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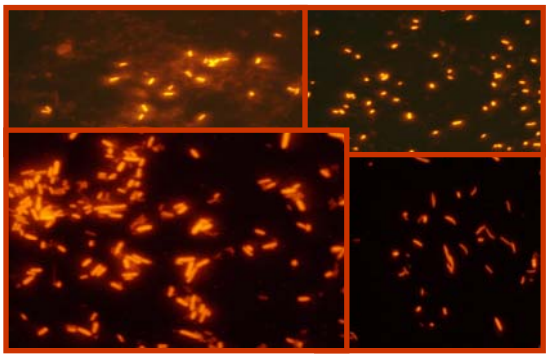

Influencia del ambiente sobre la microbiota comensal del intestino de la rata: Implicaciones en la inflamación y en mecanismos sensoriales

Memoría presentada por Demetria Evangelina Terán Ventura para optar al grado de Doctora dentro del programa de Doctorado en Farmacología, del Departamento de Farmacología, de Terapéutica y de Toxicología

Tesis Doctoral dirigida por:

Patrocinio Vergara Esteras
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Bellatera, diciembre, 2009

Universitat Autònoma de Barcelona

PATROCINIO VERGARA ESTERAS, Catedrática de Universidad, y VICENTE MARTÍNEZ PEREA, Profesor Titular de Universidad, del Departamento de Biología Celular, de Fisiología e Inmunología de la Universidad Autónoma de Barcelona

HACEMOS CONSTAR

Que la memoria titulada *"**Influencia del ambiente sobre la microbiota comensal del intestino de la rata: Implicaciones en la inflamación y en mecanismos sensoriales**"* presentada por DEMETRIA EVANGELINA TERÁN VENTURA para optar al grado de Doctora, se ha realizado bajo nuestra dirección, y al considerarla concluida, autorizamos su presentación para ser evaluada por el Tribunal correspondiente.

Y para que conste a los efectos firmamos la presente.

Bellaterra, 09 de noviembre de 2009

Dra. Patrocinio Vergara Esteras Dr. Vicente Martínez Perea

La imagen de la portada son cuatro fotografías de obtención propia, que representan un campo de microscopio con Bifidobacterias (parte superior izquierda), enterobacterias (parte superior derecha), *E.coli* (parte inferior izquierda), y bacterias totales (parte inferior derecha) fluorescentes, con la técnica de Hibridación *in situ* fluorescente.

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Bienaventurado el hombre que ha encontrado la sabiduría, el hombre que ha adquirido la inteligencia, porque adquirirla vale más que adquirir plata y poseerla más que poseer oro. Es más precioso que las perlas, y todos los tesoros que puedas desear no la igualan.

Prov 3, 13-15

Abreviaturas

DNA: ADN Ácido desoxirivonucleico

RNA: ARN Ácido ribonucléico

CB Canabinoides

cDNA ADN complementario

CpGDNA cytosine phosphate guanine ADN

CV Convencional

dNTP Deoxyribonucleotide triphosphate: tri-fosfato deoxiribonucleotido

DSS Dextran sulfate sodio: Sulfato de sodio dextrano

FISH Fluorescence *in situ* hibridization: Hibridación *in situ* fluorescente

GAPDH Glyceraldehyde 3-P-dehydrogenase: Gliceraldehido-3-deshidrogenasa

HEPA High Efficiency Particulate Air : filtros de alta eficiencia de partículas del aire.

HEPES Hydroxi-ethyl piperazine ethane sulfonic acid: Ácido Hidroxi-etil-piperacino-etano-sulfónico.

HSP60 Heat shock protein 60

IBD Inflammatory Bowel Disease: Enfermedad Inflamatoria Intestinal

IBS Irritable Bowel Syndrome: Síndrome del intestino irritable

LPS Lipopolisacárido

MPO Myeloperoxidase: Mieloperoxidasa

NOD2 nucleotide-binding oligomerization domain containing 2

PBS Phosfate buffer saline: Tampón salino fosfato

PCR Polimerase Chain Reaction: Reacción en cadena de la polimerasa

PAMPs Pathogen-associated molecular patterns: Patrón molecular asociado a patógenos.

PMSF Phenylmethylsulphonyl fluoride: Fenilmetilsulfonil fluorado.

SEM Standard error mean: Error estándar de la media

SPF Specific pathogenic free

TLR Toll Like Receptor: Receptores tipo toll

t-RFLP Terminal Restriction Fragment Length Polymorfism: Polimorfismo de la longitud de los fragmentos de restricción terminales.

Los estudios de esta tesis se han publicado o están pendientes de publicarse en los siguientes artículos y resúmenes:

Characterization of Housing-related Spontaneous Variations of Gut Microbiota and Expression of Toll Like Receptors 2 and 4 in Rats. Evangelina Terán-Ventura, Mercè Roca, Maria Teresa Martin, Maria Lourdes Abarca, Vicente Martinez and Patri Vergara. *Applied and Environmental Microbiology*. *En fase de revision*.

Susceptibility to Indomethacin-induced Enteritis in Rats Maintained Under Different Microbiological Conditions. Evangelina Terán-Ventura, Vicente Martinez and Patri Vergara. *Manuscrito.*

Gut microbiota affects cannabinoid receptors Expression in the intestine of rats. E. Terán, V. Martinez and Patri Vergara*. Manuscrito*.

Application of 16s rRNA gene-targetted fluorescence in situ hybridization (FISH) to study gut microbiota in the rat: Influence of housing conditions. Evangelina Terán-Ventura, Lourdes Abarca, Patri Vergara. Proceedings of the Tenth FELASA Symposium and the XIV ICLAS General Assembly & Conference, 11-14 Junio 2007, Cernobbio, Italia.

Influence of hygienic conditions in gut microbiota and expression of *toll like receptors* (TLR): response to inflammation. Evangelina Terán-Ventura, Mercé Roca, Lourdes Abarca, Maité Martin, Patri Vergara. Neurogastroenterology and Motility, 2008, 20: 125-126.

Gut Microbiota Affects Toll-Like Receptors (TLR) and Cannabinoid 2 Receptor (CB2) Expression in the Intestine of Rats. Evangelina Terán-Ventura, Patri Vergara, Vicente Martinez. Gastroenterology, 2009, 136 (Suppl 1): 1075.

Specific changes of gut commensal microbiota in a model of indomethacin-induced enteritis in rats. Evangelina Terán-Ventura, Vicente Martinez and Patri Vergara. Enviado para el Congreso: “Digestive Disease Week” (DDW. May 1-5, 2010), New Orleans, LA.

Gut commensal microbiota affects the expression of cannabinoid receptors (CB1 and CB2) in the intestine of rats. Evangelina Terán-Ventura, Patri Vergara, Vicente Martinez. Enviado para el Congreso: “Digestive Disease Week” (DDW. May 1-5, 2010), New Orleans, LA.

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Introducción

1 Microbiota comensal del Tracto Gastrointestinal

La microbiota intestinal comensal se define como al conjunto de microorganismos que se encuentran temporal o permanentemente, en el tracto gastrointestinal de individuos sanos (Savage D.C., 1984a). Se comenzó a estudiar en los años 70 mediante cultivos en anaerobiosis, donde se compara la microbiota humana con la de otras especies animales (Savage, 1977)**.** Está compuesta de 400 a 500 especies de bacterias, en una cantidad del orden de 10^{13} bacterias por gramo de contenido en heces, es decir que hay 10 veces más células bacterianas que el total de células de nuestro cuerpo. Entre éstas bacterias se encuentran bacterias aerobias, anaerobias facultativas, y anaerobias estrictas (Mackie *et al.*, 1999; Tannock, 1999).

La composición y cantidad de la microbiota comensal a lo largo del intestino es diferente según el área considerada; así la población del intestino delgado está compuesta por *Bacteroides*, *Lactobacillus*, *Estreptococos*, *Bifidobacterium*, *Fusobacterium* y Enterobacterias (Savage, 1977). Por su parte la población bacteriana del intestino grueso (colón) en su mayoría está compuesta por bacterias anaerobias estrictas como *Clostridium*, *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Peptostreptococcus* y *Ruminococcus* (Savage, 1977).

1.1 Composición de la microbiota intestinal comensal en roedores

El estudio de la microbiota intestinal comensal en todas las especies es muy complejo, ya que la mayoría de las bacterias del intestino son anaerobios estrictos no cultivables, hay mucha variabilidad de bacterias entre individuos, y varía con la dieta, el estrés, el tiempo y las condiciones ambientales (Eckburg *et al.*, 2005).

La composición de la microbiota intestinal comensal en roedores se ha estudiado sobretodo con cultivos. Las zonas mejor caracterizadas desde el punto de vista de la microbiota son el íleon, el ciego, y el colón. Los géneros encontrados son básicamente los mismos que en otras especies. Así, en el íleon se han encontrado fundamentalmente: *Lactobacillus*, *Streptococcus*, *Clostridium*, y coliformes. En el ciego y colón se han encontrado sobre todo: *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Propionibacterium*, *Bacteroides*, *Veillonella* y coliformes (Savage, 1977).

Recientemente se ha intentado caracterizar la microbiota intestinal comensal en heces de ratas, identificando aquellas bacterias anaerobias estrictas del intestino que son difíciles de cultivar como son el grupo de los clostridios (*Clostridium coccoides*, subgrupo de C*lostridium leptum*) y bacterias gramnegativas (*Bacteroides* y *Cytophagas*) (Brooks *et al.*, 2003)

A pesar del intento en caracterizar la microbiota intestinal comensal en roedores, propiamente en la rata, solamente se ha logrado cuantificar aproximadamente un 29% de las bacterias totales (Dinoto *et al.*, 2006).

1.2 Clasificación microbiológica de los animales de laboratorio

Los animales de laboratorio se clasifican en diferentes categorías, según la fuente de obtención, las condiciones de cría del animal, y la microbiota asociada al mismo. Si nos referimos a su caracterización microbiológica los animales de laboratorio se clasifican en: animales convencionales, animales SPF o libres de patógenos específicos, animales *germ-free*, y animales gnotobióticos.

Los animales convencionales (CV) o haloxénicos, son animales que las condiciones de mantenimiento y cría garantizan que son libres de zoonosis. Son criados y mantenidos en instalaciones abiertas, con barreras sanitarias relativas (Gustafsson, 1984).

Los animales libres de microorganismos patógenos específicos (SPF), conocidos también como animales heteroxénicos proceden de colonias obtenidas por cesárea o histerectomía aséptica, posteriormente mantenidos en barrera. Se caracterizan por poseer una microbiota asociada no patógena y específica. A éstos animales se les inocula una mezcla de bacterias que contienen los principales grupos de la microbiota intestinal (Ver como referencia la Tabla1 del Estudio 1) (Dewhirst *et al.*, 1999). Se crían en aisladores o en un sistema de barrera o zona protegida, donde se filtra el aire (filtros HEPA)**,** se esteriliza el agua, el alimento, el lecho, y cualquier otro material en contacto con los animales. Los controles sanitarios son periódicos con la finalidad de mantener éstas condiciones (Gustafsson, 1984).

Los animales *germ-free*, también llamados axénicos, están libres de bacterias, hongos, parásitos y virus, se obtienen por histerectomía (cesárea) aséptica, y se crían y mantienen en un aislador, con agua, alimento y lecho estériles (Gustafsson, 1984).

Los animales gnotobióticos son animales axénicos en los que se han introducido microorganismos específicos. Son por tanto animales con una microbiota definida. Éstos también se crían y mantienen en aisladores para prevenir la contaminación por otros microorganismos, y los controles sanitarios son periódicos. Este tipo de animales se desarrolla para poder estudiar la influencia de las bacterias específicas en el organismo (Gustafsson, 1984).

1.3 Factores que influencian la composición de la microbiota intestinal comensal

Tanto factores genéticos como factores externos, como el ambiente, el estilo de vida, la dieta, el estrés, y el tratamiento con antibióticos afectan a la composición de la microbiota comensal intestinal tanto en humanos como en roedores (Figura 1).

Un factor importante que afecta a la composición de la microbiota intestinal comensal es la genética entre individuos. Y una de las hipótesis propuestas es que determinados genotipos podrían favorecer la colonización de algunas bacterias intestinales, que son capaces de inducir ciertas enfermedades (Toivanen *et al.*, 2001).

Existen pocos estudios en humanos, de cómo afectan las condiciones ambientales a la composición de la microbiota intestinal comensal. Por ejemplo, está demostrado que el ambiente hospitalario afecta tanto a la colonización del *C. difficile* (Kim *et al.*, 2007) como a la composición bacteriana de las heces (Bartosch *et al.*, 2004). En el año 1979, ya había algunas evidencias que sugerían que las condiciones ambientales de un animal podrían alterar la composición de su microbiota (Savage, 1984b). Sin embargo, no se encuentran trabajos actuales sistemáticos que relacionen el efecto de un determinado ambiente en la composición de la microbiota intestinal comensal ni en roedores, ni en otras especies.

En varias especies, incluyendo en humanos, se ha demostrado que la dieta puede ser un factor determinante en la composición de la microbiota intestinal comensal (Cresci *et al.*, 1999; Alm *et al.*, 2002; Mai *et al.*, 2004; Castillo *et al.*, 2007b). Por otra parte, ciertas situaciones de estrés psicológico causan un efecto sobre la composición de la microbiota intestinal comensal en humanos, monos y roedores (Lizko, 1987; Bailey and Coe, 1999; Wang and Wu, 2005).
El uso de antibióticos es la causa más común y significativa de la alteración en la composición de la microbiota intestinal comensal (Verdú *et al.*, 2006). Por ejemplo, se ha demostrado que la administración de la amoxicilina en niños en los primeros días de vida afecta de manera significativa la composición de la microbiota intestinal comensal (Penders *et al.*, 2006).

De todos los factores expuestos anteriormente que afectan a la composición de la microbiota intestinal comensal, no hay uno en particular que tenga más o menos influencia sobre la microbiota. Sin embargo; debemos diferenciar entre la microbiota luminal intestinal y la microbiota de la pared intestinal que

fue colonizada en los primeros días de vida. Por tanto, al referirnos a la composición de ésta microbiota podría ser la microbiota luminal la que cambie; ya que, esta reportado que la composición de la microbiota luminal es diferente a la microbiota de la pared (Pryde *et al.*, 1999).

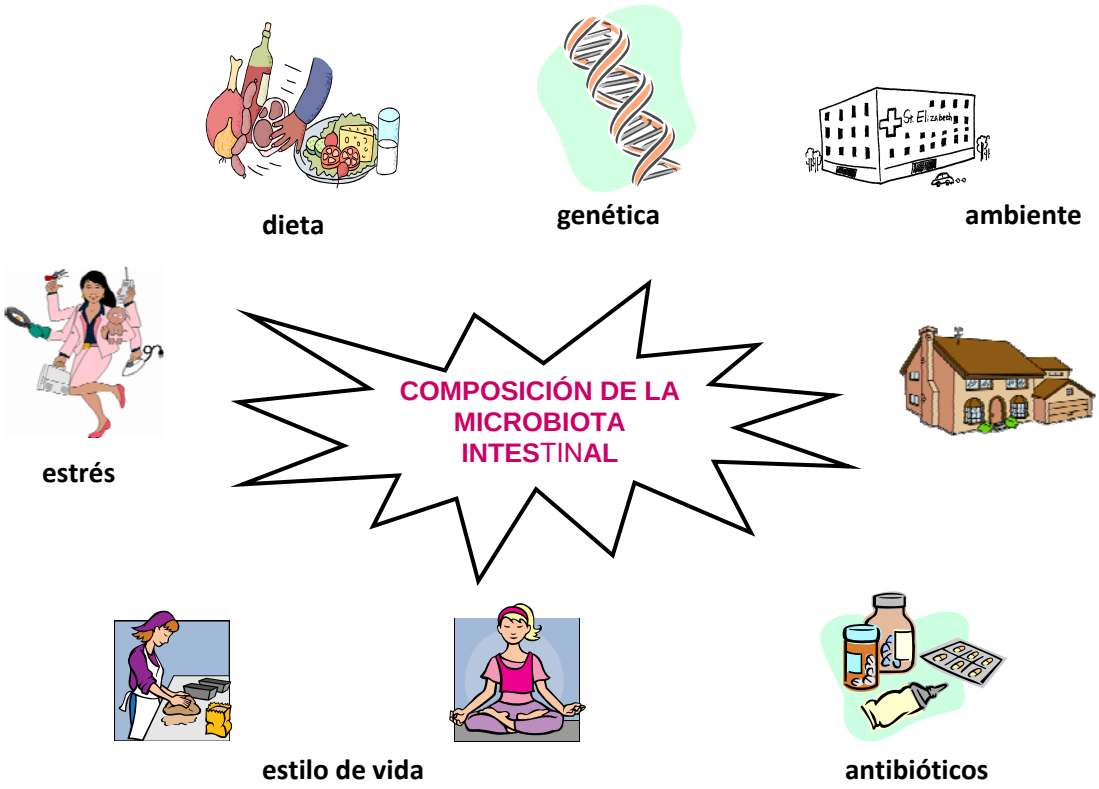

Figura 1. Factores que influyen en la composición de la microbiota comensal intestinal.

1.4 Técnicas para la caracterización de la microbiota comensal

Como se ha mencionado antes, el tracto gastrointestinal de cada individuo contiene entre 400 y 500 especies diferentes de bacterias, muchas de las cuales no son cultivables (Moter and Gobel, 2000). El aislamiento de microorganismos por cultivos es una técnica de baja sensibilidad; por tanto para una mayor caracterización de la población bacteriana intestinal de forma más sensible y fiable, se están aplicando actualmente técnicas

moleculares. A continuación se detallarán las utilizadas en este trabajo de investigación.

1.4.1 Análisis de polimorfismos de fragmentos terminales de restriction

Un buen método para el estudio cualitativo de la microbiota intestinal comensal es el empleo de la técnica *t-RFLP* (Terminal Restriction Fragment Length Polymorfism), utilizada por primera vez por Cancilla *et al.* (1992). Esta técnica es muy útil para comparar la composición de comunidades microbianas mediante el estudio del polimorfismo de diferentes fragmentos de restricción del gen 16S del rADN (Ácido desoxirribonucleico ribosomal), y permite obtener un perfil más completo de los microorganismos presentes en las muestras, ya sean de contenido cecal o de heces (Cancilla *et al.*, 1992).

La técnica t-RFLP consiste en extraer el ADN bacteriano, para su posterior amplificación mediante PCR (*Polimerase Chain Reaction*), utilizando cebadores universales marcados con fluorescencia. Posteriormente, el producto de amplificación es sometido a una o varias digestiones con diferentes endonucleasas de restricción. Para detectar las variaciones en la longitud de los fragmentos (picos) de restricción, estos fragmentos son separados por electroforesis capilar y por último, con ayuda de un lector de laser, se detectan los fragmentos marcados generando un perfil según su longitud (Levitt, 1994; Kitts, 2001).

Esta técnica es de elección para el estudio de la diversidad de los microorganismos del tracto gastrointestinal, a pesar de que no identifica los grupos taxonómicos. No obstante, existen herramientas adicionales que permiten conocer los grupos taxonómicos, por ejemplo, la “restricción enzimática teórica”, que se obtiene a partir de una base de datos TAP-RFLP del software Ribosomal Database II Project (Cole *et al.*, 2003).

La t-RFLP ha demostrado ser una herramienta importante para caracterizar cualitativamente la microbiota intestinal de diferentes especies y hasta el momento se ha aplicado en humanos (Hayashi *et al.*, 2005; Nagashima *et*

al., 2003), aves (Gong *et al.*, 2002), cerdos (Castillo *et al.*, 2007[a]) y ratas (Dinoto *et al.*, 2006).

1.4.2 Hibridación *in situ* con fluorescencia (FISH)

Esta técnica nos permite cuantificar grupos de bacterias y en función de la especificidad de los oligonucleótidos usados se llega a valorar determinadas especies bacterianas. También permite distinguir la morfología de la bacteria, así como su distribución sobre la mucosa intestinal en muestras de pared intestinal. Sobre todo nos permite cuantificar bacterias que son difíciles de cultivar, tanto en heces como en contenidos intestinales. Consiste en la utilización de oligonucleótidos marcados con diferentes fluorocromos que hibridan en el 16S rRNA. Tiene la ventaja de que se hibridan las células completas, evitándose artefactos generados por sesgos en la extracción de DNA (Moter and Gobel, 2000).

Básicamente el FISH consiste en la separación de las bacterias de la muestra problema, mediante diluciones con tampones. Posteriormente, todas las células son fijadas, y su 16S rRNA se hibrida con los oligonucleótidos marcados con fluorocromos (como pueden ser DAPI, FITC, Cy3 o Cy5, 6-FAM, TRITC). La cuantificación de las células fluorescentes se puede realizar manualmente y por citometría de flujo (Zoetendal *et al.*, 2002; Vaahtovuo *et al.*, 2005).

Actualmente la técnica de FISH está siendo utilizada para cuantificar bacterias tanto en muestras de heces humanas (Franks *et al.*, 1998; Harmsen *et al.*, 2002; Zoetendal *et al.*, 2002; Duncan *et al.*, 2003; Mai *et al.*, 2004; Vaahtovuo *et al.*, 2005), como en muestras de heces y contenidos intestinales de ratas y ratones (Hoentjen *et al.*, 2005; Swidsinski *et al.*, 2005[a]; Dinoto *et al.*, 2006; Mozes *et al.*, 2008).

1.5 Funciones de la microbiota intestinal comensal

Las bacterias que conforman la microbiota intestinal comensal proporcionan ciertos beneficios al huésped, derivados de tres funciones: metabólicas, tróficas y de protección (Guarner and Malagelada, 2003).

1.5.1 Funciones metabólicas

El metabolismo de las bacterias es responsable de la conversión de muchas sustancias energéticas en nutrientes que pueden ser absorbidos y utilizados por el huésped (Hooper *et al.*, 2002). La microbiota del intestino grueso juega un papel importante en los procesos digestivos del huésped, generando energía de los residuos alimentarios no absorbidos, de las células de descamación intestinal, y de las secreciones a través de la fermentación de los hidratos de carbono, de la putrefacción de las proteínas, y de la absorción de los ácidos grasos de cadena corta y otros productos metabólicos (Guarner and Malagelada, 2003). Éstos productos son utilizados tanto por el huésped, para suplir parte de su requerimiento de energía, como por la microbiota, para mantener su crecimiento y proliferación (Guarner and Malagelada, 2003).

1.5.2 Funciones tróficas

Posiblemente el papel más importante de la microbiota intestinal comensal sobre la fisiología del colón es su efecto trófico en el epitelio intestinal (Guarner and Malagelada, 2003). La proliferación y diferenciación de las células epiteliales está afectada en gran medida por la interacción con los microorganismos residentes (Hooper *et al.*, 2001) y sus productos metabólicos, principalmente por los ácidos grasos de cadena corta (butirato, acetato y propionato) (Guarner and Malagelada, 2003).

1.5.3 Funciones protectoras

Se conoce que las bacterias que ocupan un espacio o nicho ecológico impiden la implantación de bacterias extrañas a ese ecosistema, lo se denomina como efecto “barrera”. Este efecto de barrera se debe a la capacidad de ciertas bacterias para segregar sustancias antimicrobianas (bacteriocinas), que inhiben la proliferación de ciertas especies oportunistas que están presentes en el intestino pero con proliferación restringida, y tiene un efecto protector para el huésped (Brook, 1999). Las bacterias intestinales también protegen al huésped contra algunos patógenos, con la inducción de la producción de inmunoglobulina A secretora (IgAs), y la disminución de la

producción de enterotoxinas (Falk *et al.*, 1998; Holzapfel *et al.*, 1998; Butler *et al.*, 2000).

2 Microbiota intestinal comensal en la salud y en la enfermedad

El estudio de las bacterias consideradas como «beneficiosas» y el efecto «protector» que pueden ejercer contra ciertas enfermedades, ha despertado el interés en el uso controlado de bacterias como una forma eficaz, y segura para el tratamiento y prevención de los procesos inflamatorios intestinales.

2.1 Probióticos

Los probióticos son definidos como microorganismos vivos que cuando son administrados en dosis apropiadas confieren un efecto beneficioso para el que los consume (Galdeano *et al.*, 2007). La historia de los probióticos se remonta al año 1857, cuando las bacterias lácticas fueron descubiertas por Luis Pasteur.

Hacia 1907 el biólogo ruso Elie Metchnickoff y colaboradores sugirieron que la ingesta de bacterias podía tener un efecto positivo sobre la microbiota comensal del tracto digestivo y que el consumo de yogurt prolongaba la vida (Metchnickoff, 1908).

Los probióticos se componen típicamente de bacterias ácido lácticas (*Lactobacillus*, *Bifidobacterium*, *Streptococcus* y *Lactococcus*) (Naidu *et al.*, 1999), que son capaces de sobrevivir en el ácido del estómago y la bilis, manteniéndose viables por períodos largos de tiempo, y seguras para el consumo humano. A parte de las bacterias ácido lácticas también ha mostrado tener este efecto beneficioso la *E.coli* Nissle 1917 (Grabig *et al.*, 2006).

Entre los efectos beneficiosos de los probióticos propuestos se pueden mencionar que: favorecen la estabilización de la barrera de la mucosa intestinal (Salminen *et al.*, 1996), previenen enfermedades infecciosas (Silva *et al.*, 1999), reducen los niveles séricos del colesterol (Fukushima and

Nakano, 1996), previenen la inflamación intestinal en modelos experimentales (Setoyama *et al.*, 2003; Osman *et al.*, 2004; Herias *et al.*, 2005; Watanabe *et al.*, 2009), tienen efectos de tipo analgésico (Verdú *et al.*, 2006; Rousseaux *et al.*, 2007) y ayudan a la homeóstasis de la microbiota intestinal comensal (Alander *et al.*, 1999) (Figura 2).

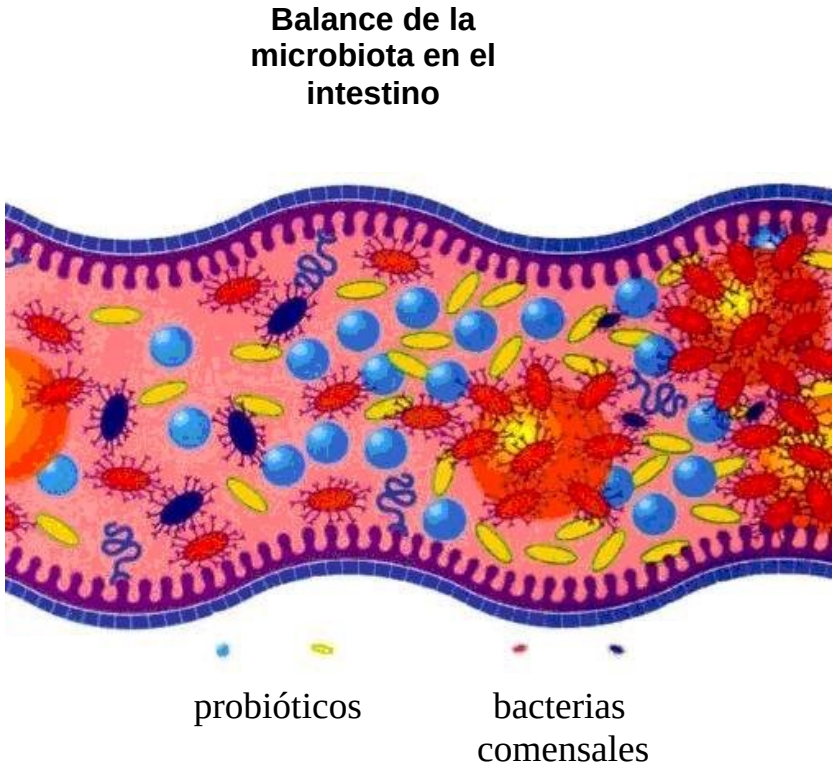

Figura 2. Imagen que muestra la interacción de la microbiota comensal con los probióticos.

2.2 Microbiota no beneficiosa para el organismo

Tanto el aislamiento de cepas de bacterias en algunos enfermos, como los estudios en animales con la microbiota intestinal restringida han llevado a buscar algunas bacterias comensales que estén asociadas con algunas enfermedades, por ejemplo: *Bacteroides*, *Escherichia coli*, y *Clostridium* (Konaka *et al.*, 1999; Basivireddy *et al.*, 2005; Swidsinski *et al.*, 2005[a, b]; Dalby *et al.*, 2006; Larrosa *et al.*, 2009; Natividad *et al.*, 2009; Watanabe *et al.*, 2009). Una hipótesis contemplada es que en condiciones normales las bacterias intestinales comensales benéficas y las bacterias intestinales

comensales asociadas con enfermedades están en equilibrio. Sin embargo, en determinadas situaciones este equilibrio se altera y componentes no beneficiosos de la microbiota podrían dar lugar a ciertas enfermedades, tales como el Síndrome del Intestino Irritable y la Enfermedad Inflamatoria Intestinal.

Así, el Síndrome del Intestino Irritable ha sido asociado con alteraciones en la microbiota intestinal comensal. Aunque su etiología es hasta el momento desconocida, se cree que aparece con frecuencia, después de una gastroenteritis o después de un tratamiento con antibióticos, justificando una relación con las alteraciones de la microbiota (Nobaek *et al.*, 2000; Pimentel *et al.*, 2000; Thornley *et al.*, 2001).

La Enfermedad Inflamatoria Intestinal también se ha asociado con alteraciones de la microbiota intestinal. Así, en las heces de pacientes con enfermedad de Crohn (EC) o colitis ulcerativa (CU) activa, se encontraron bifidobacterias y lactobacilos en cantidades reducidas, comparados con pacientes en fases inactivas y con controles sanos (Giaffer *et al.*, 1991; Hartley *et al.*, 1992). De la misma forma, en pacientes con EC y CU se encontraron algunos grupos de bacterias adheridas a la superficie del epitelio intestinal, especialmente del género *Bacteroides* (Wei *et al.*, 2001; Saitoh *et al.*, 2002; Swidsinski *et al.*, 2005[b]; Lucke *et al.*, 2006), y *E.coli* (Rolhion and Rfeuille-Michaud, 2007). En modelos experimentales de inflamación intestinal en roedores se han reproducido éstos hallazgos. Así se han encontrado asociados con respuestas inflamatorias *Bacteroides*, *Clostridium*, *Enterococcus* y enterobacterias con respuestas inflamatorias (Konaka *et al.*, 1999; Basivireddy *et al.*, 2005; Swidsinski *et al.*, 2005[a]; Dalby *et al.*, 2006; Larrosa *et al.*, 2009; Natividad *et al.*, 2009; Watanabe *et al.*, 2009).

3 La Enfermedad Inflamatoria Intestinal

La Enfermedad Inflamatoria Intestinal es una patología crónica que afecta al colon y/o al intestino delgado y comprende un grupo heterogéneo de patologías que incluyen a la EC, la CU y la colitis indeterminada (Russel and Stockbrugger, 1996).

Esta enfermedad es el resultado de la interacción de varios factores que incluyen la predisposición genética a padecer la enfermedad, la exposición a factores ambientales (dieta, estrés, el hábito de fumar, o la higiene extrema), la posible participación de agentes infecciosos y principalmente la microbiota intestinal comensal (Fiocchi, 1998).
Los factores predisponentes se hacen operativos gracias a un desencadenante, cuya naturaleza todavía desconocemos, que conduciría a la activación del sistema inmune en el intestino, y cuyo resultado final sería la producción de una gran variedad de moléculas activas, tales como: citocinas, factores de crecimiento, eicosanoides, neuropéptidos, metabolitos reactivos del oxígeno, óxido nítrico, anticuerpos y enzimas proteolíticas, cuya acción combinada produciría inflamación intestinal y daño tisular (Fiocchi, 1998).

3.1 Factores microbianos en el desarrollo de la Enfermedad Inflamatoria Intestinal

En el transcurso de los años, una larga lista de microorganismos, y particularmente la microbiota intestinal comensal han sido implicados en la patogenia tanto de la EC como de la CU (Fiocchi, 2005).

En la Enfermedad Inflamatoria Intestinal la microbiota intestinal comensal produce una respuesta inmune anormal de la mucosa, lo cual conduce a un proceso patológico que se expresa como una inflamación crónica del tracto gastrointestinal por una “pérdida de la tolerancia” hacia la microbiota entérica. El proceso resulta en una respuesta inmune inapropiada que lleva a un estado inflamatorio crónico (Figura 3) (Duchmann *et al.*, 1995; MacDonald, 1995).

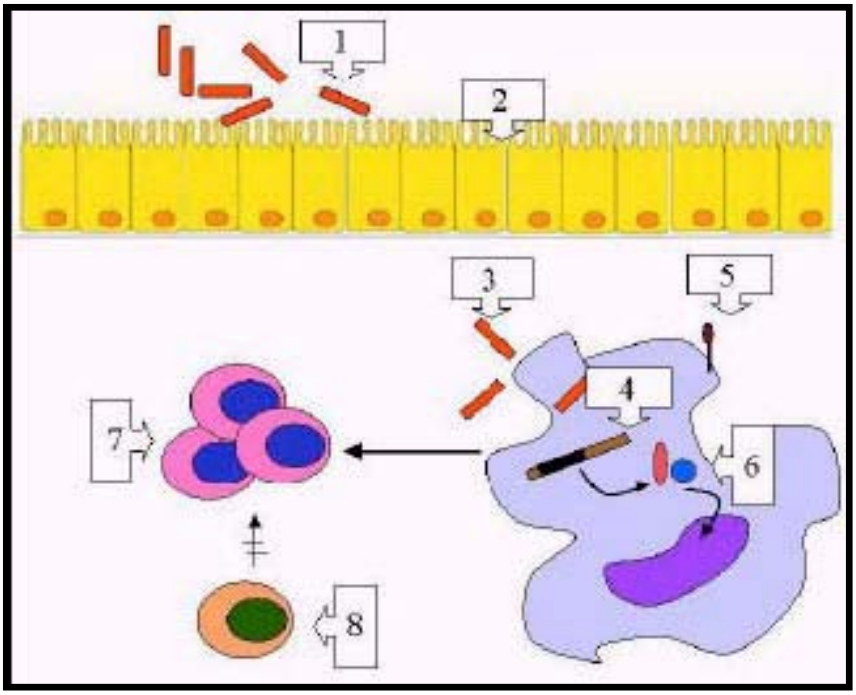

Figura 3. Papel de la microbiota comensal en la Enfermedad Inflamatoria Intestinal. Un desbalance de la microbiota intestinal (1), lleva a una traslocación de bacterias a través del epitelio (2). Así se activaría el sistema inmune de la mucosa (3) y éstas bacterias podrían ingresar en los macrófagos o células dendríticas y unirse a la proteína del gen CARD15/NOD2 (4). También podría activarse el sistema inmune si los receptores de las bacterias en la membrana celular tales como el TLR-4 (receptor tipo *toll*) estuvieran mutados (5). También existe otra posibilidad que es la mutación del gen NFkβ (6). Como resultado de estos hechos la expresión de las moléculas coestimulatorias, que normalmente se encuentran inhibidas, inducen la proliferación de células T (7) que mediante la secreción de citocinas estimulatorias de respuestas de tipo Th1 tales como IL-12, IL-23 o IL-18. Una actividad insuficiente de células T regulatorias (8) podría contribuir a una actividad de tipo Th1 prolongada que provocaría un cuadro de inflamación crónica. Adaptado de (Duchman *et al*, 1995).

Según algunos estudios, los componentes de la microbiota comensal son necesarios para desarrollar una inflamación intestinal crónica. Por ejemplo, se ha reportado que la ausencia de una inflamación intestinal en animales libres de gérmenes se debe a la falta de bacterias comensales, (Sartor, 1997ª; Matsumoto *et al.*, 1998; Veltkamp *et al.*, 2001; Strober *et al.*, 2002;

Bouma and Strober, 2003). Sin embargo, otros autores demuestran que la microbiota comensal, por ejemplo, no es esencial para la inducción de íleitis, ya que ratones libres de gérmenes, desarrollan una íleitis crónica; aunque significativamente atenuada con respecto a ratones SPF (Bamias *et al.*, 2007).

Por otro lado, pacientes con la Enfermedad Inflamatoria Intestinal tienen alterada la composición de las bacterias comensales; así tienen un incremento de *Bacteroides*, *E.coli* adherente/invasiva, Enterococos, y una disminución de *Bifidobacterium* y *Lactocillus* (Neut *et al.*, 2002; Swidsinski *et al.*, 2005[b]), Y en modelos experimentales de colitis e íleitis, también se incrementan *Bacteroides*, Enterococos, *Escherichia coli*, y *Clostridium* (Konaka *et al.*, 1999; Basivireddy *et al.*, 2005; Swidsinski *et al.*, 2005[a]; Dalby *et al.*, 2006; Larrosa *et al.*, 2009; Natividad *et al.*, 2009; Watanabe *et al.*, 2009).

A pesar de los estudios contradictorios en modelos experimentales, es importante mencionar que la mayoría de los estudios, tanto en pacientes como en animales sugieren que las bacterias intestinales comensales, estimulan una respuesta inmune anómala en la Enfermedad Inflamatoria Intestinal.

3.2 Modelos de inflamación intestinal

Durante la última década ha habido una proliferación de modelos experimentales de inflamación intestinal, la mayoría en ratón. El conocimiento adquirido a través de estos modelos nos ha permitido comprender aspectos relacionados con la aparición del brote inflamatorio y con el curso de la patología humana. Además, la mayoría de los modelos experimentales de inflamación intestinal permiten hacer aproximaciones de nuevas estrategias terapéuticas en fase pre-clínica (Mañé, 2007).

Los modelos experimentales de inflamación intestinal pueden clasificarse en inducidos y espontáneos. El modelo utilizado en este trabajo de

investigación es de tipo inducido, a partir de un agente exógeno. Otros modelos inducidos de la inflamación intestinal se resumen en la Tabla 1.

Tabla 1. Modelos experimentales inducidos de la Enfermedad Inflamatoria Intestinal (Panés, *et al*., 2002).

a) Con agentes exógenos
Vía anal con enema (Ácido acético, Ácido tri-nitrobencénico (TNBS), Formol/complejos inmunes, Oxazolona).
Vía oral (Indometacina, Carragenina, Sulfato de sodio dextrano (DSS)
Vía subcutánea (Ciclosporina A, Indometacina)
Vía intracolónica (PG/PS)
b) Por manipulación genética: animales *Knock out*
Knock out para citocinas (IL-2 -/-; IL-10 -/-; CRFB4 -/-; TNF ΔARE; Transgénico Stat4)
Knock out para la función de células T (Gai2 -/-; TCRa -/-; TCRb -/-; Transgénico HLA-B27).
c) Por transferencia de células a animales inmunodeficientes
CD45RB se transfiere a ratones SCID o ratones con deficiencia en el gen activador de la recombinación (Rag -/-)
Transplante de médula ósea a ratones Tgε26

La Indometacina puede ser administrada a los animales de forma oral o subcutánea, para inducir una inflamación intestinal. El mecanismo patogénico se basa en la inhibición de la síntesis de prostaglandinas (Whittle, 1981), lo que causa alteraciones, de tipo vascular, que junto a la presencia de bacterias en la luz intestinal (Satoh *et al.*, 1983) provoca una alteración en la permeabilidad de la mucosa (Jeffers *et al.*, 2002), confiriéndole una menor resistencia a la penetración bacteriana, y a la entrada de otros agentes luminales (Bjarnason *et al.*, 1990). Las bacterias tienen gran influencia sobre la inflamación en este modelo, ya que se ha demostrado que los animales libres de gérmenes no muestran un daño severo en el intestino delgado por la administración de Indometacina (Satoh *et al.*, 1983).

3.3 Hipótesis de la Higiene

En 1989 Strachan fue el primero que utilizó el término de "hipótesis de la higiene" (Strachan, 1989). De acuerdo con esta hipótesis, el cambio de las condiciones higiénicas en las sociedades occidentalizadas es una de las causas del incremento en la incidencia de enfermedades con una base inmunitaria, tales como eczemas, asma, rinitis, alergias y la Enfermedad Inflamatoria Intestinal (Fiocchi, 2005).

La Enfermedad Inflamatoria Intestinal podría explicarse por una disminución de la carga microbiana, asociada a unas condiciones higiénicas extremas en los primeros meses de vida. Hay evidencias que sugieren que la exposición a microorganismos no patógenos, incluyendo helmintos, transmitidos por los alimentos y por vía orofecal ejercen un impacto en la homeóstasis intestinal (Rook and Brunet, 2005). Se asume que la higiene ha reducido el contacto con patógenos que producen una respuesta Th1. Y como consecuencia, podría ocasionar un incremento compensatorio en la respuesta Th2, la cual es característica de los desordenes observados en las alergias y posiblemente también en la Enfermedad Inflamatoria Intestinal (Stene and Nafstad, 2001; Hooper, 2004; Czeresnia, 2005; Guarner *et al.*, 2006).

3.3.1 La Hipótesis de la higiene en modelos experimentales

El control de la microbiota intestinal en animales SPF y *Germ-free* (animales libre de gérmenes), nos permite estudiar el efecto de la higiene en la patogenia de procesos con una base inmune, como parece ser el caso de la Enfermedad Inflamatoria Intestinal. Sin embargo, se debe tener en cuenta que en la especie humana el efecto de la higiene, aunque ésta sea extrema, no es comparable a la de los roedores SPF y animales libres de gérmenes que se encuentran bajo condiciones higiénicas muy estrictas (Macpherson and Harris, 2004).

Precisamente, en un modelo de ratón con autoinmunidad espontánea mantenido en condiciones ambientales *Germ-free* o convencionales, se ha

demostrado que su microbiota intestinal no es un factor desencadenante de la nefritis en esta cepa de roedores (Maldonado *et al.*, 1999).
En cuanto al efecto de las condiciones higiénicas en la Enfermedad Inflamatoria Intestinal existen escasos estudios. Mañé *et al*., (2207) recientemente ha demostrado el efecto del ambiente sobre la inflamación intestinal en un modelo de ratones deficientes en IL-10. Cuando éstos animales se mantienen en condiciones convencionales desarrollan un proceso inflamatorio más grave que en condiciones SPF (Mañé, 2007).

Sin embargo, a pesar de lo expuesto anteriormente, aún no hay un modelo experimental que nos permita confirmar y estudiar la hipótesis de la higiene en la Enfermedad Inflamatoria Intestinal.

4 Microbiota intestinal y Sistema Inmune

4.1 Sistema inmune innato y microbiota intestinal

Las bacterias desempeñan también un papel esencial en el desarrollo del sistema inmunitario. El tracto intestinal constituye una interfase muy sensible para el contacto y comunicación entre el individuo y el medio externo. Para una perfecta homeóstasis del sistema tiene que distinguirse claramente entre microbios patógenos, patógenos potenciales y microbios comensales (Sartor, 1997; Macpherson and Harris, 2004; Macpherson and Uhr, 2004).

Cuando el microorganismo es patógeno o potencialmente patógeno, el huésped debe dotarse de elementos de defensa adecuados, mientras que si se trata de bacterias comensales, el huésped tiene que saber tolerarlas para obtener el beneficio de la simbiosis. Las interacciones entre los microorganismos, el epitelio y los tejidos linfoides son múltiples, continuas, y dinámicas de modo que hay una remodelación constante de los mecanismos locales y sistémicos de la inmunidad adaptándolos al ambiente microbiano (Fagarasan *et al.*, 2002).

Para verificar la interacción entre la microbiota intestinal y el sistema inmune se han utilizado animales libres de gérmenes. Éstos animales tienen un número reducido de placas de Peyer y una menor producción de IgA por las células B comparada con un animal convencional (Macpherson and Uhr, 2004). También se ha detectado que los linfonodos, como el bazo, y otros tejidos linfoides tienen un número menor de centros germinales en animales libres de patógenos comparados con animales convencionales. Otra peculiaridad es en animales libres de gérmenes el timo crece más lentamente y que nunca llega a alcanzar el tamaño que se encuentra en animales convencionales (Bealmear and Wilson, 1967).

Se ha demostrado que los animales libres de gérmenes tienen una baja concentración de células linfoides en la mucosa del intestino delgado, la estructura de los folículos linfoides esta atrofiada y la concentración de inmunoglobulinas circulantes son anormalmente bajas. Sin embargo; inmediatamente después de la exposición de los animales libres de gérmenes a la microbiota convencional aumenta el número de linfocitos en la mucosa, los centros germinales crecen en número y tamaño, y hay una aparición de células productoras de inmunoglobulinas en los folículos linfoides y la lámina propia (Yamanaka *et al.*, 2003; Helgeland *et al.*, 2004).

La microbiota intestinal comensal es importante en la defensa contra los microorganismos. Esta se produce a través de las primeras reacciones correspondientes a la inmunidad innata y las posteriores respuestas a cargo de la inmunidad adaptativa.

4.1.1 Inmunidad Innata

La inmunidad innata (también llamada inmunidad natural o espontánea) es la primera línea de defensa frente a los microbios (Figura 4). Está constituida por unos mecanismos de defensa celulares y bioquímicos ya instaurados antes de contraerse una infección y preparados para responder con rapidez una vez producida ésta. Estos mecanismos sólo reaccionan ante los microbios (y sustancias derivadas de células dañadas), y responden básicamente de la misma manera cada vez que se repite una infección.

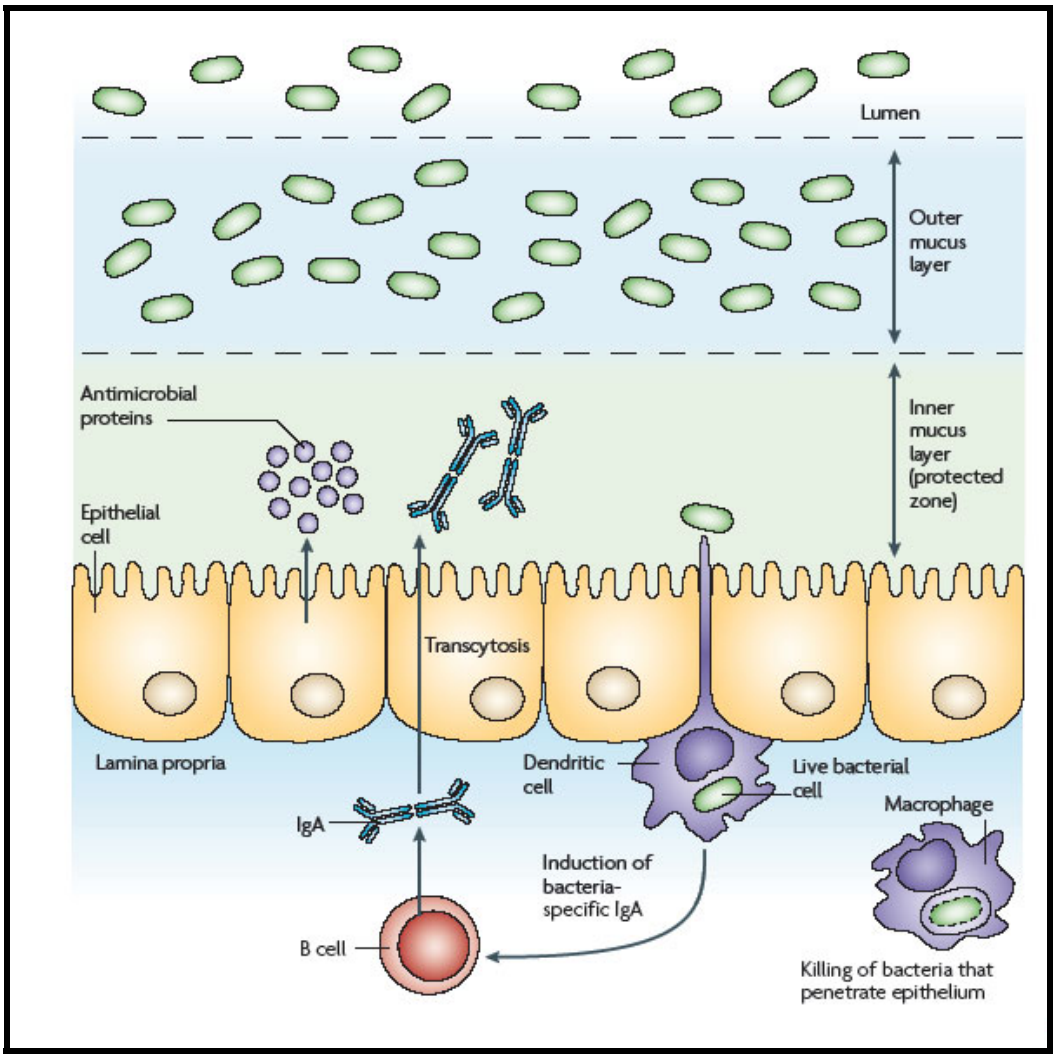

Figura 4. La inmunidad innata frente a los microorganismos, está formada por tres líneas de defensa. La primera barrera de defensa es la capa de la mucosa, la segunda son las proteínas antimicrobianas secretadas por las células epiteliales intestinales, la tercera es la IgA producida por células B contra las bacterias comensales. Las células dendriticas participan en la inducción de IgA a través de las células B, y los macrófagos son las células que eliminan a las bacterias que han penetrado en el epitelio intestinal. Adaptado de (Hooper, 2009).

Los principales componentes de la inmunidad innata son: 1) barreras físicas y químicas, como los epitelios y las sustancias antimicrobianas formadas en sus superficies; 2) células fagocíticas (neutrófilos, macrófagos) y linfocitos citolíticos naturales (NK); 3) proteínas sanguíneas, como los factores del sistema del complemento y otros mediadores de la inflamación; y 4) unas proteínas denominadas citocinas, que regulan y coordinan muchas de las actividades de las células encargadas de la inmunidad innata (Figura 5). Los mecanismos de la inmunidad innata son específicos para aquellas

estructuras comunes a los grupos de microbios afines y no tienen porque distinguir diferencias sutiles entre sustancias ajenas (Abbas *et al*. 2008).

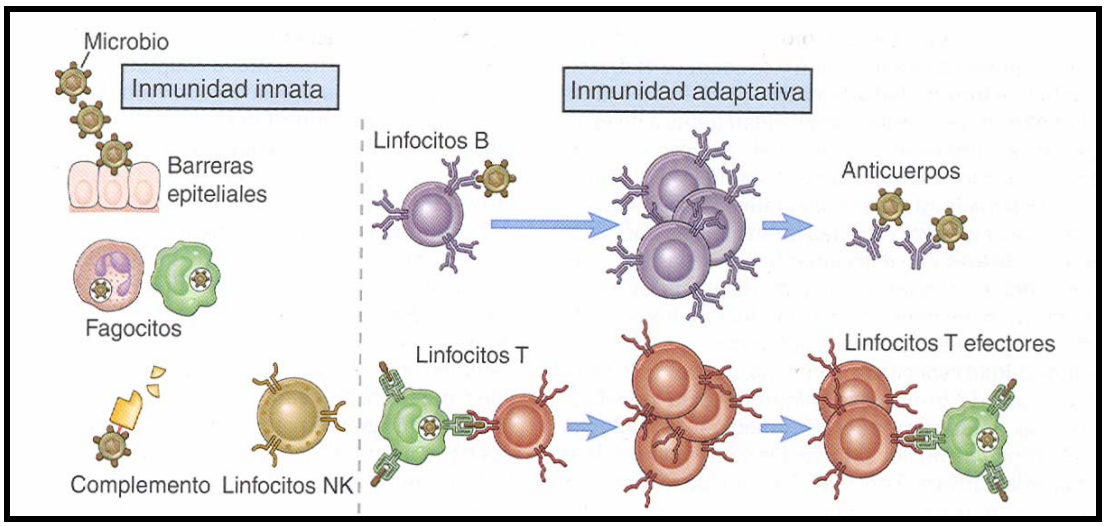

Figura 5. Componentes celulares de la inmunidad innata y de la inmunidad adaptativa (Adaptado de Abbas, 2008).

La inmunidad innata sirve para reconocer la microbiota, por ejemplo, mediante receptores para el reconocimiento de patrones asociados a las células, que se encuentran presentes sobre la superficie celular, en las vesículas endosómicas y el citoplasma. Una amplia gama de células expresan éstos receptores, entre ellas, se encuentran neutrófilos, macrófagos, células dendríticas, células endoteliales, células epiteliales, y linfocitos (Abbas *et al*. 2008).

4.1.2 *Toll like receptors* ("TLR": Receptores tipo *toll*)

Los *Toll Like Receptors* (TLR) forman una familia de receptores que reconocen bacterias patógenas, a través de patrones moleculares patógeno-específicos (Pathogen-specific molecular patterns", PAMPs), que ocupan un lugar esencial dentro de las respuestas inmunitarias innatas frente a los microbios. En un principio, el TLR se identificó a partir del gen de *Drosophila* que participa durante la embriogenia de la mosca en el establecimiento de su eje dorsoventral (Rock *et al.*, 1998), pero más tarde se descubrió que la

proteína *toll* también intervenía en las respuestas antimicrobianas. La mayoría de los TLRs han sido reportados en varias especies; por ejemplo los humanos tienen 11 TLRs diferentes **(Figura 6)** (Harris *et al*, 2006), (nombrados como TLR 1 – 11), el ratón tiene 13 TLRs (nombrados como TLR 1 – 13) (Kawai and Akira, 2006), en la rata están descritos casi los mismos TLRs que en el ratón; sin embargo los TLRs más estudiados en modelos de inflamación intestinal son el TLR-2 y el TLR-4 (Fujisawa *et al.*, 2006; Silva *et al.*, 2008; Sukhotnik *et al.*, 2008; Bai *et al.*, 2009; Biswas *et al.*, 2009; Liu *et al.*, 2009; Watanabe *et al.*, 2009).

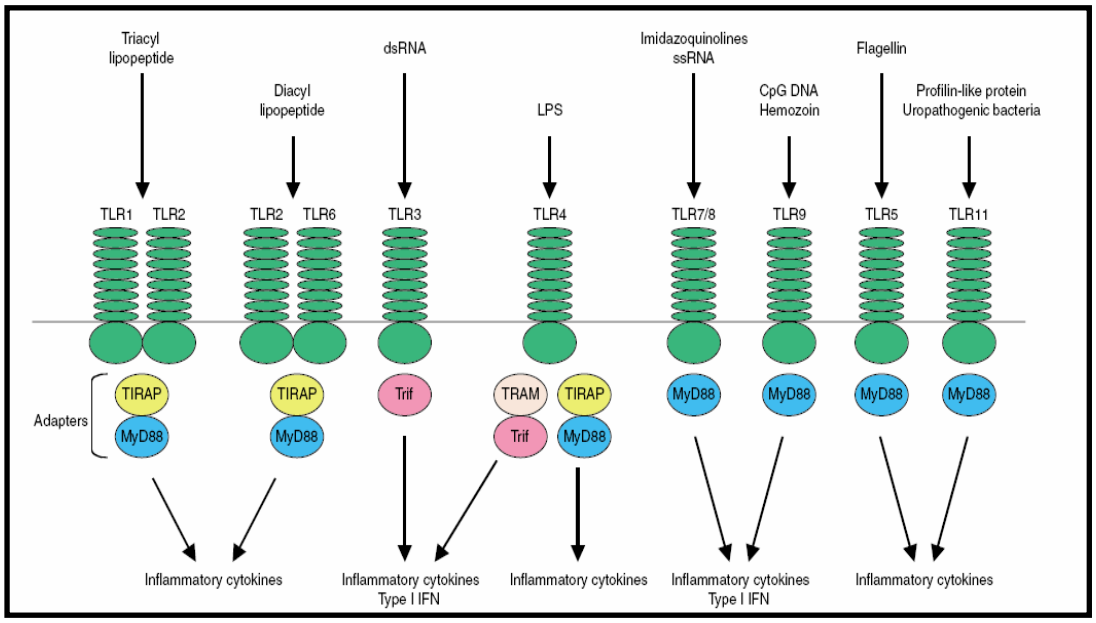

Figure 6. Muestra, para la especie humana, los diferentes Receptores tipo *toll* con sus ligandos junto a los dímeros que se unen a ellos. (Adaptada de Kawai and Akira, 2006).

Los TLRs son glucoproteínas integrales de membrana del tipo I que contienen repeticiones cargadas de leucina flanqueadas en sus regiones extracelulares por unas secuencias características con abundante cisteína y por un dominio llamado *toll:* IL-1 (TIR) que resulta fundamental para la transmisión de las señales **(Figura 7)**. Los principales tipos celulares donde se expresan los TLRs son los macrófagos, las células dendríticas, los neutrófilos, las células epiteliales de la mucosa y las células endoteliales (Takeda *et al.*, 2003; Akira, 2004).

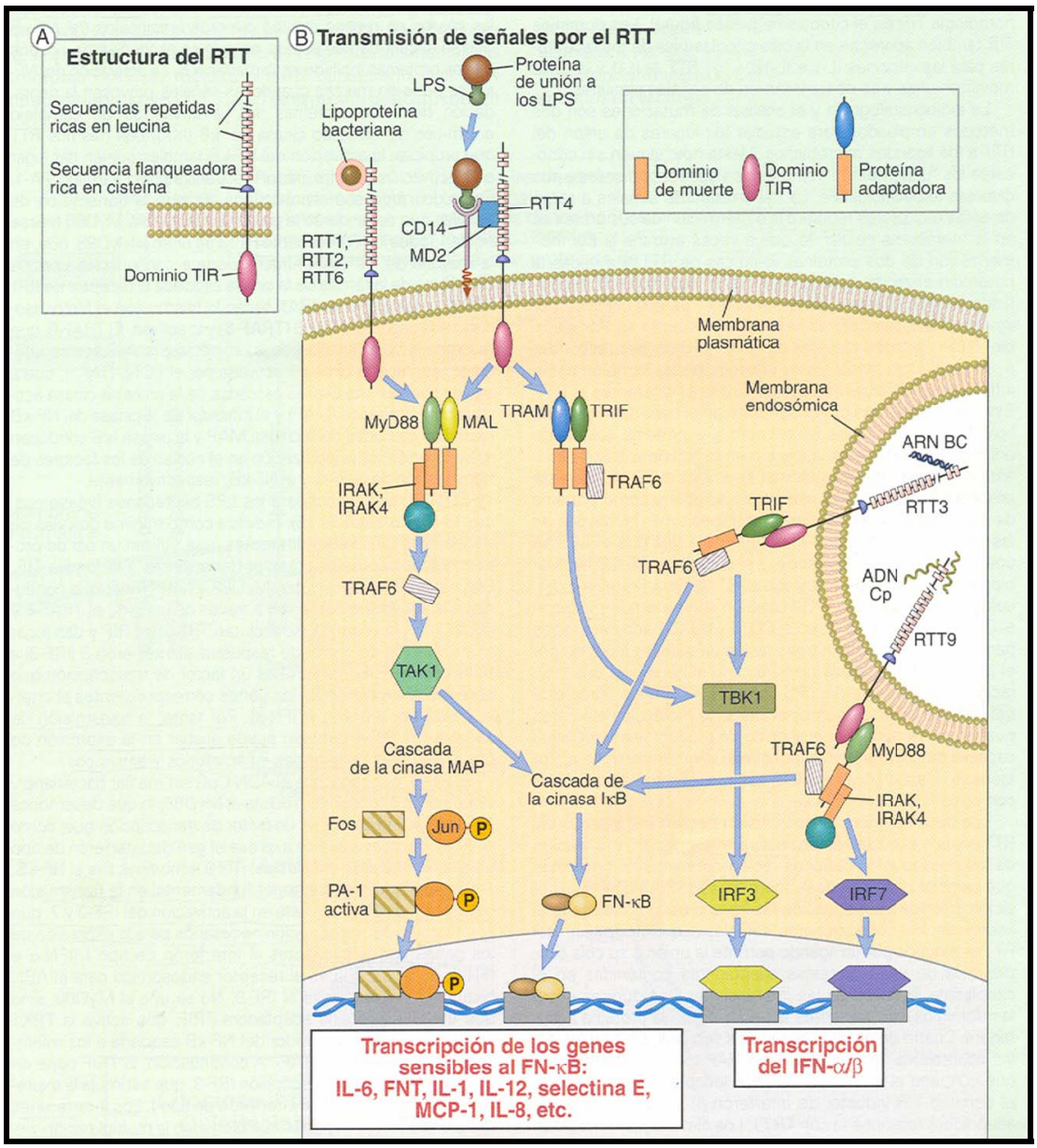

Figura 7. A) Estructura del Receptor tipo *toll* (TLR). B) Vías de transmisión de señales del TLR. (Adaptado de Abbas, 2008).

La localización de los TLRs es múltiple, varía según el tipo de células. Así, los TLRs están presentes en la superficie celular y sobre las membranas intracelulares. Esta localización le permite activarse y reconocer productos de los microbios en diversos puntos de la célula. Por ejemplo, entre los productos microbianos capaces de activar los TLRs figuran los LPS de las bacterias gramnegativas que activan el TLR-4 (Takeuchi *et al.*, 1999; Beutler,

2000; Takeda *et al.*, 2003), y los peptidoglucanos, el ácido lipoteicoico de las bacterias grampositivas que estimulan el TLR-2 (Takeuchi *et al.*, 1999; Takeda *et al.*, 2003). A parte de éstos componentes de las bacterias, también está demostrado que otros ligandos pueden activar otros TLRs, tal como se muestra en la Tabla 2 y las Figuras 6 y 7 (Kawai and Akira, 2006).

Tabla 2. TLRs y sus principales ligandos microbianos (Adaptado de Harris *et al.*, 2006).

TLRs	Ligandos microbianos
	Ligandos para lípidos
TLR1	Triacil lipopéptidos (bacterias, micobacterias)
TLR2	Lipoproteínas/lipopéptidos (una variedad de patógenos) Peptidoglicano (bacterias grampositivas) Ácido lipoteicoico (bacterias grampositivas) Lipoarabinomanano (micobacterias) Zymosan (hongos)
TLR4	Lipopolisacáridos (LPS) (bacterias gramnegativas) Proteínas de fusión (virus sincicial respiratrio) Envoltura de las proteínas (tumor mamario de virus en ratones) HSP60 (Chlamydia pneumoniae)
TLR6	Diacil lipopéptidos (micoplasmas)
	Ligandos para ácidos nucleícos
TLR3	ARN de virus
TLR7-TLR8	Uracilo de ARN
TLR9	CpG ADN de bacterias
	Ligando de proteína
TLR5	Flagelina de bacterias
	Bacterias uropatogénicas
TLR11	Bacterias uropatogénicas
	Ligando desconocido
TLR10	???????

4.2 Regulación de los TLRs en el tracto gastrointestinal

La expresión y activación de los TLRs está especialmente regulada en el tracto gastrointestinal probablemente debido a la continua presencia de microorganismos en el intestino. Es esencial que los TLRs no reaccionen con componentes de las bacterias comensales. Sin embargo, éstos deben tener la habilidad de reconocer la microbiota comensal y desencadenar una respuesta inmune efectiva contra patógenos invasores (Abreu *et al.*, 2001).

Existen diferentes estrategias para regular los TLRs en presencia de bacterias comensales. Una de ellas, por ejemplo, es que el TLR-4 intracelular conserva toda su capacidad de señalización, detecta LPS internalizados y LPS intracelulares de las bacterias gramnegativas (Hornef *et al.*, 2002; 2003). Este mecanismo permite al huésped detectar microorganismos patogénicos que han penetrado en el epitelio intestinal, sin necesidad de sobreexpresarse en la superficie del epitelio intestinal.

Hay autores que sugieren la posibilidad de que, en condiciones normales, el TLR-5 se expresa en la membrana basolateral de las células epiteliales intestinales. Por tanto, este receptor puede ser importante para el mantenimiento de la homeóstasis intestinal asociada a bacterias comensales flageladas que generalmente no han traslocado la membrana basolateral (Gewirtz *et al.*, 2001).

Está demostrado que en las criptas de las células epiteliales se expresa el TLR-2 y TLR-4. En esta zona de la mucosa no hay un contacto directo con bacterias comensales, por tanto la expresión de TLR-2 y TLR-4 en estas zonas solamente reconocería bacterias que han penetrado en las criptas. La expresión del TLR-3 en el lumen intestinal es rara, porque los ligandos víricos no están presentes de forma natural en el lumen intestinal, por tanto, normalmente no debería expresarse el TLR-3 (Furrie *et al.*, 2005).

Otra estrategia en la regulación de actividades de los TLRs en la mucosa gastrointestinal es a través de la alta expresión de antagonistas (proteínas), como son: PPAR-gama, A 20, NOD2, IRAK-M, SIGIRR, TOLLIP. Su función es controlar la activación de los TLRs en el epitelio intestinal, mediante un mecanismo regulatorio negativo para el mantenimiento del balance inmunológico. Se ha demostrado que algunas de estas proteínas, disminuyen la expresión de los TLRs en las células epiteliales intestinales. También se ha observado en algunos casos en la inflamación (Liew *et al.*, 2005[b]; Shibolet and Podolsky, 2007).

Se ha estudiado que la expresión del TLR-4 disminuye en presencia de LPS hipoacetilado comparado con LPS normalmente acetilado. Por tanto, las bacterias comensales con LPS hipoacetilado pueden contribuir a la disminución de la expresión del TLR-4, y de esta manera modular la expresión bajo condiciones fisiológicas (Backhed *et al.*, 2003).

4.2.1 TLRs en la Enfermedad Inflamatoria Intestinal

Estudios experimentales y clínicos sugieren que la sobre-expresión de ciertos TLRs y la reducción en la expresión de antagonistas de los TLRs en las células epiteliales intestinales pueden ser parte de los mecanismos patogénicos implicados en las respuestas de la microbiota comensal en la Enfermedad Inflamatoria Intestinal.

En los pacientes con EC o CU podrían estar implicados tanto el TLR-2 como el TLR-4 (Cario and Podolsky, 2000; Szebeni *et al.*, 2008). La presencia de altas concentraciones de anticuerpos específicos para flagelos en el suero de pacientes con EC, hace pensar en la posibilidad de que los flagelos de bacterias comensales podrían iniciar una respuesta inmune inapropiada en la mucosa del tracto gastrointestinal a través del TLR-5 y que este receptor también podría estar implicado en la Enfermedad Inflamatoria Intestinal (Lodes *et al.*, 2004; Sitaraman *et al.*, 2005).

La mayoría de estudios con TLRs en la inflamación intestinal se han hecho *in vitro* y han demostrado que la expresión del TLR-2 y TLR-4 esta incrementada durante la inflamación (Cario and Podolsky, 2000; Sato *et al.*, 2000; Hausmann *et al.*, 2002; Netea *et al.*, 2004; Muller-Decker *et al.*, 2005; Le Mandat *et al.*, 2007; Spiller *et al.*, 2008). De la misma manera, otros autores demuestran que la expresión del TLR-2 y TLR-4 está aumentada en modelos experimentales de inflamación intestinal (Ortega-Cava *et al.*, 2003; Liew *et al.*, 2005ª; Fujisawa *et al.*, 2006; Rakoff-Nahoum *et al.*, 2006; Le Mandat *et al.*, 2007). La mayoría de las investigaciones *in vivo* son realizadas en ratones *knockout* para TLR-2 y TLR-4 (Fukata *et al.*, 2005;

Heimesaat *et al.*, 2007). Por ejemplo, en ratones *knockout* TLR-2 y TLR-4, tratados con DSS para inducir colitis, se demuestra que la inflamación es dependiente del TLR-2 y TLR-4 (Grabig *et al.*, 2006). Estas observaciones sugieren una mayor implicación del TLR-2 y TLR-4 en la inflamación intestinal.

5 Microbiota intestinal y Sistema Nervioso Entérico

Se conoce desde hace siglos que los extractos de *Cannabis sativa* y sus derivados estimulan el apetito, inhiben la émesis, normalizan la motilidad intestinal y mejoran una serie de patologías gastrointestinales. Actualmente se conoce que el tracto gastrointestinal contiene un sistema que sintetiza localmente endocanabinoides que actúan sobre receptores específicos y modulan la actividad motora, secretora y sensorial del tracto gastrointestinal. Sin embargo se conoce poco de la posible interacción entre el sistema canabinoide y la microbiota intestinal (Duncan *et al.*, 2005).

Sin embargo, en humanos, las variaciones en la microbiota comensal del intestino se han asociado con alteraciones motoras y sensoriales y algunos probióticos son utilizados para aliviar el dolor abdominal asociado al síndrome del intestino irritable. Estos datos sugieren que la microbiota comensal del intestino es capaz de modular la actividad de los sistemas sensoriales nociceptivos intestinales, entre los cuales destaca el sistema endocanabinoide.

5.1 Receptores Canabinoides: CB_1 y CB_2

En 1990, el primer receptor canabinoide CB_1, fue clonado por Matsuda *et al.* (1990) y llamado CB_1. Luego se clonó un segundo CB_2, con una homología del 48% con el CB_1 (Howlett, 2002). Se ha sugerido la existencia de un tercer receptor, la proteína GPR_{55} (Pertwee, 2007).

Los receptores CB_1, se expresan mayoritariamente en el sistema nervioso central y periférico, incluyendo el sistema nervioso entérico (Kulkarni-Narla and Brown, 2000; Coutts *et al.*, 2002). Los receptores CB_1 se han localizado,

también en el epitelio y el músculo liso del colón (Casu *et al.*, 2003; Ligresti *et al.*, 2003).
El receptor CB_2 está presente fundamentalmente en las células inmunes y recientemente se ha demostrado su presencia en el sistema nervioso entérico (Duncan *et al.*, 2008; Storr *et al.*, 2009).

En el tracto gastrointestinal los receptores CB_1 median respuestas fundamentalmente inhibitorias, mientras que los receptores CB_2, debidos a su presencia predominante en células inmunes, se asocian a respuestas inflamatorias. Tanto el CB_1 como el CB_2 se han implicado en mecanismos sensoriales en el intestino, concretamente en mecanismos de tipo nociceptivo (Hornby and Prouty, 2004; Di Marzo and Izzo, 2006; Storr and Sharkey, 2007; Storr *et al.*, 2008[a]; Brusberg *et al.*, 2009). Ambos receptores tienen respuestas analgésicas en condiciones basales y durante los estados de inflamación inducen hiperalgesia (Sanson *et al.*, 2006).

Así ambos receptores se han implicado en variaciones de los mecanismos sensoriales que llevan a alteraciones en las respuestas al dolor, concretamente en la generación de estados hiperalgésicos. Estos mecanismos son importantes porque constituyen un componente básico de la fisiopatología de la Enfermedad Inflamatoria Intestinal y del Síndrome del Intestino Irritable (Massa *et al.*, 2005; Storr *et al.*, 2008[b]; Brusberg *et al.*, 2009).

5.2 Interacción entre receptores canabinoides y microbiota intestinal

Estudios recientes en modelos animales sugieren que las modificaciones de la microbiota intestinal comensal pueden resultar en cambios estructurales y moleculares de los sistemas de control de las funciones gastrointestinales, incluyendo respuestas nociceptivas en ratas y ratones (It-Belgnaoui *et al.*, 2006; Verdú *et al.*, 2006; Rousseaux *et al.*, 2007; Amaral *et al.*, 2008). Por ejemplo, el tratamiento con antibióticos en ratones resulta en modificaciones en los niveles de la sustancia P (mediador nervioso implicado en respuestas secretomotoras y sensoriales en el intestino) y en alteraciones de las

respuestas de dolor (Verdú *et al.*, 2006). De la misma manera, la administración de lactobacilos incrementó la expresión de receptores μ opiodes y canabinoides de tipo 2 (CB_2) y modificó las respuestas nociceptivas en ratas (Eutamene *et al.*, 2007; Rousseaux *et al.*, 2007). Confirmando al menos a nivel experimental, una posible interacción entre la microbiota intestinal comensal y el sistema endocanabinoide.

6 Hipótesis y Objetivos

De lo expuesto anteriormente se desprende que la microbiota intestinal comensal del intestino debe considerarse como un componente activo de la homeostasis intestinal y, como consecuencia, un factor a tener en consideración en estados fisiopatológicos. En particular, la microbiota intestinal comensal se ha implicado en la fisiopatología de alteraciones inflamatorias y funcionales del intestino, como son la Enfermedad Inflamatoria Intestinal y el Síndrome de Intestino Irritable. Esto ha hecho que, durante los últimos años, haya habido un interés creciente en el estudio de la microbiota comensal intestinal, tanto por su posible implicación causal en estados fisiopatológicos como por su potencial utilidad terapéutica para restaurar la homeostasis intestinal. Sin embargo, a pesar de este interés, los mecanismos por los cuales la microbiota comensal del intestino ejerce estos efectos no se conocen con precisión.

Por tanto, este trabajo de investigación parte de la **HIPÓTESIS** de que las características de la microbiota comensal del intestino en diferentes condiciones ambientales, afectan la homeostasis intestinal modificando las respuestas inflamatorias intestinales, y los mecanismos sensoriales nociceptivos intestinales.

Para demostrar esta hipótesis se ha trabajado con animales mantenidos en diferentes condiciones ambientales, caracterizándose tanto la microbiota comensal intestinal como variaciones adaptativas funcionales y moleculares. Para ello, se han planteado los siguientes **OBJETIVOS** específicos de trabajo:

- Caracterizar cambios espontáneos de la microbiota comensal del intestino en ratas criadas en diferentes condiciones ambientales, equivalentes a las empleadas habitualmente en experimentación animal: cría en condiciones estrictas de higiene (condiciones de barrera), cría en condiciones estándar de higiene (condiciones convencionales), y cría en condiciones estrictas de higiene con una adaptación a un ambiente convencional (condiciones barrera/convencionales),

- Demostrar *in vivo* como variaciones espontáneas de la microbiota intestinal comensal, según los patrones de cría descritos anteriormente, afectan la expresión intestinal del sistema de reconocimiento bacteriano dependiente de TLR-2 y el TLR-4.

- Valorar la susceptibilidad a la inflamación, empleando un modelo de enteritis inducida por Indometacina, en ratas criadas en condiciones estándar de higiene (condiciones convencionales), y condiciones estrictas de higiene adaptadas a un ambiente convencional (condiciones barrera/convencionales).

- Demostrar si los cambios espontáneos de la microbiota intestinal comensal afectan a los mecanismos sensoriales intestinales. En particular, se pretende demostrar si cambios de la microbiota afectan la expresión intestinal de receptores canabinoides (CB_1 y CB_2), como representantes significativos de los mecanismos sensoriales nociceptivos viscerales.

- Correlacionar la expresión de los receptores canabinoides CB_1 y CB_2, con la microbiota comensal del intestino en ratas criadas en condiciones estrictas de higiene (condiciones de barrera), en condiciones estándar de higiene (condiciones convencionales) y en condiciones estrictas de higiene adaptadas a un ambiente convencional (condiciones barrera/convencionales).

Capítulo 1

Characterization of Housing-related Spontaneous Variations of Gut Microbiota and Expression of Toll Like Receptors 2 and 4 in rats

Abstract

Gut microbiota has been suggested as a key component of gut homeostasis, affecting immune responses within the gut. We determined changes in intestinal commensal bacteria and expression of toll-like receptors (TLR) 2 and 4 in rats bred under microbiologically-controlled conditions (barrier), under standard conditions (conventional) and in barrier animals adapted to standard conditions (barrier/conventional). In these groups cecal microbiota was analyzed by fluorescence *in situ* hybridization and microbial profiles were assessed by terminal restriction fragment length polymorphism. Cecal expression of TLR-2 and TLR-4 was determined by RT-PCR.

Total number of cecal bacteria was similar in the three groups. However, the barrier group showed a higher number of strict anaerobic bacteria (*Bacteroides* spp and *Clostridium* spp) while *Bifidobacterium* spp were scarce. Re-housing the barrier-bred rats into conventional conditions led to a microbiota with intermediate characteristics between the barrier and the conventional groups. Richness of the cecal microbial ecosystem was similar in the three groups, although a relative time-dependent variation, with highest homogeneity in the barrier group, was observed. Expression levels of TLR-2 and TLR-4 were low, without clear correlation with the microbiota. Only TLR-4 showed a tendency to be over-expressed in the barrier group. These results show that the relative composition of the cecal microbiota in rats varies spontaneously with changes in the environmental conditions, with minor impact in the expression of TLR-2 and TLR-4. These observations might be important in the understanding of variability in animal responses, particularly to immune-related stimuli, when assessed in the context of the environmental/microbiological conditions.

Introduction

The intestinal tract harbours a complex bacterial ecosystem, which has not yet been fully characterized (Berg, 1996). It contains numerous species, most of them anaerobes, and the concentration can be as great as 1×10^{11} bacteria/g of feces (Montesi *et al.*, 2005). The presence of these commensal bacteria appears to be of crucial importance in the pathogenesis of several gastrointestinal alterations, such as inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS) (Swidsinski *et al.*, 2005[b]; Xavier and Podolsky, 2007; Collins and Bercik, 2009). In general, lactic acid bacteria are regarded as beneficial members modulating gut immune responses, on the other hand *Bacteroides*, *Clostridium* and *Enterobacteriaceae,* have been associated with the exacerbation of intestinal inflammatory responses in humans and experimental models (Setoyama *et al.*, 2003; Montesi *et al.*, 2005; Swidsinski *et al.*, 2005[a], 2005[b]; Rolhion and Rfeuille-Michaud, 2007).

Gut microbiota represents a dynamic system in continuous interaction with the host. Mammalian toll-like receptors (TLRs) play a central role in the recognition of gut microbiota and the initiation of immune responses to microbial pathogens. Among TLRs, TLR-2 and TLR-4 are considered key players in the recognition of gut microbiota. TLR-2 recognizes a variety of microbial components from gram positive bacteria, whereas TLR-4 recognizes LPS of gram-negative bacteria (Takeuchi *et al.*, 1999; Cario, 2005). It is not fully understood how TLRs distinguish between commensal and pathogen bacteria when they share the same identification groups (Rakoff-Nahoum *et al.*, 2004). However, an increase of TLRs expression has been observed after exposure to commensal bacteria, a crucial response in the maintenance of gut homeostasis and in the prevention of gastrointestinal pathophysiological alterations (Rakoff-Nahoum *et al.*, 2004).

For instance, an increase of IBD incidence in industrialized countries has been related to the improvement in standards of hygiene. Highly hygienic conditions induce changes in the composition of commensal intestinal microbiota, leading to the appearance of abnormal immune responses,

including an altered recognition of the commensal microbiota that seems to characterize IBD (Strachan, 1989; Guarner *et al.*, 2006). This agrees with the presence of changes in TLR-2 and TLR-4 expression, as described both in IBD patients and rodent models of gut inflammation (Fujisawa *et al.*, 2006; Silva *et al.*, 2008; Szebeni *et al.*, 2008). Although systematic studies have not been performed, this suggests that spontaneous variations in gut microbiota might influence the susceptibility to and course of intestinal inflammation (Hoentjen *et al.*, 2005). In the present study we characterized, for the first time, the naturally developed indigenous intestinal (cecal) microbiota in rats born and bred under barrier conditions, with a highly controlled environment and a well characterized intestinal flora at the time of inoculation, and in rats with the same genetic background but born and bred in standard (conventional) conditions. Furthermore, we also characterized spontaneous changes of gut microbiota present in rats born and bred under barrier conditions but adapted for a period of three weeks, during adulthood, to conventional conditions. Finally, we assessed changes in TLR-2 and TLR-4 expression, as two of the main components of the microbiota recognition system, in the same animals, aiming to establish a correlation between TLR expression and gut microbiota.

Materials and Methods

Animals

Twelve 6 week old and twelve 9 week old male OFA Sprague-Dawley rats bred and maintained in a barrier protected area with all materials, water, food and bedding sterilized before entering the barrier were provided by Charles River Laboratories (Lyon, France). The original microbiota inoculated when the barrier colony was established is summarized in Table 1, as provided by the breeder. Transport of barrier bred animals was made in filtered boxes to guarantee maintenance of their microbiological status.

In addition, sixteen 9 week old male OFA Sprague-Dawley rats bred in conventional conditions in the Animal Facility of the Universidad Autónoma de Barcelona were used. This conventional colony was established in 1994 from OFA Sprague Dawley rats from Charles River Laboratories. When in conventional conditions, water, food and bedding were given to animals as facilitated by the commercial provider, without any further treatment. All animals maintained at the university's animal facility were housed in standard plastic cages with stainless steel grid roofs in an environmentally controlled room (20-21ºC, 40-70% humidity, 12 hours light /dark cycle), and received a commercial pellet diet (15.4%, protein, 2.9% fat and 3.9% fibre; SASE, Panlab S.L., Barcelona, Spain) and tap water *ad libitum*. Cages and wood shavings used for bedding (Ultrasorb, Panlab S.L., Barcelona, Spain) were changed once a week. All animals were 9 week old at the time of testing.
All procedures were approved by the Ethical Committee of the Universitat Autónoma de Barcelona.

Experimental groups and time course of the studies

The following experimental groups were defined (n=12-16 for each): 1) 9 week old barrier bred rats tested immediately on arrival (barrier group); 2) 9 week old rats bred under conventional conditions (conventional group); and 3) 6 week old barrier rats maintained under conventional conditions at the animal facility of the Universidad Autónoma de Barcelona for 3 weeks

(barrier/conventional group). All procedures were performed between June 2005 and October 2008. In each experiment, at least two of the three experimental groups were included and all experimental groups were repeated at least three times during the course of the studies. For the sake of clarity, and taking into account the time course of the studies, for some of the results presented the experimental groups have been regrouped as two main subgroups, 2005-6 and 2007-8.

Samples collection

Rats were euthanized by CO_2 inhalation. Immediately, a medial laparotomy was performed, the cecum localized and exteriorized and samples of cecal content (about 0.5 g) and cecal tissue were obtained under sterile conditions and immediately frozen with liquid nitrogen. All samples were stored at -20 ºC (cecal content) or -80 ºC (tissue) until analysis.

Enumeration of bacteria using fluorescence *in situ* hybridization (FISH)

For FISH, oligonucleotide probes consisted of a single strain DNA covalently linked with Cy3 at the 5'-end. Probes used were: EUB 338 (5'GCTGCCTCCCGTAGGAGT3') to total Bacteria (Amann *et al.*, 1990); NON 338 (5'ACATCCTACGGGAGGC3') to Non bacteria (negative control) (Amann *et al.*, 1990); BAC 303 (5'CAATGTGGGGGACCTT3') to *Bacteroides* spp (Salzman *et al.*, 2002); EREC 482 (5'GCTTCTTAGTCAGGTACCG3') to Clostridium Cluster XIVa (Salzman *et al.*, 2002); LAB 158 (5'GGTATTAGCACCTGTTTCCA3') to *Lactobacillus* spp and *Enterococcus* spp (Salzman *et al.*, 2002); ENT-D (5'TGCTCTCGCGAGGTCGCTTCTCTT3') to enterobacteria (Ootsubo *et al.*, 2002); and BIF 164 (5'CATCCGGCATTACCACCC3') to *Bifidobacterium* spp (Vaahtovuo *et al.*, 2005). All probes were obtained from Tib MolBiol (Mannheim, Germany).

Frozen cecal contents were thawed and 4.5 ml of Millipore filtered PBS and 3-5 glassbeads (diameter 3mm) were added and the mixture homogenised on a vortex for 3 min. The suspension obtained was then centrifuged for 1 min at 700 g. From the supernatant, a 1 ml aliquot was collected and fixed in

3 ml of freshly prepared 4% paraformaldheyde in PBS. After overnight fixing at 4ºC, samples were divided in small aliquots of 0.4ml and stored at -20ºC until use.

At the time of analysis, aliquots of fixed samples were diluted in PBS until appropriate concentrations of cells, according to preliminary experiments: 1:1600 for the EUB338 probe, 1:160 or 1:400 for the BAC303 probe, 1:160 or 1:400 for the EREC482 probe, 1:80 or 1:160 for the LAB158 probe, 1:40 or 1:80 for the ENT-D probe, and 1:40 or 1:80 for the BIF-164 probe. Ten-well (8 mm diameter) gelatine-covered slides were used. Subsequently, 5 µl or 10 µl of the proper dilution of sample was placed in each well, air-dried at room temperature and fixed for 10 min with ethanol. For hybridization, probe solutions were further diluted in 50 ºC preheated hybridization buffer (20mM Tris-HCl, 0,9M NaCl, 0.1% sodium dodecyl sulphate pH 7.2),with 20% formamide for the LAB 158 and NON 338 probes, to a final concentration of 5 ng/µl. The hybridization mixture was added to the slides for an overnight incubation in a dark moist chamber (except for the BAC 303 probe which was hybridized for only 3 h and for the BAC 303 and LAB 158 probes which were incubated overnight at 47ºC). Samples to be hybridized with the LAB 158 probe were pre-treated with lysozime for 1 h at 37ºC prior to the hybridization process. After incubation, slides were rinsed in preheated washing buffer (20mM Tris-HCl, 0,9M NaCl, pH 7.2, 180 mM NaCl for the LAB 158 and NON 338 probe) for 30 min at 50ºC.

Thereafter, the slides were briefly rinsed with milli-Q water, air dried and mounted with Vectashield (Vector Laboratories, Peterborough, UK). Slides were viewed under oil immersion, using a Nikon Fi 60 epifluorescence microscope equipped with a filter for Cy3. Twenty five randomly selected fields were counted for each sample (in duplicate).

Terminal Restriction Fragment Length Polymorphism (t-RFLP): Procedure and data analysis

T-RFLP analysis of bacterial community was performed following the procedure described by (Hojberg *et al.*, 2005). Briefly, a 1497-pb fragment of the 16S rDNA gene was amplified using a 6-carboxy-fluorescein-labeled forward primer: S-D-Bact-0008-a-S-20 (5'-6-FAM-AGAGTTTGATCMTGGCTCAG-3') and reverse primer PH1552 (5'AAGGAGGTGATCCAGCCGCA-3'). Duplicate PCR were performed for each sample. Fluorescent-labelled PCR products were purified on QIAquick PCR purification kit columns (Qiagen, West Sussex, UK,) and eluted in a final volume of 30 µl of Milli-Q water. Then, the resultant PCR product was subjected to a restriction with *HhaI* (20,000U/µl) (Biolabs Inc., New England, USA). Fluorescent-labelled terminal restriction fragments (TRF) were analyzed by capillary electrophoresis on an automatic sequence analyzer (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) in Gene-Scan mode with a 25-U detection threshold. Determination of the TRFs sizes in the range 50-700 base pairs (bp) were performed with the size standard GS-1000-ROX (PE Biosystems).

Data obtained consisted of size (base pairs) and peak area for each terminal restriction fragment (TRF). To standardize the DNA loaded on the capillary, the sum of all TFR peak areas in the pattern was used to normalize the peak detection threshold in each sample. Following the method described by (Kitts, 2001), a new threshold value was obtained by multiplying a pattern's relative DNA ratio (the ratio of total peak area in the pattern to the total area in the sample with the smallest total peak area) by 323 area units (the area of the smallest peak at the 25 detection threshold in the sample with the smallest total peak area). For each sample, peaks with a lower area were deleted from the data set. Thereafter, a new total area was obtained by the sum of all the remaining peak areas in each pattern.

Richness was considered as the number of peaks in each sample after standardization. For pair-wise comparisons of the profiles, a Dice coefficient

was calculated and dendograms were constructed using the Fingerprinting II software (Informatix, Bio-Rad, CA, USA) and an unweighted pair-group method with averaging algorithm (UPGMA). To deduce the potential bacterial composition of the samples, *in silico* restrictions for the major rat gut bacteria with the primers and the enzyme used were obtained using the analysis function TAP-tRFLP from the Ribosomal Database Project II software. Results are presented as potential compatible bacterial species. Note also that direct attribution of species to individual peaks is not unequivocally possible unless fingerprinting is complemented with sequence analysis of clone libraries. Analysis of electropherograms was used for the visual comparison of compatible TRFs with different bacteria for the three experimental groups.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cecum tissue samples using RNAwiz (Ambion, Madison, WI) and treated with DNA-free (Ambion, Madison, WI) for 30 min at 37ºC. cDNA was synthesized from 5 µg total RNA in a reaction mixture of 50 µl containing 0.5 µg of oligo 18 (dT) primer (Ambion, Madison, WI), 2nM dNTP (Ecogen, Barcelona, Spain), and 10 Units Moloney Murine Leukemia Virus (MMLV) (Ambion, Madison, WI). The resulting cDNA was amplified in a total volume of 50 µl with 1 unit of *taq*DNA, 1 mM dNTP mixture, and 0.5 µM primers (Proligo-Sigma, Madrid, Spain) (Table 2). The PCR amplification protocol was as follows: 35 (GAPDH) or 40 (TLR-2 and TLR-4) cycles with 1 min (GAPDH) or 30 sec (TLR-2 and TLR-4) of denaturation at 95ºC, 1 min (GAPDH) or 30 sec (TLR-2 and TLR-4) of annealing at 50ºC, and 1 min of extension at 72ºC on a thermal cycler (Techno Cambridge Ltd.). Amplified products were electrophoresed on 2% agarose gel in TAE buffer, stained with ethidium bromide, photographed under ultraviolet light, and quantified using image-analyzing software (Quantity-One, Bio-Rad laboratories). For semiquantification, the ratio of the optical density of each PCR product and GAPDH was determined.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using a one-way analysis of variance (one-way ANOVA), followed when necessary, by a Student–Newman–Keuls multiple comparisons test. Results were considered statistically significant when $P<0.05$.

Results

Characterization of intestinal (cecal) microbiota by FISH

Total bacteria, determined by FISH as EUB338-positive cells, was between 1×10^{10} and 8×10^{10} cell/ml and was similar in all experimental groups, regardless the time of testing (Table 3). In the barrier group, overall characteristics of the microbiota were in agreement with the microbiological composition of the inoculus used originally to colonize the intestine of these animals (Fig. 1 and Tables 1 and 3).

Among Gram negative bacteria, the counts of enterobacteriaceae (ENT-D probe) were scarce (in most cases less than 1% of the flora quantified) and of similar magnitude in all experimental groups (Fig. 1 and Table 3). In contrast, *Bacteroides* spp (BAC 303 probe) were relatively abundant (by 19% to 26% of the microbiota quantify) and slightly, but significantly higher in the barrier group when compared with the conventional and the barrier/conventional groups ($P<0.05$; Fig. 1 and Table 3). This inter-groups variations were observed regardless the time of testing.

Gram positive bacteria represented by 80% of the FISH quantified microbiota. In particular, *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa, EREC 482 probe) accounted for the largest bacteria population in all experimental groups (Fig. 1), with higher counts in the barrier group when compared with the conventional group ($P<0.05$) and the barrier/conventional group showing an intermediate situation (Table 3). Counts for *Lactobacillus* spp and *Enterococcus* spp (LAB 158 probe) were similar in the conventional and barrier/conventional groups with lower counts in the barrier group, although statistical significance was not achieved (Table

3). As it relates to *Bifidobacterium* spp (BIF164 probe), counts were high in cecal samples from the conventional group and scarce in the barrier group ($P<0.05$) while the barrier/conventional group showed a transition towards the conventional conditions (Fig. 1, Table 3).

Table 1. Original bacteria (starter culture) implanted in the OFA Sprague Dawley barrier rat colony.*

Original implantation bacteria
• *Bacteroides distasonis* • *Lactobacillus acidophilus* • *Lactobacillus salivaris* • Schaedler fusiform-shaped bacterium • 3 strains of CRL fusiform-shaped bacterium • CRL Mouse Spirochete • *Escherichia coli* (non haemolytic) • *Streptococcus faecalis* (group D, *Enterococcus* spp)

*: Data obtained from Charles River Laboratories, France.

Table 2 . Characteristics of the primers for rat TLR-4, TLR-2 and GAPDH

Primer	Sequence	PCR products (bp)	cDNA position	NCBI. ref. seq.
TLR4				
LEFT	5'-CATAGCAGATGTTCCTAGGC-3'	561	1779-1798	NM-19178
RIGHT	5'-GGAGTCTGTAGAGTGTGTCA-3'		1197-1216	
TLR2				
LEFT	5'-CTGACCTCTCTCAACGAACT-3'	548	578-1166	NM-198769
RIGHT	5'-CGCTGAGGTCTAAGAACTCT-3'		578-597	
GAPDH				
LEFT	5'-ATGAGCCCTTCCACGATGCC-3'	140	1208-1227	NM-017008
RIGHT	5'-CCGCCCCTTCCGCTGATGCC-3'		1348-1367	

Similar results were obtained using classical plate culture techniques and standard bacteriological methods (data not shown).

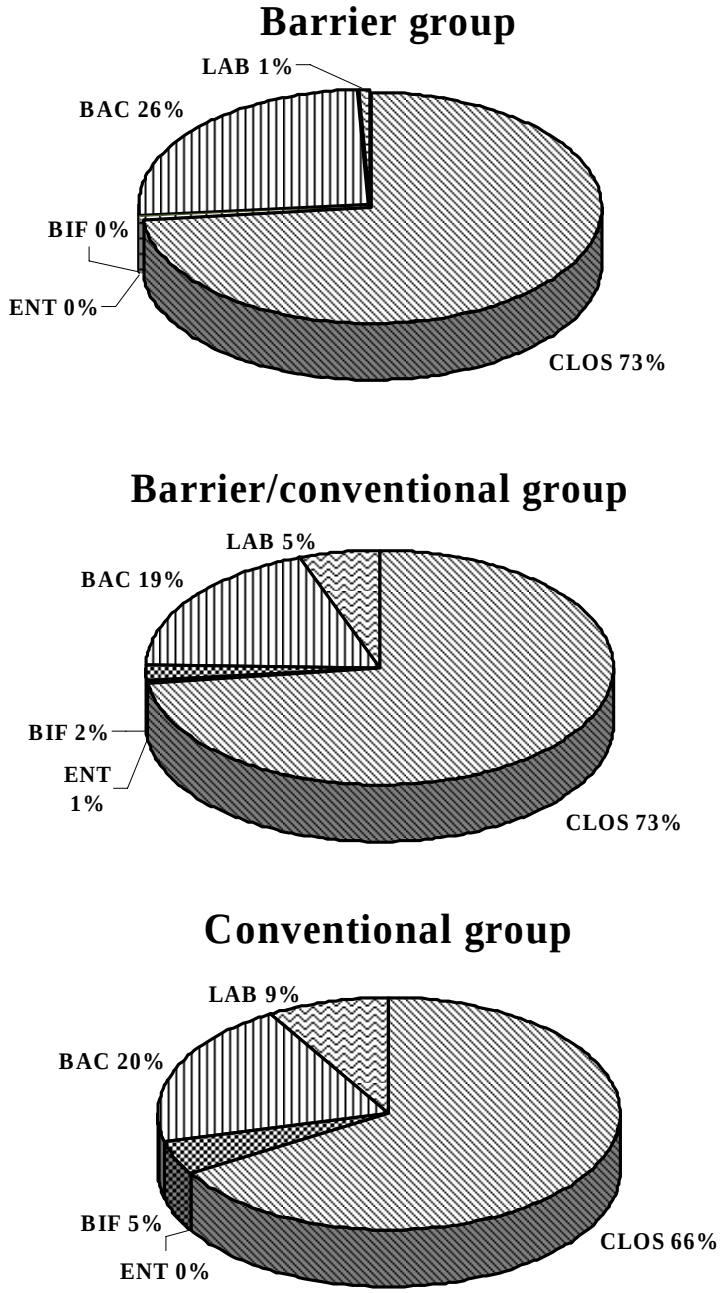

Figure 1: Relative distribution of cecal microbiota, as quantified by FISH, in the different experimental groups. Data represent the percent composition of the main bacterial groups present in the gut microbiota (*Bacteroides* spp, *Bifidobacterium* spp, Clostridium, enterobacteria, *Lactobacillus* spp and *Enterococcus* spp) quantified using FISH techniques and represent mean values from 10-16 animals per group. For exact cell counts see Table 3. BAC: *Bacteroides* spp, ENT: enterobacteria, CLO: Clostridium Cluster XIVa, LAB: *Lactobacillus* spp and *Enterococcus* spp, and BIF: *Bifidobacterium* spp.

Table 3. Bacterial counts in the different experimental groups, as determined by FISH[a]

	Barrier Group (× 10^8 cells/ml)	Barrier/conventional Group (× 10^8 cells/ml)	Conventional Group (× 10^8 cells/ml)
Total Bacteria	352 ± 191	421 ± 221	423 ± 179
Enterobacteriaceae	0.1 ± 0.2	0.6 ± 1.7	0.07 ± 0.09
Bacteroides spp	25.1 ± 7.5	16.6 ± 8.7*	13.7 ± 5.7[&]
Clostridium coccoides - Eubacterium rectale (*Clostridium* Cluster XIVa)	82.3 ± 38.6	69.6 ±43.4	44.5 ± 27.6 *
Lactobacillus and *Enterococcus* spp	1.2 ± 0.8	4.8 ± 4.6	5.4 ± 7.7
Bifidobacterium spp	0.01 ± 0.02	1.5 ± 2.5	3.1 ± 4.7 *

[a]: Data are mean±SEM, n=12-16 per group (see methods for details of the counting process).
[&]: P < 0.05 vs. barrier/conventional group (ANOVA).
*: P < 0.05 vs. barrier group (ANOVA).

Ecological characterization of the intestinal microbiota: t-RFLP analysis

The similarity indexes of the t-RFLP profiles, illustrated in the form of a dendogram, of the cecal microbiota in the different experimental groups are shown in Fig. 2A. Overall, the dendogram obtained shows a relatively high homogeneity in the microbiota of the barrier group when compared with the conventional or the barrier/conventional groups, despite the time of testing, which showed less homogeneity. Nevertheless, the overall biodiversity of the microbiota was similar in the three experimental groups, with an average number of t-RFs (taken as a measure of biodiversity) witch varied from 22 to 45 among the different experimental groups and a tendency for higher diversity in the conventional group (P=0.054, ANOVA; Fig. 2B).

Table 4 summarizes the main bacterial groups, as detected by t-RFLP analysis, with differential presence in the three experimental groups. In general, the t-RFLP analysis was concordant with the FISH data, at least for those bacterial groups identified simultaneously by FISH and by t-RFLP analysis. For instance, the general prevalence of *Clostridium* spp was

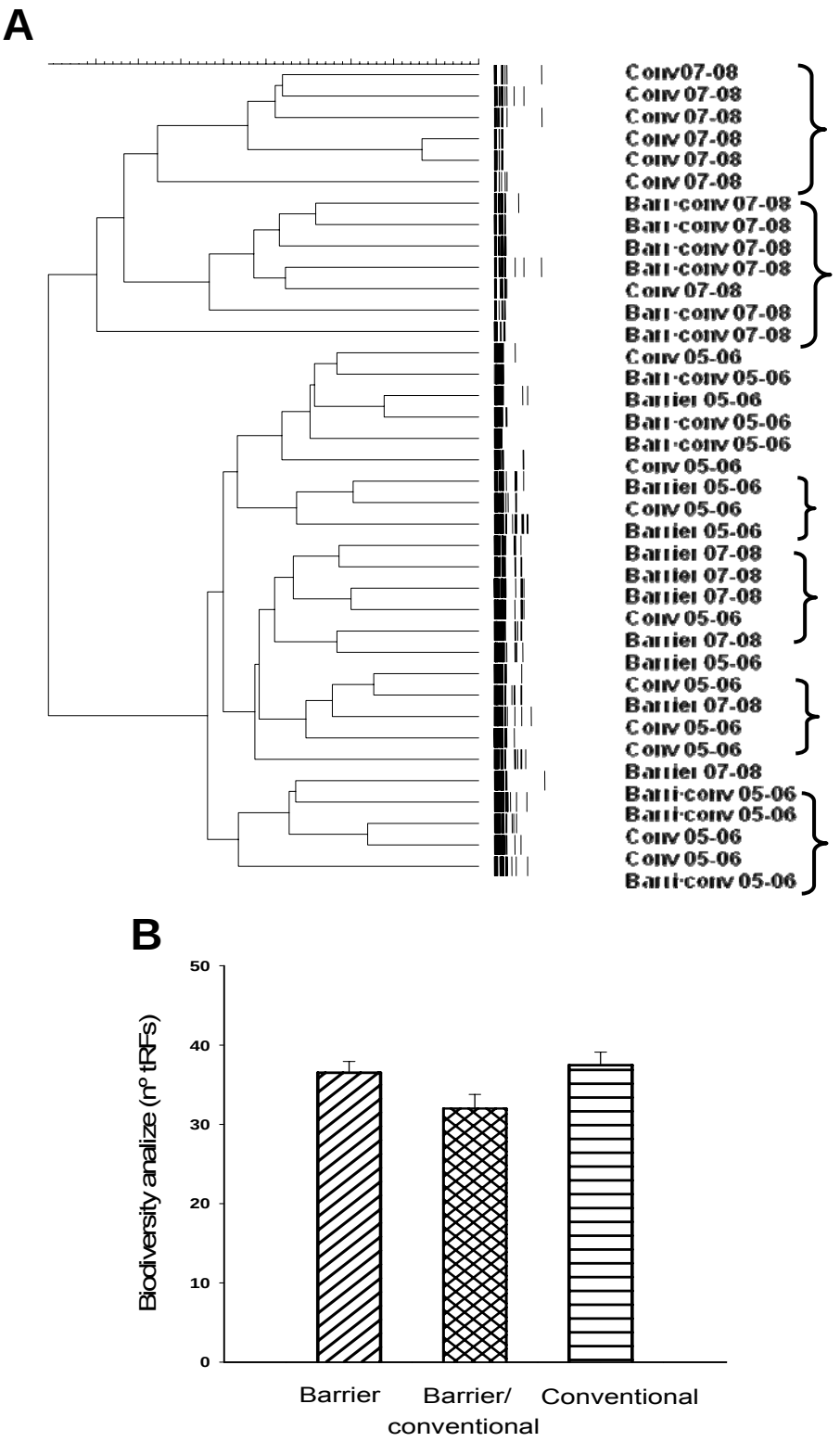

Figure 2: A: Dendogram illustrating the clustering of the different experimental groups according to the t-RFLP banding patterns obtained from the analysis of the cecum microbiota. Each line represents an animal and the code to the right identifies the experimental group and the time at which the samples were obtained. Notice how, although with some variability over time, the animals of the different experimental groups have a tendency to cluster together (Conv=conventional; Barr/conv=barrier/conventional, Barrier). The dendogram distances represent percentage of similarity. B: Bar graph showing the overall biodiversity among the different experimental groups, as determined by t-RFLP (see methods for details).

higher in the barrier group vs. the conventional and barrier/conventional groups (Fig. 1 and Table 4). Similarly, the prevalence of *Bacteroides fragilis* detected by t-RFLP was also higher in the barrier group vs. the conventional and the barrier/conventional groups, as detected by FISH (Fig. 1 and Table 4). Interestingly, in many cases the barrier/conventional group showed an intermediate situation between the barrier and the conventional groups with respect to bacterial prevalence. In general, non-identified bacterial groups (classified as “Unidentified bacterium” and “Unculture bacterium” in the t-RFLP analysis) showed higher prevalence in the barrier group than in the conventional or barrier/conventional groups.

Expression of TLR-2 and TLR-4

TLR-2 and TLR-4 transcripts were clearly identifiable, with a variable relative intensity, in all tissue samples analysed. A relatively high intra-group variability was observed in the expression of both receptors. Despite this, animals bred and maintained in barrier conditions had a clear tendency to over-express TLR-4 when compared with the conventional group (P=0.057), while the barrier/conventional group showed intermediate levels of expression (Fig. 3B). Except for 5 out of 12 animals in the barrier/conventional group and 4 out of 16 animals in the conventional group, expression levels were very low in these groups. No differences among groups were observed for TLR-2 expression; in most cases expression levels were also low, particularly in the conventional group (Fig. 3A).

Table 4. Theoretical restriction 5'-fragment (tRF) size predicted for the major rat gut bacteria and prevalence in the different experimental groups

Compatible bacterial group	tRF size	Frequency[a]		
		Barrier (n=10)[b]	Barrier/ Conventional (n=12)	Conventional (n=16)
Unidentified	54-55	1(10)	10(83)	12(75)
Bacillus spp/ Lactococcus lactis ssp	61-62	0(0)	7(58)	10(62)
Rhodoplanes spp	65	4(40)	6(50)	3(19)
Uncultured rumen bacterium/ Leptotrichia spp	71	5(50)	2(17)	3(19)
Photorhabdus sp.	74-75	6(60)	6(50)	0(0)
Uncultured bacterium	77-78	8(80)	6(50)	9(56)
Uncultured bacterium	79	0(0)	6(50)	7(44)
Erythrobacter spp/ Uncultured bacterium	82-83	3(30)	1(8)	9(56)
Desulfovibrio defluvii/ Roseiflexus spp	86-87	4(40)	6(50)	0(0)
Flavobacterium psychrophilum	88-89	7(70)	0(0)	2(12)
Flavobacteriaceae bacterium/ Uncultured rumen bacterium/ Desulfovibrio spp	93-94	7(70)	4(33)	0(0)
Desulfovibrio profundus/ Uncultured bacterium	95	6(60)	7(58)	3(19)
Uncultured bacterium	96	8(80)	6(50)	6(38)
Desulfococcus oleovorans/ Desulfomonile limimaris/ Helicobacter pylori	97-98	7(70)	11(92)	9(56)
Helicobacter pylori/ Uncultured rumen bacterium	99	2(20)	8(67)	8(50)
Bacteroides spp/ Uncultured rumen bacterium	100	1(10)	6(50)	1(6)
Bacteroides fragilis/ Uncultured rumen bacterium/ Prevotella ruminicola	101-102	9(90)	9(75)	0(0)
Uncultured rumen bacterium	103-104	6(60)	7(58)	5(31)
Desulfitobacterium hafniense	107-108	9(90)	7(58)	0(0)
Thiobacillus spp	110-111	8(80)	4(33)	0(0)
Unidentified	113	6(60)	7(58)	2(12)
Unidentified	115	7(70)	4(33)	1(6)
Unidentified	117	6(60)	2(17)	3(19)
Unidentified	123-124	3(30)	5(42)	0(0)
Streptomyces rimosus subsp. Rimosus	125	3(30)	3(25)	0(0)
Uncultured rumen bacterium	126	6(60)	0(0)	0(0)
Unidentified	127-129	7(70)	0(0)	4(25)
Uncultured rumen bacterium	134-135	4(40)	6(50)	2(12)
Unidentified	136	4(40)	0(0)	2(12)
Uncultured rumen bacterium	137	1(10)	4(33)	0(0)
Unidentified	138	3(30)	0(0)	0(0)
Microbacterium spp	144-145	0(0)	7(58)	5(31)
Unidentified	148-149	5(50)	6(50)	0(0)
Unidentified	156	4(40)	6(50)	1(6)
Unidentified	165-167	10(100)	9(75)	0(0)
Unidentified	171	3(30)	0(0)	0(0)
Micrococcus sp./ Acetobacter pasteurianus	174-175	1(10)	11(92)	3(19)
Uncultured rumen bacterium	181-182	1(10)	10(83)	1(6)
Uncultured rumen bacterium	184-185	7(70)	8(67)	3(19)
Uncultured rumen bacterium/ Clostridium spp/ Butyrivibrio fibrisolvens	189-190	9(90)	4(33)	2(12)
Psychrobacter spp/ Uncultured bacterium/ Francisella spp	194-195	4(40)	5(42)	1(6)
Clostridium spp	196-197	3(30)	8(67)	7(44)
Unidentified	199	6(60)	0(0)	0(0)
Clostridium rectum/ Uncultured bacterium/ Mycobacterium spp	201	6(60)	2(17)	2(12)
Uncultured rumen bacterium	203-204	4(40)	6(50)	0(0)
Uncultured rumen bacterium	205-206	4(40)	1(8)	1(6)
Clostridium spp	231-232	0(0)	6(50)	4(25)
Clostridium perfringens	234-235	1(10)	5(42)	3(19)
Bacillus	239-240	1(10)	4(33)	1(6)
Bacillus subtilis subsp. Subtilis/ Bacillus licheniformis/ Bacillus spp	241-242	4(40)	0(0)	1(6)
Uncultured bacterium	251-252	4(40)	1(8)	1(6)
Simkania navegensis	271-272	1(10)	4(33)	5(31)
Unidentified	391-392	4(40)	0(0)	2(12)
Unidentified	402-403	5(50)	0(0)	0(0)
Unidentified	422	3(30)	0(0)	0(0)
Unidentified	488	6(60)	0(0)	0(0)
Uncultured bacterium	500	4(40)	0(0)	0(0)

[a]: Data represent the number of animals within each group presenting the bacterial group predicted by the corresponding tRF size and the incidence, in percentage (between brackets).
[b]: Because of technical problems, only samples from 10 out of the 12 animals included in this group were analyzed.

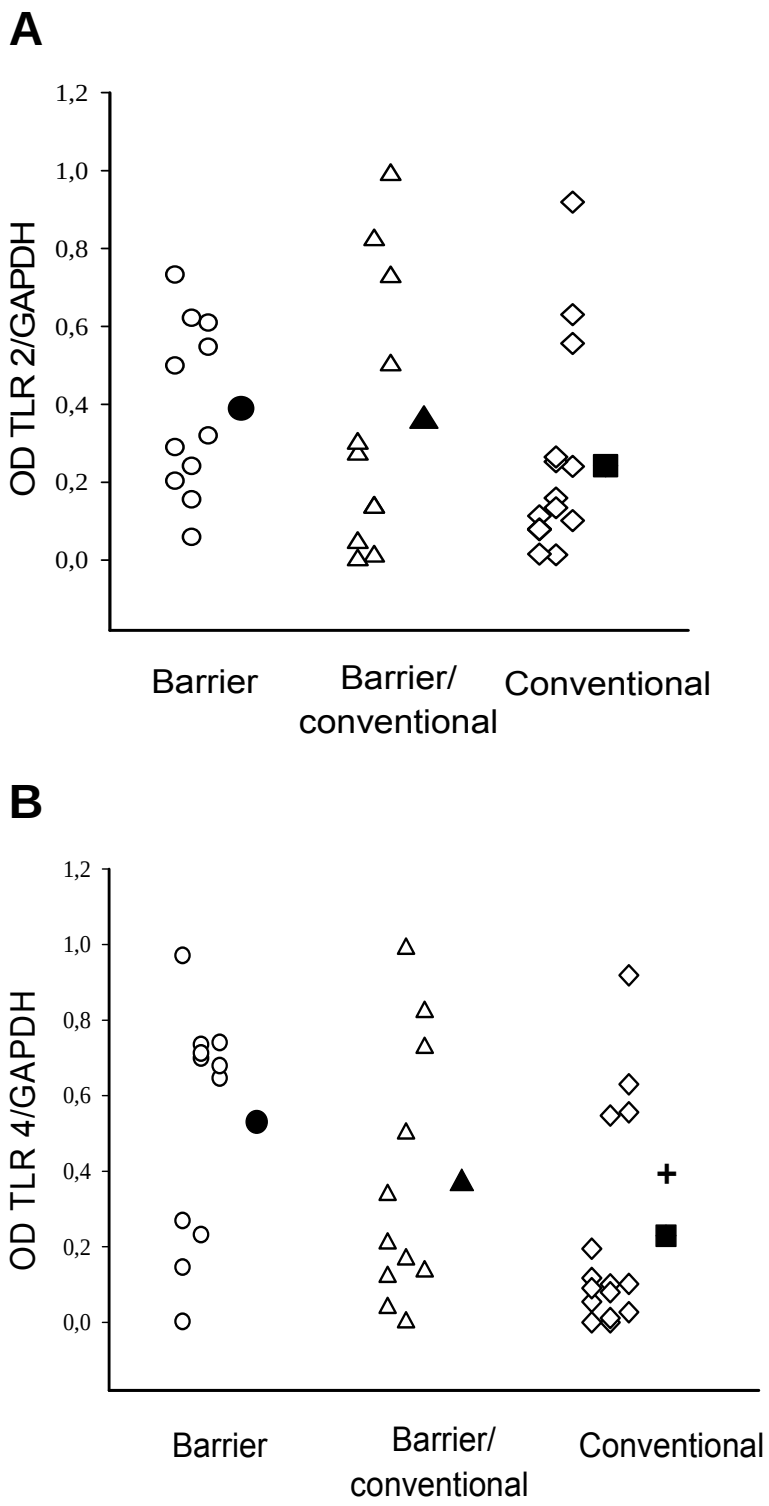

Figure 3: Relative expression of TLR-2 (A) and TLR-4 (B) in cecal tissues of the three experimental groups. Each open symbol represents an individual animal and the black symbol to the right corresponds to the mean of the group. +: P=0.057 vs. barrier group (ANOVA).

Discussion

The present results show that the relative composition of the cecal microbiota in rats varies spontaneously with changes in the environmental conditions (highly controlled environment, barrier, vs. standard conditions, conventional) although the total bacterial count seems to be unaffected. Furthermore, changes in microbiota seem to be characteristic of the environmental conditions considered with only relatively minor variations over time, at least for the main groups of bacteria analyzed here. However, these changes in bacterial flora seem to have a rather minor impact in the bacterial recognition systems present within the gut, at least as it relates to the expression of TLR-2 and TLR-4.

Although several authors have attempted to define the gut ecosystem and the number of bacteria present in the large intestine (Berg, 1996; Dinoto *et al.*, 2006), the difficult analysis of intestinal microbiota is far to be completed. Fortunately, the recent application of molecular techniques to this field, such as the use of FISH with oligonucleotide probes or t-RLFP analysis, has proven to be very useful (Duncan *et al.*, 2003; Vaahtovuo *et al.*, 2005; Dinoto *et al.*, 2006; Andoh *et al.*, 2009). In our study, the results obtained with the EUB 338 probe, designed to visualize total bacteria, give cecal cell counts similar to those previously reported (Dinoto *et al.*, 2006).

Interestingly, the total cell count was similar in animals maintained in highly controlled microbiological conditions (barrier group), animals kept in standard conditions (conventional group) or barrier animals adapted to standard conditions (barrier/conventional group). This might suggest that, in otherwise normal conditions, a highly controlled hygienic environment does not imply a lower number of bacteria colonizing the gut, but a selection in the types of bacteria conforming the commensal microbiota. Moreover, this might suggest that intestinal microbiota develops quantitatively up to a number that could be regarded as normal, at this point changes in microbiota might be generated through qualitative (characteristic and/or proportions of the bacterial groups) rather than quantitative variations. This agrees with observations suggesting

that during pathophysiological states gut microbiota changes not only qualitatively but also quantitatively (Andoh *et al.*, 2009). Overall, maintenance in barrier conditions seems to favours the settlement of strict anaerobic bacteria, particularly *Bacteroides* spp and clostridia. However, less restrictive hygienic conditions, such as a conventional housing, favour an increase of lactic acid bacteria.

This switch in bacterial flora was particularly evident for *Bifidobacterium*, very scarce in animals bred and maintained in barrier conditions while relatively abundant in conventional conditions (by 5% of the flora quantified by FISH). With these general characteristics, gut microbiota should be regarded as a relatively dynamic system that varies over time, space and environment (Camp *et al.*, 2009). In our studies, apart from the environment-related changes described above, time-related changes were also present when a relatively large proportion of cecum microbiota was analyzed by t-RLFP. As observed in the dendogram derived from the t-RLFP analysis (see Fig. 2) the animals included in the study tended to cluster according to both environment and time of testing, thus indicating the existence of time-related variations in the flora present in the same environment. Nevertheless, for the bacterial groups characterized by FISH a clear environment-related constancy was observed over time.

The present work is limited to the characterization of enterobacteria, *Lactobacillus* spp, *Bacteroides* spp, *Clostridium* spp and *Bifidobacterium* spp. In agreement with previous reports (Dinoto *et al.*, 2006), and regardless the experimental group considered, *Clostridium* spp were dominant, followed by *Bacteroides* spp, while enterobacteria, *Lactobacillus* spp and *Bifidobacterium* spp were less numerous. This was especially evident for the barrier and the conventional groups. Interestingly, barrier-bred animals housed in conventional conditions for three weeks showed, in general, an intermediate state in their microbiota, thus suggesting and spontaneous adaptation to the microbiological characteristics of the new environment. Overall, the FISH-quantified bacteria represented by 12 % to 18 % of the total cecal bacteria, as determined by FISH when using the probe EUB 338, a result in

accordance with that previously reported in rats (Dinoto *et al.*, 2006) as well as in humans (Mai *et al.*, 2004).

Although this might appear as a relative small percentage of the total cecal microbiota, these bacterial groups might have important physiological and pathophysiological significance within the gut. They have been identified as key components in determining the intestinal immune response (Galdeano and Perdigon, 2006; Xavier and Podolsky, 2007), have been implicated in different gastrointestinal physiopathological alterations (such as IBD or IBS) and/or have a potential interest as pre/probiotics (Marteau, 2002; Ruiz *et al.*, 2005; Siggers *et al.*, 2008; Takaishi *et al.*, 2008; Collado *et al.*, 2009; Collins and Bercik, 2009; Looijer-van Langen and Dieleman, 2009). Therefore, the present observations might have implications at an experimental level, indicating that the gut microbiota might be an important factor to be taken into account when performing physiological, pathophysiological and/or pharmacological studies within the gastrointestinal system. This agrees with a growing body of evidence implicating the commensal microbiota as an experimental factor influencing the outcome of both digestive and extra-digestive studies in animals (Verdú *et al.*, 2006; Rousseaux *et al.*, 2007; Wirtz and Neurath, 2007; Collins and Bercik, 2009; Tilg *et al.*, 2009).

For instance, in our conditions, although not thoroughly assessed, none of the animals showed any indication of gut inflammation at the time of euthanasia. Nevertheless, animals bred and maintained in barrier conditions, with a controlled gut microbiota, had a predominant commensal microbiota that have been associated with IBD, therefore one can speculate that these animals, although not showing spontaneous inflammation, might be more susceptible to intestinal inflammation than the other groups. This agrees with data showing that susceptibility to intestinal inflammation increases when gut microbiota has a limited development or is absent (Kim *et al.*, 2007; Wohlgemuth *et al.*, 2009).

Overall, our observations might have a relationship with the “hygiene hypothesis” (Strachan, 1989; Guarner *et al.*, 2006). As mentioned previously, qualitative characteristics of the cecal microbiota were different in animals

bred and maintained under barrier conditions, and therefore with a high hygienic standards, and in animals maintained under normal hygienic conditions (conventional group). These differences are also emphasized by the fact that barrier animals adapted to conventional conditions for three weeks (barrier/conventional group) show a switch in their intestinal flora towards the characteristics of the conventional group. As also mentioned, this can have important implications in the responses to immunological stimuli, for instance gut inflammatory responses, as initially suggested by the "hygiene hypothesis".

Immune responses to intestinal bacteria are mediated, at least partially, by the interaction of bacterial wall components with TLRs (Takeuchi *et al.*, 1999; Cario, 2005). Among TLRs, TLR-2 and TLR-4 have been reported as the main partners interacting with the gut microbiota (Fujisawa *et al.*, 2006; Szebeni *et al.*, 2008). Most of the studies performed so far investigating the interplay between TLRs and bacteria have been done either *in vitro*, by exposing cells to bacterial wall components, or, if *in vivo*, by exposing germ-free animals to specific bacterial species or to bacterial components (Takeuchi *et al.*, 1999; Karlsson *et al.*, 2002). According to these studies, Gram positive bacteria are recognized by TLR-2 while TLR-4 recognizes mainly Gram negative bacteria.

Nevertheless both receptors can interact with multiple microorganisms and are likely to be subjected to complex, multifactorial, regulatory mechanisms. Results obtained suggest a relative heterogeneity in the intra-group expression of TLR-2 and TLR-4 without clear distinctive patterns of expression between experimental groups. This agrees with the similar bacterial load in the gut, regardless the experimental group considered, and could be due to the multifactorial regulation of TLRs and the influence of multiple microbial agents not directly assessed (Heine *et al.*, 1999; Backhed *et al.*, 2003; Eckburg *et al.*, 2005). Nevertheless, TLR-4 showed a clear tendency to be over-expressed in the barrier group when compared, particularly, with the barrier/conventional group. This correlates with the higher number of Gram negative bacteria, particularly *Bacteroides* spp,

detected in the barrier group. However, as mentioned, the influence of other components of the microbiota cannot be excluded.

In summary, the present results show that the relative composition of the cecal microbiota in rats varies spontaneously with changes in the environmental conditions. However, these changes seem to have a minor impact in the expression of TLR-2 and TLR-4. This observations might have an impact in the understanding of variability in animal responses, particularly to immune-related stimuli, when assessed in the context of the environmental/microbiological conditions and support the so called “hygiene hypothesis”. These observations warrant further studies assessing spontaneous variations in other components of the intestinal microbiota and other bacterial recognition systems as well as specific studies designed to assess environment/microbiological conditions-related differences in gut immune response, particularly as it relates to intestinal inflammation, of interest to understand the role of intestinal microbiota in gut pathophysiology.

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Capítulo 2

Susceptibility to Indomethacin-induced Enteritis in Rats Maintained Under Different Microbiological Conditions

Abstract

Gut microbiota plays a major role in the pathogenesis of inflammatory bowel disease (IBD). This study assessed changes in gut commensal microbiota and expression of TLR-2 and TLR-4 and susceptibility to intestinal inflammation in a model of indomethacin-induced ileitis in rats maintained in standard microbiological conditions or breeds under barrier conditions and adapted to standard conditions. Gut microbiota was analyzed by FISH and t-RFLP and the expression of TLR-2 and TLR-4 determined by RT-PCR. Inflammation was assessed through clinical, histological and biochemical parameters. After indomethacin treatment, disease activity parameters showed a general trend to be increased in the conventional group compared with the barrier/conventional.

In control conditions, commensal ileal microbiota differed in both groups, with *Bifidobacterium*, *Bacteroides* and enterobacteriaceae populations being significantly higher in the conventional than in the barrier/conventional group; while the *Clostridium coccoides-Eubacterium rectale* group was virtually absent in both groups. During inflammation, microbiota was altered similarly in both groups. Major changes observed were a significant increase in enterobacteriacea and the appearance of the *Clostridium coccoides-Eubacterium rectale* group at high counts. During inflammation, expression levels of TLR-2 and TLR-4 were significantly increased in the cecum, with minor changes in the ileum. These results show that indomethacin-induced enteritis is more severe in animals in conventional conditions vs. animals bred under barrier and adapted to conventional conditions and that gut inflammation implies significant variations in commensal microbiota. Microbiological status of the animals in standard research conditions has an impact in the outcome of inflammation in experimental models of IBD.

Introduction

The human colon in colonized by 10^{11}-10^{12} bacteria per gram of content. This commensal microbiota is composed of 400-500 phylotypes belonging mainly to 30-40 dominant bacterial species (Hooper *et al.*, 2002; Eckburg *et al.*, 2005). Intestinal bacteria contribute to host health participating in intestinal immune responses, energy and nutrient metabolism, and tissue development and repair (Hooper *et al.*, 2001). However, in certain circumstances commensal microbiota can become detrimental to the host. For instance, it is thought that the intestinal microbiota plays a major role in the pathogenesis of inflammatory bowel disease (IBD), a pathological condition characterized by strong activation of the mucosa-associated immune system due to a complex interaction of genetic, immunological and environmental factors (Swidsinski *et al.*, 2005[a]; Xavier and Podolsky, 2007). According to this, several reports have established that the composition of the intestinal microbiota has significant impact on host immunity and influences the course of mucosal inflammation (Hooper *et al.*, 2001).

Mammalian toll-like receptors (TLRs) are members of the pattern-recognition (PRR) family and play a central role in microbial recognition and the subsequent immune responses to microbial pathogens (Rock *et al.*, 1998; Takeda and Akira, 2003). Among TLRs, TLR2 and TLR4 are considered key players in the recognition of gut microbiota. TLR-2 recognizes a variety of microbial components from gram positive bacteria, whereas TLR-4 recognizes LPS of gram-negative bacteria (Takeuchi *et al.*, 1999; Cario, 2005).

Microbial recognition by TLRs seems to be important in the initiation, course and resolution of inflammation within the gut (Iwasaki and Medzhitov, 2004). According to this, gut microbiota seems to be an active component of IBD pathogenesis. For instance, an increase of IBD incidence in industrialized countries has been related to the improvement in standards of hygiene and the subsequent changes in gut commensal microbiota (Strachan, 1989; Guarner *et al.*, 2006). These changes lead to the appearance of abnormal

gut immune responses with alterations in the microbiota recognition systems, including TLRs 2 and 4, resulting in an increased susceptibility to gut inflammation and the generation of persistent gut inflammatory states (Hoentjen *et al.*, 2005; Fujisawa *et al.*, 2006; Silva *et al.*, 2008; Szebeni *et al.*, 2008; Terán-Ventura *et al*., 2009).

We have recently described changes in the expression of TLR-2 and TLR-4 in the gut of rats and their correlation with the microbiological status of the animals and their respective gut microbiota (Terán-Ventura *et al*., 2009). Following these observations, in the present study we assessed the influence of gut microbiota in intestinal inflammation in a model of indomethacin-induced ileitis in rats. This model has been previously characterized by us and represents a valid IBD model in which a local inflammation of the ileum appears after the systemic treatment of the animals with indomethacin (Porras *et al.*, 2004, 2006, 2008). In this study we characterize the inflammatory response in animals maintained in standard microbiological conditions and in animals breed under barrier conditions (controlled microbiota) and adapted for a 3-week period to standard conditions. In these animals we assessed changes in disease activity parameters as well as characteristics of the intestinal microbiota and the expression of TLR-2 and TLR-4, as main components of the microbiota recognition systems within the gut.

Materials and methods

Animals

Adult male Sprague-Dawley rats (300-350 g) bred and maintained in a barrier protected area with all materials; water, food and bedding sterilized before entering the barrier, were provided by Charles River Laboratories (Lyon, France). Transport of barrier bred animals was made in filtered boxes to guarantee maintenance of their microbiological status. Thereafter, animals were maintained for a 3-week period in conventional conditions (referred from now on as barrier/conventional animals).

In addition, male Sprague-Dawley rats bred in conventional conditions in the Animal Facility of the *Universitat Autònoma de Barcelona* were used (refered from now on as conventional animals). This conventional colony was established in 1994 from OFA Sprague Dawley rats from Charles River Laboratories. When in conventional conditions, water, food and bedding were given to animals as facilitated by the commercial provider, without any further treatment.

All animals maintained at the university's animal facility were housed in standard plastic cages with stainless steel grid roofs in an environmentally controlled room (20-21ºC, 40-70% humidity, 12 hours light /dark cycle), and received a commercial diet (15.4%,protein, 2.9% fat and 3.9% fibre; SASE, Panlab S.L., Barcelona, Spain) and tap water *ad libitum*. All procedures were approved by the Ethical Committee of the *Universitat Autónoma de Barcelona*.

Experimental procedures

Intestinal inflammation was induced by administration of two injections of indomethacin (7.5 mg/kg, subcutaneous) 48 h apart, as previously described by us (Porras et al, 2004, 2006, 2007). Control animals received similar treatment with saline (0.3 ml/rat, subcutaneous). Animals were euthanized at day 4 after the first injection of indomethacin, corresponding to the acute

phase of inflammation (Porras *et al.*, 2004). During this time, body weight, presence of diarrhea and the general health status of the animals were monitored regularly as clinical signs of inflammation.

The following experimental groups were included (n=6-7 for each): 1) indomethacin-treated barrier/conventional rats, 2) vehicle-treated barrier/conventional rats, 3) indomethacin-treated conventional rats, and 4) vehicle-treated conventional rats.

Clinical assessment of inflammation

Clinical assessment of inflammation included monitoring of body weight, appearance of faeces, state of hydratation and general condition (including hunch posture, piloerection, motor activity and state of mucous membranes). A total score of 0-9 was assigned to each animal, corresponding to the addition of separate scores (0-3) for each parameter, except for body weight, where 0 indicates normality, 1 mild alterations, 2 moderate alterations and 3 severe alterations. A macroscopic score (0-9) was also assigned to each animal during the necropsy. The macroscopic score was based on: presence or absence of abdominal distension (0-1), presence and characteristics of edema (0-2), presence of adherences (0-2), appearance of the intestine (ileum and cecum) (0-2), appearance of bowel (ileal and cecal) contents (0-2).

Samples collection

Rats were euthanized by CO_2 inhalation. Immediately, a medial laparotomy was performed and the inflammatory state assessed macroscopically as detailed above. Thereafter, tissue samples of the cecum and ileum and fecal content of the same areas (about 0.5 g) were obtained under sterile conditions and immediately frozen with liquid nitrogen. All samples were stored at -20 ºC (fecal samples) or -80 ºC (tissue samples) until analysis.

Histological score

Paraffin sections (5µm) of ileal samples were stained with hematoxylin-eosin following standard histological procedures. Two to four coded sections for each animal were scored for inflammation, in a blinded fashion, by two

independent investigators. A histological score based on the epithelial structure (0-3), the presence of edema (0-3), the presence of ulcerations (0-3), and the inflammatory infiltrate (0-3) was assigned to each animal (maximal score of 12).

Enumeration of bacteria using fluorescence *in situ* hybridization (FISH)

For FISH, oligonucleotide probes consisted of a single strain DNA covalently linked with Cy3 at the 5'-end. Probes used were: EUB 338 (5'GCTGCCTCCCGTAGGAGT3') to total Bacteria (Amann *et al.*, 1990); NON 338 (5'ACATCCTACGGGAGGC3') to Non bacteria (negative control) (Amann *et al.*, 1990); BAC 303 (5'CAATGTGGGGGACCTT3') to *Bacteroides* spp (Salzman *et al.*, 2002); EREC 482 (5'GCTTCTTAGTCAGGTACCG3') to *Clostridium* Cluster XIVa (Amann *et al.*, 1990); LAB 158 (5'GGTATTAGCACCTGTTTCCA3') to *Lactobacillus* spp and *Enterococcus* spp (Salzman *et al.*, 2002); ENT-D (5'TGCTCTCGCGAGGTCGCTTCTCTT3') to enterobacteria (Ootsubo *et al.*, 2002); and BIF 164 (5'CATCCGGCATTACCACCC3') to *Bifidobacterium* spp (Vaahtovuo *et al.*, 2005). All probes were obtained from Biomers (Germany).

The procedures followed were identical to those described previously by us (Terán-Ventura *et al.*, 2009). Briefly, frozen cecal and ileal contents were thawed in a Millipore filtered PBS medium, the suspension obtained was centrifuged for 1 min at 700 g and a 1 ml aliquot collected from the supernatant. After an overnight fixing in 4% paraformaldehyde, at 4 ºC, samples were divided in small aliquots of 0.4 ml and stored at -20 ºC until use.

At the time of analysis, fixed samples were diluted in PBS and disposed on 10-well (8 mm diameter) gelatin-covered slides, air-dried at room temperature and fixed for 10 min with ethanol. For hybridization, probe solutions were further diluted in 50 ºC preheated hybridization buffer (20mM Tris-HCl, 0,9M NaCl, 0.1% sodium dodecyl sulphate pH 7.2), with 20% formamide for the LAB 158 and NON 338 probes, to a final concentration of 5

ng/µl. The hybridization mixture was added to the slides for an overnight incubation in a dark moist chamber (except for the BAC 303 probe which was hybridized for only 3 h and for the BAC 303 and LAB 158 probes which were incubated overnight at 47ºC).

Samples to be hybridized with the LAB 158 probe were pre-treated with lysozime for 1 h at 37ºC prior to the hybridization process. After incubation, slides were rinsed in preheated washing buffer (20mM Tris-HCl, 0,9M NaCl, pH 7.2, 180 mM NaCl for the LAB 158 and NON 338 probe) for 30 min at 50ºC. Thereafter, the slides were briefly rinsed with milli-Q water, air dried and mounted with Vectashield (Vector Laboratories, Peterborough, UK). Slides were viewed under oil immersion, using a Nikon Fi 60 epifluorescence microscope equipped with a filter for Cy3. Twenty five randomly selected fields were counted for each sample (in duplicate).

Terminal Restriction Fragment Length Polymorphism (t-RFLP)

T-RFLP analysis of bacterial community was performed in cecal contents following the procedure described by (Hojberg *et al.*, 2005). Briefly, a 1497-pb fragment of the 16S rDNA gene was amplified using a 6-carboxy-fluorescein-labeled forward primer: S-D-Bact-0008-a-S-20 (5'-6-FAM-AGAGTTTGATCMTGGCTCAG-3') and reverse primer PH1552 (5'AAGGAGGTGATCCAGCCGCA-3'). Duplicate PCR were performed for each sample. Fluorescent-labelled PCR products were purified on QIAquick PCR purification kit columns (Qiagen, West Sussex, UK,) and eluted in a final volume of 30 µl of Milli-Q water. Then, the resultant PCR product was subjected to a restriction with *HhaI* (20,000U/µl) (Biolabs Inc., New England, USA). Fluorescent-labelled terminal restriction fragments (TRF) were analyzed by capillary electrophoresis on an automatic sequence analyzer (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) in Gene-Scan mode with a 25-U detection threshold. Determination of the TRFs sizes in the range 50-700 base pairs (bp) were performed with the size standard GS-1000-ROX (PE Biosystems).

Data obtained consisted of size (base pairs) and peak area for each terminal restriction fragment (TRF). To standardize the DNA loaded on the capillary, the sum of all TFR peak areas in the pattern was used to normalize the peak detection threshold in each sample. Following the method described by (Kitts, 2001), a new threshold value was obtained by multiplying a pattern's relative DNA ratio (the ratio of total peak area in the pattern to the total area in the sample with the smallest total peak area) by 323 area units (the area of the smallest peak at the 25 detection threshold in the sample with the smallest total peak area). For each sample, peaks with a lower area were deleted from the data set. Thereafter, a new total area was obtained by the sum of all the remaining peak areas in each pattern.

Richness was considered as the number of peaks in each sample after standardization. For pair-wise comparisons of the profiles, a Dice coefficient was calculated and dendograms were constructed using the Fingerprinting II software (Informatix, Bio-Rad, CA, USA) and an unweighted pair-group method with averaging algorithm (UPGMA).

Assessment of bacterial adherence

Bacterial wall adherence was assessed following techniques described elsewhere (Garcia-Lafuente *et al.*, 1997). Briefly, a sample of ileal tissue (about 2 cm in length) was rinsed in saline solution, sonicated twice for 60 s and then fixed in 4 % paraformaldehide for 16 h at 4ºC. Thereafter tissues were paraffin embedded using standard protocols, sectioned at 5 µm and sections were hybridized following the general FISH procedures described above.

Myeloperoxidase levels

Myeloperoxidase content in ileal and cecal tissue samples was determined as previously described (Porras *et al.*, 2008). Briefly, frozen tissues were powdered, weighted and homogenized in lysis buffer (Mini complete protease inhibitor, HEPES 1M, Triton X-100, PMSF 100mM). Thereafter, the homogenates were incubated for twenty minutes at 4ºC, centrifuged at 14000 rpm for 10 minutes at 4ºC and the supernatant recuperate. MPO activity was

evaluated in the supernatants using a specific enzyme-linked immunosorbent assay (HyCult Biotechnology, Uden, The Netherlands, limit of detection 1 ng/ml),

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cecal and ileal tissue samples using RNAwiz (Ambion, Madison, WI) and treated with DNA-free (Ambion, Madison, WI) for 30 min at 37ºC. cDNA was synthesized from 5 µg total RNA in a reaction mixture of 50 µl containing 0.5 µg of oligo 18 (dT) primer (Ambion, Madison, WI), 2nM dNTP (Ecogen, Barcelona, Spain), and 10 Units Moloney Murine Leukemia Virus (MMLV) (Ambion, Madison, WI). The resulting cDNA was amplified in a total volume of 50 µl with 1 unit of *taq*DNA, 1 mM dNTP mixture, and 0.5 µM primers (Proligo-Sigma, Madrid, Spain). (Table 1). The PCR amplification protocol was as follows: 35 (GAPDH) or 40 (TLR-2 and TLR-4) cycles with 1 min (GAPDH) or 30 sec (TLR-2 and TLR-4) of denaturation at 95ºC, 1 min (GAPDH) or 30 sec (TLR-2 and TLR-4) of annealing at 50ºC, and 1 min of extension at 72ºC on a thermal cycler (Techno Cambridge Ltd.). Amplified products were electrophoresed on 2% agarose gel in TAE buffer, stained with ethidium bromide, photographed under ultraviolet light, and quantified using image-analyzing software (Quantity-One, Bio-Rad laboratories). For semiquantification, the ratio of the optical density of each PCR product and GAPDH was determined.

Statistical Analysis

Data are expressed as mean ± SEM. Comparison between multiple groups were performed using a one-way analysis of variance (one-way ANOVA), followed when necessary, by a Student–Newman–Keuls multiple comparisons test. Comparisons between two groups were performed using Students *t* test. Bacterial wall adhesion data was analysed using the chi square test. Results were considered statistically significant when $P<0.05$.

Table 1. Characteristics of the primers for rat TLR-4, TLR-2 and GAPDH

Primer	Sequence	PCR products (bp)	cDNA position	NCBI. ref. seq.
TLR4				
LEFT	5'-CATAGCAGATGTTCCTAGGC-3'	561	1779-1798	NM-
RIGHT	5'-GGAGTCTGTAGAGTGTGTCA-3'		1197-1216	19178
TLR2				
LEFT	5'-CTGACCTCTCTCAACGAACT-3'	548	578-1166	NM-
RIGHT	5'-CGCTGAGGTCTAAGAACTCT-3'		578-597	198769
GAPDH				
LEFT	5'-ATGAGCCCTTCCACGATGCC-3'	140	1208-1227	NM-
RIGHT	5'-CCGCCCCTTCCGCTGATGCC-3'		1348-1367	017008

Results

Clinical indices and macroscopic assessment of inflammation

During the 4-day period after treatment, control rats, regardless the group considered, showed no clinical signs of inflammation and a linear increase in body weight (Fig. 1A). Conversely, during the same period of time indomethacin-treated animals showed a progressive reduction in body weight (Fig. 1A). At the time of necropsy, body weight loose was higher in the barrier/conventional inflamed group compared with the conventional inflamed group (Fig. 1A). Similarly, indomethacin-treated groups showed a progressive increase in their clinical scores for the same period of time, with a trend to show higher scores in conventional animals compared with barrier/conventional, although no statistical significance was achieved (Fig. 1B).

At necropsy, macroscopic assessment of the abdominal cavity and the gut in control groups revealed no specific signs of intestinal inflammation (Fig. 1C). However, in indomethacin-treated groups, macroscopic scores increased significantly over control values, with a trend to be higher in the conventional group compared with the barrier/conventional group (Fig. 1C).

Microscopic assessment of inflammation

Vehicle-treated rats, regardless the group considered, showed no signs of intestinal inflammation, with essentially normal histological features. On the other hand, indomethacin-treated groups showed microscopic signs of inflammation and microscopic scores were significantly higher than those in control animals. The more common histopathological changes observed were alterations of the epithelial structure, with variable degree of destruction of the villi, and the presence of local or generalized inflammatory infiltrate. Ulcers were observed only in 1 out of 5 animals (20%) in the barrier/conventional group and in 3 out of 7 animals (43%) in the conventional group. Total inflammatory scores were of similar magnitude in conventional and barrier/conventional animals (Fig. 2). One animal of the

barrier/conventional group was not scored because of technical problems during the processing of the tissue.

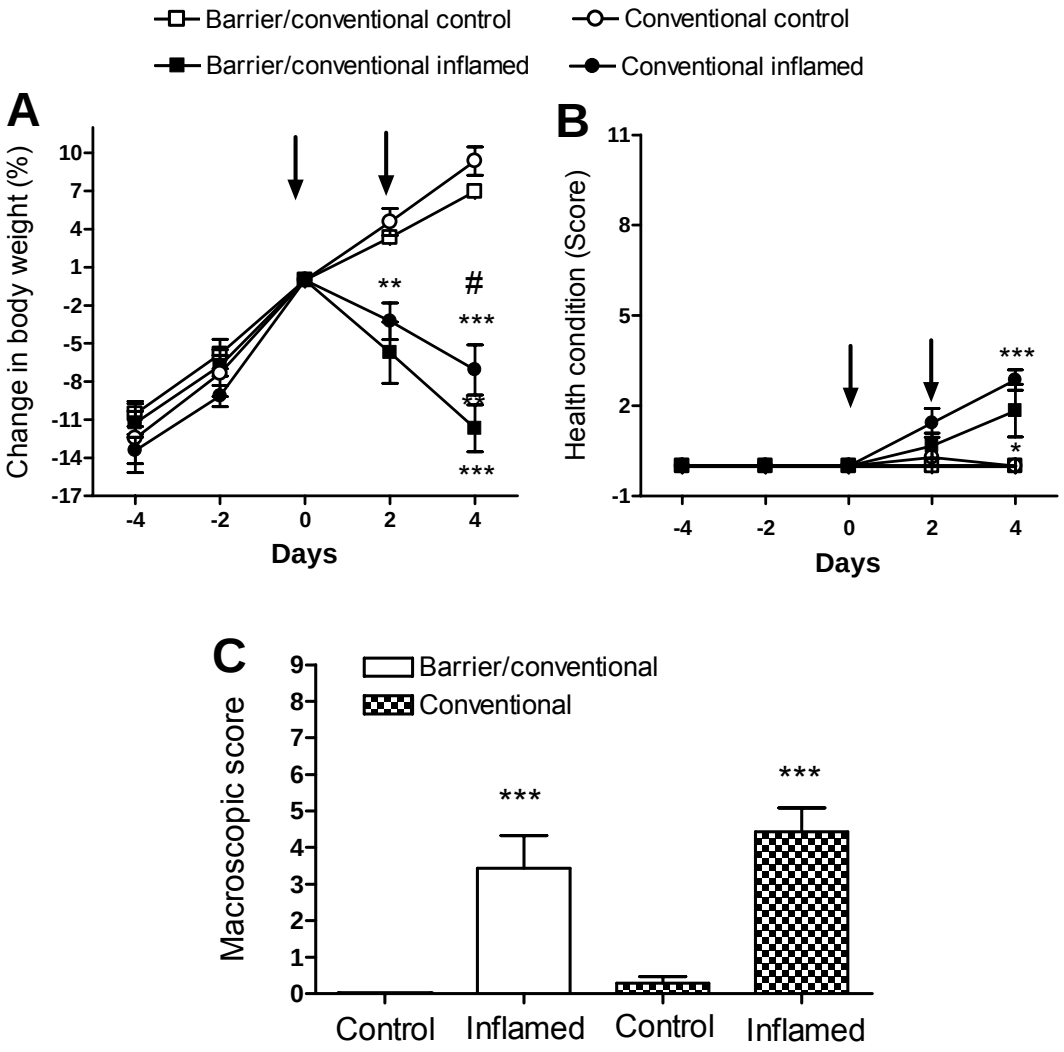

Figure 1. Effects of indomethacin on clinical symptoms (A and B) and macroscopic alterations at necropsy (C). A: Time-course changes of body weight. Body weight was assessed before the induction of inflammation (day 0) and during the development of inflammation. B: Time-course changes in general health condition in the different experimental groups. In A and B, The arrows indicate the two indomethacin or vehicle treatments (experimental days 0 and 2). Data represent mean ± SEM, n = 6-7 per group. *, **, ***: P<0.05; 0.01 and 0.001 vs. respective control group; # P<0.05 vs. inflamed barrier/conventional group (ANOVA).

Myeloperoxidase levels

As shown in Fig. 3, in cecal and ileal samples from barrier/conventional and conventional control animals MPO levels were similar, and relatively low, regardless the area considered. Indomethacin treatment resulted in significant increases in MPO levels. In the cecum, MPO levels were

increased by 5-fold (P<0.05) and 4-fold (P<0.01) over controls in the barrier/conventional and the conventional groups respectively (Fig. 3). Similarly, in the ileum, MPO levels increased by 17-fold (P<0.05) and 40-fold (P<0.001) over controls in barrier/conventional and conventional animals, respectively (Fig. 3). Interestingly, ileal levels of MPO were higher in indomethacin-treated conventional animals than in barrier/conventional indomethacin-treated animals (P<0.05) (Fig. 3).

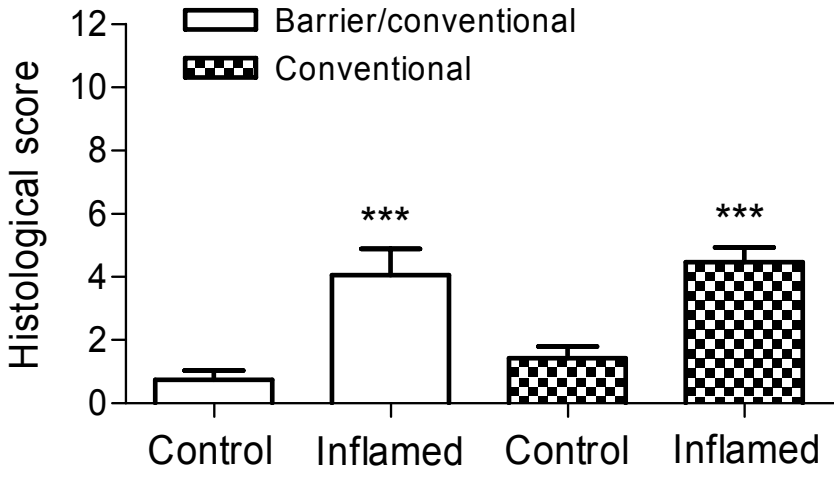

Figure 2. Histological scores for the ileum in the different experimental groups. See methods for details. Data are mean ± SEM of n = 6-7 per group. *** P< 0.001 vs. respective control (ANOVA).

Characterization of the luminal microbiota by FISH

Total cecal bacteria, determined by FISH as EUB338-positive cells, was similar in all experimental groups (Fig. 4). However, in the ileum, the total number of bacteria was higher in the control conventional group compared with the control barrier/conventional group (P<0.01) and, in both groups, showed a tendency to increase during inflammation (Fig. 5). Four days after the induction of inflammation the overall composition of the luminal microbiota changed slightly in the cecum and more strikingly in the ileum.

Composition of the luminal cecal microbiota, as determined by FISH, was similar in the conventional and de barrier/conventional control groups (Fig. 4). During inflammation, similar quantitative changes in the microbiota were

observed in conventional and barrier/conventional animals (Fig. 4). The main changes were observed in Gram negative bacteria (*Bacteroides* spp and enterobacteriaceae). The major variation was observed for Enterobacteriaceae population (ENT-D probe), which significantly increased during inflammation in both experimental groups (Fig. 4). On the other hand *Bacteroides* spp (BAC 303 probe) increased significantly in the barrier/conventional group while only a moderate increased, without

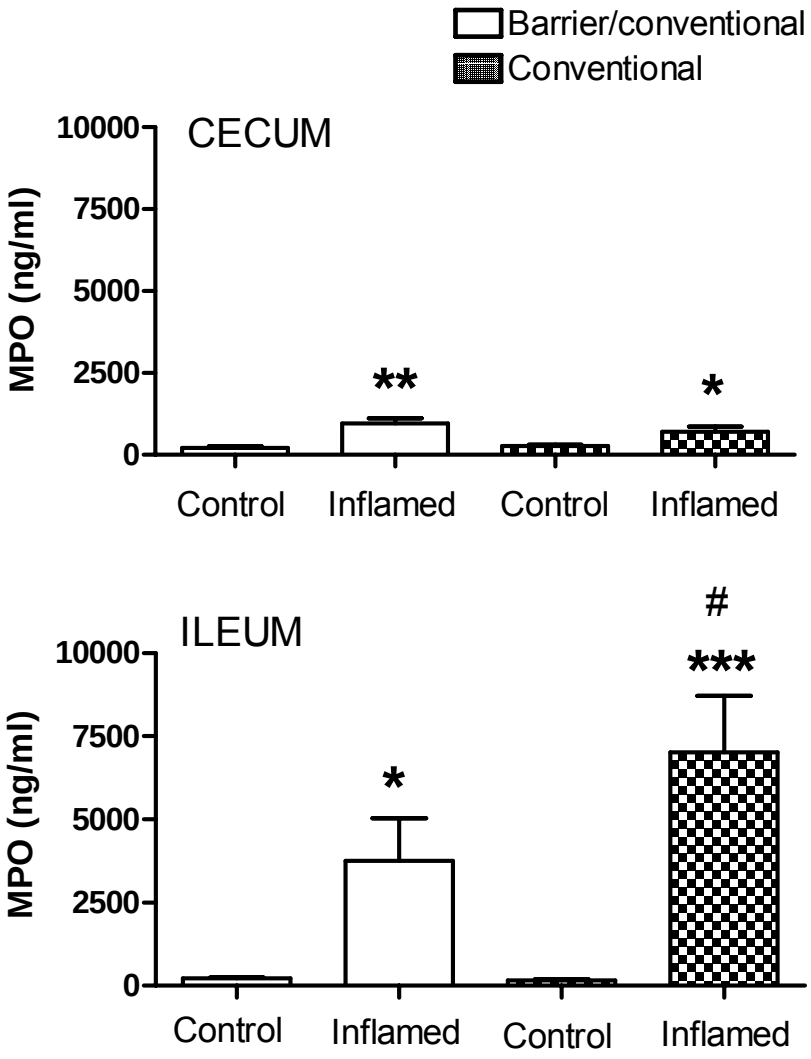

Figure 3. Tissue levels of MPO in the different experimental groups, at the time of necropsy. Data are mean ± SEM of n = 6-7 per group. *, ** P<0.05 or 0.01 vs. respective control group; #: P<0.05 vs. barrier/conventional inflamed group.

achieving statistical significance, was observed in the conventional group (Fig 4). Among Gram positive bacteria, only *Lactobacillus* spp and *Enterococcus* spp (LAB 158 probe) showed an increase during inflammation in the conventional group (Fig. 4). It is worthy to mention that variations in bacterial shape for *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa, EREC 482 probe) were observed during inflammation, particularly in the conventional group. In general, bacillary shapes, predominant in control conditions, were substituted by coccoidal shapes during inflammation. While this change was only occasionally observed in the barrier/conventional group; 6 out of the 7 animals in the conventional group showed a clear predominance of coccoidal-type organisms (Fig. 6).

As it relates to the composition of the luminal ileal microbiota in control conditions, *Bifidobacterium* spp (BIF164 probe), *Bacteroides* spp (BAC 303 probe) and enterobacteriaceae population (ENT-D probe) were significantly higher in the conventional group compared with the barrier/conventional (Fig. 5), while the *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa, EREC 482 probe) were virtually absent in both groups.

During inflammation, and regardless the experimental group considered, a general increase in all bacterial groups determined, except *Lactobacillus* spp and *Enterococcus* spp, was observed. The main finding was the appearance of the *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa, EREC 482 probe) at levels comparable to those of the other bacterial groups assessed (Fig. 5). In the ileum, changes in bacterial morphology during inflammation were less evident than in cecal samples.

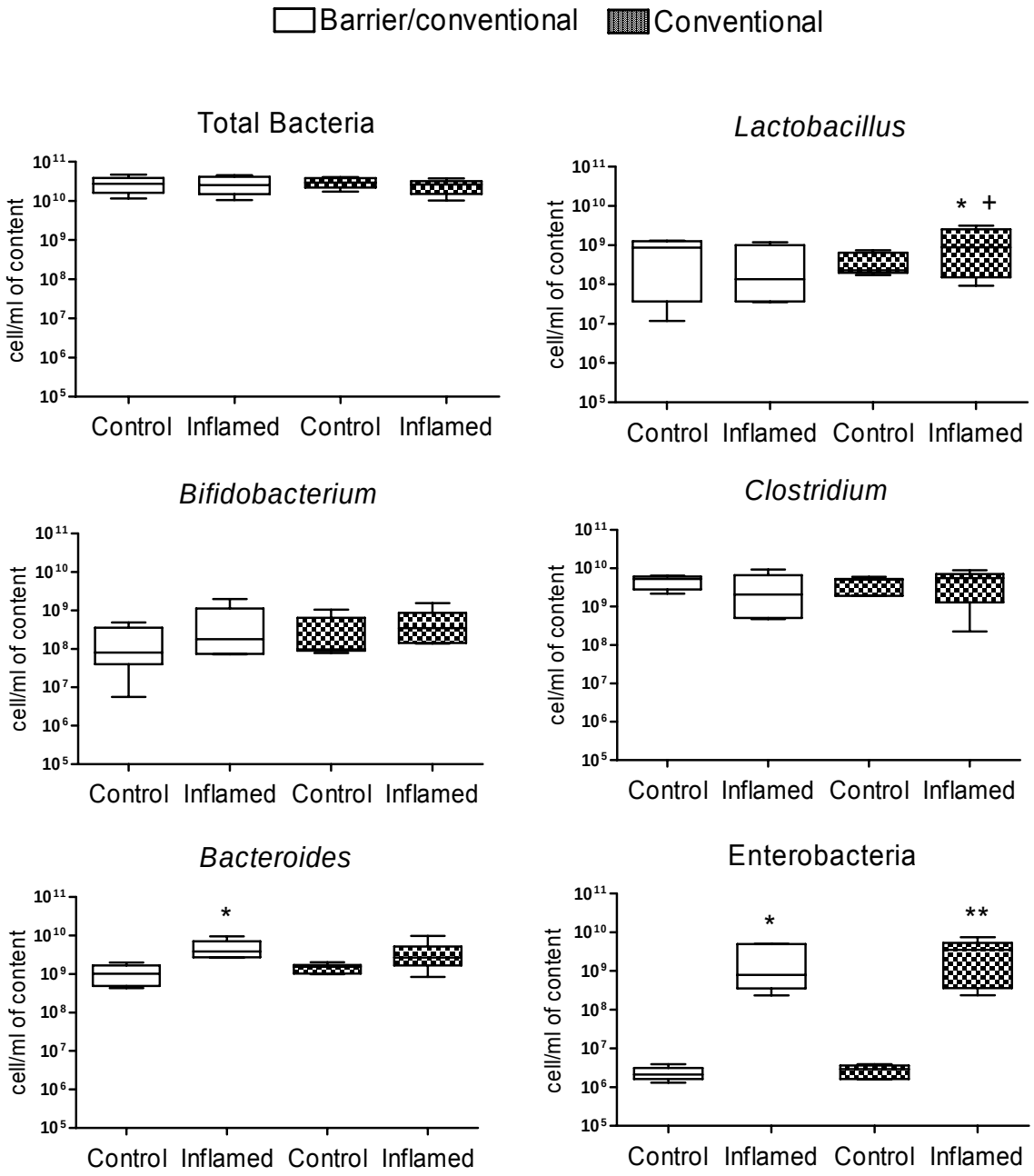

Figure 4. Cecal microbiota, as quantified by FISH, in the different experimental groups. Data are mean ± SEM, n = 6-7 per group. *, **: P<0.05 or 0.01 vs. respective control. +: P=0.06 vs. barrier/conventional inflamed group.

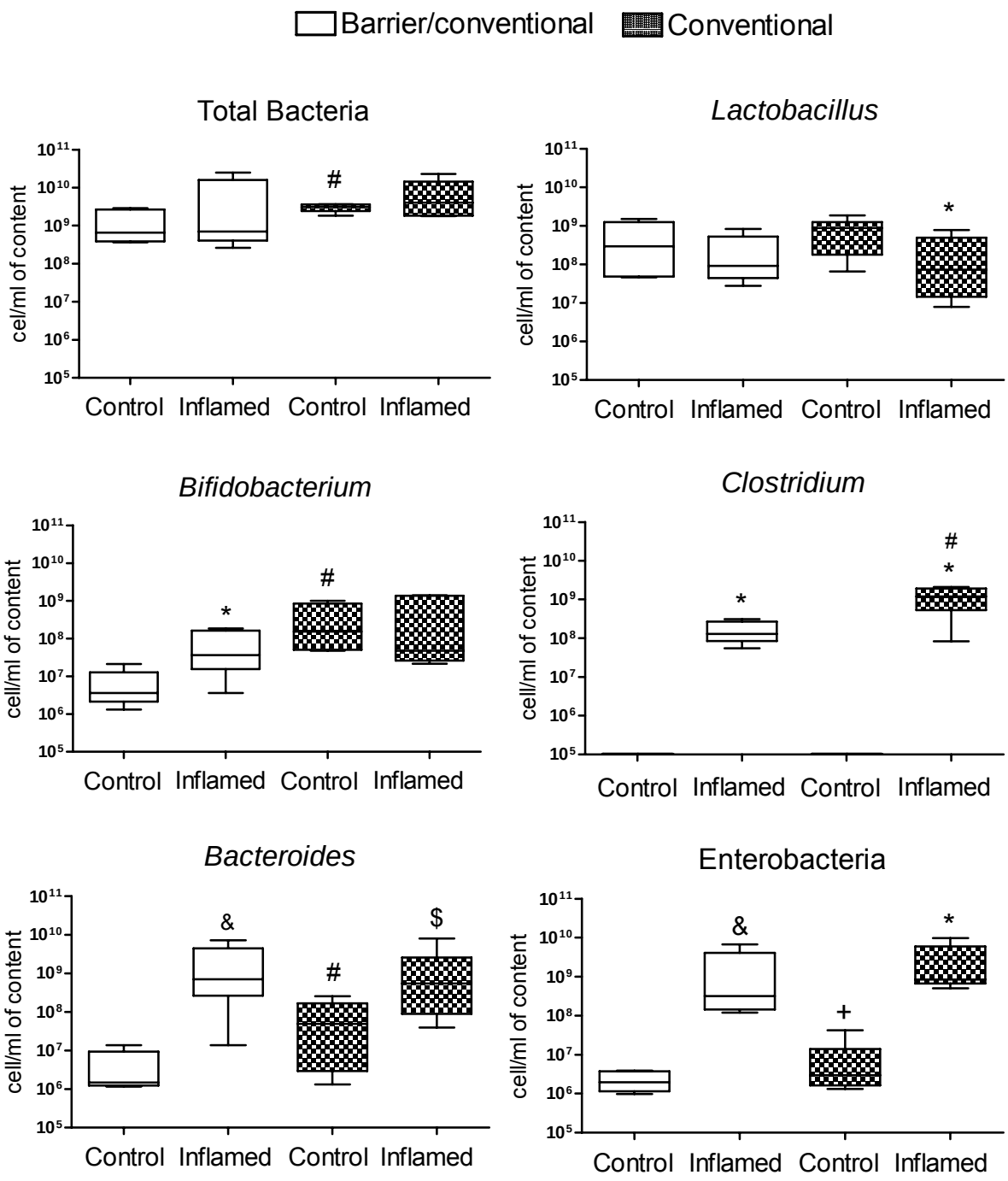

Figure 5. Ileal microbiota, as quantified by FISH, in the different experimental groups. Data are mean ± SEM of n = 6-7 per group. *: P<0.05 vs. respectively control; # P<0.05 vs seam treatment in barrier/conventional conditions; &: P=0.08 vs. barrier/conventional control; $: P=0.06 vs. conventional control; +: P=0.09 vs. barrier/conventional control.

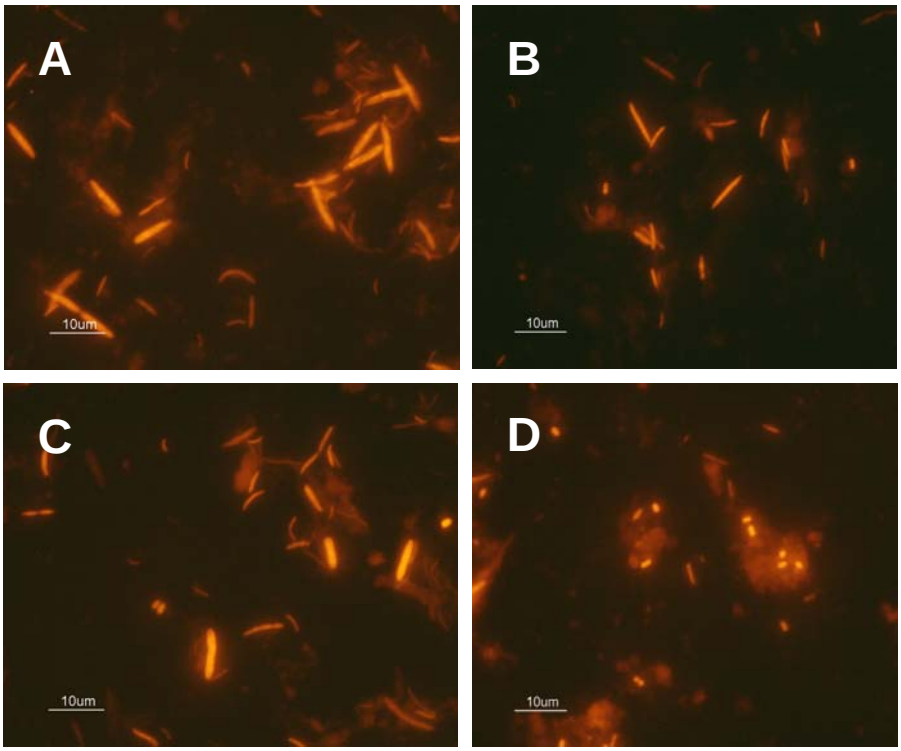

Figure 6. Representative microphotographs of cecal microbiota, as determined by FISH using the EREC 482 probe (Clostridium Cluster XIVa). (A) Barrier/conventional control, (B) Barrier/conventional inflamed, (C) Conventional control (D) Conventional inflamed. Notice how bacterial morphology changes in the conventional-inflamed group (D) to a coccobacillary shape when compared with the more typical bacillary shape observed in the other experimental groups.

Ecological characterization of the luminal microbiota: t-RFLP analysis

The similarity indexes of the t-RFLP profiles, illustrated in the form of a dendogram, of the cecal microbiota in the different experimental groups are shown in Fig 7A. Overall, the dendogram obtained showed two clearly separate clusters corresponding to the barrier /conventional and conventional groups in control conditions. The inflamed groups, either conventional or barrier/conventional, were partially overlapped forming a third, intermediate, cluster (Fig. 7A).

Nevertheless, the overall biodiversity of the microbiota was similar in the four experimental groups, with an average number of t-RFs (taken as a measure of biodiversity) witch varied from 14 to 35 among the different experimental groups (Fig 7B).

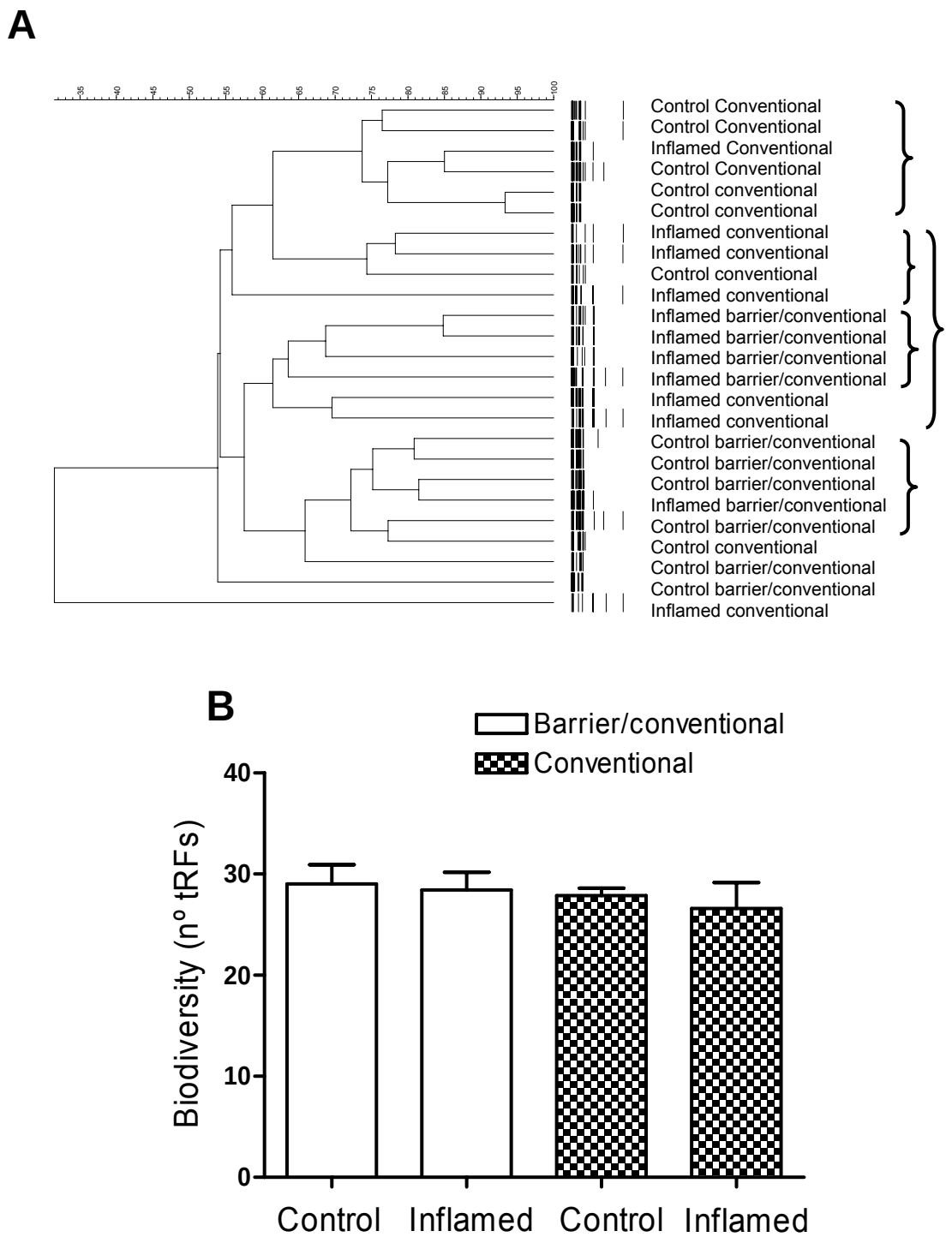

Figure 7. (A) Dendograma illustrating the clustering of the different experimental groups according to the t-RFLP banding patterns obtained from the analysis of the cecal microbiota. Notice how control groups are separated (conventional in the upper part and barrier/conventional in the lower part of the dendogram), suggestive of a different microbiota, while inflamed goups have an intermediate position, indicating similar inflammation-associated changes in microbiota. (B) Overall biodiversity among the different experimental groups, as determined by t-TRFLP.

Bacterial wall adherence

A DAPI staining showed the presence of bacteria adhered to the ileal wall in all experimental groups (data not shown). Table 2 shows the incidence of wall adherence for the bacterial groups determined by FISH in the different experimental groups. In control conditions, only *Lactobacillus* spp and *Enterococcus* spp (LAB 158 probe) were adhered to the ileal wall.

However, during inflammation the incidence of bacterial adherence increased and practically all bacterial groups assessed were found, in different degree, adhered to the ileal wall, regardless the experimental group considered. During inflammation, the more striking findings were the high incidence of adherence of enterobacteriaceae (ENT-D probe) (85-100 % of incidence) and the fact that adhesion of *Clostridium cocoides* Cluster IVa (EREC 482 probe) was only observed in the conventional group (5 out of 6 animals) (Table 2).

Table 2. Results from FISH of ileum wall specimens in acute studies after indomethacin treatment are expressed as positive cases/total number of rats.

	Barrier/ Conventional Control	Barrier/ conventional Inflamed	Conventional Control	Conventional Inflamed
Adhered Enterobacteria	0/5	5/5 **	0/6	6/7 **
Adhered *Bacteroides* spp	0/5	3/6 $^{+}$	0/7	1/7
Adhered *Clostridium cocoides* Cluster IVa	0/5	0/5 **	0/6	5/6 ** $^{\#}$
Adhered *Bifidobacterium* spp	0/6	1/5	0/5	4/6 *
Adhered *Lactobacillus* and *enterococcus* spp	2/5	1/5	4/6	5/7 $^{\$}$

Results are expressed as positive cases/total number of rats. *: P= < 0.05 vs. control conventional; **: P=<0.01 vs. respective control groups; $^{+}$: P=0.06 vs. barrier/conventional control; $^{\#}$: P<0.01 vs. Barrier/conventional inflamed; $^{\$}$: P<0.05 vs. barrier/conventional inflamed;

Expression of TLR-2 and TLR-4 in cecum and ileum

TLR-2 and TLR-4 expression was detected, with different intensities, in cecal and ileal tissues from all experimental groups. In control conditions, the levels of expression of TLR-2 and TLR-4 were lower in the cecum than in the ileum and of similar magnitude in conventional or barrier/conventional animals. In cecal tissues, expression of TLR-2 and TLR-4 increased by 3-fold during inflammation (Fig 8A-B). In the ileum, inflammation showed a similar trend to increase the level of expression of TLRs. However, statistical significance, both for TLR-2 and TLR-4, was achieved only in the barrier/conventional group (Fig 8C-D).

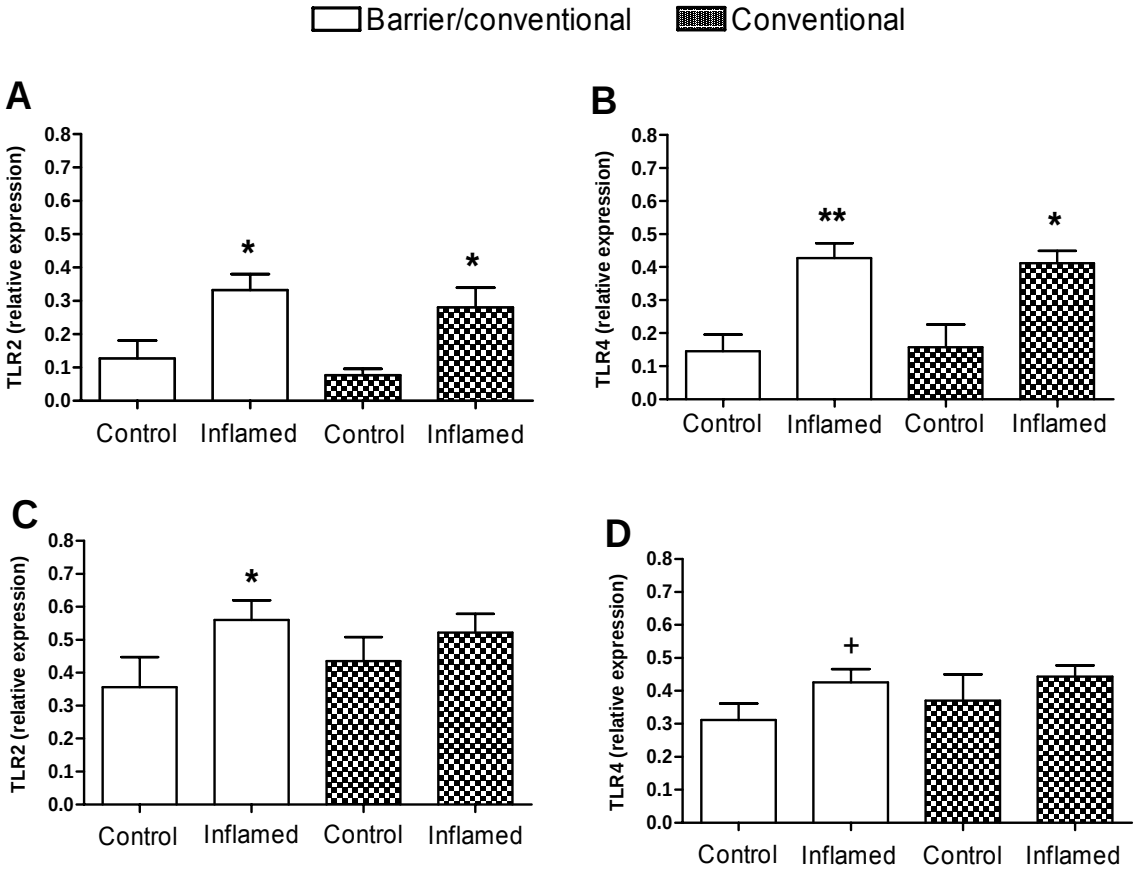

Figure 8. Expression of TLR-2 (A and C) and TLR-4 (B and D) in cecal (A and B) and ileal tissues (C and D) from the different experimental groups. *, **: P<0.05 or 0.01 vs. respective control; +: P=0.05 vs. respective control.

Discussion

In the present study we assessed gut inflammatory responses in a model of indomethacin-induced enteritis in rats bred in different microbiological conditions. Results obtained show that animals maintained in conventional conditions have a more severe acute inflammatory response to indomethacin than animals bred under barrier conditions but adapted to conventional conditions for a 3-week period. Gut commensal microbiota was different in these two experimental groups and was further modified during acute inflammation, thus suggesting a relationship between inflammation and microbiota.

During the last years the focus of microbial etiology has shifted from infectious to commensal agents. A substantial body of evidence has accumulated suggesting that commensal microbiota plays a key role in the development of intestinal immune and inflammatory responses and has, therefore, been included as an important pathogenic component of IBD (Sartor, 1997; Fiocchi, 1998; Shanahan, 2001; Danese *et al.*, 2004; Fiocchi, 2005). Total microbial count in the cecum was similar in animals bred in conventional conditions and in animals bred in barrier conditions adapted for 3 weeks to conventional conditions (barrier/conventional), and within the range previously described for the same experimental conditions (Terán-Ventura *et al*., 2009).

However, total ileal microbiota was reduced in number in barrier/conventional animals. This might reflect local differences in the adaptation process of the microbiota between ileum and cecum. As expected, adaptation to conventional conditions of barrier-bred animals implied a qualitative shift in microbiota from the original implantation bacteria towards a conventional-like situation. Again, this was particularly evident in the cecum, where microbiota was essentially identical between conventional and barrier/conventional animals. However, in the ileum, the microbiota was quantitatively different, being the relative abundance of *Bifidobacterium* spp, *Bacteroides* spp and enterobacteriaceae higher in the conventional than in the barrier/conventional

group. These differences in microbiota composition were corroborated by the t-RLFP analysis, which clearly grouped barrier/conventional and conventional animals in two separate clusters in the corresponding dendogram. In general, these variations in microbiota agree with our previous observations in similar experimental conditions (Terán-Ventura *et al*., 2009). The most interesting observation is that microbiota changed both qualitatively and quantitatively during inflammation.

Microbial changes were observed both in ileum and cecum, although were more prominent in the ileum. The main change in the cecum was an increase in the cell count of enterobacteriaceae, which was similar in conventional and barrier/conventional animals. On the other hand, in the ileum, *Bacteroides* spp and enterobacteriaceae increased and *Clostridium* cluster XIVa became a very significant component of the microbiota, while only occasionally observed in control conditions.

Interestingly, as mentioned above, during inflammation total bacteria was not altered in the cecum while in the ileum showed a tendency to increase in both conventional and barrier/conventional animals. Overall, these observations agree with data suggesting that during pathophysiological states gut microbiota changes both qualitatively and quantitatively (Andoh *et al.*, 2009). Moreover, this suggests that indomethacin-induced gut inflammation does not imply an indiscriminate bacterial overgrowth but a selective unbalance of the normal microbiota. This agrees with previous observations in other models of gut inflammation, suggesting the presence of selective changes in the microbiota (Konaka *et al.*, 1999; Hoentjen *et al.*, 2005; Larrosa *et al.*, 2009; Natividad *et al.*, 2009). In this sense, the qualitative and quantitative variations observed here are, in general, consistent with those described in rats using other models of intestinal inflammation (Basivireddy *et al.*, 2005; Dalby *et al.*, 2006; Natividad *et al.*, 2009; Watanabe *et al.*, 2009). However, it is worthy to mention that the significant increase in the counts of *Bacteroides* and *Clostridium* contrasts with previous reports indicating that these bacterial groups were relatively

rare during indomethacin-induced enteritis in rats (Dalby *et al.*, 2006; Watanabe *et al.*, 2009).

Enterobacteriaceae, and more specifically *Escherichia coli*, are associated with the aggravation of colitis (Rath *et al.*, 2001; Heimesaat *et al.*, 2007). Interestingly, the enterobacteriaceae genus was increased during inflammation, with conventional animals showing a tendency to have higher counts than barrier/conventional, which showed a rather large variability. Moreover, bacterial adherence data showed that virtually all inflamed animals were positive for Enterobacteriaceae adherence. These observations also agree with previous data demonstrating a direct involvement of *E. coli* in indomethacin-induced enteritis in rats (Konaka *et al.*, 1999; Porras *et al.*, 2004, 2008). Likewise, *Bacteroides* spp and *Clostridium* spp (cluster XIVa) have been associated with gut inflammation (Swidsinski *et al.*, 2005[a, b]). This agrees with the prominent increase observed in these groups after indomethacin treatment and also with the presence of bacterial adherence in inflamed animals.

Overall, these observations suggest a direct role of this bacterial types affecting the epithelial and immune responses during inflammation, likely acting as pathogenic factors, as previously described (Konaka *et al.*, 1999; Porras *et al.*, 2004; Swidsinski *et al.*, 2005[a, b]; Rolhion and Rfeuille-Michaud, 2007).

Immune responses to intestinal bacteria are mediated, at least partially, by the interaction of bacterial wall components with TLRs (Takeuchi *et al.*, 1999; Cario, 2005). Among TLRs, TLR-2 and TLR-4 have been reported as the main partners interacting with the gut microbiota during inflammation (Fujisawa *et al.*, 2006; Szebeni *et al.*, 2008). In the present conditions, relative expression of TLRs in control conditions, regardless the group considered, was lower in the cecum than in the ileum and similar to the expression levels previously described by us in animals maintained in similar conditions (Terán-Ventura *et al*., 2009). During inflammation, TLR-2 and TLR-4 expression increased in both cecal and ileal tissues and in similar

degree in conventional and barrier/conventional animals. Interestingly, relative changes in expression were higher in the cecum than in the ileum. This might be due to the higher basal levels of expression observed in the ileum vs. the cecum and suggest that multiple factors, likely including components of the microbiota not directly analyzed, and inflammation-dependent stimuli influence TLR expression. In this sense, it is known that regulation of TLR expression depends upon multiple microbial factors that might interact in different manner depending upon the conditions considered (Heine *et al.*, 1999; Backhed *et al.*, 2003; Eckburg *et al.*, 2005). In our conditions it is difficult to ascertain if the observed changes in expression are associated to variations of the microbiota or to the inflammatory challenge. In any case, the results suggest an interaction between flora and inflammation modulating, at least, TLR-2 and TLR-4 expression.

Indomethacin administration induced an inflammatory response that was evident in the ileum and less clear in the cecum. This agrees with the responses previously characterized by us using this model of enteritis in rats, which results in the induction of ileitis (Porras *et al.*, 2004, 2006, 2008). Disease activity parameters evaluated suggest a slightly higher inflammatory response to indomethacin in conventional than in barrier/conventional animals. Although other adaptive differences might account for this, the variations in gut microbiota, particularly within the ileum, might account for this different susceptibility to inflammation. This agrees with a growing body of evidence suggesting that gut microbiota is a key component modulating inflammatory responses within the gut (Umesaki and Setoyama, 2000; Garcia-Lafuente *et al.*, 2001; Alexopoulou and Kontoyiannis, 2005; Kelly and Conway, 2005).

In general, a restricted, or even absent (such as the case of germ free animals), gut microbiota is regarded as a protective condition against inflammation while a rich and diverse microbiota is considered as proinflammatory (Hajjar *et al.*, 2002; Backhed *et al.*, 2003; Bamias *et al.*, 2007). Our results partially agree with this view. In the present experimental conditions, conventional and barrier/conventional animals showed a similar

ecological diversity, as established by the t-RLFP analysis. However, conventional animals showed a clear tendency to have a higher number of total microbiota when compared with barrier/conventional animals, and had higher counts of the main bacterial groups characterized, namely, *Bifidobacterium* spp, *Bacteroides* spp and enterobacteriaceae. Together with the disease activity indices recorded these observation supports a major susceptibility towards inflammation when animals are bred and maintained in conventional conditions vs. animals bred in barrier conditions and adapted for a short period of time to conventional conditions. Moreover, this agrees with data showing that intestinal inflammation was aggravated in mice maintained in conventional conditions vs. those maintained under controlled conditions (Mañé, 2007).

In summary, we showed that acute inflammatory responses to indomethacin in rats bred and maintained in conventional conditions or in rats bred in barrier conditions briefly adapted to a conventional microbiological environment are associated to specific changes in gut microbiota and the microbiota recognitions systems (TLR-2 and TLR-4), at least within the ileum and cecum. Interestingly, animals bred in conventional conditions showed a tendency to have a worse inflammatory response when compared to adapted animals. These observations confirm an important role of gut microbiota modulating immune responses in the intestine and its involvement in the pathogenesis of IBD.

Furthermore, differences between conventional and barrier/conventional animals suggest a certain imprinting in barrier animals, likely associated to the characteristics of the originally implanted microbiota, that differentially modulates inflammatory responses, despite the adaptation to a new microbiological status. These observations warrant comparative studies assessing the acute and chronic inflammatory responses to indomethacin in animals maintained in strict barrier conditions. Furthermore, they suggest that the housing conditions followed in animal studies might have a significant impact in the outcome of studies on gut inflammation. Overall, these studies might help to understand the broad impact of microbiota in gut health and

might contribute to the design of effective biological treatments against inflammatory disorders of the gastrointestinal tract.

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Capítulo 3

Gut microbiota affects cannabinoid receptors expression in the intestine of rats

Abstract

Gut commensal microbiota has been suggested as a key component of gut homeostasis, implicated in the pathophysiology of inflammatory and functional gastrointestinal disorders. We determined spontaneous adaptive changes in gut commensal microbiota in rats bred under microbiologically-controlled conditions (barrier), under standard conditions (conventional) and in barrier animals adapted to standard conditions (barrier/conventional); and correlated these changes with the expression of cannabinoids receptors, CB_1 and CB_2, within the gut. Cecal commensal microbiota was analyzed using fluorescence in situ hybridization (FISH), and the expression of CB_1 and CB_2 receptors by RT-PCR. Total number of cecal bacteria was similar in the three groups. Adaptation to conventional conditions implicated a spontaneous reduction of the number of strict anaerobic bacteria (*Bacteroides* spp and *Clostridium* spp), while increasing the number of *Bifidobacterium* spp and *Lactobacillus* spp. Expression levels of CB_1 and CB_2 receptors were higher in the barrier-bred group and decreased with the adaptation to conventional conditions. Changes in cannabinoid receptors expression correlated positively with spontaneous changes in *Bacteroides* spp and *Clostridium* spp and negatively with *Bifidobacterium* spp. These observations indicate that gut commensal microbiota modulates the intestinal expression of neuro-immune modulatory systems within the gut and might influence gastrointestinal sensory functions, such are visceral pain responses.

Introduction

Gut commensal microbiota is a dynamic microbiological system comprised by numerous bacterial species (Berg, 1996; Merriam *et al.*, 2008; Camp *et al.*, 2009). During the last years, numerous evidences support a critical role for these commensal bacteria in the maintenance of gut homeostasis. For instance, gut commensal microbiota appears to be of crucial importance in the modulation of local immune responses and, through these mechanisms, to be a pathogenic component of inflammatory and functional gastrointestinal alterations, such as Inflammatory Bowel Disease (IBD) or Irritable Bowel Syndrome (IBS) (Marteau, 2002; Xavier and Podolsky, 2007; Collins and Bercik, 2009; Looijer-van Langen and Dieleman, 2009). Several reports suggest that commensal microbiota composition is altered in both IBD and IBS patients.

Therefore, commensal microbiota modulation through the administration of different probiotics and/or prebiotics has become attractive, and largely explored, approach for the treatment of these alterations. This is based in numerous studies, both in animals and humans, suggesting that specific microbial agents might modulate immune responses, local epithelial functions, motor activity, afferent sensory information associated to visceral sensitivity and even central nervous system responses associated to intestinal stimuli (Marteau, 2002; Kajander *et al.*, 2005; O'Mahony *et al.*, 2005; Fan *et al.*, 2006; Verdú *et al.*, 2006; Wildt *et al.*, 2006[b]; Rousseaux *et al.*, 2007; Diop *et al.*, 2008; Williams *et al.*, 2008; Collins and Bercik, 2009; Honda and Takeda, 2009). However, the mechanisms mediating these effects remain largely unknown.

Recent data derived from animal models of IBS evaluating sensory information arising from the gut suggest that the commensal microbiota might influence neuronal and/or neuro-immune mechanisms within the gut. In mice and rats, modificactions of the intestinal commensal microbiota result in changes in nociceptive responses that have been associated to alterations in substance P, opioid and cannabinoid dependent systems, all of them neuro-

immune mediators implicated in sensory mechanism, including pain responses, within the gut (Verdú *et al.*, 2006; Rousseaux *et al.*, 2007).

Based on these observations, and taking into account the growing interest in the endocannabinoid system as a potential target for the treatment of pain and inflammatory related alterations of the gastrointestinal function, we aimed to correlate changes in gut commensal microbiota with variations in cannabinoid receptors 1 and 2 (CB_1 and CB_2) expression within the gut. For this, we characterized spontaneous changes of gut commensal microbiota in rats born and bred under barrier conditions but adapted for a period of three weeks, during adulthood, to conventional conditions; and assessed in the same animals the intestinal expression of CB_1 and CB_2 receptors.

Materials and methods

Animals

Twelve 6 week old and eleven 9 week old male OFA Sprague-Dawley rats bred and maintained in a barrier protected area with all materials, water, food and bedding sterilized before entering the barrier were obtained from Charles River Laboratories (Lyon, France). The original microbiota inoculated in these animals (as provided by the breeder) consisted of: *Bacteroides distasonis*, *Lactobacillus acidophilus*, *Lactobacillus salivaris*, Schaedler fusiform-shaped bacterium, 3 strains of CRL fusiform-shaped bacterium, CRL mouse spirochete, *Escherichia coli* (non haemolytic) and *Streptococcus faecalis* (group D), *Enterococcus* spp. In addition, fifteen 9 week old male OFA Sprague-Dawley rats bred in conventional conditions in the Animal Facility of the Universidad Autónoma de Barcelona were used. This conventional colony was established in 1994 from OFA Sprague Dawley rats from Charles River Laboratories. When in conventional conditions, water, food and bedding were given to animals as facilitated by the commercial provider, without any further treatment. All procedures were approved by the Ethical Committee of the Universidad Autónoma de Barcelona (UAB).

Experimental groups and sample collection

Three experimental groups were defined: 1) 9 week old barrier-bred rats (barrier group) (n=12); 2) 9 week old rats bred under conventional conditions (conventional group) (n=11); and 3) 6 week old barrier rats maintained under conventional conditions at the animal facility of the UAB for 3 weeks (barrier/conventional group) (n=15). All animals were 9 week old at the time of testing.

Animals were euthanized by CO_2 inhalation followed by thoracotomy. Thereafter, the abdominal cavity was opened, the cecum localized and cecal contents (by 5 g, under sterile conditions) and a tissue sample were collected and frozen immediately with liquid nitrogen. Al samples were stored at -20 (content) or -80 ºC (tissues) until analysis.

Enumeration of bacteria using fluorescence in situ hybridization (FISH)

For FISH, general methods previously reported were followed (Amman *et al*, 1990; Terán-Ventura *et al.*, 2009). Oligonucleotide probes consisted of a single strain DNA covalently linked with Cy3 at the 5'-end. Probes used were: EUB 338 (5'GCTGCCTCCCGTAGGAGT3') to total Bacteria; NON 338 (5'ACATCCTACGGGAGGC3') to non bacteria (negative control); BAC 303 (5'CAATGTGGGGGACCTT3') to *Bacteroides* spp; EREC 482 (5'GCTTCTTAGTCAGGTACCG3') to Clostridium Cluster XIVa; LAB 158 (5'GGTATTAGCACCTGTTTCCA3') to *Lactobacillus* spp and *Enterococcus* spp; ENT-D (5'TGCTCTCGCGAGGTCGCTTCTCTT3') to enterobacteria; and BIF 164 (5'CATCCGGCATTACCACCC3') to *Bifidobacterium* spp. All probes were obtained from Tib MolBiol (Mannheim, Germany).

Hybridized slides were viewed under oil immersion, using a Nikon Fi60 epifluorescence microscope equipped with a filter for Cy3. For quantification of bacteria, 25 randomly selected fields were counted for each sample (in duplicate)

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cecal tissue samples using RNAwiz (Ambion, Madison, WI) and treated with DNA-free (Ambion, Madison, WI) for 30 min at 37ºC. cDNA was synthesized from 5 µg total RNA in a reaction mixture of 50 µl containing 0.5 µg of oligo 18 (dT) primer (Ambion, Madison, WI), 2nM dNTP (Ecogen, Barcelona, Spain), and 10 Units Moloney Murine Leukemia Virus (MMLV) (Ambion, Madison, WI). The resulting cDNA was amplified in a total volume of 50 µl with 1 unit of taqDNA, 1 mM dNTP mixture, and 0.5 µM primers for GAPDH (140 bp ; sense: 5'-ATGAGCCCTTCCACGATGCC-3'; antisense: 5'-CCGCCCCTTCCGCTGATGCC-3'), CB1 receptor (153 bp ; sense: 5'-CTACTGGTGCTGTGTGTCATC -3'; antisense: 5'-GCTGTCTTTACGGTGGAATAC-3') or CB2 receptor (75 bp ; sense5'-AGCAGGAGTTGGGAGGAGACT-3'; antisense 5'-

CTGAATCTGCCAGAGACAGCAT-3') (Merriam, et al., 2008) (Proligo-Sigma, Madrid, Spain).

The PCR amplification protocol was: 35 cycles with 1 min of denaturation at 95ºC, 1 min of annealing at 50ºC, and 1 min of extension at 72ºC on a thermal cycler (Techno Cambridge Ltd.). Amplified products were electrophoresed on 2% agarose gel in TAE buffer, stained with ethidium bromide, photographed under ultraviolet light, and quantified using image-analyzing software (Quantity-One, Bio-Rad laboratories). For semiquantification, the ratio of the optical density of each PCR product and GAPDH was determined.

Statistical Analysis

Data are expressed as mean±SEM. Comparison between multiple groups were performed using a one-way analysis of variance (one-way ANOVA), followed when necessary, by a Student-Newman-Keuls multiple comparisons test. Results were considered statistically significant when $P<0.05$.

Results

Characterization of intestinal (cecal) commensal microbiota

Total bacteria, determined by FISH as EUB338-positive cells, was between 3 × 10^{10} and 4 × 10^{10} cell/ml and was similar in all experimental groups (Table 1), within the margins previously described (Dinoto *et al*., 2006). However, t-RLFP analysis revealed significant differences among groups in commensal microbiota composition, with a good clustering of the animals included in the three experimental conditions (data not shown). These differences were also confirmed when specific bacterial groups were analyzed by FISH.

Among Gram negative bacteria, the counts of enterobacteriaceae (ENT-D probe) were scarce (in most cases less than 1% of the flora quantified) and of similar magnitude in all experimental groups (Table 1). In contrast, *Bacteroides* spp group (BAC 303 probe) were relatively abundant and significantly higher in the barrier group when compared with the conventional and the barrier/conventional groups (Table 1).

In particular, *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa, EREC 482 probe) accounted for the largest bacteria population in all experimental groups (Table1), with higher counts in the barrier group when compared with the conventional group, while the barrier/conventional group showed an intermediate situation (Table 1). Counts for *Lactobacillus* spp and *Enterococcus* spp (LAB 158 probe) and *Bifidobacterium* spp (BIF164 probe) were higher in cecal samples from the conventional group when compared with the barrier/conventional and barrier groups. Overall the *Bifidobacterium* spp was scarce in the barrier group while the barrier/conventional group showed a transition towards the conventional conditions (Table 1).

Interestingly, the number of enterobacteriaceae, *Bifidobacterium* spp, *Bacteroides* spp, *Lactobacillus* spp and Clostridium cluster XIVa group as a

whole was lower in the conventional vs. the barrier group and reduced during the adaptive process (Table 1).

Table 1. Bacterial counts in the different experimental groups, as determined by FISH[a]

	Barrier Group (× 10^8 cells/ml)	Barrier/conventional Group (× 10^8 cells/ml)	Conventional Group (× 10^8 cells/ml)
Total Bacteria[b]	352 ± 55	416 ± 64	399 ± 41
Specific total bacteria as quantified by FISH [c]	108.7 ± 12.5	91.8 ± 13.2[&]	58.8 ± 7.1**
Enterobacteriaceae	0.1 ± 0.05	0.7 ± 0.5	0.09 ± 0.02
Bacteroides spp	25 ± 2.2	17 ± 3.6*	12.5 ± 1.0**
Clostridium coccoides -Eubacterium rectale (*Clostridium* Cluster XIVa)	82.3 ± 11.1	69 ± 12.5[&]	37.6 ± 5.4**
Lactobacillus and *Enterococcus* spp	1.1 ± 0.2	4 ± 1.2*	5.5 ± 2.1*
Bifidobacterium spp	0.01 ± 0.005	1.5 ± 0.7*	3 ± 1.2 *

[a]: Data are mean±SEM, n=10-15 per group.
[b]: Total cecal bacterial counts as determined by FISH using the probe EUB 338.
[c]: Total bacterial counts for all specific genera identified by FISH (Enterobacteriaceae, *Bacteroides* spp, *Clostridium coccoides-Eubacterium rectale*, *Lactobacillus-Enterococcus* spp and *Bifidobacterium* spp).
*,**: P <0.05 and 0.01 vs. barrier group, [&]: P < 0.05 vs. conventional group (ANOVA).

Expression of CB_1 and CB_2 receptors and correlation with gut commensal microbiota

In all cases, CB_1 and CB_2 transcripts were clearly identifiable. Expression of CB_1 receptors was comparatively lower than that of CB_2, regardless the experimental group considered. Expression of CB_1 receptors was similar among groups, although a clear tendency to be increased in the barrier group was observed [F (2,35)=1.411, P=0.275] (Fig. 1). On the other hand, CB_2 expression showed a significant linear trend (P=0.0014) to decrease its expression with the adaptation from barrier to conventional conditions. Levels of expression were about 2-fold higher in the barrier group compared with the conventional group [F (2,35)=6.090, P=0.0054; barrier vs. conventional:

P<0.01], while the barrier/conventional group had intermediate levels of expression (Fig. 1).

Expression levels of CB_1 and CB_2 receptors showed a positive correlation with the total number of specific bacterial genus, as determined by FISH; however, statistical significance was only achieved for CB_2 (P=0.0032) while CB_1 showed a clear tendency (P=0.0892) (Fig. 2). In particular, expression levels of CB_1 receptors showed a positive linear correlation with the counts of *Bacteroides* spp. (P=0.02) and a tendency for negative correlation with *Bifidobacterium* spp (P=0.0948) (Fig. 2). Similarly, expression levels of CB_2 showed positive correlation with the content of *Bacteroides* spp (P=0.0002) and the *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa) (P=0.0081), while correlating negatively with *Bifidobacterium* spp (P=0.0116) (Fig. 2).

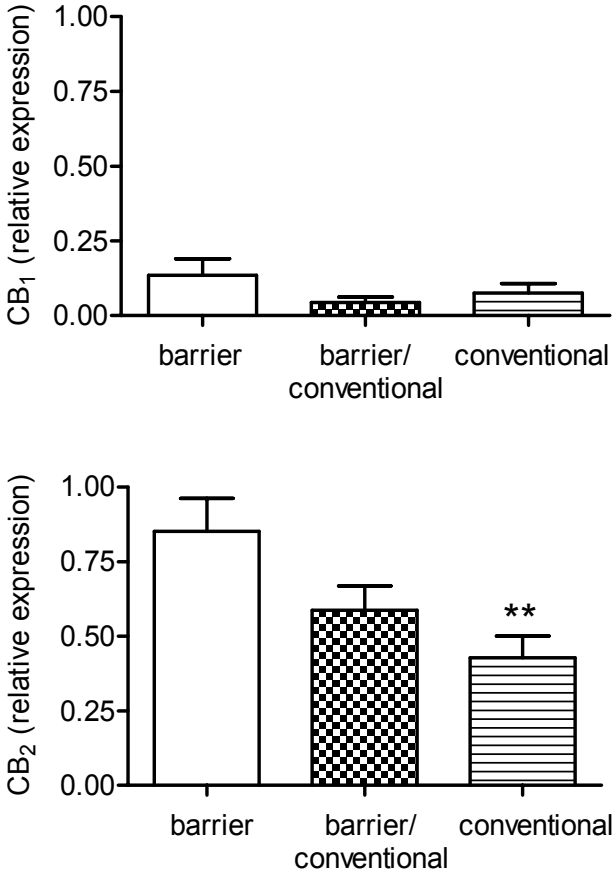

Fig. 1 Relative expression of CB_1 (upper panel) and CB_2 receptors (lower panel) in cecal tissues from animals bred and maintained under barrier conditions, barrier animals adapted for 3 weeks to conventional conditions and animals bred and maintained in conventional conditions. Data are mean±SEM of n=11-15 per group. **: P< 0.01 vs. the barrier group (ANOVA).

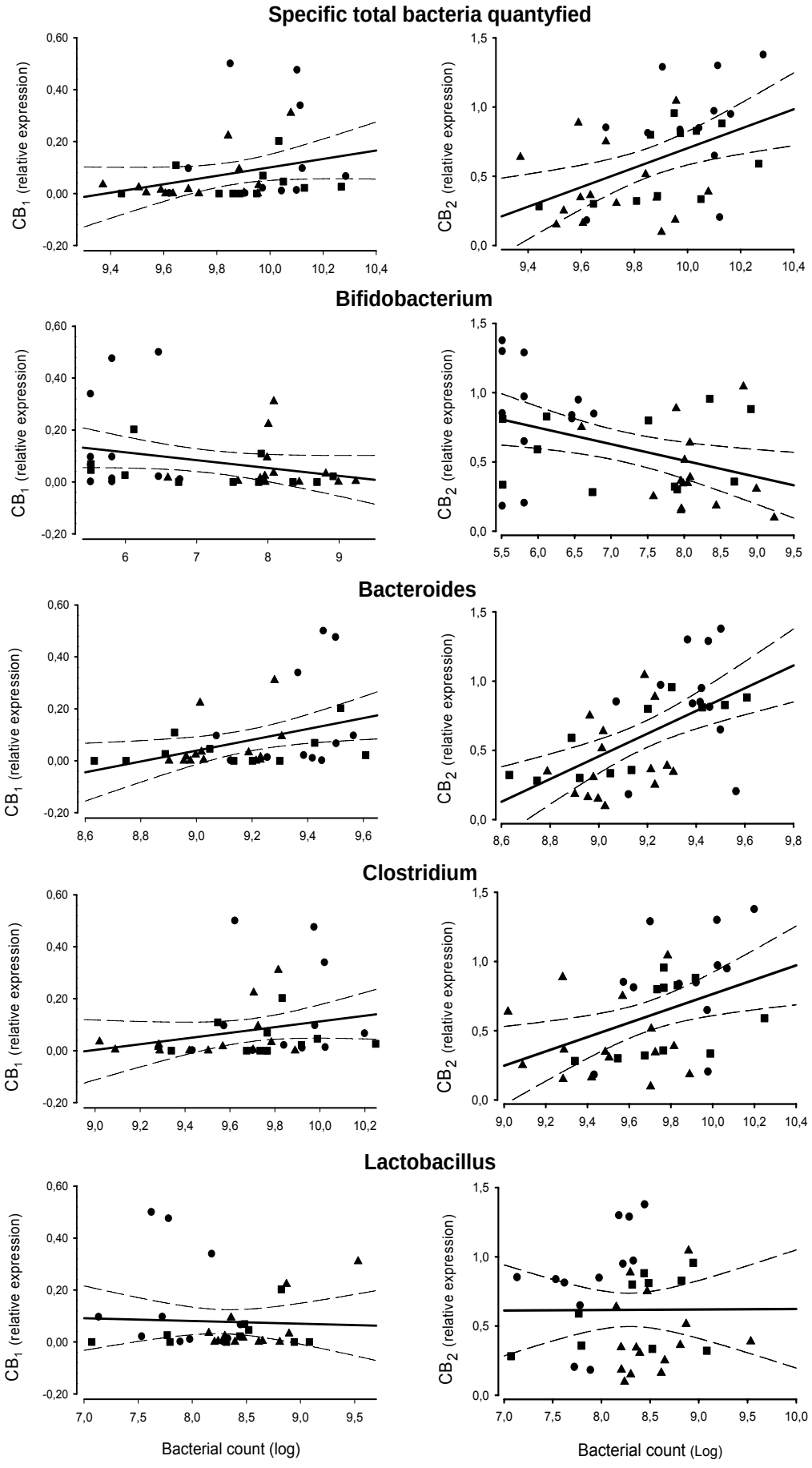
Specific total bacteria quantyfied
Bifidobacterium
Bacteroides
Clostridium
Lactobacillus
CB1 (relative expression)
CB2 (relative expression)
Bacterial count (log)
Bacterial count (Log)

Fig. 2 (Previous page) Correlation between the relative expression of CB_1 (left column) and CB_2 receptors (right column) and the bacterial counts corresponding to the main bacterial groups present in the cecal commensal microbiota, as determined by FISH. Each point represents an individual animal. Broken lines represent the 95% confidence interval.

Discussion

During the last years there is an increasing interest in the role of gut commensal microbiota in the maintenance of gastrointestinal homeostasis. Here we present, for the first time, evidence that spontaneous variations in gut microbiota, related to environmental adaptation, are associated with changes in the expression of cannabinoid receptors, CB_1 and CB_2, within the gut.

Numerous reports have implicated the intestinal commensal microbiota in the development of immune responses within the gut and have identified bacterial flora as a significant component of the pathogenesis of several intestinal disorders, including inflammatory bowel disease and irritable bowel syndrome (IBS) (Collins and Bercik, 2009), . Following these observations, several studies suggest that microbiota might influence neurally-mediated responses within the gut. In particular, bacterial flora has been implicated in the modulation of sensory mechanisms arising from the gut and, therefore to the modulation of visceral pain-related responses. Clinical and preclinical studies have shown that administration of certain probiotics may prevent abdominal symptoms in IBS patients (pain and discomfort, bloating or altered bowel habits) and also reduce visceral pain-related responses in animal models of the disease (Kajander *et al.*, 2005; O'Mahony *et al.*, 2005; Fan *et al.*, 2006; Wildt *et al.*, 2006[a]; Rousseaux *et al.*, 2007; Diop *et al.*, 2008; Williams *et al.*, 2008). Despite the clinical interest of these observations the mechanisms mediating these beneficial effects remain largely unknown. Preliminary observations suggest that specific bacterial types might modulate the endogenous expression of several mediators implicated in the neuronal mechanisms of visceral sensitivity. For instance, it has been shown that a specific strain of *Lactobacillus* (*L. acidophilus*) is able to modulate the content

of CB_2 and μ-opioid receptors in the gut and to reduce visceral pain responses in rats (Rousseaux *et al.*, 2007). These results agree with the present observations showing that spontaneous adaptive variations of the gut commensal microbiota implicated changes in the intestinal expression of CB_1 and CB_2 receptors.

In the present study, we did not correlate changes in CB receptor expression with any particular strain of bacteria, but with large spontaneous changes in gut commensal microbiota. From our observations, spontaneous changes in *Bacteroides* spp, *Bifidobacterium* spp and *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa) correlated with the expression of CB receptors. Relative counts of the *Bacteroides* spp and *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa) correlated positively with CB_2 expression, with only *Bacteroides* spp correlating positively with CB_1 expression.

On the other hand, *Bifidobacterium* spp showed a negative correlation with both CB_1 and CB_2 expression. Previous studies have pointed towards two main genera of bacteria as having beneficial effects in gut homeostasis, including the modulation of visceral pain-related responses: *Lactobacillus* spp and *Bifidobacterium* spp (Kajander *et al.*, 2005; O'Mahony *et al.*, 2005; Wildt *et al.*, 2006[a]; Fan *et al.*, 2006; Rousseaux *et al.*, 2007; Diop *et al.*, 2008; Williams *et al.*, 2008).

In our conditions, spontaneous changes in *Bifidobacterium* spp, but not in *Lactobacillus* spp, correlated with variations in cannabinoid receptors expression. This contrasts with the only previous report in which gut microbiota was related with the content of CB_2 receptors in the gut, which implicated *Lactobacillus,* but not *Bifidobacterium*, in that effect (Rousseaux *et al.*, 2007). This apparent discrepancy might be related to the fact that Rousseaux (2007) inoculated specific strains of *Lactobacillus* or *Bifidobacterium*, without assessing other changes in the gut microbiota, while here, gross and simultaneous changes in gut commensal microbiota occurred spontaneously.

Interestingly, expression levels of cannabinoid receptors in conventional conditions were rather low, particularly for CB_1 receptors. Both CB_1 and CB_2 receptors have been implicated in visceral pain mechanisms, with CB1 participating mainly in normal pain response while both receptors being involved in pain responses during inflammatory states (Sanson *et al.*, 2006; Wright *et al.*, 2008; Brusberg *et al.*, 2009). The observed variation in the expression of cannabinoid receptors might suggest differences in pain responses between animals maintained in barrier or standard conditions or during the adaptation process from one environment to the other. This agrees also with observations in mice showing that large alterations of gut microbiota associated to the treatment with antibiotics or by adaptation to a standard, non-sterile environment, developed visceral hypersensitivity (Verdú *et al*., 2006).

Although not assessed in the mentioned report, these changes in viscerosensitivity correlate with a reduction in CB_1 and CB_2 expression associated to adaptive changes in the gut microbiota, as suggested here. Interestingly, no indications of spontaneous inflammation were detected in none of the experimental groups suggesting that the variations in CB_2 expression are not linked to any state of inflammation or to immune activation within the gut. Therefore, the observed changes might represent a direct modulatory effect of the microbiota in receptor expression rather a secondary effect associated to gut inflammation.

In the present study, the exact location of cannabinoid receptors has not been evaluated. Nevertheless previous characterizations of the distribution of cannabinoid receptors within the gut suggest that epithelial and neural structures (enteric nervous system and extrinsic afferent innervation) as well as the resident immune system could be sites of expression of cannabinoid receptors within the gut (Ahluwalia *et al.*, 2000; Coutts *et al.*, 2002; Casu *et al.*, 2003; MacNaughton *et al.*, 2004; Wright *et al.*, 2008). This rather heterogeneous distribution reinforces the concept that epithelial, immune and

neural functions interact within the gut to generate physiological and pathophysiological responses.

The mechanisms through which gut commensal microbiota influences the expression of neuroimmune mediators remain largely unknown. Extensive work has demonstrated that the microbiota interacts with the internal milieu through specific bacterial recognition systems. These systems, with the so call toll-like receptors, TLR, as main exponent, recognize bacterial components throughout specific epithelial receptors which in turn modulate specific cell functions, such as the release of a variety of immune modulatory mediators (Rakoff-Nahoum *et al.*, 2004; Albiger *et al.*, 2007; Honda and Takeda, 2009). We have previously shown that spontaneous adaptive variation of the gut microbiota are associated to changes in bacterial recognition systems, particularly in the expression levels of the toll-like receptors 2 and 4 (TLR-2 and -4) (Terán-Ventura *et al.*, 2009). Therefore, it seems feasible to speculate that microbiota-dependent modulation of TLRs might be part of the underlying mechanisms mediating changes in neural and immune regulatory mechanisms, such as the expression of cannabinoid receptors, as presented here.

Overall, these observations support the view that gut commensal microbiota is able to modulate the expression of cannabinoid receptors within the gut and that this might be part of the underlying mechanisms mediating the beneficial effects of certain probiotics on gastrointestinal disorders. Moreover, the present data directly implicate large spontaneous changes in gut commensal microbiota with the modulation of endogenous systems potentially implicated in sensory responses arising from the gut. This studies support an interaction between commensal microbiota and neural enteric functions and warrant further studies assessing how spontaneous or directed changes in gut commensal microbiota affect neural functions within the gut from a functional, morphological and molecular point of view.

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Discusión general

A continuación se presenta una discusión general, que tiene como objetivo relacionar e integrar los tres trabajos de investigación, y las discusiones individuales que en cada uno se ha realizado, y que componen esta memoria.

Durante los últimos años, y en particular en países desarrollados, ha aumentado la incidencia de enfermedades tales como la obesidad, las alergias, el asma, el síndrome del intestino irritable o la enfermedad inflamatoria intestinal. En general, no se conoce con exactitud la etiología de estas enfermedades, pero se especula que sean procesos multifactoriales dependientes de factores genéticos, inmunitarios y ambientales (dieta, estrés, hábito de fumar, o la higiene extrema) (Fiocchi, 1998, 2005; Danese *et al.*, 2004).

En el caso particular de las alteraciones gastrointestinales parece que la higiene, y como ésta afecta a la microbiota comensal del intestino, es un factor clave en su desarrollo (Strachan, 1989; Stene and Nafstad, 2001; Hooper, 2004; Czeresnia, 2005; Guarner *et al.*, 2006). Por ello, ha aumentado notablemente el interés por el estudio del complejo ecosistema microbiano del tracto gastrointestinal, tanto de la especie humana como en otras especies; con especial énfasis en su posible implicación causal en estados fisiopatológicos y en su potencial utilidad terapéutica para restaurar la homeostasis intestinal.

El objetivo general de este trabajo de investigación ha sido el de demostrar cómo, la microbiota comensal del intestino experimenta cambios adaptativos espontáneos a las condiciones higiénicas de cría y mantenimiento de ratas, y cómo éstos afectan a los mecanismos intestinales de reconocimiento de la microbiota y modulan las respuestas inflamatorias y la expresión de sistemas endógenos sensoriales.

En general, nuestros resultados muestran que la microbiota intestinal es un sistema dinámico, que varía y se adapta espontáneamente a las condiciones ambientales en las cuales se mantienen los animales de experimentación. Estos cambios implican variaciones en la homeóstasis intestinal que afectan la expresión de receptores de reconocimiento bacteriano (TLR-2 TLR-4) o de receptores canabinoides (CB_1 y CB_2) y la susceptibilidad a la inflamación.

La composición de la microbiota comensal del intestino cambia espontáneamente con el ambiente

El interés por el estudio de la microbiota intestinal se ha visto reforzado con la aparición de diversas técnicas moleculares, como son el FISH o el análisis t-RTFLP, que facilitan la caracterización y el seguimiento de una enorme variedad de grupos microbianos, incluyendo poblaciones no cultivables (Amann *et al.*, 1990; Cancilla *et al.*, 1992; Levitt, 1994; Manz *et al.*, 1996; Moter and Gobel, 2000; Kitts, 2001; Harmsen *et al.*, 2002; Salzman *et al.*, 2002; Cole *et al.*, 2003). Nosotros hemos usado estas técnicas para determinar variaciones espontáneas, tanto cualitativas como cuantitativas, de la microbiota comensal del intestino en ratas mantenidas en condiciones ambientales que se corresponden con las condiciones usadas de forma estándar en experimentación animal.

En general, las poblaciones intestinales dominantes están compuestas por grupos de microorganismos anaerobios estrictos, como son *Clostridium*, *Bifidobacterium* y *Bacteroides*, con niveles muy superiores a los de las poblaciones anaerobias facultativas y aerobias, tales como Coliformes, *Lactobacillus*, y *Enterococos* (Tannock, 1999; Brooks *et al.*, 2003). Considerando esto, y al igual que en trabajos previos (Dinoto *et al.*, 2006), nuestros estudios se han basado en la cuantificación de estos grupos de bacterias. En su conjunto, estos grupos de microorganismos se ha estimado que representan sólo un 29% de la microbiota comensal total del intestino de la rata (Dinoto *et al.*, 2006). Sin embargo, se consideran representativos de la microbiota comensal por su relevancia en procesos fisiológicos y fisiopatológicos intestinales.

Si bien nuestro trabajo no caracteriza la totalidad de la microbiota intestinal de la rata, los grupos microbianos caracterizados demostraron claramente que la microbiota comensal es diferente en animales criados en condiciones de barrera, con una higiene estricta, comparada con la de animales criados en condiciones convencionales. Además, los resultados obtenidos muestran que animales criados en barrera y adaptados a las condiciones convencionales presentan una microbiota de características intermedias. Esto sugiere que la microbiota intestinal varía espontáneamente, adaptándose a las características del ambiente en que están los animales.

De lo expuesto anteriormente podemos deducir que la microbiota comensal del intestino constituye un sistema dinámico y que las condiciones ambientales son un factor determinante en su composición, aparentemente más que las condiciones de implantación de la microbiota en una edad temprana. Sin embargo, debemos diferenciar entre la microbiota luminal y la microbiota de la mucosa intestinal. Diferentes estudios muestran resultados hasta cierto punto contradictorios, sugiriendo tanto que la composición de la microbiota luminal es diferente a la microbiota de la mucosa intestinal (Pryde *et al.*, 1999), así como que ambas presentan una gran similitud (Simpson *et al.*, 1999). Los resultados obtenidos en este trabajo están de acuerdo con estudios que sugieren que las bacterias adheridas a la mucosa representan solamente un subgrupo de bacterias del lumen intestinal (Leser *et al.*, 2002).

Estas observaciones pueden tener implicaciones prácticas, ya que es razonable pensar que las características de la microbiota intestinal puedan determinar las respuestas del tracto gastrointestinal a estímulos tanto fisiológicos como fisiopatológicos. Por lo tanto, las características de la microbiota deberían ser un factor a considerar en estudios con animales y en la extrapolación de resultados a la especie humana.

Como se mencionó en la introducción, las ratas criadas en condiciones de barrera (SPF), tienen una microbiota intestinal asociada no patógena y específica, inoculada al nacimiento mediante una mezcla de bacterias que

contiene los principales grupos de la microbiota intestinal (Ver como referencia la Tabla1 del Estudio 1) (Dewhirst *et al.*, 1999). Sin embargo, en esta mezcla no se incluyen Bifidobacterias, las cuales, junto a los Lactobacilos, son consideradas beneficiosas para el organismo, y utilizadas frecuentemente como probióticos (Naidu *et al.*, 1999; Grabig *et al.*, 2006). Por lo tanto, es normal que en estos animales los contajes de Bifidobacterias sean bajos cuando se comparan con animales criados en condiciones convencionales. Por el contrario, otros grupos bacterianos se encuentran en concentraciones similares en ambos grupos (Enterobacterias) o incluso en mayor cantidad en el grupo barrera (*Bacteroides*, y *Clostridium*). Es interesante destacar que algunos de los grupos bacterianos presentes preferentemente en los animales criados en condiciones de barrera (como *Bacteroides*, *Clostridium o* Enterobacterias) se han asociado a la Enfermedad Inflamatoria Intestinal y se consideran un factor de patogenicidad en la misma (Konaka *et al.*, 1999; Basivireddy *et al.*, 2005; Swidsinski *et al.*, 2005[a, b]; Dalby *et al.*, 2006; Larrosa *et al.*, 2009; Natividad *et al.*, 2009; Watanabe *et al.*, 2009). Esta observación permite, hipotetizar que éstos animales serían más susceptibles a la inflamación intestinal que los criados en condiciones convencionales. Esta hipótesis, y las observaciones que han llevado a la misma, son la base del segundo estudio de esta memoria.

En ese estudio se ha intentado determinar si efectivamente los animales criados en barrera, con una higiene estricta y una microbiota que parece ser potencialmente pro-inflamatoria, son más susceptibles a la inflamación inducida por indometacina que los criados en condiciones convencionales, tal y como se discute más adelante.

Los cambios espontáneos de la población global de la microbiota comensal del intestino modulan la expresión intestinal de TLRs

Es razonable suponer que los cambios adaptativos en la microbiota comensal supongan, además, cambios en las respuestas inmunes intestinales implicadas en el reconocimiento de la microbiota intestinal.
La inmunidad innata es la primera línea de defensa frente a las bacterias (Abbas *et al*, 2008). En el intestino, su función básica es el reconocimiento de la microbiota. Para ello emplea un sistema de receptores específicos, entre los cuales se incluyen los receptores TLR (Rock *et al.*, 1998; Abbas *et al*, 2008).

En particular, los receptores TLR-2 y TLR-4 reconocen componentes de la pared celular de bacterias grampositivas y gramnegativas, respectivamente (Takeuchi *et al.*, 1999; Takeda *et al.*, 2003; Takeda and Akira, 2003). Varios estudios *in vitro* han demostrado la activación de estos receptores por poblaciones bacterianas específicas del tracto gastrointestinal (Cario and Podolsky, 2000; Sato *et al.*, 2000; Hausmann *et al.*, 2002; Netea *et al.*, 2004; Muller-Decker *et al.*, 2005; Le Mandat *et al.*, 2007; Spiller *et al.*, 2008), sin embargo hasta la fecha no se han realizado estudios similares *in vivo* con toda la población bacteriana del intestino.

En este trabajo de investigación, se ha analizado la expresión de éstos receptores en condiciones fisiológicas, en presencia de toda la población de bacterias cecales, y no únicamente de grupos bacterianos específicos. Estas condiciones pueden dar una indicación más cercana a la realidad que los estudios *in vitro* realizados hasta el momento sobre como el intestino y la microbiota interaccionan en su reconocimiento. En las presentes condiciones, a pesar de variaciones significativas en la microbiota intestinal entre animales criados en condiciones de barrera y en condiciones convencionales o adaptados a las mismas, tal y como se ha discutido anteriormente, únicamente se observaron cambios menores en la expresión de TLR-4.

Además, en general, no se observó ninguna correlación entre los niveles de expresión de TLR-2 o TLR-4 y la abundancia relativa de bacterias grampositivas o gramnegativas en el contenido intestinal. Esta aparente discrepancia podría tener varias explicaciones. Dada la complejidad de la microbiota intestinal, es posible que la expresión de TLRs no sólo dependa de las bacterias caracterizadas en este estudio sino de otros grupos bacterianos presentes en el tracto gastrointestinal y no caracterizados directamente (Heine *et al.*, 1999; Backhed *et al.*, 2003; Eckburg *et al.*, 2005). Alternativamente, la expresión de estos TLRs puede depender de múltiples mecanismos moduladores que, en conjunto, resultan en variaciones muy sutiles de expresión.

Además, los TLRs son un complejo de múltiples receptores, hasta 11 en la especie humana (Harris *et al*., 2006), y aunque los tipos 2 y 4 parecen ser los más importantes a nivel intestinal, otros pueden estar presentes y estar involucrados de forma más directa por las variaciones de la microbiota descritas aquí. En su conjunto, estos resultados sugieren que la expresión de TLRs puede depender de múltiples mecanismos y que, por lo tanto, no se pueden realizar extrapolaciones directas de observaciones *in vitro* a condiciones *in vivo*. De la misma forma, el análisis único de TLRs de tipo 2 y 4 puede ser insuficiente para detectar cambios en este sistema asociados a variaciones espontáneas de la microbiota comensal intestinal, que además no implican la aparición de microbiota patógena. En este sentido, podría ser de interés realizar estudios similares en los cuales se compare la expresión de otros TLRs.

Sin embargo, tal y como se describe en el segundo estudio de esta memoria, es destacable la variación en los niveles de expresión de TLR-2 y TLR-4 entre íleon y ciego; siendo, en general, los niveles de expresión superiores en el íleon que en el ciego. Lo verdaderamente interesante es que esta diferencia contradice con el hecho de que el ciego tiene una microbiota más rica y más diversa que el íleon. Por lo tanto, se podría hipotetizar que una mayor riqueza en la microbiota implicaría una mayor actividad de los mecanismos bacterianos de regulación del sistema inmune intestinal y, como

consecuencia, una supresión activa de los mecanismos intestinales de reconocimiento de la microbiota (en este caso TLR-2 y TLR-4) para permitir una mayor biodiversidad microbiana.

Es decir que, frente a diferentes áreas intestinales con una microbiota más limitada, el ciego estaría adaptado a tolerar una mayor carga y diversidad bacteriana reduciendo la expresión de los mecanismos de reconocimiento de la microbiota. Estas variaciones adaptativas en la expresión de TLRs entre diferentes áreas del intestino podrían ser uno de los factores mediando respuestas inmunes alteradas frente a variaciones y/o aumentos de la microbiota que pueden llevar, por ejemplo, a estados inflamatorios persistentes, como parece suceder en la Enfermedad Inflamatoria Intestinal.

Durante la inflamación intestinal la microbiota comensal cambia tanto cualitativa como cuantitativamente

Como se ha mencionado, numerosos estudios han relacionado la microbiota comensal del intestino con la homeostasis intestinal (Sartor, 1997; Macpherson and Harris, 2004; Macpherson and Uhr, 2004). En particular, la microbiota se ha implicado, como un factor de patogenicidad, tanto en la especie humana como en modelos animales, en las respuestas inflamatorias intestinales (Duchmann *et al.*, 1995; MacDonald, 1995; Neut *et al.*, 2002; Swidsinski *et al.*, 2005[a, b]; Dalby *et al.*, 2006; Larrosa *et al.*, 2009; Natividad *et al.*, 2009; Watanabe *et al.*, 2009). Por lo tanto, es razonable pensar que animales con características microbiológicas diferentes, como son las situaciones experimentales descritas anteriormente, pueden presentar cambios en la susceptibilidad a la inflamación intestinal y/o variaciones específicas de la microbiota asociadas al proceso inflamatorio.

Siguiendo estas hipótesis, en un modelo de enteritis (íleitis) aguda inducida por indometacina se ha demostrado que, durante la inflamación, la microbiota intestinal comensal varía. Las variaciones de la microbiota se observaron especialmente en el íleon, que corresponde al área de mayor inflamación en este modelo. Las alteraciones de la microbiota afectaron

fundamentalmente a las concentraciones de enterobacterias, *Bacteroides* spp y *Clostridium* spp. Estos resultados concuerdan con estudios previos que implican a estos grupos bacterianos en respuestas inflamatorias intestinales en diferentes modelos animales (Rath *et al.*, 2001; Porras *et al.*, 2004; Swidsinski *et al.*, 2005[a]; Dalby *et al.*, 2006; Heimesaat *et al.*, 2007; Watanabe *et al.*, 2009). Estas observaciones sugerirían que los cambios en la microbiota no son específicos del modelo inflamatorio empleado en el presente estudio (indometacina) sino que serían cambios genéricos asociados a la presencia de inflamación.

Además de estas variaciones cuantitativas de la microbiota hay que destacar dos observaciones que pueden ser de relevancia en la respuesta inflamatoria. La primera es el aumento en la adherencia bacteriana a la pared el intestino durante la inflamación y la segunda es la aparición de variaciones en la morfología bacteriana, que afectan fundamentalmente al género *Clostridium* e implican el cambio de una forma bacilar a formas cocobacilares. Estos hallazgos se observaron mayoritariamente en animales criados en condiciones convencionales. Estos factores, junto a la presencia de enterobacterias, previamente implicadas en procesos inflamatorios (Rath *et al.*, 2001; Porras *et al.*, 2004), podría sugerir que estos animales tienen una mayor respuesta a los estímulos inflamatorios que los criados en condiciones de barrera y adaptados posteriormente a condiciones convencionales; como corroboran, además, los parámetros de actividad de la inflamación (ver estudio 2 y punto siguiente).

En general, los cambios observados en la microbiota apoyan la hipótesis de que la microbiota comensal es un componente activo en la respuesta inflamatoria intestinal y que puede influir en la severidad de la misma en modelos experimentales.

Los animales criados en condiciones ambientales convencionales muestran una mayor respuesta inflamatoria aguda a la indometacina que los animales criados en condiciones de barrera y adaptados a un ambiente convencional

Actualmente un tema muy debatido es la llamada "hipótesis de la higiene". Según esta hipótesis, se asume que el incremento en la incidencia de enfermedades con una base inmunitaria (como pueden ser las alergias o la enfermedad inflamatoria intestinal) se asocia al aumento generalizado de las condiciones higiénicas en las sociedades occidentales (Strachan, 1989; Czeresnia, 2005; Guarner *et al.*, 2006).

De esta hipótesis se desprendería que una microbiota intestinal variada funcionaría como un factor de protección frente a la inflamación, mientras que al restringir la microbiota (por ejemplo con una higiene elevada) la incidencia/gravedad de la inflamación aumentaría. Por el contrario, nuestros resultados demuestran que las ratas criadas en un ambiente de barrera, con una higiene extrema, y adaptadas a un ambiente convencional tienen una menor respuesta a la inflamación intestinal que las ratas criadas en un ambiente convencional.

En general, los índices de actividad de la inflamación valorados en el modelo de enteritis inducida por indometacina mostraron que los animales convencionales tienen una tendencia general a mostrar una respuesta inflamatoria más severa que los animales criados en condiciones de barrera y adaptados posteriormente a condiciones convencionales. Estas variaciones en la respuesta a la inflamación podrían estar relacionadas con la diferente microbiota intestinal presente en ambos grupos, tal y como se demuestra con la técnica de t-RFLP, así como las diferencias en el proceso de colonización de la microbiota (adquisición original de una microbiota convencional versus adaptación a la misma tras la implantación de una microbiota restringida).

La menor respuesta inflamatoria observada en animales criados en condiciones de barrera y adaptados a condiciones convencionales sugiere que la colonización de la microbiota en fases tempranas de la vida, puede ser importante determinando las respuestas inflamatorias en la Enfermedad Inflamatoria Intestinal, independientemente de que a *posteriori* la microbiota comensal intestinal se diversifique por un mecanismo de adaptación espontánea a condiciones microbiológicas no específicas (Shi and Walker, 2004; Thompson-Chagoyan *et al.*, 2007).

Por otro lado, es importante mencionar que el modelo utilizado analiza la respuesta aguda, pero no la respuesta crónica de la inflamación o el proceso de resolución de la inflamación. Por lo tanto, cabe la posibilidad de que existan diferencias en el modo en que la inflamación progresa de aguda a crónica y en el tiempo de resolución en función de la microbiota. Puede suceder que animales criados en condiciones convencionales tengan una respuesta inflamatoria aguda más intensa que animales con una microbiota más restringida pero que resuelvan mejor el proceso inflamatorio en el tiempo (menor cronicidad de la inflamación) que éstos últimos. De ser así, nuestras observaciones serían, en general, compatibles con la "hipótesis de la higiene". Sin embargo, para evaluar esta posibilidad son necesarios estudios en fase crónica y/o de evaluación de la resolución del proceso inflamatorio en las mismas condiciones experimentales.

En cualquier caso, los resultados obtenidos, tal y como ya se ha comentado en el punto anterior, apoyan la idea de que la microbiota comensal del intestino es un factor a considerar en la patogenia de la inflamación intestinal, aunque su importancia relativa puede variar según la fase (aguda versus crónica o resolutiva) que se evalúe dentro de un proceso inflamatorio.

Cambios espontáneos de la microbiota comensal del intestino afectan a la expresión de receptores canabinoides (CB1 y CB2)

Varios autores sugieren que la microbiota intestinal podría influenciar los mecanismos sensoriales en el intestino y por tanto modular las respuestas de dolor visceral (It-Belgnaoui *et al.*, 2006; Verdú *et al.*, 2006; Rousseaux *et al.*, 2007; Amaral *et al.*, 2008). Sin embargo, sabe poco de la implicación directa de la microbiota comensal del intestino en la regulación del sistema endocanabinoide y, en particular, de la expresión de los receptores canabinoides. Los resultados obtenidos muestran que los cambios espontáneos de la microbiota comensal del intestino modulan la expresión de los receptores canabinoides, particularmente del CB_2. Además, éstos cambios espontáneos se correlacionaron positivamente con los contajes de *Bacteroides* spp y de *Clostridium* spp, y negativamente con los de *Bifidobacterium* spp.

Estos resultados contrastan con estudios previos que encuentran una influencia del *Lactobacillus*, pero no del *Bifidobacterium*, en la expresión del CB_2 (Rousseaux *et al.*, 2007). Esto podría deberse a que en el estudio mencionado se inoculan cepas específicas de *Bifidobacterium* y de *Lactobacillus* y en nuestro estudio se consideran variaciones espontáneas de toda la población microbiana comensal del intestino. Por lo tanto, estas diferencias sugieren que mútiples tipos bacterianos, además de *Bifidobacterium* y *Lactobacillus,* pueden intereaccionar modulando la expresión de receptores implicados en respuestas sensoriales y por tanto la actividad de estos mecanismos dentro de la homeostasis intestinal.

Como hemos dicho, el receptor CB_2 parece ser el más afectado por la microbiota. Se ha sugerido que estos receptores tienen una implicación preferencial en la modulación de la respuesta inflamatoria. Así, su expresión aumenta en estados inflamatorios y podrían ser importantes modulando mecanismos neuroinmunes endógenos implicados en los procesos de resolución de la inflamación (Wright *et al*., 2008; Storr *et al*., 2009). Según

esto sería razonable especular que los niveles de expresión intestinal de los receptores CB_2 podrían relacionarse con la susceptibilidad a padecer enteritis y/o a la gravedad de la misma. En este sentido, es interesante relacionar los resultados de este estudio con los obtenidos en el modelo de enteritis inducida por indometacina discutido anteriormente. En esos estudios se observó que los animales convencionales presentaban una respuesta inflamatoria más grave que los animales mantenidos en barrera y adaptados a condiciones convencionales. Aunque la expresión de CB_2 no se ha determinado en esos animales es factible asumir, como se ve en este estudio, que los animales convencionales tenían, comparativamente, niveles menores de receptores CB_2 que aquellos únicamente adaptados a esas condiciones. Como consecuencia, su respuesta a un estimulo inflamatorio, como es la indometacina, debería ser comparativamente superior, como efectivamente se observó en ese estudio.

En cualquier caso, los resultados obtenidos sugieren que la microbiota comensal, actuando globalmente, modula la actividad de los sistemas sensoriales de regulación del intestino, en particular del sistema endocanabinoide. A través de estas acciones se podrían modificar los mecanismos neuroinmunes implicados tanto en respuestas de dolor visceral como en la modulación de los mecanismos inflamatorios intestinales. Estas acciones podrían ser parte de los mecanismos por los cuales los probióticos ejercen sus efectos beneficiosos en pacientes con enfermedad inflamatoria intestinal o síndrome de intestino irritable, procesos caracterizados por alteraciones sensoriales y/o defectos en los mecanismos que determinan la cronificación y la resolución de las respuestas inflamatorias.

En su conjunto, los estudios presentados demuestran que la microbiota comensal del intestino de la rata es una variable dinámica y compleja, con capacidad de adaptarse espontáneamente a cambios en las características microbiológicas del ambiente. Lo que es tal vez más importante, los resultados obtenidos muestran que estas variaciones en la microbiota implican cambios en los mecanismos homeostáticos intestinales, en particular en la expresión de los sistemas de reconocimiento bacteriano

dependientes de los TLR-2 y TLR-4 y en la expresión de receptores para canabinoides, CB_1 y CB_2. Estas observaciones sugieren que pueden presentarse, además, cambios funcionales de relevancia en ciertas situaciones fisiopatológicas. En concreto, se han observado variaciones en la susceptibilidad a la inflamación intestinal inducida por indometacina.

Una consecuencia práctica general de estos estudios es que el estado microbiológico en el que se mantienen los animales de experimentación puede ser un factor determinante de las respuestas obtenidas en estudios funcionales gastrointestinales y, por lo tanto, determinar el valor translacional a la especie humana de los mismos.

Conclusiones

1. La microbiota intestinal comensal constituye una población dinámica y compleja, que puede variar adaptándose espontáneamente a las condiciones microbiológicas ambientales.

2. Cambios adaptativos espontáneos de la microbiota comensal del intestino al pasar de condiciones de barrera a condiciones convencionales se asocian a cambios moderados en la expresión de los sistemas de reconocimiento bacteriano dependientes de los receptores TLR-2 y TLR-4; lo que sugiere una regulación multifactorial de los mismos.

3. Durante la enteritis inducida por indometacina se producen variaciones cualitativas y cuantitativas específicas de la microbiota comensal del intestino, tanto luminal como adherida a la pared.

4. Los animales criados en condiciones ambientales convencionales muestran una mayor respuesta aguda a la indometacina como estimulo inflamatorio que los animales criados en condiciones de barrera y adaptados a un ambiente convencional.

5. Cambios adaptativos espontáneos de la microbiota comensal del intestino afectan a la expresión intestinal de los receptores canabinoides (CB_1 y CB_2). La adaptación a condiciones microbiológicas convencionales implican una reducción en la expresión de ambos receptores, pero principalmente del receptor CB_2.

Conclusión final

La microbiota comensal del intestino es un factor dinámico en la homeostasis intestinal. Las condiciones microbiológicas de cría y mantenimiento de los animales de experimentación pueden modificar los resultados de estudios experimentales gastrointestinales y deben tenerse en cuenta en la interpretación de los mismos.

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