulin and cytokines measurements

Total IgA, IgG and IgE concentrations in sera and total IgA concentration in faeces were measured by ELISA quantitation kits (BETHYL, Montgomery, TX). Cytokines concentrations in sera were measured by ELISA quantitation kits (CytoSets, BIOSOURCE, Camarillo, CA) following manufacturer's recommendations in both cases.

Statistical analysis

The data were analysed using SPSS software (version 12.0, Chicago, USA). Data are expressed as means ± SEM. P values <0·05 were considered significant.

For Gaussian variables, the longitudinal effect of each yogurt within each group at the different time points of the study was analysed by one-way repeated measures ANOVA followed by paired t test (within-group comparison). Two-way repeated measured ANOVA was used to analyse statistical differences produced by the consumption of each fermented product followed by independent t test to asses in which time-points the groups differed.

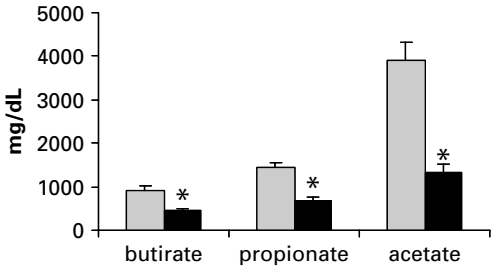

Fig. 1. SCFA concentrations (butyrate, propionate and acetate) after 24 h fermentation of the faecal samples are represented as means (± SEM) of the g/l at the beginning of the study (grey bars) and after two weeks of restricted diet (black bars). * Significant difference, $P<0·01$, between the two time points.

Results

Clinical status during the study

Throughout the study, none of the volunteers reported any adverse effect associated with the diet or the consumption of the dairy fermented products. Clinical examination by the medical staff of Puleva Biotech revealed that the health condition of all the participants was perfect during the study.

Effects of the fermented foods deprivation in diet – phase 1

Bacterial groups and quantification of SCFA in faecal samples. After two weeks of restricted diet a significant decrease in the number of faecal lactic acid bacteria was detected (Table 1). Changes in other bacterial groups such as bifidobacterium, enterobacteriaceae and clostridium could not be detected and only a significant decrease was observed in aerobes counts. With regards to SCFA a significant decrease was observed in butyric acid, propionic acid and acetic acid at the end of the deprivation phase (Fig. 1).

Effects on immune response. The phagocytic activity of granulocytes decreased significantly after the two week deprivation phase (Fig. 2). The proportion of neutrophiles was measured but no changes were observed (Table 2). In the case of monocytes the flow cytometric analysis showed a significant increase of CD14+ cells proportions (Table 2) but the phagocytic activity of these cells tended to decrease ($P=0·08$; Fig. 2).

Flow cytometric analysis of lymphocyte subsets was also performed. The proportion of cells staining positive for CD3+ (T lymphocytes), CD8+ (cytotoxic T lymphocytes), CD4+ (T helper lymphocytes), CD19+ (B lymphocytes), CD3+CD45RO+ (memory T lymphocytes), CD4+CD25+ (suppressor T lymphocytes), and CD56+ (Natural Killer cells) were in the ranges for haematologically normal Caucasian adults (Table 2). A significant increase was detected in T and B lymphocytes proportions. The T helper

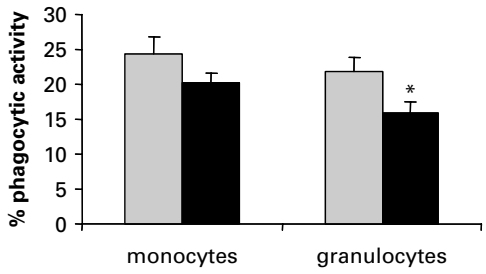

Fig. 2. Phagocytic activity of monocytes and granulocytes at the beginning of the study (grey bars) and after two weeks of restricted diet (black bars) is expressed as the mean (±SEM) of the percentage of cells containing fluoresceinated *Esch. coli* after in vitro incubation of the bacteria with fresh blood. * Statistically significant difference between the two time points $P<0{\cdot}01$.

lymphocytes increased during this period and, in contrast, a significant reduction in T memory lymphocytes was detected.

No significant changes were detected in cytokines and immunoglobulins concentrations during this period (Table 3). In faeces, however, a decrease was observed of approximately 20% in IgA concentration but differences were not statistically significant due to the variability of the samples.

Effects of the introduction of lactobacilli on diet-modified parameters – phase 2

Bacterial groups and quantification of SCFA in faecal samples. The consumption of the probiotic product caused a significant increase in faecal lactobacilli counts although these did not reach the initial values (Table 4). In contrast, the lactobacilli strains included in standard yogurt were not able to compensate for the lack of lactobacilli consumption during the period of restricted diet. The counts continued to fall during the period of supplemented diet. In the case of total aerobes, the initial values were recuperated after supplemented with both fermented products (Table 4). Regarding SCFA, the consumption of the standard yogurt did not arrest the decrease in concentrations that continued significantly during the period of supplementation. However, the probiotic product maintained the SCFA concentrations in faecal samples at the level reached at the end of phase 1 (Table 4).

Effects in immune response. With respect to immunological parameters, after consumption of both fermented products, a significant increase in phagocytic activity was observed that even overtook the values before the restricted diet began. The proportion of Natural Killer cells was not significantly modified during the two weeks of the restricted diet period, nor during the following two weeks of yoghurt consumption. However,

Table 2. Effects of the deprivation on % of white blood cells subsets and % of lymphocytes subsets

Results are expressed as mean ± SEM for $n=30$

	Day −14	Day 0
Monocytes	6·11 ± 0·35	7·10 ± 0·43*
Neutrophiles	51·97 ± 1·06	50·73 ± 1·30
Natural killer	13·61 ± 0·90	12·58 ± 0·87
T lymphocytes	59·15 ± 1·94	65·21 ± 1·66**
T helper	34·61 ± 1·54	38·98 ± 1·23*
T cytotoxic	23·55 ± 1·31	24·29 ± 1·07
T suppressor	17·82 ± 0·93	18·08 ± 0·94
T memory	43·01 ± 2·18	37·19 ± 1·57**
B lymphocytes	8·62 ± 0·84	10·40 ± 0·89**

Statistically significant difference respect to Day −14: *($P<0{\cdot}05$), **($P<0{\cdot}01$)

Table 3. Cytokines and immunoglobulins in serum or faeces

Results are expressed as mean ± SEM for $n=30$

	Day −14	Day 0
TNF (pg/ml)	38·49 ± 8·88	39·82 ± 7·70
IL-10 (pg/ml)	70·99 ± 24·2	62·70 ± 21·4
IL-12 (pg/ml)	24·87 ± 4·93	21·42 ± 3·75
IL-4 (pg/ml)	35·92 ± 7·64	30·52 ± 6·97
IgA (mg/g faeces)	237·53 ± 38·84	190·73 ± 26·19
IgA (mg/dL)	153·13 ± 15·37	143·17 ± 12·45
IgG (mg/dL)	1146·20 ± 121·6	1054·50 ± 128·3
IgE (mg/dL)	202·41 ± 40·52	191·53 ± 30·71

the consumption of the probiotic product increased the values above those obtained at the beginning of the clinical trial (Table 5). Regarding the proportions of lymphocytes subsets after the division into two groups of supplemented diet (yogurt and probiotic), the statistically significant differences were absent in some cases but in general the initial values were recovered after consumption of fermented products (Table 5).

Discussion

The effect of the diet on gut microbiota has been broadly demonstrated (Spanhaak et al. 1998; de Champs et al. 2003; Schultz et al. 2004; Valeur et al. 2004). In this work we have induced a significant decrease in lactobacillus counts by depriving fermented products from the normal diet. Intestinal colonization by lactobacilli is suggested to be a prerequisite to normal mucosa immune functions. In fact an inadequate level of lactobacilli may be involved in allergic diseases (Kalliomaki & Isolari, 2003; Savino et al. 2005). Thus, changes in lactobacillus populations in the gut could have consequences for the immune system. Microbial succession is an ongoing process influenced by numerous external and internal host-related factors. Some bacterial populations do not colonize the gastrointestinal tract permanently and need a periodic reintroduction of

Table 4. Fecal parameters. Results are expressed as mean ± SEM for $n=29$

	Yogurt group			Probiotic group		
	Day −14	Day 0	Day +14	Day −14	Day 0	Day +14
Lactobacilli (logCFU/g)	7·99 ± 0·27	6·82 ± 0·23*	6·70 ± 0·27*	8·33 ± 0·20	6·97 ± 0·15*	7·50 ± 0·17*#‡
Aerobes (logCFU/g)	7·41 ± 0·23	6·02 ± 0·29*	8·05 ± 0·13#	7·42 ± 0·19	5·98 ± 0·20*	7·80 ± 0·23#
Propionate (g/dl)	1·235 ± 0·15	0·632 ± 0·07*	0·351 ± 0·05*#	1·616 ± 0·18	0·740 ± 0·09*	0·681 ± 0·08*#‡
Butyrate (g/dl)	0·747 ± 0·10	0·463 ± 0·08*	0·221 ± 0·04*#	1·036 ± 0·01	0·431 ± 0·05*	0·505 ± 0·08*#‡
Acetate (g/dl)	3·525 ± 0·37	1·368 ± 0·23*	0·620 ± 0·10*#	4·267 ± 0·73	1·322 ± 0·22*	1·284 ± 0·20*#‡

* Statistically significant differences with respect to day −14 ($P<0·05$)
\# Statistically significant differences with respect to day 0 ($P<0·05$)
‡ Statistically significant differences between groups ($P<0·05$)

Table 5. Blood parameters. Results are expressed as mean ± SEM for $n=29$

	Yogurt group			Probiotic group		
	Day −14	Day 0	Day +14	Day −14	Day 0	Day +14
Monocytes (%)	6·24 ± 0·49	7·29 ± 0·58*	9·03 ± 1·26	5·48 ± 0·44	7·02 ± 0·53*	11·00 ± 1·40*#
Neutrophils (%)	51·44 ± 1·23	50·55 ± 1·75	57·48 ± 2·81*#	52·46 ± 1·73	50·90 ± 1·98	57·34 ± 2·37*#
Phagocytosis (%)						
monocytes	22·78 ± 2·47	19·57 ± 1·31*	33·71 ± 4·87*#	26·08 ± 4·06	22·44 ± 2·32*	37·39 ± 4·12*#
granulocytes	21·56 ± 3·06	18·19 ± 2·20*	27·39 ± 5·71*	22·24 ± 2·90	14·37 ± 2·03*	33·52 ± 4·71*#
Natural killer (%)	13·21 ± 1·10	12·22 ± 0·93	12·73 ± 1·07	14·29 ± 1·37	12·91 ± 1·41	17·29 ± 1·69*‡
T lymphocytes (%)	60·78 ± 3·04	67·65 ± 2·33	64·95 ± 2·09	57·63 ± 2·49	62·90 ± 2·36*	56·48 ± 2·68
T helper (%)	34·00 ± 2·03	37·56 ± 1·80	33·14 ± 1·89	35·21 ± 2·32	39·25 ± 1·43	36·75 ± 1·12
T cytotoxic (%)	24·70 ± 2·10	25·21 ± 1·73	25·45 ± 2·02	22·39 ± 1·53	23·74 ± 1·08	22·24 ± 1·40
T suppressor (%)	17·79 ± 1·17	17·52 ± 1·44	19·41 ± 2·09	17·85 ± 1·48	18·42 ± 1·36	18·54 ± 1·81
T memory (%)	45·26 ± 2·72	39·32 ± 2·33*	39·28 ± 4·10*	40·92 ± 3·36	35·10 ± 2·01	37·31 ± 2·69
B lymphocytes (%)	8·93 ± 1·55	11·38 ± 1·62*	11·01 ± 1·42*	8·32 ± 0·78	9·48 ± 0·70	8·74 ± 0·84

* Statistically significant differences with respect to day −14 ($P<0·05$)
\# Statistically significant differences with respect to day 0 ($P<0·05$)
‡ Statistically significant differences between groups ($P<0·05$)

bacteria by repeated oral doses or by other means. Thus, the dietary deprivation of fermented food caused the decrease observed in this bacterial population. Moreover, a decrease in SCFA faecal concentration was detected suggesting the modification in the metabolism of intestinal microbiota. SCFA, mainly butyrate, affect differentiation, maturation and function of immune cells influencing the immune response (Millard et al. 2002; Rinne et al. 2005; van Nuenen et al. 2005). We have studied how the lack in fermented foods affects the immune response. We observed that, although a slight increase was detected in monocytes proportions, the phagocytic activity of these cells was lower. Normal flora is the main bacterial stimulus of the immune system thus, the absence of stimulus by lactobacillus groups seems to induce a loss in response capabilities (Doyle et al. 2004). Toll Like Receptor (TLR) activation by bacterial components triggers the rapid differentiation of monocytes into macrophages and dendritic cells and mediates the apoptosis mechanism (Kirschnek et al. 2005; Krutzik et al. 2005). The lack of this activation could explain the observed decrease in phagocytic activity, although the proportion of monocytes increased. Monocytes and macrophages, together with dendritic cells, play a crucial role in the innate immune response against microbial antigens which precedes the development of the acquired response (Karlsson et al. 2002). Natural Killer cells were also slightly affected by the absence of lactobacillus stimulus. Natural Killer cells play an important role in recognizing and killing of virus-infected cells and tumour cells. Therefore, the deprivation of fermented foods could induce a decrease in innate immune response that might affect the capability of the organism in the defence against infections or cell transformations.

Finally probiotic, but not standard yogurt, was able to increase the faecal lactobacilli counts and counteract the decrease in SCFA concentration, however the values did not reach the initial values. The capacity for SCFA production depends on the fermentative properties of each strain. Since the probiotic supplementation did not totally recover the pre-trial SCFA concentrations, it seems that other commercial starter strains and also wild-type lactic acid bacteria, which originate from the raw material or the environment, contained in other fermented products such as cheese or fermented meat contribute in an important

way to the fermentative metabolism in gut. The lack of effect on the decreased SCFA in the yogurt group can be explained because this product was not able to compensate for the decrease in faecal lactobacilli. While the strains contained in the probiotic product have been demonstrated to survive the gastro-intestinal tract conditions (Olivares et al. 2006), the reported data about the survival of *Lb. delbruekki* sp. *bulgaricus* contained in standard yogurt are contradictory (del Campo et al. 2005; Mater et al. 2005).

Regarding the immune parameters, it was observed that the consumption of both fermented products induced the recovery of the phagocytic activity, even to a level that improved on the values at the beginning of the study. The fact that both fermented products induced similar results suggest that the viability of the lactobacillus strains is not essential to induce this activation probably because the interaction of the leukocytes with membrane components, which are also present in dead bacteria. However, in the case of Natural Killer cells, the yogurt strain did not modify their numbers, while the lactobacillus strains of the probiotic product boosted the proportion of these cells above those observed at the beginning of the study. These differences in the activation of Natural Killer cells proliferation could be dependent on the viability of the bacteria or of the intrinsic bacteria properties.

Dietary effects on acquired immune response were less evident. The dietary deprivation of fermented foods caused a slight increase in certain lymphocyte subsets, although in general, the initial levels of lymphocytes subsets proportions were recovered after consumption of both fermented products. In this sense it is known that probiotic bacteria can mediate suppression of lymphocyte proliferation (Isolauri et al. 2001). Thus the lack of suppressor effect by the decrease in lactobacillus load could lead to the increase in lymphocyte proliferation which is compensated by the administration of lactobacillus strains.

In conclusion, the dietary deprivation of fermented foods modified the gut microbiota and caused a decrease in the immune response mainly affecting the innate response that could affect the defence capacities of the immune system. These results are evidence of the importance of sustained oral ingestion of fermented products for health maintenance. Since the increasing lack of fermented foods in western diet, the capabilities of Lactobacillus strains to counteract the effect of deprivation of fermented food demonstrate the role that probiotic enriched products could play in the improving of this diet.

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8. La administración oral de *L.fermentum* CECT5716 mejora los efectos de la vacuna de la gripe

RESUMEN

OBJETIVO

La defensa frente al virus de la gripe implica tanto una respuesta innata como una respuesta adaptativa. La capacidad de las bacterias ácido lácticas para estimular ambos tipos de respuestas inmunitarias, ha sugerido la posibilidad de usarlas como coadyuvantes en procesos de vacunación. Los datos *in vitro* y en animales de experimentación mostrados en los apartados anteriores, presentan a *L.fermentum* CECT5716 como un potente inmunoestimulante que podría ser eficaz como coadyuvante.

Para demostrar el posible papel de esta bacteria como coadyuvante en un proceso de vacunación frente a la gripe, se llevó a cabo un ensayo clínico doble ciego controlado. Los voluntarios se dividieron en dos grupos de forma aleatoria. El grupo control recibió una dosis oral diaria de metilcelulosa (placebo) y, el grupo probiótico una dosis de $1x10^{10}$ ufc/día de la bacteria probiótica durante las dos semanas previas a la vacunación y las dos semanas posteriores a la vacunación.

RESULTADOS

Se produjo un incremento significativo en la proporción de células Natural Killer en el grupo probiótico detectable ya en el momento de la vacunación tras las dos semanas de tratamiento. La vacunación indujo un incremento en la concentración de citocinas Th1 y en las poblaciones de linfocitos T helper y T citotóxicos de ambos grupos; sin embargo, el grupo probiótico mostró una inducción significativa mayor en algunos de estos parámetros. Con respecto a los efectos humorales, en el grupo placebo no se pudo detectar inducción de la producción de anticuerpos. En el caso del grupo probiótico, se detectó un incremento significativo en la IgA antígeno específica frente a la vacuna de la gripe. Aunque, se observó un incremento en la IgM totales, no se observaron cambios en la IgM antígeno específica. La incidencia de síntomas gripales registrados durante los 5 meses posteriores a la vacunación (Octubre a Febrero) fue menor en el grupo que consumió la bacteria probiótica.

CONCLUSIÓN

La administración oral de la cepa *L.fermentum* CECT5716 aislada de leche materna, potencia la respuesta inmunológica frente a la vacuna de la gripe y produce una mejora en la protección sistémica frente a la infección, aumentando la respuesta Th1 y neutralizando los antígenos virales.

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Applied nutritional investigation

Oral intake of *Lactobacillus fermentum* CECT5716 enhances the effects of influenza vaccination

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Abstract

Objective: We studied the coadjuvant capability of oral consumption of the breast-milk–isolated strain *Lactobacillus fermentum* (CECT5716) for an anti-influenza vaccine.

Methods: A randomized, double-blinded, placebo-controlled human clinical trial including 50 volunteers (31 male and 19 female) was performed to address the immunologic effects of an intramuscular anti-influenza vaccine in adults (33.0 ± 7.7 y old). Fifty percent of volunteers received an oral daily dose of methylcellulose (placebo) or probiotic bacteria (1×10^{10} colony-forming units/d) 2 wk before vaccination and 2 wk after vaccination.

Results: Two weeks after vaccination there was an increase in the proportion of natural killer cells in the probiotic group but not in the placebo group. The vaccination induced an increase in T-helper type 1 cytokine concentrations and in T-helper and T-cytotoxic proportions in both groups; however, the probiotic group showed a significant higher induction in some of these parameters. Regarding the humoral effects, induction of antibody response in the placebo group could not be detected. In the case of the probiotic group, a significant increase in antigen specific immunoglobulin A was detected. Although an increase in total immunoglobulin M was observed, changes in anti-influenza antigen specific immunoglobulin M were not observed. The incidence of an influenza-like illness during 5 mo after vaccination (October to February) was lower in the group consuming the probiotic bacteria.

Conclusion: Oral administration of the strain *L. fermentum* CECT5716 potentates the immunologic response of an anti-influenza vaccine and may provide enhanced systemic protection from infection by increasing the T-helper type 1 response and virus-neutralizing antibodies.

Keywords: Lactobacillus; Immunologic response; Coadjuvant; Vaccine; Influenza

Introduction

Influenza is an acute viral respiratory infection that results in high morbidity and significant mortality mainly in older adults. Moreover, the economic burden of annual epidemics in the working population has been reported as important, with 10–20% of sick people leaving work for a mean duration of 5–7 d in an influenza season of moderate impact [1,2]. Defense against influenza infection involves innate and adaptive immune responses. After infection most influenza viruses are detected and destroyed within a few hours by innate immune mechanisms. If influenza viruses escape these early defense mechanisms, they are detected and eliminated by adaptive immune mechanisms in which cytotoxic T lymphocytes and antibodies function as antigen-specific effectors to target the virus [3].

To control influenza, protective adaptive immunity must be induced in advance by the administration of a vaccine. However, the vaccine seems to show limited clinical effectiveness,

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ranging from 20% to 86%, as reflected in main studies in the previous 20 y [2,4,5]. To improve the effectiveness of the vaccine, coadministration of the inactivated virus with adjuvants such as cholera toxin or heat-labile enterotoxin has been used [6–8]. The mechanisms by which these molecules enhance the immune response against influenza viral antigens involve stimulation of the innate immune system [9]. However, the combination of the vaccine with these kinds of coadjuvants may not be clinically safe [10].

Oral administration of lactic acid bacteria has been reported to enhance innate and adaptive immunities in the host [11–15]. It has been previously demonstrated that consumption of these bacteria induces an increase in immunoglobulin A (IgA) related to the anti-infectious properties of lactic acid bacteria in diarrhea disease [16,17]. Moreover, innate immunity is enhanced by increasing the proportion and activity of phagocytic cells, such as monocytes and neutrophils [11–18]. The function of natural killer (NK) cells is also improved by consumption of some of these bacteria [14,19]. Therefore, lactic acid bacteria have been suggested as coadjuvants in a vaccination process to gain a more efficient protective response [20–22].

In a previous work, we described that breast milk of healthy women is an important source of lactic acid bacteria to the infant gut [23]. Breast feeding provides significant protection against infections in newborns and infants [24–27]. Breast milk components such as maternal immunoglobulins, lactoferrin, lactoperoxidase, lysozymes, and oligosaccharides have been involved in this activity [28,29], but, in addition, the presence of lactic acid bacteria with probiotic potential could contribute to the protective effect of breast milk [23,30].

In this work we describe the results of a human clinical trial performed to investigate the influence of consumption of a breast milk–isolated lactobacillus strain (*Lactobacillus fermentum* CECT5716) on the immune response induced by an influenza vaccine, as the primary endpoint of this study, in healthy adults.

Materials and methods

Volunteers and study design

The recruitment of volunteers was carried out in the medical service of Puleva Food S.A. (Granada, Spain) at the beginning of the vaccination program. Sixty-four healthy adult human volunteers were approached to participate in the trial. The exclusion criteria were frequent gastrointestinal disorders (frequent diarrhea, constipation episodes, or stomach acid), gastrointestinal surgery, metabolic diseases (diabetes, food allergy, or lactose intolerance), and/or antibiotic treatment during the trial. Fifty healthy adult human volunteers (19 female and 31 male) with an age range of 22 to 56 y (33 ± 7.7) were included in the study. The study was carried out according to the Helsinki Declaration. The study protocol was approved by the ethical committee of Fundación Hospital Virgen de las Nieves (Granada, Spain) and informed written consent was obtained from all subjects. The volunteers were asked to exclude from their diet any kind of probiotic product and/or yogurt.

Table 1
Recruitment and population

	Female	Male	Total
Approached	23	41	64
Declined	3	4	7
Excluded	1*	2†‡	3
Included	19	31	50
In placebo group	9	16	25
In probiotic group	10	15	25
Age (y)	31.1±7.1	34.3±7.9	33.0±7.7
In placebo group	30.5±6.0	33.6±7.0	32.5±6.7
In probiotic group	34.5±8.6	34.1±7.3	34.3±7.7

* Excluded because of egg allergy.
† Excluded because of frequent stomach acid.
‡ Excluded because of antibiotic treatment.

Volunteers were assigned to one of two groups randomized by gender and age, and the results of this randomization are summarized in Table 1. Those in the placebo group daily consumed a capsule containing 200 mg of methylcellulose. Those in the probiotic group daily consumed a capsule containing 1×10^{10} colon-forming units of the strain *L. fermentum* CECT5716 in a matrix of the same mix of methylcellulose. The study consisted of 28 d of probiotic treatment. The intramuscular vaccination was carried out at day 14 in the medical service of Puleva Food S.A. with a vaccine containing inactivated trivalent influenza (A/New Caledonia/20/99[H1N1], A/Fujian/411/2002[H3N2], B/Shanghai/361/2002[B]) for the vaccine campaign of 2004/2005 (Chiron S.r.l. Siena, Italy). All volunteers were vaccinated in the same week (third week of September 2004).

Clinical survey and diagnosis

The primary endpoint of the study was to evaluate the immune response induced by the vaccination process and its modulation by the consumption of probiotics. We especially focused on differences in lymphocyte subpopulations and immunoglobulin levels in blood.

In addition, a survey with items concerning the presence of fever (>37°C taken at the armpit), systemic symptoms (headache, myalgia, bone/joints pain, fatigue, anorexia, and digestive disorders), and respiratory symptoms (cough, nasal symptoms, and pharyngeal symptoms) was completed daily by the volunteers during the 5-mo (October to February) survey period. Volunteers were to report the development of any of these symptoms. Volunteers were instructed how to consider positive any symptom. A diagnosis of influenza-like illness (ILI) was based on the association of fever with any systemic symptom and at least one respiratory sign that lasted for at least 3 consecutive days. The episodes of ILI were added monthly for each group.

Collection of blood samples

After an overnight fast lasting at least 10 h, blood samples were taken from the volunteers at the beginning of the study (day 0), just before the vaccination (day 14), and at the end of treatment (day 28) using Vacutainers (S-Monovette, Sarstedt, Germany) containing ethylene-diaminetetra-acetic acid.

Analysis of leukocytes in blood

Major leukocyte subset phenotypes were counted in whole blood samples treated with ethylene-diaminetetra-acetic acid by flow cytometry in a FACScalibur (Becton Dickinson, Oxford, UK) by using the following fluorochrome-conjugated monoclonal antibodies (Becton Dickinson): anti-$CD3^+$, anti-$CD19^+$, anti-$CD4^+$, anti-$CD8^+$, anti-$CD45RO^+$, and anti-$CD56^+$. The results were expressed as the percentage of mononuclear cells that stained positively.

Total immunoglobulin and cytokine measurements

Total IgA, IgG, and IgM concentrations in plasma were measured by enzyme-linked immunosorbent assay (ELISA) quantitation kits (Bethyl, Montgomery, TX, USA). Cytokine concentrations in plasma were measured by ELISA quantitation kits (CytoSets, Biosource, Camarillo, CA, USA).

Specific immunoglobulins were also measured by ELISA. Briefly, 96-well plates were coated with 500 ng/mL of the vaccine suspension in coating buffer (0.5 M Na_2CO_3). After overnight incubation at 4°C, plates were washed three times with wash solution (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin). Then plasma samples were added to the plates and incubated for 1 h at room temperature. Plates were washed three times, and 100 μL of goat anti-human IgG, IgA, or IgM (Bethyl) was added for 1 h at room temperature. Staining was performed with 3,3′,5,5′Tetramethylbenzidine (Sigma Chemical, St. Louis, MO, USA) for 20 min at room temperature in the dark. The reaction was stopped with 0.1 N H_2SO_4, and plates were read at 450 nm.

Statistical analysis

The data were analyzed with SPSS 12.0 (SPSS, Inc., Chicago, IL, USA). For the gaussian variables, the longitudinal effect of the treatment within each group at different time points of the study was analyzed by one-way repeated measures analysis of variance followed by paired *t* test (within-group comparison). Two-way repeated measures analysis of variance was used to analyze statistical differences produced by the treatment followed by independent *t* test to assess in which time points the groups differed.

The incidence of ILIs in the placebo and probiotic groups was compared using non-parametric, independent, two-sample tests (Mann-Whitney U test). Statistical significance was defined as $P < 0.05$.

Results

Tolerance and clinical observations

Throughout the entire study the capsules were well tolerated by all volunteers and none reported any adverse effect associated with its consumption. No one had or voluntarily decided to abandon the study. Compliance with the probiotic treatment was followed by fecal detection of the probiotic strain (data not shown). The detection of *L. fermentum* CECT5716 was followed by polymerase chain reaction in the feces. The bacterium was present in 92% of the volunteers (23 of 25) in the probiotic group and in 12% of the placebo group (3 of 25).

Effects on lymphocyte subsets

Flow cytometric analysis showed in all cases that cells staining positively for $CD3^+$ (T lymphocytes), $CD8^+$ (cytotoxic T lymphocytes), $CD4^+$ (T helper lymphocytes), $CD19^+$ (B lymphocytes), $CD3^+CD45RO^+$ (memory T lymphocytes), and $CD56^+$ (NK cells) were within the ranges for hematologically normal Caucasian adults (Table 2). Nevertheless, in both groups an increase in T-helper and T-cytotoxic lymphocytes was observed 2 wk after vaccination. In the case of memory T lymphocytes, the increase observed in both groups did not depend on the treatment or vaccination process because the effect was detected before vaccination. The vaccination did not cause significant changes in NK cells in the placebo group, but the consumption of probiotic bacteria induced a significant increase in the proportion of NK cells at the end of the study (Table 2).

Effects on cytokine concentration

Tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin (IL) 12 and IL-10 cytokines were measured in plasma (Table 3). The vaccination process induced an increase in serum IL-12. In the probiotic group, an increase was observed before vaccination, after 2 wk of probiotic treatment. After vaccination, although an induction was also observed and values were still higher than those in the placebo group, differences did not reach statistical significance. In the case of TNF-α, vaccination induced an increase in the cytokine concentration in both groups. However, the consumption of probiotic bacteria induced a significantly higher increase. Regarding IFN-γ and the immunoregulatory cytokine IL-10, no significant differences were detected. However, in the probiotic group, a trend to increased IFN-γ blood levels was already observed after 2 wk of probiotic consumption ($P = 0.1$; Table 3).

Table 2
Percentage of lymphocyte subsets*

	Control group			Probiotic group		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
T lymphocytes	60.16 ± 2.8	63.32 ± 2.1	58.72 ± 2.9	63.47 ± 2.9	63.91 ± 1.6	59.39 ± 2.3
T-helper lymphocytes	30.34 ± 1.7	29.47 ± 1.8	34.18 ± 1.8†‡	31.67 ± 1.5	30.46 ± 1.3	36.27 ± 1.5†‡
T-cytotoxic lymphocytes	19.19 ± 1.2	18.56 ± 1.2	25.08 ± 1.2†‡	21.48 ± 1.1	22.21 ± 1.2	26.40 ± 1.3†‡
Memory T lymphocytes	21.07 ± 2.0	29.98 ± 2.2†	33.18 ± 1.3†	22.55 ± 1.7	29.57 ± 1.8†	31.40 ± 1.9†
Natural killer cells	17.03 ± 1.6	17.41 ± 1.7	18.62 ± 1.1	16.80 ± 1.7	18.11 ± 1.6	21.64 ± 1.5†
B lymphocytes	07.36 ± 0.7	07.72 ± 0.7	07.78 ± 0.5	07.51 ± 0.4	07.95 ± 0.6	07.72 ± 0.6

* Data presented as mean ± SEM.
† Statistically significant difference with respect to week 0, $P < 0.05$.
‡ Statistically significant difference between week 2 and week 4, $P < 0.05$.

Effects on immunoglobulin concentrations

Total and anti-influenza–specific IgGs, IgAs, and IgMs were measured in plasma by ELISAs. Two weeks after vaccination, an increase in antibody response in plasma of the placebo group could not be detected but a significant decrease in IgG concentration was observed (Table 4). In contrast, in the case of the probiotic group, there was a significant increase in specific anti-influenza IgA in serum after vaccination. In addition, in the probiotic group, a significant increase in total IgM was observed, which did not reach statistical significance in the case of the specific anti-influenza IgM (Table 4).

Incidence of ILI

Episodes of ILI (defined as described in MATERIALS AND METHODS) were recorded daily by the volunteers and added monthly for each group during the 5-mo survey period (Fig. 1). During this period the number of ILI episodes in the

Table 3
Cytokine concentrations*

	Control group			Probiotic group		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
IL-10 (pg/mL)	109.17 ± 20.01	115.20 ± 12.44	122.26 ± 13.66	108.00 ± 95.15	111.14 ± 20.59	129.45 ± 15.85
IL-12 (pg/mL)	65.84 ± 7.03	72.01 ± 8.16	87.65 ± 9.70†‡	63.35 ± 6.96	89.48 ± 12.55†	102.80 ± 12.98‖
TNF-α (pg/mL)	57.48 ± 8.19	73.70 ± 9.44†	84.20 ± 10.04†‡	59.54 ± 8.84	110.15 ± 17.59†§	117.56 ± 16.11‖§
INF-γ (pg/mL)	23.65 ± 4.77	23.03 ± 4.11	23.98 ± 4.30	23.94 ± 5.88	25.25 ± 5.40	25.47 ± 5.96

IL, interleukin; INF, interferon; TNF, tumor necrosis factor
* Data presented as mean ± SEM.
† Statistically significant difference with respect to week 0, $P < 0.05$.
‡ Statistically significant difference between week 2 and week 4, $P < 0.05$.
§ Statistically significant difference between control and probiotic group, $P < 0.05$.
‖ Statistically significant difference with respect to week 0, $P < 0.01$.

Table 4
Immunoglobulin concentrations*

	Control group			Probiotic group		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
IgG (mg/dL)	814.39 ± 127.63	720.25 ± 90.15	448.28 ± 98.22†	825.00 ± 96.92	696.92 ± 107.85	824.04 ± 112.17§
IgA (mg/dL)	155.13 ± 17.06	145.03 ± 22.86	146.57 ± 20.49	147.41 ± 10.90	145.95 ± 15.78	144.31 ± 16.28
IgM (mg/dL)	260.36 ± 41.12	229.69 ± 31.31	212.66 ± 31.91	250.71 ± 24.96	241.75 ± 25.17	320.16 ± 32.90†‡§
IgG-sp (OD)	—	0.623 ± 0.05	0.549 ± 0.05	—	0.590 ± 0.04	0.538 ± 0.05
IgA-sp (OD)	—	0.445 ± 0.04	0.440 ± 0.03	—	0.488 ± 0.03	0.577 ± 0.06‡§
IgM-sp (OD)	—	0.264 ± 0.01	0.266 ± 0.00	—	0.268 ± 0.01	0.279 ± 0.00

sp, specific; Ig, immunoglobulin; OD, optical density at 450 nm
* Data presented as mean ± SEM.
† Statistically significant difference with respect to week 0, $P < 0.05$.
‡ Statistically significant difference between week 2 and week 4, $P < 0.05$.
§ Statistically significant difference between control and probiotic groups, $P < 0.05$.

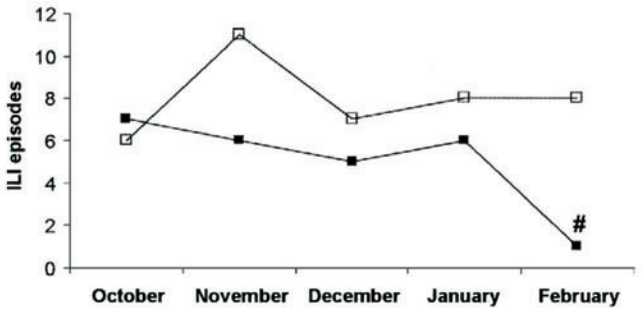

Fig. 1. Episodes of ILI were recorded monthly for the placebo group (white squares) and the probiotic group (black squares). #Statistically significant difference for control versus probiotic group ($P < 0.05$). ILI, influenza-like illness.

probiotic group was smaller than that in the placebo group, but significant differences were observed only in February. Forty ILI episodes were recorded in the placebo group, whereas 25 episodes were reported in the probiotic group. The vast majority of volunteers recorded only one episode of ILI during the 5 mo, although 3 of 25 volunteers in the placebo group and 1 of 25 in the probiotic group recorded as many as four ILI episodes during the study. Further, 36% (9 of 25) and 40% (10 of 25) of the volunteers in the placebo and probiotic groups, respectively, reported no ILI episode during the study.

Discussion

Influenza vaccination is currently recommended especially in populations at risk to prevent flu complications; however, in some annual campaigns the vaccine coverage is low [31], which calls for the requirement of new alternatives or adjuvant approaches to improve it. Cholera toxin and heat-labile enterotoxin have been used as coadjuvants because these molecules enhance the adaptive response induced by influenza vaccines by mechanisms involving stimulation of the innate immune system [6–9]. However, as previously mentioned, the use of these coadjuvants may not be clinically safe [10]. Thus, the use of other, efficient, safer coadjuvants is needed. Very recently, the capability of some lactobacilli strains to act as coadjuvants by enhancing the antibody response after polio virus vaccination has been reported [22]. Therefore, we evaluated the effect of *L. fermentum* CECT5716 during a flu vaccination process.

Two considerations must be made before discussing the results obtained. First, the population size was determined in order to obtain differences between groups regarding immune cellular and molecular parameters such as lymphocyte populations or immunoglobulin and cytokine levels, which correspond to the primary endpoint of this study. Second, the use of only two study groups (placebo and probiotic), all vaccinated 2 wk after the initiation of the study, does not allow us to clearly state whether some of the observed effects were mainly due to the vaccination process per se or to the treatment with probiotics. We assigned a probiotic effect in those differences observed between both groups, especially if they were already observed at day 14. The effect of the vaccination process per se will correspond to the differences observed in the placebo group between weeks 2 and 4. The differences in this same period that were detected only in the probiotic group in comparison with the placebo will correspond to the adjuvant effect of *L. fermentum* during the vaccination protocol.

During a natural viral infection, innate immune mechanisms constitute the first barrier against influenza infection through effector cells, molecules, and factors involved in the restriction of viral spread. For example, NK cells are detected in pulmonary lymphocytes 48 h after influenza virus infection producing IFN-γ and limiting the viral spread by virus-infected cell lysis [3,32]. In this sense, the oral administration of *L. fermentum* CECT5716 induced an increase in NK cells 2 wk after vaccination, which could not be observed in the placebo group. Vaccination induced the expression of TNF-α and IL-12 in both groups, although the increase was higher in those volunteers who consumed the probiotic bacteria. Because IL-12 is involved in NK and T-helper type 1 lymphocyte activation [33], these differences could explain the increased amount of NK cells observed in the probiotic group. Moreover, NK cells in turn are producers of IFN-γ, a fact that also correlates with the levels of this cytokine observed in the probiotic group.

Regarding cellular-specific immune responses, the vaccination induced an increase in T-helper ($CD4^+$) and T-cytotoxic ($CD8^+$) lymphocytes. T-cytotoxic lymphocytes play an important role in defense against influenza infection by killing the virus-infected cells and producing IFN-γ that inhibit virus replication [34,35]. No other clinical relevant differences in lymphocyte subtypes were observed due to the vaccination protocol or the consumption of probiotics. The differences observed in memory T lymphocytes in both groups and before the vaccination process must be related to immune modulation due to the restriction diet (volunteers were not allowed to consume fermented products during the study) [36].

The major humoral protective immunity induced by influenza virus infection is provided by S-IgA and IgG antibodies. However, parenteral inactivated vaccines have been reported to mainly induce serum IgG antibodies that are weakly cross-protective across drift viruses within a subtype [37]. Surprisingly, in this study we detected an increase in specific anti-influenza IgA antibodies in plasma of the probiotic group, whereas no increase was observed in specific IgG or IgM antibodies. A potential explanation for this could be the low response triggered by the vaccine of this current campaign. Moreover, two facts could explain the differences observed in IgA-specific antibodies. First, IgA antibodies react not only to homologous viruses but also to variant viruses in the same subtype in contrast to IgG antibodies that react mainly to homologous viruses [3].

Second, reinfection results in a secondary IgA antibody response, which is characterized by a rapid rise in the IgA antibody titer. Thus, IgA antibodies triggered by a previous natural infection or vaccinations could cross-react with the vaccine and induce a greater IgA response. Due to the high incidence of flu and the increasing number of influenza vaccination campaigns in Spain, it is impossible to obtain a test adult population without previous contact with this antigen.

Conversely, a significant increase in total IgM was detected but only in the probiotic group. Because significant changes in specific IgMs were not observed, this increase could be mainly due to the immunologic response triggered by the probiotic bacteria.

Thus far, our results suggest that the parenteral inactivated vaccine used in this study seems to induce a T-lymphocyte cell proliferation or maturation but poorly induces a complete antibody response. We have also demonstrated that the consumption of a *L. fermentum* strain during a period around vaccination could enhance the immunologic effects of the vaccine by inducing the production of specific anti-influenza antibodies and by increasing the production of T-helper type 1 cytokines and other factors involved in viral defense. The mechanisms by which lactic acid bacteria could modulate the immune response are not fully understood; however, this is not surprising because the immune system associated to the gut mucosa represents the larger immune compartment of the body [38]. In this sense, important immune disturbances have been reported to occur in germ-free animals [39].

Human studies have shown that gram-positive bacterial species are strong inducers of monocyte-derived IL-12 [40], a powerful signal to activate NK cells [41,42]. Monocytes and macrophages, together with dendritic cells, play a crucial role in the innate immune response, which in turn leads to activation of the adaptive immune system [43]. These cells recognize conserved molecular patterns of bacterial components through Toll-like receptors, the activation of which triggers the production of cytokine mediators in the development of T-cell differentiation [44]. Thus, the significantly higher values of NK cells and T-helper type 1–promoting cytokines (IL-12, IFN-γ, and TNF-α) detected in probiotic group could have led to the enhancement of the specific response against influenza triggered during the vaccination protocol. In this respect, there are several reports describing the effects of lactic acid bacteria on IgA production in rodents and humans [41].

The greater immune response observed in the probiotic group in comparison with the placebo group seems to correlate with a lower incidence of ILI during 5 mo of survey. However, these clinical data have to be taken in perspective due to the small population of this study, and more clinical studies are required to demonstrate the clinical efficacy of using probiotics in a coadjuvant approach to viral infections.

Conclusion

In this work we have demonstrated that the use of oral probiotic strains is an efficient, safe, and easy method to improve the protective immune response triggered by influenza vaccination.

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9. Efectividad de los probióticos en alergia. Un juego de niños o un asunto de adultos?

RESUMEN

En este trabajo se presentan datos tanto de estudios en animales como en humanos, que sugieren un efecto positivo de las cepas probióticas *L.gasseri* CECT5714 y *L.coryniformis* CECT5711 en alergia. Aunque los datos presentados son preliminares, en el trabajo se debate sobre el posible papel de los probióticos en la prevención y tratamiento de los procesos alérgicos.

RESULTADOS

Además del ya mencionado carácter inmunomodulador de cepas probióticas, hay otros mecanismos que podrían también ejercer un efecto beneficioso sobre la alergia. En este caso, se presentan resultados de un modelo de alergia alimentaría en ratones, donde la fermentación de alimentos llevada a cabo por los probióticos, como por ejemplo la leche en un yogurt, supone una hidrólisis parcial de las proteínas que las hace menos alergénicas.

Por otro lado, se describe también, un estudio clínico prospectivo realizado en 15 humanos adultos alérgicos al polen de olivo y de gramíneas. Se trataba de un estudio aleatorio y doble-ciego donde el grupo placebo tomó un yogurt convencional y, el grupo probiótico un producto fermentado con las cepas *L.gasseri* CECT5714 y *L. coryniformis* CECT5711, por un periodo de tres meses (meses de abril-junio) durante los cuales se alcanzan los niveles más altos de estos pólenes. Los resultados, aunque fueron discretos debido al tamaño de muestra reducido, demostraron un efecto beneficioso del consumo de probióticos en aspectos como los niveles de basófilos activos, IgE plasmáticas o liberación de mediadores inflamatorios.

CONCLUSIÓN

Si bien la mayoría de los trabajos realizados hasta la fecha hacen referencia a la prevención de la atopía en edad infantil, en este trabajo se discute sobre la posible funcionalidad de los probióticos en el tratamiento de la alergia en la edad adulta basándonos no sólo en su actividad inmunomoduladora sino también, en ciertas actividades enzimáticas que podrían tener un papel beneficioso.

Aunque son necesarios más estudios clínicos que confirmen los efectos beneficiosos del consumo de probióticos en individuos alérgicos, los resultados que se tienen hasta ahora son prometedores y sugieren que, ciertas cepas probióticas podrían ser una alternativa eficaz en la prevención y tratamiento de la alergia.

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EFFECTIVENESS OF PROBIOTICS IN ALLERGY
Child's game or adult affair?

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INTRODUCTION

During the last decades, the prevalence of allergy-related diseases have increased constantly in developed countries, affecting as much as 20-50% of the population and being among the most common chronic diseases in childhood. The emergence of clinical cases of atopic eczema, allergic rhinitis or asthma is difficult to explain only on the basis of genetic factors and, therefore, scientists have been searching for the possible involvement of environmental conditions.

Strachan (1989) proposed the 'hygiene hypothesis' after demonstrating an inverse relationship between the birth order among the members of a family and the prevalence of hay fever (1). Briefly, the hypothesis proposes that, during the perinatal and in early childhood period, contact with microorganisms that are prevalent in the family environment would be able to prevent allergic disorders. Over the past century, several factors, such as declining family size, increasing home improvement and better hygiene practices have significantly reduced opportunities for acquisition and exchange of microbes among the members of a family. Obviously, this fact has brought multiple sanitary benefits but, following this hypothesis, it has also brought a negative secondary effect: the rise of atopy and related disease (2).

Later, the hygiene hypothesis was modified to highlight the crucial role of the initial microbial colonization of the infant gut for the proper maturation of the naïve immune system (3). During the 1990's, the distinction between Th1 and Th2 lymphocyte populations, and their mutual and excluding regulation, offered a plausible immunological mechanism supporting this hypothesis since immunity to bacteria and viruses induces a Th1 pattern of cytokine release (IL-2 and IFN-γ) that has the potential to suppress the Th2 responses (IL-4, IL-5, IL-13) involved in IgE-mediated allergy (4).

From childhood food atopy to adult allergy

Food allergies occur in about 5 to 10% of the overall infant population (5). The earliest condition used to be atopic eczema, and the first sensitising antigens are frequently derived from food (6), cow´s milk protein allergy (CMPA) being the most common manifestation in young infants, with a 2-6% incidence (7,8). This atopic disease is associated with a broad spectrum of IgE-mediated reactions which are usually linked to an

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acute symptomatology, characterized by urticaria, rhinoconjunctivitis, asthma, vomiting and diarrhea, and that, in some cases, may led to anaphylactic shock and death (9).

Most children become tolerant to food antigens by approximately 2 years of age, when sensitization to air-borne allergens takes over. That is, a previous CMPA condition is often not an isolated phenomenon since it usually represents the beginning of an 'atopic career'. As a consequence, infants may subsequently suffer from other atopic diseases such as other food allergies, rhinitis, asthma or the highly prevalent early atopic dermatitis (15-20% of children) (10-12). Unlike food allergies, atopic respiratory diseases are usually not transient phenomena.

The role of food allergy in the development of atopic diseases remains far from being elucidated since the factors involved are often unclear. As mentioned above, different data suggest that infants manifesting cow's milk allergy in early infancy are at a higher risk of multiple food allergy (6) and/or asthma (13). However, prevention of food allergy by elimination diets does not result in prevention of asthma (14), suggesting distinct immunoregulatory processes.

Furthermore, it has been demonstrated that exposure to antigens does not necessarily lead to sensitization or development of atopic disease (15).

Intestinal microbiota and atopy

Initial colonization of the infant gut represents the primary microbial stimulation of the human host (16). The hygiene hypothesis suggests that exposure to commensal microbiota may represent a key modulator of the immune system against atopy and allergy-related diseases. Thus, the risk of allergy-related diseases may be reduced by modulating the gut microbiota. In fact, the composition of the gut microbiota of allergic infants is different (lower lactobacilli and bifidobacteria counts; higher coliform and staphylococci) when compared to that of healthy age-matched infants (17). Bottcher *et al* also demonstrated that there are differences in the metabolic activities of gut microflora between allergic and non-allergic infants (18). Similarly, Kalliomäki *et al* (19,20) showed that the gut microbiota of those neonates who are likely to develop atopic diseases usually displays a distinctive pattern. The 'predisposing microbiota' includes higher numbers of clostridia and lower numbers of bifidobacteria in comparison with that of healthy infants.

CHILDREN

'Bugs' for children's guts

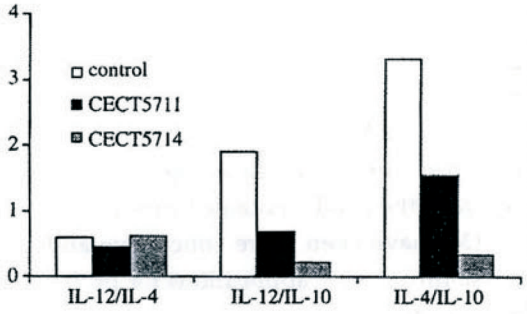
Since an aberrant gut microflora is a characteristic of the allergic infant gut, procedures aiming to its correction, such as probiotic consumption, could be useful in the prevention and/or treatment of allergy-related diseases.

Three mechanisms could be involved in the potential beneficial effects exerted by probiotics:

a regulation of the immune response,
b modification of allergenic peptides by enzymatic degradation,
c promotion of mucosal barrier maturation.

The immune response for microbial antigens, including those originated from probiotics, is accompanied by preferential expression of Th1-type cytokines and, in contrast, is inversely related to Th2 IgE-based response (4), the hallmark of allergy-related diseases. However, an absolute differentiation between Th1 and Th2 immune responder phenotypes may not always be justified since other T-cell subsets, such as T-regulatory cells and Th3 cells, have been described. Such subsets have strong immunosuppressive properties through the production of IL-10 and TGF-β, respectively, and these cytokines can counterbalance both Th1 and Th2 responses (21). In this context, we have evaluated the effect of two probiotic strains, *Lactobacillus coryniformis* CECT5711 and *Lactobacillus gasseri* CECT5714 on the cytokine pattern of spleen-derived mouse lymphocytes stimulated with 5 μg/ml concanavalin A in the presence or absence of the indicated probiotic strains ($5x10^7$ cfu/ml) for 24 h: primary T lymphocytes were induced (***Fig 1***).

Figure 1 *Cytokine ratios (expressed as pg ml-1/pg ml-1) of spleen-derived mouse lymphocytes stimulated with 5 mg/ml concanavalin A in the presence or absence of* Lactobacillus coryniformis *(CECT5711) and* Lactobacillus gasseri *(CECT5714) (5x107 cfu/ml) for 24 h*



Cytokines in the supernatants were anlyzed by specific ELISA

Although these bacteria did not modify the Th1(IL-12)/Th2(IL-4) ratio in lymphocytes, they induced a strong effect on the expression of the regulatory cytokine IL-10, reducing both the Th2 (IL-4/IL-10) and Th1 (IL-12/IL-10) responses (***Fig 1***) (manuscript in preparation).

Induction of oral tolerance or allergic sensitization to dietary antigens depends on several factors such as the dose and the degradation of antigens (**22**). Degradation of antigens could result in the formation of tolerogenic peptides that may promote the maturation of the neonatal immune system (**23**). Modification of potential allergens by the activity of bacteria, either in controlled fermentations or forming part of the gut microbiota, can alter the exposure to dietary antigens. Previously, it has been demonstrated that enzymes derived from *Lactobacillus rhamnosus* LGG contribute to degradation of antigens, rendering them non-allergenic (**24**).

We have compared the allergic sensitization potential of raw cow's milk with that of cow's milk hydrolyzed either by fermentation with *L. coryniformis* CECT5711 or by conventional enzymatic procedures. For this purpose, a murine cow's milk allergy model (**25**), consisting in an intraperitoneal challenge with cow's milk protein after 5 previous oral sensitization doses, was used. The results obtained revealed that hydrolysis of cow's milk proteins significantly reduces their allergenicity (***Table 1***), an effect that was particularly remarkable when milk proteins were hydrolyzed by the activity of the probiotic strain. Interestingly, the hydrolysis rate of the probiotic treatment was lower than in that attained by the conventional enzymatic hydrolysate (manuscript in preparation). These results demonstrate the potential of the enzymatic machinery of probiotic bacteria to generate less sensitizing or even tolerogenic peptides.

Finally, certain fermentation products derived from the metabolic activity of the gut microbiota, such as butyrate and other fatty acids, are an important energy source for the intestinal epithelium and contribute to the proper physiology and integrity of the gut mucosal barrier (**26**).

Table 1 *Allergic sensitization of mice with raw, enzymatically hydrolyzed or probiotic fermented cow's milk* (CM)

	CM	Fermented CM	Hydrolized CM
Hypersinsitivity score	4.0 ± 1.3	1.5 ± 1.1*	2.0 ± 2.0*
Serum parameters:			
CMP specific IgG1 (AU)	0.28 ± 0.21	0.05 ± 0.02*	0.11 ± 0.04*
Histamine (ng/ml)	96.0 ± 16.9	53.7 ± 24.7*	94.0 ± 31.5
IL-4 (ng/ml)	79.0 ± 12.8	13.0 ± 20.7*	12.4 ± 15.7*
Total IgE (µg/ml)	9.4 ± 4.9	0.8 ± 2.0*	1.6 ± 1.5*

* Results are expressed as the mean ± SD (n= 8)

• Statistically significant; $p<0.05$ *vs* the CM group

Hypersensitivity score was evaluated as previously described (**25**)

IgG_1 specific to cow's milk protein (CMP) were measured as described (**25**) and represented as absorbance units (AU)

Infant probiotic effectiveness, in progress

Due to the influence of the hygiene hypothesis, it is not surprising that most probiotic studies have focused on the prevention and/or treatment of allergic diseases and were performed in very early childhood. The studies performed by Erika Isolauri's group (University of Turku, Finland) have exerted a strong influence in this field and have shown that the treatment of either neonates or their lactating mothers with a probiotic strain (*Lactobacillus rhamnosus* LGG) may reduce the incidence of atopic eczema in at-risk children for at least 4 years (**20,27**). Other groups have demonstrated the involvement of a immune response modulation, such as an increase in IFN-γ (**28**) or IL-10 levels (**29**), a reduction in IgE plasma levels (**30**); or an enhancement of the intestinal permeability in atopic subjects (**31**). These studies offer a new attractive approach for the management of allergic diseases.

ADULTS

An alternative target

In the studies cited above, the probiotic targets were atopy and/or food allergies in infants.

Does this imply that probiotics can only prevent atopy if they are consumed during infancy? Can they be useful for prevention and/or treatment of such conditions when administered to adults as well? More studies are required to provide fuller answers to these questions. In contrast to infant atopy, the number of studies addressing the effects of probiotics in pollen allergy in adults is very scarce and the results are not consistent.

For example, Helin *et al* (**32**) observed that the treatment of teenagers and adults with an *L. rhamnosus* strain decreased the severity of the symptoms and the medication but did not prevent sensitization to birch pollen.

In parallel, Aldimucci *et al* (**33**) showed some immunological benefits such as a reduction in IL-4 and an increase in IFN-g levels, but neither a reduction in IgE levels nor an improvement in the allergy-related symptomatology were observed. In contrast, the results obtained in some animal studies (**34**) have been more conclusive and open the possibility of new applications for probiotics in the allergy field.

It seems clear that immunomodulation, generation of modulating peptides and the regulation of mucosal permeability and reactivity are not exclusive properties of infant mucosal immunity.

As a consequence, we designed a study to test the potential of probiotics as a preventive approach in adult allergy. More specifically, a small prospective double-blind placebo-controlled study was carried out to analyze the beneficial effect of probiotic consumption in 15 subjects with allergy to olive pollen (***Table 2***). In this 2-month study, the effect of consumption of a conventional yoghurt (6 subjects) was compared with that of a fermented dairy product containing two probiotic strains: *L. coryniformis* CECT5711 and *L. gasseri* CECT5714. For this purpose, a variety of immunological and clinical parameters were evaluated.

In contrast to yoghurt consumption, the administration of the probiotic product led to a reduction in the values of some allergic-related parameters, including the reactivity of immune cells involved in allergy, such as basophils (activated basophils) or mast cells (liberation of β-hexoaminidase), and the blood levels of T_H2 cytokines. The IgE levels were also significantly reduced but, in this case, the effect was observed in both the yoghurt and the probiotic groups. The reduction in the cited parameters after probiotic consumption could be responsible for the lower incidence of headache, although no differences were appreciated regarding rhinitis symptoms (***Table 2***).

CONCLUSIONS AND PERSPECTIVES

Although we are aware that more studies are required to demonstrate the effectiveness of probiotics in the prevention and/or treatment of allergy

Table 2 *Effects in the prevention of pollen allergy symptoms and immunologic parameters after two-months' consumption of a yoghurt or a probiotic dairy product*

	Yoghurt		Probiotic	
	Initial	**After 2 months**	**Initial**	**After 2 months**
Activayed Basophiles (%)	37.4 ± 17.2	44.6 ± 12.4	66.4 ± 9.8	60.1 ± 9.7*
IgE B cells (%)	2.53 ± 0.85	1.64 ± 0.73	2.98 ± 0.41	1.41 ± 0.25*
IgE (μg/ml)	287.98 ± 38.19	259.09 ± 38.95*	282.32 ± 37.06	264.76 ± 36.14*
b-hexoaminidase (DO)	0.75 ± 0.06	0.69 ± 0.06	0.74 ± 0.04	0.64 ± 0.03*
Il-4 (ng/ml)	93.44 ± 50.89	73.92 ± 36.46	98.78 ± 38.48	62.67 ± 27.50*
Rhinitis (score)	1.7 ± 0.95	2.9 ± 0.31	2.14 ± 0.61	2.46 ± 0.48
Headache (score)	0.89 ± 0.40	1.32 ± 0.53	0.83 ± 0.35	0.50 ± 0.17

Results are expressed as the mean ± SD (n=15)

* Statistically significant; $p<0.05$ *vs* the initial values obtained before initiation of dairy product consumption.

Rhinitis and headache scores are valued as 0 to 5, meaning 0 no symptoms and 5 strong symptomatology.

β-neuroaminidase levels were measured as described (25) and represented as absorbance units (AU).

related diseases, the studies available to the present day suggest that it is worthwhile to further explore this approach. Evidence is clear in atopy prevention in infants, where factors such as the regulation of intestinal permeability and reactivity to antigens, the regulation of the mucosal immunity and changes in the antigenic potential of allergens seem to play a major role in the beneficial effect exerted by probiotics. However, more studies are necessary since the number of studies date are small and patient numbers are small.

Probiotics could be also useful in cases of allergy in adults since the three factors cited above may also play a preventive or therapeutic role in such population segment. Further research is required in this field to demonstrate the effectiveness of probiotics in the prevention and treatment of allergic disease.

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Discusión

La microbiota intestinal está compuesta por una amplia variedad de microorganismos que intervienen en procesos tan importantes como la nutrición, la función intestinal y la maduración del sistema inmunitario. La instauración de una microbiota equilibrada, influirá de forma importante sobre la salud del individuo. De hecho, se han observado cambios singulares en la microbiota intestinal de sujetos alérgicos (Bjorksten y col., 1999; Kalliomäki y col., 2001b; Penders y col., 2007), en pacientes con Enfermedad Inflamatoria Intestinal (IBD) (Seksik y col., 2003; Sokol y col., 2006) e incluso en alteraciones metabólicas como la obesidad (Turnbaugh y col., 2006) que ponen de manifiesto la importancia de una microbiota equilibrada para la salud del individuo.

La adquisición de la microbiota del recién nacido es un largo y complejo proceso en el que influyen numerosos factores como, la carga medioambiental de microorganismos, el contacto con la madre y el entorno, dieta, medicación, etc. En este proceso, la leche materna juega un papel muy importante pues no sólo aporta componentes prebióticos que favorecen la proliferación de una flora equilibrada, sino que además, se ha demostrado que la leche materna constituye una fuente excelente y continúa de bacterias, que serán transferidas al bebé a través de la lactancia para iniciar su propia microbiota intestinal (Heikkilä y col., 2003; Martín y col., 2003). Estas características de la leche materna serían las responsables de las diferencias observadas entre, la microbiota intestinal de los niños amamantados con respecto a los niños alimentados con fórmula infantil (Benno y col., 1984; Harmsen y col., 2000a).

Entre las bacterias comúnmente aisladas de la leche materna se encuentran representantes de los géneros estafilococos, estreptococos, lactococos, enterococos y lactobacilos (Heikkilä y col., 2003; Martín y col., 2003). De todas ellas, los lactobacilos son aquellos que han despertado un mayor interés al ser considerados potencialmente probióticos. Un gran número de evidencias científicas demuestran que, las bacterias lácticas ejercen numerosos efectos beneficiosos para la salud tales como, la mejora de la intolerancia a la lactosa, la prevención y atenuación de infecciones intestinales, alergias y enfermedades inflamatorias intestinales, efectos inmunomodulares, etc. (Gill y Guarner, 2004). La presencia de bacterias lácticas en la leche materna con efectos positivos sobre estas patologías podría contribuir, al menos en parte, a los efectos beneficiosos observados en niños amamantados en los cuales se observa una menor

incidencia de infecciones o alergias en comparación con los alimentados con fórmulas infantiles (Cesar y col., 1999; Nascimento, 2003; Victoria y col., 1987).

Por tanto, nos encontramos con una fuente excelente de bacterias no explotada hasta ahora, en la que nos propusimos buscar nuevas cepas que por sus propiedades probióticas pudieran ejercer funciones beneficiosas sobre la salud, aplicables tanto a nutrición infantil como a la de adultos.

A partir de los estudios realizados por nuestro grupo (Martín y col., 2003), se llevó a cabo un amplio proceso de selección que permitió distinguir aquellas cepas que, entre la enorme variedad presente en las muestras de leche materna, presentaban el mayor potencial probiótico. Una vez finalizado el proceso de selección de los aislados de leche materna y fermentados lácticos, 5 cepas destacaron por sus propiedades potencialmente probióticas: cuatro de leche materna (*L.salivarius* CECT5713, *L.gasseri* CECT5714, *L.gasseri* CECT5715 y *L.fermentum* CECT5716) y una aislada de queso de cabra pero con capacidad para ser transferida a través de la leche materna (*L.coryniformis* CECT5711) (Martín y col., 2005a; 2005b; 2006).

Los efectos beneficiosos de las bacterias probióticas más respaldados por la bibliografía son los relacionados con la función gastrointestinal y el sistema inmunitario, como por ejemplo la prevención y/o atenuación de las infecciones intestinales y sistémicas, la mejora de la intolerancia a la lactosa, algunos efectos anticancerígenos, la prevención y el tratamiento de la alergia y la inflamación crónica (Gill y Guarner, 2004).

Durante mucho tiempo, se tendió a generalizar asumiendo que todas las cepas de lactobacilos tenían los mismos efectos probióticos. De hecho, en el mercado hay cepas que no tienen ningún estudio conocido que avale su funcionalidad y se apoyan en el conocimiento acumulado sobre otras cepas. La gran cantidad de estudios realizados en los últimos años han derrocado esta teoría y, hoy en día, no se acepta una cepa como probiótica a no ser que se demuestren sus propiedades beneficiosas. Por ello, iniciamos con las cepas seleccionadas, a las que conjuntamente denominamos como Hereditum, una extensa tarea de investigación que incluye numerosos ensayos *in vitro,* experimentación animal y ensayos clínicos destinados a la caracterización de las cepas como tal, de su tolerancia y ausencia de efectos potencialmente perjudiciales, y por supuesto de su capacidad para inducir efectos beneficiosos en la salud del hospedador, característica implícita para poder ser

denominados probióticos . Los resultados, fruto de esta investigación son discutidos a continuación y forman en parte el conjunto de esta Tesis doctoral.

1. Tolerancia y Seguridad

Las especies de lactobacilos a las que pertenecen las cepas probióticas seleccionadas en esta Tesis, están reconocidas como GRAS (*General Recognized As Safe*) (tabla 1.1), por lo que su uso en nutrición se considera seguro. Sin embargo, en las últimas décadas se han detectado casos aislados de bacteremia, y ocasionalmente, de endocarditis y abscesos, relacionados con el consumo de otras cepas pertenecientes a especies consideradas también como GRAS, aunque generalmente estaban asociados con otras patologías y no se han considerado agente causal (Aguirre y Collins., 1993; Bayer y col., 1978). El último caso fue descrito en el año 2007, en un estudio realizado con pacientes que sufrían de pancreatitis severa. En este estudio realizado con 298 pacientes, se utilizó una mezcla probiótica compuesta por 6 cepas (*L.acidophilus, L.casei, L.salivarius, L.lactis, Bf.bifidum, Bf.lactis*). Al final del estudio, se observó una mayor incidencia de mortalidad en el grupo que consumió el probiótico respecto al control, aunque dentro de los valores normales de mortalidad asociados a esta patología. A pesar de la existencia de ciertas dudas o incongruencias metodológicas en su realización, las conclusiones de dicho estudio sugirieron que había que tener precaución en el uso de tratamientos probióticos en pacientes en estado crítico y en la forma de administración de dichos tratamientos, pues en este caso, se administraron directamente por sonda oral (Besselink y col., 2008). Aunque, estudios preliminares auguraban un efecto beneficioso del tratamiento probiótico en esta patología (Besselink y col., 2004; Muftuoglu y col., 2006), los resultados negativos obtenidos demuestran que, todavía queda mucho por aprender acerca de los probióticos y que es esencial la caracterización exhaustiva de cada cepa para un uso seguro y efectivo. Por todo esto, si bien no hay todavía una normativa clara que regule la seguridad de las cepas probióticas para su uso en humanos, sí que existe un consenso acerca de ciertos criterios como son el largo historial de uso y la ausencia de mecanismos de patogenicidad, que ya se contempla en las condiciones GRAS y en los tests de toxicidad en animales de experimentación (Adams y Marteau., 1995).

Tabla 1.1: Principales especies microbianas utilizadas como probióticos

Lactobacillus spp.	*Bifidobacterium ssp*	*Lactococcus ssp.*	*Streptococcus spp.*	*Enterococcus ssp.*	*Bacillus ssp.*	Otras especies
L.acidophilus	*B. bifidum*	*L.lactis*	*S.thermophilus*	*E.faecium*	*B.subtilis*	*Saccharomyces cerevisae*
L.lactis	*B.longum*	*L.cremoris*	*S.lactis*	*E.faecalis*	*B.coagulans*	*Saccharomyces boulardii*
L.bulgaricus	*B.infantis*	*L.diacetylactis*			*B.cereus*	*Leuconostoc ssp*
L.rhamnosus GG	*B.breve*					*Escherichia coli*
L.casei	*B.lactis*					
L.kefir	*B.adolescentis*					
L.brevis						
L.reuteri						
L.helveticus						
L.plantarum						
L.johnsonii						
L.salivarius						
L.gasseri						

Fuente: Álvarez-Olmos y col (2001), Collins y col (1998), Fooks y col (1999), Salminen (2001)

A la hora de estudiar la seguridad de una cepa probiótica son numerosos los aspectos que se deben de analizar; su compleja actividad enzimática, su interacción con células del organismo o incluso con otros microorganismos e incluso su capacidad infectiva como microorganismos vivos que son. En el caso de las cepas Hereditum, se ha intentado abarcar todos estos aspectos con el fin de avalar un uso seguro de las cepas.

Entre las actividades bacterianas que podrían ser potencialmente perjudiciales por su toxicidad, se incluyen ciertas actividades enzimáticas que actúan sobre substratos que se encuentran en el lumen intestinal, dando lugar a compuestos tóxicos como son las aminas biógenas; compuestos orgánicos alcalinos que se producen en diferentes tipos de alimentos, incluidos los productos lácteos, debido a la actividad amino-descarboxilasa de algunos microorganismos presentes en los alimentos. A pesar de que las aminas biógenas (histamina, tiramina, putrescina, cadaverina..) son necesarias para ciertas funciones fisiológicas, el consumo de alimentos con altas concentraciones de estos compuestos puede derivar en graves problemas toxicológicos. La histamina y la tiramina tienen propiedades vasoactivas y psicoactivas, mientras que la putrescina y la cadaverina potencian los efectos de las anteriores y, además son precursores de nitrosaminas carcinogénicas en alimentos (Bover-Cid y Holzapfel., 1999). Dado que, algunos lactobacilos tienen capacidad para producir estas sustancias (Coton y col., 1998; de las Rivas y col., 2005) resulta conveniente evaluar esta propiedad en cualquier cepa que pretenda utilizarse con fines probióticos, especialmente en aquellas que se deseen vincular con alimentos fermentados. En el caso de las cepas Hereditum, ninguna presentó dicha actividad.

Otra actividad enzimática habitual en la flora intestinal que se descartó en las cepas Hereditum, es la actividad ß-glucuronidasa. Dicha actividad, desconjuga el ácido glucurónico que está unido a compuestos tóxicos, impidiendo la eliminación de estos compuestos tóxicos y permitiendo, por tanto, su absorción. Finalmente, los integrantes de la microbiota intestinal han desarrollado un complejo sistema de glicohidrolasas que les permite la utilización de hidratos de carbono complejos que, aportados por la dieta, constituyen el principal nutriente para su supervivencia en el tracto intestinal. Sin embargo, este complejo sistema enzimático podría degradar las mucinas que constituyen la mucosa y afectar así la integridad de la mucosa intestinal. En el caso de las cepas Hereditum, ninguna de ellas tuvo la capacidad de degradar mucinas.

Aunque todas estas actividades son intrínsecas a la microbiota intestinal, debe existir un equilibrio para evitar la posible aparición de efectos tóxicos por ello, nos pareció importante descartar la presencia de estas actividades potencialmente peligrosas en ninguna de las cepas Hereditum.

Otro aspecto importante a tener en cuenta en la evaluación de las cepas bacterianas, es el patrón de resistencia a antibióticos. Cuando las cepas probióticas llegan al intestino, están en contacto tanto con la flora intestinal común como con los posibles patógenos que puedan llegar. Por ello, se debe descartar que la cepa probiótica posea mecanismos que permitan la transferencia de resistencia a antibióticos, pues esto podría dar lugar a la aparición de patógenos multi-resistentes que provocarían infecciones difíciles de controlar. Esto implica estudiar el material genético de la cepa para descartar la presencia, por ejemplo, de plásmidos que codifiquen genes de resistencia. Estos elementos son relativamente comunes en los enterococos (von Wright y Sibakov, 1998) pero mucho menos frecuentes en otras bacterias lácticas, en las que las resistencias suelen estar asociadas a procesos catabólicos o a la composición de membranas y paredes celulares. Gevers y col. (2003) observaron que, algunos lactobacilos podían transferir genes de resistencia a ciertos antibióticos a otras bacterias lácticas pero no a bacterias patógenas como *S.aureus*. Los patrones de resistencia/sensibilidad a antibióticos de las cepas Hereditum, concuerdan con los obtenidos para otras cepas de las mismas especies de lactobacilos en estudios de referencia (Charteris y col., 1998c; Temmerman y col., 2003), no encontrándose resistencias extrañas a la especie. Dada la descripción de casos aislados de infecciones asociadas al consumo de ciertas cepas probióticas, nos

pareció importante además que, estas cepas fueran sensibles a determinados antibióticos que pudieran asegurar la resolución rápida de una posible infección.

Como ya se ha comentado, a pesar de la condición GRAS de las especies de lactobacilos de Hereditum, se recomiendan estudios de toxicidad en animales. El consumo de las cepas procedentes de leche materna ensayadas en ratones con dosis diarias 500 veces superiores a la dosis consumida por los humanos, fue bien tolerado y fue totalmente seguro no produciendo efectos adversos, ni cambios bioquímicos o hematológicos, ni síntomas de infección.

La caracterización exhaustiva de las cepas así como la ausencia de toxicidad en los modelos animales, nos animaron a realizar estudios en humanos, en los que además de analizar la efectividad de las cepas pudimos constatar la ausencia de reacción adversa alguna relacionada con su consumo.

Los resultados obtenidos en esta Tesis nos muestran datos consistentes acerca del consumo de las cepas Hereditum en humanos, datos que corresponden a estudios con un total de entre 15 y 50 voluntarios por cepa, con dosis de entre 10^7 y 10^{10} ufc/día y durante periodos que oscilan entre uno y 3 meses. Estos estudios, junto con estudios posteriores realizados en el Departamento en colaboración con pediatras y el Hospital Materno Infantil de Granada, han demostrado la seguridad de las cepas Hereditum tanto en niños como en adultos. Así por ejemplo, recientemente ha finalizado un estudio de tolerancia en bebés de 6 meses con la cepa *L.salivarius* CECT5713, los cuales han tomando diariamente y durante 6 meses una fórmula infantil que contiene dicha cepa, no observándose efecto adverso alguno. Otro estudio similar se está llevando a cabo con la cepa *L.fermentum* CECT5716, no habiéndose observado hasta la fecha ningún efecto adverso. Por último, las cepas *L.coryniformis* CECT5711 y *L.gasseri* CECT5714 salieron al mercado de forma conjunta en un preparado lácteo (MAX defensas, PulevaFood SA, España; Yox defensis, Alpina, Colombia) y hasta la fecha se han consumido más de 50 millones de dosis sin que se haya notificado ningún efecto adverso.

Por tanto, el conjunto de todos estos datos avala el consumo totalmente seguro de las cepas Hereditum siendo apto e incluso recomendable como veremos más adelante, para su uso en niños.

2. Actividad probiótica de las cepas Hereditum

Una vez seleccionadas estas cepas en base a su potencial probiótico y demostrada su seguridad y tolerancia, el siguiente paso fue demostrar su funcionalidad que justifique su uso en humanos. Para ello, a lo largo de esta Tesis se estudiaron los efectos beneficiosos de estas cepas Hereditum centrándose en los principales efectos beneficiosos demostrados tanto en ensayos preclínicos como clínicos (tabla 2.1).

Tabla 2.1: Efectos beneficiosos de las cepas Hereditum

Cepa	Efecto beneficioso	Referencia
***L.salivarius* CECT5713**	Coloniza el intestino	Martín y col., 2006
	Producción de antibacterianos	Martín y col., 2006
	No produce D-Láctico	Martín y col., 2006
	Efecto antimicrobiano	Olivares y col., 2006
	Efecto inmunomodulador	Díaz-Ropero y col., 2006
	Efecto anti-inflamatorio	Perán y col., 2005
***L.gasseri* CECT5714**	Coloniza el intestino	Martín y col., 2005 Olivares y col., 2006
	Efecto gastrointestinal	Lara-Villoslada y col., 2007 Olivares y col., 2006
	Producción de antibacterianos	Martín y col., 2005
	Efecto antimicrobiano	Olivares y col., 2006
	Efecto inmunomodulador	Olivares y col., 2006 Lara-Villoslada y col., 2007
	Efecto anti-alérgico	Olivares y col., 2005
***L.fermentum* CECT5716**	Coloniza el intestino	Martín y col., 2005 Olivares y col., 2006
	Producción de antibacterianos	Martín y col., 2005
	Efecto antimicrobiano	Olivares y col., 2006
	Efecto inmunomodulador	Díaz-Ropero y col., 2006
	Efecto adyuvante	Olivares y col., 2006
	Efecto anti-inflamatorio	Díaz-Ropero y col., 2006 Perán y col., 2006
***L.coryniformis* CECT5711**	Coloniza el intestino	Martín y col., 2005b Olivares y col., 2006
	Efecto gastrointestinal	Lara-Villoslada y col., 2007 Olivares y col., 2006
	Producción de antibacterianos	Martín y col., 2005b
	Efecto antimicrobiano	Olivares y col., 2006
	Efecto inmunomodulador	Olivares y col., 2006 Lara-Villoslada., 2007
	Efecto anti-alérgico	Olivares y col., 2005

2.1. Actividad antimicrobiana

La protección frente a infecciones víricas o frente a gastroenteritis u otras infecciones bacterianas que afectan al tracto gastrointestinal, es uno de los reclamos más frecuentes asociados al consumo de probióticos. Numerosos estudios han demostrado que, las bacterias del género lactobacilos constituyen una barrera microbiológica frente a las infecciones, poniendo de manifiesto el papel de los probióticos en la prevención y/o tratamiento de infecciones sobre el tracto gastrointestinal (Guarino y col., 1997; Huang y col., 2002; Saavedra y col., 1994; Salminen y col., 1998c). Existen diferentes mecanismos que contribuyen a esta protección, entre las cuales se incluyen la producción de sustancias antimicrobianas, como bacteriocinas o peróxido de hidrógeno, la competición por nutrientes y por la adhesión a las mucinas, etc. Además, la interacción de las bacterias con las células del organismo también influye en la defensa frente a las infecciones a través de mecanismos como la inducción de producción de mucus o la modulación de la respuesta inmune (figura 2.1).

La producción de sustancias antimicrobianas, como ácidos orgánicos, bacteriocinas, peróxido de hidrógeno o reuterina, pueden impedir la presencia y/o crecimiento de patógenos en las mucosas. De hecho, tanto los lactobacilos estudiados en esta Tesis, como otras bacterias aisladas por otros autores a partir de la leche materna (Beasly y Saris, 2004), mostraron un gran potencial para producir algunas de estas sustancias inhibidoras. Aunque no se pudo detectar actividad bacteriocinogénica en los sobrenadantes de las cepas aisladas en este trabajo, una cepa de *Lactococus lactis* aislada de leche materna resultó producir nisina, una bacteriocina activa frente a *Clostridium, Staphylococus, Listeria* y *Bacillus* (Hekkilä y Saris,2003).

Por lo que respecta a los ácidos orgánicos, todas las bacterias analizadas fueron capaces de producir ácido láctico (Martín y col., 2005; 2006). El ácido láctico, además de reducir el pH ambiental, funciona como un permeabilizador de la membrana externa de bacterias Gram-negativas y, por tanto, puede potenciar el efecto de otras sustancias antimicrobianas (Alalomi y col., 2000). En este sentido, Strus y col. (2004) observaron que el efecto bactericida del sobrenadante de bacterias productoras de peróxido de hidrógeno frente a *E.coli* y *Candida albicans* era mayor que el que provoca una cantidad equivalente de peróxido de hidrógeno puro. En el caso de las cepas Hereditum, se observó también un efecto dependiente del pH, pues el efecto

antibacteriano de los sobrenadantes bacterianos a los que se les había neutralizado el pH disminuyó significativamente respecto a los sobrenadantes a los que no se les alteró el pH.

Por otra parte, la presencia de lactobacilos productores de peróxido de hidrógeno en leche materna también resulta muy interesante, ya que, tiene una potente actividad bactericida y por ejemplo se ha descrito que en la mucosa vaginal, este metabolito representa uno de los principales mecanismos de defensa frente a patógenos (Ocaña y col., 1999; Redondo-López y col., 1990); es más, la ausencia de lactobacilos con esta propiedad esta correlacionada con una elevada tasa de infecciones vaginales (Eschenbach y col., 1989; Hawes y col., 1996). Entre nuestras cepas probióticas sólo las cepas de *L.gasseri* (CECT5714 y CECT5715) producen peróxido de hidrógeno y la cantidad producida era similar a la obtenida con *L.johnsonii* La1. La presencia en leche materna de cepas con esta propiedad pudiera tener importancia en la prevención de mastitis en madres lactantes, puesto que, los lactobacilos productores de peróxido de hidrógeno inhiben el crecimiento de *S.aureus*, microorganismo que frecuentemente causa mastitis. De hecho, aunque aún no se tienen datos concluyentes, se ha observado una mejora en madres lactantes con esta patología que han consumido las cepas estudiadas en esta Tesis (Jiménez y col., 2008).

Una peculiaridad importante de una de nuestras cepas probióticas, *L. coryniformis* CECT5711, es la producción de un agente antimicrobiano de amplio espectro, la reuterina, que no se había detectado anteriormente en esta especie. Aunque la inhibición lograda por esta reuterina resultó unas 4-5 veces inferior que la descrita para una cepa de *Lactobacillus reuteri* productora típica, podría contribuir de forma importante a la actividad antimicrobiana de esta cepa.

Todas estas actividades presentes en las cepas Hereditum, son probablemente las responsables de la actividad bactericida frente a una variedad de patógenos intestinales, como se observó en los ensayos *in vitro* (Capitulo 2 de Resultados) y que podrían tener una importante repercusión en la actividad antiinfecciosa de estas cepas.

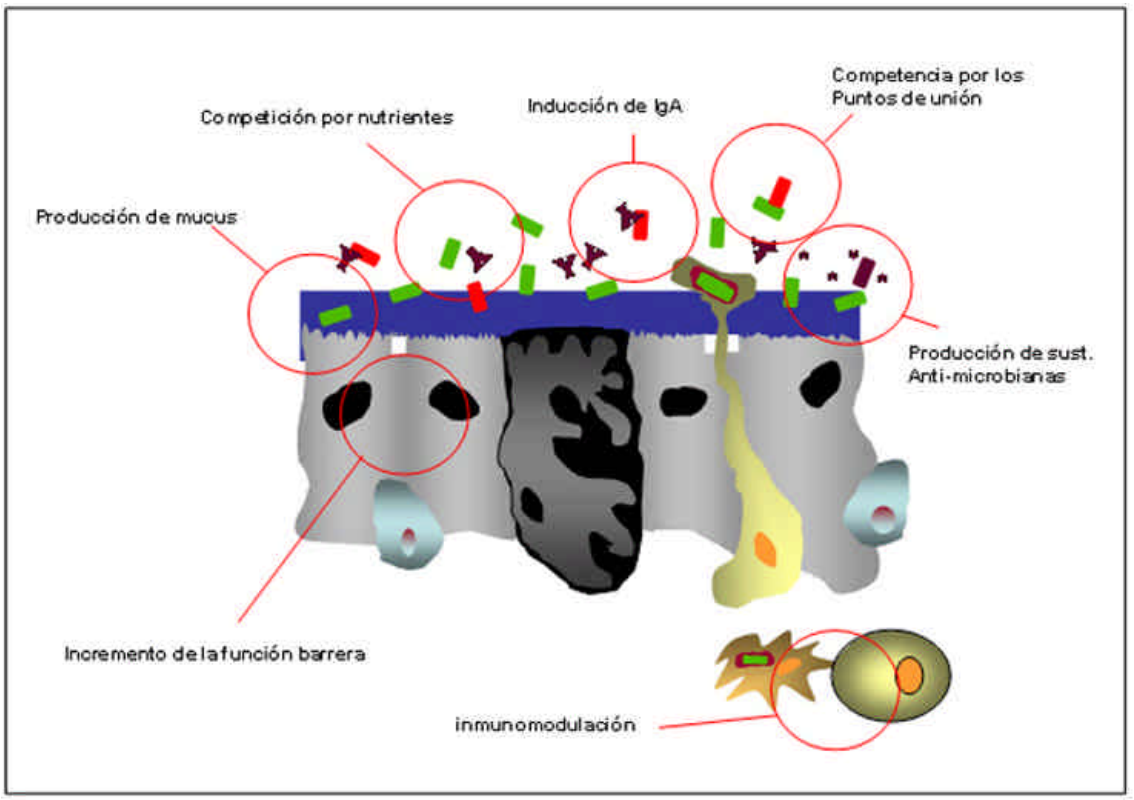

Figura 2.1: Mecanismos de acción utilizados por los probióticos para ejercer un efecto anti-infeccioso a nivel de la mucosa intestinal

Los patógenos, han desarrollado varios mecanismos para asegurar que permanecen asociados con la mucosa intestinal y que resisten ser arrastrados junto con el quimo intestinal. Numerosos experimentos *in vitro* demuestran la capacidad de varias especies de lactobacilos como *L. acidophilus*, *L. johsonii*, *L. casei*, *L. rhamnosus* o *L. gasseri* para inhibir la adhesión a células intestinales de diversos patógenos como *Salmonella* o *E. coli* (Servin, 2004). Aunque, los lactobacilos no poseen factores de adhesión de patógenos, se postula que, son capaces de inhibir la adhesión de patógenos por impedimento estérico a nivel de los sitios de los receptores en el enterocito. Este fenómeno puede producirse en varios momentos del proceso de adhesión, bien patógeno y probiótico compiten al mismo tiempo (fenómeno de competición), bien la bacteria probiótica ya adherida ocupa los lugares de unión del patógeno impidiendo su unión (fenómeno de exclusión) o bien el probiótico es capaz de desplazar al patógeno ya adherido (fenómeno de desplazamiento). Estos fenómenos son fácilmente observados en ensayos *in vitro* en los que se mide la adhesión a mucinas intestinales de patógenos marcados con fluoresceína añadido al

adhesión a mucinas intestinales de patógenos marcados con fluoresceína añadido al mismo tiempo, antes o después de la bacteria probiótica. Todos estos mecanismos se estudiaron *in vitro* con nuestras cepas aisladas de leche materna observándose que, en diferentes grados, todas ellas eran capaces de inhibir la adhesión del patógeno *Salmonella* marcado con fluoresceína a una capa de mucina intestinal. Pudimos ver que, la cepa más efectiva en este mecanismo de inhibición de la adhesión de patógenos a mucinas intestinales fue, *L. salivarius* CECT5713.

De hecho, se han observado en algunas de las cepas Hereditum fenómenos de agregación y de inducción de producción de mucinas, mecanismos que impedirán el acceso de los patógenos al epitelio intestinal.

Todas las cepas Hereditum están por tanto dotadas en mayor o menor medida, de una capacidad antimicrobiana que se lleva a cabo a través de diversos mecanismos y que podrían tener mucha importancia a la hora de prevenir o atenuar determinadas infecciones (figura 2.1). Pero estos fenómenos que se miden de forma aislada *in vitro,* podrían variar *in vivo* afectados por múltiples factores como son el flujo del bolo fecal que continuamente lava el epitelio intestinal y que, podría afectar a la capacidad de adhesión de las bacterias.

Diversos modelos de infección por *E. coli*, *Salmonella*, *Listeria*, *Helicobacter* o rotavirus desarrollados en animales, han demostrado la mayor o menor eficacia de cepas probióticas de *L. casei*, *L. rhamnosus*, *L. salivarius* (Ishida-Fujii y col., 2007; Johnson-Henry y col., 2004; Sato y col., 1988). En humanos, numerosos estudios, han demostrado la eficacia de determinadas cepas probióticas en la prevención y/o atenuación de enfermedades infecciosas intestinales siendo este efecto beneficioso de los más reconocidos para los probióticos (Van Niel y col., 2002).

En esta Tesis llevamos a cabo un modelo de infección por *Salmonella* en ratón observándose que, de acuerdo con los resultados obtenidos *in vitro*, la administración oral de las cepas Hereditum aumentaba significativamente la supervivencia de los ratones infectados con el patógeno intestinal, alcanzándose en alguno de los casos una supervivencia del 80% respecto al 10% observado en el grupo control. Se demuestra así que, las propiedades anti-infectivas de las cepas evaluadas se traducen en un efecto real de protección frente infecciones intestinales. Estudios posteriores en individuos que sufran de infección intestinal o con alto riesgo de padecerla, deberán

aclarar la repercusión real del consumo de estas cepas probióticas sobre este tipo de enfermedades.

La presencia en la leche materna de bacterias con una capacidad anti-infecciosa demostrada, tiene una importancia biológica muy importante por la repercusión que tiene sobre la salud del niño. De hecho, está demostrado que la lactancia materna está relacionada con la reducción en la incidencia y severidad de infecciones en el lactante (Gilliland y col., 1977; Ozbas y col., 1995). Además, el hecho de que la pasteurización de la leche materna le haga perder en gran parte sus propiedades protectoras, concuerdan con el papel de las bacterias probióticas en la capacidad anti-infecciosa protectora de la leche materna.

2.2. Efectos gastrointestinales: función intestinal.

Las bacterias que constituyen la microbiota intestinal son de gran importancia para el mantenimiento de la salud y el bienestar del consumidor. Además de la función nutritiva que la microbiota ejerce al metabolizar nutrientes que la maquinaria enzimática humana no puede, a nivel local, la microbiota también influye de forma muy importante en la funcionalidad intestinal estando involucrada en los procesos de regeneración de la mucosa y epitelio intestinal, motilidad y protección frente a infecciones (Falk y col., 1998).

La administración de probióticos, ha sido muy estudiada en las últimas dos décadas precisamente como, medio para modificar la microbiota intestinal e influir en las funciones fisiológicas en las que está implicada a través de la regulación de parámetros gastrointestinales y modulando la absorción de nutrientes. Trabajos tanto en animales (Perán y col., 2005), como los descritos en esta Tesis, han permitido demostrar la capacidad colonizadora intestinal de las cepas aisladas a partir de la leche materna, dando lugar a un aumento de los recuentos de lactobacilos y la subsiguiente modulación de la microbiota intestinal.

La modificación de la microbiota influirá en determinadas actividades enzimáticas y fermentativas a nivel intestinal que suponen un incremento en la producción de metabolitos funcionales como, los ácidos grasos de cadena corta tales como el butirato (Rizkalla y col., 2000). El butirato, es la principal fuente energética para los enterocitos colónicos y también uno de los principales moduladores de los hábitos intestinales al influir sobre la motilidad, absorción de agua y electrolitos y

presión osmótica (Roberfroid y col., 1995). La variación en la producción de butirato no es un parámetro muy estudiado en los estudios realizados con probióticos quizá debido a que, no son microorganismos productores de butirato *per se*. Sin embargo, sí que se ha relacionado un incremento de lactobacilos y bifodobacterias con un incremento en la producción de butirato probablemente debido a la actividad de bacterias como *M.elsdenii* que, utilizan el lactato producido por las bacterias ácido lácticas para producir butirato (Tsukahara y col., 2002).

En un estudio realizado en niños alimentados con una fórmula infantil suplementada con la cepa *L.rhamnosus* LGG, durante los seis primeros meses de vida se ha observado que, el consumo de este producto podía mejorar la tasa de crecimiento de los bebés (Vendt y col., 2006), lo cual puede estar relacionado con una mayor eficiencia en la absorción de nutrientes en estos niños.

En el caso de las cepas aisladas de leche materna en esta Tesis, su consumo indujo, un incremento en los niveles de butirato colónico en voluntarios que podría ser la causa de los beneficios observados en diversos parámetros gastro-intestinales, como pueden ser el incremento en el contenido de agua fecal o el aumento en la frecuencia y volumen de defecación, además, la mejora en estos parámetros fue considerada por parte de los voluntarios como una mejora en su habito intestinal. En un estudio realizado con la cepa *L.casei Shirota,* no se detectó un incremento en la concentración de butirato aunque sí que se observó un aumento en el tránsito intestinal y en el contenido de agua fecal (Spanhaak y col., 1998). Recientes estudios en niños, han mostrado la mejora de problemas de estreñimiento tras el consumo de cepas probióticas, sin embargo, es necesario la realización de más estudios que corroboren los resultados y que además estudien los mecanismos implicados en el fenómeno (Bekkali y col., 2007; Bu y col., 2007).

El aumento del bolo fecal, un mayor contenido en agua que diluye las heces así como el aumento de la velocidad del tránsito intestinal, se consideran positivos en la prevención de fenómenos cancerígenos al disminuir la concentración de agentes carcinogénicos y su tiempo de contacto con la mucosa intestinal (Cummings y col., 1992, Weisburger y col., 1993). El aumento en el contenido de agua de las heces de los voluntarios que consumieron las cepas Hereditum junto con, las posibles alteraciones en el metabolismo enzimático fruto de la modificación de la flora, podría explicar la reducción en la toxicidad de las aguas fecales observada en los voluntarios que consumieron las cepas probióticas Hereditum. Este efecto protector es difícil de

evaluar en estudios clínicos pues sería observable a largo plazo, lo que requiere estudios muy largos y con un gran número de participantes con el fin de analizar un efecto sobre el riesgo de padecer este tipo de fenómenos cancerígenos.

Estudios posteriores realizados por nuestro equipo de investigación, no incluidos en la presente Tesis, en niños de 3 a 12 años, principales consumidores de este tipo de productos, demuestran que las cepas de leche materna probadas son capaces de colonizar y modular los hábitos intestinales también en este grupo poblacional (Lara-Villoslada y col., 2006). Además, en este estudio se demuestra que la modulación de la microbiota intestinal puede ejercer un efecto beneficioso, reduciendo la toxicidad de las aguas fecales de aquellos voluntarios que consumen dichas cepas probióticas.

2.3. Efectos inmunomoduladores

La microbiota intestinal está en estrecho y continuo contacto con células epiteliares y del sistema inmunitario, resultando en una estimulación esencial para el correcto funcionamiento de la respuesta inmunitaria. De hecho, el tipo de bacterias que colonizan el intestino de los recién nacidos va a determinar la modulación de su sistema inmunitario, pues actúan como importantes estímulos antigénicos en el proceso de maduración de la respuesta (Holt, 1995; Sudo y col., 1997). En las últimas décadas, se ha detectado un gran incremento en la incidencia de enfermedades derivadas de un desequilibrio de la respuesta inmunológica como pueden ser las alergias, inflamaciones crónicas o enfermedades de tipo autoinmune (figura 2.2). Para intentar explicar este fenómeno surgió “la hipótesis de la higiene”. Dicha hipótesis achaca este fenómeno, a un menor contacto de los niños con antígenos bacterianos y virales debido a los actuales hábitos de higiene, alimentación y médicos (Strachan, 2000). Se argumenta que los avances en la higiene personal (junto al avance de la medicina anti-infecciosa y la prevención) han llevado a una disminución en la incidencia de infecciones en los estadios iniciales del desarrollo. Dicha disminución de la tasa de infección, ha coincidido con el aumento progresivo en el último siglo de enfermedades como las alergias y enfermedades relacionadas con una desregulación del sistema inmunitario, como la enfermedad inflamatoria intestinal (figura 2.2).

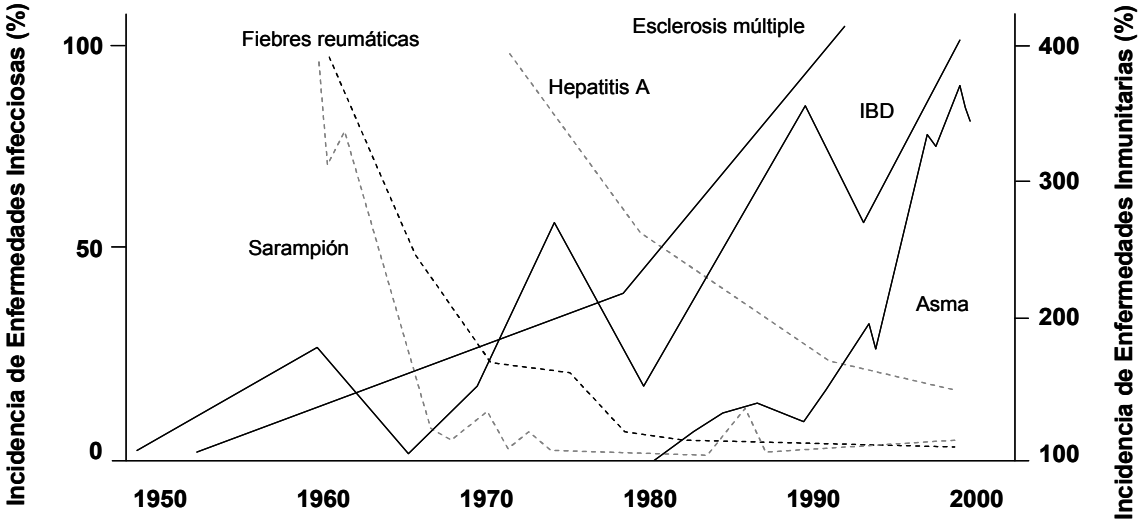

Figura 2.2: Evolución de la incidencia de enfermedades infecciosas y de enfermedades relacionadas con alteraciones del sistema inmunitario.

Aunque dicha teoría pueda parecer rocambolesca, la explicación inmunológica a dicha observación inicial apareció con el descubrimiento de la dualidad en las respuestas linfocitarias Th1 y Th2 (Mosmann y Coffman, 1989). En los bebés, el perfil de citocinas está polarizado hacia las respuestas de tipo Th2, la producción de IgE y la posible sensibilización hacia procesos atópicos (Holt y col., 1990), debido a las hormonas y factores reguladores que se producen durante la gestación, entre ellos la prostaglandina E2. Además, la mayor permeabilidad intestinal durante el periodo neonatal, el retraso en la generación de IgA y la inmadurez de la tolerancia oral en el recién nacido, son factores que pueden favorecer el paso de antígenos que puedan escapar de la tolerancia y desencadenar una respuesta de tipo alérgico (Isolauri, 1997).

La activación de la respuesta inmunitaria de tipo Th1 por la microbiota y otros antígenos medioambientales, va a equilibrar la respuesta evitando ese predominio Th2 característico de los neonatos. A pesar de esto, la conclusión de dicha teoría no es permitir que los niños se infecten para evitar esas enfermedades en el futuro, aunque si ampliásemos el término “infección”, nos acercaríamos a una posible alternativa. Lo que concluye dicha teoría es que los estilos de vida modernos han provocado una modificación de la microbiota intestinal, la cual es directamente responsable de la modulación del sistema inmunitario neonatal. Por tanto, una mejora en la calidad de la microbiota intestinal podría ser una alternativa terapéutica válida para reducir el riesgo de determinadas enfermedades en el adulto.

Avalando la importancia de la microbiota intestinal sobre el correcto funcionamiento de la respuesta inmunológica, en los últimos años se han detectado determinados cambios en los grupos bacterianos en la microbiota de niños alérgicos respecto a niños sanos (Bjorksten y col., 1999; Kalliomaki y col., 2001; Penders y col., 2007), así como también en pacientes de enfermedad inflamatoria intestinal (IBD) (Seksik y col., 2003; Sokol y col., 2006).

El mecanismo completo a través del cual la microbiota ejerce su efecto sobre el sistema inmunitario no es totalmente conocido aunque, se sabe que células del sistema inmunitario como dendríticas, monocitos y macrófagos y los propios enterocitos participan en el desarrollo de la respuesta (figura 2.3). Estas células, reconocen patrones moleculares conservados en estructuras bacterianas a través de los receptores TLR (*Toll-like Receptors*). La activación de estos receptores de membrana, induce la activación de factores de transcripción que conducen a la producción de citocinas, que son responsables de la diferenciación de las células T hacia Th1, Th2 o T reguladoras (D´Andrea y col., 1993; Takeuchi y col., 1999).

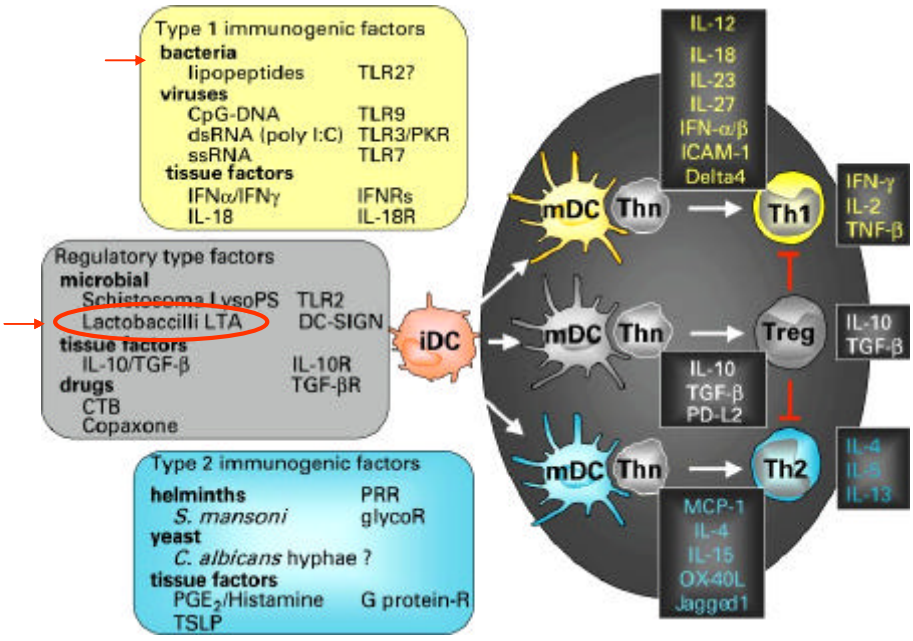

Figura 2.3: Las células dendríticas controlan los fenómenos de tolerancia e inmunidad. Las células dendríticas maduran de diferente manera dependiendo del antígeno que las active. Ya en los ganglios linfáticos activan a los linfocito Th naïve induciendo su maduración hacia linfocitos Th1, Th2 o Treg.

Como ya se ha comentado en la Introducción, la colonización bacteriana es un largo y complejo proceso que se inicia en el nacimiento del niño o incluso antes como se revela de las últimas investigaciones realizadas sobre, la presencia de bacterias en sangre de cordón umbilical y meconio (Jiménez y col., 2005; Jiménez y col., 2008). Durante los dos primeros años de vida es cuando se producen los mayores cambios en la microbiota y ya a partir de los dos años se mantiene relativamente estable hasta la vejez, donde se producen importantes alteraciones. Sin embargo, a pesar de esta relativa estabilidad existen factores moduladores ya sean intrínsecos al individuo o extrínsecos tales como la carga ambiental, dieta o medicación, que pueden inducir una cierta variación. Entre estos factores externos resalta la dieta, que a través de los productos fermentados supone una fuente importante de bacterias, su importancia queda claramente reflejada en el estudio que describimos en esta Tesis, en el que observamos como, la eliminación de productos fermentados en la dieta provocaba importantes cambios en la respuesta inmunitaria de individuos sanos. Estos resultados

evidencian que, el descenso en el consumo de alimentos fermentados que las nuevas tecnologías y modas están imponiendo en la dieta occidental podrían llegar a, suponer un problema en la salud y debería ser tomado en cuenta.

Los lactobacilos son un componente importante de la microbiota del intestino y numerosos estudios en humanos han demostrado su capacidad para modular la respuesta inmunitaria, pudiendo afectar a la incidencia y/o a la gravedad de diversas condiciones patológicas, entras las que se pueden incluir procesos alérgicos o inflamatorios (Björksten y col., 1999; Cross, 2002a;2002b; Gill, 1998; Salminen y col., 2000).

Aunque las respuestas inmunitarias son respuestas muy complejas resultado de la activación de diferentes tipo celulares, estudios *in vitro* de estos mecanismos en macrófagos se han utilizado como instrumento para dar idea del carácter inmunomodulador de las bacterias probióticas pues, dependiendo del patrón de citocinas inducidas por la bacteria, se puede predecir el tipo de respuesta que inducirá dicha bacteria en el organismo. Sin embargo, el modelo tiene sus limitaciones pues el efecto de una cepa probiótica va a depender no solo de sus características intrínsecas sino también del contexto de la microbiota en la que se encuentre y del contexto del individuo sobre el que actúa, factores que deberán tenerse en cuenta a la hora de interpretar los resultados obtenidos *in vitro*. Así, diversos estudios han mostrado como diferentes especies de lactobacilos ejercen muy diferentes patrones de activación e incluso en tratamientos conjuntos se producen efectos agonistas o antagonistas. Es el caso de los estudios realizados por Christensen y col. (2002) que muestran que la capacidad de una cepa de *L. casei* para inducir la producción de citocinas pro-inflamatorias como IL-12 y TNF-a es inhibida por *L. reuteri* DSM12246, probablemente debido a la inducción de IL-10. En base a esta alta producción de IL-10 ellos sugieren que esta cepa de *L. reuteri* podría ser beneficiosa en situaciones de inflamación crónica como IBD.

En el caso de las cepas Hereditum, los resultados obtenidos en esta Tesis muestran que las cepas aisladas de leche materna son capaces de modificar la respuesta de macrófagos derivados de médula ósea (BMDM) de ratón tanto en condiciones basales como en presencia de un activador como el LPS. Los resultados muestran cómo el efecto producido es de nuevo dependiente de la cepa, observándose efectos dispares. Así, *L.salivarius* CECT5713 destaca por su capacidad para activar los macrófagos induciendo una respuesta en la que predomina la

producción de IL-10 respecto a citocinas pro-inflamatorias como IL-12 y TNF-alpha incluso tras la estimulación con LPS. La respuesta inducida por *L.fermentum* CECT5716 tiene sin embargo un mayor carácter pro-inflamatorio, aunque en presencia de LPS es capaz también de atenuar la respuesta inflamatoria inducida por este compuesto en los macrófagos. Esta capacidad para activar la respuesta inmunitaria de forma diferente según el estado de activación, ya ha sido descrito anteriormente en otras cepas probióticas en las que se han observado efectos contrapuestos en individuos sanos y enfermos (Isolauri y col., 2001).

Se pone de manifiesto así, la singularidad de cada cepa con diferentes propiedades cualitativas y cuantitativas que además se verán afectadas por la microbiota presente en el intestino y por el estado de activación del organismo con el que interactúen. La peculiaridad de cada cepa en cuanto a su actividad, debería ser valorada y usada para el diseño de tratamientos específicos en los que la combinación de cepas pueda significar un efecto más amplio o potente. Esto es importante puesto que, una tendencia actual en el mercado es la de usar mezclas de probióticos pero en estas mezclas se debería valorar la posible interacción entre las cepas que puede ser positiva o no.

La presencia en leche materna de bacterias con diferentes perfiles inmunomoduladores, podría tener además un papel interesante en el proceso de maduración de la respuesta inmunitaria del niño muy influenciado por la flora presente en su intestino y que requiere una fina regulación para la correcta maduración del sistema inmunitario.

Los resultados inmunomoduladores de las cepas Hereditum in vitro, se han visto también confirmados en los estudios realizados en animales. Estos estudios nos han permitido estudiar la respuesta global del organismo al contacto con la bacteria, tanto en condiciones de salud como en condiciones patológicas en modelos de inflamación crónica o infección. Así, coincidiendo con el perfil observado *in vitro*, *L.salivarius* CECT5713 mostró en animales sanos un carácter anti-inflamatorio/regulatorio mediado por el incremento significativo de la citocina reguladora IL-10 que se tradujo en, un efecto beneficioso en un modelo de inflamación intestinal en ratas provocada por TNBS, donde la administración de dicha cepa protegió del daño inflamatorio característico de este modelo (Perán y col., 2005). La capacidad de esta cepa para inducir la producción de IL-10, será probablemente la responsable del efecto ya que en este tipo de enfermedad el papel de IL-10 es muy

importante para intentar atenuar la fuerte respuesta inflamatoria y, de hecho, es una de las terapias biológicas que se están estudiando en la actualidad (Li y He, 2004).

Por otro lado, *L.fermentum* CECT5716, actuó como un agente inmunoestimulante en ratones sanos evidenciado por un incremento tanto en la respuesta innata como en la específica. En un estudio posterior no presentado en esta Tesis realizado en el modelo de inflamación intestinal, se observó el carácter anti-inflamatorio de esta cepa previniendo en gran medida la respuesta inflamatoria provocada por el TNBS caracterizada por un claro predominio de citocinas proinflamatorias (Perán y col., 2006). Probablemente, el efecto de esta cepa sea consecuencia de la capacidad inmunomoduladora de *L.fermentum* CECT5716 que en situación de sobre-estimulación, al contrario que en una situación basal, reduce la respuesta inflamatoria tal y como se observó en los estudios realizados *in vitro* con macrófagos sobre-estimulados con LPS. También a este hecho se podría sumar la habilidad de esta cepa para producir glutation, un antioxidante natural importante en el mantenimiento de la integridad de las mucosas y que jugaría un papel fundamental en prevenir el daño producido por las especies reactivas de oxígeno que se producen en este modelo y que son responsables del daño en los tejidos (Perán y col., 2006).

Cepas de *L.casei* o *L.fermentum* o la mezcla probiótica VSL#3® han dado resultados muy prometedores en modelos de inflamación crónica en animales de experimentación (Bibiloni y col., 2005; Chapman y col., 2006; Fitzpatrick y col., 2007; Gaudier y col., 2005; Geier y col., 2007), sin embargo, los resultados en estudios con humanos no han sido tan claros. Esta variabilidad en los resultados podría ser causada, al menos en parte, a que la etiología de la Enfermendad Inflamatoria Intestinal no está muy clara y a que engloba a un grupo heterogéneo de enfermedades que tienen una manifestación final común: la presencia de inflamación (Podolsky, 2002). Esto hace que dentro de un mismo estudio haya mucha variabilidad en cuanto a la patología se refiere. Se necesita avanzar más en cuales son los mecanismos de la enfermedad y de qué manera están actuando aquellas cepas en las que se observa un efecto beneficioso para llegar a definir un tratamiento efectivo.

A la vista de los resultados obtenidos tanto *in vitro* como en modelos animales, el último paso es la demostración del efecto beneficioso de cada cepa en humanos. En adultos sanos las cepas probióticas estudiadas han demostrado un efecto inmunomodulador con un aumento en la respuesta innata, que abarca desde un incremento en la proporción de células *Natural Killer* a un aumento en la capacidad

fagocítica de monocitos y granulocitos. También, hemos podido ver un aumento en la respuesta específica evidenciada por un incremento en la concentración de inmunoglobulinas tales como la IgA. Todo apunta, por tanto, a que el consumo de estas cepas mantendría las defensas inmunológicas en un nivel de activación mayor que debería mejorar la respuesta en el caso de que se produjese la amenaza de una infección. En los probióticos no se ha estudiado con mucho detalle la relación entre una estimulación de la respuesta inmunitaria y la mayor protección frente a infecciones. Sin embargo, se ha observado que en individuos sanos el consumo de cepas de *L.casei* y *L.rhamnosus* con efectos inmunoestimulantes tiene efectos moderados en la prevención o atenuación de enfermedades banales (Hatakka y col., 2001; Turchet y col., 2003), así como, una relación entre el incremento de IgA y el efecto protector de bacterias probióticas en diarreas (Valeur y col., 2004; Yasui y col., 1999). La combinación de los efectos inmunomoduladores con los efectos antibacterianos y de mejora de la barrera física en las mucosas supone que, las cepas probióticas pueden ejercer un potente efecto anti-infeccioso protegiendo de la infección desde varios frentes. Futuros estudios en humanos susceptibles o en riesgo de infección deberán corroborar esta hipótesis.

Otras evidencias que apuntan al efecto beneficioso de la inmunoestimulación ejercida por el consumo de probióticos, es la mejora de la respuesta a vacunas. Puesto que, la administración oral de bacterias ácido lácticas eleva las respuestas inmunitarias innata y adaptativa implicada en la defensa frente a infecciones (Isoulari y col., 2001; Schiffrin y col., 1995) se ha empezado a considerar el posible uso de estas bacterias como coadyuvantes en procesos de vacunación (de Vrese y col., 2005b; Hori y col., 2002; Perdigón y col., 1991). E incluso en los últimos años, se ha prestado gran atención a la posibilidad de emplear bacterias lácticas como vectores vacunales (Hanniffy y col., 2004; Mercenier, 1999; Pouwels y col., 1998; Wells y col., 1996). *L.fermentum* CECT5716 mostró, tanto *in vitro* como *in vivo* en ratones, unas características inmunoestimulantes que podrían ser útiles en un proceso de vacunación. Una vacuna de uso muy extendido pero con una efectividad limitada susceptible de ser mejorada, es la vacuna de la gripe, por lo que nos pareció un modelo adecuado para estudiar la capacidad coadyuvante de la cepa probiótica. En esta Tesis pudimos ver como la administración de la cepa *L fermentum* CECT5716 como coadyuvante sobre la vacuna de la gripe, potenció la respuesta inmunitaria desencadenada en un proceso de vacunación frente a la gripe, activando tanto la respuesta innata como la específica. La respuesta innata, es la primera que tiene lugar frente a la entrada de un antígeno y conduce hacia la activación de la respuesta

específica. Probablemente, el incremento en la respuesta innata en los individuos que tomaron la cepa probiótica contribuyó a una mejor respuesta celular que se manifestó en un patrón de respuesta de tipo Th1 y en una más efectiva producción de anticuerpos antígeno-específicos frente a los antígenos virales.

Como ya se ha comentado anteriormente, se ha observado una relación entre microbiota y desequilibrios de la respuesta inmune como la alergia caracterizada por un exceso en la respuesta Th2 (Björksten y col., 1999). La relación de la microbiota con esta enfermedad también se ha puesto en evidencia, al comprobar alteraciones en la composición de la microbiota intestinal de niños alérgicos y que el tratamiento con determinadas cepas probióticas consigue buenos resultados en la prevención de la aparición de esta enfermedad en niños con riesgo de padecerla (Kalliomaki y col., 2001a). En este sentido, el estudio presentado en esta Tesis con las cepas *L.gasseri* CECT5714 y *L.coryniformis* CECT5711 en adultos alérgicos al polen de gramíneas y de olivo, mostró que el consumo de estas dos cepas probióticas atenuó la respuesta alérgica a través del efecto sobre alguno de los parámetros inmunológicos involucrados en este tipo de respuesta como una reducción en los niveles de IgE y en la liberación de mediadores alérgicos así como de citocinas inductoras de la respuesta alérgica como la IL-4. Recientemente, se ha realizado un estudio con niños alérgicos con estas dos cepas Hereditum en el cual se observa que, si bien la respuesta inmunitaria que media la reacción alérgica es atenuada, las defensas inmunológicas no disminuyen, observándose un aumento tanto de la respuesta innata como de la respuesta específica mediada por anticuerpos (Martínez-Cañavate y col., 2008).

Además, del carácter inmunoregulador de las cepas probióticas que como hemos visto es capaz de modular la respuesta alérgica, las bacterias lácticas pueden también intervenir degradando antígenos alergénicos e impidiendo así la activación de la respuesta alérgica frente a ellos (Sütas y col., 1996). En el Capitulo 9 de Resultados de esta Tesis Doctoral, se expone cómo la hidrólisis de proteína de leche de vaca por *L.coryniformis* CECT5711 redujo sensiblemente la alergenicidad de la leche en un modelo murino. A pesar de que el grado de hidrólisis fue menor que el producido por una hidrólisis enzimática convencional, la alergenicidad fue menor en el hidrolizado bacteriano probablemente debido al efecto inmunomodulador aportado por la bacteria probiótica. Esta capacidad proteolítica de las bacterias probióticas junto con su carácter inmunomodulador, es la base de los estudios que se están llevando a cabo en el campo de la enfermedad celiaca donde ya hay estudios que reclaman un efecto beneficioso para determinadas cepas probióticas (Di Cagno y col., 2004).

Como hemos visto, la microbiota intestinal ha ido adquiriendo a lo largo de su coexistencia con el hombre diversas funciones que a día de hoy son fundamentales para el correcto funcionamiento del organismo. La posibilidad de influir sobre esta microbiota mediante el uso de bacterias probióticas, ha abierto un campo de investigación que nos está descubriendo las múltiples y variadas posibilidades que ofrecen las bacterias probióticas no sólo en nutrición si no también en el campo de la farmacia. Los estudios realizados en los últimos años junto con los presentados en esta Tesis demuestran la singularidad de cada cepa, indicando la necesidad de estudiar en profundidad las características propias de cada una, de forma que su selección para una determinada aplicación esté basada en sus propiedades particulares y en las condiciones de salud del consumidor al que vaya dirigido su consumo.

A lo largo de esta Tesis se ha evaluado el potencial probiótico de los lactobacilos seleccionados entre los aislados de leche materna y queso de cabra, mediante pruebas *in vitro* ampliamente aceptadas (FAO/WHO, 2002) y que, en general, se suelen correlacionar con efectos probióticos *in vivo* (Dunne y col., 2001). Sin embargo, a pesar de ser de gran utilidad en las primeras etapas de selección, los estudios *in vitro* o con animales de experimentación no se consideran suficientes para asegurar la eficacia y la seguridad de estas bacterias, resultando imprescindible la realización de ensayos clínicos. Los ensayos clínicos realizados con las cepas Hereditum y desarrollados en esta Tesis, han demostrado efectos beneficios sobre la función intestinal, la función inmunológica así como sobre la barrera de protección frente a infecciones intestinales mostrando las peculiaridades de cada cepa.

La presencia de estas bacterias probióticas en la leche materna, parece ser un mecanismo más que la madre usa para proteger al niño en sus primeros meses de vida cuando es más susceptible a las infecciones. Además, puesto que la maduración del sistema inmunitario es dependiente de la flora instaurada en el niño, la transferencia de bacterias inmunomoduladoras a través de la leche materna influiría en este proceso de maduración, ayudando a prevenir la aparición de futuras enfermedades en el niño desencadenadas por desequilibrios de la respuesta inmunológica causados por, un deficiente proceso de maduración o aprendizaje del sistema inmunitario. En los últimos años, con el fin de minimizar los problemas derivados de desequilibrios en la flora observados en los niños alimentados con fórmula infantil que, se acompañan de un mayor riesgo de infecciones y de alergia, se

está empezando a introducir en las fórmulas infantiles bacterias probióticas de diversos orígenes. El uso de cepas probióticas aisladas de leche materna con demostrado efecto beneficioso, se presenta como una excelente alternativa para la suplementación de estas fórmulas infantiles con el fin de asemejarlas aún más a la leche materna.

Por otro lado, hemos visto como una dieta deficiente en productos fermentados puede disminuir la capacidad de respuesta inmunológica. Los actuales hábitos de alimentación y nuevas tecnologías de preparación y conservación de los alimentos implican un menor consumo de bacterias. El uso de alimentos funcionales ricos en bacterias con demostrados efectos beneficiosos o de suplementos probióticos, ayudaría a contrarrestar dicho fenómeno.

Conclusiones

Las conclusiones generales que se pueden obtener a partir de los resultados mostrados en esta Tesis Doctoral pueden resumirse como:

1. La administración oral prolongada de dosis muy altas de *L.salivarius* CECT5713, no conlleva ningún riesgo para los animales. Además, la simulación de una eventual translocación no indujo enfermedad en los animales. Estos resultados junto con las características intrínsecas de la bacteria, avalan la seguridad de *L.salivarius* CECT5713 para su uso en humanos.

2. Las cepas Hereditum presentan efectos beneficiosos en modelos preclínicos tanto en estudios *in vitro* como *in vivo.* Así:

   2.1. La capacidad antibacteriana de cada cepa Hereditum se debe a un proceso multifactorial en el que se implican diferentes mecanismos tales como, la producción de sustancias antimicrobianas, inhibición de adhesión a mucinas y el incremento de la expresión de mucinas. La conjunción de estas actividades son probablemente las responsables del efecto protector de estas cepas en el modelo de infección por *Salmonella choleraesuis* CECT4155 en ratones.

   2.2. Todas las cepas Hereditum son capaces de modular la respuesta inmunitaria aunque el efecto difiere de unas cepas a otras, siendo una característica propia de cada una de ellas. El efecto es incluso dependiente del estado de activación del sistema inmune con el que interactúan, observándose en todas las cepas una tendencia a equilibrar la respuesta en condiciones de sobre-estimulación.

   2.3. El tratamiento con *L.salivarius* CECT5713 previene la inflamación en los tejidos provocada en un modelo de colitis experimental por TNBS en ratas. El efecto es probablemente debido a la capacidad de la cepa para modular la respuesta inmunitaria, evidenciada por una disminución significativa en la producción de mediadores inflamatorios como el TNF-a.

3. Las cepas Hereditum son capaces de ejerces efectos beneficiosos sobre voluntarios.

3.1. La importancia del aporte de bacterias ácido lácticas en la dieta es evidenciada por, la alteración en la función intestinal y en la respuesta inmunitaria observada tras la eliminación de productos fermentados en la dieta de voluntarios sanos. La incorporación a la dieta de un producto fermentado que contiene, *L.gasseri* CECT5714 y *L.coryniformis* CECT5711, fue capaz de contrarrestar dichos efectos.

3.2. La incorporación a la dieta de individuos sanos de un producto fermentado que contiene, *L.gasseri* CECT5714 y *L.coryniformis* CECT5711, modificó positivamente algunos parámetros gastrointestinales que se tradujeron en la mejora del hábito intestinal de los voluntarios.

3.3. La incorporación a la dieta de individuos sanos de un producto fermentado que contiene, *L.gasseri* CECT5714 y *L.coryniformis* CECT5711, activó tanto la respuesta inmunitaria innata como la específica.

3.4. La administración oral de *L.fermentum* CECT5716 en adultos sanos, potenció la respuesta frente a la vacuna de la gripe incrementando tanto la respuesta Th1 principal involucrada en la defensa frente al virus, como la producción de anticuerpos específicos frente a antígenos virales.

3.5. Las bacterias probióticas pueden ser efectivas en el tratamiento y prevención de la alergia a través de diferentes mecanismos tales como, la hidrólisis de proteínas antigénicas o la modulación de la respuesta inmunitaria. La administración del producto fermentado con la mezcla de probióticos, *L.gasseri* CECT5714 y *L.coryniformis* CECT5711 a individuos alérgicos, redujo la respuesta alérgica evidenciada por la reducción en parámetros inmunológicos involucrados en este tipo de respuesta.

Como conclusión general podemos decir que, las cepas Hereditum han demostrado a nivel preclínico y clínico su funcionalidad, mejorando parámetros relacionados con la función intestinal y la del sistema inmune que podrían traducirse en una mejora del estado de bienestar y de salud del consumidor. Cada cepa es sin embargo singular y su uso para una determinada aplicación deberá basarse en el conocimiento de las capacidades que presenta dicha cepa.

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