UNIVERSIDAD DE GRANADA

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DEPARTAMENTO DE FARMACOLOGIA

EFECTO ANTIINFLAMATORIO INTESTINAL DE PROBIÓTICOS EN EL MODELO DE COLITIS EXPERIMENTAL INDUCIDA POR ÁCIDO TRINITROBENCENOSULFÓNICO EN RATAS

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Tesis doctoral para aspirar al Grado de Doctor en Farmacia que presenta la Licenciada Dña. Laura Perán Montero

2007

Departamento de Farmacología

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Certifica: Que el trabajo de Tesis Doctoral titulado: "Efecto antiinflamatorio intestinal de probióticos en el modelo de colitis experimental inducido por ácido trinitrobencenosulfónico en ratas" ha sido realizado por la Licenciada en Farmacia Laura Perán Montero en los laboratorios de este departamento.

Y a los efectos legales se firma la siguiente constancia en Granada, a veintisiete de Marzo de 2007.

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Certifican: Que la Tesis Doctoral titulada: "Efecto antiinflamatorio intestinal de probióticos en el modelo de colitis experimental inducido por ácido trinitrobencenosulfónico en ratas" presentada por la Licenciada en Farmacia Laura Perán Montero, ha sido llevada a cabo bajo su dirección y reúne todos y cada uno de los requisitos necesarios para ser defendida y optar al grado de Doctor.

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Dr. Antonio Zarzuelo Zurita

Dr. Julio Gálvez Peralta

Dr. Jordi Xaus Pey

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INTRODUCCIÓN

1. ENFERMEDAD INFLAMATORIA CRÓNICA DEL INTESTINO.

1.1. ASPECTOS GENERALES.

La enfermedad de Crohn (EC) y la colitis ulcerosa (CU) son enfermedades inflamatorias intestinales (EII) de etiología desconocida y de curso crónico y recurrente, con períodos de exacerbación de los síntomas seguidos de intervalos más o menos prolongados de remisión de los mismos.

Aunque se ha progresado en la caracterización de la patogenia de estas enfermedades, su causa primaria sigue siendo desconocida. La hipótesis genérica actual es que la EII engloba a un grupo heterogéneo de enfermedades que tienen una manifestación final común: la presencia de inflamación, y que varios factores genéticos, ambientales e inmunológicos están implicados en la fisiopatología de estas enfermedades (Podolsky, 2002).

Tanto la EC como la CU se caracterizan por tratarse de trastornos inflamatorios del intestino pero presentan diferencias en cuanto a lo que anatomía patológica y manifestaciones clínicas se refiere (*Tabla 1*).

La EC puede afectar a cualquier segmento del tracto gastrointestinal, desde la boca hasta el ano, si bien es más frecuente en la región ileocecal (Gassull y Cabre, 1994). La inflamación, de carácter transmural, se propaga a través de toda la pared intestinal, favoreciendo la aparición de perforaciones, estenosis y fístulas con órganos adyacentes (Gasche, 2000; Levine, 1994). Las lesiones pueden ser focales (úlceras aftoides), segmentarias o difusas (Levine, 1994) y con frecuencia afecta de forma discontinua y simultánea a distintas zonas del aparato digestivo, separadas entre sí por segmentos intactos.

En contraste con la EC, la afectación de la CU se limita al colon, fundamentalmente a la región distal (recto/ano), y se extiende progresivamente en dirección proximal. La inflamación afecta predominantemente a las capas superficiales de la pared intestinal, normalmente mucosa y submucosa, y se caracteriza por infiltración de neutrófilos, eosinófilos y células plasmáticas, con

formación frecuente de abscesos de las criptas (Obrador y Riera, 1994; Stenson y McDermott, 1991), consistentes en un acúmulo de neutrófilos adyacentes a las criptas, la necrosis del epitelio, y la presencia de edema y hemorragia. La mucosa tiene un aspecto granuloso, consecuencia de la irregularidad de la inflamación, y con frecuencia aparecen pólipos inflamatorios (Geller, 1994). La enfermedad suele manifestarse con diarrea, generalmente sanguinolenta, acompañada o no de síntomas sistémicos: fiebre, malestar general, pérdida de peso, etc. (Sutherland, 1994).

***Tabla 1**. Características diferenciales entre la EC y la CU.*

Enfermedad de Crohn (EC)	*Colitis ulcerosa (CU)*
Desde la boca hasta el ano	Recto +/– colon
Afectación discontinua	Afectación continua
Transmural (afecta a todas las capas del intestino)	Implica sólo la mucosa
Diarrea pastosa	Diarrea líquida con sangre, moco y pus
Fístulas y estenosis intestinal frecuentes	Fístulas y estenosis intestinal infrecuentes
Anatomía patológica:	Anatomía patológica:
Granulomas	Abscesos de criptas
Agregados linfoides	Depleción de mucina
Fibrosis	Distorsión glandular

En un 10-15% de los pacientes con EII es imposible poder establecer un diagnóstico definitivo de CU o EC de colon. La presencia de granulomas constituye el único carácter patognomónico de la EC frente a la CU, pero tan sólo se detectan en un 25% de las biopsias y no son específicos de ésta, ya que se han observado también en enfermedades como la tuberculosis colónica y la esquistosomiasis (Geboes, 1994).

En ambas entidades patológicas pueden aparecer complicaciones de tipo autoinmune que afecten a órganos extraintestinales como las articulaciones, el ojo o la piel y que se presentan hasta

en un 40% de los casos (Lichtman y Balfour Sartor, 1994). Entre las complicaciones no autoinmunes cabe destacar la aparición de episodios tromboembólicos, anemia y osteoporosis (Gasche, 2000; Szulc y Meunier, 2001). Además, el riesgo de cáncer se incrementa de modo acumulativo en los pacientes de EII, siendo factores predisponentes la duración de la enfermedad, extensión de la misma, complicaciones extraintestinales y aparición de la enfermedad a edades tempranas; el riesgo de cáncer es superior en pacientes de CU que en EC (Pohl *et al*., 2000).

1.2. EPIDEMIOLOGÍA.

A pesar de que la incidencia y prevalencia de la EC y la CU comienzan a estabilizarse en áreas de alta incidencia, son 2,2 y 1,4 millones de personas, en Europa y Estados Unidos respectivamente, los que sufren estas enfermedades (Loftus, 2004).

En general, las tasas más altas de incidencia y prevalencia tanto para la EC como la CU se han descrito en el norte de Europa, Reino Unido y Norteamérica, que son regiones geográficas asociadas históricamente con la EII. Sin embargo, existe una incidencia y prevalencia crecientes en otras áreas como el sur y centro de Europa, Asia, África y Latinoamérica, indicando que la EII es un proceso dinámico (Loftus, 2004).

La frecuencia de la enfermedad se encuentra influenciada por una serie de factores demográficos como son el género, la edad o las diferencias étnicas.

En la incidencia de la EII parece existir una leve diferencia entre géneros. En general, se encuentra un ligero predominio de la EC en la mujer, aunque en ciertas áreas de baja incidencia es más frecuente en el varón. Este predominio, especialmente entre mujeres en la adolescencia tardía y la edad adulta temprana, sugiere que los factores hormonales pueden jugar un papel importante en la expresión de la enfermedad. Por otra parte, si existe algún tipo de influencia del sexo en la CU, parece ser que afecta al varón (Loftus *et al.*, 2000).

Clásicamente se ha mostrado una distribución bimodal de la incidencia de la EII en cuanto a la edad (es decir, un primer pico de incidencia aparece entre la segunda y tercera décadas de la vida, seguido por un segundo pico menor en décadas posteriores). La EC y la CU normalmente son diagnosticadas en la adolescencia tardía y la edad adulta temprana, aunque el diagnóstico puede realizarse a todas las edades. Así, el pico de incidencia máximo para la EC se encuentra entre los 15-

30 años, mientras que para la CU es, en general, de 5 a 10 años más tardío que el asociado a la EC (Bjornsson y Johannsson, 2000; Loftus *et al.*, 2000).

Teniendo en cuenta que hay que interpretar con mucha cautela los datos epidemiológicos de que se dispone, parece claro que la EII es más frecuente en individuos de raza blanca. La EII es inusual en los individuos de raza negra, así como en los hispanoamericanos y asiáticos, sin embargo es previsible que con la progresiva culturización y adquisición de hábitos occidentales por parte de estos grupos de población, las cifras de incidencia de EII se aproximen, en algunos casos, a las de los individuos de raza blanca. Finalmente, numerosos estudios reflejan un aumento en el riesgo de padecer EII en sujetos de etnia judía. La CU es tres veces y la EC hasta seis veces más frecuente en judíos que en el resto de la población en sus mismas áreas (Roth *et al.*, 1989).

1.3. ETIOLOGÍA.

La etiología de estas enfermedades continúa siendo desconocida, aunque se propone que se trataría de una respuesta inmunitaria incontrolada frente a un estimulo no identificado en la actualidad que se desarrolla en un individuo genéticamente predispuesto (*Figura 1*).

***Figura 1**. Componentes implicados en la etiopatogénesis de la EII*

1.3.1. Factores genéticos.

Son muchas las evidencias de la contribución de los genes en la EII. Así, los familiares en primer grado de individuos afectados por EII muestran un riesgo 25-50 y 10-20 veces mayor de desarrollar EC y CU, respectivamente, comparados con la población general. Además, los parientes afectados de una misma familia presentan proporciones de concordancia del 80% a edades similares para el sitio específico de afectación, comportamiento y manifestaciones extraintestinales (Zheng *et al*., 2003). Estudios realizados en gemelos muestran una concordancia de un 20-44% en univitelinos y 3,8-6,5% en bivitelinos para la EC; el porcentaje de concordancia para la CU es de 6-16% y 3%, respectivamente (Thompson *et al*., 1996).

Varios grupos de investigadores han identificado al menos 7 loci (*inflammatory bowel disease* 1-7, IBD1-7) en los cromosomas que se relacionen con genes de susceptibilidad, centrándose en las mutaciones de los genes NOD2/CARD15 (*intracellular nucleotide oligomeration domain 2/caspase recruitment domain 15*), del MHC-II (complejo mayor de histocompatibilidad-II), de citocinas, de receptores de citocinas y de moléculas de adhesión (Duerr, 2003; Sartor, 2003; Zheng *et al*., 2003) (*Figura 2*). Algunos loci han mostrado ser específicos para la CU (como IBD2) (Bonen y Cho, 2003) o para EC (como IBD1) (Cho, 2001; 2003), mientras que otros confieren una susceptibilidad común a ambas.

El gen NOD2/CARD15 se encuentra localizado en el cromosoma 16q12 (IBD1) (Hugot *et al*., 1996), y tres de sus variantes confieren 15-20% de riesgo para la EC (Cho, 2001; Cho, 2003). Además, la presencia de un alelo de riesgo NOD2 se asocia con el fenotipo fibrosante obstructivo de la EC (Sartor, 2003), con la enfermedad ileal y con un debut temprano de la enfermedad (Cho, 2003; Gasche *et al*., 2003).

Este gen se expresa principalmente en las células inmunitarias de la línea monocítica (monocitos, macrófagos y células dendríticas), pero también, aunque en niveles bajos, en los granulocitos y algunos linfocitos (Gutierrez *et al*., 2002; Ogura *et al*., 2001) tanto de la lámina propia intestinal como de sangre periférica (Berrebi *et al*., 2003). Existen evidencias de su expresión, en bajas cantidades, en células epiteliales, siendo fuertemente inducida por estímulos inflamatorios, incluidos algunos componentes bacterianos (Berrebi *et al*., 2003, Rosenstiel *et al*., 2003). De hecho,

su expresión epitelial es más marcada en las células de Paneth, células epiteliales intestinales con funciones de defensa frente a patógenos entéricos (Lala *et al*., 2003).

La proteína NOD2/CARD15, es una proteína citosólica (Berrebi *et al*., 2003; Ogura e*t al*. 2001) cuya activación por componentes bacterianos produce un cambio conformacional, haciendo exponer ciertos dominios que se asociarían a proteínas quinasas específicas (Chamaillard *et al*., 2003; Rosenstiel *et al*., 2003). Este complejo proteico, a su vez, activaría también al factor de transcripción nuclear κB (*nuclear factor κB*, NFκB), factor que promueve la liberación de citocinas proinflamatorias (Inohara *et al*., 1999; Inohara *et al*., 2002; Ogura *et al*., 2001).(Figura 2)

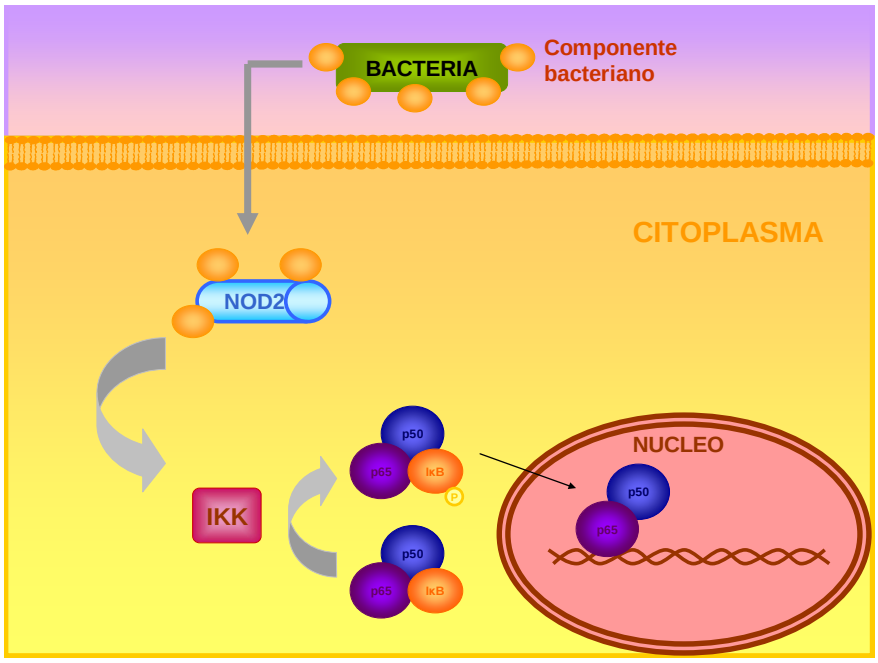

Figura 2. *Influencia de la proteína NOD2/CARD15 en la EII. Las bacterias o sus componentes celulares penetran en macrófagos o células epiteliales y se unen a la proteína, que a su vez activa al factor de transcripción NF-κB. p50/p65: forma activa del NF-κB; IκB: proteína inhibidora del NF-κB; IKK: kinasa del IκB.*

Muy recientemente, han sido identificados dos nuevos genes asociados con la EC. El primero de ellos se encuentra localizado en el cromosoma 5 y codifica para el transportador de cationes orgánicos, OCTN, y sus mutaciones afectan a la capacidad de los transportadores para bombear xenobióticos y aminoácidos a través de las membranas celulares (Peltekova *et al*., 2004). En el cromosoma 10 se encuentra el segundo gen, el de un miembro de la familia de la guanilato quinasa, DLG5, cuya mutación dificulta la capacidad de DLG5 para mantener la polaridad en la célula epitelial (Stoll *et al*., 2004). Ambos genes pueden ser importantes en la permeabilidad epitelial, y un fallo en su función podría provocar una exposición inapropiada del sistema inmunitario de la mucosa a productos bacterianos.

1.3.2. Factores ambientales.

Es evidente la influencia de factores ambientales en el desarrollo de la EII dado el gran incremento de la incidencia, tanto de la EC como de la CU, durante la segunda mitad del siglo XX como consecuencia de profundos cambios en el estilo de vida en los países desarrollados. Este hecho se ve reforzado por el aumento de estas patologías en los países en vías de desarrollo que han adquirido hábitos occidentales. De acuerdo con la llamada "hipótesis de la higiene", se ha producido un cambio fundamental desde un estilo de vida "sucio" con una alta exposición a microbios, a un estilo de vida "limpio" con una exposición baja a éstos (Wills-Karp *et al*., 2001). Cambios ambientales tales como una mejor vivienda y nutrición, alimentos y agua más seguros, una mejora en la higiene y la sanidad, y el uso extendido de antibióticos, han conducido a un descenso progresivo de las enfermedades infecciosas, aunque a expensas de un aumento paralelo de las enfermedades alérgicas y autoinmunes, incluidas la EC y CU, como resultado de un desarrollo reducido del sistema inmunitario en edades tempranas (Bach, 2002).

El hecho de que se hayan observado cambios en la incidencia de la EII en las poblaciones con idéntica etnia que viven en lugares diferentes, junto con la falta de concordancia absoluta existente entre gemelos monozigóticos respecto a la enfermedad, refuerza la importancia de los factores ambientales en la patogenia de la EII crónica.

Son numerosos los factores ambientales reconocidos como de riesgo para la EII: tabaco, dieta, fármacos, el estrés y microorganismos (Danese *et al.*, 2004).

1.3.2.1. Tabaco.

El mejor ejemplo de la influencia del ambiente en la EII es el consumo de tabaco. El tabaco presenta un llamativo efecto contrario en la EC y la CU, apoyando la idea de que son distintos mecanismos los implicados en la patogénesis de cada forma de EII (Thomas *et al*., 1998a). Es un importante factor de riesgo para la EC, aumentando la frecuencia de recidivas y la necesidad de cirugía, y cuya interrupción en su consumo mejora el curso de la enfermedad (Rubin y Hanauer, 2000). Por el contrario, el hecho de que los pacientes de CU son con frecuencia no fumadores, y que el dejar de fumar aumenta el riesgo de desarrollo de CU, sugiere su papel protector en esta enfermedad (Bridger *et al*., 2002).

Los mecanismos de este efecto diferencial del tabaco en la EC y la CU aún no están claros, aunque sí se ha comprobado que el tabaco afecta tanto a la inmunidad sistémica como a la de la mucosa intestinal, alterando numerosas funciones inmunológicas tanto innatas como adquiridas (Sopori, 2002): altera la relación entre células T colaboradoras (Th, *helper*) y T supresoras, reduce la proliferación de células T, modula la apoptosis, y disminuye significativamente los niveles de inmunoglobulinas en suero y mucosas. Además, provoca un incremento en la producción colónica de moco (Cope *et al*., 1986), alteraciones en el flujo sanguíneo (Srivastava *et al*., 1990) y, al igual que la nicotina, una reducción de la motilidad colónica (Coulie *et al*., 2001).

Es sabido que el consumo de tabaco puede generar aproximadamente unos 4.000 compuestos; cualquiera de ellos podría tener acciones biológicas responsables de su acción en la genésis o el mantenimiento de enfermedad, aunque es probable que la nicotina sea el agente activo más importante. A este respecto, la nicotina transdérmica muestra un efecto beneficioso en pacientes con CU (Guslandi y Tittobello, 1996; Pullan *et al*., 1994). En distintos modelos experimentales de colitis, se observa cómo tras la administración de nicotina el proceso inflamatorio mejora, coincidiendo con una disminución local de la concentración de varias de las citocinas proinflamatorias (Agrawal y Rhodes, 2003). Por otra parte, la nicotina podría ser perjudicial en la EC a través de la contribución al estado de hipercoagulación presente en esta condición.

1.3.2.2. Factores dietéticos.

Dado que la EII se trata de una patología digestiva, es lógica la posibilidad de que pudiera haber productos de la dieta implicados en su patogenia. Sin embargo, son pocos los datos objetivos contundentes, principalmente porque proporcionan sólo una evidencia indirecta de la posible relación causa-efecto entre factores dietéticos específicos y EII. Se ha sugerido que, en la EC, ciertos alimentos podrían actuar como antígenos, con un efecto desencadenante de la sintomatología. En este sentido, se han utilizado con fines terapéuticos dietas de exclusión en las que los pacientes evitan comer los alimentos supuestamente culpables. Esta maniobra terapéutica tiene efectos positivos en un pequeño porcentaje de enfermos (Jones *et al*., 1985); sin embargo, no se ha demostrado que, después de lograda la remisión de la enfermedad mediante este planteamiento, la reintroducción de los alimentos excluidos induzca una recaída. No obstante, teniendo en cuenta que la mayor incidencia de EII se puede asociar con los cambios en los hábitos de vida (incluidos los dietéticos) que conlleva el bienestar económico de los países occidentales, y que el intestino es la principal localización del proceso inflamatorio, sería muy probable que algunos nutrientes presentes en la luz intestinal pudieran actuar como antígenos, o que incluso pudieran influir en los mecanismos inmunitarios y reparadores de la mucosa intestinal. Un estudio en el cual se determinó el flujo de sangre rectal y la proliferación de linfocitos tras la exposición a productos alimenticios específicos demostró sensibilización a algunos antígenos dietéticos en pacientes con EC (Van Den Bogaerde *et al*., 2002).

Estudios dirigidos a establecer una relación causal entre dieta y EII hacen frente a dificultades importantes, como definir la verdadera composición de cada dieta. A pesar de todo ello, se ha sugerido que el consumo de azúcar refinado puede ser un factor de riesgo para la EC, pero no para la CU (Sonnenberg, 1988); el consumo de grasa ha sido asociado con la aparición de CU. El consumo de fruta, vegetales y fibra parece descender el riesgo de EII (Reif *et al*., 1997). Además esta relación entre dieta y EII está apoyada por el beneficio en la EC de dietas elementales tanto como terapia primaria como adyuvante, aunque en algunos estudios este planteamiento fue menos efectivo que las terapias convencionales, como esteroides o aminosalicilatos (Lochs *et al*., 1991). Finalmente, algunas deficiencias nutricionales pueden estar ligadas a una disfunción del sistema inmunitario, hecho que favorecería la aparición o incluso agravaría la EII.

1.3.2.3. Fármacos.

Los anticonceptivos orales y los antiinflamatorios no esteroídicos (AINEs) son los dos principales grupos de fármacos que han sido mejor estudiados acerca de la posible relación etiológica entre su uso y el mayor riesgo de desarrollar la enfermedad.

En un meta-análisis realizado por Godet *et al.* (1995) parecía confirmarse una asociación epidemiológica entre el uso de anticonceptivos orales y la EII, algo más intensa en la EC (riesgo relativo de 1,44) que en la CU (riesgo relativo de 1,29). Las conclusiones de este estudio se vieron reforzadas en un trabajo multicéntrico italiano publicado posteriormente (Corrao *et al.* 1998), demostrando además que el riesgo era significativamente mayor en las pacientes que los continuaban utilizando frente a las que ya habían abandonado su uso.

Ha sido motivo de controversia el hecho de si las mujeres que usan anticonceptivos orales presentan una peor evolución clínica de la EII. Los anticonceptivos orales a bajas dosis no afectan significativamente a la actividad clínica de la enfermedad, al menos en EC. Sin embargo, considerando el estado de hipercoagulación presente en la EII activa, el uso concomitante de anticonceptivos orales puede agravar el riesgo de procesos tromboembólicos, aunque son necesarios datos definitivos relacionando estos factores (Alstead, 1999). Por otra parte, si bien se había sugerido que los anticonceptivos podrían afectar a la evolución de la EII a través de estos mecanismos trombogénicos, no se pueden descartar otros efectos inmunomoduladores, como algunos relacionados con la supresión del factor de transcripción NFκB que están empezando a describirse recientemente (Evans *et al*., 2001), y podrían variar según el tipo y dosificación del fármaco. Estos efectos inmunomoduladores podrían producirse, además, con dosis inferiores o ser específicos de determinadas formas moleculares.

La situación es menos ambigua en el caso de los AINEs, porque su uso está claramente asociado con un mayor riesgo de EII. Pacientes de EII en remisión clínica pueden recaer tras la administración de AINEs (Evans *et al*., 1997; Hanauer y Sandborn, 2001). Sin embargo, recientemente se ha publicado un estudio retrospectivo en el cual se sugiere que, en general, los inhibidores de la ciclooxigenasa-2 (COX-2) son seguros en los pacientes con EII (Mahadevan *et al*., 2002).

1.3.2.4. Estrés.

Son importantes las evidencias que asocian el estrés y la enfermedad (CU), probablemente relacionado con el deterioro de la respuesta inmunológica (Herbert y Cohen, 1993). A pesar de que esta creencia es popular entre aquellos que padecen EC y CU, es más probable que el estrés module las manifestaciones de la enfermedad más que ser un factor iniciador. Observaciones clínicas, modelos experimentales de colitis, y estudios de interacciones neuroinmunológicas en animales de laboratorio han demostrado que el estrés puede agravar el curso de la EII (Collins, 2001).

La duración del estrés puede también ser importante, ya que el riesgo de exacerbación de la actividad clínica de la enfermedad parece estar asociado con un estado de estrés prolongado (Levenstein *et al*., 2000). Esta relación presenta semejanzas con los "cotton-top tamarins", primates que viven en la jungla tropical de Sudamérica y que desarrollan colitis espontánea tipo CU sólo cuando se mantienen en cautividad en climas más fríos a largo plazo (Maunder *et al*., 2000). Aún son desconocidos los mecanismos específicos que expliquen la exacerbación de la enfermedad inducida por estrés, aunque probablemente esté implicada una compleja interacción entre factores nerviosos, endocrinos e inmunes (Hart y Kamm, 2002).

1.3.2.5. Factores microbianos.

Durante muchos años se ha tratado de establecer una relación entre un agente infeccioso específico y la EII sin obtener resultados concluyentes. Sin embargo, actualmente es cada vez mas importante el papel que se le atribuye a la flora intestinal comensal en el desarrollo de estas patologías.

Agentes infecciosos específicos

Los agentes infecciosos específicos de tipo microbiano que se han propuesto como responsables de la EC y la CU son muy variables: *Listeria monocytogenes, Chlamydia tracomatis, Escherichia coli, Mycobacterium paratuberculosis.* El papel etiológico de éste último en la EC ha sido centro de gran controversia, ya que esta bacteria es el agente causante de la enfermedad de Johne, una ileítis granulomatosa crónica en rumiantes que se asemeja mucho a la EC. *M. paratuberculosis* fue inicialmente aislado de varios tejidos con EC (Chiodini *et al*., 1984), sin

embargo en estudios posteriores se intentó cultivar este microorganismo, buscar secuencias específicas de ADN en tejidos intestinales o medir anticuerpos en suero frente al mismo, alcanzando resultados conflictivos o no concluyentes. Además, distintos ensayos han determinado una falta de efecto terapéutico de la terapia antituberculosa en pacientes con EC (Thomas *et al*., 1998b).

Una etiología viral también ha sido propuesta como la causa de EII, en particular para la EC. La presencia de partículas semejantes a paramixovirus en granolumas endoteliales de EC sugiere que esta enfermedad podría ser debida a una vasculitis crónica causada por la persistencia del virus del sarampión en la mucosa (Wakefield *et al*., 1993). Como apoyo a esta hipótesis, algunos datos epidemiológicos y serológicos establecieron una asociación entre el sarampión perinatal y la predisposición a la EC (Ekbom *et al*., 1996). Sin embargo estas observaciones preliminares no fueron confirmadas por estudios posteriores (Fisher *et al*., 1997). El descenso progresivo de la infección por el virus del sarampión en las últimas décadas con el incremento de EC durante el mismo período de tiempo, habla en contra de un papel etiológico del sarampión en la EC.

La hipótesis de que la vacunación del sarampión, más que la propia infección, puede ser un factor de riesgo para la EC también fue sugerida, pero de nuevo estudios posteriores no logran confirmar esta asociación (Ghosh *et al*., 2001).

Flora intestinal comensal

Antes del nacimiento, el tracto gastrointestinal es estéril, y durante el nacimiento se produce la primera exposición microbiana por la flora fecal y vaginal de la madre. Durante los meses después del nacimiento, se establece una flora comensal estable (Fanaro *et al*., 2003). La diversa microflora intestinal establece una relación simbiótica con las células epiteliales de la mucosa. Las células bacterianas se benefician en el intestino de un constante fluido de nutrientes, de la temperatura estable y de un nicho para vivir. De igual manera, el hospedador se beneficia de las bacterias por su capacidad de sintetizar vitamina K, obtener energía de los nutrientes no absorbidos en forma de ácidos grasos de cadena corta (AGCC), inhibir el crecimiento de patógenos y mantener la integridad y homeostasis inmunológica en la mucosa. De hecho, hay estudios en animales libres de gérmenes que revelan que la ausencia de microflora intestinal provoca alteraciones significativas en la estructura y función intestinal, como reducción de villi, criptas poco profundas, bajo recuento de

leucocitos (Sharma *et al*., 1995; Szentkuti *et al*., 1990), reducción del número y densidad de las placas de Peyer (Maeda *et al*., 2001) y una disminución de la estimulación de la migración de complejos motores (Husebye *et al*., 2001).

En su convivencia con las bacterias, los vertebrados desarrollan receptores de reconocimiento de indicadores específicos de bacterias, hongos y virus que no se encuentran en eucariotas (lipopolisacaridos, peptidoglucano, dipéptido muramilo, flagelinas…). Estos receptores incluyen los TLR (*toll-like receptor*) y los NOD (dominios de oligomerización de unión de nucleótidos), que son imprescindibles para el inicio de la respuesta inmunitaria innata y cuya activación genera unas cascadas de señalización que acaban en la producción de citocinas proinflamatorias. Las cascadas de señalización del TLR proporcionan un enlace entre la respuesta inmunológica innata y adaptativa, ya que la primera acaba en maduración de células dendríticas, las cuales activan la respuesta inmunológica adaptativa (Medzhitov, 2001). Aunque la estimulación de estos receptores se traduzca en una producción de citocinas proinflamatorias, son también esenciales en la adaptación de las bacterias intestinales y el mantenimiento de la homeostasis (Sansonetti 2004). Una de las características mas importantes de la flora comensal es su incapacidad de atravesar la barrera epitelial, y si alguna de las bacterias penetra, son fagocitadas rápidamente por la respuesta inmunológica innata de la mucosa del individuo sano (Macpherson *et al*., 2000). El objetivo de la respuesta inmunológica de la mucosa es mantener la tolerancia a estas bacterias intestinales, resultando lo contrario en efectos perjudiciales para el hospedador.

En la actualidad se sabe que las bacterias intestinales influyen en el inicio y perpetuación de la EII. La teoría actual sobre el desarrollo de la EII comprende una respuesta inmunitaria exacerbada hacia la microflora comensal en individuos genéticamente susceptibles (Bamias *et al*., 2005), hipótesis sustentada por distintas observaciones: la mayor inflamación se produce en áreas con mayor densidad de bacterias intestinales, el uso de antibióticos mejora la inflamación intestinal crónica, y la desviación quirúrgica del flujo fecal puede prevenir la recurrencia de la enfermedad de Crohn. En pacientes con EII se ha observado que las bacterias adherentes con capacidad de penetrar en la mucosa, como *Bacteroides* ssp, *Escherichia coli* y *Enterobacterium*, son mas abundantes en comparación con los sujetos sanos (Swidsinski *et al*., 2002; Seksik *et al*., 2003) (*Figura 3*). El sobrecrecimiento bacteriano y la disbiosis se asocian también con el desarrollo de pouchitis,

consistente en la inflamación del íleo como consecuencia de una colectomia en pacientes con colitis ulcerosa (CU) (Ruseler-van Embden *et al*., 1994).

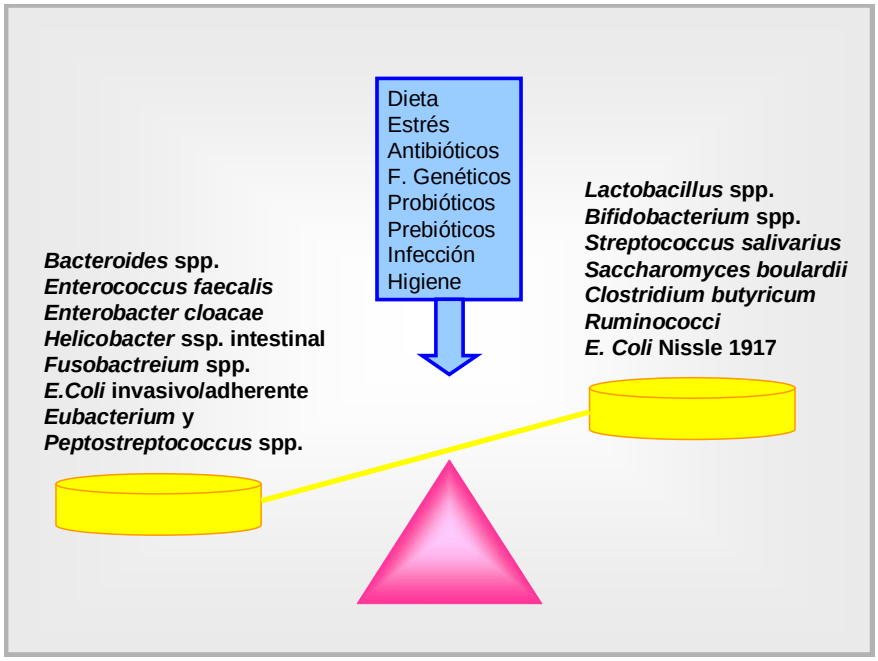

Figura 3. *Balance microbiano y disbiosis. En la enfermedad inflamatoria intestinal, las bacterias luminales desencadenan una respuesta inmunológica anormal. El equilibrio entre las bacterias beneficiosas y las agresivas regula la homeostasis en la inflamación crónica, influenciado éste por diferentes factores genéticos y ambientales (Ewaschuk y Dieleman, 2006)*

La hipótesis de que la flora normal de algún modo funcione como un modulador de la "inflamación fisiológica" ha sido consolidada por las observaciones de Duchmann *et al*. (1995; 1999) que han demostrado que las células mononucleares de la mucosa de pacientes con EII, pero no de la sangre periférica, proliferan cuando son expuestas a bacterias intestinales autólogas. Por el contrario, células de mucosa no afectada de estos mismos pacientes o de pacientes en remisión no proliferan frente a la flora EII autóloga. Esto indica que existe una pérdida de tolerancia durante la inflamación (Duchmann *et al*., 1995).

Probablemente el hecho más convincente es que en la mayoría de los modelos animales de EII la inflamación intestinal no se desarrolla cuando son mantenidos en un ambiente libre de gérmenes, como fue demostrado inicialmente en ratas transgénicas HLA-B27 (Taurog *et al*., 1994). Esta observación ha conducido al ampliamente aceptado paradigma "no bacteria, no colitis". La causa de una respuesta "anormal" a bacterias intestinales "normales" en la EII no está clara, pero el descubrimiento reciente de que la EC está asociada genéticamente con mutaciones del gen NOD2, cuyo producto son proteínas reconocedoras de bacterias, apunta a una relación entre la inflamación intestinal y el reconocimiento bacteriano (Girardin *et al*., 2003).

Por tanto, la asociación entre la microflora intestinal y el desarrollo de la EII ha conducido a la abundancia de estudios que investigan el potencial terapéutico de la alteración de bacterias luminales con el uso de probióticos.

2. PROBIOTICOS EN LA ENFERMEDAD INFLAMATORIA INTESTINAL

2.1. CARACTERÍSTICAS Y CONCEPTO DE PROBIOTICO

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. Esto 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 un concepto 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 pueden considerarse como alimentos funcionales.

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. No obstante, no fue hasta el siglo XX cuando 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 numerosos alimentos, sino que además existían algunas bacterias que ejercían efectos beneficiosos para la salud de los hospedadores que las consumían. En 1906, Cohendy tras administrar leche fermentada por *Lactobacillus bulgaricus* (actualmente *Lb. delbrueckii* subsp. *bulgaricus*) a pacientes con alteraciones en sus "fermentaciones intestinales", observó una notable mejoría tras 8-12 días de tratamiento. Paralelamente, Tissier 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.

Un año después, el premio Nobel Elie Metchnikoff publicó un libro con una gran influencia en la comunidad científica: *Prolongation of Life,* en él que postulaba que el consumo de 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. 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. Desde entonces, se han descrito y comercializado numerosas bacterias con propiedades probióticas.

Posiblemente, el término "probiótico" fue empleado por primera vez por Vergio en 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 microorganismos que promovían el crecimiento de otros microorganismos. Fuller (1989) redefinió probióticos como "aquellos suplementos alimenticios integrados por microorganismos vivos que

afectan beneficiosamente al hospedador que los consume 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). Más 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 *et al*., 2002; Ouwehand y Salminen, 1998).

Entre los microorganismos considerados como probióticos, las bacterias lácticas y las bifidobacterias ocupan el lugar más destacado, 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 *et al*., 2002). 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); las cuales 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).

Para que las cepas potencialmente probióticas puedan ejercer sus efectos beneficiosos deben ser capaces de resistir las condiciones ambientales existentes durante el tránsito por el aparato digestivo y de colonizar el tracto gastrointestinal. Para su estudio, se ha recurrido tanto a métodos *in vitro* como a métodos *in vivo*, los cuales han sido contradictorios en algunas ocasiones (Mattila-Sandholm *et al.*, 1999). En cualquier caso, la capacidad de los probióticos para sobrevivir a las condiciones gastrointestinales es una característica específica de cepa (Charteris *et al.*, 1998a; 1998b; Xanthopoulos *et al.*, 2000; Zárate *et al.*, 2000; Zavaglia *et al*., 1998).

La concentración de probióticos viables que debe llegar al intestino para obtener un efecto beneficioso es de aproximadamente $\geq 10^6$ CFU/ml en el intestino delgado y $\geq 10^8$ ufc/g en el colon (Marteau y Shanahan, 2003). Para establecerse como habitante permanente del tracto

gastrointestinal, deben adherirse a las células epiteliales intestinales o a la capa de mucus, siendo el primer paso en la colonización, y que muchos autores lo consideran como un prerrequisito para ejercer efectos beneficiosos en el hospedador.

Finalmente, cabe decir que la seguridad de los productos actuales es excelente, pero teóricamente, los probióticos, al ser organismos vivos, pueden ser responsables de diversos efectos secundarios en individuos susceptibles (VIH, trasplantados, post quirúrgicos…): infecciones por desplazamiento bacteriano intestino-sangre; efectos metabólicos indeseables como desconjugación y deshidroxilación de sales biliares, excesiva estimulación inmunitaria y transferencia de genes como por ejemplo de resistencia a antibióticos.

2.2. EFECTOS DE LOS PROBIÓTICOS EN LA ENFERMEDAD INFLAMATORIA INTESTINAL EN HUMANOS

En la actualidad, el uso de probióticos se ha asociado con un gran número de efectos beneficiosos en humanos, muchos de ellos establecidos de forma empírica, como la mejora de la intolerancia a la lactosa, la modulación del sistema inmunitario, la reducción de la hipercolesterolemia y la protección frente a enfermedades infecciosas, inflamatorias y alérgicas (Gill, 2003). Sin embargo, no se debe asumir, que todos los probióticos posean las mismas propiedades beneficiosas. De igual manera, cuando se adscribe un efecto beneficioso a una cepa, este no se puede extrapolar a las restantes cepas de la misma especie. Incluso el efecto que una cepa puede presentar depende de las condiciones de su empleo y, muy particularmente, de la dosis.

Los resultados obtenidos de varios estudios animales y diversos ensayos clínicos con probióticos en la enfermedad inflamatoria intestinal son bastante prometedores. Estos estudios muestran la capacidad de los probióticos de prevenir las recaídas de la enfermedad inflamatoria intestinal, incluso algunos tienen actividad sobre la EII activa (Fedorak y Madsen, 2004; Sartor, 2004). Sin embargo aun hacen falta más ensayos que verifiquen su actividad.

2.2.1. Colitis ulcerosa.

El primer estudio que se realizó con un pequeño numero de pacientes evaluó la actividad de *E. coli* Nissle 1917 en comparación con dosis bajas de mesalamina, mostrando que el cociente

remisión/recaídas en el caso del probiótico fue del 16%/67% frente al 11%/73% de la mesalamina (Kruis *et al*., 1997). Kruis *et al*. (2004), ampliaron estos estudios valorando la efectividad de una preparación oral de *E. coli* Nissle 1917 frente a mesalamina en un estudio doble ciego aleatorizado con 327 pacientes durante 12 meses, obteniendo como resultado que no había diferencias significativas entre los dos grupos, siendo el valor de las recaídas del 36,4% para el probiótico, y del 33,9% para el caso de la mesalamina. Recientemente, Zocco *et al*. (2006), estudiaron la eficacia de la asociación del probiótico *L. rhamnosus* GG con mesalamina en el mantenimiento de la remisión de la colitis ulcerosa en comparación con mesalamina sola, no obteniendo diferencias en el número de recaídas después de 6 y de 12 meses. Sin embargo, si se obtuvieron diferencias en el tiempo de remisión ($P<0,05$). Otro estudio con una mezcla de probióticos denominado VSL#3* demostró que 15 de 20 pacientes no sufrieron recaídas durante 1 año (Venturi *et al*., 1999).

La eficacia del tratamiento probiótico en la colitis ulcerosa activa también se evaluó mediante un estudio que demuestra la equivalencia entre *E. coli* Nissle 1917 y mesalamina en la inducción de la remisión de la CU (Rembacken *et al*., 1999). Ishiwaka *et al*. (2003) probaron la actividad de una leche fermentada con *Bifidobacterium* en el tratamiento de la colitis ulcerosa durante 1 año, observándose después del mismo una exacerbación de los síntomas en sólo 3 de 11 pacientes tratados con la leche en comparación con 9 de 10 del grupo control ($P=0,01$). Sin embargo no se observaron diferencias en el índice de la actividad endoscópica de la enfermedad. Después se realizó otro estudio usando un placebo como control con la leche fermentada con *Bifidobacterium* durante dos semanas en pacientes con colitis ulcerosa activa, en el que se redujo de manera significativa tanto el daño histológico como el índice de la actividad endoscópica de la enfermedad en comparación con el placebo (Kato *et al*., 2004).

*VSL#3: mezcla probiótica compuesta por: *Lactobacillus casei, Lactubacillus plantarum, Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. bulgaricus, Bifidobacterium longum, Bifidobacterium breve* y *Bifidobacterium infantis.*

Tabla 2. *Efectos de los probióticos en la inducción de la remisión de la colitis ulcerosa*

Inducción de la remisión de colitis ulcerosa				
Kato *et al*. 2004	DC, A, C	Leche fermentada *Bifidobacterium*	Placebo	Reducción índice de actividad de CU
Rembacken *et al*. 1999	DC, A, C	*E. coli* Nissle 1917 (10^{11} CFU)	Mesalamina	Tan efectivo como mesalamina en la remisión de CU
Bibiloni *et al*. 2005	Abierto	VSL#3 ($3,6*10^{9}$ CFU)	Ninguno	77 % de remisión de CU
Ishikawa *et al*. 2003	A, C	Leche fermentada con *Lactobacillus* y *Bifidobacterium*	Placebo	Disminución de la exacerbación de los síntomas (P<0,01)
Borody *et al*. 2003	Enema fecal	Enema fecal	Ninguno	100 % de remisión

DC: doble ciego; A: aleatorizado; C: controlado.

Tabla 3. *Efecto de los probióticos en el mantenimiento de la remisión de la colitis ulcerosa*

Mantenimiento de la remisión de colitis ulcerosa				
Kruis *et al*. 2004	DC, A, C	*E. coli* Nissle 1917 ($2,5\text{-}25*10^9$ CFU)	Mesalamina	Tan efectivo como mesalamina en la remisión de CU
Zocco *et al*. 2006	Abierto	*Lactobacillus* GG ($1,8*10^{10}$ CFU)	Mesalamina Mesalamina + LGG	No hay diferencias en numero de recaídas a los 12 meses, pero si en el tiempo de remisión
Shanahan *et al*. 2006	DC, A, C	*Lactobacillus salivarius* o *Bifidobacterium infantis* (10^9 CFU)(52/grupo)	Placebo	No mejora el tiempo de remisión
Venturi *et al*. 1999	Abierto	VSL#3 ($1*10^{12}$ CFU)	Ninguno	Remisión del 75%

DC: doble ciego; A: aleatorizado; C: controlado.

2.2.2. Enfermedad de Crohn.

Existe un menor número de trabajos que describen el uso de probióticos en la prevención y tratamiento de la enfermedad de Crohn. En un ensayo se probó la eficacia de *Saccharomyces boulardii* en el mantenimiento de la remisión de la EC. A los 6 meses la incidencia de recaídas era del 37,5 % en el grupo administrado solo con mesalamina, y del 6,3 % en el grupo tratado con la mesalamina y el probiótico (Guslandi *et al*., 2000). En otro estudio, McCarthy *et al*., mostraron que la administración oral de *Lactobacillus salivarius* UCC118 reducía de manera significativa el índice de la enfermedad en pacientes con EC leve y moderada. Aunque estos resultados son prometedores, es importante indicar la existencia de numerosos estudios en los que distintos probióticos no han demostrado tener eficacia. Un estudio randomizado controlado por placebo con 98 pacientes mostró que el probiótico *L. johnsonii* LA1 no previno la recurrencia de EC postoperatoria (Marteau *et al*., 2006). De igual manera *L. rhamnosus* GG tampoco la previno, en pacientes con EC post-operatoria y resección intestinal (Prantera *et al*., 2002).

***Tabla 4**. Efectos de los probióticos en la inducción de la remisión de la enfermedad de Crohn*

Inducción de la remisión de la Enfermedad de Crohn				
Schultz *et al*. 2004	DC, A, C	*Lactobacillus* GG ($2*10^9$ CFU)	Placebo	Sin diferencias en la capacidad de remisión
McCarthy *et al*. 2001	Abierto	*Lactobacillus salivarius* ($1*10^{10}$ CFU)	Ninguno	Reducción de la actividad de la enfermedad comparado con niveles basales
Gupta *et al*. 2000	Abierto	*Lactobacillus* GG ($2*10^{10}$ CFU)	Ninguno	Mejora del IAEC en comparación basales ($P<0,05$)

DC: doble ciego; A: aleatorizado; C: controlado; IAEC: índice de actividad de la enfermedad de Crohn

***Tabla 5**. Efectos de los probióticos en el mantenimiento de la remisión de la enfermedad de Crohn*

Mantenimiento de la remisión de la Enfermedad de Crohn				
Prantera *et al*. 2002	DC, A, C	*Lactobacillus* GG ($1,2*10^{10}$ CFU)	Placebo	Sin diferencias significativas de remisión
Campieri *et al*. 2000	A, C	VSL#3 ($3*10^{11}$ CFU)	Mesalamina	Igual eficacia que mesalamina en la prevención de recaídas
Marteau *et al*. 2006	DC, A, C	*L. .johnsonii* LA1 ($2*10^{9}$ CFU)	Placebo	Sin diferencias en las recaídas
Malchow *et al*. 1997	DC, A, C	*E. coli* Nissle 1917 ($5*10^{10}$ CFU)	Placebo	Sin diferencias en remisión de los síntomas
Bousvaros *et al*. 2005	DC, A, C	*Lactobacillus* GG ($2*10^{10}$ CFU)	Placebo	Sin diferencias en el tiempo de recaídas
Guslandi *et al*. 2000	A, C 6 meses	*Saccharomyces boulardii* (1 g/d) + mesalamina (2g)	Mesalamina	Prolongación de la remisión s ($P<0,05$)

DC: doble ciego; A: aleatorizado; C: controlado.

2.2.3. Pouchitis crónica.

Es en esta patología donde los probióticos han demostrado un beneficio indiscutible al comprobarse en distintos estudios que éstos son capaces de mantener la remisión inducida con antibióticos en pacientes con pouchitis crónica tras resección del colon debido a una colitis ulcerosa refractaria. En este sentido, Gionchetti *et al*. (2000), han completado los ensayos usando la mezcla probiótica VSL#3 en pacientes con pouchitis crónica recurrente, la cual redujo la incidencia de recaídas tras 9 meses a un 15%, frente al 100% del grupo placebo. Otro estudio con los mismos grupos también demostró que tras un año, solo desarrollaron pouchitis un 10% frente a un 40% del grupo placebo después de la cirugía por colitis ulcerosa (Gionchetti *et al*., 2003). También se llevó a cabo un estudio doble ciego, aleatorizado, usando un placebo como control, en 20 pacientes tratados con *L. rhamnosus* GG vs. placebo durante 3 meses (Kuisma *et al*., 2003). Sin embargo, en contraste al estudio con la mezcla VSL#3, no se observaron diferencias significativas en la pouchitis crónica durante el tratamiento con *L. rhamnosus* GG.

***Tabla 6**. Efectos de los probióticos en la pouchitis crónica*

Inducción de la remisión de la pouchitis				
Kuisma *et al*. 2003	DC, A, C	*Lactobacillus* GG ($1*10^{10}$ CFU)	Placebo	Sin diferencias en el IAP
Laake *et al*. 2004	Abierto	Leche fermentada con *L. acidophilus* y *Bifido-bacterium lactis* (500 mL)	Ninguno	Mejora del IAP, pero sin diferencias en la histología
Gionchetti *et al*. 2000	DC, A, C	VSL#3 (6g)	Placebo	Aumento del tiempo de remisión (P< 0,001)
Mimura *et al*. 2004	DC, A, C	VSL#3 (6g)	Placebo	Aumento del tiempo de remisión (P< 0,001)
Gionchetti *et al*. 2003	DC, A, C	VSL#3 ($1*10^{11}$ CFU)	Placebo	Aumento del tiempo de remisión (P< 0,05)

DC: doble ciego; A: aleatorizado; C: controlado; IAP: índice de actividad de la pouchitis

2.3. MECANISMO DE ACCION DEL EFECTO ANTIINFLAMATORIO INTESTINAL

Clásicamente se ha atribuido el efecto de los probióticos a su capacidad de modificar la composición de la microflora intestinal de potencialmente dañina, a beneficiosa para el hospedador. Sin embargo el mejor conocimiento de la biología de estos microorganismos ha permitido establecer diferentes mecanismos de acción posibles para ejercer sus efectos beneficiosos (*Figura 4*)

1.- Competición con bacterias nocivas por:

a) desplazamiento de su sitio de unión al epitelio y

b) inhibición de su crecimiento y/o promoción de su muerte mediante la producción de compuestos antibacterianos o reducción del pH;

2.- Mejora de la función de barrera intestinal;

3.- Producción de nutrientes importantes para la función intestinal y

4.- Modulación de la respuesta inmune de la mucosa del hospedador.

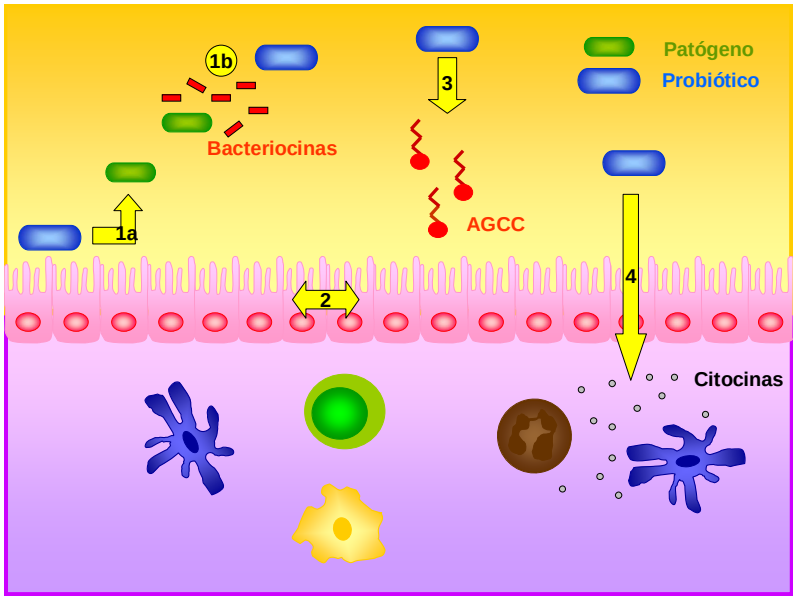

***Figura 4**. Diferentes mecanismos de acción ejercidos por las bacterias probióticas.*

2.2.1. Competición con bacterias patógenas.

Los probióticos son bacterias sin capacidad patógena, capaces de prevenir la adherencia, establecimiento, replicación y/o la acción de las bacterias patógenas. Los mecanismos posibles pueden implicar modificación del pH, mediante producción de ácidos grasos de cadena corta como consecuencia de su capacidad fermentativa sobre la fibra dietética; o producción de compuestos antibacterianos como peroxido de hidrogeno o bacteriocininas (Jack *et al*., 1995; Liévin *et al*., 2000).

El desplazamiento de bacterias nocivas no necesariamente implica actividad bacteriostática o bactericida, sino que también puede ser consecuencia de la competición física por unirse al epitelio, consumiendo también los sustratos disponibles para las bacterias patógenas.

Hay diversos estudios que demuestran el efecto competitivo ejercido por los probióticos, por ejemplo, estudios *in vitro* e *in vivo* han demostrado que *B. infantis* inhibe el crecimiento de *Bacteroides vulgatus* (Shiba *et al*., 2003). La mezcla probiótica VSL#3 es capaz de inhibir la invasión de *Salmonella dublin* en células T-84 (Madsen *et al*., 2001). *Lactobacillus salivarius ssp salivarius* UCC118 inhibe el crecimiento de numerosos patógenos como *Listeria monocytogenes* o *Sthaphilococcus aureus* meticilin resistentes, efecto derivado de su factor antimicrobiano ABP118 (Dunne et al., 1999). En otro estudio *Escherichia coli* Nissle 1917 fue capaz de reducir al adhesión del patógeno *Escherichia coli* enteroinvasivo en un 97 %, sugiriendo un posible mecanismo en el mantenimiento de la remision de la colitis ulcerosa (Malchow, 1997; Rembacken *et al*., 1999).

2.2.2. Mejora de la función de barrera intestinal.

La monocapa epitelial y el revestimiento de moco que la recubre, junto con las uniones estrechas (del inglés *tight junction*) que mantienen unidos a los enterocitos, forman una barrera física que previene a los patógenos potenciales y a antígenos luminales de pasar libremente a la lámina propia. Por otro lado, la inmunoglobulina (Ig) A, además de bloquear sus uniones al epitelio, previene su internalización, y también es capaz de aglutinar bacterias y virus en unos grandes complejos que son atrapados en la barrera de moco y eliminados en las heces.

En la EII, la integridad de la barrera epitelial está comprometida, lo que permite el paso de antígenos luminales a la lámina propia, y contribuye a la perpetuación del proceso inflamatorio

(Plevy, 2002). Así, se ha demostrado la existencia de una permeabilidad intestinal incrementada en pacientes con EC (Teahon *et al*., 1992), y se ha descrito como un factor temprano predisponente a la patogénesis de esta enfermedad.

Los probióticos podrían normalizar la permeabilidad intestinal incrementada mejorando las funciones de barrera intestinal, y secundariamente la respuesta inflamatoria intestinal. Son diferentes los procesos que pueden intervenir en dicha actividad: a) Se ha demostrado que la incubación con *L. plantarum 299v* aumenta los niveles de expresión de mRNA de las proteínas MUC2 y MUC3 en células HT-29 (Mack *et al*., 1999), y b) *L. casei* o *Clostridium butyricum* aumentan de forma muy marcada la proliferación de las células epiteliales intestinales (hasta 200% en el colon) en ratas mejorando así la protección del tejido intestinal (Ichikawa *et al*., 1999).

2.2.3. Producción de nutrientes importantes para la función intestinal.

Los ácidos grasos de cadena corta, AGCC, (principalmente acetato, propionato y butirato) son los productos finales de la descomposición de los carbohidratos de la dieta por parte de las bacterias anaerobias en el intestino grueso. Son la principal fuente de energía para los colonocitos regulando su desarrollo y diferenciación (Cummings, 1981). Además, y en íntima relación con las propiedades normalizadoras de la función de barrera intestinal, tienen efectos tróficos sobre el epitelio intestinal, lo que es de gran importancia para la recuperación de la inflamación y para la reducción del riesgo de translocación bacteriana durante la alteración de la barrera intestinal (Urao *et al*., 1999). En concreto, el butirato, tiene la capacidad de inducir enzimas (por ejemplo transglutaminasas) que promueven la restitución de la mucosa (D' Argenio *et al*., 1999)

Se ha postulado que la deficiencia en la cantidad de estos AGCC puede estar relacionada con la aparición de colitis (Roediger, 1980); como consecuencia, la administración de preparaciones probióticas que contienen *Bifidobacterium, Enterococcus* o *Lactobacillus*, que aumentan la cantidad total de AGCC, contrarrestarían la aparición de estas enfermedades (Sakata *et al*., 1999)

2.2.4. Modulación de la respuesta inmunitaria de la mucosa del hospedador.

Los efectos antiinflamatorios de los probióticos en la enfermedad de Crohn y colitis ulcerosa pueden incluir señales que ejercen sobre el sistema inmunológico del epitelio intestinal,

incluyendo la producción de anticuerpos (Ig A, capaz de promover la barrera inmunológica) (Kaila *et al*., 1992; Rinne *et al*., 2005), el aumento del número de fagocitos (Shu y Gill, 2002) y células natural killer (Gill *et al*., 2001; Gill *et al*., 2001; Ogawa *et al*., 2006; Sheih *et al*., 2001), modulación de las vías de señalización del NF-κB (Jijon *et al*., 2004; Petrof *et al*., 2004; Tien *et al*., 2006) y la inducción de la apoptosis de células T (Di Marzio *et al*., 2001). Además, algunos probióticos como *Escherichia coli* no patógeno o *Lactobacillus sakei*, tienen la capacidad de aumentar la producción de citocinas antiinflamatorias tales como IL-10 y TGF-β, y de reducir la producción de citocinas proinflamatorias por ejemplo TNF-α, IFN-γ o IL-8 (Haller *et al*., 2000; Maassen *et al*., 2000; Madsen *et al*., 1999; Morita *et al*., 2002).

Lammers *et al*. 2005, administró una mezcla probiótica a pacientes con anastomosis ileo-anal mostrando una reducción de los niveles de mRNA de IL-1β, IL-8 y IFN-γ, y del número de células polimorfonucleares en comparación con los pacientes que recibieron el placebo. Ulisse *et al*. (2001), describió una reducción de la expresión de las citocinas IFN-γ y IL-1α, y de la actividad iNOS en biopsias de pacientes con pouchitis tratados con probióticos. Por último, en explantes de la mucosa iliaca de pacientes con enfermedad de Crohn, el tratamiento con *Lactobacillus casei* y con *Lactobacillus bulgaricus* redujo la liberación de TNF-α y el número de células CD4 (Borruel *et al*., 2002).

OBJETIVOS

La enfermedad inflamatoria intestinal es una de las patologías con una alta prevalencia en los últimos años. Aunque no se conoce exactamente su etiología, es cada vez mayor la evidencia de que en su inicio y progresión intervienen antígenos de la dieta, posiblemente producidos por bacterias. Una hipótesis bastante aceptable para la prevención y el tratamiento de estas enfermedades podría ser la modificación de la flora bacteriana con el objeto de interferir en la producción de estos agentes antigénicos.

Los probióticos, definidos como agentes nutricionales vivos, que consumidos en cantidades adecuadas, ejercen un efecto beneficioso para el hospedador, podrían constituir una estrategia terapéutica muy atrayente ya que modifican el equilibrio hacia bacterias carentes de este potencial antigénico. Sin embargo, no todos los probióticos han manifestado efectos beneficiosos en los diversos modelos experimentales y en humanos. En esta variabilidad del efecto pueden intervenir diferentes factores:

- Especie y/o cepa probiótica ensayada, ya que aunque todos los probióticos tienen una capacidad innata de adherirse al epitelio, no todos modifican la respuesta inmune
- Dosis usada
- Tipo de patología
- Momento en el cual se inicia el tratamiento, de hecho han mostrado ser mas eficaces en el tratamiento de la colitis ulcerosa que en la enfermedad de Crohn, y mas activos en el mantenimiento de la remisión que en el tratamiento de la enfermedad aguda

El objetivo general de esta Tesis Doctoral es ratificar que el efecto antiinflamatorio intestinal depende de la cepa/especie seleccionada y encontrar un probiótico con características ideales para el tratamiento de estas patologías. Para llevarlo a cabo, se plantearon los siguientes objetivos concretos:

1. Comparar el efecto antiinflamatorio intestinal de *Lactobacillus casei*, *Lactobacillus acidophilus* y *Bifidobacterium lactis*, probióticos con actividad demostrada en estudios previos

2. Estudiar el efecto antiinflamatorio de *Lactobacillus salivarius* ssp. *salivarius* y *Lactobacillus fermentum*. Éstos, fueron aislados en la empresa Puleva Biotech, y su actividad antiinflamatoria intestinal ha sido estudiada por primera vez en esta Tesis.
3. Comparar la eficacia del probiótico con el mejor perfil antiinflamatorio de todos los anteriores con *Lactobacillus reuteri*, probiótico ampliamente utilizado tanto en modelos de colitis experimental como en humanos

MATERIAL Y MÉTODOS

1. ENSAYOS *IN VIVO*.

Estos estudios se realizaron de acuerdo con las directivas de la *Convención para la protección de los animales vertebrados usados en experimentación y con otros fines científicos* establecidas por la Unión Europea (85/ETS123; 86/609/EEC).

1.1. Preparación del probiótico

Los probióticos utilizados fueron: *Lactobacillus salivarius* ssp. *salivarius, Lactobacillus fermentum, Lactobacillus reuteri, Lactobacillus casei, Lactobacillus acidophilus y Bifidobacterium lactis*

Los probióticos fueron suministrados por Puleva Biotech (Granada, España). Se hicieron crecer normalmente en medio MSR a 37º C en condiciones anaerobias usando el sistema Anaerogen (Oxoid, Basingstoke, UK), se suspendieron en leche desnatada (5.10^8 CFU/mL) y se almacenaron a –80º C hasta su uso.

1.2. Animales de experimentación.

Los animales utilizados en este estudio fueron ratas albinas hembra de la cepa Wistar de 180-200 g de peso, suministradas por el Servicio de Animales de Laboratorio de la Universidad de Granada. Los animales se mantuvieron en el estabulario del laboratorio al menos 7 días antes de iniciar los experimentos, a una temperatura de 22±2 º C y con un ciclo de luz-oscuridad de 12 horas. Las ratas fueron alojadas en cubetas de makrolon con lecho de viruta, dispuestas en estante de acero inoxidable, alimentadas con la correspondiente dieta para roedores, y agua corriente *ad libitum*.

1.3. Inducción de la colitis experimental por TNBS y administración del probiótico

La colitis se indujo utilizando el método descrito por Morris *et al.* (1989) con algunas modificaciones incorporadas por nuestro grupo de investigación. Los animales fueron sometidos a un período de ayuno de 24 horas, después del cual fueron anestesiados ligeramente con halotano y se procedió a la administración rectal de 0,25 ml de una solución de 10 mg de ácido trinitrobencenosulfónico (TNBS) en etanol al 50 % (v/v). La instilación se realizó introduciendo un catéter de teflón (2 mm de grosor) 8 cm desde el ano, manteniendo a los animales en posición supina hasta la recuperación de la anestesia. La solución de TNBS se preparó a partir de un liofilizado de la solución comercial de origen consistente en una solución acuosa al 5 % p/v. Dado que se han descrito variaciones en la respuesta en función del lote comercial de TNBS (Yamada *et al.*, 1992), todos los experimentos se realizaron con TNBS de un mismo lote.

Los animales se distribuyeron de modo aleatorio en diferentes grupos, uno control sano y los demás sometidos a inflamación intestinal (*Figura 5*):

- Grupo control sano: se les administró por vía rectal 0,25 ml de solución salina isotónica de ClNa (0,9 %), y recibieron 0.5 ml de leche desnatada (vehiculo) mediante sonda gastroesofágica.

- Grupo control colítico: se les indujo la colitis con TNBS y también recibieron 0.5 ml de leche desnatada mediante sonda gastroesofágica.

- Grupo tratado con probióticos: se les administró por vía oral el probiótico correspondiente a cada grupo a una concentración de 5.10^8 vehiculizado en leche desnatada durante los catorce días previos a la inducción del proceso inflamatorio intestinal con TNBS, así como durante los siete días posteriores mediante sonda gastroesofágica.

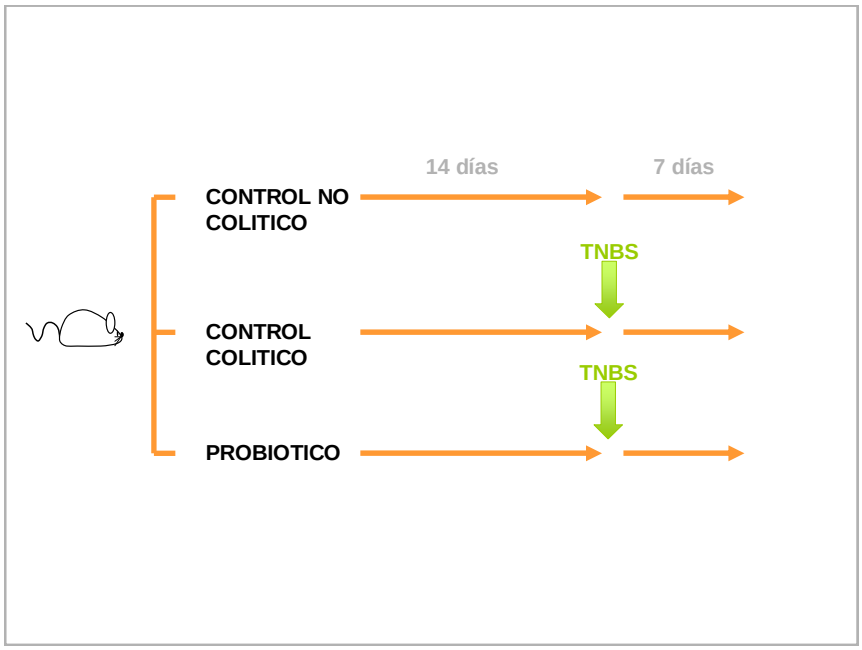

***Figura 5**. Diseño experimental*

2. VALORACIÓN DEL PROCESO INFLAMATORIO INTESTINAL.

Durante el desarrollo de estas experiencias se controló diariamente el peso y el consumo de agua y comida de los animales, así como la aparición de diarrea por visualización de restos perianales (Bell *et al*., 1995). Todos los animales fueron sacrificados a los siete días de la administración del agente inflamatorio intestinal

Una vez sacrificados los animales, se les extrajo el colon en su totalidad, observándose la existencia o no de adhesiones entre el intestino grueso y los órganos adyacentes. Seguidamente se procedió a la limpieza de los distintos segmentos intestinales, retirando los restos de grasa y las adhesiones mesentéricas, sobre una placa Petri con hielo.

El colon se abrió longitudinalmente y se extrajeron los contenidos colónicos, que fueron pesados y procesados para las determinaciones microbiologicas y de AGCC posteriores.

El colon se lavó con solución salina isotónica y se determinó su longitud bajo una tensión constante de 2 g, así como su peso. Tras abrirlo longitudinalmente, un observador ajeno al desarrollo del experimento valoró el daño macroscópico de acuerdo con el criterio descrito por Bell *et al.* (1995) (*Tabla 7*) poniéndose de manifiesto la presencia o no de las distintas características del proceso inflamatorio.

Tabla 7. *Escala de valoración del índice de daño macroscópico (IDM) en el modelo de colitis experimental por TNBS en ratas*

0 puntos	Colon normal
1 punto	Hiperemia localizada, sin ulceras
2 puntos	Ulceración sin hiperemia ni engrosamiento en la pared intestinal
3 puntos	Ulceración con un punto de inflamación
4 puntos	Dos o mas sitios de ulceración e inflamación
5 puntos	Zonas grandes de daño, inflamación y ulceración con una extensión mayor de 1 cm
6-10 puntos	Zonas grandes de daño tisular con una extensión mayor de 2 cm, añadiéndose 1 punto (hasta 10) por cada cm adicional de extensión

Para la realización de los correspondientes estudios histológicos, se obtuvieron muestras de colon (0,5 cm de longitud) de la zona proximal inmediatamente adyacente al daño.

Por último, el colon se dividió en distintos fragmentos longitudinales que, a excepción de uno, fueron congelados inmediatamente a –80º C para la realización posterior de las determinaciones bioquímicas.. El segmento destinado a la determinación de glutation total, se congeló en 1 ml de

ácido tricloroacético (TCA) (Fluka, Madrid) al 5 % (p/v), con objeto de inhibir su degradación por la γ-glutamil transpeptidasa (Anderson, 1985). El fragmento no congelado fue procesado en el momento de su obtención para la determinación de la producción de LTB_4 y de citocinas (IL-1β y TNFα). Todas las determinaciones se realizaron en las dos semanas siguientes al sacrificio de los animales.

2.1 Determinación de la actividad mieloperoxidasa colónica.

La determinación de la actividad mieloperoxidasa colónica (MPO) se realizó por el método descrito por Krawisz *et al.* (1984). Esta enzima se utiliza como marcador de la infiltración de neutrófilos, aunque no es una enzima estrictamente específica de estos fagocitos.

Los fragmentos de colon fueron dispuestos sobre una placa Petri enfriada con hielo y picados con tijeras durante aproximadamente 15 segundos. A continuación, se homogeneizaron en tampón de bromuro de hexadeciltrimetilamonio (HTAB) al 0,5% (p/v) en tampón fosfato salino (50 mM, pH 6,0), con una dilución final de 1:20 (p/v) en un homogeneizador Heidolph hasta obtener un aspecto uniforme. El HTAB actúa como detergente, lo cual facilita la liberación del enzima MPO de los gránulos azurófilos de los neutrófilos, donde se encuentra almacenada. Seguidamente, el homogenado se sonicó durante 10 segundos y se sometió a un triple proceso de congelación-descongelación que facilitó la ruptura de estructuras celulares, y en consecuencia la liberación de la enzima. Tras la última descongelación se centrifugó el homogenado a 7000 G durante 10 minutos a 4º C y se procedió a la determinación de la actividad MPO siguiendo la cinética de la reacción frente al agua oxigenada. Para ello, en una placa de 96 pocillos se añadieron 50 µL del sobrenadante a 150 µL del reactivo de coloración preparado de forma extemporánea, y compuesto por clorhidrato de o-dianisidina (0,167 mg/mL) y peróxido de hidrógeno al 0,066% en tampón fosfato (50 mM, pH 6,0). El incremento de absorbancia se determinó a 450 nm en un espectofotómetro Microplate Reader (Bio-Rad). La actividad MPO se calculó por interpolación en una curva patrón, realizada con mieloperoxidasa procedente de neutrófilos humanos. Una unidad de MPO (U) se define como la cantidad necesaria para degradar 1 mmol/minuto de peróxido de hidrógeno a 25º C. Los resultados se expresan como U/g tejido fresco.

2.2. Determinación del contenido colónico de glutation total.

La determinación del contenido colónico de glutation total se realizó de acuerdo con el método de la reducción cíclica del DTNB-GSSG (Akerboom y Sies, 1981).

Se basa en la oxidación del glutatión reducido (GSH) presente en una muestra a su forma oxidada (GSSG) mediante incubación con el ácido 5,5'-ditiobisnitrobenzoico (DTNB), el cual es reducido y adquiere una coloración amarillenta que puede ser determinada espectrofotométricamente. El GSSG generado es reducido por acción de la enzima glutatión reductasa (GSSGrd) en presencia de nicotinamida adenín dinucleótido fosfato (NADPH). El GSH resultante se oxida de nuevo, dando lugar a un ciclo continuo en el cual la velocidad de reducción del DTNB (con el consiguiente incremento de la absorbancia) es proporcional a la cantidad total de glutatión.

Para efectuar esta determinación se utilizaron los fragmentos de colon congelados en solución de TCA al 5% (p/v). Se picaron con tijeras durante 15 segundos aproximadamente sobre una placa Petri con hielo y posteriormente se homogeneizaron en la solución de TCA en una proporción 1:20 (p/v) en un homogeneizador automático Heidolph. A continuación, se centrifugó el homogenado a 2000 G durante 5 minutos a 4º C y se recogió el sobrenadante, para someterlo a una segunda centrifugación a 10000 G durante 5 minutos a 4º C. Para la determinación del glutation total se mezclaron, por este orden, 20 µL de sobrenadante, 5 µL de tampón fosfato salino con ácido etilendiaminotetraacético (EDTA) (PBS-EDTA) (solución acuosa de fosfato sódico 143 mM y EDTA 6,3 mM, pH 7,5), 140 µL de solución de β-NADPH (0,298 mM) en PBS-EDTA y 20 µL de DTNB (6 mM) en PBS-EDTA, en una placa de 96 pocillos, que se incubó a 30ºC durante 5 minutos. Tras adicionar 15 µL de solución de GSSGrd (Boehringer-Mannheim, Barcelona) (266 UI/mL) en PBS-EDTA, se agitó y se registró el incremento de absorbancia a 412 nm en un dispositivo Microplate Reader (Bio-Rad). La concentración de glutation se calculó por interpolación en la curva patrón realizada con GSH. Los resultados se expresan como nanomoles de glutation total/g de tejido fresco.

2.3 Determinación de los niveles colónicos de LTB_4, TNFα, IL-1β e IL-10

Para la determinación de estos parámetros bioquímicos se realizó un procedimiento simple de extracción de forma inmediata (<1 h) a la obtención del órgano (McCafferty *et al.*, 1992). Los fragmentos de colon se pican con unas tijeras sobre una placa Petri con hielo durante 15 segundos y se incuban en tampón fosfato sódico 10 (mM, pH=7,4) en proporción 1:5 (p/v) a 37º C durante 20 minutos con agitación constante.

A continuación se centrifugan las muestras a 9000 G durante 30 segundos a 4º C y se congela el sobrenadante obtenido a –80º C. Para la determinación de la concentración de LTB_4 y TNFα se utilizaron kits comerciales de enzimoinmunoensayo suministrados por Amersham Pharmacia Biotech (Little Chalfont, Reino Unido). Los resultados se expresan como ng/g tejido fresco en el caso del LTB_4 y como pg/g tejido fresco en el caso del TNFα. IL-1β e IL-10 se expresaron como pg/mg de proteina.

2.4. Determinación de la expresión de iNOS y COX-2 en tejido colónico.

La determinación de la expresión de iNOS y COX-2 se realizó mediante Western blot. Para ello, los fragmentos colónicos fueron descongelados, picados y homogeneizados en proporción 1:5 (p/v) durante 1 minuto en PBS con dodecil sulfato sódico (*sodium dodecyl sulfate*, SDS) al 0,1%, desoxicolato sódico al 0,1% y Tritón X-100 al 1%, conteniendo inhibidores de proteasas (aprotinina, 1,10-fenantrolina, iodoacetamida, fluoruro de fenilmetilsulfonilo). Seguidamente, los homogenados fueron centrifugados a 10000 G durante 10 minutos. Tras determinar el contenido en proteínas por el método del BCA, los sobrenadantes (100 μg) fueron hervidos durante 5 minutos en tampón de carga Laemli 5x (Tris-HCl 0,2 M, pH 6,8, SDS 5%, 2-mercaptoetanol 8,8% (v/v), glicerol 37,5%, azul de bromofenol 60 μg/mL) y sometidos a electroforesis en un gel desnaturalizante de poliacrilamida (SDS-PAGE) al 6%. Como indicadores de peso molecular, se utilizó una mezcla de proteínas previamente marcadas con un colorante (BioRad, Hercules, CA, EEUU). Las proteínas fueron transferidas a una membrana de nitrocelulosa (PROTAN©, Schlecher & Schuell, Germany), la cual posteriormente fue bloqueada durante al menos 1 hora a temperatura ambiente con leche desnatada al 5% (p/v) en tampón Tris salino (*tris buffer saline*, TBS) conteniendo Tween-20 al 0,1% (v/v)

(TBS-T). Transcurrido el bloqueo, las membranas se sometieron a tres lavados con TBS-T durante 5 minutos, y fueron expuestas a las correspondientes diluciones de anticuerpo incorporado en albúmina de suero bovino al 5% (p/v) en TBS-T. Las incubaciones con dichas diluciones de anticuerpo primario, 1:2000 frente a iNOS (Transduction Laboratories, Becton Sickinson Biosciences, Madrid, España) y 1:1000 frente a COX-2 (Cayman Chemical Company, Montigny le Bretonneux, France), se realizaron durante toda una noche a 4º C. Un anticuerpo primario frente a β-actina se empleó como control de carga.

Tras tres lavados de 5 minutos con TBS-T, las membranas fueron incubadas con anticuerpo conjugado con peroxidasa IgG anti-conejo (1:3000) durante 1 hora. Las bandas resultantes fueron detectadas por quimioluminiscencia (NEM Life Science Products, Zaventem, Bélgica) y cuantificadas por densitometría mediante el programa informático Scion Image (Scion Corporation, U.S.A.).

2.5. Determinación del contenido de proteínas: Método del Ácido bicinchonínico (BCA).

Se siguió la técnica descrita por Smith *et al*. (1985). Este método se utiliza cuando en el tampón de homogeneización puede haber agentes detergentes o de otro tipo que presentan reacciones de interferencia con el reactivo azul Coomassie del método convencional de Bradford. El fundamento de la técnica se basa en la capacidad de las proteínas para reducir el Cu^{2+} a Cu^{1+}, de forma que el Cu^{1+} reacciona con el ácido bicinchonínico formando un complejo púrpura con un máximo de absorbancia a 562 nm.

En este método se emplea un colorante que se obtiene mezclando dos reactivos A y B en proporción 50:1 (v/v). El reactivo A consiste en una disolución acuosa de ácido bicinchonínico (25,8 mM) en forma de sal sódica (ácido 4,4'-dicarboxi-2,2'-biquinolínico), $Na_2CO_3 \cdot H_2O$ (0,16 M), tartrato sódico-potásico (5,7 mM), NaOH (0,1 M) y $NaHCO_3$ (0,11 M) (pH 11,25). El reactivo B es una disolución acuosa al 40% de $CuSO_4 \cdot 5H_2O$. Ambos reactivos son estables a temperatura ambiente de forma indefinida. Para realizar la determinación, se añadieron a 2 µL del homogenado 200 µL del reactivo de coloración y, tras incubar a 37ºC durante 30 minutos, se procedió a la lectura

espectrofotométrica a 560 nm. El cálculo del contenido de proteínas se realizó por interpolación en una curva patrón de albúmina sérica bovina fracción V.

2.6. Estudio histológico.

Las muestras destinadas para el estudio histológico se fijaron en una solución tamponada de formaldehído (al 4% en tampón fosfato, pH 7,2) durante tres días. Después se deshidrataron con alcohol a concentraciones crecientes hasta alcohol absoluto y se incluyeron en parafina.

Con ayuda de un microtomo se obtuvieron cortes de 3-5 µm de grosor que fueron posteriormente teñidos con hematoxilina-eosina para su evaluación histológica mediante microscopía óptica. Los cortes histológicos se evaluaron según el criterio establecido por Stucchi *et al.* (2000) (*Tabla 8*).

2.7. Determinación del pH y humedad del contenido colónico

Los valores de pH del contenido colónico se midieron usando un medidor de pH GLP21-21 (Crisol, Barcelona, Spain), después de su suspensión en agua (1:5 p/v). El contenido de agua de las heces se calculo por diferencia entre el peso de las mismas frescas (inmediatamente después de su extracción) y secas (mantenidas en una estufa a 65º C durante 24 horas).

Tabla 8. *Escala de valoración del daño histológico en el modelo de colitis experimental por TNBS en ratas.*

Epitelio de la mucosa
Ulceración: ninguna (0); leve (1); moderada (2); intensa transmural (3)
Criptas
Actividad mitótica en: tercio inferior (0); tercio medio leve (1); tercio medio leve (1); tercio medio moderada (2); tercio superior (3)
Infiltrado leucocitario
Depleción de moco
Lamina propia
Infiltrado plasmocítico
Infiltrado leucocitario
Vascularización
Deposición de fibrina: ninguna (0); mucosa (1); submucosa (2); transmural (3)
Submucosa
Infiltrado leucocitario
Edema

3. ESTUDIOS MICROBIOLÓGICOS

Las muestras del contenido luminal se pesaron, se homogeneizaron, se resuspendieron a una concentración de 50-100 mg/mL en agua de peptona estéril, y se hicieron diluciones seriadas 1/10 (de 10^{-1} a 10^{-5}). Se inocularon en una placa Petri 100µL de las diluciones 10^{-4} y 10^{-5} en medio especifico para *Lactobacillus* (medio MSR, Oxoid) o *Bifidobacterium* (medio MSR suplementado

con 0.5 mg/L de dicloxacilina, 1g/L de LiCl, y 0.5 g/L de L-cisteína) y se incubaron en cámaras de anaerobiosis durante 24-48 horas a 37º C. En algunos ensayos también se determinaron coliformes y enterobacterias usando placas de contaje especificas petrifilm (3M, St. Paul, MN). Después de la incubación, el recuento final de colonias se expresó como $\log_{10}$ unidades formadoras de colonias/ gramo de material.

4. CUANTIFICACIÓN DE AGCC EN EL CONTENIDO INTESTINAL POR CROMATOGRAFÍA DE GASES.

Los AGCC son los principales productos derivados de la fermentación de la fibra por la flora bacteriana intestinal, principalmente acético, propiónico y butírico. Por este motivo, y en algunos ensayos procedimos a su determinación en los contenidos colónicos de los animales utilizados en nuestros estudios.

Los contenidos colónicos se homogeneizaron inmediatamente tras su obtención en una solución de $NaHCO_3$ (150 mM, pH 7,8) en atmósfera de argón y en una proporción 1:5 (p/v). Las muestras se incubaron durante 24 horas a 37º C con agitación constante. Transcurrido ese tiempo, las muestras se congelaron a –80º C hasta su procesamiento.

Tras descongelar los homogenados, los AGCC generados como consecuencia de su incubación previa durante 24 horas, se extrajeron con acetato de etilo. Para ello, a una alícuota de 1 mL de homogenado fecal se le adicionaron 50 µL del estándar interno, acido 2-metilvalérico (100mM), y una gota de acido sulfúrico (=10uL). Se agitó bien y se centrifugó a 10000 G durante 5 minutos a 4º C. Se recogió el sobrenadante (0.5 mL), y se le adicionaron 0.3 mL de acetato de etilo. Se agitó suavemente evitando la formación de micelas, y se volvió a centrifugar a 14000 RPM durante 5 minutos a 4º C para separar una fase inferior acuosa y otra superior de acetato de etilo donde se encuentran los AGCC. Se recogió la capa superior y tras deshidratarla con sulfato sódico anhidro se centrifugó por última vez a 10000 G durante 5 minutos a 4º C. Se recogió el extracto orgánico exento de agua, y se pasó a un vial cromatográfico sellado y mantenido a –80º C hasta su análisis. Se empleó un cromatógrafo de gases (Varian CP-3800) equipado con una ID CPWAX 52CB de 60 m de longitud y 0,25 mm de diámetro interno, y un detector FID (Varian, Lake Forest,

CA). El helio fue el gas usado como transportador y mezclador de la muestra con un flujo de 1.5 mL/min. La temperatura de inyección fue 250º C. Las concentraciones de butirato, acetato y propionato se calcularon automáticamente mediante el área de los picos usando el programa Chromatography Workstation (versión 5.5), que estaba conectado on-line al detector FID

5. ENSAYOS *IN VITRO*

5.1. Determinación de la producción endógena de glutation y γ-Glu-Cys por los probióticos

Para la determinación de glutation, los probióticos se cultivaron en medio MSR a 37º C durante 24 horas y se inocularon en tubos Falcon de 50 mL que contenían medio MSR. Se incubaron durante 24 horas, y se cogió una muestra de 1 mL en la que se midió el contenido de glutation. Las células se lavaron con agua destilada y se suspendieron en 300 μL de TCA al 7,5 %. La mezcla se centrifugó a 10500 G durante 2 minutos y 10 μL del sobrenadante se mezcló con 300 μL de agua miliQ en un nuevo tubo. 20 μL de esta solución se mezcló con 340 μL de de tampón fosfato 0,6 M (pH 7,8), y 340 μL de Tris (carboxietilo) fosfina HCl (TCEP) en HCl 20 mM. La muestra se mantuvo en oscuridad durante 15 minutos, se le añadió 800 μL de orto-ptalaldehido 12 mM en acetato sódico 50 mM, y se mantuvo a 4º C durante 15 minutos. Las muestras se analizaron por HPLC usando una columna Spherisorb S3 ODS a 0,8 ml/min en modo isocrático usando como fase móvil acetato sodico (pH 7,7)/acetonitrilo (96:4).

5.2. Determinación de la producción de citocinas

Para la determinación de la producción de citocinas se obtuvieron macrófagos de medula ósea de ratón, según el método descrito por Comalada *et al*., (2003). Se estimularon con 100 ng/mL de LPS en presencia y ausencia del probiótico a una concentración de 10^6 CFU/mL durante 2 horas. Pasado este tiempo, se lavaron con medio de cultivo para eliminar las bacterias no adheridas y seguidamente se cultivaron en nuevo medio de cultivo durante 12 horas. La producción de TNF-α, IL-12 e IL-10 se cuantificó por ELISA (CytoSets™; Biosource Internacional, Nivelles, Belgium) en los sobrenadantes celulares según las instrucciones del fabricante.

6. ESTUDIO ESTADÍSTICO.

Todos los resultados están expresados como la media aritmética ± error estándar de la media, excepto los datos no paramétricos (índice de daño macroscópico y microscópico, valoración de las características de las heces), que se expresan como mediana (rango). La significancia estadística de las diferencias entre las medias se determinó realizando un análisis de varianza de una vía (ANOVA). Los datos no paramétricos se evaluaron mediante el test de Mann-Whitney. Las diferencias entre porcentajes se analizaron con el test de χ^2. El umbral de significación se estableció en $p<0,05$. El programa estadístico utilizado fue SIGMA STAT.

RESULTADOS

1. Estudio comparativo de los efectos preventivos ejercidos por tres probióticos: *Bifidobacterium lactis, Lactobacillus acidophilus* y *Lactobacillus casei* en el modelo de colitis experimental por TNBS en ratas

RESUMEN

OBJETIVO

Los probióticos constituirían una posible terapia para el tratamiento de la EII, sin embargo el efecto y mecanismo de acción de los mismos difiere de unas cepas a otras, y además depende de las características del hospedador; por esta razón, el objetivo del presente estudio fue probar los efectos preventivos de diversos probióticos *Lactobacillus casei, Lactobacillus acidophilus,* y *Bidifobacterium lactis* en el modelo de colitis experimental por TNBS en ratas, modelo de inflamación intestinal con características histológicas y bioquímicas similares a la EII humana. La selección de estos probióticos se basó en anteriores estudios que muestran la eficacia de los mismos.

RESULTADOS

Los resultados obtenidos en este estudio revelaron el efecto antiinflamatorio intestinal ejercido por los tres probióticos. Desde el punto de vista macroscópico todos los probióticos redujeron de manera significativa el cociente colónico peso/longitud (*B. lactis*: 156.7 ± 15.8 mg/cm; *L. acidophilus*: 148.6 ± 15.8 mg/cm; *L. casei*: 189.8 ± 22.2 mg/cm; $p<0.01$ vs. Control: 249.7 ± 26.1 mg/cm), sin embargo solo las ratas tratadas con *Bifidobacterium lactis*, mostraron una menor incidencia de diarrea en comparación con el grupo control.

El análisis bioquímico mostró que solo el tratamiento con *Lactobacillus acidophilus* redujo la producción de MPO (77.4 ± 9.0 U MPO/g vs. Grupo Control: 133.5 ± 15.7 U MPO/g; $p<0.01$); sin embargo, todos los probióticos restauraron los niveles colónicos de glutation, reducidos a consecuencia del estrés oxidativo producido en el colon por el proceso inflamatorio (*B. lactis*: 1454 ± 63 nmol/g; *L. acidophilus*: 1357 ± 78 nmol/g; *L. casei*: 1428 ± 65 nmol/g; $p<0.05$ vs. Control: 1075 ± 100 nmol/g). El grupo de ratas tratadas con *Bifidobacterium lactis*, experimentaron una reducción de los niveles colónicos de TNFα (45.5 ± 7.9 pg/mg prot vs. Grupo control: 68.4 ± 10.3 pg/mg prot; $p<0.05$), así como una reducción en la expresión colónica de iNOS y COX-2; la administración del probiótico *Lactobacillus acidophilus* redujo los niveles colónicos de LTB_4 (66.2 ± 16.2 pg/mg prot vs. Grupo control: 136.3 ± 35.4 pg/mg prot; $p<0.05$) y la expresión de iNOS; por

ultimo, el tratamiento con *Lactobacillus casei* se asoció a una disminución en la expresión colónica de COX-2. Finalmente, ninguno de los probióticos modificó los niveles de lactobacilos ni bifidobacterias, sin embargo *B. lactis* aumento de manera significativa la relación *Bifidobacterium*/patógeno y *L. acidophilus* y *L. casei* lo hicieron sobre la relación *Lactobacillus*/patógeno

CONCLUSIÓN

Los tres probióticos ensayados mostraron cierta actividad antiinflamatoria intestinal en el modelo de colitis experimental por TNBS en rata, siendo el perfil antiinflamatorio de cada probiótico diferente, pudiendo ser considerados para su futuro desarrollo en el tratamiento de la enfermedad inflamatoria intestinal en seres humanos.

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ORIGINAL ARTICLE

A comparative study of the preventative effects exerted by three probiotics, *Bifidobacterium lactis*, *Lactobacillus casei* and *Lactobacillus acidophilus*, in the TNBS model of rat colitis

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cyclo-oxygenase 2, eicosanoid, experimental colitis, inducible nitric oxide synthase, probiotic, pro-inflammatory cytokine, rat.

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Abstract

Aims: The intestinal anti-inflammatory effects of three probiotics with immunomodulatory properties, *Lactobacillus casei*, *Lactobacillus acidophilus* and *Bifidobacterium lactis*, were evaluated and compared in the trinitrobenzene-sulphonic acid (TNBS) model of rat colitis.

Methods and Results: Colitis was induced in rats by intracolonic administration of 10 mg of TNBS dissolved in 0·25 ml of 50% ethanol. Each probiotic was administered orally (5×10^8 CFU suspended in 0·5 ml of skimmed milk) for 3 weeks, starting 2 weeks before the administration of TNBS. Colonic damage was evaluated histologically and biochemically 1 week after TNBS instillation. The results obtained revealed that all probiotics assayed showed intestinal anti-inflammatory effects, macroscopically evidenced by a significant reduction in the colonic weight/length ratio. Only *B. lactis* showed a lower incidence of diarrhoea in comparison with untreated rats. Biochemically, all probiotics restored colonic glutathione levels, depleted as a consequence of the oxidative stress of the inflammatory process. *Bifidobacterium lactis* treatment reduced colonic tumour necrosis factor (TNF)-α production, and inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2) expression; *L. acidophilus* administration reduced colonic leukotriene B_4 production and iNOS expression and *L. casei* intake was associated with a decrease in colonic COX-2 expression.

Conclusion: The three probiotics assayed have shown intestinal anti-inflammatory activity in the TNBS model of rat colitis, although each probiotic shows its own anti-inflammatory profile.

Significance and Impact of the Study: These probiotics could be considered as potential adjuvants in the treatment of inflammatory bowel disease, although more studies are required in order to demonstrate their efficacy in humans.

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, which are characterized by chronic and spontaneously relapsing inflammation. Although the aetiology of IBD remains unknown, there is increasing experimental evidence to support that abnormal regulation of the mucosal immune system against enteric bacteria is a key event underlying inflammatory mechanisms that lead to intestinal injury in these intestinal conditions (Fiocchi 1998;

Guarner 2005). Among the different factors that may contribute to this aberrant immune response, an imbalance between aggressive and protective micro-organisms within the gut lumen can play an important role. In consequence, a possible therapeutic approach in IBD therapy is the administration to these patients of probiotic micro-organisms (Sartor 2004), defined as live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance (Macfarlane and Cummings 2002).

In fact, it has been reported that the administration of a mixture of bifidobacteria and lactobacilli (Venturi *et al.* 1999) or *Escherichia coli* (Nissle 1917) (Rembacken *et al.* 1999) prevents the relapse of ulcerative colitis, the latter being as effective as low-dose 5-amionosalicylic. Moreover, there are reports on successful induction and maintenance of the remission of chronic pouchitis and ulcerative colitis after oral bacteriotherapy, being the most convincing evidence of the clinical efficacy of probiotics in clinical IBD (Gionchetti *et al.* 2003; Mimura *et al.* 2004; Furrie *et al.* 2005). However, treatments of Crohn's disease with probiotic preparations have reported conflicting results (Prantera *et al.* 2002; Bousvaros *et al.* 2005).

Although the results obtained after probiotic treatment in both human IBD and experimental colitis are promising, it is clear that all probiotics are not equally beneficial; each may have individual mechanisms of action, and the host characteristics may determine which probiotic species and even strain may be optimal. In fact, several studies have shown that not all probiotics exert constant intestinal anti-inflammatory effects in experimental models of intestinal inflammation (Shibolet *et al.* 2002). For this reason, it would be interesting to compare different probiotics in the same experimental model, in order to establish the best profile in a given setting and to further understand this new concept for the therapy of IBD. The aim of the present study was to test the preventative effects of different probiotics, two lactobacilli, *Lactobacillus casei* and *Lactobacillus acidophilus*, and one bidifobacteria, *Bifidobacterium lactis*, in the trinitrobenzenesulphonic acid (TNBS) model of rat colitis, a well-established model of intestinal inflammation that has some histological and biochemical features of the human disease (Jurjus *et al.* 2004). The selection of these probiotic strains was based on previous *in vitro* and *in vivo* studies that would support their potential beneficial effect in these intestinal conditions: *L. casei* has been described to downregulate proinflammatory cytokines, such as IL-6 and IFN-γ in lipopolysaccharide (LPS)-stimulated lamina propria mononuclear cells (Matsumoto *et al.* 2005); *L. acidophilus* treatment to mice has been shown to stimulate regulatory cytokine, like IL-10 and transforming growth factor (TGF)-β, in the colon of mice (Chen *et al.* 2005); and finally, *B. lactis* has been reported to ameliorate bacterial translocation (Eizaguirre *et al.* 2002) and to modulate intestinal immune functions (Roller *et al.* 2004; Ruiz *et al.* 2005). In addition, previous studies have also demonstrated the intestinal anti-inflammatory activity of *L. acidophilus* and *L. casei* in experimental models of colitis (Chen *et al.* 2005; Llopis *et al.* 2005).

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.

Preparation and administration of the probiotics

Probiotic strains *L. casei* (LAFTI® L26), *L. acidophilus* (LAFTI® L10) and *B. lactis* (*Bifidobacterium animalis lactis* LAFTI® B94) were provided by DSM Food Specialties (The Netherlands). Lactobacilli strains were cultured in MRS media at 37°C in anaerobic conditions using the Anaerogen system (Oxoid, Basingstoke, UK). *Bifidobacterium lactis* was grown in de Man Rogosa Sharpe (MRS) media supplemented with 0·5-mg l^{-1} dicloxacilin, 1-g l^{-1} LiCl and 0·5 g l^{-1} L-cysteine hydrochloride and incubated under anaerobic conditions. For probiotic treatment, bacteria was suspended in skimmed milk at the final concentration of 10^9 colony-forming units (CFU) per ml and stored at -80°C until usage. Freezing in milk and storage at -80°C of probiotic samples did not modify their viability, at least up to six months (data not shown).

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 five groups ($n = 10$); two of them (noncolitic and control groups) received no probiotic treatment and the remaining groups (treated groups) received orally every probiotic (5×10^8 CFU suspended in 0·5 ml of skimmed milk) daily for 3 weeks. Both noncolitic 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.* (1989). Briefly, they were anaesthetized with halothane and given 10 mg of TNBS dissolved in 0·25 ml of 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm through the anus. Rats from the noncolitic group were administered intracolonically 0·25 ml of phosphate buffered saline instead of TNBS. All rats were killed with an overdose of halothane 1 week after induction of colitis.

Histological and biochemical assessment of colonic damage

The body weight, water and food intake, and the presence of diarrhoea (as detected by perianal fur soiling), were recorded daily throughout the experiment. Once the rats were sacrificed, the colon was removed aseptically and the existence of adhesions between the colon and adjacent organs stated. Then, it was placed on an ice-cold plate, longitudinally opened and the luminal contents were collected for the microbiological studies (see later). 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.* (1995), which takes into account the extent and the severity of colonic damage. The colon was subsequently divided into four segments for biochemical determinations. Two fragments were directly frozen at -80°C, and the latter used for myeloperoxidase (MPO) activity and inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2) expressions, as these enzymatic activities were not modified by the freezing procedure. Another sample was weighed and frozen in 1 ml of 50-g l^{-1} trichloroacetic acid for total glutathione content determinations. The remaining sample was immediately processed for the measurement of colonic tumour necrosis factor (TNF)-α and LTB_4 levels. All biochemical measurements were completed within 1 week from the time of sample collection and were performed in duplicate.

MPO activity, as a marker of neutrophil infiltration, was measured according to the technique described by Krawisz *et al.* (1984); 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 per minute at 25°C. Glutathione (reduced and oxidized) concentrations, as markers of the colonic oxidative status, were assayed by HPLC with fluorimetric detection of oxidized and reduced glutathione, according to the method proposed by Martin and White (1991); the results were expressed as nmol of glutathione per mg wet tissue. Colonic samples for TNFα and LTB_4 determinations were immediately weighed, minced on an ice-cold plate and suspended in a tube with 10-mmol l^{-1} sodium phosphate buffer (pH 7·4) (1 : 5 w/v). The tubes were placed in a shaking water bath (37°C) for 20 min and centrifuged at 9000 ***g*** for 30 s at 4°C; the supernatants were frozen at -80°C until assay. TNFα was quantified by enzyme-linked immunoabsorbent assay (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the results were expressed as pg mg^{-1} protein. LTB_4 was determined by enzyme-immunoassay (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the results expressed as pg mg^{-1} protein.

The colonic expression of iNOS and COX-2 was analyzed by western blotting as previously described (Camuesco *et al.* 2004). The dilutions of each primary antibody were 1 : 2000 for iNOS (Transduction Laboratories, Becton Dickinson Biosciences, Madrid, Spain) and 1 : 1000 for COX-2 (Cayman Chemical Company, Montigny le Bretonneux, France), and incubated overnight at 4°C followed by peroxidase-conjugated anti-rabbit IgG antibody (1 : 3000) for 1 h. Control of protein loading and transfer was conducted by the detection of the β-actin levels.

Microbiological studies

Luminal content samples were weighed, homogenized and serially diluted in sterile peptone water. Serial 10-fold dilutions of homogenates were plated on MRS media (Oxoid, Madrid, Spain) for *Lactobacillus* or MRS media supplemented with 0·5-mg l^{-1} dicloxacilin, 1-g l^{-1} LiCl and 0·5-g l^{-1} L-cysteine hydrochloride for *Bifidobacterium* and incubated under anaerobic conditions in an anaerobic chamber for 24 h (lactobacilli) or 48 h (bifidobacterias). Although MRS media is not selective for lactobacilli, their growth at 37°C and for only 24 h limits the growth of other potential lactic acid bacteria. However, it is impossible to eliminate the possibility that other lactic acid bacteria will be able to grow in these conditions. Coliforms and entherobacteria were also determined by using specific Count Plates Petrifilm (3M, St. Paul, MN, USA). After incubation, the final count of colonies was reported as $\log_{10}$ CFU per gram of material.

Statistics

All results are expressed as the mean ± SEM. The differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and *post hoc* least significance tests. Nonparametric data (score) are expressed as the median (range) and were analyzed using the Mann–Whitney *U*-test. Differences between proportions were analyzed with the chi-squared test. All statistical analyses were carried out with the

Statgraphics 5.0 software package (STSC, Maryland, USA), with statistical significance set at $P < 0{\cdot}05$.

Results

The administration of probiotics for 2 weeks before colitis induction did not affect weight evolution compared with untreated rats. The intracolonic administration of TNBS resulted in an intestinal inflammatory status in the rats characterized by anorexia, loss of weight and diarrhoea, which gradually increased with time during the 7 days after instillation (not shown). These parameters were not significantly modified by any probiotic treatment, with the exception of the group treated with *B. lactis*, which showed an amelioration in the diarrhoeic process, and resulting after 7 days in a significantly lower incidence of diarrhoea when compared with TNBS control rats ($P < 0{\cdot}05$, Fig. 1). Furthermore, at 1 week after TNBS treatment, the animals that were treated with probiotics had fewer signs of mucosal inflammation. This beneficial effect was evidenced macroscopically by a significant reduction of the colonic weight/length ratio in all probiotic treated groups (Table 1). However, only the group of colitic rats treated with *L. acidophilus* showed a significantly lower colonic damage score than that of TNBS control rats ($P < 0{\cdot}05$). Animals treated with *L. acidophilus* had a significant decrease in the extent of colonic necrosis and/or inflammation induced by the administration of TNBS. Furthermore, this group of rats also showed a significant reduction in the incidence of adhesions of the colon to adjacent organs (intestinal loops, stomach, etc.) in comparison with untreated colitic control rats ($P < 0{\cdot}05$, Fig. 1).

The biochemical analysis of the colonic specimens confirms the intestinal anti-inflammatory effect exerted by the different probiotics, although their effects on the different biochemical parameters assayed were not homogeneous among groups. Thus, colonic MPO activity was only reduced after treatment with *L. acidophilus*, which indicates reduced neutrophil infiltration of the inflamed tissue after this probiotic treatment (Fig. 2). The treatment of colitic rats with any probiotic showed a significant increase in colonic glutathione content (Fig. 3), 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 (Galvez *et al.* 2003). The colonic inflammation induced by TNBS was also characterized by increased levels of colonic TNFα and LTB_4 (Figs 4 and 5). When *B. lactis* was administered to colitic rats, a significant reduction in colonic TNFα production was observed, whereas *L. acidophilus* administration significantly reduced colonic LTB_4 production in the inflamed colon in comparison with untreated colitic rats (Figs 4 and 5).

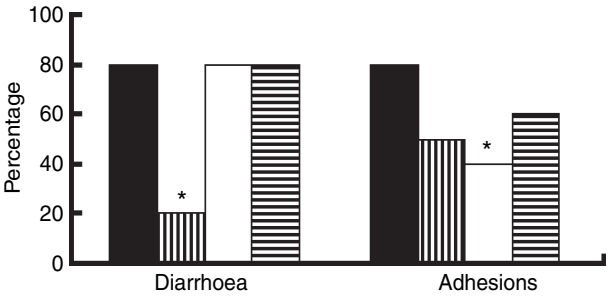

Figure 1 Effects of probiotic treatment (5×10^8 CFU per rat per day) on the incidence of diarrhoea and of adhesions of the colon to adjacent organs in trinitrobenzenesulphonic acid (TNBS) experimental colitis in rats. $*P < 0{\cdot}05$ *vs* TNBS control group. (■) TNBS-control; (▥) *B. lactis*; (□) *L. acidophilus*; (▤) *L. casei*.

Table 1 Effects of probiotic treatment on macroscopic damage score, extent of the inflammatory lesion along the colon and changes in colon weight in trinitrobenzenesulphonic acid (TNBS) experimental colitis in rats

Group	Damage score (0–10)	Extent of damage (cm)	Colon weight (mg cm^{-1})
Noncolitic	0	0	71·0 ± 2·5
TNBS control	6·5 (5·5–8·5)	3·5 ± 0·3	249·7 ± 26·1
Bifidobacterium lactis	6 (4–7)	2·8 ± 0·3	156·7 ± 15·8†
Lactobacillus acidophilus	5·5 (4–7)*	2·5 ± 0·3*	148·6 ± 15·8†
Lactobacillus casei	6 (4–7·5)	2·7 ± 0·4	189·8 ± 22·2†

Damage score for each rat was assigned according to the criteria described previously by Bell *et al.* (1995); data are expressed as median (range) ($n = 10$). Extent of damage and colon weight data are expressed as mean ± SEM ($n = 10$).

$*P < 0{\cdot}05$.

†$P < 0{\cdot}01$ *vs* TNBS control.

All colitic groups differ significantly from noncolitic group ($P < 0{\cdot}01$, not shown).

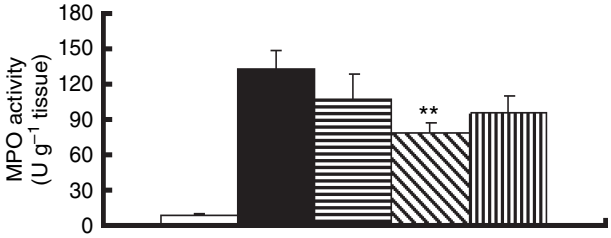

Figure 2 Effects of probiotic treatment on colonic myeloperoxidase (MPO) activity in TNBS experimental colitis in rats. One unit of MPO activity was defined as that degrading 1-μmol hydrogen peroxide per min at 25°C. Data are expressed as mean ± SEM ($n = 10$). All groups differ from noncolitic group ($P < 0{\cdot}01$; not shown). $**P < 0{\cdot}01$ *vs* trinitrobenzenesulphonic acid (TNBS) control group. (□) non-colitic; (■) TNBS-control; (▤) *B. lactis*; (▧) *L. acidophilus*; (▥) *L. casei*.

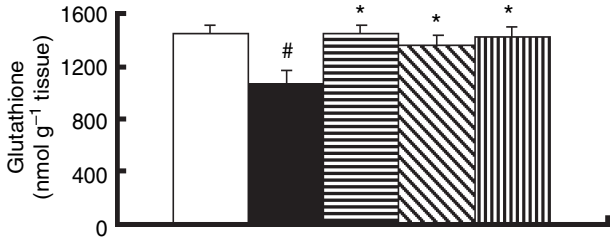

Figure 3 Effects of probiotic treatment on colonic glutathione (GSH) content in trinitrobenzenesulphonic acid (TNBS) experimental colitis in rats. Data are expressed as mean ± SEM (n = 10). $^{\#}P < 0{\cdot}01$ *vs* noncolitic group; $^{*}P < 0{\cdot}05$ *vs* TNBS control group. (□) non-colitic; (■) TNBS-control; (▤) *B. lactis*; (▧) *L. acidophilus*; (▥) *L. casei*.

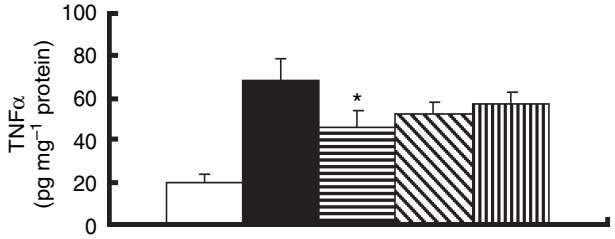

Figure 4 Effects of probiotic treatment on colonic tumour necrosis factor (TNF)-α levels in trinitrobenzenesulphonic acid (TNBS) experimental colitis in rats. Data are expressed as mean ± SEM (n = 10). All groups differ from noncolitic group ($P < 0{\cdot}01$; not shown). $^{*}P < 0{\cdot}05$ *vs* TNBS control group. (□) non-colitic; (■) TNBS-control; (▤) *B. lactis*; (▧) *L. acidophilus*; (▥) *L. casei*.

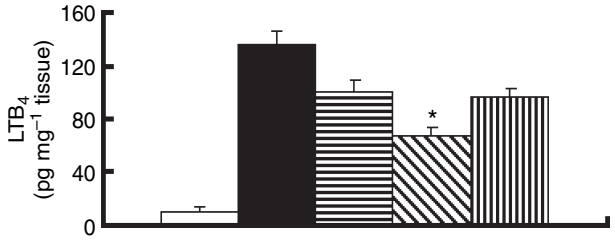

Figure 5 Effects of probiotic treatment on colonic leukotriene B_4 (LTB_4) levels in trinitrobenzenesulphonic acid (TNBS) experimental colitis in rats. Data are expressed as mean ± SEM (n = 10). All groups differ from noncolitic group ($P < 0{\cdot}01$; not shown). $^{*}P < 0{\cdot}05$ *vs* TNBS control group. (□) non-colitic; (■) TNBS-control; (▤) *B. lactis*; (▧) *L. acidophilus*; (▥) *L. casei*.

Finally, the inflammatory process in the colonic tissue was also associated with higher expression of both iNOS and COX-2 (Fig. 6), in comparison with noncolitic animals. The treatment of colitic rats with *Bifidobacterium lactis* resulted in a significant reduction of the expression of both induced enzymes; *Lactobacillus casei* was able to significantly reduce COX-2 expression, whereas *L. acidophilus* only reduced iNOS expression (Fig. 6).

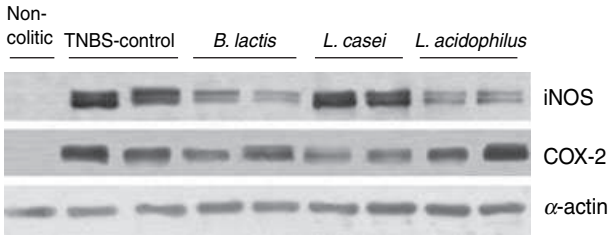

Figure 6 Effects of probiotic treatment (5×10^8 CFU per rat per day) on colonic inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2) expression in trinitrobenzenesulphonic acid (TNBS) experimental colitis in rats. iNOS and COX-2 expression were analysed by western blot using colonic homogenates as described in materials and methods. One hundred and fifty micrograms of protein were load in each lane. Only two representative samples of each experimental group (n = 10) were represented in this figure, but all of them were analysed. α-actin expression was used as control for loading and transfer.

Effects of probiotic administration on colonic bacterial profile

TNBS colitis resulted in a significant reduction in faecal lactobacilli count in comparison with normal rats ($P < 0{\cdot}05$), which was returned to normal values in probiotic-treated colitic rats (Fig. 7a). No statistical differences were observed in bifidobacteria counts among three groups ($P > 0{\cdot}1$; Fig. 7a) nor in the amount of other faecal potential

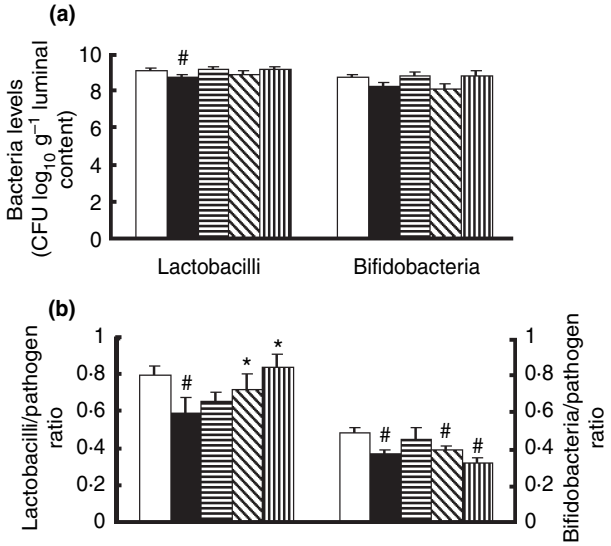

Figure 7 Effects of probiotic treatment (5×10^8 CFU per rat per day) on (a) bacteria levels (lactobacillus and bifidobacteria) and on (b) lactobacilli/pathogen ratio or bifidobacteria/pathogen ratio in trinitrobenzenesulphonic acid (TNBS) experimental colitis in rats. The values are represented as the mean ± SEM (n = 10). $^{*}P < 0{\cdot}05$ *vs* TNBS control group; $^{\#}P < 0{\cdot}01$ *vs* noncolitic group. (□) non-colitic; (■) TNBS-control; (▤) *B. lactis*; (▧) *L. acidophilus*; (▥) *L. casei*.

pathogenic bacteria, such as entherobacteria or coliform bacteria (data not shown). However, when the ratio of lactobacilli/pathogen or bifidobacteria/pathogen was evaluated, the inflammatory process did result in a significant decrease in comparison with normal rats. The administration of probiotic resulted, in all cases, in a normalization of their corresponding ratio, that is *B. lactis* restored bifidobacteria/pathogen ratio, whereas both lactobacilli significantly increased lactobacilli/pathogen ratio (Fig. 7b).

Discussion

The results obtained in the present study support previous studies describing the efficacy of probiotic therapy in the treatment of intestinal conditions, like inflammation and diarrhoea (Doron and Gorbach 2006). However, these studies have also reported that not all probiotics are equally beneficial, and these differences can be derived from different mechanisms of action among micro-organisms. For this reason, it is interesting to evaluate and compare the effects of different probiotics in the same experimental conditions in order to establish which micro-organisms can show the best profile as anti-inflammatory agents, or even to know if different probiotics may act synergistically to downregulate the intestinal inflammation by acting on different targets in the inflammatory response.

In this study, we have described the preventative intestinal anti-inflammatory activity of three probiotics *B. lactis*, *L. casei* and *L. acidophilus*, in the TNBS model of rat colitis. The results obtained confirm previous studies performed with these micro-organisms in different experimental models of colitis. The beneficial effect exerted by *B. lactis* was initially observed by a significant reduction in the number of rats with diarrhoea in comparison with the colitic untreated group, suggesting the preservation of the barrier integrity in the colon. This may be of interest as a barrier disruption by inflammation leads to increased stimulation by luminal antigens, such as food antigens, bacterial toxins and micro-organisms, of the different types of cells in the lamina propria, including immune cells (Heyman *et al.* 1994). In fact, a previous study has reported the ability of *B. lactis* in preserving the integrity of intestinal mucosa in a murine short bowel model and preventing bacterial translocation (Eizaguirre *et al.* 2002). In addition, a recent study has proposed that *B. lactis* promotes the activation of the TLR2 system in epithelial cells (Ruiz *et al.* 2005), which may contribute to the preservation of the barrier integrity in the colon (Cario *et al.* 2004). The evaluation of the colonic specimens from colitic rats treated with this probiotic showed a significant decrease in the weight/length ratio, an index of colonic oedema, which increased significantly as a consequence of the inflammatory process. This beneficial effect was also associated with a restoration in colonic glutathione content, which is depleted as a result of the intense oxidative stress that takes place after TNBS administration (Galvez *et al.* 2001). This is an important mechanism for tissue damage during chronic intestinal inflammation. In fact, previous studies have shown that the antioxidant and/or radical scavenging properties of different compounds, including flavonoids and 5-aminosalicylic acid (5-ASA) derivatives, may contribute to their beneficial effects in these intestinal conditions (Grisham 1994; Camuesco *et al.* 2004). When the different inflammatory mediators were evaluated, *B. lactis* was able to significantly reduce colonic TNFα production, a cytokine that has been reported to play a key role in intestinal inflammation (Rutgeerts *et al.* 2004). This effect can be 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, as it has been also proposed to occur with *B. lactis*, either alone (Ruiz *et al.* 2005) or in combination with other probiotics or prebiotics (Roller *et al.* 2004). The intestinal anti-inflammatory effect of this probiotic was also associated with a reduced expression of iNOS and COX-2, which are induced in intestinal epithelium during active intestinal inflammation (Kimura *et al.* 1997; Singer *et al.* 1998). It is interesting to note that this effect may be associated with the aforementioned ability of this probiotic to preserve glutathione content and to exert antioxidant properties given the relationship previously established between oxidative stress and upregulation of iNOS and COX-2 expression (Hecker *et al.* 1996; Lu and Wahl 2005). The final consequence of these interventions would be the downregulation of the production important proinflammatory mediators in IBD, i.e. reactive nitrogen metabolites and prostaglandins, which have been reported to play a key role in the pathophysiology of these intestinal conditions (Fiocchi 1998).

Previous reports have shown that *L. acidophilus* can exert immunomodulator properties (Galdeano and Perdigon 2004; Haghighi *et al.* 2005) that make this probiotic bacteria suitable for the treatment of pathologies with an altered immune response, including IBD. In fact, the inoculation of this probiotic in mice inhibits murine colitis induced by *Citrobacter rodentium* infection (Chen *et al.* 2005). The present study reveals that *L. acidophilus* partially prevents TNBS-induced colonic damage in rats, as is evidenced both macroscopically, by a significant decrease in the colonic damaged area and in the weight/length ratio, and biochemically, by a significant reduction in MPO activity, in comparison with untreated control rats. The decrease in this enzyme activity reveals a lower infiltration of neutrophils in the inflamed intestine, probably derived from the reduction in the colonic production

of the chemotactic eicosanoid LTB_4 observed in the rats treated with *L. acidophilus*. The inhibitory effect on neutrophil activity can result in an attenuation of the colonic oxidative stress, as observed by the partial restoration in colonic glutathione content elicited after the treatment of colitic rats with this probiotic treatment. Consequently, an inhibitory effect on LTB_4 synthesis and/or release could be considered as a mechanism involved in the beneficial effect exerted by the probiotic *L. acidophilus* in this model of experimental colitis, similar to what has been previously proposed for different drugs used in the treatment of IBD, like sulphasalazine and 5-ASA (Travis and Jewel 1994), or for dietary manipulations, like ω-3 polyunsaturated fatty acids (Camuesco *et al.* 2005a) or prebiotics (Camuesco *et al.* 2005b). Finally, the intestinal anti-inflammatory effect showed by this probiotic was associated with a reduced expression of iNOS expression, which would result in a lower NO production, thus avoiding the deleterious effect of this free radical when produced in large amounts (Grisham 1994).

The third probiotic used was *L. casei*, a probiotic that has been reported to attenuate mucosal injury and inflammatory response in the TNBS experimental model of rat colitis, and the effect associated with a partial prevention in bacterial translocation to mesenteric lymph nodes, spleen and liver (Llopis *et al.* 2005). The results obtained in the present study confirm the beneficial effect of this probiotic in this experimental model of rat colitis. This was evidenced macroscopically by a significant reduction in the colonic weight/length ratio, and biochemically by significant amelioration of the glutathione depletion. The effect was associated with a significant reduction in COX-2 expression, probably derived from the amelioration in the colonic oxidative stress that takes place in this intestinal condition. However, when the efficacy of *L. casei* is compared with the other two probiotics assayed in the present study, it shows a modest intestinal anti-inflammatory effect.

A common feature in the three probiotics assayed is their ability to modify colonic microflora, which was altered as a consequence of the inflammatory process induced by TNBS, similar to that described previously (Peran *et al.* 2005). In fact, the probiotic treatment resulted in a restoration in the ratio between pathogenic bacteria and bifidobacteria (*B. lactis*) or lactobacilli (*L. acidophilus* and *L. casei*). This effect can definitively contribute to the beneficial effect exerted by these probiotics in this model of experimental colitis. In fact, it has been previously described that the increase in *Lactobacillus* sp. or *Bifidobacteria* sp. levels reduces the concentration of adherent and translocated bacteria and attenuates the colitis in IL-10 gene-deficient mice (Madsen *et al.* 1999), thus preventing the pathogenic effect of other species that may play an important role in the generation of the exacerbated immune response in intestinal inflammation, as proposed, both in experimental models (Garcia-Lafuente *et al.*, 1997) and in humans (Cummings *et al.* 2003).

In conclusion, the three probiotics assayed (*B. lactis*, *L. acidophilus* and *L. casei*) have shown intestinal anti-inflammatory activity in the TNBS model of rat colitis. However, each probiotic shows its own anti-inflammatory profile, confirming previous observations that not all the probiotics present the same efficacy as anti-inflammatory agents, and do not share the same mechanisms of action in exerting the beneficial effect.

Acknowledgements

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

RESUMEN

OBJETIVO

Los probióticos ensayados anteriormente mostraron solamente un efecto beneficioso moderado, por lo que decidimos ensayar otros probióticos con propiedades añadidas cuya selección atendió a diferentes criterios como su capacidad para regular la respuesta inmunológica en la mucosa intestinal mediante la producción de citocinas. En este sentido, varias especies de *Lactobacillus* tienen la capacidad de afectar a la producción del factor de necrosis tumoral α (TNFα), citocina proinflamatoria con un papel relevante en la patogénesis de la EII.

El objetivo del presente estudio fue ensayar el efecto preventivo de *Lactobacillus salivarius* ssp. *salivarius* en el modelo de colitis experimental por TNBS en ratas. La selección de *Lactobacillus salivarius ssp. salivarius* se basó en su capacidad de reducir la producción de citocinas proinflamatorias, de adherirse a las células de la mucosa intestinal e inhibir el crecimiento de bacterias patógenas.

RESULTADOS

El tratamiento con *Lactobacillus salivarius* ssp. *salivarius*, 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 del grupo control, $P<0.01$) 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 *Lactobacillus salivarius* también logro restaurar de manera significativa los niveles colónicos de glutation (1252 ± 42 nmol/g vs. 1087 ± 51 nmol/g, $P<0.05$), que se depleciona a causa del estrés oxidativo provocado por el proceso inflamatorio. Además, el tratamiento probiótico redujo también de manera significativa los niveles de TNFα

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 el probiótico *Lactobacillus salivarius ssp. 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 TNFα, y NO. Este efecto beneficioso se podría atribuir a su efecto sobre la respuesta inmunitaria alterada característica 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 was 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 the 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 pro-inflammatory 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.

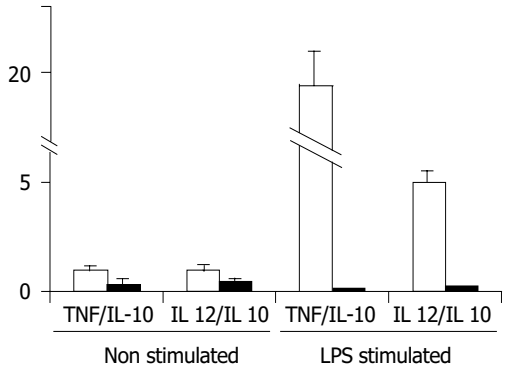

Figure 1 Production of inflammatory cytokines by bone marrow-derived macrophages (BMDM). TNF-α, IL-12, and IL-10 production was analyzed by ELISA in the supernatants of BMDM stimulated or not with LPS (100 ng/mL) and incubated with 10^6 CFU/mL of *L. salivarius ssp. salivarius* (CECT5713) (black bars)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

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 bacterial 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

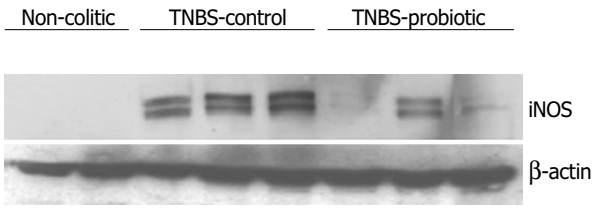

Figure 3 Effects of *L. salivarius ssp. salivarius* treatment (5×10^8 CFU/rat·d) on colonic nitric oxide synthase (NOS) expression in TNBS experimental colitis in rats.

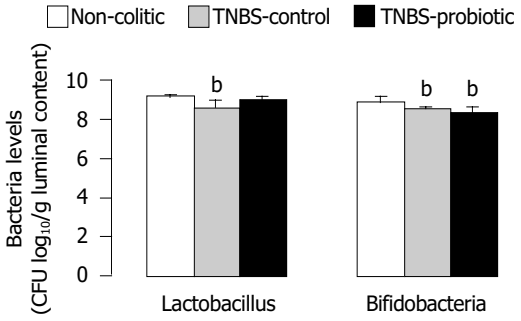

Figure 4 Effects of *L. salivarius ssp. salivarius* (5×10^8 CFU/rat·day) treatment on bacteria levels (Lactobacillus and Bifidobacteria) in TNBS experimental colitis in rats. [b]P<0.01 *vs* non-colitic group.

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·d)

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±SE. [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 pro-inflammatory cytokine TNF-α, thus reducing the activity of other pro-inflammatory 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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3. Efectos preventivos del probiótico *Lactobacillus fermentum*, capaz de liberar glutation en el modelo de colitis experimental por TNBS en ratas

RESUMEN

OBJETIVO

El desequilibrio entre la formación de especies reactivas de oxigeno y de compuestos antioxidantes juega un papel muy importante en la patogénesis y mantenimiento de la EII, por tanto una estrategia terapéutica podría ser su capacidad de contrarrestar la producción de especies reactivas de oxigeno mediante la restauración de los niveles de productos antioxidantes como el glutation, antioxidante endógeno al que se le atribuye un papel esencial en el mantenimiento de la integridad de la mucosa y por tanto en la patogénesis de la enfermedad inflamatoria intestinal.

El objetivo de este estudio fue probar los efectos preventivos del probiótico *Lactobacillus fermentum*, capaz de producir glutation, en el modelo de colitis experimental por TNBS en ratas.

RESULTADOS

Los ensayos in vitro ratificaron la capacidad de *Lactobacillus fermentum* de producir glutation (1.4 ± 0,3 mM en medio de cultivo), y su precursor γ-Glu-Cys per se (2.3 ± 0,2 mM en medio de cultivo) frente a valores basales.

El tratamiento con *Lactobacillus fermentum* mejoró la respuesta inflamatoria en las ratas colíticas evidenciado mediante la longitud del daño (2.6 ± 0.5 cm vs. 4.0±0.3 cm $P<0.01$) y la medida del peso /longitud (149.7 ± 13.0 mg/cm vs. 226.5 ± 17.6 mg/cm $P<0.01$) y bioquímicamente mediante una reducción significativa de la actividad MPO (193.7 ± 25.9 U MPO/g vs. 313.0 ± 6.1 U MPO/g $P<0.01$); un aumento del glutation, agotado en el colon como consecuencia del proceso inflamatorio (1614 ± 85 nmol/g vs. 1331 ± 62 nmol/g $P<0.05$); reducción de los niveles de la citocina proinflamatoria TNFα (469.5 ± 67.6 pg/g vs. 680.9 ± 52.8 pg/g $P<0.05$); y finalmente una reducción de la expresión de la iNOS en comparación con el grupo control.

Los estudios de los AGCC en el contenido colónico, muestran el aumento significativo de los mismos en las ratas tratadas con el probiótico con respecto al grupo control (Acetato: 13.3 ± 1.9 mg/g heces vs. 6.2 ± 1.6 mg/g heces $P<0.05$; Butirato: 2.6 ± 0.7 mg/g heces vs. 0.8 ± 0.2 mg/g

heces; Propionato: 5.3 ± 0.7 mg/g heces vs. 2.7 ± 0.6 mg/g heces). Finalmente, los animales tratados mostraron un mayor recuento de *Lactobacillus* en el contenido colónico, sin existir diferencias en el recuento de *Bifidobacterium*.

CONCLUSIÓN

La administración del probiótico *Lactobacillus fermentum* mejora la recuperación de la colitis inducida por TNBS en rata, atribuido a un aumento en los niveles colónicos de glutation, y a una reducción de la producción de ciertos mediadores implicados en la respuesta inflamatoria intestinal tales como TNFα y oxido nítrico. Este efecto beneficioso también se podría atribuir a un aumento en los niveles colónicos de AGCC, así como a un mayor recuento de *Lactobacillus* en el contenido colónico.

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ORIGINAL ARTICLE

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Lactobacillus fermentum, a probiotic capable to release glutathione, prevents colonic inflammation in the TNBS model of rat colitis

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Abstract *Background and aims:* Inflammatory bowel disease is associated with intestinal oxidative stress. In the present study we test the preventative effect of *Lactobacillus fermentum*, a probiotic that produces per se glutathione, in the trinitrobenzenesulphonic acid (TNBS) model of rat colitis. *Methods:* Colitis was induced in rats by intracolonic administration of 10 mg of TNBS dissolved in 0.25 ml of 50% ethanol. *L. fermentum* was administered orally (5×10^8 CFU suspended in 0.5 ml of skim milk) to a group of rats for 3 weeks, starting 2 weeks before colitis induction. Colonic damage was evaluated both histologically and biochemically, and the colonic luminal contents were used for bacterial studies as well as for short chain fatty acid (SCFA) production. *Results: L. fermentum* treatment resulted in an amelioration of the inflammatory response in colitic rats as evidenced histologically and by a significant reduction of colonic MPO activity ($P<0.05$). The probiotic partially counteracted the colonic glutathione depletion induced by the inflammatory process. In addition, probiotic-treated colitic rats showed significant lower colonic tumour necrosis factor (TNF)α levels ($P<0.01$) and inducible nitric oxide synthase (iNOS) expression when compared to non-treated rats. Finally, the probiotic induced growth of *Lactobacilli* species and production of SCFA in colonic contents in comparison with control colitic rats. *Conclusion:* Administration of the probiotic *L. fermentum* facilitates the recovery of the inflamed tissue in the TNBS model of rat colitis, an effect associated with increased levels of glutathione as well as with amelioration of the production of some of the mediators involved in the inflammatory response of the intestine, such as TNFα and NO.

Keywords *Lactobacillus fermentum* · TNBS experimental rat colitis · Glutathione · Oxidative stress

Introduction

Inflammatory bowel disease (IBD) is a chronic disease of the digestive tract, and usually refers to two related conditions, ulcerative colitis and Crohn's disease, which are characterised by chhronic and spontaneously relapsing inflammation. Although the aetiology 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]. A possible therapeutic approach in IBD therapy is the administration 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 of successful induction and maintenance of remission of chronic pouchitis after oral bacteriotherapy [7, 8].

Different mechanisms have been proposed to participate in the therapeutic effects exerted by probiotic microorganisms. Firstly, these microorganisms could 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, 9]; secondly, probiotics have been proposed to modulate the host defenses by influencing the intestinal immune system [10, 11]; and thirdly, these microorganisms have been reported to positively affect the intestinal barrier function [12, 13]. Moreover, an interesting approach in IBD treatment is the administration of probiotics capable of delivering in the intestinal lumen compounds that have been reported to exert beneficial effects in these intestinal conditions. Thus, the use of genetically modified *Lactococcus lactis* able to promote the delivery of either the anti-inflammatory cytokine mIL-10 [14] or trefoil factors [15] in the intestine cures or prevents experimental enterocolitis in mice. In addition, nitric oxide released by *Lactobacillus farciminis* improves experimental colitis in rats [16].

It is well reported that IBD is characterised by an unbalanced formation of reactive oxygen species and antioxidant micronutrients, and this may be important in the pathogenesis and/or perpetuation of the tissue injury in IBD [17, 18], which may provide a rationale for therapeutic modulation of these intestinal conditions with antioxidants. Thus, antioxidant therapy has been shown to be beneficial in experimental models of colitis [19–21], having been proposed that the beneficial effects exerted by 5-aminsalycilic derivates in human IBD are derived from their antioxidant properties [22]. In addition, glutathione, the major component of the endogenous nonprotein sulfhydryl pool, is an endogenous antioxidant that is essential in maintaining mucosal integrity; and some experimental data confirm this important role. Firstly, the inflammatory status in experimental colitis is associated with its depletion [17, 18]; secondly, when the sulfhydryl blocker iodoacetamide is administered intracolonically to rats, they develop colonic inflammation [23]; and thirdly, glutathione supplementation improves colonic damage in experimental colitis [24, 25]. Considering all of the above, a probiotic strain able to directly produce or promote the intestinal release of glutathione could have potential use in the treatment of IBD.

The aim of the present study was to test the preventative effects of a *Lactobacillus fermentum* strain in the trinitrobenzenesulphonic acid (TNBS) model of rat colitis, a well-established model of intestinal inflammation with some resemblance to human IBD [26]. The selection of this lactobacilli strain was based on its capacity to produce glutathione, an uncommon feature amongst lactobacilli strains. Special attention was paid to its effects after oral administration to colitic rats on the colonic glutathione levels and on the production of some of the mediators involved in the inflammatory response, such as tumour necrosis factor α (TNFα), leukotriene B_4 (LTB_4) and nitric oxide (NO). In addition, the correlation between the intestinal anti-inflammatory effect of *L. fermentum* and the modifications induced on colonic flora and on SCFA production in the luminal contents 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, and was approved by the Animal Research and Ethic Committee of the University of Granada (Spain).

Reagents

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

Glutathione production in bacteria

Puleva Biotech lactic acid bacterial collection was screened for Lactobacilli bacteria with the ability to produce glu-

tathione. Cultures were grown in MRS medium at 37°C for 24 h and used to inoculate 50-ml Falcon tubes containing MRS medium. Cells were incubated for 24 h and 1-ml samples were taken to analyse glutathione content. Cells were washed with distilled water, suspended in 300 μl of TCA 7.5% (w/v) and disrupted by stirring. The mixture was centrifuged (at 10,500 *g* for 2 min) and 100 μl from the supernatant was transferred to a new tube containing 300 μl of MilliQ water. A portion (20 μl) from this solution was mixed with 340 μl of 0.6 M phosphate buffer (pH 7.8) and 340 μl of 1.25 mM Tris (carboxyethyl) phosphine HCl (TCEP) in 20 mM HCl. The sample was placed in the dark for 15 min, and then 800 μl of 12 mM *ortho*-pthalaldehyde in 50 mM sodium acetate was added and samples were placed at 4°C for 15 min. Samples were analysed by HPLC using a Spherisorb S3 ODS column at 0.8 ml/min in isocratic mode using 50 mM sodium acetate (pH 7.7)/acetonitrile (96:4) as mobile phase.

Preparation and administration of the probiotic

L. fermentum 5716, a human breast milk derived strain [27], was obtained from Puleva Biotech (Granada, Spain) and was normally growth in MRS media at 37°C under anaerobic conditions using the Anaerogen system (Oxoid, Basingstoke, UK). For probiotic treatment, bacteria was suspended in skim milk (10^9 CFU/ml) and stored at −80°C 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 under 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 the probiotic orally (5×10^8 CFU suspended in 0.5 ml of skim milk), daily for 3 weeks. Both non-colitic and control groups were given daily administration of the vehicle used to administer the probiotic (0.5 ml of skim milk). Two weeks after the treatment was started, 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. [28]. Briefly, they were anaesthetised with halothane and given 10 mg of TNBS dissolved in 0.25 ml of 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm through the anus. Rats from the non-colitic group were administered intracolonically with 0.25 ml of phosphate-buffered saline instead of TNBS. All rats were killed with an overdose of halothane 1 week after induction of colitis.

Assessment of colonic damage

Body weight, water and food intake were recorded daily throughout the experiment. After the rats were sacrificed, the colon was removed aseptically and placed on an ice-cold plate, and longitudinally opened; then the luminal contents were collected for microbiological studies and for SCFA quantification (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 who were unaware of the treatment, according to the criteria described by Bell et al. [29] and Camuesco et al. [30], which take 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 taken at different levels and stained with haematoxylin and eosin. The histological damage was evaluated on a 0–27 scale by two pathologist observers (A.N. and A.C.), who were blinded to the experimental groups, according to the criteria described previously [30]. The colon was subsequently divided into four segments for biochemical determinations. Two fragments were frozen at −80°C 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 glutathione content determination. The remaining sample was immediately processed for the measurement of TNFα and leukotriene B_4 (LTB_4) levels. All biochemical measurements were performed in duplicate and completed within 1 week of sample collection.

MPO activity was measured according to the technique described by Krawisz et al. [31]; the results were expressed as MPO units per gram of wet tissue; 1 unit of MPO activity was defined as that degrading 1 μmol hydrogen peroxide/min at 25°C. Total glutathione content was quantified with the recycling assay described by Anderson [32], and the results were expressed as nmol/g wet tissue. Colonic samples for TNFα and LTB_4 determinations were immediately weighed, minced on an ice-cold plate and suspended in a tube with 10 mM sodium phosphate buffer (pH 7.4) (1:5 w/v). The tubes were placed in a shaking water bath (37°C) for 20 min and centrifuged at 9,000 *g* for 30 s at 4°C; the supernatants were frozen at −80°C until assay. TNFα was quantified by enzyme-linked immunoabsorbent assay (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the results were expressed as pg/g wet tissue. LTB_4 was determined by enzyme immunoassay

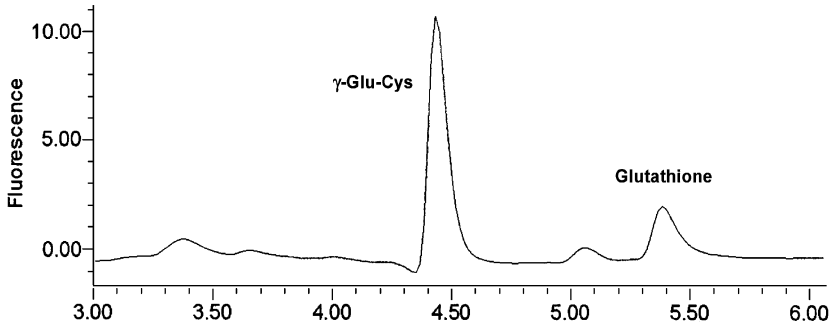

Fig. 1 HPLC analysis of glutathione and γ-Glu-Cys dipeptide production by *L. fermentum* 5716

(Amersham Pharmacia Biotech) and the results expressed as ng/g wet tissue. iNOS expression was analysed by Western blotting as previously described [20], and control of protein loading and transfer was conducted by detection of the β-actin levels.

Microbiological studies

Luminal content samples were homogenised in peptone physiological saline (100 mg faeces/ml). Tenfold serial dilutions were made in the same medium and aliquots of 0.1 ml of the appropriate dilution were spread onto the following agar media: MRS agar for lactobacilli, MRS agar supplemented with 0.5 mg/l dicloxacillin, 1 g/l LiCl and 0.5 g/l L-cysteine hydrochloride for *Bifidobacterium*; reinforced clostridial containing 20 μg/ml de polymyxin for Clostridium. All media were obtained from Oxoid (Basingstoke, UK), whereas antibiotics and other supplements were obtained from Sigma (St. Louis, MO). Culture plates were incubated in absence of oxygen at 37°C for 24–48 h. Similarly, 1 ml of suitable dilution was spread onto specific Count Plates Petrifilm (3M, St. Paul, MN) for coliforms, 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 faecal (CFU/g) was calculated. The mean and standard error per group were calculated from the log values of the CFU/g.

SCFA quantification in colonic contents

To quantify the SCFA concentration in the colonic luminal contents, samples were homogenised 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 the internal standard 2-methylvaleric acid (100 mM), 10 μl of sulphuric acid and 0.3 ml of ethyl acetate were added to 1 ml of the homogenate and then centrifuged at 10,000 *g* for 5 min at 4°C. The supernatants were dehydrated with sodium sulphate anhydrous and centrifuged 10,000 *g* for 5 min at 4°C. Later, 0.5 ml of the sample was splitless inoculated into a gas chromatograph (Varian CP-3800) equipped with an ID (CPWAX 52CB 60 m×0.25 mm), and connected to a FID detector (Varian, Lake Forest, CA). Helium was used as the carrier and the make-up gas, with a flow rate of 1.5 ml/min. Injection temperature was 250°C. Acetate, propionate and butyrate concentrations were automatically calculated from the areas of peaks using the Star Chromatography WorkStation program (version 5.5), which was on-line connected to the FID detector.

Statistics

All results are expressed as the mean±SEM. 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 analysed using the Mann–Whitney *U*-test. Differences between propor-

Table 1 Effects of *L. fermentum* (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	60.4±2.8
TNBS control	7 (6–9)	4.0±0.3	226.5±17.6
TNBS probiotic	5.5 (2–7)*	2.6±0.5**	149.7±13.0**

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±SEM

*$P<0.05$, **$P<0.01$ vs TNBS control. All colitic groups differ significantly from non-colitic group ($P<0.01$, not shown)

tions were analysed with the chi-square test. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Maryland), with statistical significance set at $P<0.05$.

Results

Intestinal anti-inflammatory activity of *L. fermentum* administration in rats with TNBS-induced colitis

More than 50 strains of lactic acid bacteria belonging to Puleva Biotech's collection were screened for their ability to produce glutathione. The results confirmed that the ability to produce glutathione is not a common feature in the lactobacilli group, although it has been reported in other prokaryotic microorganism [33, 34]. In fact, the production of glutathione was detectable in the strain *L. fermentum* 5716, which, in addition to its ability to produce glutathione (1.4±0.3 mM in culture media), was also able to generate the antioxidant dipeptide γ-Glu-Cys (2.3±0.2 mM in culture media) (Fig. 1). For this reason, we decided to use this strain to test its ability to prevent the inflammatory response in the in vivo assay of experimental colitis.

L. fermentum 5716 administration for 2 weeks failed to induce any symptoms of diarrhoea or effect in the weight evolution (data not shown). However, once 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 of the extent of colonic necrosis and/or inflammation (Table 1). This anti-inflammatory effect was also associated with a significant reduction of the colonic weight/length ratio between both colitic groups, an index of colonic oedema that is increased significantly as a consequence of the inflammatory process (Table 1). The histological studies confirmed the intestinal anti-inflammatory effect exerted by *L. fermentum* (Fig. 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 21.6±2.3 (mean±SEM). Colonic samples were characterised by severe oedema, interstitial microhaemorrhages and diffuse leucocyte 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

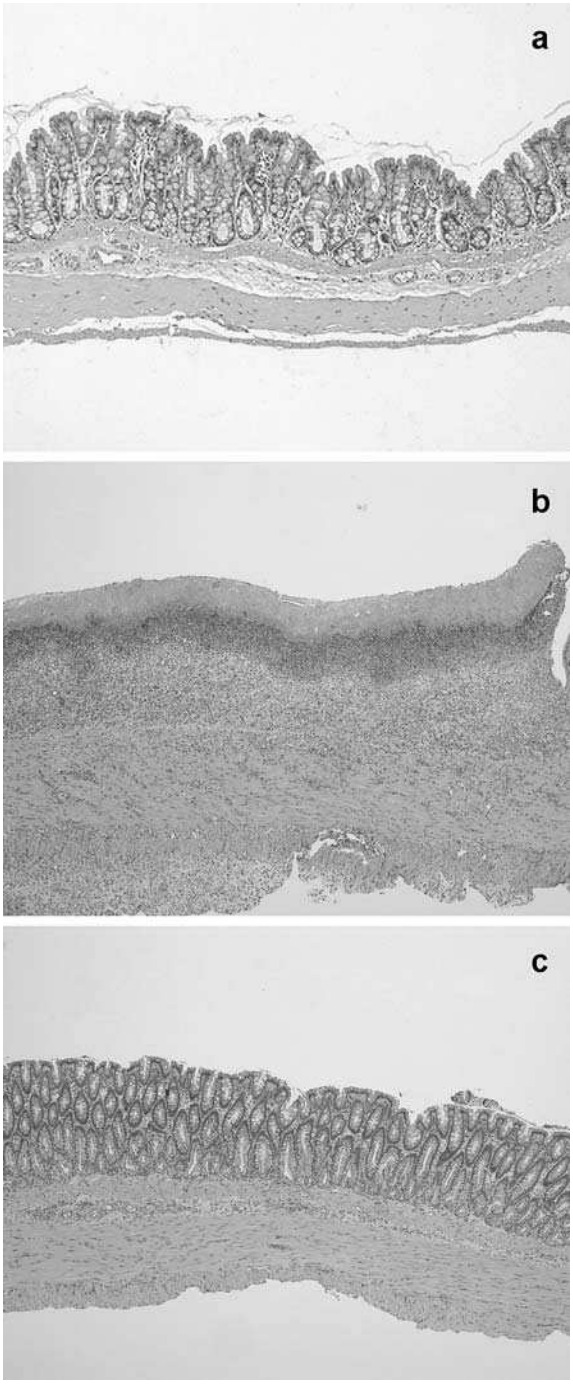

Fig. 2 Histological sections of colonic mucosa from colitic rats 1 week after TNBS instillation stained with haematoxylin and eosin. **a** Non-colitic group showing the normal histology of the rat colon (original magnification ×20). **b** TNBS control group showing complete destruction of the mucosa, which has been substituted by inflammatory granulation tissue. There is evident edema and intense diffuse transmural inflammatory infiltrate (original magnification ×100). **c***L. fermentum*-treated group showing amelioration in the inflammatory process and 'restoration' of the mucosal tissue with the presence of mucin-replenished goblet cells (original magnification ×100)

Table 2 Myeloperoxidase (MPO) activity, total glutathione (GSH) content, TNFα and LTB_4 levels in colon specimens from non-colitic rats, TNBS control colitic rats and TNBS colitic rats treated with *L. fermentum* (5×10^8 CFU/rat·day)

Group	MPO activity (units MPO/g)	GSH (nmol/g)	LTB_4 (ng/g)	TNFα (pg/g)
Non-colitic (n=10)	8.3±1.8	1,937±37	3.7±0.4	331.6±25.7
TNBS control (n=10)	313.0±6.1$^{\#\#}$	1,331±62$^{\#\#}$	14.6±1.7$^{\#\#}$	680.9±52.8$^{\#}$
TNBS probiotic (n=10)	193.7±25.9$^{\#\#,**}$	1,614±85$^{\#,*}$	13.2±1.6$^{\#\#}$	469.5±67.6$^{*,\#}$

Data are expressed as mean±SEM
$^{*}P<0.05$, $^{**}P<0.01$ vs TNBS control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs non-colitic group

moderate to severe goblet cell depletion. However, histological analysis of the colonic specimens from rats treated with the probiotic revealed a more pronounced recovery of the intestinal architecture than controls, with a score of 9.4±1.9 (mean±SEM) ($P<0.01$ vs TNBS control group). Thus, most of the samples (nine of ten) 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-epithelisation. 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 slight to moderate with a patchy distribution, although neutrophils were the predominant cell type.

The lower leucocyte infiltration was also assessed biochemically by the reduction of colonic MPO activity, a marker of neutrophil infiltration that was enhanced in the TNBS control group (Table 2). In addition, probiotic-treated colitic rats showed a significant increase of colonic glutathione 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 [35] (Table 2). Finally, the colonic inflammation induced by TNBS was characterised by increased levels of colonic TNFα and LTB_4 (Table 2) as well as by a greater colonic iNOS expression (Fig. 3) in comparison with non-colitic animals. Treatment of colitic rats with *L. fermentum* resulted in a significant reduction of colonic TNFα levels (Table 2), but no significant modification of colonic LTB_4 levels was obtained between both colitic groups (Table 2). Finally, a lower colonic iNOS expression was also observed in colitic animals that received the bacteria suspension when compared to TNBS control animals (Fig. 3).

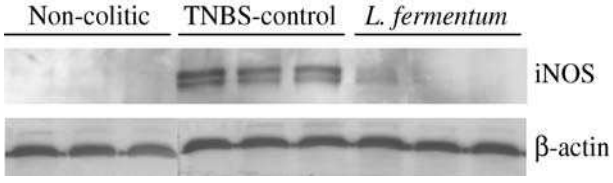

Fig. 3 Effects of *L. fermentum* treatment (5×10^8 CFU/rat·day) on colonic nitric oxide synthase (*NOS*) expression in TNBS experimental colitis in rats

Effects of *L. fermentum* administration on colonic bacterial profile

TNBS colitis resulted in a significant reduction of faecal lactobacilli count in comparison with normal rats (P=0.003). Probiotic-treated colitic rats showed significantly higher counts of *Lactobacilli* species in colonic contents in comparison with control colitic rats (P=0.039), without showing statistical differences with non-colitic control group (Fig. 4). No statistical differences were observed in Bifidobacteria counts amongst three groups ($P>0.1$; Fig. 4) or in the amount of other faecal potential pathogenic bacteria such as entherobacteria or coliforms (data not shown).

Effects of *L. fermentum* administration on SCFA production

When the colonic contents from TNBS control rats were incubated for 24 h, a reduction of the levels of SCFA was

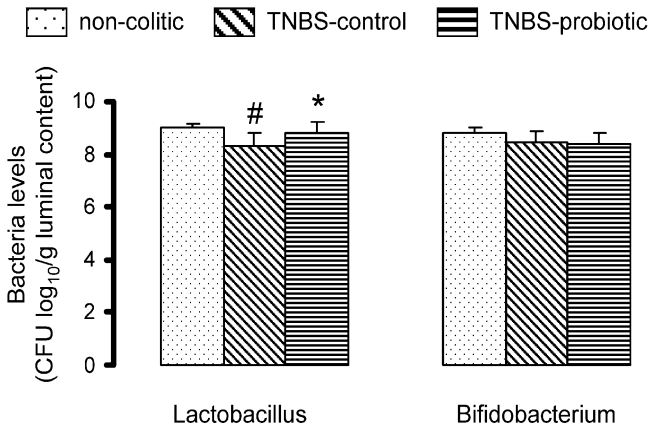

Fig. 4 Effects of *L. fermentum* (5×10^8 CFU/rat·day) treatment on bacteria levels (Lactobacillus and Bifidobacteria) in TNBS experimental colitis in rats. $^{*}P<0.05$ vs TNBS control; $^{\#}P<0.01$ vs non-colitic group

Table 3 Effects of *L. fermentum* (5×10^8 CFU/rat·day) treatment on short chain fatty acid production in colonic contents in TNBS experimental colitis in rats

Group	Acetate (mg/g faeces)	Propionate (mg/g faeces)	Butyrate (mg/g faeces)
Non-colitic (n=8)	25.3±2.9	11.5±1.0	6.3±1.1
TNBS control (n=9)	6.2±1.6	2.7±0.6	0.8±0.2
TNBS probiotic (n=10)	13.3±1.9*	5.3±0.7**	2.6±0.7**

Data are expressed as mean±SEM
*P<0.05, **P<0.01 vs TNBS control. All colitic groups differ significantly from non-colitic group (P<0.01, not shown)

observed compared to non-colitic rats (P<0.01, Table 3), similar to that described previously in this model of experimental colitis [36]. However, the intestinal contents obtained from the colitic treated rats showed greater acetate, butyrate and propionate production than those from TNBS control rats without probiotic treatment (P<0.01, Table 3).

Discussion

IBD is characterised by the abnormal production of free radicals with resultant oxidant-induced tissue injury and reduced antioxidant defenses [17, 18]. For this reason, antioxidant therapy can constitute an interesting approach in the downregulation of this inflammatory condition. In fact, the beneficial effects exerted by the 5-aminosalicylic derivatives in the treatment of IBD have been partially attributed to their antioxidant and free radical scavenger properties [22]. Moreover, several antioxidant compounds, such as flavonoids or vitamin E, have been reported to exert anti-inflammatory activity in experimental models of rat colitis [19–21], which was associated with restoration of glutathione colonic mucosal levels. Glutathione is a sulfhydryl-derived compound that actively participates in the antioxidant mechanisms of the intestinal mucosa, preserving it from oxidant-induced tissue damage. Different studies have reported diminished glutathione content in these intestinal conditions, both in humans [37] and in experimental models of rat colitis [19–21], and that glutathione supplementation results in beneficial effects in experimental colitis [24, 25]. All these facts prompted us to evaluate the intestinal anti-inflammatory effect of the probiotic *L. fermentum*, a microorganism that has been demonstrated in vitro to produce antioxidant compounds, such as glutathione and its precursor the dipeptide γ-Glu-Cys, in the TNBS model of rat colitis.

The results obtained in the present study reveal the efficacy of *L. fermentum* in this experimental model of colitis, thus incorporating a new microorganism to the probiotics that have been reported to attenuate the development of colonic injury in experimental and human IBD [38]. This beneficial effect was histologically evidenced with a significant reduction of the extent and severity of inflamed tissue achieved after probiotic treatment in comparison with non-treated colitic rats. The anti-inflammatory effect was also stated biochemically, since its administration to colitic rats resulted in a significant inhibition of colonic MPO activity, a marker of neutrophil infiltration previously described to be upregulated in experimental colitis [31], and widely used to detect and follow intestinal inflammatory processes. In consequence, a reduction of the activity of this enzyme can be interpreted as a manifestation of the anti-inflammatory activity of a given compound [39]. The ability of the probiotic to reduce granulocyte infiltration was confirmed histologically since the level of leucocyte infiltrate in the colonic mucosa was lower in treated colitic animals than in the corresponding TNBS control groups. This may account for the beneficial effect showed by this probiotic because margination and extravasation of circulating granulocytes contributes markedly to the colonic injury in this model of IBD [40]. These results are in agreement with other studies that describe the attenuation exerted by several probiotics in leucocyte–endothelial cell adhesion in this experimental model of rat colitis [16]. The inhibitory effect on leucocyte infiltration may be the consequence of the preventative effect exerted by the probiotic against the free radical derived oxidative injury that takes place after TNBS instillation in the colonic tissue [25, 41], since the intestinal anti-inflammatory effect was associated with a restoration of the colonic glutathione levels in comparison with non-treated colitic rats. The production of γ-Glu-Cys, precursor of glutathione, by this Lactobacilli strain may play a key role, since it has been described to be more efficiently uptaken than glutathione in the intestine. Although γ-Glu-Cys can be also substrate for other enzymes, like gamma-glutamylcyclotransferase, glutathione synthesis is increased in animal cells because of its higher affinity for the enzyme glutathione synthetase [42]. The free radical scavenger properties attributed to both compounds, γ-Glu-Cys and glutathione, produced by this probiotic seem to be crucial in its anti-inflammatory effect. In fact, it has been proposed that free radical generation in the inflamed tissue constitutes an early signal that promotes the infiltration of neutrophils into colonic tissue, which in turn produce a large amount of free radicals that actively participate in the perpetuation of the inflammatory response [43]. For this reason, the rapid neutralisation of these reactive oxygen species would result in the inhibition of neutrophil infiltration, as observed in the present

study. The inhibitory effect of the probiotic on the production and/or release of others mediators with chemotactic properties, like LTB_4, can be ruled out because the probiotic treatment was not associated with a significant modification of the colonic levels of this eicosanoid in comparison with non-treated colitic rats.

Moreover, this inhibitory effect on neutrophil infiltration attributed to the probiotic may also justify the inhibition of the synthesis and/or release of NO, another mediator that participates in the inflammatory process, since probiotic treatment of colitic rats was associated with a reduction in colonic iNOS expression. During the last decade, it has become increasingly clear that chronic colonic inflammation, both in human IBD and in experimental colitis, is associated with enhanced NO production, mainly via iNOS activity [44–46]. The simultaneous overproduction of NO and reactive oxygen metabolites, like superoxide anion, can yield the highly toxic radical peroxynitrite in the inflamed intestine [17]. Since neutrophils have been also considered as an important source of NO [47, 48], the effect exerted by *L. fermentum* in decreasing the neutrophil infiltration may in turn contribute to preserve colonic mucosa from peroxynitrite insult.

The present study also reveals that probiotic treatment promotes the downregulation of TNFα, a pro-inflammatory mediator that has been proposed to play a key role in colonic inflammation [49]; in fact, different drugs capable of interfering with the activity of this mediator are being developed for IBD therapy [50]. The ability of probiotic bacteria to downregulate TNFα 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 [51]. 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 [51]. The results obtained in the present study show that, in the case of *L. fermentum*, this relationship between bacteria and mucosal cells may be driven by SCFA, mainly butyrate. In fact, when colonic contents were incubated for 24 h, SCFA production was increased in probiotic-treated colitic rats in comparison with the corresponding control rats without probiotic treatment. Thus, the inhibitory effect of probiotic administration on cytokine production may be related to the ability of SCFA to interfere with transcription factors. Nuclear factor-kappa B (NF-κB) is a transcription factor that, in combination with others, plays a central role in regulating the expression of genes encoding numerous cytokines in immune and inflammatory responses [52]. Thus, it has been previously reported that butyrate decreases TNFα production by intestinal biopsies and by isolated lamina propria mononuclear cells via inhibition of NF-κB activation and IκBα degradation [53]. The inhibitory effect of butyrate on NF-κB activation in HT-29 cells, probably derived from its ability to inhibit deacetylases, has also been reported [54]. However, the amelioration of the colonic oxidative stress observed after probiotic treatment to colitic rats may also account for its effect on cytokine production, since NF-κB is a redox-sensitive transcription factor activated by oxidant stress in the inflamed intestinal mucosa [55].

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 by other probiotics, mainly a role in prevention in the imbalance in the intestinal microflora, given the relative predominance of aggressive bacteria and insufficient amount of protective species that has been reported to occur 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 contributes to the transmural inflammation [56]. 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 probiotic treatment without showing statistical differences with non-colitic rats.

In conclusion, administration of the probiotic *L. fermentum* 5716 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, including TNFα and NO. This beneficial effect could be ascribed to its ability to prevent oxidative stress that occurs in this inflammatory condition, through the increased production of glutathione, which might attenuate the exacerbated immune response evoked by the colonic instillation of the hapten TNBS in the rats.

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4. Estudio comparativo de los efectos preventivos de dos probióticos *Lactobacillus fermentum* y *Lactobacillus reuteri* en el modelo de colitis experimental por TNBS en ratas

RESUMEN

OBJETIVO

El probiótico con mejor perfil antiinflamatorio de todos los ensayados fue *Lactobacillus fermentum*, por lo que lo quisimos comparar con *Lactobacillus reuteri* en el modelo de colitis experimental por TNBS en ratas. La selección de *Lactobacillus reuteri* se realizó atendiendo a los estudios anteriores que muestran su gran eficacia.

RESULTADOS

El tratamiento con *Lactobacillus fermentum* y *Lactobacillus reuteri* durante las dos semanas anteriores a la inducción de la colitis no afectó al peso corporal en comparación con el grupo control, sin embargo una semana después de la inducción del daño, ambos probióticos normalizaron levemente el peso de los animales.

La actividad antiinflamatoria intestinal de *Lactobacillus fermentum* se manifestó mediante una reducción significativa de la incidencia de diarrea (20% vs. 80%, $P<0,05$), del cociente peso/longitud y del índice de daño macroscópico en comparación con el grupo control, mostrando *Lactobacillus reuteri* solo una tendencia. Desde el punto de vista bioquímico, únicamente *Lactobacillus fermentum* redujo significativamente la MPO y aumentó los niveles de glutation, que se depleciona en el colon como consecuencia del proceso inflamatorio; sin embargo, ambos mostraron diferencias significativas sobre la producción de la citocina proinflamatoria TNF-α ($P<0,05$), pero no sobre la producción de IL-1β, IL-10 y LTB_4 en comparación con el grupo control. Finalmente, *Lactobacillus fermentum* y *Lactobacillus reuteri* redujeron de manera significativa la expresión de la iNOS, pero solo *Lactobacillus fermentum* lo hizo también sobre la expresión de la ciclooxigenasa-2.

Los estudios microbiológicos del contenido colónico mostraron que tanto *Lactobacillus fermentum* como *Lactobacillus reuteri* aumentaron el crecimiento de *Lactobacillus* en el lumen intestinal en comparación con las ratas no tratadas, sin embargo solo *Lactobacillus fermentum* aumento la producción de ácidos grasos de cadena corta.

CONCLUSIÓN

Lactobacillus fermentum, probiótico con características inmunomoduladoras beneficiosas en la enfermedad inflamatoria intestinal (EII), es más eficaz que *L. reuteri*, cuya eficacia sobre la colitis tanto en humanos como en modelos experimentales ha sido ampliamente demostrada.

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A comparative study of the preventative effects exerted by two probiotics, *Lactobacillus reuteri* and *Lactobacillus fermentum*, in the trinitrobenzenesulfonic acid model of rat colitis

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The intestinal anti-inflammatory effects of two probiotics isolated from breast milk, *Lactobacillus reuteri* and *L. fermentum*, were evaluated and compared in the trinitrobenzenesulfonic acid (TNBS) model of rat colitis. Colitis was induced in rats by intracolonic administration of 10 mg TNBS dissolved in 50 % ethanol (0·25 ml). Either *L. reuteri* or *L. fermentum* was daily administered orally (5×10^8 colony-forming units suspended in 0·5 ml skimmed milk) to each group of rats (n 10) for 3 weeks, starting 2 weeks before colitis induction. Colonic damage was evaluated histologically and biochemically, and the colonic luminal contents were used for bacterial studies and for SCFA production. Both probiotics showed intestinal anti-inflammatory effects in this model of experimental colitis, as evidenced histologically and by a significant reduction of colonic myeloperoxidase activity ($P<0·05$). *L. fermentum* significantly counteracted the colonic glutathione depletion induced by the inflammatory process. In addition, both probiotics lowered colonic TNFα levels ($P<0·01$) and inducible NO synthase expression when compared with non-treated rats; however, the decrease in colonic cyclo-oxygenase-2 expression was only achieved with *L. fermentum* administration. Finally, the two probiotics induced the growth of Lactobacilli species in comparison with control colitic rats, but the production of SCFA in colonic contents was only increased when *L. fermentum* was given. In conclusion, *L. fermentum* can exert beneficial immunomodulatory properties in inflammatory bowel disease, being more effective than *L. reuteri*, a probiotic with reputed efficacy in promoting beneficial effects on human health.

Probiotics: Inflammatory bowel diseases: Immunomodulation: Anti-inflammatory activity

Several studies have proposed that breast-feeding protects against many immune-mediated diseases, including those related to inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease (Klement *et al.* 2004). These observations confirm previous studies in which breast-milk feeding limited the development of colitis in IL-10 knockout mice. This finding was explained by a change in the intestinal flora of the developing mice from pathogenic bacteria to non-adherent bacteria, promoted by milk oligosaccharides that stimulate *Bifidobacterium* and *Lactobacillus* growth (Kunz *et al.* 2000). In addition, the presence of lactic bacteria in breast milk could also account for its preventative effect against intestinal inflammation (Martin *et al.* 2003).

In fact, the administration of probiotic micro-organisms has been proposed to promote a balanced colonic microbial environment and thus probably help in both prevention and control of IBD. Previous studies have reported that the administration of a mixture of bifidobacteria and lactobacilli (Venturi *et al.* 1999) or *Escherichia coli* Nissle 1917 (Rembacken *et al.* 1999) prevents the relapse of ulcerative colitis, showing the latter to have an equivalent effect to mesalazine in maintaining remission. The studies performed both in human subjects and in animal models of intestinal inflammation have provided some clues about the different mechanisms involved in the therapeutic effects exerted by probiotic micro-organisms. First, probiotics could suppress the growth or epithelial binding and invasion of enteric pathogenic bacteria, maybe due to their ability to decrease luminal pH via production of SCFA (Sakata *et al.* 2003), promote the secretion of bactericidal proteins (Boris *et al.* 2001; Collado *et al.* 2005) and/or stimulate mucin production (Mack *et al.* 1999). Second, probiotics have been reported to exert immunoregulatory activities, either by inducing protective cytokines, such as IL-10 and transforming growth factor-β, or by suppressing pro-inflammatory cytokines, such as TNFα, in the intestinal mucosa (Borruel *et al.* 2002; Schultz *et al.*

Abbreviations: COX-2, cyclo-oxygenase-2; IBD, inflammatory bowel disease; iNOS, inducible NO synthase; LTB_4, leukotriene B4; MPO, myeloperoxidase; TNBS, trinitrobenzenesulfonic acid.

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2003; Pathmakanthan *et al.* 2004; Chen *et al.* 2005). And third, these micro-organisms positively affect the intestinal barrier function by decreasing mucosal permeability (Madsen *et al.* 2001). However, the detailed mechanisms by which these bacteria mediate their effects are not fully understood.

The aim of the present study was to compare the preventative effects of *Lactobacillus fermentum* CECT5716 and *L. reuteri* ATCC55730, two hetero-fermentative bacteria found in breast milk (Martin *et al.* 2005; BioGaia, 2006), in the trinitrobenzenesulfonic acid (TNBS) model of rat colitis. This is a well-established model of intestinal inflammation with some resemblance to human IBD (Jurjus *et al.* 2004). The selection of the probiotics was based on previous *in vitro* and *in vivo* studies that make them suitable candidates for the treatment of these intestinal conditions. In a previous study, we have reported that *L. fermentum* CECT5716 showed intestinal anti-inflammatory activity in the TNBS model of rat colitis (Peran *et al.* 2006). That effect was attributed, at least partially, to its ability to release glutathione and the antioxidant dipeptide γ-Glu-Cys, thus counteracting the damaging effects derived from the intestinal oxidative stress generated (Grisham *et al.* 1991), similarly to what occurs in human IBD (Grisham, 1994). This effect was also associated with a reduction in TNFα production and in inducible NO synthase (iNOS) expression in the inflamed tissue (Peran *et al.* 2006). On the other hand, different strains of *L. reuteri* have been described to show beneficial effects in several experimental models of colitis, both in mice (IL-10 and $CD4^{+}$T cell-induced colitis in the severe combined immunodeficient mouse) (Madsen *et al.* 1999; Moller *et al.* 2005), and in rats (acetic acid- and methothrexate-induced) (Mao *et al.* 1996; Holma *et al.* 2001). *In vitro* studies have shown that *L. reuteri* DSM12246 is able to down regulate the stimulated production of the pro-inflammatory cytokines IL-12 and TNFα in dendritic cells while inducing the anti-inflammatory cytokine IL-10 (Christensen *et al.* 2002). Similarly, another strain of *L. reuteri* inhibited mRNA up regulation, cellular accumulation and secretion of the chemokine IL-8 induced by TNFα in intestinal epithelial cells (Ma *et al.* 2004).

Materials and methods

The present 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 (Bethesda, MD, USA).

Reagents

All chemicals were obtained from Sigma Chemicals (Madrid, Spain), unless otherwise stated.

Preparation and administration of the probiotics

L. fermentum CECT5716 was provided by Puleva Biotech (Granada, Spain), *L. reuteri* ATCC55730 was obtained from a commercial dairy product licensed by BioGaia AB (Stockholm, Sweden). Lactobacilli strains were normally grown in De Man–Rogosa–Sharpe (MRS) media at 37°C in anaerobic conditions using the Anaerogen system (Oxoid Ltd, Basingstoke, Hants, UK). For probiotic treatment, bacteria were suspended in skimmed milk (10^9 colony-forming units/ml) and stored at −80°C until usage.

Experimental design

Female Wistar rats (180–200 g) were obtained from the Laboratory Animal Service of the University of Granada (Granada, Spain), maintained in standard conditions and fed the Panlab A04 diet (Panlab, Barcelona, Spain) *ad libitum*. The composition of the diet was: 17·2 % protein, 2·7 % fat, 59·7 % carbohydrates, 3·9 % fibre (mainly cellulose), 4·4 % minerals and 12 % humidity. The rats were randomly assigned to four groups (n 10); two of them (non-colitic and control groups) did not receive probiotic treatment and the remaining groups (treated groups) received orally each probiotic (5×10^8 colony-forming units suspended in 0·5 ml skimmed milk) daily for 3 weeks. Both non-colitic and control groups received orally the vehicle used to administer the probiotic (0·5 ml daily). At 2 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.* (1989). Briefly, they were anaesthetised with halothane and given 10 mg TNBS dissolved in 0·25 ml ethanol (50 %, v/v) by means of a Teflon cannula inserted 8 cm through the anus. Rats from the non-colitic group were administered intracolonically 0·25 ml PBS instead of TNBS. All rats were killed with an overdose of halothane 1 week after induction of colitis. After killing, the following tissues were quickly removed and weighed: spleen, thymus, kidneys, liver and soleus muscle. Also the colon was obtained for the assessment of colonic damage.

Assessment of colonic damage

The body weight, water and food intake, as well as stool consistency, 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 the luminal contents were collected for the measurements of faecal moisture, pH and microbiological and SCFA production studies (see later). 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.* (1995), 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 taken at different levels and stained with haematoxylin and eosin. The histological damage was evaluated on a 0–27 scale by two pathologist observers (A. N. and A. C.), who were blinded to the experimental groups, according to the criteria described previously (Camuesco *et al.* 2005). The colon was subsequently divided into four segments for biochemical determinations. Two

fragments were frozen at −80°C for myeloperoxidase (MPO) activity and iNOS and cyclo-oxygenase-2 (COX-2) expressions, and another sample was weighed and frozen in 1 ml TCA (50 g/l) for total glutathione content determinations. The remaining sample was immediately processed for the measurement of colonic TNFα, IL-1β, IL-10 and leukotriene B4 (LTB_4) levels. All biochemical measurements were completed within 1 week from the time of sample collection and were performed in duplicate.

MPO activity was measured according to the technique described by Krawisz *et al.* (1984). The results are expressed as MPO units per g wet tissue; one unit MPO activity was defined as that degrading 1 μmol H_2O_2/min at 25°C. Glutathione (reduced and oxidised) concentrations were assayed by HPLC with fluorimetric detection of oxidised and reduced glutathione, according to the method proposed by Martin &White (1991); the results are expressed as nmol glutathione/mg wet tissue. Colonic samples for cytokine and LTB_4 determinations were immediately weighed, minced on an ice-cold plate and suspended in a tube with 10 mM-sodium phosphate buffer (pH 7·4) (1:5, w/v). The tubes were placed in a shaking water-bath (37°C) for 20 min and centrifuged at 9000 ***g*** for 30 s at 4°C; the supernatant fractions were frozen at −80°C until assay. TNFα, IL-1β and IL-10 were quantified by ELISA (Amersham Pharmacia Biotech, Amersham, Bucks, UK) and the results were expressed as pg/mg protein; the detection limits were 31–2500 pg/ml for TNFα, 25·6–2500 pg/ml for IL-1β and 16–500 pg/ml for IL-10. LTB_4 was determined by enzyme immunoassay (Amersham Pharmacia Biotech) and the results expressed as pg/mg protein; the detection limits were 6·2–800 pg/ml.

The colonic expression of iNOS and COX-2 was analysed by Western blotting as previously described (Camuesco *et al.* 2004). The dilutions of each primary antibody were 1:2000 for iNOS (Transduction Laboratories, Becton Dickinson Biosciences, Madrid, Spain) and 1:1000 for COX-2 (Cayman Chemical Company, Montigny le Bretonneux, France), and incubated overnight at 4°C followed by peroxidase-conjugated anti-rabbit IgG antibody (1:3000) for 1 h. Control of protein loading and transfer was conducted by detection of the β-actin levels.

pH, moisture and short-chain fatty acid quantification in colonic contents

The pH values in the colonic contents were measured using a GLP21-21 pH-meter (Crison, Barcelona, Spain) after their suspension in water (1:5, w/v). The water content of the luminal stools was calculated by weight differences between fresh (immediately after collection) and dried (kept during 24 h at 65°C) samples.

To quantify the SCFA concentrations in the colonic luminal contents, the samples were homogenised with 150 mM-$NaHCO_3$ (pH 7·8) (1:5, w/v) in an Ar atmosphere. Samples were incubated for 24 h at 37°C and stored at −80°C until the extraction. To extract the SCFA, 50 μl of the internal standard 2-methylvaleric acid (100 mM), 10 μl sulfuric acid and 0·3 ml ethyl acetate were added to 1 ml of the homogenate and, then, centrifuged at 10 000 ***g*** for 5 min at 4°C. The supernatant fractions were dehydrated with sodium sulfate anhydrous and centrifuged at 10 000 ***g*** for 5 min at 4°C. Later, 0·5 ml of the sample was splitless inoculated into a gas chromatograph (Varian CP-3800) equipped with an ID (CPWAX 52CB 60 m × 0·25 mm), and connected to a FID detector (Varian, Lake Forest, CA, USA). The carrier and the make-up gas was He, 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 peaks using the Star Chromatography WorkStation program (version 5.5; Varian Inc., Palo Alto, CA, USA), which was on-line connected to the FID detector.

Microbiological studies

Luminal content samples were weighed, homogenised 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 dicloxacilin (0·5 mg/l), LiCl (1 g/l) and L-cysteine hydrochloride (0·5 g/l)) and incubated under anaerobic conditions in an anaerobic chamber for 24–48 h at 37°C. Coliforms and enterobacteria were also determined by using specific Count Plates Petrifilm (3M, St Paul, MN, Canada). After incubation, the final count of colonies was reported as $\log_{10}$ colony-forming units per g material.

Statistics

All results are expressed as means with their standard errors. Differences between means were tested for statistical significance using a one-way ANOVA and *post hoc* least significance tests. Non-parametric data (scores) are expressed as medians and ranges and were analysed using the Mann–Whitney *U* test. Differences between proportions were analysed with the χ^2 test. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Rockville, MD, USA), with statistical significance set at $P<0{\cdot}05$.

Results

Effects of probiotic administration on body and tissue weight in colitic rats

The administration of probiotics for 2 weeks before colitis induction did not affect rat weight gain compared with untreated rats (data not shown). The intracolonic administration of TNBS resulted in an intestinal inflammatory status in the rats characterised by anorexia, loss of weight and diarrhoea, which gradually increased. Thus, 1 week after colitis induction, body weight was reduced by 4·5 (SEM 1·9) % in the TNBS-treated rats, whereas in saline-treated rats it was increased by 4·8 (SEM 0·7) % ($P<0{\cdot}01$). Although none of the probiotics were able to inhibit the anorexia and the loss of weight in the acute phase of the inflammation (data not shown), both lactobacilli restored the animals' weight at the end of the study, since it was increased by 0·6 (SEM 2·5) and by 0·88 (SEM 2·6) % in the colitic rats that received *L. fermentum* or *L. reuteri*, respectively, without showing statistical differences with control groups.

The anorexia and the inflammatory response caused an important modification in the weight of some tissues such as muscle, thymus, spleen, while liver and kidneys did not

show any significant changes (Table 1). Soleus muscle weight was reduced in colitic rats in comparison with non-colitic rats, although the statistical differences were only obtained in the rats treated with *L. reuteri*. Moreover, the inflammatory process provoked a reduction in thymus weight and an increase in spleen weight. None of the probiotics were able to counteract the increase in spleen weight, and only *L. fermentum* was able to partially restore the thymus weight.

Effects of probiotic administration on colonic inflammation

L. fermentum administration showed an amelioration of the diarrhoeic process, resulting in a significantly lower incidence of diarrhoea (20 %) after 7 d when compared with untreated control rats (80 %; $P<0{\cdot}05$) (Table 2). The macroscopic evaluation of the colonic segments 1 week after colitis induction revealed the preventative effect exerted by probiotics. This was evidenced by a significant reduction of the colonic weight:length ratio ($P<0{\cdot}01$) in both cases (Table 2), as well as by a significantly lower colonic damage score in comparison with control colitic rats, derived from a decrease in the extent of colonic necrosis and the presence of intestinal adhesions induced by the administration of TNBS (Table 2). However, only the group of colitic rats treated with *L. fermentum* showed significant reduction in these inflammatory parameters in comparison with untreated colitic control rats; *L. reuteri* showed only a tendency to decrease them ($P=0{\cdot}07$; Table 2).

The histological studies revealed that *L. fermentum* was more efficient in promoting the recovery of colonic tissue than *L. reuteri*. Histological assessment of colonic samples from the TNBS control group showed 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 15·9 (SEM 2·5). The histological analysis of the colonic specimens from rats treated with *L. fermentum* revealed a more pronounced recovery of the intestinal architecture than controls, with a score of 9·4 (SEM 1·9) ($P<0{\cdot}05$ *v.* TNBS control group). Thus, most of the samples (eight out of ten) showed almost complete restoration of the epithelial cell layer, in contrast to the extensive ulceration observed in non-treated animals. The improvement in colonic histology was accompanied by a reduction in the inflammatory infiltrate, which was slight to moderate with a patchy distribution, although neutrophils were the predominant cell type. The colonic specimens from colitic rats treated with *L. reuteri* also showed a higher recovery than the intestinal segments from control colitic rats, and they were assigned a score value of 10·8 (SEM 2·5), lower than in the control group, but without showing statistical differences ($P=0{\cdot}14$). Thus, four out of ten samples showed evident restoration of the epithelial cell layer, while in the rest of the samples the epithelial ulceration of the mucosa affected over 40–50 % of the surface, lower than in most of the specimens from control colitic rats. Similarly, the goblet cell depletion was also attenuated in this group, and the presence of mucin content was evident, together with an absence of dilated crypts. Finally, the inflammatory infiltrate was also attenuated, being moderate with a patchy distribution.

The biochemical analysis of the colonic specimens confirmed the intestinal anti-inflammatory effect exerted by the probiotics, although again some differences were observed in their effects on the different parameters assayed. Colonic MPO activity was reduced after treatment with *L. reuteri* or *L. fermentum* by approximately 40 % although only *L. fermentum* treatment reached significance (Table 3). Since colonic MPO activity is considered as a biochemical marker of neutrophil infiltration (Krawisz *et al.* 1984), these results confirm the lower leucocyte infiltration into the inflamed tissue after probiotic treatment observed in the histological studies. Furthermore, treatment of colitic rats with the probiotics showed an increase in colonic glutathione content (Table 3), depleted in colitic rats as a consequence of the colonic oxidative stress caused by the TNBS-induced inflammatory process (Galvez *et al.* 2003). However, although both probiotics restored the values observed in non-colitic rats, only the group of rats treated with *L. fermentum* showed statistical differences in comparison with control colitic rats ($P<0{\cdot}01$). The colonic inflammation induced by TNBS was also characterised by increased levels of colonic TNFα (Table 3), IL-1β (339·5 (SEM 43·9) *v.* 28·4 (SEM 3·4) pg/mg protein in the non-colitic group; $P<0{\cdot}01$) and LTB_4 (146·6 (SEM 33·1) *v.* 9·8 (SEM 2·5) pg/mg protein in the non-colitic group; $P<0{\cdot}01$), and a reduction in IL-10 production (5·1 (SEM 1·2) *v.* 18·3 (SEM 3·1) pg/mg protein in the non-colitic group; $P<0{\cdot}01$). Only TNFα production was significantly reduced after treatment with either *L. reuteri* or *L. fermentum* (Table 3). No statistical differences were observed in the other pro-inflammatory mediators assayed (data not shown).

Finally, the inflammatory process in the colonic tissue was also characterised by higher expression of both iNOS and COX-2 in comparison with non-colitic animals (data not

Table 1. Effects of probiotic treatment on tissue weights in trinitrobenzenesulfonic acid (TNBS) experimental colitis in rats (Mean values with their standard errors for ten rats per group)

	Muscle (mg/g rat)		Liver (mg/g rat)		Kidneys (mg/g rat)		Spleen (mg/g rat)		Thymus (mg/g rat)	
Group	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Non-colitic	6·6	0·1	31·3	1·3	5·9	0·1	2·2	0·2	1·9	0·1
TNBS control	6·3	0·2	31·9	1·1	6·2	0·1	3·0*	0·2	0·9*	0·1
Lactobacillus reuteri	6·0*	0·1	35·1	1·3	5·9	0·2	3·3*	0·4	0·9*	0·1
L. fermentum	6·5‡	0·1	34·9	1·1	6·1	0·2	2·9*	0·2	1·2*†‡	0·1

* Mean value was significantly different from that of the non-colitic group ($P<0{\cdot}05$).
† Mean value was significantly different from that of the TNBS control group ($P<0{\cdot}05$).
‡ Mean value was significantly different from that of the *L. reuteri* group ($P<0{\cdot}05$).

Table 2. Effects of probiotic treatment on diarrhoea, adhesions, damage score, extent of the inflammatory lesion along the colon and changes in colon weight in trinitrobenzenesulfonic acid (TNBS) experimental colitis in rats
(Percentages, medians and ranges, and mean values with their standard errors for ten rats per group)

			Damage score (0–10)§		Extent of damage (cm)		Weight/length (mg/cm)	
Group	Diarrhoea (%)	Adhesions (%)	Median	Range	Mean	SEM	Mean	SEM
Non-colitic	0	0	0	0	0	0	71·0	2·5
TNBS control	80*	80*	7*	6–8·5	3·6*	0·3	249·7*	22·1
Lactobacillus reuteri	50*	50*	6*	4–8	2·8*	0·3	175·9*	11·9
L. fermentum	20†	10*†‡	5·5*‡	4–6·5	2·4*†	0·3	145·7*†‡	7·6

* Percentage or mean value was significantly different from that of the non-colitic group ($P<0·05$).
† Percentage or mean value was significantly different from that of the TNBS control group ($P<0·05$).
‡ Percentage or mean value was significantly different from that of the *L. reuteri* group ($P<0·05$).
§ Damage score for each rat was assigned according to the criteria described previously by Bell *et al.* (1995).

shown). Treatment of colitic rats with *L. fermentum* resulted in a significant reduction of the expression of both inducible enzymes in eight out of ten rats, whereas *L. reuteri* was only able to significantly reduce iNOS expression, and this was achieved in seven out of ten rats.

Effects of probiotic administration on colonic short-chain fatty acid production and bacterial profile

No clear differences were observed in the pH values of the colonic contents among the different groups of rats (Table 4). Moreover, although a tendency to increase the faecal water content was observed in all the colitic rats, only those treated with *L. reuteri* showed a significant difference in the faecal moisture (Table 4).

When the colonic contents from colitic control rats were evaluated for SCFA production, no significant reduction in any of their levels was observed compared with non-colitic rats (Table 4). However, a significant reduction in all the analysed SCFA was observed in the *L. reuteri*-treated group in comparison with all the other experimental groups (colitic or not). In contrast, colitic rats treated with *L. fermentum* showed similar values to those observed in non-colitic rats (Table 4).

Table 3. Effects of probiotic treatment on colonic myeloperoxidase (MPO) activity, glutathione content and tumour necrosis factor α levels in trinitrobenzenesulfonic acid (TNBS) experimental colitis in rats
(Mean values with their standard errors for ten rats per group)

	MPO activity (units/g)§		Glutathione (nmol/g)		TNFα (pg/mg protein)	
Group	Mean	SEM	Mean	SEM	Mean	SEM
Non-colitic	80	12	1479	51	17·8	2·4
TNBS control	1325*	144	1093*	85	74·0*	9·6
Lactobacillus reuteri	989*	206	1351	135	42·0*†	7·0
L. fermentum	882*†‡	114	1490†	112	53·1*†	11·0

* Mean value was significantly different from that of the non-colitic group ($P<0·05$).
† Mean value was significantly different from that of the TNBS control group ($P<0·05$).
‡ Mean value was significantly different from that of the *L. reuteri* group ($P<0·05$).
§ One unit of MPO activity was defined as that degrading 1 μmol H_2O_2/min at 25°C.

TNBS colitis also resulted in a significant reduction in colonic lactobacilli and bifidobacteria counts ($P<0·05$; Fig. 1), together with an increase in coliforms and enterobacteria ($P<0·05$; data not shown) in comparison with normal rats. Probiotic-treated colitic rats showed higher counts of lactobacilli and bifidobacteria species in the colonic contents than in control colitic rats, without showing statistical differences with the non-colitic control group (Fig. 1 (A)). No statistical differences were observed in the amount of faecal potential pathogenic bacteria such as enterobacteria or coliforms among the three colitic groups (data not shown). As expected, when the lactobacilli:pathogen ratio was evaluated, the inflammatory process did result in a significant decrease in comparison with normal rats; the administration of *L. fermentum* or *L. reuteri* resulted in the normalisation of this ratio (Fig. 1 (B)).

Discussion

The results obtained in the present study are supportive of the helpfulness of the dietary incorporation of probiotics in IBD therapy (Sartor, 2004). Furthermore, they confirm the intestinal anti-inflammatory activity previously shown by this strain of *L. fermentum* (CECT5716) (Peran *et al.* 2006) as well as by other strains of *L. reuteri* (Mao *et al.* 1996; Madsen *et al.* 1999; Holma *et al.* 2001; Moller *et al.* 2005), although the present study is the first that describes the efficacy of *L. reuteri* ATCC55730 in the TNBS model of rat colitis.

Both probiotics ameliorated some of the clinical manifestations of this colitis experimental model such as anorexia or diarrhoea and the macroscopic colonic damage; however, *L. fermentum* treatment seemed to be more effective. In fact, this probiotic significantly attenuated the incidence of diarrhoea and adhesions, increased thymus weight and reduced the colonic weight:length ratio as well as the damage score and extension. On the contrary, *L. reuteri* treatment did not show significant modifications on most of these parameters; only the colonic weight:length ratio was significantly reduced in comparison with untreated colitic rats.

The reduction in the diarrhoeic process exerted by *L. fermentum* can be a consequence of an improvement of the gut epithelial cell barrier function, thus contributing to its intestinal anti-inflammatory effect, as has been proposed to occur with other probiotics (Gionchetti *et al.* 2005). In fact,

Table 4. Effects of probiotic treatment on faecal pH and moisture, and on colonic short-chain fatty acid production in trinitrobenzenesulfonic acid (TNBS) experimental colitis in rats

(Mean values and standard deviations for ten rats per group)

	Faecal pH		Faecal moisture (%)§		Total SCFA (mg/l)		Acetate (mg/l)		Propionate (mg/l)		Butyrate (mg/l)	
Group	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Non-colitic	7·05	0·06	68·9	3·5	10 388	3655	6922	2917	2335	708	1130	267
TNBS control	7·26	0·04	72·2	1·4	7662	1290	4978	847	1858	322	825	177
Lactobacillus reuteri	7·32	0·06	78·5*	1·1	2821*†	75	1822*†	44	556*†	40	299*†	20
L. fermentum	7·31	0·03	76·1	1·1	9659‡	2298	6830‡	1888	2896‡	908	1028‡	279

*Mean value was significantly different from that of the non-colitic group ($P<0·05$).
†Mean value was significantly different from that of the TNBS control group ($P<0·05$).
‡Mean value was significantly different from that of the *L. reuteri* group ($P<0·05$).
§Faecal moisture was expressed as the proportion in water content expressed in %.

microscopic evaluation showed that the restoration in the epithelial lining was more evident in the rats administered *L. fermentum* (80 % of the samples showed complete restoration) than in those that received *L. reuteri* (40 %). This may be interesting since a barrier disruption leads to increased stimulation by luminal antigens. In this regard, mucosal inflammation can be considered a self-perpetuating process in which the disruption of the epithelial layer plays a central role (Heyman *et al.* 1994).

L. fermentum and *L. reuteri* were able to reduce neutrophil infiltration in the inflamed colon, as was observed in the microscopic analysis, although only *L. fermentum* treatment significantly decreased colonic MPO activity. The inhibition of neutrophil infiltration can account for their intestinal anti-inflammatory effect, given the important role attributed to these cells in the inflammatory process.

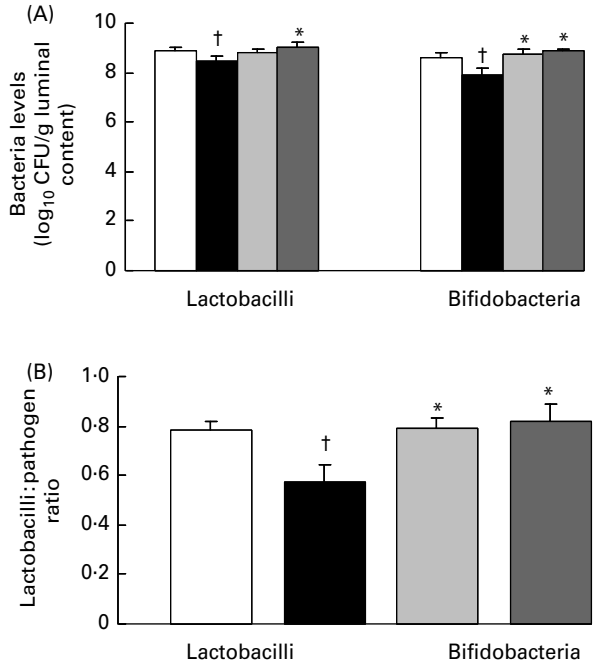

Fig. 1. Effects of probiotic treatment (5×10^8 colony-forming units (CFU)/rat·per d) on (A) bacteria levels (lactobacilli and bifidobacteria) and on (B) lactobacilli:pathogen ratio in trinitrobenzenesulfonic acid (TNBS) experimental colitis in rats. (□), Non-colitic group; (■),TNBS control group; (▩), *Lactobacillus reuteri*-treated group; (■), *L. fermentum*-treated group. Values are means, with their standard errors represented by vertical bars. *Mean value was significantly different from that of the TNBS control group ($P<0·05$). †Mean value was significantly different from that of the non-colitic group ($P<0·01$).

L. fermentum treatment of TNBS colitic rats counteracted the depletion of colonic glutathione levels that took place in control colitic animals. This activity may play a crucial role in the intestinal anti-inflammatory effect of the probiotic because a situation of intense oxidative insult is an important mechanism for tissue damage during chronic intestinal inflammation and thus a common feature in human IBD (Grisham, 1994) as well as in the different experimental models of rat colitis, including the TNBS (Galvez *et al.* 2003) and the dextran sodium sulfate (Camuesco *et al.* 2004) models. The effect exerted by this probiotic could be due to its ability to release glutathione and the antioxidant dipeptide γ-Glu-Cys (Peran *et al.* 2006).

When other pro-inflammatory mediators were evaluated, *L. fermentum* and *L. reuteri* were able to significantly reduce colonic TNFα production. This may be relevant since this cytokine plays a key role in intestinal inflammation, and different drugs capable of interfering with the activity of this mediator are being developed for IBD therapy (Rutgeerts *et al.* 2004). Previous *in vitro* studies have also shown the ability of different probiotic, including *L. casei*, *L. bulgaricus*, *L. fermentum* or *L. salivarius* ssp. *salivarius*, to down regulate TNFα production (Borruel *et al.* 2002; Peran *et al.* 2005, 2006).

A common feature of both probiotics assayed is their ability to modify colonic microflora, which was altered as a consequence of the TNBS-induced inflammatory process (Peran *et al.* 2006). In this regard, the probiotic treatment restored the pathogenic bacteria:lactobacilli ratio. This effect could definitively contribute to the beneficial effect exerted by these probiotics in the TNBS model of experimental colitis. In fact, it has been previously described that the increase in *Lactobacillus* sp. levels reduces the concentration of adherent and translocated bacteria and attenuates the colitis in IL-10 gene-deficient mice (Madsen *et al.* 1999). This could prevent the pathogenic effect of other species that may contribute to the generation of an exacerbated immune response in intestinal inflammation, as proposed both in experimental models (Garcia-Lafuente *et al.* 1997) and in human subjects (Cummings *et al.* 2003).

However, the colonic SCFA content profiles shown by the two probiotics were different. Thus, *L. fermentum* was able to significantly counteract the decrease in colonic SCFA production observed in TNBS colitic rats, whereas *L. reuteri* treatment reduced even more the SCFA production despite its effect on colonic microbiota. The effect of *L. fermentum* on butyrate production is very interesting since it has been proposed that the inflammatory process results in an alteration of the intestinal epithelial cell function, including colonic SCFA utilisation, mainly butyrate, which is considered the most important SCFA for colonocyte metabolism (Mortensen & Clausen, 1996; Rodriguez-Cabezas *et al.* 2002).

In conclusion, *L. fermentum* and *L. reuteri* have shown intestinal anti-inflammatory activity in the TNBS model of rat colitis. However, each probiotic shows its own anti-inflammatory profile, confirming that not all probiotics present the same efficacy as anti-inflammatory agents, and do not share the same mechanisms of action. Of note, *L. fermentum* can be considered more effective than *L. reuteri*, a probiotic with reputed efficacy in promoting beneficial effects on human health (Valeur *et al.* 2004). Both probiotics can be found in breast milk, and although the doses administered to rats in the present study are higher than those probably incorporated in the infant by breast milk, the present results suggest that the colonisation of these probiotics in the colonic lumen would result in beneficial preventative effects in these intestinal conditions, probably derived from their immunomodulatory properties. Human clinical studies will be required in order to confirm these results.

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DISCUSIÓN

Durante los últimos años se ha puesto de manifiesto un incremento gradual de la incidencia de patologías intestinales relacionadas con procesos inflamatorios (Loftus, 2004), lo que justificaría el importante esfuerzo que se viene realizando por parte de la comunidad científica para la búsqueda de nuevos fármacos eficaces en el tratamiento de la EII y la prevención de las recidivas que la caracterizan. En la actualidad, prácticamente todos los fármacos utilizados presentan importantes reacciones adversas, hecho que adquiere una especial relevancia dado que el tratamiento farmacológico en la mayoría de pacientes se realiza durante un prolongado período de tiempo (Enns y Sutherland, 1998; Stein y Hanauer, 2000), por lo que resulta de gran interés el establecimiento de nuevas estrategias terapéuticas que, dotadas de actividad frente a estas patologías, presenten menos efectos secundarios (Kho *et al.*, 2001).

Aunque la causa de la EII es todavía desconocida, es un hecho bien aceptado que en su iniciación y progresión intervienen las bacterias del lumen intestinal, probablemente provocado por un desequilibrio entre las bacterias potencialmente patógenas y las protectoras (Fiocchi, 1998; Shanahan, 2000). Por lo tanto, una alternativa terapéutica sería el uso de microorganismos probióticos. De hecho, hay diversos estudios que avalan la eficacia de los mismos en la EII en humanos.

Uno de los primeros estudios en colitis ulcerosa se realizó con un pequeño numero de pacientes donde se evaluó la actividad de *Escherichia coli* Nissle 1917 en comparación con dosis bajas de mesalamina (500 mg tres veces al día), mostrando que el número de recaídas era menor en el caso del grupo tratado con el probiótico (Kruis *et al*., 1997). Esto se vio posteriormente ratificado con otro estudio realizado con la mezcla de probióticos VSL#3* (Venturi *et al*., 1999). Recientemente, Zocco *et al*. (2006), estudiaron la eficacia de la asociación del probiótico *Lactobacillus rhamnosus* GG (LGG) con mesalamina en el mantenimiento de la remisión de la colitis ulcerosa en comparación con mesalamina sola, no obteniendo diferencias en el número de recaídas después de 6 y de 12 meses; sin embargo, sí que fue capaz de prolongar significativamente el tiempo de remisión ($P<0,05$).

*VSL#3: mezcla probiótica compuesta por: *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. bulgaricus, Bifidobacterium longum, Bifidobacterium breve* y *Bifidobacterium infantis.*

Existe un menor número de estudios que describen el uso de probióticos en la prevención y tratamiento de la enfermedad de Crohn. En un ensayo se probó la eficacia de *Saccharomyces boulardii* en el mantenimiento de la remisión de la EC. A los 6 meses la incidencia de recaídas era mucho menor en el grupo tratado con mesalamina y el probiótico en comparación con el grupo tratado con mesalamina sola (Guslandi *et al*., 2000). En otro estudio, McCarthy *et al*. (2001), mostraron que la administración oral de *Lactobacillus salivarius* UCC118 reducía de manera significativa el índice de la enfermedad en pacientes con EC leve y moderada.

No obstante, es en la pouchitis donde los probióticos han demostrado un beneficio indiscutible; se ha comprobado en distintos estudios que son capaces de mantener la remisión inducida con antibióticos en pacientes con pouchitis crónica tras resección del colon debido a una colitis ulcerosa refractaria. En este sentido, Gionchetti *et al*. (2000), han realizado un estudio usando la mezcla probiótica VSL#3 en pacientes con pouchitis crónica recurrente, la cual redujo el numero de recaídas tras 9 meses a un 15% frente al 100% del grupo placebo. Otro estudio con los mismos grupos también demostró que tras un año, solo desarrollaron pouchitis un 10% frente a un 40% del grupo placebo después de la cirugía por colitis ulcerosa (Gionchetti *et al*., 2003).

Además de estos estudios en humanos, se han realizado otros ensayos en modelos experimentales de inflamación intestinal que ratifican estos efectos beneficiosos (*Tabla 9*).

Para explicar el efecto antiinflamatorio intestinal ejercido por los probióticos sobre estas patologías intestinales, se ha propuesto la participación de distintos mecanismos, entre los cuales se incluyen la competición con bacterias nocivas por el sitio de fijación al epitelio, la inhibición de su crecimiento y/o la promoción de su muerte mediante la producción de compuestos antibacterianos o reducción del pH. Recientemente se le está dando una gran importancia a la modulación de la respuesta inmunitaria de la mucosa del hospedador por parte de los probióticos, hecho que ha sido ratificado en distintos ensayos *in vitro*. De hecho, *Lactobacillus casei* y *Lactobacillus bulgaricus* son capaces de modular la respuesta inmunitaria mediante la reducción de los niveles de TNFα en explantes intestinales de individuos con enfermedad de Crohn (Borruel *et al*., 2002). *Lactobacillus plantarum* 299v también revirtió la producción de citocinas proinflamatorias por parte de *E. coli* enteropatógena en la mucosa colónica de pacientes con colitis ulcerosa, induciendo la producción de

Tabla 9. *Efectos beneficiosos de algunos probióticos en modelos experimentales de inflamación intestinal*

Probiótico	Modelo	Eficacia	Cita bibliográfica
RATAS			
L. reuteri	Acido acético	Reducción de la MPO y de la permeabilidad de la mucosa	Fabia *et al*., 1993
L. reuteri	Metotrexato	Recuperación del peso animal, Reducción de la permeabilidad y MPO	Mao *et al*., 1996
LGG/ VSL#3	Iodoacetamida	Reducción del peso colónico, MPO, PGE2 y NOS	Shibolet *et al*., 2002
L. plantarum NCIMB8826	TNBS	Inhibición de la traslocación bacteriana a los nódulos linfáticos mesentéricos y bazo	Pavan *et al*., 2003
L. rhamnosus GG + Antibióticos	Ratas transgénicas HLA-B27	Reducción del score histológico, MPO, IL-1β, TNF-α. Aumento de IL-10	Dieleman *et al*., 2003
RATONES			
L. reuteri	IL-10 KO	Reducción de la adherencia a la mucosa y la traslocación bacteriana	Madsen *et al*., 1999
L. salivarius ssp. Salivarius UCC118	IL-10 KO	Reducción de la prevalencia de cáncer de colon. Reducción de Coliformes y Enterococos	O'Mahony *et al*., 2001
VSL#3	IL-10 KO	Normalización de la función colónica, y la integridad de la barrera mucosa. Reducción del TNFα y del INFγ	Madsen *et al*., 2001
Ag. solubles *E coli* cepa *Laves*	DSS	Reducción de IL-1β, TNF-α e INFγ	Konrad *et al*., 2003
Ag solubles *B. breve*, *B. longum*	DSS + *Bacteroides vulgatus*	Reducción de la densidad de *Bacteroides vulgatus*	Setoyama *et al*., 2003

la citocina antiinflamatoria IL-10 por células T y macrófagos de la misma, mostrando así su posible efecto antiinflamatorio intestinal (Pathmakanthan *et al*., 2004).

Aunque estos resultados son prometedores, es importante indicar la existencia de algunos estudios en los que los probióticos no han demostrado tener eficacia tanto en ensayos clínicos como en modelos experimentales. Esto sugiere que la eficacia de los mismos puede variar dependiendo de diferentes factores:

- Especie y cepa probiótica
- Dosis
- Momento de comienzo del tratamiento
- Características del hospedador
- Características del modelo

Por esta razón, es interesante evaluar y comparar los efectos beneficiosos de diferentes probióticos en el mismo modelo experimental y con la misma dosis, y así establecer qué probióticos muestran el mejor perfil antiinflamatorio, datos que incluso podrían predecir una actuación sinérgica tras su asociación. Con este objetivo la presente tesis se ha desarrollado en tres apartados:

1.- Valoración del efecto antiinflamatorio intestinal de *Lactobacillus casei, Lactobacillus acidophilus*, y *Bifidobacterium lactis*, probióticos con eficacia demostrada en estudios anteriores. Estos resultados nos permitirán ratificar las afirmaciones anteriores, estableciendo sus características diferenciales en el modelo de colitis experimental seleccionado.

2.- Ensayo de la actividad de los probióticos *Lactobacillus salivarius ssp. salivarius* y *Lactobacillus fermentum*, probióticos ensayados por primera vez en un modelo de colitis experimental y que en estudios *in vitro* presentaron propiedades añadidas que podían conferirle propiedades antiinflamatorias, como la modificación de la relación citocinas antiinflamatorias/citocinas proinflamatorias y la liberación de glutation respectivamente

3.- Estudio comparativo de los efectos preventivos ejercidos por los dos probióticos: *Lactobacillus fermentum* y *Lactobacillus reuteri*, siendo este último un probiótico con elevada eficacia demostrada tanto en humanos como en modelos experimentales de enfermedad inflamatoria intestinal.

Para llevar a cabo nuestros estudios, el modelo experimental utilizado ha sido el del ácido trinitrobenecenosulfónico (TNBS), uno de los más utilizados para conocer los mecanismos involucrados en el proceso inflamatorio intestinal, así como para valorar inicialmente nuevos tratamientos potencialmente útiles en la EII en humanos. Su amplio uso se debe a su gran similitud con las características de la EII en humanos, su fácil reproducción y su bajo coste. Este modelo consiste en la administración intracolónica de una solución de TNBS en etanol al 50%. El etanol da lugar a la ruptura de la barrera intestinal facilitando el acceso del TNBS a la mucosa, donde éste actúa como hapteno (Morris *et al*., 1989) siendo el responsable de la instauración del proceso inflamatorio (Elson *et al*., 1995). Se ha postulado que el TNBS, además de actuar como hapteno, ejerce un efecto citotóxico directo sobre el epitelio colónico, como consecuencia de su capacidad para generar especies reactivas derivadas del oxígeno tras su metabolismo por parte de los colonocitos (Grisham *et al*., 1991), así como por producir una disminución de los niveles colónicos de glutation (Ardite *et al*., 2000). Estos hechos facilitarían la desorganización de la citoarquitectura colónica, el aumento de la permeabilidad intestinal y la entrada de productos luminales, promoviendo la activación del sistema inmunológico intestinal con la consecuente hiperproducción de diferentes mediadores proinflamatorios (*Figura 6*) (Yamada *et al*., 1992). En cualquier caso, la presencia de la flora bacteriana colónica es determinante en la colitis crónica (Garcia-Lafuente *et al*., 1998), de manera que, cuando el TNBS/etanol es administrado a animales libres de gérmenes (o bajo tratamiento antibacteriano), la respuesta inflamatoria obtenida es cuantitativamente inferior.

Como consecuencia del daño colónico inducido por el TNBS tiene lugar la activación e infiltración de neutrófilos y monocitos (inicialmente), así como linfocitos (posteriormente) en la lámina propia, que contribuyen de forma clave al daño y cronicidad de proceso inflamatorio (Palmen *et al*., 1995; Yamada *et al*., 1992). El proceso inflamatorio intestinal generado se mantiene entre 3 y 8 semanas (dependiendo de la dosis del TNBS), lo que supone un periodo de tiempo adecuado para la realización de estudios farmacológicos (Raban *et al*., 1996). Concretamente, este modelo presenta una gran similitud con la enfermedad de Crohn en humanos, tanto con relación a las modificaciones histomorfológicas, como al perfil de citocinas que se genera en el colon de los animales de experimentación. Así, en distintos estudios con TNBS se ha demostrado que existe un predominio de células T con predominio Th1, que se caracterizan por la producción de grandes cantidades de INF-γ y pequeñas cantidades de IL-4 (Elson *et al*., 1996; Neurath *et al*., 1995); este tipo de respuesta esta

promovida por una hipersecreción de IL-12 que actúa como un potente inductor del desarrollo de células del tipo Th1, productoras de INF-γ (Trinchieri, 1994), al igual que ocurre en la EC (Breese *et al*., 1993; Fuss *et al*., 1996; Monteleone *et al*., 1997; Parronchi *et al*., 1997). Del mismo modo la colitis por TNBS reproduce otras características de esta enfermedad como la fibrosis y la formación de adherencias mesentéricas, obstrucción intestinal e impacto fecal (Morris *et al*., 1989; Sánchez de Medina *et al*., 1996)

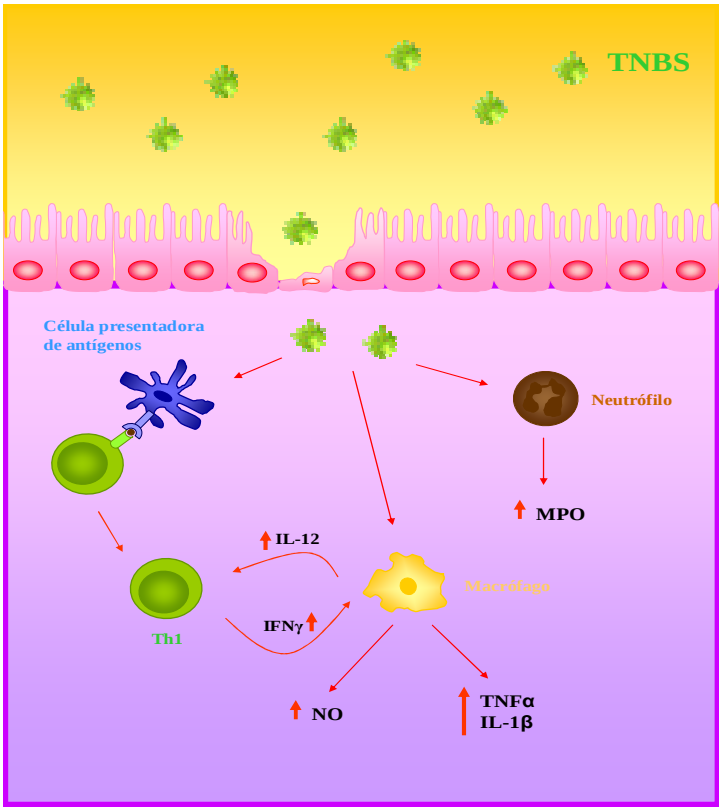

Figura 6. *La administración del TNBS por vía intracolónica origina un proceso colítico caracterizado por la liberación de numerosos mediadores proinflamatorios, así como la inducción de ciertas enzimas.*

El modelo descrito inicialmente por Morris *et al*. (1989) consiste en la inducción del daño colónico mediante la administración de una dosis única de 30 mg de TNBS en etanol al 50% (v/v). En el presente estudio la dosis administrada se ha reducido a 10 mg, con el objeto que el proceso inflamatorio inducido sea mas fácil de modular mediante el correspondiente tratamiento, tal y como ha sido propuesto anteriormente (Allgayer *et al*., 1989; Veljaca *et al*., 1995). De hecho, y en comparación con resultados previos de nuestro grupo de investigación y con los obtenidos por otros autores, la administración de esta dosis de TNBS (10 mg) desarrolló un daño colónico cualitativamente similar al inducido con 30 mg, aunque cuantitativamente menor (Camuesco *et al*., 2005; Morris *et al*., 1989).

1.- **Ensayo de los probióticos *Bifidobacterium lactis, Lactobacillus acidophilus y Lactobacillus casei* en el modelo de colitis experimental por el acido trinitrobencenosulfónico (TNBS) en ratas.**

La selección de estos tres probióticos se realizó atendiendo a los estudios anteriores que muestran su eficacia en distintos modelos de inflamación intestinal.

En este sentido, *Bifidobacterium lactis* ejerce un efecto protector sobre la permeabilidad intestinal (y en consecuencia, un efecto beneficioso sobre la EII) al reducir la traslocación bacteriana a los nódulos linfáticos mesentéricos en un modelo de resección del 80% del intestino delgado, tanto en ratas Wistar como en ratones (Eizaguirre *et al.*, 2002; Garcia-Urkia *et al*., 2002).

Estas observaciones son confirmadas en nuestro estudio, ya que *B. lactis* ejerce un efecto beneficioso que se observó inicialmente por una reducción significativa de la incidencia de diarrea en las ratas tratadas en comparación con el grupo colítico sin tratamiento, sugiriendo así la preservación de la integridad de la mucosa colónica. Ésto es de gran importancia ya que la alteración de esta barrera conlleva un incremento en el trasporte de antígenos luminales, toxinas bacterianas y microorganismos hacia la lámina propia, estimulando los distintos tipos celulares de la misma, incluyendo las células inmunes, provocando así la inflamación del tejido (Heyman *et al*., 1994). El análisis macroscópico del colon de las ratas tratadas con este probiótico mostró una reducción significativa del cociente peso/longitud, indicador de edema colónico, que se encuentra aumentado en el grupo colítico sin tratamiento probiótico como consecuencia del proceso inflamatorio. Éste efecto beneficioso se asoció con una restauración del contenido colónico de glutation cuyo

agotamiento constituye un mecanismo importante de daño tisular en la enfermedad inflamatoria intestinal. De hecho, estudios previos han mostrado que compuestos con propiedades antioxidantes, como los derivados del ácido 5 aminosalicílico o los flavonoides, contribuyen de manera beneficiosa en la remisión de éstas patologías intestinales (Camuesco *et al.*, 2004; Grishman 1994). El análisis bioquímico mostró que *B. lactis* es capaz de disminuir de manera significativa la producción de TNFα colónica, citocina con un papel clave en la inflamación intestinal (Rutgeerts *et al.*, 2004). Por último, el efecto antiinflamatorio de este probiótico también se acompañó por una reducción de la expresión de la iNOS, inducida en el epitelio durante la inflamación intestinal activa (Kimura *et al*., 1997; Singer *et al.*, 1998). Este efecto puede estar asociado con su capacidad de reducir los niveles de TNFα, citocina que promueve la expresión de la iNOS mediante la activación del NF-κB; o de preservar los niveles de glutation. ya que existe una relación directa entre el estrés oxidativo y la regulación de la expresión de iNOS (Hecker *et al*., 1996; Lu y Wahl 2005). Por tanto, como consecuencia del tratamiento probiótico, se inhibe la producción de mediadores proinflamatorios importantes derivados del NO, como el peroxinitrito, agente que juega un papel muy importante en la patogénesis de la enfermedad (Fiocchi 1998)

El siguiente probiótico utilizado fue *Lactobacillus acidophilus*. Chen et al., (2005), pusieron de manifiesto su actividad inmunomoduladora inhibiendo la producción de citocinas antiinflamatorias como IL-6, IL-12, y TNF-α y estimulando la de citocinas antiinflamatorias como IL-10 en un modelo de colitis por *Citrobacter rodentium*, modelo específico de ratones con hiperproliferación de células epiteliales. Además, demostraron un efecto protector sobre la mucosa colónica al aumentar la secreción intestinal de IgA y limitando la infección por bacterias entéricas en el mismo modelo (Chen *et al*., 2005). También, cabe destacar que *L. acidophilus* forma parte de la mezcla probiótica VSL#3 con gran actividad en distintos modelos de inflamación intestinal.

En el presente estudio *Lactobacillus acidophilus* previene la colitis inducida por TNBS en ratas, evidenciado macroscópicamente, por una reducción significativa del daño colónico, y del cociente peso/longitud; y bioquímicamente por una disminución de la MPO en comparación con las ratas colíticas no tratadas. Esta bajada en la actividad enzimática muestra la menor infiltración de neutrófilos, probablemente derivado de una reducción en la producción colónica de LTB_4, eicosanoide con capacidad quimiotáctica. Este efecto también se puede relacionar con la atenuación del estrés oxidativo en el colon, ya que el tratamiento con este probiótico recupera de manera

significativa los niveles colónicos de glutation. El efecto inhibidor sobre la síntesis y/o liberación del LTB_4 puede considerarse un mecanismo de acción bastante característico del efecto antiinflamatorio intestinal ejercido por *L. acidophilus*, siendo de gran interés ya que se ha descrito para diferentes fármacos para el tratamiento de la EII como sulfasalazina o 5-ASA (Travis y Jewel 1994). Finalmente, el efecto antiinflamatorio ejercido por este probiótico también se asoció a una menor expresión de la iNOS, posiblemente derivado de la restauración de los niveles de glutation o de la reducción de los niveles de LTB_4

Por último, estudiamos la actividad antiinflamatoria de *Lactobacillus casei*, microorganismo que también forma parte de la mezcla probiótica VSL#3. Éste ha mostrado su eficacia en varios modelos experimentales, mediados por diferentes mecanismos de acción. Se ha demostrado que *Lactobacillus casei* DN-114001 es capaz de reducir la permeabilidad intestinal alterada como consecuencia del proceso inflamatorio inducido por el TNBS en ratas, disminuyendo así la traslocación bacteriana a los nódulos linfáticos mesentéricos, al hígado y al bazo (Llopis *et al*., 2005). Su efecto antiinflamatorio ha sido apoyado también por su capacidad de aumentar los niveles de IgA (inmunoglobulina capaz de promover la barrera inmunológica) en la mucosa colónica en un modelo de colitis por DSS en ratones BALB-c (Kokesova *et al*., 2006). La misma cepa también ha inhibido la adhesión e invasión de *Escherichia coli* enteropatógena en células epiteliales Caco-2, hecho que se puso de manifiesto en un estudio *in vitro* incubándose el probiótico, a la vez, antes y después del patógeno (Ingrassia *et al.*, 2005). *L. casei* shirota ha demostrado su actividad antiinflamatoria ejerciendo un efecto inmunomodulador al inhibir la producción de citocinas proinflamatorias: IFNγ e IL-6 en células mononucleares de la lámina propia del intestino grueso estimuladas con LPS (Matsumoto *et al.*, 2005). Finalmente, *L. casei* inmunitas es capaz de inhibir la migración de leucocitos al epitelio colónico al inhibir su adherencia al endotelio vascular, como consecuencia de la inhibición de la ICAM-1 (molécula de adhesión intercelular-1), en un modelo de colitis experimental por TNBS en ratas (Angulo *et al*., 2006).

Los resultados obtenidos del tratamiento con el tercer probiótico, *Lactobacillus casei* LAFTI L26, muestran su efecto beneficioso desde el punto de vista macroscópico mediante la reducción significativa del cociente peso/longitud; y bioquímico por una recuperación de los niveles de glutation. Su actividad beneficiosa también se asoció a una reducción de la expresión de la COX-2, enzima que cataliza la conversión del acido araquidónico dando lugar a las prostaglandinas PGE_2

y PGI_2, que se encuentran implicadas en procesos patológicos del tracto gastrointestinal, y es inducida en macrófagos, fibroblastos y células vasculares, endoteliales y de músculo liso por varias citocinas, endotoxinas, factores de crecimiento o promotores de tumores (Smith y Langenbach, 2001)

Una característica interesante que nos gustaría destacar es que solo los probióticos del género *Lactobacillus* fueron capaces de modificar la flora colónica, mejorando el cociente *Lactobacillus*/Bacterias patógenas. Este efecto contribuye de manera significativa al efecto beneficioso ejercido por los probióticos en este modelo de colitis experimental. De hecho, se ha demostrado que el aumento en la concentración de *Lactobacillus* sp. reduce la adherencia y traslocación de bacterias patógenas en ratones IL-10 KO (Madsen *et al.*, 1999), previniendo así sus efectos perjudiciales sobre la respuesta inmunitaria exacerbada en la inflamación intestinal, según lo propuesto tanto en modelos experimentales (Garcia-Lafuente *et al.*, 1997) como en humanos (Cummings *et al.*, 2003).

Para poder comparar en conjunto los probióticos, realizamos la *Tabla 10*. Analizando estos resultados, podemos observar que si bien los tres probióticos presentan actividad antiinflamatoria (evidenciado por la relación peso/longitud y la restauración de los niveles de glutation), tienen marcadas diferencias en su comportamiento desde el punto de vista macroscópico, bioquímico y microbiológico. En este sentido, podemos destacar que:

- *B. lactis*, es el único probiótico capaz de modular la producción de TNFα , modulando de manera beneficiosa la respuesta inmune alterada en la inflamación.
- Solamente *L. acidophilus*, inhibió los niveles de LTB_4, eicosanoide quimiotáctico que juega un papel muy importante en los estadios iniciales del proceso inflamatorio. La menor infiltración leucocitaria derivada de esta inhibición viene avalada por la disminución en los niveles de la actividad MPO. Otro mecanismo importante es el efecto sobre el cociente *Lactobacillus*/patógeno.
- Por último, *Lactobacillus casei*, ademas de disminuir el cociente *Lactobacillus*/Patógeno, redujo la producción enzimática de la COX-2, probablemente derivado de la reducción del estrés oxidativo como consecuencia del tratamiento probiótico, ejerciendo un efecto positivo sobre la inflamación intestinal.

***Tabla 10**. Resumen de resultados del tratamiento con B. lactis, L. acidophilus, L. casei y en el modelo de colitis experimental por TNBS en ratas.*

	B. lactis	*L. acidophilus*	*L. casei*
IDM		+	
Longitud daño		+	
Peso/longitud	++	++	++
MPO		++	
Glutation	+	+	+
TNFα	+		
LTB_4		+	
iNOS	+	+	
COX-2			+
***Lactobacillus*: Patógeno**		+	+

+: p<0,05 vs. control; ++: p<0,01 vs. control.

2.- **Ensayo de los probióticos *Lactobacillus salivarius ssp. salivarius* y *Lactobacillus fermentum* en el modelo de colitis experimental por el acido trinitrobencenosulfónico (TNBS) en ratas.**

El segundo objetivo planteó la búsqueda de otros probióticos con características idóneas para el tratamiento de la EII. Surgió una colaboración con Puleva Biotech, empresa que aisló un gran número de probióticos de diferentes fuentes, incluída la leche materna. De esta colección de probióticos, se seleccionaron dos, que poseían características añadidas determinadas en estudios *in vitro*:

Lactobacillus salivarius ssp. salivarius es capaz de modificar el perfil de citocinas proinflamatorias/citocinas antiinflamatorias, reduciendo los niveles de citocinas proinflamatorias liberadas por macrófagos (TNFα e IL-12), y aumentando los niveles de la citocina antiinflamatoria IL-10.

El segundo probiótico fue *Lactobacillus fermentum*, que procede de la leche materna, produce compuestos antioxidantes como el glutation y su precursor, el dipéptido γ-Glu-Cys.

Los resultados obtenidos en el presente estudio revelan que el tratamiento con ambos probióticos mostró una clara eficacia en este modelo de colitis experimental. La administración oral de los mismos facilitó la recuperación del tejido, evidenciado histológicamente, por una reducción significativa del tamaño y severidad del tejido inflamado, mostrando las zonas ulceradas un proceso de reepitelización; y macroscópicamente, mediante la disminución del tamaño de la lesión y del cociente peso/longitud.

Este efecto beneficioso además se constató desde el punto de vista bioquímico por una menor actividad MPO. Ambos probióticos fueron capaces de reducir los niveles de esta enzima, hecho confirmado histológicamente mediante una disminución de la infiltración leucocitaria en los grupos tratados con los probióticos en comparación con el grupo control, lo que demuestra su efecto beneficioso, ya que la extravasación de leucocitos contribuye de forma muy marcada al daño colónico en este modelo de inflamación intestinal (Ajuebor *et al*., 2004).

Sin embargo, el mecanismo por el que estos probióticos inhiben la migración leucocitaria puede diferir en función de las características descritas *in vitro* para estas bacterias.

Así, el efecto inhibidor de *Lactobacillus fermentum* sobre la infiltración leucocitaria puede ser consecuencia del efecto preventivo ejercido contra los radicales libres derivados del daño oxidativo provocado por el TNBS, mediante la producción de glutation y de su precursor γ-Glu-Cys (Ardite *et al*., 2000; Grishman *et al*., 1991). Este dipéptido tiene un papel muy importante, de hecho, diversos estudios han descrito que es un antioxidante aún más efectivo que el glutation en el intestino. Aunque γ-Glu-Cys puede ser también el sustrato de otras enzimas, como γ-glutamilciclotransferasa, la síntesis de glutation esta aumentada en las células animales debido a su alta afinidad por la glutation sintetasa (Wu *et al*., 2004). Las propiedades antioxidantes de estos dos compuestos (glutation y γ-Glu-Cys), parecen ser cruciales para el efecto beneficioso de este probiótico. De hecho, se ha propuesto que la generación de radicales libres en el tejido inflamado constituye una señal temprana que promueve la infiltración de neutrófilos en el tejido colónico, que a su vez produce una gran cantidad de radicales libres que participan de manera activa en la perpetuación de la respuesta inflamatoria (Guo *et al*., 1999). Por esta razón, la neutralización de estas especies reactivas del oxigeno llevaría consigo la inhibición de la infiltración de neutrófilos, hecho observado en este estudio.

En relación con la actividad antiinflamatoria ejercida por *Lactobacillus salivarius ssp. salivarius*, se caracterizó por una reducción muy marcada de los niveles de TNFα, mediador muy importante, ya que actúa como un potente quimioatrayente, contribuyendo así al reclutamiento de neutrófilos en la mucosa. Este hecho concuerda con los estudios *in vitro* que demuestran que *L. salivarius* es capaz de reducir los niveles de las citocinas proinflamatorias liberadas por macrófagos (TNFα y IL-12) y aumentar los de la citocina antiinflamatoria IL-10. Hay una gran diversidad en las características inmunomoduladoras de las cepas de *Lactobacillus* ya que hay trabajos previos que indican que ciertas cepas de *Lactobacillus* tienen la capacidad de aumentar los niveles de TNFα (Miettinen *et al*., 1996), sin embargo otras, como *Lactobacillus rhamnosus GG* (LGG) lo reducen (Pena y Versalovic, 2003). LGG también reduce el cociente TNFα/IL-10, y ha demostrado ejercer un efecto antiinflamatorio tanto en humanos (Schultz *et al*., 2004) como en modelos experimentales (Dieleman *et al*., 2003). Estos efectos sobre la respuesta inmunitaria tienen especial relevancia ya que podría promover el cambio de una respuesta inmunitaria mediada por Th1 a una respuesta inmunitaria mediada por Th2/Th3, según lo propuesto para *Lactobacillus* GG (Schultz *et al*., 2003).

Finalmente, es muy interesante destacar la capacidad de *Lactobacillus fermentum* de modificar la flora colónica, aumentando de manera significativa los niveles de *Lactobacillus*. Sabiendo que los *Lactobacillus* son los responsables de la fermentación de la fibra dando lugar a la producción de los ácidos grasos de cadena corta, decidimos medir su producción colónica. De hecho, se comprobó que se encontraba incrementada en las ratas colíticas tratadas con *Lactobacillus fermentum* en comparación con el grupo control sin tratamiento probiótico. Ésto nos hizo pensar que el efecto inhibidor del tratamiento probiótico sobre la producción de citocinas se podía deber a la actividad de los AGCC sobre distintos factores de transcripción, como el factor nuclear-κB (NFκB), el cual, juega un importante papel en la regulación de la expresión de genes que codifican numerosas citocinas en la inflamación (Schottelius y Baldwin, 1999). De hecho, se ha publicado que el butirato reduce la producción de TNFα en células mononucleares de la lamina propia de biopsias intestinales mediante la inhibición de la activación del NFκB y de la degradación del IκBα (Segain *et al*., 2000). También se ha señalado el efecto del butirato sobre el NFκB en células HT-29, probablemente derivado de su capacidad de inhibir deacetilasas (Inan *et al*., 2000). Finalmente hay que destacar que la reducción del estrés oxidativo colónico puede reducir la producción de citocinas, ya que el NFκB es un factor de transcripción que se activa por el estrés oxidativo en la inflamación intestinal (Rogler *et al*., 1998).

De este estudio podemos deducir (*Tabla 11*) que ambos probióticos muestran actividad antiinflamatoria. Aunque existen ciertas diferencias, como en cuanto a su capacidad inmunomoduladora, ya que *Lactobacillus salivarius* baja de manera más marcada los niveles de TNFα en comparación con *Lactobacillus fermentum*. Por otro lado, solamente *L. fermentum* es capaz de aumentar de forma significativa los niveles de *Lactobacillus* en el colon, y en consecuencia los niveles de ácidos grasos de cadena corta, sustratos del colonocito, ejerciendo un efecto beneficioso en su recuperación.

***Tabla 11**. Comparación del efecto antiinflamatorio de Lactobacillus salivarius ssp. salivarius y Lactobacillus fermentum en el modelo de colitis experimental por TNBS en ratas.*

	L. salivarius	*L. fermentum*
IDM	+	+
Long daño	++	++
Peso/longitud	++	++
MPO	+	++
Glutation	+	+
TNFα	++	+
iNOS	+	+
Lactobacillus		+

+: $p<0,05$ vs. control; ++: $p<0,01$ vs. control.

Analizando los resultados, *L. fermentum* aporta una serie de características que lo hacen un probiótico ideal para el tratamiento de la enfermedad inflamatoria intestinal, bien solo o asociado, ya que es uno de los pocos capaces de generar compuestos antioxidantes per se. Además, de todos los probióticos ensayados, es el único que incrementó el recuento de bacterias saprofitas beneficiosas.

3.- Estudio comparativo de los efectos preventivos ejercidos por los dos probióticos: *Lactobacillus fermentum* y *Lactobacillus reuteri* en el modelo de colitis experimental por TNBS en ratas.

Teniendo en cuenta los resultados obtenidos en el estudio de la eficacia de los anteriores probióticos, decidimos comparar la actividad antiinflamatoria de *Lactobacillus fermentum* y de *Lactobacillus reuteri*, probiótico con eficacia sobradamente demostrada en anteriores estudios (Holma *et al*. 2001; Madsen *et al*. 1999; Mao *et al*. 1996; Moller *et al*. 2005), sin embargo éste es el primero que la describe en este modelo de colitis experimental.

Los dos probióticos redujeron algunos de los parámetros clínicos característicos de este modelo de colitis experimental como la anorexia, diarrea, y parámetros macroscópicos; sin embargo, el tratamiento con *Lactobacillus fermentum* resultó, de nuevo, ser mas efectivo. De hecho, este probiótico disminuyó la incidencia de diarrea y adhesiones, el cociente peso/longitud así como la extensión y el índice de daño macroscópico. Y por el contrario, el tratamiento con *Lactobacillus reuteri* sólo modificó el cociente peso/longitud en comparación con las ratas colíticas no tratadas.

Desde el punto de vista bioquímico, únicamente *Lactobacillus fermentum* redujo la actividad enzimática MPO, y contrarrestó la reducción de los niveles de glutation como consecuencia de la colitis. Sin embargo, si disminuyeron de manera significativa los niveles de TNFα ambos probióticos

Finalmente, el estudio microbiológico mostró que tanto *Lactobacillus fermentum* como *Lactobacillus reuteri* restauraron el cociente *Lactobacilli*/Bacterias patógenas, contribuyendo al efecto beneficioso ejercido por los dos probióticos. Es interesante destacar que *Lactobacillus fermentum* es capaz de aumentar la producción de AGCC, especialmente butirato, que es la fuente de energía fundamental del colonocito. Sin embargo, diversos autores han propuesto que la EII podría ser consecuencia de una falta de butirato en el colon o de una alteración de la utilización del mismo por parte del colonocito (Mortensen y Clausen, 1996; Rodríguez-Cabezas *et al*. 2002).

Tabla 12. *Resumen de resultados del tratamiento con L. fermentum y L. reuteri en el modelo de colitis experimental por TNBS en ratas.*

	L. fermentum	*L. reuteri*
IDM	*	
Longitud daño	+	
Peso/longitud	+*	+
MPO	+*	
Glutation	+	
TNFα	+	+
Acetato	*	
Butirato	*	
Propionato	*	
Lactobacillus	+	
Bifidobacterium	+	+
***Lactobacillus*:** **Patogeno**	+	+

+: p<0,05 vs. control; *: p<0,05 vs. *Lactobacillus reuteri.*

Basándonos en la *Tabla 12*, y comparando los resultados de este estudio, ambos probióticos tienen en común la capacidad de reducir el edema colónico evidenciado por el cociente peso/longitud; la reducción de los niveles de la citocina proinflamatoria TNFα, mostrando por tanto

ambos un efecto modulador de la respuesta inmunitaria; y el cociente *Lactobacillus*/Patógeno, parámetro clave para su actividad antiinflamatoria intestinal. Sin embargo, *Lactobacillus fermentum* posee propiedades añadidas como la producción de glutation, que podría explicar la menor infiltración de neutrófilos evidenciado por unos reducción de los niveles de MPO. Por tanto, nuestro probiótico es mas eficaz que *L. reuteri*, probiótico con eficacia más que demostrada sobre la salud humana (Valeur *et al*., 2004).

También cabe destacar que estos probióticos se encuentran en la leche materna, aunque las dosis administradas a las ratas en este estudio probablemente son mucho mayores a las presentes en la misma. Sin embargo, estos resultados sugieren que la colonización de estos probióticos en el lumen colónico, podría resultar en efectos beneficiosos para estos sujetos, probablemente derivados de sus propiedades inmunomoduladoras.

Finalmente, en este estudio hemos ratificado el gran potencial terapéutico de *Lactobacillus fermentum* sobre estas patologías intestinales, superando incluso a probióticos como Lactobacillus *reuteri*, que ha demostrado su eficacia en distintas patologías en un gran número de estudios. *Lactobacillus fermentum*, además de ser uno de los pocos que es capaz de producir glutation per se, posee actividad inmunomoduladora y es capaz de preservar la función de barrera intestinal al aumentar los niveles de lactobacilos y bifidobacterias. No obstante, hay que hacer más estudios tanto experimentales como en humanos para poder confirmar estos resultados.

CONCLUSIONES

1. *Lactobacillus casei, Lactobacillus acidophilus* y *Bifidobacterium lactis* muestran actividad antiinflamatoria intestinal en el modelo de colitis experimental por TNBS en ratas, siendo su eficacia diferente tanto desde el punto de vista cualitativo como cuantitativo.

2. El tratamiento con *Lactobacillus salivarius* ssp. *salivarius* también muestra eficacia antiinflamatoria en el mismo modelo. Ésta se basa en su capacidad de modular la respuesta inmune, evidenciada por una disminución significativa de la citocina TNFα.

3. *Lactobacillus fermentum* mostró un claro efecto beneficioso en esta colitis experimental debido a su capacidad de generar sustancias antioxidantes. Además, este probiótico fue el único de todos los ensayados que elevó la población de lactobacilos y bifidobacterias, lo que repercutió en un aumento de los niveles de ácidos grasos de cadena corta, especialmente butirato.

4. Por ultimo, *Lactobacillus fermentum* puede ser una especie con un gran interés en el tratamiento de la enfermedad inflamatoria intestinal utilizándose bien solo o asociado ya que es uno de los pocos probióticos capaces de generar sustancias antioxidantes.

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ANEXOS

ABREVIATURAS

Ag.	Antígeno
AGCC	Ácido graso de cadena corta
AINE	Antiinflamatorio no esteroídico
BCA	Ácido bicinchoninico
CARD	Dominio de recruimiento de caspasas (*Caspase recruitment domain*)
CFU	Unidad formadora de colonias
CMH-II	Complejo mayor de histocompatibilidad de clase II
COX-2	Ciclooxigenasa-2
CU	Colitis ulcerosa
DSS	Sulfato de dextrano sódico
EC	Enfermedad de Crohn
EDTA	Ácido etilendiaminotetraácetico (*ethylenediaminetetraacetic acid*)
EII	Enfermedad inflamatoria intestinal
E. coli	*Escherichia coli*
FID	Detector de ionización de llama (*Flame ionization detector*)
GSH	Glutation reducido
GSSG	Glutation oxidado
HPLC	Cromatografía líquida de alta definición (*High Performance Liquid Chromatography*)
IAEC	Índice de actividad de la Enfermedad de Crohn
IAP	Índice de actividad de la pouchitis
IBD	Inflammatory bowel disease
IDM	Índice de daño macroscópico
Ig	Inmunoglobulina
I κB	Subunidad inhibidora κB
I κK	I κB kinasa
IL	Interleucina

IFN-γ	Interferon-γ
iNOS	Óxido nítrico sintasa inducible
LGG	*Lactobacillus rhamnosus* GG
LPS	Lipopolisacarido
LTB_4	Leucotrieno B_4
MPO	Mieloperoxidasa
NADPH	Nicotinamida adenin dinucleotido fosfato reducido
NF-κB	Factor de transcripción nuclear κB
NO	Óxido nítrico
NOD	Dominio intracelular de oligomerización de nucleótidos (*intracellular nucleotide oligomeration domain*)
NOS	Óxido nítrico sintasa
PBS	Tampon fosfato salino (*Phosphate buffer saline*)
PGE2	Prostaglandina E2
SDS	Dodecil sulfato sódico (*sodium dodecyl sulfate*)
SDS-PAGE	Electroforesis en gel desnaturalizante de poliacrilamida
TBS	Tampón tris salino (*Tris buffer saline*)
TCA	Ácido tricloroacético
TGF-β	Factor de crecimiento transformante-β
Th	Célula T colaboradora (*T helper*)
TLR	Receptor tipo Toll (*Toll like receptor*)
TNBS	Ácido trinitrobencenosulfónico
TNF-α	Factor de necrosis tumoral α

ÍNDICE DE TABLAS

ÍNDICE DE FIGURAS